CELLULAR IMMUNOLOGY 96, 12–25 (1985)

# Immune Responses to T-Dependent and T-Independent Antigens during Visceral Leishmaniasis in Mice: Evidence for Altered T-Cell Regulation of Immune Responses to Non-parasite Antigens[1]

STEVEN G. REED,*,[2] SILENE B. ROTERS,† JILL A. INVERSO,*
THOMAS C. JONES,* AND EDMOND A. GOIDL‡

*Cornell University Medical College, New York 10021; †The Federal University of Bahia, Salvador, Brazil; and ‡University of Maryland School of Medicine, Baltimore, Maryland 21201

Received April 1, 1985; accepted June 27, 1985

Antibody responses to T-dependent and T-"independent" antigens were studied in disease-susceptible (BALB/c and C57BL/10) and disease-resistant (A/J) mice infected with *Leishmania donovani chagasi.* Disease-susceptible mice but not disease-resistant mice showed a transient decrease in PFC responses to TNP on a T-dependent carrier (BGG) during the period of 4–8 weeks after infection. Infected disease-susceptible animals also showed increased responses to TNP on a type II T-independent carrier (Ficoll), which persisted until at least 14 weeks after infection. The increased responses were associated with a significant increase in anti-TNP antibody of the IgG2b subclass. When T-enriched spleen cells from infected mice and B-enriched spleen cells from uninfected mice were transferred to irradiated recipients immunized with TNP–Ficoll, increased anti-TNP PFC were observed over numbers seen in irradiated recipients which received both B and T cells from uninfected mice. Increased responses to TNP–Ficoll were also induced by prior administration of soluble leishmania extract in CFA. Infected mice immunized with TNP-LPS, a T-independent type I antigen, also had increased anti-TNP antibody responses, but had normal anti-LPS antibody responses. The elevated antibody production which occurred in response to the T-"independent" antigens could not be attributed to the relatively low polyclonal response which occurred in both disease-resistant and disease-susceptible mice infected with *L. donovani chagasi.* The observations are consistent with leishmania induced, transient alterations in some T-cell functions including response to haptens on T-dependent carriers, and a lack of down regulation of T-"independent" responses. Subtle lesions in immunoregulation may be important correlates of successful protozoal infection and may be responsible for some of the immunologic manifestations of the disease. © 1985 Academic Press, Inc.

## INTRODUCTION

The leishmania are a diverse group of intracellular protozoan parasites responsible for both visceral and cutaneous infections in humans. Inbred strains of mice have been useful as models for several of the clinical forms of the disease. Mice display varying degrees of susceptibility to either visceral (*Leishmania donovani*) (1, 2) or

[1] This work was supported by NIH Program Project AI 16282. These data were presented in part at the annual meeting of the Federation of American Societies Experimental Biology and Medicine, 1982.

[2] Present address: Issaquah Health Research Institute, 1595 N.W. Gilman Blvd., Issaquah, Wash. 98027.



0008-8749/85 $3.00
Copyright © 1985 by Academic Press, Inc.
All rights of reproduction in any form reserved.

cutaneous (*L. mexicana* and *L. tropica*) (3–5) leishmaniasis. Infection with species responsible for human visceral leishmaniasis is characterized in BALB/c and C57BL mice by the development of high parasite burdens which then fall to low levels after several months (1, 6). Other mouse strains, such as A/J, develop and maintain low parasite levels throughout the infection consistent with relative resistance to infection. Genes associated with these variations in response to infection have been suggested (2, 7).

Intact T-cell function is essential for the development of resistance to leishmaniasis. An important correlate of the control of leishmania infection in mice is the generation of lymphocyte products which lead to killing of leishmania within macrophages (8, 9). Recovery from visceral leishmaniasis is strongly associated with the appearance of a positive delayed hypersensitivity to leishmania (10, 11). In contrast, a protective role for antibody has not been established. Humans and experimental animals with fatal leishmania infections invariably have high levels of specific antibody. The regulation of lymphocyte function is therefore of great interest in understanding immunological control of leishmaniasis. Specific (12) and nonspecific (13, 14) suppression of the immune response has been reported in BALB/c mice during nonhealing cutaneous infections. The details of immunoregulatory dysfunction associated with progressive disease have not been well established, although suppressor-T-cell activity has been identified in infected BALB/c mice (12).

One method which has been used extensively for studying immune responses is the analysis of antibody production by isolated spleen lymphocytes to defined haptens on T-dependent and T-independent carriers. This technique, originally described by Jerne (15), has proved useful in studies of the kinetics of the normal immune response (16, 17) as well as those defining immunoregulatory problems in aging (18) and during infection (19). Application of this method to the study of response to antigens which are strongly or weakly T-cell-dependent by animals with leishmaniasis has allowed us to define subtle aspects of immune dysfunction. These alterations are being explored as potentially important correlates of successful parasitism. This paper describes the T-cell regulatory dysfunction during leishmania infection in disease-susceptible mice, in terms of alterations in antibody production to heterologous antigens by spleen cells. We suggest that decreased or altered T-cell regulatory function may play an important role in the hypergammaglobulinemia characteristic of acute visceral leishmaniasis.

## MATERIALS AND METHODS

*Animals.* Eight- to 12-week-old female A/J, BALB/c and C57BL/10 (B10) mice, originally obtained from Jackson Laboratories (Bar Harbor, Me.) were used for all experiments. They were reared in the central animal facility in Fiocruz, Salvador, Bahia, maintained in a temperature-controlled environment with water and a standard mouse chow provided *ad libitum.*

*Parasites.* A strain of *L. donovani chagasi* (MHOM/BR/79/Imperatriz) isolated from the bone marrow of a patient with acute visceral leishmaniasis from northern Brazil was used for all studies. The parasite was characterized by isoenzymes and by kinetoplast-DNA analysis (courtesy of Dr. Peter Jackson, Walter Reed Army Institute of Research) and maintained in hamsters by syringe passage of splenic amastigotes. Infections were fatal in hamsters. Mice were infected by intravenous (iv) inoculation of amastigotes separated from hamster spleen by a modification of the method of

Dwyer (20). Briefly, spleens were homogenized in phosphate-buffered saline, (PBS), pH 7.2, with a tissue grinder, passed twice through a 20-gauge needle, and centrifuged twice at 250*g*. Cells were washed three times in PBS with DNAase (25 $\mu$g/ml) (Sigma Chemical Co., St. Louis, Mo.), treated with tris-ammonium chloride to lyse erythrocytes, washed twice more in PBS, and separated on a Ficoll–Hypaque gradient (density 0.1077). The parasite band was removed, washed twice, and resuspended in PBS. Mice were infected with $2 \times 10^6$ amastigotes.

*Determination of parasite burden.* At various times after infection liver imprints were stained with Giemsa and the number of amastigotes was determined after counting at least 1000 host cells on different oil-immersion fields. Total liver parasite burdens were determined by the method of Stauber (21). Spleen index was calculated as:

$$\frac{\text{mean spleen wt infected group}}{\text{mean spleen wt uninfected group}}.$$

*Reagents and antigens.* Trinitrophenyl (TNP) derivatives of bovine $\gamma$-globulin (BGG, Miles-Yeda, Kankakee, Ill.) were prepared by the reaction of 2,4,6-trinitrobenzene sulfonic acid ($TNBSO_3$, Sigma) with protein as described (22). TNP–Ficoll was prepared by coupling TNP–lysine (Sigma) to Ficoll 400 (Pharmacia Fine Chemicals, Piscataway, N.J.) as described (23). TNP-LPS was purchased from Calbiochem, (La Jolla, Calif.). Sheep red blood cells (SRBC) were obtained from a select animal in the Fiocruz herd.

*Cell separation.* T-Enriched spleen cells were obtained by nylon wool purification followed by treatment with rabbit anti-mouse Ig (Cappel, Malvern, Pa.) plus lyophilized rabbit serum as a source of complement (C). Cells obtained by this procedure were approximately 92% Thy 1.2 positive and 96% Ig negative as determined by immunofluorescence. T-Depleted (B-enriched) spleen cells were obtained by two cycles of treatment with a cocktail consisting of monoclonal anti Thy 1.2 and anti Lyt 1.2 (both from New England Nuclear, Boston, Mass.) followed by C. These cells were approximately 98% Thy 1.2 negative and 91% Ig positive.

*Irradiation.* BALB/c mice were lethally irradiated using a $^{60}$Co source, calculated to deliver a total dose of 600 R.

*Immunizations.* Mice were immunized at different intervals postinfection by iv injection of 10 $\mu$g TNP–Ficoll or intraperitoneal (ip) injection of 500 $\mu$g TNP-BGG emulsified in complete Freund's adjuvant (CFA), or 40 $\mu$g of TNP-LPS. Animals were killed by cervical dislocation and their spleens assayed for the number of plaque-forming cells (PFC).

*Parasite extract.* Promastigotes of *L. donovani chagasi* were grown in RPMI 1640 (GIBCO) supplemented with hemin (10 $\mu$g/ml), essential and nonessential amino acids (100× concentrates from GIBCO), and 20% fetal bovine serum. Parasites were harvested in early stationary phase, washed three times in phosphate-buffered saline, pH 7.0, with 2% glucose, resuspended in sterile $H_2O$, and subjected to 10–15 rapid freeze–thaw cycles. The suspension was centrifuged at 5000*g* for 30 min at 4°C, supernatant collected, adjusted to 0.8% saline, and diluted to the desired protein concentration as determined by the Bio-Rad protein assay (Bio-Rad, Richmond, Calif.).

*PFC assay.* A modification of the Jerne method was used to determine the number of anti-TNP PFC (15, 24). The indicator cells were sheep red blood cells (SRBC) which

had been conjugated with $TNBSO_3$ by the method of Rittenberg and Pratt (25). Cell suspensions were obtained from individual spleens of experimental animals, washed once, and resuspended in Hank's balanced salt solution. Lyophilized guinea pig serum (Grand Island Biological Co., Grand Island, N.Y.) was dissolved in Millipore-filtered, deionized $H_2O$ and used at 1/20 dilution as a source of C. Rabbit anti-mouse $\gamma$-globulin (Cappel) and goat anti-mouse $\mu$, our own preparation, were assayed for optimal plaque development and used at the appropriate dilution to develop the indirect plaques.

*ELISA.* To determine IgG subclass levels of anti-TNP antibody, the indirect ELISA assay was performed in 96-well flat-bottom, polystyrene MicroTest III plates (Falcon, Oxnard, Calif.) according to methods previously described (26). One microgram TNP-BGG was used to sensitize each well. Test sera were diluted 1:50 for the assay. Goat anti-mouse subclass antibodies, peroxidase conjugates (Serotec, Oxon, England) were titrated against purified mouse myeloma proteins (Bionetics, Charleston, S.C.) to determine optimal titers at which no cross-reactivity with heterologous subclass proteins occurred. This predetermined optimal concentration of conjugate was used in the assay. Following substrate addition, plates were incubated in the dark until the standard positive sera attained a predetermined value when examined at 490 nm on the ELISA microplate reader (Dynatech, Alexandria, Va.). Absorbance values reflect actual values minus background levels obtained with pooled sera from unimmunized mice.

Student's $t$ test was used to analyze the data.

## RESULTS

### *Course of Infection*

The course of the leishmania infection in susceptible and resistant strains of mice was first determined. Figure 1 shows the total liver parasite burdens following intravenous inoculation of $2 \times 10^6$ amastigotes of *L. donovani chagasi* in BALB/c and A/J mice. In BALB/c mice, liver parasite burdens gradually increased through 10 weeks after infection, and decreased thereafter. At peak infection, a mean of $2.5 \times 10^8$ amastigotes per liver was obtained in these mice. After 22 weeks of infection, the BALB/c mice had reduced parasite numbers to approximately $3 \times 10^3$ amastigotes per liver. The development of splenomegaly paralleled the course of infection in the BALB/c mice (Fig. 1). Infection patterns in B10 mice were similar to those seen in BALB/c mice, except that the B10 mice usually developed higher parasite burdens. The A/J mice had low parasite numbers throughout the period of observation, not exceeding $1.5 \times 10^6$ amastigotes per liver. Significant increase in spleen size was not noted in A/J mice during the course of infection. To evaluate correlates of immune dysfunction during infection, time points between 2 and 14 weeks in BALB/c and B10 mice were selected for comparison with A/J mice.

To determine the role of polyclonal activation by leishmanial antigens during infection, the number of direct anti-TNP PFC per spleen was recorded in control animals and in BALB/c and A/J mice 4 and 14 weeks after infection (Table 1). There was an increase in the number of PFC in infected animals compared to controls. The BALB/c mice at 4 weeks and 14 weeks showed a polyclonal response slightly higher, but not significantly different from that of the A/J mice. Therefore, though polyclonal activation was noted, the changes did not significantly correlate with either mouse strain or time

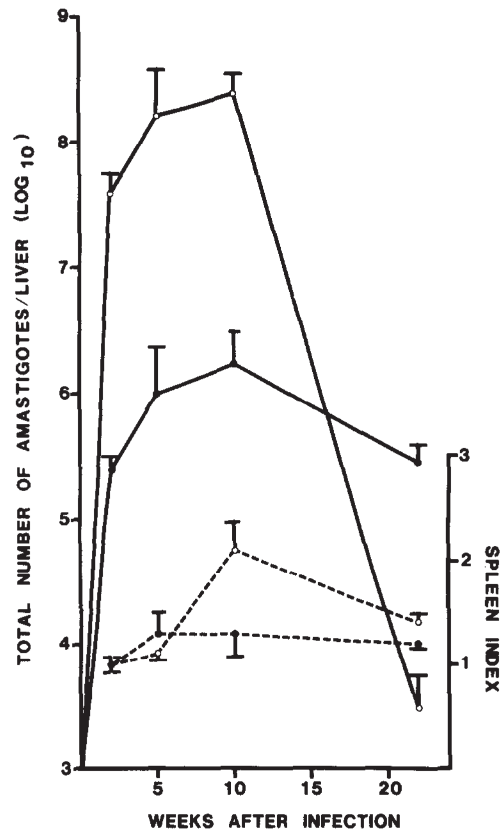


FIG. 1. Course of infection (O—O) and spleen index (O---O) in BALB/c (O) and A/J (●) mice infected intravenously with $2 \times 10^6$ amastigotes of *L. d. chagasi*. Six-eight mice/group. $\bar{x} \pm$ SD.

after infection and could not account for the changes seen in the strain-related responses following infection to primary immunization with the T-dependent and T-independent antigens described below.

TABLE 1

Polyclonal Activation following Infection with *L. donovani chagasi* as Measured by Anti-TNP-SRBC PFC Responses[a]

| | Direct anti-TNP PFC/spleen ($\bar{x} \pm$ SD) | |
|---|---|---|
| | BALB/c | A/J |
| 4 weeks after infection | 570 ± 450 | 200 ± 230 |
| uninfected | 92 ± 78 | 95 ± 71 |
| 14 weeks after infection | 920 ± 790 | 560 ± 180 |
| uninfected | 108 ± 75 | 78 ± 52 |

[a] Mice were infected with $2 \times 10^6$ amastigotes iv; 5 mice/group. BALB/c vs A/J, $P > 0.05$ at all points. BALB/c infected vs. uninfected, $P > 0.05$ at 4 and 14 weeks. A/J infected vs uninfected, $P > 0.05$ at 4 weeks; $P < 0.05$ at 14 weeks.

TABLE 2

Anti-TNP PFC in Mice Infected with *L. donovani chagasi*[a] and Immunized with TNP-BGG[b]

| | | Anti-TNP PFC ($\bar{x} \pm$ SE) | | | |
|---|---|---|---|---|---|
| | | /Spleen ($\times 10^{-3}$) | | /$10^6$ Cells | |
| Mouse strain | Group | Direct | Indirect | Direct | Indirect |
| BALB/c | Uninfected | 1.8 ± 0.8 | 17.3 ± 3.3 | 15 ± 0.5 | 259 ± 70 |
| | Infected | 2.3 ± 0.2[c] | 4.6 ± 1.2[d] | 12 ± 4[c] | 56 ± 20[d] |
| C57BL/10 | Uninfected | 4.3 ± 0.3 | 31.4 ± 5.3 | 22 ± 2 | 155 ± 27 |
| | Infected | 2.7 ± 0.4[c] | 9.1 ± 2.1[d] | 6 ± 1[d] | 20 ± 2[d] |
| A/J | Uninfected | 1.9 ± 0.5 | 18.6 ± 4.0 | 9.1 ± 1 | 80 ± 19 |
| | Infected | 2.1 ± 0.4[c] | 20.7 ± 3.6[c] | 9 ± 3[c] | 99 ± 25[c] |

[a] Mice were infected with $2 \times 10^6$ amastigotes iv; 10 mice/group.

[b] Mice were immunized ip with 500 μg TNP-BGG in CFA, 4 weeks after infection. PFC assay performed 9 days after immunization.

[c] Uninfected mice vs infected $P > 0.05$.

[d] Infected vs. uninfected $P < 0.05$.

### *Analysis of the Immune Response to TNP-BGG during L. donovani chagasi Infection*

Table 2 describes the results of immunizing infected and uninfected mice with TNP-BGG. At 4 weeks after infection the number of indirect PFC in BALB/c and B10 mice was significantly lower than in uninfected controls, both on a per spleen and per $10^6$ cell basis. Direct (IgM) anti-TNP PFC were not significantly altered in infected BALB/c or B10 mice. There was no decrease in the IgM or IgG anti-TNP PFC numbers in spleens from infected A/J mice. When the time course of the responses was evaluated it was found that the anti-TNP response to TNP-BGG in susceptible mice (BALB/c) was lowest at 4 weeks after infection, but gradually returned to normal levels by 16 weeks (Fig. 2). The splenic anti-TNP PFC response to TNP-BGG in infected A/J mice remained similar to uninfected controls at all points examined.

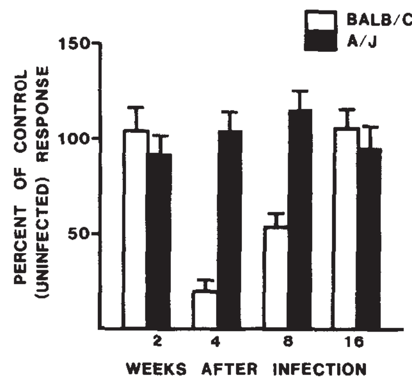


FIG. 2. Indirect anti-TNP-PFC responses in BALB/c and A/J mice immunized with TNP-BGG during infection with *L. d. chagasi*. Results expressed as number of PFC/spleen of infected mice as percentage of PFC/spleen in uninfected (control) mice $\bar{x} \pm$ SD. Five-seven mice/group.

In order to determine the kinetics of responses following immunization of infected mice, we examined PFC responses to TNP at 6 and 9 days after TNP-BGG immunization. Suppressed IgG responses occurred in infected BALB/c and B10 mice at both 6 and 9 days after immunization. The Day 9 data are shown in Table 2. At Day 6 both direct and indirect anti-TNP PFC responses were reduced by $\geq$80% in infected mice, on either a per spleen or per $10^6$ cells basis. The lowered PFC responses observed at Day 9 after TNP-BGG immunization of infected mice did not appear to be the result of an earlier peak in response. We could, therefore, not document any change in the kinetics of the PFC response to TNP-BGG antigen in disease-susceptible mice. In these studies, infected mice immunized with CFA alone had anti-TNP-SRBC PFC numbers on the order of 500–1000/spleen, not significantly different from those seen in unimmunized infected mice.

### *Analysis of Immune Responses to T-independent Antigens during L. donovani chagasi Infection*

Using TNP–Ficoll and TNP-LPS, the responses to T-independent antigens were studied in disease susceptible and resistant mice during *L. donovani chagasi* infection. Unlike the transient suppression of TNP-BGG responses seen in infected BALB/c mice, no suppression of responses to TNP presented on classic T-independent type I (LPS) or type II (Ficoll) antigens was noted in these animals.

Mice were infected with *L. donovani chagasi* and immunized with TNP–Ficoll at 2, 4, 8, and 14 weeks after infection. At all time points, direct anti-TNP PFC were significantly elevated in infected BALB/c mice (Fig. 3). No significant alterations in anti-TNP antibody responses were seen in A/J mice. Experiments were performed to analyze the anti-TNP responses in infected BALB/c mice at various times after immunization with TNP–Ficoll. In both infected and uninfected mice responses decreased after 4 days postimmunization (Table 3). Infected mice had significantly higher than normal numbers of anti-TNP PFC at 4, 7, and 9 days after immunization. Significantly elevated anti-TNP PFC numbers were also seen in infected BALB/c mice following immunization with TNP-LPS (Table 4). However, anti-LPS PFC numbers were not elevated in these mice.

Further experiments were performed to examine mechanisms responsible for increased TNP–Ficoll responses in leishmania-infected mice. Although TNP–Ficoll was previously defined as an antigen not requiring T-cell participation, it has been shown that T cells do contribute to optimal responses (27). Experiments with TNP-BGG suggested a possible defect in T-cell participation in infected mice. Such a defect was also suggested by elevated responses to TNP–Ficoll in infected mice, since elevated responses to TNP–Ficoll have been observed in athymic nu/nu mice (28). Responses to TNP–Ficoll were chosen for further study because these responses were altered early and the alteration persisted until late in infection.

Lethally irradiated BALB/c mice were reconstituted with B-enriched cells and/or T-enriched cells from uninfected or infected BALB/c mice, and immunized with TNP–Ficoll. As expected, irradiated mice receiving no cells had no splenic PFC (Fig. 4). Mice which received only B cells from infected mice produced lower numbers of PFC than mice which received equal numbers of B cells from normal mice. This indicated that on a per cell basis, B cells from infected mice did not display abnormally high activity in TNP–Ficoll responses. However, irradiated mice which received B cells

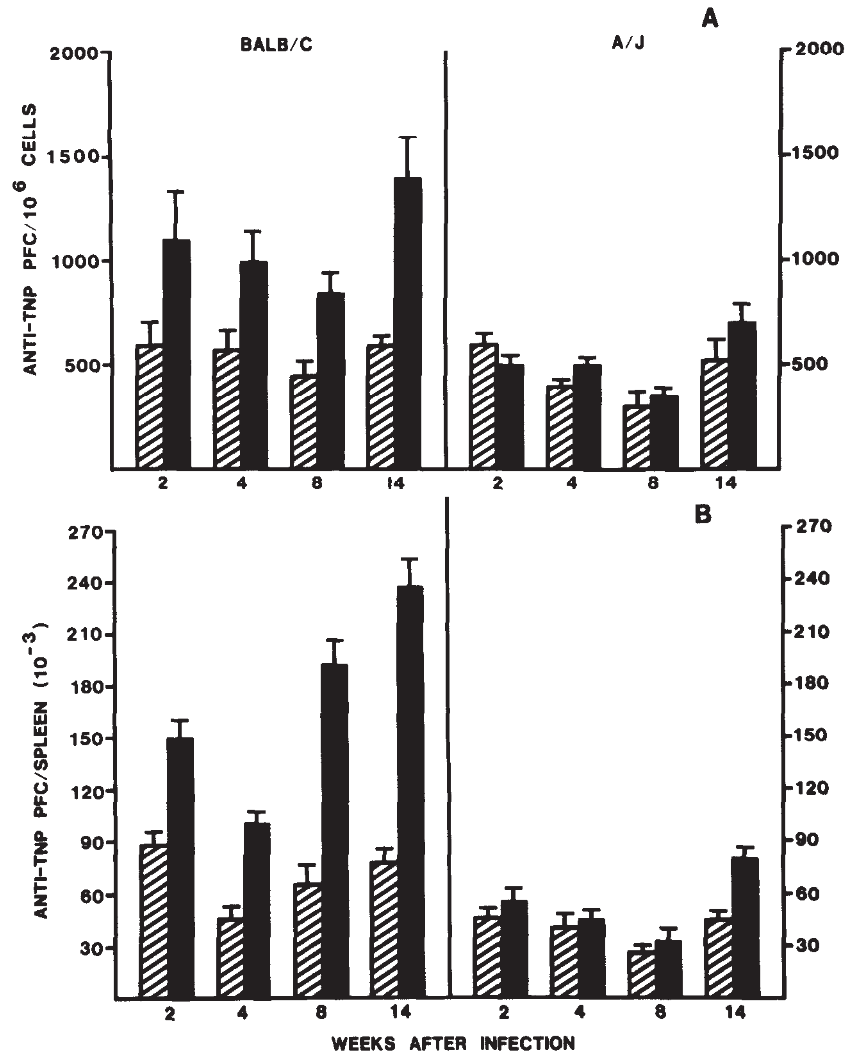


FIG. 3. Direct anti-TNP-PFC/$10^6$ cells (A) and /spleen (B) in BALB/c and A/J mice, uninfected (stripes) or infected (solid bars) with *L. d. chagasi*. Five mice/group. $\bar{x} \pm$ SE.

from uninfected mice plus T cells from infected mice had significantly ($P < 0.05$) higher anti-TNP PFC numbers than those receiving T and B cells from uninfected mice. It was shown that T cells from uninfected mice did not contribute significantly to PFC numbers with the regimen used. However, T cells from infected mice did significantly increase PFC numbers.

Since anti-TNP IgG subclass levels in mice immunized with TNP–Ficoll have been shown to be under T-cell control (16, 29) we determined relative amounts of $IgG_1$, $IgG_{2a}$, $IgG_{2b}$, and $IgG_3$ anti-TNP antibody in mice infected with *L. donovani chagasi* and immunized with TNP–Ficoll (Table 5). Levels of anti-TNP $IgG_1$, $IgG_{2a}$, and $IgG_3$ were not significantly different between infected and uninfected mice. $IgG_{2b}$ levels were significantly ($P < 0.05$) elevated in infected mice.

In order to determine whether leishmania antigens have the ability to alter im-

TABLE 3

Kinetics of Anti-TNP PFC Responses to TNP–Ficoll in Mice Infected with *L. donovani chagasi*

| | Direct anti-TNP PFC/Spleen ($\times 10^{-3}$) $\bar{x} \pm$ SE | |
|---|---|---|
| Day of assay[a] | Uninfected | Infected[b] |
| 4 | 69.6 ± 18.8 | 201.9 ± 25.7 |
| 7 | 37.5 ± 11.2 | 166.8 ± 0.8 |
| 9 | 5.6 ± 1.5 | 54.9 ± 2.7 |

[a] Day after TNP–Ficoll immunization (10 μg iv).
[b] BALB/c mice were infected iv with $2 \times 10^6$ amastigotes 4 weeks before immunization with TNP–Ficoll. Uninfected vs infected, $P < 0.05$ at all time points. Five mice/group.

mune responses in the absence of active infection, mice were injected with a soluble promastigote extract prior to administration of TNP–Ficoll. Intravenous administration of soluble parasite material did not alter anti-TNP PFC numbers (Table 6). However, mice immunized with 500 μg soluble parasite extract in CFA prior to TNP–Ficoll immunization had significantly more anti-TNP PFC than did untreated or CFA-treated mice.

## DISCUSSION

In the present study we observed alterations in the immune responses to hapten-carrier complexes during experimental visceral leishmaniasis in strains of mice which differed in their susceptibility to leishmania infection. These alterations included a transient decrease in the response to hapten on a T-dependent carrier (BGG), particularly noted as a reduced IgM to IgG switch, and increased responses to hapten on a T-independent carrier (Ficoll) in two susceptible mouse strains, BALB/c and B10. These alterations were not observed in infection-resistant A/J mice, and were not due to differences in the kinetics of immune responses in infected mice. The increased responses to TNP–Ficoll observed in BALB/c mice appeared to be due at least in part

TABLE 4

Anti-TNP and Anti-LPS PFC in BALB/c Mice Infected with *L. donovani chagasi* and Immunized with TNP-LPS

| | PFC/Spleen ($\times 10^{-3}$) $\bar{x} \pm$ SE | |
|---|---|---|
| | Anti-TNP | Anti-LPS |
| Uninfected | 47.2 ± 2.8 | 44.8 ± 5.5 |
| Infected[a] | 93.3 ± 4.3 | 47.5 ± 14.9 |

[a] Mice were infected with $2 \times 10^6$ amastigotes iv 8 weeks before immunization with 40 μg TNP-LPS ip. PFC assay performed 5 days later. Uninfected vs infected $P < 0.05$ for anti-TNP PFC, $>0.05$ for anti-LPS PFC. 5 mice/group.

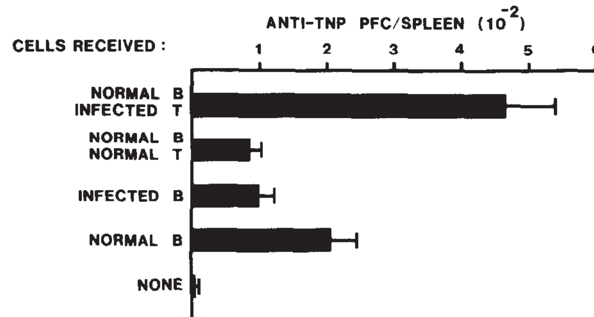


FIG. 4. Direct anti-TNP-PFC/spleen in reconstituted irradiated BALB/c mice immunized with TNP-Ficoll. Mice received $2 \times 10^7$ T-depleted (B) or B-depleted (T) cells from infected (I) or normal uninfected (N) mice iv within 24 hours after lethal whole body irradiation (650 r). TNP-Ficoll (10 μg ip) was injected immediately after cell transfer, and the PFC assay was performed 4 days later. BN vs. none (no cells), $P < 0.01$; BN vs. BI or BNTN, $P > 0.05$; BNTI vs. all other groups, $P < 0.05$. $\bar{x} \pm$ SD. Six recipient mice/group.

to the activity of T cells in the infected mice. Furthermore, soluble extract from leishmania promastigotes, delivered in CFA prior to TNP–Ficoll immunization, mimicked the effect produced by active infection by producing an increase in anti-TNP PFC.

Acute visceral leishmaniasis is characterized clinically by hepatosplenomegaly, hyperglobulinemia, transient suppression of specific delayed-type hypersensitivity, and decreased antigen-specific lymphocyte proliferative responses *in vitro* (10, 30–32). In certain rodent models, including the BALB/c mouse, the acute disease period is followed by spontaneous healing (6) whereas humans with acute disease require drug therapy to initiate reversal of the process. In our epidemiological studies in Brazil we have found that the majority of humans with *L. donovani chagasi* infections do not develop acute disease, but appear to undergo spontaneous healing (Badaro *et al.*, in preparation). In this regard, the mouse model may seem to be useful for the study of some aspects of visceral leishmaniasis, including the sites of the various immunologic lesions that may exist in the responses of infected hosts.

TABLE 5

Anti-TNP ELISA Titers of Normal and *L. donovani chagasi*[a] Infected BALB/c Mice Immunized with TNP–Ficoll[b]

| | IgG Subclass titer ($\bar{x} \pm$ SD) | | | |
|---|---|---|---|---|
| | $IgG_1$ | $IgG_{2a}$ | $IgG_{2b}$[c] | $IgG_3$ |
| Normal | 500 ± 100 | 1200 ± 462 | 300 ± 57 | 150 ± 86 |
| Infected | 680 ± 120 | 880 ± 196 | 900 ± 225 | 120 ± 38 |

[a] Mice were infected with $2 \times 10^6$ amastigotes of *L. donovani chagasi* 4–6 weeks before TNP–Ficoll immunization.

[b] Mice received 10 μg TNP–Ficoll ip 10 days prior to assay.

[c] For $IgG_{2b}$, normal vs infected $P < 0.05$.

TABLE 6

Anti-TNP PFC Responses in Mice Pretreated with Leishmania Extract[a] and Immunized With TNP–Ficoll

| Treatment group | Direct anti-TNP PFC/spleen ($\times 10^{-3}$) ($\bar{x} \pm$ SE) |
|---|---|
| 1. None | 86.6 ± 19.8 |
| 2. CFA | 85.2 ± 22.8 |
| 3. CFA + leishmania extract | 303.7 ± 22.3 |
| 4. leishmania extract iv | 110.8 ± 25.8 |

[a] Mice received 500 μg protein soluble leishmania extract either iv or ip in CFA 7 days before immunization with TNP–Ficoll. Five mice/group. Groups 1 vs 2, $P > 0.05$; 1, 2 vs 3, $P < 0.05$; 1, 2 vs 4, $P > 0.05$.

Depressed T-cell function in responses to homologous and heterologous antigens has been observed in BALB/c mice with cutaneous leishmaniasis. Arrendondo and Perez (13) noted lowered responses to sheep erythrocytes in mice infected with *L. mexicana*. Suppression of specific cellular responses to leishmania antigens were also observed in BALB/c mice during cutaneous leishmaniasis (12). This has been attributed to the emergence of suppressor T cells during infection, the effects of which could be eliminated by sublethal irradiation or thymectomy (34). There is similar evidence for parasite-specific suppressor T cells developing in mice with *L. donovani* infection (35). The T cells involved in specific suppression in mice with cutaneous leishmaniasis appear to be Lyt 1+2− cells (36, 37).

In a model of visceralized cutaneous leishmania infections in highly susceptible BALB/c mice, *in vitro* antibody responses to SRBC were found to be suppressed (38) although *in vivo* responses to SRBC (38) and to TNP–Ficoll and TNP-LPS (39) were augmented, emphasizing that experimental leishmania infections do not lead to generalized suppression of immune responsiveness. Furthermore, it was reported that BALB/c mice infected with *L. donovani* had increased levels of in response antibody to pneumococcal polysaccharide, another T-independent antigen, and were capable of mounting delayed hypersensitivity responses to T-dependent sperm whale myoglobin (40). These same animals, however, had decreased spleen cell proliferative responses to phytohemagglutinin.

We have observed defective regulation of the immune response to heterologous hapten-carrier conjugates in leishmania-susceptible BALB/c and B10 mice during infection with *L. donovani chagasi*. This was evident by lowered responses to TNP-BGG (T-dependent) and elevated responses to TNP–Ficoll and TNP-LPS (T-independent). The depressed TNP-BGG responses were also expressed as a decrease in the IgM to IgG switch. The basis of the observed alterations in T-dependent response in infected BALB/c and B10 mice remains unclear. Although infected BALB/c and B10 mice showed T-dependent and T-independent antigen responses in some ways characteristic of athymic mice, some aspects of T-cell function were apparently intact in these animals. Further, we have observed significant augmentation of *in vivo* PFC responses to SRBC in both BALB/c or B10 mice during acute infection with *L. donovani chagasi*, similar to findings by others (38). It appears paradoxical that responses to one T-dependent antigen TNP-BGG, are suppressed in infected BALB/c mice while

responses to SRBC, also strongly T-cell dependent, are enhanced. However, SRBC share properties with both T-dependent and T-independent antigens (41), and differences exist in the mechanisms of antibody production to haptenated proteins or to SRBC. At least two functional types of helper T cells were identified as necessary for optimal responses to haptenated proteins whereas only one was required for responses to SRBC (42).

The pattern of T-cell dysfunction in leishmania-infected mice resembles the alterations seen in aged animals rather than in those with generalized immunosuppression (17, 18). Suppressor cells are more readily induced in aged than in younger mice (18), and cellular responses in aged mice were shown to be reversed by interleukin 2 (43). Interleukin 2 deficiencies have also been reported in mice with visceral leishmaniasis (44). Alterations in immunoregulation have been described in mice infected with *Toxoplasma* (19) and *Trypanosoma cruzi* (45) infections, raising the possibility that infection with these intracellular protozoa rapidly initiates lesions in the immune response similar to those which develop gradually with age.

We were able to produce heightened responses to TNP–Ficoll in BALB/c mice by prior administration of leishmania antigen in CFA. It is apparent from these and previous studies that leishmania possess molecules capable of altering heterologous immune responses. Weintraub *et al.* (46) showed that an extract of *L. tropica* promastigotes could stimulate division of normal mouse B cells *in vitro.* These authors also reported that BALB/c mice with *L. tropica* infections had increased anti-TNP PFC responses following immunization with TNP-SRBC or TNP-LPS. Anti-SRBC responses were increased in infected mice. They postulated that the increased responsiveness and polyclonal activity observed *in vivo* (46, 47) may be due to mitogenic activity of a parasite molecule(s). The occurrence of parasite products which can augment or suppress immune responses *in vivo* has also been reported for trypanosomes (48, 49).

It is probable that the lowered responses to TNP-BGG in infected BALB/c mice involved a specific defect at the level of T cell function. From the cell transfer experiments reported here it is evident that T cells from infected mice also played a role in increased TNP–Ficoll responses. This may be of interest in further defining cellular interactions involved in immune responses to T-"independent" antigens. T cells or products of T cells have been shown to be important in TNP–Ficoll responses, even though this antigen has been considered to be relatively T independent (26, 50). It is possible that increased activity of one or more T-cell populations could produce an increase in responses to TNP–Ficoll. One possibility is the absence of down-regulation of the normal anti-TNP–Ficoll response, an activity shown to be dependent on T-cell function (28). The impairment of this regulatory activity could be due to increased activity in a population of suppressor T cells which could inhibit normal T-cell-mediated regulation. An association between suppressed-T-cell function and polyclonal B-cell activation in hamsters with *L. donovani* infections has been suggested (51).

Results obtained by analyzing IgG subclasses of anti-TNP-antibody in mice immunized with TNP–Ficoll were also useful in assessing T-cell function. T cells can influence the relative amounts of subclasses of anti-TNP IgG produced in response to TNP–Ficoll. Mongini *et al.* (29) showed that $IgG_{2a}$ and $IgG_{2b}$ were the most dependent on T-cell activity. In the present study, one of these subclasses of anti-TNP antibody, $IgG_{2b}$, was significantly elevated in infected mice, reinforcing the concept

of heightened T-cell activity. The immune deregulation may be mediated by suppressor T cells which are present in experimental cutaneous leishmania infections. However, it is important to emphasize that a rather selective and not generalized suppression occurs in mice with nonfatal visceral leishmaniasis.

## ACKNOWLEDGMENT

We thank Dr. W. Alfonso for irradiation and Anne Lamoreux for preparation of the manuscript.

## REFERENCES

1. Bradley, D. J., and Kirkley, J., *Trans. R. Soc. Trop. Med. Hyg.* **66,** 527, 1972.
2. Bradley, D. J., *Clin. Exp. Immunol.* **30,** 130, 1977.
3. Perez, H., Arrendondo, B., and Gonzales, M., *Infect. Immun.* **22,** 301, 1978.
4. Perez, H., Labrador, F., and Torrealba, J. W., *Int. J. Parasitol.* **9,** 27, 1979.
5. Nasseri, M., and Modabber, F. T., *Infect. Immun.* **26,** 611, 1979.
6. Murray, H. W., Masur, H., and Keithly, J. S., *J. Immunol.* **129,** 344, 1982.
7. Skameme, E., Gros, P., Forget, A., Kongshower, P. A. L., St. Charles, C., and Taylor, B. A., *Nature (London)* **294,** 506, 1982.
8. Murray, H. W., *J. Immunol.* **129,** 351, 1982.
9. Chang, K. P., and Chiao, J. W., *Proc. Natl. Acad. Sci. USA* **78,** 7083, 1981.
10. Manson-Bahr, P. E. C., *East Afr. Med. J.* **38,** 165, 1961.
11. Southgate, B. A., and Manson-Bahr, P. E. C., *J. Trop. Med. Hyg.* **70,** 29, 1967.
12. Howard, J. G., Hale, C., and Liew, F. Y., *J. Exp. Med.* **152,** 607, 1980.
13. Arrendondo, B., and Perez, H., *Infect. Immun.* **35,** 16, 1979.
14. Perez, H., Pocine, M., and Malare, I., *Infect. Immun.* **32,** 415, 1981.
15. Jerne, N. K., and Nordin, A. A., *Science (Washington, D.C.)* **140,** 405, 1963.
16. Mongini, P. K. A., Stein, K. E., and Paul, W. E., *J. Exp. Med.* **153,** 1, 1981.
17. Goidl, E. A., Innes, J. B., and Weksler, M. E., *J. Exp. Med.* **144,** 1037, 1976.
18. Doria, G., Mancini, C., and Adorini, L., *J. Immunol.* **79,** 3803, 1982.
19. Goidl, E. A., Ramstedt, U., and Jones, T. C., *In* "Host Responses to Intracellular Pathogens" (T. K. Eisenstein, P. Actor, and H. Friedman, Eds.), *Adv. Exp. Med. Biol.* Vol. 162, 145, Plenum, New York, 1983.
20. Dwyer, D. M., *J. Immunol.* **117,** 2081, 1976.
21. Stauber, L. A., Franchino, E. M., and Grun, J., *J. Protozool.* **5,** 269, 1958.
22. Eisen, H. N., Belman, S., and Carsten, M. E., *J. Amer. Chem. Soc.* **75,** 4583, 1953.
23. Mitchell, G. F., Humphrey, J. H., and Williamson, A. R., *Eur. J. Immunol.* **2,** 460, 1972.
24. Dresser, D. W., and Greaves, M. F., *In* "Handbook of Experimental Immunology" (D. M. Weir, Ed.), P271, Blackwell Scientific Publications, Ltd., Oxford, 1967.
25. Rittenberg, M. B., and Pratt, K. L., *Proc. Soc. Exp. Biol. Med.* **132,** 575, 1969.
26. Voller, A., Bidwell, D. E., and Bartlett, A., "The Enzyme Linked Absorbent Assay" (ELISA). Dynatech Laboratories Publications, 1979.
27. Endres, R. O., Kushnir, E., Kappler, J. W., Marrack, P., and Kinsky, S. C., *J. Immunol.* **130,** 781, 1983.
28. Schrater, A. F., Goidl, E. A., Thorbecke, G. J., and Siskind, G. W., *J. Exp. Med.* **150,** 808, 1979.
29. Mongini, P. K. A., Paul, W. E., and Metcalf, E. S., *J. Exp. Med.* **155,** 884, 1982.
30. Carvalho, E. M., Teixeira, R. S., and Johnson, W. D., *Infect. Immun.* **33,** 498, 1981.
31. Haldar, J. P., Ghose, S. A., Saha, K. C., and Ghose, A. C., *Infect. Immun.* **42,** 702, 1983.
32. Andrade, T. M., Teixeira, R., Andrade, A. F., Pereira, C., and Carvalho, E. M., Rev. *Inst. Med. Trop. Sao Paulo* **24,** 298, 1982.
33. Wyler, D. J., *Trans. R. Soc. Trop. Med. Hyg.* **76,** 304, 1982.
34. Howard, J. G., Hale, C., and Liew, F. Y., *J. Exp. Med.* **153,** 557, 1981.
35. Ulczak, O., and Blackwell, J. M., *Parasit. Immunol.* **5,** 449, 1983.
36. Mitchell, G. F., Curtes, J. M., Scollay, R. G., and Handman, E., *Aust. J. Exp. Biol. Med. Sci.* **59,** 539, 1981.
37. Liew, F. Y., Hale, C., and Howard, J. G., *J. Immunol.* **128,** 1917, 1982.

38. Leclerc, C., Modabber, F., Deriand, E., Djoko-Tannon, J., and Chedid, L., *Infect. Immun.* **37,** 895, 1982.
39. Colle, J. H., Truffa-Bachi, P., Chedid, L., and Modabber, F., *J. Immunol.* **131,** 1492, 1983.
40. Reiner, N. E., *Infect. Immun.* **38,** 1223, 1982.
41. Mosier, D. E., and Subbarao, B., *Immunol. Today* **3,** 217, 1982.
42. Keller, D. M., Swierkosz, J. E., Marrack, P., and Kappler, J. W., *J. Immunol.* **124,** 1350, 1980.
43. Thoman, M. L., and Weigle, W. O., *J. Immunol.* **128,** 2358, 1982.
44. Reiner, N. E., and Finke, J. H., *J. Immunol.* **131,** 1487, 1983.
45. Reed, S. G., Inverso, J. A., and Roters, S. B., *J. Immunol.* **133,** 1558, 1984.
46. Weintraub, J., Gottlieb, M., and Weinbaum, F. I., *Exp. Parasitol.* **53,** 87, 1982.
47. Campos-Neto, A., and Bunn-Moreno, M., *Infect. Immun.* **38,** 871, 1982.
48. Corsini, A. C., Costa, M. G., Oliveira, O. L. P., Carmargo, I. J. B., and Rangel, H. A., *Immunology* **40,** 505, 1980.
49. Sacks, D. L., Bancroft, G., Evans, W. H., and Askonas, B. A., *Infect. Immun.* **36,** 160, 1982.
50. Mond, J. J., Farrar, J., Paul, W. E., Fuller-Farrar, J., Schaefer, M., and Howard, M., *J. Immunol.* **131,** 633, 1983.
51. Bunn-Moreno, M. M., Madeira, E. D., Miller, K., Menezes, J. A., and Campos-Neto, A., *Clin. Exp. Immunol.* **59,** 427, 1985.