INFECTION AND IMMUNITY, Nov. 1978, p. 548-554
0019-9567/78/0022-0548$02.00/0

Vol. 22, No. 2
Printed in U.S.A.

Contact Sensitivity Responses in Mice Infected with *Trypanosoma cruzi*

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Received for publication 27 April 1978

Mechanisms of depression of contact sensitivity responses in C57BL/10 mice infected with *Trypanosoma cruzi* were studied. Cellular involvement during sensitization with oxazolone was investigated in mice acutely infected with *T. cruzi*. Contact sensitivity was not expressed in mice during the latter stages of the acute infection. Spleen cells from sensitized, infected mice which were unable to respond to oxazolone could confer contact sensitivity upon normal syngenic mice as effectively as spleen cells from uninfected, sensitized donors. The ability of mice infected with *T. cruzi* to respond to an eliciting dose of oxazolone was significantly improved when macrophages from normal syngenic donors were administered to them at the time of skin test. When either normal or infected mice were used as recipients of lymphocytes from sensitized donors, the normal mice responded significantly better than did infected mice after administration of an eliciting dose of oxazolone. An increase in pyroninophilic cells was observed in draining lymph nodes after application of a sensitizing dose of oxazolone to the ears of either normal or acutely infected mice. These results indicate that suppression of contact sensitivity during acute *T. cruzi* infection is directed toward the efferent arm rather than the afferent arm of the response.

Various protozoan infections produce depression of host immune responses. Experimentally, suppression of immune responses to antigens other than those of the parasite has been observed in animals with African trypanosomiasis, Chagas' disease, leishmaniasis, malaria, and toxoplasmosis (1, 3-5, 9, 10, 13, 20, 21, 23). Immunosuppression has been observed in malaria patients (8).

Mechanisms of parasite-induced immunosuppression appear to vary with the host-parasite system involved. *Trypanosoma cruzi*, an intracellular flagellated protozoan and the causative agent of Chagas' disease, has been shown to cause suppression of both humoral (5) and cellular (20) responses in infected hosts. Similar observations have been made in experimental African trypanosomiasis (11, 17). Suppression of humoral responses occurs during malaria infections (7). Loose et al. (16) concluded that malarial immunosuppression was due to a defect in antigen processing by macrophages and that lymphocyte responses appeared to be normal. Phagocytosis as determined by colloidal carbon clearance is reportedly enhanced during experimental malaria (16) and *T. cruzi* (5) infections. We have observed an increase in in vitro phagocytosis of killed yeast cells by macrophages from mice infected with *T. cruzi* (unpublished data).

The present study was undertaken to determine the effects on lymphocyte and macrophage responses produced by acute *T. cruzi* infections in mice. Oxazolone, a potent contact sensitizing agent, was used to test delayed hypersensitivity (DH). Mice sensitized with this substance develop lymphoblasts in the draining lymph nodes and spleen as part of the induction of contact sensitivity. Formation of these cells in selected areas of secondary lymphoid tissue is indicative of T cell stimulation. Expression of DH occurs after restimulation of sensitized T cells with specific antigen, resulting in release of lymphokines and infiltration by characteristic mononuclear cells at the skin test site.

The effects of *T. cruzi* upon the induction and expression of DH in acutely infected mice is reported herein.

MATERIALS AND METHODS

Animals. Six- to eight-week-old male C57BL/10 mice were obtained from the Rocky Mountain Laboratory, Hamilton, Mont. They were housed in plastic cages, four animals per cage, and supplied with food and water ad libitum.

Parasites. The Tulahuén strain of *T. cruzi*, obtained from Robert G. Yaeger, Department of Parasitology, Tulane University, New Orleans, La., was maintained in our laboratory by weekly passage of whole blood from infected to normal mice. Mice were

infected by intraperitoneal injection of 1×10^3 blood forms.

MEM. Powdered minimal essential medium (MEM) (Eagle) with Earle salts, L-glutamine, and nonessential amino acids (GIBCO, Santa Clara, Calif.) was reconstituted to 90% of its final volume with triply distilled water. The medium was buffered with 1.0 g of $NaHCO_3$ and 15 mM HEPES (*N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid [Sigma, St. Louis, Mo.]) per ml, pH adjusted to 7.3 with 1 N NaOH, and filter sterilized.

Tris-ammonium chloride. One part of 0.17 M tris(hydroxymethyl)aminomethane (Tris) (20.59 g of Tris/liter) buffer (Sigma) (pH 7.65) was combined with nine parts of 0.83% (wt/vol) ammonium chloride immediately before use. This hypotonic solution was adjusted to pH 7.2 with 1 N HCl and used to lyse erythrocytes in spleen or lymph node preparations. Approximately 1 ml of packed cells was suspended gently in 10 ml of Tris-ammonium chloride and allowed to incubate at room temperature for 10 to 15 min. The cells were then washed three times in MEM and counted.

Testing for contact sensitivity. Sensitization was achieved by rubbing 0.1 ml of a freshly prepared 3% (wt/vol) solution of oxazolone (4-ethoxymethylene-2-phenyl oxazolone; BDH Chemicals Ltd., Pool, England) in absolute ethanol on the preshaven abdomen of mice. On day 7 postsensitization, oxazolone in a 1:1 mixture of absolute ethanol and olive oil was applied to the inner surface of one ear. The other ear was treated similarly with an equal volume of the diluting mixture only. The difference in thickness between the two ears was determined 24 h later with a "Schnell-taster" dial gauge micrometer.

Separation of B and T lymphocytes. Spleen cells were separated into B and T cell-enriched fractions with a nylon wool column as described by Julius et al. (15). The B and T cell-enriched fractions were each washed twice with MEM. Cell surface immunoglobulin as a B cell marker was identified by fluorescein-conjugated rabbit antimouse immunoglobulin (Colorado Serum Co., Denver, Colo.). Fluorescent cells were identified as B cells and nonfluorescent cells were identified as T cells.

Transfer of contact sensitivity. Mice were sensitized to oxazolone as described above and skin tested with oxazolone after 7 days to ascertain sensitivity. Spleens or lymph nodes were removed and teased apart with sterile forceps into MEM. After lysis of erythrocytes, the remaining cells were washed three times, counted, and injected intravenously (i.v.) into recipient mice. After 48 h, an eliciting dose of oxazolone was applied to the ears of each recipient, and ear thickness was measured 24 h after application of oxazolone.

Transfer of peritoneal exudate cells. Donor cells were collected from the peritoneal cavities of normal mice. These cells were washed twice with cold MEM and adhered to plastic plates at 37°C for 45 min in 5% CO_2–95% air. Nonadherent cells were then removed by spraying the plates vigorously with MEM. Adherent cells were obtained by scraping the surface of each plate with a rubber policeman. These cells were suspended in MEM and injected i.v. into recipient mice. On day 8 postinfection with 10^3 trypomastigotes, recipient mice were sensitized with oxazolone. On day 7 after sensitization (15 days after infection), the mice received 2×10^7 adherent peritoneal exudate cells from normal mice and were skin tested immediately. A group of uninfected, unsensitized mice received similar treatment.

Histology of induction of contact sensitivity in draining lymph nodes. Auricular lymph nodes were stimulated by applying 0.05 ml of a 3% solution of oxazolone in absolute ethanol to the inner surface of each pinna. Normal mice and mice infected 13 days earlier with 10^3 blood forms were sensitized in this manner. Three days after application of oxazolone to the ears, both auricular lymph nodes were removed and placed in Plutznik fixative, composed of 91 parts absolute ethanol, 4.5 parts formaldehyde, and 4.5 parts acetic acid. The tissue was sectioned in paraffin at 5 μm, stained with methyl-green pyronin, and photographed with a Zeiss Universal microscope. Lymph nodes of eight mice from each group were examined microscopically. A total of 10 fields (625×) were examined from each mouse. The ratios of lymphoblasts to smaller lymphocytes were determined.

Statistics. The Mann-Whitney significance test was used where applicable.

RESULTS

Mice infected with *T. cruzi* and sensitized with oxazolone did not express DH to this agent when sensitized after day 3 and skin-tested after day 10 of infection. Similarly, mice sensitized to oxazolone before infection lost this sensitivity after day 12 of infection (20). To determine whether lymphocyte or macrophage populations were selectively affected, the following experiments were performed.

Transfer of delayed hypersensitivity to oxazolone. Delayed hypersensitivity to oxazolone was readily transferred between normal syngenic mice (Table 1). Lymphocyte populations from spleens of mice sensitized 7 days earlier with oxazolone were divided into B and T cell-enriched fractions. The B and T cell-en-

TABLE 1. *Transfer of contact sensitivity with separated spleen cell populations from oxazolone-sensitized C57BL/10 mice to normal syngenic mice*

Group	Population of spleen cells received[a] (1.25×10^7 cells i.v.)	Recipient response (mm)[b]
1	B cell enriched	0.21 ± 0.06[c]
2	T cell enriched	0.46 ± 0.12[d]
3	Mixed lymphocytes[e]	0.38 ± 0.12

[a] Cells were from syngenic mice that were sensitized 7 days earlier with oxazolone and were skin test positive ($\bar{x} = 0.60 \pm 0.10$).

[b] Recipients (five mice per group) were skin tested with oxazolone 48 h after cell transfer. Values are $\bar{x}$ ± standard deviation.

[c] Group 1 vs. group 2 ($P < 0.05$); group 1 vs. group 3 ($P < 0.05$).

[d] Group 2 vs. group 3 ($P > 0.05$).

[e] A 1:1 mixture of T and B cell-enriched populations.

riched fractions contained approximately 85 and 15% fluorescing cells, respectively. Five normal syngenic recipients each received 1.25×10^7 cells from either B or T cell-enriched fractions, or from a 1:1 mixture of the two fractions. The T cell-enriched fraction was significantly more effective in transferring contact sensitivity than the B cell-enriched fraction, whereas the 1:1 mixture was intermediately effective.

Lymphocyte transfer from sensitized donors infected with *T. cruzi* to normal recipients. Mice infected with *T. cruzi* lost their ability to respond to oxazolone as the infection progressed beyond day 10 (Table 2). However, spleen cells from *T. cruzi*-infected and oxazolone-sensitized donors were able to confer DH to oxazolone upon normal recipients, regardless of the stage of infection of the donors (Table 2). Transfer experiments could not be conducted beyond day 17 of the infection since mice usually died 18 days after infection with 10^3 blood forms. Sensitized mice that were unable to respond to eliciting doses of oxazolone had spleen cells that were able to confer sensitivity to this agent as effectively as cells of uninfected sensitized mice. No significant differences were seen in the responses of recipient mice whether the donor cells were from sensitized uninfected mice or from sensitized mice infected for varying lengths of time.

Lymphocyte transfer from normal donors to recipients infected with *T. cruzi*. In another experiment involving transfer of sensitivity to oxazolone with lymphocytes, spleen cells were obtained from normal mice sensitized with oxazolone 7 days earlier (Table 3). Macrophages were removed by adherence to plastic, and the nonadherent cells were injected i.v. into either normal mice or mice infected with *T. cruzi* 16 days previously. After transfer of nonadherent spleen cells from uninfected sensitized donors, recipient mice infected for 16 days with *T. cruzi* failed to respond as well to eliciting doses of oxazolone as uninfected recipients.

TABLE 2. *Transfer of contact sensitivity to C57BL/10 mice from mice infected with T. cruzi and sensitized to oxazolone*

Group	No. of days postinfection[a]	Donor response (mm)	Recipient response (mm)[b]
1	0	0.60 ± 0.10	0.50 ± 0.07[c]
2	10	0.55 ± 0.10	0.45 ± 0.10[c]
3	14	0.28 ± 0.09	0.42 ± 0.11[d]
4	16	0.05 ± 0.05	0.40 ± 0.09[d]
5	17	0.05 ± 0.05	0.44 ± 0.12[d]

[a] Sensitivity was transferred by i.v. inoculation of 3×10^7 spleen cells taken from donor mice at various days postinfection with *T. cruzi*. Donors were sensitized to oxazolone 8 days and skin tested 24 h before cell transfer.

[b] Recipients (six mice per group) were skin tested with oxazolone 48 h after cell transfer. No significant differences were noted in responses of recipient groups. Values are $\bar{x}$ ± standard deviation.

[c] Donor vs. recipient ($P > 0.05$).

[d] Donor vs. recipient ($P < 0.01$).

TABLE 3. *Transfer of contact sensitivity with spleen cells from C57BL/10 mice sensitized to oxazolone to normal or T. cruzi-infected syngenic mice*

Group	Received spleen cells from oxazolone-sensitized mice[a]	Infected for 16 days	Response (mm)[b]
1 (donors)	−	−	0.60 ± 0.10[c]
2	+	+	0.22 ± 0.05[d]
3	+	−	0.47 ± 0.06
4	−	+	0.05 ± 0.05

[a] Donor mice sensitized with oxazolone 7 days previously were skin tested 24 h before transfer. Donor cells were incubated on plastic for 45 min to remove adherent cells before transfer. Nonadherent spleen cells (3×10^7) were injected i.v. into each recipient.

[b] Recipients (six mice per group) were skin tested with oxazolone 48 h after cell transfer. Values are $\bar{x}$ ± standard deviation.

[c] Group 1 vs. group 3 ($P > 0.05$); group 1 vs. group 4 ($P < 0.01$).

[d] Group 2 vs. group 3 ($P < 0.01$); group 2 vs. group 4 ($P < 0.05$).

Transfer of contact sensitivity with lymph node cells from infected and uninfected mice sensitized to oxazolone. Donors were sensitized with oxazolone by application of this agent to both ears 3 days before harvest of auricular lymph node cells. One group of donor mice was not infected and another group was infected with *T. cruzi* 13 days before they were sensitized with oxazolone. Groups of normal recipient mice were injected i.v. with 4×10^7 lymph node cells obtained from either of the two groups of donors. Cells obtained from either group of donors were first allowed to adhere to glass at 37°C for 45 min, and the nonadherent cells were harvested. Nonadherent cells from either group of donors were injected into designated recipients which were tested for DH to oxazolone 48 h later. Lymphocytes from either group of donors were equally effective in transferring specific DH to normal syngenic mice (Table 4).

Transfer of normal mouse peritoneal exudate cells to mice sensitized with oxazolone and infected with *T. cruzi*. Mice infected 15 days previously with 10^3 blood forms of *T. cruzi* and sensitized to oxazolone on day 8 postinfection were given adherent peritoneal exudate

cells i.v. from normal mice immediately before the skin test dose of oxazolone was applied. These animals responded to oxazolone significantly better than did infected sensitized mice that did not receive peritoneal cells (Table 5).

Histology of the induction of contact sensitivity in draining lymph nodes. To further study the induction of delayed hypersensitivity, oxazolone was applied to the ears of infected or uninfected mice. After 3 days, the auricular lymph nodes were removed, fixed, and stained with methyl-green pyronin. Follicular and interfollicular areas of the lymph nodes were observed by light microscopy for the presence of lymphoblasts. These large pyroninophilic cells are readily distinguishable from smaller lymphocytes. Localized adenopathy was observed in the auricular nodes of infected mice (see Fig. 3 and 4). Other lymph nodes were not examined for such degenerative changes. Auricular nodes from infected mice not sensitized to oxazolone had significantly more large pyroninophilic cells in the follicular and interfollicular areas than did those from normal mice (Fig. 1 and 3; $P < 0.05$). This difference may have been from immunological stimulation due to infection.

Mice sensitized to oxazolone on the ears had significantly more lymphoblasts in the follicular and interfollicular areas of the auricular lymph nodes than normal mice (Fig. 1 and 2; $P < 0.05$). Similarly, mice infected with *T. cruzi* for thirteen days before sensitization in this manner had significantly more lymphoblasts than infected unsensitized mice (Fig. 3 and 4; $P < 0.05$). Lymph node cells from either infected or uninfected mice which had been sensitized to oxazolone transferred contact sensitivity to normal mice (Table 4).

TABLE 4. *Transfer of contact sensitivity with lymph node cells to normal C57BL/10 mice from syngenic mice infected with T. cruzi and sensitized to oxazolone*

Source of donor[a] lymph node cells	Recipient response (mm)[b]
Infected, sensitized mice	0.33 ± 0.07
Uninfected, sensitized mice	0.32 ± 0.08

[a] The lymph node cells of mice infected with *T. cruzi* 16 days previously were compared with those from uninfected mice in their ability to transfer contact sensitivity to normal recipients. Oxazolone was applied to both ears of each donor 3 days before removal of their auricular lymph nodes.

[b] Recipients (five mice per group) were injected i.v. with 3.0×10^7 donor lymph node cells 48 h before skin testing with oxazolone. Values are $\bar{x}$ ± standard deviation. $P > 0.05$.

TABLE 5. *Transfer of adherent peritoneal exudate cells to C57BL/10 mice infected with T. cruzi and sensitized to oxazolone*

Group	Infected for 15 days	Received peritoneal exudate cells[a]	Sensitized to oxazolone[b]	Response (mm)[c]
1	+	+	+	0.20 ± 0.05[d]
2	+	−	+	0.04 ± 0.05[e]
3	−	+	−	None
4	−	−	+	0.48 ± 0.09

[a] Adherent cells from unstimulated peritoneal cavities of normal syngenic mice were administered i.v. immediately before skin test.

[b] Mice were sensitized to oxazolone 8 days before skin test.

[c] Recipients (six mice per group) were skin tested with oxazolone 48 h after cell transfer. Values are $\bar{x}$ ± standard deviation.

[d] Group 1 vs. group 2 ($P < 0.01$); group 1 vs. group 4 ($P < 0.05$).

[e] Group 2 vs. group 4 ($P < 0.01$).

DISCUSSION

Oxazolone is a hapten that binds to epithelial proteins to become antigenic. Application of oxazolone to the skin results in rapid sensitization and normally induced DH. Rapid proliferation of lymphocytes occurs in draining lymph nodes as part of the process of induction of the immune response (2, 22). These cells confer lasting sensitivity to oxazolone when transferred to syngenic recipients (2, 19).

Experiments were performed to determine whether lymphoblasts were formed after administration of oxazolone to mice infected with *T. cruzi*. Since production of lymphoblasts after antigenic stimulation indicates induction of an immune response, the absence of these cells in a sensitized, infected animal may represent interference with this process. We found that infected mice which were exposed to oxazolone but which failed to express DH were capable of forming lymphoblasts after sensitization with oxazolone and that these cells were capable of transferring contact sensitivity to normal syngenic recipients. Therefore, the absence of DH in mice infected with *T. cruzi* suggests that the defect is not due to lack of a lymphocyte response.

Cell transfer studies were made to determine if infection with *T. cruzi* interfered with the afferent or the efferent arm of the immune response and to examine the nature of the response to oxazolone. Spleen cells from sensitized uninfected mice were divided into T cell-enriched and B cell-enriched populations. Nearly all latex-ingesting cells were eliminated from these preparations by passage through a nylon wool column (12, 15). Transfer of the spleen cell

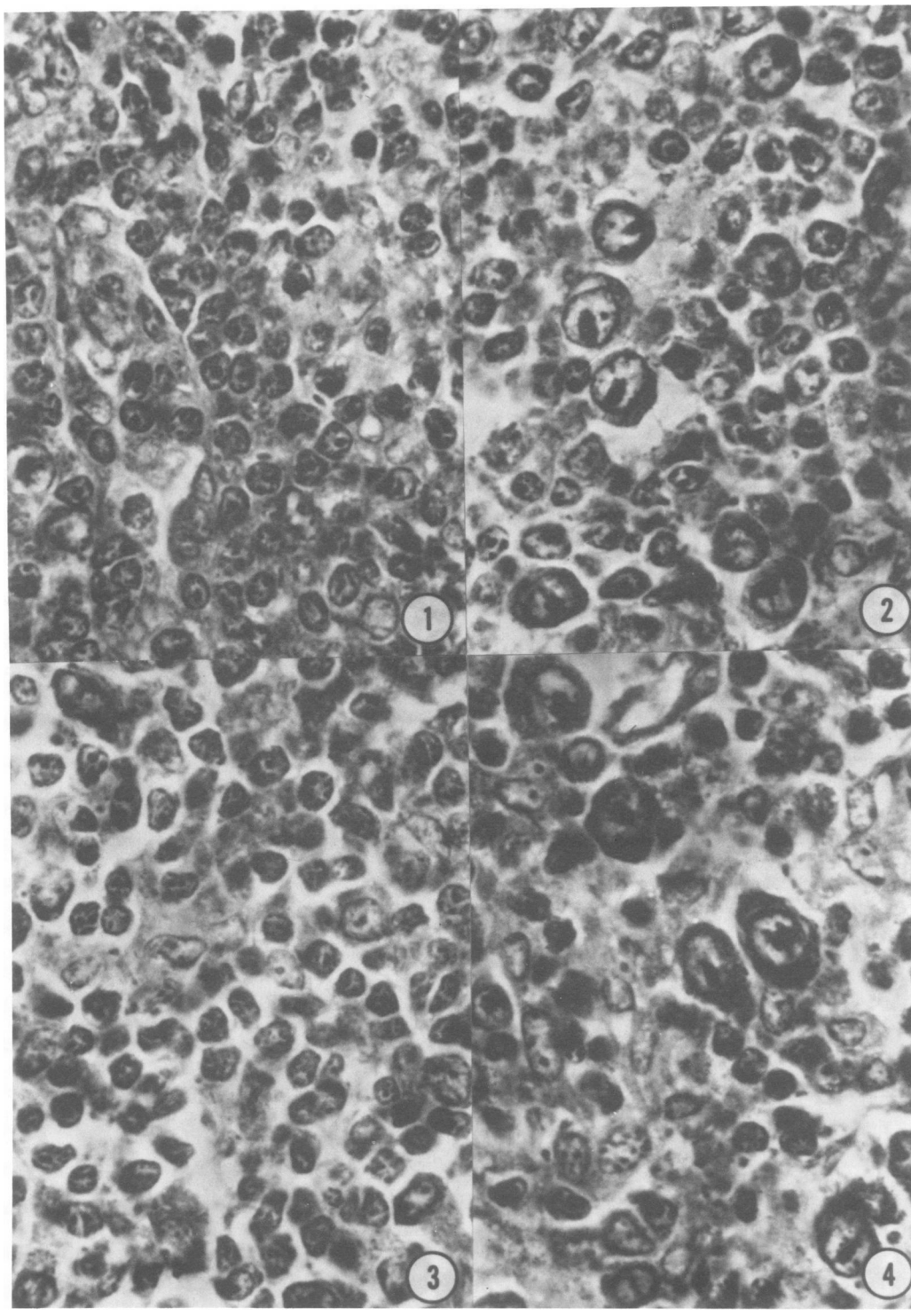

FIG. 1–4. *Photomicrographs of the follicular and interfollicular regions of mouse auricular lymph nodes after various treatments; methyl-green pyronin; phase-contrast; ×1,400.*

FIG. 1. *Unsensitized, uninfected.*

FIG. 2. *Sensitized with oxazolone.*

FIG. 3. *Infected with T. cruzi.*

FIG. 4. *Infected with T. cruzi and sensitized with oxazolone.*

populations confirmed the importance of T cells in the response to oxazolone. A significantly greater DH response occurred in normal mice that received T cell-enriched populations than in those receiving only B cell-enriched populations (Table 1).

Contact sensitivity was readily transferred from sensitized, infected mice to normal mice, even though the infected animals themselves were unable to respond to the antigen. The sensitivity was successfully transferred even when donor mice were in the final days of the infection and were immunodepressed (Table 2). These results indicated that the infected mice, although unable to respond to oxazolone, had sensitized T cells in their spleens.

Approximately 6 days were required for production of optimum numbers of sensitized cells in the donor spleen. Since the mice survive only about 18 days after infection with the Tulahuén strain of *T. cruzi*, it is best not to sensitize an infected mouse after day 11 of infection to ensure the survival of the animal throughout the test period. Immunosuppression was most evident between days 12 and 18 postinfection. Therefore, analysis of the induction of DH during this period was limited when spleen cells were used for transfer experiments. Lymphoblasts develop in the draining auricular nodes 3 days after oxazolone is applied to the ears of guinea pigs (22) or mice. This occurred even when oxazolone was applied to both ears of mice during the period of peak parasitemia (Table 4). This more rapid sensitization made it possible to test for induction of DH during the final days of infection. Induction of cellular immunity as shown by transfer experiments and histological observation was observed in mice infected for 16 days.

Based on these observations, immunosuppression during acute Chagas' disease appeared to be due to a defect at the macrophage level. To examine this possibility, adherent peritoneal exudate cells from normal mice were given i.v. to infected, sensitized mice immediately before application of the eliciting dose of oxazolone to the ear. The addition of adherent peritoneal exudate cells from normal animals before skin testing significantly improved the recipients' response (Table 5).

This indicates that perhaps a deficiency of available macrophages in the infected mice was responsible for immunosuppression. Since there does not seem to be a significant drop in any single circulating leukocyte population during acute infection in mice (personal observation), and since there is an increased clearance rate of colloidal carbon in infected mice, a decrease in the number of macrophages did not appear to be responsible. However, macrophages may not have responded to products of stimulated lymphocytes, due to the presence of intracellular parasites within macrophages. The presence of many parasites, both inside and outside of macrophages, may have interfered with the ability of the macrophage to recognize and respond to another antigen or to lymphokines. Mice immunized with attenuated trypanosomes and infected with virulent forms respond well to oxazolone (20). This might provide an argument against antigenic competition, since much antigen is present in the immunized, infected mice. However, extensive parasite invasion of macrophages and high parasitemia usually do not occur when specifically immunized mice are infected with virulent organisms. It is possible that the intracellular presence of virulent parasites is chiefly responsible for the failure of the macrophages to respond. Our data indicate that suppression of DH during acute *T. cruzi* infection in mice is not a defect at the lymphocyte level but rather is associated with unresponsiveness of macrophages.

Although lymphocyte sensitization occurred in infected mice, we have found that spleen cells from infected mice show a lowered response to T and B cell mitogens (unpublished data). Decreased immune responsiveness in infected mice may be due to suppressor cells or factors. Considering the effectiveness with which spleen cells from infected mice transferred contact sensitivity, a suppressor T cell population is not apparent by this criterion. Recently, suppressor cells have been reported in animals with experimental African trypanosomiasis (6, 14). Further studies are currently underway to determine if suppressive factors or cell populations occur in mice infected with *T. cruzi* which may interfere with normal immunological responses.

ACKNOWLEDGMENTS

We wish to express appreciation to J. Green, B. Hyde, and J. Inverso for assistance in preparation of the manuscript. This investigation was supported in part by Public Health Service Research Career Award 4 KO6 AI 16502-16 from the National Institute of Allergy and Infectious Diseases.

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