Adoptive Transfer of Resistance to Acute *Trypanosoma cruzi* Infection with T-Lymphocyte-Enriched Spleen Cells

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Inbred C57BL/10 mice immunized with live culture forms of *Trypanosoma cruzi* were resistant to acute infection after challenge with bloodstream forms. Splenic leukocytes or serum from immunized mice were transferred to syngeneic recipients 2 days before or 2 days after challenge. Protection was not observed in recipients of serum, although the serum contained high levels of agglutinating antibody. Unfractionated splenic leukocytes from immunized donors conferred partial protection, and preparations enriched for T lymphocytes were significantly more effective than preparations enriched for B lymphocytes. Recipients of T-lymphocyte-enriched spleen cells had significantly higher survival times and significantly lower parasitemias than did recipients of B-lymphocyte-enriched spleen cells.

Several studies have been made to characterize the immune response to the intracellular protozoan parasite *Trypanosoma cruzi* (Chagas 1909). It is well documented that resistance to a second acute infection follows recovery from a first (6) and that animals may be experimentally protected against acute *T. cruzi* infection by prior inoculation with live culture forms or with avirulent strains (21, 24, 30). Specific antibodies occur after acute infection (6). Delayed hypersensitivity responses and other parameters of cell-mediated immunity have been detected in humans and experimental animals previously infected with *T. cruzi* (7, 28, 29, 33, 35). Resistance to acute infection has been passively transferred with serum (5, 8, 12) and cells (4, 16, 26) from donors recovered from inoculation of bloodstream trypomastigotes. The thymus has been shown to be important in mediating resistance to acute *T. cruzi* infections (2, 15, 25, 27).

The present study was designed to examine the ability of spleen cells or serum from immunized donors to confer protection against acute *T. cruzi* infection upon syngeneic recipients. Donors were immunized with live culture forms of *T. cruzi* and did not experience acute infection. Splenic leukocytes were administered whole or as purified populations enriched for B or T lymphocytes to examine the relative importance of these cell types in the mediation of resistance to acute *T. cruzi* infections.

MATERIALS AND METHODS

Mice. Male inbred C57BL/10 mice, 8 to 12 weeks old, were used in all experiments. Breeding stock was obtained from the Rocky Mountain Laboratory, Na-

tional Institute of Allergy and Infectious Diseases, Hamilton, Mont., and the mice were subsequently reared in our laboratory.

Parasites. The Tulahuén strain of *T. cruzi* was used in all experiments. Both bloodstream and culture forms were obtained through the courtesy of Robert G. Yaeger, Department of Parasitology, Tulane University. Bloodstream forms were maintained by weekly passage in mice. Culture forms were maintained by monthly passage in diphasic medium (34) or dialysate medium (22). The parasites were passaged more than 60 times in our laboratory and had previously been maintained in culture for ca. 20 years.

Immunization. Parasites used for immunization were cultured in dialysate medium, harvested 4 weeks after culture inoculation, washed three times in phosphate-buffered saline (PBS) (pH 7.2) and resuspended in PBS. Mice were immunized intraperitoneally (i.p.) with 10^6 motile parasites in 0.2 ml. The parasites were ca. 97% epimastigotes as revealed by counting a minimum of 10^3 parasites in different fields of Giemsa-stained slides.

Challenge. Parasites used for challenge were obtained from the blood of acutely infected mice. The blood was diluted in PBS and centrifuged at $200 \times g$ for 10 min to remove blood cells. The supernatant containing parasites was harvested and centrifuged as before. The parasites were then washed twice and resuspended in PBS. Mice were challenged i.p. with 2×10^5 motile bloodstream forms in 0.2 ml of PBS.

Spleen cells. Spleens were minced through a stainless-steel screen into minimal essential medium (MEM) with Earle salts, L-glutamine, and nonessential amino acids (GIBCO Laboratories, Santa Clara, Calif.) buffered with N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid (GIBCO). After removal of particulate debris, the cells were treated with a buffered ammonium chloride solution to lyse erythrocytes and washed three times in MEM. Preparations enriched for B or T lymphocytes were obtained as described by Julius et al. (11).

The separated populations were each adjusted to 5

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$\times 10^7$ cells/ml in MEM plus 5% inactivated fetal calf serum (MEM-FCS), and 5 ml was added to glass wool columns prepared by packing 2.4 g of acid-washed glass wool to the 20-ml mark of 30-ml plastic syringes and preincubating with MEM-FCS at 37°C for 30 min. Cells were incubated in the columns at 37°C in a humidified chamber with 5% CO₂ for 60 min. Nonadherent cells were collected, washed twice in MEM and resuspended in MEM at 5×10^7 cells/ml. The cell populations were further purified by incubating them first with antisera directed against cell surface markers and then with complement for selective lysis of B or T lymphocytes. Rabbit anti-mouse immunoglobulin (Colorado Serum, Denver, Colo.) was added to the nylon-wool nonadherent cells at a final concentration of 1 part serum:20 parts cell suspension. Rabbit anti-mouse brain serum (Bionetics, Kensington, Md.) was added to the nylon-wool adherent cells at a final concentration of 1 part serum:400 parts cell suspension. The cells were incubated for 45 min at room temperature before the addition of fresh guinea pig serum previously adsorbed with mouse spleen leukocytes (10^6 cells per ml of serum) at a concentration of 1 part serum:20 parts cell suspension. The cells were incubated for 30 min at 37°C + 5% CO₂, washed three times in MEM, and resuspended in MEM at 10^8 cells/ml. Fluorescein-conjugated rabbit anti-mouse immunoglobulin (Colorado Serum) was used to detect immunoglobulin-positive cells. At least 10^3 cells were counted from each enriched population. Ingestion of polystyrene latex (1.0- μ m diameter, Dow Chemicals, Midland, Mich.) was used to determine the numbers of phagocytic cells. Cell viability was determined by trypan blue exclusion. All counts were performed with an improved Neubauer chamber.

Immunological assays. Antigens were prepared from parasites cultured in dialysate medium. Sera were collected and tested individually for agglutinating antibodies as described by Allain and Kagan (1). Delayed hypersensitivity tests were performed by using antigen from culture forms. Parasites were washed three times in PBS resuspended in PBS at 5×10^8 parasites per ml and lyophilized. Mice were injected with 10 μ g (dry weight) of the resuspended parasites in 0.03 ml in one hind footpad and with 0.03 ml of

saline in the other footpad. Differences in footpad thickness were compared 24 h later by using a Schnell-taster dial guage micrometer. The Mann-Whitney *U* test, two-tailed, was used for data analyses.

RESULTS

Specific immune responses in mice immunized with *T. cruzi* culture forms. Mice were tested for responses to *T. cruzi* antigen or challenge with bloodstream forms at progressive intervals after immunization (Table 1). Animals challenged 12 or more days after immunization all survived, and all but one challenged 8 days after immunization survived. Mice immunized 12 or more days before challenge had positive (≥ 0.2 -mm increase) skin test responses to *T. cruzi* antigen, but agglutinating antibodies were not detected until after a second immunization was given. Agglutinating antibodies were consistently low or undetectable in mice given a single immunizing dose even after 24 days. Mice challenged 4 days after immunization all died, with survival times not significantly different from unimmunized controls. Agglutinating antibodies and delayed hypersensitivity responses were consistently low or undetectable in unimmunized mice infected with *T. cruzi* throughout the course of infection.

Adoptive transfer of serum or cells from specifically immunized mice. Unfractionated spleen leukocytes from immunized mice conferred partial protection against acute *T. cruzi* infection when given 2 days before or 2 days after challenge (Table 2). Serum from immunized mice did not confer protection. Survival times of mice given serum from immunized donors did not differ significantly from unimmunized controls, and both groups developed higher parasitemias than did recipients of unfractionated spleen leukocytes from immunized mice (Fig. 1 and 2).

TABLE 1. Onset of protection and of specific immune responses to *T. cruzi* antigens in mice after immunization with *T. cruzi* culture forms

Group	Day after immunization ^a	Antibody titer ^b ($\bar{x} \pm$ SD)	Skin test response (mm) ^c ($\bar{x} \pm$ SD)	Deaths	
				No./total	$\bar{x} \pm$ SD (days)
1	4	0	0	8/8	20.1 $\pm$ 3.6
2	8	0	0	2/8	22.1 $\pm$ 1.5
3	12	0	0.21 $\pm$ 0.04	0/8	—
4	16	0	0.23 $\pm$ 0.08	0/8	—
5	24	1,664 $\pm$ 768	0.29 $\pm$ 0.06	0/8	—
6	Unimmunized	0	0	8/8	19.2 $\pm$ 3.4

^a C57BL/10 mice were given 10^6 live culture forms of *T. cruzi* i.p.; one group (5) was given a second identical immunization 17 days after the first. Days represent the time of immunological assay or i.p. challenge with 2×10^3 blood forms of *T. cruzi*.

^b Average direct agglutination titer of eight mice. SD, Standard deviation.

^c Average footpad delayed hypersensitivity responses of eight mice to soluble antigen prepared from lyophilized culture forms of *T. cruzi*.

TABLE 2. The effects of adoptive transfer of spleen cells or serum from specifically immunized mice on the course of infection with *T. cruzi* in syngeneic recipients^a

Group	Treatment	Deaths	
		No./total	$\bar{x} \pm SD^a$ (days)
1	Spleen cells ^b from immunized donors, 2 days before challenge	1/7	27.0
2	Spleen cells from immunized donors, 2 days after challenge ^c	0/7	
3	Serum ^d from immunized donors beginning 2 days before challenge	7/7	20.1 $\pm$ 1.9
4	Serum from immunized donors beginning 2 days after challenge	7/7	19.6 $\pm$ 0.4
5	Spleen cells ^b from normal donors, 2 days before challenge	7/7	19.3 $\pm$ 1.2
6	Serum ^d from normal donors beginning 2 days before challenge	7/7	18.4 $\pm$ 1.6
7	None	7/7	18.8 $\pm$ 0.9

^a SD, Standard deviation.

^b Intravenous inoculation of 2×10^7 viable unfractionated leukocytes from the pooled spleens of normal mice or mice immunized with 10^6 live culture forms of *T. cruzi* on both 14 and 7 days before; C57BL/10 mice.

^c Mice were challenged with 2×10^3 blood forms of *T. cruzi* i.p.

^d A 0.2-ml amount of pooled sera from normal or immunized (direct agglutination titer of 1024) mice was administered i.p. in each of six injections given on alternate days. Groups 3, 4, 5, and 6 versus 7, $P \geq 0.05$.

Adoptive transfer of fractionated spleen cell populations from specifically immunized mice. The final preparations of nylon-wool-adherent cells were ca. 89% immunoglobulin positive and 0.8% latex ingesting. Nylon-wool-nonadherent cells were ca. 8.0% immunoglobulin positive and 0.2% latex ingesting. Mice that received spleen cell populations enriched for immunoglobulin-negative (referred to as T lymphocytes) cells had significantly higher survival rates (Table 3) and lower parasitemias (Fig. 1 and 2) than did mice that received populations enriched for immunoglobulin-positive (referred to as B lymphocytes) cells. All mice that received unfractionated spleen leukocytes, populations enriched for T lymphocytes, or equal numbers of B- and T-lymphocyte-enriched preparations from immunized mice survived acute infection. Mice that received only B-lymphocyte-enriched preparations 2 days after challenge all died, and 3 of 7 mice that received these cells 2 days before challenge survived. Recipients of spleen cells from normal mice all

died, with survival times similar to those of untreated controls. Mice which survived acute infection generally had decreasing parasitemias after 36 days after challenge. All of these animals survived for 120 days after which the experiment was terminated.

Parasitemias generally correlated well with survivals. The lowest parasitemias were consistently seen in mice that received either T-lymphocyte-enriched or both B- and T-lymphocyte-enriched preparations from immunized mice (Fig. 1 and 2). Higher parasitemias occurred in recipients of B-enriched lymphocytes or of serum from immunized mice. The parasitemias in these groups did not differ significantly ($P \geq 0.05$) from those in unimmunized controls.

Donor mice had no parasitemias detectable by microscopic observation, nor was splenomegaly present in any of the donors. Spleen cell preparations were cultured in diphasic medium or in human embryonic kidney cell cultures to test for the presence of parasites. Each type of

FIG. 1. Parasitemias in C57BL/10 mice which received spleen cells or sera from immunized donors before i.p. challenge with *T. cruzi*. Cells were given intravenously 2 days before challenge, and a total of six injections of sera were given i.p. on alternate days beginning 2 days before challenge. Points represent mean value for seven mice except those after day 20 for recipients of B lymphocytes when only three survivors remained. Points ending on day 20 indicate death of entire group. Recipients of B lymphocytes versus the recipients of T lymphocytes, $P \leq 0.05$ at all points. Recipient group: Δ , B cells; $\blacksquare$, T cells; $\blacktriangle$, B + T cells; $\bullet$, whole spleen cells; $\circ$, serum; $\square$, untreated.

FIG. 2. Parasitemias in C57BL/10 mice which received spleen cells or sera from immunized donors after i.p. challenge with *T. cruzi*. Cells were given intravenously 2 days after challenge, and a total of six injections of sera were given i.p. on alternate days beginning 2 days after challenge. Points represent mean value for 7 mice. Points ending on day 20 indicate death of entire group. Recipients of B lymphocyte versus recipients of T lymphocytes, $P \leq 0.05$ at all points. Recipient group: Δ , B cells; $\blacksquare$, T cells; $\circ$, B + T cells; $\blacktriangle$, whole spleen cells; $\bullet$, serum; $\square$, untreated.

culture medium was inoculated in replicates of six with 2×10^7 cells each from fractionated or unfractionated spleen cell preparations. Cell cultures were examined for parasites daily for 8 days. Diphasic cultures were examined weekly for 10 weeks. Cultures were uniformly negative for *T. cruzi*. Eight recipients of 2×10^7 unfractionated spleen leukocytes from immunized donors were left unchallenged. None of these animals developed detectable *T. cruzi* infection.

Efforts were made to correlate agglutinating antibody and delayed hypersensitivity responses with protection in spleen cell recipients. In one experiment mice were tested 6 days after cell transfer. In another, mice were challenged 6 days after cell transfer and tested 6 days after challenge. Neither delayed hypersensitivity responses nor agglutinating antibodies were consistently detected, although strong responses were present in the donors (Table 1).

DISCUSSION

Resistance to acute *T. cruzi* infection was passively transferred with lymphocytes from syngeneic donors previously immunized with

TABLE 3. Effects of adoptive transfer of separated spleen cell populations from specifically immunized mice on the course of infection with *T. cruzi* in syngeneic recipients

Group	Cells received ^a	Day of challenge ^b relative to receiving cells	Deaths	
			No./total	$\bar{x} \pm SD^c$ (days)
1	B (from immunized ^d donors)	+2	4/7	19.4 $\pm$ 1.5
2	B (from immunized ^d donors)	-2	7/7	16.0 $\pm$ 1.2
3	T (from immunized ^d donors)	+2	0/7	
4	T (from immunized ^d donors)	-2	0/7	
5	B + T ^e (from immunized ^d donors)	+2	0/7	
6	B + T (from immunized ^d donors)	-2	0/7	
7	B + T (from normal donors)	+2	7/7	18.5 $\pm$ 1.2
8	B + T (from normal donors)	-2	7/7	16.8 $\pm$ 1.4
9	None		7/7	17.6 $\pm$ 0.5

^a A total of 2×10^7 viable B- or T-lymphocyte-enriched spleen cells given intravenously.

^b A total of 2×10^3 blood forms of *T. cruzi*.

^c SD, Standard deviation.

^d C57BL/10 mice were immunized i.p. with 10^6 live culture forms of *T. cruzi* 21 and 7 days before cell transfer.

^e A total of 2×10^7 viable cells of each cell population given intravenously in separate injections. Groups 1, 2, 7, and 8 versus 9, $P \geq 0.05$.

culture forms of *T. cruzi*. The donors were immune to acute *T. cruzi* infection. T-lymphocyte-enriched preparations were significantly more effective in conferring protection than were those enriched for B lymphocytes.

It has previously been reported that lymphoid cells from animals which survived acute infection with blood forms conferred protection upon syngeneic recipients (4, 16, 26). In the present study donors were immunized with culture forms and did not develop patent infections. Thus culture forms, predominantly epimastigotes, were able to induce the production of cells that could confer protection against challenge with bloodstream trypomastigotes. Hungerer et al. (10) reported failure to transfer protection to C57BL mice with spleen or thymus cells from syngeneic donors previously immunized with live culture forms. The recipients were challenged 1 day before, simultaneously with, or 1 day after cell transfer. However, the immunizing dose, time between immunization and cell harvest, number of cells transferred, and challenge dose were not specified.

In the present study, protection against acute infection was not transferred with serum from

immunized donors. Previous reports of successful transfer of protection with serum have involved donors which had recovered from acute infection (5, 8, 12). Voller and Shaw (38) were unsuccessful in transferring protection to mice with heterologous antiserum produced after epimastigote inoculation. In reference to this study, Hanson (8) suggested that immunoglobulin directed against epimastigotes may not be protective against the trypomastigotes and amastigotes which are responsible for infection. If this is the case it may have to be assumed that serum factors were of little importance in the protection achieved by antigens prepared from epimastigotes and administered with adjuvants (18-20, 23) or without (13). It is evident that immunoglobulin produced against blood forms during infection reacts with determinants on epimastigotes which are used in direct agglutination tests (1, 36). Conversely, serum from mice immunized with live culture forms agglutinated Formalin-killed trypomastigotes (personal observation). In vitro complement-mediated lysis of trypomastigotes by serum from mice immunized with killed epimastigotes has been demonstrated (3), and I have observed evidence of enhanced killing of trypomastigotes by macrophages preincubated with anti-epimastigote serum. Therefore antiserum raised against epimastigotes is effective against trypomastigotes, although in vivo evidence for this is lacking.

Protection may have been transferred with serum by using larger amounts of serum or a different transfer schedule. It is unlikely that idiotypic responses against the transferred immunoglobulin were responsible for the lack of protective effects, as not even transient decreases in parasitemias were noted in recipients after each serum injection (Fig. 1 and 2). Hanson (8) also used a schedule of repeated injections to transfer protection to infected recipients with serum from donors recovered from acute infection.

Spleen cells from mice immunized with live culture forms conferred resistance against acute infection upon syngeneic recipients when given 2 days before or 2 days after challenge. Cells enriched for T lymphocytes were significantly more effective in conferring protection than were those enriched for B lymphocytes. Both preparations were depleted of phagocytic cells. These observations provide direct evidence for the importance of T lymphocytes in resistance to acute *T. cruzi* infection. Roberson and Hanson (25) and Vilches et al. (37) have reported increases in mortality and higher parasitemias in mice treated with antithymocyte serum and injected with *T. cruzi*. Neonatal thymectomy of mice (2, 27) or rats (25) led to exacerbated *T. cruzi*

infections. Athymic (nude) mice have been shown to be more susceptible to acute infection than were thymic littermates (15).

The use of live parasites, especially blood forms, to obtain immune animals as donors in cell transfer experiments may complicate interpretation of the results. This is especially true when transferred cells include those that may harbor intracellular parasites. In the present study, the possibility that recipients may have become immunized actively rather than passively was carefully considered. Donors showed no sign of acute infection at any time. Neither splenomegaly nor circulating parasites were detected. Unchallenged recipients of spleen cells from immunized donors never developed detectable *T. cruzi* infections. Furthermore, spleen cells used for transfer were inoculated into both cell culture and axenic media, and the cultures were uniformly negative. Both media readily support the growth of *T. cruzi*. Hanson et al. (9) reported that *T. cruzi* were recovered from the majority of Vero cell cultures after inoculation of the cultures with as few as one trypomastigote. It is possible that parasites would have eventually become evident in the immunized donors. Detailed long-term analyses were not made. Schmunis et al. (28) observed parasites in the blood of mice 28 days after immunization with 10^5 epimastigotes grown in diphasic medium, but not in mice immunized with 10^3 or 10^4 epimastigotes. If undetected parasites were present in the spleen cell preparations the numbers would have been too small to consistently confer protection. For example, mice inoculated with fewer than 10^3 to 10^4 culture forms do not consistently develop resistance to challenge with blood forms (31; personal observation). McHardy (18) found that an antigen mass equal to more than 10^4 freeze-thawed epimastigotes administered with saponin was necessary to protect mice against acute infection. Further, the time between immunization and challenge is an important factor in determining whether immunization with *T. cruzi* culture forms will be effective. Mice immunized with a protective dose of *T. cruzi* culture forms and challenged 4 days later were not protected (Table 1). In the passive transfer experiments, mice were challenged 2 days before or 2 days after cell transfer. Thus it is unlikely that active immunization could have occurred with this schedule.

Spleen cell preparations enriched for T lymphocytes transferred resistance more effectively than did those enriched for B lymphocytes. If active immunization had occurred, it must be assumed that only T lymphocyte-enriched populations contained parasites. If intracellular parasites were present in phagocytic cells, they

would have been more likely to have been in nylon-wool-adherent cells. However, both T- and B-lymphocyte-enriched populations were depleted of phagocytic cells by passage through glass wool.

The resistance to acute infection achieved by transfer of lymphocytes from immunized donors was incomplete in that high numbers of circulating parasites were present after challenge. In some survivors parasites were detected up to 60 days after challenge, but individuals varied greatly after day 36. In contrast, circulating parasites were not detected in actively immunized mice. The recipients of T-lymphocyte-enriched populations had consistently lower parasitemias than did recipients of B-lymphocyte-enriched populations. Thus parasitemias were correlated with survivals, although this is not always true in experimental *T. cruzi* infections (16; personal observation).

Delayed hypersensitivity responses and agglutinating antibodies were not detected in recipients of spleen cells from immunized donors. The donors readily developed delayed hypersensitivity responses to *T. cruzi* antigen after immunization and, unlike spleen cell recipients, these animals did not develop detectable parasitemias after challenge. It is likely that other factors, such as antigen processing and presentation by macrophages, are necessary for more complete resistance to develop. Macrophages undoubtedly are important in resistance to *T. cruzi* (4, 14, 32). An essential role for T lymphocytes in mediating resistance to *T. cruzi* is firmly established. Studies of this nature are necessary to evaluate the nature of the protective responses evoked by immunizing preparations of both live and killed parasites.

ACKNOWLEDGMENTS

This paper is dedicated to the memory of the late Carl L. Larson, who provided the guidance and facilities for the initiation of these studies.

Support for this work was received from the Stella Duncan Memorial Institute, University of Montana.

I acknowledge and express sincere gratitude to Jill Inverso for excellent technical assistance and to Mari Otsuka for assistance in manuscript preparation.

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