1977

Trypanosoma cruzi in mice | studies on resistance and effects of acute infection on the immune response

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TRYPANOSOMA CRUZI IN MICE.
STUDIES ON RESISTANCE AND EFFECTS
OF ACUTE INFECTION ON THE IMMUNE RESPONSE.

by

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B. A., Whitman College, 1973

Presented in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

UNIVERSITY OF MONTANA

1977

Chairman, Board of Examiners

Dean, Graduate School

Date Mar. 23, 1977

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789 East Eisenhower Parkway
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6-28-77

Reed, Steven G., M. S., March 1977 Microbiology

Trypanosoma cruzi in Mice. Studies on Resistance and Effects of Acute Infection on the Immune Response. (132 pp)

Director: Carl L. Larson C.L.L.

Specific immunization with live attenuated trypanosomes and stimulation of non-specific resistance by viable Mycobacterium tuberculosis strain BCG were used to examine mechanisms of induced immunity against Chagas' disease in mice. In addition, the effects of acute infection on the immune response of mice to antigens unrelated to the trypanosomes were observed.

Resistance to T. cruzi in mice appears to be cell-mediated and to require specific activation of the immune system. BCG alone did not protect against acute infection but live attenuated trypanosomes did if given at least 12-14 days in advance of challenge. Killed trypanosomes were not protective. Administration of silica, a macrophage toxin, prior to challenge exacerbated infection. Activation of the immune system with BCG actually interfered with the induction of specific resistance by attenuated trypanosomes.

Mice lost their ability to mount an immune response to other antigens during the period of acute infection. Suppression of humoral and cellular responses occurred during the stages of marked parasitemia. Infected mice unable to respond to oxazolone could confer sensitivity to this substance via spleen cell transfer. The ability of infected mice to respond to oxazolone was significantly improved when they received syngeneic macrophages at the time of skin test.

Mice became increasingly susceptible to bacterial endotoxin as the infection progressed, also suggesting impairment of their immune response.

ACKNOWLEDGMENTS

I would like to express sincere appreciation to Dr. Carl L. Larson for his support and guidance throughout this study.

I wish to thank the other members of my degree committee: Drs. Nakamura, Speer, Tibbs, and Ushijima.

Special thanks is given to Ms. Kelly, Green, and Sanderson for their expert technical assistance on several aspects of this work and for their invaluable efforts in typing and preparing this thesis.

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ABBREVIATIONS

B-cell	bursa-equivalent lymphocyte
BCG	Bacillus Calmette-Guèrin
h	hours
ip	intraperitoneal
iv	intravenous
LD_{50}	lethal dose fifty; minimum number of parasites required to kill at least 50% of the animals in a given period of time
LPS	endotoxin; bacterial lipopolysaccharide
MEM	minimal essential medium
PBS	Dulbecco's phosphate buffered saline
sc	subcutaneous
SRBC	sheep red blood cells
T-cell	thymus-dependent lymphocyte

Chapter 1

INTRODUCTION

Classification of Trypanosoma cruzi Hoare, 1966

Phylum PROTOZOA Goldfuss, 1818; emend. Siebold, 1845

Subphylum SARCOMASTIGOPHORA Honigberg and Balamuth, 1963

Superclass MASTIGOPHORA Diesing, 1866

Class ZOOMASTIGOPHORMA Calkins, 1909

Order KINETOPLASTIDA Honigberg, 1963

Suborder TRYPANOSOMATINA Kent, 1880

Family TRYPANOSOMATIDAE Doflein, 1901; emend. Grobben, 1905

Genus TRYPANOSOMA Gruby, 1843

Species CRUZI Chagas, 1909

General

Chagas' disease, or American trypanosomiasis, is the result of infection with Trypanosoma (Schizotrypanum) cruzi, a protozoan parasite of man and many other mammals. It is an extremely important public health problem in many Ibero-American countries. The disease is widely distributed, occurring in portions of the southern United States and most of the South American countries. An estimated seven million persons are believed to harbor the parasite (41) and the mortality rate is high, especially among young children. It is most often a severe chronic infection leading to

debilitation of the victim through a variety of pathological changes.

The disease is transmitted by many species of reduviid bugs (subfamily Triatominae), also known as cone-nosed or kissing bugs. In Latin America, they are known popularly as barbeiros, hitas, pitos, or vinchucas. These blood-sucking insects feed at night and defecate immediately after feeding. The excreta contains the infective stages of the parasite, which have completed the final stages of development in the digestive tract of the bug. Contamination of the mucous membrane of the eye or of the wound made by the bite leads to infection. This method of transmission is referred to as posterior station. The main reservoirs of the disease appear to be armadillos and opossums, but many other terrestrial mammals are infected. Other means by which humans may become infected include blood transfusion and transplacental passage.

Trypanosoma cruzi has two main stages in its life cycle. One takes place in man or a reservoir host, and the other in the insect vector. The parasite is pleomorphic, occurring in different forms during the course of its life cycle. The insect ingests the trypanosomes with a blood meal, and the insect forms develop to the infective forms found in the feces. The parasites, having gained entrance into a suitable host, multiply rapidly at

the site of infection and a local inflammatory reaction ensues. This consists of cellular infiltration characteristic of a local inflammation, with both polymorphonuclear and mononuclear phagocytes present. The regional lymph nodes become enlarged. After a brief incubation period the organism enters the blood stream and initiates the acute phase of Chagas' disease. The organism will not divide in the blood stream, but does so only within cells which either engulf the parasite or are invaded by the organism. Some strains have greater affinity for muscle tissue, especially cardiac, while others preferentially invade nervous or reticuloendothelial tissue. Most strains divide readily within host macrophages (6, 91). Characteristic features of this phase of Chagas' disease include splenomegaly and hepatomegaly, generalized adenopathy, and fever. Parasites are readily isolated from the blood during the acute phase. If the host survives, the chronic phase is initiated, which is characterized by few circulating trypanosomes and progressive tissue destruction by the intracellular forms. The chronic phase may extend for a period of many years but spontaneous recovery may occur. Death often results from severe damage to the heart, or from the destruction of nerve plexi responsible for maintaining muscle tone in the alimentary canal leading to megacolon or megaesophagus. When few circulating

parasites are present, diagnosis may be made by hemoculture or by xenodiagnosis. The latter is a procedure utilizing a non-infected vector which is allowed to feed on the patient and is subsequently examined for the presence of parasites. Trypanosoma cruzi is the only major pathogen of the Stercoraria group of trypanosomes.

History

Accounts of a disease, "Bicho", peculiar to the area of the natural distribution of the reduviid bugs began to appear in American chronicles as early as the 16th century. The disease was described as having a long and chronic course, with a characteristic feature of rectal distension (34). Infestation of the diseased rectum by worms led the early observers to believe the disease was caused by helminthic infection, and they named it "Mal de Culo" in Spanish, or "Maculo" in Portuguese. This means simply disease of the colon. Later reports referred to "Mal de Engasgo", or megaesophagus, as being a serious disease in Brasil. A common treatment was to apply juice from the tobacco leaf to the affected area.

Observations of the reduviid vectors were recorded long before the identification of the parasite. A passage from Charles Darwin (1809 - 1882) describing his encounter with the insects appeared in the "Journal of researches

into... various countries visited by H. M. S. Beagle", London, 1839 (after Guerra, 1970).

(1839 ed., pp. 403-404) "... At night I experienced an attack (for it deserves no less a name) of the 'Benchuca' (species of Reduvius) the great black bug of the Pampas. It is most disgusting to feel soft wingless insects, about one inch long, crawling over one's body. Before sucking they are quite thin, but afterwards become round and bloated with blood, and in this state they are easily crushed. They are also found in the northern parts of Chile and in Peru. One which I caught at Iquiques was very empty. When placed on the table, and though surrounded by people, if a finger was presented, the bold insect would immediately draw its sucker, make a charge, and if allowed, draw blood. No pain was caused by the wound. It was curious to watch its body during the act of sucking, as it changed in less than ten minutes, from being as flat as a wafer to a globular form. This one feast, for which the 'Benchuca' was indebted to one of the officers, kept it fat during four whole months; but, after the first fortnight, the insect was quite ready to have another suck." This account is especially interesting, for it is generally agreed that Darwin contracted Chagas' disease on this expedition, and suffered from the infection, until his death some forty years later (57).

In 1907, Carlos Chagas, a young medical officer, was assigned to investigate measures to be taken to safeguard the health of railroad workers in the Minas Gerais state in the United States of Brasil. The main concern in the area was malaria. While investigating local insects, he noticed flagellated protozoa in the hindgut of the cone-nosed bugs. It should be remembered that this was a time of great interest in insects and their role as vectors, for in 1893, Theobald Smith had associated a tick with Texas Cattle Fever (85). Chagas sent specimens to his supervisor, Oswaldo Cruz in Rio de Janeiro. Shortly after his initial findings of the organisms in bugs, Chagas found them in the blood of monkeys and in a small girl suffering from an unknown illness. (It is interesting to note that this girl was found to be harboring the parasite some sixty years after being examined by Chagas). These investigations were followed by numerous findings of the parasite in human patients, and it was soon associated with a disease known to be prevalent in that area. This is an unique example of the identification of an etiological agent before that agent was associated with a disease.

Chagas went on to describe the life cycle of the **parasite** (11, 12) but mistakingly thought the organism was passed to mammals directly through the bite of the bug (anterior station transmission), and that multiplication

occurred in the mammal by a form of schizogony. Hence the misnomer Schizotrypanum cruzi. These mistakes concerning the transmission and life cycle of T. cruzi were corrected by Brumpt (9).

Immunity

The course of infection with T. cruzi is influenced by a number of factors. The numerous strains capable of causing infection vary greatly in their virulence for different hosts. This variation is affected by their histotropism, as well as a variety of host factors. Among the most virulent strains is the Tulahuen, a reticulotropic strain first isolated in Chile from a human patient. This organism shows a special affinity for host macrophages (90). As with most virulent strains, the Tulahuen shows a preference for a certain tissue but is capable of invading and dividing in others.

Among the host factors influencing the course of the disease are age and sex. In general, young males are most susceptible to infections. The mortality rate is especially high in children.

Natural immunity to Chagas' disease is not uncommon, as evident from the large number of asymptomatic carriers. Both humoral and cellular immune responses occur in T. cruzi infection (64, 65, 72). The acute phase, characterized by a progressive rise in parasitemia can be established (61).

Both IgM and IgG classes of antibody are present during the acute stage (28, 99).

The role of these antibodies in immunity is not well established. Conference of protection by passive transfer of immune serum has been generally unsuccessful (19, 38). Complement-mediated lysis of culture forms occurs *in vitro*, and complement depletion in mice has been reported to exacerbate infection (73). Some workers have reported passive transfer of protection with immune serum (46), but with most strains of high virulence this is not possible. The value of antibodies in protection was supported by the observation (52) that mother rats could transfer immunity to their offspring, but in human cases, infants with high antibody titers at birth often have severe parasitemia. Much of the apparent contradiction of the results concerning protection and antibody in Chagas' disease may be due to differences in strains used, and in the interpretation of results, since much of the work was done before many of the immunological processes were understood.

To support the view that antibodies are not directly responsible for the destruction of *T. cruzi in vivo*, Pizzi put forth the following arguments (41): a) immunized rats can be shown to have high antibody titers but little or no protection, b) the serum of immune animals has no effect on virulent trypanosomes *in vitro*, c) administration of

hyperimmune serum to non-immune animals fails to protect them against infection, d) splenectomy does not alter the course of infection, and e) newborns with high titers of specific antibody may be born with acute parasitemia.

The importance of cell-mediated immunity in Chagas' disease is well established. Delayed skin reactions can be demonstrated in immunized and chronically infected individuals (95), and passive transfer of sensitivity to T. cruzi antigens can be made using lymphocytes from sensitized donors (93). In vitro correlates of cellular immunity, such as blast cell transformation and inhibition of macrophage migration have also been observed (79, 80, 81, 95, 107).

Immune protection against lethal challenge involves cellular immunity, as shown by the passive transfer of protection with spleen cells from infected animals (53, 76). Animals that have been thymectomized or treated with anti-thymocyte serum prior to challenge with virulent T. cruzi develop higher parasitemias and die more rapidly than do normal infected animals (75, 78).

Animals with acquired immunity mobilize inflammatory macrophages when challenged with T. cruzi as do non-immune animals, but they do so more rapidly and with higher numbers (26). Phagocytosis appears to be very important in defense against T. cruzi infection. Some workers (47)

have reported enhancement of infection when experimental animals were pretreated with silica particles to destroy phagocytic cells. Correspondingly, non-specific stimulation of the reticuloendothelial system with diethylstilbesterol or complete Freund's adjuvant reportedly enhances resistance when used in the presence of specific antigen (3, 49). Non-specific activation of the reticuloendothelial system with BCG is not a useful method to protect against virulent challenge, however. Although one group of investigators has reported achieving non-specific resistance using viable BCG (69), the great majority of attempts to immunize with BCG have been unsuccessful (42, 53).

The fact that virulent T. cruzi can multiply within macrophages suggests that they are generally resistant to phagocytic process of these cells. Some in vivo studies have shown the parasites are destroyed in macrophages of immune animals, but survive and multiply in normal macrophages (90). This is consistent with the idea of the importance of phagocytosis in immune protection against Chagas' disease. However, inconsistencies arise from studies using BCG-activated macrophages. Hoff (42) has shown that macrophages from BCG-immunized animals were able to limit parasite multiplication and to resist destruction better than normal macrophages when cultured

with virulent T. cruzi in vitro. In fact, the macrophages from BCG-immunized mice were as resistant as those from mice specifically immunized with live avirulent T. cruzi. Interestingly, when either of the groups of activated macrophages were incubated with immune serum, their ability to destroy the parasites was significantly increased, strongly suggesting an important role for cytophilic antibody. Immune serum did not alter the resistance of normal macrophages to T. cruzi. Although BCG-activated macrophages were more resistant than normal ones in vitro, BCG-immunized mice were not protected against lethal challenge, even when the parasites were first incubated with immune serum.

Infection alone, without prior immunization, appears to activate the host reticuloendothelial system. Macrophages from infected animals spread more on glass than do normal cells, and the clearance of colloidal carbon particles from the bloodstream is more rapid in infected than in normal mice (15).

Immunization

Further implicating the importance of cell-mediated immunity in Chagas' disease is the proven superiority of live vaccines to produce protection against virulent infection (60, 106). Animals that survive acute infection

are usually resistant to subsequent infection (19, 105). In some cases, this is not a sterile immunity, but relies on the presence of parasites in the host. This condition is known as premunition.

Protection is often produced experimentally with live attenuated trypanosomes. Repeated passage in culture media is a useful way to produce organisms of lowered virulence (62). X-irradiated organisms may also be used for immunization. The use of live vaccines for prevention of Chagas' disease in man is not advocated at this time due to the limited information available regarding possible reversion of attenuated organisms to the virulent state and the potential of producing chronic infection by such vaccines.

Killed organisms are far less effective immunizing agents. In a few instances, incorporation of a killed vaccine with an adjuvant provided some degree of resistance (44). Methods that kill the organism but leave it intact are desirable. Fernandes et al. (22) were able to protect animals against lethal challenge by first incubating the organisms with actinomycin D. This procedure left the organism motile, but unable to divide due to blockage of nucleic acid synthesis. Recently, culture forms killed with a mild solution of sodium perchlorate have proven effective for immunization of experimental animals (49). To achieve good protection with killed organisms, multiple

doses are usually necessary (106). Animals effectively immunized, especially with organisms of low virulence, typically demonstrate good cell-mediated responses to T. cruzi antigen (27, 79).

All of the reticuloendothelial organs may undergo change during infection, due to the presence of large numbers of organisms in macrophages. The most dramatic changes occur in the spleen and lymph nodes. In the spleen, hyperplastic and degenerative changes occur concommitantly. The hyperplasia predominates initially, and is followed by degeneration (90). In general, an overall reduction of lymphocytes is seen in spleens of severely infected hosts. Macrophages appear activated, and are often engorged with parasites and cellular debris. As the infection progresses, an increase in the ratio of large lymphocytes to small is noted.

Lymphocyte depletion is also noted in certain areas of the lymph node, along with mobilization of reticular cells. Whether or not the overall lymphocyte population of the nodes declines is doubtful, for intense mitotic activity takes place until just before death or recovery from the acute phase. This activity predominates throughout the node, and especially in the B-lymphocyte-rich cortical areas.

In the liver of an infected animal, Kupffer cells are often seen to contain many parasites. Acute and

chronic hepatitis develops, with local inflammatory lesions throughout. Although often filled with parasites, the Kupffer cells apparently do not lose their ability to phagocytose other particles as demonstrated by the increased rate of carbon clearance in infected animals.

Tissue destruction and inflammation occurs in many other areas; particularly in muscle, nervous, and gonad tissue. The damage is generally caused by invasion and multiplication by the parasite, although the possibility of damage from toxins may exist (25, 51). Evidence for toxic damage stems from observations of destruction of uninfected cells. Furthermore, a trypanosomal toxin has been postulated to explain inhibitory effects on certain malignancies seen in animals with T. cruzi infection (36, 37). An extract from cultured T. cruzi is sold commercially in Europe under the trade name "Trypanosa" for use as an antitumor agent.

Some workers suggest that a state of allergy is produced during infection by sensitization to antigens released by degenerated parasites. Muniz and Azevado (63) were able to produce lesions of myocarditis in monkeys indistinguishable from those seen in Chagas' disease by the injection of dead antigens of T. cruzi. Some of the pathological changes are due to destruction of neurons throughout the peripheral nervous system.

Allergic type reactions may have a role in this aspect of the pathology.

Immunosuppression in Protozoal Infections

An interesting phenomenon associated with certain protozoal infections is the suppressive effect these infections have on the ability of the host to respond to other antigens. This has been observed in malaria, leishmaniasis, toxoplasmosis, African trypanosomiasis, and Chagas' disease (1, 7, 14, 15, 16, 17, 29, 30, 31, 32, 39, 66, 77, 98). Affects on both humoral and cellular immunity have been noted, though not always in the same disease. The mechanisms involved are not understood, and appear to be varied and complex. It is probable that active infection is necessary for the immunosuppressive effect to occur (59, 109).

There is evidence for direct lymphocyte involvement, since histological aberrations of the spleen and lymph nodes appear in animals with malaria, toxoplasmosis, Chagas' disease, and African trypanosomiasis (30, 39, 59, 90). Many of the parasites involved with immunosuppression are harbored within blood cells (*Toxoplasma*, *T. cruzi*, *Leishmania*, *Plasmodium*), but others are not (African trypanosomes). Many differences in the host-parasite relationships involved in these diseases make it difficult

to postulate a common mechanism for immunosuppression. Possible mechanisms at work in immunosuppression associated with protozoal infection will be discussed later. Interest in this aspect of the infectious process lies in a better understanding of the immune system, with special reference to cell cooperation. Clinically, immunosuppressive effects stemming from such infections could predispose patients to other disease.

Suppression of cell-mediated immunity may be directed against the afferent or efferent arm of the response. Delayed hypersensitivity reactions may be considered to occur in two stages: the induction of the response and the expression or manifestation of the response. During the inductive phase, T-lymphocytes become "primed" by the antigen and are stimulated to begin producing clones. After sufficient time has elapsed for significant numbers of these cells to be produced, the host is able to respond to eliciting doses of the sensitizing substance, and thus to express delayed hypersensitivity. This stage is characterized by a visible reaction at the site of injection of specific antigen and the typical cells observed in histological preparations of the lesion.

When oxazolone, a chemical contact sensitizing agent, is applied to the skin, large pyroninophilic cells, termed immunoblasts, appear in the lymph node draining the site

of application. Expression of the response involves induration and erythema at the site of skin test, and an abundance of lymphocytes and macrophages in the lesion. There is typically a relative paucity of polymorphonuclear leukocytes (97).

Statement of Problem

This research was designed to examine: 1) procedures for immunization against *Trypanosoma cruzi* and 2) the effects of acute infection with this parasite on the immune system of the host.

Chapter 2

MATERIALS AND METHODS

Animals

Conventional outbred male Swiss mice, weighing 30-40 g were used in most experiments. In the passive transfer studies, inbred C57Bl/10 mice, adult males, were used. All mice were obtained from the Rocky Mountain Laboratory in Hamilton, Montana, housed in plastic cages, and given Purina Lab Chow and water ad lib.

Parasites

Blood and culture forms of the Tulahúen strain of Trypanosoma cruzi were obtained from Dr. Robert G. Yaeger (Dept. of Tropical Medicine, Parasitology Division, Tulane University, New Orleans, La.).

Blood forms were maintained by weekly passage of whole blood from infected to normal mice. Culture forms were maintained in the dialysate media of Nakamura (1967). These organisms have been passed in culture more than 300 times. Dialysate media was chosen to minimize foreign proteins in the cultures. Culture forms were used for immunization and blood forms for infecting mice.

Media

Powdered MEM (Eagle) with Earle's Salts, L-glutamine, and non-essential amino acids was purchased from GIBCO (Santa Clara, Ca.) and was reconstituted to 90% of its final volume with triple distilled water. Streptomycin sulfate and penicillin G were added during preparation to final concentrations of 100 µg/ml and 100 U/ml respectively. The pH was adjusted to 7.3 with NaOH. The media was filter-sterilized before use.

Determination of the Lethal Dose-fifty (LD_{50})

Determination of the LD_{50} for the Tulahúen strain in Swiss and C57Bl/10 mice was performed by challenging mice with different numbers of blood forms obtained from infected mice. Blood was drawn into a heparinized syringe by severing the brachial artery of an etherized mouse in which the parasitemia was high. Approximately 1.0 ml of blood was obtained in this manner from a 30 g mouse. The blood was diluted with sterile Locke's solution and the trypanosomes were counted with a hemocytometer. The parasite suspension was adjusted to concentrations ranging from 5×10^6 to 1×10^2 parasites/ml and 0.2 ml of each concentration was injected into groups of 8 mice each. The Swiss mice were injected with various concentrations of parasites by intravenous (iv) or intraperitoneal (ip)

routes. Deaths were recorded on a daily basis. Survivors were killed after forty days.

Immunization Studies

Adult male conventional Swiss mice were used in this and all subsequent experiments with the exception of the cell transfer studies.

BCG vaccine

Attenuated Mycobacterium bovis, or BCG (bacille Calmette-Guérin), was obtained from stock maintained in the Stella Duncan Institute. Stock cultures were diluted with Dubos broth to an optical density of 200 ("Klett 200") on a Klett-Summerson photoelectric colorimeter equipped with a blue filter (Klett-Summerson no. 42, spectral range 400-465nm). These were stored at -70 C and prepared for use as follows: After thawing, the stock was diluted to a "Klett 100" suspension by adding an equal volume of Dubos broth. This was inoculated ip into mice in 0.2 ml volumes. To determine the number of colony-forming units (CFU) of BCG administered, 3 0.1 ml volumes of 10^{-4}, 10^{-5}, 10^{-6} dilutions were plated on duplicate Dubos agar plates. The plates were sealed in plastic bags and incubated at 37 C for 28 days. The visible colonies were counted, making it possible to calculate the number of viable BCG organisms/dose. This number was found to be 5×10^7 - 10×10^7.

T. cruzi vaccine

To prepare trypanosomes for use in immunization, dialysate media was inoculated with culture forms of T. cruzi and incubated at 28 C for 14-16 days. This was sufficient time to achieve prolific growth in media seeded with approximately 5 x 10^6 organisms/ml. After incubation, cultures were centrifuged at 5,000 x g for 10 minutes and the pellet was resuspended in sterile Locke's solution. The parasites were washed 3 times and adjusted to a concentration of approximately 1 x 10^7/ml. Mice injected ip with 0.02 ml of this suspension of live trypanosomes 14 and 7 days before challenge with virulent blood forms showed transient parasitemias but survived infection.

Effects of specific and non-specific immunization on the course of infection with T. cruzi

Mice were injected ip with various combinations of BCG and live attenuated T. cruzi prior to challenge with 5 LD_{50} doses of virulent T. cruzi. The challenge was given 8 days after the last immunizing dose. An unimmunized control group was infected with the same dose at the same time.

Effects of iv immunization with BCG on the course of infection with T. cruzi

Mice were given one or two doses of BCG (0.2 ml of a Klett 100 preparation) iv 11 days and 1 day prior to

infection. Groups of mice treated in this way were then challenged with 2 or 50 LD_{50} doses of T. cruzi. Separate groups of unimmunized control mice were infected with either of the 2 doses of trypanosomes. In addition, a group of mice receiving only a primary dose of BCG, 11 days before infection, was challenged with 2 LD_{50} doses of T. cruzi. Deaths were recorded daily.

Effects of immunization with live and killed culture forms on the course of infection with virulent T. cruzi

Live attenuated culture forms were administered as described previously. Killed parasites were prepared by subjecting washed culture forms to 5 cycles of alternate freeze-thawing, using an acetone-dry ice bath and a 37 C water bath. The organisms were counted before the freeze-thaw treatment, and equivalent numbers of killed and live parasites were used for the immunizations. All injections were given ip.

Effect of Treatment with Silicon Dioxide Particles on the Course of Infection with T. cruzi

Silicon dioxide (SiO_2) ("Min-U-Sil, Whittaker, Clark and Daniels, N.Y. 5 μ) were suspended in sterile phosphate-buffered saline (PBS) at concentrations of 100, 200, and 300 mg/ml. Two-tenths of one of the suspension were injected ip into the various groups of mice. Groups of 8

mice each were given one of these doses on the day before challenge, the day of challenge, or on both days. A control group receiving no SiO_2 was challenged at the same time in a similar manner. Deaths were recorded daily.

Course of Infection with T. cruzi

Reticuloendothelial clearance of colloidal carbon particles in mice infected with T. cruzi (after Stuart, (88))

A solution of colloidal carbon was prepared as follows: black ink, "Spezialtusche" (Gunther Wagner, Pelikan Werke, Hanover, Germany), in a stock solution of 64 mg carbon/ml was diluted in saline to a final concentration of 16 mg/ml. The diluting saline contained approximately 0.5% concentration of gelatin. Normal mice and mice acutely ill with Chagas' disease were injected iv with the carbon suspension using doses of 100 mg/kg body weight. The infected animals had received 1 LD_{50} dose of virulent T. cruzi 16 days prior to administration of the carbon. Blood samples of approximately 0.05 ml were taken from the retro-orbital sinus with a heparinized capillary pipette at intervals of 2, 6, 10 and 20 minutes after injection of the carbon suspension. The size of the blood sample was constant at each bleeding. The blood was mixed with 2.0 ml distilled water immediately after being drawn and the optical density of the suspension was read in a Coleman Jr. II, Model 6/35 spectrophotometer at a wavelength of

490 nm. Control readings of blood alone from normal and infected mice were also made.

Changes in spleen weight during the course of infection with T. cruzi

Spleens were removed from groups of mice, 5 mice/group, at various days after ip injection of 1×10^3 blood forms of T. cruzi and placed in a beaker containing MEM.

The spleens were trimmed of foreign tissue, blotted dry, and weighed on a top-loading Mettler balance. The weights of each group of spleens were averaged.

Changes in total blood cell count during the course of infection with T. cruzi

The blood of groups of five mice was pooled with a small amount (0.5 ml) of heparinized saline at intervals of 5, 8, 10, 12, 14 and 16 days after infection with 1×10^3 virulent T. cruzi. The total number of red blood cells for each group and for an uninfected control group were determined with a hemocytometer.

Similar procedures were used to compare total and differential white blood cell counts.

Course of parasitemia during infection with T. cruzi

Male Swiss mice were inoculated ip with 1 LD_{50} dose of T. cruzi blood forms. Groups of mice were exsanguinated every other day following infection, their blood was pooled, and parasite counts were made with a hemocytometer.

Effect of infection with T. cruzi on the inflammatory response

Groups of 5 normal mice and of mice infected for 16 days with 1 LD_{50} dose of T. cruzi blood forms received 0.05 ml of a 1:5 mixture of turpentine in olive oil subcutaneously (sc) in the right ear and 0.05 ml of saline in the left. Difference in thickness between the ears were measured with a "Schnelltaster" dial guage micrometer after 24 h.

Tests for Delayed Hypersensitivity (DH) in Normal Mice and Mice Infected with T. cruzi

Oxazolone (4-ethoxymethylene-2-phenyl oxazolone, BDH Chemicals, Ltd. Poole, England), a potent skin-sensitizing agent, and complete Freund's adjuvant (CFA) were used to compare DH responses in normal and infected mice.

Oxazolone

One-tenth ml of a freshly prepared 3% (wt/vol) solution of oxazolone in absolute ethanol was rubbed on the pre-shaven abdomen of mice. Seven days after sensitization, 1 drop (from a 25 ga. needle) of a 1.5% solution of oxazolone in a 1:1 mixture of absolute ethanol and olive oil was applied to the inner surface of each ear. The thickness of each ear was measured with a "Schnelltaster" immediately before applying the solution and again at 24 h.

CFA (Complete Freund's Adjuvant)

Two parts of Arlacel A (Sigma, St. Louis) and 3 mg of dried tubercle bacilli were ground together with mortar and pestle. Eight parts light mineral oil were added and mixed. Ten parts sterile saline, pH 7.0, were added dropwise and an even emulsion was obtained. The preparation was drawn back and forth between two 10 ml syringes joined with a double-hubbed needle. Mice were injected sc with 0.2 ml of this preparation, which contained 0.1 mg of dried tubercle bacilli.

Fourteen days after immunization with CFA, mice were inoculated sc with 12.5 µg of BCG protoplasm suspended in 0.05 ml saline in one footpad. The other footpad was injected with the same volume of saline. After 24 h, the thickness of each footpad was measured with a "Schnell-taster" and the differences in measurements were recorded. The response was expressed as the thickness of the footpad injected with the protoplasm minus that of the footpad injected with saline only.

Mice were sensitized with 1 of these antigens at various times before or during injection and subsequently skin tested. The responses were compared to those of uninfected animals.

The ability of mice to either develop or express sensitivity to oxazolone was tested. To determine the

effect of trypanosome infection on the afferent arm of the reaction, oxazolone was administered to mice at various intervals after infection and the animals were subsequently skin tested for delayed hypersensitivity. To determine the effect of infection on the efferent arm of the response, the animals were first sensitized with oxazolone, and after sensitivity was attained the mice were infected with 1 LD_{50} dose of T. cruzi. The infected animals and an uninfected control group were then skin tested as before at various intervals. The results were expressed as the percentage of the second reaction as compared to the first.

Tests for Humoral Responses in Normal Mice and Mice Infected with T. cruzi

Two antigens, sheep red blood cells (SRBC) and alkaline-detoxified bacterial lipopolysaccharide (dLPS), were used to test the humoral response in normal and infected mice.

SRBC (Sheep Red Blood Cells)

Whole sheep blood was obtained from the Rocky Mountain Laboratory, Hamilton, Montana. The blood was centrifuged at 2000 x g for 5 minutes and the packed layer of red cells retained. These were washed three times in sterile phosphate-buffered saline (PBS), pH 7.2, and adjusted to a concentration of 2×10^8 red cells/ml, or 4×10^7/0.2 ml. Groups of 6 mice each were given iv

injections of 0.2 ml of a freshly-prepared suspension of SRBC at various days after infection with 1 LD_{50} dose of T. cruzi blood forms. The animals were exsanguinated 4 days later and their sera pooled. Groups of mice were bled on days 6,8, 10, 14 and 16 post infection. An uninfected, unsensitized control group was handled similarly. The sera was frozen and inactivated at 56 C for 30 minutes prior to testing. The sera was tested for antibodies by passive hemagglutination, using a 1% solution of normal SRBC for the antigen.

dLPS (detoxified lipopolysaccharide)

The dLPS was obtained from Dr. Jon A. Rudbach, Department of Microbiology, University of Montana, Missoula, Montana. Ten µg of dLPS, suspended in sterile PBS, pH 7.0, was administered in 0.2 ml volumes. The same experimental procedure was used as in the assays for antibodies against SRBC. For the antigen in the passive hemagglutination assay, SRBC with adsorbed dLPS were used.

Effects of Infection with T. cruzi on the Development of Lymphoblasts in Local Lymph Nodes Following Exposure to Oxazolone

At various days after infection with 1 LD_{50} dose of T. cruzi, groups of 4 mice each received 3 drops (from a 25 ga needle) of 3% oxazolone in absolute ethanol on the inner surface of each ear. The solution was rubbed

into the skin with a cotton applicator stick. Four days later the auricular lymph nodes were removed from each side of the animal's neck. Nodes were removed 10, 12, 14 and 16 days post infection, as well as from an uninfected control group which had received the same oxazolone treatment. Immediately after excision, the nodes were put in Plutznik's fixative and stored until all nodes for the experiment were obtained. The minimum time in the fixative was 24 h. The nodes were then embedded in paraffin and sectioned with a Spencer Model F20 microtome (7 microns). The sections were mounted and stained with methyl-green pyronin, a RNA stain. The slides were examined microscopically and the percentage of pyroninophilic (RNA-rich) cells, termed lymphoblasts, was determined. The lymphoblasts in the nodes from sensitized, infected mice were compared to those in the nodes of sensitized, uninfected mice. As a control, nodes from normal mice were observed as well as nodes from infected unsensitized mice.

Transfer of Contact Sensitivity

In this and the other cell-transfer studies, C57Bl/10 adult male mice were used. Groups of 6 mice each were sensitized with oxazolone on the abdominal region as described previously. Seven days later, they were

killed by cervical dislocation. Their spleens were removed and forced through a fine wire mesh into petri plates containing tissue culture media. To avoid formation of clots by spleen cells, no more than 2 or 3 spleens were combined in the same petri dish, in approximately 20 ml of media. The number of spleens combined was largely a function of spleen size. Animals infected for 12 or more days with virulent T. cruzi generally have greatly enlarged spleens. The cells were allowed to settle in a 40 ml centrifuge tube to remove large particles. The cell suspension was poured off, and centrifuged at 250 x g for 5 minutes, resuspended in media, and washed 2 times. The cells were counted using Turk's solution to lyse red blood cells and were adjusted to a concentration of 2.5×10^8 white blood cells/ml. Recipient mice were injected ip with 0.02 ml of this suspension (a total of 5×10^7 cells/recipient). Twenty-four h after receiving cells, the recipient animals were skin tested with oxazolone by applying 1.5%oxazolone in ETOH:olive oil to 1 ear and diluent only to the other ear. This procedure was followed in infected sensitized and uninfected sensitized donor mice. Infected sensitized donors were used at 8, 10, 14, 16 and 17 days post infection. To assay for oxazolone response in the donors, they were skin tested 24 h before being killed. The oxazolone

response was compared in groups of recipient mice, 5 mice/group, receiving cells from different groups of donors.

Transfer of contact sensitivity with separate lymphocyte populations

Mice were sensitized with oxazolone as described earlier, and their spleens removed 7 days later. The spleen cells were separated by forcing the spleen through a wire mesh as before. The cells were then separated into populations of B and T lymphocytes as described by Hoades et al., (40), using a nylon wool column.

Twelve spleens were removed and divided into tubes containing 40 ml MEM with 2 spleens per tube. The erythrocytes were lysed in ammonium chloride and the remaining cells were washed 3 times with MEM. One and seven-tenths g of nylon wool (FT-242, Fenwal Laboratories, Morton Grove, Ill) which had been prewashed in glass-distilled water was packed into the barrel of a 30 ml disposable syringe to the 18 ml mark and autoclaved. Before cell application, the nylon in the column was saturated and rinsed with MEM containing 10% fetal calf serum (MEM-FCS), and the column was incubated for 1 h at 37 C. After this preincubation, 1.6×10^9 cells, in 15 ml warmed MEM-FCS were added to the column, and the preparation incubated at 37 C in a humidified air-5% CO_2 incubator.

After the incubation the non-adherent cells were removed by adding warmed MEM-FCS to the column, the first 30 ml of effluent, the T-cell enriched fraction, was collected. The column was washed with 100 ml warmed MEM-FCS and this wash was discarded. Adherent cells were removed by compressing the nylon wool with the syringe plunger, removing the plunger and teasing up the wool with sterile forceps. The wool was resaturated with MEM-FCS and compressed again. The process was repeated until about 50 ml containing the B-cell enriched fraction were collected.

Cell surface immunoglobulin as a B-cell marker was identified by fluorescein-conjugated rabbit antimouse immunoglobulin. Fluorescent cells were identified as B-cells and non-fluorescent cells as T-cells.

Transfer of normal mouse peritoneal exudate cells prior to skin testing mice sensitized with oxazolone and infected with T. cruzi

Mice were sensitized with 3% oxazolone 8 days after infection with 1 LD_{50} dose of virulent T. cruzi. Seven days following sensitization (15 days after infection) the mice received 2 x 10^7 peritoneal exudate cells from normal donor mice and were skin tested immediately. An infected, sensitized control group received no cells, and a group of normal, unsensitized mice received the same dose of peritoneal exudate cells prepared as follows:

Normal mice were killed by cervical dislocation. A lengthwise incision was made with a sharp scissors from the groin to the chest, taking caution to avoid puncture of the peritoneal membrane. Five ml of cold tissue culture media was injected with a 26 ga needle into the peritoneal cavity. The distended abdomen was massaged for about 30 seconds and the media was then withdrawn with a 20 ga needle, taking care to keep blood from the exudate suspension. The exudate suspension was put in cold siliconized glass centrifuge tubes and centrifuged at 250 x g for 5 minutes. The white blood cells were counted and resuspended in MEM to a concentration of 1×10^8 cells/ml.

Attempts to transfer sensitivity to oxazolone to normal and infected mice

Spleen cells were harvested as before from groups of 7 mice/group which had been previously sensitized and skin tested with oxazolone. The cells were washed, placed in a plastic petri dish and incubated for 30 minutes. The non-adherent cells were removed by spraying the dish with media from a syringe and were again incubated on a plastic surface. The non-adherent cells were removed and adjusted to a concentration of 2×10^8 cells/ml and 0.02 ml of this suspension was injected into mice ip. The recipient animals consisted of normal mice and of mice that had been infected with

1 LD_{50} dose of virulent T. cruzi 15 days previously. Twenty-four h after receiving the spleen cells, the animals were skin tested with oxazolone, and the results were read after another 24 h. As a control, normal spleen cells, handled in the same manner, were injected into other groups of normal and infected animals. There were 5 recipient mice/group.

Effects of LPS on Mice Infected with T. cruzi

On various days following ip challenge with 1 LD_{50} dose of virulent T. cruzi, different groups of Swiss mice were injected iv with 1.0, 0.5, or 0.1 µg LPS (J. A. Rudbach, University of Montana). Mice were given LPS on days 9, 12, 14, 16 and 18 days after infection. Deaths within 12 h after LPS injection were quantified and used for comparative data.

Statistics

The Mann-Whitney test was used to analyze the data.

Chapter 3

RESULTS

Determination of the LD_{50} of the Tulahuen Strain of T. cruzi in RML Mice

Mice consistantly died about 18 days following infection with 1 x 10^3 blood forms of T. cruzi. The average time of death was less predictable when lower doses were used, so 1 x 10^3 parasites were chosen as the challenging dose. This provided the longest working period after infection within a predictable course of infection. Challenge with only 10-20 parasites would often kill mice by 30 days or more, but survivors were often noted when low challenge doses were used. Route of infection was found to make little difference; iv and sc challenge produced similar results. C57Bl mice were found to approximate the Swiss mice in their susceptibility. In all cases, males were more susceptible than females, and young animals were more susceptible than older ones (Fig. 1).

Immunization Studies

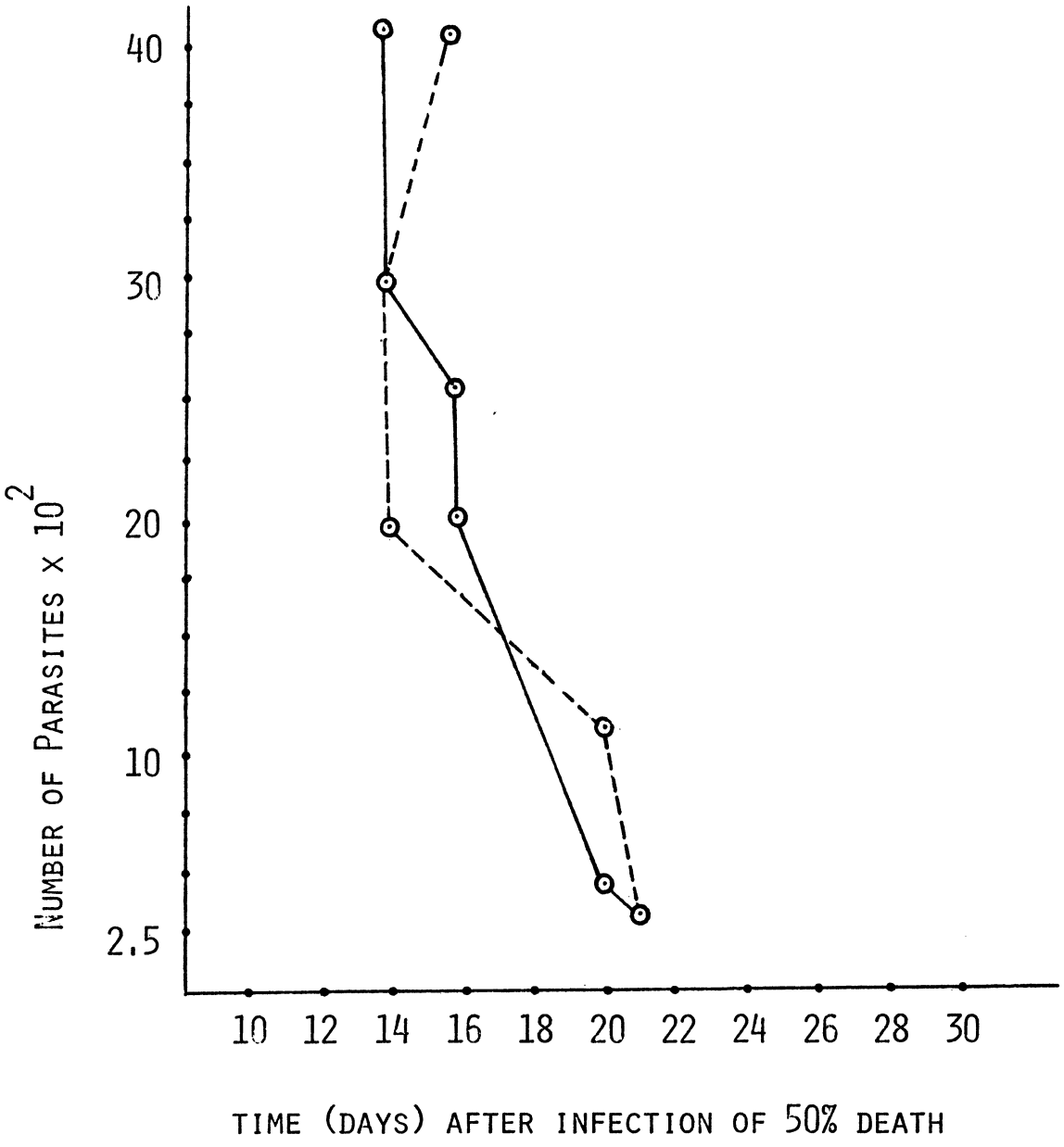
Effects of immunization with BCG and/or live attenuated T. cruzi on the course of infection in mice challenged with virulent T. cruzi

Mice were protected against lethal challenge with blood forms of T. cruzi by ip inoculation of one dose of attenuated T. cruzi given 18 days prior to challenge or by

Fig. 1. Determination of the LD_{50} in RML mice. Mice were six weeks old, and injected ip.

Symbols: male ————

female- - - - - -


40
30
20
10
2.5
NUMBER OF PARASITES X 10^2
10 12 14 16 18 20 22 24 26 28 30
TIME (DAYS) AFTER INFECTION OF 50% DEATH

two doses of the same preparation give 18 and 7 days prior to challenge (Table 1). A single inoculation of the attenuated organisms given 7 days before challenge did not give good protection. Immunization with BCG gave little or no protection, although some protection was noted in mice receiving BCG 40 and 18 days before challenge.

A loss of effectiveness of protection due to specific immunization was noted in mice immunized with both attenuated T. cruzi and BCG. Good protection was apparent in mice receiving 1 or 2 doses of attenuated trypanosomes (groups 6 and 8) but animals were not completely protected by the same treatment if they had been previously immunized with BCG (groups 2,3,4,5,7). This was true in all cases except that in which mice were treated with 2 doses of BCG and 2 doses of attenuated trypanosomes.

Effects of BCG immunization iv on the course of infection with T. cruzi

Mice immunized iv with BCG 1 and 11 days prior to challenge with 50 LD_{50} doses of T. cruzi survived significantly longer than did normal mice, although 7 of 8 were dead by the 19th day of infection (Table 2). Mice challenged with 2 LD_{50} doses showed a similar trend, although the differences were not significant. Very little difference in survival time was seen between groups receiving 1 or 2 doses of BCG.

Table 1. Effects of Immunization with BCG and Attenuated *Trypanosoma cruzi* Trypomastigotes on the Survival Time of Mice Infected with Virulent *T. cruzi*.[a]

Group	Immunizations d-40[b] BCG[e]	d-18[c] BCG	d-7[d] ATC[f]	d-7[d] ATC	Number Dead/Total	Survival Times (days) $\bar{x}$	Range
1	+	+	+	+	0/8	--	--
2	-	+	+	+	2/8	16.0	15-17
3	+	+	+	-	2/8	28.5	17-30
4	-	+	+	-	4/8	20.5	17-26
5	+	-	+	+	3/8	26.3	17-38
6	-	-	+	+	0/8	--	--
7	+	-	+	-	4/8	20.0	17-26
8	-	-	+	-	0/8	--	--
9	-	-	-	+	6/8	28.0	17-32
10	+	-	-	-	8/8	17.9	16-30
11	+	+	-	-	5/8	20.2	19-25
12	-	-	-	-	8/8	17.8	16-20

[a] challenged with 5×10^3 virulent blood forms (5 LD_{50} doses) ip

[b] 40 days prior to challenge

[c] 18 days prior to challenge

[d] 7 days prior to challenge

[e] Paris strain; approx. 1×10^7 viable organisms/dose ip

[f] attenuated live *T. cruzi* culture forms; approx. 1×10^6/dose ip

Table 2. Effects of iv Immunization with BCG on the Survival Time of Mice Challenged with Virulent Trypanosoma cruzi.

Group	BCG Immunizations[a] d-11[c]	BCG Immunizations[a] d-11, d-1[d]	Number Challenge doses[b]	No. Dead/ Total	Survival Times (days) $\bar{x}$	Survival Times (days) Range
1	-	+	50	7/8	16.2	13-19
2	-	-	50	8/8	13.4	13-15
3	-	+	2	8/8	20.3	17-30
4	-	-	2	8/8	17.2	16-20
5	+	-	2	8/8	20.3	17-27

[a] Paris strain of BCG; approx. 1×10^7 viable organisms/dose

[b] No. LD_{50} doses of virulent blood forms, ip

[c] 1 injection of BCG 11 days before challenge

[d] 2 injections of BCG 11 and 1 days before challenge

Effects of immunization with live and killed culture forms on the course of infection with virulent T. cruzi

All mice given 2 x 10^6 live attenuated T. cruzi 20 days prior to challenge survived infection, regardless of the route of immunization (Table 3). Killed culture forms offered little protection with any of the immunizing methods employed.

Effect of Treatment with Silicon Dioxide Particles on the Course of Infection with T. cruzi

Mice receiving 2 doses of silica, at any of the concentrations used, died significantly faster when infected with T. cruzi than did infected control mice (Table 4). The mice treated with a single dose of 10 or 40 mg of silica also differed significantly from the controls in their survival times. The group treated with one 20 mg dose did not differ significantly from controls in survival time. There were 4 survivors in this group, as compared to 2 in the group given one 40 mg dose of silica, and 1 in the control group.

Course of Infection with T. cruzi

Reticuloendothelial clearance of colloidal carbon particles in mice infected with T. cruzi

Mice infected with T. cruzi (16 days after receiving 1 LD_{50} dose) were able to remove colloidal carbon from their bloodstream significantly faster than normal animals

Table 3. Effects of Immunization with Live or Killed Attenuated Trypanosoma cruzi on the Survival Time of Mice Challenged with Virulent Trypanosoma cruzi.

Group	Immunization[a]	Days Before Challenge[b]	No. Dead/ Total	Survival Times (days) $\bar{x}$	Range
1	L, iv[c]	20	0/8	--	--
2	L, ip[d]	20	0/8	--	--
3	K, iv[e]	20	5/8	21.0	18-26
4	K, ip	20	4/8	20.5	19-22
5	--	--	8/8	18.3	17-19

[a] approx. 2×10^6 attenuated T. cruzi, live or killed per dose

[b] approx. 2×10^3 virulent blood forms (2 LD_{50} doses), ip injection

[c] live T. cruzi, iv injection

[d] live T. cruzi, ip injection

[e] T. cruzi killed by freeze-thaw method

Table 4. Effects of Treatment with Silica on the Survival Time of Mice Infected with Virulent Trypanosoma cruzi.[a]

Group	Dose	Silica Treatment[b] d-1	d 0	d-1,d 0	No. Dead/ Total	Survival Times (days) $\bar{x}$	Range	p ≤ 0.05[c]
1	10mg	+	-	-	8/8	18.0	15-20	+
2	"	-	+	-	8/8	18.3	17-20	+
3	"	-	-	+	8/8	17.8	16-20	+
4	20mg	+	-	-	5/8	18.6	16-26	-
5	"	-	+	-	7/8	20.7	18-26	-
6	"	-	-	+	8/8	17.8	16-20	+
7	40mg	+	-	-	7/8	17.4	16-19	+
8	"	-	+	-	7/8	17.4	16-19	+
9	"	-	-	+	8/8	17.5	16-19	+
10	none	-	-	-	7/8	19.7	16-21	

[a] all mice challenged ip with 1 x 10^3 T. cruzi blood forms

[b] silica particles given ip the day before challenge (d-1), the day of challenge (d 0), or on both days (d-1, d 0)

[c] p value for Mann-Whitney significance test; level of significance when compared to control group (10)

(Fig. 2, Table 5). Control readings of blood from normal and infected mice were approximately the same, indicating that the presence of trypanosomes in the blood samples did not alter the results.

Changes in spleen weights during the course of infection with T. cruzi

Individual spleen weights increased dramatically as the infection progressed (Fig. 3, Table 6). This increase in the weights of individual spleens was apparent 8 days after infection was induced.

Changes in total blood cell count during the course of infection with T. cruzi

Total erythrocyte and leukocyte counts were lower in acutely infected mice than in normal mice. Erythrocyte numbers delined from 11-12 x 10^6/ml to about 6 x 10^6/ml as the infection approached the terminal stage. Leukopenia was also noted; the total white cell counts decreased from 2-3 x 10^4/ml to 0.9-1.4 x 10^4/ml. There was no apparent selective decline of a single leukocyte population (Fig. 4&5).

Course of parasitemia during infection with T. cruzi (Fig. 6, Table 7)

The number of circulating trypanosomes rose logrithmically following ip injection of 1 LD_{50} dose of T. cruzi. Eighteen days after infection, parasite levels ranged from 6-20 x 10^6/ml blood.

Table 5. Reticuloendothelial Clearance of Colloidal Carbon in Mice Infected with Trypanosoma cruzi.

Time (minutes) After Injection	% Transmittance[b] Normal	Infected[a]	$p \leq 0.05$[c]
2	16.2	30.2	+
6	17.8	30.6	+
10	20.2	29.8	+
20	21.2	32.4	+

[a] infected with 1×10^3 virulent blood forms T. cruzi ip 16 days prior to carbon injection

[b] average value for 5 mice; % transmittance spectrophotometrically

[c] p value for Mann-Whitney significance test, comparing normal group to infected group

Fig. 2. Reticuloendothelial clearance of colloidal carbon in mice infected with T. cruzi. Mice were tested 16 days after infection with 1×10^3 blood forms. Clearance was determined by evaluating blood withdrawn at intervals for spectrophotometric transmittance.

Symbols: infected mice - - - - - -

normal mice, ——————

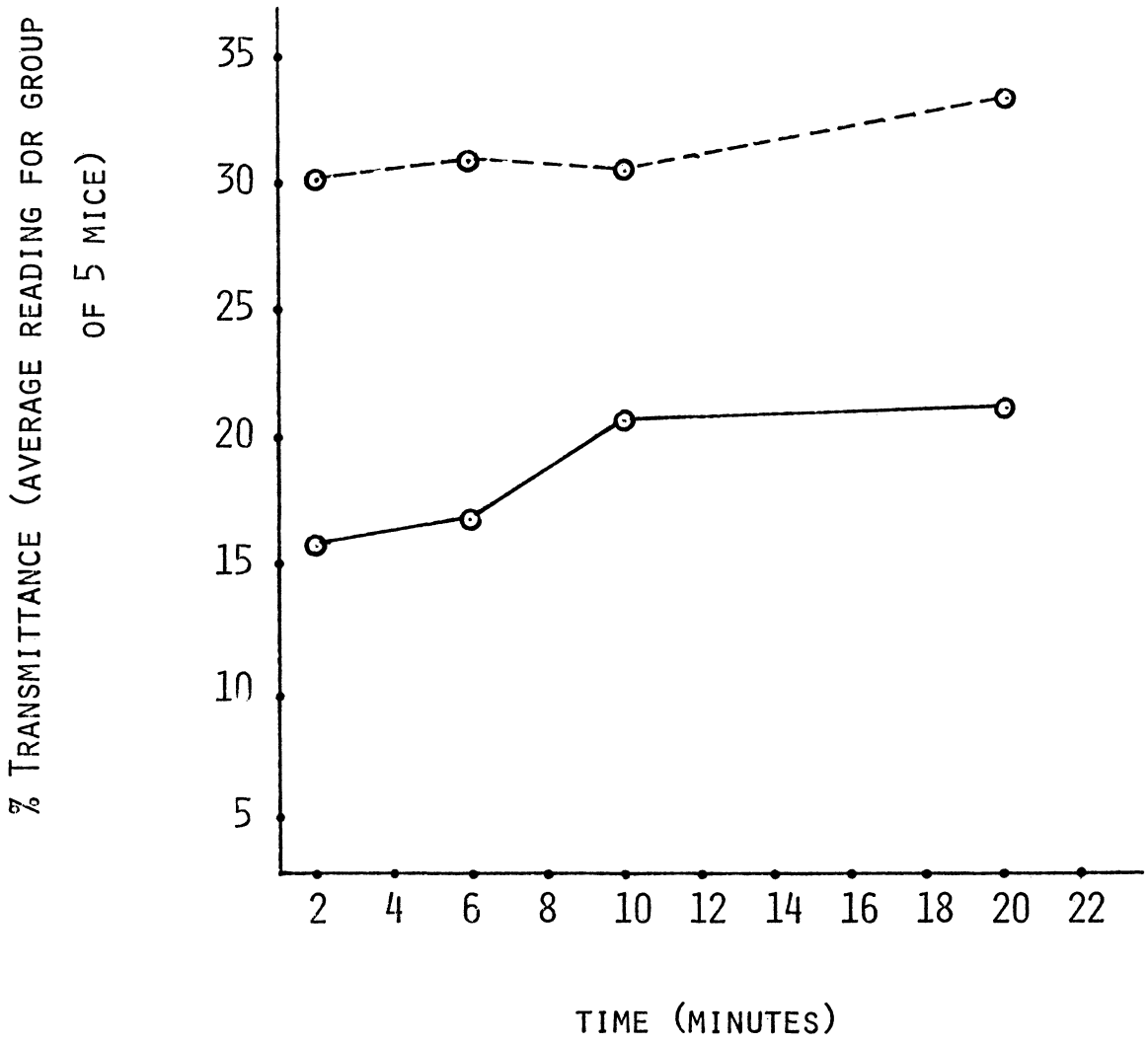

% TRANSMITTANCE (AVERAGE READING FOR GROUP OF 5 MICE)
35
30
25
20
15
10
5
2
4
6
8
10
12
14
16
18
20
22
TIME (MINUTES)

Table 6. Changes in Spleen Weight During the Course of Infection with Trypanosoma cruzi.

No. Days Infected[a]	Ave. Spleen Wt. (g)[b]
--	0.12
2	0.12
4	0.13
6	0.17
8	0.28
10	0.54
12	0.55
14	0.68
16	0.68
18	0.74

[a] infection with 1 LD_{50} dose of T. cruzi blood forms ip

[b] average value of 5 mice

Fig. 3. Changes in spleen weights during the course of infection. Mice were infected ip with 1 x 10^3 blood forms.

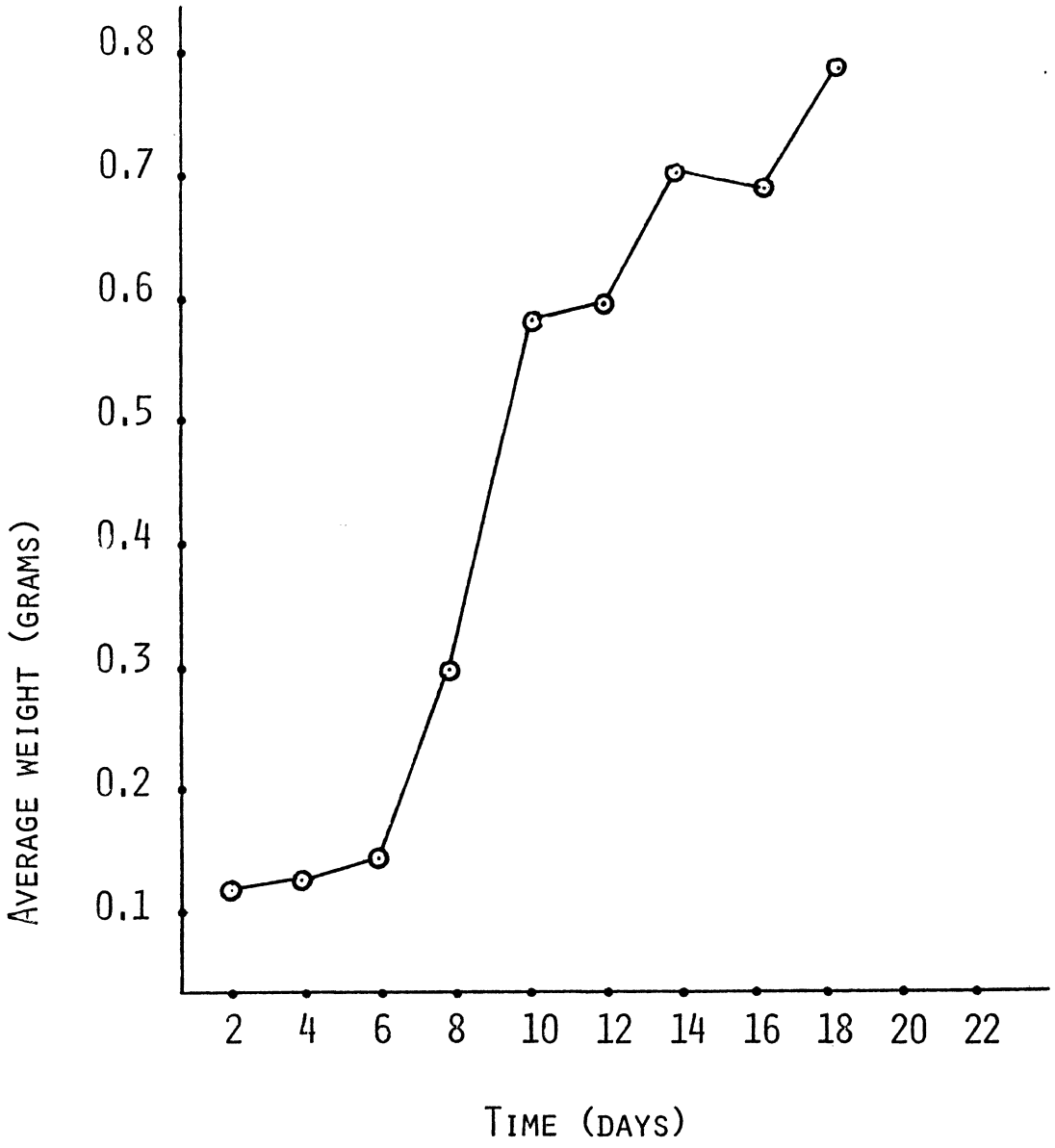

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
AVERAGE WEIGHT (GRAMS)
2
4
6
8
10
12
14
16
18
20
22
TIME (DAYS)

Fig. 4. Changes in blood cell numbers in mice infected with T. cruzi. Mice were infected ip with 1×10^3 blood forms. Cell counts for groups of 5 mice each were averaged. N represents the average value for uninfected male RML mice, 6 weeks of age.

Symbols: leukocyte counts ———

erythrocyte counts - - - - - -

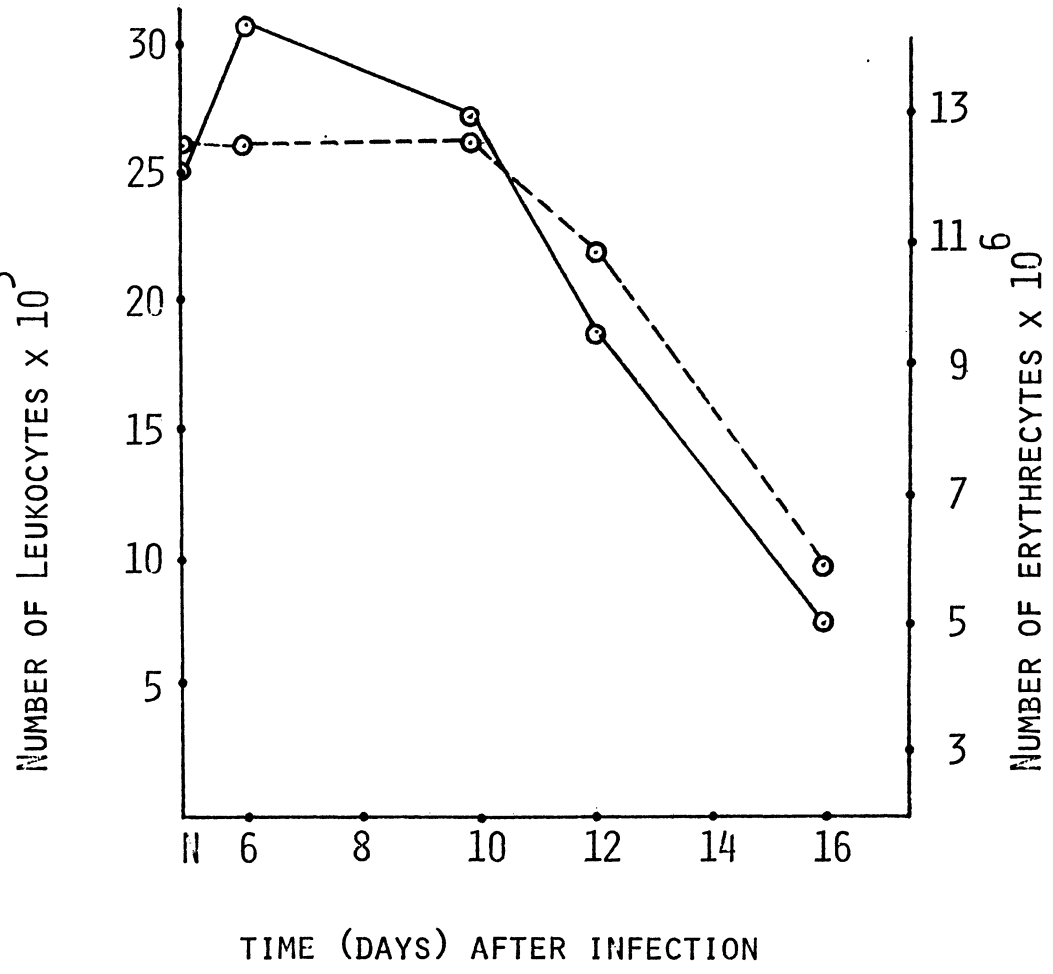

30
25
20
15
10
5
13
11
9
7
5
3
N
6
8
10
12
14
16
NUMBER OF LEUKOCYTES X 10 3
NUMBER OF ERYTHRECYTES X 10 6
TIME (DAYS) AFTER INFECTION

Fig. 5. Differential leukocyte counts during the course of infection with T. cruzi in RML mice. Mice were infected ip with 1 x 10^3 blood forms. N represents the average value for uninfected mice.

Symbols:

neutrophils	—.—.—.—
lymphocytes	————
monocytes	- - - - - -
eosinophils	·——·——·——

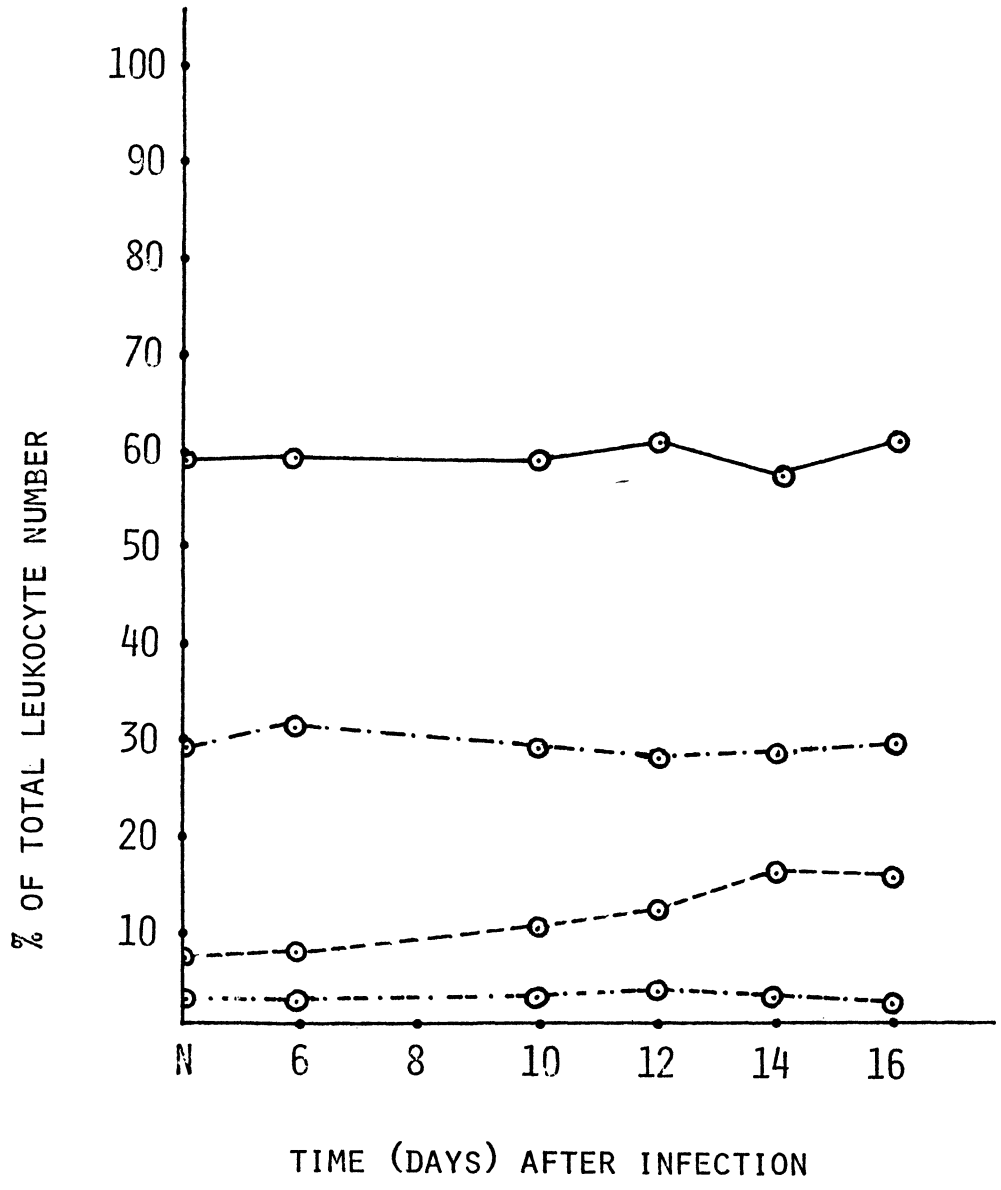
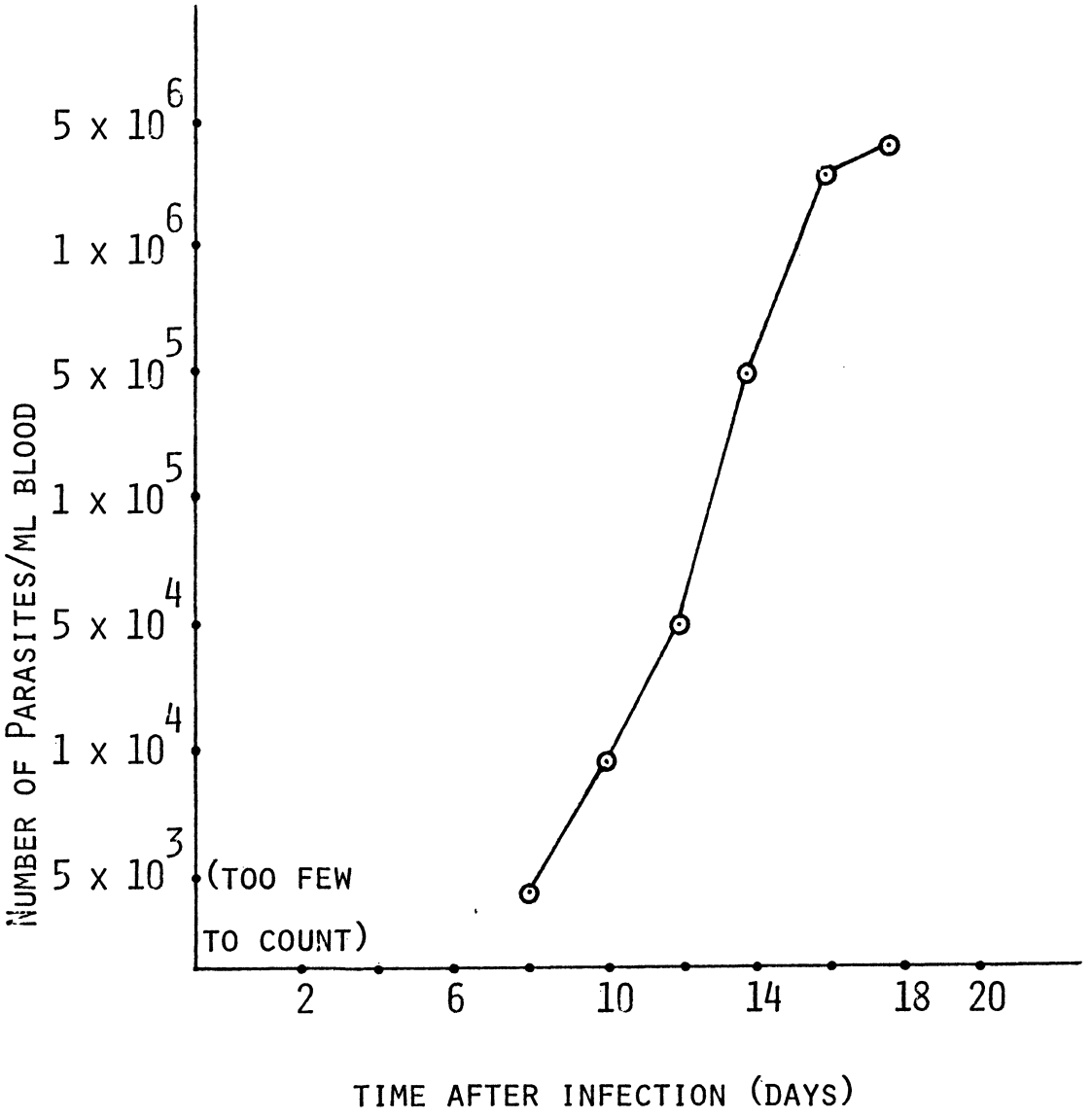

100
90
80
70
60
50
40
30
20
10
% OF TOTAL LEUKOCYTE NUMBER
N
6
8
10
12
14
16
TIME (DAYS) AFTER INFECTION

Table 7. Course of Parasitemia During Infection with Trypanosoma cruzi.

No. Days Infected[a]	No. Trypanosomes/ ml Blood
8	6×10^3
10	2×10^4
12	6×10^4
14	5×10^5
16	3×10^6
18	4×10^6

[a] infected with 1×10^3 virulent blood forms of T. cruzi ip

Fig. 6. Course of parasitemia during infection with *T. cruzi*. Mice were infected ip with 1×10^3 blood forms.


NUMBER OF PARASITES/ML BLOOD
5 x 10^6
1 x 10^6
5 x 10^5
1 x 10^5
5 x 10^4
1 x 10^4
5 x 10^3
(TOO FEW
TO COUNT)
2
6
10
14
18
20
TIME AFTER INFECTION (DAYS)

Effect of infection with T. cruzi on the inflammatory response

Significant differences in ear thickness were seen between infected and control mice 24 h after injection of a turpentine:olive oil mixture. The mice treated with cortisone developed a lowered inflammatory response. It was less than those of the other 2 groups of animals tested; and cortisone-treated mice showed a more rapid regression of inflammation than did normal animals (Table 8).

Tests for Delayed Hypersensitivity (DH) in Normal Mice and Mice Infected with T. cruzi

CFA

Groups of 8 mice each were infected with T. cruzi either 4 days before, on the same day, 4 days after or 9 days after sensitization. The animals were subsequently tested for delayed hypersensitivity 14 days after sensitization. Thus the mice were tested 5, 10, 14 or 18 days after infection with T. cruzi. As shown in Fig. 7, mice tested on days 5 and 10 after infection had delayed hypersensitivity reactions roughly equivalent to those of uninfected mice. Those tested 14 or 18 days after infection had markedly decreased delayed responses. These 2 groups differed significantly from the uninfected mice.

Table 8. Effects of Infection with *Trypanosoma cruzi* on the Inflammatory Response in Mice.

Group	Treatment	Inflammatory Response (mm)[a]	$p \leq 0.05$[d]
1	none	0.42	-
2	*T. cruzi*[b]	0.32	+
3	cortisone[c]	0.25	+

[a] 0.05 ml of a 1:5 mixture of turpentine:olive oil was injected in one ear; 0.05 ml saline was injected into the other. Difference between thickness of the 2 ears was recorded 24 h later.

[b] 1×10^3 blood forms injected ip 16 days prior to test

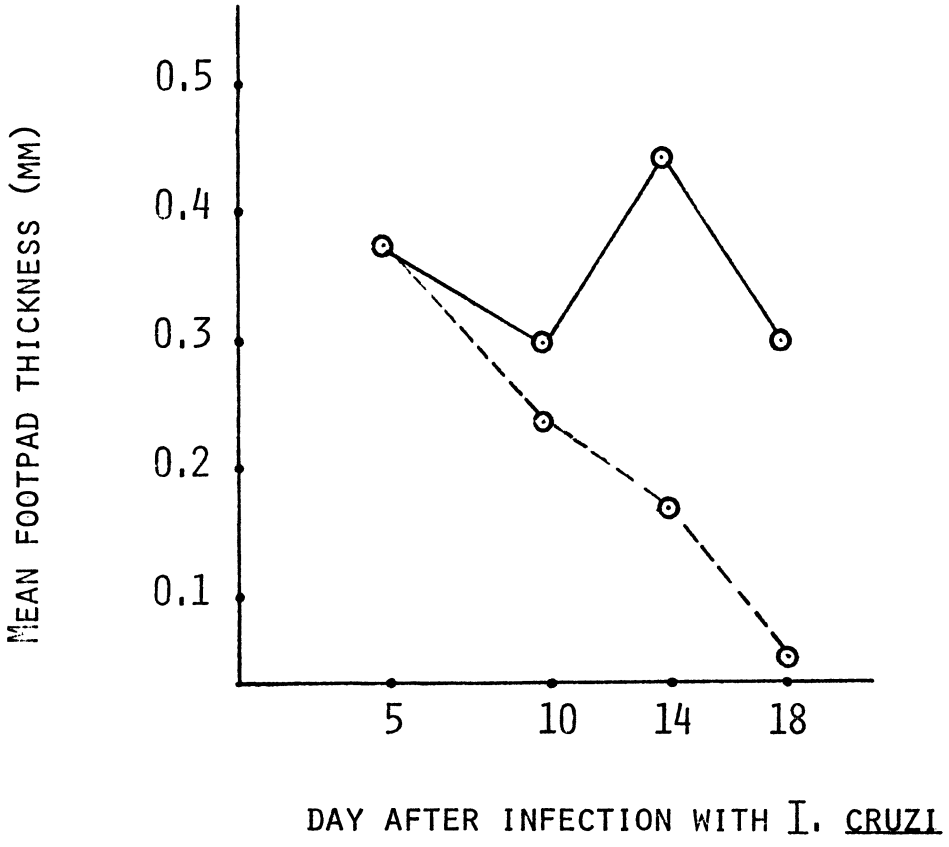
[c] hydrocortisone acetate, 50 mg/kg, given ip 24h prior to test

[d] Mann-Whitney significance test used to analyze the data compared to normal control (group 1)

Fig. 7. Suppression of delayed hypersensitivity to 12.5 µg BCG protoplasm injected into the footpad of mice immunized with BCG and infected with T. cruzi. Each point represents the mean response of 8 mice, skin tested 14 days after sensitization with CFA.

Symbols: infected mice ------

normal mice ————————


MEAN FOOTPAD THICKNESS (MM)
0.5
0.4
0.3
0.2
0.1
5
10
14
18
DAY AFTER INFECTION WITH I. CRUZI

Oxazolone

Influence of infection with T. cruzi on the induction of delayed hypersensitivity. In the experiment shown in Fig. 8, groups of mice were sensitized with oxazolone on days 0, 4, 8 and 10 after infection with T. cruzi and tested for sensitivity to oxazolone 8 days after sensitization (8, 12, 16 and 18 days after infection). Mice sensitized with oxazolone on the day of infection displayed delayed hypersensitivity to oxazolone when tested 8 days later, whereas animals given oxazolone on the fourth day after infection and tested 12 days after infection showed a marked decrease in the response. The response to oxazolone was further decreased in mice sensitized 8 and 10 days after infection and tested 16 and 18 days after infection, respectively. The 3 latter groups of infected animals differed significantly in their response from the uninfected controls.

Influence of infection with T. cruzi on the expression of delayed hypersensitivity. A rise in response to oxazolone was noted in uninfected mice as the time following the first skin test increased (Fig. 9). However, infected mice that had been sensitive to oxazolone before infection became less responsive as the infection progressed. This loss of sensitivity was first apparent by the fourteenth day

Fig. 8. Suppression of delayed hypersensitivity to oxazolone applied to the ears of mice sensitized with oxazolone and infected with T. cruzi. Each point represents the mean response at 8 days after sensitization with oxazolone.

Symbols infected mice ———

normal mice ———

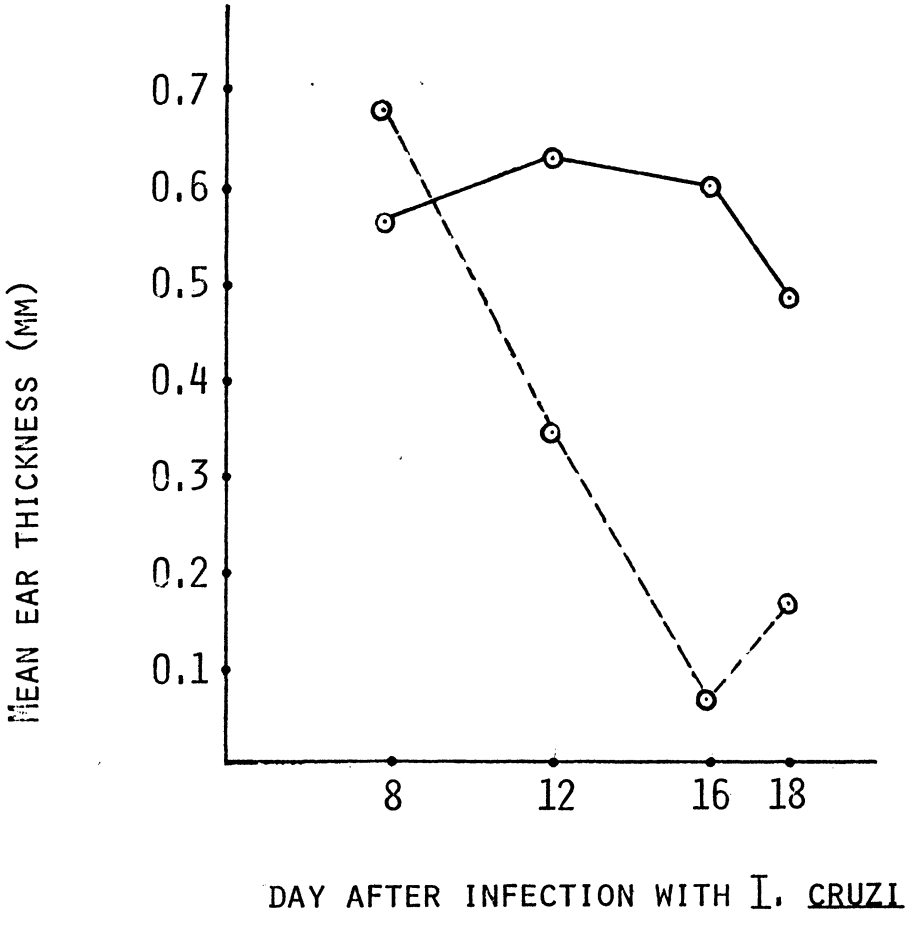
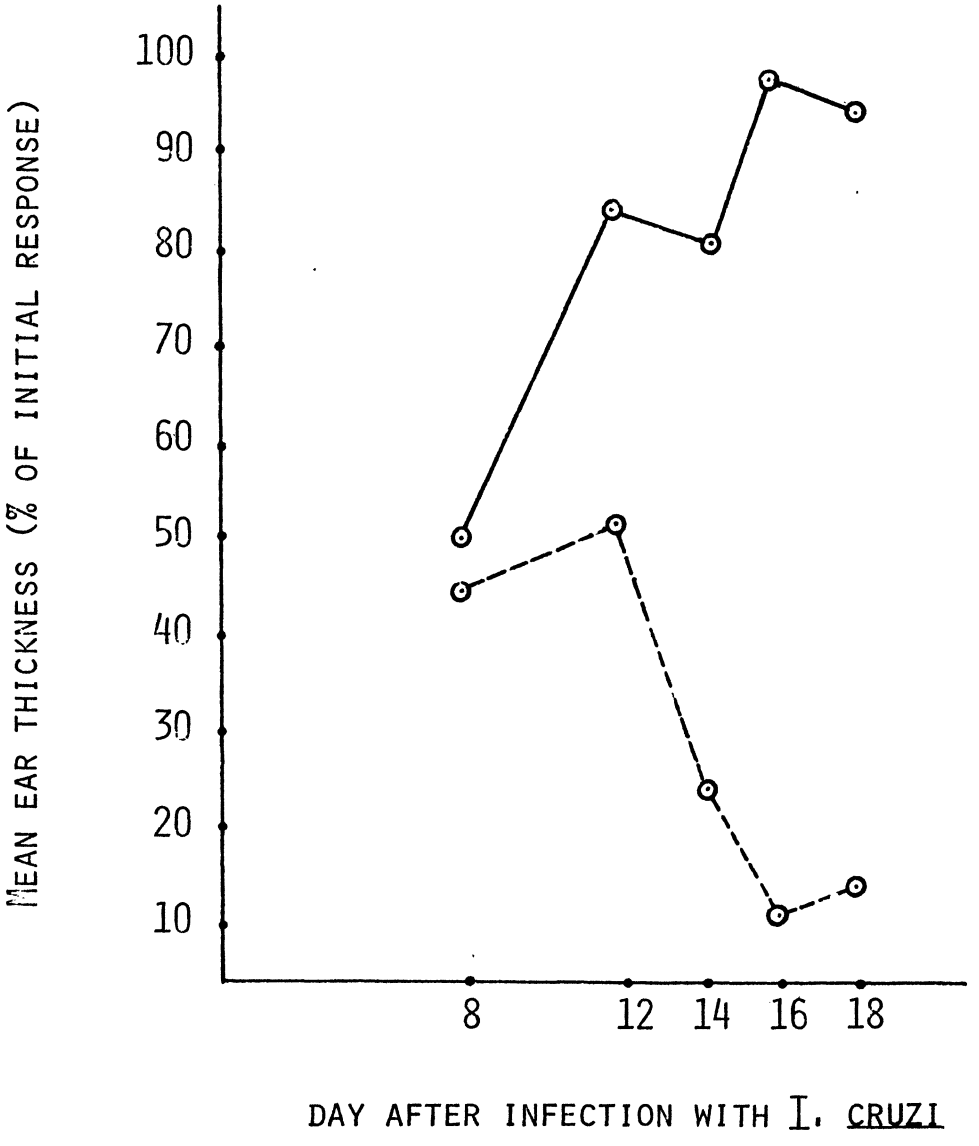

MEAN EAR THICKNESS (MM)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
8
12
16
18
DAY AFTER INFECTION WITH I. CRUZI

Fig. 9. Decreased ability of mice infected with T. cruzi to express delayed hypersensitivity to oxazolone. Each point represents the mean response of 8 mice. All mice were sensitive to oxazolone prior to infection.

Symbols: infected mice – – – – –

normal mice ————————

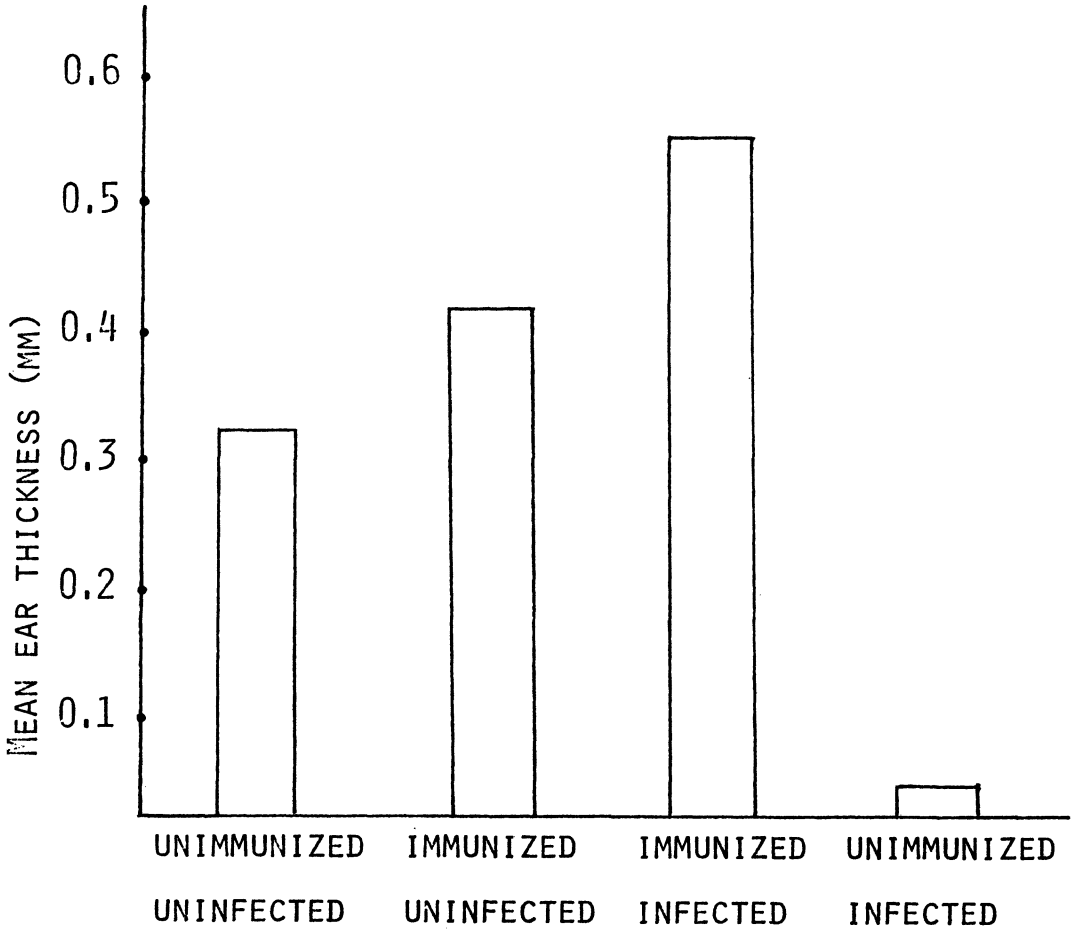

MEAN EAR THICKNESS (% OF INITIAL RESPONSE)
100
90
80
70
60
50
40
30
20
10
8
12
14
16
18
DAY AFTER INFECTION WITH I. CRUZI

after infection. Again, the 3 groups of mice infected the longest (14, 16 and 18 days) differed significantly in their responses from the uninfected mice.

Effects of immunization with attenuated T. cruzi on delayed hypersensitivity to oxazolone. Mice immunized with 2 doses of live attenuated T. cruzi 14 and 7 days prior to challenge with virulent trypanosomes developed greater sensitivity than did unimmunized animals (Fig. 10). Both groups of mice were infected 8 days prior to sensitization with oxazolone and tested for DH 16 days after infection. Mice immunized with the attenuated trypanosomes showed consistently higher levels of sensitivity to oxazolone than did normal mice. This state of increased responsiveness occurred in both challenged and unchallenged mice. The responses differed significantly only between the sensitized, infected animals and the other groups.

The cellular response in auricular lymph nodes of normal and infected mice sensitized with oxazolone. The medullary areas of the auricular lymph nodes from normal mice sensitized 3-4 days earlier by applying oxazolone to the ear showed a large number of lymphoblasts. These are large, pyroninophilic cells easily distinguished from unstimulated cells. Auricular nodes taken from unsensitized mice that had been infected with T. cruzi, 1 LD_{50} dose for 16 days,

Fig. 10. Effects of immunization with attenuated T. cruzi on delayed hypersensitivity to oxazolone in mice infected with T. cruzi. Each bar represents the mean response of 8 mice.


MEAN EAR THICKNESS (MM)
0.6
0.5
0.4
0.3
0.2
0.1
UNIMMUNIZED UNINFECTED
IMMUNIZED UNINFECTED
IMMUNIZED INFECTED
UNIMMUNIZED INFECTED

also had high numbers of large, pyroninophilic cells. However, in these animals, the occurance of these cells was noted in both the medullary and cortical areas. The nodes from infected animals are also enlarged, as are those from normal sensitized animals, due to the generalized adenopathy resulting from infection.

The nodes from infected, sensitized mice had a proportionally higher content of lymphoblasts than those from infected, unsensitized mice. These differences were apparent in the medulla, but the cortical areas showed little difference in the number of lymphoblasts (p. 71).

Transfer of Contact Sensitivity

Delayed hypersensitivity to oxazolone is readily transferrable between inbred mice (Table 9). Spleen cells were used in these studies, although better results may be often obtained using peritoneal exudate cells. Fewer cells are required if peritoneal exudate cells are used, and a 24 h delay from the time of transfer to the time of skin test is not necessary.

Lymphocyte populations were separated from spleens removed from mice sensitized 7 days earlier with oxazolone. The lymphocytes were divided into B-cell enriched and T-cell enriched populations by passage through a nylon wool column. Groups of syngeneic recipient mice received equal numbers of cells from either the T-cell or B-cell fraction or a

Table 9. Spleen Cell Transfer of Contact Sensitivity from Infected to Uninfected C57Bl/10 Mice.

Number of Donors[a]	Days Infected	Donor Response (mm)[b] $\bar{x} \pm SD$	Recipient Response (mm)[c] $\bar{x} \pm SD$	p[d]
0		0.60 ± 0.10	0.50 ± 0.07	>0.05
10		0.55 ± 0.10	0.45 ± 0.10	>0.05
14		0.28 ± 0.09	0.42 ± 0.11	$\leq$0.01
16		0.05 ± 0.05	0.40 ± 0.09	$\leq$0.01
17		0.05 ± 0.05	0.44 ± 0.12	$\leq$0.01

[a] sensitized with oxazolone 7 days before cell transfer

[b] oxazolone skin test immediately before transfer

[c] oxazolone applied at 24 h and skin tested at 48 h after receiving 3 x 10^7 spleen cells from infected, sensitized donors

[d] p value from Mann-Whitney significance test; donor vs. recipient

comparable number of a 50-50 mixture of the 2 fractions. The T-cell fraction was significantly more effective in transferring contact sensitivity than was the B-cell fraction. The mixture was intermediate in effectiveness.

Spleen cells from infected, sensitized donors were able to confer specific sensitivity upon normal recipients, regardless of the stage of infection of the donors. Sensitized mice that were unable to respond to eliciting doses of oxazolone possessed spleen cells capable of conferring sensitivity to oxazolone as effectively as cells of uninfected animals. No significant differences were seen in the responses of the recipient animals when donor cells from normal mice or mice at different stages of infection were used. (Table 10)

Transfer of normal mouse peritoneal exudate cells to mice sensitized with oxazolone and infected with T. cruzi

Mice infected 15 days previously with 1 LD_{50} dose of T. cruzi and sensitized to oxazolone on the eighth day following infection were given peritoneal exudate cells iv from normal mice immediately before skin testing on the fifteenth day. These mice were able to respond to oxazolone significantly better than infected, sensitized mice which did not receive peritoneal cells (Table 11).

Table 10. Transfer of Contact Sensitivity in C57Bl/10 Mice with Separated Spleen Cell Populations.

Population of Cells Received	Response (mm) of Recipients[a] $\bar{x} \pm SD$	Statistical Analysis Groups Compared	$p \leq 0.05$[e]
B[b]	0.21 ± 0.06	B vs. B + T	+
B + T[c]	0.38 ± 0.12	T vs. B + T	+
T[d]	0.46 ± 0.12	T vs. B	+
none	0.10 ± 0.02		

[a] 5 recipients/group, 1.25×10^7 cells/recipient; skin tested with oxazolone (1.5%) 24 h after cell transfer, read 48 h after cell transfer

[b] B-cell enriched; shown to be approx. 95% B-cells by immunofluorescence

[c] 50% B-cell fraction and 50% T-cell fraction

[d] T-cell enriched; shown to be approx. 80% T-cells by immunofluorescence

[e] Mann-Whitney significance test applied

Table 11. Restoration of Skin Test Response to Oxazolone by Transfer of Normal Peritoneal Exudate Cells to Mice Sensitized with Oxazolone and Infected with T. cruzi.

Treatment	Response (mm) $\bar{x} \pm SD$	p[b]
Peritoneal Exudate Cells[a]	0.28 ± 0.08	≤ 0.01
None	0.04 ± 0.05	

[a] 2×10^7 cells given iv at time of application of skin test dose of oxazolone

[b] the Mann-Whitney test was used to analyze the data

Transfer of spleen cells from mice sensitized with oxazolone to normal mice and infected mice

Mice infected for 16 days with 1 LD_{50} dose of T. cruzi responded significantly less to eliciting doses of oxazolone than did normal mice following transfer of spleen cells from uninfected sensitized donors (Table 12).

Lymph node cell transfer of contact sensitivity from infected to uninfected mice

Auricular lymph nodes from normal and infected mice which had been sensitized by application of oxazolone to both ears 3 days before, were removed and transferred to normal recipients. This experiment was done to ensure that sensitization was carried out during the stages of high parasitemia and greatest immunosuppression. Both infected and uninfected mice transferred contact sensitivity to normal recipients. No significant difference was observed between the two groups of recipients (Table 13).

Effects of LPS iv on Mice Infected with T. cruzi

Normal Swiss mice inoculated iv with 1.0, 0.5 or 0.1 µg of LPS displayed no visable effects (Table 14). However, many of the infected mice inoculated with the same amount of LPS died shortly after administration of this material. Deaths due to inoculation with LPS were first noted 9 days after the mice were infected with 1 LD_{50} dose of T. cruzi, and became more frequent as the

Table 12. Spleen Cell Transfer of Contact Sensitivity to Infected and Uninfected C57Bl/10 Mice.

	Skin test Response (mm) $\bar{x} \pm SD$	p[d]
Donors[a]	0.60 ± 0.10	
Recipients[b,c] infected	0.22 ± 0.05	<0.01
Recipients[b] uninfected	0.47 ± 0.06	<0.01
Infected Control	0.05 ± 0.05	<0.01

[a] uninfected mice sensitized with oxazolone 7 days before

[b] oxazolone applied 24 h and skin tested 48 h after receiving 3 x 10^7 spleen cells from sensitized donors

[c] peak parasitemia stage, 16 days following ip injection of 1 LD_{50} dose

[d] data analyzed with Mann-Whitney significance test; comparisons made to donor values. The recipient groups also differed significantly from each other at the 0.01 level.

Table 13. Lymph Node Cell Transfer of Contact Sensitivity from Infected to Uninfected C57Bl/10 Mice.

No. of Days Donors[a] Infected	Recipient Response $\bar{x} \pm SD$	p[b]
0	0.30 ± 0.08	>0.05
16[c]	0.26 ± 0.10	

[a] oxazolone (2%) applied to both ears three days prior to removal of auricular lymph nodes and cell transfer (5 mice/group)

[b] p value from Mann-Whitney significance test comparing recipient groups

[c] infected with 1×10^3 blood forms ip 16 days before cell transfer of 5×10^6 lymphocytes

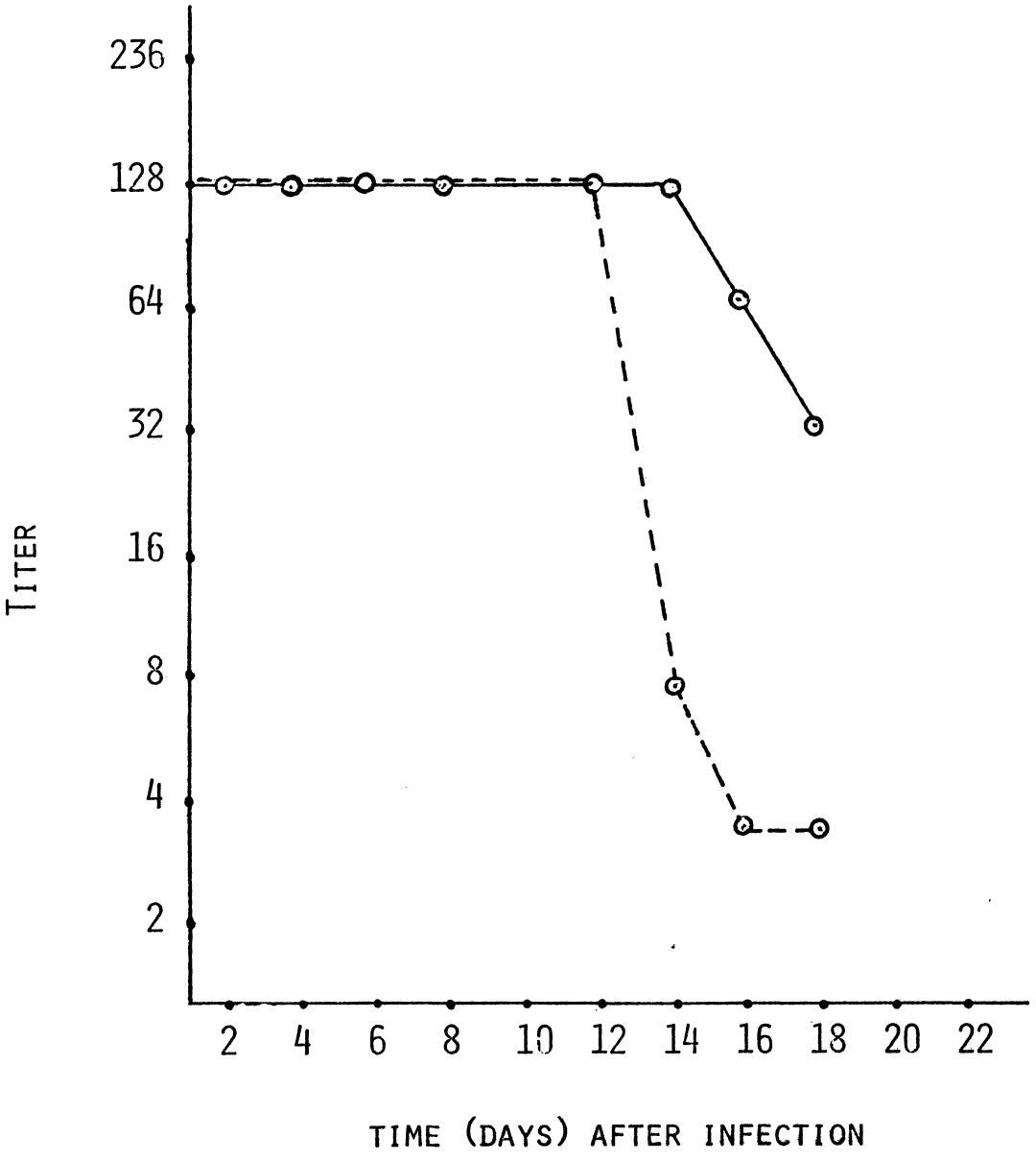
Table 14. Increased Susceptibility to Endotoxin in Mice Infected with Trypanosoma cruzi.

Number of Days Infected at Time of Endotoxin Administration	Number of Deaths/Total Following iv Injection of Endotoxin		
	0.1µg	0.5µg	1.0µg
6	0/4	0/4	0/4
9	0/4	2/4	0/4
12	0/4	2/4	2/4
14	1/4	2/4	1/4
16	2/4	4/4	4/4
18	4/4	4/4	4/4

infection progressed. Deaths were usually directly proportional to the number of days the animals had been infected and the amount of LPS administered.

Humoral Responses to SRBC and dLPS

Mice showed a decreased ability to produce antibodies against both SRBC and dLPS as the infection progressed (Fig. 11). Decreased production of antibodies against dLPS was first noted on the fourteenth day after infection, and was most marked just before death. On the other hand, antibodies against SRBC were not decreased until late in the course of infection.


236
128
64
32
16
8
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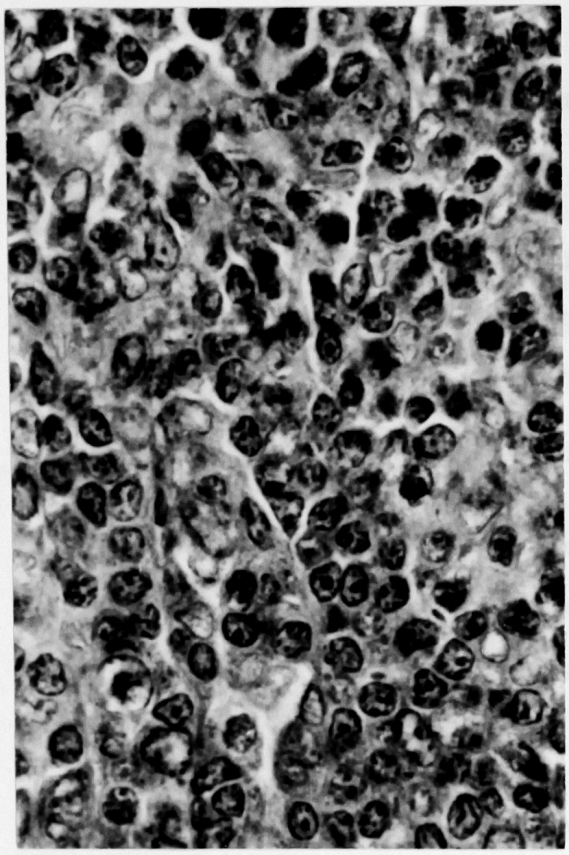
22
TIME (DAYS) AFTER INFECTION



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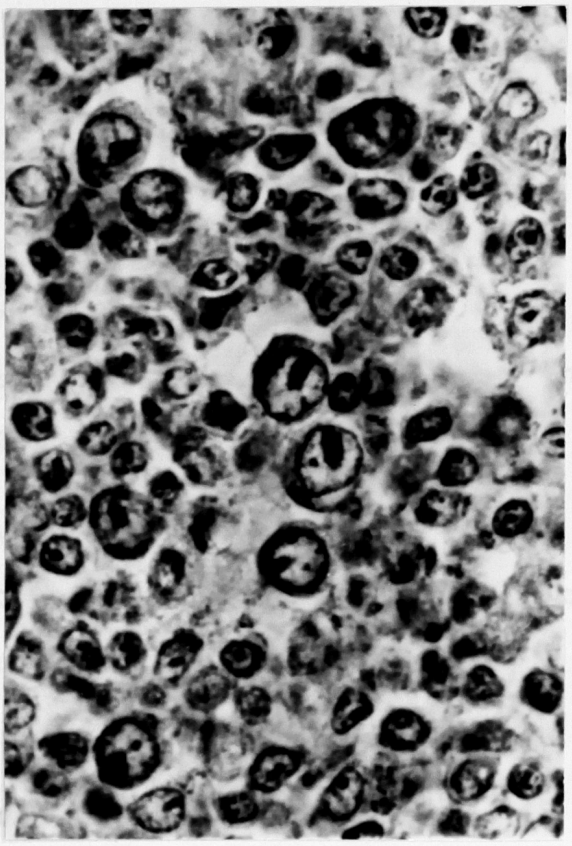

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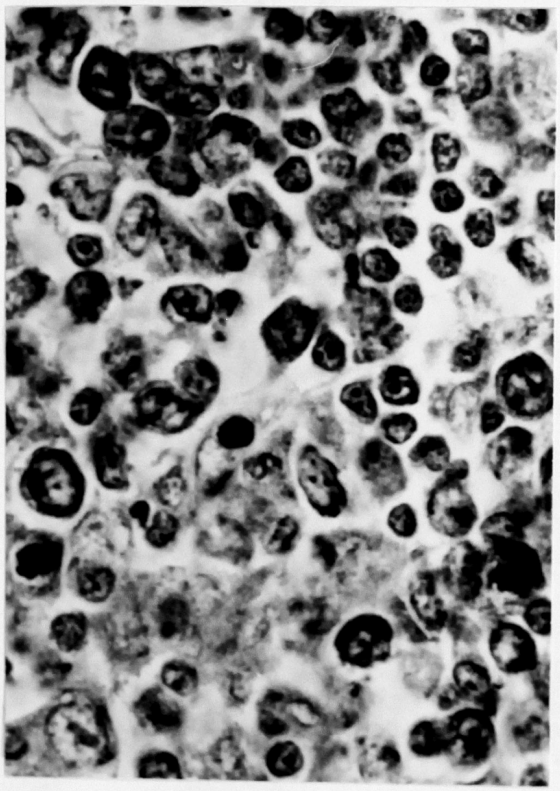

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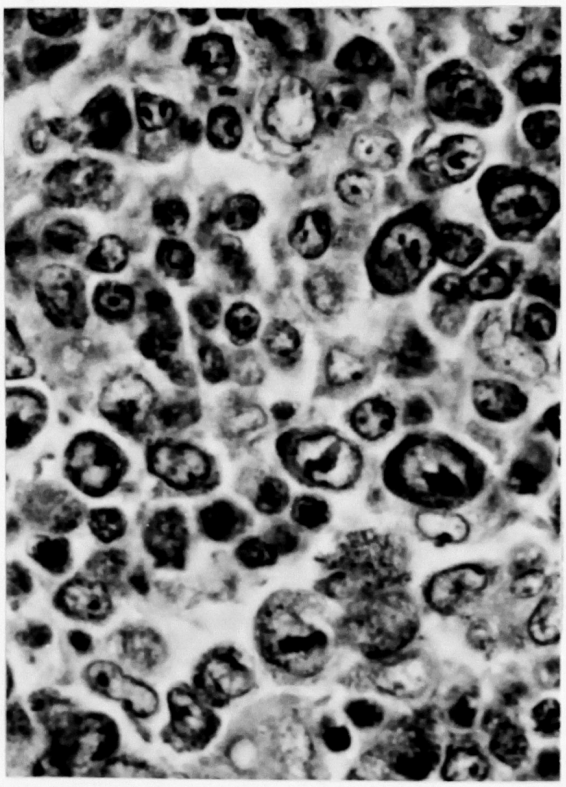

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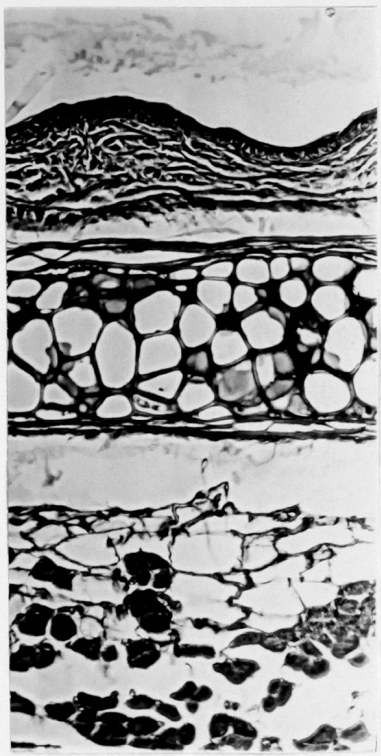
Illus. 5. Ear, normal mouse. hematoxylin and eosin (H & E) 224X.

Illus. 6. Ear, normal mouse sensitized to oxazolone. Mouse sensitized to oxazolone 8 days and skin tested 1 day prior to removal of ear. Note extensive infiltration of monocytic cells. H & E 224X.

Illus. 7. Ear, infected mouse sensitized to oxazolone. Mouse infected ip with 1 x 10^3 blood forms 16 days prior to removal of ear. Mouse was sensitized andskin tested as above. H & E 224X.

Illus. 8. Ear, infected mouse sensitized to oxazolone, with macrophages administered prior to skin test (Table 11). H & E 224X.

Illus. 9. Ear, oxazolone sensitized, monocyte infiltration. Typical cells of the sensitized ear. H & E 1400X.



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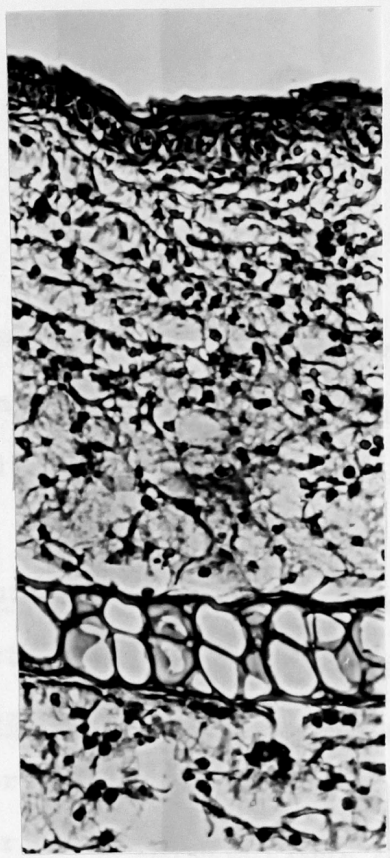

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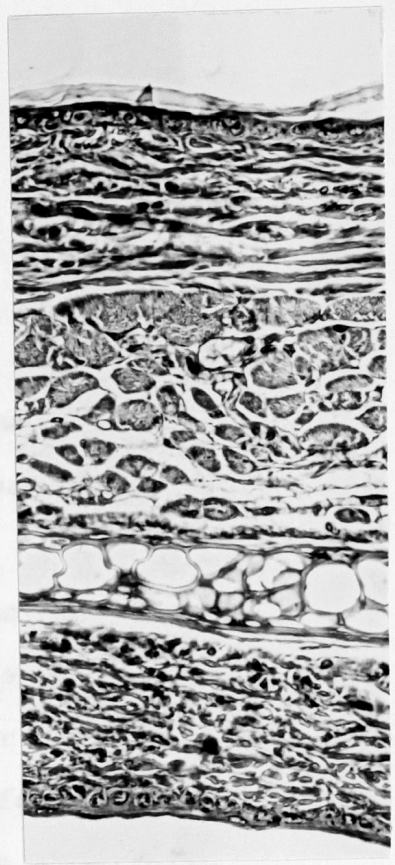

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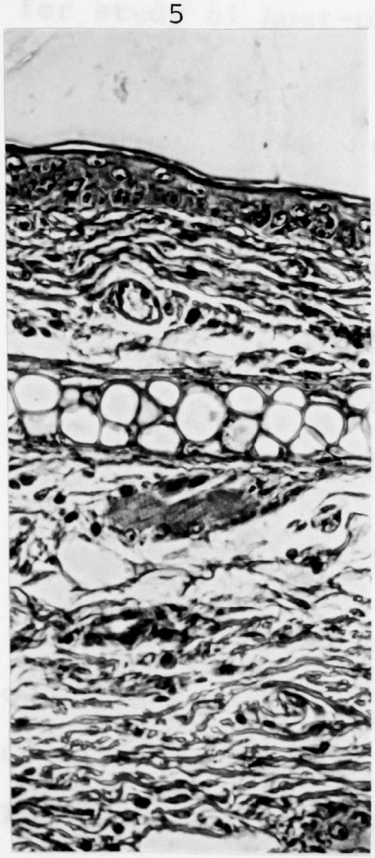

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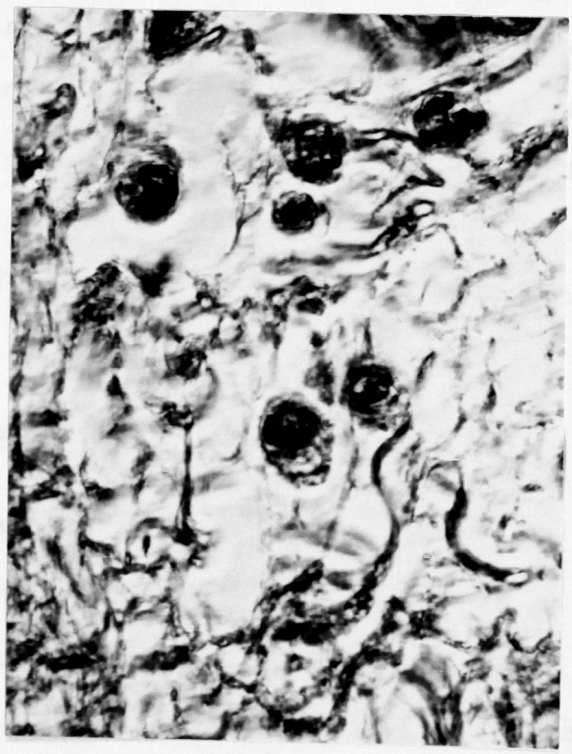

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Chapter 4

DISCUSSION

The results presented in this study demonstrate a number of interesting aspects of the immune responses occurring in mice with acute Chagas' disease. The information can be divided into two main areas: 1) studies on resistance to T. cruzi infection in the mouse, and 2) effects of acute infection on the immune responses of the mouse. Since T. cruzi is an intracellular parasite, infections with this organism provide an informative model for study of host-parasite interactions on a cellular level. In addition, the presence of virulent T. cruzi in a susceptible host affects the host's ability to mount an immune response to antigens unrelated to those of the parasite. Thus, studies of T. cruzi infection can be used to obtain specific knowledge of the immune system as well as specific information on the host-parasite relationship in this particular disease.

Immunization Studies

Both specific and non-specific immunization procedures were used in attempts to protect mice against virulent challenge with T. cruzi. Mice were immunized with live attenuated Mycobacterium bovis (BCG), a non-specific stimulator of the cellular immune response, or with

preparations of either live attenuated or killed attenuated T. cruzi. The parasites are attenuated by repeated passage in culture. These organisms are viable when introduced, but are not able to produce disease or persistent infection in mice.

Since the Tulahuen strain of T. cruzi is an intracellular parasite preferring cells of the reticuloendothelial system, it appeared likely that BCG, which activates phagocytic cells, might be useful for protecting mice against the intracellular trypanosomes. However, all reports (69) but one have indicated that this is not the case. In the present study, as in most others, BCG was of little or no value in protecting mice against virulent challenge with T. cruzi. This appears to be a rather unusual situation, for BCG has been proven successful in protecting against a wide variety of intracellular parasites, including many viruses and bacteria, as well as Leishmania (86) and Plasmodium (13) infections.

It is apparent that the route of immunization influences the effectiveness of BCG as a protecting agent. Clark et al. (13) have reported successful immunization with BCG only if administered iv for protection of experimental animals against malaria. Similarly, Ortiz-Ortiz (19) reported successful immunization utilizing iv

administration of BCG prior to challenge of mice with virulent T. cruzi. In the present study, an attempt to repeat this experiment was less successful than that reported by Ortiz-Ortiz et al. This may be due to differences in virulence of the strain used. However, in the present study, mice receiving iv treatment with BCG did survive significantly longer than unimmunized controls, although all but one of the immunized mice died from the parasitic infection. Kuhn et al. (54) have reported complete lack of protection against challenge with the virulent Brazilian strain in mice pretreated with high doses (3 mg wet weight) of BCG iv. In Kuhn's experiments, similar numbers of trypanosomes were used for challenge as in the experiments reported here.

In contrast to the slight degree of protection seen in mice immunized with BCG iv, none was seen in mice which received BCG ip. This is in agreement with the results reported by Hoff (42) who found that macrophages from mice injected ip with BCG were able to destroy the trypanosomes in vitro, but that protection was not seen. Hoff suggests that, although BCG may non-specifically activate macrophages to increase their phagocytic and cytolytic capabilities, specific stimulation may be required in vivo to produce a population of lymphocytes directed against the parasites.

Mackaness (58) has shown that specific immunological mechanisms may activate macrophages, but once they are activated they show an increased ability to engulf and kill organisms unrelated antigenically to the original immunogen. Organisms such as BCG, Corynebacterium parvum, or Listeria monocytogenes are known to non-specifically activate macrophages (2, 35, 87). Animals stimulated immunologically by these agents are more resistant to infection with many bacteria, viruses, and tumors. Treatment of mice with C. parvum has been shown to be effective in protecting them against malaria infections, even when the immunizing organisms were first killed (68).

The only proven method of obtaining consistent protection against T. cruzi is by injection of specific T. cruzi antigen, preferably in the form of live avirulent organisms. In addition to attenuated culture forms shown to be effective in this study, avirulent strains obtained from non-human hosts may be used (42). Parasites treated with irradiation and, more recently, those killed with sodium perchlorate have been shown to be of protective value (48, 49). It should be remembered that studies of this type may not always be reliable due to the tremendous variation in the virulence of parasites in any given host species.

In this study, it was repeatedly demonstrated that treatment of mice with one or two doses of attenuated culture forms rendered mice resistant to challenge with one LD_{50} dose of virulent trypanosomes, provided that the antigen was given sufficiently ahead of challenge (Table 1). Mice given this treatment 18 days prior to challenge were completely protected, but were not if given the antigen only seven days before challenge. It is suggested that cell-mediated immunity is involved in this protection. Culture forms killed by freeze-thawing failed to confer protection (Table 3). With the single exception noted above, nearly all attempts to immunize mice with killed trypanosomes have been unsuccessful. In general, cell-mediated immunity is best produced by administering a preparation of live, attenuated organisms as opposed to killed preparations. Even though the attenuated trypanosomes do not persist after introduction into mice, they cause a slight and transient parasitemia, and provide a more prolonged antigenic stimulation. A similar effect may be achieved by the use of certain adjuvants combined with specific trypanosome antigen (44), but killed preparations alone are generally ineffective.

An interesting aspect of the immunization experiments was the observation that mice treated with BCG

prior to the administration of live culture forms were not completely protected against virulent challenge. In contrast to this, mice which were not first treated with BCG were always protected after immunization with live culture forms. This phenomenon has since been verified by Kiersenbaum (48) who found that mice pre-treated with C. parvum were not completely protected by a preparation of T. cruzi antigen proven effective in normal mice. It may be that non-specific activation of macrophages is not a useful procedure for increasing in vivo killing of T. cruzi, and, in fact, may even be deleterious. As pointed out previously, Hoff (42) has found non-specifically activated macrophages to be more effective in vitro in destroying T. cruzi. However, in our laboratory we have seen consistently that mice immunized with BCG are not protected against challenge with T. cruzi, and often die more rapidly. It might be postulated that, in vivo, macrophages are capable of taking up the parasite, but cannot destroy it. Since the organism is an intracellular one, activated macrophages may simply be more accommodating in providing an environment in which the parasites can divide. This argument is not supported by the in vitro studies, which show that the activated macrophages are capable of killing the parasites after ingestion. Perhaps

in vivo other factors are important. One possible explanation might be directed toward the T-cell population, with the postulation of a group of suppressor T-cells. Such cells are seen in animals with tumors or immunodepressed aged animals (82). However, evidence for their presence in this instance is quite weak, especially in view of all the instances in which BCG provides nonspecific protection. It may be simply that macrophages can more easily engulf parasites in an in vitro situation, perhaps due to artificially concentrating the phagocytes on a plastic surface enabling them to trap and break down the parasite more effectively.

A decrease in the ability of the attenuated trypanosomes to protect mice immunized previously with BCG may be explained on the basis of reticuloendothelial stimulation by the BCG. Perhaps administration of BCG stimulates the macrophages to kill and degrade the immunizing trypanosomes. This could prevent production of specific cell-mediated immunity by the attenuated trypanosomes since they may not persist as long in the BCG-stimulated host.

The effects of treating mice with BCG and attenuated trypanosomes can be seen in Table 1. Of the groups of mice that received both organisms, only those mice in

group one, which had two injections of each preparation, survived totally. It appears that in this case, the effect of two doses of trypanosomes overcame any adverse effect due to the BCG. The only other groups in which all survived were those that received only attenuated trypanosomes, either 18 or 18 and 7 days beofre challenge. A single immunizing dose of trypanosomes given less than 12-14 days prior to challenge is not effective in providing complete protection. Groups of mice that received immunizing doses of trypanosomes sufficiently ahead of challenge, but which were previously treated with BCG, showed only about 50 per cent survival. BCG alone, given ip, did not significantly increase numbers of survivors or survival time, although two doses were consistently more effective than one. When BCG was given iv, somewhat better results were obtained as far as increasing survival time. Mice given two iv injections of BCG 11 and 1 day prior to challenge survived significantly longer than their unimmunized counterparts (Table 2). This was consistent when challenge was with either two or fifty LD_{50} doses. In addition, a single iv injection of BCG given 11 days before challenge increased survival time significantly.

These results indicate that non-specifically activated macrophages may be able to confer at least partial

protection. Perhaps iv administration of the BCG allows for better contact of the organisms with the phagocytic cells of the liver, spleen and lungs, the most important organs of the reticuloendothelial system. Activation of cells in these areas may be useful against the circulating blood forms, providing increased contact between activated host cells and the parasite. It should be noted, however, that increased survival time does not always indicate protection. The mice treated with BCG nearly all died, averaging about eight days longer survival than the unimmunized groups.

Not only was a live vaccine preparation found to be effective in producing immunity, but, as indicated previously, a minimum time of 12 to 14 days was necessary between immunization and challenge for the preparation to be effective. This time period corresponds to that required for macrophage activation following a primary stimulus (70). In most experiments a single immunizing dose protected as effectively as two.

Further evidence of the importance of the macrophage in producing resistance was seen in experiments in which silicon dioxide, a macrophage toxin, was used in conjunction with infection with T. cruzi. The results generally agreed with those obtained by Kiersenbaum et al. (47) who found that depression of reticuloendothelial

activity by silica resulted in decreased resistance to infection with T. cruzi. In addition, these workers reported that stimulation of the reticuloendothelial system (RES) by diethylstilbesterol before infection reduced both mortality rates and levels of parasitemia.

An interesting situation exists in this system, since the Tulahuen strain of T. cruzi multiplies within macrophages. It might therefore be expected that selective killing of macrophages prior to infection could actually lead to a lowered parasitemia by decreasing the number of available cells in which the parasites can divide. Initially we believed this was the case, since there were often more survivors in those groups receiving the highest doses of silica. However, repeated experimentation with varying doses of silica and timesof administration proved these instances to be isolated and inconsistent in occurrence. Overall, the data indicated a significantly longer survival time in the untreated mice.

Some of the inconsistencies observed with macrophage activation studies and resistance of animals to T. cruzi infection may be due to the preference of many strains for cells of the RES. Studies of macrophage activation with BCG may be complicated by the involvement of two intracellular organisms trying to inhabit the same

population of cells. Data using non-living RES activators and depressors may be more useful in determining the roles of the RES in resistance against T. cruzi infection.

Kuhn and Durum (53) have shown with adoptive transfer experiments that the onset of the ability of mice infected with T. cruzi to transfer immune protection via lymphocytes occurs about nine days after infection. Recipients of spleen cells from infected donors were resistant to subsequent challenge given three hours after the cells. It was noted that high parasitemias did not correlate with survival times, since the passively immunized mice survived infection but developed high parasite levels. This immunity appears to differ from that actively conferred with attenuated trypanosomes as described in these experiments, for high parasitemias did not develop following challenge of actively immunized mice.

To explain their observations, Kuhn and Durum (53) postulated the presence of a specific antibody response to a particular product of the trypanosome that may be important in immunity. Koeberle (51) has postulated that trypanosomes invade cells by secreting a lytic enzyme. An antibody to such a product was suggested by Kuhn and Durum to explain the disparity between high parasitemias and survival of adoptively immunized mice. They reasoned that circulating antibody to such a factor could limit

cell invasion and cause an accumulation of parasites in the blood, resulting in "increased longevity and an increase in parasitemia in these immune manipulated mice." They suggested that increased longevity could be due to decreased tissue damage since fewer cells are invaded and destroyed. The weakness of this argument, aside from the fact that no such lytic product or antibody to such a product has been found, is that T. cruzi has never been observed to multiply outside of host cells. Since Kuhn and Durum observed an increase of over 100 fold in the number of parasites from the challenge dose to the peak of parasitemia, cell invasion apparently occurs. Also, there has been consistent failure in attempts to transfer protection with serum from infected animals. In summary, there have been a number of indications that parasitemia levels are not good indices of pathological state. There apparently is more than the activated macrophage involved in the immune status of the experimental animal, since the phagocytes would seemingly tend to decrease the numbers of extracellular forms rather than to allow them to increase. However, in the present study involving protection with live, attenuated organisms, specifically activated macrophages probably play a very important role in protection. High numbers of parasites did not develop in immunized mice, and macrophages from acutely ill animals were observed to appear activated when cultured in vitro.

This observation indicates that the RES may be stimulated during acute Chagas' disease. Peritoneal exudate cells removed from mice 15 days after infection with one LD_{50} dose appeared larger than peritoneal exudate cells from normal animals, with more and larger pseudopodia (personal observation). Infected mice show an increased ability to clear colloidal carbon from the blood stream (Fig. 1, Table 5). Similar reports have been made by Clinton et al. (15). Associating increased carbon clearance with an increase in the activity of individual phagocytes may not be entirely correct. This study shows that a dramatic increase in spleen size occurs in infected animals, beginning on about day seven or eight after infection with one LD_{50} dose (Fig. 2, Table 6). Increased carbon clearance could be merely a function of the relatively huge spleen possessed by these mice. The rate of carbon clearance and course of changes in spleen size in immunized, infected animals is not known. This information might be very useful in interpreting the mode of action of the live trypanosome vaccine.

Of possible significance along these lines are the observations by Grogan (33) that treating animals with anti-thymocyte serum (ATS) did not decrease their phagocytic activity as measured by carbon clearance. This indicates that immunosuppressed animals can show normal

abilities to phagocytose, a situation somewhat analogous to that existing in mice acutely infected with T. cruzi.

Immunosuppression in Experimental Chagas' Disease

Studies were undertaken to determine the effects of acute infection with T. cruzi on the immune response of mice to antigens unrelated to those of the parasite. It was found that acute parasitemia was accompanied by pronounced alterations in the immune responses of the unprotected host. In all cases, there were direct correlations between the degree of parasitemia and the degree of immunosuppression. Responses in mice to various antigens were found to be decreased as the infection progressed. Various physical changes that accompanied the infection were also studied.

Within eight days following ip challenge with one LD_{50} dose of virulent trypanosomes, the organisms could be seen in the peripheral circulation. After that time, the numbers rose logarithmically until death of the host on about the eighteenth day following infection (Fig. 3, Table 7). Accompanying this rise in parasitemia was a corresponding increase in spleen size (Fig. 2, Table 6).

Alterations in blood counts were noted in mice as the infection progresses. Marked anemia occurred by the twelfth day of infection.

Leucopenia was noted by the sixteenth day after infection. Differential counts indicated that the major populations of white blood cells were depressed, without one population being severely depleted over the others. This would correspond to the observations of Taliaferro and Pizzi (90) who noted lymphocyte depletion in the spleen. It does not appear that the observed immunosuppression can be wholly attributed to lymphocyte depletion, especially since infected animals have been shown to mount a good immune response against T. cruzi antigens (99). Also, a good inflammatory response was produced in infected mice following application of an alive oil-turpentine mixture (Table 8). Measurable induration and erythema appeared in normal and acutely infected mice by 24 hours after application of the irritant. This suggests that the observed suppression of response to certain antigens involves a rather specific mechanism directed against cells of the immune system rather than a non-specific depression of white blood cell populations. The slightly lowered inflammatory response in infected animals is probably due to the decrease in circulating leukocytes that accompanies infection.

Mice showed a decrease in their primary response to SRBC and LPS during the terminal stages of infection when parasitemia was at its peak. The antibody levels were

measured by passive hemagglutination procedures. It would probably have been better to use a more sensitive method such as assaying for numbers of plaque-forming cells, so that sharper distinctions between antibody levels could have been made.

Depression of the immune response to heterologous erythrocytes has been noted before in animals with parasitic infections. Clinton (15) observed a depression of antibody to burro erythrocytes in mice during the acute stages of infection with a "myo- and reticulotropic strain of T. cruzi." Numbers of plaque-forming cells were assayed during the primary response to the erythrocytes. Their results closely paralleled those obtained in this study. In both studies, it was found that infected mice not given erythrocytes had negligible amounts of non-specific agglutinins in their serum.

Perhaps the parasitic disease most often associated with immunosuppression is malaria. Wedderburn (103) observed a reduced number of hemolytic plaque-forming cells in the spleens of mice infected with Plasmodium berghei yoelli and given SRBC. These animals were infected with a non-pathogenic Plasmodium that produces a high parasitemia but is a self-limiting disease. It is probable that a different mechanism is at work in this system than in acute Chagas' disease, a rapidly fatal

infection. Also, the malaria parasite infects red blood cells rather than cells of the reticuloendothelial system. However, like Chagas' disease, the peak of immunosuppression corresponds to the peak of parasitemia.

Malarial-induced immunosuppression has been demonstrated using a variety of antigens including heterologous RBC (5, 29, 30, 77, 83), human gamma globulin (29), Salmonella typhimurium (45), and certain viruses (77). However, responses to human serum albumin, Keyhole limpet hemocyanin (KLH), and bacteriophage ϕ x 174 are reportedly normal in mice with malarial infections (5, 29, 30, 101).

These results indicate that responses to T-cell independent antigens are normal, while those to T-cell dependent antigens are depressed. Presumably, many of the T-cell independent antigens, such as bacteriophage, KLH, and LPS, do not require macrophage processing or T-cell cooperation for antibodies to be produced. In contrast, T-cell dependent antigens, such as erythrocytes, do require macrophage processing and T-cell-B-cell interaction for the production of antibodies. These findings concerning malarial immunosuppression suggest a defective immune mechanism at the T-cell or macrophage level. Further, it has been noted that P. berghei infection has no suppressive effect on an already established response to SRBC, and that malarial antibodies are produced at the time of

maximal immunosuppression. These findings suggest that there is not a non-specific inhibition of immunoglobulin synthesis (29, 30).

No other reports of the effects of infection with T. cruzi on antibody response to T-cell independent antigens are available. This study indicates that the response to dLPS, an antigen not requiring T-cell processing (102), is depressed during infection. Here again it would be of interest to perform more sensitive tests for antibody levels.

Although decreased response to SRBC may indicate a T-cell or macrophage involvement, a reduced response to dLPS cannot be explained in these terms. It does not seem likely that a decrease in immunoglobulin synthesis occurs since trypanosomal antibodies are produced during acute infection. However, lymphocyte depletion in the spleen and lymph nodes does occur. Perhaps this prevents de novo immunoglobulin synthesis to an antigen administered when the trypanosomal antigen is already present in high amounts.

An interesting observation was made by Perkins and Makinodan (71) who have shown in vitro that an excess of macrophages has an inhibitory effect on the SRBC response. Presumably, increased macrophage activity removes the antigen making it less accessible to the lymphocyte

populations. However, it would seem that increased uptake of antigen processing by macrophages is required for antibody production against erythrocytes. Nevertheless, consistent observations associate increased macrophage activity with decreased antibody production. As mentioned earlier, mice with acute T. cruzi infection appear to have activated macrophages. Perhaps increased phagocytosis of parasites affects antibody production to SRBC in infected mice. However, such an explanation is not sufficient to explain a decreased response to dLPS if macrophage processing is not required by plasma cells to produce antibodies against endotoxin.

This study demonstrates that a significant depression of the cellular immune response develops in mice suffering from acute infection with T. cruzi. Groups of mice were immunized with CFA and challenged with T. cruzi on the same day or on the fourth or ninth day after immunization. Animals tested five days after infection responded as well as did the normal sensitized mice but animals tested 14 days after infection with T. cruzi had markedly reduced delayed hypersensitivity reactions. Animals sensitized with CFA on the fourth day after infection and tested on the eighteenth day of infection failed to express delayed hypersensitivity. It is

evident that as the disease progresses the animals have less ability to express delayed hypersensitivity.

Studies of induction of delayed hypersensitivity to oxazolone in mice infected with T. cruzi appeared to indicate an inability to develop a delayed response as the disease progressed. This interpretation may be incorrect, however, since the tests for delayed hypersensitivity were performed at least eight days after the animals were sensitized. This prevented sensitization past the tenth day of the eighteen day duration of the disease. The disease had progressed to a marked extent by the time of skin test in the animals sensitized four or eight days after infection.

Animals sensitized with oxazolone, skin tested, and infected two days later, were skin tested again at various days after infection. By the twelfth or fourteenth day of infection the animals were unable to express a delayed response. These data indicate that the efferent rather than the afferent arm of the response is suppressed.

Immunosuppression during acute Chagas' disease has been previously reported. Clinton et al. (15) observed a lowered antibody response to burro erythrocytes in mice infected with a "myo- and reticulo-tropic strain" of T. cruzi. Observations made by these workers, also supported by our own observations, indicated that

an increased phagocytic and cytolytic activity of macrophages occurred in infected animals.

Many other protozoal infections are associated with immunosuppressive effects. Mansfield and Wallace (59) noted a decreased response to CFA in rabbits chronically infected with T. congolense. The rabbits were immunized with CFA after infection in these experiments. Decreased antibody production to sheep red blood cells occurred in mice infected with Plasmodium berghei yoelii (29), but their response to oxazolone was not affected, even during peak parasitemia. In this instance, mice develop an acute but non fatal disease. Other workers have observed immunosuppression in malarial infections (31, 77), including depression of certain antibody responses in children with malaria. A diminished antibody response to ovalbumin in hamsters infected with Leishmania donovani has been observed by Clinton et al (14).

Inasmuch as oxazolone sensitivity is an indicator of a cell-mediated response (4, 96, 97), the failure of animals to respond to eliciting doses of this substance indicates an involvement of the macrophage and/or the thymus-dependent lymphocyte populations. The histological appearance of contact sensitivity reactions is characterized by an abundance of these cell types (97). Spleen damage and lymphocyte depletion is found in mice infected

with T. cruzi (90, 92) and depressed lymphoid cell populations have been reported in animals with Toxoplasma gondii infections (39, 43). Thus lymphocyte depletion may be the key to the observed immunosuppressive effects in acute Chagas' disease. However, if it is the efferent arm of the response that is depressed, as these results indicate, alteration of macrophage function may be responsible for immunosuppression.

The Tulahuen strain of T. cruzi is reticulotropic, showing a preference for macrophages as the host cell. Although the macrophage may be functioning normally in phagocytosis (15), harboring the parasite might in some way interfere with the receptor sites on the cell surface that interact with T-lymphocytes in cellular responses. The T-lymphocyte itself may be the key to the immunosuppressive effects, since this cell is involved in cellular responses and is required for production of antibodies to erythrocytes. However, a similar argument may be made for implicating the macrophage as the primary cell involved. Infection of the macrophage may affect the macrophage-T-cell interaction, or might interfere with processing of the test antigen by the macrophage.

Although the response to oxazolone in mice acutely infected with malaria is not depressed, there is evidence that cell-mediated immunity may be depressed in such

animals. Sengers et al., (83), observed that infected mice were unable to develop cell-mediated immunity against a skin heterograft. Observations of this type of immunosuppression in malaria is rare, however. It seems that there are different mechanisms involved in malarial and American trypanosomal-induced immunosuppression.

Loose et al. (56) has called malarial immunosuppression a macrophage mediated defect. This suggestion is based on the observation that macrophages from infected mice showed decreased capability to invoke antibody formation when transferred to syngeneic recipients. The macrophages were first allowed to phagocytose heterologous erythrocytes in vitro, then passed to normal and infected recipients. Macrophages from mice with malaria were apparently less effective at processing and/or presenting the antigen for the production of an immune response. The authors concluded that malaria was immunosuppressive through action on the macrophage by interfering with antigen processing. This would indicate that the inductive phase of the immune response is interrupted, in that the T-cells are not stimulated by macrophage-associated antigen.

To determine whether the depressed cellular response during T. cruzi infection was directed toward the induction or expression of delayed hypersensitivity, several experiments were done using oxazolone as the test antigen.

Oxazolone is a hapten that binds to epithelial proteins to become antigenic. It invokes a good cell-mediated response when applied to the skin and is convenient for rapid sensitization and easy elicitation.

Administration of oxazolone to the skin produces rapid proliferation of lymphocytes in draining lymph nodes (97). This process is part of the induction of a cellular immune response. Three to four days following sensitization with oxazolone, draining lymph nodes contain large numbers of lymphoblasts. These are relatively large cells, rich in RNA. These cells are able to transfer sensitivity to oxazolone when injected into syngeneic animals (4). Several experiments were done in the present study to determine whether lymphoblasts were formed following oxazolone administration in an infected mouse. Since formation of these lymphoblasts following antigenic stimulation indicates the induction of an immune response, their presence or absence in an infected animal would indicate whether or not this phase of the response was being interfered with.

Quantification of lymphoblasts in sensitized infected mice is difficult, since the infectious process alone leads to adenopathy and increased numbers of large dividing cells in the lymph nodes. However, by comparing draining lymph nodes from infected mice, both sensitized with

oxazolone and unsensitized, it was found that there were proportionally more lymphoblasts in the sensitized animals. Furthermore, cells from draining nodes of sensitized infected mice were able to transfer contact sensitivity to normal mice (Table 13).

It is also of interest to note that in infected animals not sensitized with oxazolone, lymphoblast-like cells appear in both the cortical and paracortical regions. However in mice sensitized with oxazolone, the paracortical areas and not the cortex show large numbers of lymphoblasts. This observation made it easier to assess the induction of contact sensitivity in infected mice. The results obtained from histological observations and cell transfer studies indicate that induction of contact sensitivity does occur in infected mice, as measured by the ability of lymphocytes to become sensitized following administration of oxazolone. No experiments of this nature have been reported in association with immunosuppression in other diseases. It would be of interest to determine whether induction of the immune response occurs in other parasitic diseases that lead to immunosuppression.

To further examine whether the afferent or efferent arm of the immune response was interfered with, a series of cell transfer studies was undertaken.

A primary criterion for the existence of cell-mediated immunity is whether or not the immunity or sensitivity can be passively transfered with sensitized T-lymphocytes. To demonstrate the nature of the response to oxazolone, transfers of different populations of lymphocytes from sensitized to normal mice were made (Table 10). By using a nylon wool column, spleen cells from sensitized mice were divided into three groups; a B-cell enriched population, a T-cell enriched population, and a mixture of the two. Macrophages are nearly all eliminated from the preparation by the nylon column. The three preparations were injected into syngeneic recipients which were skin tested 48 hours later and read 24 hours after skin test. The results confirmed the cell-mediated nature of the response to oxazolone. The T-cell enriched populations transferred sensitivity much more effectively than the B-cell enriched populations.

Asherson & Ptak (4) showed that contact sensitivity was more effectively transferred with peritoneal exudate cells than with lymph node cells, although both types were effective. This observation has been confirmed in our laboratory. This is probably due to the presence of large numbers of macrophages in the peritoneal cell preparations. The macrophages are already primed to the oxazolone, so when they are transferred along with sensitized T-cells, all the requirements for expressing a

delayed hypersensitivity response are met. If only the lymphocytes are transferred, it is best to wait 24 to 48 hours before skin testing to allow the sensitized T-cells to interact with the recipient's macrophages. It cannot be reasonably argued that this delay produces active sensitization rather than passive transfer, for at least four to five days are required to bring about active sensitization to oxazolone (97)

Contact sensitivity was readily transferred from infected to normal mice, even though the infected animals were unable to respond to the agent. The sensitivity was successfully transferred even when the donor mice were in the final days of infection, at the height of immunosuppression. These experiments indicate that the infected mice, although unable to respond to oxazolone, have sensitized T-cells.

One shortcoming of the spleen cell transfer experiments results from the limited number of days animals can be sensitized with oxazolone following infection. Since the mice usually die by eighteen days after infection, and because about six days are required to obtain sensitized spleen cells following oxazolone application, it is not possible to sensitize animals in this manner when they are in the peak infection period. Oxazolone must be applied to the abdomen by the eleventh or twelfth day

after infection to allow the spleen cells to become sensitized before the mice succumb. As seen earlier, the peak period of immunosuppression occurs between days 14-18.

To overcome this problem, the experiment mentioned earlier was performed. Mice were sensitized by applying oxazolone to both ears during the period of peak parasitemia (Table 13). The successful transfer of sensitivity with lymph node cells indicates that mice are able to become sensitized to oxazolone during the height of immunosuppression.

Based on these observations, it was postulated that the immunosuppression might be due to a macrophage defect. The first approach to examine this possibility was to administer normal peritoneal exudate cells, which had been adhered to glass, to infected sensitized mice immediately before applying the eliciting dose of oxazolone to the ear. The addition of normal macrophages to infected animals prior to skin testing improved their response significantly. This indicates that perhaps a deficiency of available macrophages in the infected mice could explain the immunosuppression. Since there doesn't seem to be a significant drop in any single circulating leukocyte population during acute infection (Fig. 6), and there is an increased clearance rate of colloidal carbon in infected

mice (Table 5), a decrease in macrophage number is probably not responsible. However, the macrophages may be unable to respond to an antigen such as oxazolone, possibly due to the large numbers that are infected with parasites. The presence of many parasites, both inside and outside of macrophages, may interfere with the ability of the macrophage to recognize and respond to another antigen. Evidence against this possibility comes from the observation that mice immunized with attenuated trypanosomes and infected with virulent forms respond well to oxazolone (Fig. 10). 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 does not occur when specifically immunized mice are infected. It could be the intracellular presence of the virulent parasites that is chiefly responsible for the failures of the immune response to other antigens.

Potentiation of Bacterial Lipopolysaccharide (LPS) Toxicity in Mice Infected with T. cruzi

When studying the antibody response to bacterial LPS in infected mice, it was noted that many of the animals died suddenly, often within fifteen minutes, after receiving an iv injection of the endotoxin. Subsequently, detoxified endotoxin was used for antibody assays. The

degree to which the toxicity of LPS was potentiated was quantitated in further experiments.

Mice in the acute stages of infection with T. cruzi were found to die of what appeared to be toxemia or endotoxin shock when given iv injections of very small amounts of LPS. Death generally occurred within twelve hours or less after the mice received the endotoxin. In mice near the terminal stages of infection, deaths occurred in one hour or less. In general, mice infected the longest were most susceptible to injection of endotoxin (Table 14). Whereas 200 µg or more are usually required to kill a normal RML adult mouse (personal observation), as little as 0.1 µg was toxic to acutely infected mice.

It is difficult to suggest a mechanism to explain the potentiation of LPS toxicity, since the mode of action of LPS is not clearly understood. Filkens (23) observed that sonicates of rat peritoneal, alveolar, and hepatic macrophages detoxified endotoxin from Salmonella enteriditis, and he suggested that macrophage lysosomes were important in the detoxification of LPS. Evans and Alexander (21) have reported that endotoxin activates peritoneal exudate cells so that they non-specifically kill target cells. In addition, Gery and Waksman (24) have reported that macrophages treated with endotoxin release a soluble factor that stimulates T-lymphocytes. It is apparent that

LPS effects macrophages, which in turn can presumably detoxify LPS.

Studies on the relationship between endotoxin and the RES have indicated that the liver and spleen are the major organs responsible for the removal of endotoxin from the blood (20) and its subsequent detoxification (94). Stimulation of the RES by BCG or glucan has been shown to increase sensitivity to endotoxin (18, 89). In addition, Loose et al., (55) found increased toxicity of endotoxin in mice infected with Plasmodium berghei: a 41-fold increase was reported. They gave iv injections of from 200-500 μg of endotoxin, much higher doses than found necessary to kill mice infected with T. cruzi.

In malaria, as in T. cruzi infections, there appears to be stimulation of the RES (16, 50). In the studies of Loose et al. (55) it was concluded that the ability to detoxify LPS was reduced in infected mice. Evidence indicates that activation of the RES has an effect on the lysosomes, preventing them from detoxifying the LPS. This effect could be due to depletion of lysosomal contents upon activation, possibly resulting from the presence of intracellular parasites.

In view of the apparent stimulation of the RES during T. cruzi infection, it seems more likely that failure to detoxify endotoxin, rather than failure to

phagocytose it might explain the increased susceptibility to LPS at the macrophage level. We have observed that agents that block RES activity, such as colloidal carbon and silica also potentiate LPS toxicity in mice. Lysosomal depletion could explain these situations as well. Although the RES is not activated by such agents, they act to fill the phagocytes with particles, as with carbon, or to rupture them, as with silica. Both situations cause release of lysosomal contents.

The presence of very large numbers of circulating trypanosomes can deprive the host of a tremendous amount of glucose. The parasites are extremely active, and they use serum glucose in their metabolism. Several workers have reported that blood glucose concentrations fall to below a fasting level subsequent to administration of endotoxin (84). Cumulative effects of infection and endotoxin could cause hypoglycemic shock. Shands et al. (84) tested for this by administering glucose to infected animals, but they had variable results and were not able to draw any definite conclusions.

Chapter 5

SUMMARY

Resistance to Trypanosoma cruzi in mice is cell-mediated and requires specific activation of the immune system. Several lines of evidence support this hypothesis. 1) BCG alone does not protect against acute infection. 2) Live preparations of attenuated trypanosomes provide good protection whereas killed organisms do not. 3) To obtain protection, live preparations must be administered at least 12-14 days in advance of challenge. 4) Administration of silica particles, a macrophage toxin, prior to challenge exacerbates infection. Activation of the immune system with BCG not only fails to confer immunity to T. cruzi, but actually interferes with induction of specific resistance by attenuated trypanosomes. Mice sensitized with BCG prior to immunization with attenuated trypanosomes were not protected by the immunization procedure as well as normal mice.

Although the phagocytic system is apparently activated in mice infected with T. cruzi, and the inflammatory response appears to be largely unaffected, the ability of infected animals to mount a response against antigens other than those of the trypanosome is decreased. Mice infected with T. cruzi and sensitized with heterologous

erythrocytes at various intervals after infection lost their ability to produce antibodies as the infection progressed. Similarly, there was a decrease in the animals' response to detoxified bacterial lipopolysaccharide during the latter stages of infection.

The effect of acute infection on the cellular immune response was also studied. Mice were immunized with either Freund's complete adjuvant or oxazolone, a chemical sensitizing agent, and were subsequently skin tested with either BCG protoplasm or oxazolone to detect delayed hypersensitivity. Depression of the response to these antigens was observed in infected mice during the stage of marked parasitemia. Mice which were responsive to oxazolone before infection lost their ability to respond as the infection progressed. When immunized with live attenuated T. cruzi, mice developed a greater than normal sensitivity to oxazolone and survived infection. Infected mice unable to respond to oxazolone were capable of conferring sensitivity to this substance in normal syngeneic mice via spleen cell transfer. The ability of infected mice to respond to oxazolone was significantly improved when macrophages from normal syngeneic donors were administered at the time of skin test. When normal and infected mice were used as recipients of lymphocytes from syngeneic oxazolone-sensitized donors, the normal

mice responded significantly better than did the infected mice. These results indicate that immunosuppression due to infection with T. cruzi is directed toward expression rather than induction of the cell-mediated immune response to the antigens employed.

Decrease in the capability of the immune response of infected mice was also shown by their increased susceptibility to the toxic effects of bacterial lipopolysaccharide. Mice became increasingly susceptible to minute quantities of the toxin as the infection progressed, leading to death shortly after administration of the substance.

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APPENDIX

Bouin's fixative

Picric acid (saturated aqueous, filtered)	75 ml
Neutral formalin (filtered) 20 g sodium acetate plus 100 ml 40% formalin	25 ml
Glacial acetic acid	5 ml

Giemsa stain for mouse blood cells

Stock giemsa:
1. Dissolve 0.5 g giemsa powder in 33 ml glycerine.
2. Heat at 55-60°C for 1 1/2 to 2 h.
3. Add 33 ml absolute methanol.

0.15 M phosphate buffered saline pH 6.4 (PBS):

0.85% saline	100 ml
0.15 M Na_2HPO_4	32.2 ml
0.15 M KH_2PO_4	67.8 ml

1. Fix smears in absolute methanol for 10 seconds or longer.
2. Allow slides to air dry.
3. Rinse a small test tube with 5 ml distilled water and add another 5 ml distilled water to the tube. Add a few crystals of hematoxylin to the test tube. If the water appears yellow the pH must be adjusted. Add about 0.1 ml 1% KCO_3/200 ml distilled water. The color of the water in the 5 ml tube should be light pink in color when hematoxylin is added.
4. Use 0.2 ml stock giemsa/ml PBS pH 6.4. Flood smear with 3 ml/slide for 30 seconds.
5. Float off stain with adjusted distilled water.

Turk's white blood cell diluting fluid

Glacial acetic acid	2 ml
Aqueous gentian violet 1% w/v	1 ml
Distilled water	100 ml

Mix and filter