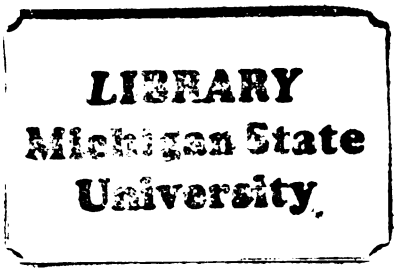

This is to certify that the

dissertation entitled

Mechanisms of Alteration of Cellular Immune Responsiveness during Experimental Trypanosoma cruzi Infection.

presented by

James Ray Maleckar

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Microbiology and Public Health

Major professor

Date May 20, 1983

MECHANISMS OF ALTERATION OF CELLULAR IMMUNE RESPONSIVENESS DURING EXPERIMENTAL *Trypanosoma cruzi* INFECTION

By

James Ray Maleckar

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Microbiology and Public Health

1983

ABSTRACT

MECHANISMS OF ALTERATION OF CELLULAR IMMUNE RESPONSIVENESS DURING EXPERIMENTAL TRYPANOSOMA CRUZI INFECTION.

BY

JAMES RAY MALECKAR

Chagas' disease, caused by the hemoflagellate protozoan Trypanosoma cruzi, usually presents an acute and a chronic phase. Reports of normal immune responses have often conflicted with other studies which have described an immunologically deficient state in Chagas' disease. A survey of the literature on this subject has suggested that immunosuppression is limited to the acute phase. The present work delineates the kinetics of the suppression by examining the host's ability to produce a delayed-type hypersensitivity reaction both in vitro and in vitro to T. cruzi antigens in the mouse model system of Chagas' disease. The measured parameters, footpad swelling and inhibition of macrophage migration, did not differ significantly from those of uninfected mice during the initial 40 days postinfection. Removal of Lyt 2.1-bearing cells from an acute mouse spleen cell suspension did not restore responsiveness to that population. Furthermore, addition of cells from acutely infected mice to chronic mouse spleen cells did not affect the ability of the latter to respond to stimulation with the trypanosomal antigen. Thus, this work indicated that suppressor T cells were not involved in production of immunosuppression. The ability of the parasite to modulate mitogen-induced lymphoproliferation was next examined. The responses of

normal mouse spleen cells to either concanavalin A (Con A) or a bacterial lipopolysaccharide (LPS) were reduced in the presence of sufficient numbers of either culture (epimastigote) or virulent bloodstream (trypomastigote) forms of T. cruzi. This suppressive action was found to be dependent on the parasite dose and not to be due merely to removal of the mitogen by absorption onto the parasite surface. Non-living preparations of T. cruzi were shown to be suppressive, indicating that parasite viability was not a requirement for production of suppression and that the phenomenon was not due to competitive consumption of essential nutrients by the parasites. Suppressed lymphoproliferation was observed when only the non-adherent spleen cells from uninfected mice were incubated with the trypanosomes. Treatment of adherent spleen cells with the parasite had no significant effect on the ability of these cells to support lymphocyte responses to mitogens. These results demonstrate that immunosuppression in an infected mammalian host is restricted to the acute phase of Chagas' disease and suggest that the parasite itself contributes to this effect by modulating lymphocyte function by an as yet undefined mechanism.

ACKNOWLEDGEMENTS

I would like to express my deepest gratification to Dr. Kierszenbaum whose wisdom and patience has played a major part both in my development as a scientist and as a person. Although the path I followed was not always the straightest or the quickest his enthusiasm and input never let me lose sight of my goal.

I would like to express my thanks to my wife, Veronica. Even though I received the degree, I feel that she earned it as well as I did. Her support, patience, and understanding as well as her humor and willingness has made the journey much easier to travel.

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ABBREVIATIONS

ADCC	Antibody dependent cell-mediated cytotoxicity
CMI	Cell mediated immunity
Con A	Concanavalin A
cpm	Counts per minute
CSA	Crude sonicated antigen (Kuhn, 1973)
DTH	Delayed type hypersensitivity
FTE	Freeze-thawed epimastigote antigen
ip	intraperitoneal
LPS	Bacterial lipopolysaccharide
MEM	Minimal essential medium
MEMS	Minimal essential medium supplemented with 10% fetal calf serum
MIF	Macrophage inhibition factor
NK	Natural killer cells
PBS	Phosphate buffered saline
PHA	Phytohemagglutinin
p.i.	Post infection
STA	Soluble trypanosome antigen (epimastigote origin)
STC	Sonicated *T. cruzi* preparation (trypomastigote origin)
T. cruzi	*Trypanosoma cruzi*

INTRODUCTION

Trypanosoma cruzi, a hemoflagellate protozoan first described by Carlos Chagas in 1909 (1,2), is the causative agent of American trypanosomiasis or Chagas' disease. It is primarily found in rural and underdeveloped areas and human cases have been reported from Argentina to the southern United States (3). Currently at risk are approximately 50 million people whereas it has been estimated that over 7 million people have previously contracted the disease (4). Cardiopathology (mainly chronic myocarditis), megacolon, and megaesophagus are not uncommon manifestations of the chronic phase of Chagas' disease (5, 6). In endemic regions of Chagas' disease, chronic myocarditis is the leading cause of heart failure and sudden death (7). Therefore, it is not surprising that Chagas' disease represents a major health problem in vast areas of the American continent.

T. cruzi is transmitted to man and other mammals by several species of blood-sucking reduviid bugs. In the invertebrate host, the parasite multiplies in the epimastigote form while migrating down the gut. In the hind gut, epimastigotes transform into the infective metacyclic trypomastigote stage and are passed out with the feces. Man acquires the parasite by rubbing contaminated feces into broken or intact skin, mucosa, or the lesion left by the bite of the reduviid bug. Organisms invade local tissue cells and multiply intracellularly as amastigotes. This form then transforms into the trypomastigote stage just prior to the rupture of the cell. Following lysis, trypomastigotes are transported via the bloodstream, body fluids and lymphatics thus reaching other types of tissues and cells. The cycle is completed when trypomastigotes in the

blood are ingested by the vector.

Chagas' disease often starts with an acute, subacute or subclinical acute phase in which the parasites may or may not be readily detectable in the circulation. Any of these forms of the disease is followed by the chronic phase in which the parasites are frequently not detected by routine screening methods. The immunology of Chagas' disease has been the subject of several reviews in recent years (8-11). Whereas a more detailed discussion of past and current progress in this area will be presented in the Literature Review, below is a brief outline of the approaches made in the present research program.

In the host, T. cruzi is both exposed to and sequestered from the effector mechanisms of the immune system. Resulting from these conditions is a delicate balance between parasite destruction and survival. During the acute phase of the disease relatively low levels of serum antibodies occur which are specific for T. cruzi that may play an important role in controlling the course of the infection. Concomitantly, the humoral response to other antigens and the cellular immunity are severely suppressed. Responses to both T. cruzi and unrelated antigens return to levels comparable with those of uninfected individuals during the chronic phase of the disease. Our understanding of the mechanisms involved in host resistance to the parasite, production of immunosupression and control of the infection is far from being complete.

At the time that this research program was undertaken there was confusion in the literature as to the meaning of impaired immunological alterations occurring during experimental Chagas' disease in the light

of other reports describing normal responsiveness. Careful screening of the apparently conflicting evidence revealed that suppressed responses had been documented in experimental models of the acute phase of the infection whereas normal responses were obtained with chronic chagasic patients. The possibility that suppression and immune responsiveness could represent two distinct phases of the disease was construed as the first working hypothesis to be explored in this work. Results of a study in which delayed-type hypersensitivity to a crude trypanosomal antigen was monitored both in vivo and in vitro during the entire course of an experimental T. cruzi infection will be presented in Chapter I (12).

The subject of induction of immunosuppression was addressed in the second phase of this research. The possible role of T. cruzi in modulation and/or impairment of immune responses was examined by using a protocol in which either the parasite or its products altered mitogen-induced lymphocyte proliferation. The results of these studies are presented in Chapters II (13) and III (14).

A final but important consideration in this program was to obtain information about the cell type(s) that, influenced by the parasite, contribute to the establishment of the suppressed state. Incubation of the parasite with the main cell types involve in the mounting of a lymphoproliferative response and the effect of such treatment on the response itself was the selected approach. The results of these efforts will be presented in Chapter IV.

Closing this Thesis will be a Summary highlighting the conclusions derived from this work and their significance.

LITERATURE REVIEW

The complexity of the disease caused by Trypanosoma cruzi, Chagas' disease, has been recognized since Carlos Chagas first reported its existence as a clinical entity related to an etiological agent (1). In examining various tissues of chagasic patients, Vianna (16) reported intense inflammation, including lesions in the heart, in infected individuals whether or not the parasite was present at the site. Many cell types were found to be vulnerable to invasion by the parasite, muscle, reticuloendothelial tissues, and glial cells being the primary target cells (16-19). Extensive information about parasite localization in the infected host contrasts with our limited understanding of the mechanisms of defense against infection.

Natural Immunity

T. cruzi has no known mammalian host specificity. It is capable of parasitizing a wide range of wild and domestic animals but amphibians and birds are naturally resistant [reviewed by Brener (20)]. Kierszenbaum et al. (21,22) demonstrated that avian resistance was antibody independent but complement dependent. Thus, blood forms of the parasite were readily detectable in the circulation of chickens previously depleted of complement by intravenous injections of cobra venom factor 24 hr after intravenous injection whereas in normal animals the flagellates could not be detected within a few minutes after inoculation. Lysis of the trypanosomes by avian serum in vitro required the presence of magnesium ions but not calcium ions, indicating that the alternative

pathway of complement activation was involved. Similar results were obtained when trypomastigotes grown in irradiated mice or in tissue culture were used, clearly indicating that antibodies were not required (22). Whereas, trypomastigotes are not lysed by normal mammalian sera, epimastigotes are sensitive to these sera (23,24).

Many non-specific factors affect resistance and susceptibility to T. cruzi. Among these are age, sex, and nutritional aspects of the host along with environmental conditions, such as temperature (reviewed in 6). Resistance also varies substantially between species of animals as well as within a given species. Studies performed in inbred strains of mice have demonstrated variations in the degree of susceptibility to T. cruzi infection, ranging from the very susceptible to the highly resistant (25). Trischmann and Bloom (26) have suggested that host resistance in mice may be governed by multiple genetic factors not including those coded for at the major histocompatibility complex (H-2).

Recent evidence has indicated the presence of natural killer (NK) cells capable of lysing trypomastigotes and unrelated tumor cell lines in uninfected as well as T. cruzi-infected mice (27,28). This lytic activity was demonstrated within 48 hr of infection. Both spleen and peritoneal exudate cells exhibited anti-T. cruzi lytic activity which was increased after T. cruzi infection or sensitization with polyinosinic-cytodylic acid and decreased by treatment with anti-NK 1.2 antiserum plus complement. However, the significance of this activity to host resistance is not known.

Humoral Immunity

Non-Specific Antibody Responses. The acute phase of experimental Chagas' disease is accompanied by an inability to produce antibodies against non-related antigens *in vitro*. Clinton *et al.* (29) did not observe normal production of antibody-forming cells (plaque forming cells, PFC) following immunization with optimal doses of burro red blood cells. This deficiency extended to the production of either 19S or 7S immunoglobulins. These workers also reported depressed *in vitro* or *in vivo* immune responses to aggregated human gamma globulin and soluble T-independent antigens such as DNP-Ficoll (30). Deficient responsiveness to sheep red blood cells has also been shown (31-33) as well as reduced levels of IgG1 and IgE antibodies to an unrelated antigen as measured by passive cutaneous anaphylaxis (34). On the other hand, investigators have been unable to find depressed antibody responses during the chronic phase (34, 35).

Specific Antibody Responses. *T. cruzi* has been described as being immunogenic (10). Evidence has accumulated which indicates the presence of circulating antibodies directed against the parasite throughout the course of infection in rabbits, mice, and humans (8, 36-39); these include complement-fixing, hemagglutinating and precipitating antibodies.

The importance of specific antibodies in resistance to *T. cruzi* was first elucidated by Culbertson and Kolodny (40) when they found increased resistance in mice after passive transfer of anti-*T. cruzi* serum. Kierszenbaum and Howard (41) showed a role for specific antibodies in their work with genetically-selected lines of mice, Biozzi Ab/L and Ab/H, differing only in their ability to produce humoral immune responses.

When compared to infected Ab/H mice, T. cruzi-infected Ab/L mice had higher parasitemia and mortality levels and shortened survival times. Furthermore, mere administration of passive anti-T. cruzi antibodies eliminated the difference. Krettli and Brener (42) found that bloodstream forms of the parasite were rapidly agglutinated in vitro by sera from chronically infected mice or humans and that the number of trypanosomes was reduced after such treatment. Circulating forms of T. cruzi were thought to be insensitive to immune lysis for many years. This concept was revised in 1975 when bloodstream forms of the parasite were described to be lysed in vitro by specific antibodies from chronic patients or mice with Chagas' disease (43) and later on confirmed in other laboratories (44-48). Complement was an absolute requirement for the lytic reaction and both the classical and alternative pathways of complement activation provided lytic activity (43).

Cell Mediated Immunity (CMI)

Protective effects of CMI. The importance of the cellular arm of the immune system in resistance to T. cruzi became apparent when the lack of a functional thymus was found to result in marked exacerbation of the course of the infection (47-49). Administration of anti-thymocyte serum (47), neonatal thymectomy (48) and use of congenitally athymic mice were the selected means for disabling thymic function.

CMI has also been shown to be important in controlling the infection during the later phase of the disease. Thus, when chronic mice were immunosuppressed with cyclophosphamide (50,51) or by lethal irradiation (50), parasitemias, which had become undetectable, re-occurred and other acute symptoms reappeared.

Delayed Type Hypersensitivity (DTH). Two methods have been commonly used to establish the presence of DTH reactions in T. cruzi-infected mice. Namely, skin reactivity to parasite antigen and the in vitro correlate of DTH, the macrophage migration inhibition test (MIF). Presence of DTH has been shown by both methods in both man and in mice with chronic Chagas' disease by several investigators (35,37,39,52-56). The most extensive report concerning humoral and cellular reactivity in chagasic patients is that by Montufar et al. (35). They found that during the chronic phase leukocyte migration was inhibited (as it would occur in normal individuals). Skin sensitization with non-T. cruzi antigens lead to a detectable immune response and the relative levels of peripheral blood T- and B-lymphocytes were not significantly different from normal subjects.

Comprehensive studies to evaluate variations in immunological status from the beginning of the infection have not been feasible with humans owing to difficulties in dating the origin of the infection. Such studies can be undertaken with laboratory animals causing infection under conditions that lead first to an acute and then to a chronic stage. The results of such an experiment is a subject of a part of this thesis.

Cytotoxicity Mechanisms. Involving Unsensitized Cells. In recent years, a considerable amount of work has focused on the ability of unsensitized cells to lyse parasitic agents in the presence of specific antibodies. These cytotoxic mechanisms have been referred to as antibody-independent cell-mediated cytotoxicity or ADCC. Eosinophils, neutrophils, macrophages, and lymphocytes from rats, mice, and humans have been shown to have the capability to lyse culture forms (epimastigotes) of

T. cruzi (57-62). The unfortunate drawback of this vast amount of research was the use of epimastigotes, a form rarely found in the mammalian host. Kierszenbaum and Hayes (63,64) and Okabe et al. (65) were able to demonstrate that bloodstream forms were also susceptible to lysis by human and mouse cells via ADCC mechanisms. The former group was able to show effector activity in this system for eosinophils, neutrophils, and lymphoid cells of human and mouse origin.

Cytotoxicity Mechanisms. Involving Sensitized Cells. The ability of sensitized cells to specifically lyse T. cruzi remains a controversy. Kuhn and Murnane (66) described the specific cytolysis of parasitized syngeneic fibroblasts in vitro by spleen cells from acutely infected mice. The rate of cytolysis was estimated by assaying ^{51}Cr- release. Normal, uninfected fibroblasts were not affected, suggesting an immune response directed against parasite antigens expressed by host cells during the acute phase. On the other hand, Hanson (67) could not find significant lymphocyte-mediated lysis of T. cruzi-infected kidney cells or syngeneic macrophages.

Destruction of T. cruzi by Macrophages. Macrophages have been a central target for study for many years. In 1959, Taliaferro and Pizzi (68) demonstrated that these cells were both a target for and an adversary of the parasite. Thus, epimastigotes were shown to be readily destroyed within macrophages. More recent studies have demonstrated the ability of macrophages to kill virulent blood forms in vivo and in vitro. Kierszenbaum et al. (69) injected mice with silica, a macrophage-killing agent, and infected them with trypomastigotes.

Resistance was reduced as measured by mortality rates and parasitemia. Macrophage stimulation had the opposite effect.

Activated macrophages demonstrate a greater-than-normal capacity to take up and destroy T. cruzi. Mouse macrophages stimulated in vivo with BCG, Corynebacterium parvum, or other activators of mononuclear phagocytes readily kill the parasites (70-72). Similar results have been reported for activated human macrophages (73).

Suppression of CMI

Altered Responsiveness to Mitogenic Stimulation. Several investigators have reported suppression of responses to mitogens. In mice immunized with a crude sonicated preparation of T. cruzi (CSA) and infected with bloodstream forms of T. cruzi, lymphocyte responses to phytohemagglutinin (PHA) were markedly reduced when compared to mice which received only CSA (74). A similar disparity in responses to CSA was shown by using a dermal test. Ramos et al. (15) reported decreased responses in acutely infected mice to concanavalin A (Con A) and bacterial lipopolysaccharide (LPS). Kierszenbaum and Hayes (51, 75) evaluated lymphocyte responsiveness to PHA, Con A, and LPS in inbred mice during the different stages of the disease. Responses to all three mitogens were severely inhibited during the acute phase but normal responses were seen during the chronic stage. It was also noted that during the acute period splenic T lymphocytes were reduced both in absolute number and in percentage. Whereas B lymphocytes increased in absolute numbers but their percentage varied within narrow limits. Normalization of the lymphocyte population in the spleen during the chronic period suggest that immunological events may

play a role in supporting the transition from acute to chronic Chagas' disease.

Depressed Drug-Induced Contact Sensitivity. In studies of contact sensitivity Reed et al. (76,77) examined the ability of T. cruzi-infected mice to respond to the sensitizing drug oxazolone. Mice which had been sensitized prior to infection lost their responsivess after infection. Spleen cells from infected mice transferred to normal mice responded to the agent; transfer of sensitized non-adherent cells to uninfected mice effected responses but non-adherent cells transferred to infected mice could not increase responses. In contrast, the transfer of normal adherent cells significantly improved responsiveness. From these observations, these investigators concluded that macrophages from infected mice were altered. The possibility that the macrophages themselves could have been suppressive was not addressed.

Mechanisms of Immunosuppression

Suppressor T Lymphocytes. Ramos et al. (15) reported that spleen cells from infected mice were able to suppress mitogen-induced responses of normal mice. The cell responsible was claimed to be non-adherent and removable by treatment with anti-mouse thymocyte serum plus complement. These results could not be confirmed by several other studies in which removal of Lyt 2.1-bearing cells -which include the suppressor T cells- failed to restore responsiveness (12, 76-78).

Adherent Suppressor Cells. Macrophages have been implicated as mediators of the suppression seen during the acute phase of infection (76,79). In mitogen-induced mouse spleen cell response assays,

Cunningham and Kuhn (74) noted that macrophage-enriched spleen cells were suppressed during acute infection. Macrophage-depleted spleen cells had greater responses, though responses did not return to normal. Kierszenbaum (76) reported that reduced mitogen-induced responses were seen when purified adherent spleen cells from infected mice were co-cultured with normal spleen cells. The responses were enhanced by exchanging the adherent cells for those from uninfected mice. In addition, treatment of infected spleen cells with indomethacin -a drug known to block the cyclooxygenase pathway of biosynthesis of prostaglandin products- improved their responses to T and B cell-specific mitogens.

Parasite-Induced Suppression. A role for T. cruzi in directly modifying cell-mediated responses was initially disqualified by Ramos et al. (15). In contrast, a more in-depth study of this phenomenon is presented in Chapters II and III.

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CHAPTER I

VARIATIONS IN CELL-MEDIATED IMMUNITY TO *TRYPANOSOMA CRUZI* DURING EXPERIMENTAL CHAGAS' DISEASE

ABSTRACT

Mice infected with bloodstream forms of Trypanosoma cruzi were found unable to respond significantly to subcutaneous stimulation with parasite antigens with a delayed-type hypersensitivity skin reaction during the acute phase of the disease. Consistent with this observation, peritoneal cells collected from infected mice during the acute period were unresponsive in in vitro assays for production of macrophage migration inhibitory factor. By contrast, both specific skin reactivity and significant inhibition of macrophage migration were noted during the chronic stage, i.e., when parasitemias were on the decline or undetectable and mortality no longer occurred. Given the similarity between the kinetic changes in cell-mediated immunity monitored by in vivo tests and their in vitro correlate, the latter was used to explore the possible role of suppressor T cells in the causation of immunodeficiency recorded during the acute phase. Peritoneal cells from acutely infected animals failed to alter production of macrophage migration inhibitory factor by chronically infected mouse cells when present in mixtures at proportions representing up to 75% of the total number of cells. Furthermore, the dilution effect of cell addition on the in vitro reaction to the trypanosomal antigens was comparable whether normal or acutely infected mouse cells were mixed with those from chronically infected animals. Removal of Lyt 2.1 bearing cells from suspensions of acutely infected mouse cells did not restore lymphocyte responsiveness to the T. cruzi antigens in the macrophage migration inhibition assay. These results show that the impairment of specific cell-mediated

immunity of mice infected with T. cruzi is circumscribed to the acute phase of the disease and do not support a role for suppressor T lymphocytes in the lack of host responsiveness to the parasite's antigens.

INTRODUCTION

The deficient status of lymphocytes from mice infected with Trypanosoma cruzi has been documented in terms of altered reactivity to mitogenic stimulation (1-3), impaired ability to mount antibody responses to non-trypanosomal antigens in vitro (4-6) and by depressed drug-induced contact sensitivity (7). More recently, these observations have been extended to specific in vitro lymphoproliferative responses triggered by T. cruzi antigens derived from host (bloodstream) forms of the parasite (8). However, the available evidence for the deficient immunological status has been reported to occur in vitro during the chronic period of Chagas' disease both in man (9-13) and in mice (8). Furthermore, exacerbation of the disease in chronically infected mice following immunosuppressive treatment with cyclophosphamide (14,15), suggests an important role of the immune system in controlling the course of T. cruzi infection. On this basis, the study of variations in the specific immunological status of the host assumes importance in understanding better the delicate balance of host-T. cruzi interaction. In this work, we have examined the pattern of cell-mediated immune reactivity of mice to trypanosomal antigens during the course of an experimental infection produced in inbred mice which first leads to an acute phase and then to the chronic stage of Chagas' disease.

MATERIALS AND METHODS

Four-week-old inbred, female CBA/J mice used in this work were purchased from the Jackson Laboratory (Bar Harbor, ME).

Bloodstream trypomastigote forms of Tulahuen strain T. cruzi were maintained by serial intraperitoneal (ip) passages in mice. Infected blood was drawn from the retro-orbital venous plexus and collected in heparinized tubes. Dilutions were made with sterile RPMI-1640 medium (Flow Laboratories, Rockville, MD). The doses of T. cruzi used for parasite maintenance and for production of experimental infection were 10^5 and 25 organisms, respectively, and were administered ip in a volume of 0.1 ml. Parasite concentrations were measured by a standardized microscopic method described elsewhere (16) and expressed as number of T. cruzi ml^{-1}. Cultured (mainly epimastigote) forms were maintained in a biphasic medium consisting of agar, sheep blood, glucose, liver and brain-heart infusions (17) at 26°C. Epimastigotes used for the preparation of antigenic material were grown in the same medium except for the absence of sheep blood.

Epimastigotes were harvested during their logarithmic phase of growth (on day 5 of culture) and washed three times with sterile phosphate-buffer saline, pH 7.0 (PBS) by centrifugation at 2500 rpm for 15 minutes at 4°C. The pelleted flagellates were resuspended in ten volumes of PBS (final concentration of 5×10^8 T. cruzi

cells ml^{-1}), frozen with a mixture of dry ice-acetone and thawed in a 37°C water bath. After five cycles of freezing and thawing, the organisms were disrupted by sonication (for 45-second periods of exposure at 30 watts) in a Cell Disrupter (Heat Systems, Plainview, NY). The suspension was maintained in an ice bath during this treatment. After centrifugation at 35,000g for 30 minutes at 4°C, the supernatant was separated, aliquoted and stored under liquid nitrogen until used. This preparation will be referred to in the text as the soluble trypanosome antigen (STA).

Infected and uninfected mice were sacrificed at predetermined time intervals starting on day 5 post infection (p.i.). An aseptic operation was maintained throughout. Peritoneal lavages with serum-free Eagle's Minimum Essential Medium (MEM, Flow Laboratories) were collected from five animals as described by Stuart and co-workers (18) and pooled. The cells were washed three times with the same medium and finally resuspended in MEM supplemented with 10% heat inactivated fetal calf serum (Microbiological Associates, Walkersville, MD)(MEMS). Cells were counted using a Neubauer hemacytometer and the concentration was adjusted to 3×10^7 viable, trypan blue-excluding cells ml^{-1} in MEMS. Capillary tubes (1.4 mm internal diameter x 100 mm long) were filled with the cell suspension and plugged at the end with Critoseal (A. H. Thomas, Co., Philadelphia, PA). After centrifugation at 700 rpm for eight minutes at 4°C, the tubes were cut at the cell-fluid interface. The end containing the cells was placed in a culture chamber (Sterilin,

London, England) which was then filled with MEM. Cultures were set up in duplicate or quadruplicate both in the presence and absence of 100 µl of STA and incubated at 37°C for 24 hr. This amount of STA was selected because of optimal responses obtained with it in preliminary tests for production of macrophage migration inhibitory factor (MIF) performed with cells from chronically infected mice. MIF assays were also performed with mixtures containing cells from chronically infected mice and cells from either normal or acutely infected animals. The composition of the tested cell mixtures will be described under Results. The sensitivity of the assay was increased by measuring uniformly enlarged (by projection at constant magnification) areas of cell migration using a compensating polar planimeter (Keuffel and Esser model 620005, New York, NY). Results were expressed in terms of percentage migration inhibition calculated by the equation % MI = 100(1 - Mi) where Mi is the migration index (Mi = area of migration in the presence of antigen/area of migration in the absence of mitogen).

One half ml of cell suspension containing 1.6×10^7 cells in MEM was mixed with 0.2ml of monoclonal anti-Lyt 2.1 antibody (New England Nuclear, Boston, MA; titer 10^{-4}) diluted 1/30 in MEM and incubated at 37°C for 15 minutes. The source of complement (C) activity was guinea pig serum diluted 1/8 in MEM; 0.5 ml of this reagent was added to the mixture which was incubated further for 30 minutes. The cells were then washed twice with 50 ml volumes of MEM, counted in the presence of trypan blue and finally suspended to 3×10^7 viable cells ml^{-1}.

Determinations of footpad reactivity to STA were made on a time schedule similar to the one followed for the MIF tests but separated groups of animals were used. Five mice were tested on any given day, each receiving a subcutaneous injection of 0.05 ml of STA into the right hind footpad and 0.05 ml PBS into the contralateral footpad. Footpad thickness was measured with a micrometric caliper 24 hr after the injections and the difference between the thicknesses of the right and left footpads calculated. Groups of five non-infected mice receiving STA and PBS were used to test each batch of STA. No significant difference in footpad size or swelling was observed in these animals.

Each set of data presented in the figures of this paper is typically representative of two or more separate experiments with identical protocols.

RESULTS

Course of T. cruzi infection in CBA/J mice

A typical course of infection produced in CBA/J mice injected with 25 T. cruzi is depicted in Fig. 1 to serve as reference for the studies described below. All of the infected mice developed parasitemias which attained significant levels on days 13-15 pi, increased thereafter up until days 23-26 pi and then declined until becoming undetectable. Separate groups of mice were infected to determine the mortality rate produced by infecting with 25 organisms. Forty to 60 percent of these animals survived in the various experiments in which 100-150 mice were used; a representative mortality curve is shown in Fig. 1. The survivors were considered to be chronically infected. This criterion is supported not only by the observations of Laguens et. al. (19) who established histologic and electrocardiographic similarities between chronic Chagas' in humans and mice but also by the previous finding that drug-induced immunosuppression exacerbates the infection in surviving CBA/J mice whose parasitemias and other signs of the infection have become undetectable (15).

Cellular reactivity to STA during T. cruzi infection

Animals which received STA and PBS during the first 25 days pi showed no significant differences in footpad swelling (Fig. 2). Positive reactions denoted by swelling of the footpads injected with STA were recorded on day 40 and persisted up until day 60 pi when the determinations were discontinued.

FIGURE 1

Course of T. cruzi infection in CBA/J mice. Mice received 25 trypomastigotes i.p.

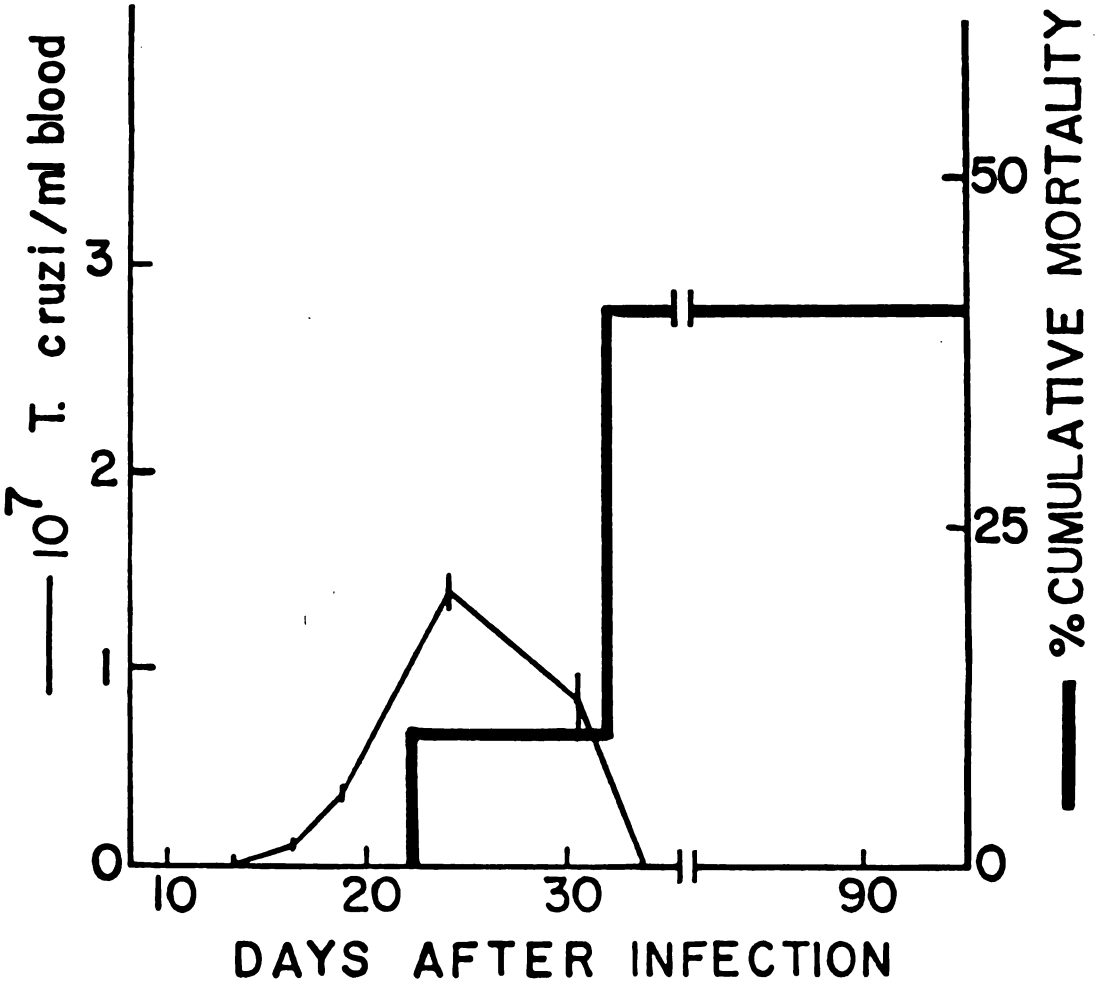
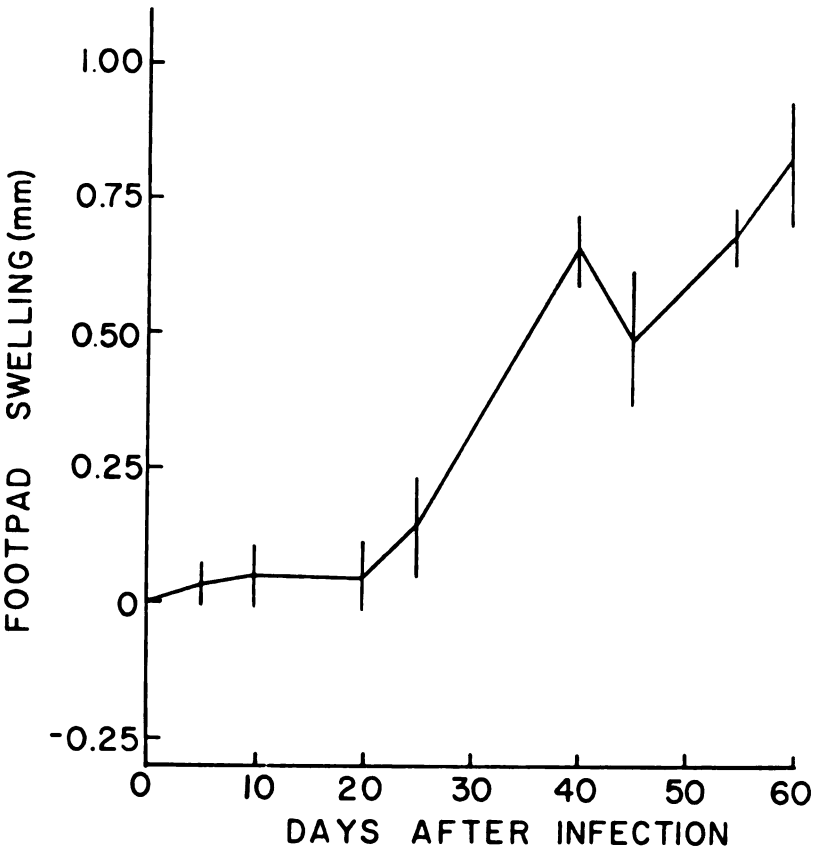


FIGURE 2

Delayed-type hypersensitivity reaction elicited by STA during the course of experimental *T. cruzi* infection. Points represent the average swelling difference between footpads of five animals. Vertical lines are the standard deviations. There was no significant difference in thickness between the footpads of uninfected animals receiving STA and PBS (not shown).

FIGURE 2


1.00
0.75
0.50
0.25
0
-0.25
FOOTPAD SWELLING (mm)
0
10
20
30
40
50
60
DAYS AFTER INFECTION

MIF assays did not reveal significant inhibition of macrophage migration until days 40-45 pi when a relatively small but reproducible inhibitory phenomenon was observed in culture chambers containing STA (Fig. 3). The percentage of inhibition of macrophage migration followed a trend of continuous ascent with time after this initial occurrence.

MIF tests were set up using cell mixtures containing varying portions of leukocytes from mice sacrificed on their 20th and 60th day of infection. This was done to find out if acutely infected mouse cell suspensions contained a population of suppressor cells inhibiting the production of MIF. Control assays performed with each of the cell suspensions prior to mixing readily reproduced the capacity and incapacity of chronically and acutely infected mouse cells, respectively, to produce MIF following in vitro stimulation with STA. However, cell cultures containing 50 or 75% peritoneal cells from mice sacrificed on day 20 pi failed to significantly alter the responsiveness of cells derived from animals sacrificed on day 60 pi (Fig. 4). The effect of dilution of the chronically-infected mouse cells with cells from either normal or acutely infected mice were comparable. Similar results were obtained when cells from mice sacrificed on day 15 pi were used (data not shown).

Using an independent approach we tested responsiveness of acutely infected mouse cells in the MIF assay before and after removal of the Lyt 2.1-bearing cells, known to include the suppressor T lymphocyte

FIGURE 3

Variations in the % MI during the course of experimental *T. cruzi* infection. Solid line, infected mouse cells; dashed line, normal mouse cells. Points represent the average of four separate experiments, each of which was performed in quadruplicate with pooled cells from five mice. Vertical lines are the standard deviations.

FIGURE 3

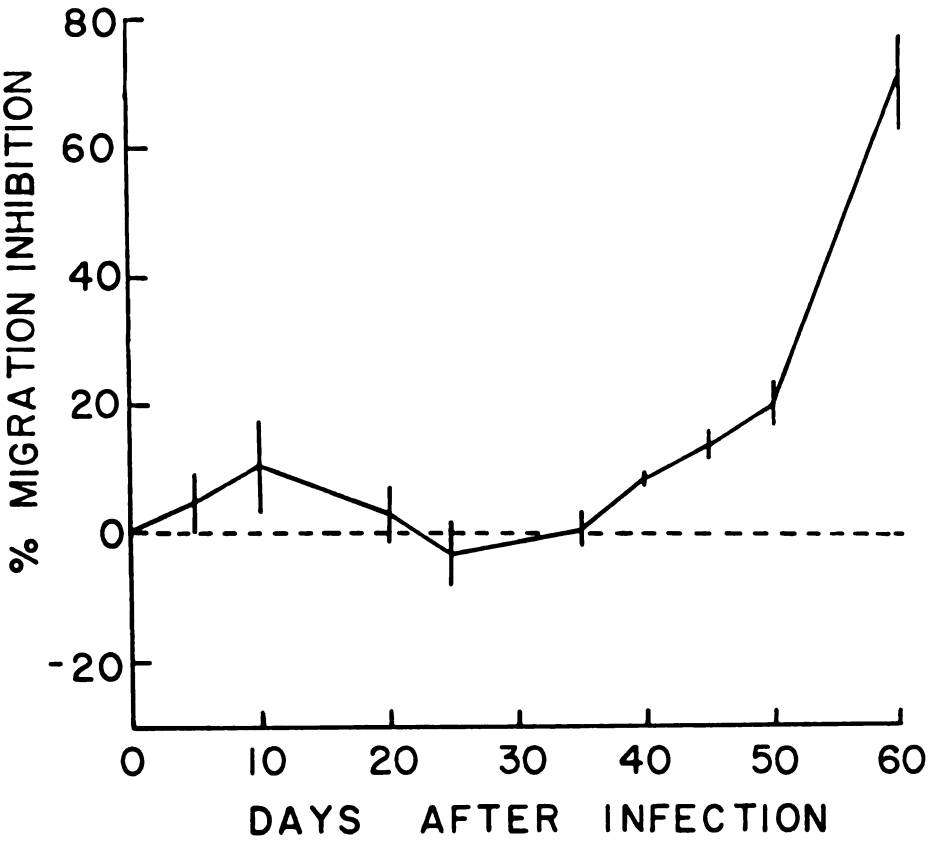
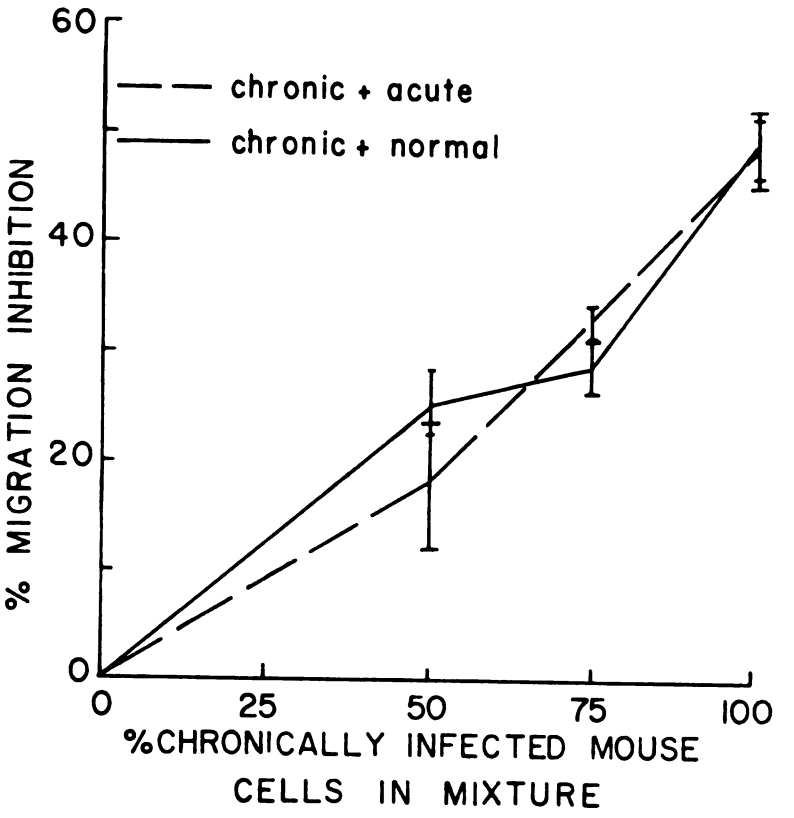

80
60
40
20
0
-20
% MIGRATION INHIBITION
0
10
20
30
40
50
60
DAYS AFTER INFECTION

FIGURE 4

Effects of mixing chronically infected mouse cells with cells from either normal or acutely infected mice on the % MI. Cells from acutely and chronically infected mice were obtained on day 20 and 60 p.i., respectively. The total number of cells in the assay was constant. The difference between 100% and the percentage of chronic mouse cells given in the abscissa corresponds to the percentage of normal or acute mouse cells present in the reaction mixture. Points represent the average of duplicate determinations and the vertical lines the standard deviation. Note that addition of up to 50% cells from acutely infected mice did not affect the capacity of chronically infected mouse cells to produce inhibition of macrophage migration.

FIGURE 4


60
40
20
0
chronic + acute
chronic + normal
% MIGRATION INHIBITION
0
25
50
75
100
%CHRONICALLY INFECTED MOUSE
CELLS IN MIXTURE

subset. As can be seen in Table 1., treatment with monoclonal anti-Lyt 2.1 antibody plus C had no consequences on the unresponsive status of the cells. We considered the possibility that cells responsible for MIF production in our system could have been Lyt 2.1 positive in that their removal might have abrogated MIF production in the absence of suppressor T cells. For this reason, we carried out our parallel control experiments with cells from chronically infected mice and found that their response to the antigen was not significantly altered after treatment with anti-Lyt 2.1 plus C.

It is noteworthy that the results of the experiments described in this paper, performed with pools of cells from several animals, were systematically confirmed when spot checks were conducted by using cells from individual mice (data not shown).

TABLE 1

Effect of removal of Lyt 2.1-positive lymphocytes on the responsiveness of acutely and chronically infected mouse cells in the MIF test.

Cells from	Treatment of cells	% Migration ± S.E.M.*
Uninfected mice	none	100
Acutely infected mice+	none	111 ± 17
Acutely infected mice	anti-Lyt 2.1 + C	108 ± 4
Chronically infected mice‡	none	61 ± 2
Chronically infected mice	anti-Lyt 2.1 + C	68 ± 9

* Results are the average of the two separate experiments in each of which the test was performed in duplicate.

+ Cells from 5 mice sacrificed on day 15 postinfection.

‡ Cells from 5 mice sacrificed on day 60 postinfection.

DISCUSSION

While evidence for impaired nonspecific lymphocyte reactivity during experimental Chagas' disease has been presented in the literature, these results show that the immunological deficiency also applies to *in vivo* specific responses to *T. cruzi* antigens. The impaired expressions of specific cell-mediated immunity were limited to the early, acute period of the disease, whereas the chronic phase was characterized by significant cellular responsiveness to trypanosomal antigens detected *in vivo* and *in vitro*. This kinetic pattern of variation of *T. cruzi* antigen-induced lymphocyte reactivity closely resembled that previously described for infected mice with respect to the ability of their spleen cells to mount proliferative responses to stimulation with T (concanavalin A and phytohaemagglutinin P) or B (endotoxic lipopolysaccharide) cell-specific mitogens (3,15). It follows from this correlation that nonspecific polyclonal activators may be used to both indirectly evaluate the immunologic status of the infected host and derive information about the course of the disease.

Tests of inhibition of leukocyte migration have proven useful in the detection of specific cellular reactivity in chronic chagasic patients before and after chemotherapy (11). In our work, results of MIF assays performed during the acute and chronic periods of the infection correlated well with the variations in host reactivity noted in *in vivo* delayed-type hypersensitivity reactions elicited with STA. This

justified the use of the MIF test in subsequent work to explore the cause of the immunological deficiency. In human Chagas' disease, chronically-infected patients are those who, remaining infected, survive the acute phase. We have used in this work a similar criterion by considering as chronically infected those mice which survived for extended periods of time. Since approximately 50% of the infected animals survived and animals sacrificed during the acute period were randomly chosen, we assumed that one half of these mice would have survived had they not been used. Lack of responsiveness in the MIF assay during the acute but not the chronic stage of the infection was a systematic and consistent finding, suggesting not only that this phenomenon was characteristic of the acute phase of experimental Chagas' disease but also independent on the fate of the animals.

Presence of T. cruzi in chronically-infected mice can be readily demonstrated by the exacerbating effects of immunosuppression with cyclophosphamide (14,15) evidenced by the reappearance in the circulation of significant numbers of trypomastigotes and renewed manifestation of signs of the disease. It is clear, therefore, that mere presence of the parasite in the host is not the only requirement for production of the immunodeficient condition. On the other hand, with injection of 25 parasites relatively high levels of circulating flagellates were recorded on days 12 through 35 p.i. These should have constituted an effective antigenic stimulus both in length of time and dose of stimulation; yet responses to STA were insignificant during this period of time whether measured in vivo or in vitro. It is

conceivable that induction of the immunodeficient state may be dependent on the parasite concentration as suggested by the results of Schmunis and coworkers (20) who performed MIF tests with cells from outbred mice infected with different doses and forms of T. cruzi.

The precise nature of the phenomenon resulting in impaired immunologic reactivity in experimental Chagas' disease is not known. Ramos and coworkers (2) have proposed a role for suppressor T lymphocyte in the inhibition of mitogen-induced proliferative responses by spleen cells from mice infected with T. cruzi. However, presence of up to 50% peritoneal cells from acutely-infected mice failed to cause any significant diminution of the reactivity of chronically mouse cells in MIF assays. This observation suggests that suppressor T lymphocytes are unlikely to be relevant to the inhibition of STA-induced responses. In keeping with this concept was the observation that removal of Lyt 2.1 bearing cells from suspensions of acutely infected mouse cells had no consequence on the unresponsive status of these cells to T. cruzi antigens. It should be noted that the use of monoclonal anti-Lyt 2.1 antibody in our work precluded the removal of lymphocytes other than those bearing the Lyt 2.1 marker. Lyt 2.1-positive cells are known to include the suppressor T lymphocyte subpopulation in CBA/J mice (reviewed in 21). Nevertheless, a suppressive role for another type of regulatory cell is not negated by the present results and we are currently addressing this possibility. It should be noted that adherent cells have been proposed as having a suppressive activity in African trypanosomiasis (22). Also relevant in this context is the recent

finding that an immunosuppressive factor present in the sera of mice infected with T. cruzi requires macrophage-like cells to display its activity (23). In our own research, the removal of adherent cells from suspensions of infected mouse spleens has been shown to partially correct the deficient responsiveness to T and B cell-specific mitogens. Furthermore, the purified adherent cell population from acutely infected mouse spleen preparations curtailed the response of uninfected mouse lymphocytes (24). Although these observations support a suppressor role for phagocytic cells, whether or not this is the only cause for the immunological deficiency noted during the acute stage of the disease remains to be examined.

Recent kinetic studies performed with CBA/J mice revealed a severe depletion of T lymphocytes in the spleen in the acute phase of the disease (14). Therefore, there is a possibility that the impaired immunologic responsiveness of the acutely infected mice may be due, in part, to a reduction in the number of responder cells.

The present results do not reveal what mechanisms produce the altered immunologic status of the infected host and the subject deserves further attention. Presumably, the parasite benefits from this condition, evading specific host defenses. A delicate balance between the capacity of the parasite to induce suppression (directly or indirectly) and the ability of the immune system to counteract seems to determine whether or not the infected host will survive, and enter the chronic stage.

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CHAPTER II

SUPPRESSION OF MOUSE LYMPHOCYTE RESPONSES TO MITOGENS *IN VITRO* BY *TRYPANOSOMA CRUZI*

Abstract

The ability of T. cruzi to inhibit mitogen-induced mouse lymphocyte responses was studied to find out if the organism itself is involved in the production of the immunosuppression that occurs during the acute phase of Chagas' disease. Significant suppression of normal spleen cell responses to concanavalin A (a T cell-specific mitogen) or to bacterial lipopolysaccharide (a B cell-specific mitogen) was seen when the concentration of epimastigote forms of the parasite reached or exceeded 2.5×10^6 organisms/ml in the cultures. The inhibitory effect was noted over wide ranges of concentrations of either mitogen. Since spleen cells stimulated with mitogenic solutions that had been absorbed with 1×10^7 parasites/ml produced significant responses, the suppressive effect could not be attributed just to mitogen removal by the parasites. Preparations of T. cruzi disrupted by freezing and thawing also inhibited mitogen-induced responses. This indicated that production of suppression was not a result of parasite competition for essential medium nutrients and that trypanosome viability was not required. Suppression was demonstrable only when the parasites were incorporated into the cultures within 12 hr after mitogenic stimulation. These results taken together indicate that T. cruzi has the ability to modulate directly or indirectly lymphocyte function by interfering with the initial stages of commitment to lymphoproliferation.

INTRODUCTION

As is the case in many other parasitic infections (reviewed by 1), suppression of immune responses during the acute period of Trypanosoma cruzi infection (2-7) has been viewed as a biological phenomenon fostering the establishment and persistence of the flagellate in the host. Several mechanisms have been postulated for the production of immunosuppression, including a role for suppressor T lymphocytes (8), suppressor macrophages (9) and suppressive factors found in the serum of infected mice (10). Ramos et al. (8) concluded that T. cruzi could not adversely influence lymphocyte responses because addition of parasites to mouse spleen cell cultures had no significant consequence on mitogen-induced responses. At odds with this observation are preliminary results from our laboratory revealing that marked suppression does occur when the parasite is incorporated into the cultures. In view of this difference, we examined in this work the suppressive phenomenon with the double aim of defining the requirements and kinetics of the effect and explaining the discrepancy.

MATERIALS AND METHODS

Animals. Five- to six-week-old female CBA/J mice were purchased from Jackson Laboratory (Bar Harbor, ME).

Parasites. Epimastigote forms were grown in Warren's medium (12) supplemented with 10% heat-inactivated (56°C, 60 min) newborn calf serum (KC Biological, Kansas, MO) at 28°C. The cultures were harvested during the logarithmic phase of growth (day 5) and centrifuged at 800 x g for 10 min. The parasites were then washed 4 times and resuspended at the appropriate concentrations with medium (see Results). Concentrations of epimastigotes and trypomastigotes were determined microscopically using a Neubauer haemacytometer.

Non-living T. cruzi preparations (FTE). Suspensions containing 5×10^7 epimastigotes per ml in medium were subjected to 5 cycles of freezing (dry ice-ethanol) and thawing (37°C waterbath). No intact organisms were detectable in any preparation, referred to in the text as FTE.

Cells. CBA/J mouse spleen cell suspensions were prepared in a sterile Ten-Broeck tissue grinder (two strokes only) using cold medium. Tissue debris was removed by filtration through a sterile nylon gauze. The cells were washed 3 times with medium by centrifugation (240 x g, 10 min, 4°C) and finally adjusted to 1×10^7 viable, trypan-blue-excluding cells per ml. All cell preparations used in this study contained >95% viable cells.

Mitogens. Solutions of concanavalin A (Con A, Sigma Chemical Co., St. Louis, MO) or bacterial lipopolysaccharide extracted from

Escherichia coli 055:B5 by the phenol-water method (LPS, Difco, Detroit, MI) were prepared in medium and filtered through a sterile 0.45-μm pore size filter (Millipore, Bedford, MA). Concentrations of these mitogens are given in the Results section.

Mitogen-induced DNA synthesis. Cell cultures were set up in triplicate in flat-bottom microculture plates (Limbro, New Haven, CT). The basic culture system consisted of 0.025 ml of spleen cell suspension, 0.025 ml of medium supplemented with 10% heat-inactivated fetal bovine serum (Sterile Systems, Logan, UT), 0.025 ml of the appropriate mitogen solution and 0.025 ml of medium. When living parasites, or FTE were incorporated into the culture system, 0.025 ml of the corresponding preparation substituted for the same volume of medium. The cultures were incubated at 37°C in a 5% CO_2-in-air atmosphere saturated with water vapor for 72 hr. Each culture received 1 μCi of ^{3}H-thymidine (specific activity 2 Ci/mmole, New England Nuclear, Boston, MA) in 0.025 ml of medium 24 hr prior to its termination by processing in a cell harvester. Radioactivity representing ^{3}H-thymidine incorporation into synthesized DNA was measured in a liquid scintillation spectrometer.

Cell and parasite viability. Concentrations of intact, motile parasites and trypan-blue-excluding spleen cells were measured microscopically at various times in cultures initiated with 2.5×10^6 cells/ml in the presence or absence of 2.5×10^6 trypanosomes/ml. The final volume of the cultures was 0.1 ml.

Kinetics of parasite effects on spleen cell responses to mitogens. Cultures were set up as described under Mitogen-induced DNA synthesis except for the omission of 0.025 ml medium. After 0, 12, 24, 36 or 48 hr

of incubation, experimental cultures received epimastigotes (in 0.025 ml) so that the concentration would be 2.5 x 10^6 organisms/ml. Control cultures received medium instead. Cultures of parasites alone in the presence or absence of the mitogens as well as mitogen-free spleen cell cultures were also included as controls. Culture conditions and processing were also as described above.

Absorption of mitogen solutions with epimastigotes. Solutions containing varying concentrations of Con A or LPS were incubated with 1 x 10^7 epimastigotes/ml in a 5% CO_2 incubator at 37°C for 24 hr. The parasites were then removed by centrifugation (800 x g, 10 min, 20°C) followed by filtration through a sterile 0.45-μm pore size filter. The supernatants were tested for residual mitogen activity as described above.

Presentation of results. All sets of data presented in the figures and tables of this paper are typically representative of two or more experiments of identical design. Radioactive counts (cpm) represent the mean of triplicate determinations ± standard deviation. Differences were considered to be significant if $P<0.05$ as calculated by Student's t test.

RESULTS

Inhibition of T and B lymphocyte responses by T. cruzi

Spleen cell responses to Con A and LPS were markedly inhibited by epimastigote forms of T. cruzi when the parasite concentration reached or exceeded 2.5×10^6 organisms/ml (Fig. 1). Similar suppressive effects were seen when parasites used at this concentration were co-cultured with up to 1×10^7 spleen cells/ml, i.e., four times as many cells as present in the cultures of the experiment depicted in Fig. 1 (data not shown). This increased number of splenocytes was, however, capable of mounting significant responses to the mitogens, denoting that culture crowding had not occurred at these cell concentrations.

T and B lymphocyte responses were inhibited by T. cruzi epimastigotes over a wide range of mitogen concentrations (Fig. 2 and 3). The typical supraoptimal zone effect, produced by high concentrations of Con A, occurred whether or not the parasites were present in the cultures. However, a shift of peak responses toward higher concentrations of Con A was often seen when epimastigotes were present. Although LPS-induced responses are not characterized by a supraoptimal zone effect, suppression was observed even when the dose of the mitogen was 100 times greater than the minimal stimulatory concentration. It is noteworthy that actual cell responses to either Con A or LPS in cultures containing T. cruzi had to be smaller than those depicted in Fig. 2 and 3 because the recorded values include the contribution of the flagellates.

To find out if reduced responses were due to unusual cell killing in the presence of T. cruzi, cell concentrations were measured at various

FIGURE 1

Effect of addition of T. cruzi epimastigotes to spleen cell cultures on their ability to respond to Con A and LPS. Dashed curve, responses by cell cultures containing the indicated concentration of parasites. Solid curve, parasites alone. Points represent the mean of triplicate determinations and the vertical bars the standard deviation. The concentrations of Con A and LPS were 2.5 and 50 µg/ml, respectively. Only the differences between the values obtained with epimastigote concentrations equal or greater than 2.5×10^6 organisms/ml and the control value (no parasites present, ordinate) were statistically significant ($P < 0.05$), after the contribution of the parasites was subtracted.

FIGURE 1

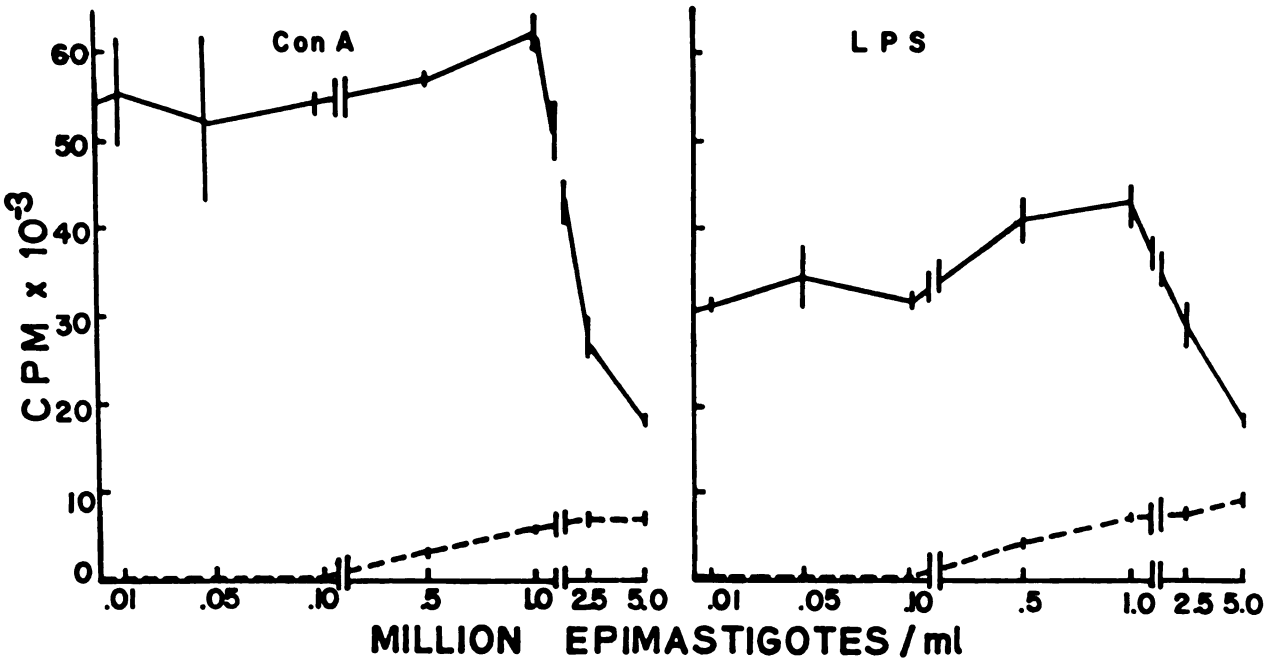
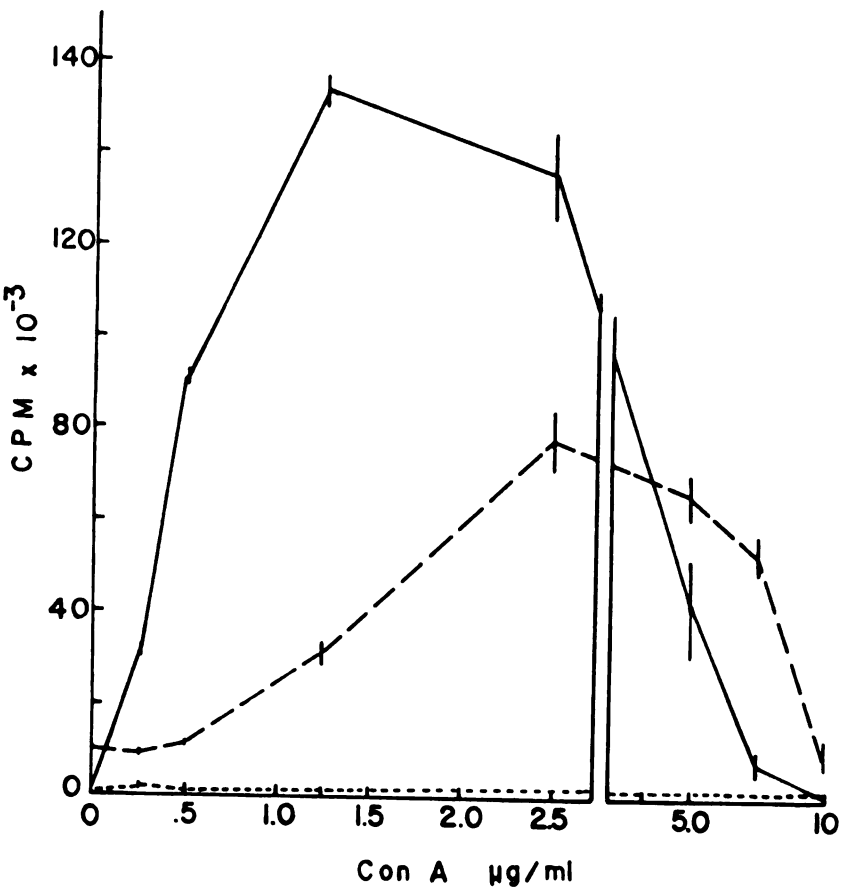

Con A
L P S
60
50
40
30
20
10
0
CPM x 10-3
.01
.05
.10
.5
1.0
2.5
5.0
MILLION EPIMASTIGOTES / ml

FIGURE 2

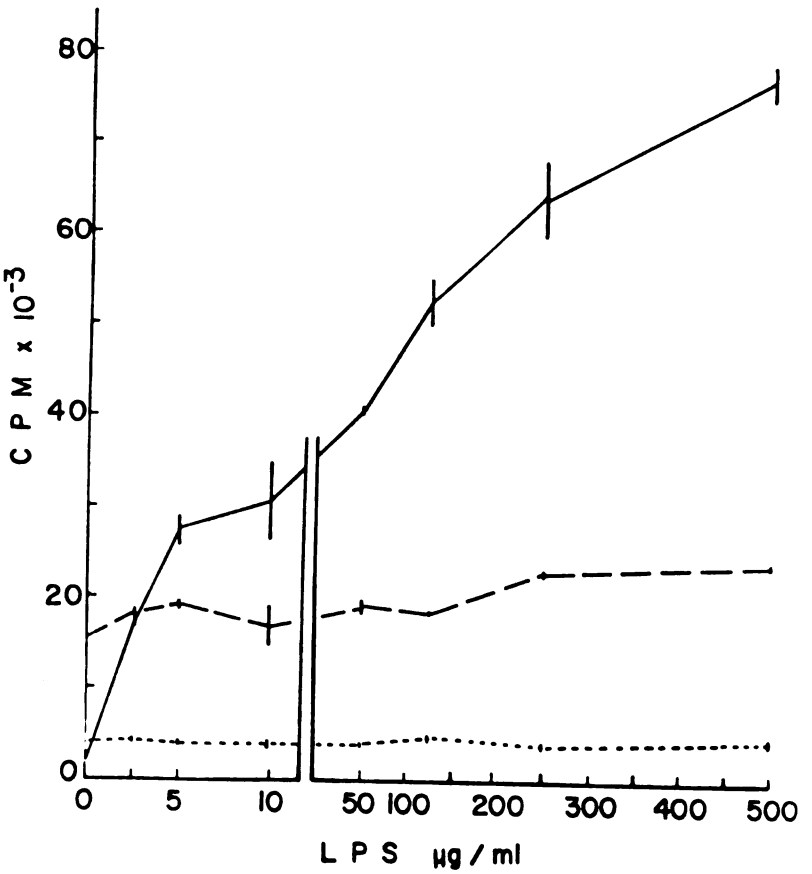


Suppressive effect of T. cruzi epimastigotes at varying concentrations of Con A. Solid curve, spleen cells alone; dashed curve, spleen cells plus parasites; dotted curve, parasites alone. Cultures contained 2.5 x 10^6 spleen cells/ml and/or 2.5 x 10^6 epimastigotes/ml.

FIGURE 3

Suppressive effect of *T. cruzi* epimastigotes at varying concentrations of LPS. Symbols and conditions are as described in the legend to Figure 2. Statistically significant ($P < 0.05$) differences existed between the responses of spleen cell cultures containing and lacking the parasites at LPS concentrations of 5.0 μg/ml or greater.

FIGURE 3



time intervals in cultures containing or lacking the trypanosomes. The concentration of viable spleen cells declined with time in both cases and the rates of such decline were comparable (data not shown).

Inhibition of lymphoproliferation is not due to mitogen absorption by T. cruzi

To establish whether inhibition of cell responses was merely due to mitogen absorption by the parasites, solutions containing the concentrations of Con A and LPS used in most experiments were tested before and after incubation with up to 4 times the parasite concentration used in the experiments described above. The results, presented in Table 1, indicated the occurrence of mitogen absorption in terms of a shift of maximal responses to Con A towards initially higher mitogen concentrations and a slight reduction in LPS-induced responses at some of the tested concentrations. However, significant and even maximal proliferative responses were readily induced by the absorbed solutions, revealing that stimulatory concentrations of Con A and LPS had remained after absorption.

Kinetics of T. cruzi-induced inhibition of lymphoproliferation

Results of experiments in which 2.5×10^5 epimastigotes were added to cell cultures at various times after mitogen stimulation revealed that T and B cell responses were significantly suppressed only when the flagellates were incorporated within the first 12 hr of culture (Fig. 5). It is of interest that the concentration of epimastigotes, added at time 0, increased threefold over the 3-day incubation period of co-culture with the spleen cells (data not shown).

TABLE 1

Mitogenic capacity of Con A and LPS before and after absorption with epimastigotes.*

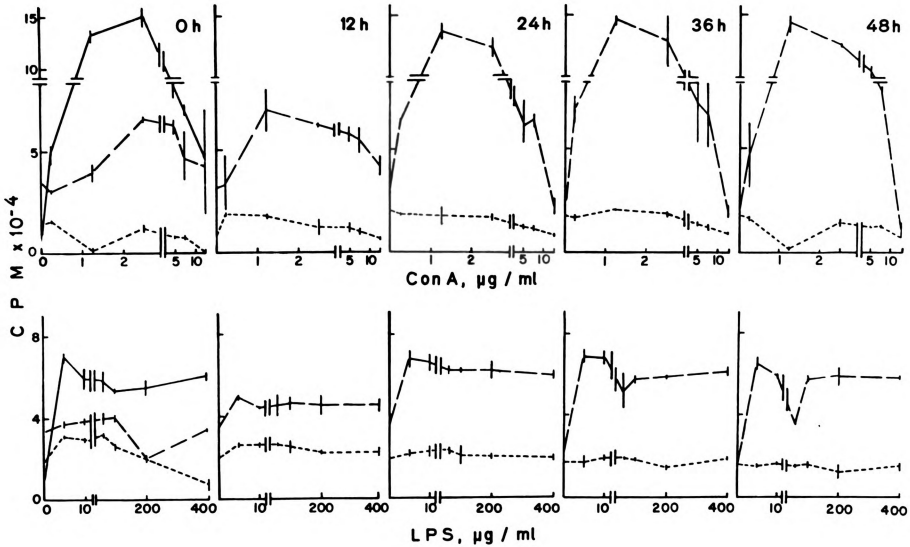
Mitogen	Initial mitogen concentration µg/ml	Mitogen-induced responses (cpm x 10^{-3})	
		Before absorption	After absorption
Con A	0	5.6 ± 1.0	5.6 ± 1.0
Con A	0.5	43.3 ± 11.7	4.4 ± 0.4
Con A	1.25	90.9 ± 5.8	5.8 ± 0.3
Con A	2.5	77.6 ± 5.8	29.6 ± 2.6
Con A	5.0	68.4 ± 3.4	90.1 ± 5.1
LPS	0	2.0 ± 0.6	2.0 ± 0.6
LPS	5.0	21.0 ± 0.9	14.0 ± 0.9
LPS	20.0	27.7 ± 1.2	21.6 ± 1.8
LPS	100.0	28.9 ± 4.3	27.8 ± 4.5
LPS	400.0	26.1 ± 0.5	27.0 _ 4.4

* Results are expressed as the mean of triplicate values ± standard deviation.

FIGURE 4

Kinetics of suppression of Con A- and LPS-induced responses by T. cruzi epimastigotes. Symbols are as described in the legend to Figure 2. The solid curves (cells alone) shown only on the left hand side panels are the control applicable to the other panels in the same row. The times indicated in the upper right had corners represent the time of addition of parasites to mitogen-stimulated cultures. Statistically significant ($P < 0.05$) suppressive effects were recorded only when the parasites were added to the cultures within 12 hr after mitogenic stimulation.

FIGURE 4



Considering the possibility that inhibited lymphocyte responses could have been the result of competitive use of essential medium nutrients by the parasite, experiments were set up in which FTE was substituted for the viable organisms. As can be seen in Table 2, addition of FTE to the spleen cell cultures resulted in significant suppression of Con A- and LPS-induced responses. Two concentrations of each mitogen near optimal stimulatory levels were systematically used in our experiments. Although results are shown for one concentration of each mitogen, similar reductions were observed when the concentrations were doubled.

TABLE 2

Suppressive effects of preparations of nonliving T. cruzi epimastigotes (FTE).

Mitogen	Mitogen Concentration ug/ml	T. cruzi preparation FTE	Response cpm x 10^{-3}	% Reduction*
None	0	None	2.4 ± 0.8	
Con A	1.0	None	51.9 ± 0.7	
Con A	1.0	FTE	18.3 ± 7.5+	64.7
LPS	50.0	None	16.8 ± 5.1	
LPS	50.0	FTE	7.1 ± 4.2+	57.7

* Percentages of reduction were calculated with respect to the corresponding response to the mitogen in the absence of T. cruzi material.

+ The difference between this value and that obtained with the same mitogen in the absence of parasite material was statistically significant ($P < 0.05$).

DISCUSSION

These results highlight the ability of T. cruzi epimastigotes to suppress murine T and B lymphoproliferative responses. The effect was dependent upon the concentration of flagellates and was produced by levels comparable with the usual parasitaemias occurring during the acute phase of the disease, i.e., when immunosuppression is seen (6,13). Although epimastigotes are not found in infected mammalian hosts, the suppressive effect of this form of T. cruzi was similar to that produced by bloodstream forms both in magnitude and parasite concentration requirement (data not shown). Ramos et al. (8), did not observe suppression of mouse spleen cell responses to either Con A or LPS using parasite concentrations up to 5×10^5 organisms/ml. Since suppression in our culture system was not seen until the concentration of epimastigotes reached 2.5×10^6 organisms/ml, the negative results obtained by these investigators might relate to insufficient numbers of flagellates in their cultures.

To be noted, the increased DNA synthesis observed in the absence of mitogen (Figs. 3-5) represented increased parasite multiplication in the presence of cells and not the reverse (data not shown). Mitogen-induced responses were diminished despite such increase, indicating that the extent of suppression of was greater than that represented by the differences in cpm values.

The present results do not disclose whether living organisms and the tested preparations of parasite components (FTE) induced suppression by the same mechanism but do indicate that viability was not a requirement for production of the phenomenon. Whether parasite components present in

FTE absorbed Con A could not be ascertained due to inability to separate soluble parasite material from the mitogen solution after absorption.

Even when mitogen absorption was evidenced by a shift in maximal stimulatory activity following incubation with epimastigotes, sufficient activity remained in the solutions to produce optimal lymphocyte stimulation. Since the absorptions were performed with four times the flagellate concentration present in cell cultures it appears unlikely that the suppressive effect of T. cruzi resulted merely from mitogen removal. This demonstration of Con A binding by the parasite is in keeping with reports by other investigators that Con A agglutinates all forms of T. cruzi (14-16). Also against a possible role for mitogen absorption in the production of suppression were the markedly inhibited lymphocyte responses that were consistently seen when the concentrations of Con A and LPS were increased to levels well beyond those producing optimal responses. In fact, a supraoptimal zone effect was observed at high concentrations of Con A whether or not T. cruzi was present in the cultures, indicating that excess mitogen was present. If mitogen absorption by the parasites had been a major factor in causing suppression, optimal responses would have been recorded before occurrence of a supraoptimal dose effect but this was not the case.

Although epimastigote growth occurred under our culture conditions, three observations rendered unlikely the possibility that reduced lymphocyte responsiveness was due to crowding of the culture by the parasites. First, in experiments carried out with 1×10^7 cells/ml -i.e., the same number of cells as the combined number of cells and

parasites present in co-cultures at the end of the incubation period-optimal responses to Con A or LPS were recorded. Second, living and nonliving (FTE) T. cruzi preparations were equally suppressive. Third, incorporation of ^{3}H-thymidine by the parasites alone was relatively small (Fig. 1-5) and was included in the responses mounted by cultures containing both parasites and spleen cells. These combined responses were significantly lower than those produced by the cells alone.

Significant suppression occurred only when T. cruzi was added to the cultures during the initial 12 hr, suggesting that effective lymphocyte triggering or activation had been impaired. The parasites appeared to affect mechanisms leading to DNA synthesis and not DNA synthesis itself since their addition after 12 hr had no consequence on T or B lymphocyte responses represented by DNA synthesis during the third day of culture. The rate of decline of splenic cells in cultures lacking mitogen but containing parasites was comparable to that of similar cultures to which T. cruzi had not been added. Therefore, reduced responses are unlikely to result from accelerated cell death as a direct result of the presence of the trypanosomes.

Although the present results support the concept that the parasite plays an important role in curtailing immune responses in the infected host, the mechanisms whereby the effect is exerted remain a subject for further study.

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CHAPTER III

INHIBITION OF MITOGEN-INDUCED PROLIFERATION OF MOUSE T AND B LYMPHOCYTES BY BLOODSTREAM FORMS OF *TRYPANOSOMA CRUZI*

ABSTRACT

The role of virulent forms of Trypanosoma cruzi in modulating mitogen-induced lymphocyte responses was investigated in this work. Bloodstream forms of T. cruzi inhibited normal mouse spleen cell responses to Con A and LPS in a dose-dependent manner. Reduced responses were observed over relatively large ranges of concentration of Con A (50-fold) and LPS (160-fold). The inhibitory action of the parasites could not be overcome by increasing the mitogen dose beyond optimal levels. Furthermore, absorption of mitogen solutions with four times as many parasites are used in the proliferation assays revealed that sufficient mitogen activity remained to produce optimal lymphocyte responses. Therefore, reduced lymphocyte responsiveness was not due to absorption of mitogen by the parasite. Inhibited responses were also seen when a sonicated T. cruzi preparation was used, indicating that parasite viability was not required to produce suppression. Inhibition of Con A- or LPS-induced responses by the parasites occurred only when the trypanosomes were incorporated into the system during the first 24 hr of culture. These results show that virulent forms of T. cruzi can induce suppression of T and B cell responses in vitro and suggest that the parasite affects lymphocyte commitment to blastogenesis during the early stages of lymphocyte activation.

INTRODUCTION

A marked immunologic deficiency occurs during the acute period of Chagas' disease or American trypanosomiasis, caused by the unicellular hemoflagellate Trypanosoma cruzi (1-7). The immunosuppressed status that ensues is believed to benefit the parasite during the period of its establishment in host tissues. Several possible mechanisms have been proposed for this immunosuppression. Suppressor T lymphocyte activity has been described in acutely infected mice (1) but has not been confirmed in some laboratories (2-5). Depletion of T lymphocytes (3, 6), presence of a soluble suppressive substance in the serum of infected animals (7), and suppressor macrophage activity (2,8) have also been reported. The possibility that the parasite itself could somehow alter lymphocyte reactivity was initially dismissed by the apparent inability of circulating forms of T. cruzi to affect mitogen-induced responses in vitro(1). However, preliminary results from our laboratory indicated that the flagellates do induce suppression when present in the cultures at appropriate concentrations. In view of this new development, we have re-examined the possibility that virulent bloodstream (trypomastigote) forms of T. cruzi affect proliferative T and B lymphocyte responses.

MATERIALS AND METHODS

Animals. Cultures were set up with cells from 5- to 6-week-old inbred (female) CBA/J mice (The Jackson Laboratory, Bar Harbor, ME), whereas in vivo transfers of the parasite were made in 4-week-old stock CD1 mice (Charles River, Wilmington, MA).

Parasites. The Tulahuen strain of T. cruzi was used. Bloodstream forms were maintained by serial intraperitoneal (ip) passages. Two weeks after infection with 1.5×10^5 organisms, mice were anesthetized with ether and bled by cardiac puncture. The blood was aseptically collected into heparinized tubes and centrifuged at 200 x G for 10 min at 20°C The supernatant was applied on top of a Ficoll-Hypaque mixture of density 1.007 (Lymphoprep, Nyegaard, Oslo) and centrifuged at 400 x G for 45 min at 20°C (9) to separate the free-swimming flagellates (interface). The recovered trypomastigotes were washed three times by centrifugation at 800 x G for 10 min with RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 100 U/ml penicillin and 100µg/ml streptomycin (RPMI 1640A) and adjusted to the appropriate concentrations with the same medium. Parasite concentrations were measured microscopically by using a Neubauer hemacytometer.

Sonicated T. cruzi preparation (STC). Suspensions containing 1×10^7 purified T. cruzi/ml in RPMI 1640A were subjected to four 45-sec sonication pulses while maintained refrigerated in a dry ice bath. After centrifugation at 800 x G for 15 min at 4°C , the supernatant was filtered through a 0.45-µm pore-size filter (Millex;

Millipore Corp., Bedford, MA) and used immediately. When added to cell cultures, this preparation provided an amount of trypanosomal material equivalent to 2.5×10^6 organisms/ml of culture. In some experiments STC was used after dialysis against 500 vol of RPMI 1640A at 4°C.

Cells. The spleens of CBA/J mice were aseptically removed and converted to single-cell suspensions in ice-cold RPMI 1640A by using a Ten Broeck tissue grinder (two strokes only). The preparations were filtered through a sterile nylon gauze to remove tissue debris, and the cells were washed three times with RPMI 1640A by centrifugation. Cells were counted by using a hemacytometer and were adjusted to the appropriate concentrations of viable trypan blue-excluding nucleated cells per ml.

Mitogens. Concanavalin A (Con A; Sigma Chemical Co., St. Louis, MO) and endotoxic lipopolysaccharide (LPS) extracted from Escherichia coli O55:B5 by the phenol-water method (Difco, Detroit, MI) were used to stimulate cell cultures. Repeated purchases of Con A and LPS during the period of this study resulted in the use of different batches. Solutions of these mitogens were prepared in RPMI 1640A and sterilized by filtration through Millipore filters of 0.45μm pore size.

Mitogen assays. Duplicate or triplicate cultures were set up in flat-bottom microculture wells (Linbro, New Haven, CT). Each culture consisted of 0.1 ml containing either 2.5×10^5 or 5.0×10^5 cells alone or cells plus varying concentrations of parasites (see Results), and the appropriate mitogen concentration. All cultures contained 2.5% heat-inactivated fetal bovine serum (Sterile Systems, Logan, UT). The cultures were incubated at 37°C in a 5% CO_2-in-air incubator

saturated with water vapor for 72 hr. One μCi of ^{3}H-thymidine (specific activity 2 Ci/mmol; New England Nuclear, Boston, MA) was added to each well 24 hr before termination of the cultures. An automated culture harvester (MASH II, Microbiological Associates, Walkersville, MD) was used to process the cultures for measurement of ^{3}H-thymidine incorporation into synthesized DNA in a liquid scintillation counter. Results were expressed as the mean counts per minute ± standard deviation. Control cultures were simultaneously set up to which mitogens were not added. Additional controls consisted of parasites alone cultured with and without mitogen.

Kinetic studies. Spleen cell cultures containing 2.5 x 10^{6} cells/ml were stimulated with various concentrations of Con A or LPS and received trypomastigotes to provide a final concentration of 2.5 x 10^{6} organisms/ml at predetermined times after mitogenic stimulation. Cell cultures were set up at 12 hr intervals but received the parasites at the same time, i.e., at different times after initiation of the cultures. Additional cultures were set up at each time interval that received RPMI 1640A instead of the parasite suspension. Cultures of the parasites alone in the presence or absence of mitogen as well as mitogen-free cell cultures were also included. After 48 hr the cultures received 1 μCi of ^{3}H-thymidine and were terminated 24 hr later as described above.

Absorption of mitogen by T. cruzi. Solutions of Con A and LPS whose concentrations were the same as those used in the mitogenic assays were incubated with 1 x 10^{7} trypomastigotes/ml at 37°C for 24 hr in a 5% CO_2 incubator. After removal of the parasites by centrifugation, the supernatants were tested for residual mitogenic activity.

Presentation of results and statistics. Sets of data presented in this paper are typically representative of two or more experiments of identical design. Differences were considered to be statistically significant if $P<0.05$ as calculated by Student's "t" test.

RESULTS

Inhibition of mitogen-induced lymphoproliferation by T. cruzi trypomastigotes. Although optimal mitogen-induced responses were mounted by cultures containing up to 1 x 10^7 spleen cells/ml (data not shown), 2.5 x 10^6 cells/ml was the concentration selected for most subsequent experiments. When these cultures were stimulated with optimal concentrations of Con A (2.5 µg/ml) or LPS (50 µg/ml), addition of T. cruzi trypomastigotes significantly reduced H-thymidine incorporation. The minimal concentration of parasites that induced such an effect was 2.5 x 10^6 parasites/ml (Figs. 1 and 2). Therefore, subsequent experiments were performed by incorporating into the culture system parasites at 2.5 x 10^6 organisms/ml. This number of trypanosomes consistently suppressed mitogen responses by cultures containing up to 1.0 x 10^7 cells/ml, i.e., four times as many cells as present in our standard culture system (data not shown). In experiments in which the concentrations of Con A or LPS were varied over wide ranges, the inhibitory effects of the trypomastigotes were readily reproduced (Figs. 3 and 4). Similar results were obtained when the number of cells and parasites were doubled, whereas other conditions remained unchanged (data not shown). Supraoptimal responses were produced with Con A whether the parasites were present or not. It should be noted that the actual lymphocyte responses mounted in the presence of T. cruzi would have to be smaller than those depicted in Figures 2 and 3 because these data represent the combined uptake of ^3H thymidine by cells and parasites.

FIGURE 1.

Dose dependence of trypomastigote-induced inhibition of mouse lymphocyte responses to Con A. (——) cells plus parasites (-----) parasites alone. The concentration of Con A was 2.5 μg/ml.

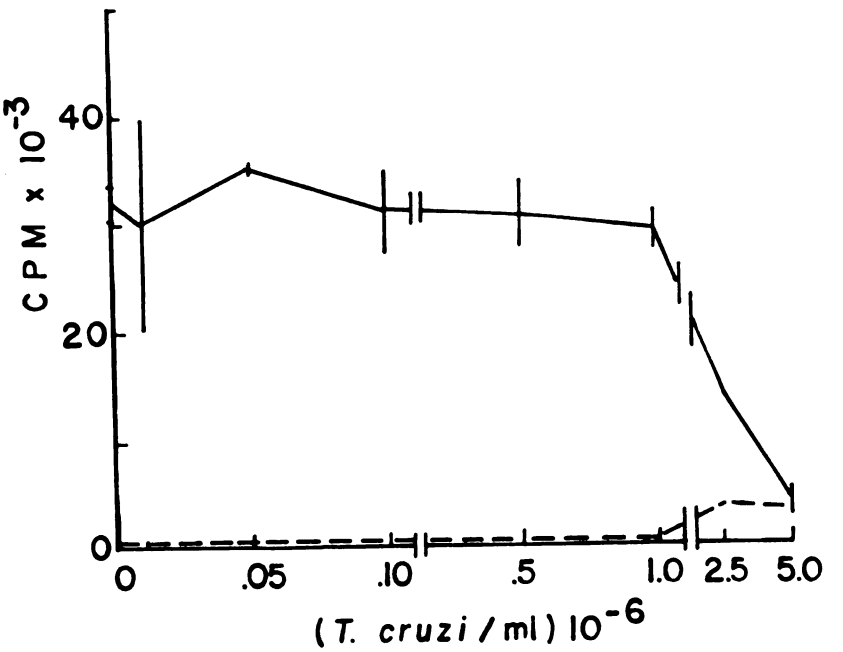


FIGURE 2

Dose dependence of trypomastigote-induced inhibition of mouse lymphocyte responses to LPS. (——) cells plus parasites; (----) parasites alone. The concentration of LPS was 50 μg/ml.

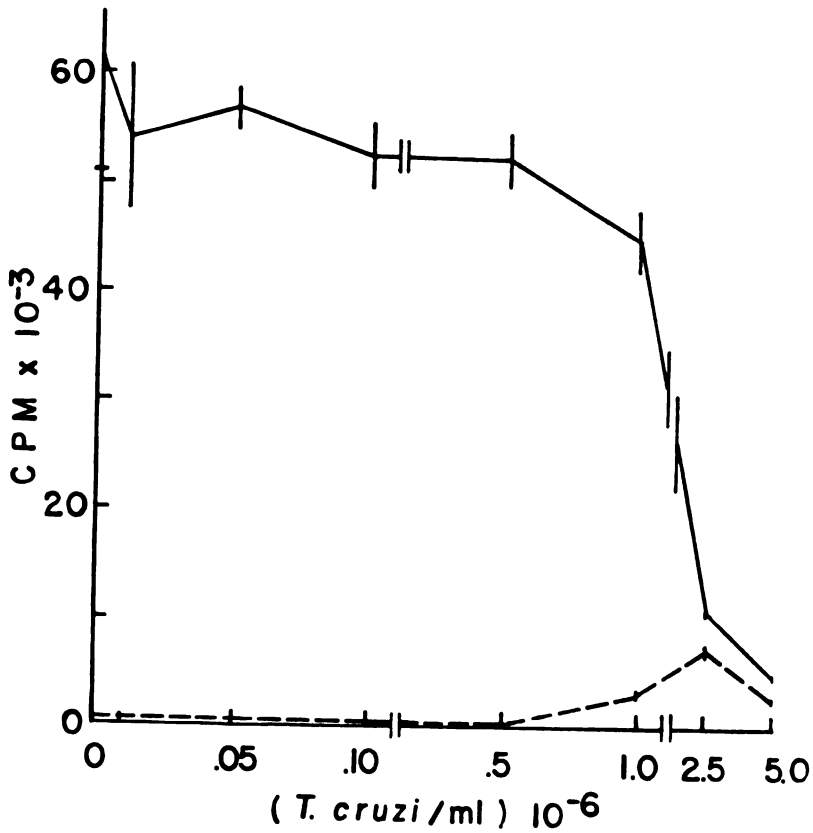


FIGURE 3

T. cruzi-induced inhibition of mouse lymphocyte responses to Con A over a full dose range. (——) cells alone; (----) cells plus parasites; (.....) parasites alone. Differences between the responses of cells in the presence and absence of parasites were statistically significant ($P < 0.05$) for all mitogen concentrations.

FIGURE 3

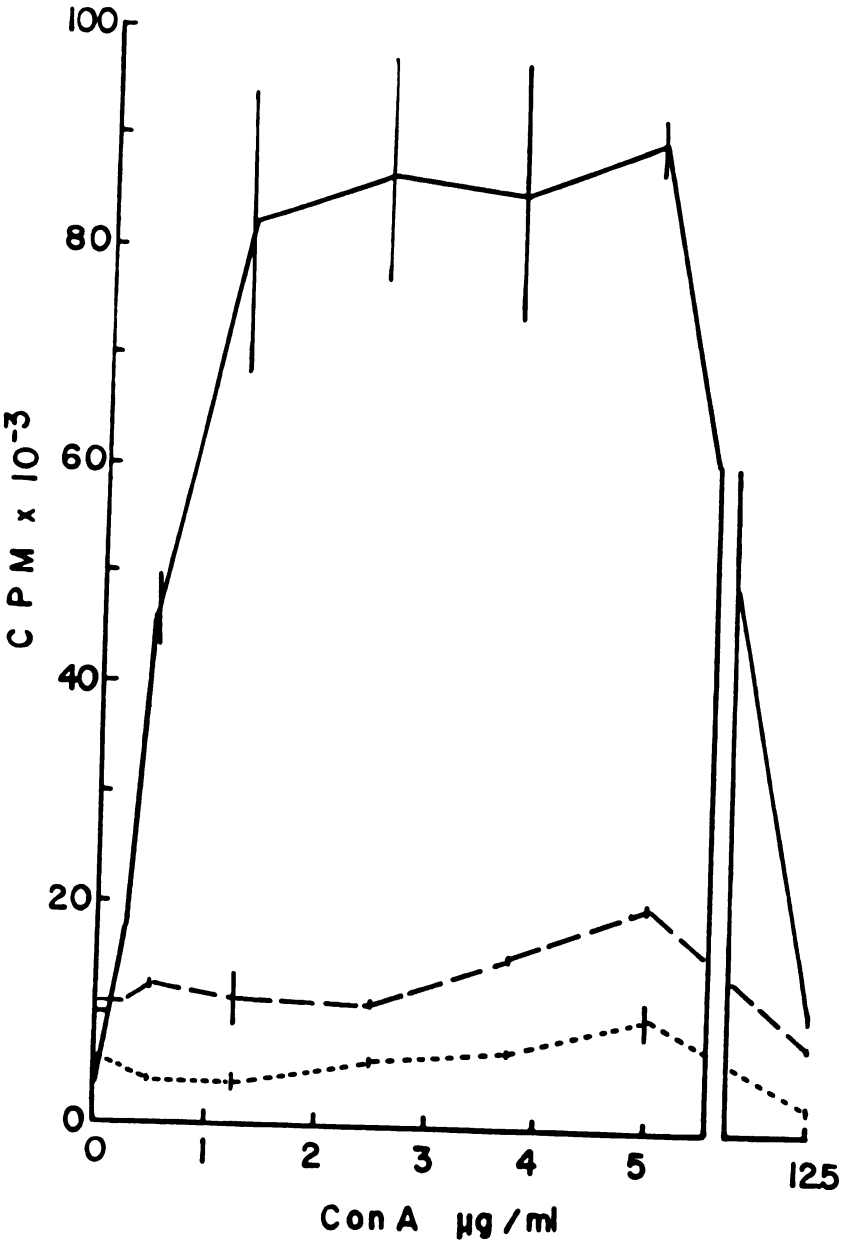
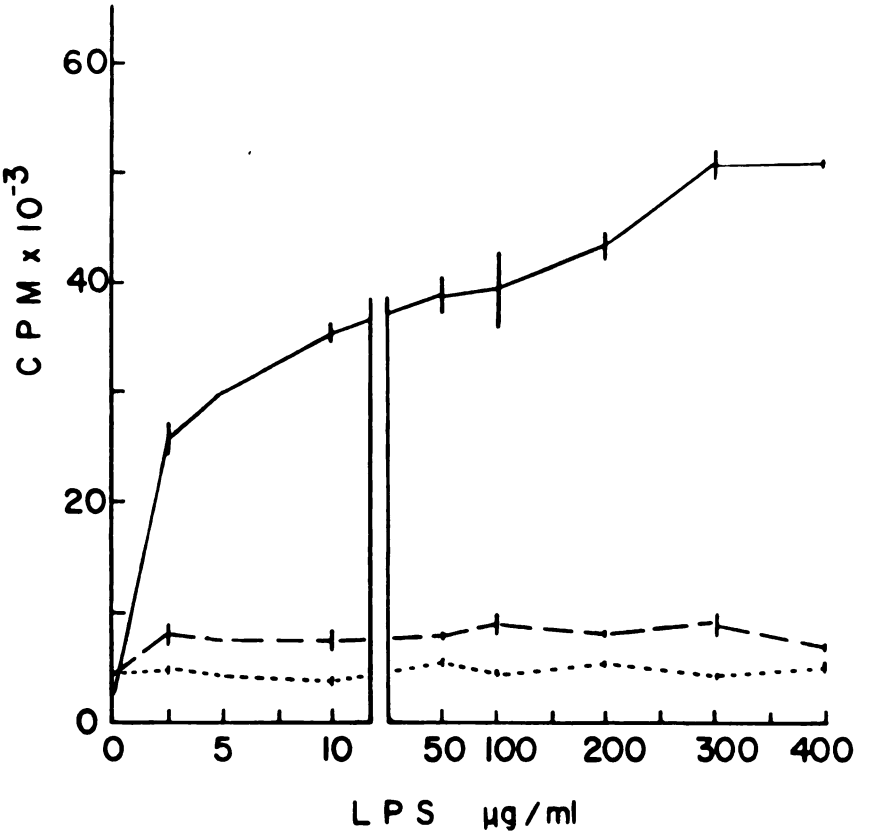


FIGURE 4



T. cruzi-induced inhibition of mouse lymphocyte responses to LPS over an extended dose range. Symbols and statistics are as described in the legend to Figure 3.

The concentrations and viability of spleen cells measured after 24, 48, and 72 hr of incubation in cultures containing or lacking T. cruzi were comparable (data not shown). Therefore, impaired responsiveness was not likely to be due to production of cell death by the parasites.

Because T. cruzi is known to bind Con A (9-11), the possibility that reduced cell responsiveness might have resulted from absorption of mitogen by the parasite was examined. Results presented in Table 1 showed that despite absorption of Con A solutions with 1×10^7 trypomastigotes/ml (i.e., four times the concentration of T. cruzi present in the cultures), significant mitogenic activity remained to induce maximal responses. However, maximal responses were produced with lower dilutions of the absorbed mitogen solution, indicating that a certain amount of Con A had been removed by the parasites.

Marked reductions in parasite concentration (75 to 85%) were recorded 24 hr after initiation of the cultures whether spleen cells were present or not (data not shown). No additional loss of living organisms was observed thereafter. Considering the possibility that parasite products might be suppressive and that viability of the flagellate may not be a requirement for production of suppression, we tested the effect of addition of STC to spleen cell cultures. Spleen cells incubated with an amount of STC equivalent to 2.5×10^6 trypomastigotes/ml showed significantly reduced responses to either Con A of LPS with respect to those of cultures free of STC (Table 2). Similar results were obtained with dialyzed STC (data not shown).

TABLE 1

Mitogenic capacity of solutions of Con A before and after absorption with trypomastigote forms of T. cruzi.

Con A Dilution	Mitogen-Induced Responses[b] (cpm x 10^{-3})	
	Before absorption	After absorption
None	0.9 ± 0.3	0.9 ± 0.3
1/10	32.0 ± 0.5	1.1 ± 0.6
1/4	125.3 ± 12.7	3.8 ± 3.4
1/2	149.4 ± 15.2	61.8 ± 3.1
Undiluted[a]	13.1 ± 5.8	149.6 ± 4.5

[a] The highest culture concentration of Con A (before absorption) was 5.0 µg/ml.

[b] Results expressed as mean cpm ± one standard deviation.

TABLE 2

Inhibition of Con A- and LPS-induced mouse lymphocyte responses by STC.

Mitogen	STC[a]	Mitogen-Induced Responses[b] (cpm x 10-4)	Percent Reduction
Con A (μg/ml)			
1.25	Absent	7.1 ± 0.2	
1.25	Present	3.1 ± 0.1	56
LPS (μg/ml)			
10.0	Absent	5.3 ± 0.1	
10.0	Present	0.3 ± 0.06	94

[a] Amount equivalent to 2.5 x 10^6 trypanosomes/ml.

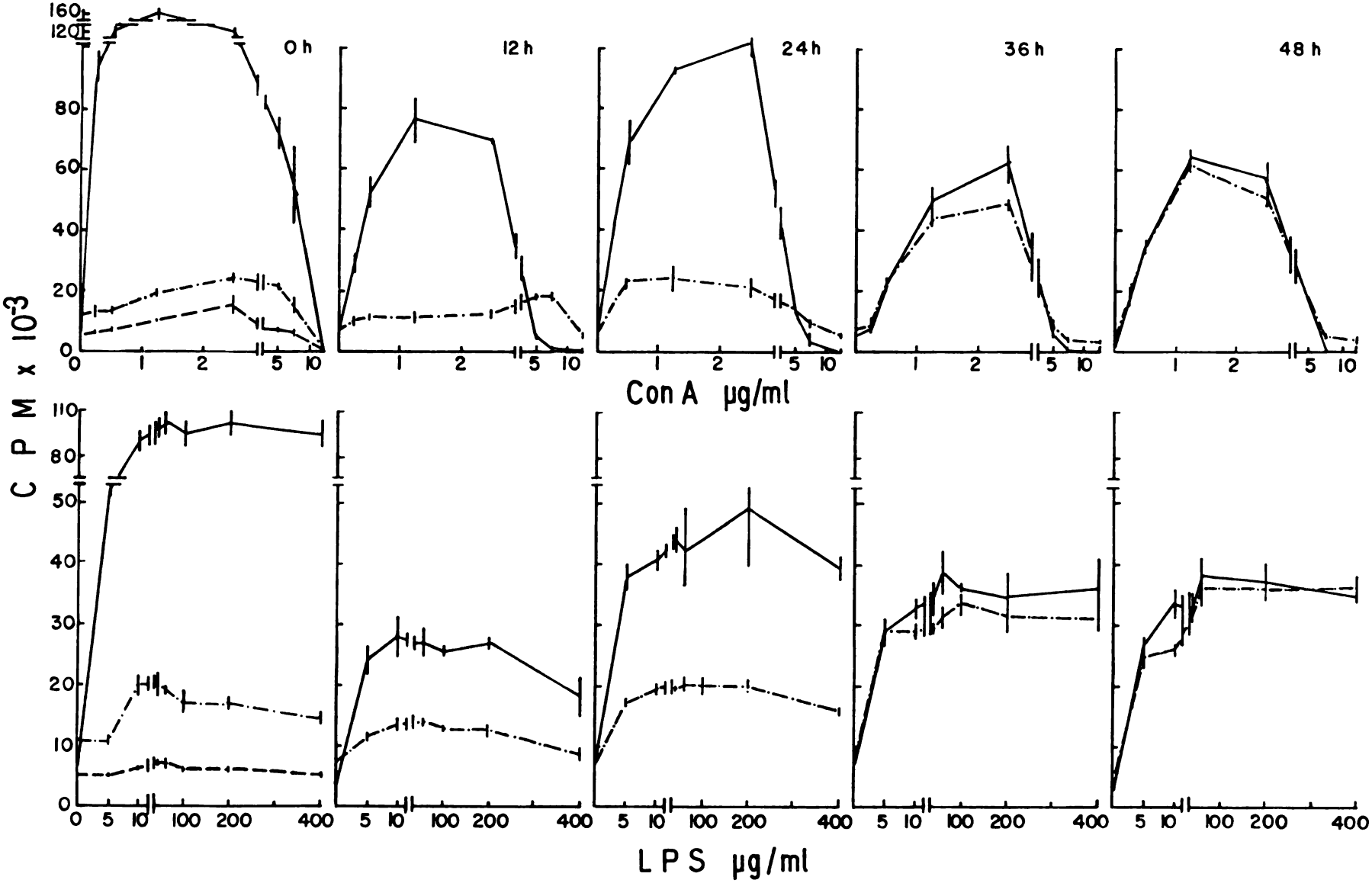
[b] Results expressed as means cpm ± one standard deviation.

In experiments designed to study the kinetics of parasite-induced inhibition of lymphocyte responses to Con A and LPS, significant decreases were seen only in cultures that received the trypanosomes within 24 hr of initiation (Fig. 5).

FIGURE 5

Kinetics of T. cruzi-induced inhibition of mouse lymphocyte responses to Con A and LPS. The times indicated in the upper right corners represent the time of addition of parasites to mitogen-stimulated cultures. Cells from different groups of mice were used for each time condition. (——) cells alone; (-·-) cell cultures receiving parasites; (-----) parasites alone. The "parasite alone" control is applicable to all time conditions. Differences between the responses of cell cultures containing and lacking T. cruzi were statistically significant ($P < 0.05$) for all concentrations of LPS and all concentration of Con A except 5 through 12.5 μg/ml for the experiments in which the trypanosomes were added at 0, 12, and 24 hr. None of the other differences (36 hr and 48 hr) were statistcally significant.

FIGURE 5



DISCUSSION

The present results demonstrate the inhibitory effect of virulent bloodstream forms of T. cruzi on mitogen-induced proliferation of mouse T and B lymphocytes. Ramos and co-workers (1) did not observe significant reduction in responses to either Con A or LPS when they added up to 1 x 10^5 organisms to 0.2-ml mouse spleen cell cultures (i.e., 5 x 10^5 parasites/ml). In our work, the minimal concentration of trypanosomes that caused a significant reduction in spleen cell responses to the mitogens was 2.5 x 10^6 organisms/ml. This minimal effective concentration of parasites falls within the usual range of parasitemias seen in acutely infected CBA/J mice (6). This implies that this condition for occurrence of parasite-induced suppression exists in infected hosts and may contribute to the severe immunosuppression that is seen during the acute period of the disease (13-16).

Whether LPS binds to circulating forms of T. cruzi is not known, but the binding of Con A to these forms has been reported (10-12). Two lines of evidence denied that absorption of Con A to the parasites may have caused an apparent lack of lymphocyte responsiveness. First, supraoptimal zones (decreased responses) were produced with the identical concentration of Con A whether the parasites were present in the cultures or not, demonstrating the presence of excess mitogen in both systems. If significant binding of Con A by the parasites had taken place, increasing responses would have been expected to occur as the concentration of Con A increased. However, this was not the case (Fig. 3 and Fig. 5, upper left

panel). With LPS, the parasites inhibited responses even after a 160 fold increase in mitogen concentration (Fig. 4). It is therefore unlikely that absorption of LPS to T. cruzi, if occurring at all, was responsible for the inhibitory effect. Second, the solutions of Con A used in the mitogenic assays displayed suboptimal and optimal stimulatory activity even after being absorbed with four times as many parasites as present in the inhibition assay system. Therefore, stimulatory levels of mitogen should have been present in the cultures. Crowding of the cell cultures by the parasites and competition between cells and parasites for essential nutrients were considered as hypothetical alternatives to a suppressive mechanism. Crowding by the trypanosomes (2.5×10^6 parasites/ml) seemed unlikely in view of the optimal responses that were mounted by cultures containing up to 1×10^7 cells, i.e., twice the total number of cells and trypanosomes present in the mitogenic assays. Furthermore, H-thymidine incorporation by the parasites was relatively small (Figs. 1-5) and was included in the responses mounted by cultures containing both cells and parasites. These combined responses were nevertheless significantly lower than the mitogenic responses produced by the cells alone. Also against crowding was the finding that STC, which did not contain living trypomastigotes, was also suppressive. This observation ruled out possible competition for nutrients as a cause for the noted inhibition. Of interest, suppression of responses to sheep erythrocytes after intravenous injection of a freeze-thaw preparation of T. cruzi has been seen in mice (17). The suppressive action of STC also suggested that presence of live trypanosomes may not be a requirement for production of immunosuppression.

T. cruzi induced significant suppression only when added to the cultures during the initial 24 hr period. This is the time during which mitogens must be present to commit lymphoid cells to blastogenesis (18-20). Thus, it is conceivable that the parasites are interfering with effective lymphocyte triggering or activation. Our results also suggest that reduced responsiveness is not a consequence of direct interference with DNA synthesis by the parasites, because flagellates added after 24 hr remained in the culture until termination but were unable to cause significant alterations in cell responses to the mitogens.

These results highlight a potentially important role for bloodstream forms of the parasite or their products in inhibiting lymphocyte proliferation, which may be relevant to the immunosuppression that ensues during acute infection with *T. cruzi*.

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SUMMARY AND CONCLUSIONS

Immunosuppression during acute Chagas' disease is both severe and complex in its expression. Reactivity to all of the immunologic stimuli tested to-date. Therefore, it has been difficult for researchers to elucidate the mechanisms that control and protect against the infection.

The first part of this study analyzed the cellular immune responses for T. cruzi antigen during the course of infection with this parasite. This work provided a chronological occurrence, duration, and extent of the immunodeficiency state. Significant differences in footpad swelling or inhibition of peritoneal macrophage migration were observed until day 40 post infection. The reappearance of responsiveness correlated with the disappearance of parasitemias and mortalities. Effective immune responsiveness was maintained in the chronic phase of the disease. This report does not support a major role for suppressor T lymphocytes because a) mixing cells from chronically infected mice with up to 50% cells from acutely infected mice failed to alter the responses of chronic mouse spleen cells. Furthermore, removal of Lyt 2.1-bearing cells did not restore responsiveness to cells from acutely infected mice.

The second part of this research was concerned with exploring the possibility that T. cruzi can alter lymphocyte responses. It was established that both culture (epimastigote) and virulent bloodstream (trypomatigote) forms are capable of reducing mouse splenic cell responses to mitogens. Of particular interest was the initial finding that production of the suppression was directly linked to parasite concentration. The minimal concentration necessary to demonstrate

suppression was well within the levels of parasites present in the acutely infected animal. Therefore, the conditions for production of suppression are given in the infected host. It could also be speculated that these findings may illustrate a possible reason why immunosuppression is not seen in chronically-infected animals.

Two pieces of evidence indicated that the immunosuppression was not solely due to mitogen removal by the parasites. First, mitogen solutions absorbed with either epimastigotes or trypomastigotes were still stimulatory. Second, the suppression could not be overcome with concentrations of mitogen far exceeding those necessary to produce significant responses.

Non-living preparations of both forms of T. cruzi were also demonstrated to be suppressive. It can be concluded from these findings that parasite viability is not a requirement for production of suppression. Secondly, suppression was not merely due to competition for essential nutrients in the culture media.

Presence of epimastigotes and trypomastigotes was required within the first 24 hr of incubation of the cultures for suppression to be seen, suggesting that the action of the parasites is realized during the early (induction) phase of lymphocyte activation.

Recent work, described in the Appendix, focused on the cell(s) which are sensitive to the suppressive effect of the trypanosomes. Results indicated that macrophage-depleted cultures were subject to suppression induced by the parasite. Addition of increasing numbers of adherent cells to cultures containing non-adherent cells did not decrease responsiveness. Adherent cells treated with trypomastigotes were unable

to negatively affect non-adherent cell responses. These results, taken together, indicated that the parasites are able to regulate lymphocyte function directly and that macrophages are not a requirement for suppression to be demonstrated. These results do not rule out the possibility that macrophage are the mediators of parasite-induced suppression *in vivo*.

In conclusion, the significance of this work is several fold. It is the first definitive report to describe host CMI to a *T. cruzi* antigen during the course of experimental infection with the parasite. Second, it advanced the viewpoint that suppressor T lymphocytes are not involved in production of immunosuppression. Finally, it established a role for the parasite in directly modulating lymphocyte functions and gave evidence to the cells involved. Thus, this research gives insight into the understanding of the mechanisms involved in host-*T. cruzi* interactions underlying Chagas' disease.

APPENDIX I.

Occurrence of immunosuppression during acute Chagas' disease has been well documented. Several mechanisms have been proposed to clarify this phenomenon. Ramos and coworkers (1) described the presence of suppressor T lymphocytes. However, several other studies have not confirmed their presence (2-5). Adherent cells as mediators of suppression in this system have been reported some workers (2,6). Recently our laboratory established a role for Trypanosoma cruzi in modulating lymphocyte function (7,8). Culture forms (7) or virulent bloodstream forms (8) of T. cruzi when incorporated into mitogen-induced DNA synthesis assays significantly decrease the normal mouse spleen cell response. In this work we advance the understanding of this mechanism by examining the involvement of the two major populations of cells known to interact in the production of immune responses, non-adherent cells and adherent cells and their possible roles in parasite-induced immune suppression.

Spleen cell suspensions were prepared from six-week-old CBA/J mice (Jackson Laboratories, Bar Harbor, Me) as described elsewhere (8) but with the following modifications. Erythrocytes were removed by density gradient separation over Ficoll-Hypaque (Lymphoprep, Nyegaard, Oslo). Bloodstream forms of Tulahuen strain of T. cruzi were isolated from acutely infected CD1 mice at the time of maximal parasitemias and purified by a two-step method described previously (8). Cell responsiveness was assayed by measuring the uptake of radiolabeled thymidine in response to stimulation with either concanavalin A (Con A) or a bacterial lipopolysaccharide (LPS) (8). Non-adherent cells were

TABLE 1

The effect of addition of varying numbers of treated or un-treated adherent cells on the non-adherent cell response to Con A and LPS in the presence or absence of T. cruzi.

Mitogen*	% AD	TC	TC Treatment**	Mean cpm x 10^{-3}	% Decrease***
Con A	0	-	-	56.9 ± 6.7	-
	0	+	-	10.1 ± 0.8	82.2
	0.1	-	-	58.8 ± 4.8	-
	0.1	+	-	9.0 ± 0.6	84.7
	0.1	-	+	53.8 ± 4.0	8.5
	1.0	-	-	68.1 ± 6.8	-
	1.0	+	-	8.2 ± 0.3	88.0
	1.0	-	+	55.8 ± 3.0	18.1
	10.0	-	-	82.6 ± 8.2	-
	10.0	+	-	8.7 ± 0.2	89.5
	10.0	-	+	80.5 ± 0.0	2.5
LPS	0	-	-	23.8 ± 0.2	-
	0	+	-	4.1 ± 0.5	82.8
	0.1	-	-	22.5 ± 1.1	-
	0.1	+	-	8.4 ± 1.4	62.2
	0.1	-	+	19.9 ± 0.7	11.6
	1.0	-	-	23.6 ± 0.4	-
	1.0	+	-	7.7 ± 0.6	67.4
	1.0	-	+	22.8 ± 3.9	3.4
	10.0	-	-	26.9 ± 0.0	-
	10.0	+	-	7.9 ± 0.1	70.6
	10.0	-	+	25.6 ± 0.7	4.8

* Con A, 1.0 ug/ml, LPS 50 ug/ml.

** Adherent cells treated for 1.5 hr with T. cruzi (ratio of cells to parasite was 5:1).

*** % decrease with respect to untreated cells in the absence of parasites.

purified by three steps of plastic adherence at 37°C. Cells attached to plastic petri dishes after the first step of adherence were rinsed with RPMI 1640 medium (GIBCO, Grand Island, NY) and either treated with parasites (the ratio of parasites to adherent cells was 5:1) or medium for 1.5 hr at 37°C. T. cruzi-treated or medium-treated adherent cells were harvested, washed, and added at varying concentrations (see Table 1) to mitogen-stimulated cultures containing 2.5×10^6 non-adherent cells/ml.

As can be seen in Table 1, non-adherent cells produced significantly lower responses to either Con A or LPS in the presence of parasites than cultures incubated in the absence of parasites. Increasing levels of medium-treated adherent cells significantly enhanced the response of normal non-adherent cells to the mitogens only when T. cruzi was not present in the system. The effect of adherent cells which had been pretreated with T. cruzi on the response of normal lymphocytes did not differ significantly from that caused by medium-treated adherent cells.

These results indicate that bloodstream forms of T. cruzi are able to modulate lymphocyte responses to mitogens in the absence of macrophages. This is the first report indicating a direct effect by the parasites on lymphocytes. These results do not demonstrate or rule out the possibility that the trypanosomes induce their suppressive effect on lymphocytes through macrophage modulation.

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