# STUDIES ON THE IMMUNOBIOLOGY OF *TRYPANOSOMA LEWISI* INFECTIONS IN RATS

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## ABSTRACT

The immunological responses in hosts infected with Trypanosoma lewisi were examined during the course of infection and after recovery. Peak antibody levels coincided with the time of parasite elimination, but remained significantly elevated for over one year after the end of the infection. The antigen repertoire recognized by antibodies demonstrated that some were revealed only by sera taken during the infection, and other antigens were revealed for the first time only by post-recovery sera. Immunomodulatory protective and suppressive factors were demonstrated in the plasma of irradiated, infected rats. These factors were identified as parasite-derived exoantigens which are shed in vivo and in vitro; exoantigens are complexes of proteins, lipids and polysaccharides and are membrane-surface coat associated, as shown by phase-partitioning and surface-labeling studies. The suppressive activity of the exoantigens was dose-dependent, probably mediated by a suppressor substance(s) produced by macrophages that subsequently inhibits production of interleukin 2 by T helper cells.

ABREGE

La réponse immune chez l'hôte infecté à Trypanosoma lewisi fût etudiée durant l'infection ainsi qu'àpres la guérison. Une corrélation temporelle fût mise en évidence entre le taux maximum d'anticorps et l'élimination du parasite, bien que ces taux restèrent élevés plus d'un an après la guérison. Le répertoire antigénique reconnu par les anticorps a mîs en évidence le fait que certaines spécificités antigéniques n'étaient reconnues que par des sérums d'animaux en cours d'infection, tandis que d'autres antigènes distincts étaient reconnus par des sérums d'animaux guéris. En outre, des facteurs immunomodulatoires (suppresseurs ou protecteurs) fûrent démontrés dans le plasma de rats infectés après irradiation. Ces facteurs fûrent identifiés comme étant des exoantigènes du parasite qui sont libérés in vivo et in vitro: les exoantigènes sont des complexes de protéines, lipides et polysaccharides et sont associés à la membrane, tel que démontré par des études de marquage et de séparation de phases. De plus, il a été démontré que l'activité suppressive des exoantigènes était dépendante de la dose, médiée probablement par des substances suppressives produites par les macrophages qui inhibent subséquemment la production d'interleukine-2 par les cellules T "helper".

To Njoki and Wangu

Suggested short title:

Immunobiology of *Trypanosoma lewisi* infections

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## CLAIM OF ORIGINALITY

The present studies are original and contribute to scientific knowledge for the following reasons:

1. Although humoral responses play a major role during the course of infection with Trypanosoma lewisi, studies presented in Chapter III are the first to demonstrate the kinetics of this response in relation to the level of infection. The persistence of these antibodies at significantly high levels is implicated with protective immunity in recovered animals. These studies are also the first to demonstrate the functional kinetics of protective immunity to an induced secondary parasitemia in recovered animals: the protective immunity was unaffected by the duration post-recovery nor by treatment with tolerable doses of the immunosuppressive agent cyclophosphamide.

2. Animals that have recovered from Trypanosoma lewisi infections are known to be solidly immune; this immunity is thought to develop only after recovery from a conventional primary infection. Studies presented in Chapter IV are the first to show, by drug-abbreviated infections, that protective immunity develops very early during the epimastigote phase of the infection, and probably before the appearance of the parasites in the peripheral circulation; protective immunity does not require the trypomastigote phase to be fully functional.

3. Although exoantigens have been demonstrated in the sera of animals infected with Trypanosoma lewisi, their role and the amount of circulating exoantigens during the course of infections has not been

previously established. Studies in Chapter V are the first to demonstrate *in vivo* that plasma from irradiated, infected rats, which contains high levels of exoantigens, can induce suppression or protection in naive normal animals, depending on the time of challenge with the parasite following plasma treatment. *In vitro* studies indicate that a high concentration of exoantigens suppress normal spleen cell stimulation by mitogens, whereas, low levels enhance; these results correlate with the level of circulating exoantigens *in vivo* and the pattern of infection.

4. Studies presented in Chapter VI are also the first to demonstrate, to isolate and to characterize the exoantigens of *Trypanosoma lewisi* derived from *in vitro* culture system.

5. Surface glycoproteins have been shown to play a major role in the induction of immunological responses during parasitic infections Studies in Chapter VII are the first to isolate and characterize membrane-associated glycoproteins by phase partitioning, SDS-PAGE, Western blotting and surface iodination. This study is the first to determine not only the effect of phase-partitioned hydrophilic (protein-rich) and amphiphilic (glycolipoprotein-rich) fractions on mitogenic responses of lymphocytes *in vitro* but also their effect to immunize naive animals.

## PREFACE

The results described in these studies are presented in five chapters after the general introduction (Chapter I) and the literature review (Chapter II), followed by the summary and general discussion. Figures and tables are numbered separately for each chapter and are presented at the end of each chapter.

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# CHAPTER I: INTRODUCTION

Trypanosomes were first described nearly 300 years ago by the Dutch biologist Antony van Leeuwenhoek who reported numerous small animacules in the fluid contents taken from the gut of a horsefly, these animacules were probably the bovine parasite Trypanosoma theileri. By mid nineteenth century, several observations of trypanosomes were recorded by scientists, and the generic term Trypanosoma was used for the first time by Gruby (1843). Since the association of trypanosomes with the disease nagana was first established late in the nineteenth century by David Bruce (after whom Trypanosoma brucei was named), extensive research has been done worldwide in a bid to understand the host-parasite relationship and the control of these parasite infections. Today, several species of the genus Trypanosoma have been incriminated as the causative agents of disease in both humans and animals. Chagas' disease (American trypanosomiasis), occurring exclusively in South America, is caused by the intracellular trypanosome Trypanosoma cruzi; this disease is mainly manifested by extensive cardiac and gastro-intestinal lesions and may occur either in an acute or in a chronic form. African trypanosomiasis (Sleeping sickness) on the other hand is caused by the extracellular zoonotic parasite Trypanosoma brucei and is a disease characterized by acute febrile lymphadenopathy during the early stages and chronic meningoencephalomyelitis at the terminal stages. Up to 10,000 cases of clinical sleeping sickness are reported annually in man in Africa while 35 million are at risk (Roelants 1986); an estimated 3 million cattle are reported also to die (Bloom 1979) every year from

this parasite. The economic impact of animal trypanosomiasis cannot, therefore, be underscored, especially with the knowledge that up to 7 million square kilometers (Roelants 1986) of the vast savannah land south of the Sahara are infested with the tse tse fly vectors of Trypanosoma brucei, T. congolense and T. vivax, the most common parasites causing nagana. Although these trypanosomes are constantly in close association with the immune system of the host, infections are invariably fatal, unless chemotherapy is instituted at the early stages of the disease. African trypanosomes have evolved mechanisms that effectively evade the immune responses of the host in the peripheral circulation by altering the surface coat glycoprotein (VSG) and by inducing a severe immunodepression to specific and non-specific antigens; T. cruzi survives inside macrophages, rendering these phagocytic cells infective by suppressing the digestive enzymes of the cell.

To date, immunological studies pertaining to trypanosome infections has mainly been done in laboratory animal models. While these studies have provided information regarding the immunological responses of the infected hosts, their validity has been constantly questioned as to their representation of the natural host-parasite relationship. In support of this argument is the observation that immunodepression in vivo, or the suppression in vitro of stimulation by mitogens, characteristic of mice infected with pathogenic African trypanosomes (Bancroft and Askonas 1985), is inapparent in cattle infected for upto 5 1/2 months (Masake and Morrison 1981).

Trypanosoma lewisi and other related rodent trypanosomes are host specific, show moderate pathogenicity and complex biological and immunological equilibria that must have developed through a long period

of association with their hosts. Although Trypanosoma lewisi may differ from the pathogenic trypanosomes by their limited antigen repertoire, the immunological and pathological manifestations which occur in infected rodent hosts are essentially similar, with the exception that they are more severe in infections by salivarian trypanosome. It is because of these characteristics that T. lewisi has been used as a laboratory model for the study of the sophisticated concepts of modern trypanosome immunology.

## CHAPTER II: LITERATURE REVIEW

### 1. HISTORY AND TAXONOMY

Trypanosoma lewisi was first reported by Chaussat (1850) more than a century ago in the blood of rats, where it was mistakenly regarded as nematode larvae. However, Lewis (1878) correctly identified this organism as a hemoflagellated protozoa, and it was later placed in the genus Herpetomonas by Kent (1880) under the name Herpetomonas lewisi; Laveran and Mesnil (1901) showed that it conformed in all essential characteristics to the genus Trypanosoma and therefore renamed it Trypanosoma lewisi, the name by which it has been known to date.

Taxonomically, T. lewisi is classified as follows (Cox 1981):

| | | |
|---|---|---|
| Subkingdom | - | Protozoa |
| Phylum 1 | - | Sarcomastigophora |
| Sub-phylum | - | Mastigophora |
| Class 2 | - | Zoomastigophora |
| Order | - | Kinetoplastida |
| Sub-order | - | Trypanosomatina |
| Family | - | Trypanosomatidae |
| Genus | - | Trypanosoma |
| Species | - | lewisi |

### 2. LIFE CYCLE

Trypanosoma lewisi is a parasite with a cosmopolitan distribution occurring in the blood of black or brown rats (Rattus rattus and R. norvegicus, respectively). The intermediate host is the rat flea

Nosopsyllus fasciatus in the temperate zones and Xenopylla cheopis in tropical areas. Although transmission of this rat trypanosome was first described by Rabinowitch and Kemper (1899), our present knowledge of the developmental cycle of T. lewisi in the rat flea (Figure 1.1) was given by Minchin and Thompson (1915) and has been thoroughly reviewed by Hoare (1972).

When the flea takes a blood meal from an infected rat, the trypanosomes undergo physiological changes in the lumen of the gut of the arthropod and lose their power to infect rats within 30 minutes after being ingested. In the next 6-7 hours, they penetrate the epithelial cells of the stomach where the trypanosomes reproduce by repeated multiple division; the parasites are then released into the lumen of the stomach, either actively or after rupturing the cells of the host. At this stage, trypanosomes are long with a swollen posterior end; Minchin and Thomson (1915) referred to them as "crithidiomorphic forms". These trypanosomes then pass through the colon into the rectum of the flea where they undergo other morphological changes, eventually transforming into epimastigote forms. Some of the epimastigotes are free-swimming in the rectum, whereas others are attached to the wall of the posterior gut where they actively divide by repeated binary fission. The kinetoplast of these transitional epimastigote forms shifts to the posterior extremity of the parasite, transforming into small club-shaped trypomastigotes, or metatrypanosomes, the final stage of development of T. lewisi in the vector. The rat vertebrate host becomes infected when it ingests an infected flea, or its feces; the metacyclic trypanosomes penetrate the oral mucus membrane (Hoare 1972) and eventually enter into the capillaries where they reproduce extracellularly.

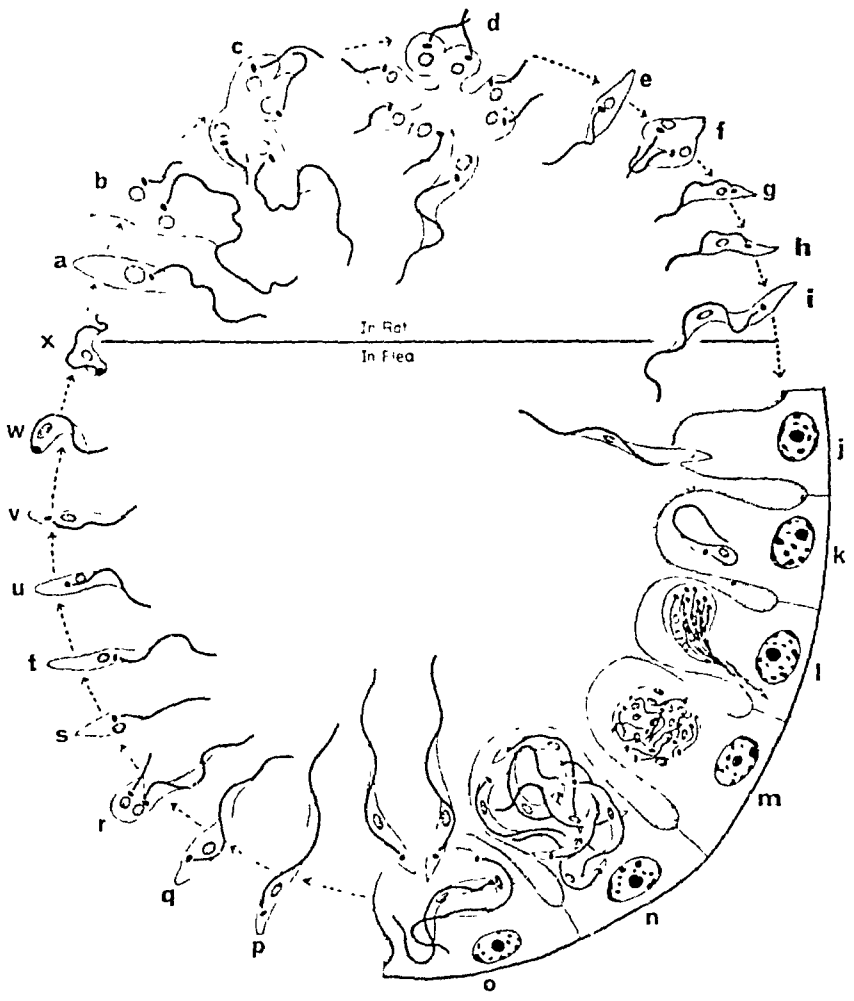
Fig. 2.1. The life cycle of Trypanosoma lewisi (Hoare 1972).

a - f : represents the dividing epimastigotes in the bloodstream of the rat.

g - i : represents the non-dividing trypomastigotes in the bloodstream of the rat.

j - o : represents the intracellular multiplication of the trypomastigotes in the mid-gut epithelial cells of the flea.

p - x : represents the transitional stages in the hind-gut of the flea from the stomach trypomastigotes forms through epimastigotes to the final infective metacyclic stages or the metatrypanosomes.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## 3. IMMUNOBIOLOGY OF TRYPANOSOMA LEWISI

Trypanosoma lewisi and other rodent trypanosomes induce changes in their host that compare very well with those induced by the pathogenic trypanosomes. The effectiveness of the immunological response of the host to infections by rodent trypanosome is shown in the fact that antibodies are produced during the infection which clear the parasitemia. A unique characteristic of these infections is the production of ablastin, an antibody that has no lethal action but which inhibits the reproduction of epimastigotes. This antibody was first described by Taliaferro (1924). Despite this well-balanced host-parasite association, pathological changes, like anemia, thrombocytopenia, glomerulonephritis, splenomegaly, (Thoongsuwan and Cox 1978), and immunodepression (St. Charles et al 1981) occur in T. lewisi infections, especially during the peak of parasitemia. These changes also occur in laboratory animals infected with pathogenic trypanosomes, except that they are more severe. It should be noted that, although rodent trypanosomes have been often referred to as non-pathogenic, salivarian trypanosome infections with T. brucei are less severe in cattle and wild animals; these animals tolerate the infection without harmful consequences and wild animals will often serve as reservoirs for the infections of more susceptible mammalian hosts. While the tolerance of non-pathogenic trypanosomes has been attributed to an effective immunological response, it is not clearly known what mediates a similar tolerance of the wild animals to pathogenic trypanosome infection. However, factors associated with innate resistance like, the control of trypanosome growth, the development of effective immune responses and a resistance to development of anemia

(Murray and Black 1985) probably play an important role since they are a feature in the trypanotolerant breeds of cattle and of the wild bovidae (Murray et al 1982). It is interesting that these are also characteristic of infections with non-pathogenic trypanosomes, and they may indeed be the key factors which underlie host susceptibility to trypanosomiasis.

### 3.1 The course of infection

Following ingestion of either an infected flea or the syringe inoculation of blood parasites in the laboratory, the trypanosomes multiply in the capillaries of the kidneys (Ormerod 1963) for a period which depends on the size of the inoculum (Augustine 1941); the parasites then appear in the peripheral circulation. They then actively reproduce in the peripheral blood, producing a sharp rise in the parasitemia which reaches a peak between the seventh and tenth day of the infection. During this time the intensity of reproduction is measured by determining the coefficient of variation (C.V.) which is the ratio of the number of dividing epimastigotes and newly formed parasites (Taliaferro and Taliaferro 1922). The C.V. is high in the early stage of the infection, but falls to almost zero by the tenth day due to the effect of the reproduction-inhibiting antibody ablastin (Taliaferro 1924, 1932; Coventry 1925). At about the time when reproduction ceases, the so- called "first crisis" occurs in which most of the immature trypanosomes are killed by a trypanocidal antibody (Coventry 1930). Those trypanosomes that survive this crisis are unable to resume multiplication due to the continued presence of ablastin in circulation; these trypomastigotes are eliminated either

gradually or suddenly by a second trypanocidal antibody which terminates the infection in a "second crisis" (Coventry 1930). Following recovery, the state of immunity that ensues is absolute, specific and probably life-long (Corradetti 1963). Although immunity in mice recovered from infections with Trypanosoma musculi has been attributed to a persistence of small numbers of trypanosomes in the vasa recta of kidneys (Viens et al 1972; Target and Viens 1975a); such forms of T. lewisi have not been found, either in the kidneys or in other tissues (Wilson et al 1973). The responsibility of acquired humoral antibodies for the arrest of reproduction and the trypanocidal crisis has been well established by passive transfer experiments with the serum of recovered rats. Such experiments were first reported by Rabinowitch and Kemper (1899) who showed that serum of recovered rats was protective for normal infected animals; these studies were confirmed by Laveran and Mesnil (1900). Although, immunity to T. lewisi is largely mediated by humoral responses, a cellular involvement was reported by Laveran and Mesnil (1901) and later by Regendanz and Kikuth (1927) who considered that the disappearance of the trypanosomes from infected animals was due to phagocytosis by the reticulo-endothelial system. Indeed, the active participation of the reticulo-endothelial system in the control of infections by T. lewisi has been reported, (Patton 1972b; Ferrante and Jenkin 1978, 1979).

### 3.2 Humoral responses

The humoral control of rodent trypanosomiasis was first attributed to trypanocidal antibodies and ablastin as the most important immunological responses by Taliaferro (1932). Ablastin, which only inhibits reproduction and has no apparent effect on the viability or

the motility of the parasite, is produced early during the course of infection and the arrest of division coincides with the lytic crisis of the first trypanocidal antibody. The elimination of the mature non-dividing trypomastigotes is attributed to a second trypanocidal antibody which finally terminates the infection.

### 3.2.1 Ablastin

The most peculiar immunological response of the rodent trypanosomiasis is the induction of ablastin, a phenomenon that does not occur in African, nor in American trypanosomiasis; the importance of ablastin in infections with rodent trypanosomes is shown by the fact that stress factors which interfere with the production of ablastin (D'Alesandro 1970) can result in an uninhibited reproduction that will produce fulminating infections comparable to those occurring with salivarian trypanosomes. Since the original description of the reproduction inhibiting antibody as a "reaction product" by Taliaferro (1924) which he later renamed ablastin (Taliaferro 1932), there has been extensive research on this controversial and most unusual antibody (Chandler 1952; D'Alesandro 1970; Sanchez 1973; Greenblatt 1975; Dusanic 1975; Targett and Viens 1977 ). Taliaferro (1932) first demonstrated that ablastin is precipitated with the globulin fraction of immune serum. More recent studies by gel filtration, electrophoresis and immunoabsorbent technics have identified ablastin as an IgG molecule (D'Alesandro 1959, 1975; Dusanic 1975; Hsu and Dusanic 1982).

At present, ablastin activity has only been reported in the serum of animals infected with T. lewisi and T. musculi through passive

transfer studies (Taliaferro 1924, 1932, 1938a), although recent evidence shows that the serum of normal uninfected rats contains low levels of ablastic activity (Balber and Sturtevant 1986; Giannini 1987). Initial studies suggested that ablastin was a non-adsorbable antibody (Taliaferro 1932; D'Alesandro 1959, 1966, 1970, 1975, 1976) since repeated adsorptions of immune serum by living trypanosomes failed to reduce ablastic activity. However, the reports which suggested that ablastin, in fact was an avid antibody were by Giannini and D'Alesandro (1979) who showed by immunofluorescence the presence of surface IgG on intact trypanosomes, as early as the fifth day of infection. The intensity of fluorescence on the parasite increased concomitantly with rising titres of parasite-specific antibody, while trypanosomes obtained from immunosuppressed animals lacked detectable surface IgG (Giannini and D'Alesandro 1979). The appearance of specifically adsorbed IgG as early as the fifth day of infection, when only ablastic activity is demonstrable, suggested that some of the IgG is indeed ablastin. D'Alesandro and Clarkson (1980) suggested that the failure to reduce ablastin titres after several adsorptions in earlier experiments was not due to a lack of avidity and adsorbability of ablastin but due to low cell: serum ratios. Similar studies by Brooks and Reed (1980) also indicated that ablastin could be adsorbed by T. musculi. Reports by Trudel et al (1982) and Desbiens and Viens (1981) have shown however that, the presence of adsorbable IgG on the surface of T. musculi could also be attributed to trypanocidal antibodies; they have suggested that the initial control of parasitemia was mediated by trypanocidal antibodies, rather than by ablastin. Earlier studies by Viens and Targett (1971) had shown that T cell deprived mice could control an initial parasitemia with T.

musculi as efficiently as either newborn mice (Brenier and Viens 1980) or mice treated with antithymocyte serum (Viens et al 1974) or with cyclophosphamide (Pouliot 1975).

The effect of ablastin on the physiology of the parasites was first elucidated by Moulder (1948) who showed that this antibody lowers glucose metabolism to maintenance levels. This was later confirmed by Pizzi and Taliaferro (1960) who observed that dividing epimastigotes incorporated more $^{14}C$ from labeled glucose than non-dividing trypomastigotes. Ablastin also has been reported to affect enzymes associated with carbohydrate metabolism (Moulder 1948; D'Alesandro and Sherman 1964; D'Alesandro 1966; Patton 1975). The arrest of cell division and the changes in enzyme levels consequently results in the inhibition of nucleic acid synthesis and protein synthesis also drops to maintenance levels (Taliaferro and Pizzi 1960). There has been some other controversy over the role of ablastin in T. lewisi infections. Drew and Jenkin (1982) have reported that, even after removal of over 90% of the immunoglobulins in immune serum, they could not reduce the inhibitory effect of ablastin, contrary to an earlier report by D'Alesandro and Clarkson (1980). While Drew and Jenkin (1982) respect the existence of ablastic phenomenon, these authors doubt whether ablastin is an antibody, since earlier studies by Ferrante et al (1978a) had shown a non-immunoglobulin factor in the serum that inhibited DNA synthesis of the trypanosome. This factor was found in the serum four days after infection, in the absence of specific antibody (Ferrante and Jenkin 1978). In support of this argument are the reports by Perla and Marmorston - Gottesman (1930) and Hanson and Chapman (1974) who showed that neonatal thymectomy

either reduced or had no effect on the course of parasitemia, compared to control animals. Similar results were obtained by Drew and Jenkin (1984) who after thymectomy and X-irradiation of weanling rats prior to infection, observed that there was no difference in the ability to eliminate the infection with T. lewisi between the control and the T lymphocyte-depleted rats. These authors reported similar ablastin titres in the serum of the thymectomized and control animals. They further argue that if ablastin is indeed an IgG molecule, then it should not be produced in the T lymphocyte-depleted rats as IgG is a T cell dependent antibody (Ebersole et al 1979). These reports further deepen the controversy over the nature of ablastin, but, whichever school of thought is right, it is evident that infections with rodent trypanosome induce some serum components which inhibit the perpetual replication of epimastigotes.

### 3.2.2 Trypanocidal antibodies

The induction of agglutinating and lytic antibodies in rats infected with T. lewisi was first demonstrated by Coventry (1930) and later Taliaferro (1932) found that these antibodies occur in the globulin fraction of immune serum. Studies by D'Alesandro (1959, 1970, 1976) indicated that the trypanocidal antibody mediating the "first crisis" was a small 6S-gamma G-globulin (IgG) and the second crisis was effected by a 16S gamma M-macroglobulin (IgM) class of antibody. The conventional immune response to a primary antigen challenge in rats and other mammals is the production of IgM and a switch to IgG (Harris 1965; Santos and Owen 1966; de Carvallo et al 1967). It is difficult to explain the response in the opposite manner by rats infected with T. lewisi, although mice and rats immunized with bovine serum albumin have

been reported to produce only IgG during their primary response (Cushing and Johnson 1966; Banovitz and Ishizaka 1967) and the possibility of an initial low level of IgM which was undetected by the filtration methods used cannot be ruled out. However, reports by Viens et al (1975) indicate the first trypanocidal antibody in mice infected with T. musculi is an IgM molecule and that the late trypanocidal effect was mediated by IgG antibodies. It is interesting to note that lactating rats produce a protective anti T. lewisi rheumatoid-like IgM factor which stimulates the IgG-mediated ablastic response (Clarkson and Mellow 1981). The rheumatoid factor stimulates an ablastic response (IgG) which confers resistance to previously uninfected rats and their pups to infections with T. lewisi (Clarkson and Mellow 1981). A similar anti-immunoglobulin rheumatoid-like factor has also been reported in rabbits infected with T. equiperdum (Klein et al 1970; Mattern et al 1980); this anti-immunoglobulin antibody could be suppressed by therapy, suggesting a specific association with the parasite antigens.

The dividing and the mature forms of T. lewisi differ antigenically (D'Alesandro 1976). It has been reported that the first IgG trypanocidal antibody acts on immature forms at the peak of parasitemia; this antibody, however, has no effect on adult forms, but the second IgM trypanocidal antibody can lyse and agglutinate both forms (D'Alesandro 1976).

Infections of humans, cattle or experimental animals with African trypanosomes induce IgM antibodies primarily directed toward the variant surface glycoprotein (VSG), followed by IgG. Studies indicate that T cell functions are not important at least in T. brucei brucei

and T. b. rhodesiense, since athymic mice producing only IgM antibodies against the parasite are able to control parasite multiplication (Campell 1978). Sacks and Askonas (1980), Sack et al (1982) observed that, during chronic infections with T. brucei, IgG antibody was rapidly suppressed but IgM responses to the parasite declined more gradually, providing evidence that IgM production is sufficient to control the waves of variant trypanosomes (Hudson et al 1976). In T. cruzi, there is evidence that IgG (Takehara et al 1981) can confer immunity to naive animals; this class of antibody increases in the serum of infected animals when the numbers of parasites are rapidly decreasing and peak level is reached when parasites disappear from the blood and the organs of infected mice.

### 3.3 Cellular Immunity

There have been few studies relating to the cellular responses to infections with T. lewisi; this is probably due to the fact that most workers strongly believe that the immune response to infections with this rodent trypanosome is principally antibody-mediated (reviewed D'Alesandro 1970). However, the participation of mononuclear phagocytes in the elimination of trypanosomes was first suggested by Laveran and Mesnil (1901), who observed phagocytosis of T. lewisi in the peritoneal cavity of actively and passively immunized rats. Later, Brown (1915) reported the agglutination and phagocytosis of trypanosomes in infected rats and also noticed disintegrating parasites that were probably destroyed by lytic antibodies. Later, Augustine (1943), and Lange and Lysenko (1960), having made similar observations, suggested that phagocytosis of trypanosomes only occurred in the presence of immune rat serum. The latter authors observed that

whether agglutination or trypanolysis occurred, macrophages were eventually involved in the removal of parasite debris; their role, they concluded, though secondary, was an important one.

### 3.3.1 Role of mononuclear phagocyte system

Changes in the reticulo-endothelial system during infections with T. lewisi in rats were first noted by Marmorston-Gottesman et al (1930) who showed that infected animals had enlarged spleens. Ferrante et al (1978b) further observed that there was a marked increase in the weight of the liver and spleen during infection with T. lewisi; associated with these changes, there was a marked hyperactivity of the reticulo-endothelial system demonstrated by the rapid elimination of colloidal carbon. The rate of elimination of carbon was higher in animals during the course of infection and lower in recovered rats. These authors observed an increase in phagocytic cells which accounted for the rapid clearance of colloidal carbon and which was evident as early as the 4th day post-inoculation of the parasite. Similar results had been reported by Greenblatt and Tyroler (1971), who noted that macrophages became activated as early as the second day after incubation in vitro with T. lewisi; this phenomenon was maximal at the peak of infection.

The mechanisms involved in the elimination of T. lewisi infections has been rather controversial (D'Alesandro 1970) and may indeed be due to several immunological mechanisms. Agglutination of the parasite was first demonstrated by Laveran and Mesnil (1900) and later confirmed by Tempelis and Lysenko (1965) and Lincicome and Watkins (1965) Complement fixation (Marmorston-Gottesman et al 1930), lysis (Taliaferro 1932, 1938a) and opsonization (Lange and Lysenko 1960)

have also been demonstrated. However, direct evidence implicating macrophages with immunity in T. lewisi-infected rats was first reported by Ferrante and Jenkin (1978). These authors showed that, although the weight of the spleen increased dramatically during the infection, the liver played a major role in removing the trypanosomes from the circulation; the removal, however, required the presence of opsonic antibody and was not by a lytic process involving specific antibody and complement as proposed by some workers (Marmorston-Gottesman et al 1930; Taliaferro 1932, 1938a). In vitro studies by Ferrante and Jenkin (1979) have further shown that the removal of the parasites is by phagocytosis mediated by specific antibody. Similar studies have been reported by Takayanagi and Nakatake (1974) and Takayanagi et al (1974) who showed similar interaction between the peritoneal macrophages and T. gambiense as those reported in T. lewisi. Reduction in the level of parasitemia has been reported in rats infected with T. lewisi after activation of peritoneal macrophages with carrageenan (Sirand 1983) or by lysozyme (Bierman et al 1979), while Ackerman (1977) found that systemic administration of BCG intravenously inhibited the development of peak parasitemia. These results further suggest that macrophages play a significant role during the early and late periods of infection with T. lewisi.

Studies in T. musculi have shown that macrophages become activated and that their number, size and the release of peroxidases are increased (Vincendeau et al 1981). In vitro phagocytosis of T. musculi is usually enhanced in the presence of immune serum (Chang and Dusanic 1976), indicating the dependence of antibody-mediated phagocytosis in the elimination of this parasite. More recently, Viens et al (1983) have described the adherence of platelets onto trypanosomes with

subsequent lysis; this lytic process was also shown to be antibody-dependent and was observed to occur with blood from animals at late infection and in recovered mice. Antibody-mediated phagocytosis has been reported to mediate the clearance of $^{75}$Se-labeled T. brucei by hepatic macrophages from the circulation of immune mice (MacAskill et al 1980). Studies with T. cruzi have also provided evidence that macrophages play a significant role in the control of infections with this parasite (Milder and Kloetzel 1980; Bertelli et al 1981; Abrahamsohn et al 1981).

### 3.3.2 Role of T cells

The relation of the T-cells to infections with T. lewisi has received very little attention compared to similar studies in T. musculi. However, treatment of rats with anti-thymocyte serum and anti-lymphocyte serum produces severe and fulminating infections with T. lewisi ( Spira and Greenblatt 1970; Tawil and Dusanic 1971; Dusanic 1975), but neonatal thymectomy had no apparent effect on the infection (Hanson and Chapman 1974). Its believed that treatment with antiserum interferes with ablastin production, resulting in uncontrolled replication of the parasite (Dusanic 1975); a fai'ure to enhance parasitemia after neonatal thymectomy (Hanson and Chapman 1974) may have been due to an incomplete removal of thymus tissue (Viens 1985), although Drew and Jenkin (1984) have recently reported that complete thymectomy has no effect on ablastic titres and on parasitemia. However, the initial control of parasitemia in T. musculi is resistant to all forms of immunosuppressive agents except irradiation (Viens et al 1974), and this may be due to the fact that trypanocidal antibody,

rather than ablastin, appears to control the parasitemia. Viens et al (1974) and Targett et al (1975) have also shown that the late elimination of T. musculi from the blood is clearly thymus-dependent; both splenic B and T cell populations (Albright et al 1977) and IgG secreting cells (Hirokawa et al 1981) increase in number during the elimination phase. Available evidence indicates that thymus dependent lymphocytes are involved in protective immunity in T. cruzi since neonatal thymectomy of mice (Shmunis et al 1971) or rats (Robertson et al 1973) or treatment of mice with antithymocyte serum inhibits the development of acquired immunity to this parasite. It has been established more recently that depression of T- dependent mechanism results in an enhancement of T. cruzi infections (Rodriguez et al 1983). Interestingly T-cell responses appear to play no role in the control of infections with salivarian trypanosomes since athymic nude mice are as able to control the first wave of parasitemia as are normal mice (Campell et al 1978; Clayton et al 1979a),

### 3.4 Role of cellular mediators

Infections with trypanosomes are often accompanied by a release of mediators from cells. Macrophages from mice infected with T. brucei clone NIM6 have been found to secrete $PGE_2$ prostaglandins, the maximum release of which occurs, at the height of parasitemia (Fierer et al 1986). Prostaglandin secretion may indeed affect the host's immune system since these mediators are thought to inhibit lymphocyte activity (Bray 1980) and to modulate the expression of Ia by macrophages (Snyder et al 1982). Interleukin 1 (IL-1) production by macrophages is also enhanced in mice infected with T. brucei; it can be further increased by activation with lipopolysaccharide (Bancroft and Askonas 1985).

However, macrophages obtained from mice some days before death also show increased production of IL-1 which cannot be enhanced by LPS, indicating that cells cannot be further stimulated during the terminal stages of infection (Bancroft and Askonas 1985). Besides the induction of interferon (IFN) by viruses, intracellular protozoa like Toxoplasma (Freshman et al 1966; Pestka 1981), Plasmodium berghei (Huang et al 1968) and T. cruzi (Sonnenfeld and Kierszenbaum 1981) enhance the production of IFN in vivo; African trypanosome infection in mice also enhances serum levels of IFN (Bancroft and Askonas 1985). The actual role of IFN in infections with African trypanosomes is not clear, although treatment with poly I:C, an interferon inducer has resulted in a decrease in parasitemia without, however, altering the mortality rate of the host (Bancroft and Askonas 1985). The mitogen-induced production of interleukin 2 (IL-2), a lymphokine that plays a central role in T cell activation and proliferation, is suppressed in infections with T. brucei (Sileghem et al 1986), T. cruzi (Tarleton and Kuhn 1984; Beltz and Kierszenbaum 1987), Leishmania donovani (Reiner and Finke 1983) and more recently in T. lewisi (Proulx 1988).

## 4. TRYPANOSOME ANTIGENS

Trypanosoma lewisi antigens have been suggested to originate from three different compartments (Bawden 1975).

### 4.1 Intracellular antigens

In this category, the antigens are derived from the cytoplasm or from the cellular organelles and are only released upon disruption of the trypanosome; they represent the bound antigens of T. lewisi (Etner

1968; Oelerich 1969). Antibodies against intracellular antigens probably have no effect on the trypanosomes since the antibody cannot penetrate the cells in an active form (D'Alesandro (1970).

### 4.2 Plasma membrane antigens

Antigens located on the plasma membrane may be either structural components or enzymes with membrane transport functions. Antibodies against plasma membrane antigens are induced during infections with T. lewisi since a membrane ATPase is inhibited by immune serum (Patton 1970, 1972). A second subdivision of plasma membrane antigens is the surface coat. The surface coat is apparently attached onto the outer side of the plasma membrane and represents the variant antigens of salivarian trypanosomes (Vickerman and Luckins 1969; Allsopp et al 1971) and the two antigenic variants of T. lewisi (D'Alesandro 1976). Antibodies against the variant antigens of African trypanosomes (Allsopp et al 1971; Herbert and Macadam 1971; Cross 1975) and the surface antigens of T. lewisi (Giannini and D'Alesandro 1984) and T. cruzi (Araujo and Remington 1981) are protective. However, in the case of African trypanosomiasis, antibodies are only protective against those organisms that bear homologous surface antigens.

### 4.3 Extracellular antigens

These comprise enzymes which are shed by trypanosomes into flagellar pocket (Jadin and Creemers 1972) where they digest nutrients that are eventually assimilated by the parasite. If these enzymes are immunogenic, they may induce antibodies that would interfere with the nutrition of the trypanosome as suggested by Chandler (1958).

The reaction of trypanosome antigens with specific antibodies has been extensively studied mainly by gel diffusion. Etner and Gonzalez (1966) reported changes in the antigenicity of T. lewisi during the course of infection; their antigenic patterns revealed at least three distinct stages of development (Etner 1968), although the differences between the young and adult forms was primarily quantitative. Through agglutination techniques, D'Alesandro (1976) reported the existence of two antigenic variants in T. lewisi. A comparative analysis of the antigens of T. musculi and T. lewisi has shown these two related trypanosomes share a minimum of eight cell antigens, only three of which appeared to be unique to T. lewisi and only one to T. musculi (Dusanic 1979)

4.4 The exoantigens

Thillet and Chandler (1957) and later Wilson (1962) reported "metabolic products" of Trypanosoma lewisi released by bloodstream forms incubated in vitro at room temperature; these products were referred to as "ablastinogen" and normal rats treated with ablastinogen were protected from T. lewisi infection. Although, these authors named these "metabolic products" as ablastinogen, Bawden (1970) observed that these metabolic products contained antigens that elicited both ablastic and trypanocidal antibodies. At about the same time as the release of "metabolic products" by T. lewisi was being reported, Weitz (1960a, 1960b) described the release of a soluble antigen ("exoantigen") in the serum of rats infected with T. brucei. He observed that the exoantigen could induce protective and agglutinating antibodies. Several studies have since then reported a correlation between the appearance of a new antigenic variant with the release by salivarian

trypanosomes of the corresponding exoantigen (Weitz 1963; Seed 1963; Miller 1965; Clarkson and Awan 1968, 1969; Vickerman and Luckins 1969; Takayanagi et al 1970).

Exoantigens are probably released in the form of 'filopodia' or "plasmanemes" which are long filamentous extensions of the trypanosome membrane and the surface coat (Wright et al 1970). These filopodia are rapidly shed by the trypanosome and are found as aggregates in the parasite-free serum of mice infected with T. brucei (Macadam and Herbert 1970). The continuous production and shedding of filopodia allow the trypanosome to escape the lytic effects of specific antibodies (Macadam and Herbert 1970) and may eventually appear in the form of circulating immune complexes (Terry 1976). It may be that T. lewisi "metabolic products" are also released in the form of filopodia, and that the ablastinogen and the antigens which induce trypanocidal antibodies are themselves derived from the plasma membrane antigen compartment (Bawden 1975). The release of ablastinogen and the antigens which induce trypanocidal antibodies (collectively the exoantigens) in vivo was demonstrated by Bawden (1970a, 1970b, 1974) in the plasma of rats which had been immunosuppressed with hydrocortisone and infected with T. lewisi. D'Alesandro (1972) showed that the exoantigens present in the plasma of untreated rats infected with T. lewisi were not detectable with sera obtained during a typical primary infection, but only with hyperimmune rat sera or immunized rabbit serum; more recently Long and Dusanic (1983) have shown precipitin reactions with exoantigens using infected sera from untreated rats. Exoantigens in the plasma obtained from immunosuppressed rats after infection with T. lewisi are however, readily precipitated with sera

from animals undergoing a typical infection (Long and Dusanic 1978). Since immunosuppression causes reticuloendothelial blockade (Wiener et al 1967) resulting in an impairment of antigen clearance, exoantigen levels would be expected to be high in an unbound form in the plasma of treated infected rats, in contrast to the possible formation of immune complexes between the exoantigens and antibodies in untreated animals, as suggested by Terry (1976). This latter phenomenon may account for the failure of exoantigens of T. lewisi to precipitate readily with the serum from untreated rats.

Production of exoantigens has however been reported in several other parasitic infections, such as T. musculi (Dusanic 1978), Babesia (Sibinovic et al 1965, 1967; Smith et al 1979, 1981; James 1984; James et al 1987; Montenegro-James 1987), Plasmodia (Eaton 1939; Corwin and McGhee 1966; Jepsen and Anderson 1981; Thelu 1981, 1985), Leishmania (Aldler 1965; El-On et al 1979), Anaplasma (Amerault and Roby 1964) and Schistosoma mansoni (Berggren and Willer 1967; Gold et al 1969).

## 4.5 Antigenic variation

The phenomenon of antigenic variation has been reported in several protozoans: Babesia (Currow 1973; Cohen 1982), Plasmodia (Corwin et al 1970; Voller and Rossan 1969; Butcher and Cohen 1972; Brown 1977; McBride et al 1982; Hommel et al 1983), Paramecium (Sommerville 1969) and in viral glycoproteins (Waterfield et al 1979); its largely complicated mechanisms of induction has made this phenomenon, one of the most studied and extensively reviewed field in African trypanosomiasis (Vickerman 1978; Englund et al 1982; Bernards 1984; Donelson and Rice-Ficht 1985; Barry 1986). Antigenic variation is the

the ability of African trypanosomes to change their surface coat, each variant being antigenically unique from the ones previously expressed, and the ones to come subsequently. In this context, these bloodstream trypanosomes can present the immune system of the mammal with unendless antigenically-distinct types of parasites. In contrast, the bloodstream T. lewisi seems to manifest only two antigenic types in the dividing and adult forms (D'Alesandro 1976). No antigenic variation has ever been reported in T. cruzi (Nussenzweig and Goble 1966; Snary 1980; Scott and Snay 1982). Vickerman (1969) has reported that T. lewisi has a loosely organized, diffuse and filamentous coat compared to the dense, compact coat of the bloodstream salivarian trypanosomes; whether this structural difference may account for the limited antigenic variant repertoire of T. lewisi is still not clear.

Initially, antigenic variation was thought to be triggered by antibodies directed towards the antigens of the surface glycoprotein, but this phenomenon is currently known to be a spontaneous process occurring at a low frequency in the bloodstream of mammals (Van Meirvenne 1975) and with metacyclic trypanosomes in the salivary glands of tse tse fly ( Hajduk and Vickerman 1981; Crowe et al 1983). It has also been demonstrated that new antigenic types can occur in vitro in the total absence of antibody (Doyle et al 1980), further supporting the view that antigenic change may occur due to a perturbation of the trypanosome membrane (Jenni 1977). However, although antibodies do not induce antigenic variation, Vickerman (1978), Nantulya et al (1979) and Donelson and Rice-Ficht (1985) claim that antibodies play a significant role in indirectly selecting new variants that will give rise to the next parasitemia. The appearance of new antigenic variants

in the bloodstream has been a controversial issue, and most workers have observed the random appearance of the variable surface glycoprotein (VSG; Campell et al 1979; Miller and Tunner 1981; Kooter et al 1984), although some VSGs seem to occur more frequently than others (Gray 1965). It has been reported that some VSGs in T. equiperdum have a tendency to appear early in the infection whereas other will most likely occur late in the infection (Longacre et al 1983). Donelson and Rice-Ficht (1985) suggested that the genomic location of a VSG gene in the telomere influences the frequency at which the VSG is expressed. Surely, the unpredictable appearance of new antigenic variants with distinct antigenic reactivities has been the subject of continued frustrating efforts directed towards the development of a vaccine against African trypanosomiasis, although the studies of Esser et al (1982) and Jenni and Brun (1981) indicate that the metacyclic variable antigen repertoire may be limited and, should this always be the case, protection against a metacyclic challenge may be possible.

## 5. PATHOLOGY OF TRYPANOSOME INFECTIONS

Infections with trypanosomes are usually accompanied by lesions manifested in the hemopoeitic and immuno-committed organs such as the spleen and in the peripheral circulation (Jenkins and Facer 1985). In T. cruzi the pathological changes are characterized by cardiac and gastrointestinal disturbances (Taylor 1986) in the chronic disease and severe myocarditis with subepicardial and endocardial petechiae in the acute form (Santos-Buch and Acosta 1985). Although rodent trypanosomes have been referred to as non-pathogenic, moderate pathological lesions occur during infections, mainly manifesting as

anemia, splenomegaly and glomerulonephritis (Thoogsuwan and Cox 1978); these pathological changes have also been reported in infections with experimental African trypanosomes (Rickman and Cox 1979). These symptoms are thought to be induced by autoantibodies in the form of cold active hemagglutinins (CAH) directed towards the erythrocyte stroma (Soni and Cox 1974, 1975) and fibrinogen/fibrin products (Boreham and Facer 1974) of the infected host.

5.1 Anemia, splenomegaly and glomerulonephritis

In infections causing the anemia, splenomegaly and glomerulonephritis syndrome characteristic of T. brucei (Rickman and Cox 1979), T. lewisi (Thoongsuwan and Cox 1978) and avian malaria caused by Plasmodium gallinaceum (Soni and Cox 1974), autoantibodies in the form of cold active hemagglutinin (CAH), immunoconglutinin (IC) and anti-fibrinogen/fibrin (anti-F) are often produced; the titres of these autoantibodies are highest when anemia, splenomegaly and glomerulonephritis are most severe, usually at the peak of parasitemia (Rickman and Cox 1979). Although the mechanisms behind these pathological features are still not clearly known, it is thought that immunoconglutinin enhances the sequestration of both erythrocytes and the parasites by the spleen; this phenomenon would also produce to a blockade of the microvasculature leading to disseminated intravascular coagulation (DIC), a condition commonly encountered in trypanosome infections (Isoun 1968; Losos and Ikede 1972; Van Dijk 1973; Thoongsuwan and Cox 1978; Rickman and Cox 1979; Masake 1980; Rickman et al 1981; Thoongsuwan and Cox 1981; Cox 1982). Rickman and Cox (1979) suggested that anemia and splenomegaly could result from this

phenomenon, although sequestration of such cells and parasites have also been reported to occur in the liver of animals suffering from severe anemia (Valli et al 1979; Anosa and Isoun 1980). It has been suggested that the destruction of red cells by liver macrophages is not parasite-specific and that activation of these reticulo-endothelial cells may lead to increased erythrophagocytosis, thus enhancing anemia (Vincendeau et al 1981; Fierer and Askonas 1982). There is evidence that trypanosome antigen(s) can attach to the red cell membrane rendering them susceptible to phagocytosis by the reticulo-endothelial system (Herbert and Inglis 1973). Trypanosoma brucei antigens readily adsorb onto the surface of rabbit red blood cells in vitro; the antigen-coated cells can then be lysed by the addition of trypanosome-specific antibody and complement (Woo and Kobayashi 1975). It has also been found that an intravenous dose of the homologous antigen of T. brucei given to rabbits previously immunized with sonicated T. brucei antigen resulted in a marked decrease in hemoglobin and hematocrit (Jenkins and Facer 1985). The presence of a hemolysin that causes direct lysis of circulating erythrocytes has been described in suspensions of T. brucei, T. congolense, T. gambiense and T. vivax (Jenkins and Facer 1985) indicating that anemia in protozoal infections may be caused by several processes.

Glomerulonephritis in T. lewisi (Thoongsuwan and Cox 1978; Cox 1982), experimental African trypanosomiasis (Rickman and Cox 1979) and in avian malaria (Soni and Cox 1974, 1975a, 1975c) has been associated with a deposition of autoantibodies (Thoongsuwan and Cox 1978) or antigen-antibody complexes (Soni and Cox 1975b) on the basal membranes of glomerular capillaries. The presence of antibodies to fibrin/fibrinogen and fibrinogen degradation products have been

detected in rabbits with experimental trypanosomiasis (Boreham and Facer 1974) and Rickman and Cox (1979) also reported similar autoantibodies to fibrinogen (anti-F) in rats which had been infected with T. brucei rhodesiense. The latter authors observed that high anti-F titres correlated with anemia, splenomegaly and glomerulonephritis. Anti-F reacted with erythrocytes and trypanosomes suggesting that they become coated with fibrin, fibrinogen or fibrinogen degradation products as immune complexes. These complexes on erythrocytes have also been observed to occur in the kidneys and urinary wastes suggesting that antibody-antigen complexes mediate nephropathy associated with trypanosome infections (Soni and Cox 1975b; Musoke et al 1977).

5.2 Immunodepression

Immunodepression in parasite infections is a complex phenomenon which is manifested as either a depression of humoral responses to specific and non-specific antigens, depression of cell-mediated immune responses or an alteration of susceptibility to other diseases Immunodepression has been reported in African trypanosomiasis where it has also been widely studied (Mansfield and Wallace 1974; Murray et al 1974; Ackerman and Seed 1976; Hudson et al 1976; Sacks and Askonas 1980; Vickerman and Barry 1982); it has also been reported in infections with T. lewisi (St. Charles et al 1981; Proulx 1988), T. musculi (Albright et al 1977, 1978; Hazlett and Tizard 1978; Albright and Albright 1980, 1981), T. cruzi (Clinton et al 1975; Brener 1980; Cunningham and Kuhn 1980; Maleckar and Kierszenbaum 1983; Harel-Bellan et al 1983; Tarleton and Kuhn 1983), Leishmania donovani (Ghose et al

1979; Carvalho et al 1981; Reiner 1982), Toxoplasma gondii (Strickland et al 1975), Plasmodium falciparum (Greenwood et al 1972; MacDermott et al 1980; Druilhe et al 1983; Brasseur et al 1983), and Trichinella spiralis (Faubert 1976). In African trypanosomiasis, immunodepression may occur as result of the generation of suppressor T cells (Jayawardena and Waksman 1977; Eardley and Jayawardena 1977; Jayawardena et al 1978), suppressor macrophages (Charoenvit et al 1981), polyclonal B-cell activation (Diffley 1983), or by active suppression of interleukin 2 (Sileghem et al 1986).

The present knowledge about the hyporesponsiveness in trypanosome infections in animals has been obtained mainly through studies in laboratory animals; immunosuppression is demonstrable in mice infected with T. brucei by around the tenth day of infection (Corsini et al 1977; Charoenvit et al 1981) and, although this phenomenon has been described in humans (Greenwood et al 1973) and in bovine trypanosomiasis (Scott et al 1977; Whitelaw et al 1979), suppression in cattle is relatively mild and trypanosome-specific antibodies are produced for several months after the initial infection (Nantulya 1979). Infected cattle show less evidence of immunosuppression to unrelated antigens and less disruption of lymphoid organ architecture than that observed in laboratory animals (Masake and Morrison 1981). In view of this, it is difficult to assess the significance and the level at which studies in murine trypanosomiasis can be extended to represent the natural situation.

### 5.2.1 Generation of suppressor cells

The induction of suppressor cell activity is a well-studied phenomenon in African trypanosomiasis (Jayawardena and Waksman 1977;

Pearson et al 1979; Wellhausen and Mansfield 1979). Following infections in laboratory animals, it has been observed that primed T-cells fail to proliferate in the presence of trypanosome antigens in vitro (Campell et al 1978; Gasbarre et al 1980), a phenomenon that has been attributed to infection-induced immunosuppression mediated by suppressive T-cells in conjunction with defective macrophages. Interestingly, this dysfunction can be restored by treatment of the infected animals with the trypanocidal drug Berenil (Gasbarre et al 1980). Although T-cell functions are also greatly affected during murine trypanosomiasis, athymic nude mice control parasitemia with T. brucei or T. rhodesiense just as well as normal mice (Campell et al 1978; Clayton et al 1979) and T-cell numbers are less affected in euthymic mice than B or null cells during the course of the infection (Mayor-Withey et al 1978). The suppressor T cells generated in the spleen in experimental African trypanosomiasis are however, not antigen specific and they affect the in vitro responses of T and B cells to mitogens and heterologous antigens (Jayawardena et al 1978). The latter authors observed that T cells that suppressed normal responses in vitro are insensitive to treatment with anti-lymphocyte serum and are lost after thymectomy. Presently, there is no evidence that antigen-specific T suppressor cells are generated during the course of the infection, nor is it clear how antigen-nonspecific T suppressor cells arise (Bancroft and Askonas 1985).

The first indication of the existence of independent suppressor cells was reported after the observation that spleen cells from T. b. rhodesiense-infected nude mice suppressed the LPS response of normal spleen cell cultures (Mansfield et al 1981). Further studies showed

that the suppressive cells were radiation-resistant and could be abrogated by silica, but resisted lysis with anti-thy 1, anti-lyt +2, implicating macrophage involvement (Wellhausen and Mansfield 1980). More evidence has shown that upon uptake of trypanosomes by macrophages in the presence of antibodies and subsequent transfer into normal hosts, macrophages are able to mediate suppression of a primary IgG antibody response to sheep erythrocytes *in vitro* and *in vivo* (Grosskinsky and Askonas 1981). At present, there is no evidence of a direct interaction between *T. brucei* components and the lymphoid cells as has been reported in *T. musculi* by Albright and Albright (1981) where trypanosome-derived substances bind to host cells to effect immunosuppression. In view of the present evidence, it appears that macrophages are the most important cells in mediating immunosuppression in African trypanosomiasis (Bancroft and Askonas 1985) but it is yet unclear whether macrophages become immunodepressive because they release parasite products after phagocytosis or whether suppression is a result of mediators released by activated macrophages that are generated during the course of the infection (Sacks and Askonas 1980).

### 5.2.2 *Polyclonal B cell activation*

Hypergammaglobulinemia, the marked elevation of IgM in individuals infected with African trypanosomes is the earliest feature which indicates a defect in B cell function (Greenwood 1974; Greenwood and Whittle 1980). Autoantibodies, heterophile agglutinins and IgM rheumatoid factor are mainly found in the serum while free light chains are found in the cerebrospinal fluid and the urine of infected patients (Houba *et al* 1969; Klein *et al* 1970; Greenwood and Whittle 1975).

Increased production of IgM and IgG occur in mice infected with T. rhodesiense, T. congolense, T. gambiense, T. brucei and T. equiperdum (Hudson et al 1976; Corsini et al 1977; Askonas et al 1979; Baltz et al 1981; Morrison et al 1982) and the production of idiotype, anti-idiotype antibodies and immune complexes (Rose et al 1982) have consistently been reported to accompany infections with African trypanosomes. Evidence has accumulated that the trypanosome-mediated B cell stimulation is polyclonal and is not antigen-specific; it is probably induced by a B-cell mitogen since only a small proportion of the immunoglobulin molecules are directed towards the parasite (Corsini et al 1977). Corsini and coworkers (1977) observed that less than ten percent of the immunoglobulins secreted in vivo in T. brucei- infected mice could be adsorbed by homologous parasites.

Clayton et al (1979) showed both polyclonal B-cell activation and immunosuppression in mice injected with a crude trypanosome fraction. However, Diffley (1983) later demonstrated that intravenous treatment of normal mice with purified variant antigen at concentrations occurring in infected hosts induced splenomegaly, polyclonal lymphocyte responses and the production of non-specific antibodies. The consequence of non-specific stimulation eventually leads to exhaustion of B cell clones and a loss of B and T cell memory (Corsini et al 1977; Askonas et al 1979) or to suppression of the potential of B cell (Diamanstein et al 1976; Jayawardena and Waksman 1977; Jayawardena et al 1978) to mount an antigen-specific immunity to subsequent variants.

### 5.2.3 Suppressor factors

While immunosuppression characterizes most parasitic infections,

the host or parasite products that induce this hyporesponsiveness of the immune system has been a subject of great interest. It has been shown that a host-specific, antigen non-specific soluble suppressor substance is present in the sera of mice infected with T. cruzi which can passively immunosuppress normal recipients (Cunningham et al 1978). Corsini et al (1980) also demonstrated suppression of both the delayed hypersensitivity reaction and antibody formation by a fraction derived from cultured epimastigotes. Further studies by the former authors indicated that suppressive substances are produced in vivo in hosts infected with T. cruzi and that they can passively suppress the response to heterologous antigens by uninfected mice; this suppression is probably mediated by macrophages, since removal of cells with macrophage-like properties ablated suppression (Cunningham and Kuhn 1980). More recently Serrano and O'Daly (1987) have shown that culture supernatants from spleen cells of T. cruzi- infected mice are immunosuppressive. These authors found that the in vitro suppression of the blastogenic response to mitogens was induced by a hydro-insoluble protein with a relative molecular weight of 14-15 kDa. An immunosuppressive protein was also detected in the serum of infected animals and these same investigators observed that this protein probably acts by interacting with normal macrophages, rendering them immunosuppressive. St. Charles et al (1981) reported the depression of spleen cells to a secondary response to sheep erythrocytes by soluble products from T. lewisi. Sheep erythrocyte-primed spleen cells co-cultured with the parasite in chambers in which the cells and the trypanosomes were separated by a 0.45 μm Millipore membrane, were depressed in the ability to form hemolytic plaques in vitro; these authors suggested that soluble exoantigens released by the parasite

mediated the suppression. Studies by Albright and Albright (1981) have also shown that soluble substances derived from T. musculi inhibit the humoral responses to T-dependent and independent antigens; the immunosuppressive activity, however, does not involve typical suppressor cells (Albright et al 1977, 1978), but probably acts through a direct action on B or T lymphocytes by the binding of trypanosome-derived products on the cells (Albright and Albright 1981). They also observed that spleen cell preparations from mice infected with T. musculi contain trypanosome-derived products on their surfaces and that treatment of these cells with specific T. musculi antibody eliminated the inhibitory effect of these cells on co-cultured normal spleen cells. Cox et al (1984) have also reported the presence of suppressor factor(s) in the plasma of rats infected with Trypanosoma brucei rhodesiense. Incubation of sheep erythrocyte-primed spleen lymphocytes with plasma from T. b rhodesiense infected rats suppressed the formation in vitro of hemolytic plaques; this effect was maximal at a time when high levels of circulating soluble immune complexes and high titres of immunoconglutinins were present in the plasma. From this latter study, the authors suggested that soluble antigen-antibody complexes may have interacted with Fc receptors of activated lymphocytes to suppress antibody production.

Membrane fractions have also been incriminated in the immunosuppression of animals infected with African trypanosomes. Clayton and coworkers (1979b) demonstrated both the immunosuppressive and mitogenic effects of membrane fractions from T. brucei that could mimic active trypanosome infections by inducing polyclonal B cell activation and immunosuppression in vivo. Further studies on the

membrane fractions by Sacks et al (1982) showed that the immunosuppressive and mitogenic components are complexes of proteins, glycoproteins and lipid; the extracted lipid component could significantly suppress a primary IgG anti-sheep erythrocyte response. The passive mediation of immunosuppression in normal hosts by macrophages that had ingested trypanosomes (Grosskinsky and Askonas 1981) was also obtained when macrophages were incubated with the macromolecular complex of proteins, glycoproteins and lipids (Sacks et al 1982); the complex did not however, enrich these activities.

## 6 RATIONALE FOR THE PRESENT STUDY

From the preceding literature review it is clear that antibodies play a major role in the control of parasitemia by trypanosomes, once the infection has established in the host. It is also evident that parasite-derived products are released by trypanosomes and may therefore contribute to the regulation of these infections.

The objectives of this study were (1) to present a more comprehensive understanding on the host responses during the permissive and non-permissive phases of infection by Trypanosoma lewisi, (2) to isolate and characterize the parasite-derived products that regulate the immune system of the host and (3) to study the mechanism through which immunoregulation to protect or to suppress is achieved.

# CHAPTER III: HOST RESPONSES TO INFECTIONS WITH TRYPANOSOMA LEWISI

## INTRODUCTION

The immunological responses by hosts following infections with T. lewisi were first established by Taliaferro (1924, 1932) and Coventry (1925, 1930) when they identified the production of ablastin, the reproduction - inhibiting antibody and trypanocidal antibodies in rats infected with this parasite. Control of T. lewisi infections are primarily mediated by these antibodies (D'Alesandro 1970), and recovered animals remain solidly immune to re-infection (Corradetti 1963). The first description of a permissive (multiplicative) and two non-permissive (eliminative) phases of infection with T. lewisi is that of Taliaferro and Taliaferro (1922) and studies demonstrating the effect of these antibodies on the parasites or on protective immunity have mainly focused on the permissive phase of the infection. Rabinowitch and Kemper (1899), Taliaferro (1924, 1932) and Coventry (1925) showed that it was impossible to induce an infection when epimastigotes and immune serum were introduced simultaneously into susceptible animals. Coventry (1930) subsequently reported that the infection disappeared when rats were treated in the permissive phase on day 3, or 4 with serum taken as early as the 10th day, or as late as day 96. Taliaferro (1932) similarly found that the serum of hyperinfected rats is curative in that it eliminated a parasitemia of dividing epimastigotes. The present study was done to investigate the kinetics of the humoral responses during the permissive and non-permissive phases and to determine, whether protection of the treated animals correlates with the level of circulating antibodies in the sera

used in the treatment experiments and whether heat-inactivation of the serum can alter the capacity to protect or cure infected animals.

The effect of various immunosuppressing strategies in enhancing the permissive phase of T. lewisi has been reviewed by Dusanic (1975); in this context, it was of interest to examine the effect of well-tolerated doses of the alkylating agent cyclophosphamide on the course of infection with this parasite. Preliminary results from such treatment indicated that such doses of this drug can have profound effects on the cellular and immunological responses of the rats, as shown by the depression of leucocyte counts, activation by Concanavalin A, total globulins, plaque-forming cells to sheep erythrocytes and phagocytosis by macrophages. It was also of interest to determine whether cyclophosphamide-treated rats that had recovered from the infection would retain the ability to withstand a super-infection (secondary infection), as compared to recovered non-treated animals.

## MATERIALS AND METHODS.

### 1.1 Parasite maintenance

The strain of Trypanosoma lewisi used in these experiments and in subsequent studies was originally obtained from the parasite bank of London School of Hygiene and Tropical Medicine and was provided by Dr. Pierre Viens of Department of Microbiology and Immunology, University of Montreal. The parasite has since been maintained by intra-peritoneal passage in inbred Fischer CDF (F-344/CrlBR) rats (Charles River, Canada Inc., St Constant, Quebec). Infected rats were anesthetized with $CO_2$ and heparinised infected blood for passage of the parasites was obtained by cardiac puncture; the blood was centrifuged

at 500 x g for 8 minutes to sediment erythrocytes. Trypanosomes obtained after a second centrifugation of the supernatant plasma at 1000 x g for 15 minutes were resuspended in phosphate-buffered saline containing 1% glucose (PBSG, pH 8.0) and then counted under phase-contrast illumination in a Neubauer hemocytometer.

1.2 Treatment with cyclophosphamide

The alkylating agent cyclophosphamide monohydrate (CPA) is an effective immunosuppressant when administered before or after the introduction of antigen (reviewed by Dusanic 1975). Cyclophosphamide (Aldrich Chemical Company, Milwaukee, WI) was suspended in distilled water to a concentration of 10mg/ml and the drug was administered per os at a dose of 10 mg/kg total body weight; the animals were treated daily for 10 days. This treatment regimen was effective in inducing significant immunosuppression as determined by, among other things, cells counts, and the ability of treated rats to respond to antigen and mitogen stimulation. A single intra-peritoneal injection of 70 mg/kg of CPA used by El-On and Greenblatt (1971) in their studies produced fulminating lethal parasitemias when given 2 days after the inoculation of the parasite. The severe treatment used by these authors could not allow the effect of this immunosuppressant to be determined on the normal course of infection, the production of antibodies and subsequently, its effect on the protective immunity. In this study, treated animals were infected 24 hours after the last oral treatment dose of cyclophosphamide.

## 1.3 Infection of experimental animals

In four separate experiments, a total of 44 twelve-week old female inbred Fischer rats were inoculated intraperitoneally with $10^7$ day 15 trypomastigotes in phosphate-buffered saline containing 1% glucose (PBSG, pH 8.0).

## 1.4 Treatment with plasma

Plasma for the treatment experiments (Figures 3.3 and 3.4) was obtained from the donor animals either during the course of infection, after recovery or from normal uninfected rats. The rats were anesthetized with $CO_2$ and heparinised blood obtained by cardiac puncture was immediately transferred into centrifuge tubes in an ice bath. The blood was then centrifuged at $0^oC$ to separate the plasma. Heat inactivation of the plasma was done at $56^oC$ for 30 minutes. In one experiment animals were treated intravenously with one ml of non-heat inactivated day 15 infected rat plasma and either heat or non-heat inactivated day 42 rat plasma. The animals were then infected 10 minutes after the treatment. Animals in the second experiment were treated intravenously on the 8th day of infection with non- and heat-inactivated infected plasma taken on days 15 and 34 post-parasite inoculation and the parasitemia was determined for the next 48 hours. Controls in both experiments were treated with normal rat plasma.

## 1.5 Parasitemia

The level of infection was determined every 4 days by diluting 10 $\mu$l of tail blood with 1.99ml of 2% acetic acid in PBSG (pH 8.0) in

Unopets (Beckton-Dickinson, Rutherford, NJ). The trypanosomes in the resulting dilution of 1:200 were counted in a hemocytometer under phase-contrast illumination.

1.6 Serum collection

One ml of blood was obtained for the determination of antibodies from the retro-orbital plexus under light $CO_2$ anesthesia at 5-day intervals from day 5 to 60 and also on days 69, 84, 127, 185 and 318 post-inoculation. The blood was allowed to clot at room temperature before centrifugation at 1000 x g for 20 minutes; the serum obtained was dispensed into 1.5 ml Eppendorf centrifuge tubes and stored at -70°C until analyzed for antibodies.

1.7 Antigen preparation

Trypanosomes from day 15 infected rats were isolated from heparinised blood as described above before filtering through a DE 52 cellulose column equilibrated with PBSG (pH 8.0) as described by Lanham (1968), to remove all the erythrocytes and leucocytes present in the trypanosome suspension. The parasites obtained from the DE 52 column were centrifuged at 1000 x g for 15 minutes at 4°C and resuspended in 10 mM phosphate buffer (pH 8.0), containing 4 mM of the enzyme inhibitor phenylmethyl-sulfonyl-fluoride (Sigma, St. Louis, Mo). The trypomastigotes were then disrupted at 0°C with 150kc ultrasounds (Blackstone Ultrasonic Inc., Shieffield, PA). The trypanosome extract was centrifuged at 120,000 x g for 1 hour in a Beckman L3-5 Ultracentrifuge (Beckman Instruments Inc. Palo Alto, CA). The supernatant obtained was used as antigen for the determination of antibody levels in rat sera. The protein content of the antigen

extract was determined by the protein-dye binding assay, as described by Bradford (1976).

### 1.8 Enzyme-linked immunosorbent assay (ELISA)

Sera from infected rats were analyzed by ELISA, as described by Voller et al (1979). The extract of trypomastigotes was diluted in carbonate buffer (pH 9.6) to give a solution of 5 $\mu$g protein/ml; 100 $\mu$l of this preparation was then dispensed into each of the 96 wells of flat-bottomed microtiter plates (Beckton-Dickinson Labware, Oxrand, CA) for sensitization overnight at 4°C. The plates were washed three times with phosphate-buffered saline containing 0.05% Tween-20 (PBST, pH 7.2). The plates were then incubated for 15 minutes at 37°C with PBS containing 1% bovine serum albumin (BSA) to block any remaining binding sites in the wells. After a further wash with PBST, 100 $\mu$l of serum diluted 1:80 with PBST containing 1% BSA were then added to each sensitized well and the plates were incubated for 30 minutes at 37°C. Following another wash with PBST, goat peroxidase-conjugated anti-rat IgM (heavy-chain specific; Miles Yeda Ltd., Israel) and or goat anti-rat IgG (heavy and light chain specific; Miles-Yeda Ltd., Israel) were diluted 1:500 and 1:1000 respectively in PBST-1% BSA, and 100 $\mu$l of this preparation were added to appropriate wells before incubation at 37°C for 30 minutes. The plates were then washed and the peroxidase substrate ABTS (Sigma) was added. The results were read after 10 minutes incubation at room temperature with a Titertek Multiscan MC microplate reader (Flow Laboratories, McLean VA) at a wavelength of 405nm. Each serum sample was tested in triplicate. Sera from the three groups of recovered animals in the challenge experiment were

serially diluted to determine the antibody titer prior to the challenge inoculum.

### 1.9 Challenge of recovered rats

Three groups of recovered rats, one group at day 56 post-inoculation and previously treated with cyclophosphamide, and two non-treated groups at day 56 and 109 post-parasite inoculation were initially bled from the retro-orbital plexus to determine the antibody titer by ELISA before the challenge. The animals were then inoculated intravenously with a super-inoculum of $2.6 \times 10^9$ trypomastigotes from day 15 infected rats and the ensuing secondary parasitemia determined from the first minute after the challenge until no parasite could be detected in the peripheral circulation.

### 1.10 Statistics

Data were analyzed using Student's t-test (Snedecor and Cochran 1967) and considered significant at $p < 0.05$ level.

## RESULTS

The parasitemia in the rats treated with cyclophosphamide (CPA) and in control animals which had received distilled water is shown in Figure 3.1 (a). The level of infection rose sharply in both the treated and control groups, reaching a peak on day 8 in the control animals and on day 12 in the CPA-treated rats. The parasitemia then declined gradually until the infection was eliminated from the peripheral circulation by day 32 in the control animals, producing the characteristic normal Trypanosoma lewisi infection in Fischer - 344

rats. However, the parasites persisted at relatively low level for the next 24 days in the CPA-treated rats until day 56 when the infection was resolved in these animals.

The relationship between the specific IgG and IgM antibody responses to the level of parasitemia and to the normal termination of the infection in both treated and untreated animals is shown in Figure 3.1 (b) and 3.1 (c) respectively. The results of this experiment indicate that specific IgG and IgM responses were detectable as early as the fifth day post infection. However, IgG levels in the CPA-treated animals were lower than normal within the first 15 days post-infection. The levels of circulating IgG antibodies then rose during the course of parasitemia in the untreated and in the CPA-treated animals, reaching a peak at around day 30, the time when the parasitemia was being cleared from the peripheral circulation in untreated animals. After the end of the infection in the untreated animals, specific IgG persisted and remained significantly high at least until day 318 post-infection.

In contrast, IgM responses fluctuated during the course of the infection. In untreated animals, two major peaks were observed: the first occurred around the fifth day of infection and was followed by a sharp decline through day 20; a second peak then appeared at the time of parasite elimination (day 30) and a third minor rise occurred at day 50; thereafter, the IgM antibodies declined to insignificant levels. However, in the CPA-treated animals the peak observed on the 5th day of infection in untreated animals was absent, and the first IgM peak did not occur in these animals until day 30; the second peak occurred at day 55, when the infection was cleared in these animals.

The ability of the recovered CPA-treated and untreated rats to

eliminate a secondary parasitemia after a super-inoculation of 2.6 x $10^9$ is shown in Figure 3.2. Recovered untreated animals 24 or 77 days after they had cleared the infection or 56 or 109 days, respectively, after they had been inoculated, rapidly eliminated the trypanosome challenge from the circulation in 3 or 6 hours; the anti-trypanosome titer in these animals was the same (1:980). When challenged on day 56, just as the parasitemia cleared, the CPA-treated rats took, however, up to 48 hours to eliminate the challenge.

The protective role of antibodies in the sera obtained at mid-parasitemia (day 15) and ten days after animals had recovered (day 42) is shown in Figure 3.3. Four groups of animals were treated intravenously with 1 ml of normal rat plasma, day 15 infected rat plasma, or day 42 infected heat or non-heat-inactivated rat plasma prior to inoculating $10^7$ parasites 10 minutes later. The results of this experiment indicate that infection failed to develop in animals treated with plasma taken after recovery on day 42, whether it was heat-inactivated or not. Plasma taken during the course of infection on day 15 significantly reduced the level of parasitemia and accelerated the termination of the infection by 12 days, compared with the control group which was treated with normal plasma; the duration of the infection in the control animals lasted the normal 32 days. In animals treated with day 15 plasma, however, dividing forms were not observed during the entire course of infection.

The curative role of the antibodies in the immune plasma of the infected animals was investigated by determining whether the plasma can affect an on-going parasitemia. Four rats were each treated intravenously on day 8, at the peak of the infection, with 2 ml of non-

and heat-inactivated plasma taken on days 34 and 15 post-parasite inoculation. The results of this experiment shown in Figure 3.4 (a) and 3.4 (b) indicate that the plasma from recovered or infected rats can produce a transient but significant depression (up to 40%) in the parasitemia of infected animals, whether the plasma was heat-inactivated or not. The maximum depression occurred 3 or 12 hours after treatment with the day 15 or the day 34 plasma, respectively. The initial effect of the plasma waned and the level of parasitemia could not be distinguished statistically from that of the controls 24 hours after treatment, the treatment did not shorten the normal course of the infection.

## DISCUSSION

It is well known that humoral responses (Seed and Gam 1966; Murray and Urquhart 1977) play a significant role in protective immunity against specific variants in African trypanosomiasis (Stevens and Moulton 1978; MacAskill et al 1980). Similarly, antibody-dependent cellular cytotocity has been shown to participate in protection against Trypanosoma cruzi epimastigotes ( Sanderson and co-workers 1977, 1978; Abrahamson and Dias de Silva 1977; Olabuenaga et al 1979) and trypomastigote forms (Kierzenbaum and Hayes 1980). Evidence has also accumulated over the years that antibodies play a significant role in the control of primary infections with rodent trypanosomes (Taliaferro 1924, 1925; Giannini and D'Alesandro 1979; Wechsler and Kongshavn 1984). The results presented here add to this evidence and demonstrate the persistence of parasite-specific IgG and, to a lesser extent, IgM antibodies after the parasitemia has been cleared from the peripheral circulation. This study shows that two major peaks of IgM occur during

the course of parasitemia: the first occurs during the multiplication phase of the infection and the second occurs during the mature, non-dividing trypomastigote phase.

The production of specific IgM antibodies is an important response in African trypanosomiasis and is a result of the primary stimulation of the immune system by variant antigen types (Campbell et al 1978; Sacks and Askonas 1980; Morrison et al 1985). D'Alesandro (1976) reported that two antigenic variants (the dividing epimastigotes and the non-dividing mature trypomastigotes) occur during the course of infection in T. lewisi: these variants were claimed to elicit the first and second trypanocidal antibodies. Through gel filtration techniques, the latter author found that the agglutinin which was specific for the dividing forms was IgG and that which was specific for the non-dividing mature trypomastigote was IgM. The IgM class of antibodies are, classically, responses to a primary antigen sensitization (Klein 1982); the reproducing forms early in the course of the infection would be expected, therefore, to induce first an IgM response, followed by a switch to IgG in the classical manner. Our results suggest that this is, indeed, the case in infection by T. lewisi since IgM appeared as early as the fifth day of the infection, when the IgG was at its lowest level. Since the elimination of the parasitemia in infected animals coincided with peak levels of both IgM and IgG antibodies, it may be that resolution of the infection is a function of both IgG and IgM antibodies and not to IgM alone, as suggested by D'Alesandro (1976). Indeed, the IgG antibody levels were about three times higher than those of IgM suggesting that IgG is probably the major antibody that mediates elimination of the primary infection. This proposition is

strengthened by the observation that IgG levels remained significantly high for at least 318 days post-infection, while the IgM levels declined to insignificant levels after the 55th day of infection. Its likely that the three IgM peaks observed here probably represent stimulation by cryptic variant-specific antigen(s) produced during the course of infection and after animals had recovered. Ferrante and Jenkin (1977) have shown that some of the immunoglobulins produced during infection with T. lewisi are not opsonic nor complement-fixing and may therefore not play a major role in imparting resistance to infection by phagocytic mechanisms. Since our studies assessed for total IgG, some of the antibodies were certainly ablastic, which have been shown to be non-agglutinating (D'Alesandro 1966), can be demonstrated in the serum of infected rats as early as the fifth day of infection (Giannini and D'Alesandro 1979) and curiously, also in the serum of uninfected rats (Giannini 1987). Ablastin titres have been shown to be high during the early phase of the infection but decline in the later phases of parasitemia (Coventry 1925). Studies by Ferrante and Jenkin (1978) further showed that opsonic antibody, which is primarily IgG (Roitt, 1984), does not appear in the serum of infected rats until day 8 following infection, when the rapid removal of the parasites by the mononuclear phagocyte system could be demonstrated for the first time (Ferrante and Jenkin 1977). The level of opsonic antibody increases during the course of infection and reaches a maximum during the time that the rats eliminate the parasitemia. These results are in line with the evidence shown in this study and suggest an important role for IgG antibody in the elimination of the infection.

Cyclophosphamide is an immunosuppressant that has been shown to mainly affect B cells, and has been used to study the role of B cells

in African trypanosomiasis (Esuruoso 1976) and in T. musculi (Pouliot 1978). Treatment of mice with cyclophosphamide renders B cells unresponsive to mitogens (Esuruoso 1976) and, when administered prior to infection, initial parasitemia is more severe and the production of IgG is delayed (Pouliot 1978). Fulminating parasitemia and a delay in IgG also occurred when rats infected with T. lewisi were treated with high doses of cyclophosphamide two days after infection (El-On and Greenblatt 1976). Using tolerable doses of cyclophosphamide (CPA) that significantly immunosuppressed the rats without causing any fatalities, we were able to monitor the kinetics of the production of both IgG and IgM, in relation to the level of infection. These studies indicate that, although the level of parasitemia was not significantly enhanced in the treated animals the duration of the infection was greatly prolonged and the initial production of IgM and IgG was delayed. While two IgM peaks were observed in normal as well as in CPA-treated animals, it is interesting that the maximum levels of IgG and the second IgM peak coincided in the treated and untreated infected rats with the elimination of parasitemia. It is certainly also very interesting that the level of IgG antibodies in recovered animals remained significantly elevated at least up to 318 days after the initial inoculation of the parasites, 286 days after elimination of the parasitemia, in the absence of any demonstrable parasites in the peripheral circulation. Its persistence strongly implicates this antibody with protective immunity in recovered rats.

Infections with rodent trypanosomes have long been known to induce a solid immunity (Taliaferro and Paulinova 1936; Corradetti 1963). The protective immunity in recovered hosts can be demonstrated by the

ability of these animals to clear a secondary trypanosome challenge, as has been shown in T. musculi (Viens et al 1975), where the persistence of IgG (Olivier and Viens 1985) induced by kidney forms of this parasite, have been shown to confer immunity after recovery from the parasitemia. Viens et al (1975) observed that the ability to clear a secondary trypanosome challenge depended on the duration of the infection: mice that had recovered from their parasitemia one month previously eliminated the challenge within 48 hours, whereas mice that had recovered 11 months previously took 96 hours to clear the challenge. Antibodies which persisted in the serum of the recovered mice were thought by these authors to be responsible for mediating protective immunity by enhancing phagocytosis by macrophages. Our study, however, indicates that rats that had recovered 24 or 77 days previously from a parasitemia with T. lewisi eliminated a heavy challenge inoculum of $2.6 \times 10^9$ trypanosomes within 3 or 6 hours, respectively; elimination of the challenge was, however, delayed to 48 hours in the CPA-treated rats that had just recovered from an infection. Kloetzel and Deane (1970) have demonstrated that antibodies present in the immune sera of rats infected with T. lewisi mediate in vitro, the phagocytosis of this parasite by macrophages. These studies have been further extended by Ferrante and Jenkin (1977, 1978) and Ferrante et al (1978a, 1978b). These latter authors have shown that elimination of the trypanosomes by phagocytic cells in vivo coincides with the appearance of an opsonic antibody in the infected rats; in vitro macrophages from either normal or infected animals were equally efficient in the phagocytosis of T. lewisi in the presence of immune sera (Ferrante and Jenkin 1978). Since recovered animals have high levels of IgG, its conceivable that this antibody may have mediated

elimination of the secondary parasitemia in our study by opsonizing the parasite, thus enhancing phagocytosis by macrophages in a manner similar to that described by Ferrante and Jenkin (1978) and Ferrante et al (1978b). These authors have shown that more than 80% of the parasites are removed by the liver during the elimination phase of a primary infection. The delay in the elimination of the secondary parasitemia in CPA-treated animals, although the antibody level was similar as in the untreated animals, suggests that this treatment interfered with the cells of mononuclear phagocytic system and that the phagocytic capacity of these cells had not fully recovered by the time the parasite was eliminated. An effect of cyclophosphamide on mononuclear phagocytes may also account in part for the prolonged persistence of a low level of parasitemia in the CPA-treated rats before complete elimination of the infection was effected. Indeed, preliminary studies showed that phagocytosis by macrophages was one of the cellular functions that was depressed by this treatment regimen. Antibody-mediated phagocytosis in the liver has been observed in mice infected with T. brucei or in normal mice passively immunized with hyperimmune serum (MacAskill et al 1980); the activity of parasite-specific antibodies in the phagocytosis of T. brucei by macrophages from normal animals has also been demonstrated (Takayanagi et al 1974; Stevens and Moulton 1978).

Having determined the antibody level during the course of infection, we were interested in determining whether immune plasma obtained during the parasitemia and after recovery would protect naive animals, and whether the protection would be affected by heat-inactivation of the plasma. Protection mediated by the intraperitoneal

passive transfer of immune serum mixed with T. lewisi has been reported by Rabinowitsch and Kemper (1899); protection mediated by the passive transfer of immune sera has also been demonstrated in T. cruzi (Culbertson and Koloday 1938; Kagan and Norman 1961; Ryckman 1965) and in infection by T. musculi (Wechsler and Kongshavn 1984). Our studies suggest that protection is dependent on the level of antibodies the plasma used in treatment, since animals treated with day 15 immune plasma exhibited a lowered parasitemia and a shorter infection in contrast to complete protection in rats treated with day 42 immune plasma. The protective capacity of the immune plasma was not affected by heat-inactivation: heat-inactivated day 42 immune plasma protected just as well as day 42 immune plasma that had not been inactivated. The incomplete protection shown by animals treated with day 15 immune plasma correlated well with lower level of IgG in the animals at mid-infection.

Recently, Wechsler and Kongshavn (1984) reported that the passive transfer of plasma from mice that had recovered from T. musculi rapidly induced elimination of the parasitemia. These authors suggested that the curative property, but not the protective role, of the plasma was mediated by a heat-labile immunoglobulin since cure was ablated by heat-inactivation. Our studies indicate that passive transfer of day 15 and 34 immune plasma into animals with an on-going parasitemia effected a transitory cure, whether the plasma was heat inactivated or not. It is interesting to note that Coventry (1930) reported a curative property associated with the passive transfer of immune serum; Taliaferro (1932) conceded, however, that Coventry's (1930) success in curing infected animals by T. lewisi was due to the low level of parasitemia, since the treatment was done within the first few days of

the infection. Wechsler (1987) made a similar observation with T. musculi: cure of T. musculi infection only occurred in the genetically resistant strains of mice which exhibited a low parasitemia: cure did not occur in the more susceptible strain that showed a high infection. The failure to completely cure an ongoing infection in this study may have been due to the presence of large numbers of parasites at the time when treatment was done at peak parasitemia.

The studies presented here show that the specific antibody response induced during the course of an infection with T. lewisi persists for long periods after resolution of the parasitemia. These studies further implicate these persisting antibodies in protective immunity and the delay in eliminating of a secondary challenge in CPA-treated rats indicates the participation of another component with the antibodies in conferring absolute immunity; this additional component is most probably, the reticulo-endothelial phagocytes. Antibodies are protective and the degree of protection depends on the level of antibodies present in immune plasma. The antigens which these antibodies recognize during the course of the parasitemia and after the end of infection and the chemical nature of these antigens are presented in Chapter VII. The study which follows investigates using drug-abbreviated infections, the relationship between the length of infection and the induction of immunity by Trypanosoma lewisi.

Fig. 3.1(c). Specific IgM responses in the serum of untreated (■) and cyclophosphamide-treated (□) rats infected with Trypanosoma lewisi. The absorbance and the mean range of ELISA absorbance in normal uninfected rats is shown by (▲) and (▨) respectively. Each point is the mean ± S.E. ELISA absorbance of triplicate readings from six animals. The astericks represent significant difference by Student's t-test between the IgM levels of untreated and cyclophosphamide-treated animals: *** - $p<0.001$.

Fig. 3.1(b). The legend for the specific IgG production is as described in Fig. 3.1(c). The asterick represents significant difference by Student's t-test between the IgG levels of untreated and cyclophosphamide-treated animals: * - $p<0.05$.

Fig. 3.1(a). The course of parasitemia in untreated (■) and cyclophosphamide-treated (□) Fischer CDF rats. Each point represents the mean ± S.E. of parasite count from six animals. There was no significant difference in the level of infection by Student's t-test between the cyclophosphamide-treated and untreated rats.

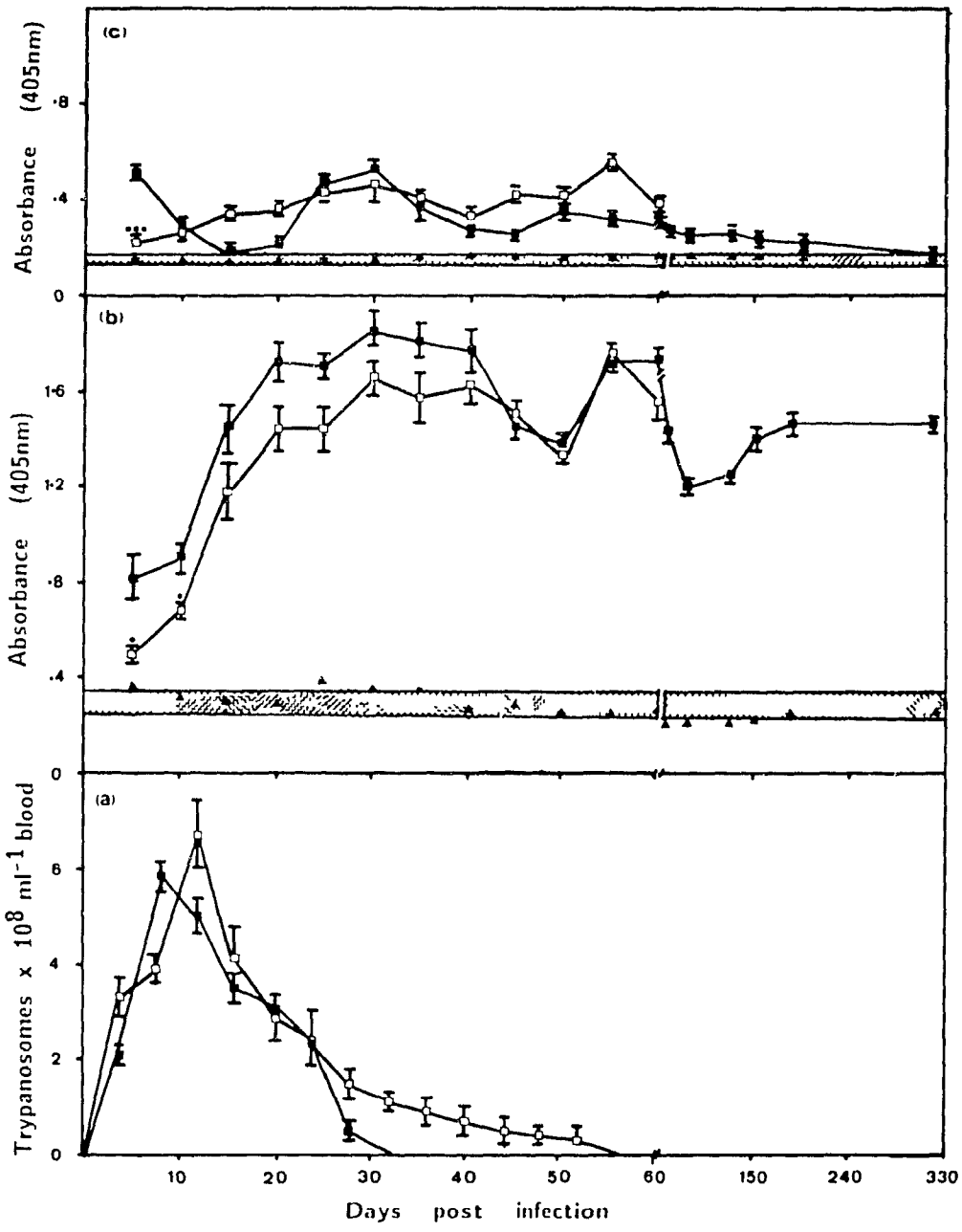

(c)
Absorbance (405nm)
·8
·4
0
(b)
Absorbance (405nm)
1·6
1·2
·8
·4
0
(a)
Trypanosomes x $10^8$ $ml^{-1}$ blood
6
4
2
0
10
20
30
40
50
60
150
240
330
Days post infection

Fig. 3.2. The elimination of a secondary parasitemia in recovered untreated rats at day 56 (●), day 109 (■) and day 56 cyclophosphamide-treated recovered rats (□) following an intravenous inoculum of 2.6 x $10^9$ trypomastigotes. Each point represents the $\log_{10}$ mean ± SE parasite count from three to four animals.

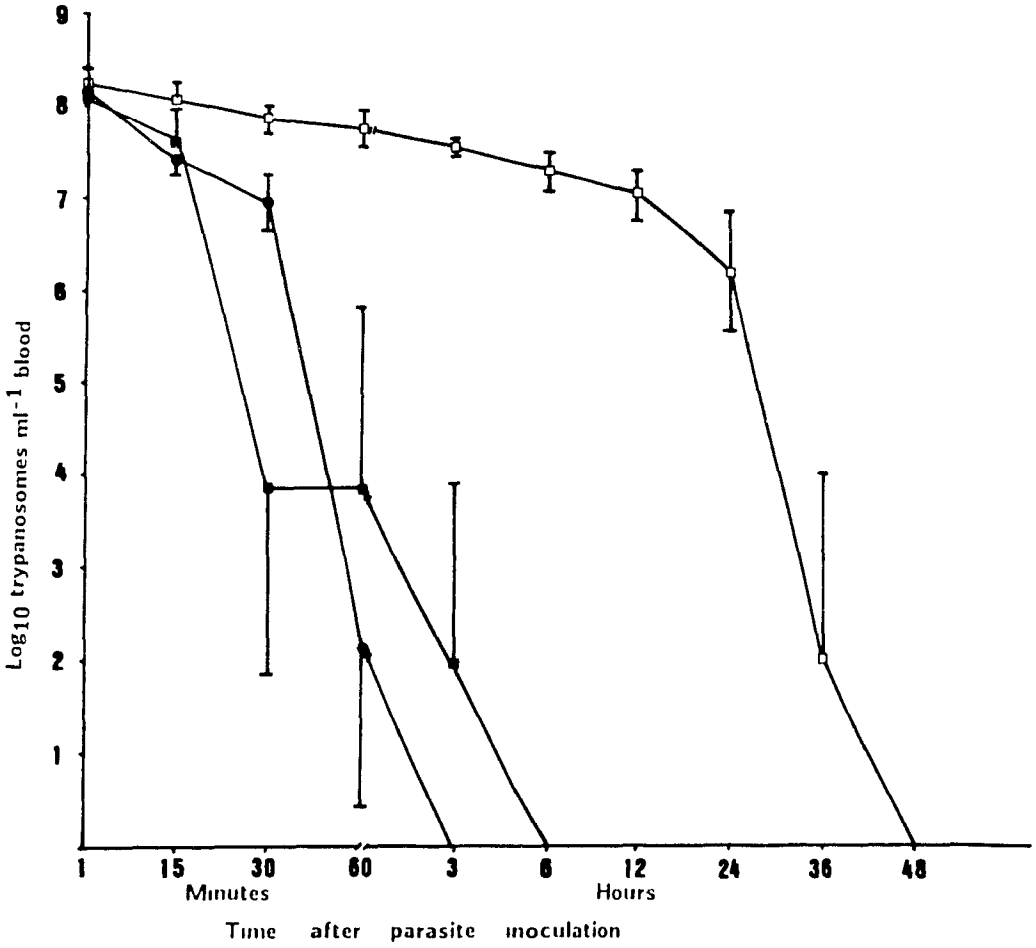

Log10 trypanosomes ml-1 blood
9
8
7
6
5
4
3
2
1
1
15
30
60
3
6
12
24
36
48
Minutes
Hours
Time after parasite inoculation

Fig. 3.3. The effect of treating naive rats with day 42 non- (▲) or heat-inactivated (Δ) infected rat plasma, plasma from mid-infection at day 15 (O) or controls that received normal rat plasma (●) prior to inoculation with $10^7$ trypomastigotes. Each point represents the mean ± S.E. parasite count of four animals. The asterick represents significant difference by Student's t-test from the parasitemia of the normal rat plasma treated controls: *-$p<0.05$. Complete protection occurred in animals treated with day 42 immune serum.

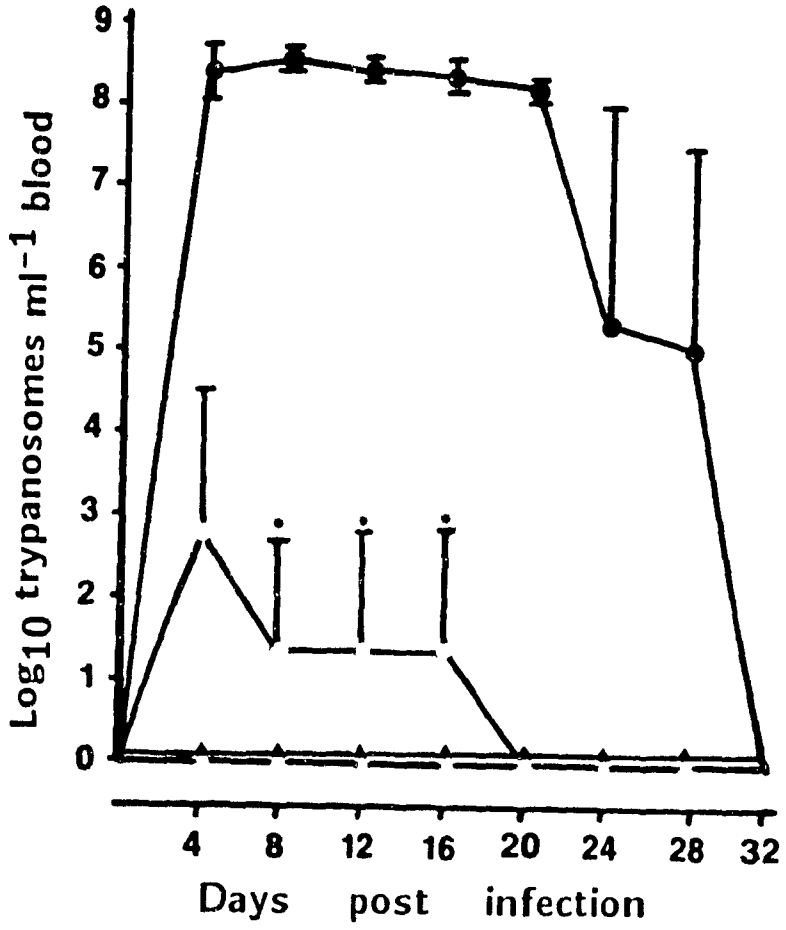
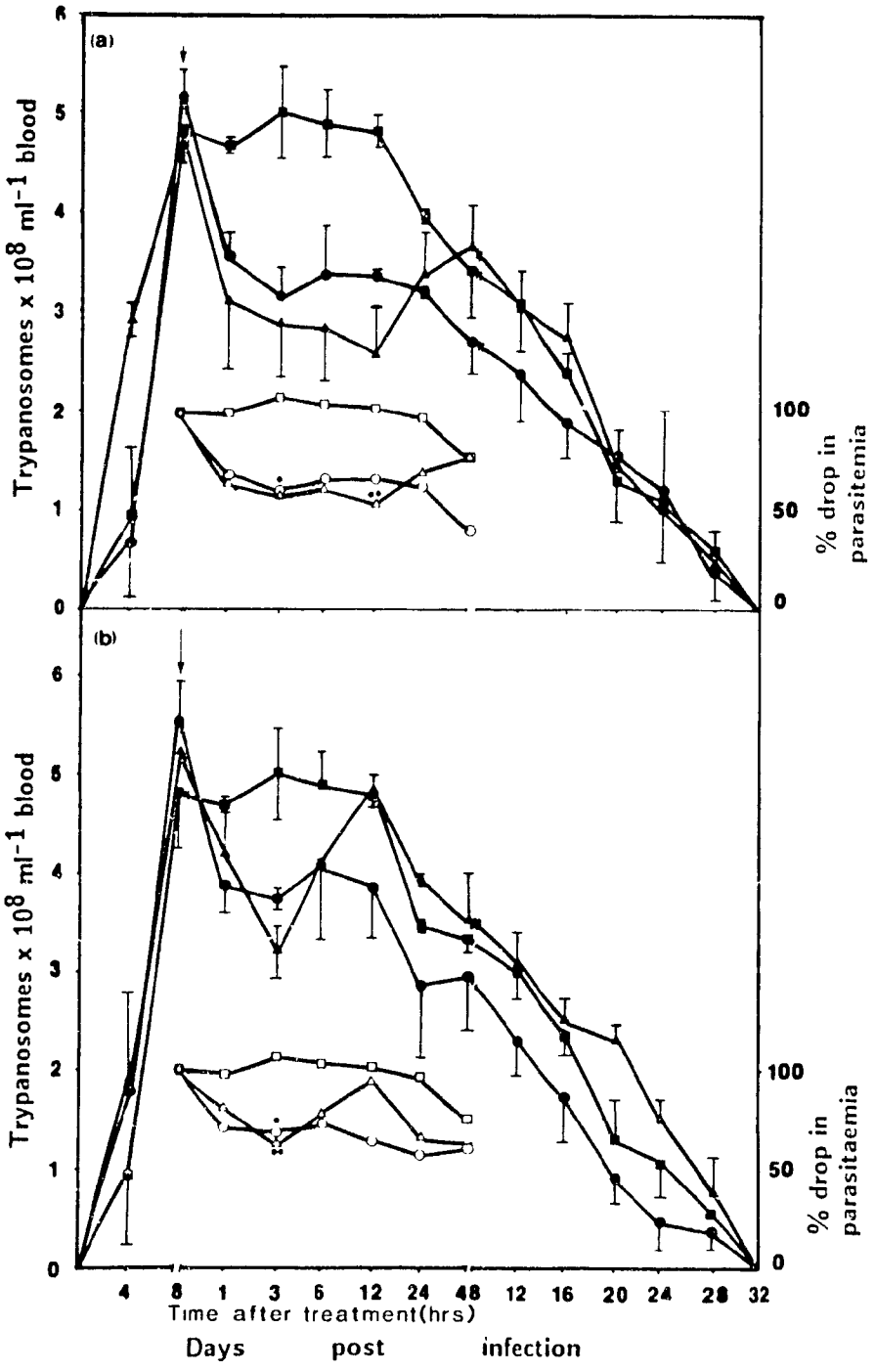

Log10 trypanosomes ml-1 blood
0
1
2
3
4
5
6
7
8
9
4
8
12
16
20
24
28
32
Days post infection

Fig. 3.4(a). The effect of the transfer of day 34 non- (●) or heat-inactivated (▲) plasma, or normal rat plasma (■) at the peak of infection on the level of infection (↓). Their corresponding % drop after treatment is indicated by the corresponding open symbols. The level of parasitemia is represented by mean ± S.E. parasite counts from four rats. The asterick represents significant difference by Student's t-test at maximal % drop in parasitemia compared to the control animals treated with normal rat plasma: *-$p<0.05$, **-$p<0.01$.

Fig. 3.4(b). The effect of the transfer of day 15 non- (●) or heat-inactivated (▲) plasma, or normal rat plasma (■) at the peak of infection on the level of infection (↓). Their corresponding % drop after treatment is indicated by the corresponding open symbols. The level of parasitemia is represented by mean ± S.E. parasite counts from four rats. The asterick represents significant difference by Student's t-test at the maximal % drop in parasitemia compared to the control animals treated with normal rat plasma: *-$p<0.05$, **-$p<0.01$.


(a)
(b)
Trypanosomes x $10^8$ $ml^{-1}$ blood
% drop in parasitemia
% drop in parasitaemia
0
1
2
3
4
5
6
100
50
0
4 8 1 3 6 12 24 48 12 16 20 24 28 32
Time after treatment(hrs)
Days post infection

# CHAPTER IV: THE EFFECT OF DRUG-ABBREVIATION THE INDUCTION OF SPECIFIC ANTIBODY AND PROTECTIVE IMMUNITY IN INFECTIONS BY TRYPANOSOMA LEWISI.

## INTRODUCTION

Infections with Trypanosoma lewisi induce a solid, sterile and probably lifelong immunity (Corradetti 1963). This immunity has been attributed to the presence of trypanocidal antibodies which prevent reinfection and depends on the relative titers of trypanocidal and, in some cases, ablastin antibodies (D'Alesandro 1970). In infections with the related rodent trypanosome, Trypanosoma musculi, actively reproducing forms occur on the vasa recta of the kidneys (Viens et al 1972) and it has been shown that these forms are responsible for inducing antibodies that mediate protection in recovered mice. The destruction of the parasite in the kidneys with the trypanocidal drug Berenil was followed by a decline in the level of antibodies and the treated mice re-acquired a classical parasitemia, as in normal uninfected mice when secondarily challenged (Olivier and Viens 1985). Trypanocidal drugs could effectively reveal the relationship of parasitemia and the protective immunity that ensues after animals have recovered from Trypanosoma lewisi. Occult forms of this parasite have not been reported, yet our studies indicate a prolonged persistence of parasite-specific antibodies after resolution of the parasitemia. The objectives of this study were, therefore, to investigate the effectiveness of four commonly used trypanocidal drugs (ethidium bromide, Berenil, Lampit and Radanil) to reduce infections with T. lewisi, and to determine whether abbreviation alters the specific anti-trypanosome antibody response and the protective immunity that develop after infection.

## MATERIALS AND METHODS

### 1.1 Animals and infections

Twelve-week old female inbred Fischer CDF (F-344/CrlBr) rats (Charles River, Canada, Inc., St. Constant, Quebec) were used in this study. The animals were inoculated with trypomastigotes obtained from day 15 infected rats and the ensuing parasitemia was assessed as described previously (Chapter III).

### 1.2 Treatment with trypanocidal drugs

Six groups consisting of five animals each were used in this study. Two of these groups were treated on the fifth day of the infection with a single intramuscular (i.m.) dose of either 35 mg/kg body weight of ethidium bromide (EB; Sigma Chemical Co. St. Louis, MO) or 100 mg/kg body weight of Berenil (BE; Hoechst AG, West Germany), dissolved in 0.85% saline. The control group for the EB- and BE-treated animals was treated i.m. with saline at the same time as drug treatment. Two other groups of five rats each were treated intraperitoneally for four consecutive days (Jennings and Gary 1982; Dusanic 1983), starting from the fifth day of the infection, either with a suspension of 350 mg/kg body weight of Lampit (LA; Bayer, Leverkusen, West Germany) in saline or the same dose of Radanil (RA; F. Hoffman-La Roche and Co. Ltd. Basle, Switzerland) dissolved in saline. The control group for both the LA- and RA-treated animals received saline on similar days.

Serum, antigen preparation and serology were done as described previously (Chapter III).

### 1.3 Challenge of treated recovered rats

The effect of the treatment with trypanocidal drugs on the protective immunity was determined 240 days after the initial parasite inoculation by an intra-peritoneal challenge with $10^7$ trypomastigotes. The presence of trypanosomes after the challenge was determined daily by the examination of wet blood smears of tail blood; the smears were scored as positive or negative for the presence of the trypanosomes.

### 1.4 Statistics

Data was analyzed by Student's t-test and considered significant at $p < 0.05$ level. Data for antibody titers were converted to their logarithmic values before the analyses.

## RESULTS

The level of parasitemia in the animals treated with either a single intramuscular 35 mg/kg dose of ethidium bromide (EB) or with Berenil (BE) at a dose of 100 mg/kg body weight on the fifth day after inoculation of the parasite is shown in Figure 4.1. After EB administration, the parasitemia rose sharply through day 8 and then declined sharply thereafter to be effectively cleared by day 12. Treatment with Berenil (BE), even at a dose of 100 mg/kg body weight, which is two and half times the dose used to clear Trypanosoma brucei infections in mice (Jennings and Urquhart 1983), failed to eliminate the parasitemia; the infection was cleared normally as in the saline-treated control animals.

The effect of four consecutive treatments with Radanil (RA) and

Lampit (LA) on the course of infection in rats is shown in Figure 4.2. These results indicate that treatment with LA (Figure 4.2) did not have an effect until after day 8 and the parasitemia continued to rise during the four days that the drug was being administered. The level of infection in the LA-treated rats reached a peak on the 8th day, as in the control animals, and then declined sharply; the parasitemia disappeared, however, by day 16 of the infection. In contrast, parasitemia in RA-treated rats declined rapidly after the start of drug treatment through day 12 but the animals failed to eliminate a persistently low level of infection until day 20.

The effect of treatment of infected animals with EB and BE on the specific antibody response is shown on Table 4.1. The results, given as the $\log_2$ antibody titers, indicate that the rats treated with EB produced lower total IgG antibody than the control animals whose specific IgG titers remained elevated long after the parasitemia had been cleared (Table 4.1). The antibody titers in animals treated with Lampit (Table 4.2) were also significantly depressed, as in the EB-treated rats, through day 240 post-infection. Treatment with Berenil (Table 4.1) had no effect on the specific antibody responses; Radanil (Table 4.2) induced a transitory depression of antibodies 20 and 30 days after the initial parasite inoculation but the level of specific immunoglobulins was as in the control animals 240 days later.

To determine the effect of the treatment with these trypanocidal drugs on protective immunity, the treated animals were challenged with $10^7$ trypomastigotes 240 days after the initial inoculation of the parasite. Except for the animals treated with ethidium bromide, the other treated and control rats completely resisted the challenge. Trypanosomes persisted for only four days in the circulation of three

out of the five challenged rats which had been and treated with ethidium bromide; these animals had antibody titers lower than 1:160 when they were challenged, in contrast to pre-challenge titers of 1:320 or higher in the drug-treated and the control animals which resisted the infection.

## DISCUSSION

The effect of trypanocidal drugs on various trypanosomes is well documented: T. cruzi (Richle et al 1980; Malanga et al 1981; Gutteridge 1982; Krettli et al 1982), T. brucei (Jennings et al 1977; 1980; Zweygarth and Rottcher 1987b), T. congolense, T. simiae, T. evansi (Zweygarth and Rottcher 1987a) and T. vivax (Schonefeld et al 1987). The effect of the drug therapy on T musculi has also been reported, especially in the ability of the treatment to clear a parasitemia (Jennings et al 1982; Dusanic 1983) and to depress the specific antibody response in the treated animals (Dusanic 1983; Olivier and Viens 1985).

The present study shows that T. lewisi like other trypanosomes, is susceptible to the trypanocidal effect of most of the commonly used anti-trypanosome drugs, except Berenil. Ethidium bromide was the most effective in clearing the parasitemia since a single intramuscular dose on the fifth day of the infection rapidly eliminated the parasite from the peripheral circulation. Treatment of infected animals with ethidium bromide on the second day after initiation of the infection, the phase of the infection characterized by active multiplication in the capillaries of the kidneys (Hoare 1972), prevented the trypanosomes from appearing in the peripheral blood (results not

shown). The antibody titers of the animals treated with ethidium bromide on the second day (results not shown) of infection were also significantly depressed. These animals were, however, also transitorily re-infected for only four days when challenged 40 days later with $10^7$ trypomastigotes, indicating that protective immunity is induced very early during the course of an infection by T. lewisi. Ethidium bromide intercalates with the DNA and inhibits its biosynthesis (Delain et al 1972); this mode of action may, therefore, explain its dramatic effect on the rapidly-dividing trypanosomes. The lower antibody response in animals treated with this drug would thus be due to the action to remove the bloodstream trypanosomes and, therefore, reducing the antigenic stimulation of the immune system. It is interesting to note that, even at the relatively high dose of 100 mg/kg body weight, Berenil failed to eliminate the infection. Subsequently, the antibody level in infected rats treated with this drug was not statistically different from that in control animals and the treated rats effectively cleared the infection normally.

Berenil has successfully been used to treat animals suffering from infections with African trypanosomes (Wellde and Chumo 1983; Gutteridge 1985) and, more recently, in humans suffering from the Gambian and Rhodesian forms of the sleeping sickness, although some relapses have subsequently occurred (Gutteridge 1985). Jennings et al (1982) have, on the other hand, reported the failure of Berenil to eliminate the bloodstream forms of T. musculi but to effectively clear the kidney forms in recovered mice. The mode of action of Berenil and other diamidines is still not clearly known although these drugs markedly affect the biosynthesis of nucleic acids and readily bind to DNA (Gutteridge 1969). Thus, the selectivity of the action of Berenil

appears to be due to a differential permeability of the compound between the cells of the host and the parasite (Gutteridge 1985). Susceptible trypanosomes have a transport system for Berenil (and other diamidines) which raises the intracellular concentration of the drug to many times the concentration in the plasma (Damper and Patton 1976). It is conceivable that bloodstream forms of T. lewisi lack this transport system and are, thus, unaffected by even higher doses than 40 mg/kg previously reported by Jennings et al (1983) to effectively clear T. brucei from the circulation.

Lampit treatment cleared the parasitemia effectively and shortened the duration of infection and, although this was accompanied by a significant depression of specific antibody responses, these animals and those in the Radanil-treated group resisted a re-infection when challenged 240 days later. Lampit has successfully been used in eliminating infections with T. musculi in mice, with subsequent depression of specific antibody (Dusanic 1983). Lampit binds to DNA, alters oxygen consumption and induces abnormal changes in the cell morphology of T. cruzi. The mechanism of the trypanocidal activity of this drug is, however, still not well understood (Huezo et al 1973; Villata et al 1979), although Docampo and Stoppani (1979) have also reported the generation of superoxide ions and hydrogen peroxide which accumulate to cytotoxic levels in T. cruzi due to the absence of catalase (Docampo and Moreno 1984) in animals treated with Lampit. The trypanocidal effect of Radanil has been mainly attributed to its inhibition of nucleic acid and protein synthesis (Polak and Richle 1978; Sims and Gutteridge 1979). Although these two drugs were effective in clearing the parasitemia of T lewisi with kinetics that

resembled that of ethidium bromide, it is difficult to explain why Radanil and Lampit did not have the same effect on antibody titers and protection as treatment with ethidium bromide had.

The maintenance of high levels of specific antibody after animals have cleared infection with T. lewisi suggests a continued antigenic stimulation which could be due to occult forms of the parasite, as it has been shown in T. musculi (Viens et al 1972). However treatment of recovered animals with ethidium bromide (results not shown) which cleared the parasitemia by day 12 did not result in a lowering of the antibody titre and did not render the animals susceptible to a challenge re-infection (results not shown) suggesting that occult forms of the parasite may not persist after the parasitemia has resolved. A more likely possibility is the persistence of antigen residues after the resolution of the parasitemia which are slowly released to continue stimulating the antibody response long after the parasite has disappeared from the circulation. This phenomenon of antigen persistence in animals immunized with protein antigens has been reported by Bystryn et al (1971) and Tew et al (1980, 1984). It has also been shown that protein antigens persist in lymphoid tissue on large cells with dendritic morphology; these antigen-retaining dendritic cells are located in lymphoid follicles of draining lymph nodes and spleens of immune animals (Nossal et al 1968; Szakal and Hanna 1968; Hanna and Szakal 1968). More recently Tew et al (1984) have reported the persistence for years of antigen retained by follicular dendritic cells in the lymphoid tissues of immune animals which participates in the induction and maintenance of B cell memory, as well as the maintenance of serum antibody levels. Our observation that inoculation of naive immunosuppressed rats with blood or with

homogenates of tissues from recovered animals has never resulted in patent infection is more in favor of antigen-retention than the existence of occult forms in recovered animals. It is interesting to note that naive rats, immunized with living non-infective culture forms of T. lewisi develop a life-long solid immunity within ten days, similar to that occurring in the animals that have recovered from a normal infection (Novy et al 1912). The failure to induce a normal infection in animals that did not show parasites in peripheral circulation after the treatment on the second day of infection with EB, or in rats that effectively cleared the infection either by day 12 (EB) or by day 16 and 20 (LA and RA respectively) indicates that protective immunity to T. lewisi is established very early in the infection; this protection is probably established before the appearance of the parasite in the peripheral circulation during the epimastigote phase, and may not require the stimulation by trypomastigotes forms for development to occur. Only animals that showed antibody titers of less than 1:160 acquired an infection (if only transitorily) from the challenge inoculation, indicating a correlation between the antibody titers and the resistance to the challenge.

Previous studies have concerned the description and effector functions of the antibody response in infection with Trypanosoma lewisi (Chapter III) and an investigation of the therapeutic effect of several drugs and their effect on the induction of protecting responses in the host (Chapter IV). The chapter that follows describes an investigation of the role that soluble factors from the parasite itself have to modulate the infection.

Fig 4.1. The effect of treatment with ethidium bromide (▲) and Berenil (●) on the fifth day (↓) of infection at dosage rates of 35mg/kg and 100mg/kg body weight respectively on the course of parasitemia in rats infected with Trypanosoma lewisi, compared with saline treated control animals (■). Each point represents the mean ± S.E. parasite count of five infected rats.

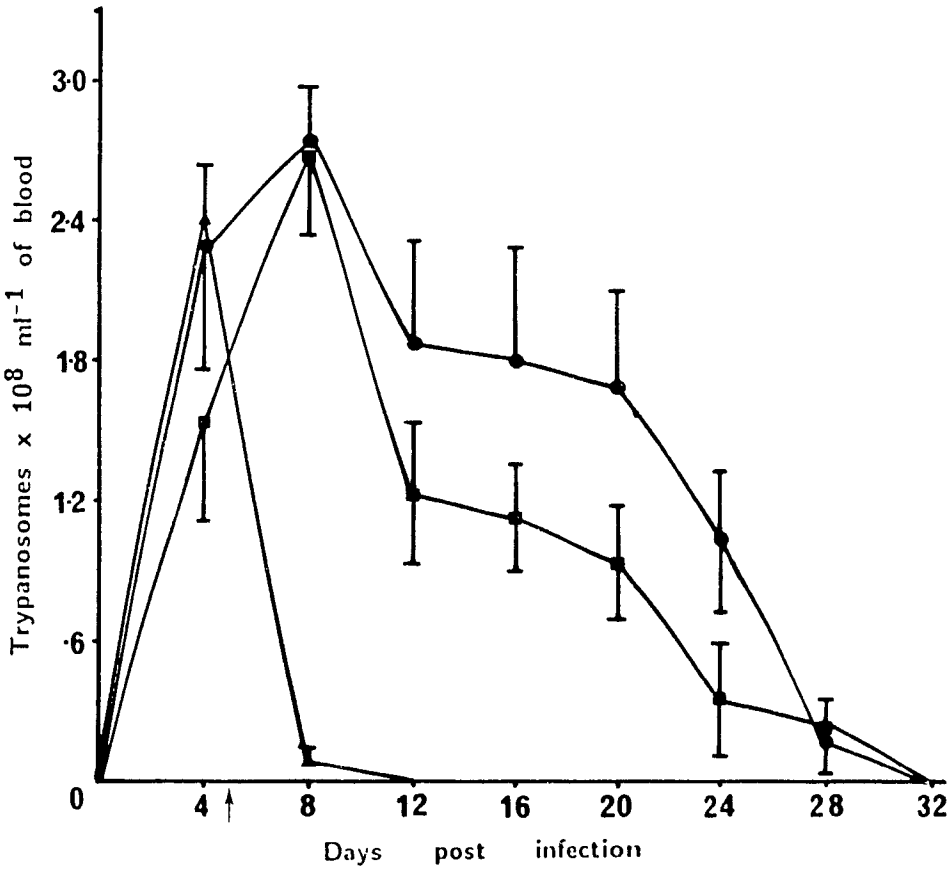
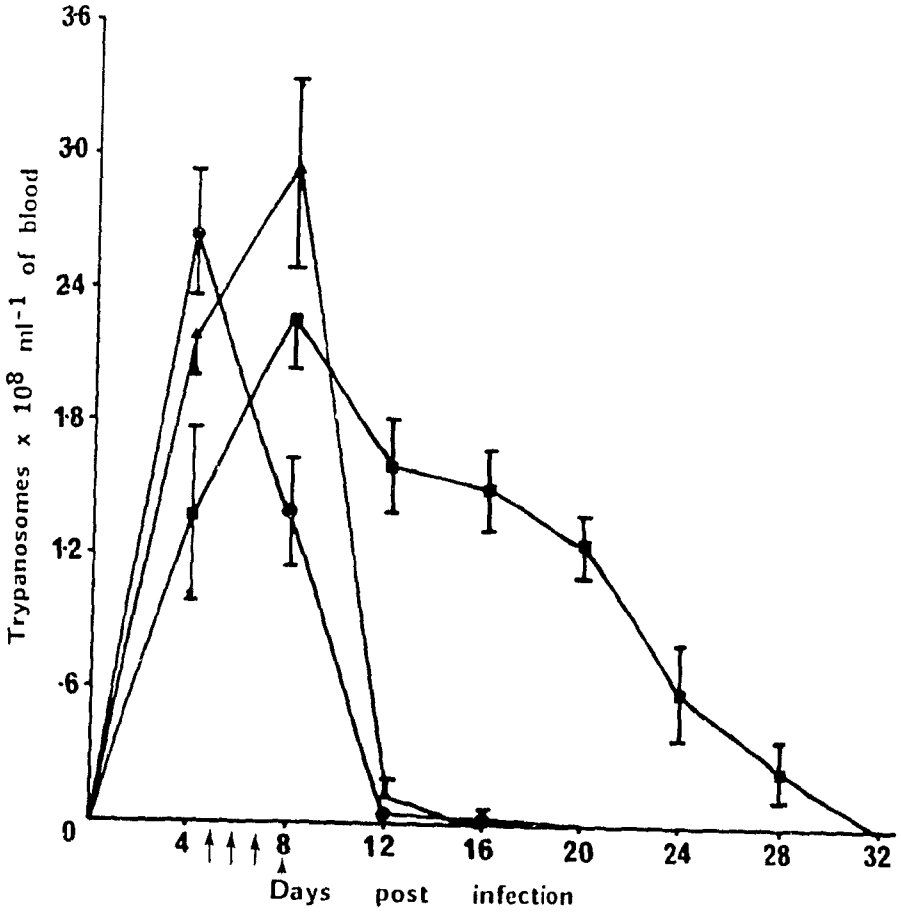

3·0
2·4
1·8
1·2
·6
0
Trypanosomes x $10^8$ $ml^{-1}$ of blood
4
8
12
16
20
24
28
32
Days post infection

Fig 4.2. The effect of treatment with Lampit (●) and Radanil (▲), starting on the fifth day of infection for four consecutive days (↑) at dosage rate of 350mg/kg body weight respectively, on the course of parasitemia in rats infected with Trypanosoma lewisi, compared with saline treated control animals (■). Each point represents the mean ± S.E. parasite count of five infected rats.


3·6
3·0
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Trypanosomes x $10^8$ $ml^{-1}$ of blood
4
8
12
16
20
24
28
32
Days post infection

Table 4.1. The effect of treatment with ethidium bromide and Berenil on the IgG production in rats infected with Trypanosoma lewisi[a].

| | Days post infection | | | |
|---|---|---|---|---|
| Drug | 10 | 20 | 30 | 240 |
| Ethidium bromide | 4.9 ± 0.2 | 4.3 ± 0.4* | 5.9 ± 0.5** | 6.7 ± 0.5** |
| Control | 6.8 ± 0.8 | 8.2 ± 0.8 | 11.4 ± 0.4 | 11.1 ± 0.1 |
| Berenil | 6.3 ± 0.4 | 5.9 ± 0.4 | 11.7 ± 0.2 | 10.7 ± 0.2 |

a. Data expressed as mean ± SE of $Log_2$ antibody titer of triplicate determinations of sera from five animals.

*. Astericks indicate a significant difference by Student's t-test in antibody titer between the drug-treated animals and the saline-treated controls: * - $p<0.05$, ** - $p<0.01$.

Table 4.2. The effect of treatment with Lampit and Radanil on the IgG production in rats infected with Trypanosoma lewisi[a].

| | Days post infection | | | |
|---|---|---|---|---|
| Drug | 10 | 20 | 30 | 240 |
| Lampit | 6.3 ± 0.7 | 5.5 ± 0.2** | 6.3 ± 0.5** | 9.1 ± 0.3* |
| Control | 5.3 ± 0.3 | 7.4 ± 0.2 | 10.8 ± 0.2 | 10.9 ± 0.2 |
| Radanil | 4.9 ± 0.2 | 5.5 ± 0.4* | 8.5 ± 0.4* | 10.7 ± 0.2 |

a. Data expressed as mean ± SE of $Log_2$ antibody titer of triplicate determinations of sera from five animals.

*. Astericks indicate a significant difference by Student's t-test in antibody titer between the drug-treated animals and the saline-treated controls: * - $p<0.05$, ** - $p<0.01$.

# CHAPTER V: PROTECTING AND ENHANCING FACTORS DERIVED FROM TRYPANOSOMA LEWISI.

## INTRODUCTION

Parasite infections are largely accompanied by a hyporesponsive immune period which begins soon after the establishment of the organism in the host. In this context, the pathogenic trypanosomes have received considerable attention and the phenomenon of immunosuppression has been widely studied in these infections (Pearson et al 1979; Mansfield 1981; Bancroft and Askonas 1985). This phenomenon has also been reported in animals mainly infected with other protozoal parasites: Plasmodia (Greenwood et al 1971; Williamson et al 1978, Ballet et al 1982), T. cruzi (Cunningham et al 1978, 1980; Corsini et al 1981), T musculi (Albright and Albright 1980, 1981; Albright et al 1978) and T. lewisi (St. Charles et al 1981; Proulx 1988). Immunosuppression, however, has also been documented in helminth infections: Schistosoma mansoni (Pelley et al 1976; Colley 1976; Coulis et al 1978; Attalah 1979), Hemonchus contortus (Adams 1978; Shubber et al 1984) and Nematospiroides dubius (Behnke et al 1983; Losson et al 1985).

The mechanisms underlying the induction of immunological unresponsiveness are not fully understood and the induction of this phenomenon has been a subject of great controversy. Several mediator mechanisms have been suggested to explain this phenomenon: polyclonal activation of immunocompetent cells (Mansfield and Bagasra 1978; Azzoku et al 1979; Diffley 1983; Langhorne et al 1983), alteration of lymphocyte function by the parasite in the absence of suppressor cells

(Albright et al 1977; Kierszenbaum and Badzko 1982; Maleckar and Kierszenbaum 1983, 1984), lymphocyte depletion (Kierszenbaum 1981), generation of T suppressor cells (Jayawardena and Waksman 1977; Eardley and Jayawardena 1977; Jayawardena et al 1978; Pearson et al 1979), suppressor macrophages (Mansfield and Bagasra 1978, Grosskinsky and Askonas 1981) or a depression of interleukin 2 production (Sileghem et al 1986; Beltz and Kierszenbaum 1987).

It is not clear at present whether these immunological anomalies are precipitated as a result of a direct contact between immune cells and the parasite or soluble parasite products, or both; the answer to these questions is at present the subject of extensive research. A soluble parasite product has been incriminated in the suppression of the in vitro secondary responses to sheep erythrocytes of spleen cells from rats infected with T. lewisi (St. Charles et al 1981). In this study we identify soluble parasite exoantigens in the plasma of both immunocompetent and irradiated infected rats, and we also show the immunoregulatory functions of these exoantigens in vivo and in vitro in their ability either to enhance or to suppress infections by T. lewisi.

## MATERIALS AND METHODS

The animals used in this study, the induction and the assessment of the infections have been been described above.

### 1.1 Irradiation

Normal Fischer rats were exposed to whole-body lethal irradiation with 850 Rads from $^{60}Co$ in a Gamma-Cell (Atomic Energy of Canada, Ottawa, Ontario) prior to initiation of the infection. These animals were used as the source of infected rat plasma.

## 1.2 Preparation of culture-derived soluble exoantigens

Exoantigens were prepared in vitro from either dividing day 7 epimastigotes from animals which had been immunosuppressed with 70 mg/kg cyclophosphamide at the time of parasite inoculation, and from non-dividing day 15 trypomastigotes from non-immunosuppressed rats. Trypanosomes obtained from immunosuppressed or normal rats as described previously were washed three times with PBSG (pH 8.0); the numbers of the parasite were adjusted to 5 x $10^7$ cells/ml and incubated in PBSG for 3 hours at 37°C in a 5% $CO_2$ humidified atmosphere. The trypanosome suspension was then centrifuged at 1000 x g for 15 minutes at 4°C to sediment the parasites; the culture of epimastigote or trypomastigote supernatants were then filtered through 0.8 and 0.22 $\mu$m-pore membrane filters (Millipore Corporation, Bedford, MA) before concentration with Amicon concentrators fitted with PM 10 filters under nitrogen pressure. The buffer containing the exoantigens was then adjusted to 10 mM Tris-HCl (pH 8.0) containing 4 mM of phenylmethyl-sulfonyl-fluoride (PMSF, Sigma, St. Louis, MO), by several washings of the exoantigens with buffer in the concentrators; the exoantigens were stored at -70°C until analyzed.

## 1.3 Preparation of plasma or serum

Infected irradiated rats were bled by cardiac puncture on the 8th day post-infection and the heparinised blood from these animals was processed as previously described. The trypanosome-free plasma from ten day 8 infected irradiated rats (D8IIrRP), from 10 non-infected rats 8 days after irradiation (D8IrRP), as well as from non-infected,

non-irradiated normal rats were obtained separately and each plasma was pooled and stored separately at -70° until used in the treatment experiments.

Infected rat plasma for quantifying the amount of exoantigens produced during the course of the infection was obtained from the retro-orbital plexus with Pasteur pipettes containing 10% of the anticoagulant sodium citrate in PBSG (pH 8.0); the plasma was obtained at 4 days intervals starting from the 4th to 32nd day, and then every 30 days from the 60th to 210 days post-parasite inoculation. Serum was also prepared from rats immunized with either the D8IIrRP or with the culture-derived soluble exoantigens. Blood was obtained by cardiac puncture, clotted and was then centrifuged at 1000 x g for 20 minutes; the serum obtained was stored at -70°C until analyzed.

1.4 Production of antibodies

Two groups of animals, each consisting of three normal Fischer rats, were immunized with either culture-derived exoantigens from epimastigotes (Eexoag) or trypomastigotes (Texoag). The first immunization was done by emusifying 70 μg of each exoantigen in complete Freund's adjuvant (CFA; Difco, Grand Island, NY) and injected subcutaneously at multiple sites on the back of the animals. The subsequent four immunizations were also done with 70 μg of each antigen but at a single subcutaneous site every 7 days in incomplete Freund's adjuvant; the last immunization was given intraperitoneally without adjuvant and the animals were sacrificed 10 days later for serum. Sera for ablastic assays were obtained from five Fischer rats immunized intraperitoneally once without adjuvant with 2 mls of D8IIrRP (1.5 mg total protein of exoantigens); the animals were sacrificed 7 days later

to obtain the serum. This latter protocol was carried out also to determine the role of immunization in protection against infection; the treated animals were inoculated with $10^7$ trypomastigotes 7 days after the single injection of the antigen.

1.5 Treatment of normal Fischer rats with plasma

Animals were treated intra-peritoneally with either day 8 infected-irradiated rat plasma (D8IIrRP), plasma from non-infected rats 8 days after irradiation (D8IrRP) or normal rat plasma (NRP). Five groups of animals, each consisting of four rats were studied: two groups of animals were treated with 2 mls of D8IIrRP (1.5 mg protein of exoantigens), and infected with $10^7$ trypomastigotes either 2 hours or 7 days after the treatment with the plasma. Two control groups of four rats each received 2 mls of D8IrRP intraperitoneally (i.p.) and inoculated with $10^7$ trypomastigotes either 2 hours or 7 days after treatment. One other control group was inoculated i.p. with 2 mls of NRP 2 hours prior to infection with the parasite.

1.6 Trypanostatic and trypanolytic assays

Trypanostatic (ablastic) and trypanolytic assays were performed on sterile serum obtained from animals 7 days after immunization with D8IIrRP, or from non-infected rats as controls. Ablastic activity was assessed in vitro in 96-well tissue culture plates (Flow Laboratories, McLean, VA) by determining the inhibition of the normal transformation in vitro of non-dividing day 15 trypomastigotes into dividing epimastigotes. After separation of the trypomastigotes aseptically from plasma, they were washed three times

in sterile M199 containing 10% heat-inactivated (56°C for 30 minutes) fetal calf serum, 60 mg penicillin and 100 mg streptomycin. The parasites were then adjusted to a final concentration of $3 \times 10^7$ trypomastigotes/ml. One hundred $\mu$l each of the five test sera from the immunized rats, and the trypanosome suspension were added into each well and incubated at 37°C for 17 hours in 5% $CO_2$. Each test serum and the control normal rat serum was tested in triplicate. Cytospin smears of each culture were prepared; they were stained with Giemsa and examined under oil immersion for the percentage of dividing and small forms in a total of 300 trypanosomes.

Trypanolysis assays of the serum of rats immunized with D8IirRP were done on dividing epimastigotes obtained from day 8 infected. irradiated rats and on trypomastigotes of day 15 infected non-irradiated rats. The two stages of the parasite were separated from blood and washed as described above; the trypanosome suspension was then adjusted to contain $10^8$ organisms/ml. Fifty $\mu$l of each suspension, the test serum and whole guinea pig serum (Cedarlane Laboratories, Hornby, Ontario) diluted 1:5 in the complete medium 199 were added into each well of 96-well culture plates; controls consisted of normal rat serum, complement and the trypanosome suspensions or complement and trypanosomes. Each serum was tested in triplicate. The trypanosomes in each well were counted immediately after they were mixed with complement and or test or control serum and after incubation at 37°C for 1 hour in 5% $CO_2$; the percentage lysis was calculated from the difference between the initial and the final count.

## 1.7 Antigen preparation

Epimastigotes from day 8 irradiated rats and trypomastigotes from day 15 infected animals were recovered separately from blood as described above and filtered through DE52 cellulose columns equilibrated with PBSG (pH 8.0), as described by Lanham (1968). The two stages of the trypanosome obtained from the DE52 column were centrifuged at 1000 x g for 15 minutes at 4°C and re-suspended in 10 mM Tris-HCl buffer (pH 8.0) containing 4 mM of the enzyme inhibitor phenylmethyl-sulfonyl-fluoride (PMSF, Sigma, St. Louis, MO). The parasites were then disrupted at 0°C with 150 kc ultrasounds (Blackstone Ultrasonic Inc. Sheffield, PA); the resulting trypanosome extract was centrifuged at 120,000 x g at 4°C for 1 hour in a Beckman L3-5 ultracentrifuge (Beckman Instruments Inc. Palo Alto, CA). The supernatant obtained was used as antigen in immunodiffusion analysis and to titrate by ELISA (Chapter III) the antibodies in the sera of rats immunized with D8IIrRP and soluble exoantigens from the *in vitro* culture systems.

Epimastigotes were also solubilized in 1% Triton X-114 in 10 mM Tris-HCl (pH7.4) containing 150 mM NaCl and 4 mM of PMSF and phase-partitioned according to the method of Bordier (1981). The detergent-rich fraction obtained was used in the immunodiffusion analysis with the exoantigens in D8IIrRP, and in culture supernatants of the epimastigotes and trypomastigote and the soluble antigens released by ultrasounds from the two forms of *T. lewisi* to detect antibodies in rats immunized with TexoAg, EexoAg and D8IIrRP.

## 1.8 Mitogenic stimulation of normal rat spleen cells

Exoantigen-containing supernatants of epimastigote and trypomastigote cultures were extensively dialyzed against distilled water, lyophilized and then reconstituted in RPMI 1640 medium (Flow Laboratories), containing 10% fetal calf serum, 100 mg streptomycin and 60 mg penicillin. Epimastigotes and trypomastigotes obtained aseptically were washed twice with sterile PBSG (pH 8.0) and then in RPMI 1640 containing 10% fetal calf serum, 60 mg penicillin and 100 mg streptomycin. These cells were then lysed by three cycles of freezing and thawing from -196° to room temperature and the lysate were then used in blastogenic assay without further processing. The test samples were filter-sterilized and the protein concentration was determined by the method of Bradford (1976) before the blastogenic assay.

Cell suspensions were prepared from the spleens of three normal rats by passing each organ through a 60 gauge, 80 mesh sterile stainless steel screen into RPMI 1640 containing FCS and antibiotics, using a glass pestle. The cell suspension was placed in sterile plastic 15 ml centrifuge tubes and large particles and tissue debris were allowed to settle at 0°; the supernatant cell suspension was transferred into fresh 15 plastic tubes. The cell suspension was centrifuged at 400 x g for 10 minutes, and the supernatant was discarded, the pellet was resuspended in the complete RPMI 1640 medium and the numbers of cells were counted in a hemocytometer under phase-contrast illumination. The cells were adjusted to $10^7$ cells/ml; 50 $\mu$l of this suspension (5 x $10^5$ cells) were dispensed into wells which contained 50 $\mu$l of the exoantigen preparation, or the trypanosome

lysate each at a final protein concentration of 25, 12.5, 6.3, 3.1, 1.6, 0.8, 0.2, 0.1, 0.05 or 0.03 μg/ml. The mitogens Concanavalin A (Con A, Sigma) or lipopolysaccharide (LPS), prepared by phenol-water extraction from Escherichia coli E145, were added to each well at an optimal final concentration of 10 μg/ml for each mitogen. The cultures were then incubated at 37°C in 5% $CO_2$ and 48 hours later they were labeled with 1 μCi $^3$H-thymidine ($^3$H-TdR 6.7 Ci/mM; ICN Radiochemicals, Irvine, CA). The cells were harvested 24 hours later on a glass fiber filter using a MASH II cell harvester (Microbiological Associates, Bethesda, MD) and the incorporation of the thymidine into the DNA of cells was measured in triplicate with an LKB 1219 Rack Beta liquid scintillation counter (Wallac Oy, Turku, Finland) spectrophotometer. The mitogenic response was expressed as percentage suppression (negative response), or enhancement (positive response) of the control cultures calculated as follows:

$$\frac{\text{Test CPM - Control CPM}}{\text{Control CPM}} \times 100$$

1.9 Addition of exogenous interleukin 2

Normal rat spleen cells prepared as described above were added into each well of 96 well culture plates containing 25, 12.5, 6.3, 3.1, 1.6, 0.8 and 0.4 μg/ml of the in vitro exoantigen preparation from epimastigotes, or the epimastigote lysate. Cultures were either stimulated with 10 μg/ml Con A or with Con A in the presence of 25% final optimal concentration of the IL 2-rich supernatant from the T cell line MLA 144. The cultures were then treated as described above.

The data of the test cultures was transformed into the percentage blastogenic response of the control cultures before the analysis.

1.10 Stimulation of normal spleen cells incubated with suppressing concentrations of the exoantigen preparation

Normal rat spleen cells ($5 \times 10^5$ cells/ml) were incubated with 25, 12.5, and 6.3 μg/ml of the epimastigote exoantigens for 24 hours; the cells were then washed with RPMI 1640 medium three times prior to stimulation with Con A, as described above. The data of the test cultures was transformed into the percentage blastogenic response of the control cultures before the analysis.

1.11 Quantification of exoantigens in the plasma of infected rats

The levels of circulating exoantigens in vivo in the plasma of infected rats was determined by radial immunodiffusion, as described by Diffley et al (1980). Anti-exoantigen serum, produced by the immunization of normal rats with the culture-derived exoantigen preparation, was added to a final concentration of 10% to melted 1% Noble agar (Difco Laboratories, Detroit, MI) solution at 50°C. The mixture was placed on slides pre-coated with an agar film and allowed to gel at room temperature before cutting 3 mm diameter wells in the agar; the bottom of each well was then sealed with a 1% agar solution. The wells were loaded with 15 μl of plasma freshly obtained from the retro-orbital plexus, every 4 days, from the 4th to 32nd day, and then every 30 days from 60th to 210th day post-parasite inoculation. The slides were incubated in a humidified chamber at room temperature for 24 hours; the diameter of the precipitation rings were then measured in millimeters (mm) and the amounts of exoantigens in the plasma was

determined by comparison with a standard curve made by diffusing known concentrations of pure rat IgG in 1% agar gels containing 10% of goat anti-rat IgG (Miles Laboratories, Elkhart, IN).

1.12 Agar gel immunodiffusion

The ability of antisera to precipitate exoantigens in the D8IIrRP, in culture supernatant, and in sonicate preparations from the epimastigote and trypomastigotes and their respective detergent-phases of Triton X-114 (glycoprotein-rich) was tested by the Ouchterlony technique, as described by Hudson and Hay (1980). The antigen preparations were tested against sera raised in rats immunized with D8IIrRP, Texoag and Eexoag. Slides were pre-coated with 1% Noble agar (Difco Laboratories, Detroit, MI) in barbital buffer (pH 8.2). Melted agar was then poured onto each pre-coated slide; 3 mm diameter center and four satelite wells were then made with a gel punch once the agar had solidified. The antibody-containing antisera was applied into the central well and the various antigen preparations applied into the outer wells and allowed to diffuse overnight at room temperature in a humidified enviroment. After three washes in phosphate buffered saline (pH 7.2), the slides were air-dried, stained with amido black and destained in 2% acetic acid before photography.

1.13 Determination of circulating immune complexes

Immune complexes in the plasma of infected and normal control rats were precipitated with 2% polyethylene glycol, as described by Hudson and Hay (1980). The precipitated complexes were redissolved in Veronal buffered saline (pH 8.8) at 37° for 1 hour prior to measuring the

absorbance at 280 nm in a Beckman DU-7 spectrophotometer (Beckman Instruments Inc. Fullerton, CA).

## Statistics

Data was analyzed by the Student's t-test (Snedecor and Cockran, 1967); in some cases, original data was transformed into percentages of the controls before analysis and they were considered significant at the $P < 0.05$ level.

## RESULTS

The relationship between the level of parasitemia in rats infected with Trypanosoma lewisi to that of circulating exoantigens is shown in Figure 5.1. Following parasite inoculation, the level of parasitemia rose sharply through day four, reaching a peak on the eight day. Thereafter, the number of trypanosomes declined gradually until the infection was eliminated from the peripheral circulation by day 32. The levels of exoantigens in the infected plasma show that these parasite products could be detected as early as the fourth day of infection, and increased sharply thereafter reaching a peak (35 $\mu$g/ml) on day eight, at the time of peak parasitemia. There was only a slight drop in the levels of exoantigens through day 12, but thereafter, there was a gradual decline until the end of infection, when concentrations of less than 12 $\mu$g/ml were maintained at least until day 210.

The results of the analyses for circulating immune complexes in infected rats are shown in Figure 5.2. High levels of immune complexes were detected as early as the 4th day , and at least two peaks were observed during the course of infection; the first appeared on day 8 after parasite inoculation, this was followed by a slight decline

through days 12 and 16 but gradually the immune complexes increased in the plasma of infected rats, reaching a second peak towards the end of the infection. Thereafter, immune complexes remained significantly high, with occasional fluctuations that formed at at least two more peaks by day 210 when the experiment was terminated.

A comparative immunodiffusion analysis of the exoantigens from D8IIrRP (no. 1), epimastigote supernatant (no. 2), epimastigote (no. 3) and trypomastigote (no. 4) sonicates and the glycolipoprotein-rich fraction of epimastigotes (no. 5), after developing with antiserum obtained from rats immunized with <u>in vitro</u> exoantigen preparation from either trypomastigote (no. 7), epimastigote (no. 8) or D8IIrRP (no. 9) are shown in Figures 5.3A, B and C. Precipitin reactions obtained with exoantigens in D8IIrRP (Figure 5.3A, no.1), Eexoag (Figure 5.3A, no. 2) and trypomastigote sonicate (Figure 5.3A, no. 4) show a line of identity between the three antigen preparations after developing with the serum from rats immunized with trypomastigote exoantigens (anti-Texoag, Figure 5.3A, (i) no. 7). Similar precipitation reactions were observed when the antibody was the serum of rats immunized with <u>in vitro</u> epimastigote exoantigens (anti-Eexoag, Figure 5.3B, (i) no. 8). Antiserum from D8IIrRP-immunized rats (Figure 5.3C, (i) no. 9) also recognized similar antigens, but more faintly. When the components of the amphiphilic fraction from epimastigote (Figure 5.3A, no. 5) D8IIrRP (Figure 5.3A, no. 1) and Eexoag (Figure 5.3A, no. 2) were diffused against anti-Texoag, (Figure 5.3B, (ii) no. 7) a line of identity was formed. Two precipitation lines were developed with epimastigote glycolipoprotein-rich fraction (Figure 5.3B, (ii) no.5), as compared to the one line in D8IIrRP (Figure 5.3A, (ii), no. 1) and

Eexoag (Figure 5.3B, (ii) no. 2). These same reactions were however more intense when they were developed with anti-Eexoag serum (Figure 5.3 C, (ii) no. 8) and were only faintly revealed with the anti-D8IIrRP serum (Figure 5.3B, (ii) no. 9). Using anti-Texoag serum (Figure 5.3A, (iii) no. 7), the reactivity against with D8IIrRP ( Figure 5.3C, (iii) no. 1), epimastigote ( Figure 5.3A, (iii) no. 3) and trypomastigote antigens (Figure 5.3A, (iii) no. 4), again showed an identical continuous precipitation line. With anti-Eexoag (Figure 5.3B, (iii) no. 8), a similar line of identity was formed, but an outer stronger precipitation line developed with Eexoag (Figure 5.3B, (ii) no. 2), which otherwise was only faintly revealed when anti-Texoag (Figure 5.3A, (iii) no. 7) was used. Reactions obtained with anti-D8IIrRP (Figure 5.3C, (iii) no. 9) were however faint, except for that against Eexoag (Figure 5.3C, (iii) no. 2).

The effect of previous treatment with D8IIrRP on the parasitemia with T. lewisi, when animals were inoculated with the parasite 2 hours or 7 days post-treatment is shown in Figure 5.4. These results show a significantly higher than normal level of parasitemia ($p<0.01$) when the animals were, inoculated with the parasite 2 hours after the treatment with the D8IIrRP. These infections showed peak parasitemia on days 8 to 12 and remained higher than that in infected control animals that had been treated with normal rat plasma (NRP). The immunosuppressed animals were, however, able to clear their infection by the 36th day post-inoculation. In contrast, delaying inoculation of the parasite until 7 days after treatment with the D8IIrRP significantly reduced ($P<0.01$) the level of parasitemia, in comparison to infected control rats that had received NRP. The infection in the protected animals was terminated 8 days earlier than in controls. The suppression and the

protection phenomena were not observed when plasma from normal uninfected rats, 8 days after an identical (850 rads) irradiation (D8IrRP) was used to treat normal animals 2 hours or 7 days before inoculation of the parasite (Figure 5.5); the parasitemia was identical to that in normal controls treated with NRP.

The capacity of the exoantigens present in the D8IIrRP to induce antibodies that can mediate ablastic and trypanolytic activity (hence protection in vivo) against trypomastigotes or epimastigotes was studied in the serum obtained 7 days after immunizing rats intraperitoneally with 2 mls of this plasma. The results presented in Table 5.1 (iii) show that anti-D8IIrRP serum significantly inhibited ($P<0.01$) the transformation in vitro of trypomastigotes into dividing epimastigote forms. The antiserum also had trypanolytic activity in the presence of guinea pig complement (Table 5.1 [i]) which was directed significantly to epimastigote than trypomastigote targets. There was no lysis of either stage of the parasite when the trypanosome suspension was incubated with NRS and complement, or with complement alone. Titration of the same sera by ELISA using antigen derived from the epimastigotes (Table 5.1[ii], a) or trypomastigotes (Table 5.1[ii], b) indicates that significantly higher antibody titres ($P<0.001$) were obtained when epimastigote rather than trypomastigote antigen was used to coat the ELISA plates.

The stimulation of the normal rat spleen cells with the mitogens Concanavalin A (Con A) and lipopolysaccharide (LPS) in the presence of Eexoag (Figure 5.6) or Texoag (Figure 5.7) shows that the Con A responses were suppressed in the presence of high concentrations of the parasite-derived soluble products. The suppression of Con A occurred

with concentrations of 1.6-25 μg/ml of the Eexoag (Figure 5.6) preparation. At the lower concentrations of 0.03 and 0.05 μg/ml of Eexoag (Figure 5.6) and 0.05-1.6 μg/ml of Texoag (Figure 5.7) of the exoantigens, the responses to Con A were enhanced. Both Eexoag (Figure 5.6) and Texoag (Figure 5.7) enhanced the normal response of normal splenocytes to activation by LPS.

The results of Con A and LPS -induced mitogenesis of normal cells grown in the presence of epimastigote lysate shown in Figure 5.8 indicate that suppression to Con A and LPS responses occurred at high concentrations of the lysate (12.5-25 μg/ml), whereas enhancement of LPS response occurred with lower concentrations of 0.4-1.6 μg/ml; at even lower concentrations the responses of the splenocytes were normal. Concanavalin A responses were suppressed at all the concentrations used in test cultures. Similar results were obtained when normal splenocytes were stimulated with the mitogens Con A and LPS in the presence of trypomastigote lysed cells (Figure 5.9). Con A responses were suppressed significantly by 12.5-25 μg/ml of the lysate in cultures, whereas the lower concentrations of 0.05-0.8 μg/ml induced enhancement. These same preparation of trypomastigote lysate induced enhancement of LPS mitogenesis. The effect of the addition of interleukin 2-rich supernatant from the MLA 144 T-cell line on the spleen cells suppressed by relatively high concentrations of the Eexoag and epimastigote lysate are shown in Table 5.2 and Table 5.3 respectively; addition of the IL 2 restored normal responses to the spleen cells. However, the response in those cultures which contained IL 2 gradually increased with decreasing concentrations of the exoantigens or the lysate and reached a peak at concentrations of 0.4 μg protein/ml and 3.1 μg protein/ml of the exoantigens and epimastigote

lysate respectively; thereafter the response declined with lower concentrations of the preparations from the parasite that otherwise enhanced Con A stimulation. Washing normal spleen cells co-cultured with suppressing concentrations of Eexoag or epimastigote lysate (6.3-25 μg/ml) for 24 hours before the stimulation with Con A and LPS restored the normal responses of splenocytes to these mitogens (Table 5.4).

## DISCUSSION

The results presented in this study show that Trypanosoma lewisi produces soluble factors in vivo that can protect or suppress normal animals after the passive transfer of plasma from infected irradiated rats. These parasite-derived products are also shed during in vitro culture. The phenomenon of immune suppression is a fascinating one and has been well studied in trypanosome infections (Hudson et al 1976; Hazlett and Tizard 1978; Cunningham and Kuhn 1980; Charoenvit et al 1981; St. Charles et al 1981), although there is much that is still unknown. Immunosuppression induced by a soluble suppressor substance that can passively render normal recipients immunologically hyporesponsive has been reported in the serum of Trypanosoma cruzi (Cunningham et al 1978) and Cox et al (1984) have also shown that plasma of rats infected with T. b. rhodesiense can suppress the humoral responses to sheep erythrocytes, probably by the interaction of soluble immune complexes with the Fc or C3b receptors of activated lymphocytes.

It is apparent that parasites induces complex changes in the host immune system usually to its own benefit. Such changes are reflected in the generation of suppressor cells (Eardley and Jayawardena 1977; Jayawardena et al 1978; Pearson et al 1979), decreased responses to

mitogen stimulation *in vitro* (Hazlett and Tizard 1978; Ramos *et al* 1979; Pearson *et al* 1979; Sacks *et al* 1982), or by an active suppression of mediators necessary for cellular cooperation (Ptak and Gershon 1975). The factors that induce suppression are a subject of much investigation and whether the hyporesponsiveness is the result of the direct or indirect interaction of parasite-derived products with the immune system is still unclear although at present there is evidence suggesting that both mechanisms may be operable (Hudson and Terry 1979; Selkirk 1981; Albright and Albright 1981; Serrano and O'Daly 1987).

In African trypanosomes, a crude membrane fraction has been shown to mimic the *in vivo* immunosuppressive and mitogenic effects of living parasites (Clayton *et al* 1979; Sacks *et al* 1982). St. Charles *et al* (1981) have studied the effects of living *T. lewisi* on the secondary responses of spleen cells to sheep erythrocytes in a culture system that separated the parasites from the cells. The secondary responses to sheep erythrocytes were markedly suppressed and parasite-derived products, probably exoantigens, were incriminated in the induction of the suppression. Our present results indicate that exoantigens shed during the course of infection correspond well with and mirror the level of infection. Diffley *et al* (1980) observed that the variant specific antigen (VSA), the principal constituent of the surface coat of salivarian trypanosomes, is shed into the circulation of infected animals in a proportional relationship to parasitemia; it appears to be a mitogen for murine splenic T-lymphocytes *in vitro* and induces polyclonal lymphocyte activation (Diffley 1983). Proulx (1988) has shown that the response to Con A of spleen cells from rats infected with *T. lewisi* and the production of IL 2 are maximally suppressed at

with T. lewisi and the production of IL 2 are maximally suppressed at the peak of infection; the Con A response and the IL 2 production, however, gradually recover as the level of infection declines and are fully restored by the time the infection is cleared. The results of exoantigen quantification in the plasma of infected rats in this study indicate that maximal production of these products occurs on days 8 to 12, (Figure 5.1), which coincides with the period of peak immunosuppression and the maximal IL 2 depression in these infections (Proulx 1988).

It has been reported that the immunosuppression of rats infected with T. lewisi results in the production of large quantities of free exoantigens in uncomplexed form (Long and Dusanic 1978; Dusanic 1975). Such plasma has been shown to induce an ablastic response when transferred to normal rats (Bawden and Stauber 1974; D'Alesandro 1986). The concentration of exoantigens in the plasma of irradiated-infected rats used in our treatment studies was 750 $\mu$g protein/ml. Treatment of rats with 2 mls (1.5 mg total protein of exoantigen) of this plasma, followed by parasite inoculation within 2 hours induced an enhancement of the parasitemia; delaying inoculation of the parasite by 7 days in similarly-treated animals resulted in the protection of the recipient animals. These results suggest the coexistence of immunomodulating factors in the plasma of infected rats. The fact that suppression occurred only when parasite inoculation followed shortly after treatment with D8IIrRP indicates that the stimulus to enhance parasitemia must operate before the infected animals can produce the protecting antibodies which eventually eliminate the parasite and, thus, the production of the suppressor logically occurs early in the infection. Analysis of sera obtained 7 days after immunization of

normal rats with D8IIrRP suggests that protection in vivo is mediated by exoantigen-elicited antibodies since such sera demonstrated ablastic activity and stage-specific trypanolysis in vitro. D'Alesandro (1986) has shown the induction of an ablastic response in rats treated with the plasma of cortisone-treated rats infected with T. lewisi. Our results indicate that these exoantigens exhibit some stage specificity in that lysis (in presence of complement) and the ELISA titres given by antibodies induced by epimastigote antigen were significantly greater than those induced by trypomastigotes. However, immunodiffusion analysis indicated that the Eexoag, D8IIrRP, the sonicate from epimastigote and trypomastigote forms or the epimastigote glycolipoprotein-rich fraction had similar exoantigens, as shown by the lines of identity (Figure 5.3); the difference in the reactivity of anti-Eexoag and anti-Texoag sera was, however, qualitative rather than quantitative. These results indicate that both stages of the parasite contain similar antigens, but that the affinity of antibody-binding differs slightly. The reactivity of the anti-Eexoag antibody against the trypomastigote sonicate was less intense than the reaction with homologous antigen (Figure 5 3 A [i] and [ii]). The failure of the anti-D8IIrRP serum to show as intense a reaction as that observed with the anti-Eexoag and anti-Texoag sera may be attributed to the low antibody titer in the anti-D8IIrRP (1:320) compared to the latter two antisera (1:1280).

The response of spleen cells to mitogen stimulation has been used to study the mechanisms by which parasite products mediate their effect on the immune system (Esuruoso 1976; Harrel-Bellan 1983). We therefore also used this assay to study the effect of exoantigens obtained from

*in vitro* cultures on normal spleen cells on their capacity to be stimulated by Con A and LPS. The observation that high levels of exoantigens, or parasite lysates, suppress the *in vitro* blastogenic response to Con A whereas low concentrations enhanced this response is consistent with reports by Hazlett and Tizard (1978) who showed that lysates of $3 \times 10^7$ *T. musculi* enhanced while $6 \times 10^8$ suppressed the mitogenic response of normal mouse spleen cells. These studies and those by Proulx (1988), suggest that immunosuppression of rats infected with *T. lewisi* occurs with relatively high concentrations of the parasite-derived substances, or at peak parasitemia *in vivo* which is the period of the infection when, as is shown here, high levels of exoantigens occur in the circulation. There is thus, correlation between the levels of these parasite products and the competence of the immune system of infected rats. It is interesting to note that the lysate and the exoantigens of the dividing epimastigotes (Eexoag) forms were more suppressive (Figure 5.8 and 5.6) than the lysate and exoantigens (Texoag) from the mature non-dividing trypomastigotes (Figure 5.9 and 5.7) forms. The Eexoag suppressed the Con A response at low protein concentrations that otherwise enhanced the response to Con A with Texoag. Suppression of the immune system of the host may be regarded as a mechanism that ensures the establishment and the propagation of the parasite. In view of this, it is not surprising that the epimastigotes of *T. lewisi* play an important role in the establishment of infections in the host and, since they are the first developmental forms of the parasite, it is logical that this stage of the parasite, or its products, are the more suppressive. The ability of the trypomastigote form, or its products, to enhance mitogen response is also logical since this stage of the parasite induces the

effector immune mechanisms that eventually eliminate the infection. This latter phase of the infection corresponds with lower levels of circulating exoantigens which, from the evidence we obtained *in vitro*, probably induces the effector mechanisms which are responsible for eliminating the infection. The concentrations of exoantigen in the plasma were slightly higher than the concentrations that were observed to suppress mitogenic response *in vitro*; it is likely, however, that the levels detected in the plasma of infected animals consisted of both free exoantigens and exoantigens in immune complexes as the latter are also significantly high in infected rats (Figure 5.2). Such an association of exoantigens and specific immunoglobulins producing immune complexes is well documented (Fruit *et al* 1977; Diffley 1978; Tizard *et al* 1978) and has been reported to play a major role in immune dysfunctions especially in African trypanosomiasis.

Interleukin 2 (IL 2) is a mediator which plays a key role in the regulation of cellular immune responses (Smith *et al* 1980). It is produced by T lymphocytes in the presence of interleukin 1 (IL 1) and mitogen or antigen and sustains the proliferation of helper and effector T-cells, thus amplifying the effector phase of the immune response (Smith *et al* 1980); inhibition of this cell mediator interferes with normal immune responses. In this study, the normal responses of splenocytes to Con A in the presence of suppressing concentrations of epimastigote exoantigens, or lysate, were restored by the addition of exogenous IL 2, suggesting that exoantigens mediate their suppressive activity in part by inhibiting IL 2 production. Proulx (1988) has observed a reduced production of IL 2 by spleen cells from *T. lewisi* infected rats which was maximal at the peak of

parasitemia; a similar correlation has been reported by Nickol and Bonventre (1985) in Leishmania donovani and in T. cruzi by Harel-Bellan et al 1983. Depressed production of this mediator appears to be an important modification of the immune system induced during parasite infections (Harel-Bellan et al 1983; Sileghem et al 1986; Beltz and Kierszenbaum 1987), and may therefore play a key role in immunosuppression. The restoration of normal mitogenic response by washing normal splenocytes incubated with suppressing concentrations of the epimastigote indicates that suppression induced by these parasite substances is temporary and requires the continued presence of the exoantigens in the cell cultures; and relatively high levels of the exoantigens are mandatory to induce suppression. This fact suggests that the exoantigens of T. lewisi induce suppression by a direct effect on the cells without the participation of typical suppressor cells, as occurs in African trypanosomiasis (Jayawardena et al 1978; Pearson et al 1979). Although Proulx (1988) reported suppressor activity by adherent macrophages from T. lewisi-infected animals on the normal rat splenocyte response stimulated with Con A, it should be noted that the contamination of these cultures with trypanosomes could have been responsible for the suppression of the normal splenocyte response. Furthermore, as we have shown in Chapter VII, the adherent cell population appears to play a key role in mediating suppression only in presence of parasite derived products. Indeed, ingestion of trypanosomes by macrophages has been reported to render these cells immunosuppressive (Sacks et al 1982); if such mechanisms are also operable in T. lewisi, the incubation of unfractionated normal splenocytes with suppressing doses of the parasite products would have resulted to suppression of mitogenic responses, even after the washing

of the cells.

The amounts of T. lewisi exoantigens in the infected rats are dependent on the level of parasitemia, and their ability to suppress mitogen stimulation. The possibility that these parasite-derived products play the major immunoregulatory role of inhibiting the production of lymphokines indicate that they may effectively determine the disease state of infected hosts. The obvious importance of the exoantigens in regulating infection by T. lewisi stimulated a study to characterize the components of this parasite-derived product. The following two chapters report on the results of these characterization studies.

Fig 5.1. The course of parasitemia (■) in rats infected with Trypanosoma lewisi, relative to the level of circulating exoantigens ( □ ) in the plasma of infected animals. The level of circulating exoantigens declined below 12 μg/ml after day 60, the minimum detection level of the radial immunodiffusion assay. Each point represents mean ± S.E. of parasite count, or exoantigen determination from four rats.

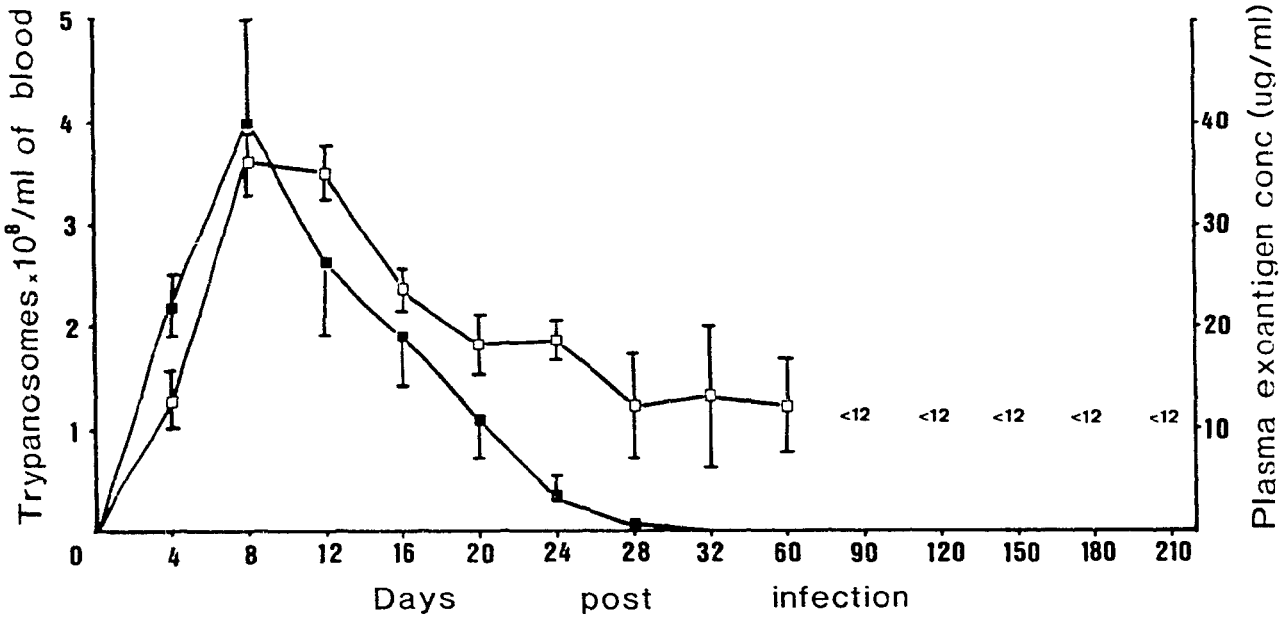

Trypanosomes x $10^8$/ml of blood
Plasma exoantigen conc (ug/ml)
0
1
2
3
4
5
10
20
30
40
4
8
12
16
20
24
28
32
60
90
120
150
180
210
<12
<12
<12
<12
<12
Days post infection

Fig 5.2. The level of circulating immune complexes (■) in the plasma of rats infected with Trypanosoma lewisi and in normal uninfected animals (▣). Each point represents mean ± S.E. absorbance from four animals.

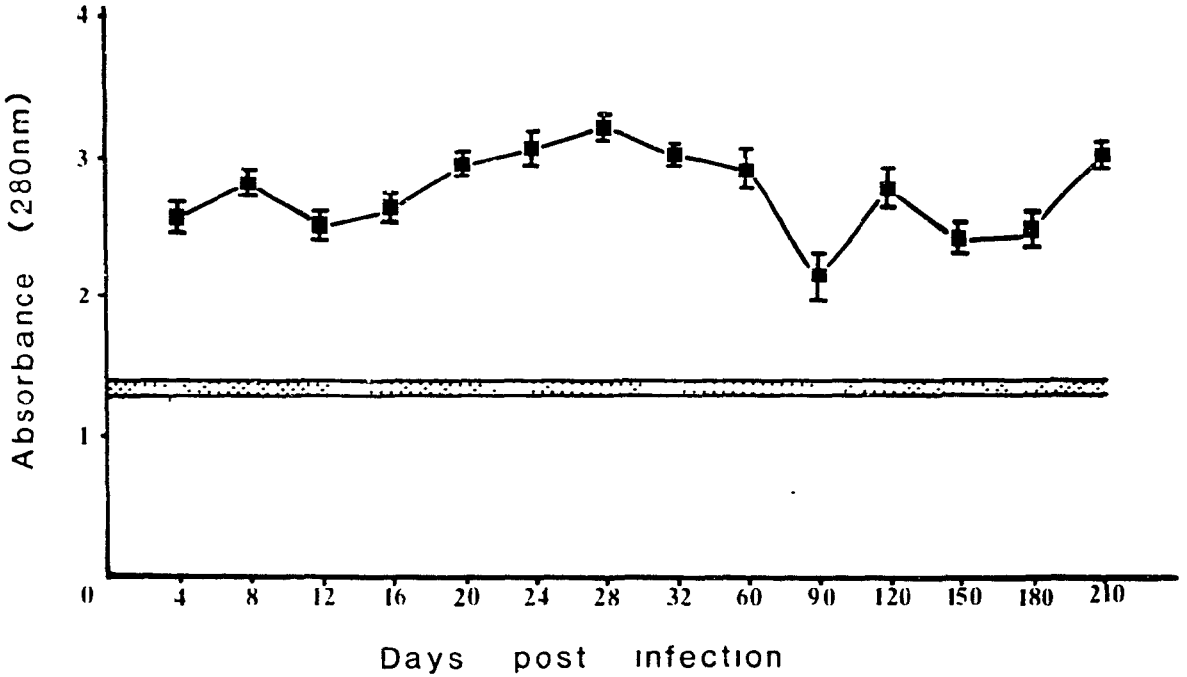

Absorbance (280nm)
4
3
2
1
0
4
8
12
16
20
24
28
32
60
90
120
150
180
210
Days post infection

Fig 5.3. Immunodiffusion analysis of the exoantigens from day 8 infected irradiated (850 rads) rat plasma (D8IIrRP, 1) and the in vitro cultures of epimastigote forms (2), or of sonicate preparations from the epimastigotes (3) and trypomastigotes (4), or the components of amphiphilic fraction of Triton X-114 solubilized epimastigotes (5). The antigen preparations were allowed to diffuse against serum (in central wells) obtained from rats immunized with the in vitro culture-derived exoantigens from trypomastigotes (7, [A, i-iii), epimastigotes (8, [B, i-iii]), and D8IIrRP (9, [C, i-iii]).

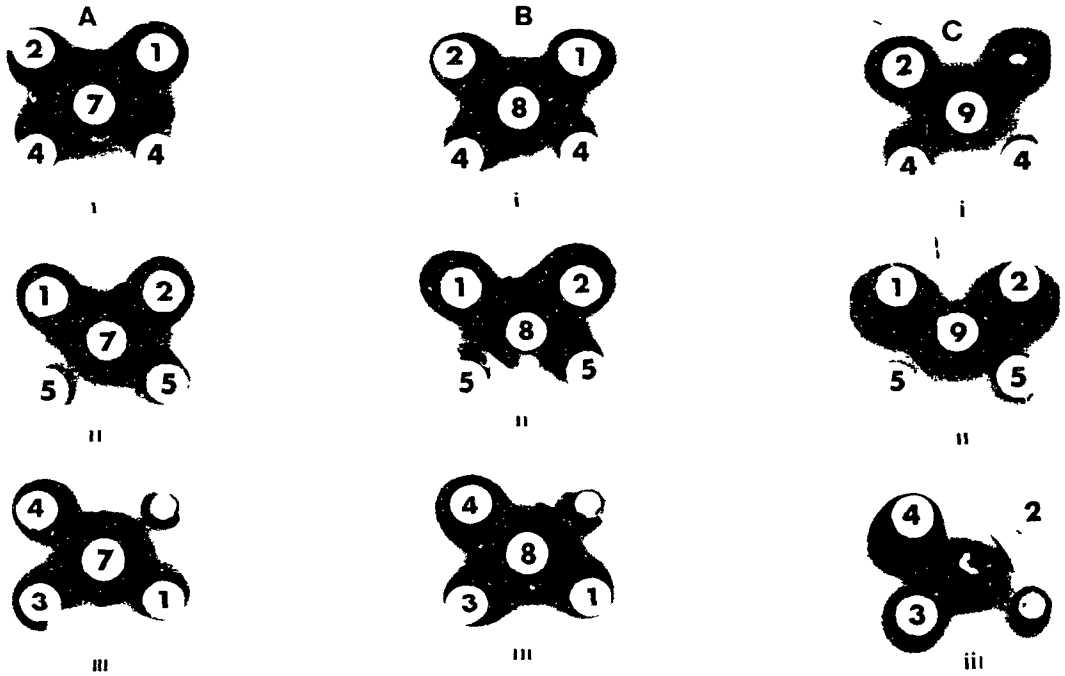

A
2
1
7
4
4
i
1
2
7
5
5
ii
4
7
3
1
iii
B
2
1
8
4
4
i
1
2
8
5
5
ii
4
8
3
1
iii
C
2
9
4
4
i
1
2
9
5
5
ii
4
2
3
iii

Fig. 5.4. The course of parasitemia in rats inoculated with $10^7$ trypomastigotes 2 hours (■) or 7 days (□) after an intraperitoneal treatment with 2 mls of day 8 infected irradiated rat plasma (D8IIrRP). Control animals were infected 2 hours after a similar treatment with normal rat plasma (●). Each point represents, the mean ± S.E. of trypanosome count from four animals.

$a^1$. indicates, a statistically lower ($p<0.01$) level of parasitemia by Student's t-test in animals infected 7 days after treatment with D8IIrRP compared to control animals which received normal rat plasma.

$a^2$. indicates, a statistically higher ($p<0.01$) level of parasitemia by Student's t-test in animals infected 2 hours after treatment with D8IIrRP compared to control animals which received normal rat plasma.

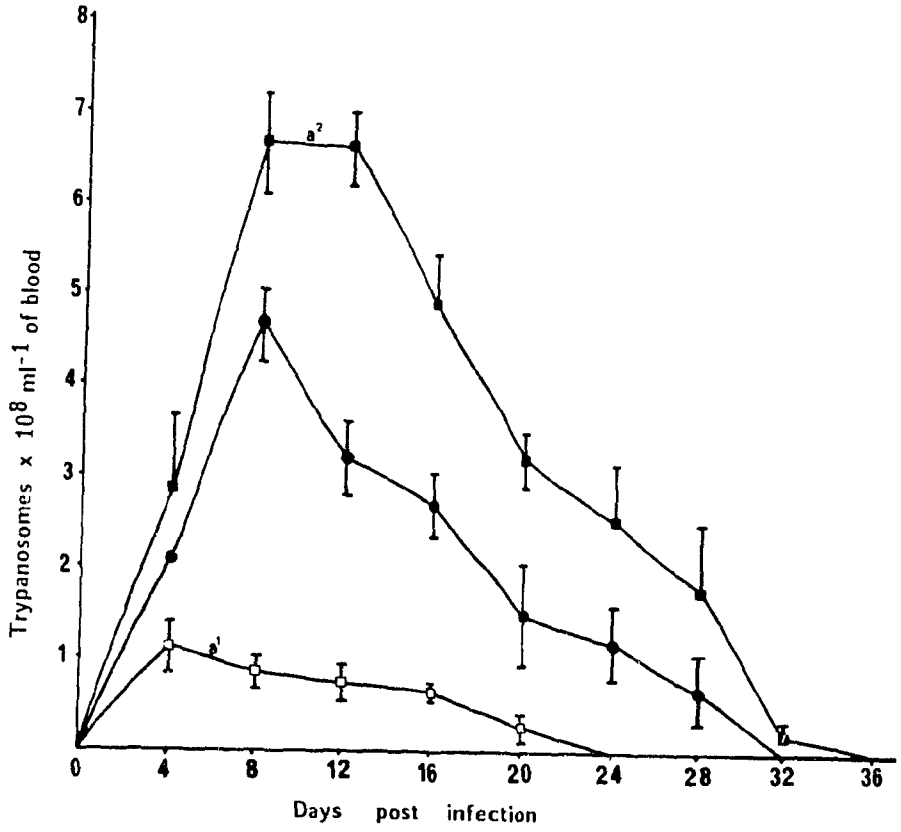

Trypanosomes x $10^8$ $ml^{-1}$ of blood
8
7
6
5
4
3
2
1
0
4
8
12
16
20
24
28
32
36
Days post infection

Fig. 5.5. The course of parasitemia in rats inoculated with $10^7$ trypomastigotes 2 hours (▲) or 7 days (△) after an intraperitoneal treatment with 2 mls of day 8 irradiated rat plasma (D8IrRP). Control animals were infected 2 hours after a similar treatment with normal rat plasma (●). Each point represents the mean ± SE of trypanosome count from four animals. There was no significant difference in the level of infection between the three groups of animals.

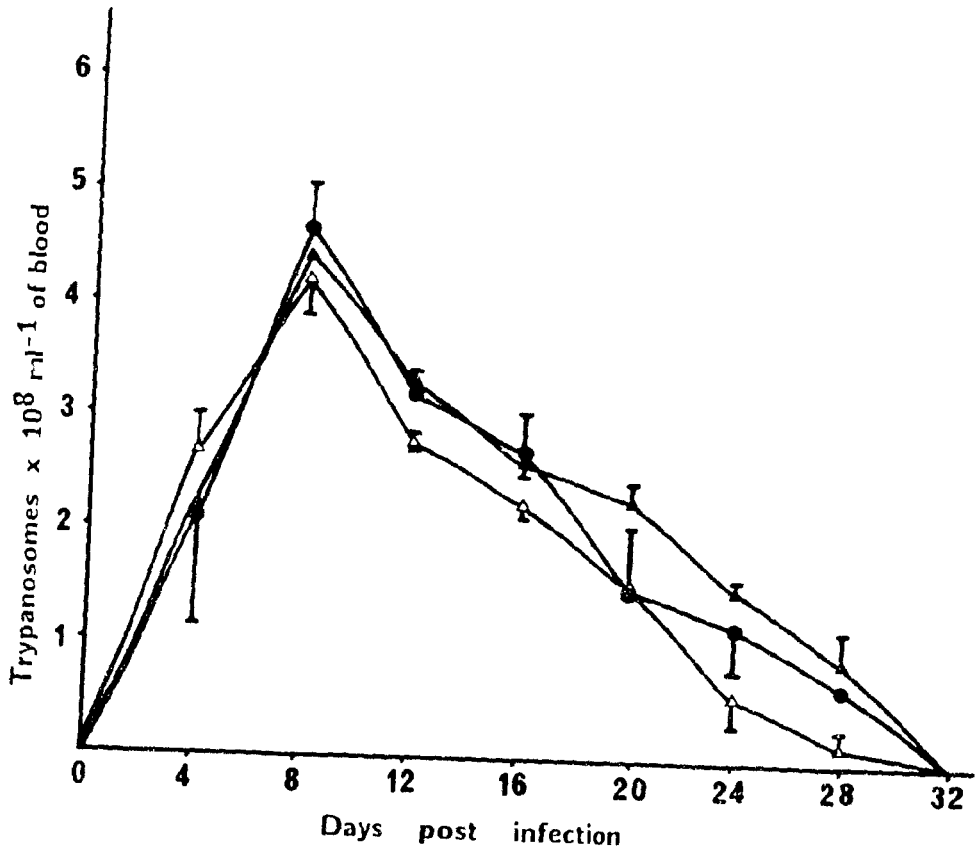

Trypanosomes x $10^8$ $ml^{-1}$ of blood
6
5
4
3
2
1
0
4
8
12
16
20
24
28
32
Days post infection

Fig. 5.6. The mitogenic response of normal rat spleen cells to Concanavalin A (▩) and lipopolysaccharide (□) stimulation in cultures containing varying concentrations of the soluble culture-derived epimastigote exoantigens. Each bar represents the % mean ± S.E. of triplicate cultures from three rats. The astericks represent significant differences in either suppression or enhancement of mitogenesis by Student's t-test from the stimulated cultures lacking the exoantigen preparation: *-$p<0.05$, **-$p<0.01$, ***-$p<0.001$.

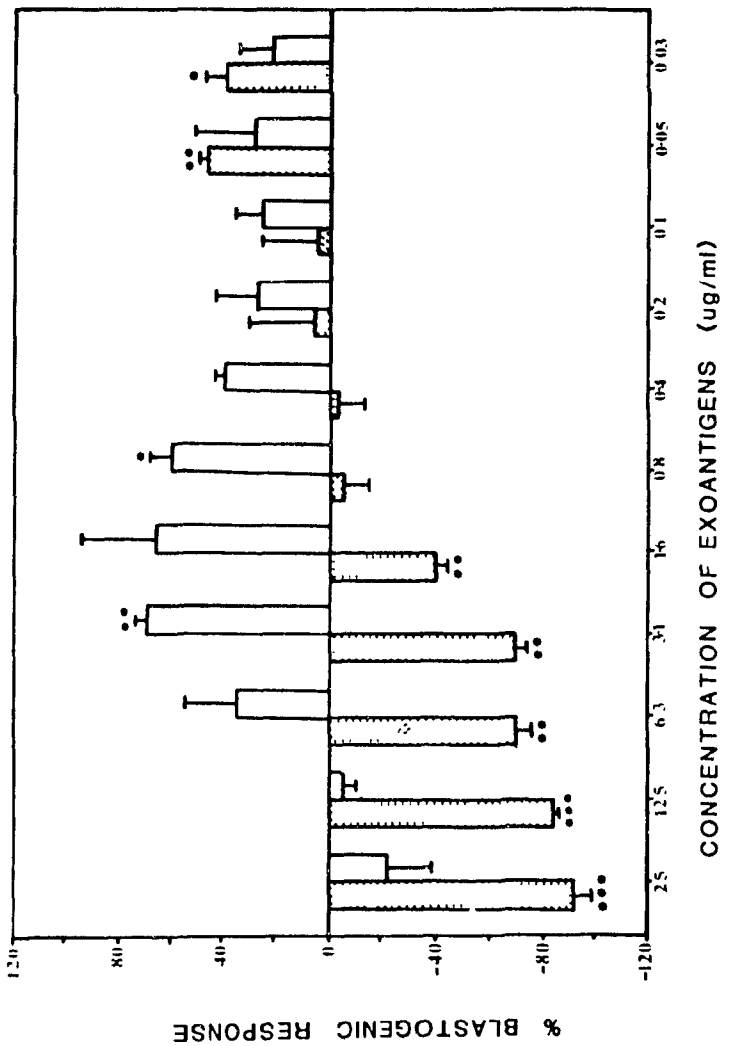

% BLASTOGENIC RESPONSE
120
80
40
0
-40
-80
-120
25
12.5
6.3
3.1
1.6
0.8
0.4
0.2
0.1
0.05
0.03
CONCENTRATION OF EXOANTIGENS (ug/ml)

Fig. 5.7. The mitogenic response of normal rat spleen cells to Concanavalin A (▨) and lipopolysaccharide (□) stimulation in cultures containing varying concentrations of the soluble culture-derived trypomastigote exoantigens. Each bar represents the % mean ± S.E. of triplicate cultures from three rats. The astericks represent a significant difference in either suppression or enhancement of mitogenesis by Student's t-test from the stimulated cultures lacking the exoantigen preparation: *-$p<0.05$, **-$p<0.01$, ***-$p<0.001$.

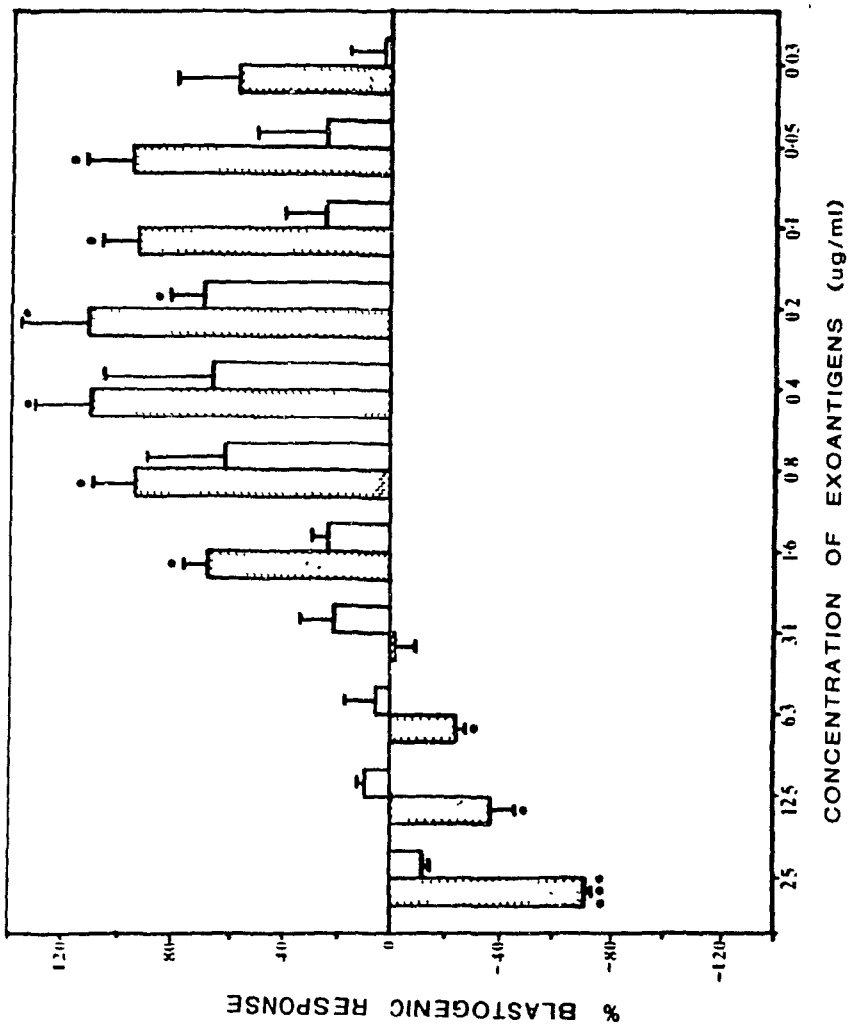

% BLASTOGENIC RESPONSE
CONCENTRATION OF EXOANTIGENS (ug/ml)

Fig. 5.8. The mitogenic response of normal rat spleen cells to Concanavalin A (▩) and lipopolysaccharide (□) stimulation in cultures containing varying concentrations of the epimastigote lysate. Each bar represents the % mean ± S.E. of triplicate cultures from three rats. The astericks represent significant difference in either suppression or enhancement of mitogenesis by Student's t-test from the stimulated cultures lacking the lysate preparation: *-$p<0.05$, **-$p<0.01$, ***-$p<0.001$.

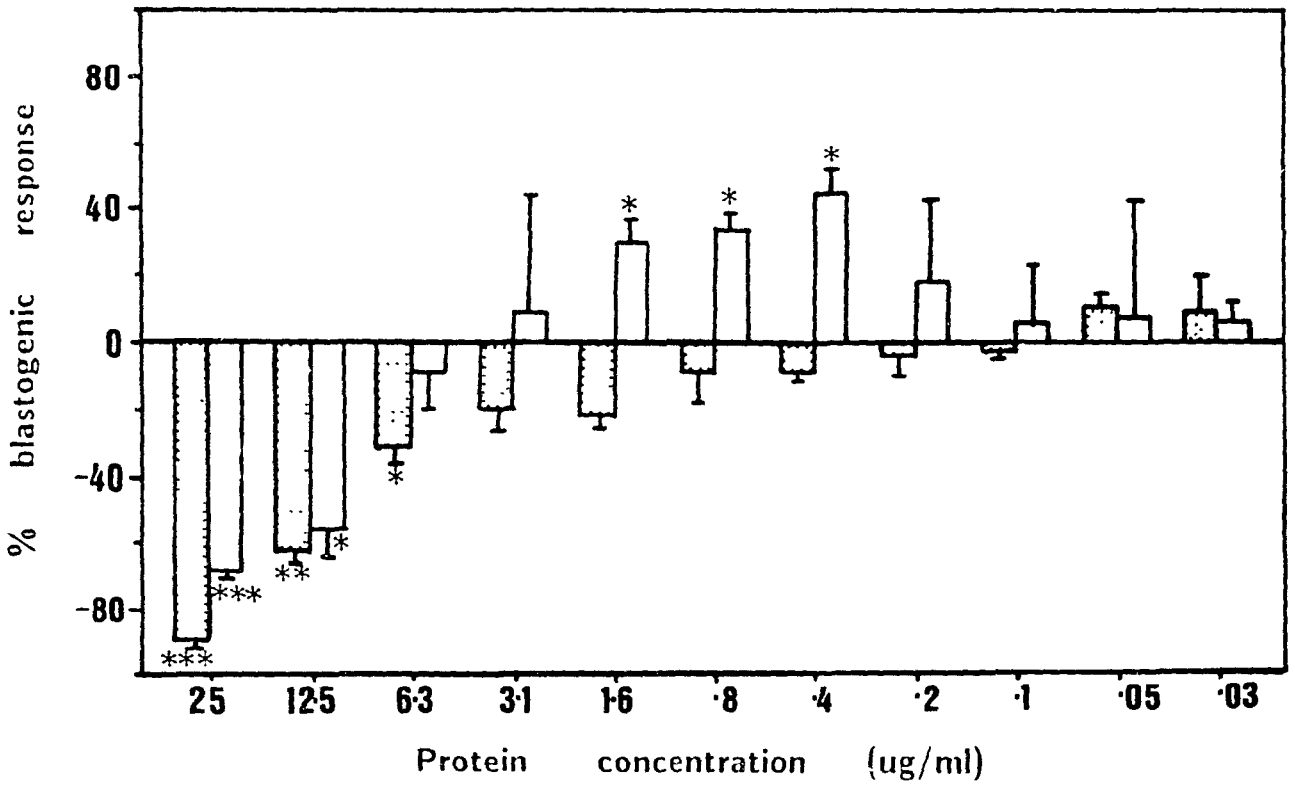

% blastogenic response
80
40
0
-40
-80
25
12·5
6·3
3·1
1·6
·8
·4
·2
·1
·05
·03
Protein concentration (ug/ml)

Fig. 5.9. The mitogenic response of normal rat spleen cells to Concanavalin A (▨) and lipopolysaccharide (□) stimulation in cultures containing varying concentrations of the trypomastigote lysate. Each bar represents the % mean ± S.E. of triplicate cultures from three rats. The astericks represent significant difference in either suppression or enhancement of mitogenesis by Student's t-test from the stimulated cultures lacking the lysate preparation: *-$p<0.05$, **-$p<0.01$, ***-$p<0.001$.

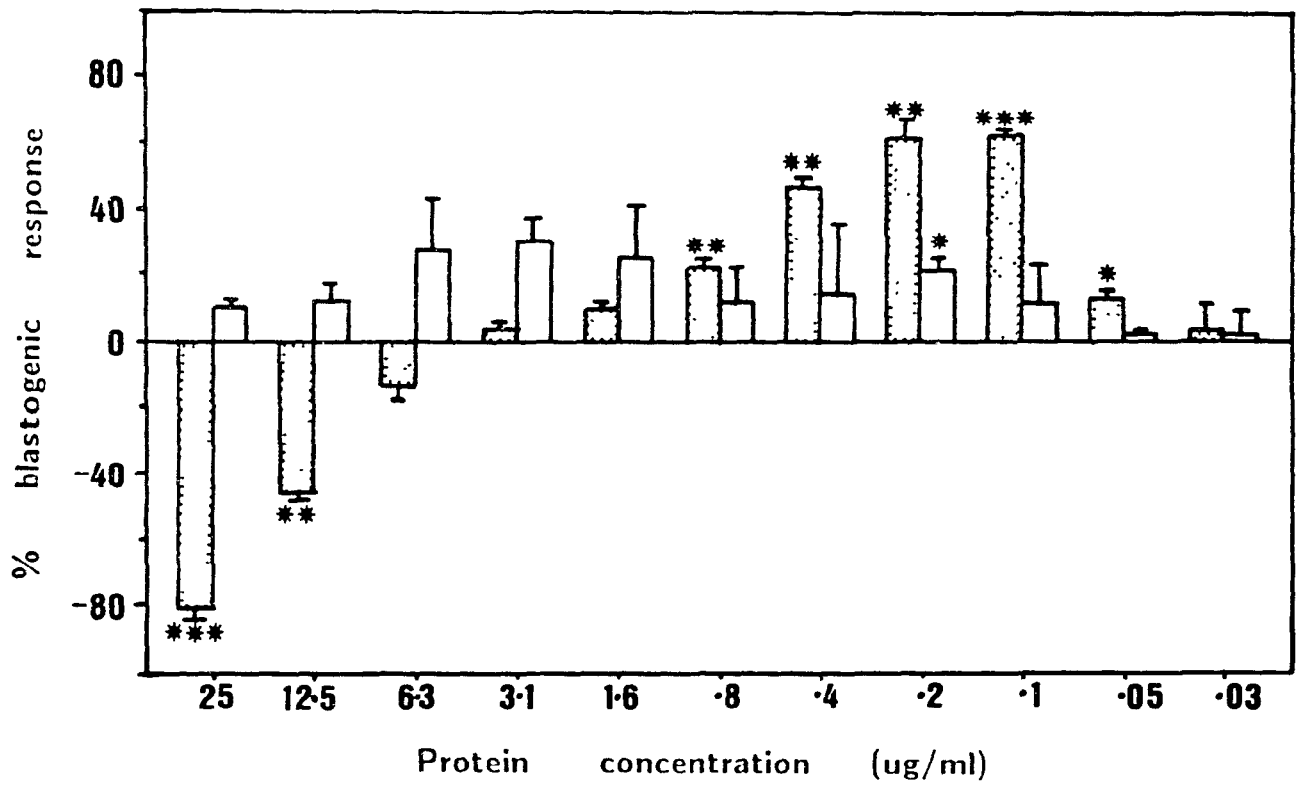

80
40
0
-40
-80
% blastogenic response
25
12·5
6·3
3·1
1·6
·8
·4
·2
·1
·05
·03
Protein concentration (ug/ml)

Table 5.1. Trypanolytic, trypanostatic and antibody activity in the serum of rats immunized with day 8 infected plasma from irradiated (D8IIrRP) rats[a].

| Assay | Test serum[b] | Control serum[c] |
|---|---|---|
| i) Trypanolysis | | |
| a) epimastigotes | 35.8 ± 2.5*** | NL |
| b) trypomastigotes | 5.3 ± 1.4 | NL |
| ii) Antibody titers ($Log_2$) by ELISA | | |
| a) epimastigotes | 6.4 ± 0.4*** | NT |
| b) trypomastigotes | 1.2 ± 0.3 | NT |
| iii) Ablastic activity (% dividing forms) | 21.7 ± 2.5** | 95.0 ± 1.0 |

a. Data from triplicate determinations from five rats is expressed as mean ± SE.

b. Sera from immunized rats.

c. Normal rat serum

NL. No lysis

NT. No antibody titers.

*. Astericks indicate a significant difference by Student's t-test in trypanolysis or antibody titers compared with trypomastigote forms or from cultures containing normal rat serum: ** - $p<0.01$, *** - $p<0.001$.

Table 5.2 The effect of washing cells and adding exogenous IL 2 on Concanavalin A stimulated normal rat spleen cell cultures containing suppressing concentrations of epimastigote exoantigens[a].

| Exoantigen concentration ($\mu$g/ml) | % Blastogenic response | | |
|---|---|---|---|
| | (i) Con A | (ii) Con + 1L 2 | (iii) Con A[b] |
| 25 | 11.6 ± 7.0 | 101.7 ± 5.6** | 100.7 ± 0.9[ns] |
| 12.5 | 16.5 ± 0.5 | 107.0 ± 6.9** | 97.3 ± 0.9[ns] |
| 6.3 | 29.0 ± 5.5 | 129.0 ± 2.1** | 98.2 ± 3.2[ns] |
| 3.1 | 30.0 ± 3.5 | 150.0 ± 5.8** | ND |
| 1.6 | 60.1 ± 3.1 | 152.6 ± 11.0* | ND |
| 0.8 | 95.0 ± 11.0 | 159.0 ± 7.0* | ND |
| 0.4 | 97.0 ± 10.0 | 169.0 ± 9.0* | ND |
| 0.2 | 106.0 ± 25.0 | 137.0 ± 10.0 | ND |
| 0.1 | 105.0 ± 15.0 | 130.0 ± 27.0 | ND |

a. Data from triplicate cultures from three animals is expressed as % mean ± SE..

b. Normal rat splenocytes were stimulated with Concanavalin A, 24 hours after incubation with epimastigote exoantigens followed by three washes with RPMI 1640 complete medium.

ND. Not done.

*. Astericks indicate a significant difference by Student's t-test from cultures stimulated with Concanavalin A alone: * - $p<0.05$, ** - $p<0.01$.

ns. Not significant by Student's t-test ($p>0.05$) from normal rat splenocyte response (normal response 100%).

Table 5.3. The effect of adding exogenous IL 2 on Concanavalin A stimulated normal rat spleen cells in cultures containing the epimastigote lysate[a].

| Conc. of epimastigote lysate ($\mu$g/ml) | Blastogenic response | |
|---|---|---|
| | Con A | Con A + IL 2 |
| 25.0 | 10.8 ± 1.4 | 103.9 ± 3.4** |
| 12.5 | 38.4 ± 2.8 | 107.8 ± 6.1* |
| 6.3 | 68.9 ± 4.3 | 137.4 ± 11.1* |
| 3.1 | 77.6 ± 4.4 | 145.3 ± 4.2* |
| 1.6 | 79.9 ± 7.3 | 123.8 ± 16.9 |
| 0.8 | 90.5 ± 11.2 | 105.4 ± 3.0 |

a. Data from triplicate cultures from three animals is expressed as % mean ± SE.

*. Astericks indicates a significant difference by Student's t-test from cultures stimulated with Concanavalin A: * - $p<0.05$, ** - $p<0.01$.

Table 5.4. The effect of washing normal rat splenocytes on the mitogen stimulation in cultures containing suppressing concentrations of the epimastigote lysate[a].

| Mitogen | % Blastogenic response | | |
|---|---|---|---|
| | Conc. of epimastigote lysate | | |
| | 25μg/ml | 12.5μg/ml | 6.3μg/ml |
| i) Con A | 10.8 ± 1.4 | 38.4 ± 2.8 | 68.9 ± 4.3 |
| ii) Con A[b] | 102.0 ± 1.5*** | 99.3 ± 1.8** | 101.2 ± 2.1** |
| iii) LPS | 32.1 ± 0.7 | 42.9 ± 7.9 | 91.5 ± 11.5 |
| iv) LPS[b] | 101.0 ± 3.5** | 98.7 ± 1.5* | 112.5 ± 1.8 |

a. Data from triplicate cultures from three animals is expressed as % mean ± SE.

b. Normal rat splenocytes were stimulated with Concanavalin A, 24 hours after incubation with epimastigote lysate, followed by three washes with RPMI 1640 complete medium.

*. Astericks indicate a significant difference by Student's t-test from unwashed cultures stimulated with the corresponding mitogen: * - $p<0.05$, ** - $p<0.01$, *** - $p<0.001$.

# CHAPTER VI: CHARACTERIZATION OF TRYPANOSOMA LEWISI EXOANTIGENS

## INTRODUCTION

Weitz (1960) described the release of a soluble antigen, which he called exoantigen, into the serum of rats infected with Trypanosoma brucei which stimulated the formation of agglutinating and protecting antibody; these same exoantigens also occurred in trypanosome homogenates. Since Weitz' reports, several other studies have demonstrated a correlation of new antigenic variants with the appearance of a corresponding exoantigen (Seed 1963, Miller 1965; Clarkson and Awan 1969; Vickerman and Luckins 1969; Takayanagi et al 1970; Diffley et al 1980).

The release of exoantigens by Trypanosoma lewisi was initially described by Thillet and Chandler (1957), who observed the presence of "metabolic products" during in vitro cultures of the parasite in a normal rat serum-saline preparation. Chandler (1958) gave the name of "ablastinogen" to these "metabolic products" since they induced ablastic antibodies in normal rats treated with culture supernatants. The presence of exoantigens in the serum of infected rats was subsequently reported by D'Alesandro (1972), although Bawden (1970) had earlier reported the presence of "ablastinogen" in the plasma of hydrocortisone-immunosuppressed rats infected with T. lewisi. The production of exoantigens has also been reported in other parasitic infections: Plasmodium falciparum (Wilson 1974), Anaplasma marginale (Amerault and Roby 1964), Babesia bovis (James 1984) and Angiostrcngylus cantonensis (Fujii 1988). Since the reports that exoantigens are shed into the circulation of animals infected with T. lewisi, the nature of these parasite products has not been

characterized and their demonstration has mainly been confined to gel diffusion studies (D'Alesandro 1972; Long and Dusanic 1983). In this study, we characterized by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting the exoantigens shed by actively motile trypanosomes into the supernatant of a short-term culture system.

## MATERIALS AND METHODS

The animals, the parasite and its collection and counting have been described previously.

### 1.1 Preparation of culture-derived soluble exoantigens

Exoantigens were obtained from short-term cultures in vitro of dividing day 7 epimastigotes and non-dividing day 15 trypomastigotes. The trypanosomes obtained from infected rats were washed three times with phosphate-buffered saline containing 1% glucose (PBSG, pH 8.0), then adjusted to 5 x $10^7$ parasites /ml and incubated for 3 hours in PBSG at 37°C in a 5% $CO_2$ atmosphere. The trypanosome suspension was then centrifuged at 1000 x g for 15 minutes at 4°C to sediment the trypanosomes; the culture supernatant obtained was then filtered through 0.8 and 0.22 $\mu$m membrane filters (Millipore Corporation, Bedford, MA) before concentration with Amicon concentrators fitted with PM 10 filters under nitrogen pressure. The supernatant was finally washed in the concentrators with 10 mM Tris-HCl (pH 8.0) buffer containing 4 mM of phenylmethyl-sulfonyl fluoride (PMSF; Sigma, St. Louis, MO) and stored at -70°C until analyzed.

## 1.2 Electrophoretic analysis of the exoantigens

The culture-derived exoantigens obtained from the epimastigotes (Eexoag) and trypomastigotes (Texoag) were analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), as described by Laemmli (1970) using 12% gels. Each well was loaded with a total of 3 μg protein of the exoantigen preparation. The samples were treated with 2-mecarptoethanol before being separated in a Mini-Gel electrophoresis apparatus (Bio-Rad Laboratories, Richmond, CA) for 1 hour at 200 volts. The separated exoantigens were transferred from the gels onto nitrocellulose membranes in a Transphor cell (Hoefer Scientific Instruments, San Francisco, CA), containing 25 mM Tris-HCl (pH 8.3), 150 mM glycine and 20% (v/v) methanol, as described by Towbin et al (1979). The Eexoag blots were washed twice for 10 minutes in 10 mM Tris-HCl (pH 7.4), containing 150 mM NaCl and 0.1% (v/v) of the non-ionic detergent Tween-20 (TBST), as described by Birkett et al (1985). The nitrocellulose sheets were finally washed for another 10 minutes in high salt Tween (HST) buffer which consisted of 10 mM Tris-HCl (pH 7.4), 1.0 M NaCl and 0.5% (v/v) Tween-20 prior to probing the blots with a 1:10 dilution of day 30 immune serum in the same buffer. Control blots were either probed with normal rat serum diluted 1:10 as above or developed with peroxidase-labeled anti-rat IgG, (Miles Yeda Ltd., Israel) diluted 1:1000 in the HST buffer. The nitrocellulose membranes were incubated at 37°C for 1 hour with the probes and subsequently washed for 1 hour with each of six changes of TBST, with a final 10 minute wash in the HST buffer. Peroxidase-labeled anti-rat IgG, diluted as above, was applied onto the blots probed with immune and normal rat sera and then incubated at 37°C for 1 hour. Peroxidase

activity, and the antigen with which the antibody probe had reacted was revealed with the substrate 4-chloro-1-naphthol. The molecular weights of the components separated in the SDS-PAGE and Western blot analysis were determined from a calibration curve of the log molecular weights against the relative mobility prepared from standard molecular weight markers (Bio-Rad Laboratories, Richmond, CA).

1.3 Phase separation of culture-derived exoantigens

The culture supernatant containing epimastigote exoantigens (Eexoag) was washed three times with 10 mM Tris-HCl (pH 7.4) containing 150 mM NaCl and 4 mM PMSF in an Amicon concentrator. Pre-condensed Triton X-114 was added to ice-cold culture-derived Eexoag to a final concentration of 1% and phase partitioning was carried out, as described by Bordier (1981). The exoantigen supernatant was overlaid onto a 6% sucrose solution in 10 mM Tris-HCl (pH 7.4) containing 150 mM NaCl and 4 mM PMSF. The solution was allowed to separate into aqueous (hydrophilic; HP) and detergent (amphiphilic; AP) phases in a water bath at 32°C for 5 minutes, prior to centrifugation at 1500 x g for 15 minutes at similar temperatures to sediment detergent aggregates. The AP and the HP phases were both precipitated with ice-cold acetone and the precipitate dissolved in 10 mM Tris-HCl (pH 8.0) buffer containing 4 mM PMSF before the SDS-PAGE analysis under reducing conditions.

1.4 Protein estimation

The amount of protein in the exoantigen preparations was determined by the protein dye-binding assay as described by Bradford (1976).

## 1.5 Staining of gels for polysaccharides and lipids

The determination of polysaccharide-containing exoantigens in the polyacrylamide gels was done by staining with the periodic acid Schiff (PAS) method, according to Zacharius et al (1969) with the slight modifications of Carter and Colley (1978). The polyacrylamide gels were immersed for 15 minutes in 12.8% trichloroacetic acid immediately after the electrophoretic separation to fix the proteins. The gels were then rinsed with distilled water for one minute before incubation for two hours in a saturated solution of 2, 4, dinitrophenylhydrazine, as described by Carter and Colley (1978). The treated gels were then rinsed in distilled water to remove excess phenylhydrazine, prior to incubation in 1% periodic acid for 50 minutes. Before staining in the dark for 50 minutes with the periodic acid Schiff (PAS) reagent, the gel was extensively washed in distilled water until the washings were free from periodate ions, as monitored by precipitation reactions with silver nitrate solution. After the PAS, the gels were washed thrice for 10 minutes in 0.5% potassium metabisulphite and then immersed in one litre of distilled water overnight in the dark to remove excess PAS reagent.

Staining the gels for lipids was done according to the method of Prat et al (1969). The gels were stained overnight in a solution of 0.4% (w/v) Sudan black in distilled water, containing 10% acetic acid and 15% acetone. The destaining solution was prepared with similar proportions of acetic acid and acetone in distilled water; the gels were then dried onto Whatman paper with a Bio-Rad gel-drier (Bio-Rad Laboratories, Richmond, CA) prior to photography.

### 1.6 Analysis for mannose sugars

The exoantigens containing mannose sugars were determined by probing the blots of epimastigote and trypomastigote preparations with 1 $\mu$g/ml of peroxidase-labeled Concanavalin A (Sigma, St. Louis, MO). The specificity for Con A binding onto the mannose sugars of the blotted exoantigens was determined by incubating 1 $\mu$g/ml of peroxidase-labeled Con A in the HST buffer containing 0.2 M of $\alpha$-methyl-$\delta$-mannopyrannoside (Sigma) for 30 minutes at room temperature, prior to incubating the blots at 37$^{o}$C for 1 hour in the same buffer. After washing the blots as described above, the peroxidase substrate, 4-chloro-1-naphthol was added to reveal the mannose containing glycoproteins.

## RESULTS

The results of SDS-PAGE analysis of the culture-derived exoantigens from the epimastigotes (Eexoag) and trypomastigotes (Texoag), stained for proteins with Coomasie brilliant blue, are shown in Figure 6.1 (lane 1) and (lane 2), respectively. Two major bands of 84 and 72kDa stained strongly for proteins in exoantigens from both stages of the parasite; other minor bands were also revealed with molecular weights of 48, 30 and 18 kDa.

The periodic acid Schiff (PAS) stain for polysaccharides (Figure 6.2) of the Eexoag and Texoag in lanes 1 and 4, respectively, indicate that bands with relative mobilities ranging from 30-185 kDa stained for polysaccharides. The PAS stain of the amphiphilic fraction (Figure 6.2, lane 2) after phase partitioning of the Eexoag (Figure 6.2, lane

1) shows that the polysaccharide-containing components were enriched to 15 bands from 11 with molecular weights of 185, (a), 165 (b), 160 (c), 155 (d), 140 (e), 130 (f), 84 (g), 72 (h), 48 (j), 30 (k), 26 (l), 22 (m), 18 (n) and 16 kDa (o); fewer bands (with molecular weights from 130-185 kDa) were revealed in the hydrophilic phase (Figure 6.2, lane 3). The 165 kDa (b) polysaccharide-positive band in the Texoag (Figure 6.2, lane 4) stained more intensely that its equivalent in the Eexoag (Figure 6.2, lane 1).

Staining of gels containing the electrophoretically separated exoantigen preparations with Sudan black indicated that only the 185 kDa band of the Eexoag stained for lipids (Figure 6.3, lane 1); no lipids were detected in the Texoag (Figure 6.3, lane 2) preparation.

Analysis for mannose sugars in the exoantigens with the peroxidase-labeled Con A probe (Figure 6.4) shows that three major bands of molecular weights of 84, 72 and 48 kDa stained intensely for mannose sugars in both the Eexoag (lane 3) and Texoag (lane 4), although the 48 kDa was component not as clearly defined in the Eexoag. Two other minor mannose-containing components were also revealed: a 30 kDa component of both exoantigen preparations and an 18 kDa component which only occurred in the Eexoag preparation (Figure 6.4, lane 3). Control blots of Eexoag (Figure 6.4, lane 1) and Texoag (Figure 6.4, lane 2), incubated with HST buffer containing 1 $\mu$g/ml of peroxidase-labeled Con A and 0.2 M $\alpha$-methyl-$\delta$-mannopyrannoside, failed to show any mannose-containing bands.

The antigenicity of the glycosylated exoantigens, as revealed by the reaction of the blots with immune sera, show that three major antigens of 84, 72 and 42 kDa and minor ones of 40, 30, 26, 22 and 18

kDa were recognized in both the Eexoag (Figure 6.5, lane 1) and in the Texoag (Figure 6.6, lane 1). Normal rat serum (NRS) probes of the same blots indicated that nearly all the antigens revealed by immune serum in the Texoag (Figure 6.6, lane 1) were also revealed by the NRS (Figure 6.6, lane 2); the 72 kDa band, the component that stained for proteins and polysaccharide (thus a glycoprotein), bound most of the normal rat IgG as shown by its intense reaction; the other bands were however, less intense than they were with the immune serum probes. Only the 72 and 48 kDa glycoproteins bound rat IgG in the Eexoag (Figure 6.5, lane 2). Fewer reactions were revealed in the blots of Texoag probed only with the peroxidase-labeled anti-rat IgG (Figure 6.6, lane 3) than those probed by NRS (Figure 6.6, lane 2) or by immune serum (Figure 6.6, lane 1); the 84 and 72 kDa components did not react with the anti-rat IgG. Only the 48 kDa glycoprotein in the Eexoag reacted with the peroxidase-labeled anti-rat IgG Eexoag (Figure 6.5, lane 3), the 72 kDa was, however, not revealed by the peroxidase-labeled anti-rat IgG. In both exoantigen preparations, the 185, 165, 160, 155, 140, 130 and 16 kDa glycoprotein bands failed to react with immune serum and for presence of mannose sugars.

## DISCUSSION

Exoantigens are associated either directly or indirectly with immunoregulatory functions during parasite infections. In infections with African trypanosomes, they have been reported to induce cellular dysfunctions (Diffley 1983) and since exoantigens are also bound in immune complexes, they may play an important role in the immunopathology (Nydegger 1979; Boreham and Facer 1974) and in immunosuppression (Urquhart 1980) in infected hosts. Immune complexes

generated from circulating antigens in hosts infected with schistosomes (Okabe and Akusawa 1971; Bawden and Weller 1974) have also been associated with renal pathology (Carlier et al 1975; Bout et al 1977). However, it is only in African trypanosomes where these products have been well studied and it is now evident that the circulating exoantigens correspond with the antigenic variant type (Takayanagi et al 1970; Diffley et al 1980). Despite the constant demonstration of these circulating parasite antigens in infected hosts (D'Alesandro 1972; Bout et al 1977; Montenegro-James et al 1987; James et al 1987), the nature of these exoantigens has not been determined in most parasitic infections. The present study analyzed exoantigens from culture supernatants shed by actively motile parasites and we have found them to be complexes of proteins, polysaccharides and lipids; the results presented in Chapter V suggest that some of the exoantigens play an important immunoregulatory role to enhance parasitemia during infections. The additional ability of some exoantigens to protect naive animals following immunization has also been demonstrated in Chapter V where it was shown that protection was mediated by exoantigen-elicited trypanolytic and ablastic antibodies. Thillet and Chandler (1957) and D'Alesandro (1986) have also reported the production of ablastic antibodies following immunization of normal animals with culture supernatants or infected rat plasma. In this study, Western blot analysis was used to determine the antigenicity of the culture-derived exoantigens, after probing the blots with infected rat serum. At least eight antigens, all glycoprotein in nature were recognized by the immune serum, with the 48, 72, and 84 kDa being the major antigens which were most intensely revealed in the blots. Interestingly, these

strongly antigenic bands also have a large component of mannose sugars, as revealed by the intense reaction which they gave with the peroxidase-labeled Con A probe; the antigens that reacted less strongly appeared to correspondingly react less strongly for mannose sugars. The significance of this observation is at present difficult to explain, but these results suggest a correlation of mannose-containing glycoproteins and immunogenicity. In support of this observation is the fact that the variant surface glycoprotein (VSG) of African trypanosomes is highly immunogenic and is the target of the host's immune response (Vickerman and Luckins 1969; Vickerman, 1974; Fruit et al 1977); VSGs have been classified as 'high mannose' oligosaccharide, since they contain a high proportion of this sugar relative to their content of N-acetylglucosamine and galactose (Holder and Cross 1981; Carrol and McCrorie 1987). It is interesting to note that only the high mannose glycoproteins of T. cruzi can effectively mediate protection; these protecting glycoproteins also constitute the major antigens detected by immune sera obtained from infected patients (Nogueira et al 1981; Snary et al 1983). Lectin-mediated agglutination studies of bloodstream forms of T. lewisi have shown Con A to be the most potent inducer of the agglutination of this parasite, whether the parasite are trypsinized or not; this fact indicates that mannose is the main sugar of the surface coat and membranes of T. lewisi (Dwyer 1976), with other sugars (N-acetylgalactosamine, N-acetylglucosamine and L-Fucose) present in lesser amounts (Dwyer 1976). Although no attempts were made to analyze for the other sugars, it is evident that not all PAS-staining glycoproteins contain mannose: it was, particularly, not revealed in the high molecular weight glycosylated proteins (130, 140, 155, 160, 165, 185 kDa) which were not recognized

by immune serum on Western blots. Control blots probed with normal rat serum (NRS) revealed nearly all the bands recognized in blots probed with immune serum, more so with Texoag than with Eexoag, although considerably less intensely. The 72 kDa component was the major glycoprotein that bound normal rat IgG most strongly. These results are consistent with the report by Balber and Sturtevant (1986) who also noted a similar reaction in trypanosome extracts probed with normal rat serum, or peroxidase-labeled anti-rat IgG, which were attributed to presence of natural antibodies; more recently Giannini (1987) has demonstrated that ablastic antibodies are present in the serum of uninfected normal rats.

Although the protein-staining profile of both the Eexoag and Texoag were remarkably similar, there was a qualitative difference in the staining for polysaccharide which was evident mainly with the 185 kDa band, which stained more prominently in the Texoag than in the Eexoag, although the protein concentration loaded into the wells of the polyacrylamide gels was always the same. A positive stain for lipids was only obtained with the 185 kDa band in the epimastigote exoantigen, suggesting that some changes in the trypanosome's constituents accompany the morphological transformation of this parasite.

Following phase-partitioning of culture-derived exoantigens from epimastigotes, these fractions were analyzed by SDS-PAGE and stained for polysaccharides. The majority of the polysaccharide-containing bands occurred in the amphiphilic fraction, with an enrichment of the number of bands in comparison to the number found in the original unfractionated exoantigen preparation. These results suggest that hydrophobic exoantigens of T. lewisi are actively shed and that they

consist of surface coat and surface membranes components, which are constantly replaced at the membrane level as the outer exoantigen components are shed. This phenomenon is unlike the enzymatic release of the VSG by T. brucei: the hydrophobic membrane form of the VSG (mfVSG) is converted by an endogenous phospholipase c (Herold et al 1986) to a hydrophilic form (sVSG); after phase partitioning, the mfVSG occurs in the amphiphilic phase and the sVSG in the aqueous phase (Turner et al 1985; Ward et al 1987). The high molecular weight glycoproteins (130, 140, 155, 160, 165 and 185 kDa) in our study partitioned into both the amphiphilic (detergent) and hydrophilic phases; this result suggests that the high molecular weight components are released both with and without the hydrophobic transmembrane domains; whether the absence of these domains is the result of enzymatic cleavage (as occurs in T. brucei) is presently unknown.

Having characterized some of the physicochemical properties of the exoantigens of the epimastigotes and the trypomastigotes of Trypanosoma lewisi, it was of interest to determine their source in the intact trypanosomes by isolation and characterization of whole solubilized parasite components and to assess the role they play in regulating infections with this parasite.

Fig. 6.1. SDS-PAGE analysis of the culture-derived soluble exoantigens from epimastigotes (lane 1) and trypomastigotes (lane 2) stained for proteins with Coomasie brilliant blue to reveal the components of the culture supernatant obtained after incubation of the parasites for 3 hours at 37°C in phosphate buffered saline glucose (PBSG, pH 8.0).

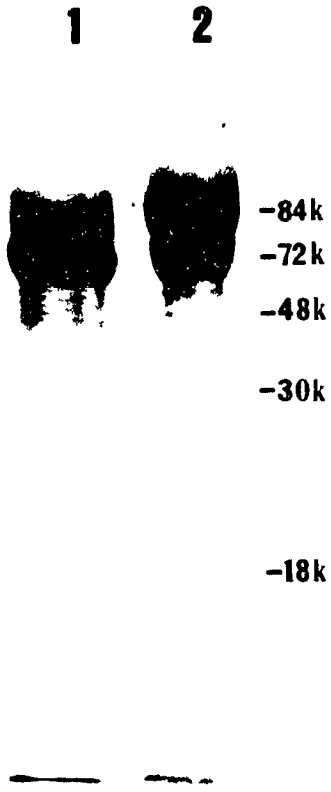

1
2
-84k
-72k
-48k
-30k
-18k

Fig. 6.2. Polyacrylamide gels stained with the periodic acid Schiff reagent (PAS) to reveal the polysaccharide-containing proteins of the culture-derived soluble exoantigens of the epimastigotes (lane 1) and trypomastigotes (lane, 4). Enhancement of up to 15 PAS reacting bands of relative molecular weights of 185 (a), 165 (b), 160 (c), 155 (d), 140 (e), 130 (f), 84 (g), 72 (h), 48 (i), 40 (j), 30 (k), 26 (l), 22 (m), 18 (n) and 16 kDa (o) occurred in the amphiphilic fraction (lane 2) after phase partitioning of the epimastigote culture supernatant (lane 1), compared to the six 130-185 kDa bands which also occurred in the hydrophilic (lane 3) fraction.

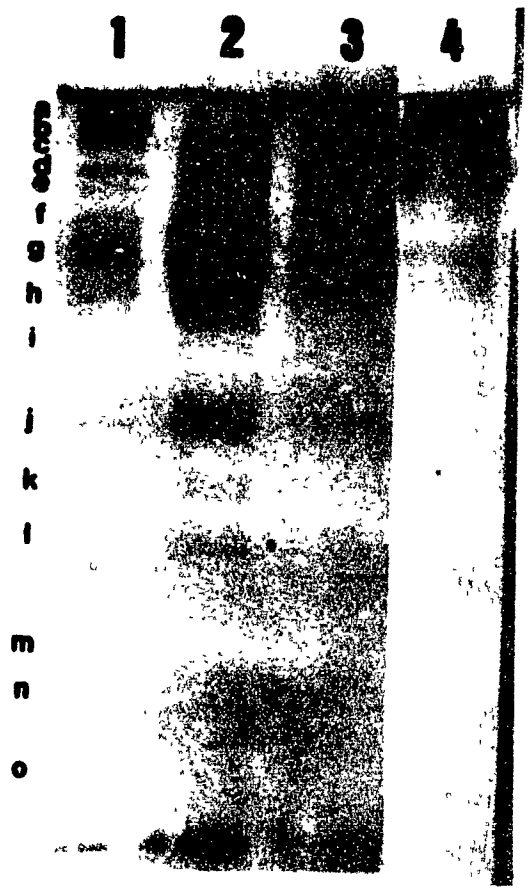

1
2
3
4
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o

Fig 6.3. Polyacrylamide gels stained with Sudan black to reveal the the components of the culture-derived exoantigens from the epimastigotes (lane 1) and trypomastigotes (lane 2) that contain lipids.

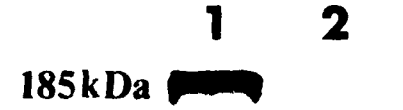

1
2
185kDa

Fig. 6.4. Blots of epimastigote (lane 1 and 3) and trypomastigote (lane 2 and 4) culture-derived soluble exoantigens probed with 1 μg/ml of peroxidase-labeled Concanavalin A (lanes 3 and 4) to reveal the exoantigen glycoproteins containing mannose sugars. Control blots (lanes, 1 and 2) were probed with 1 μg/ml of peroxidase-labeled Concanavalin A in 0.2 M α-methyl-δ-mannopyrannoside.


1
2
3
4
84k
72k
48k
30k
18k

Fig. 6.5. Western blots showing the antigenic soluble exoantigens from epimastigotes revealed by day 30 immune serum (lane 1). Control blots were probed with normal rat serum (lane 2) and peroxidase-labeled anti-rat IgG (lane 3). The relative molecular weights of the reactivities are shown in kDa.

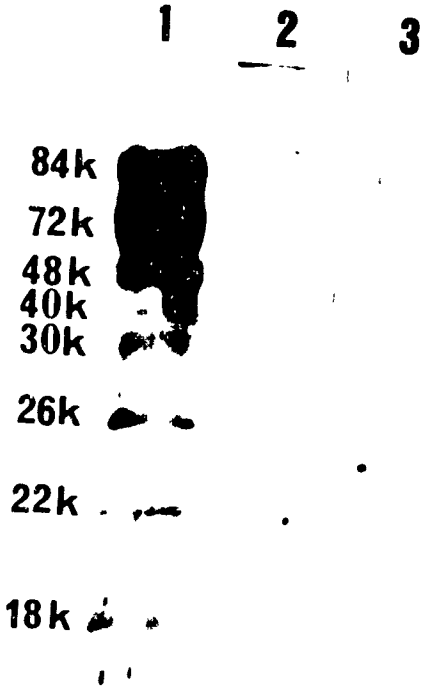
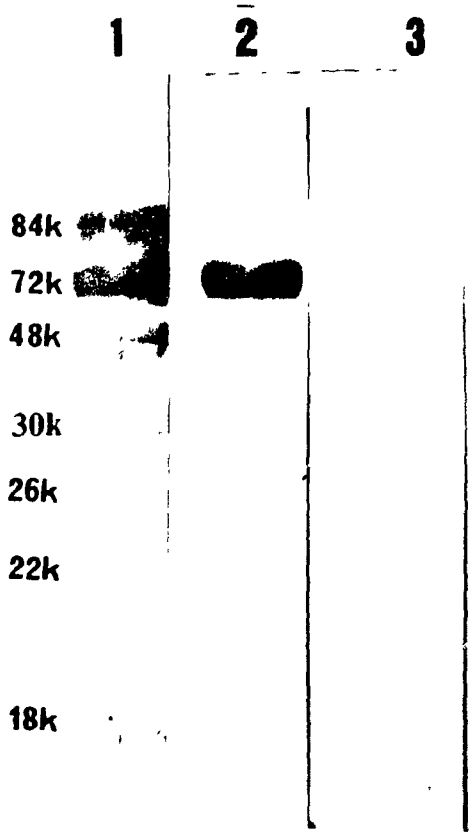

1
2
3
84k
72k
48k
40k
30k
26k
22k
18k

Fig. 6.6. Western blots showing the antigenic soluble exoantigens from trypomastigotes revealed by day 30 immune serum (lane 1). Control blots were probed with normal rat serum (lane 2) and peroxidase-labeled anti-rat IgG (lane 3). The relative molecular weights of the reactivities are shown in kDa


1
2
3
84k
72k
48k
30k
26k
22k
18k

# CHAPTER VII: CHARACTERIZATION OF THE ROLE OF PHASE-PARTITIONED FRACTIONS AND THE COMPONENTS IN TRYPANOSOME LYSATE WHICH CONTROL INFECTION BY TRYPANOSOMA LEWISI.

## INTRODUCTION

In recent years there have been extensive studies on the surface antigens of parasites to establish their nature and to evaluate their biological role during infections, or for immunodiagnostic purposes (Mackenzie et al 1978; Philipp et al 1980; Nogueira et al 1981; Maizels et al 1982; Snary 1983; Giannini and D'Alesandro 1984). Most surface antigens are glycoprotein in nature (Ogilvie et al 1982) and among the most studied is the variant surface glycoprotein (VSG) of salivarian trypanosomes, which is highly immunogenic and protective to homologous variants (Baltz et al 1977, Vickerman 1978; Cross 1975, 1979, 1984).

In T. lewisi infections, specific antibodies have been studied by gel diffusion to analyze antigenic differences between the dividing epimastigote and the non-dividing trypomastigote forms (Etner and Gonzalez 1966; Etner 1968). D'Alesandro (1976) reported that agglutinins specific to the dividing forms could not agglutinate the non-dividing mature trypomastigote, indicating stage-specificity in the antibody response against this parasite. More recently, Long and Dusanic (1983) have reported variations in the number of exoantigen precipitin lines which developed in gel diffusion with sera obtained from different rats at various days of the infection. These previous studies were limited to the general characterization of the antigen components involved in these antigen-antibody reactions. The present studies of the immunobiological role of the antigens of T. lewisi were

done using the more precise Western blot analysis of lysed epimastigotes and trypomastigotes probed with antisera obtained during and after infection. One of the objectives of this study was, therefore, to determine the antigen repertoire recognized by immune sera obtained at various days of the infection. The source of the antigens in the parasite was determined after surface radio-iodination and phase partitioning in Triton X-114. It was also of interest to determine the biological role of the phase-partitioned fractions by establishing their effect on the susceptibility of normal splenocytes to stimulation by mitogens and to determine the function of these fractions to change the parasitemia in naive infected animals after immunization.

## MATERIALS AND METHODS

With the exception of the procedure used for surface iodination, all the methods used for the studies in this chapter have been described previously.

### 1.1 Surface iodination

Surface labeling with $^{125}I$ was done as described by Howard et al (1982). Epimastigotes from cyclophosphamide-treated rats and trypomastigotes from day 15 infected non-immunosuppressed animals were obtained as described above and then purified by filtering through a DE52 cellulose column. The trypanosomes were counted under phase contrast illumination and adjusted to $10^8$ cells/300 $\mu$l of PBSG; this cell suspension was then added to 300 $\mu$l of PBSG containing 1 mCi of carrier-free $Na^{125}I$ (ICN Radiochemicals Irvine, CA) and four Iodobeads (Pierce Chemical Co., Rockford, IL). The suspension was mixed

thoroughly and the iodination was allowed to proceed for 15 minutes at room temperature with occasional agitation; iodination was terminated by removing the Iodobeads. The parasites were then washed four times in ice-cold PBSG before solubilization in pre-condensed Triton-X-114 and phase partitioned as described (Bordier 1981) or dissolution in sample buffer containing SDS and 2-mecarptoethanol. The hydrophilic and amphiphilic fractions of the iodinated epimastigotes and trypomastigotes were then analyzed by SDS-PAGE under reducing conditions; radioautographs of the dried gels were performed by exposing Kodak X-OMAT film at -70°C, under an intensifying screen.

## RESULTS

The protein profile of the the hydrophilic (HP) and amphiphilic (AP) fractions on nitrocellulose sheets were obtained after staining with 0.1% (w/v) amido black; these profiles are shown in Figure 7.1 and in Figure 7.2. Several protein bands with molecular weights ranging from 16-84 kDa, were revealed in both HP and AP fractions of the two morphological forms of the parasite. The 16, 48, 72 and 84 kDa bands of the epimastigote forms (Figure 7.1) and the 20, 72, and 84 kDa of the trypomastigote preparations (Figure 7.2) stained more strongly in the AP fraction (lane 2) of both forms of the parasite than in the HP fraction (lane 1). The periodic acid Schiff (PAS) stain for polysaccharides revealed at least 12 bands in the amphiphilic fractions of the epimastigote form (Figure 7.3) and ten in the trypomastigote AP (Figure 7.4); the 155 and 16 kDa bands of the epimastigote AP were not observed in the trypomastigote preparation The high molecular weight (185 and 165 kDa) PAS bands of the AP fraction of epimastigotes and

trypomastigotes also occurred in the epimastigote and trypomastigote HP preparations (Figures 7.3 and 7.4); an additional 130 kDa PAS-positive component was evident in the trypomastigote HP fraction. Interestingly, the 165 kDa component of the trypomastigote forms was more prominent in the HP than in the AP fraction.

Sudan black staining for lipids of both the HP and AP fractions of epimastigotes and trypomastigotes is shown in Figure 7.5. A major fast moving component occurred at the bottom of the gel exclusively in the AP fraction of both epimastigotes (lane 2) and trypomastigotes (lane 4). A slow moving constituent of 185 kDa also stained for lipids in both the HP and AP fractions of the epimastigotes; this band was not apparent in either fraction of the trypomastigotes

Western blot analysis by day 30 immune serum of the phase-partitioned fractions indicate that major antigens of molecular weight 16, 26, 30, 72, and 84 kDa were revealed in the AP fraction while only minor antigen components were recognized in the HP fraction (Figure 7.6) in the epimastigote preparation. These same major antigen bands were revealed in the AP and HP fractions in the trypomastigote preparation (Figure 7.7), although the HP fraction also contained, in addition, several minor antigens; the 72 kDa antigen was more intensely revealed in the HP fraction than in the AP fraction. Control blots, for the epimastigote and trypomastigote preparations respectively probed with normal rat serum (NRS), or developed with peroxidase-labeled anti-rat IgG (Miles Yeda Ltd. Israel), are shown in Figure 7.8 and Figure 7.9. Minor reactions occurred when the epimastigote HP and AP fractions were probed with NRS or with peroxidase-labeled anti-rat IgG: NRS reacted mainly with the AP fraction, with the 72 kDa glycoprotein revealed most prominently. This band was not revealed

when the AP fraction was developed with the peroxidase-labeled anti-rat IgG; low molecular weight components of 18, 20, and 26 kDa were, however, identified, in this, but not in the hydrophilic fraction (Figure 7.8). The reactions obtained with NRS were stronger in the trypomastigote preparation, especially in the AP fraction, with the 72 kDa glycoprotein being the most prominent component. Peroxidase-labeled anti-rat IgG also revealed low molecular weight components in both fractions of the trypomastigote preparation; as with the epimastigote preparation, the 72 kDa was not revealed with this probe

A comparative analysis of the antigen repertoire recognized by Western blots of paired lysed preparations of epimastigotes (EE, lane 1) and trypomastigotes (TE, lane 2) probed with immune sera taken at various days of the infection are represented in Figures 7.11 and 7.12. SDS-PAGE analyses of EE and TE, stained with amido black for proteins, are shown in Figure 7.12. The EE antigens showed a stronger reaction than the TE preparation when the Western blots were probed with day 5 and the 15 sera. The number of bands and the intensity of the reaction increased when the blots were probed with antiserum taken on day 30 and thereafter; the 72 kDa component stained most intensely with the day 30 and 70 probes. The 40 kDa and the 18 kDa antigen bands were recognized for the first time by the day 30 antiserum; the intensity of the 18 kDa component increased with each post-recovery serum and was most strongly revealed by the day 70 probe The 18 and 30 kDa components were the two antigens which were revealed most intensely and consistently by the post-recovery serum probes, but not by serum probes obtained during the course of the infection. The 40 kDa antigen, which was identified in the paired preparations by day 30,

70, 160 and 220 antisera, was only weakly revealed by the day 386 probe; the intensity of the reaction of the 30 kDa component was also weaker with the last antiserum, when compared with reactivities obtained with the day 70, 160 and 220 probes. Except for the slight difference in the reaction between the EE and TE preparations with the day 5 and 15 probes, the antigens and the polypeptide patterns of both preparations of the trypanosomes were remarkably similar; and with the exception of a few bands, the NRS and the peroxidase-labeled anti-rat IgG probes revealed as many antigens in the lysates of the two trypanosome stages as had been revealed by the immune sera, although very faintly Most of the antigens revealed by the NRS and the peroxidase-labeled anti-rat IgG control probes were of low molecular weight (16-40 kDa), a 72 kDa component which reacted with NRS did not appear in blots probed with peroxidase-labeled anti-rat IgG.

Surface labeling of epimastigotes (Figure 7.13) and trypomastigotes (Figure 7.14) indicates that at least nine surface components were iodinated on epimastigotes while six surface constituents were labeled on trypomastigotes. The 16 and 165 kDa polypeptides were the most strongly labeled surface components on the epimastigotes, the 48 and the 72 kDa were not labeled as intensely Similarly, the 16, 72, and 165 kDa were the most intensely labeled fractions of the trypomastigote preparation; the 48 kDa polypeptide, which was prominently revealed in the epimastigote preparation, was not labeled on the trypomastigotes.

Phase-partitioning of surface-iodinated epimastigotes (Figure 7.15) and trypomastigotes (Figure 7.16) by solubilization in Triton X-114 show that the parasite constituents which took the label occurred mainly in the AP fractions of both stages of the trypanosome. The fast

moving constituent at the bottom of the gel and the slow moving component of 185 kDa were the most strongly labeled products of the epimastigote surface (Figure 7.15); only the 72 kDa peptide was intensely labeled as a surface constituent of trypomastigotes (Figure 7.16). Bands of 185 and 130 kDa appeared in radioautographs of the AP fraction of trypomastigotes, although they were not revealed in the crude lysate of the organism (Figure 7.14). The 84, 72, and 48 kDa constituents in AP fraction of epimastigotes were moderately-labeled while the 40, 30, 22, and 18 kDa fractions were only lightly labeled. The 165, 72, 48 and 16 kDa components of the hydrophilic fraction of epimastigotes were very light in the radioautographs while no bands were obtained in the same fraction of the trypomastigotes (Figure 7.14).

To investigate a possible biological role of the exoantigens, normal rat spleen cells were incubated in presence of the amphiphilic and hydrophilic fractions of the epimastigotes and trypomastigote The effect of the fractions was assessed by stimulating the cells subsequently with the mitogens Con A and LPS, and determining the incorporation of the triated thymidine. Suppression of the Con A-induced mitogenesis of normal rat spleen cells was produced by relatively high (6.3-25 $\mu$g/ml) concentrations of the glycolipoprotein-rich amphiphilic (AP) fraction of the epimastigotes (Figure 7.17); low concentrations (0.05-0.1 $\mu$g/ml) of the same fraction enhanced the mitogenic response. The protein-rich HP fraction of epimastigotes enhanced Con A-induced mitogenesis at all concentrations tested. Suppression by the AP fraction could not be demonstrated when macrophages were removed from target spleen cell population, before the

activation by Con A (Table 7.1). It was most interesting that the cultures where the adherent cells had been depleted, the enhancing abilities of the phase-partitioned epimastigote exoantigens were significantly increased, even at concentrations that otherwise suppressed Con A-induced mitogenesis in spleen cell cultures containing the adherent cell population Lipopolysaccharide-induced blastogenesis (Figure 7.18) was also suppressed by high concentrations of the glycolipoprotein-rich (AP) fraction of epimastigotes and enhanced at lower concentrations. As for Con A, the protein-rich HP fraction induced only enhanced response to activation of splenocytes by LPS.

Similar results were obtained with phase-partitioned fractions of trypomastigotes (Figure 7.19): enhancement of Con A stimulation of normal rat spleen cells occurred with lower concentrations of the glycoprotein-rich AP fraction of the trypomastigotes, while suppression was obtained with the maximum concentration (25 $\mu$g/ml) of the same fraction; the protein-rich HP fraction induced only enhanced Con A responses. Similar results were obtained for LPS-induced mitogenesis (Figure 7.20): the glycoprotein-rich AP fraction suppressed the incorporation of thymidine at high and enhanced the incorporation by spleen cells at low concentrations; the protein-rich HP fraction stimulated an enhanced response to LPS-induced activation of splenocytes at all concentrations tested.

Since it was of interest to determine whether suppression require the continued presence of the parasite exoantigens, spleen cells were washed with medium after 24 hours incubation with 6.3, 12.5 and 25 $\mu$g/ml concentrations of the epimastigote AP fraction, before stimulation of the cells with Con A or LPS. Washing of the cells restored normal Con A and LPS responses (Table 7.2) compared to

cultures where the AP fraction was present throughout the period of the study. It was also of interest to determine whether the suppressor action could be ascribed to a deficiency in the production of IL 2 by lymphocytes. A significant enhancement of the normal response was obtained when normal spleen cells were incubated, in the presence of IL 2 with concentrations of the epimastigote AP fraction (Table 7.1) that suppressed Con A-induced blastogenesis. Interleukin 2-induced enhancement increased with a decline in the concentration of the AP fraction in the culture, reaching a peak at 1.6 μg/ml.

The possible function *in vivo* of the exoantigens of the *T. lewisi* was determined by the infection of animals 7 days after treatment with an intraperitoneal injection of one ml of phase-partitioned fractions of epimastigotes, each containing 150 μg total protein. Naive animals were protected by the HP and AP fractions, since the level of parasitemia was depressed in both groups (Figure 7.21). Although the level of infection was significantly lowered by treatment with the HP fraction, the parasitemia persisted until day 24 before it was completely eliminated. In contrast, AP-treated animals had considerably lower parasitemias than HP-treated group and the infection was completely cleared from these animals by day 12.

## DISCUSSION

The distinct morphological changes that some parasites undergo during the course of infection in the host are usually associated with changes in their cellular (Araujo and Remington 1981) or antigenic constituents (Nogueira *et al* 1981, 1982; Lanar and Manning 1984). These phenomena have been well established with *T. cruzi* and Sacks *et al*

(1985) have reported antigenic changes in Leishmania major during the development of promastigotes from the non-infective to the infective forms.

The results presented in this study show that the polypeptide pattern obtained with lyzed epimastigotes and trypomastigotes is essentially similar and that, in contrast to a similar study in T. cruzi by Rangel-Aldoa et al (1986), stage-specific polypeptides do not exist in T. lewisi. These result indicate that the transformation of the parasite from dividing epimastigotes to non-dividing "mature" trypomastigotes is not associated with any discernible expression of specific polypeptides. However, slight differences occurred with different sera in the antigens recognized by different serum probes since the intensity and the number of antigens identified by immune sera increased with sera taken later in the course of infection. The fact that some antigens (the 18 and 30 kDa polypeptides) were only recognized by post-recovery sera and not by sera obtained during the course of patent infection suggest that these antigens are probably involved in the maintenance of protective immunity. Thus, specific antibodies to these two antigens do not appear in the circulation of infected animals until the time of the elimination of the parasitemia and persist thereafter in recovered animals; it appears that most of the antibodies in the post recovery sera are induced by and directed to these two antigens (18 and 30 kDa). The induction of this immunity is difficult to explain since parasites are not demonstrable in the circulation of rats that have recovered from a T. lewisi infection, unlike the well-documented persistence of T. musculi in the kidneys of mice that have recovered from infection (Viens et al 1972).

The immunogenicity of internal and surface antigens of T. lewisi was assessed by Western blots probed with day 30 immune serum, after phase partitioning into the water-soluble HP and lipid-soluble AP fractions of both the epimastigotes and trypomastigotes; the HP consisted of internal and non-membrane associated constituents of the parasite, whereas AP fraction represented constituents associated with the membrane and the surface coat. The majority of the antigens released from the lysed trypanosomes were also identified in the AP fraction, the lipid-soluble phase that contains a complex of protein, polysaccharide and lipid (glycolipoproteins), indicating that they are surface-membrane associated antigens.

Since only the products associated with membrane hydrophobic molecules separates into the AP phase (Bordier, 1981), these components therefore are part of the membrane and the surface coat of T. lewisi; the Western blot experiments indicate that they constitute the major antigens recognized by the host's immune system during infection by the parasite. The fact that components in the HP fraction also reacted with immune sera suggests either that the surface-membrane antigens share antigenic determinants with intracellular antigens or that the immune system produces antibodies against internal components released as a result of immunolysis. Although D'Alesandro (1970) suggested that antibodies against intracellular antigens could have no effect on viable trypanosomes, the results presented here clearly indicate that naive animals immunized with 150 $\mu$g of the HP fraction, were significantly protected when challenged 7 days later; the AP fraction was, however, superior in protective ability. These results strongly suggest that intracellular antigens share determinants with the surface coat and they may indeed represent internal components of the coat

prior to their mobilization to their final location on the surface of the parasite.

It is most interesting that the majority of the bands recognized by the immune serum probes were also obtained in Western blots probed with normal rat serum (NRS) although immune sera produced very much stronger reactions; most of these reactivities occurred in the AP fraction of phase-partitioned epimastigotes and trypomastigotes. Reactions with NRS and peroxidase-labeled anti-rat IgG have recently been demonstrated by Balber and Sturtevant (1986) who suggested that the binding of components of T. lewisi by NRS is due either to the presence of natural antibodies in uninfected rats, or to a non-specific binding of normal serum components onto the surface glycoproteins of the trypanosome. More recently, Giannini (1987) has shown that the serum of uninfected rats contains low levels of ablastic activity and she has suggested that normal animals are primed by "environmental" antigens. It has been reported that ablastin adsorbs onto the surface coat of trypanosomes (D'Alesandro and Clarkson 1980) and this property has been used to isolate protective surface coat antigens of T. lewisi (Giannini and D'Alesandro 1984). It is conceivable, therefore, that ablastic antibody induced by "environmental" antigens is cross-reactive with trypanosome surface components and that the reactivities observed in this study are not due to non-specific binding of the normal serum components.

The similarity in the number of the components of extracts of epimastigotes and trypomastigotes in Western blots probed with normal and immune serum suggests that the difference between the two is essentially quantitative. Etner (1968), studying changes in the

antigenicity of the two stages of T. lewisi by immunodiffusion analysis, reached a similar conclusion. The increase in the intensity of the reaction in blots probed with infected serum taken as the infection progressed may have been due in fact to an anamnestic immune response, initially primed by "environmental" antigens. The fact that the 18, 30, 40, 72, 84, 130 and 145 kDa antigen bands were recognized more strongly by immune serum probes obtained at the time of parasite elimination and thereafter, indicates that the bands produced on the blots were due to antibodies which appeared later in the course of the infection; these antibodies are probably not ablastin since this antibody appears 2-4 days after the start of the initial infection (D'Alesandro 1962, 1975). Bloodstream T. lewisi has been reported to bind host serum proteins onto its surface coat (Etner 1968; D'Alesandro 1972; Dwyer 1976; Sturtevant and Balber 1986) and the accretion of host serum proteins has also been reported in Trypanosoma vivax (Desowitz and Watson 1953; Kettridge 1970); T. b. gambiense (Seed 1974; Bogucki and Seed 1978; Diffley 1978), T. b. brucei (Vickerman 1972; Le Ray 1975; Diffley 1978) and in T. congolense (Diffley and Honiberg 1977). Most of the host proteins have been demonstrated by these other authors by gel diffusion analysis; the reactions obtained on Western blots developed with peroxidase-labeled anti-rat IgG in this study also revealed host components present in the parasite preparation. The appearance of these reactivities mainly in the AP fraction suggests that they are associated with the membrane-surface coat of the parasite.

Trypanosoma lewisi has a membrane-associated surface coat (Vickerman 1969, 1971; Dwyer 1975) which contains carbohydrate moeities; as shown by cytochemical staining techniques (Dwyer 1975a)

and lectin-mediated agglutination studies (Dwyer 1976), the presence of glycopeptides and glycoproteins (Dwyer and D'Alesandro 1974, 1980) on the membrane has been indicated. The present study has isolated and characterized membrane-associated trypanosome products by solubilizing the parasites in Triton X-114 (Bordier 1981); such phase partitioning allowed the amphiphilic integral membrane proteins to be identified and to be isolated from the soluble and cytoskeletal proteins. Under these conditions, only those cellular proteins associated with the hydrophobic portion of the lipid bilayer were recovered in the detergent (Triton-rich) phase after partitioning. The hydrosoluble HP fraction contained mostly proteins except for two high molecular weight PAS staining bands (protein-rich). The detergent, lipid soluble, AP fraction contained 10-12 bands with molecular weight ranging from 16-185 kDa which stained positive both for proteins and for polysaccharides, indicating they are glycopeptides and glycoproteins. Although the high molecular weight glycoproteins of the AP fraction failed to stain for proteins, they, however, stained intensely with PAS and the 185 kDa band, which occurred in the HP and AP fraction of epimastigote but not in trypomastigotes, stained with Sudan black. The component which stained most intensely for lipids was the fast moving constituent at the bottom of the gel which occurred in the AP fraction of both epimastigotes and trypomastigotes. This and the 185 kDa components of epimastigotes are glycolipoproteins since both of them also stained for proteins and polysaccharides; the 185 kDa component of the trypomastigotes was however, deficient in lipids.

Phospholipids are the most abundant form of lipids in trypanosomes and Dixon and Williamson (1970) have shown by thin layer chromatography

that T. lewisi extracts also contain linoleic, oleic, palmitic and stearic acids, which constitute up to 10-12% of the dry weight of this protozoa. The present study indicates that lipids are associated in epimastigotes with both low and high molecular weight membrane components, whereas the glycolipoprotein is found only in the fast moving component of trypomastigotes. The fact that the 185kDa band in trypomastigote preparation was devoid of lipids suggests that the transformation of epimastigotes to trypomastigotes is accompanied by an inability to utilize or to incorporate some of the exogenous lipid sources into the glycoproteins since the lipids of the trypanosome compare well with those of its enviroment in the host (Dixon and Williamson 1970).

It is apparent from the results of this study that the the two high molecular weight glycoproteins (165 and 185 kDa) occur in both hydrophilic (HP) and amphiphilic (AP) phases of Triton X-114 solubilized epimastigotes and trypomastigotes; the former phase is devoid of the hydrophobic domains. Since surface labeling of both stages of the parasite with $^{125}I$ indicated that the major surface glycoproteins partitioned only into the AP phase, it is possible that the unlabeled glycosylated proteins of the same molecular weight in the hydrophilic (HP) fraction are endogenous precursors of the surface glycoproteins; if they are precursors, the HP glycoproteins would not be accessible to the isotope and, would therefore thus fail to take the surface label. Another possibility is that an enzyme cleaves off the hydrophobic domains of the glycoproteins in the AP fraction thus converting them to hydrosoluble form in a fashion similar as occurs in T. brucei (Jackson and Voorheis 1985; Hereld et al 1986) There was however, no difference in this study in the relative migration in SDS-

PAGE of the hydrosoluble and the AP form of the high molecular glycoproteins of T. lewisi, as has been observed after the conversion of the membrane form of the variant surface glycoprotein (mfVSG) of the brucei trypanosome to the water soluble sVSG (Cardoso de Almeida and Turner 1983; Turner et al 1985; Ward et al 1987; Balber and Ho 1988).

The biological functions of parasite surface glycoproteins are diverse and in T. cruzi (Snary 1985, Carroll and McCrorie 1987) they act as receptors during the penetration of the cell. Chakraborty and Das (1988) have also reported that the attachment and penetration of Leishmania donovani into hepatic macrophages is mediated by mannose/acetylglucosamine sugar receptors, while a major glycoprotein (gp63) helps in the attachment of Leishmania mexicana mexicana promastigotes to macrophages (Russel and Wilhelm 1986). Besides these functions, surface glycoproteins also form the major antigens which the immune system of the host recognizes (Nordens et al 1982; Zodda and Phillips 1982) and are therefore important in protective immunity (Soulsby 1985). Although parasite infections induce specific antibody responses, most parasites cause immunological hyporesponsiveness in the host at some stage of the infection. Indeed, the phenomenon of immunosuppression is expressed at a time when the host is developing immunity against the parasite resulting, perhaps, in a reduced ability of the host to reject the parasite (Mansfield 1981). The ability of parasite-derived products to induce blastogenic changes in cells in the presence of mitogens has been extensively used to study the role of parasite-derived immunoregulatory mechanisms during the course of infections in the host (Hazlett and Tizard 1978; Cunningham and Kuhn 1980; Grosskinsky and Askonas 1981; Charoenvit et al 1981).

Trypanosoma lewisi, like other trypanosome infections, has been reported to induce immunosuppression (St. Charles et al 1981) and more recently, Proulx (1988) has shown that the mitogenic response of rat spleen cells obtained from infected animals is depressed. The present study has demonstrated that depression of the mitogenic response of normal splenocytes occurs only with the AP fraction, which contains complexes of glycosylated lipoproteins; no suppression occurred with the protein-rich HP fraction In a similar study with T. musculi, Hazlett and Tizard (1978) reported suppression of mitogenesis with large numbers of autolyzed trypanosomes; these authors also reported enhancement of $^{3}$H-thymidine incorporation with lower numbers of autolyzed parasites This same phenomenon was obtained in this study only with the amphiphilic fraction, whereas the protein-rich HP fraction enhanced the mitogenic response of spleen cells Clayton et al (1979) have shown that the immunosuppressive and mitogenic effects of living T. brucei were restricted to a membrane fraction collected after high speed centrifugation. This report was later expanded by Sacks et al (1982) who demonstrated that the heterogeneous endoplasmic reticulum fraction that mediated the suppression or enhancement of mitogenesis was a complex of lipids and glycosylated proteins

The suppression of the action of the mitogen Con A indicates an interference in T helper cell function (Harel-Bellan et al 1983). In this present study, suppression of Con A-induced blastogenesis by glycolipoproteins was ablated by the addition of exogenous IL 2; an optimal peak response was obtained which declined subsequently with lower concentrations of AP glycolipoproteins. The suppression phenomenon may, therefore, be due to an inadequate production of IL 2 in the cultures containing high concentrations of the parasite

glycolipoproteins; lower concentrations of the glycolipoproteins in the cultures probably permitted the Con A to stimulate higher levels of IL 2, leading to a decreased incorporation of $^{3}$H-thymidine. Removal of the adherent cell population prior to the blastogenic assay restored Con A responses in a manner similar to that obtained after adding exogenous IL 2 The fact that the washing of normal splenocytes incubated with suppressing concentrations of the glycoproteins restored normal responses to activation by Con A and LPS indicates that the continued presence of these parasite products in contact with the cells is mandatory for the induction of suppressive activity. The restoration of normal Con A responses by the removal of adherent cells (macrophages) suggests that it is the action of suppressor macrophages, generated when normal unfractionated splenocytes are incubated with the exoantigens of the parasite which are responsible for the suppressive activity. Macrophages are thus, the prime targets of the suppressing action of T. lewisi exoantigens. How exoantigens induce these suppressor macrophages and how these cells prevent the activation of T helper cells by Con A are critical questions for future studies.

Macrophages significantly affects the ability of immunologically-competent cells to respond to antigen in vitro (Ptak and Gershon 1975). These cells release soluble factors which promote the viability of T cells (Chen and Hirsch 1972; Pierce et al 1974) and they process, handle and present antigen to the lymphocytes (Unanue 1972, Kartz and Unanne 1973). Macrophages release a number of immunoregulatory factors into supernatants of the cultures (Bach et al 1970; Dutton et al 1970; Hoffmann and Dutton 1971; Shortman and Palmer 1971; Gery et al 1972)

among which are a low molecular weight inhibitory factor and a non-dialysable stimulatory factor (Calderon and Unanue 1975). The actual role played by macrophages in immunosuppression in T. lewisi infections has not been clearly determined, although the present and an earlier study by Proulx (1988) strongly implicates these cells as playing a central role in mediating hyporesponsiveness. Since washing of unfractionated cells restored Con A stimulation, the suppressor activity only occurs in the presence of the trypanosome preparation, unlike in African trypanosomiasis where ingestion of the living trypanosome (Grosskinsky and Askonas 1981) or incubation with membrane fraction of glycosylated proteins and lipids (Sacks et al 1982) render the macrophage immunosuppressive to naive animals in vivo or in vitro The results of this study indicate that the suppressing component in the exoantigens of T. lewisi is similar to that of the brucei group of trypanosomes since both are identified as complexes of lipids and glycosylated proteins. It appears that the parasite-derived products of T. lewisi have a direct inhibitory effect on macrophages in a manner that has been shown for T musculi (Albright et al 1977, 1978; Albright and Albright 1981). This effect probably inhibits the production of interleukin 1 (IL 1) by the macrophages, the mediator necessary in the proliferation of and secretion of interleukin 2 (IL 2) by T helper cells in presence of either mitogen or antigen (Farrar et al 1980; Smith et al 1980; Larsson et al 1980) One other possible mechanism is the induction by the exoantigens of the production of a suppressor factor by the macrophages (Calderon and Unanne 1975) which inhibits the proliferation of the lymphocytes. The latter possibility is the more likely since the removal of adherent cells (which also deprives the source of IL 1) did not render the non-adherent cells unresponsive to

mitogen or antigen, but, instead, restored and enhanced mitogenic responses in the presence of suppressor parasite glycolipoproteins.

Fig 7.1. SDS-PAGE analysis of the hydrophilic (lane 1) and amphiphilic (lane 2) fractions obtained after the phase-partitioning of Triton X-114 solubilized epimastigote forms. The relative molecular weight of the major components that stained for proteins with amido black on nitrocellulose membranes are shown in kilodaltons (kDa).

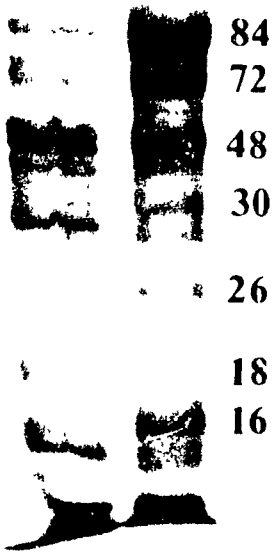

1
2
Mol.wt.(kDa)
84
72
48
30
26
18
16

Fig 7.2. SDS-PAGE analysis of the hydrophilic (lane 1) and amphiphilic (lane 2) fractions obtained after the phase-partitioning of Triton X-114 solubilized trypomastigote forms. The molecular weights of the major components staining for proteins with amido black on nitrocellulose membranes are shown in kilodaltons (kDa).

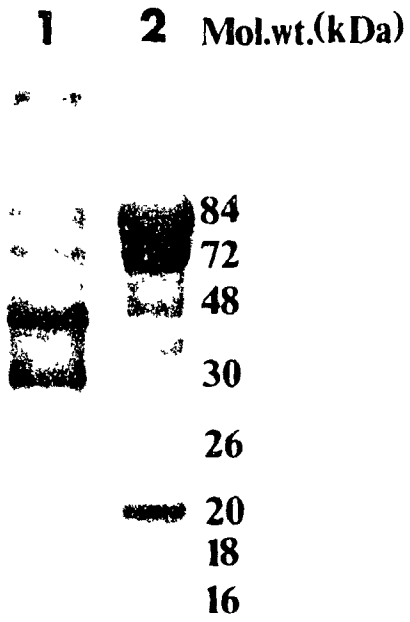

1
2
Mol.wt.(kDa)
84
72
48
30
26
20
18
16

Fig 7.3. The relative molecular weights of the polysaccharide-containing proteins of the phase-partitioned fractions of the epimastigote forms, the hydrophilic (lane 1) and amphiphilic (lane 2) fractions, revealed by the periodic acid Schiff (PAS) reagent on polyacrylamide gels are indicated in kDa: 185(a), 165(b), 155(c), 130(d), 84(e), 72(f), 48(g), 30(h), 26(i), 22(j) 18(k) and 16 (l).

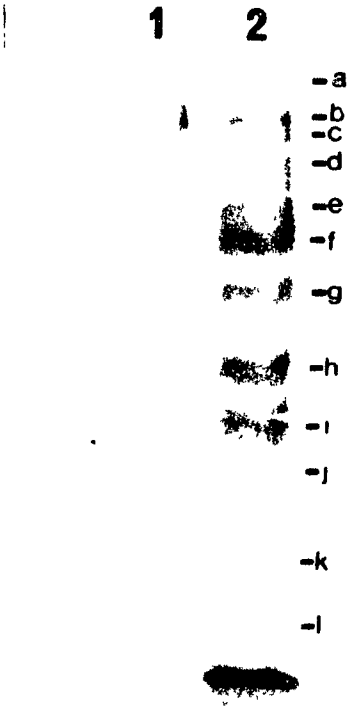

1
2
a
b
c
d
e
f
g
h
i
j
k
l

Fig 7.4. The relative molecular weights of the polysaccharide-containing proteins of the phase-partitioned fractions of trypomastigote forms, the hydrophilic (lane 1) and amphiphilic (lane 2) fractions, revealed by the periodic acid Schiff (PAS) reagent on polyacrylamide gels are indicated in kDa: 185(a), 165(b), 130(c), 84(d), 72(e), 48(f), 30(g), 26(h), 22(i) and 18(j).

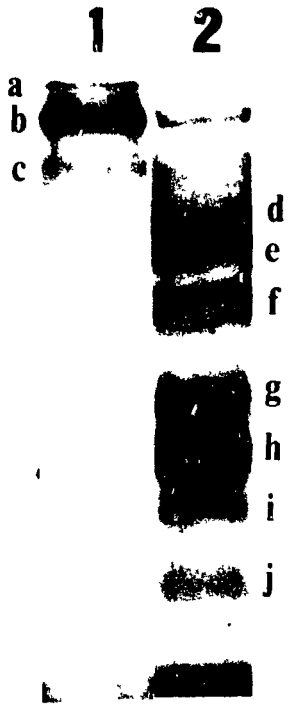

1
2
a
b
c
d
e
f
g
h
i
j

Fig 7.5. The epimastigote and trypomastigote hydrophilic (lanes 1 and 3, respectively) and the amphiphilic ( lanes 2 and 4, respectively) fractions stained with Sudan black on polyacrylamide gels to reveal the lipid-containing proteins.


kDa
1
2
3
4
185▶

Fig 7.6. Western blot analysis of the antigens in the phase-partitioned fractions of epimastigote forms (shown in Fig. 7.1), revealed by day 30 immune serum. The molecular weights of the major antigens are given in kilodaltons (kDa).

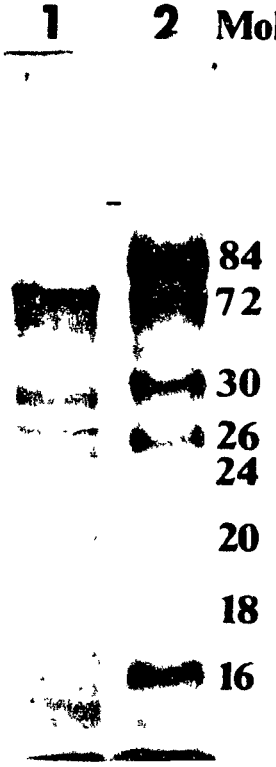

1
2
Mol.wt.(kDa)
84
72
30
26
24
20
18
16

Fig 7.7. Western blot analysis of the antigens in the fractions shown in Fig 7.2, revealed after probing with day 30 immune serum. The molecular weight of the major antigens are given in kilodaltons (kDa).

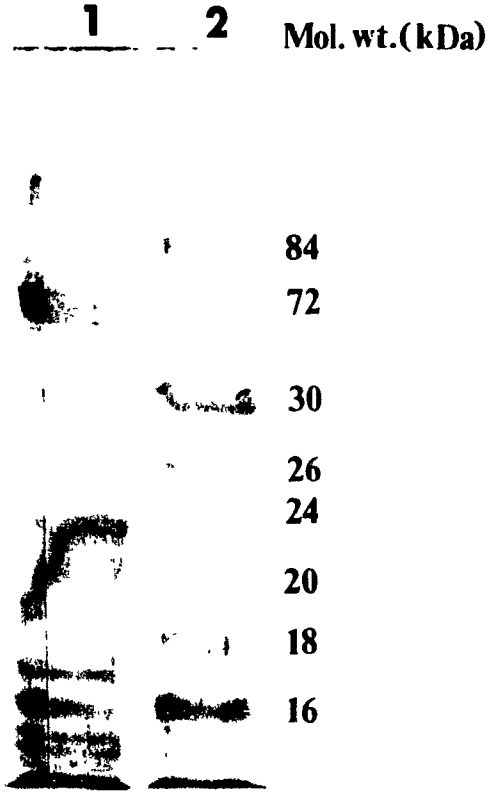

1
2
Mol. wt.(kDa)
84
72
30
26
24
20
18
16

Fig 7.8 Control blots of hydrophilic (lanes 1 and 3) and amphiphilic (lanes 2 and 4) fractions from epimastigote forms probed with normal rat serum (lanes 1 and 2) or with peroxidase-labeled anti-rat IgG (lanes 3 and 4).

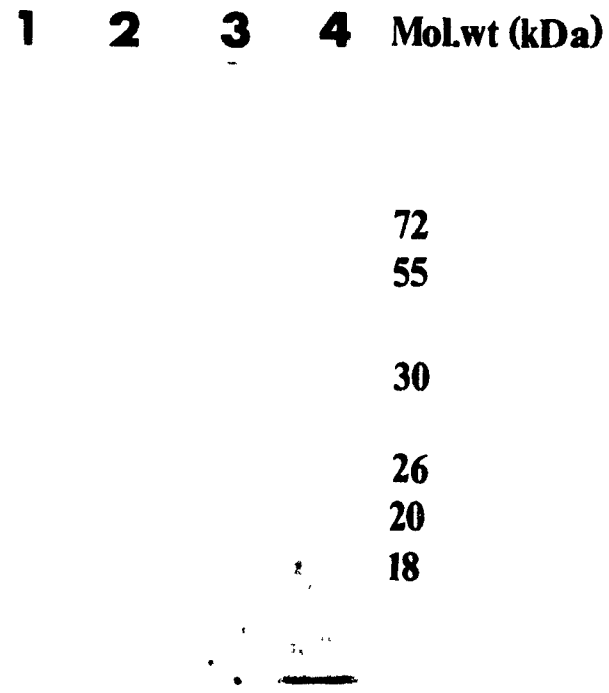

1 2 3 4 Mol.wt (kDa)
72
55
30
26
20
18

Fig 7.9. Control blots of hydrophilic (lanes 1 and 3) and amphiphilic (lanes 2 and 4) fractions from trypomastigotes probed with normal rat serum (lanes 1 and 2) or with peroxidase-labeled anti-rat IgG (lanes 3 and 4).

1 2 3 4 Mol.wt.(kDa)

72
55

30

26
20
18

Fig. 7.10. The blots of protein-stained epimastigote (lane 1) and trypomastigote (lane 2) lysates and the molecular weight designations in kDa (lane 3).

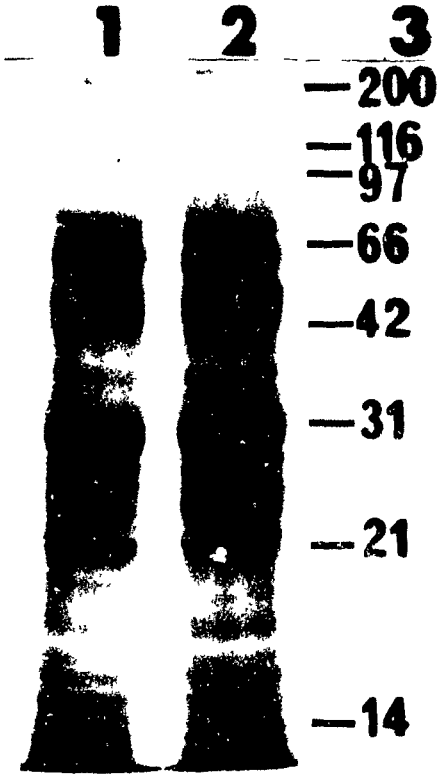

1
2
3
200
116
97
66
42
31
21
14

Fig. 7.11. SDS-PAGE analysis of epimastigote (EE, 1) and trypomastigote (TE, 2) lysates and the antigens revealed in the Western blots by sera taken at different times after inoculation of Trypanosoma lewisi. The EE and TE are presented as pairs probed with day 5 (A), 15 (B), 30 (C), and 70 (D) antisera. The relative molecular weights of the antigens are indicated in kDa: 145 (a),130 (b), 84 (c), 72 (d), 40 (e), 30 (f), 22 (g), 18 (h), and 16 (i).

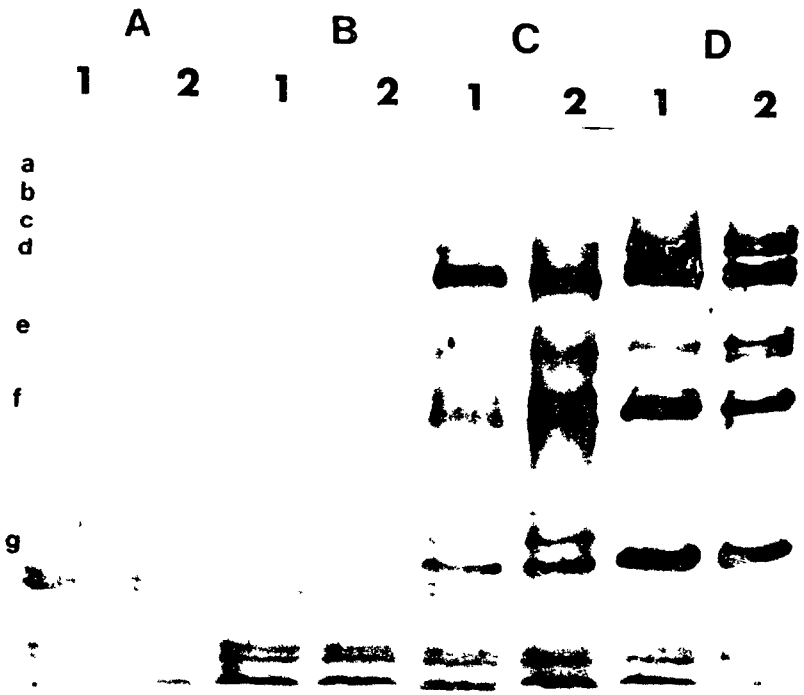

A
B
C
D
1
2
1
2
1
2
1
2
a
b
c
d
e
f
g

Fig. 7.12. The figure legend is as given in Fig. 7.11, except that the paired lysates were probed with day 160 (E), 220 (F) and 386 (G) antisera. Control paired blots were either probed with normal rat serum (H) or with peroxidase-labeled anti-rat IgG (I).

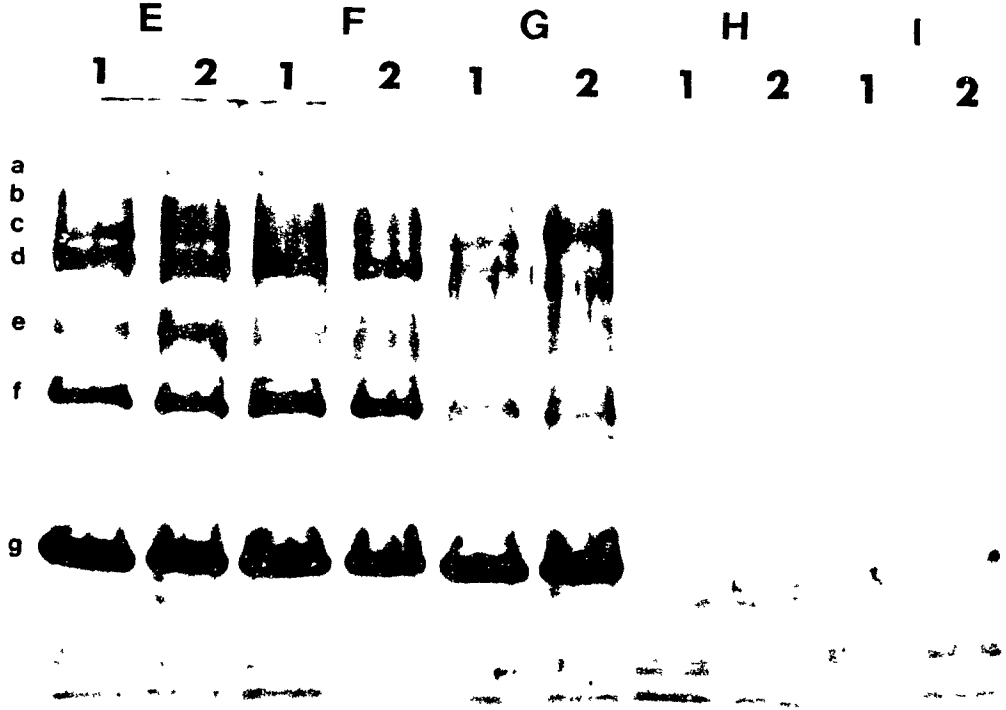
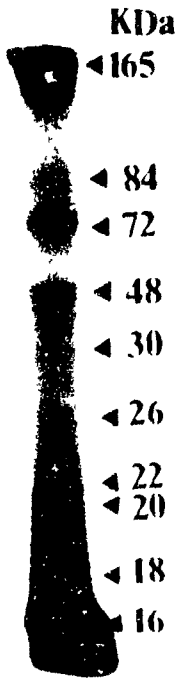

E
F
G
H
I
1 2 1 2 1 2 1 2 1 2
a
b
c
d
e
f
g

Fig. 7.13. Radioautography of $^{125}I$ surface-labeled epimastigote forms. The relative molecular weight of the labeled polypeptides are given in kilodaltons (kDa).

Fig. 7.14. Radioautography of $^{125}I$ surface-labeled trypomastigote forms. The relative molecular weight of the labeled polypeptides are given in kilodaltons (kDa).


KDa
165
84
72
48
30
26
22
20
18
16

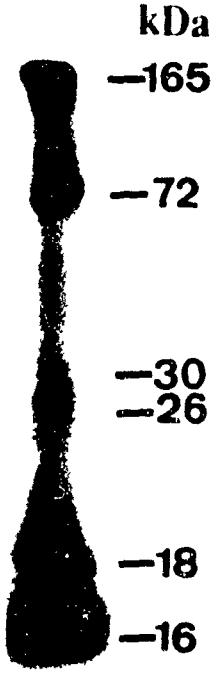
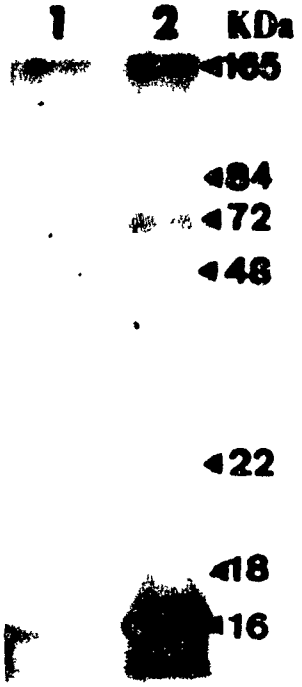
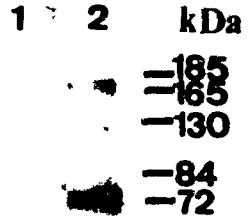

kDa
165
72
30
26
18
16

Fig 7.15. Radioautographs of hydrophilic (lane 1) and amphiphilic (lane 2) fractions of surface-labeled epimastigotes, after phase partitioning in Triton X-114. The molecular weights of the surface-labeled components are given in kilodaltons (kDa).

Fig 7.16. Radioautographs of the hydrophilic (lane 1) and amphiphilic (lane 2) fractions of surface-labeled trypomastigotes, after phase partitioning in Triton X-114. The molecular weights of the labeled components are given in kilodaltons (kDa).


1
2
KDa
165
84
72
48
22
18
16


1
2
kDa
185
165
130
84
72


18

Fig 7.17. The response of normal rat splenocytes to Concanavalin A - induced mitogenesis in cultures containing varying concentrations of amphiphilic ( ▨ ) and hydrophilic (□) fractions of Triton X-114 phase- partitioned epimastigote forms. Each bar represents the mean ± S.E. of triplicate cultures from three animals. The astericks represent statistical differences in either suppression or enhancement of mitogenesis by Student's t-test from stimulated cultures lacking the parasite preparation: *-$p<0.05$, **-$p<0.01$, ***-$p<0.001$.

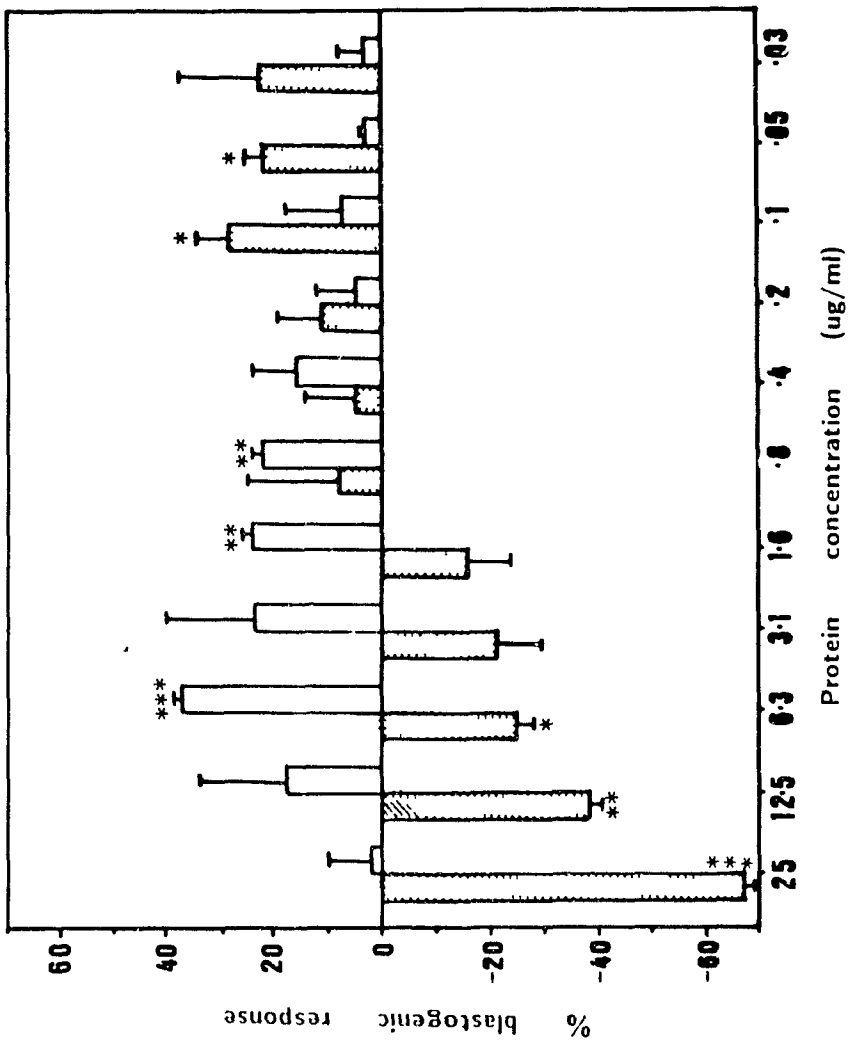

% blastogenic response
60
40
20
0
-20
-40
-60
25
12·5
6·3
3·1
1·6
·8
·4
·2
·1
·05
·03
Protein concentration (ug/ml)

Fig 7.18. The response of normal rat splenocytes to lipopolysaccharide- induced mitogenesis in cultures containing varying concentrations of amphiphilic ( ▣ ) and hydrophilic (□) fractions of Triton X-114 phase-partitioned epimastigote forms. Each bar represents the mean ± S.E. of triplicate cultures from three animals. The asterick represents statistical differences in either suppression or enhancement of mitogenesis by Student's t-test from stimulated cultures lacking the parasite preparation: *-$p<0.05$.

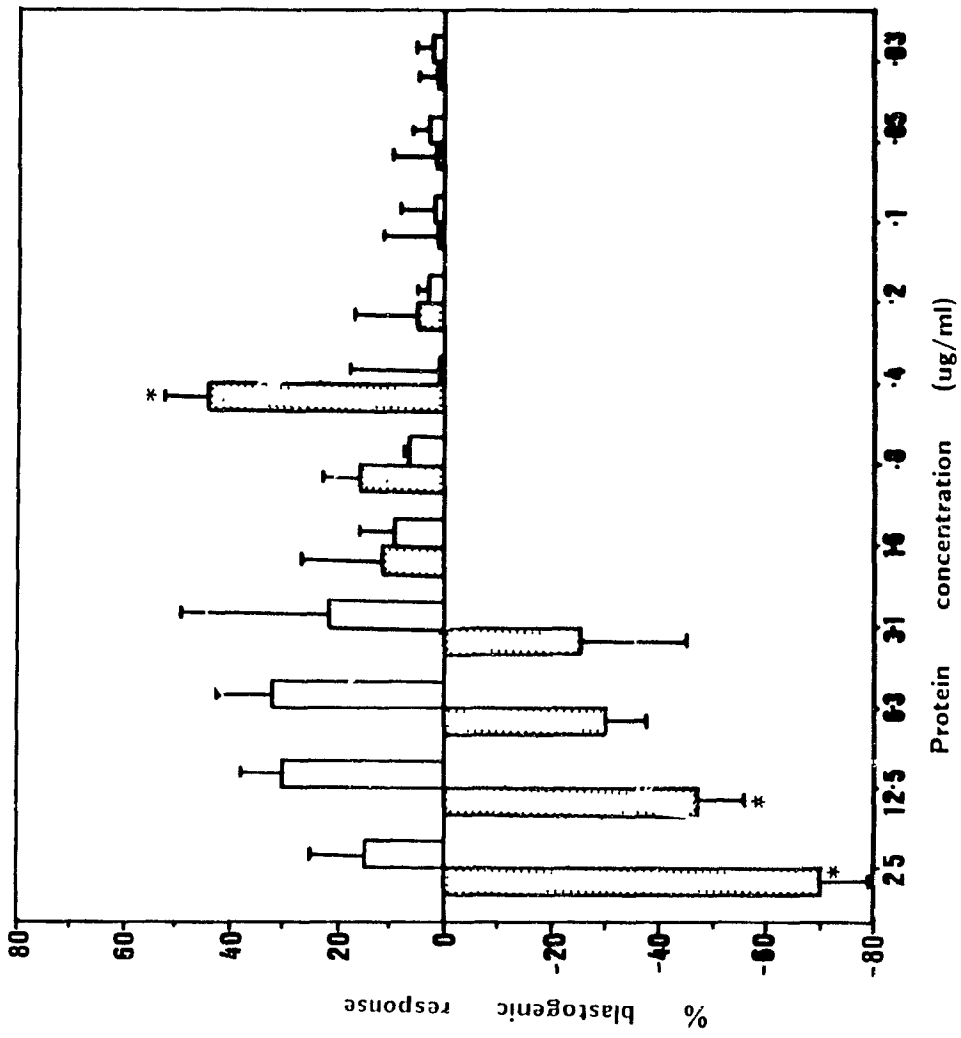

% blastogenic response
80
60
40
20
0
-20
-40
-60
-80
25
12·5
6·3
3·1
1·6
·8
·4
·2
·1
·05
·03
Protein concentration (ug/ml)
*
*
*

Fig 7.19. The response of normal rat splenocytes to Concanavalin A - induced mitogenesis in cultures containing varying concentrations of amphiphilic ( ▩ ) and hydrophilic (□) fractions of Triton X-114 phase-partitioned trypomastigote forms. Each bar represents the mean ± S.E. of triplicate cultures from three animals. The astericks represent statistical differences in either suppression or enhancement of mitogenesis by Student's t-test from stimulated cultures lacking the parasite preparation: *-$p<0.05$, **-$p<0.01$.

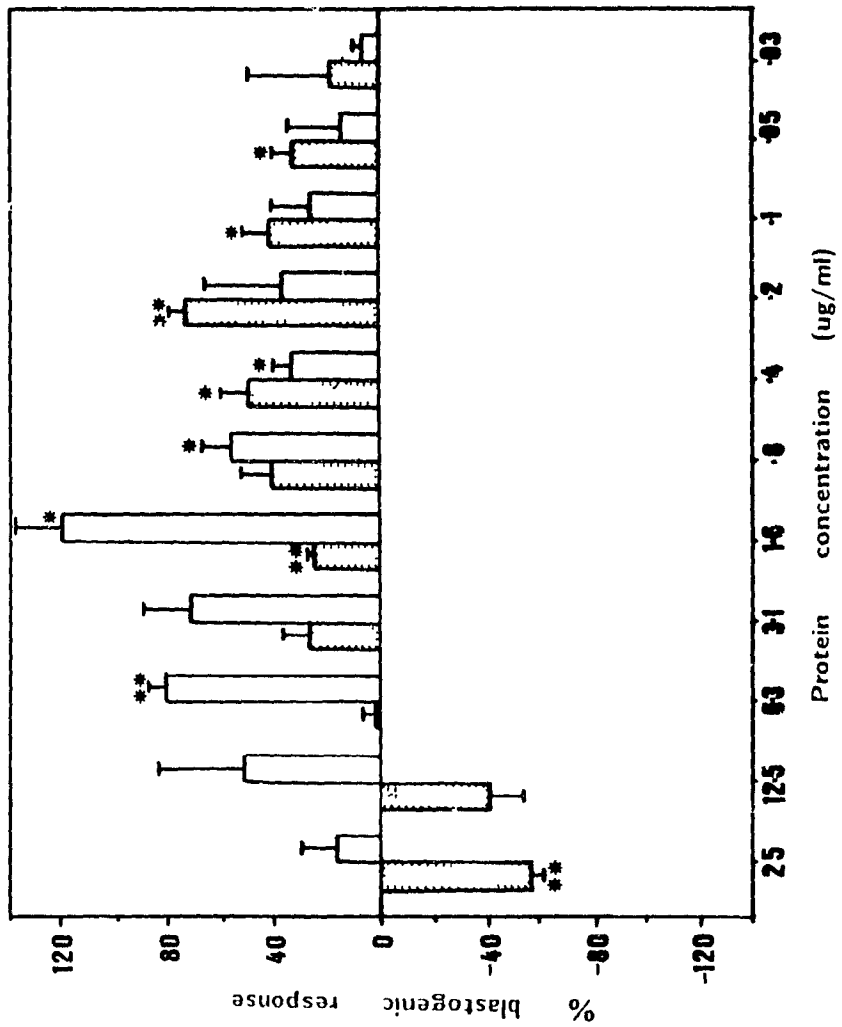

% blastogenic response
120
80
40
0
-40
-80
-120
25
12.5
6.3
3.1
1.6
.8
.4
.2
.1
.05
.03
Protein concentration (ug/ml)

Fig 7.20. The response of normal rat splenocytes to lipopolysaccharide- induced mitogenesis in cultures containing varying concentrations of amphiphilic ( ▩ ) and hydrophilic (□) fractions of Triton X-114 phase-partitioned trypomastigote forms. Each bar represent the mean ± S.E. of triplicate cultures from three animals. The astericks represents statistical differences in either suppression or enhancement of mitogenesis by Student's t-test from stimulated cultures lacking the parasite preparation: *-$p<0.05$, **-$p<0.01$.

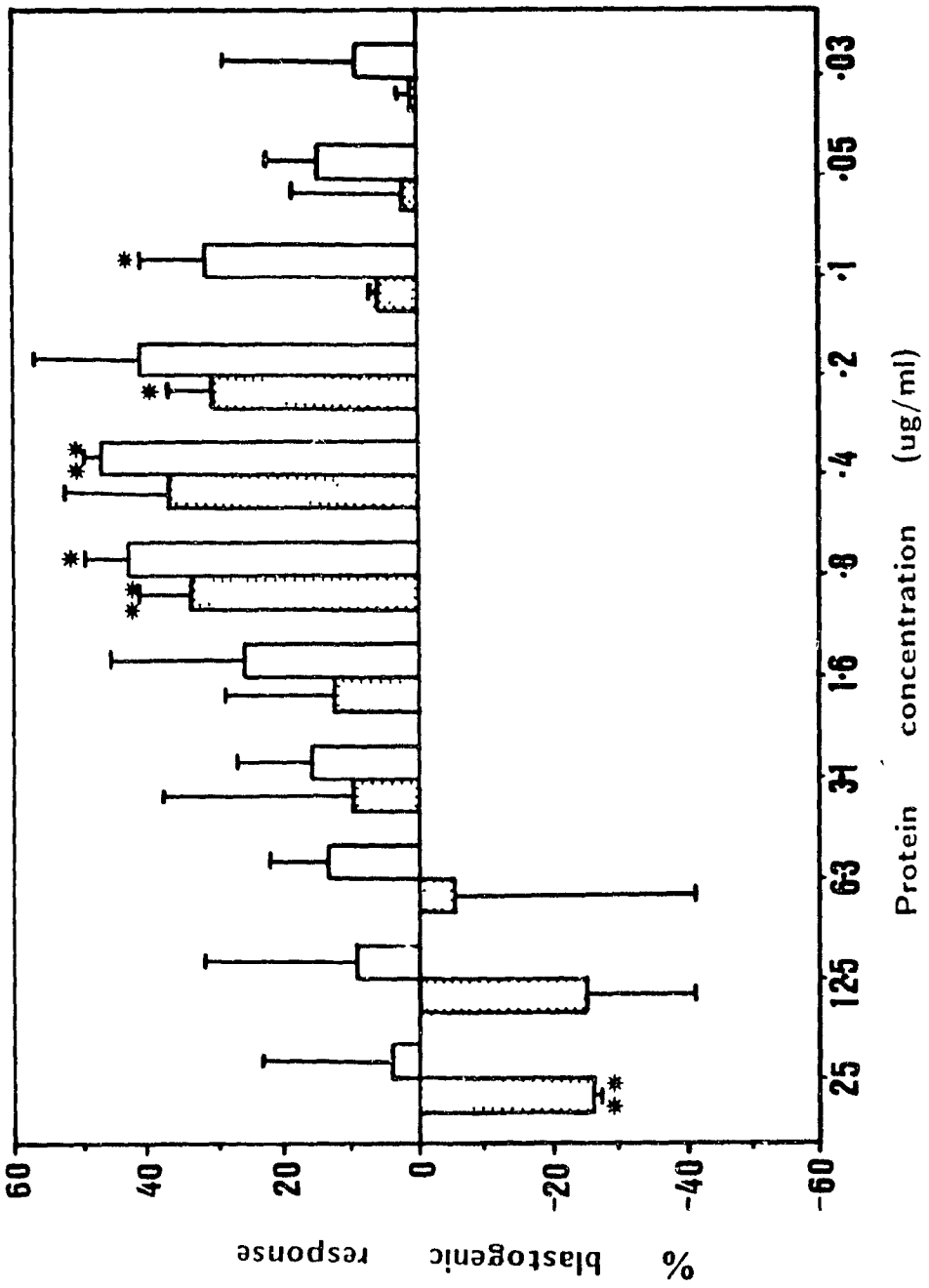

60
40
20
0
-20
-40
-60
% blastogenic response
25
12·5
6·3
3·1
1·6
·8
·4
·2
·1
·05
·03
Protein concentration (ug/ml)

Fig 7.21. The effect of treating naive animals with the constituents of the hydrophilic ( ■ ) and amphiphilic ( □ ) fractions or with buffered saline (●) in the control group, one week prior to parasite inoculation. Each point is the mean ± S.E. of trypanosome counts from five infected rats.

*[1]. designates statistical difference ($p < 0.05$) from the hydrophilic treated group by Student's t-test.

**[2]. designates statistical difference ($p<0.01$) from the saline-treated controls by Student's t-test.

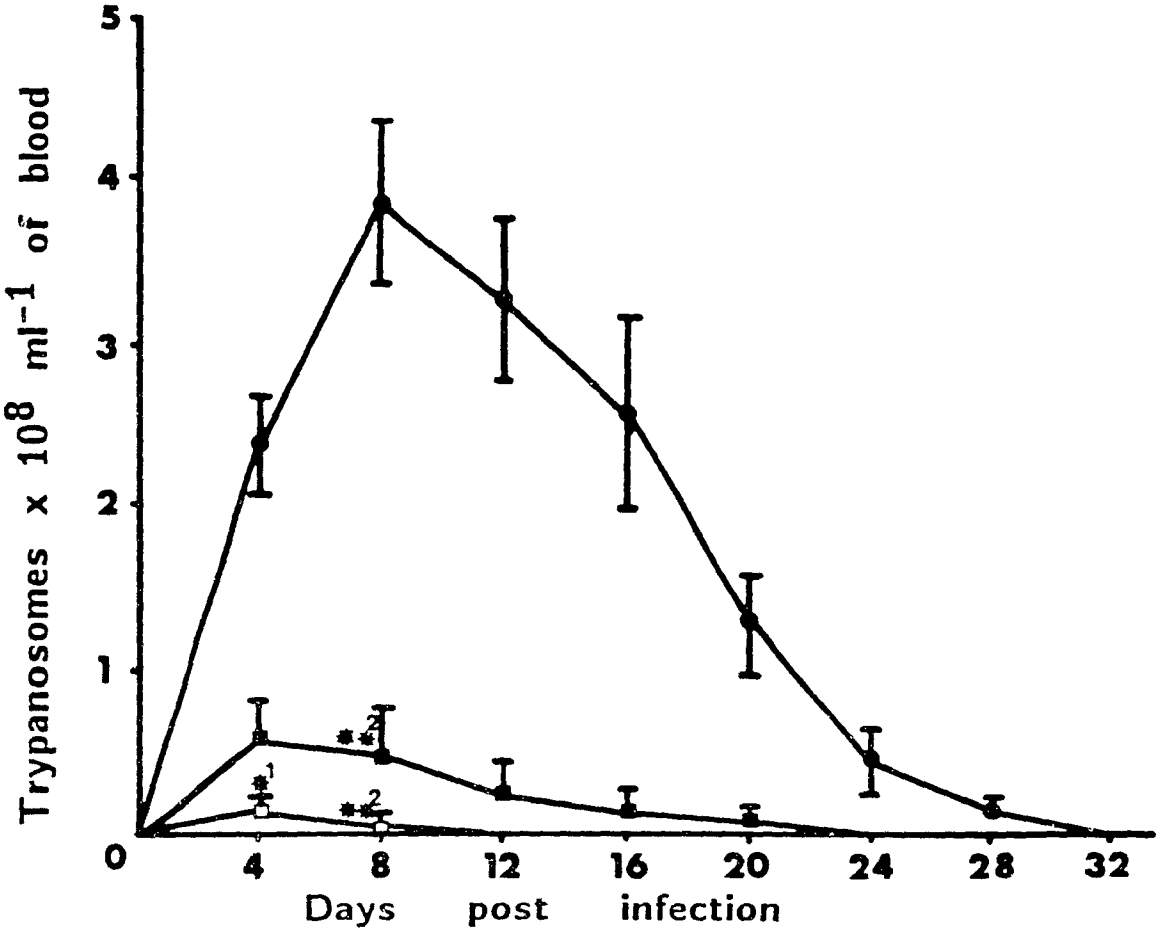

Trypanosomes x $10^8$ $ml^{-1}$ of blood
5
4
3
2
1
0
4
8
12
16
20
24
28
32
Days post infection
1
2
1
2

Table 7.1. The effect of adding exogenous IL 2 on Concanavalin A-stimulated normal rat spleen cells in cultures containing epimastigote glycolipoproteins[a].

| Conc. of glycolipo-proteins ($\mu$g/ml) | % Blastogenic response | |
|---|---|---|
| | Con A | Con A + IL 2 |
| 25.0 | 33.9 ± 1.8 | 103.8 ± 2.9** |
| 12.5 | 62.9 ± 2.5 | 117.0 ± 4.7* |
| 6.3 | 75.8 ± 8.3 | 129.9 ± 12.9* |
| 3.1 | 79.3 ± 2.2 | 133.2 ± 1.6* |
| 1.6 | 85.0 ± 6.8 | 145.1 ± 7.8* |
| 0.8 | 109.1 ± 17.8 | 134.9 ± 20.6 |

a. Data from triplicate cultures from three animals is expressed as % mean ± SE.

*. Asterick indicates significant difference by Student's t-test from cultures stimulated with Concanavalin A: * - $p<0.05$, ** - $p<0.01$.

Table 7.2. The effect of washing normal rat splenocytes on mitogen stimulation in cultures containing suppressing concentrations of the epimastigote glycolipoproteins[a].

| Mitogen | % Blastogenic response | | |
|---|---|---|---|
| | Conc. of epimastigote glycolipoproteins | | |
| | 25μg/ml | 12.5μg/ml | 6.3μg/ml |
| i) Con A | 33.0 ± 1.8 | 62.5 ± 2.5 | 75.8 ± 2.3 |
| ii) Con A[b] | 101.3 ± 6.7* | 101.0 ± 1.6* | 99.0 ± 1.2 |
| iii) LPS | 30.0 ± 9.4 | 53.1 ± 8.7 | 70.2 ± 7.5 |
| iv) LPS[b] | 117.3 ± 2.3** | 112.7 ± 13.1* | 108.0 ± 7.2 |

a. Data from triplicate cultures from three animals is expressed as % mean ± SE.

b. Normal rat splenocytes were stimulated with the mitogens 24 hours after incubation with epimastigote glycolipoproteins, followed by three washes with RPMI 1640 complete medium.

*. Asterick indicates significant difference by Student's t-test from unwashed cultures stimulated with corresponding mitogen: * - $p<0.05$, ** - $p<0.01$.

Table 7.3. The effect of fractionation of normal rat splenocytes on Concanavalin A stimulation in cultures containing the epimastigote glycolipoproteins[a].

| Conc. of glycolipo-proteins ($\mu$g/ml) | Concanavalin A stimulation | |
|---|---|---|
| | adherent and non-adherent cells | Non-adherent cells |
| 25.0 | 25.8 ± 0.4 | 99.8 ± 4.7** |
| 12.5 | 35.2 ± 1.5 | 151.5 ± 8.9** |
| 6.3 | 78.4 ± 1.1 | 127.9 ± 1.6*** |
| 3.1 | 96.2 ± 9.2 | 124.3 ± 16.3 |
| 1.6 | 122.8 ± 22.2 | 102.6 ± 8.3 |

a. Data from triplicate cultures from three animals is expressed as % mean ± SE.

*. Asterick indicates significant difference by Student's t-test from cultures stimulated with Concanavalin A: ** - $p<0.01$, *** - $p<0.001$.

# CHAPTER VIII: SUMMARY AND GENERAL DISCUSSION

The relationship between the host and the parasite is usually a complex biological association which, despite an effective immune response, the parasite always establishes and perpetuates itself. In order to achieve this goal, the parasite has to evade the host's responses by either the accretion of host components onto its surface membranes, or by suppressing the immune system to render the host incapable of destroying the parasite. The outcome of the infection depends on the balance between the effectiveness of the immune system to destroy the parasite and the level of the suppressor factor(s) produced by the parasite directly, or indirectly.

It is within the above understanding of the interplay between the host and the parasite that the present study was developed. The main objective of the study was directed to a better understanding of the interaction that occurs between Trypanosoma lewisi and its rat host during the course of infection and after the parasitemia has resolved, in relation to specific immune responses and to levels of suppressor substances produced by the parasite. It was of interest to isolate, identify and characterize the immunomodulatory parasite products and, further, to establish the possible mechanisms through which the suppressor substances mediate these activities.

In Chapter III it was shown that specific IgG antibody levels increase during the course of the infection and reach a peak at the time of parasite elimination. The circulating antibody levels remain significantly elevated for at least 318 days and are immunoreactive on Western blots at 386 days post parasite inoculation. There were three peaks of IgM responses during the course of infection: the first one

during the epimastigote phase, second one during the trypomastigote phase of infection and the third peak occurred immediately after the resolution of the infection; the IgM levels, unlike the IgG, declined subsequently to insignificant levels. While the study did not resolve the source of the antigen stimulus that maintained the IgG response elevated, it is certain that at least the first two IgM responses reflect the start of an immune response to each of the two stages of the parasite. Tolerable doses of the immunosuppressant cyclophosphamide (CPA) only induced a delay in the appearance of the IgG and IgM peaks, and prolonged the of duration of the infection. It was confirmed that the protection of naive animals, after treatment with immune serum, was dependent on the antibody levels and that post-recovery sera was superior to sera taken at mid-infection. The curative and protective properties of the sera was unaffected by heat inactivation.

The trypanocidal experiments in Chapter IV established that even early drug abbreviation of the infection failed to depress antibody levels significantly to allow the establishment of a secondary challenge infection. This result confirmed that protective immunity in T. lewisi is indeed established very early during the epimastigote phase of the infection, probably even before the parasites can be detected in the peripheral circulation; the trypomastigote phase is not necessary for the development of protective immunity.

The presence of two distinct functional immunomodulatory factors were demonstrated in the plasma of infected irradiated rats (Chapter V). Enhancement of parasitemia occurred when the parasite was inoculated 2 hours after plasma treatment, while protection, mediated by exoantigen-elicited antibodies was demonstrated when the parasite

was inoculated 7 days after treatment with the plasma. The exoantigen-elicited antibodies had ablastic and trypanolytic activities and showed a high degree of stage specificity. Quantification of exoantigens in the plasma of infected rats by immunodiffusion analysis showed that these parasite products could be demonstrated as early as the fourth day of the infection, increasing to reach a peak on days 8-12, and declined thereafter with the level of parasitemia, but low levels persisted after the end of infection. Culture-derived exoantigens at high concentrations suppressed the activation in vitro of normal splenocytes by the T cell mitogen Concanavalin A; epimastigote-derived exoantigens were more suppressive than trypomastigote derived exoantigens. Suppression mediated by exoantigens was ablated either by washing the cells prior to stimulation with the mitogen, or by the addition of exogenous IL 2-rich supernatant from the MLA 144 T cell line. Lysates of epimastigotes and trypomastigotes produced a similar suppression of the ability of the normal splenocytes to respond to activation by mitogen although this phenomenon was significantly higher with the epimastigote than trypomastigote lysate. The characterization of culture-derived exoantigens in Chapter VI demonstrated that they are a complex of proteins, polysaccharides and lipids. Most of these glycolipoproteins occur in the amphiphilic phase after partitioning culture supernatant with Triton X-114; the high molecular weight glycolipoproteins (136-185 kDa) separated into both the hydrophilic and amphiphilic phases. Lipid was present only in the 185 kDa component of the epimastigotes; it was not present in a similar component of trypomastigotes. Western blot analysis of the exoantigens showed that their reaction with immune serum could be categorized into two groups: low molecular weight immunogenic exoantigens of 16-84 kDa and high

molecular weight non-immunogenic exoantigens of 130-185 kDa. The immunogenicity of each specific glycoprotein correlated with its levels of mannose, as determined in Western blots developed with peroxidase-labeled Con A.

In Chapter VII, a comparative study of the polypeptide pattern and the antigens recognized in epimastigote and trypomastigote lysates showed that the polypeptide pattern of these two stages of the parasite are essentially similar. Some antigens were identified in the lysate only by post-recovery serum and not by serum obtained during the course of infection. Other antigens, such as the 72 and 84 kDa component were only recognized significantly by sera obtained at the end of the infection. The 18 and 30 kDa antigens were the most intensely and consistently recognized by post-recovery sera. Characterization of phase-partitioned fractions of epimastigotes and trypomastigotes revealed that glycolipoproteins occurred exclusively in the amphiphilic phase, except for those of high molecular weight that also separated into the hydrophilic phase. Staining with Sudan black showed that most of the lipids were associated with a fast moving component at the bottom of the gel occurred exclusively in the amphiphilic phase of both stages of the parasite, while the slow-moving 185 kDa component of epimastigotes (but not of trypomastigotes) stained for lipids in both hydrophilic and amphiphilic phases. Surface-labeling with $^{125}I$ confirmed that most of the glycosylated proteins are surface coat in origin; the labeled components separated into the amphiphilic phase after partitioning in Triton X-114, with the 16, 72. and 165 kDa components labeled most intensely. Western blot analysis categorized these glycolipoproteins into low molecular weight antigens (16-84 kDa)

and high molecular weight non-antigenic components (130-185 kDa).

Studies of the blastogenic response of normal splenocytes, also described in Chapter VII, confirmed that the surface coat glycolipoproteins of the amphiphilic fraction suppressed stimulation by Concanavalin A, while the hydrophilic fraction enhanced Con A mitogenesis, confirming that the exoantigens of Trypanosoma lewisi can either suppress or enhance immune responses depending on which fraction is in contact with lymphoid cells. Suppression was ablated by washing of normal splenocytes incubated with suppressing concentrations of the glycolipoproteins before the addition of the mitogen, or by the addition of exogenous IL 2. Depriving splenocytes cultures of the adherent cell (macrophages) population also ablated Con A-induced suppression.

From the present study, it is apparent that some antigens induce antibodies that have a role to terminate the infection, while other antigens are important in protective immunity by inducing an immune response only after the animals have recovered from the parasitemia. The fact that some antigens were intensely recognized by antibodies produced during the infection (mainly, the 72 kDa antigen) or only after the end of infection (the 18 and 30 kDa antigens) testifies to this fact and indicates that most of the antibodies in post-recovery sera are mainly directed to the 18 and 30 kDa antigens. The phenomenon whereby some antigens induce specific immune responses at different times of the infection may account for the appearance of the 2nd and 3rd IgM peaks. However, the ability of the infected host to mount an effective immune response may be regarded to be "indirectly proportional" to the level of parasitemia. The antibody response and IL 2 production (Proulx 1988) are lowest during the epimastigote

phase, and the peak antibody levels and IL 2 responses are obtained at the time the parasitemia is being cleared. An "indirect proportion" phenomenon is also observed with the level of circulating exoantigens and the IL 2 and antibody responses. Maximum levels of circulating exoantigens were observed during the epimastigote phase and were lowest during the elimination phase of the parasitemia and in post-recovered rats. The time of maximal exoantigen levels (epimastigote phase) coincides with the time of infection when maximal depression of IL 2 production and low antibody levels occur; the latter two responses are restored or reach peak levels when exoantigens in the serum of infected animals are at the lowest. which coincides with the period when the parasitemia is eliminated. Suppression of the production of IL 2 in vivo therefore appears to occur with high levels of exoantigens and, indeed, these parasite-derived products suppress Con A responses in a dose-dependent manner in in vitro splenocyte cultures; the Con A-induced suppression could be ablated by the addition of exogenous IL 2 suggesting an interference by the parasite in T cell function. Such an interference in the activity of the lymphocytes is probably induced by a suppressor substance produced by suppressor macrophages generated during the infection or in culture, when these cells (macrophages) are in contact with the parasite products. Since IL 2 is an important regulatory mediator in the production of antibodies and other immunological functions, it is conceivable that the high levels of circulating exoantigens during the epimastigote phase suppress the production of IL 2. This suppression results subsequently in reduced antibody production; the decline in the level of these parasite-products would therefore restore IL 2

production and the antibody production. Although, many questions still remain unanswered, the present study has shown a correlation between the levels of parasite-derived products and the immunological response that is induced during and after infection with Trypanosoma lewisi. These immunomodulatory products have been characterized and identified as a complex of proteins, lipids and polysaccharides which are membrane-surface coat associated and are shed during in vitro cultures and probably also in vivo; they separate into one fraction after phase-partitioning, which also contains all the surface-labeled components. Characterization was not pushed further to isolate the specific component(s) in the amphiphilic fraction that was responsible for the induction of suppression. It is conceivable, however, that the high molecular weight glycolipoproteins mediated suppressive activity since they were non-immunogenic on Western blot analysis; suppressor activity can be attributed to either the lipid, the polysaccharide moeities or both in the amphiphilic fraction, since the protein-rich hydrosoluble fraction was non-suppressive. The present studies indicates that these glycosylated parasite products are critical in the regulation and maintenance of immunity in animals infected with Trypanosoma lewisi.

There is no doubt that trypanosomiasis is a major health problem in both man and animals and that there are no prospects for vaccine prophylaxis in the future. The phenomenon of antigenic variation has made the development of a vaccine bleak and the massive suppression of the host's immune system which is mediated by parasite-derived complexes of proteins, lipids and polysaccharides adds to the problem of controlling the pathogenic trypanosomes. Similar components were found which mediate suppression in infections with Trypanosoma lewisi. It is my hope that the present studies have provided a better

understanding on the immunological responses in relation to the suppressive and protecting factors produced by Trypanosoma lewisi and that it will stimulate other workers to address more rationally the phenomenon of immunosuppression, as an approach to the control of African trypanosomiasis.

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