IMMUNE MECHANISMS OF CURE

IN

TRYPANOSOMA MUSCULI INFECTION

© by Daniel Steven Gary Wechsler

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To My Parents

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ABSTRACT

Trypanosoma musculi is a protozoan parasite which produces a characteristic, self-limiting murine infection of approximately three weeks duration; the infection comprises a growth phase, a plateau phase and an elimination phase. Following clearance of parasitaemia, a mouse is cured and immune to reinfection. The present studies examine the immune mechanisms which operate during the elimination phase.

Passive transfer of plasma from an immune mouse to an infected recipient brings about rapid and complete clearance of parasitaemia in C57BL/6 mice. This curative activity is labile to heat treatment for 30 minutes at 56°C. A protein A- derived immunoglobulin fraction of immune plasma (IP) shares these properties. Further purification shows that the curative activity resides primarily in the IgG2a subclass, and that this antibody is intrinsically heat-labile. Complement component C3 (but not the lytic C5-C9 sequence)

is necessary for antibody-mediated cure of infection. Cellular elements (macrophages) are also essential for elimination of parasitaemia to occur. The ultimate T. musculi effector mechanism thus requires the interaction of both humoral and cellular components.

LES MECANISMES IMMUNITAIRES DANS LA CURE D'UNE INFECTION TRYPANOSOMA MUSCULI

de

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Thèse de Ph.D.
mars 1987

CONDENSÉ

Trypanosoma musculi est un parasite protozoaire qui produit chez la souris une infection caractéristique, auto-limitative, d'une durée approximative de trois semaines. L'infection comprend une phase de croissance, une phase de stabilization, et une phase d'élimination. Après la disparition de l'infection parasitaire, une souris est guérie et à l'ábri de la contagion. Notre étude examine les mecanismes immunitaires qui fonctionnent au cours de la phase d'élimination.

Un transfert passif de plasma d'une souris immunisée à une souris infectée aboutit à une élimination rapide et complète de la parasitémie chez les souris C57βL/6. Cette activité curative est thermo-labile à 56°C pour une durée de 30 minutes. Une fraction, dérivée de protein A, de plasma immunisé contenant d'immunoglobuline est aussi douée de ces propriétés. Une purification plus approfondee démontre que l'activité curative réside essentiellement dans la

sous-classe IgG2a, et que cet anticorps est intrinsequement thermo-labile. La componante complémentaire C3 (mais non pas la séquence lytique C5-C9) est nécessaire pour la cure de l'infection par anticorps. Des elements cellulaires (macrophages) sont tout autant essentiels pour que l'élimination de l'infection parasitaire ait lieu. Ainsi l'ultime mécanisme effecteur du Trypanosoma musculi exige l'interaction des composantes humorales et cellulaires.

STATEMENT OF INDEPENDENCE

All work described in this thesis has been carried out by the candidate, with the exception of the immunoblots shown in Figure 7.6 (b,c). I am indebted to Paula Ryan for making these immunoblots available to me. Although I introduced the technique of immunoblotting to our laboratory and carried out the initial studies to demonstrate that this appproach was indeed feasible, a photographic record of these preliminary results was not made. Further refinement of the technique by Ms. Ryan yielded the results shown in the Figure.

Some of the studies described in this thesis have been published as the folowing articles:

Wechsler, D.S., and P.A.L. Kongshavn. 1984. Cure of Trypanosoma musculi infection by heat-labile activity in immune plasma. Infect. Immun. 44(3): 756-759.

Wechsler, D.S., and P.A.L. Kongshavn. 1985. Characterization of antibodies mediating protection and cure of Trypanosoma musculi infection in mice. Infect. Immun. 48(3): 787-794.

Wechsler, D.S., and P.A.L. Kongshavn. 1986. In vitro assay for curative activity in blood of mice infected with Trypanosoma musculi. Infect. Immun. 53(2): 240-244.

Wechsler, D.S., and P.A.L. Kongshavn. 1986. Heat-labile IgG2a antibodies effect cure of Trypanosoma musculi infection in C57BL/6 mice. J. Immunol. 137(9): 2968-2972.

Wechsler, D.S., and P.A.L. Kongshavn. 1986. Strain differences in the cure of murine trypanosomiasis by passively transferred immune plasma. Submitted.

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Bub, Zaid, Rob, Mike, and Leora, for loving support and tolerance.

My parents -- I love you.

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PREFACE

Tropical diseases caused by parasitic infections remain among the major causes of human sickness and death in the world today. Malaria, trypanosomiasis, leishmaniasis, schistosomiasis, filariasis and other parasitic diseases are responsible for morbidity and mortality in over one billion people (David, 1983). During the past decade, the social and economic impact of these conditions have become more apparent, and there has been a resurgence of interest in tropical diseases and in approaches to their control. One of these approaches is immunologic, and it focuses on several of the interactions between the parasite and its host. It is believed that an understanding of the mechanisms used by parasites to evade the immune response of the host, combined with a comprehension of those immune mechanisms which are successful in parasite elimination, will make it possible to artificially induce a stronger protective immunity against these parasites, thereby reducing the magnitude of their deleterious effects.

Trypanosomiasis in man and animals is produced by a large number of species of the genus Trypanosoma. Two distinct pathological entities are associated with trypanosomes: Chagas' disease (American trypanosomiasis), caused by T. cruzi, is distinguished by cardiac manifestations (ultimately leading to heart failure) and

gastrointestinal disturbances. African trypanosomiasis (sleeping sickness) is caused by the extracellular T. brucei and is characterized by an acute febrile lymphadenopathy followed by a chrcnic lethal meningoencephalomyelitis. Sleeping sickness affects 250,000 humans in Africa (Katz, 1982): ten thousand new cases are officially reported annually, and 35 million humans are at risk (Roelants, 1986). In addition, sleeping sickness results in an estimated annual mortality of 3 million cattle (Bloom, 1979). This effectively prevents the exploitation of the vast forest grasslands south of the Sahara for cattle production. The economic impact of the animal disease is substantial when one considers that an area of approximately 7 million (Roelants, 1986) to 11 million square miles in Africa is not populated because of the impossibility of keeping animals in sites where tsetse flies, the vectors of T. brucei, are infected (Plorde, 1983).

Infections with trypanosomes present such an acute problem to public health in Africa, Central and South America (Acha & Szyfres, 1980), that the World Health Organization has recognized trypanosomiasis as one of its major priorities in its special program for research and training in tropical medicine. A primary goal of this program is to find a means of enhancing mechanisms of natural resistance and acquired immunity against trypanosomes. A number of attempts have been made to control trypanosomal disease in humans and cattle by the development

of effective vaccines and new drugs. These efforts have been largely unsuccessful, as a result of the difficulty in counteracting the different mechanisms used by the parasite to evade the immune response, including antigenic variation and immunodepression. Interest has, therefore, turned towards examining the reasons for the increased resistance to infection seen in certain breeds of cattle. To this end, a great deal of work has been carried out to examine the immune response generated by inbred strains of mice infected with trypanosomes of man and cattle. While these studies have provided much information regarding the immunologic means by which parasites are eliminated from the host, they may be criticized for representing unnatural host-parasite interactions. Therefore, they are not necessarily indicative of, nor can they be readily extrapolated to effector mechanisms in natural situations.

Trypanosoma musculi is a natural parasite of the house mouse (Mus musculus). Like T. lewisi in the rat, T. musculi in the mouse constitutes a naturally occurring laboratory model of sleeping sickness (Viens, 1985). Long-established host-parasite relationships have led to a state of low-pathogenicity, complex biological and immunological equilibria and strict host restriction (Hoare, 1967; Molyneux, 1976). Although T. musculi differs in significant ways from the pathogenic trypanosomes of man and cattle, it shares a considerable number of characteristics with these parasites. Morphologically similar, both T.

musculi and T. brucei are solely extracellular parasites, confined to the bloodstream. Although T. musculi does not undergo the antigenic variation characteristic of its pathogenic counterpart (Vargas, 1981), the rates of increase and decrease of parasitaemia and peak parasitaemias observed in T. musculi-infected mice are similar to those seen in cattle infected with T. brucei, suggesting that control mechanisms of these parasites might be similar. The overall pathological effects of T. brucei and T. musculi are strikingly alike: both infections are characterized by anemia, hypocomplementemia, hepatosplenomegaly and altered lymphoid histoarchitecture (Duffey et al., 1985). Thus, studies in this natural host-parasite model may be regarded as highly valid tests of some of the sophisticated concepts of modern trypanosome immunology (Viens, 1985; Mitchell, 1982), and results obtained from this model may be extrapolated to natural conditions (Playfair, 1980).

The present studies have been carried out to clarify the role of humoral immunity and its interactions with cellular components of the immune system in the elimination of T. musculi infection in mice. It is hoped that a better understanding of the mouse-trypanosome relationship will shed light on some of the reasons for the failure of host defenses in infections caused by pathogenic trypanosomes of man and cattle.

CHAPTER I

HISTORICAL REVIEW

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1. HISTORY

The first report of the trypanosome of the house mouse (Mus musculus) was likely made in Gambia by Dutton and Todd (1903). When these investigators examined the blood of mice, they found "flagellated Protozoa" closely resembling Herpetomonas (Leptomonas). In Senegal, Thiroux (1905a) described what he considered to be the mouse trypanosome and named it Trypanosoma duttoni. It was subsequently found that the host which Thiroux identified as the house mouse was not a mouse but a rat, and it is likely, therefore, that his T. duttoni was in fact T. lewisi.

This confusion arises because of the difficulty in differentiating rodent trypanosomes from one another: they are morphologically indistinguishable. They may, however, be separated on the basis of particular biological properties: the ability to reproduce in specific hosts (host-restriction), and differences in the course of infection in their respective hosts. There are considerable contradictions in the data concerning the specificity of T. duttoni. Whereas Thiroux (1905b) claims to have used this trypanosome to infect Mus musculus but not the rat, Roudsky

(1912a,b) was apparently successful in infecting rats with T.duttoni. It is thus not possible to know whether Thiroux and Roudsky were dealing with a trypanosome of mice or rats. Because of these doubts, Hoare (1972) suggests that the name T. duttoni be discarded and replaced by one assigned indisputably to a trypanosome of the house mouse.

Kendall (1906) was the first to ascribe the name T. musculi to the mouse trypanosome, and Hoare (1972) proposed that this become the valid name thereof. He cautions, however, that since subsequent authors used T.duttoni to refer to the true mouse trypanosome, T. duttoni should be considered to be synonymous with T. musculi.

2. TAXONOMY AND DISTRIBUTION OF TRYPANOSOMA MUSCULI

Taxonomically, T. musculi has been classified as follows:

Phylum	:	Protozoa
Subphylum	:	Sarcomastigophora
Superclass	:	Mastigophora
Class	:	Zoomastigophora
Order	:	Kinetoplastida
Suborder	:	Trypanosomatina
Family	:	Trypanosomatidae
Genus	:	Trypanosoma
Subgenus	:	Herpetosoma
Species	:	musculi

Although the house-mouse is found in various parts of

the world, the geographical distribution of T. musculi, in contrast with that of the allied rat parasite T. lewisi, is not cosmopolitan but appears to be confined mainly to warm countries of the Mediterranean basin and the West Coast of Africa (Hoare, 1972). Its occurrence in the Western Hemisphere is attributed to the relatively recent introduction of infected mice through human agency (Krampitz, 1969a).

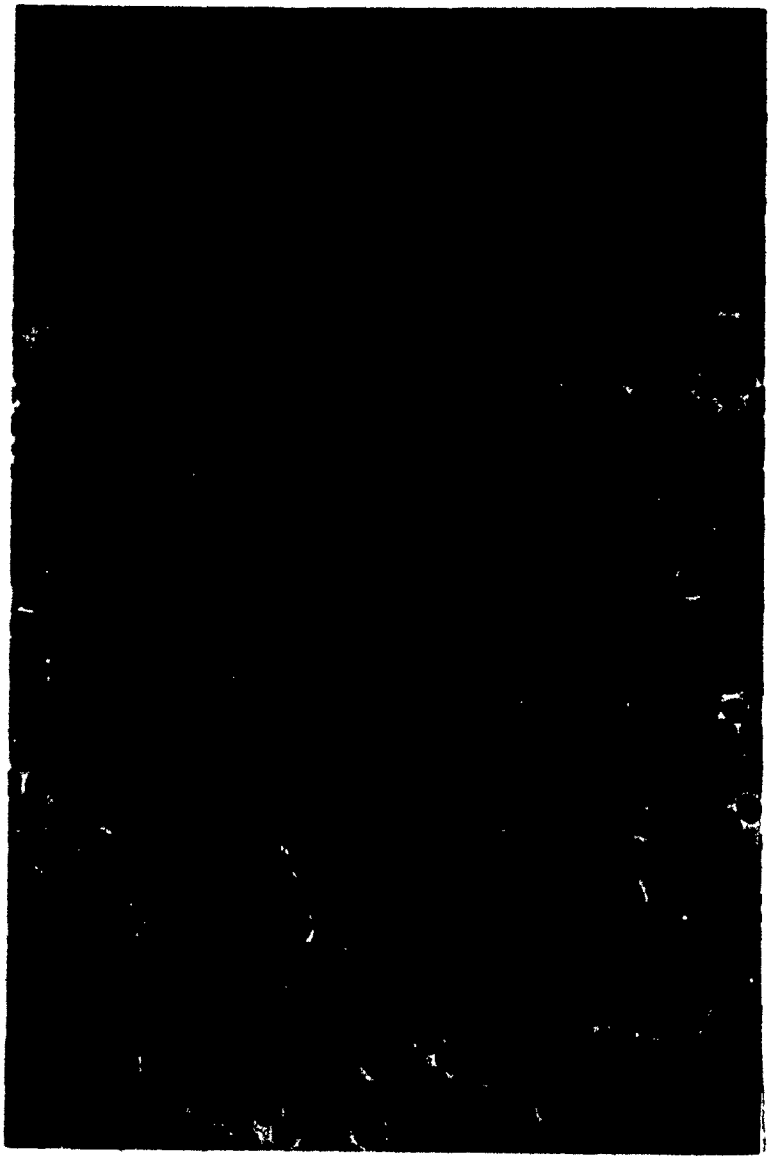
3. MORPHOLOGY AND LIFE CYCLE OF TRYPANOSOMA MUSCULI

3.1 Morphology

The forms of T. musculi found in the blood of infected mice may be grossly categorized into two types: 1) Adult forms are typical slender flagellates with a pointed anterior end, a blunt posterior extremity and a long flagellum projecting from the anterior end after passing along the edge of the undulating membrane (Figure 1.1). A large, oval nucleus is centrally positioned, and a kinetoplast is located at the base of the flagellum. The dimensions of the mouse trypanosome were estimated by Davis (1952) to be as follows: the total length of the trypanosome is approximately 30 microns; the width of the body is about 2 microns; the free flagellum is approximately 6 microns in

FIGURE 1.1

Adult forms of T. musculi in bloodstream of infected mouse. Spheres are red blood cells (400x, no stain).



length, and the kinetoplast at the base of the flagellum is about 5 microns from the posterior end of the body.

2) Young/Dividing forms are described as short and stumpy and are found to possess shorter flagella (Figure 1.2). They are pleomorphic as shown in Figure 1.3. The transitions between the forms are described below. Movement of both morphologic types is accomplished by rapid to-and-fro beating of the flagellum, resulting in a wavy, spiraling forward motion.

3.2 Development in the Insect Vector

The true intermediate host of T. musculi is unknown, but it is likely that under natural conditions, it is transmitted by one of the mouse fleas (Ctenophthalmus, Nosopsyllus fasciatus, Leptopsylla segnis) (Roger & Viens, 1986). Pricolo (1906) observed stages of trypanosome development in fleas collected from infected mice in Italy. Brumpt (1913) infected Ceratophyllus hirundinis (the swallow-flea) with T. musculi, which subsequently developed in the hindgut giving rise to epimastigotes (Figure 1.3 e,f,g). He was also successful in infecting mice with the rectal contents of such infected fleas, and was led to the conclusion that under natural conditions, the infection is transmitted by the contaminative method when mice eat infected fleas or their droppings (Khazindar & Dusanic, 1982). This allowed for the description of T.

FIGURE 1.2

Reproductive form of T. musculi (arrow) among adults in bloodstream of infected mouse (600x, no stain).

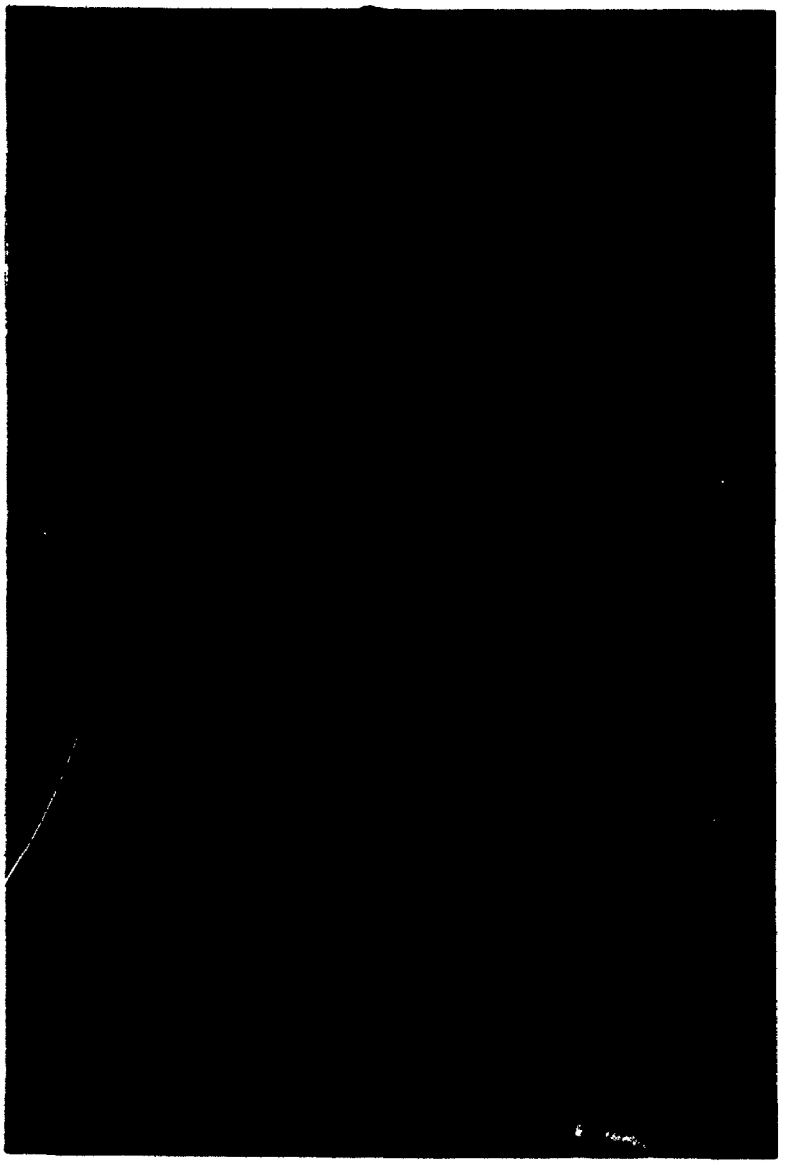


FIGURE 1.3

Trypanosoma musculi (= T.duttoni) (1600x):

a,b: Adult blood trypomastigotes;

c,d: Trypomastigotes in blood of mouse during reproductive phase;

e: Pre-division epimastigote;

f,g: Binary division in epimastigote stage;

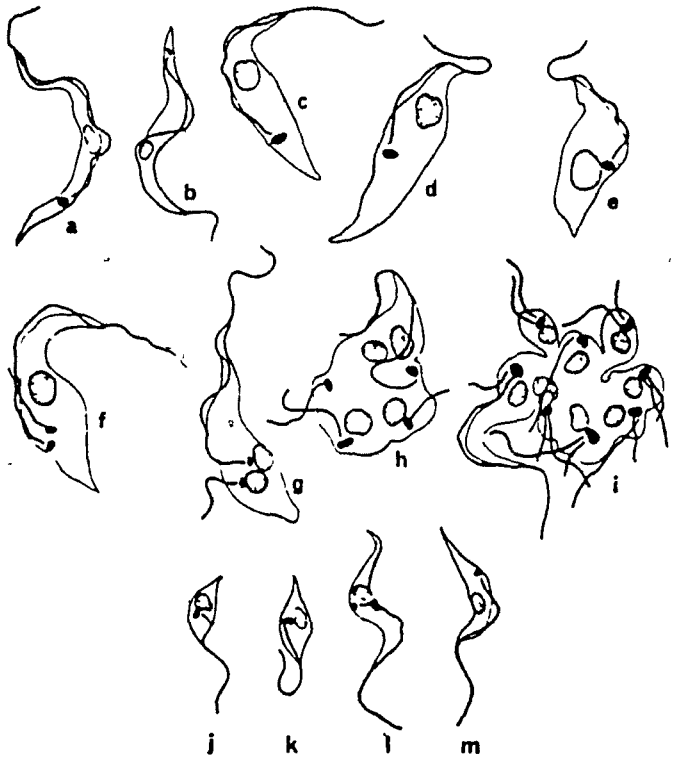
h,i: Stages of multiple division;

j,k: Young epimastigotes resulting from division (i);

l,m: Transition from epimastigote to trypomastigote forms.

(After Taliaferro & Pavlinova, 1936)

STAGES OF TRYPANOSOMA MUSCULI



musculi as a stercorarian trypanosome, in contrast to the salivarian African trypanosomes, transmitted by direct bites of Glossina species.

3.3 Development in the Mammalian Host

It is generally agreed that the forms of T. musculi in the blood of infected mice are indistinguishable from those of T. lewisi (Hoare, 1972). As such, it is not surprising that the mouse trypanosome proliferates in the blood in a manner similar to that described for the rat trypanosome (Galliard, 1934; Taliaferro & Pavlinova, 1936). During the reproductive phase of the infection, the first stage to appear in the blood is the metacyclic trypomastigote (Fig. 1.3c,d), whose body is broader than the adult trypomastigote (Fig. 1.3a,b). Like other stercorarian parasites, T. musculi does not multiply in the trypomastigote stage, but rather assumes the epimastigote (crithidial) stage in which the kinetoplast has migrated to the nuclear region, and the body has broadened further (Fig. 1.3e). The epimastigote stage divides several times in succession (by unequal binary and multiple fission (Hoare (1972)) without complete cytoplasmic separation (Fig. 1.3f,g). The kinetoplast and nucleus are first duplicated, and a new flagellum appears near the old one; the cytoplasm subsequently undergoes incomplete fission until eight or more partially distinct entities are formed. This results in rosette forms in which the daughters remain

attached to one another prior to breaking apart (Fig. 1.3h,i). Finally, segmentation occurs, causing liberation of the daughters as small epimastigote forms. These young forms may again divide by multiple or binary fission, or they may undergo a series of morphological changes in which the kinetoplast gradually migrates backwards, generating small trypomastigotes (Fig 1.3j-m). These increase in size and eventually transform into long adult trypanosomes. The ability of adult trypomastigotes to become metacyclic, pre-epimastigote forms is undetermined, although it is almost certain that they can do so; inoculation of a population of adult form trypomastigotes obtained from an infected mouse into a naive mouse results in the subsequent appearance of young and dividing forms (see below).

The time required for the generation of new trypanosomes (i.e. the division time) has been estimated to be 6 h 40 min - 14 h 20 min in vitro (Vincendeau, 1986).

4. IMMUNOBIOLOGY OF TRYPANOSOMA MUSCULI

T. musculi is a member of the subgenus Herpetosoma, a group which contains organisms considered to be benign or nonpathogenic (D'Alesandro, 1970; Molyneux, 1976; Viens, 1985). In addition, these organisms are host-restricted, being uninfective to other rodents (Albright & Albright,

1981a). These features distinguish T. musculi from the African and American trypanosomes, which are described as pathogenic (Hoare, 1972), and which are able to infect a considerable number of different hosts (Mansfield, 1977). Although the terms "pathogenic" and "nonpathogenic" are convenient designations for these groups of trypanosomes, they are not strictly correct. Rodent trypanosomes have been shown to cause important histological modifications in immuno-committed organs or tissues (Hirokawa et al., 1981; Duffey et al., 1985), in the liver and kidney (Molyneux, 1976; Viens, 1985), and under certain conditions, they may cause death of the rodent host. Conversely, the African and American trypanosomes, while frequently lethal in man and certain domestic animals, are tolerated in some species of wild animals which serve as reservoir hosts (Roelants, 1986), although they likely cause a mild degree of the histopathological changes referred to above. Thus, apparently nonpathogenic infections may occur with any trypanosomes studied. The factors involved in determining the pathogenicity of a species of Trypanosoma within a given host are not well understood, although they are considered to be at least partly immunologic in nature (D'Alesandro, 1970).

Despite the relatively large number of rodent trypanosomes (Hoare, 1972), the only ones to have been studied to any significant extent are T. lewisi of the rat and T. musculi of the mouse. These models of natural

host-parasite interactions offer certain advantages: laboratory strains of rats and mice are easily maintained; infections in these animals may be monitored daily without sacrificing the host; infections are highly reproducible; the relatively simple course of infection as compared to that with African or American trypanosomes enables a dissection of the host factors involved in parasite control. Studies of a natural host-parasite relationship are more likely to be indicative of the interactions between pathogenic trypanosomes and their natural hosts.

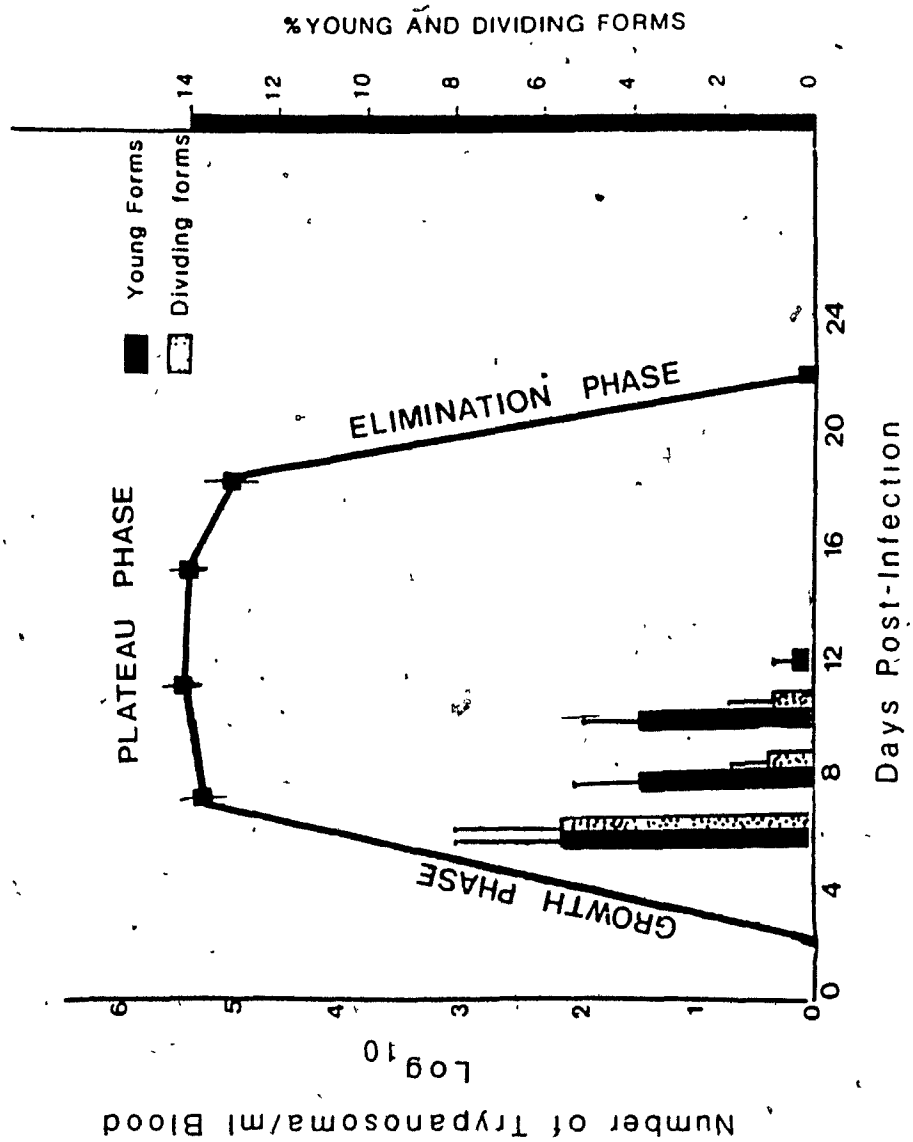
4.1 Course of Infection with T. musculi

In an immunocompetent host, T. musculi produces a self-limiting infection that lasts approximately three weeks (Viens, Targett, et al., 1974). The course of infection is characterized by a series of distinct phases (Figure 1.4). Following inoculation with parasites, there is a period of 3-5 days during which no parasites are detected in the blood (prepatent phase). The length of this phase is inversely proportional to the number of parasites in the inoculum (Targett & Viens, 1975a). Over the next few days, the parasitaemia increases in an exponential fashion, and a relatively high proportion of young/dividing forms (Figure 1.3) is seen in the blood. It has been determined, however, that the most active reproduction of trypanosomes occurs in the peritoneal cavity of infected mice (Lajeunesse et al.,

FIGURE 1.4

Course of parasitaemia in an immunocompetent mouse. The figure demonstrates the three phases seen during the course of infection with T. musculi, namely the growth phase, the plateau phase, and the elimination phase. It also demonstrates the two crises that bring the infection under control.

(modified from Vargas, 1981)


%YOUNG AND DIVIDING FORMS
14
12
10
8
6
4
2
0
Young Forms
Dividing forms
ELIMINATION PHASE
PLATEAU PHASE
GROWTH PHASE
24
20
16
12
8
4
0
Days Post-Infection
6
5
4
3
2
1
0
Log10
Number of Trypanosoma/ml Blood

1975; Duffey et al., 1985). The rate of reproduction declines between 7-10 days post inoculation (p.i.), and the parasitaemia stabilizes for a period of approximately 10 days (plateau phase). The onset of this phase has been termed the "first crisis", analogous to a similar event in T. lewisi infected rats (Taliaferro, 1932). During the plateau phase, the blood forms are essentially monomorphic, consisting of long, slender adult trypomastigotes (Viens & Targett, 1971). The plateau phase is terminated between 18 and 22 days p.i. by the so-called "second crisis", at which time the parasites are eliminated from the blood within 24-48 hours (elimination phase). Following clearance of parasitaemia, a mouse becomes immune to reinfection. This immunity is absolute, specific and probably life-long (Viens et al., 1975). In spite of this observed immunity, Viens et al. (1972) demonstrated the persistence of multiplicative forms of T. musculi in the vasa recta of kidneys of mice which had recovered from infection. These parasites persisted in the kidneys for at least one year and probably for the life of the host (Dusanic, 1985); the infectivity of these parasites was demonstrated by inoculation into the peritoneal cavities of naive mice (Wilson et al., 1973). Olivier & Viens (1985) suggest that persistence of T. musculi in the kidneys of the immune host plays an important role in the maintenance of host immunity, since cured mice treated with an antitrypanosomal drug 8 months after infection become susceptible to reinfection. In contrast,

Dusanic (1985), using a similar approach, found that kidney forms were not required for sustained protective immunity to reinfection up to 25 weeks after initial infection. The discrepancy between these findings might result from differences in the strain of mouse used, or simply to different sampling times.

It is generally considered that the relatively benign nature of T. musculi infection in mice is related to the effective immune response that they generate against the parasite. Taliaferro (1938) first suggested the importance of humoral immunity against T. musculi when he described antibodies inhibiting parasite reproduction ("ablastin") and antibodies which destroy the parasite ("trypanocidal"). The cellular immune response has also been implicated in the control of T. musculi infection -- Jaroslow (1959), Chang & Dusanic (1976), Brooks & Reed (1979), Vincendeau et al. (1981, 1986) and Ferrante (1986) have pointed to the importance of macrophages in the elimination of T. musculi from the blood of infected mice. It is, therefore, not surprising that effective immunodepression of the host will allow the normally non-pathogenic parasites to produce a fulminating, usually fatal, infection strikingly similar to that seen with pathogenic species of trypanosomes (D'Alesandro, 1970). A detailed account of the immune response to T. musculi and the effects of immunodepression will be provided in Section 5. It is important to point out at this stage, however, that the course of infection

described above is subject to modification if the host is stressed.

It is interesting that reports of the course of T. musculi infection in different strains of mice (Magluilo et al., 1983; Albright & Albright, 1981b) are much the same, differing only in the level of the plateau phase; the times of the first and second crises, as well as the rates of parasite growth and elimination, are strikingly similar. This suggests that comparable immunologic phenomena are responsible for the control of infection in the different strains.

4.2 Pathology of T. musculi Infection

Although T. musculi infection is grossly nonpathogenic, important histological modifications of immuno-committed organs or tissues (blood cells, spleen and thymus) as well as changes in the liver and kidneys have been described. In addition, trypanosomes are able to induce significant immunodepression and mitogenic responses in their murine hosts.

4.2.1 Anemia and Thrombocytopenia

The appearance of anemia and thrombocytopenia has been well documented in T. musculi-infected mice (Jarvinen & Dalmasso, 1977b; Hirokawa et al., 1981; Duffey et al., 1985;

Viens, 1985). Since suppression of hematopoiesis has not been shown to occur in infected mice, it has been suggested that these phenomena develop as a result of the generation of nonspecific immunoconglutinins which are found in many of the trypanosomiases (Cox, 1982). Jarvinen & Dalmasso (1977b) showed that intravascular hemolysis was not the process by which these cells were eliminated. They postulate that the immune complexes adhere nonspecifically to the surface of erythrocytes and platelets, leading to their elimination by splenic phagocytes. Davis (1982) suggests that thrombocytopenia *in vivo* may arise as a result of platelet aggregation, as he was able to induce platelet aggregation *in vitro* with trypanosome-derived products. The increase in phagocytosis is likely to contribute to the marked splenomegaly observed in trypanosome-infected mice (Albright et al., 1977; Hirokawa et al., 1981).

4.2.2 Spleen, Liver and Thymus

Splenomegaly is consistently observed in *T. musculi* infection (Viens, 1985). The condition develops rather quickly, persists throughout the infection, and returns to normal relatively rapidly following the termination of infection. *T. musculi*-infected mice show a 10-fold increase in spleen size (Albright et al., 1977), as compared to the 20-fold increase observed in the spleens of mice infected with the more pathogenic *T. rhodesiense* (Mansfield

& Bagasra, 1978). The spleens of T. musculi-infected mice show a rapid increase in the amount of white pulp, resulting partly from formation of germinal centers and partly from the marked proliferation of immunoglobulin G (IgG)-producing plasma cells around the central arterioles i.e. in the T-cell-dependent areas (Robinett & Rank, 1979; Hirokawa et al., 1981). There is also hyperplasia of the red pulp resulting from erythropoiesis, and an increase in lymphoid elements (Duffey et al., 1985). Approximately one week after the infection ends, the spleen returns to its normal size, and regains its normal structure (Albright et al., 1977). It is likely that the marked lymphoid hyperplasia and consequent splenomegaly result from the intense antigenic stimulation that occurs during trypanosome infection. Mitogens of parasite origin may also be involved, leading to polyclonal B-lymphocyte activation (Hazlett & Tizard, 1978). In addition, the erythrophagocytosis mentioned above, and the consequent erythroid hyperplasia (Duffey et al., 1985) likely contribute to the increase in spleen size and cellularity.

Hirokawa et al. (1981) described enlargement of the T. musculi-infected mouse liver to twice its normal size, with a return to normal approximately 6 weeks following trypanosome inoculation. Light microscopy, however, revealed no obvious histological changes in the liver. It should be pointed out that these investigators did not perform their histopathological analyses on specimens removed from mice

immediately prior to and following clearance of parasitaemia. In the livers of rats infected with T. lewisi, however, degenerative changes characterized by cloudy swelling of parenchymal cells, as well as a marked round cell infiltration are observed (Ferrante et al., 1978). Further, Lee & Barnabas (1974) demonstrated the appearance of residual lysosomes as well as phagocytosed trypanosomes within Kupffer cells of the rat liver, coincident with the general decline and termination of parasitaemia. It is possible that the hepatomegaly results, at least partially, from increased macrophage activity involved in the removal of trypanosomes from the circulation.

Hirokawa et al. (1981) also noted a temporary thymic involution of unknown significance in T. musculi-infected mice. The normal thymic architecture was restored within a week of parasite inoculation.

4.2.3 Kidney

Viable, infective T. musculi may be found in the kidneys of immune mice for as long as one year following the termination of parasitaemia (Viens et al., 1972). The relationship of this persistence of parasites in the kidney to pathological changes is presently unclear, because the kidneys, although enlarged as much as twice their normal size, return to their normal dimensions approximately one week following termination of the infection (Albright et

al., 1977). Molyneux (1976) demonstrated that at the peak of parasitaemia, there was an infiltration of the glomeruli by eosinophils, neutrophils and other leukocytes, which later evolved into glomerulonephritis. These glomerulonephritic changes were not observed until 21 days after infection, when electron-dense material and a thickening of the basement membrane were observed. It is likely that the non-specific immune complexes referred to above, as well as trypanosome-specific complexes appearing following elimination, are at least partially responsible for these changes.

4.2.4 Immunodepression and Mitogenicity

It has been well documented that at the peak of *T. musculi* infection, the mouse immune response to sheep red blood cells (SRBC) and other T-dependent antigens is depressed (Albright et al., 1977, 1978; Hazlett & Tizard, 1978; Lajeunesse & Viens, 1981; Vargas et al., 1984; Viens, 1985). The mechanism of this depression has been attributed to a direct, negative effect of trypanosome-derived substances on B-cells, rather than to the induction of classical suppressor cells: Albright et al. (1978) demonstrated that serum from infected mice, saline extracts of blood trypanosomes, and living blood trypomastigotes strongly inhibited the humoral response of spleen cells *in vitro*; marked inhibition also occurred when such cultures

were separated from the parasites by membranes. Paradoxically, Hazlett & Tizard (1978) and others have shown an increase in the background levels of antibody in infected mice, and that an antigenic extract of T. musculi was mitogenic in vitro. Similar observations have been made with regard to the mitogenicity of T. brucei (Viens, 1985). As Viens (1985) points out, although the concept of parasitic modulation of host immune response via immunodepression is "academically attractive", the data must be reviewed with caution, lest they represent epiphenomena. Much of the work has been done in vitro, and the biological significance of these findings should be reexamined following in vivo confirmation. Nevertheless, the importance of trypanosome-mediated immunodepression in prolonging parasite elimination should not be underestimated.

5. HUMORAL AND CELLULAR IMMUNE RESPONSES AGAINST T. MUSCULI

Although T. musculi is generally considered to be nonpathogenic in mice, it is able to produce severe, sometimes fatal infections in newborn and young animals (Breniere & Viens, 1980), in pregnant hosts (Krampitz, 1969b, 1975; Viens et al., 1983; Viens, 1985), and in mature animals with concomitant infections (D'Alesandro, 1970; Cox,

1975; Bungener, 1975a; Bell et al., 1984a,b). In all of these cases, the severity of the infection seems to result from inadequacy or impairment of the anti-trypanosome immune response. It is likely that in normal, non-compromised hosts, the rapidity with which the immune response is generated attenuates the pathogenicity of T. musculi and is the basis for the favorable host-parasite relationship. The immune system is likely to be involved in mediating control of the parasite at the major turning points during the course of infection, viz. the first and second crises. It is probable that the mechanisms involved in mediating each crisis are different, and they will therefore be considered separately.

5.1 Serology of T. musculi Infection

Using the indirect immunofluorescence technique, Viens, Targett, et al. (1974) demonstrated the presence of specific anti-trypanosome antibodies (IgM, IgG1, IgG2) in the sera of infected and recovered mice. These investigators showed that IgM appears early, and reaches maximum titers more rapidly than IgG antibodies. This IgM-IgG shift in response to T. musculi was validated by Brooks et al. (1982) using a sensitive enzyme-linked immunosorbent assay (ELISA). In addition, Viens, Targett, et al. (1974) found that total immunoglobulin and IgG2 titers fall after recovery from infection, but that subsequently, relatively high and

constant antibody levels are detectable for many months. This finding is consistent with the strong, long-lasting immunity to T. musculi found in normal, recovered mice. However, Viens et al. (1975) found no correlation between fluorescent antibody titers and the degree of protection in mice challenged with T. musculi 11 months after initial cure. In addition, Targett & Viens (1975b) reproduced the observation of Kendall (1906), noting the absence of agglutinating antibodies in the sera of infected, immune or immunized mice. It is noteworthy, however, that sera from mice which had recovered from infection were found to have a marked neutralizing in vitro effect on the infectivity of homologous parasites; this neutralization occurred without an apparent reduction in the motility or the number of live organisms (Viens et al., 1975). This finding raised the possibility that the neutralizing antibody was an opsonin; it has been demonstrated that phagocytosis of parasites by macrophages occurs in vitro in the presence of immune serum (Chang & Dusanic, 1976; Vincendeau et al., 1986; Ferrante, 1986). The possibility that the antibody is an opsonin is strengthened by the finding of Targett & Viens (1975b) that lytic antibodies are absent in T. musculi infection.

5.2 The First Crisis

5.2.1 Ablastin and Early Trypanocidal Antibodies

In 1924, Taliaferro described a "reaction product" in the serum of T. lewisi-infected rats whose properties were to selectively inhibit T. lewisi reproduction without affecting the parasite in other ways. In 1932, he provided a detailed description of the biological characteristics of this product, and renamed it "ablastin", from the Greek a (not) and blastos (a sprout, germ or offspring). Ablastin conferred a reproduction-inhibiting immunity, controlling parasite replication without harming the parasite.

Coventry (1930) was the first to demonstrate the presence of two distinct trypanocidal antibodies in the rat. She discovered that serum taken from infected rats after the first crisis was trypanocidal for dividing forms but not for adults (first trypanocidal antibody), and that serum taken after the second crisis was trypanocidal for all stages of the parasite (second trypanocidal antibody).

In 1938, Taliaferro repeated the protocols that had led to the understanding of T. lewisi infection in the rat, and concluded that initial control of T. musculi infection in the mouse was mediated by ablastin and a first trypanocidal antibody, cooperating in a process similar to that observed in rats. Briefly, he hypothesized that ablastin was synthesized in response to the presence of young and

dividing forms of T. musculi, leading to a cessation of parasite replication. Young and dividing forms would then be eliminated by the trypanocidal activity of the first antibody. Again, by analogy with T. lewisi, Taliaferro believed that final elimination of T. musculi from the blood was effected by a second trypanocidal antibody (see Section 5.3).

It should be pointed out that Ormerod (1963) criticized the duality of the ablastin-first trypanocidal antibody concept, proposing instead that dividing and adult trypanosomes were different in terms of their surface antigen makeup. He believed that when the number of dividing parasites initially increased, a first trypanocidal antibody destroyed them without the need for ablastin. At the same time, some of the progeny of the dividing forms would mature into adults, thereby becoming resistant to the first trypanocidal antibody; a second set of trypanocidal antibodies generated later in the course of infection would clear the blood of adult trypomastigotes. The plateau phase would, therefore, be due to an automatic killing of newly formed parasites fed into the bloodstream from the site of their generation, i.e. the peritoneal cavity. Ormerod proposed this scheme for T. lewisi infection. However, the theory was all but abandoned when several of its basic assumptions failed to be verified experimentally in the rat, since it was believed that T. musculi infection of the mouse was similar to T. lewisi in the rat.

The hypothesis that the mouse-T. musculi host-parasite relationship was similar to that of the rat and T. lewisi was accepted for many years (Viens, 1985). Thus, many investigators have endeavoured to evaluate the nature of ablastin in mice: its synthesis has been shown to be thymus-dependent (Viens, Targett, et al., 1974; Targett & Viens, 1975a; Brooks & Reed, 1980), and its physicochemical characteristics are consistent with its being an immunoglobulin, likely IgG1 (Dusanic, 1975b). Finally, it has been shown to be absorbable by young and dividing forms of T. musculi (Brooks & Reed, 1980), although this work has been criticized (Viens, 1985).

Despite this extensive characterization of ablastin in the mouse, there is currently a great deal of controversy over the existence of the ablastic phenomenon (Viens, 1985). Desbiens & Viens (1981) demonstrated rapid clearance of young and dividing forms administered intravenously to mice infected for 10-15 days. They hypothesized that if elimination was due to ablastin, the transferred dividing parasites would have persisted in the blood for at least the first few hours of observation. The fact that the dividing trypanosomes were cleared immediately (within 5 minutes) suggested to these investigators that the initial control of parasitaemia was due to a trypanocidal mechanism directed against the young and dividing forms rather than to ablastin. Because of the earlier findings of Viens, Targett, et al. (1974) that T-cell deprivation did not abrogate

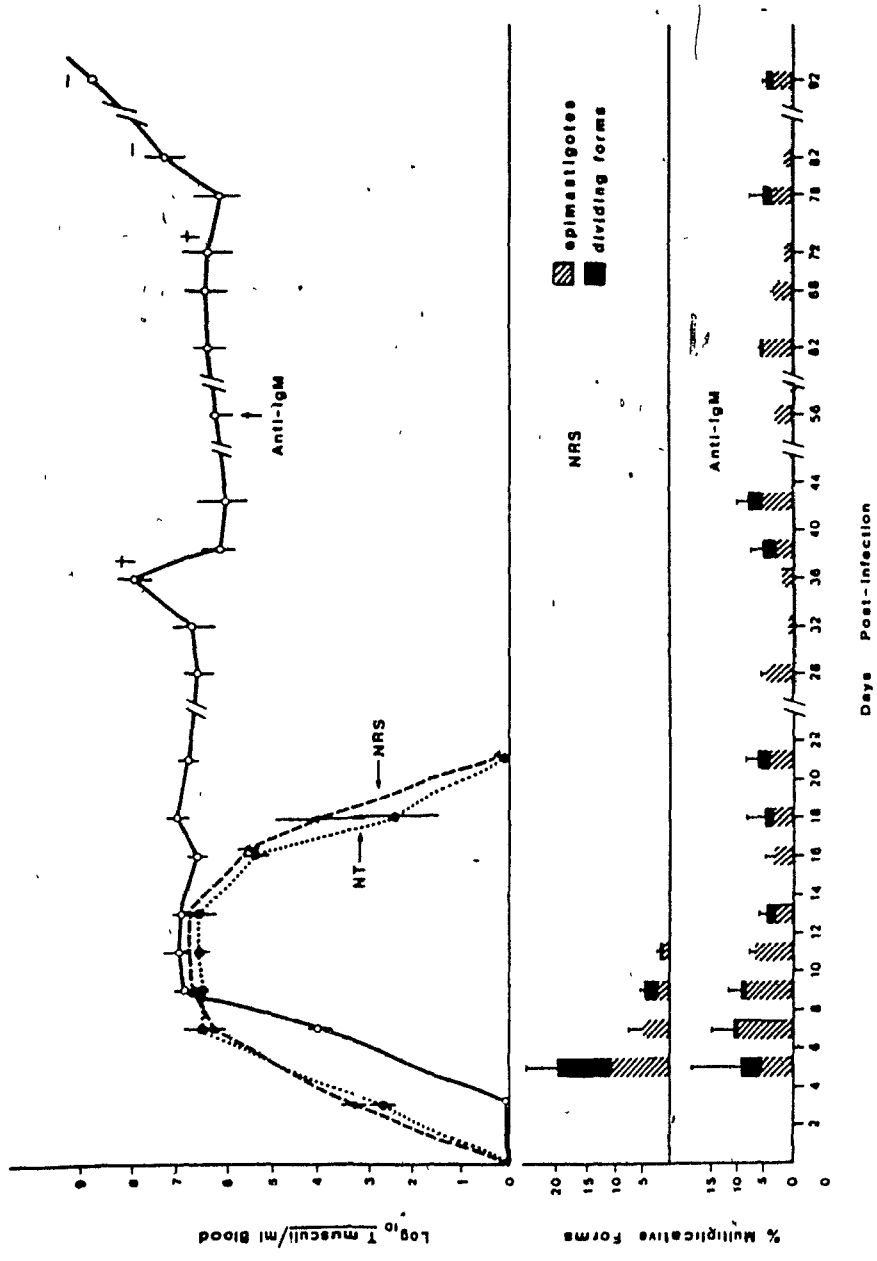
control at the first crisis, they concluded that the first trypanocidal antibody was a thymus-independent IgM. The large size of the IgM molecule would, to a large extent, prevent its appearance in the peritoneal cavity, thereby allowing for the very active trypanoblastic activity therein, while at the same time removing young and dividing forms from the bloodstream. Thus, Ormerod's theory would appear to have been vindicated.

Recent findings from our laboratory have cast doubt on the importance of this first trypanocidal antibody (Vargas, 1981; Vargas, et al., 1984). When mice rendered B-cell deficient (by anti-IgM treatment from birth onwards) are infected with T. musculi, parasitaemia is established at the same plateau level and at the same time as that in normal, nontreated, infected mice (Figure 1.5). In contrast to the findings in normal mice, however, young forms persist in the blood in significant numbers over the entire period of infection in these B-cell deficient mice, indicating that a first trypanocidal antibody may well be the normal mechanism of destruction of the newly formed parasites. The relatively steady percentage of dividing forms in the blood of anti-IgM treated mice suggests that the major mechanism controlling reproduction of the parasite is still intact in these hosts. In support of these findings, Olivier et al. (1986) report that IgG2b antibodies appear to be involved in the initial control of T. musculi infection based on passive transfer of immunoglobulin fractions early in the course of

FIGURE 1.5

Percentage of young and dividing forms of T. musculi in B-cell-deficient mice (rabbit anti-mouse IgM treated), and in normal rabbit serum (NRS)-treated mice. Values for non-treated mice were similar to those for NRS-treated mice. Each point represents the mean of five mice +/- 1 standard error of the mean. Each histogram represents the mean value of 5 mice.

(From Vargas, 1981, with permission)


Log10 T musculi/ml blood
% Multiplicative Forms
Days Post-Infection
Anti-IgM
NRS
NT
epimastigotes
dividing forms

infection. In summary, it is evident that although antibodies may assist by removing young/dividing forms from the blood, a mechanism other than antibody is primarily responsible for controlling parasite growth in the initial phase of infection.

5.2.2 Cellular Immunity

The initial control of parasitaemia, manifested by the first crisis, appears to be resistant to most immunosuppressive measures: T-cell deprived mice (Viens & Targett, 1971; Viens, Targett, et al., 1974), antithymocyte-treated mice (Viens, Targett, et al., 1974), congenitally athymic nude mice (Brooks & Reed, 1977; Rank et al., 1977), newborn mice (Breniere & Viens, 1980) and cyclophosphamide-treated mice (Viens, 1985) all show signs of a first crisis approximately one week following inoculation, although plateau level parasitaemias are different under these different conditions.

Total body irradiation, however, prevents this initial phase of control. Jaroslow (1955) reported that radiation administered 24 h after T. musculi inoculation produced a higher parasitaemia and an increase in reproductive forms. Albright & Albright (1981b) reported that exposure of mice to sub-lethal doses of ionizing radiation resulted in a marked elevation of parasitaemia, with no apparent plateau. These results were confirmed by Rappatoni (1984). Thus,

ionizing radiation treatment during the early part of infection interferes with innate immunity and enhances the host's susceptibility to infection. This suggests the involvement of a radiosensitive cell in the initial control of parasite growth.

Taliaferro & Pavlinova (1936) showed that the initial rate of proliferation of T. musculi was partly associated with the state of the "lymphoid-macrophage" system. Jaroslow (1955) suggested that ionizing radiation caused injury to this "lymphoid-macrophage" system, and further examined its involvement by the administration of India Ink, a macrophage blocker. While he observed no increase in the plateau level of parasitaemia, he did detect an increase in the number of dividing forms in the blood (1959). Administration of trypan blue, an inhibitor of macrophage function (Brooks & Reed, 1979), gave rise to a significant elevation in plateau parasitaemia, as a result of increased reproductive activity. Studies with dextran sulfate (Vargas, 1981) and silica (Albright & Albright, 1981b; Rappatoni, 1984), potent inhibitors of macrophage function, showed minor increases in parasitaemia in susceptible strains of mice. Thus, the innate reproduction-inhibiting capacity of the host seems to be regulated by the mononuclear phagocyte system, since impairment of this system is reflected in an increased level of reproductive activity of the parasite.

Taliaferro (1938) suggested that the level of parasitaemia is determined by two independent factors: the

rate of reproduction and the rate of killing. The implicit assumption in this hypothesis is that if one of these two factors is changed, the level of parasitaemia will be altered accordingly. Vargas (1981) demonstrated that this, in fact, is not the case, as she and others have shown that abnormal macrophage function does not seem to affect the development of the first crisis. Macrophages are, however, important in controlling parasite reproduction.

5.2.3 Summary

The following findings emerge: the first crisis occurs despite deprivation of B-cells, T-cells, or impairment of mononuclear phagocyte function. Only in a situation in which all the cells are destroyed, i.e. irradiation, does the first crisis fail to occur. This leads to the conclusion that one of several mechanisms may be sufficient for the host to bring parasitaemia under control at the first crisis. Under normal circumstances, both mononuclear phagocyte activity and antibody production may play a role, either independently or via a complex interaction. In the absence of one mechanism, however, the other may compensate sufficiently to mediate the first crisis (Vargas, 1981). The magnitude of the compensatory effect might be dependent on the plateau parasitaemia level, a function of the strain of mouse.

5.3 The Second Crisis

5.3.1 Cellular Involvement

Inasmuch as the first crisis is dependent on an intact murine immune system, the second crisis, too, is affected in immunodeprived mice. Irradiation of infected mice during the plateau phase prevents elimination of parasitaemia (Rappatoni, 1984). Thus, it seems that some radiosensitive cell(s) is (are) involved in effecting parasite removal from the blood. By analogy with the first crisis, cellular involvement in the second crisis may be evaluated by depleting the mouse of certain cell populations.

In 1901, Laveran & Mesnil proposed that removal of parasites from the infected animal was due to phagocytosis of living trypanosomes. That this is, in fact, a plausible mechanism for trypanosome elimination has been shown by Ferrante & Jenkin (1978, 1979) for T. lewisi, by Greenblatt et al. (1983) for T. rhodesiense, and by others for different trypanosome species (Liston & Baker, 1978; Ngaira et al., 1983). Thus, Brooks & Reed (1979) administered trypan blue to T. musculi-infected mice, and found that clearance of parasitaemia was significantly delayed, by approximately 10 days, compared to nontreated mice. Since trypan blue is an inhibitor of macrophage function, these results suggest the involvement of macrophages in the elimination of T. musculi from the blood.

Rappatoni (1984) demonstrated that silica, selectively cytotoxic to macrophages (Kessel et al., 1963; Allison et al., 1966), also delays parasite clearance from the blood. In addition, it has been found that macrophages become activated during the course of T. musculi infection; their number, size, protein content and release of peroxides are all augmented (Vincendeau et al., 1981). Furthermore, Chang & Dusanic (1976), Vincendeau et al. (1986) and Ferrante (1986) have demonstrated the in vitro phagocytosis of T. musculi by macrophages. Thus, it is possible that the mononuclear phagocyte system is somehow involved in terminating T. musculi infection.

Elimination of T. musculi from the blood of infected mice at the time of the second crisis has been shown to be dependent on an intact T-cell system. Viens, Targett, et al., (1974) were the first to demonstrate this dependence, using antithymocyte serum-treated mice, as well as adult mice which were thymectomized, irradiated, and then reconstituted with bone marrow (ATxBM). In both groups of mice, the first crisis occurred as in nontreated mice, and the parasitaemia stabilized at a level higher than that in control, infected animals. The striking finding in these mice was the complete inability to terminate the infection. Parasitaemias continued at the plateau level for as many as 110 days p.i. Similar findings were reported for T. musculi infected nude mice (Brooks & Reed, 1977; Rank et al., 1977); although these mice were able to mediate an initial control

of the parasitaemia, they were unable to effect clearance of trypanosomes from their blood. It was therefore concluded that the termination of T. musculi infection requires the presence of T-cells in either a helper or an effector capacity.

Subsequent experiments involving transfer of normal and immune spleen cells to infected animals showed that immune, non-adherent cells were able to effect termination of the infection significantly earlier (within 6 days) than normal spleen cells (Viens, Pouliot & Targett, 1974; Targett & Viens, 1975b). Removal of theta-positive (i.e. T) cells from the inoculum did not, however, impair the ability of the inoculum to restore immunocompetence to the recipient (Pouliot et al., 1977). In addition, thymus graft reconstitution of T. musculi-infected, T-cell-deprived mice allowed for only partial restoration of immunocompetence after prolonged (>100 days) periods of time (Targett et al., 1981). It was therefore proposed that, although T-cell-dependent, the immune process responsible for the second crisis was mediated by theta-negative cells.

5.3.2 Humoral Immune Response

Given the above findings, it seemed logical to propose that the elimination of trypanosomes from the blood of infected mice was mediated by a T-cell-dependent antibody. It was known that mice are able to generate specific anti-

T. musculi antibodies (Section 5.1), and it was suggested that these antibodies should be present in the blood of mice which had naturally cleared their infections. It was reasoned that, if this were true, it should theoretically be possible to a) protect mice by passive transfer of immune serum prior to inoculation with trypanosomes, and b) cure T. musculi infected mice of their parasitaemias by passive transfer of immune serum. A number of investigators have been able to protect naive mice from becoming infected by administering small amounts of immune serum prior to inoculation with T. musculi. Thus, Taliaferro (1938) showed that by taking serum from mice whose infections had terminated, and injecting it together with trypanosomes into normal mice, he could either completely protect mice from infection, or at least significantly delay the onset and reduce the magnitude of infection. Viens, Targett, et al. (1974) reported that passive transfer of antiserum just before infection prolonged the prepatent period, and that the subsequent parasitaemia was markedly reduced. Further, Viens et al. (1975) demonstrated that immune serum was able to neutralize the infectivity of T. musculi when incubated together in vitro. Finally, Bungener (1975b) was able to completely protect mice from infection by administering immune serum just prior to inoculation; the degree of protection was, however, dose-related. Thus, it was apparent that immune serum contained a protective activity, an activity which Taliaferro (1938) suggested was mediated by a

direct trypanocidal antibody.

The curative activity, on the other hand, has been much more difficult to demonstrate. Although Taliaferro (1938) claimed that up to 4.6 ml of immune serum injected into mice infected for 3-7 days will rid the mice of their parasitaemias, his scant data and lack of quantitative evidence make it difficult to substantiate his claim. Indeed, he qualified his findings by suggesting that the recipient mice could "not contain too many trypanosomes" in order for him to observe the curative effect. Viens, Targett, et al. (1974) showed that parasite development was checked by administration of immune serum on day 6 of infection, although it resumed 7 days subsequently. Immune serum had no effect at all when administered during the plateau phase. Similarly, Bungener (1975b) stated that parasitaemia may be cleared by immune serum administration. This, however, was based on a single mouse which was injected intraperitoneally with 6 ml of serum in two doses over a 24 h period. For comparison, the normal blood volume of a mouse is approximately 1.6 ml. Other attempts to effect clearance of parasites during the plateau phase have met with complete failure. Viens, Pouliot & Targett (1974) and Targett & Viens (1975b) state unequivocally that the course of parasitaemia is unaffected by immune serum transfer. This view is echoed by Brooks et al. (1982) who state: "the 2nd crisis does not depend on a direct (trypanocidal/ trypanolytic) antibody effect, but appears to be dependent

on cellular mechanism (sic)". Albright & Albright (1982) also failed to alter the course of an established infection by passive transfer of immune serum, and thus concluded that "the elimination of T. musculi during the establishment of immunity is not a humoral antibody-dependent process." Therefore, it seemed quite clear to most investigators that antibody did not play a major role in the elimination of T. musculi from the blood.

Thus, it was provocative to find that mice which had been rendered B-cell-deprived, and therefore antibody-deficient, were unable to effect T. musculi elimination (Vargas, 1981; Vargas et al., 1984) (Figure 1.5). This finding strongly suggested that antibody was essential to the process of trypanosome removal during the elimination phase. This was substantiated by the finding of Pouliot (1978) that cyclophosphamide, which selectively depresses humoral immunity, abrogated elimination of parasites when administered to mice near the end of the plateau phase. These findings seem to indicate the necessity of antibodies in effecting parasite elimination.

It appears, then, that a paradox exists: if antibody has been shown indirectly to be important in parasite removal, and if such antibody has been demonstrated in immune serum, why is it not possible to effect clearance of parasitaemia during the plateau phase by passive transfer of immune serum? This failure has led some investigators (Viens, 1985) to place little emphasis on the results

obtained with B-cell deprived mice.

5.3.3 Summary

It is apparent that the mechanisms involved in the removal of parasites from the blood of infected mice are not well understood. Although macrophages seem to play a role, their exact function requires further study. The process of elimination is dependent on functioning B- and T-lymphocyte systems, suggesting that a T-dependent antibody is required, possibly together with a cytotoxic T-cell. Yet, the inability to effect clearance by passive transfer of immune serum is not consistent with the concept of a humoral immune process. It is evident, therefore, that the mechanisms mediating immunity of the second crisis have yet to be fully elucidated.

5.4 The Role of Complement

The varying influence of complement on the outcome of infections has been demonstrated with many protozoa. Whereas complement may or may not be important in immunity to *T. cruzi* (Ferrante, 1985), complement components have been shown to play an insignificant role in the recovery from infection with *T. lewisi* (Jarvinen & Dalmasso, 1976). However, Albright & Albright (1985) and Desai et al. (1987) have demonstrated the importance of complement component C3

in elimination of T. lewisi from its xenogeneic host, the mouse. The role of complement in host resistance to infection with T. musculi has been examined in normal, C5-deficient and C3-depleted mice (Dusanic, 1975; Jarvinen & Dalmasso, 1977a). Infections in normocomplementemic strains of mice were not significantly different from those in strains genetically deficient in component C5 -- there were no differences in inhibition of reproduction, duration of infection, persistence of parasites in the kidneys, or resistance to infection. There was, however, a slight increase in plateau parasitaemia of some C5-deficient mice (B10.D2/o) compared to their congenic, normocomplementemic counterparts (B10.D2/n). This suggests that trypanolysis mediated by the terminal complement components is not a mechanism of major importance in T. musculi elimination. Although reports of complement-dependent trypanolysis have been presented (Viens, 1985), the fact that C5-deficient mice are able to clear their infections at the same time and in the same manner as normocomplementemic mice implies that C5 is not essential for elimination.

Additional experiments by these investigators involved the use of cobra venom factor to deplete mice of C3 and the late-acting complement components. This treatment resulted in slightly greater peak parasitaemias, as well as in prolonged infections, secondary to a significant delay of the second crisis and the subsequent elimination of parasites. It therefore seems likely that C3 is involved in

parasite elimination, possibly via a C3-mediated function such as phagocytosis.

Thus, although certain complement components are important in controlling T. musculi infection, their exact role is presently unclear.

CHAPTER II

MATERIALS & METHODS

CHAPTER II

MATERIALS & METHODS

1. THE PARASITE

The strain used throughout this study was isolated by Krampitz in Sicily in 1962 from Mus musculus brevirostis and named "Partinico II" (Krampitz 1969b). After years of syringe passage it was obtained by the Liverpool School of Tropical Medicine and stabilated as LUMP 136 (Lumsden & Hardy, 1965). A cryopreserved stock of this clone was given to our laboratory by Dr. Pierre Viens (Département de Microbiologie, Université de Montréal) in September 1978.

In our laboratory, propagation of the parasite was initially achieved by T. musculi inoculation of lethally irradiated C3H/HeN mice, which were subsequently exsanguinated during the plateau phase of infection. Trypanosome-infected blood thus obtained was made 10% in glycerin and aliquoted in sealed 75 microliter heparinized capillary tubes. These were stored in methanol at $-70^{\circ}C$.

In 1982, following some difficulties in experimental reproducibility, this mode of propagation was abandoned in favor of biweekly in vivo intraperitoneal passage in male A/J mice.

2. THE ANIMALS

2.1 Mice

Male C57BL/6 and A/J mice were raised by successive brother-sister matings from parents obtained from Jackson Laboratories (Bar Harbor, ME) in the breeding colony of the Montreal General Hospital Research Institute, Montreal, QUE and used at 6-8 wk for experiments. Male 6-8 wk old DBA/2, BALB/c, C3H/HeN, and CBA/N mice were obtained from Charles River Breeding Laboratories, Inc., St. Constant, QUE. Retired female C57BL/6, A/J, DBA/2, and C3H/HeN breeders 6-9 months old (Charles River Breeding Laboratories, Inc., St. Constant, QUE; and Jackson Laboratories, Bar Harbor, ME) were used in the preparation of plasma. Mice were housed in groups of five in 11 1/2 " x 7 1/2 " x 5 " polycarbonate cages (Lab Products, NJ); all were maintained under identical conditions, being provided with Purina Mouse Chow #5015 and water ad libitum. Following inoculation with T. musculi, cages of infected mice were kept in a separate "infected" room under a negative airflow cabinet (Lab Products, NJ).

2.2 Rabbits

New Zealand White male rabbits, 1.5 kg (Claude Leonard, QUE until May 1984, and subsequently from Laka, QUE) were used to prepare antisera.

3. INOCULATION OF MICE & MONITORING OF THE INFECTION

3.1 Inoculum

Trypanosome-infected blood obtained from a) a frozen stabilate or b) infected A/J "passage" mice was diluted in sterile normal saline (0.85 % NaCl) so that the final trypanosome concentration was 2.5×10^4 - 5×10^4 parasites/ml. A 0.2 ml inoculum of this dilution (containing 5×10^3 - 10^4 trypanosomes) was then administered to each mouse. Injections were initially given intraperitoneally (i.p.), but following the demonstration that intraperitoneal and intravenous (i.v.) routes of infection give rise to identical courses of parasitaemia (Rappatoni, 1984), subsequent infections have been by the i.v. route alone.

3.2 Measurement of Infection

3.2.1 Blood Samples

The course of infection was monitored by obtaining blood samples from the retro-orbital sinus in heparinized capillary tubes. Samples were taken every 2-3 days at approximately the same time each day; this varied with each infection.

3.2.2 Retro-orbital Bleeding

To obtain blood, the technique of Sorg & Buckner (1964) was used. The head of the mouse is firmly held with the thumb and forefinger of the left hand, stretching back the skin, thereby allowing the eyeball to protrude. A heparinized 50 microliter capillary tube or Pasteur pipette is introduced into the medial canthus at a 45^{o} angle with the midline both laterally and superiorly, and is gently pushed inwards with a slight rotatory motion until blood begins to flow. Following removal of the tube, the eye is gently compressed with a sterile gauze square to stop bleeding. Repeat bleedings may be performed for many consecutive days on the same eye if care is taken not to damage the eyeball.

3.2.3 Wet Films & Haemocytometer Counts

If the level of parasitaemia was low (e.g. at the beginning or end of infection, or in certain strains throughout the course of infection), the degree of infection was assessed by counting parasites on wet blood films. Blood (5 microliters) was dispersed on a glass slide with a 22 x 40 mm glass coverslip. Parasites were enumerated microscopically with a Nikon Labophot microscope (Japan) under phase contrast with a 40x objective and a 10x ocular.

When more than five parasites per high power field of the wet film were present, haemocytometer counts were performed. Infected blood was diluted 1:100 with formol saline (0.85% NaCl containing 0.02% formalin), and immobilized trypanosomes in this mixture were counted in an American Cptical Neubauer Haemocytometer (Buffalo, NY) under phase contrast with the 40x objective and 10x ocular of the Nikon microscope.

In both cases, the parasitaemias were expressed per ml of blood and converted to $\log_{10}$ values. Counts were made in individual mice, and the mean of the $\log_{10}$ values from 3-5 mice was calculated.

4. PLASMA PREPARATION & INJECTION

4.1 Plasma Preparation

4.1.1 Normal Mouse Plasma (NMP)

Normal Mouse Plasma (NMP) was prepared from noninfected retired breeders of different strains which were bled out by cardiac puncture with heparinized syringes. Plasma was separated from cellular components by centrifugation and stored at -20°C.

4.1.2 Immune Plasma (IP)

Immune Plasma (IP) was obtained from T. musculi infected C57BL/6 retired breeders which were exsanguinated by cardiac puncture with heparin 28-30 days post-inoculation (p.i.). Plasma was prepared as described above. IP was also prepared from retired breeders of other strains (A/J, DBA/2, C3H/HeN), but unless otherwise indicated, IP refers to plasma obtained from C57BL/6 mice.

4.1.3 Other Plasma Samples

Blood was obtained from C57BL/6 mice infected 7, 14 and 19 days before collection, and plasma was prepared therefrom in the manner indicated above.

4.2 Heat-Treatment (HT)

If required, plasma samples were heat-treated (HT) by incubation in a 56°C water bath for 30 min to 2 h.

4.3 Plasma Injection

Unless otherwise indicated, all injections of plasma were i.v., and were administered as a volume of 0.4 ml per mouse. Plasma was administered either before trypanosome inoculation or during the course of an established infection.

5. CHROMATOGRAPHY

5.1 Molecular Sieve Chromatography

Plasma was applied to an LKB Ultrogel ACA34 (IBF, Villeneuve-La-Garenne, France) column (1.6 x 90 cm) equilibrated in phosphate buffered saline, pH 7.4 (PBS). Fractions of 2.5 ml were collected, and the protein content of the fractions was calculated by measurement of absorbance at 280 nm, assuming an extinction coefficient of 1.4 optical density Units/mg of protein per ml. Appropriate fractions were pooled, concentrated to original volumes in a protein

concentrator (Amicon Corporation, Danvers, MA), and dialyzed versus PBS before injection into mice to assay activity.

5.2 Protein A-Sepharose

The procedures described by Ey et al. (1978) and Seppala et al. (1981) were followed with minor modifications. Staphylococcal protein A covalently linked to Sepharose 4B was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden), swollen in phosphate-buffered saline (pH 8.0) with 0.02% sodium azide, and packed into a 10 ml glass pipette. The column (bed volume, 5 ml) was stored and used in a cold room at 4°C. A two ml volume of 0.1 M phosphate buffer (pH 8.0) was added to 5 ml of plasma, and the pH was adjusted to 8.1. This material was applied to the column and the column was rinsed with the pH 8.0 buffer until the optical density at 280 nm (O.D. 280 nm) was less than 0.01. Material bound to the column was successively eluted with 0.1 M citrate buffers of pHs 6.0, 4.5 and 2.5; each time, the column was rinsed until no further material was eluted. Fractions (2.5 ml) were collected, absorbances at 280 nm were measured, and fractions eluted with a given buffer were pooled and neutralized to pH 7.0 with 1 M Tris hydrochloride (pH 8.5). These pooled fractions were designated by the pH of elution, concentrated to the original plasma volume in a protein concentrator, and dialyzed versus PBS.

Each fraction was tested for the presence of immunoglobulin G (IgG) or immunoglobulin M (IgM) by the Ouchterlony double immunodiffusion technique, with rabbit anti-mouse immunoglobulin reagents (Litton Bionetics, Willowdale, ONT, and Miles, Rexdale, ONT) in the center wells and serial doubling dilutions of fraction in the outer wells. The endpoint was read as the weakest titer of fraction which gave a detectable precipitin line.

5.3 Immunoglobulin Subclass Fractionation

Rabbit anti-mouse IgG2a (2 mg/ml gel) and rabbit anti-mouse IgG3 (1 mg/ml gel) were obtained already coupled to separate quantities of sepharose beads (Southern Biotechnology Associates, Birmingham, AL). Beads were packed into two columns and washed with PBS pH 7.4 until the O.D. 280 nm was zero. Two 1 ml samples of IP were diluted 1:3 with PBS, and one sample was applied to each column. The columns were rinsed with PBS until the O.D. 280 nm was zero, and the material which flowed through (FT) the columns was pooled and stored at 4°C (labelled G2a-FT or G3-FT, respectively). Bound material was eluted from the columns with a pH 2.8 glycine-HCl buffer, neutralized with dilute NaOH, and stored at 4°C (labelled G2a-ELU or G3-ELU, respectively). The columns were stripped with 0.1 M HCl, reequilibrated with PBS, and the FT fractions were again passed over the respective columns. The cycle was

repeated 3-4 times until no more protein appeared in the eluate. The FT and ELU fractions from each column were pooled separately, dialyzed versus PBS, and concentrated down to 0.5 ml (i.e. 1/2 original volume) using a stirred ultrafiltration cell followed by Centricon-30 microconcentrators (Amicon Corporation, Danvers, MA). Three 1 ml aliquots of IP were made with each column in order to obtain sufficient material for testing.

5.4 Injection of Fractions

Mice were injected with the aforementioned column fractions either before T. musculi inoculation or during an established infection. Injections were i.v. in a volume of 0.8 to 1.0 ml (2 to 2.5 times the volume of IP normally active) to compensate for losses during fractionation. Parasitaemia levels were monitored immediately prior to and for several days following fraction administration. If required, fractions were heat treated (HT) by incubation in a 56^{o}C water bath for 30 min to 2 h.

6. IN VIVO ASSAY

Plasma samples (0.4, 0.8, or 1.2 ml) or concentrated fraction samples (0.8 to 1.0 ml) were administered to infected mice during the plateau phase of infection (10-15 d

p.i.). Parasitaemias were monitored immediately prior to and for several days following plasma or fraction administration.

7. IN VITRO ASSAY

Heparinized blood was obtained by cardiac puncture from infected mice 10-15 d p.i. Triplicate aliquots of 0.2 ml of this blood were placed in wells of a 96 well flat-bottom assay plate (Linbro, Flow Laboratories Inc., McLean, VA) and 0.05 ml of plasma or fraction was added to each well. The contents of each well were mixed with a 0.1 ml Eppendorf pipettor, and a small sample was immediately removed for parasite enumeration. The assay plate was placed in a 5% CO_2 37°C incubator and, 1 and 2 h later, the contents of the wells were admixed, and samples were removed for parasite enumeration.

8. COBRA VENOM FACTOR (CVF)

Cobra venom factor (CVF; Cedarlane Laboratories, Hornby, ONT), a decomplementing agent (Cochrane et al., 1970; Bottger et al., 1986), was administered to mice in two injections. Mice received one dose of 10 Units (U) of CVF i.p. 24 h before injection of IP and another 10 U

immediately before IP administration. Plasma was tested for the presence of C3 by the Ouchterlony double diffusion technique with rabbit anti-mouse C3 (Cappel Laboratories, Cochranville, PA) in the center well and dilutions of plasma in the outer wells. Agar immunodiffusion plates (Hyland Diagnostics, Malvern, PA) were placed in an incubator at 37°C for 2h, followed by overnight incubation at 4°C.

9. MONOCLONAL ANTIBODIES

Monoclonal antibodies were prepared according to a protocol synthesized from the methods of Gefter (1977) used at the Harvard Biological Laboratories, Cambridge, MA and those of Fuks et al. at the McGill Cancer Center, Montreal, QUE (Wechsler, 1982). Cell fusions were performed using Sp2/O Ag cells derived from the P3X63Ag8 BALB/c myeloma line, and graciously provided by Dr. Joyce Rauch, Montreal General Hospital Research Institute, Montreal, QUE. These cells are azaguanine-resistant i.e. HAT (Hypoxanthine-Aminopterin-Thymidine) sensitive; normal lymphocytes are HAT resistant, but they will not grow under the conditions of culture. If HAT is added to the culture medium, only those cells which have undergone successful fusion (i.e. hybridomas) will survive. Sp2/O cells do not synthesize any immunoglobulin chains, allowing for easy

isolation of pure antibody made by the secreting partner.

Using sterile technique, spleen cells are isolated from C57BL/6 mice infected with T. musculi 7 to 35 days previously; certain mice were rechallenged with inocula of T. musculi up to 1 wk prior to harvest of spleen cells. Spleen cells are resuspended in serum-free RPMI 1640 medium (RPMI) (Gibco, Grand Island, NY), and 10^8 cells are combined in a 5-10:1 ratio with myeloma cells in a round bottomed test tube. The mixture is washed 3x in serum free RPMI, following which the supernatant is aspirated. One ml of 35% Polyethylene Glycol (PEG) in serum free RPMI is slowly dripped down the side of the tube. After 2 min, the pellet is gently flicked; after an additional min, the tube is spun for 3 min at the lowest speed of a desk-top centrifuge. One min later, 2 ml of fetal calf serum (FCS) followed by 8 ml of serum-free RPMI are slowly added to the tube without disturbing the pellet. The tube containing this mixture is gently inverted; and split into two 50 ml conical tubes each containing 45 ml of RPMI with 15% FCS. These tubes are left in a 5% CO_2 37°C incubator for 1 h, following which 0.5 ml aliquots are transferred to eight 24-well plates (Costar, Cambridge, MA). Twenty four h later, 1.0 ml of HAT medium is added to each well, and cells are allowed to grow until macroscopic clones are visible. Medium is replaced with 1x HAT every few days as necessary. Once macroscopic clones are visible, supernatants of wells containing such clones are assayed for activity by

enzyme-linked immunosorbent assay (ELISA). Supernatants used for assay are replaced with HT- (no aminopterin) supplemented medium until subcloning.

Once a well is scored as being positive, it becomes necessary to separate the cells producing the desired antibody from those making irrelevant antibody so that a pure preparation of antibody may be achieved. The technique of limiting dilution is employed to isolate individual hybridoma cells producing the desired antibody. The cells in a well of a 24 well plate screened as positive are resuspended, and several drops are transferred to a new plate as "backup". Remaining cells are counted and diluted in HT-supplemented culture medium to concentrations of 10, 5 and 1 cell/0.1 ml (i.e. 100, 50 and 10 cells/ml), and at least one 96 well plate is prepared from each dilution (with 0.1 ml/well). BALB/c thymocytes serving as a feeder cell layer to help support growth of small numbers of hybridoma cells are harvested and prepared in culture medium at a concentration of 10^6 thymocytes/ml. 0.1 ml of this thymocyte preparation is added to each well. Ten d to two wk later, macroscopic clones become visible. Wells with growth are screened for trypanosome antigen binding by ELISA, and positives are allowed to grow until they cover the well bottom. At this point, cells are transferred to a well of a 24 well plate to grow. Finally, hybridomas are grown in 100 mm x 15 mm Petri dishes; every few days, cells are split 1:10 with culture medium to maintain healthy growth.

Once a hybridoma secreting a particular antibody is subcloned, it is desirable to set aside a portion of cells to form a permanent supply of cells. This is done by selecting a Petri dish of healthy appearing cells in the growth phase, harvesting the cells and pelleting them. Cells are resuspended in 1 ml of freezing medium: RPMI 1640 with 10% FCS and 10% DMSO (Fisher, Montreal, QUE) to prevent rupture of cell organelles upon freezing of water. This aliquot is sterilely placed in a freezing vial, stored in a -70°C freezer for 24 h, and then transferred to a -160°C liquid nitrogen tank. To regrow the frozen cells, a vial is quickly thawed, washed in culture medium, and plated in 10 ml of medium.

When cells of a subclone growing in a Petri dish are deemed healthy and relatively dense, that particular dish is harvested and resuspended in 1 ml of PBS. This suspension is used to inject 0.3 ml intraperitoneally into BALB/c x C57BL/6 mice "primed" with 1 ml of pristane 5 d previously. Ten days to 2 wk later, such mice are observed to have swollen abdomens, indicating the presence of ascitic fluid. The ascitic fluid is tapped at 2 day intervals using an 18 gauge, 1 1/2" needle inserted into the peritoneal cavity. Ascites fluid collected is spun down at 250 x g in a desk-top centrifuge. The supernatant is saved and stored at -20°C until further studies are performed.

10. ISOLATION OF TRYPANOSOMES

Isolation of trypanosomes was carried out by a process of differential centrifugation. Briefly, trypanosome-containing blood or culture medium was subjected to successive cycles of centrifugation (100-200 x g, 10 min/spin), in which the heavier red and white blood cells formed a pellet at the bottom of the tube, leaving the trypanosomes in suspension. Once the supernatant was deemed to be free of such contaminating cells, it was spun at 2000 x g to obtain a pellet of packed trypanosomes, which was resuspended as necessary.

Alternatively, the method of Lanham & Godfrey (1970) utilizing DEAE-cellulose was occasionally used to purify *T. musculi* from blood samples.

11. TRYPANOSOME ANTIGEN

In order to obtain a pure population of parasites for preparation of antigen, a modification of the trypanosome culture technique described by Albright & Albright (1980) was used. Briefly, peritoneal exudate cells (PEC) were obtained from C57BL/6 mice injected intraperitoneally 3 d previously with 2.5 ml of 10% thioglycollate broth. These cells were washed, suspended at a concentration of 10^6/ml in RPMI 1640 tissue culture medium

supplemented with 20% fetal calf serum, antibiotics, and 5 x 10^{-5}M 2-mercaptoethanol, and 1 ml aliquots were plated in wells of a 24 well tissue culture plate (Costar, Cambridge, MA). Parasites were obtained from infected mice during the plateau phase, diluted in the above medium to 5 x 10^4/ml, and 1 ml aliquots of this mixture were added to the plated PEC. The tissue culture plates were placed in a 37°C 5% CO_2 incubator for 5-7 d until parasite growth was dense. The trypanosomes were harvested from the wells, and processed according to a modification of the method of Brooks et al. (1982) for preparation of antigen. The trypanosome suspension was spun several times at 100-200 x g (10 min/spin) at 4°C to remove any contaminating cells. Once the trypanosome-containing supernatant was deemed free of such cells by microscopy, it was spun at 2000 x g for 30 min at 4°C to obtain a pellet of packed trypanosomes. The pellet was washed in serum-free RPMI 1640 (Gibco, Grand Island, NY), the packed trypanosomes were diluted 1:6 in 0.1 M bicarbonate buffer, and this suspension was sonicated (50W for 30 min) at 4°C (Artek Systems Corp., Farmingdale, NY). The sonicated preparation was rocked overnight at 4°C, and the entire preparation was analyzed for protein concentration (by optical density at 280 nm) and adjusted to a concentration of 1 mg/ml in 0.1 M bicarbonate buffer. Samples of this trypanosome antigen were aliquoted and stored at -20°C.

12. ELISA

Round-bottom, 96-well, Immulon-treated plates (Dynatech, Alexandria, VA) were coated with a) goat anti-mouse IgG (Cappel/COOPERBiomedical, Malvern, PA) or rabbit anti-mouse IgG2a or rabbit anti-mouse IgG3 (Southern Biotechnology Associates, Birmingham, AL) at concentrations of 10 micrograms/ml or b) T. musculi antigen (see above) at a concentration of 0.1 mg/ml and incubated overnight at 4°C. The remaining steps were carried out at room temperature. Wells were washed 3 times with PBS-Tween 20 (0.05%), then blocked with the same for 30 min, and dilutions of plasma or fraction samples were added for a minimum of 2 h. After 3 washes, beta-galactosidase-labelled anti-mouse immunoglobulin antibodies (BRL, Bethesda, MD) or alkaline phosphatase-labelled rabbit anti-mouse IgG subclass antibodies (Southern Biotechnology Associates, Birmingham, AL) were added to wells for a minimum of 1.5 h. Wells were again washed 3 times, and either p-nitrophenyl-beta-D-Galactoside (BRL, Bethesda, MD) 1 mg/ml in 0.05 M phosphate buffer (pH 7.2) with 1.5 mM $MgCl_2$ (for beta-galactosidase-labelled antibodies) or p-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, MO) 1 mg/ml in 0.1 M glycine pH 10.4 with 1 mM $MgCl_2$ and 1 mM $ZnCl_2$ (for alkaline phosphatase-labelled antibodies) was added for 30 min. Absorbance at 405 nm was read in an ELISA plate reader

(Dynatech Laboratories Inc., Alexandria, VA).

13. POLYACRYLAMIDE GEL ELECTROPHORESIS

Polyacrylamide gel electrophoresis (PAGE) was carried out using a Bio-Rad Slab Gel Apparatus (Bio-Rad, Mississauga, ONT). Samples and standards of known molecular weight (Pharmacia, Uppsala, Sweden) were run on 5-20% gradient SDS gels, as well as non-gradient SDS gels. Gels were either stained for protein with Coomassie Blue (Bio-Rad, Mississauga, ONT), or used in immunoblotting.

14. IMMUNOBLOTTING

Immunoblotting is an enzyme immunoassay optimized for detection of nanogram-picogram levels of specific antigens. The technique involves electrophoretic transfer of separated antigen from a polyacrylamide gel to a nitrocellulose membrane support matrix (Bio-Rad, Mississauga, ONT). The nitrocellulose with bound antigen may be: a) stained by immersion in 0.5% amido black 10B in aqueous methanol-acetic acid (45%-45%-10%) for 2-3 min, followed by destaining in aqueous methanol-acetic acid (45%-45%-10%), or b) may be treated as follows: After binding of antigen, remaining unbound sites on the membrane are blocked with gelatin, a

non-reactive protein. The membrane with bound antigen is then incubated with first antibody (at 1:50 to 1:100 dilution) specific for the antigen to be detected, washed to remove unbound antibody, and incubated with either horseradish peroxidase (HRP)-labelled (Hyclone, Logan, UT) or alkaline phosphatase (AP)-labelled (Southern Biotechnology Associates, Birmingham, AL) anti-mouse (second) antibody (at a dilution of 1:2000 to 1:4000). The membrane is again washed, and a color development reagent (3,3' diaminobenzidine tetrahydrochloride dihydrate for HRP-labelled antibodies or naphthol phosphate and Fast Red for AP-labelled antibodies) is used to bring out the antigen bands to which the antibodies have bound.

15. IRRADIATION

Radiation was delivered by a Linear Accelerator X-Ray source, therapy 4 SHM, at the Radiotherapy Department of the Montreal General Hospital, Montreal, QUE. Mice were placed in individual 3 cm x 3 cm x 8 cm chambers of a lucite box, and exposed to the desired dose for total body irradiation at a dose rate of 100 rad/minute.

16. ANTI-PLATELET ANTISERUM

Rabbit anti-mouse platelet antiserum was prepared according to a protocol kindly provided by Dr. William Soper, Division of Transplantation Surgery, Abraham Lincoln School of Medicine, Chicago, IL. Whole blood from adult male and female C57BL/6 mice was collected into 3.8% sodium citrate at a final ratio of 1 part sodium citrate to 9 parts blood. The citrated blood was mixed with one half volume of 4% dextran in normal saline and centrifuged at room temperature (RT) for 20 min at 400 x g. The supernatant was removed and recentrifuged at 400 x g for 15 min; this pelleted most of the contaminating WBC. One half volume of normal saline was then added to the supernatant, and this was again centrifuged at 400 x g for 15 min producing a platelet suspension with less than 0.1% WBC contamination. Platelets in the supernatant were pelleted by centrifugation at 1200 x g for 15 min, washed 2x in normal saline and then resuspended in normal saline with complete Freund's adjuvant for immunization. Rabbits were immunized intradermally on the back with approximately 6×10^8 platelets; a booster injection of 1.5×10^9 platelets was given 2 wk later. Rabbits were bled out by cardiac puncture 1 wk after boosting. Blood was clotted, serum collected, and absorbed with normal mouse spleen, thymus and liver cells; these cells were washed thoroughly to remove possible contaminating platelets prior to absorption. The serum was

aliquoted and stored at -20°C for further use.

Experimental mice received 0.1-0.2 ml intraperitoneal injections of the antiserum.

17. TRYPAN BLUE

The azo dye trypan blue has been reported to inhibit both the *in vivo* and *in vitro*, specific and nonspecific, cytotoxic effector function of activated macrophages (Hibbs, 1975; Brooks & Reed, 1979; Hall et al., 1982). Mice were injected with 1 mg or 4 mg of trypan blue (Sigma Chemical Co., St. Louis, MO) in saline at different intervals. Injections of 4 mg were intraperitoneal or intravenous; 1 mg injections were subcutaneous.

18. SILICA

Silica is an agent which displays selective cytotoxicity to macrophages (Kessel et al., 1963; Allison et al., 1966). Silica (#216, min-u-sil: Whittaker, Clarke & Daniels, Inc. (Plainfield, NY)) for inoculation was prepared by the technique of Allison (1966). Briefly, a suspension of 12 mg/ml of silica particles (less than 5 microns in size) in saline was sonicated immediately prior to administration, to prevent settling of particles. Mice received 0.25-0.5 ml

of suspension by the intravenous route on the day prior to assay. One h prior to use of blood from silica-treated mice in the in vitro assay, mice were given a suspension of latex particles by the intravenous route.

19. LABELLED SHEEP RED BLOOD CELLS (SRBC)

Five ml of sheep red blood cells (SRBC) (Inst. Armand Frappier, Laval, QUE) were washed with sterile saline 3x for 5 min at speed #5 in a desktop centrifuge. An appropriate volume of ^{51}Cr (New England Nuclear, c/o Dupont, Lachine, QUE) (calculated from decay chart) was added to the 1 ml SRBC pellet to a concentration of 100 microCurie/ml. This mixture was incubated in a shaking water bath at RT for 30 min, then again washed 3x with sterile saline. The SRBC were sensitized with rabbit anti-sheep hemolysin at a subagglutinating titer, and incubated in a shaking water bath at 37°C for 30 min. The SRBC were washed 3x with sterile saline, resuspended to 5×10^9 cells/ml, and 0.2 ml was given to each mouse. Mice were sacrificed 1 h later, and radioactivity (counts per minute (cpm)) in various organs was determined using a Gamma 310 counter (Beckman, USA).

20. STATISTICAL ANALYSIS

In all experiments in which parasitaemia was monitored, the mean value of a group of 3-5 samples +/- the Standard Error of the Mean was calculated.

Statistical analysis was performed by using the Student t test (two-tailed). P values of <0.05 were considered significant.

CHAPTER III

THE PHENOMENON OF PASSIVELY TRANSFERRED IMMUNITY

CHAPTER III

THE PHENOMENON OF PASSIVELY TRANSFERRED IMMUNITY

It is clear from the Historical Review that the immune mechanisms involved in mediating parasite elimination at the second crisis are not well understood. In both T-cell- (Brooks & Reed, 1977; Rank et al., 1977; Viens, Targett, et al., 1974) and B-cell- (Vargas et al., 1984) deprived mice, the parasitaemia stabilizes after the initial growth phase, yet elimination of trypanosomes does not occur. These observations suggest that the development of T-cell-dependent humoral immunity is important for host defense, and essential for parasite elimination and cure of the mouse. Indeed, serum from immune hosts passively transferred to naive recipients has been shown to have a transient protective effect on the latter (Vargas et al., 1984; Viens, Targett, et al., 1974) or to be fully protective (with no ensuing parasitaemia) (Albright & Albright, 1982); mice born to normal mothers and nursed by immune ones are also initially completely protected upon trypanosome challenge (Breniere & Viens, 1980). It follows also, that the passive transfer of serum from an immune host to an infected mouse should have the capacity to terminate the infection, and cure the mouse. Reports on the success of this procedure, however, have been equivocal. Although

Taliaferro (1938) reported a curative effect of immune serum when administered to mice infected 3 to 7 d previously, the scant data make it difficult to interpret his exact findings. Similarly, the data on which Bungener (1975b) based his cure of infected mice are somewhat ambiguous. More recently, investigators have been unable to cure an infected mouse with the passive transfer of serum from mice naturally recovered from T. musculi infection (Albright & Albright, 1982; Targett & Viens, 1975; Viens, Targett, et al., 1974). Thus, although fairly good evidence could be obtained for an activity in immune serum that fully or partially protected a naive recipient from becoming infected, a role for humoral immunity in eliminating the parasites in T. musculi infection has been seriously questioned (Albright & Albright, 1982; Viens, 1985).

We chose to begin our studies of the mechanism(s) of parasite elimination by repeating the passive transfer experiments using mice of the C57BL/6 strain.

RESULTS

1. CURATIVE EFFECT OF IMMUNE PLASMA ADMINISTERED DURING THE PLATEAU PHASE

Groups of four to five adult male or female C57BL/6 mice were infected by the intraperitoneal or intravenous

route with 5 x 10^3 - 10^4 trypanosomes in 0.2 ml of buffered saline. The normal course of parasitaemia was followed in one group of mice which received no further treatment (Figure 3.1). The typical phases of parasitaemia were seen, namely, the growth period (0-8 d p.i.), the plateau phase (8-18 d p.i.), and the elimination phase (18-22 d p.i.). Two other groups of mice were treated with a single dose of 0.4 ml of immune plasma (IP) or normal mouse plasma (NMP), respectively, on d 13 p.i. (Figure 3.2a). Although administration of normal plasma had no effect on the course of infection, the parasitaemia cleared completely within 24 h of injection of immune plasma, and the blood remained aparasitaemic. A 0.2 ml dose of IP was ineffective, whereas a 0.8 ml dose produced a result similar to that seen with a 0.4 ml dose (data not shown). IP has been demonstrated to clear parasitaemia in as little as 3 h. Intraperitoneal administration of immune plasma was as effective as intravenous administration (data not shown), but subcutaneous administration was significantly less effective (Figure 3.3). When the IP was heated at 56°C for as little as 30 min, its ability to eliminate parasitaemia in an infected mouse was abolished (Figure 3.2b). Even 0.8 ml of heat treated (HT) IP was ineffective in curing parasitaemia (data not shown).

Intravenous administration of IP at any time during the plateau phase cleared the parasitaemia. Thus, a single 0.4 ml dose of immune plasma was given intravenously to

FIGURE 3.1

Course of parasitaemia in C57BL/6 mice inoculated on day 0 with 10^4 T. musculi. Each point represents the mean value of four to five mice +/- 1 standard error of the mean.

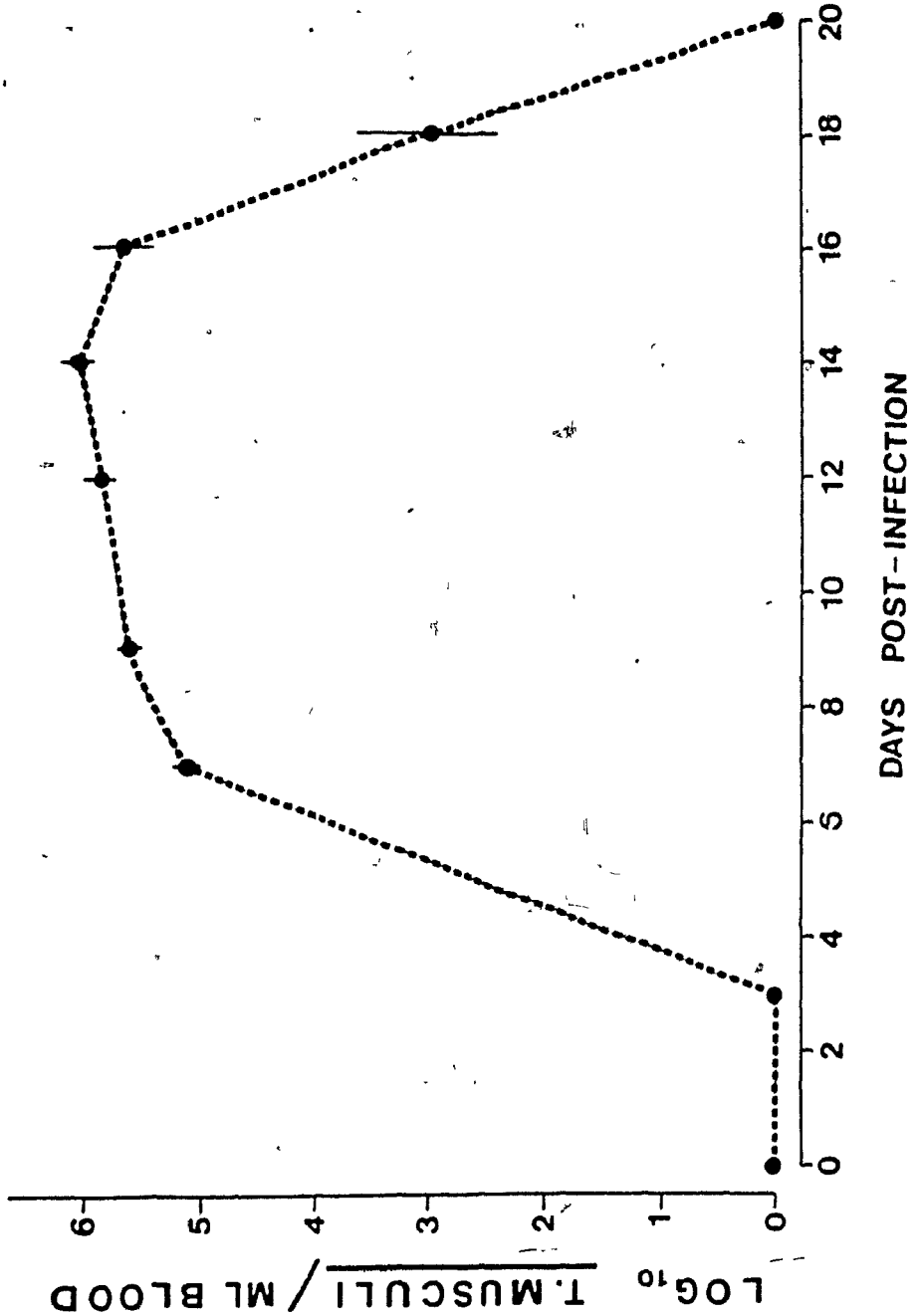

LOG10 T.MUSCULI / ML BLOOD
0
1
2
3
4
5
6
0
2
4
5
8
10
12
14
16
18
20
DAYS POST-INFECTION

FIGURE 3.2.

Effect of administration of immune plasma to infected C57BL/6 mice. (a) On day 13 post-infection (arrow), T. musculi-infected C57BL/6 mice received 0.4 ml of immune plasma (IP,■) or normal mouse plasma (NMP,O) intravenously; shaded line shows no-treatment group. (b) On day 13 post-infection (arrow), T. musculi-infected C57BL/6 mice received intravenously 0.4 ml of immune plasma, heat inactivated for 30 min at 56°C (HI-IP,Δ); shaded line shows no-treatment group. Each point represents the mean value of four to five mice +/- 1 standard error of the mean.

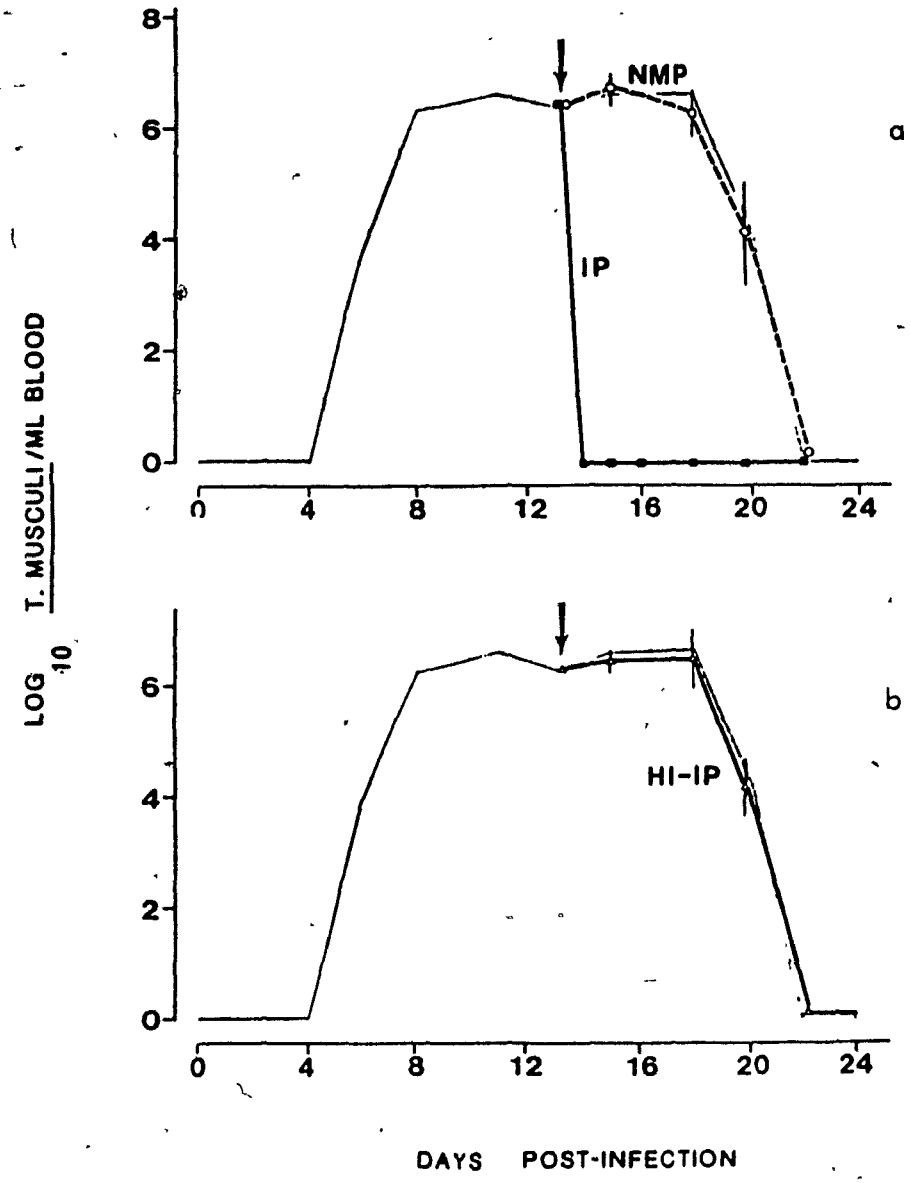
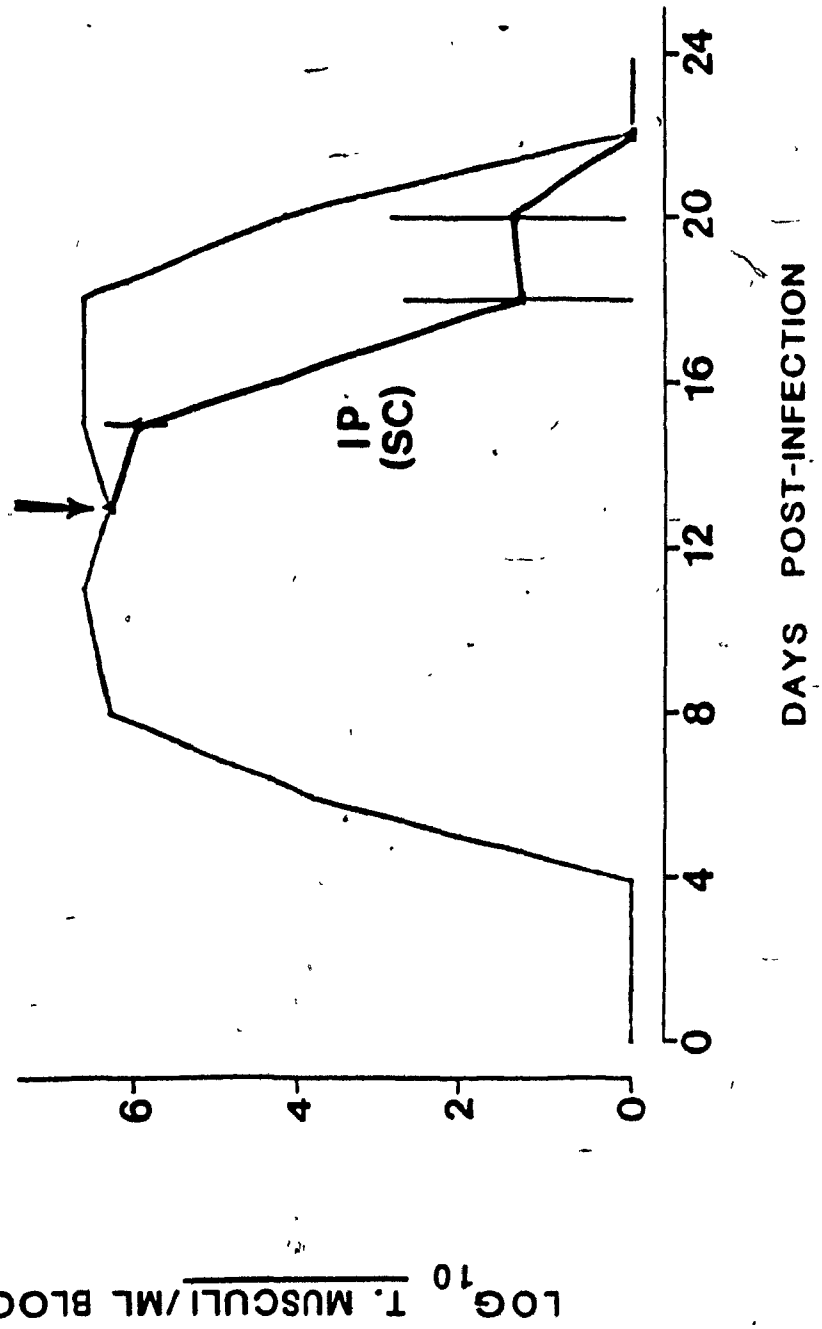

LOG 10 T. MUSCULI/ML BLOOD
8
6
4
2
0
NMP
IP
a
0
4
8
12
16
20
24
6
4
2
0
HI-IP
b
0
4
8
12
16
20
24
DAYS POST-INFECTION

FIGURE 3.3

Effect of route of administration on curative activity of immune plasma. On day 13 post-infection (arrow), T. musculi-infected C57BL/6 mice received 0.4 ml of immune plasma subcutaneously (IP(SC); shaded line shows no-treatment group. Each point represents the mean value of four to five mice +/- 1 standard error of the mean.


IP
(SC)
0
4
8
12
16
20
24
DAYS POST-INFECTION
0
2
4
6
LOG10 T. MUSCULI/ML BLOOD

individual groups of mice 7, 10, 12, 14 and 16 d p.i.; 24 h later, the parasitaemia counts fell from mean values which ranged between 6.3 and 6.6 $\log_{10}$ trypanosomes per ml of blood to zero values, and counts remained at zero in all cases. Normal mouse plasma given to control groups had no effect.

2. KINETICS OF DEVELOPMENT OF CURATIVE ACTIVITY IN DONOR PLASMA FROM INFECTED MICE

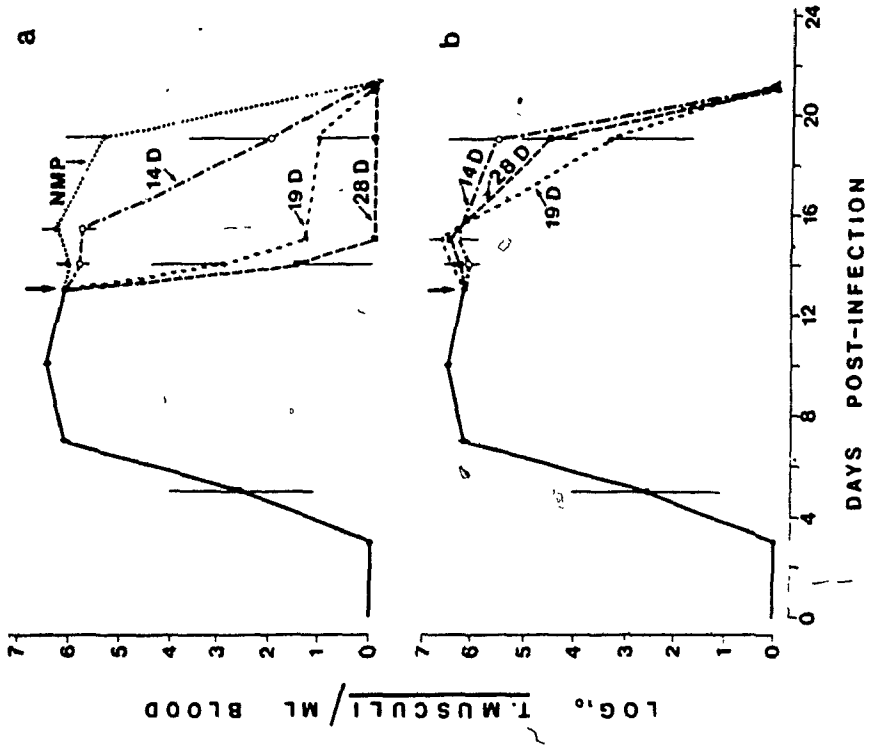
Plasma preparations from donors infected 14, 19 and 28 d previously were given to groups of recipient mice on d 13 of infection. The plasma from donors infected 14 and 19 d previously conferred some ability to eliminate the parasites, whereas the 28-d IP cleared the blood of trypanosomes in 24 to 48 h (Figure 3.4a). When the plasma preparations were heat-treated for as little as 30 min at 56°C and tested as before, the curative activity was lost (Figure 3.4b).

3. PROTECTIVE EFFECT OF IMMUNE PLASMA ADMINISTERED TO NAIVE MICE

Immune plasma was then tested for its ability to protect a naive mouse from developing trypanosomal infection when the plasma was given before inoculation with the parasite. Accordingly, groups of mice were either nontreated,

FIGURE 3.4

Thermolabile curative activity in donor plasma from infected mice that was given to recipient mice during the course of T. musculi infection. C57BL/6 mice were inoculated intraperitoneally with 10^4 trypanosomes and given 0.4 ml of plasma i.v. on day 13 post-infection (arrow). Plasma was taken from mice infected 14 (O), 19 (▲), or 28 (■) days previously, or from noninfected mice (▽). (a) Freshly thawed plasma. (b) plasma was heat-treated at 56°C for 30 min. Each point represents the mean value of four to five mice +/- 1 standard error of the mean.


a
b
NMP
14 D
19 D
28 D
14 D
28 D
19 D
0
1
2
3
4
5
6
7
0
4
8
12
16
20
24
DAYS POST-INFECTION
LOG10 T.MUSCULI/ML BLOOD

or treated with IP, NMP or 30 min heat-treated IP; all of the mice were inoculated with T. musculi 1 h later. IP, both nontreated and heat-treated, completely protected the mice from developing parasitaemia (Figure 3.5). Heat treatment for up to 2 h was equally ineffective in abolishing this protective activity of IP. NMP did not alter the normal course of infection.

4. KINETICS OF DEVELOPMENT OF PROTECTIVE ACTIVITY IN PLASMA DURING THE COURSE OF T. MUSCULI INFECTION

Groups of mice were inoculated with trypanosomes after administration of NMP or plasma taken from mice infected 7, 14, 19 or 28 d previously. A control group of infected mice received no treatment (NT). Parasitaemias were followed in all mice, and the results are shown in Table 3.1. Plasma obtained from mice infected 7 d previously had no effect, but when taken at later times p.i., it lowered or completely abolished the parasitaemia. The longer the duration of infection of the plasma donors, the greater the degree of protection obtained, and IP (28 d) was fully protective, i.e., no parasites were detected in the recipients at any time.

When the above plasma preparations were HT for 2 h at 56°C and tested in the same manner, essentially the same results were obtained as before, with the parasitaemias becoming patent on the same d p.i. When analyzed for

FIGURE 3.5

Protective activity of immune plasma. Course of parasitaemia in C57BL/6 mice inoculated on day 0 with 10^4 trypanosomes 1 h after the following treatments: no treatment (normal,●), 0.4 ml of i.v. normal mouse plasma (NMP,O), 0.4 ml of i.v. immune plasma (IP,■), 0.4 ml of i.v. heat-treated (56^{o}C for 30 min) immune plasma (HT-IP,▲). Each point represents the mean value of four to five mice +/- 1 standard error of the mean.

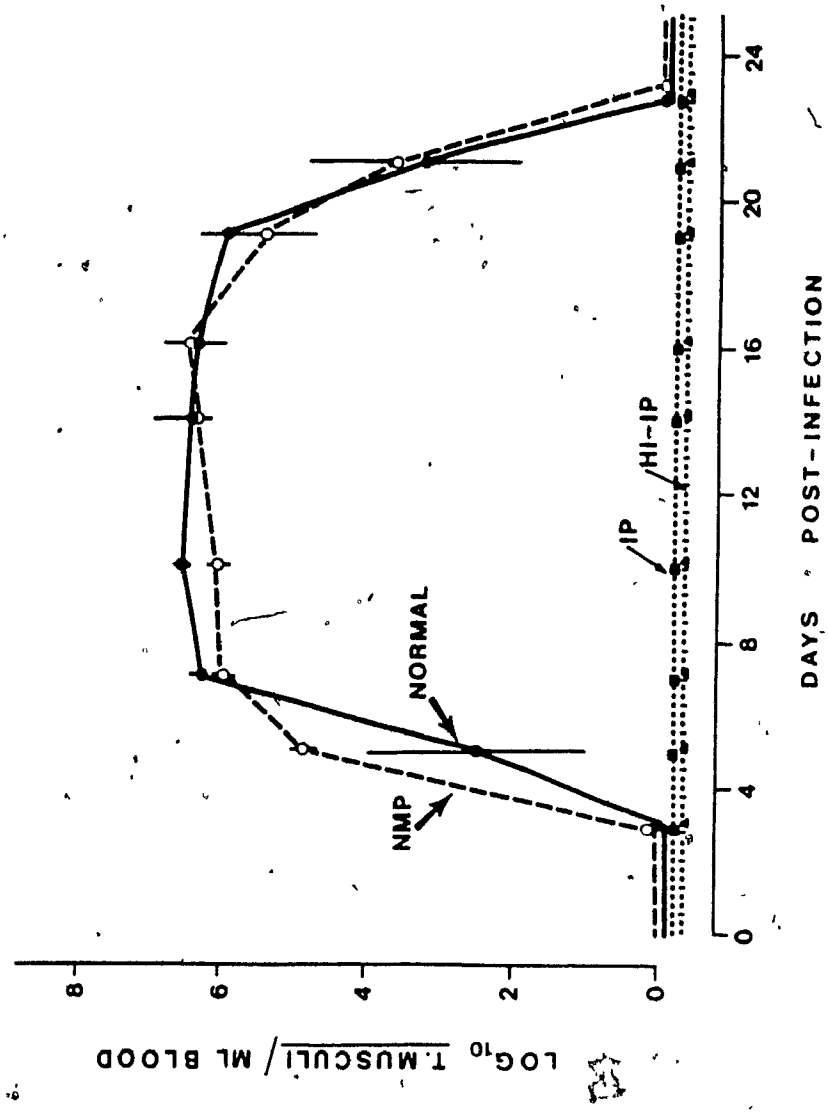

LOG10 T.MUSCULI / ML BLOOD
0
2
4
6
8
DAYS POST-INFECTION
0
4
8
12
16
20
24
IP
HI-IP
NORMAL
NMP

TABLE 3.1: Effect of heat-treatment on protective activity in plasma taken from mice at various times post-infection and used t pre-treat mice prior to infection with T. musculi

Donor plasma used to pre-treat mice[a]	Log_{10} Trypanosomes/ml blood (mean of 4-5 mice ± 1 s.e.m.)						
	5[b]	7	10	12	14	18	20
None	3.23 ± 1.52	4.90 ± 0.02	5.55 ± 0.09	5.55 ± 0.09	5.64 ± 0.06	4.35 ± 0.35	4.70 ± 0.10
7-day	4.00 ± 1.40	4.83 ± 0.11	5.60 ± 0.12	5.67 ± 0.03	5.64 ± 0.06	5.40 ± 0.00	4.70 ± 0.26
7-day, HT	3.08 ± 1.03	3.72 ± 1.24	5.79 ± 0.13	5.70 ± 0.17	5.76 ± 0.24	5.63 ± 0.55	5.26 ± 0.36
14-day	0	3.00 ± 1.00	4.65 ± 0.06	4.79 ± 0.17	5.07 ± 0.08	4.35 ± 0.22	4.13 ± 0.16
14-day, HT	0	3.85 ± 0.09	4.77 ± 0.17	5.17 ± 0.16	4.85 ± 0.16	4.75 ± 0.19	4.70 ± 0.40
19-day	0	0	4.00 ± 0.17	4.16 ± 0.08	4.43 ± 0.22	4.63 ± 0.15	4.30 ± 0.38
19-day, HT	0	0	1.93 ± 1.11	3.16 ± 1.05	3.35 ± 1.13	4.50 ± 0.07	4.50 ± 0.40
28-day	0	0	0	0	0	0	0
28-day, HT	0	0	0	0	0	0	0
NMP	2.77 ± 1.34	4.82 ± 0.06	5.53 ± 0.09	5.65 ± 0.05	5.58 ± 0.06	5.23 ± 0.15	5.18 ± 0.23
NMP, HT	3.90 ± 0.10	4.71 ± 0.18	5.33 ± 0.13	5.40 ± 0.14	5.28 ± 0.12	4.54 ± 0.54	4.15 ± 0.45

[a] Donor plasma was collected from mice 7, 14, 19 and 28 days following infection, as indicated. Half of the pooled plasma sample were heat-treated (HT) at 56°C for 2 hours. A dose of 0.4 ml plasma was given to each recipient mouse i.v., one hour prior to infection with 10^4 trypanosomes i.p. One group received normal mouse plasma (NMP).

[b] Days post-infection

statistical significance, it was found that the numbers of trypanosomes in the blood of mice that had received either freshly thawed or HT plasma were not significantly different ($P > 0.05$). This was true for any day on which the parasitaemia was measured.

5. KINETICS OF DISAPPEARANCE OF PROTECTIVE ACTIVITY OF TRANSFERRED IMMUNE PLASMA

Groups of mice were pretreated with IP at different times before trypanosome infection. IP was only fully protective when given on the day of infection (Figure 3.6). However, partial protection was clearly evident when IP was administered 3 d before infection, and some protective activity remained even when IP was administered 2 weeks before *T. musculi* inoculation, as evidenced by the decreased plateau level of parasitaemia.

6. PROTECTIVE EFFECT OF PLASMA ON INFECTION WITH DIFFERENT DOSES OF T. MUSCULI

Table 3.2 demonstrates that the protective activity of IP was considerably diminished when the infecting dose of trypanosomes was increased by two orders of magnitude. As before, the parasitaemia observed in mice pretreated with heat-treated IP was not significantly different from that seen in mice pretreated with IP ($P > 0.05$) for any day on

FIGURE 3.6

Kinetics of disappearance of transfused protective activity in plasma during the course of T. musculi infection. C57BL/6 mice were pretreated i.v. with 0.4 ml of IP either 14 (●), 7 (■), or 3 days (▲) before infection or on the day of infection. A dose of 10^4 trypanosomes was given i.p. Infected mice receiving no pretreatment served as controls. Each point represents the mean value of four to five mice +/- 1 standard error of the mean.

LOG10 T. MUSCULI/ML BLOOD
DAYS POST-INFECTION
0
1
2
3
4
5
6
7
0
2
4
6
8
10
12
14
16
18
20
22
24
IP DAY 0
IP DAY -3
IP DAY -7
IP DAY -14
INT

TABLE 3.2: Effect of administering plasma to mice immediately prior to infection with different doses of T. MUSCULI

re-Treatment of mice[a]	Inoculating Parasite dose	Log_{10} Trypanosomes/ml blood (mean of 5 mice ± 1 s.e.m.)							
		0[b]	2	4	6	8	14	18	22
NT	10^4	0	0	0.93 ± 0.93	4.55 ± 0.41	6.07 ± 0.15	6.14 ± 0.25	5.37 ± 0.03	0
IP	10^4	0	0	0	0	0	0	0	0
HTIP	10^4	0	0	0	0	0	0	0	0
NT	10^6	0	0.70 ± 0.70	4.98 ± 0.08	5.50 ± 0.14	5.48 ± 0.17	6.54 ± 0.36	0	0
IP	10^6	0	0	2.57 ± 1.29	3.95 ± 1.39	5.23 ± 0.62	5.97 ± 0.52	2.23 ± 2.23	0
HTIP	10^6	0	0	4.79 ± 0.06	4.98 ± 0.19	5.37 ± 0.38	4.30 ± 0.27	1.85 ± 1.85	0

Mice were either non-treated (NT) or injected i.v. with 0.4 ml of immune plasma (IP) or heat-treated immune plasma (HTIP) 1 h prior to i.p. infection with 10^4 or 10^6 parasites.

Days post-infection

which the parasitaemia was measured.

DISCUSSION

The findings described above demonstrate that passive transfer of immune plasma from cured mice, when given in adequate amounts (0.4 ml) at any time during the plateau phase, brings about a permanent cure of the blood parasitaemia in infected recipients. This curative activity presumably represents the same immune process whereby elimination of parasites develops in the final phase of normal infection. Two reasons for the success of our experiments in curing parasitaemia are proposed. First, in some of the earlier experiments, heat-inactivated serum reportedly was used (Targett & Viens, 1975); also, the serum was given subcutaneously (Targett & Viens, 1975; Viens, Pouliot & Targett, 1974). Second, our experiments were done in genetically resistant C57BL/6 mice (Albright & Albright, 1981b; Magluilo et al., 1983) in which the parasitaemia plateau is 1-2 $\log_{10}$ lower than in the genetically susceptible mouse strains used by others. Albright & Albright (1982) collected sera on d 8, 12, 16 and 20 of *T. musculi* infection and administered them to infected recipient mice. They were, however, unable to effect any form of cure by passive transfer of any of these sera. The discrepancy between their results and the present findings

may be explained by considering that the efficacy of IP in curing infection depends on the relative amounts of curative factor therein and the level of parasitaemia. MacAskill et al., in studies on T. brucei (1981) and T. congolense (1983; Whitelaw et al., 1983), suggested that acute fatal infections of trypanosomiasis are the result of the inability of the host to achieve effective levels of circulating antibody against a rapidly replicating trypanosome clone. Indeed, they too were unable to cure T. brucei-infected mice by passive transfer of hyperimmune serum, attributing the failure to inadequate levels of antibody. Their results indicate that the trypanotolerance of C57BL/6 mice (to T. congolense) depends on the superior humoral response of this mouse strain. Albright & Albright (1982), on the other hand, performed their serum transfer experiments in A/J and C3H mice, known to have higher plateau levels of parasitaemia than C57BL/6 mice (Albright & Albright, 1981b). Moreover, their injections were performed on d 7 and 10, and with smaller amounts of serum. Presumably, the transferred plasma or serum is more efficacious when given later in the infection, at a time when endogenously produced curative activity would have an additive effect. Incidentally, the experiments of Viens and his group were performed in CBA mice, also genetically susceptible to T. musculi, and therefore characterized by higher parasitaemias.

The results presented in Figure 3.4 demonstrate the

gradual appearance of the curative activity during the course of infection. As mentioned above, indirect evidence strongly suggests that the elimination process is brought about by an immunoglobulin, particularly since parasitaemia is not eliminated in B-lymphocyte-deprived mice; such mice have normal T-cell function but lack B-cell function and fail to develop specific anti-trypanosomal antibodies (Vargas et al., 1984). Thus, it seems reasonable to assume that the curative activity observed in IP is due to a specific anti-trypanosomal antibody; yet, the curative mechanism apparently is not a complement-mediated lytic process, since trypanosome elimination occurs spontaneously in mice deficient in complement component C5 (Dusanic, 1975a).

The class of immunoglobulin purportedly mediating cure of parasitaemia is not apparent from these experiments, but is presumably T-cell dependent (Brooks & Reed, 1977; Rank et al., 1977; Viens, Targett, et al., 1974). Immunoglobulin M (IgM) seems an unlikely candidate, particularly since its production was found to be normal in T-cell-deprived infected mice (Brooks et al., 1982; Viens, Targett, et al., 1974).

The curative activity is unstable when heated and less effective when administered subcutaneously, suggesting that it could be homocytotropic. Reaginic antibody of the IgE class is distinctive in being thermolabile and retained in the tissues (Lehrer & Bozelka, 1982). However, until

purified, it cannot be assumed that the putative immunoglobulin in question is intrinsically thermolabile. Indeed, the heat lability of the curative activity immediately suggests that it is complement which is destroyed by heat treatment (see Chapter IV). It is possible that the curative activity is mediated by one of the subclasses of IgG, all of which have been implicated in antibody-dependent cellular cytotoxicity and antibody-mediated phagocytosis (Ralph et al., 1980). Certain subclasses of human (Solomon et al., 1978) and mouse (Banda et al., 1983) IgG are sensitive to the proteolytic activity of proteases; for example, mouse IgG1 and IgG3 are cleaved by macrophage-derived elastase (Banda et al., 1983). The loss of curative activity observed upon heating or subcutaneous administration of IP may be a result of proteolytic cleavage of an IgG subclass, rendering it incapable of eliminating parasites, e.g. through an Fc receptor-mediated mechanism.

The protective activity observed in IP (Figure 3.5) is presumably the same as that reported previously by others, and is attributed to the activity of specific anti-trypanosomal antibody. Immune serum administered shortly before inoculation with parasites provided either partial protection (Taliaferro, 1938; Viens, Targett, et al., 1974), or else completely prevented the establishment of an infection (Albright & Albright, 1982). This putative immunoglobulin is stable to heat treatment, suggesting that

it is different from that which mediates the curative activity. In a manner similar to the curative activity, the protective activity develops over a period of time (Table 3.1). Here also, the protective activity is not labile to heat treatment. The fact that there is still some protective activity 2 weeks after IP injection (Figure 3.6) suggests a fairly long biological half-life of the protective activity.

The above results demonstrate the presence of an apparently heat-labile curative activity, and an apparently heat-stable protective activity in IP. Although the two activities may be distinguished on the basis of their sensitivity to heat treatment, this does not necessarily imply that the factors responsible are different entities. Rather, the observed differential heat sensitivity of the activities may be a quantitative phenomenon. It is likely that heat inactivation is a gradual process, in which more and more of the factor is rendered ineffective with increasing exposure to heat. If so, it might be expected that after 30 min of treatment, there could still be some residual amount of active factor present, sufficient to eliminate 10^4 trypanosomes and protect the host, but insufficient to cure the latter of the plateau load of 10^6 parasites. Indeed, when mice were infected with 10^6 trypanosomes, the protective effect previously seen even with non-HTIP was severely diminished (Table 3.2). Again, this may be explained by a lack of sufficient factor

to cope with the higher number of parasites.

In summary, the present chapter has delineated the phenomenon of passively transferred immunity to mice infected with Trypanosoma musculi. The curative activity is observed when 0.4 ml of IP is administered to mice at any time during the plateau phase of infection; parasitaemia clears within 24-48 h (although it may occur in as little as 3 h). This activity appears gradually over the course of infection and is labile to heat treatment. The protective activity may be seen when mice are treated with IP prior to inoculation with T. musculi; these mice do not develop a parasitaemia. The protective activity appears gradually over the course of infection, has a fairly long biological half-life (5 to 6 d), and is apparently not labile to heat treatment.

With an initial evaluation of the kinetic parameters of the biological activities of curing and protection, the next logical step was to determine the molecular nature of these activities.

CHAPTER IV

THE ROLE OF COMPLEMENT

CHAPTER IV

THE ROLE OF COMPLEMENT

With an appreciation of the phenomenon of IP-induced trypanosome clearance, we set out to investigate the molecular components involved, as well as the mechanism(s) by which parasites are eliminated from the blood. The heat lability of the curative activity immediately suggested to us that the curative activity was mediated primarily by complement, since the conditions used for heat treatment are exactly those used routinely for the inactivation of complement. We therefore attempted to restore the curative activity to HTIP by reconstitution with fresh complement. We then investigated the effect of complement depletion on the ability of passively transferred IP to mediate cure.

RESULTS

1. RECONSTITUTION OF HTIP WITH COMPLEMENT

To ascertain whether the heat lability of IP was related to complement inactivation, HTIP was reconstituted with fresh NMP as a source of complement and tested for curative activity. Parasitaemia was cleared within 24 h in

control groups receiving non-HTIP, whereas no such clearance occurred in mice receiving HTIP, even when supplemented with NMP (Table 4.1). Thus abolition of curative activity in IP by heat treatment is apparently due to inactivation of a plasma component other than complement.

2. DECOMPLEMENTATION OF MICE BEFORE IP ADMINISTRATION

Cobra venom factor (CVF) is known to bring about in vivo depletion of complement component C3 as well as late-acting components (Cochrane et al., 1970; Bottger et al., 1986). Groups of mice received CVF before IP administration on d 12 of T.musculi infection, and parasitaemia was subsequently monitored. Control mice receiving IP alone cleared the infection within 24 h, whereas mice receiving IP preceded by CVF failed to eliminate the trypanosomes (Table 4.2). Analysis of plasma by Ouchterlony double immunodiffusion analysis revealed that the plasma from CVF-treated mice was severely depleted of C3, showing only a faint line at a dilution of 1:2. Plasma from non-CVF-treated mice, on the other hand, showed a line at a dilution of 1:64 (data not shown).

DISCUSSION

As detailed in the previous chapter, the in vivo

TABLE 4.1: Effect of supplementing heat-treated plasma with complement

Treatment[a]	Log_{10} Trypanosomes/ml blood ± 1 s.e.m.		
	0[b]	1	2
IP (7)	5.61 ± 0.07	0	0
HTIP (5)	5.80 ± 0.13	5.33 ± 0.32	5.47 ± 0.41
HTIP + NMP (11)	5.66 ± 0.07	5.33 ± 0.16	5.75 ± 0.13
IP + HTNMP (4)	5.61 ± 0.05	0	0

[a] Mice were treated with a 0.4 ml i.v. injection of immune plasma (IP) or normal mouse plasma (NMP). 0.4 ml of heat treated plasma (HTIP or HTNMP) was mixed with 0.4 ml of non-heat-treated plasma (NMP or IP respectively) and administered in a 0.8 ml i.v. dose. All injections were performed on day 12 post-infection. The number in parentheses indicates the number of mice per group.

[b] Days post-plasma administration

TABLE 4.2: Effect of CVF treatment on curing activity of IP

Treatment[a] (no. of mice)	Log_{10} Trypanosomes/ml blood ± 1 s.e.m.		
	0	1	2
NT (7)	5.68 ± 0.08	0	0
CVF (5)	5.78 ± 0.08	5.87 ± 0.18	6.06 ± 0.26

[a] Mice were infected with T. musculi and either NT or treated with 20 U of CVF. IP was administered on day 12 p.i.

curative activity of IP is sensitive to heat treatment. The question naturally arises: what is the nature of the lability? Is the activity (presumably antibody) intrinsically sensitive to heat treatment, or is the labile factor an accessory component necessary for elimination (i.e. complement)? The addition of fresh NMP as a source of complement did not reverse the effects of heat treatment, indicating that heating likely affected some other component (Table 4.1). This leads to the conclusion that the activity is intrinsically heat-labile (see following chapter).

The results presented in Table 4.2 demonstrate that, although the curative activity (presumably antibody) of IP is necessary to bring about trypanosome elimination, it alone is not sufficient. Our data indicate that C3 must be present for IP to bring about clearance of parasitaemia. Jarvinen & Dalmasso (1977a) have shown that rendering mice C3-deficient during the plateau phase prolongs T. musculi infections by interfering with parasite elimination. Similar findings were obtained with T. brucei by MacAskill et al. (1980), who showed that CVF treatment prevented immune clearance in passively immunized mice. Although CVF also depletes the later complement components, it is unlikely that these are involved in parasite elimination; Dusanic (1975a) and Jarvinen & Dalmasso (1977a) have shown that the course of T. musculi infection in inbred, C5-deficient mice is not significantly different from that in normocomplementemic mice and have concluded that

complement-dependent, antibody-mediated cytolysis is probably not a major mechanism for the elimination of T. musculi by the infected mouse. Again, MacAskill et al. (1980) demonstrated similar findings for T. brucei infections in mice. Jones & Hancock (1983) reached the same conclusion with T. rhodesiense using mouse strains B10.D2/o (C5-deficient) and B10.D2/n (C5-sufficient), stating that the lytic function of complement is not necessary for controlling trypanosomiasis. Ferrante & Jenkin (1978) showed that T. lewisi was not lysed in diffusion chambers implanted in the peritoneal cavity of rats immune to this parasite (where, presumably, complement and antibody could freely attach to the trypanosome surface). Finally, IP obtained from C5-deficient mice is able to cure C5-deficient recipients (see Chapter VIII). Thus, even though lysis may be demonstrated in vitro (Viens et al., 1983), these findings argue against Taliaferro's original suggestion (1938) that trypanosome infections are terminated by a complement-mediated, lytic event.

If this is the case, how is it that CVF treatment prevents IP-induced trypanosome elimination? A likely explanation relies on the fact that complement component C3 may act as an opsonin. This finding, together with the assumption that the curative activity is antibody in nature, suggest a murine model for the elimination of T. musculi based on the cooperative interactions of both antitrypanosomal antibody and C3. Other data from our

laboratory and elsewhere (Albright & Albright, 1982; Vincendeau et al., 1981, 1986; Vincendeau, 1986; Ferrante, 1986) suggest that a cell (macrophage?) is involved in the elimination process (see Chapter IX). For instance, it is well known that antigens complexed with both antibody of the IgG class and C3b form complexes that bind avidly to macrophages by means of multiple Fc and C3b receptor interactions (Hahn & Kaufmann, 1981; King et al., 1986). Indeed, if the assumption is made that opsonization of the trypanosome represents the first step in its elimination, it is conceivable that parasite-bound complement molecules, notably C3 and its derivatives, assist parasite-specific immunoglobulin in improving cell-cell interactions, ultimately leading to parasite elimination (Erdei et al., 1984). Blackwell et al. (1985) have suggested that the binding of parasites to macrophages is dependent on C3 receptors at the macrophage surface, indicating that $\overline{C3}$ on the parasite surface might improve binding. Finally, Griffin & Mullinax (1985) propose a model of phagocytosis in which activation of macrophage C3 receptors is the primary factor responsible for promoting phagocytosis of opsonized microorganisms.

A number of researchers have demonstrated the requirement for complement in the <u>in vitro</u> and <u>in vivo</u> killing of a number of trypanosome species: <u>T. lewisi</u> (Ferrante & Jenkin, 1978; 1979); <u>T. rhodesiense</u> (Greenblatt et al., 1983); <u>T. brucei</u> (Holmes et al., 1979; MacAskill et

al., 1981); and T. musculi (Ferrante 1986). Interestingly, Townsend and Duffus (1985) showed that complement does not seem to be required for in vitro killing of T. theileri. In addition, Schmitz et al. (1984) found that a complement-mediated cytotoxic reaction does not play a significant role in terminating T. congolense infection.

Finally, the necessity for C3 opsonization has been well documented in the immune response to other parasites. Stefani et al. (1983) have found that C3 acts to facilitate the antibody-dependent cellular cytotoxicity directed against T. cruzi. Aime et al. (1984) noted the requirement for complement in the granulocyte-mediated clearance of the microfilaria Dipetalonema viteae. Finally, Egwang et al. (1985) refer to the importance of C3 in termination of infections caused by Nippostrongylus brasiliensis as well as Schistosoma mansoni. It is, therefore, clear that the proposal of complement involvement in the resolution of T. musculi infection is a reasonable one.

CHAPTER V

IN VITRO ASSAY FOR CURATIVE ACTIVITY

CHAPTER V

IN VITRO ASSAY FOR CURATIVE ACTIVITY

The previous chapters have demonstrated that IP is able to effect rapid clearance of parasitaemia when administered *in vivo* to recipient mice during the plateau phase of infection. Furthermore, the component of IP necessary for elimination is heat-labile (likely antibody in nature), and requires complement in order to be active. Assuming that the observed rapid clearance of parasitaemia is a reflection of the natural elimination process, the *in vivo* assay affords an opportunity to examine other factors involved in mediating parasite removal. Impairment of the fixed mononuclear phagocyte system (see Chapter IX) did not prevent the IP-induced clearance of trypanosomes, suggesting that parasite elimination might take place in the blood stream itself. We therefore chose to examine the effect of IP on trypanosome-infected blood *ex vivo*.

The present chapter describes an *in vitro* assay designed to enable further analysis of components involved in the process of parasite elimination. The assay is rapid, economical, and correlates well with all our *in vivo T. musculi* studies.

RESULTS

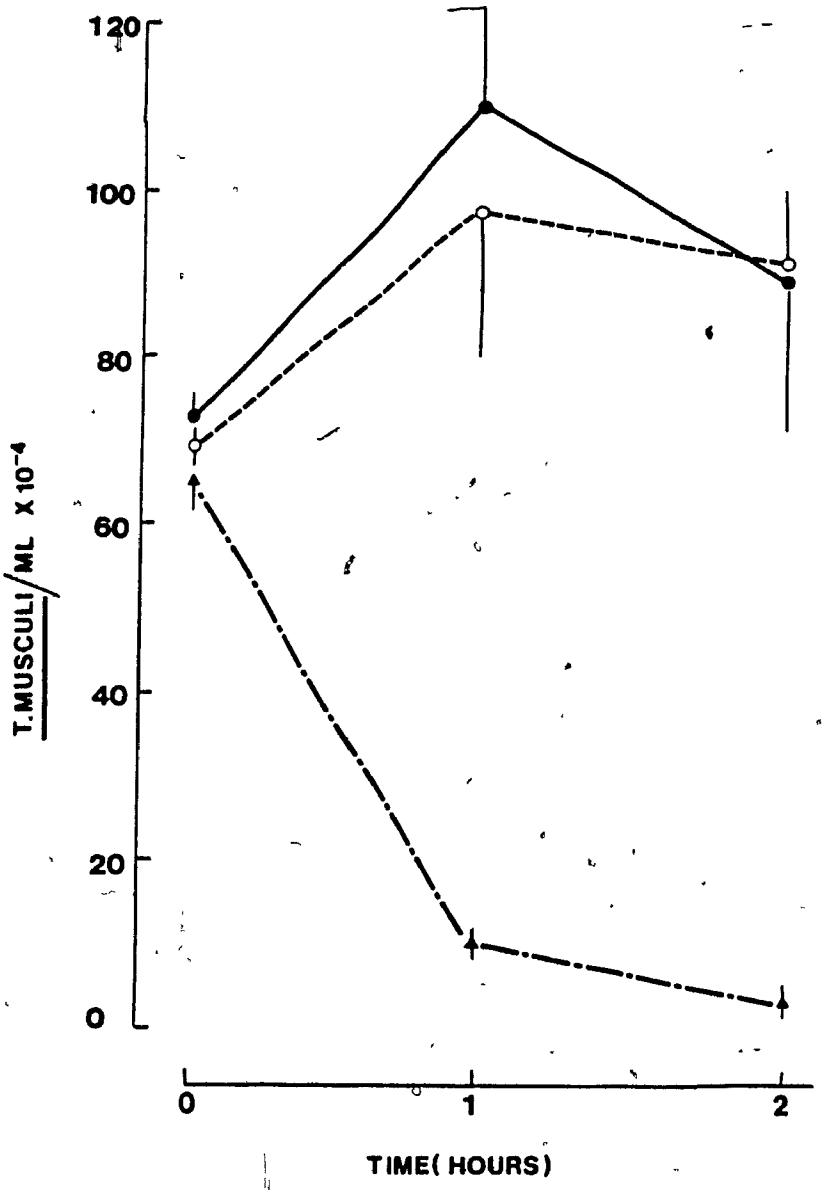
Blood collected from trypanosome-infected C57BL/6 mice at different times during the plateau phase of infection (10-15 d p.i.) was tested in the *in vitro* assay (page 57). It was determined that the particular day chosen for bleeding did not affect the results of the assay, as long as parasitaemias were in the range of 40-80 x 10^4 parasites/ml blood. The trypanosome numbers recorded below are less than the actual parasitaemias by a factor of approximately 0.8, as the infected blood was diluted 4:1 with plasma.

1. PHENOMENON OF IN VITRO IP-INDUCED TRYPANOSOME ELIMINATION

Figure 5.1 shows the results obtained from a representative *in vitro* assay of the curative activity present in NMP, IP and HTIP. Whereas initially the parasite counts in all wells are approximately the same, by 1 h there is an increase in the number of trypanosomes in those wells to which NMP has been added; by 2 h, the number of trypanosomes has returned to the initial value. This result has been reproduced on at least ten separate occasions, with the trypanosome count increasing by 30-70% after 1 h, and returning to the original value by 2 h. In contrast, addition of IP to the wells leads to a highly significant

FIGURE 5.1

Phenomenon of *in vitro* IP-induced trypanosome elimination. Fractions (0.05 ml) of NMP (●), IP (▲), or HT-IP (O) obtained from C57BL/6 mice were added to triplicate aliquots of 0.2 ml of *T. musculi*-infected C57BL/6 blood, and parasite numbers were determined 1 and 2 h later. The data shown are representative of numerous experiments (see text). Each point represents the mean value of a triplicate sample +/- 1 standard error of the mean.


120
100
80
60
40
20
0
T.MUSCULI/ML X 10⁻⁴
0
1
2
TIME(HOURS)

($p < .001$) reduction in the number of parasites after 1 h, a reduction that is maintained at 2 h. Many dead trypanosomes are visible in the blood sample during enumeration under the microscope (Figure 5.2). This result has been reproduced at least twenty times, with an initial (1 h) decrease of 60-95% of the original parasite count, followed by either a stabilization or a further decrease in trypanosome number at 2 h. The variation in the number of parasites removed initially appears to depend on the batch of IP used. Finally, heat treatment of IP abolishes its ability to effect any parasite removal; there is no significant difference between the trypanosome counts in wells to which NMP or HTIP have been added, although, in all experiments performed to date, the 1 h rise is less for HTIP wells than for NMP wells.

2. DETAILED TIME COURSE OF IN VITRO TRYPANOSOME ELIMINATION

Figure 5.3 presents a more detailed examination of the changes in trypanosome number which occur over the first hour of the *in vitro* assay. In those wells to which IP is added, the trypanosome number does not change immediately; it is only after 30 min ($p < .02$) that a decrease is first observed, a decrease that is continued in a linear fashion. In NMP wells, the parasite count increases substantially over the first 20 min, after which it stabilizes and then

FIGURE 5.2

Trypanosoma musculi attached to a cell in the in vitro assay (600x, Diffquik stain)

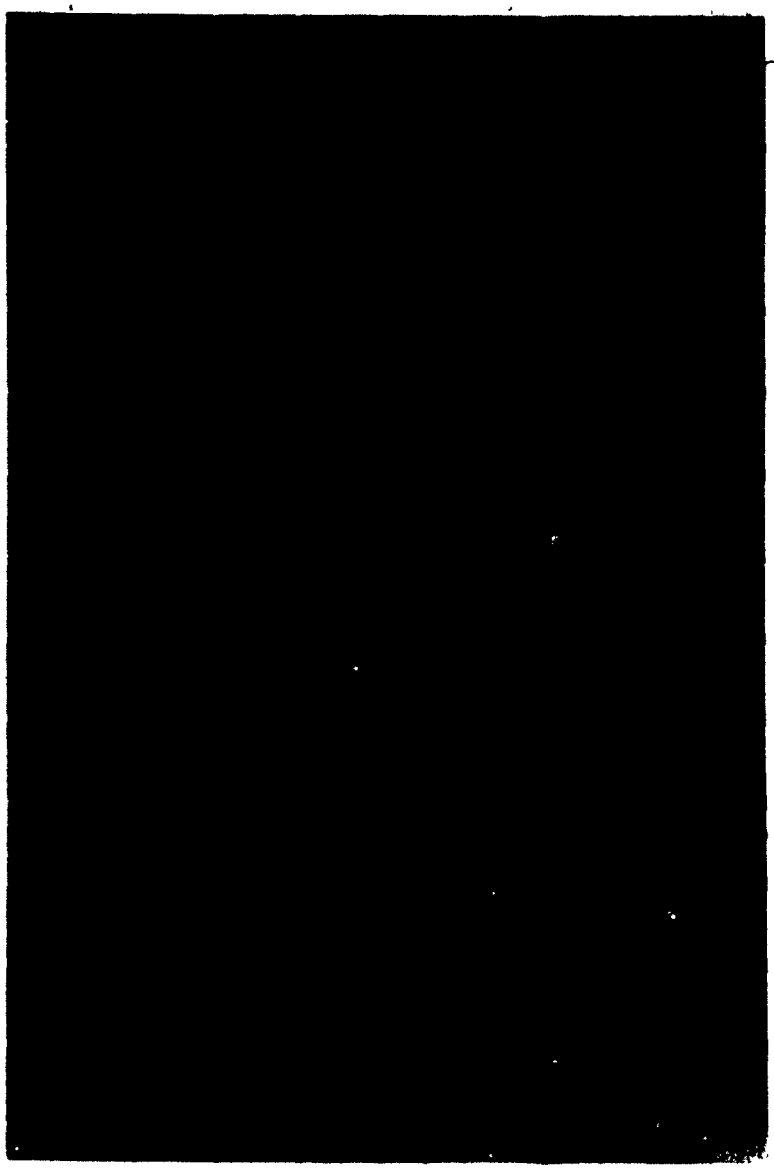
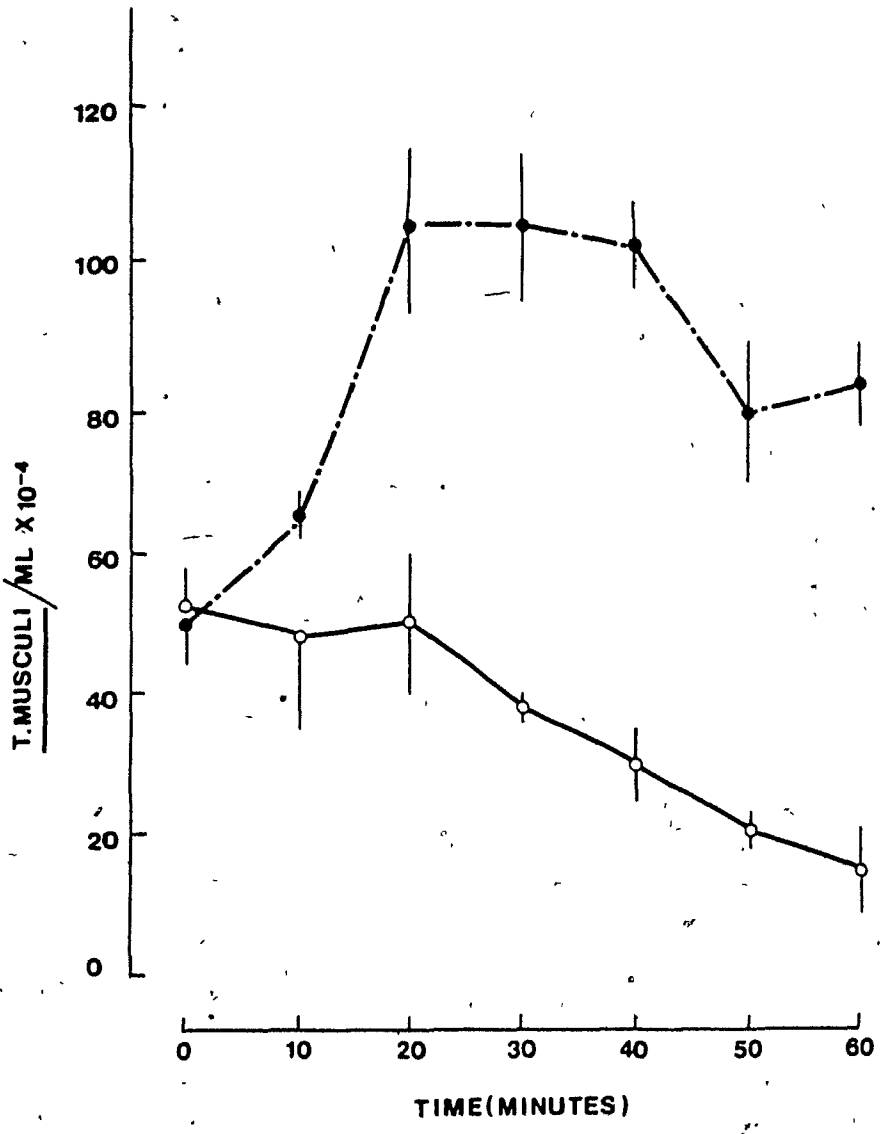


FIGURE 5.3

Time course of *in vitro* trypanosome elimination. NMP (●) or IP (O) was used in the *in vitro* assay as described in the text. Each point represents the mean value of two triplicate samples +/- 1 standard error of the mean.


120
100
80
60
40
20
0
T.MUSCULI /ML X 10⁻⁴
0
10
20
30
40
50
60
TIME(MINUTES)

gradually decreases after 40 min.

3. DOSE-RESPONSE OF IP IN THE IN VITRO ASSAY

The results depicted in Figure 5.4 demonstrate that elimination of trypanosomes is quite sensitive to the amount of IP present. Non-diluted IP shows the characteristic ability to reduce trypanosome number. A 1/2 dilution of IP, however, results in a diminished, although still significant ($p < .025$), ability to eliminate trypanosomes. Further dilution of IP to 1/4 or 1/8 completely abrogates the trypanosome elimination effect.

4. EFFECT OF CVF TREATMENT OF MICE (PRIOR TO BLOOD COLLECTION) ON PARASITE ELIMINATION

Blood used for this experiment (Figure 5.5) was obtained from mice pre-treated with CVF (to deplete complement). In this assay, IP was unable to effect any reduction in trypanosome number, even after 2 h. NMP and HTIP addition showed characteristic changes in trypanosome number over the 2 h period.

FIGURE 5.4

Dose-response of IP in the in vitro assay. The assay was performed with the following different dilutions of IP in phosphate-buffered saline: nondiluted (▲), 1/2 (O), 1/4 (●), and 1/8 (■). Each point represents the mean value of two triplicate samples +/- 1 standard error of the mean.

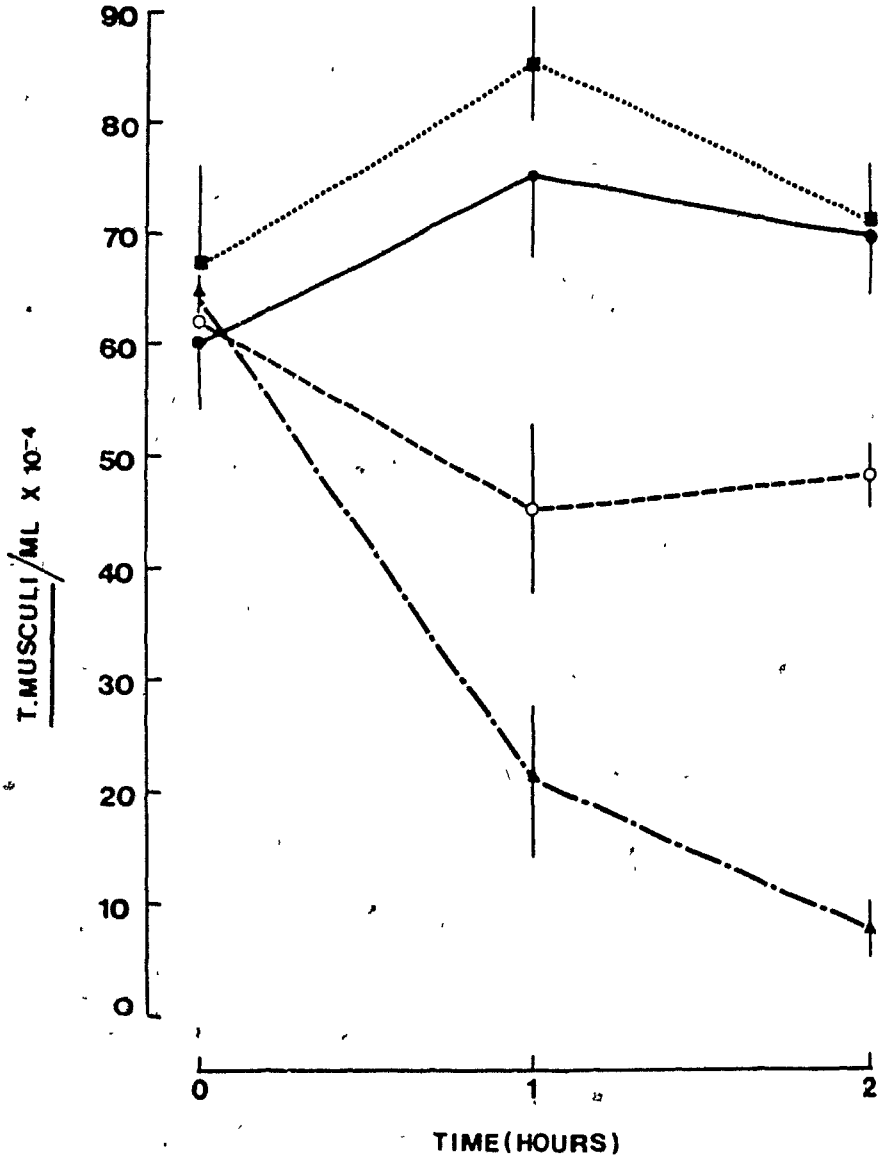
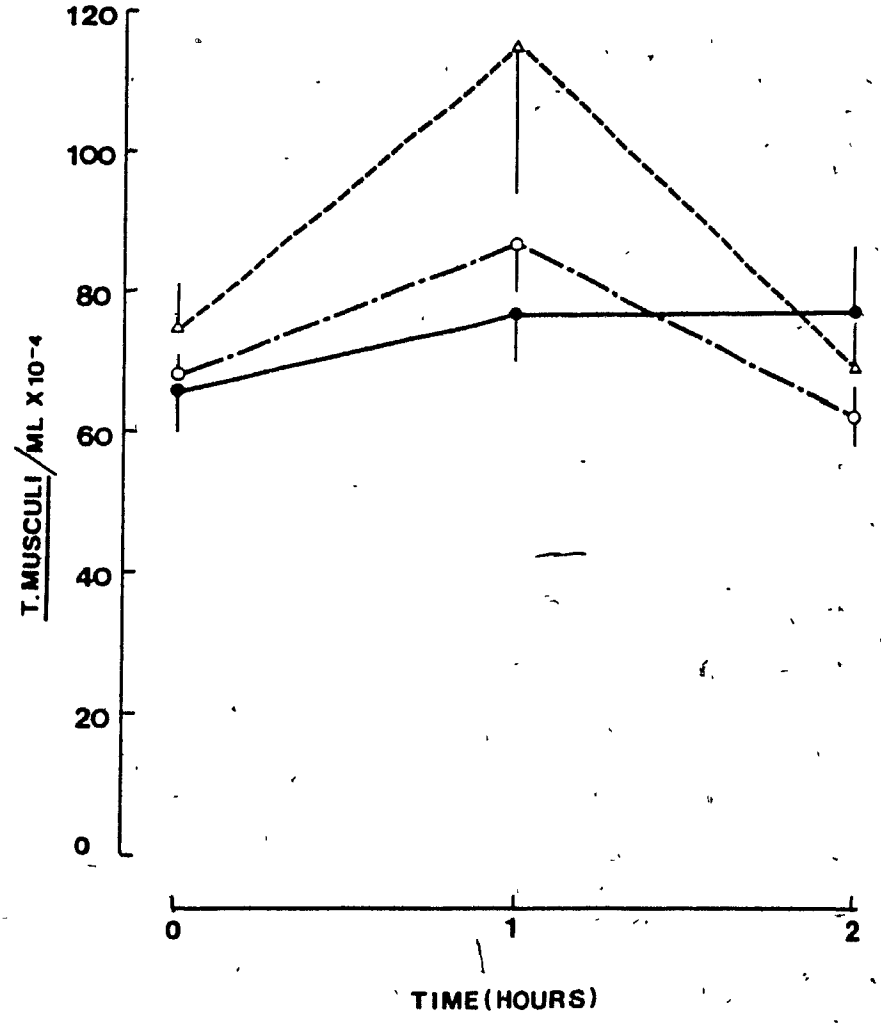

90
80
70
60
50
40
30
20
10
0
T.MUSCULI/ML X 10⁻⁴
0
1
2
TIME(HOURS)

FIGURE 5.5

Effect of CVF treatment of infected blood on activity of IP in the *in vitro* assay. Mice were treated with CVF prior to collection of *T. musculi*-infected blood. This blood was used in the *in vitro* assay with NMP (Δ), IP (●), and HTIP (○). Each point represents the mean value of two triplicate samples +/- 1 standard error of the mean.


120
100
80
60
40
20
0
T. MUSCULI/ML X10⁻⁴
0
1
2
TIME (HOURS)

DISCUSSION

Chapter III delineated the phenomenon of IP-induced T. musculi clearance which likely reflects the process of trypanosome removal during the elimination phase of infection. The present chapter describes an in vitro assay which demonstrates the same phenomena observed in vivo, viz. a parasite count which falls rapidly in the presence of IP but not NMP. The ability of IP (and not NMP) to induce a rapid decrease in parasite number (Figure 5.1) exactly parallels our earlier in vivo findings. More important, however, is the complete abrogation of in vitro assay activity upon heat treatment of IP. The fact that heat treatment also abolishes the curative activity of IP in vivo strongly suggests that the in vitro assay reflects this IP-induced trypanosome clearance.

The results shown in Figure 5.3 reveal that the IP-dependent decrease in trypanosome number is not immediate; approximately 30 min must elapse before a significant decrease is detected, following which the parasite count falls in a linear fashion. This suggests that the trypanosome elimination process requires a certain period of time to become activated, part of which is presumably devoted to opsonization of the parasite prior to elimination. It is not surprising that IP has such a rapid effect -- even in vivo IP has been shown to effect clearance in as little as 3 h in some cases (page 74).

In vivo, IP-induced trypanosome clearance is exquisitely sensitive to the amount of IP administered; whereas 0.4 ml effects complete clearance, 0.2 ml has no demonstrable effect on the course of infection (page 74). Thus, IP-induced clearance seems to be an all-or-none effect. In the in vitro assay (Figure 5.4), a 1/2 dilution of IP is significantly less effective than non-diluted IP. Dilutions of 1/4 or 1/8 have no activity whatsoever in decreasing the number of trypanosomes.

Cobra venom factor (CVF) has been used as an effective decomplementing agent (Cochrane et al., 1970; Bottger et al., 1986). Chapter IV showed that CVF treatment of infected mice prior to IP administration prevents IP-induced trypanosome clearance in vivo. When blood used in the in vitro assay was obtained from mice previously treated with CVF, IP was no longer able to mediate a reduction in trypanosome number (Figure 5.5). Thus, in parallel with the in vivo results, it seems that complement is necessary for in vitro assay activity to be observed.

The reason for the increase in the number of trypanosomes 1 h after NMP addition is unclear. It is not likely to reflect parasite reproduction, as the doubling time for trypanosomes, 6 h 40 min to 14 h 20 min in vitro (Vincendeau, 1986), is significantly longer than the total assay time (2 h). It is possible that a proportion of the trypanosomes in infected blood is initially sequestered by attachment to a) blood cells, b) each other or c) the assay

plate surface, and that a factor in NMP promotes their release, leading to the observed increase in number. In the case of IP, this effect might be masked by its anti-trypanosome activity. Regardless of the actual mechanism of the NMP-induced rise in trypanosome number, the importance of the *in vitro* assay lies in the clear difference in the activities of IP and NMP.

In summary, the *in vitro* assay correlates exactly with the observed *in vivo* phenomena. Since the *in vitro* assay involves minimal manipulation of components, it seems reasonable to assume that the activity of IP measured in the *in vitro* assay is indicative of its ability to effect trypanosome removal *in vivo*. There are three major advantages to using the *in vitro* assay as an initial step in evaluating the curative activity of an unknown plasma sample. First, the *in vitro* assay is rapid: whereas it is usually necessary to wait 18-24 h to determine whether a plasma sample is curative *in vivo*, results of the *in vitro* assay are obtained in 2 h. Second, the *in vitro* assay is economical: an *in vivo* assay uses 0.4 ml of plasma injected into one mouse; the *in vitro* assay requires 0.05 ml of plasma, and a single mouse usually provides enough blood for 5 wells in an *in vitro* assay. Finally, the *in vitro* assay offers the opportunity to examine the individual components involved in trypanosome elimination, using purified trypanosomes (Lanham & Godfrey, 1970), a defined antibody preparation, complement components, and defined cell

populations. This should lead to a better understanding of the mechanism of trypanosome removal at the elimination phase.

CHAPTER VI

THE ANTIBODY NATURE OF THE ANTI-TRYPANOSOMAL ACTIVITY OF IMMUNE PLASMA

CHAPTER VI

THE ANTIBODY NATURE OF THE ANTI-TRYPANOSOMAL ACTIVITY OF IMMUNE PLASMA

Given an initial evaluation of the kinetic parameters of the biological activities of cure and protection, we next proceeded to examine the molecular nature of these activities. The fact that immunity could be conferred by simple plasma transfer implies that a humoral factor in IP is involved in mediating parasite elimination. From the discussion of the preceding chapter, the most likely candidate for the mediator of protective and curative immunity is antibody in nature. We therefore attempted to purify an immunoglobulin fraction of IP by molecular sieve chromatography. Following the demonstration that the activities resided in a fraction with molecular weight of less than 200,000, we took advantage of an affinity chromatography technique proposed by Ey et al. (1978) and modified by Seppala et al. (1981) for the isolation of IgG subclasses with Protein A-Sepharose. Protein A is a constituent of the outer cell wall of Staphylococcus spp., and it has the capacity to bind to the Fc portion of IgG (but no other immunoglobulin subclasses). The different subclasses of IgG bind to Protein A with different affinities, and as demonstrated by Ey et al. (1978), buffers

of decreasing pH may successively elute the different subclasses from Protein A which has been covalently linked to a Sepharose support matrix. Using this technique we have investigated the curative and protective activities.

RESULTS

1. MOLECULAR SIEVE CHROMATOGRAPHY

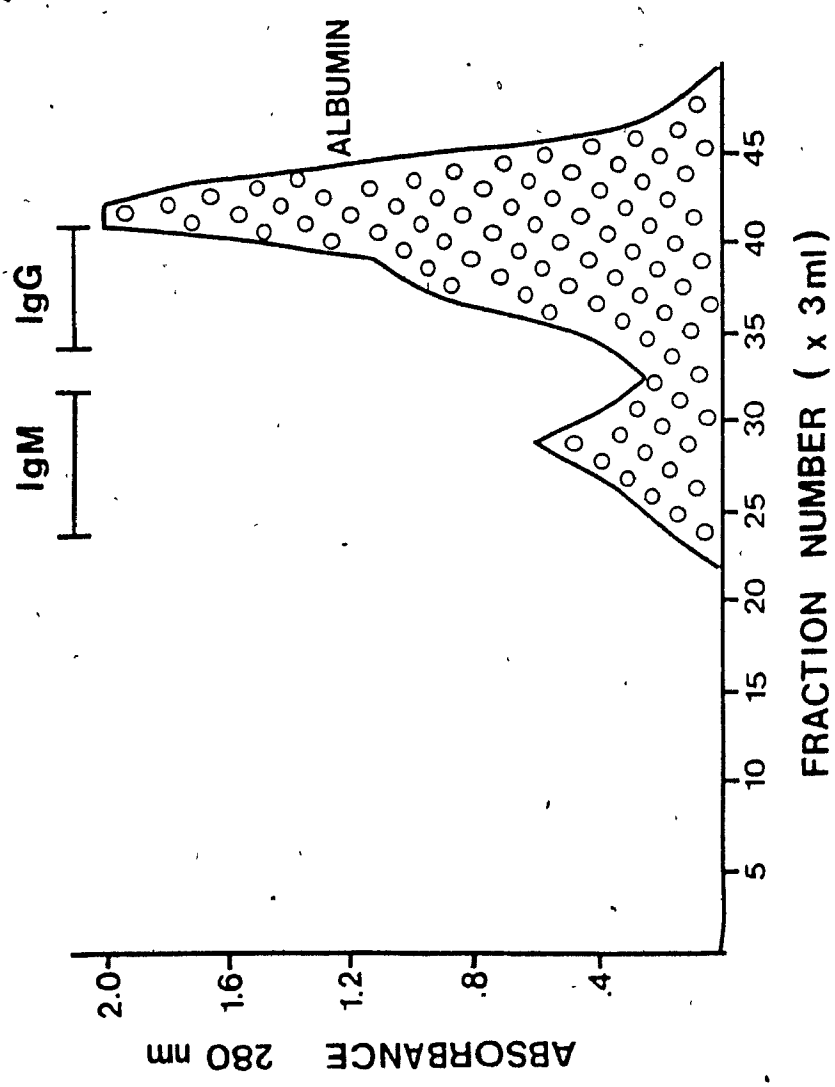
Initial partial purification was performed by passage of IP over an Ultrogel ACA34 molecular sieve chromatography column. This produced the separation profile of Figure 6.1. Standard marker proteins were used to calibrate the column and confirmed that the first peak corresponded to IgM (MW 900,000) and that the prominent second peak was primarily albumin (MW 68,000); the shoulder to the left of the second peak presumably contained IgG. The fractions marked "IgM" and "IgG" were pooled separately, concentrated and dialyzed prior to assay *in vivo*. Fraction IgM had no effect on the subsequent course of infection when given to infected mice on d 13 p.i.; on the other hand, fraction IgG cleared the parasitaemia within 24 h (data not shown).

2. PROTEIN A CHROMATOGRAPHY

Further purification of activity was obtained by

FIGURE 6.1

Profile of IP fractionation by gel filtration chromatography (Ultrogel ACA34). Markings indicate fractions pooled for assay *in vivo*. IgM = Immunoglobulin M; IgG = Immunoglobulin G.


ALBUMIN
IgG
IgM
2.0
1.6
1.2
.8
.4
ABSORBANCE 280 nm
5
10
15
20
25
30
35
40
45
FRACTION NUMBER (x 3 ml)

applying a sample of plasma to a column of protein A-Sepharose and collecting fractions containing material eluted by citrate-phosphate buffers of decreasing pH. The elution profiles of Figure 6.2 compare the protein A-Sepharose separations of IP and NMP, demonstrating a significantly larger amount of protein in the pH 4.5 peak. Following concentration to original volumes and dialysis, the relative concentrations of immunoglobulin isotypes in each fraction were assessed by Ouchterlony immunodiffusion analysis (Table 6.1). IgM was found only in the pH 8.0 fraction (flow-through); IgG1 was found primarily in the pH 6.0 fraction; the pH 4.5 fraction contained almost all of the IgG2a and IgG3, and no other fractions had significant amounts of these immunoglobulin subclasses present; finally, nearly all of the IgG2b was eluted with the pH 3.5 buffer.

When tested in vivo (Figure 6.3), the pH 4.5 eluate brought about a dramatic reduction in parasitaemia (curative effect) within 24 to 48 h of administration, whereas the pH 8.0, 6.0 and 3.5 fractions did not markedly affect the course of infection. Heat treatment of the fractions before administration abolished any curative effect.

Figure 6.4 shows the effect of administering fractions to naive mice before T. musculi inoculation to assay the protective activity of the fractions. It is evident that the pH 4.5 fraction significantly delayed the onset of parasitaemia, as well as decreased the plateau level, whereas the other fractions had marginal, if any, effect on

FIGURE 6.2

Protein A elution profile of IP (——) and NMP (----). Elution was stepwise with buffers of decreasing pH. Fractions were collected and pooled as indicated and concentrated to original plasma volumes. At 280 nm, 1.4 absorbance units represent 1 mg of protein per ml. O.D. = optical density.

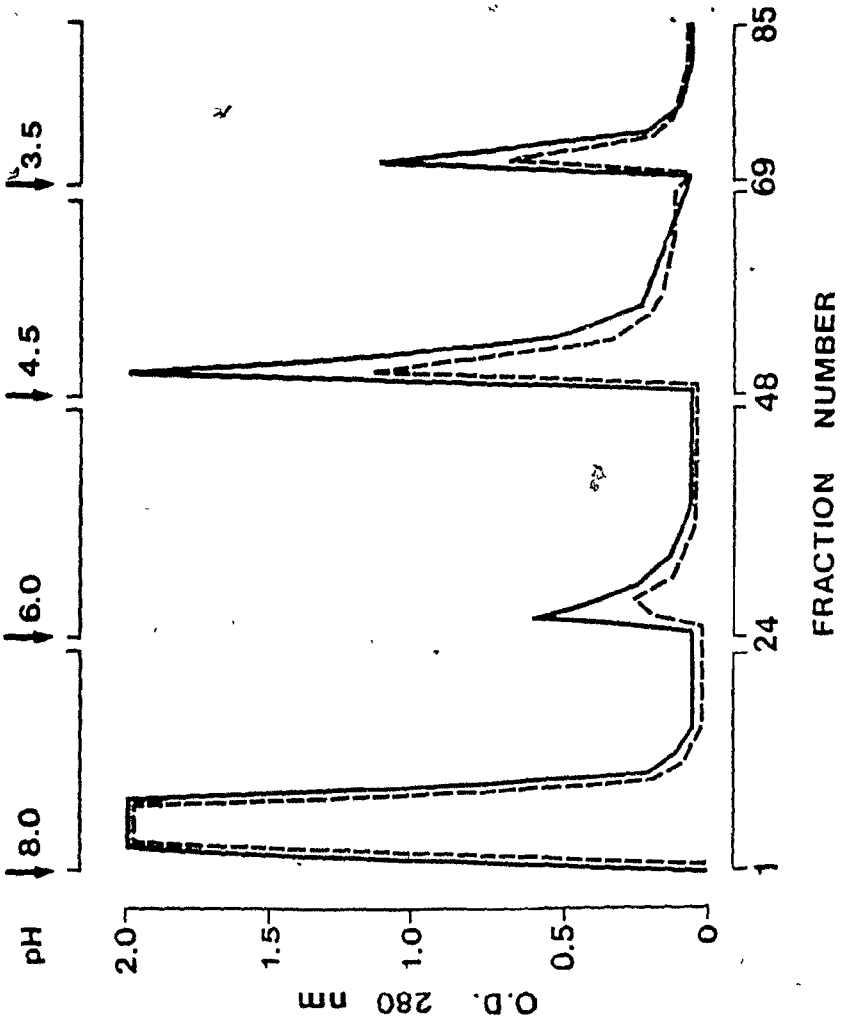

pH
8.0
6.0
4.5
3.5
O.D. 280 nm
2.0
1.5
1.0
0.5
0
1
24
48
69
85
FRACTION NUMBER

TABLE 6.1: Analysis of IP fractions by Ouchterlony double immunodiffusion

	Titer of Isotype[a]				
pH of fraction	IgM	IgG1	IgG2a	IgG2b	IgG3
8.0	1:16[a]	-	-	-	-
6.0	-	1:128	-	-	-
4.5	-	1:8	1:128	1:1	1:32
2.5	-	1:1	1:2	1:128	1:1

[a] End-point read as weakest dilution of fraction giving a precipitin line.

FIGURE 6.3

Curative activity of Protein A-eluted IP fractions. On day 12 p.i. (arrow), C57BL/6 mice were given 0.8 ml i.v. injections of IP fractions eluted from protein A with buffers of decreasing pH (8.0 (▲), 6.0 (△), 4.5 (○), 3.5 (●)). Heat treatment of fractions before injection abolished any curative effect. Each point represents the mean value of five or six mice +/- 1 standard error of the mean.

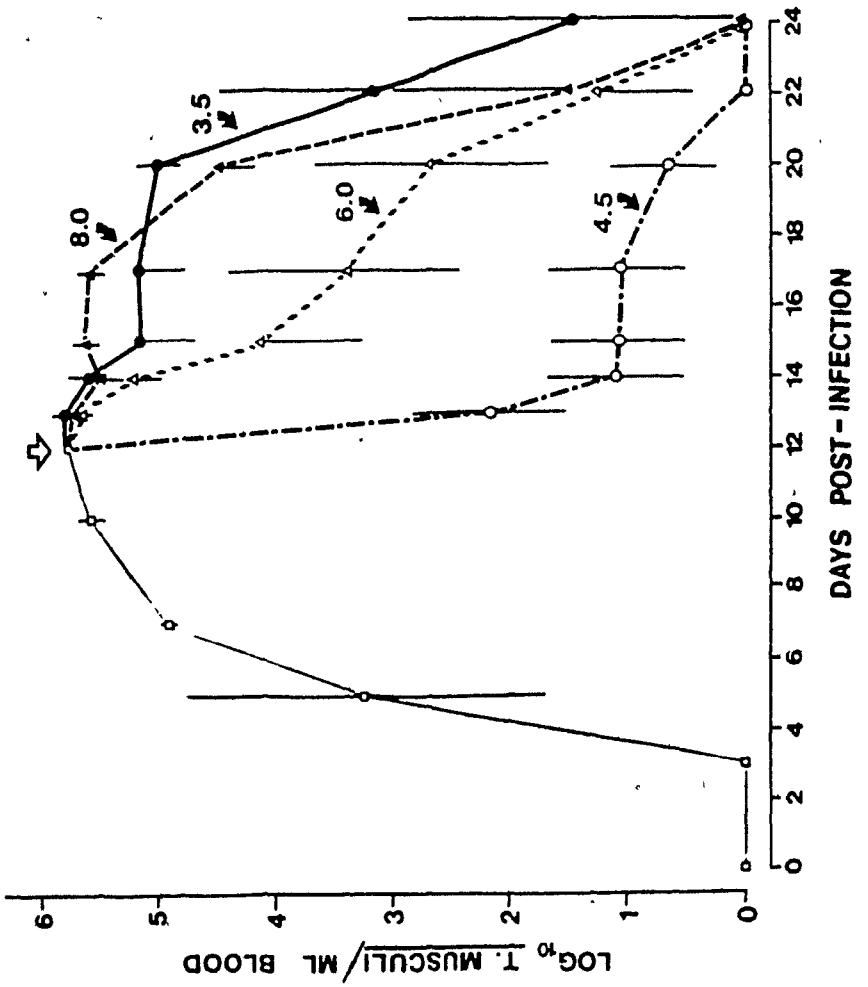
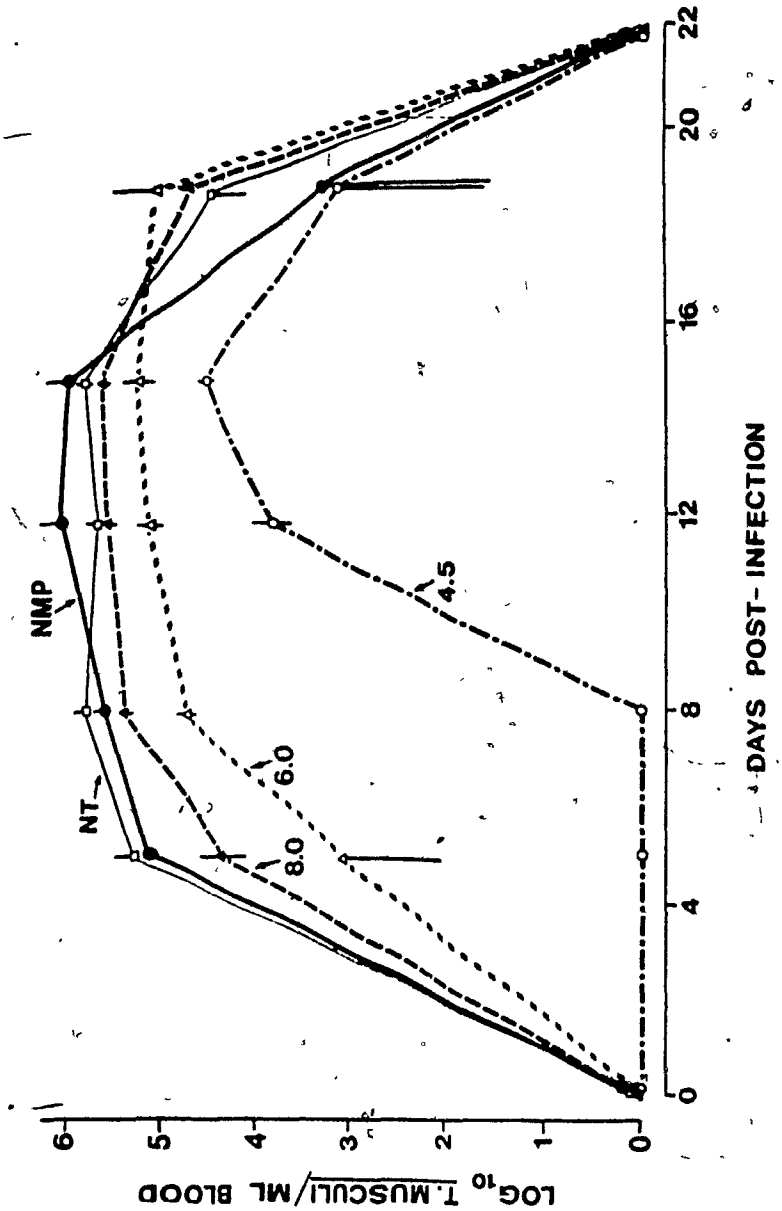

3.5
8.0
6.0
4.5
DAYS POST-INFECTION
LOG10 T. MUSCULI/ML BLOOD

FIGURE 6.4

Protective effect of protein A-eluted IP fractions on the course of infection in naive recipients. C57BL/6 mice were inoculated i.p. with 10^4 trypanosomes 1 h after i.v. administration of 0.8 ml plasma equivalents of IP fractions eluted from Protein A with buffers of decreasing pH (8.0 (▲), 6.0 (△), 4.5 (○)). NMP (●) was administered and there was a no-treatment (NT,□) control. Each point represents the mean value of four or five mice +/- 1 standard error of the mean.


LOG10 T.MUSCULI/ML BLOOD
0
1
2
3
4
5
6
DAYS POST-INFECTION
0
4
8
12
16
20
22
NMP
NT
4.5
6.0
8.0

the course of parasitaemia.

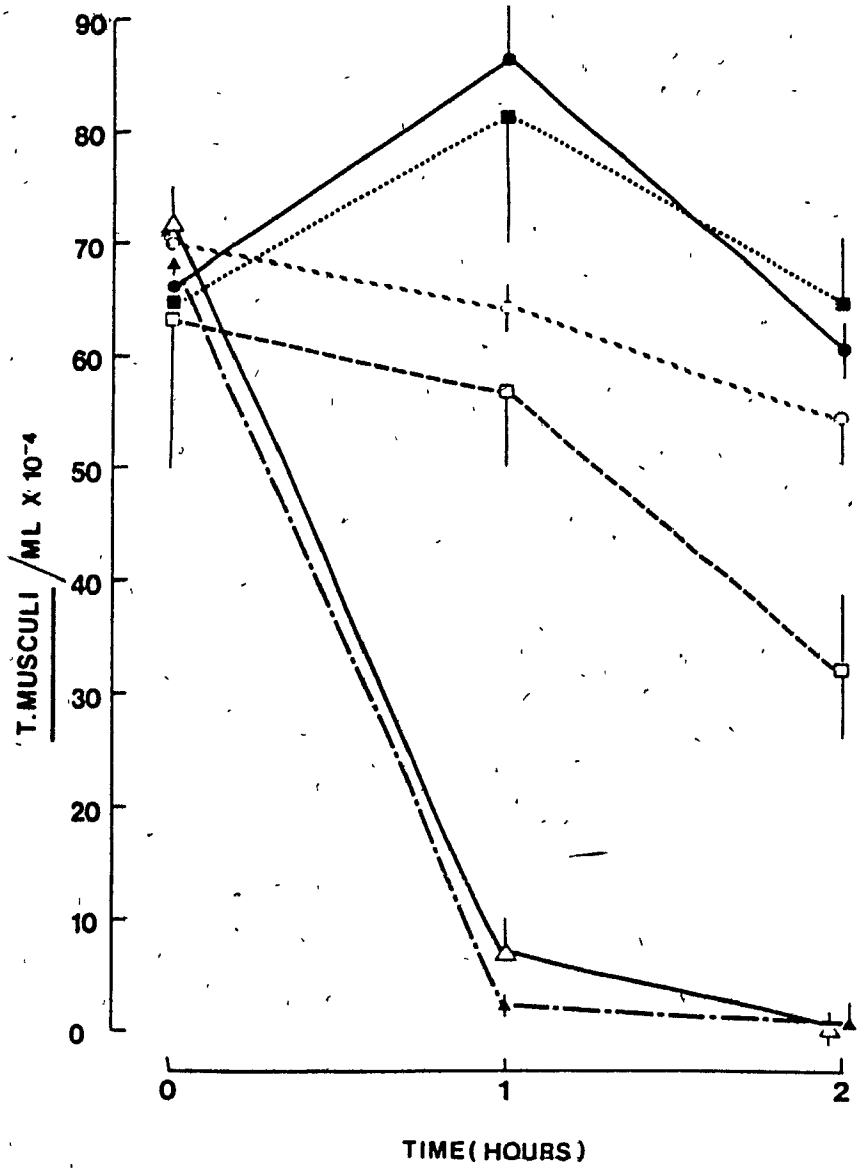
Finally, the fractions were tested in the in vitro assay, and the results shown in Figure 6.5 were obtained. It is apparent that the pH 4.5 fraction is the only one to have had a significant effect ($p < 0.05$) in reducing the number of trypanosomes. The activity of the pH 4.5 fraction was lost upon heat treatment (data not shown).

DISCUSSION

An initial partial purification of IP was carried out using molecular sieve chromatography. The curative activity was found to be associated with a fraction of molecular weight of less than 200,000, which is consistent with the size of IgG. In subsequent studies, advantage was taken of the technique proposed by Ey et al. (1978) and modified by Seppala et al. (1981) for the isolation of IgG subclasses with Protein A-Sepharose. These workers and others (Lindmark et al., 1983; Sarvas et al., 1983) have shown that mouse IgG is adsorbed by Protein A-Sepharose at pH 8.0, whereas other proteins do not bind to any significant extent. The different subclasses of IgG may be sequentially eluted with buffers of decreasing pH, viz., IgG1 elutes at pH 6.0, IgG2a and IgG3 elute at pH 4.5, and IgG2b elutes at pH 3.5. IP was thus fractionated, and Figure 6.3 shows that the curative activity does not appear in the flow-through fraction but is

FIGURE 6.5

Protein A-Sepharose-derived immunoglobulin fractions of C57BL/6 IP in the in vitro assay. NMP (●), IP (▲), pH 8.0 (■), pH 6.0 (□), pH 4.5 (△), and pH 3.5 (○) fractions were used. Each point represents the mean value of two triplicate samples +/- 1 standard error of the mean.


90
80
70
60
50
40
30
20
10
0
T.MUSCULI / ML X 10⁻⁴
0
1
2
TIME(HOURS)

associated with the pH 4.5 fraction. This provides persuasive evidence that the curative factor is antibody in nature. Moreover, it was confirmed that the pH 4.5 fraction contained mostly subclasses IgG2a and IgG3 and that no other fraction had significant amounts of either subclass (Table 6.1). Based on these observations and on the aforementioned elution characteristics, the curative antibody seems to be associated with either IgG2a or IgG3. The slight decrease in parasitaemia observed with the pH 6.0 fraction may be attributed to a partial effectiveness of IgG1 in causing parasite elimination, or it may be the result of contamination of the fraction with IgG2a or IgG3. Indeed, although IgG2a was not detected in this fraction by the Ouchterlony technique, small amounts were detected in the pH 6.0 fraction by the Mancini radial immunodiffusion technique (unpublished observations). Both Ey et al. (1978) and Seppala et al (1981) pointed out a similar slight contamination problem.

Figure 6.5 demonstrates that in the <u>in vitro</u> assay, only the pH 4.5 fraction is able to reduce the parasitaemia to any significant extent. Further, the activity of this fraction is heat-labile. These observations are consistent with the <u>in vivo</u> findings, and corroborate the suggestion that antibody of the IgG2a or IgG3 subclasses is responsible for parasite elimination in the infected mouse. In addition, these results show that a heat-labile fraction containing antibody alone is able to mediate reduction in trypanosome

number. This is further evidence that antibody per se is the heat-labile component of IP.

The protective activity was also associated with the pH 4.5 fraction of IP (Figure 6.4), indicating that it too may be mediated by IgG2a or IgG3. As discussed in Chapter III, the same antibody may be responsible for both the curative and protective activities.

The apparent restriction of functional anti-trypanosome curative activity to one or two subclasses is of interest in light of findings by Perlmutter et al. (1978) that murine anti-group A streptococcal carbohydrate antibodies are largely restricted to the IgG3 subclass, as well as the work of Majarian et al. (1984), demonstrating the curing of Plasmodium yoelii infections with an IgG3 monoclonal antibody.

As mentioned previously, the in yivo curative activity of IP is sensitive to heat treatment. Initially, the nature of the lability was unclear; either the activity was intrinsically heat sensitive or some accessory plasma component was labile. The heat lability of the isolated pH 4.5 activity confirms that the curative antibody therein is intrinsically heat sensitive; polyacrylamide gel electrophoresis of this fraction demonstrates no contamination by other components, i.e., only immunoglobulin is present (data not shown). Although IgE is generally assumed to be the sole heat-labile murine immunoglobulin (Ishizaka et al., 1967), it has not been shown to bind to

Protein A-Sepharose; it is thus unlikely to be present in the pH 4.5 fraction and is unlikely to be responsible for the observed specific cure.

With the knowledge that the active fraction contained IgG2a and IgG3 the next logical step was to isolate antibodies of each subclass to determine their relative contributions to trypanosome clearance.

CHAPTER VII

HEAT-LABILE IgG2a CURES T. MUSCULI INFECTION

CHAPTER VII

HEAT-LABILE IgG2a CURES T. MUSCULI INFECTION

Armed with sensitive *in vitro* and *in vivo* assays with which to evaluate the curative activity present in affinity purified IP fractions, we set out to determine which of the two IgG subclasses present in the active pH 4.5 fraction (IgG2a or IgG3) was primarily responsible for effecting clearance of trypanosomes. To this end, we employed separation columns containing rabbit anti-mouse IgG2a and rabbit anti-mouse IgG3 antibodies covalently linked to Sepharose beads to isolate the respective subclasses from IP. After determining (by enzyme-linked immunosorbent assay (ELISA)) that the columns were effective in depleting IP of the respective subclasses, the flow-through (subclass-depleted) and eluate (subclass-enriched) fractions of each column were tested for curative activity both *in vitro* and *in vivo*. The levels of the IgG subclasses in plasma throughout the infection were determined by ELISA in C57BL/6 mice. In addition, immunoglobulin levels were determined in *T. musculi*-infected CBA/N mice, known to be impaired in their immune responses to certain thymus-independent antigens as a result of an X-chromosome-linked recessive B-cell defect (Xid defect) (Scher *et* al., 1975; Mond et al., 1982), and in which

clearance of parasitaemia is delayed by approximately one week (Vigeant et al., 1986). Finally, studies were begun to delineate the nature of the antigen(s) recognized by the antibody.

RESULTS

1. DEMONSTRATION OF EFFECTIVENESS OF ANTI-SUBCLASS COLUMNS

Following passage of IP over the anti-IgG2a and anti-IgG3 columns, the resultant Flow-Through (FT) and Eluate (ELU) fractions were tested for subclass composition using the ELISA. The results of Figure 7.1 demonstrate that the anti-IgG2a column effectively depletes IP of IgG2a, and that the anti-IgG3 column removes essentially all IgG3 from IP. Further ELISA analysis showed that although there was some slight contamination of the G2a-ELU fraction with other subclasses, there were at least 50-100 times more of these contaminating subclasses in the G2a-FT fraction than in the G2a-ELU fraction (Figure 7.2).

2. IN VITRO AND IN VIVO ACTIVITY OF THE SUBCLASS-DEPLETED AND -ENRICHED FRACTIONS

The data recorded in Table 7.1 show the effectiveness

FIGURE 7.1

Effectiveness of anti-subclass columns in depleting IP of respective subclasses as shown by ELISA. Wells of a 96-well assay plate were coated with rabbit anti-mouse IgG2a (Panel A) or rabbit anti-mouse IgG3 (Panel B), and serial dilutions of fractions obtained from anti-IgG2a and anti-IgG3 columns were incubated as described. Alkaline phosphatase-labelled rabbit anti-mouse IgG was added to all wells before development with p-nitrophenyl phosphate. Eluate from anti-IgG2a column (G2a-ELU,Δ), flow-through from anti-IgG2a column (G2a-FT,▲), eluate from anti-IgG3 column (G3-ELU,□), and flow-through from anti-IgG3 column (G3-FT,■) were assayed. Each point represents the mean of triplicate samples, and the data shown are from a representative column run.

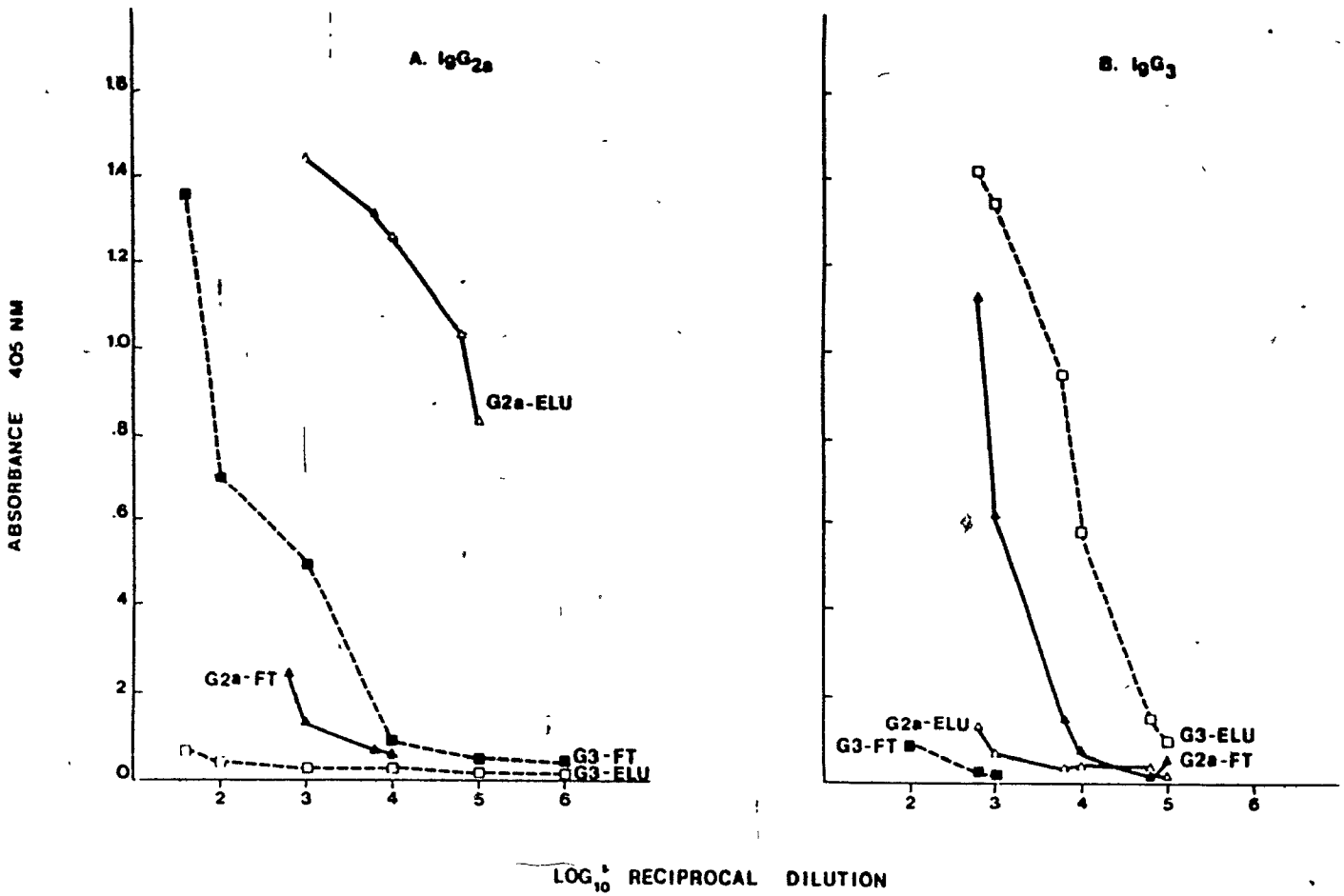

A. IgG2a
B. IgG3
ABSORBANCE 405 NM
1.8
1.4
1.2
1.0
.8
.6
.4
.2
0
G2a-ELU
G2a-FT
G3-FT
G3-ELU
2
3
4
5
6
LOG10 RECIPROCAL DILUTION

FIGURE 7.2

Specificity of anti-IgG2a column for IgG2a. Wells of a 96-well plate were coated with rabbit anti-mouse IgG1 (Panel A) or rabbit anti-mouse IgG2b (Panel B), and serial dilutions of fractions obtained from the anti-IgG2a column were incubated as described. Alkaline phosphatase-labelled rabbit anti-mouse IgG was added to all wells before development with p-nitrophenyl phosphate. Eluate from anti-IgG2a column (G2a-ELU,O) and flow-through from anti-IgG2a column (G2a-FT,●) were used. Each point represents the mean of triplicate samples, and the data shown are from a representative column run.

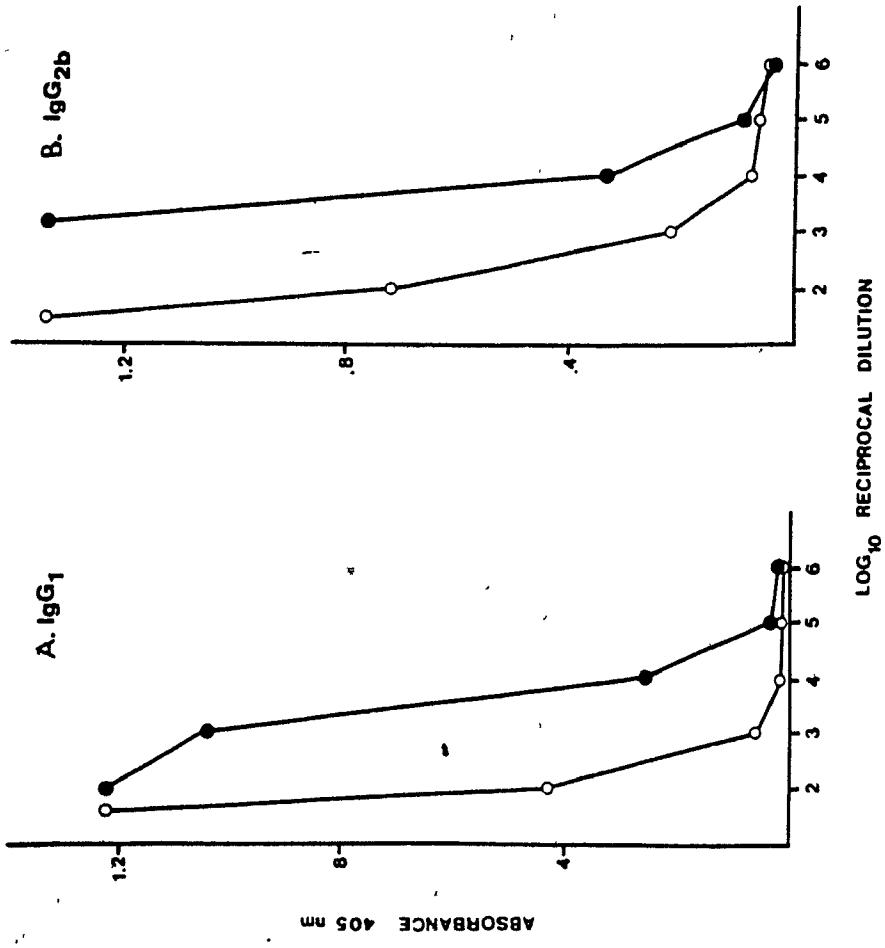

B. IgG2b
A. IgG1
1.2
.8
.4
2
3
4
5
6
ABSORBANCE 405 nm
LOG10 RECIPROCAL DILUTION

TABLE 7.1: Activity of subclass-enriched and -depleted fractions[a]

		in vitro (T. musculi x 10^4/ml)[b]			in vivo (T. musculi x 10^4/ml)[c]			
		0h	1h	p Value[d]		d0	d1	p Value[e]
1	IP	50.0 ± 3.6	7.5 ± 2.7	.001	IP	45	0	
						53	0	
						47	0	.001
						50	0	
2	G2a-FT	52.7 ± 2.8	45.9 ± 3.9	NS	G2a-FT	40	39	
						48	34	
						41	41	NS
						51	47	
3	G2a-ELU	52.6 ± 2.8	11.8 ± 2.7	.001	G2a-ELU	42	6	
						46	12	
						53	12	.001
						51	3	
4	G3-FT	42.0 ± 6.0	8.3 ± 2.1	.001	G3-FT	44	0	
						54	0	.001
						45	0	
5	G3-ELU	39.0 ± 2.7	46.0 ± 1.2	NS	G3-ELU	43	37	
						51	47	NS
						47	46	
6	HT-IP	44.0 ± 8.0	47.0 ± 5.0	NS	HT-IP	50	46	
						47	52	NS
						48	48	
7	HT-G2a-ELU	40.0 ± 2.6	41.3 ± 3.7	NS	HT-G2a-ELU	51	45	
						57	51	NS
8	HT-G2a-FT	37.5 ± 4.5	35.0 ± 2.0	NS	HT-G2a-FT	47	42	
						46	48	NS

[a] IP = immune plasma; G2a = anti-IgG2a column; G3 = anti-IgG3 column; ELU = eluate, FT = flow-through; HT = heat treated; NS = not significant.

[b] Plasma or fraction was used in the *in vitro* assay described in the text. Each value represents the mean of at least three separate triplicate experiments ± S.E.M.

[c] Plasma or fraction was tested *in vivo* as indicated in the text. Because of the relatively large amount of plasma or fraction necessary for the assay, the values shown are from individual mice, each of which received fractions pepared from separate passages over the columns.

[d] Comparison of 0 h trypanosome count to 1 h trypanosome count.

[e] Comparison of mean of d 0 trypanosome count to mean of d 1 trypanosome count.

of the different fractions as measured by the *in vitro* and *in vivo* assays. IP (positive control) causes parasite removal both *in vitro* and *in vivo*. IgG2a-depleted IP (G2a-FT) has no effect in either assay system, whereas the IgG2a-enriched fraction (G2a-ELU) mediates a highly significant decrease in trypanosome number in both assays. IP depleted of IgG3 (G3-FT) is able to mediate parasite elimination, but the IgG3-enriched fraction (G3-ELU) has no effect. Thus, only those fractions which contain IgG2a are able to bring about trypanosome elimination.

3. HEAT LABILITY OF CURATIVE ANTIBODY

As seen in row 6 of Table 7.1, heat treatment of IP completely abrogates its effectiveness in both assays. Similarly, heat treated IgG2a-enriched fraction (HT-G2a-ELU) has no significant effect on trypanosome number either *in vitro* or *in vivo* (row 7). Aliquots of G2a-ELU were heated for different periods of time and samples were run on a 6% nonreducing polyacrylamide gel, subsequently stained with Coomassie Blue. The gel shown in Figure 7.3 demonstrates that with increasing time of exposure to 56°C, increasing amounts of a high molecular weight substance appeared.

FIGURE 7.3

Kinetics of appearance of high molecular weight substance (arrow) in anti-IgG2a column eluate (G2a-ELU) heated for different periods of time. Aliquots of G2a-ELU were run on a nonreducing 6% polyacrylamide gel after no treatment (lane b) or heating at 56°C for 15 min (lane c), 30 min (lane d) or 60 min (lane e). Standards (lane a) consisted of phosphorylase B (94,000), albumin (67,000) and ovalbumin (43,000). Gel was stained in Coomassie Blue.

4. APPEARANCE OF IgG2a OVER THE COURSE OF INFECTION

The ELISA titers of Figure 7.4 demonstrate the appearance and gradual increase in concentration of trypanosome-specific antibody in C57BL/6 plasma over the course of T. musculi infection. It is clear that IgG2a builds up gradually, as do antibodies of other subclasses. Of the subclasses measured, IgG2a and IgG2b decrease in concentration at the time of clearance of parasitaemia, only to rebound back shortly thereafter. In the experiment shown, trypanosomes were still present in the blood on d 20 but had completely disappeared by d 21.

5. ANTIBODY TITERS IN CBA/N MICE

CBA/N x DBA/2 (B-cell-defective) mice as well as normal (DBA/2 x CBA/N) mice were infected with T. musculi, and antibody levels were determined in plasma samples collected at different times during and following infection. As may be seen in Figure 7.5, the peak IgG2a antibody response develops significantly later in the B-cell-defective mice; in these mice, clearance occurs approximately 10 days after that in normal mice. The peak levels of other IgG subclasses in plasma samples of infected B-cell-defective mice are reached later than the peak IgG2a level (data not shown).

FIGURE 7.4

ELISA titers of different subclasses of IgG in C57BL/6 plasma over the course of T. musculi infection. Wells of a 96-well assay plate were coated with T. musculi antigen, and 1/50 dilutions of plasma samples were incubated in coated, washed wells. Alkaline phosphatase-labelled anti-subclass reagents were added to wells before development with p-nitrophenyl phosphate. Anti-IgG1 (△), anti-IgG2a (▲), anti-IgG2b (■), and anti-IgG3 (●) were used. Each point represents the mean value of triplicate samples, and the data shown are from a representative course of infection in which parasitaemia was cleared on d 21.

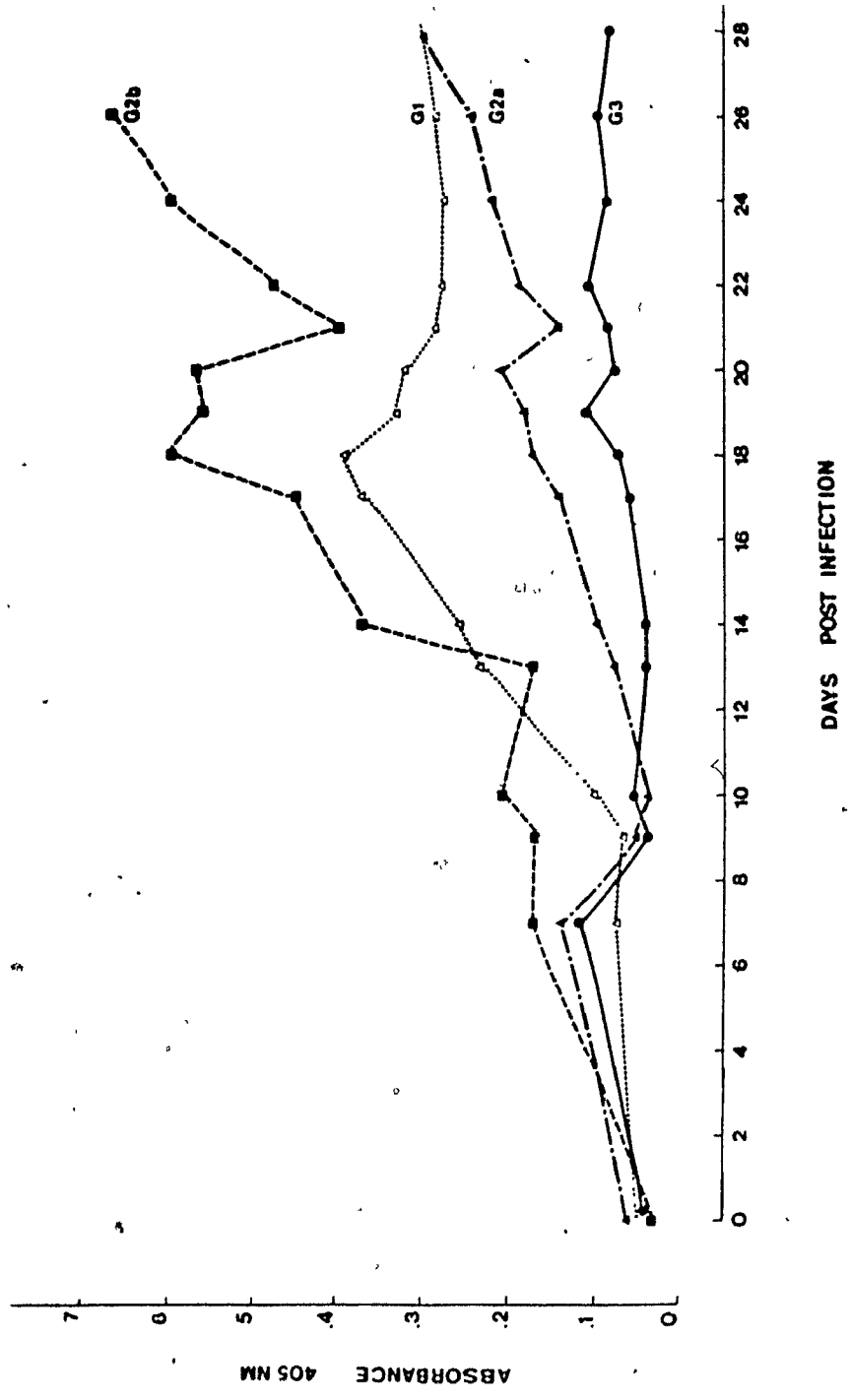
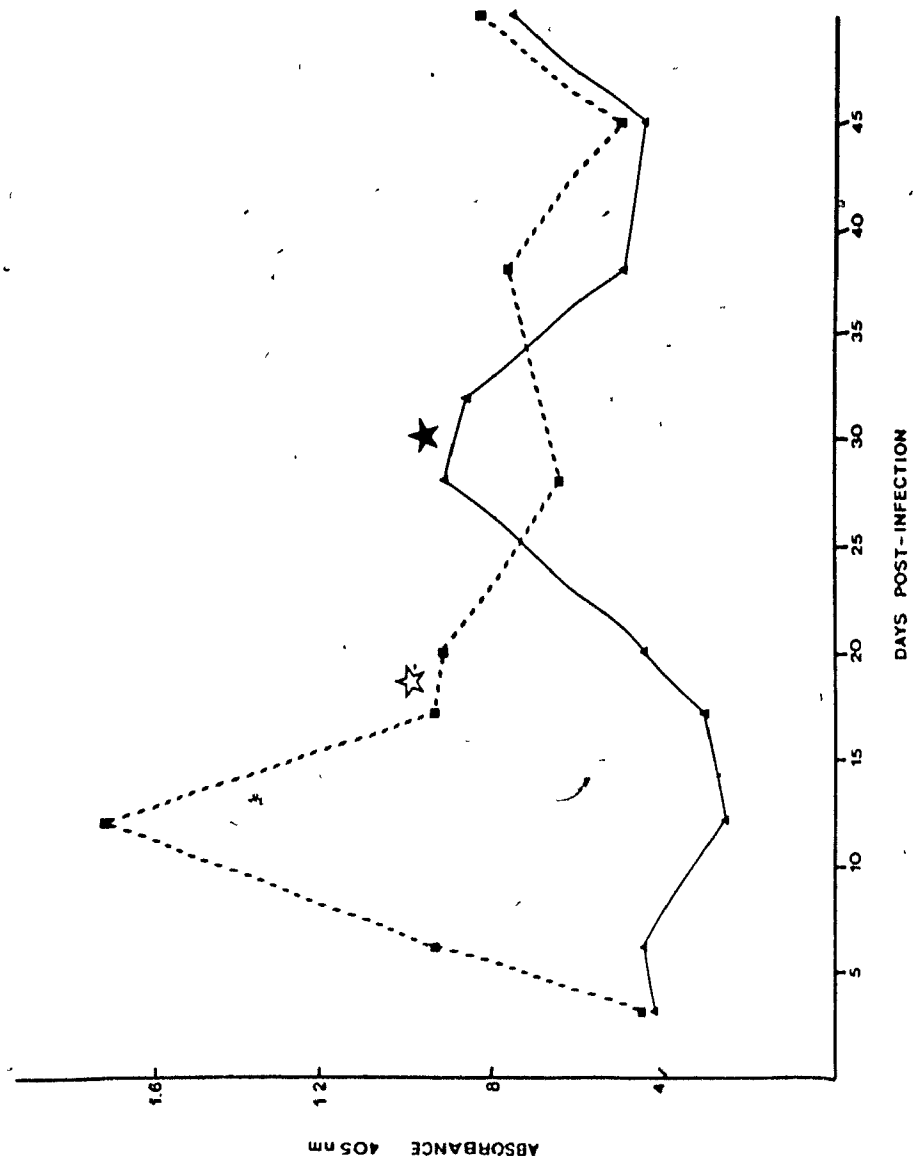

G2b
G1
G2a
G3
ABSORBANCE 405 NM
0
.1
.2
.3
.4
.5
.6
.7
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
DAYS POST INFECTION

FIGURE 7.5

ELISA titers of IgG2a in plasma of CBA/N x DBA/2 (Xid defect) and DBA/2 x CBA/N (normal) mice over the course of T. musculi infection. Wells of a 96-well assay plate were coated with T. musculi antigen, and 1/50 dilutions of plasma samples were incubated in coated, washed wells. Alkaline phosphatase-labelled rabbit anti-mouse IgG2a was added to wells prior to development with p-nitrophenyl phosphate. Each point represents the mean value of triplicate samples. In DBA/2 x CBA/N (normal,■) mice, parasitaemia was cleared around d 18 (open star), while in CBA/N x DBA/2 mice (▲), parasitaemia ended at approximately d 28 (closed star).


ABSORBANCE 405 nm
1.6
1.2
.8
.4
5
10
15
20
25
30
35
40
45
DAYS POST-INFECTION

6. IMMUNOBLOTTING

With the knowledge that an antibody fraction of IP is essential to the process of trypanosome elimination from the blood, we set out to investigate its specificity. It is clear that the active fraction of IP contains molecules which bind to the crude T. musculi antigen used in the ELISA. We therefore took advantage of the technique of immunoblotting to specifically define those trypanosome antigens to which the putative active antibody binds. Figure 7.6a shows the electrophoretic separation of a trypanosome antigen preparation on a 10% polyacrylamide gel under reducing conditions; there is clearly a large number of antigens in the preparation. Figure 7.6b is a nitrocellulose strip incubated first in IP followed by labelled anti-mouse IgG, and it demonstrates that IP contains antibodies directed against numerous trypanosome antigens. The last strip in the series (Figure 7.6c) was first incubated in a protein A-derived pH 4.5 fraction of IP, and then in labelled anti-mouse IgG2a; it clearly shows a limited number of bands as compared to IP, indicating that IgG2a present in a curative fraction of IP recognizes a limited number of T. musculi antigens.

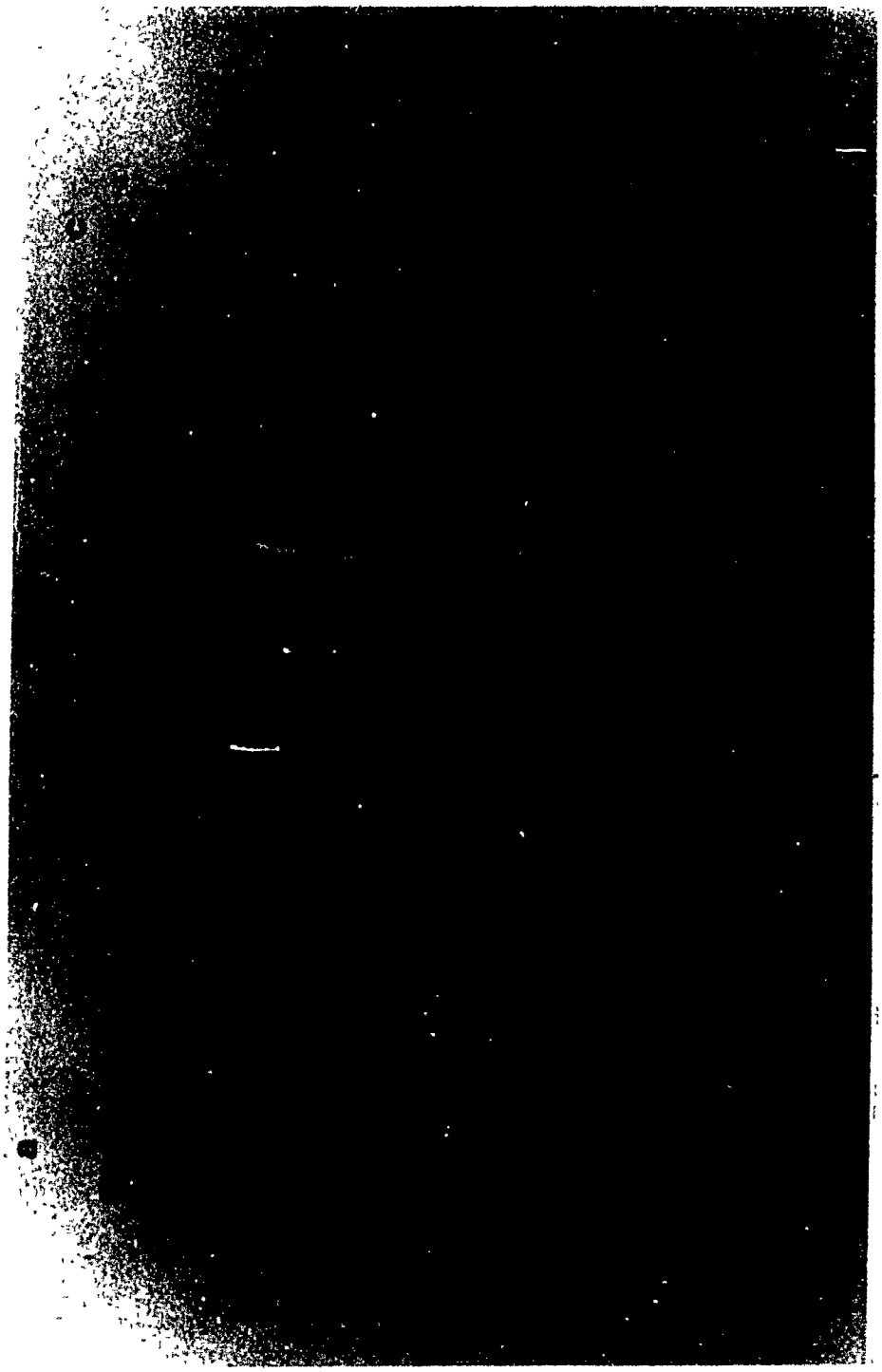
7. MONOCLONAL ANTIBODIES

With the understanding that immunoglobulin is essential

FIGURE 7.6

Polyacrylamide gel electrophoresis and immunoblotting with T. musculi antigen. (a) 0.01 ml of T. musculi antigen was run on a reducing 10% polyacrylamide gel, a portion of which was stained with Coomassie Blue and is shown here. Standards included phosphorylase b (94,000), albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (21,000) and lactalbumin (14,400), and their positions are indicated to the right of the gel. (b) Strip of nitrocellulose paper containing transferred T. musculi antigens. This strip was incubated in IP prior to horseradish peroxidase (HRP) -labelled rabbit anti-mouse IgG, followed by developing reagent. (c) Strip of nitrocellulose paper containing transferred T. musculi antigens. This strip was incubated in a pH 4.5 protein A-derived fraction of IP prior to HRP-labelled rabbit anti-mouse IgG2a, followed by developing reagent.

(Nitrocellulose strips provided by Paula Ryan)



to T. musculi elimination, we set out to generate monoclonal antibodies with the characteristics of the curative antibody (IgG2a isotype, thermolability, biological curative activity) in order to more easily determine which trypanosome antigen(s) is (are) recognized by curative antibody in IP. Using standard techniques of cell fusion, numerous attempts were made to generate anti-trypanosome antibody-secreting hybridomas: mouse spleen cells were harvested from T. musculi-infected mice at different times prior to and following elimination of parasites, as well as after hyperimmunization with both trypanosomes and trypanosome antigen. Unfortunately, exceedingly low yields of antibody-secreting cells were obtained. On several occasions, T. musculi-specific (as determined by ELISA) antibodies were obtained: these, however, consistently belonged to the IgG2b subclass, and had no anti-trypanosome effect in vivo or in vitro. Attempts to generate specific monoclonal antibodies are in progress in our laboratory.

DISCUSSION

We have previously demonstrated (Chapter VI) that a heat-labile, antibody-enriched fraction of IP is able to effectively clear trypanosomes from the blood of infected mice. In view of the findings that: a) certain humoral immune responses are restricted to particular immunoglobulin subclasses (Der-Balian et al., 1980; Hammarstrom et al.,

1984; Perlmutter et al., 1978) and b) different subclasses are associated with different effector functions (Fahey et al., 1964; Grey et al., 1971; Potter, 1983), it was of interest to determine whether the curative activity of IP was restricted to a single immunoglobulin subclass.

The results of Figures 7.1 and 7.2 and Table 7.1 indicate that heat-labile IgG2a antibodies seem to be responsible for effecting cure of Trypanosoma musculi infection in C57BL/6 mice. In vitro, IP, G2a-ELU, and G3-FT are equally effective in mediating reduction in parasite number; in contrast, G2a-FT and G3-ELU (neither of which contains a significant amount of IgG2a) are unable to effect any significant change. Furthermore, heat treatment of both IP and G2a-ELU abolishes their ability to effect an in vitro cure.

In vivo, although G2a-ELU is unable to effect complete elimination of trypanosomes from the blood, it brings about a highly significant reduction in parasite number. Again, similar to the results of the in vitro assay, heat treatment of G2a-ELU renders it unable to effect any reduction in trypanosome number. The inability of G2a-ELU to completely remove T. musculi in vivo, unlike IP, indicates that other additional factors present in IP may be necessary to effect cure. For example, it is possible that other opsonizing subclasses of antibody act in synergy with the curative IgG2a antibody and thereby render the elimination process more effective. This may be the reason for the

decrease in titer of other subclasses around the time of clearance. Alternatively, the preparation of fractions may be associated with losses in activity, so that the amount of purified material necessary to bring about complete parasite elimination would be larger than that used in the present experiments. The relatively large amounts of plasma required for the *in vivo* assay, together with the relatively small column volumes and the limited usages thereof, make it difficult to obtain large amounts of fraction for *in vivo* testing. However, the fact that removal of IgG2a from IP (G2a-FT) completely abolishes its ability to effect cure provides persuasive evidence that IgG2a is the antibody subclass essential in mediating cure of *T. musculi* infection.

The restriction of curative activity to IgG2a is interesting in that the predominant immunoglobulin subclass in C57BL/6 serum is considered to be IgG2b (Natsuume-Sakai et al., 1977; Potter, 1983). Indeed, subclass-specific ELISAs have shown increased titers of IgG2b compared to IgG2a in C57BL/6 plasma samples from *T. musculi*-infected mice (Figure 7.4), although the use of subclass-specific reagents with possibly different affinities and sensitivities renders the direct comparison of subclass levels invalid. In addition, we and others (Brooks et al., 1982) have used *T. musculi* antigen-specific ELISAs to demonstrate the presence of anti-trypanosome activity in *all* immunoglobulin subclasses. Despite this finding, only IgG2a

is able to effect cure of T. musculi infection. Taken together, these observations indicate that there is evidently something distinctive about IgG2a molecules in IP which enable them to mediate parasite elimination. In this light, it is interesting that mice chronically infected with T. cruzi show predominantly IgG2 (sic) antibodies in their sera (Scott & Goss-Sampson, 1984), and that protective antibodies against T. cruzi are found to be associated with the IgG2 (sic) isotype (Scott & Goss-Sampson, 1984; Stefani et al., 1983).

Antibody levels in CBA/N mice provide supportive, though not confirmatory evidence of the involvement of IgG2a in cure of T. musculi infection. CBA/N mice, because of their B-cell defect, are impaired in their antibody responses to type 2 thymus-independent antigens (TI-2), possibly as a result of an arrest in B-cell maturation (Mond et al., 1982). Figure 7.5 shows that peak IgG2a levels are delayed in such mice in comparison to normal mice; elimination of parasitaemia in these mice is similarly delayed. Vigeant et al. (1986) demonstrated that IgM, IgG1 and IgG2 (sic) antibodies appeared later in T. musculi-infected CBA/N mice than in normal mice. Thus, the delay in IgG2a production in CBA/N mice is associated with delayed clearance in such mice.

It is possible that a particular antigenic determinant of the trypanosome surface coat becomes exposed late in the infection, and selectively induces the production of IgG2a

molecules. Indeed, Perlmutter et al. (1970, 1978) have described the restriction of murine anti-bacterial carbohydrate antibodies to a particular subclass, and others have corroborated this finding (Der-Balian et al., 1980; Slack et al., 1980; Basta & Briles, 1984; Majarian et al., 1984). Hammarstrom et al. (1984) suggest that the repertoire of V genes may be different in each subclass. In order to evaluate this hypothesis, a detailed categorization and characterization of the different trypanosome surface antigens is necessary.

Given the antigenic complexity of the trypanosome surface (Viens, 1985), however, it is more likely that the ability of IgG2a to mediate parasite elimination is related in some way to its interaction with effective effector mechanisms. Circumstantial evidence from our laboratory (see Chapter IX) and elsewhere (Vincendeau, 1986) suggests that macrophages are intimately involved in the T. musculi elimination process. Indeed, it has been shown that macrophages are important in immunity to many trypanosome species (Viens, 1985; Rappatoni et al, 1984; Dempsey & Mansfield, 1983; Ferrante & Jenkin, 1978, 1979; Liston & Baker, 1978; Ngaira et al., 1983; Thorne et al., 1979; Townsend & Duffus, 1985; Vincendeau et al., 1981, 1986; Ferrante, 1986; Grosskinsky et al., 1983; Greenblatt et al., 1983). It has been demonstrated that the Fc portion of monomeric IgG2a is uniquely recognized by trypsin-sensitive receptors (FcRI) present on the surface of mouse macrophages

(Heusser et al., 1977; Ralph et al., 1980; Unkeless et al., 1975; Unkeless, 1979; Walker, 1976; Vetvicka et al., 1986). Walker (1977) and others (Ralph et al., 1980) suggest that IgG2a selectively mediates phagocytosis by macrophages. Furthermore, IgG2a-mediated, macrophage-dependent cell damage or destruction has been elegantly demonstrated in the control of murine adenocarcinoma (Haagensen et al., 1978; Matthews et al., 1981; Langlois et al., 1981), and in the destruction of human tumors (Herlyn & Koprowski, 1982; Steplewski et al., 1983a,b; Herlyn et al., 1985; Johnson et al., 1985, 1986). Ezekowitz et al. (1983) have shown that activation of macrophages selectively enhances the expression of FcR for IgG2a, while depressing FcR for other subclasses. It is tempting to postulate that the activation of macrophages seen during T. musculi infection (Vincendeau et al., 1980) occurs in order to more effectively eliminate trypanosomes in association with IgG2a.

It must be pointed out that IgG2a may not be the subclass of antibody which mediates T. musculi elimination in all strains of mice (see Chapter VIII). Moreno & Esdaile (1983) indicate that the isotype distribution of anti-polysaccharide antibodies (first described by Perlmutter et al. (1970; 1978)) is strain-dependent. In relation to macrophage function, Daeron et al (1982) and others (Ralph et al., 1980) suggest that there may not be a strict association between immunoglobulin isotype and effector function.

The heat-lability of the curative activity is an interesting finding. To our knowledge, this is the first description of a non-IgE, heat-labile antibody. Earlier work has mentioned the aggregation of immunoglobulins on exposure to heat or alkali (Henney & Stanworth, 1965; Soltis et al., 1979), yet no loss of biological activity was reported. (In this light, the results of Figure 7.3 may be interpreted as showing the gradual accumulation of high molecular weight aggregates of IgG2a). Indeed, serum samples are routinely heat inactivated to eliminate the effects of complement without subsequent losses of immunoglobulin activity. Although heating at 63°C for 30 min may cause aggregation of IgG1 and IgG2a (Daeron et al., 1982), heating at 56°C is only considered to affect IgE molecules.

A clue to the understanding of the mechanism of heat inactivation is provided by the work of Rousseaux-Prevost et al. (1983) on rat IgE inactivation. They showed that heating induces non-disulfide-linked polymerization of IgE molecules and proposed that steric hindrance does not allow interaction between C-terminal domains of the H chain and the IgE receptor. Henney & Stanworth (1965) proposed a similar aggregation of IgG molecules via formation of intermolecular disulfide bonds in the Fc region. Assuming that an antitrypanosomal antibody molecule bound to a parasite via the antigen-binding site must then interact with a cell via the Fc region to effect clearance, it is evident that blocking the Fc region will lead to a loss of

biological activity. It should be pointed out that the loss of biological activity will not necessarily be paralleled by a loss of immunoreactive activity (agglutination, enzyme-linked immunosorbent assay (ELISA)) if the antigen-combining region is left unaltered. Although the heat lability presumably has no biological significance, it is useful in that it distinguishes the anti-trypanosome activity of curative IgG2a from other isotypes.

The studies depicted in Figure 7.6 constitute only the beginning of a detailed characterization of the trypanosome antigens recognized by the curative antibody. There is evidently an enormous number of trypanosome proteins, many of which may be recognized by many different antibodies in IP. The fact that the IgG2a fraction of IP appears to recognize but a small portion of this antigen repertoire is significant, and it raises two important questions: why is it that IgG2a alone is able to mediate cure, and why does it take three weeks before parasites are cleared from the blood? This problem is discussed at length in Chapter X. The apparent restriction of the curative activity to IgG2a raises the possibility of using the IgG2a-specific antigen(s) to vaccinate a naive host against development of trypanosomiasis. Several groups have used different strategies in attempts to vaccinate mice against various trypanosome species (El-Ridi et al., 1985; Morrison et al., 1982). If the relevant T. musculi antigen(s) could be isolated with monoclonal antibodies (as has been elegantly

performed by Parish et al. (1985) for T. congolense), a number of studies could be performed to elucidate the means by which infection might be controlled. Unfortunately, however, we have not yet been successful in eliciting hybridomas which produce appropriate antibody.

In summary, we have shown that IgG2a antibodies mediate clearance of parasites from the blood of T. musculi infected C57BL/6 mice. These antibodies are distinguished by their heat lability, and their gradual increase in concentration until a critical concentration is reached. This may occur simultaneously with activation of macrophages at the time of the second crisis, culminating in elimination of parasitaemia. We have also begun an initial characterization of the antigens recognized by the curative antibody.

Before examining the nature of the cell involved in T. musculi elimination, we chose to investigate the efficacy of IP-mediated clearance in different inbred strains of mice which vary in their ability to generate immune responses.

CHAPTER VIII

STRAIN DIFFERENCES IN THE CURE OF T. MUSCULI INFECTION

CHAPTER VIII

STRAIN DIFFERENCES IN THE CURE OF T. MUSCULI INFECTION

Different inbred strains of mice vary in their susceptibility to infection with T. musculi, and may be classified as either susceptible or resistant (Magluilo et al., 1983; Albright & Albright, 1981b; Kongshavn et al., 1985). Whereas the total duration of infection is similar in all mouse strains studied to date, genetically susceptible strains (e.g A/J, C3H/HeN) develop plateau phase parasitaemias which are 1-2 $\log_{10}$ higher than those in genetically resistant strains (e.g. C57BL/6, DBA/2, BALB/c). The present studies examine the efficacy of IP obtained from susceptible and resistant strains in effecting trypanosome elimination in different inbred strains of mice.

RESULTS

1. IP TREATMENT OF RECIPIENT STRAINS DURING THE PLATEAU PHASE

IP from donor strains (C57BL/6, C3H/He) was able to cure resistant recipients (C57BL/6, DBA/2) using doses of 0.8 ml or less (Figures 8.1 & 8.2). Plateau parasitaemias

FIGURE 8.1

Effect of treating various recipient strains of T. musculi-infected mice with C57BL/6 IP. On d 12 p.i. (arrows), T. musculi-infected mice were treated with 0.4 ml (▲), 0.8 ml (O) or 1.2 ml (□) of IP obtained from C57BL/6 donor strain mice, or 0.4 ml C57BL/6 NMP (●). Each point represents the mean value of four mice +/- 1 standard error of the mean.

TREATMENT OF VARIOUS RECIPIENT STRAINS WITH IMMUNE PLASMA FROM C57BL/6 DONOR STRAIN MICE

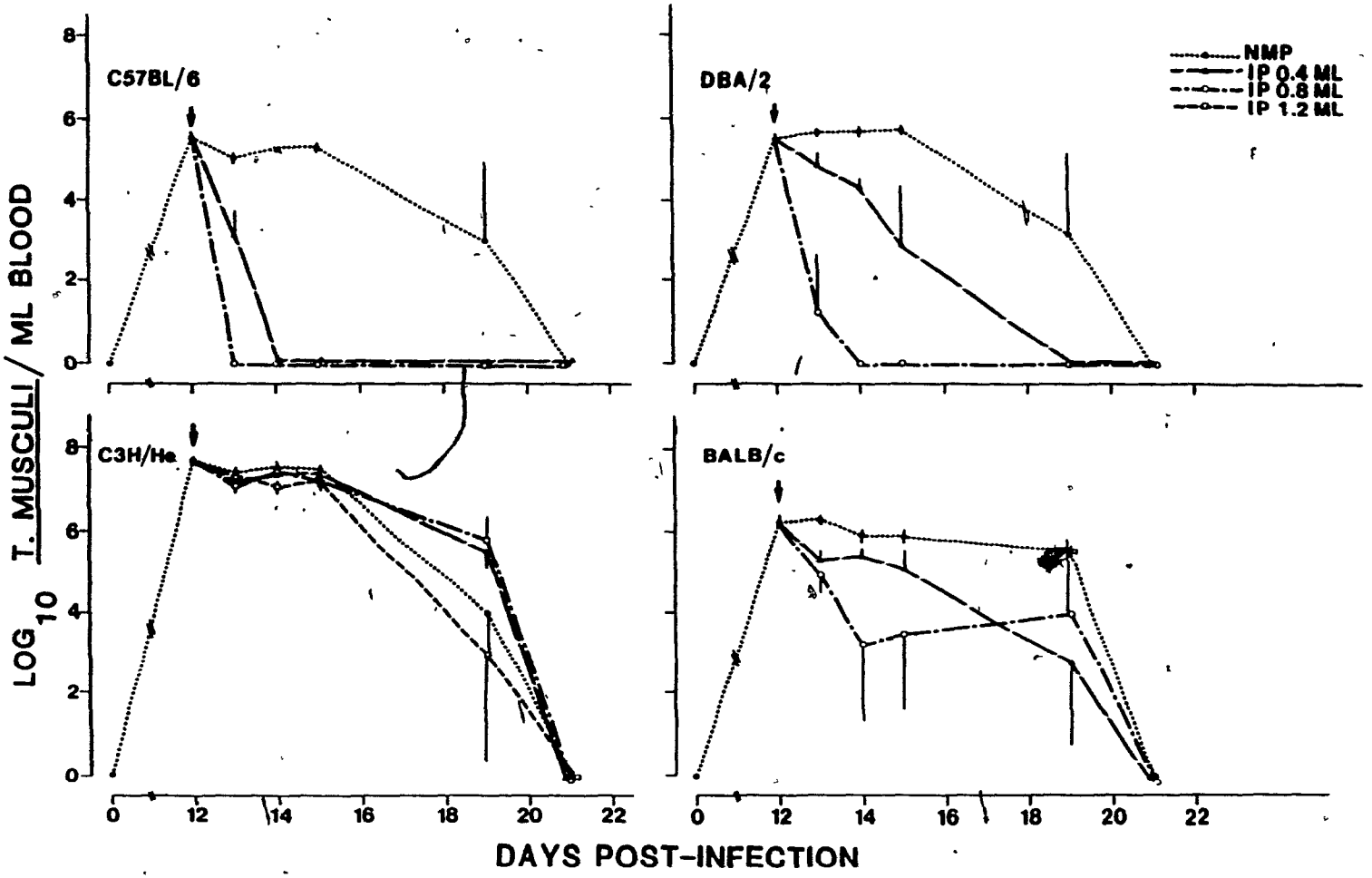
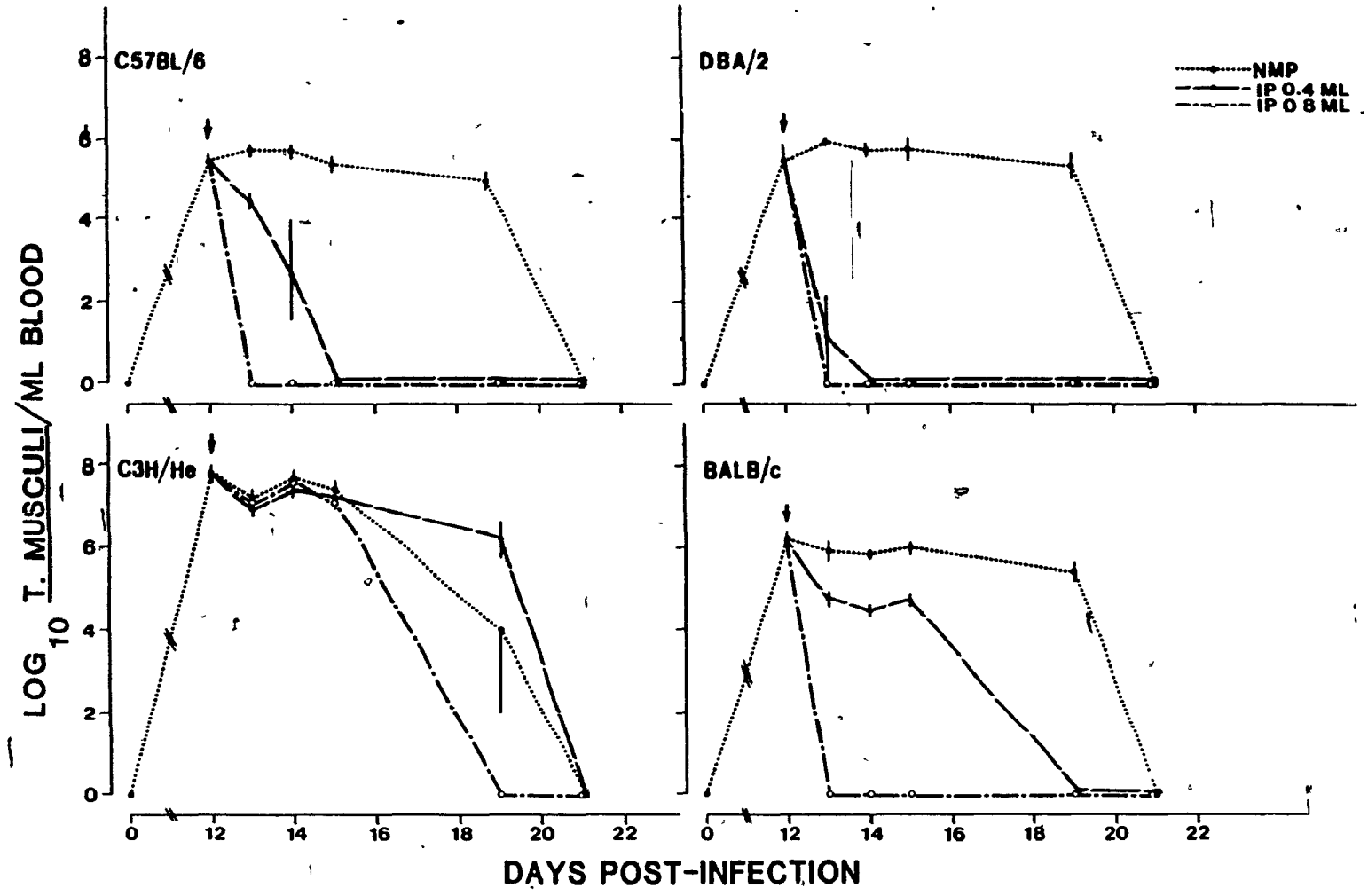


FIGURE 8.2

Effect of treating various recipient strains of T. musculi-infected mice with C3H/He IP. On d 12 p.i. (arrow), T. musculi-infected mice were treated with 0.4 ml (▲) or 0.8 ml (O) of IP obtained from C3H/He donor strain mice, or 0.4 ml C3H/He NMP (●). Each point represents the mean value of four mice +/- 1 standard error of the mean.

TREATMENT OF VARIOUS RECIPIENT STRAINS WITH IMMUNE PLASMA FROM C3H/He DONOR STRAIN MICE



were less than 10^6 parasites/ml blood in these mice. BALB/c recipients, whose plateau trypanosome loads were slightly elevated (approx. 10^6 parasites/ml blood) relative to those of C57BL/6 and DBA/2, could not be cured completely with C57BL/6 IP, and required 0.8 ml of C3H/HeN IP for clearance. Neither type of donor was able to effect clearance of parasitaemia in C3H/HeN recipients (approx. 10^8 parasites/ml blood), even when 1.2 ml was administered. NMP from either donor strain had no demonstrable effect on the course of infection in any of the recipient mouse strains.

2. IP TREATMENT OF RECIPIENT STRAINS DURING EARLY INFECTION

In order to ascertain whether the inability of IP to clear susceptible recipients was related to a defective defense mechanism in such recipients or to their high levels of parasitaemia, IP was administered to susceptible recipients during the exponential growth phase, when their parasitaemias were comparable to the plateau levels of resistant strains (Table 8.1). Resistant recipients were given IP at the same time as controls. In each of the strains tested, initial parasitaemias were $< 10^6$ parasites/ml blood, and administration of C57BL/6 IP was able to effect a temporary elimination or reduction of

TABLE 8.1: Treatment of various recipient mouse strains with C57BL/6 immune plasma (IP) or normal mouse plasma (NMP) at time when parasitaemia was 10^6/ml blood

MOUSE STRAIN	TREATMENT	T. MUSCULI/ml BLOOD[a] BEFORE TREATMENT[b]	AFTER TREATMENT DAY 1	DAY 2	DAY 3	DAY 6
A/J	IP	5.74 ± .06	0.00	0.00	5.03±.14	5.2 ±.10
	NMP	5.62 ± .04	6.32±.06	7.35±.06	7.67±.05	7.66±.02
C3H/HeN	IP	5.82 ± .01	4.02±.20	4.23±.22	5.44±.26	5.96±.07
	NMP	5.75 ± .03	6.43±.05	7.41±.06	7.78±.05	7.30±.11
C57BL/6	IP	5.64 ± .17	0	0	0	0
	NMP	5.59 ± .07	5.55±.07	5.61±.07	5.72±.04	5.70±.13

[a] Mean ± SEM of 4 mice/group.

[b] Immune plasma given on day 4 post-infection to A/J and C3H/HeN mice, and on day 6 post-infection to C57BL/6 mice.

circulating trypanosomes for at least two days following treatment in all cases ($p < .05$). Plateau parasitaemias were subsequently reduced. NMP had no significant effect on the course of infection.

3. EFFICACY OF IP ADMINISTRATION IN EFFECTING CURE IN DIFFERENT RECIPIENT STRAINS

To examine the possible role of complement component C5 in the process of IP-mediated trypanosome clearance, IP obtained from C5-sufficient (C57BL/6, C3H/HeN) and C5-deficient (DBA/2, A/J) mouse strains was tested for curative activity in different recipient strains (Table 8.2). It is apparent that C5-deficient IP is as effective at curing mice of T. musculi infection as normocomplementemic IP. In addition, it is again noted that only those strains of mice with low plateau parasitaemias can be cured by IP in the amounts given, since A/J and C3H/HeN mice, whose plateau parasitaemias are approx. 10^8/ml blood, were not cured of infection.

4. T. MUSCULI-SPECIFIC ANTIBODY LEVELS IN DIFFERENT STRAINS OF MICE

Table 8.3 shows the levels of anti-trypanosome antibody in IP obtained from different strains of mice. Although the total levels of anti-trypanosome antibody appear to be

TABLE 8.2: Ability of donor IP from different strains to effect clearance of parasitaemia in recipient inbred strains

		DONOR[a]			
		C3H/HeN	C57BL/6	A/J	DBA/2
RECIPIENT	C3H/HeN (C5+, high parasitaemia)	-	-	ND[b]	ND
	C57BL/6 (C5+, low parasitaemia)	+	+	+	+
	A/J (C5-, high parasitaemia)	-	-	-	-
	DBA/2 (C5-, low parasitaemia)	+	+	+	+

[a] A positive sign indicates that complete clearance of parasitaemia was observed with either 0.4 or 0.8 ml of IP within 24-48 h; a negative sign indicates that no clearance was obtained with up to 1.2 ml of IP. Each immune plasma transfer was performed in at least 4 recipients on at least 2 separate occasions.

[b] Not done.

TABLE 8.3: T. musculi-specific antibody levels in IP from mice of different strains

Strain	Immunoglobulin levels[a]					
	IgG	IgG1	IgG2a	IgG2b	IgG3	IgM
A/J	0.12	0.47	0.74	0.36	0.22	0.15
DBA/2	0.18	0.63	0.95	1.54	0.19	0.12
C3H/HeN	0.14	0.31	0.59	1.08	0.19	0.08
C57BL/6	0.15	0.21	0.16	1.02	0.13	0.05

[a] Levels were determined by ELISA. Wells of a 96-well assay plate were coated with T. musculi antigen, and 1/50 dilutions of IP were incubated in coated, washed wells. Alkaline phosphatase-labelled anti-subclass reagents were added to wells before development with p-nitrophenyl phosphate. Each value in the table represents the mean absorbance (405 nm) from 2-3 samples of IP obtained from different strains of mice; each sample was assayed in triplicate.

similar in the strains tested, marked differences are seen in the different IgG subclasses. Specifically, the titer of T. musculi-specific IgG2a is higher in A/J, DBA/2 and C3H/HeN mice as compared to C57BL/6 mice. The levels of IgG2b are higher in DBA/2, C3H/HeN and C57BL/6 mice than in A/J mice.

DISCUSSION

We have previously demonstrated the phenomenon of clearance of trypanosomes in C57BL/6 mice following passive transfer of plasma obtained from syngeneic mice cured of T. musculi infection (Chapter III). Administration of as little as 0.4 ml of IP during the plateau phase effects a complete and rapid elimination of parasites from the blood of infected mice. Inbred strains of mice may be classified as either susceptible or resistant to infection with T. musculi (Albright & Albright, 1981b; Magluilo, Viens & Forget, 1983; Kongshavn et al., 1985). The present studies confirm this finding, demonstrating that A/J and C3H/HeN mice are susceptible to T. musculi, while C57BL/6, BALB/c and DBA/2 mice are relatively resistant. In addition, these studies evaluate the ability of IP obtained from various donor strains to effect trypanosome clearance in different recipient strains. Figures 8.1 and 8.2 demonstrate that C57BL/6 and DBA/2 strains of mice are easily cleared of trypanosomes by IP, BALB/c mice require somewhat more IP to

be cured, and parasitaemias in C3H/HeN mice cannot be eliminated by passive transfer of IP. Thus, it appears that resistant strains of mice are more easily cleared of trypanosomes when compared to susceptible mice. A likely reason for this finding is related to the lower plateau parasitaemias in resistant strains as compared to susceptible mice. Thus, the number of trypanosomes in C57BL/6 and DBA/2 mice is sufficiently low relative to the amount of anti-trypanosome antibody in IP for clearance to occur. The finding that BALB/c mice are more difficult to clear than C57BL/6 or DBA/2 mice likely reflects the fact that BALB/c plateau parasitaemias are approximately 0.5 $\log_{10}$ higher than those of other genetically resistant mouse strains. The inability of C3H/HeN mice to eliminate the trypanosomes may reflect the increased parasite load supported by these mice, i.e. the number of parasites is too high to be eliminated by a single dose of IP. It should be pointed out that the failure of other investigators to effect clearance of trypanosomes by passive transfer of plasma may be due to their use of strains of mice with elevated parasitaemias such as C3H/Anf (Albright & Albright, 1982) and CBA/cbi (Viens et al., 1974) as discussed in chapter III.

Another possible explanation for the inability of susceptible strains to be cleared by IP administration is that these strains may have a defective defense mechanism which renders them unable to effect elimination subsequent

to IP administration. This seems rather unlikely, given the fact that in susceptible strains the infection is terminated at essentially the same time as in resistant strains, the only difference in the pattern of infection being in the height of the plateau (Magluilo et al., 1983). To test this possibility, however, IP was administered to susceptible mice during the exponential growth phase of infection, at a time when parasitaemias were comparable in height to those of the plateau level in resistant mice. Table 8.I shows that IP was able to effect a temporary elimination, or at least a significant reduction in parasitaemias, not only in resistant C57BL/6 mice, but also in susceptible A/J and C3H/HeN mice; the subsequent plateau levels were also significantly lower than control, NMP-treated mice. This demonstrates that IP is able to effect trypanosome removal in genetically susceptible mouse strains, when the level of parasitaemia is the same as that of resistant mice. The failure of IP to cure these mice is thus attributed to the much higher (100-fold) number of parasites in their blood, as compared to that of the resistant strains. The subsequent reappearance of trypanosomes in the susceptible mice, but not in the resistant C57BL/6 mice, may be due to an inherent ability of young and dividing trypanosome forms to grow in the susceptible host, i.e. the conditions for trypanosome growth and reproduction may be more favorable in susceptible hosts than in resistant ones (Albright & Albright, 1981b), and elimination of most of the trypanosomes in susceptible

hosts may not be sufficient to prevent their reappearance. Thus, it appears that resistance or susceptibility to T. musculi infection per se does not directly determine whether or not IP administration will cure a given strain of mice. The primary factor is the magnitude of the parasitaemia, this in turn being determined by the relative susceptibility of the mouse strain in question.

It should be pointed out that IP collected from C3H/HeN mice is more potent in effecting clearance than that obtained from C57BL/6 mice. For example, in BALB/c mice, parasitaemia was completely eliminated by C3H/HeN IP but not C57BL/6 IP; similarly, less C3H/HeN IP was required to cure DBA/2 mice. We have demonstrated similar findings in vitro in which 1/8 dilutions of A/J IP were still partially effective in reducing parasite number, whereas no trypanosome elimination was seen with dilutions of C57BL/6 IP greater than 1/2 (data not shown). Presumably, the increased number of parasites in C3H/HeN and A/J mice would require more antibody to be produced in order for complete elimination to occur at the second crisis; therefore, there may be a higher effective antibody concentration in C3H/HeN and A/J IP relative to C57BL/6 IP, accounting for the differential potency. Indeed, as Table 8.3 shows, the levels of trypanosome specific IgG2a are higher in IP of A/J, DBA/2 and C3H/HeN mice as compared to C57BL/6 mice. These data support the hypothesis that IgG2a is indeed the curative antibody in different strains of mice (but this remains to

be evaluated rigorously).

In light of the above, it is interesting that a number of investigators have noted "the superior humoral immune response of C57BL/6 mice in overcoming infections with T. congolense" (MacAskill et al., 1980, 1983; Whitelaw et al., 1983; Mitchell & Pearson, 1983, 1986). Morrison & Murray (1985) demonstrated that there was no significant difference in the humoral immune response of C57BL/6 (resistant) and A/J (sensitive) mice to doses of irradiated, non-infective T. congolense, indicating that an inherent difference in immune responsiveness to the trypanosomal antigens is not the major factor determining susceptibility. Moreover, they showed that A/J mice which received infective and irradiated trypanosomes simultaneously showed depressed antibody responses as compared to those receiving only irradiated parasites, suggesting that active infection of A/J mice with T. congolense impairs their ability to initiate an appropriate immune response to the trypanosome. This finding was corroborated by Black et al. (1986) working with T. brucei brucei (sic). This group found that the inability of susceptible C3H/HeN mice to control parasitaemia resulted from an impaired ability of parasite-induced antibody-containing cells to secrete immunoglobulin. This block in antibody secretion was reversible, being maintained by living parasites or short-lived components of degenerating parasites. Selkirk & Sacks (1980) initially pointed out that C3H/HeN mice mount

less efficient parasite-specific antibody responses than infected C57BL/6 mice, as well as becoming more readily suppressed in their capacity to respond to unrelated antigens. Albright & Albright (1980, 1981c) describe a T. musculi-induced immunodepression correlated with a decreased responsiveness to unrelated antigens, and Hirokawa et al. (1981) hypothesize that this is related to the temporary inhibition of the normal maturation of less mature precursor cells by trypanosome-derived substances (TDS); they also state that the polyclonal B cell activation in trypanosome infected animals relates to a positive effect of TDS on the proliferation and terminal maturation of more mature lymphoid cells.

Thus, the increased sensitivity of susceptible mouse strains to parasite-derived inhibitory substances seems to be involved in the differential ability of mice to effectively combat trypanosome infection. These immunodepressive phenomena may be responsible for the prevention of clearance of T. musculi from the blood of infected mice until 3 weeks post-inoculation; specifically, suppression of T. musculi-specific IgG2a production may be a reason for the duration of the infection. These issues will be discussed at length in the remaining chapters.

Finally, an assumption central to the phenomenon of IP-induced clearance of parasitaemia is that it is representative of the immune process involved in parasite elimination at the second crisis. To demonstrate that this

is in fact the case, it is essential to show that clearance of parasitaemia following IP administration is in all ways similar to the process of parasite elimination that normally occurs at the end of infection. Jarvinen & Dalmasso (1977a) showed that the course of T. musculi infection in inbred, C5-deficient mice is not significantly different from that in normocomplementemic mice, i.e. termination of infection in both types of mice occurs at the same time and rate. The data in Table 8.2 demonstrate that trypanosomes in C5-deficient DBA/2 mice may be eliminated by IP obtained from both C5-sufficient and C5-deficient donors, indicating once again that IP-induced clearance of parasites can (and in fact, must) involve a trypanocidal mechanism other than the one mediated through C5. Thus, administration of IP to an infected mouse during the plateau phase appears to reflect the same process which occurs at the second crisis in a normal infection.

With an appreciation of the different humoral factors involved in the immune response to T. musculi, we may finally progress to an evaluation of the cellular component(s) essential to the control of murine trypanosomiasis.

CHAPTER IX

CELLULAR INVOLVEMENT IN TRYPANOSOME ELIMINATION

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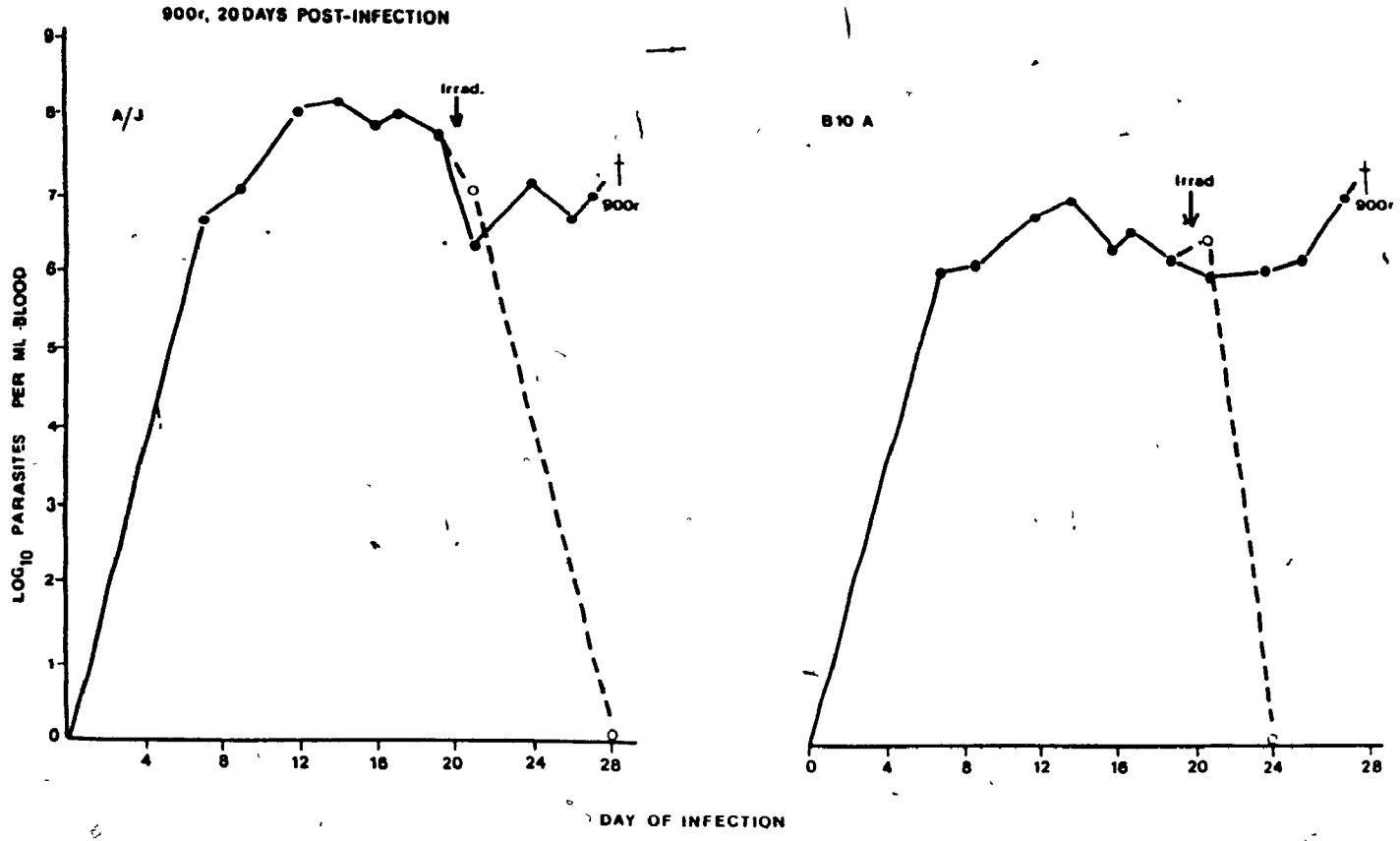
CELLULAR INVOLVEMENT IN TRYPANOSOME ELIMINATION

Previous chapters have demonstrated the essential roles of a heat-labile antitrypanosomal IgG2a immunoglobulin as well as early complement components in the elimination of T. musculi from the infected mouse. Experiments performed by other investigators suggest that cellular mechanisms are also at play during the second crisis: Rappatoni (1984) showed that lethal irradiation (900 R) of mice just prior to the second crisis results in an inability to clear parasitaemia (Figure 9.1), and concluded that a radiosensitive cell population was involved in the clearance of T. musculi from its host. Further, she demonstrated that administration of silica (an inhibitor of macrophage function (Kessel et al., 1963; Allison et al., 1966)) to mice prior to inoculation led to a slight (2 d) prolongation of infection. Albright & Albright (1982) and Brooks & Reed (1979) used trypan blue, another macrophage inhibitor, to show that clearance of parasitaemia was slightly prolonged in treated mice. Vincendeau et al. (1981) investigated the changes in macrophage function which occur during T. musculi infection and found that macrophages become activated during the plateau phase of infection. Chang & Dusanic (1976) demonstrated in vitro phagocytosis of T. musculi by

FIGURE 9.1

Course of parasitaemia in A/J mice following irradiation on day 20 post-infection. This figure demonstrates the effect of radiation upon the course of infection in lethally irradiated (900 R), infected A/J and B10.A mice. Ionizing radiation was given on day 20 post-infection (arrows). The effect of radiation upon the infected mice is shown by the solid lines. The course of infection in control mice is shown by the dotted lines.

(From Rappatoni, 1984, with permission)


900r, 20 DAYS POST-INFECTION
A/J
Irrad.
900r
B10 A
Irrad
900r
LOG10 PARASITES PER ML BLOOD
DAY OF INFECTION
0
1
2
3
4
5
6
7
8
9
0
4
8
12
16
20
24
28

peritoneal macrophages. Similarly, using *in vitro* techniques, other investigators have shown that peritoneal macrophages as well as cultured J774 murine macrophages are able to mediate *T. musculi* phagocytosis in the presence of heat-labile factors in immune mouse serum (Ferrante, 1986) or trypanosome-specific IgG1 and IgG2a (Vincendeau et al., 1986). As described in Chapter VII, the involvement of macrophages in immunity to a number of different trypanosome species has been shown; specifically, it is believed that liver macrophages (Kupffer cells) are important in trypanosome elimination (MacAskill et al., 1980, 1981). Thus, it seems possible that the macrophage participates in *T. musculi* elimination. This does not, however, rule out the possibility that other cells are also involved in elimination: a recent paper by Viens et al. (1984) showed that platelets may be able to mediate trypanosome killing *in vitro*.

We thus undertook to investigate the effects of a number of treatments on IP-induced clearing, to determine the nature of cellular involvement in trypanosome elimination.

RESULTS

1. EFFECT OF IRRADIATION ON THE ABILITY OF IP TO EFFECT PARASITE REMOVAL

T. musculi-infected C57BL/6 mice were subjected to 900 R (lethal dose) of irradiation 10-12 d p.i. IP was administered to groups of irradiated mice at different times post irradiation. Figure 9.2 demonstrates that IP was able to effect trypanosome elimination in mice up to 3 days following irradiation, but subsequently could not bring about clearance. The differential cell counts on corresponding days are shown in Table 9.1.

2. EFFECT OF PLATELET DEPLETION ON IP-INDUCED PARASITE REMOVAL

In order to assess the contribution of platelets to the trypanocidal process, we prepared a rabbit anti-mouse platelet antiserum (APAS) and injected it into infected mice prior to IP administration. As Table 9.2 shows, the antiserum was highly effective at reducing the number of circulating platelets; the reduction in platelet number was achieved within 3 h of APAS administration (data not shown). These platelet-depleted animals, however, were still able to effectively clear their parasitaemias following IP administration. IP also functioned in the in vitro assay

FIGURE 9.2

Effect of irradiation on the ability of IP to induce clearance of parasitaemia in infected C57BL/6 mice. Mice were irradiated (900 R) on day 10-12 p.i. (open arrow). IP (0.4 ml i.v.) was administered to irradiated, infected mice 1 (--), 2 (···), 3 (-·) and 4 (··) days after irradiation (solid arrows). One group of irradiated mice received no plasma (IRR,○), and a group of mice received no treatment (NT,---). All mice died within 8 days of irradiation. Each point represents the mean value of 4 to 5 mice +/- 1 standard error of the mean.

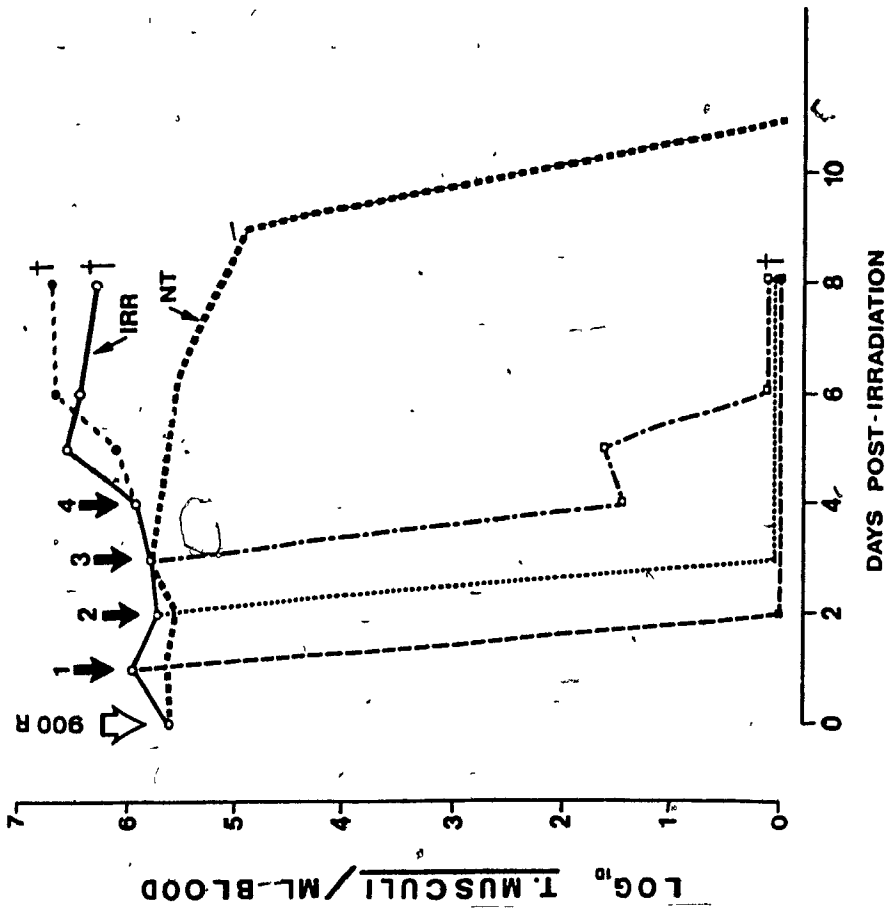

LOG10 T. MUSCULI/ML-BLOOD
7
6
5
4
3
2
1
0
900 R
1
2
3
4
IRR
NT
0
2
4
6
8
10
DAYS POST-IRRADIATION

Table 9.1: Differential cell counts in blood of irradiated mice

	Days post-irradiation[a]				
	0	1	2	3[b]	4
WHITE BLOOD CELLS (x 10^5/ml)	83.0	5.2	4.0	2.50	2.60
GRANULOCYTES	12.5	0.5	ND[c]	0.03	0
LYMPHOCYTES	67.2	4.6	ND	1.53	0.91
MONOCYTES/MACROPHAGES	3.3	0.1	ND	0.95	1.69
PLATELETS (x 10^8/ml)	1.2	1.0	1.4	1.65	1.54

[a] T. musculi-infected C57BL/6 mice were irradiated (900 R) on d 10-12 p.i. Blood samples were drawn, and cell counts performed. Each value represents the mean of 4-5 mice. The values tabulated are compiled from 3 separate experiments.

[b] These values represent the means from 3 mice.

[c] Not done.

Table 9.2: Effect of anti-platelet antiserum (APAS) on IP-induced trypanosome clearance *in vivo*

	APAS-treated[a]		NRS-treated	
	T. musculi ($x10^4$)	platelets ($x10^6$)	*T. musculi* ($x10^4$)	platelets ($x10^6$)
day 0	63 ± 4	685 ± 33	58 ± 5	574 ± 45
day 1	0	2.3 ± 0.3	0	748 ± 74
day 2	0	3.0 ± 0.6	0	637 ± 9

[a] On d 12 p.i., trypanosome and platelet counts were performed in infected C57BL/6 mice. Four mice then received 0.1 ml APAS i.v.; an additional 4 mice received 0.1 ml of normal rabbit serum i.v.. Three h later (after determining that platelet counts had indeed fallen in APAS-treated mice), all mice received 0.4 ml IP i.v. Trypanosome and platelet counts were determined on 2 successive days. Each value represents the mean value of 4 mice ± 1 standard error of the mean.

using blood obtained from platelet-depleted C57BL/6 mice (data not shown).

3. EFFECT OF SILICA ON IP-MEDIATED PARASITE ELIMINATION

Infected C57BL/6 mice were treated with silica intravenously 1 d prior to IP administration. In order to assess the degree of mononuclear phagocytic system (MPS) impairment, silica-treated mice were given 10^9 ^{51}Cr labelled sheep red blood cells (SRBC) intravenously 24 h after silica treatment; 1 h later, mice were sacrificed and radioactivity was measured in liver, spleen and blood samples. Table 9.3 shows that silica-treated mice had markedly decreased (but not zero) liver uptake, and markedly increased spleen and blood levels of labelled SRBC, as compared to untreated controls. Note that the total uptake in non-treated mice appears to be higher than that in silica-treated mice ($p < .05$).

The effect of IP on infected, silica-treated mice is shown in Figure 9.3. It is evident that IP was still able to effectively eliminate trypanosomes from the bloodstream, despite the marked reduction in MPS function. The curative activity of IP *in vitro* was assessed using blood obtained from infected mice which were irradiated (900 R) and treated with silica 1 d later. Table 9.4 shows that there is no difference between blood obtained from silica-treated and

TABLE 9.3: Effect of silica on ^{51}Cr-labelled sheep red blood cell (SRBC) uptake by various organs

	Radioactivity-counts per minute (% of total)			
	Blood[a]	Liver	Spleen	Total
NONTREATED	51,218 ± 8,486 (8.7%)	521,340 ± 37,256 (88.3%)	17,870 ± 3,406 (3.0%)	590,429 ± 31,089 (100%)
SILICA TREATED[b]	114,114 ± 14,487 (27.7%)	103,991 ± 20,097 (26.0%)	185,596 ± 20,756 (46.3%)	400,702 ± 49,183 (100%)

[a] Counts were normalized to a blood volume of 1.5 ml/mouse.

[b] Six C57BL/6 mice received 3 mg of silica i.v. one day prior to challenge with 10^9 labelled and sensitized SRBC; 6 non-treated mice served as controls. One h following i.v. administration of SRBC, mice were sacrificed. Individual livers, spleens and 0.15 ml blood samples were placed in scintillation vials and counted in a gamma counter. Values shown are means for 6 mice ± 1 standard error of the mean.

FIGURE 9.3

Effect of silica administration on the ability of IP to induce clearance of parasitaemia in infected C57BL/6 mice. Infected mice received 5 mg of silica i.v. on day 10 p.i. (open arrow). The following day, silica-treated mice received 0.4 ml IP i.v. (Si+IP); control mice received silica alone (Si), IP alone (IP), or no treatment (NT). Each point represents the mean of 4 mice +/- 1 standard error of the mean.

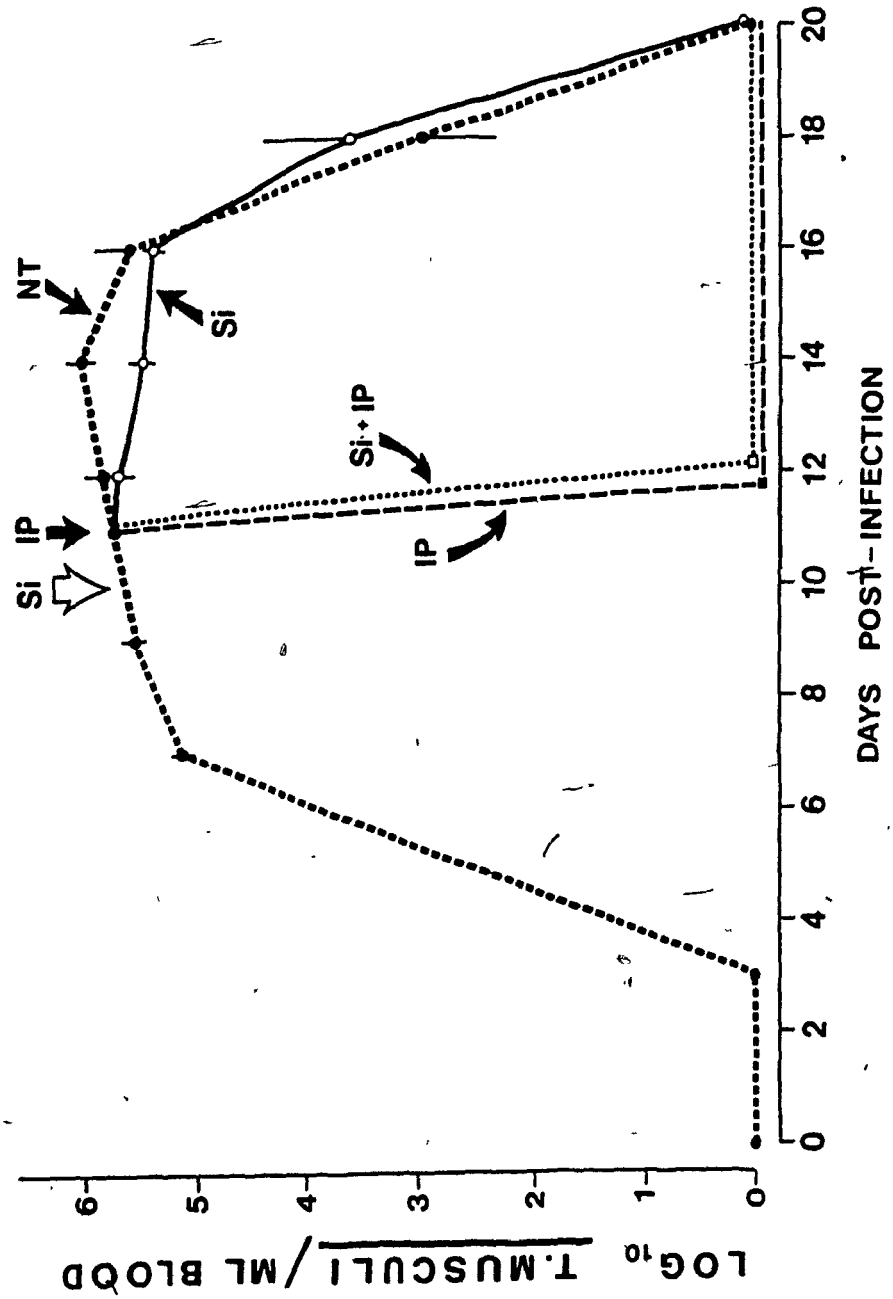

LOG10 T.MUSCULI/ML BLOOD
0
1
2
3
4
5
6
DAYS POST-INFECTION
0
2
4
6
8
10
12
14
16
18
20
NT
Si
Si IP
Si + IP
IP

TABLE 9.4: Effect of silica on IP-induced trypanosome clearance *in vitro*

	T. musculi counts ($x10^4$)	
	Silica-treated[a]	Non-treated
0h	76.3 ± 5.2	88.0 ± 7.2
1h	0.7 ± 0.7	0.3 ± 0.3
2h	0.3 ± 0.3	0.3 ± 0.3

[a] C57BL/6 mice infected 1 d previously with *T. musculi* were irradiated (900 R). One d later, mice were treated with 2 x 3 mg doses of silica i.v. On the following day, blood was collected from these mice and used in the *in vitro* assay. The data are from a representative experiment. Each point represents the mean value of a triplicate sample ± 1 standard error of the mean.

non-treated mice. Figure 9.4 shows a trypanosome attached to a macrophage laden with latex particles (used to demonstrate the phagocytic capability of macrophages). Over a period of approximately 1 h, the trypanosome gradually moved less and less, finally dying and becoming partially engulfed by the macrophage.

4. EFFECT OF TRYPAN BLUE ON IP-INDUCED TRYPANOSOME ELIMINATION

Administration of a course of trypan blue to infected mice was the only treatment shown to have a significant effect on parasite elimination; mice treated with 6 mg of trypan blue were cleared of parasites by IP, although it took somewhat longer as compared to control mice (Figure 9.5). *In vitro*, trypan blue did not interfere with elimination; IP reduced the number of parasites from 47 x 10^4/ml to 0 within 1 h (data not shown).

DISCUSSION

Results obtained from our laboratory have suggested the existence of a radiosensitive cell which mediates cure of parasitaemia at the time of the second crisis in *T. musculi* infection. Work by other investigators with *T. musculi* and other trypanosome species has suggested that the macrophage

FIGURE 9.4

Trypanosoma musculi attached to a latex-laden cell in the *in vitro* assay (600x, Diffquik stain).

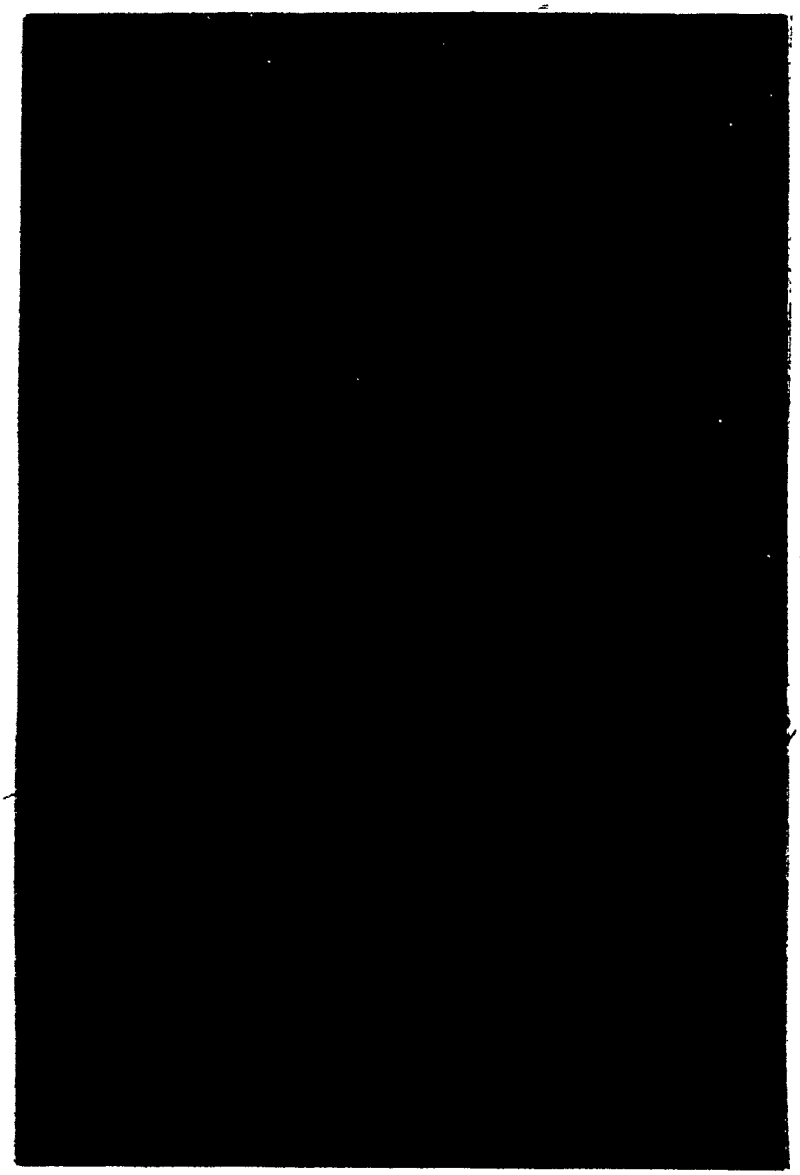
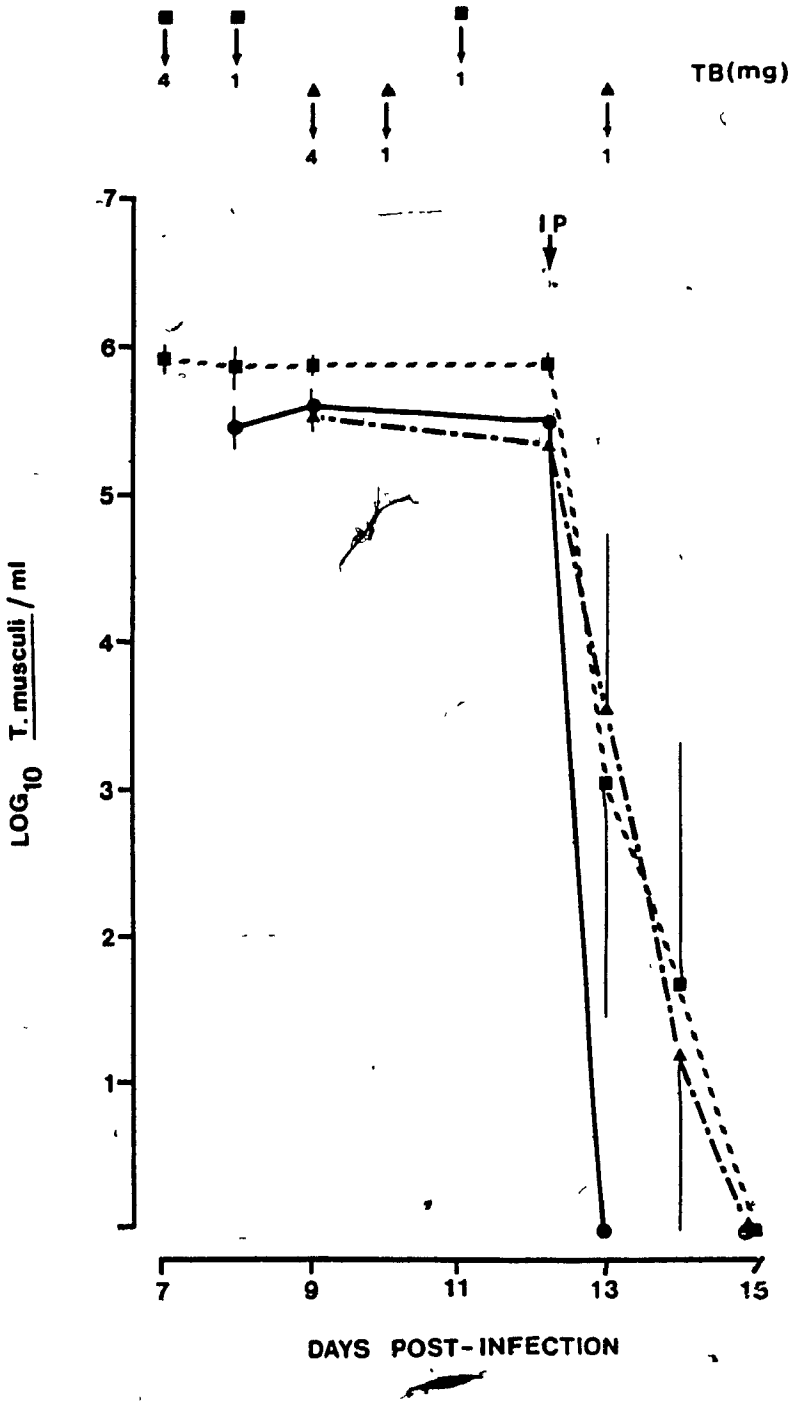


FIGURE 9.5

Effect of trypan blue administration on the ability of IP to induce clearance of parasitaemia in infected C57BL/6 mice. Infected C57BL/6 mice received trypan blue in saline starting on either day 7 (■) or day 9 (▲) p.i. as indicated in the top panel of the Figure. In each schedule, the first two injections were i.p., the last one being s.c. On day 12 p.i., mice from both groups as well as control, non-treated mice received 0.4 ml IRBC i.v. Each point represents the mean of 3-4 mice +/- 1 standard error of the mean.


TB(mg)
4
1
4
1
1
1
IP
7
6
5
4
3
2
1
LOG10 T. musculi / ml
7
9
11
13
15
DAYS POST-INFECTION

is intimately involved in parasite elimination. Since we also demonstrated phagocytosis of trypanosomes in vitro (Figure 5.2) by "macrophage-like" cells, we undertook studies to examine the role of the macrophage in IP-induced clearance of parasitaemia.

The results of the irradiation studies (Figure 9.2) show that IP is able to mediate parasite elimination as long as 4 days following a lethal dose of irradiation. This seems to be at variance with the data of Rappatoni (1984), in which irradiation prevented mice from spontaneously clearing their parasitemias at the second crisis. The difference may be explained, however, if it is considered that irradiation at the second crisis affects primarily the antibody-producing cell; the trypanocidal effector cell may only be affected secondarily. If this is the case, then the conclusion to be derived from Figure 9.2 is that a relatively radioresistant effector cell is involved in trypanosome elimination. If it is assumed that the production of white blood cells (WBC) by the bone marrow completely ceases following irradiation, the radioresistant cell must be one which has a lifespan of approximately 4 days. Polymorphonuclear cells are considered to be particularly radiosensitive; in fact, their numbers decreased substantially just 1 d post-irradiation (Table 9.1), and are therefore unlikely candidates for the effector cell. A direct role for T lymphocytes as effector cells in the control of T. musculi infection is unlikely, considering

that removal of theta-positive T-cells from spleens of mice immune to T. musculi did not impair the ability to transfer immunity to deprived animals (Pouliot et al., 1977). Albright et al. (1984) demonstrated conclusively that natural killer (NK) cells do not play a major role in cure of T. musculi infection. Thus, the possible effector cell candidates remaining include platelets and the cells of the reticuloendothelial system.

A recent paper by Viens et al. (1985) suggests that platelets are involved in the killing of T. musculi by a mechanism termed "platelet activated trypanolysis" (PATL). To determine whether this phenomenon was operative in vivo, we treated mice with anti-mouse platelet antiserum. After demonstrating that the antiserum was effective in reducing platelet number, we showed that IP was still able to bring about parasite elimination both in vivo and in vitro (Table 9.2), thereby indicating the unlikelihood of platelet involvement in T. musculi removal from the blood. It must be pointed out, however, that multiple effector mechanisms may be operative in vivo, and the platelet may still be one of several possible effector cells.

We therefore next turned to the cells of the reticuloendothelial system (RES), attempting to demonstrate that they play a key role in the elimination of T. musculi

Silica is a macrophage toxin, known to produce dysfunction of the RES by inducing morphological and biochemical modifications in macrophages (Kessel et al.,

1963; Allison et al., 1966). These changes result in interference with the uptake and processing of antigens (Pearsall & Weiser, 1968) as well as a significant reduction of the phagocytic capacity of macrophages (O'Brien, 1979). Rappatoni (1984) previously showed that silica administration caused a mild but significant prolongation of infection. Albright & Albright (1981b) treated mice with a single intravenous injection of silica, and found an increase in parasitaemia only in susceptible strains of mice. The data of Figure 9.3 indicate that silica had no significant effect on the ability of IP to effect complete parasite elimination in vivo. Again, the discrepancy between these results and those of Rappatoni may be explained by hypothesizing an incomplete blockade of the MPS by silica administration. Table 9.3 shows a marked reduction in macrophage function as measured by ^{51}Cr-labelled SRBC uptake. It must be noted that the liver (Kupffer cell) uptake is not reduced to zero. A sizeable number of labelled SRBC are still taken up by the liver macrophages, i.e. 10-25% of Kupffer cell function is still maintained. This may still be sufficient to eliminate trypanosomes. Interestingly, the total uptake of SRBC in nontreated mice appears to be significantly higher than that in silica-treated mice. This is curious since the mice were given identical doses of SRBC. The discrepancy might be explained by postulating that SRBC are taken up in other sites (e.g. lungs) by macrophages whose function becomes

apparent only when Kupffer cells are overwhelmed. Our failure to measure radioactivity in these other sites could account for the differential total uptake. Thus, it is possible that we were unable to achieve a complete macrophage block using silica, and therefore IP was still able to effect a clearance of parasitaemia.

Table 9.4 indicates that silica did not impair IP-induced trypanosome removal in vitro. Indeed, the ability of macrophages from such mice to phagocytose latex particles and trypanosomes (Figure 9.4) suggests that the dose of silica administered did not affect blood macrophages. The fact that trypanosomes were still eliminated in such mice with apparently intact blood macrophage function raises the possibility that the final effector process might take place in the blood.

The trypan blue studies were slightly more convincing, in that a small, but significant, impairment of IP-induced trypanosome clearance was seen. Previous workers have used trypan blue to ablate macrophage function. Trypan blue is actively taken up by macrophages but it is not ingested by viable lymphocytes, neutrophils, basophils or eosinophils (Beck et al., 1967; Guckian et al., 1978); the dye is thought to act selectively on macrophages, although at high concentrations, it reduces lymphocyte blastogenesis (Kripke et al., 1977). Interestingly, trypan blue is reported to exhibit trypanocidal activity, although it is not used clinically (Williamson, 1962). Thus, Brooks & Reed (1979)

showed that mice injected with trypan blue were less capable of controlling infection and the data of Figure 9.5 support these earlier studies, indicating that macrophage function is likely to be necessary for T. musculi elimination. In studies investigating the different mechanisms used by macrophages to inflict damage to target cells, Hall et al. (1982) found that trypan blue depletes macrophages of their ability to kill by spontaneous macrophage-mediated cytotoxicity, but not by antibody-dependent cytotoxicity. Thus, the data presented here support but do not confirm the importance of macrophages in the clearing of T. musculi from the blood of infected mice.

It has been mentioned on several occasions that other investigators have demonstrated the importance of macrophages in effecting trypanosome removal. Recent work by Vincendeau et al. (1986) has elegantly shown that macrophages are effective in antibody-mediated phagocytosis of T. musculi both in vitro and in vivo. These investigators infected mice intraperitoneally with T. musculi and showed that at the time of natural decline of trypanosomes, phagocytosis of parasites by peritoneal macrophages was obvious. They observed moving parasites, engulfed in phagocytic vacuoles, with flagella lashing until completely enveloped by the macrophages. Identical observations were made within 40 min of intraperitoneal administration of anti-T. musculi hyperimmune serum. Furthermore, using inhibition studies with mouse monoclonal

antibodies and anti-FcR antibodies, this group showed that IgG1 and IgG2a antibodies mediated macrophage cytotoxicity via distinct Fc receptors. It was concluded that antibody-mediated phagocytosis, followed by killing by peritoneal macrophages is of prime importance in the in vivo elimination of parasites in infected mice.

In summary, although additional data have been presented regarding cellular involvement in T. musculi elimination, it is far from clear as to which cell(s) is/are involved in effecting parasite removal from the blood. Although peritoneal macrophages have been observed to bind to and phagocytose trypanosomes both in vitro and in vivo, this does not explain how parasites are removed from the blood, the major locus of infection following intravenous inoculation. Much work involving cell depletion in vivo as well as in vitro remains to be performed before the macrophage (blood or tissue) can be unequivocally identified as the final effector cell mediating trypanosome elimination in vivo.

CHAPTER X

GENERAL DISCUSSION

CHAPTER X

GENERAL DISCUSSION

Infection of mice with Trypanosoma musculi is characterized by a well-defined and reproducible pattern of parasitaemia in which three phases are clearly distinguished: the initial growth phase, the plateau phase, and the elimination phase. Previous studies have demonstrated that control of parasitaemia is determined by the host rather than by the parasite. Although considerable efforts have been devoted to elucidating the mechanisms underlying this control, many aspects of the host-parasite interaction remain unclear. The major purpose of the work embodied in this thesis was to investigate the immune mechanisms involved in the elimination of T. musculi from the blood of infected mice.

Other investigators have demonstrated conclusively the thymus-dependency of trypanosome elimination (Viens, Targett, et al., 1974; Brooks & Reed, 1977; Rank et al., 1977). In addition, work in our own laboratory has pointed to the the necessity of intact B-cell function for trypanosome elimination to occur (Vargas et al., 1984). The most obvious deduction, therefore, is that trypanosome elimination is an immune process in which a T-cell dependent anti-trypanosomal antibody plays a role. If so, the passive

transfer of serum from an immune host to an infected mouse should have the capacity to terminate an infection. Yet, the procedure has been ineffective in the hands of recent investigators, and the role of antibody in T. musculi elimination has been seriously questioned: as recently as 1982, it was affirmed that antibody does not play a significant role in the elimination phase (Albright & Albright, 1982). We have clearly demonstrated (Chapter III) that it is, in fact, possible to cure mice via passive transfer of immune plasma, and that the agent mediating clearance of parasitaemia is labile to heat-treatment. The apparent heat lability of the active agent in immune plasma suggested a possible role for complement -- itself heat-labile -- in the mechanism of cure. The experiments presented in Chapter IV show that complement is necessary for clearance to take place, although it does not appear to be the only heat-sensitive plasma constituent involved. By deduction, since mice genetically deficient in C5 are still able to effect trypanosome clearance, the complement component required for cure must be C3, rather than the late-acting lytic sequence (C5-C9).

In order to better understand the mechanisms involved in parasite elimination, we undertook the development of an in vitro assay (Chapter V) in which we could better control the different parameters involved. This assay proved to correlate exactly with all in vivo studies, and had the advantages of rapidity, economy and allowance for dissection

of the components involved.

With these tools in hand, we embarked on an investigation of the heat-labile plasma component involved in cure of infected mice. Initial purification suggested, and Protein A chromatography confirmed, the immunoglobulin nature of the activity (Chapter VI), and pointed to its apparent restriction to one or two subclasses of IgG. The affinity chromatography studies described in Chapter VII demonstrate unequivocally that the curative activity lies principally in the IgG2a fraction. The same Chapter begins a characterization of those antigens to which the curative antibody binds, using the technique of immunoblotting; we have demonstrated that IgG2a antibodies present in an immunoglobulin fraction of immune plasma recognize a small number of proteins in a trypanosome homogenate, suggesting that the IgG2a response is selective.

Finally, given the involvement of antibody and complement in elimination and the apparent lack of involvement of the complement lytic pathway, cellular involvement in the immune effector mechanism was considered. The studies of Chapter IX begin an evaluation of the cellular components involved in the elimination mechanism, and point to the likelihood of macrophage participation in trypanosome removal.

Thus, we have demonstrated that the effector mechanisms operative in T. musculi elimination involve at least

antibody (IgG2a), complement component C3, and a cellular element (macrophage). Together, these findings suggest the following sequence of events occurring at the time of trypanosome clearance: (1) Antibody binds to antigen at the trypanosome surface. (2) The Fc moiety of bound antibody initiates the classical complement cascade, leading to deposition of component C3b on the trypanosome surface. Alternately, the parasite itself initiates the alternative pathway of complement activation, with the same result. (3) The trypanosome, opsonized by both antibody and complement, binds to an effector cell via Fc and C3b receptors. (4) The parasite-cell interaction leads to activation of cellular effector mechanisms which result in death of the trypanosome.

This postulated sequence of events raises a number of questions: Why is it that the curative activity resides primarily in a single immunoglobulin subclass? What is the nature of the effector cell? How does it bring about death of the trypanosome? Finally, why does parasite elimination consistently occur three weeks following inoculation? The work presented in this thesis clarifies some of these issues.

Given the representation of anti-trypanosome activity in all classes and subclasses examined, why should only IgG2a antibodies be able to effect cure? Evidence has already been presented for the restriction of certain

antibody responses to particular subclasses, so that this phenomenon is not a completely novel finding. It is, however, the first instance (to our knowledge) of a restriction of a functional activity to a subclass of IgG in a natural, in vivo, host-parasite interaction. It is possible that IgG2a antibodies are the only ones to recognize a specific antigen on the surface of the parasite. This antigen may be a structural one, in which case the antibody simply serves as an opsonin. If, however, the antigen were a transport protein or an enzyme, antibody binding could interfere with the viability of the parasite, rendering it more susceptible to host effector mechanisms. The nature of the antigen should be amenable to clarification by a refinement of the blotting procedure.

A more likely possibility is that IgG2a molecules are the only ones whose Fc portions are recognized by appropriate effector cells. A number of studies have been described (Chapter VII) in which only IgG2a antibodies effectively mediate cytotoxicity directed against tumor cells. This capacity is believed to be related to the expression of Fc receptors (FcRI) specific for IgG2a on the surface of effector macrophages. It has been shown, however, that receptors for IgG1, IgG2b and IgG3 are also present on such cells. The failure of these other subclasses of IgG to mediate parasite elimination may result from: (a) low affinity of binding to antigenic determinants of the trypanosome; (b) low affinity of binding to effector cell Fc

receptors; (c) low receptor density on the effector cell surface; or (d) failure of these receptors to trigger the appropriate effector mechanism(s). Alternatively, the Fc portions of IgG1, IgG2b or IgG3 may be unavailable for binding to effector cell Fc receptors (Vincendeau et al., 1986): they might be degraded by parasite-derived proteases (shown to be produced by T. cruzi -- Krettli et al., 1980), or they might bind to parasite FcR (found on Schistosoma mansoni -- Torpier et al., 1979). It is likely, however, that antibodies of other subclasses do participate in in vivo trypanosome clearance by providing additional sites of contact between the cell and the parasite.

The nature of the cell involved in effecting trypanosome elimination is still uncertain, although much evidence points to the macrophage. Chang & Dusanic (1976), Vincendeau et al. (1986) and Ferrante (1986) have demonstrated that peritoneal macrophages are able to mediate phagocytosis of T. musculi in the presence of immune serum and complement. Our own in vitro observations of trypanosomes attached to macrophages make this cell a likely candidate. Given the mobility of trypanosomes and the inherent difficulty in forming a cohesive bond between two mobile components, it would seem reasonable that the macrophage involved is a resident, non-circulating cell. Although resident macrophages are present in all tissues, it is in the liver (as Kupffer cells) that macrophages are

present in High numbers. Indeed, Kupffer cells have been demonstrated to be responsible for removal of circulating foreign cells, including trypanosomes of different species: Scott & Moyes (1982) showed that antibody facilitates liver uptake of infective T. cruzi trypomastigotes; Holmes et al. (1979), MacAskill et al. (1980, 1981) and Anderson & Banks (1982) demonstrated that radioactively labelled T. brucei are removed principally by the liver. It was puzzling to find, therefore, that blockade of the reticuloendothelial system (RES) by doses of silica which markedly reduced its efficacy in removing sensitized circulating SRBC, did not appear to affect the elimination of trypanosomes. It is possible, as discussed, that the results may reflect a quantitative phenomenon. In C57BL/6 mice, a maximum of 10^7 trypanosomes must be cleared, and although SRBC uptake was significantly reduced in silica-treatd mice, the liver was still able to remove 20% of the 10^9 SRBC (i.e. it was still capable of removing 10x more cells than required). Such an hypothesis is easily testable. The alternative is that the Kupffer cells of the liver are, in fact, not primarily involved in the elimination phase. For instance, it is possible that endothelial cells might function in the effector mechanism. Morrison et al. (1978) report that a significant number of T. congolense remain bound to vascular endothelium. MacAskill et al. (1980) and Anderson & Banks (1982) found trapping of T. brucei in capillary beds of the lungs. Endothelial cells have Fc

receptors and C3 recptors which are normally masked in tissue culture, but which may be exposed in response to certain stimuli e.g. immune complex deposition (Ryan et al., 1981). Such cells have also been shown to possess microbicidal function: Ryan (1986) describes endothelial cells of the lung that are able to participate in "vigorous phagocytosis". The hypothesis of endothelial cell involvement could be tested in vivo by the histological demonstration of parasites bound to endothelial cells, and by using them in the in vitro assay following isolation of such cells.

The means by which trypanosomes are killed also remains unclear. Effector cell-parasite contact presumably leads to activation of trypanocidal mechanisms in the effector cell. This might simply be phagocytosis, with death of the parasite intracellularly upon exposure to lysosomal contents (superoxide or HOCl (Vincendeau, 1986), or polyamine oxidase-stimulated hydrogen peroxide or aldehydes (Ferrante et al., 1984)). Alternatively, an antibody-dependent cellular cytotoxic (ADCC) mechanism could be involved, in which the parasite is killed extracellularly by released products and subsequently phagocytosed. It is conceivable that more than one type of effector cell is involved in mediating parasite death. The opsonized trypanosome might be bound to one type of cell (endothelial cell, platelet) while another cell (macrophage) effects its demise via release of

toxic metabolites. Clearly, a great deal of work remains to be done in order to elucidate the actual mechanism of parasite disposal.

It is generally accepted that the IgG response reaches a peak 10-14 days following initial exposure to an antigen. It is, therefore, curious that plasma collected 14 days following inoculation with parasites has no effect on the course of infection. If IgG2a is intimately involved in trypanosome elimination, it is interesting to speculate on the reasons for the three week duration of parasitaemia. One possibility is that a population of trypanosomes (adult forms?) bearing a distinct antigenic determinant appears 7-10 days following infection; IgG2a (and other) antibodies are then produced in response to this "new" determinant, reaching appropriate concentrations just prior to clearance of parasitaemia. Verification of this hypothesis requires a comparison of trypanosome antigen preparations made from different populations, as well as a clear understanding of the relationships among the different morphological forms of T. musculi in the bloodstream of the mouse.

It is equally likely that the IgG2a response, and in fact, the entire normal sequence of events in the murine humoral immune reponse is depressed during the early stages of infection. The phenomenon of immunodepression has been extensively described in all species of trypanosomes, including T. musculi. Pattison et al. (1983) have

suggested that trypanosome-derived substances affect the ability of the mouse to produce high affinity antibody. This might occur via an interference with antigen presentation and macrophage processing, or by generation of T-suppressor cells which lead to preferential tolerization directed against certain antigens. T. musculi infection has been shown to delay the normal process of antibody affinity maturation (Pattison & Steward, 1985). Only after the host escapes the immunodepression is it able to generate an adequate anti-trypanosome immune response, consisting of high affinity IgG2a at an appropriately elevated concentration. If it is primarily young and dividing forms which mediate this immunodepression, it is conceivable that a normal immune response may again be generated following disappearance of these forms 7-10 days after inoculation.

It is also possible that the involved effector cells require a significant amount of time to become sufficently activated to mediate trypanosome elimination. Vincendeau et al. (1981) showed that macrophages attain maximal changes in metabolic and phagocytic activity 14 days post inoculation. Ezekowitz et al. (1983a,b) have found that macrophage activation is associated with an increased capacity to bind IgG2a because of increased receptor number and affinity for IgG2a. It is conceivable that the delay in effector cell activation is also a result of parasite-derived immunodepressive molecules. Escape from the effect of such molecules might be coincident with a decline in the

percentage of young and dividing forms. Thus, a number of factors might contribute to the three week duration of T. musculi infection.

Numerous questions remain unanswered. It is evident that the murine model of trypanosomiasis, though relatively simple in comparison with human or cattle disease, is far from completely understood. There are probably several immune mechanisms which operate in concert to control the course of infection; more work is required to clarify these interactions. Despite this, if it could be shown that curative activity in humans or cattle is primarily restricted to one immunoglobulin subclass, this particular subclass could be selectively administered, or its production selectively stimulated, to effect parasite elimination earlier on in the course of infection.

Trypanosomiasis is a major health problem. To reduce the magnitude of the deleterious effects of this disease, a better comprehension of trypanosome biology, supported by studies of experimental infection, might lead to the discovery of factors critical to improvement of the efficacy of host defense against the parasite. The immune mechanisms of cure in Trypanosoma musculi infection are more complicated than was previously thought. We have clearly shown that antibody of the IgG2a subclass is involved, as is complement and an as yet undetermined (though likely

macrophage) effector cell. The present studies point the way to a more complete understanding of the manner in which an organism defends itself against invasion by a parasite. It is hoped that this understanding will contribute to a reduction in the morbidity and mortality of trypanosomiasis.

SUMMARY

(Note: Summary points marked with a '*' indicate original work presented in this thesis)

* 1. Passive transfer of plasma from a mouse cured of *Trypanosoma musculi* infection (immune plasma -- IP) to an infected host rapidly eliminates parasitaemia (curative activity).

* 2. The curative activity is sensitive to heat treatment (56°C, 30 min).

* 3. The curative activity develops gradually over the course of infection.

4. Pretreatment with IP prevents the development of a patent parasitaemia in a naive host (protective activity).

* 5. The protective activity is heat-stable.

* 6. The protective activity develops gradually over the course of infection.

* 7. The protective activity is diminished when the infective dose of trypanosomes is increased.

* 8. Treatment of recipient mice with cobra venom factor (CVF) prevents occurrence of IP-induced trypanosome clearance.

* 9. The *in vitro* assay is rapid, economical, and correlates well with *in vivo* studies.

* 10. The curative activity is immunoglobulin in nature, and is associated with a heat-labile protein A-

derived fraction of IP eluted at pH 4.5 that contains IgG2a and IgG3.

* 11. IgG2a-enriched fractions of IP mediate complete parasite removal *in vitro* ; they effect a highly significant reduction in trypanosome number *in vivo*. IP depleted of IgG2a is devoid of curative activity.

* 12. The curative activity of IgG2a is heat-labile.

* 13. Trypanosome-specific IgG2a appears gradually over the course of infection, reaching a peak immediately prior to clearance of parasitaemia.

* 14. Infected CBA/N mice (B-cell defective) clear their parasitaemias approximately 10 days later than those in normal mice; IgG2a levels in CBA/N mice reach their peak 10 days after those in normal mice.

* 15. IgG2a antibodies in a protein A-derived pH 4.5 fraction of IP recognize a limited number of antigens compared with those recognized by IgG antibodies in IP.

16. Mouse strains vary in susceptibility to *T. musculi* infection.

* 17. The ability of IP to mediate cure in different strains of mice appears to be related to the level of parasite burden rather than to a defective defense mechanism in susceptible recipients.

* 18. The absence of C5 in both donor and recipient

strains of mice does not impair the ability of IP to effect trypanosome removal, reinforcing the idea that complement-dependent trypanolysis is not essential for clearance.

19. A relatively radioresistant cell is involved in T. musculi elimination.

* 20. Platelets do not appear to be essential for trypanosome elimination in vivo or in vitro.

* 21. Silica does not significantly affect IP-induced trypanosome removal in vivo or in vitro.

* 22. Trypan blue has only a minimal effect on IP-induced clearance.

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