This is to certify that the

thesis entitled

IMMUNOCONGLUTINATION IN EXPERIMENTAL
AFRICAN TRYPANOSOMIASIS

presented by

William J. Rickman

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Microbiology

Herbert W. Cox
Major professor

Date 7 April, 1981

IMMUNOCONGLUTINATION IN EXPERIMENTAL AFRICAN TRYPANOSOMIASIS

By

William J. Rickman

A DISSERTATION

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

DOCTOR OF PHILOSOPHY

Department of Microbiology and Public Health

1981

ABSTRACT

IMMUNOCONGLUTINATION IN EXPERIMENTAL
AFRICAN TRYPANOSOMIASIS

By

William J. Rickman

Rats infected with *Trypanosoma brucei rhodesiense* developed a syndrome of anemia, thrombocytopenia and coagulopathy accompanied by reductions in parasitemia. Onset of the syndrome was associated with elevated titers of cold-active hemagglutinin (CAH), antibody to fibrinogen related products (Anti-F), antibody against parasite antigen (Anti-Tryp) and antibody against the third component of fixed complement or immunoconglutinin (IK). Soluble parasite antigen and fibrinogen related antigen were also detected and soluble immune complexes of the antigens were demonstrated. Association of the immune factors with anemia, thrombocytopenia and reduced parasitemia, accompanied by consumption of complement, suggested a causal relationship. Prolonged partial thromboplastin times indicated that the coagulation defect involved the Hageman factor dependent pathway. Terminal signs consistent with those of disseminated intravascular coagulation (DIC) were accompanied by consumption of immune factors and complement. Blood cells of these rats autoagglutinated at 4 C. Plasma from anemic rats agglutinated both normal rat and sheep erythrocytes at 4 C, and when the washed agglutinated cells were warmed to 37 C, soluble immune complexes, Anti-F and IK were eluted. These eluates in turn agglutinated sheep and rat erythrocytes at 4 C. Injection of IK, produced in rats by autostimulation, and Anti-Tryp, stimulated in rats by immunization with trypanosome homogenate, into rats infected

with *T. b. rhodesiense* caused anemia with reduced parasitemia to appear earlier than in infected control rats. Injection of the antibodies into uninfected rats did not affect erythrocyte counts and injection of IK or Anti-Tryp alone into infected rats did not produce the early anemia and reduced parasitemia.

In interpretation, it is suggested that an antigenemia consisting of soluble trypanosomal products and of fibrinogen products resulted from infection. With the stimulation of antibodies, soluble immune complexes were formed and complement was fixed. In addition to stimulating IK, the complement-fixing complexes became bound to erythrocytes, platelets and trypanosomes. These cells, coated with the antigen for IK, were immunoconglutinated, or if free complement was present, they may have been lyzed. Cells immunoconglutinated by IK may have been sequestered in the filter organs, e.g. the spleen, where they were phagocytized, or trapped in the capillary beds such as those of the lungs, kidneys, etc., where they act as thrombi occluding the microvasculature. It is also considered possible that platelets were injured by complement-fixing immune complexes which lead to the activation of the Hageman factor pathway of coagulation. This action could contribute to the hypoclottable state associated with the onset of anemia and thrombocytopenia. While DIC signs could have been a consequence of secondary fibrinolysis, thrombi formation could have also been a result of immunoconglutination.

To
my wife
Lisa

ACKNOWLEDGEMENTS

I wish to express my sincere appreciation to my academic advisor, Dr. Herbert W. Cox, for his guidance and encouragement throughout these studies and during the preparation of this dissertation.

Advice and criticism of the dissertation received from my guidance committee, Drs. Gordon R. Carter, Harold D. Newson, and Scott N. Swisher are gratefully acknowledged. I wish also to acknowledge Drs. Robert J. Moon and Leland F. Velicer who served on my examination committees.

The companionship and moral support received from my fellow students, Mr. Fred DeGraves, Miss Nancy Wilcox and Miss Charlotte Bay will be remembered with affection. A special thanks to Miss Mary Ufford for her moral support and helpful criticism during my teaching experience.

This program of advanced studies at Michigan State University in the Department of Microbiology and Public Health was made possible by a departmental graduate assistantship which is gratefully acknowledged. I wish to thank the College of Veterinary Medicine at Michigan State University for a fellowship award in my last year of matriculation.

I wish to acknowledge the support received from funds from Grant NO. R01-A1-17018-01 from the National Institutes of Health, Bethesda, Maryland, from the Michigan Heart Association and from the General Research Funds from the College of Veterinary Medicine of Michigan State University which made it possible to carry on this research.

TABLE OF CONTENTS

LIST OF TABLES

INTRODUCTION

Disease resulting from infections with parasites of the *Trypanosoma brucei* species complex has hindered the settlement and economic development in over 35 countries on the African continent. The agents of human trypanosomiasis, *Trypanosoma brucei gambiense* and *T. b. rhodesiense*, are transmitted by blood sucking dipterans of the genus *Glossina*, commonly known as tsetse flies. An estimated 35 million persons remain at risk of infection with at least 10,000 cases reported annually. Rhodesian and Gambian trypanosomiasis remains well-established in tropical Africa, surviving either in undetected human cases or in an animal reservoir of wild ungulates (Duke, 1978).

At a recent international symposium on The Relevance of Parasitology to Human Welfare Today, Griffiths (1978) emphasized the urgency of a long-term program for the control of African animal trypanosomiasis. "In addition to direct losses arising from disease *per se*, as well as the cost of control operations, the socio-economic impact of animal trypanosomiasis makes itself felt upon: (i) human health, through protein deficiencies caused by shortage of meat and milk; (ii) livestock production, since trypanosomiasis prevents the introduction of improved breeds; (iii) agricultural production, through the lack of draught animals and manure; (iv) the rural economy, by preventing integrated agriculture and livestock production; and (v) the national economy, since the deficit in animal production compels affected countries to import meat and dairy products." Even with support in excess of 50 million dollars per annum, some four million square miles of land south of the Sahara Desert remain unfit for cattle production because of tsetse-borne trypanosomiasis.

The clinical aspects of human and animal trypanosomiasis have been well documented; however, the mechanisms by which these symptoms arise remain unclear. With the increased interest of concerned governments in the prevention of the disease by vaccination, these deficiencies in our knowledge of the pathogenesis of trypanosomiasis need correction. In this respect, laboratory models of infection have been valuable, shedding light on the mechanisms of disease in both human and bovine trypanosomiasis.

Anemia is considered one of the cardinal signs of the disease yet the complex interactions of the host-parasite relationship resulting in clinically recognized anemia remain unresolved. In addition, it has been recently documented that disturbances in the clotting mechanisms of man and animals is a serious complication of trypanosomiasis and may account for the high mortality of the disease. Barrett-Connor and associates (1973) first recognized the existence of several coagulation abnormalities detectable by conventional laboratory tests in patients suffering from Rhodesian trypanosomiasis. On the basis of their laboratory findings, the authors classified this abnormality as disseminated intravascular coagulation (DIC). Robins-Browne et al. (1975) confirmed this association of clotting disturbances with the Rhodesian form of the disease. Greenwood and Whittle (1976) reported similar findings in human cases of Gambian sleeping sickness and Wellde and coworkers (1978) presented evidence indicating DIC as a consequence of bovine trypanosomiasis. To date, the proposed explanations of the pathogenesis of DIC have been speculative and without experimental documentation.

Rickman and Cox (1979) reported on the pathogenesis of a syndrome characterized by anemia, splenomegaly and nephritis in rodents experi-

mentally infected with *T. b. rhodesiense*. It was noted in this investigation that the terminal events leading to the death of infected animals were accompanied by symptoms consistent with pulmonary insufficiency and vascular shock. The authors postulated that the mechanism they associated with the pathogenesis of the syndrome may also be operative in the pathogenesis of the overt clotting abnormalities observed. To test the hypothesis that the mechanisms of anemia and DIC may be interrelated, the experiments reported in this dissertation were designed to ascertain the coagulation status of infected rodents and to determine the role of complement and immune factors in the development of blood pathology during acute *T. b. rhodesiense* infection. The results of this investigation are presented.

REVIEW OF LITERATURE

The Agents of African Trypanosomiasis

Trypanosomes are protozoan flagellates belonging taxonomically to the class *Zoomastigophora*. The family *Trypanosomatidae* is comprised of nine genera of which only two are of medical and veterinary importance; namely, *Leishmania* and *Trypanosoma*. A comprehensive review of the trypanosomes of mammals given by Hoare (1972), outlined 125 species of trypanosomes as parasites of 12 of the 19 orders of mammals with 218 genera being parasitized. Curiously, whales, seals, aardvarks and flying lemurs are among the few mammalian examples lacking parasitic trypanosomes.

Trypanosomes of the genera *Leishmania* and *Trypanosoma* require two hosts to complete their life cycle, an appropriate mammal and blood sucking arthropod. In their cyclic development up to four stages can be recognized: amastigote or leishmanial, promastigote or leptomonad, epimastigote or crithidial, and trypomasitgote or trypanosomal. The amastigote stage is usually intracellular found within cells of a phagocytic nature, whereas the other forms are extracellular parasites found in either the intravascular or extravascular compartments of the hosts (Levine, 1973).

Based on the nature of transmission, trypanosomes have been grouped into one of two sections, the *Stercoraria* or *Salivaria*. Members of the section *Stercorcaria* develop into infective forms in the hindgut of the arthropod vector. While the arthropod obtains a blood meal, it deposits fecal material containing the infective forms, which is rubbed into the site of the bite or transferred to mucous membranes thus establishing infection in the host. On the other hand, members of *Salivaria* develop

in the salivary glands of the vector host with emergence of infective forms via the proboscis. Thus transmission of some trypanosomes occurs through the bite of the blood sucking arthropod (anterior station development), while others are passed in the feces of the feeding arthropod being rubbed into wounds, oral mucosa or conjunctiva (posterior station development; Noble and Noble, 1976).

Some trypanosomes appear to have evolved to the point where the biological requirement for an arthropod host has been lost. The trypanosome responsible for a disease in horses called surra is transmitted mechanically by the contaminated mouth parts of biting flies, primarily those of the *Tabanus spp.*, and *Stomoxys spp. Trypanosoma equiperdum*, the agent of equine dourine, is transmitted venerally (Levine, 1973).

The trypanosomes of the *brucei*-species complex, *Trypanosoma brucei gambiense*, *T. b. rhodesiense* and *T. b. brucei*, all have similar developmental cycles in the tsetse fly (*Glossina spp.*). As members of the section *Salivaria*, these parasites have anterior station development. When trypomastigotes in the blood circulation of a mammalian host are ingested by the fly during a blood meal, the parasites accumulate in the midgut, where they undergo extensive multiplication for about ten days. Migration to the proventriculus occurs, then they pass through the esophagus and pharynx to the proboscis. They enter the hypopharynx and proceed through the salivary ducts into the salivary glands, where they lie freely or attached to the epithelial wall of the salivary glands. At this site, the parasites transform into the epimastigote stage and further multiply. Within a short time the parasites transform into the infective metacyclic trypomastigote stage. When the

metacyclic forms are introduced into a suitable host during a subsequent blood meal, transmission is accomplished. This cycle in the fly spans anywhere from 15 days up to a month (Hoare, 1972). Development in the mammalian host is extensive and is associated with a considerable degree of pathology (See Clinical Manifestation of African Trypanosomiasis).

The distribution of the Gambian and Rhodesian forms of trypanosomiasis coincides closely with the geographic distribution of their respective vectors. Tsetse flies of the *Glossina morsitans* group (also considered a species complex), are the primary vectors of *T. b. rhodesiense*. This group of flies inhabits the savanna-like woodlands of eastern tropical Africa. In this area game animals are plentiful, especially antelopes, on which the fly feeds (Hoare, 1972). Investigations by Weitz (1970) demonstrated that many of the antelopes in this region are infected with trypanosomes of the *brucei*-complex. In this respect, the disease of East African origin is considered a zoonosis.

Transmission of Gambian sleeping sickness in West and Central Africa is accomplished by tsetse flies of the *Glossina palpalis* group. The ecological peculiarities of this group of flies indicate that Gambian sleeping sickness is essentially an anthroponosis, maintained by man-to-man transmission. As a group, the vectors are of a riverine nature living among the trees lining the rivers of tropical Africa. Serologic studies have demonstrated that these flies depend primarily on reptiles as their source of blood meals (Weitz, 1970). The inhabitants of the zones occupied by these flies frequently become infected since dependence on the waterways is essential as a means of livelihood and travel. The distribution of the Gambian form of the disease throughout Africa is discontinuous and corresponds to that of the vector.

An historical account of the discoveries and investigations that lead to the modern day classification of the trypanosomes of the *brucei*-complex can be found in a recent review (Rickman, 1978).

The primary agents of animal trypanosomiases in Africa are *T. b. brucei*, *T. congolense*, and *T. vivax*. The latter two species account for the greatest incidence of morbidity and mortality associated with the disease (Levine, 1973). Mornet (1954) reported the following data on the incidence of bovine trypanosomiasis in West Africa: *T. congolense*, 48 percent; *T. vivax*, 34 percent, and *T. b. brucei*, 14 percent. These figures may fluctuate in this and other areas, but Hoare (1972) considers that the relative order of incidence is essentially the same regardless of geographic considerations. Transmission requiring biological development within the vector host is accomplished by a wide variety of *Glossina* species. The developmental cycle in these flies is classified as anterior station development, characteristic of the salivarian trypanosomes (Hoare, 1972). In areas devoid of *Glossina spp.*, flies of *Tabanus spp.* and *Stomoxys spp.* maintain the infection by mechanical transmission. Under natural conditions, mechanical transmission depends on interrupted feeding, when the fly starts its meal on an infected animal but completes it on a healthy one. Transmission succeeds only if the interval between the two blood meals is very short. Such a mechanism has accounted for significantly large epizootics of *T. congolense* in tsetse-free areas in Sudan during 1946 (Buxton, 1955).

Wild game animals, particularly ruminants, serve as the reservoir hosts of animal trypanosomiases. Bruce (1895) produced the first scientific evidence that wild ungulates are the reservoir hosts from

which domestic animals acquired infection. Wild animals listed by Hoare (1972) as reservoir hosts included elands, waterbucks, reedbucks, giraffes, impalas, wild pigs and antelopes, with the latter accounting for the highest incidence of naturally acquired infection. In a survey conducted by Mackenzie and Boreham (1974b) little in the way of pathology could be demonstrated in the reservoir animals. However, the sampling size of the investigation appeared too small to be considered applicable to the general population of wild animals in Africa.

Clinical and Histopathologic Features of African Trypanosomiasis

Human Trypanosomiasis

It is generally recognized that human infections with trypanosomes of *brucei*-species complex present with clinical manifestations of varying degrees and duration depending on the species of the infecting agent. The Gambian or West African form, caused by *Trypanosoma brucei gambiense,* is subacute in its early stages progressing to a more chronic debilitating disease lasting up to 20 years. Patients infected with the Rhodesian or East African variety, *T. b. rhodesiense,* develop an acute disease which may result in death with a few weeks or months. In either case, there are three clinically recognized stages or phases of infection: the incubation period, the febrile or glandular period and the cerebral period (Strong, 1944; Greenwood and Whittle, 1980).

Within one to two weeks following the bite of an infected tsetse fly a small chancre develops at the site of the bite. There is a moderate degree of inflammation at the site associated with the presence of the parasite. After an incubation period ranging from a few weeks

to a month, trypanosomes can be detected in the peripheral blood circulation. This event marks the initiation of the second stage of the infection. The incubation period is generally shorter with the Rhodesian form and infection with the Gambian form can occasionally proceed unrecognized until the appearance of the cerebral stage.

The febrile stage of the infection is characterized by irregular bouts of fever, enlargement of lymph nodes, headache, backache, and stiff neck. A rash may be evident presenting as irregular erythematous patches with a clear center distributed primarily on the trunk and thighs. Local edema of the hands or feet may occur along with edematous swellings about the eyes or joints. The enlargement of the lymphatic glands, particularly those in the posterior cervical region, is considered a classic diagnostic sign hence referred to as "Winterbottom's Sign." The spleen and liver may also be enlarged. At times, there is spontaneous improvement in the symptoms followed by another febrile episode. The Rhodesian form can be severe enough in this early stage to result in death.

The third stage of the disease comes about with the invasion of the central nervous system by trypanosomes. The syndrome is commonly referred to as "sleeping sickness" since patients exhibit a great deal of lethargy, sometimes sleeping continuously. The cerebral manifestations accompanying this stage includes tremors, severe headaches, delusions, mania and hysteria. Emaciation can become extreme in the advanced stages. Convulsions leading to a comatous state usually follow and results in death. The progressed stages of trypanosomiasis may be accompanied by secondary bacterial infections with pneumonia being most common.

The laboratory abnormalities of African trypanosomiasis are numerous. The most characteristic findings are anemia, thrombocytopenia, hypergammaglobulinemia, and coagulation abnormalities implicating disseminated intravascular coagulation - DIC (Greenwood and Whittle, 1980; Barrett-Connor et al., 1973). The laboratory findings are usually most marked in the Rhodesian cases. The anemia is normochromic, normocytic, and normoblasts are sometimes present. The white blood count is normal yet a relative increase in the mononuclear population is frequently observed. The thrombocytopenia can be severe and is usually accompanied by elevated fibrin degradation products consistent with the clinical picture of DIC. Maintenance of the patient is critical when DIC is indicated and death will follow frequently.

The most striking lesions seen upon autopsy involve the lymphatic and central nervous systems. The lymph node alterations are characterized by marked hypercellularity of the germinal regions with a generalized infiltration of lymphocytes and mononuclear phagocytes (Greenwood and Whittle, 1980). With progression of the disease, affected lymph nodes become atrophic and depleted of lymphoid cells. Histologically, the lesions of the central nervous system are consistent with a diffuse meningoencephalitis. Infiltration of the tissues by lymphocytes, plasma cells and morular cells is common. Trypanosomes are usually distributed in an irregular pattern throughout the brain. Perivascular cuffing of mononuclear cells can be observed about the blood vessels supplying the brain. In advanced cases, neuronal degeneration can be observed particularly in the region of the basal ganglia. This finding may account for the characteristic neurological signs that are present during the terminal stages of the disease (Greenwood and Whittle, 1980).

Abnormal electrocardiograms have been associated with the febrile stage of trypanosomiasis yet only a few patients exhibit overt signs of cardiac pathology (Poltera, 1980). The histopathology of cardiac tissue is variable but most commonly characterized as myocarditis. Poltera et al., (1976) in a review of fourteen Ugandan cases were able to find a generalized involvement of all cardiac layers. The changes observed were marked cellular infiltrates similar to that described for the brain. Involvement of heart layers containing the elements of the conducting and autonomic nervous systems are thought to explain the abnormal electrocardiograms (Poltera, 1980).

Although damage to the small blood vessels is a prominent feature of experimental trypanosomiasis (Goodwin, 1970), this aspect has not been given attention in descriptions of human cases. Additional information regarding the morbid anatomy of human trypanosomiasis can be found in Binford and Connor (1976).

Animal Trypanosomiases

Several African trypanosomes produce serious diseases in domestic animals. The common name given to these infections is nagana, a Zulu term meaning weak. The two species of tsetse-borne trypanosomiasis of major economic importance affecting livestock are *Trypanosoma congolense* and *T. vivax*. Infections with the close relative of the human trypanosome, *Trypanosoma brucei*, are not as severe but are very commonly associated with economic losses with regards to decreased milk and meat productivity. Together these parasites constitute a major obstacle in the development of vast areas of rich agricultural and grazing lands south of the Sahara Desert. Infections of cattle

are probably the most important economically with sheep, goat, pig, and dog infections following closely. Most of the information regarding the clinical manifestations of nagana is reflected by this hierarchy.

In an extensive series of studies conducted by Valli and coworkers (Valli, et al., 1978a; 1978b; 1979a; 1979b), *Trypanosoma congolense* infections in Holstein calves were most severe in young animals of five to six months of age. The disease was insidiously chronic and without severe clinical signs other than debility, occasional diarrhea, anorexia corresponding with fever peaks and a poor hair coat. Gains in body weight of infected calves were much slower than the control group. Pulse and respiratory rates rose with each fever spike. Precapular and prefemoral lymph nodes and hemal nodes in the flank were enlarged and palpable.

The gross postmortem changes consisted of moderate to extreme enlargement of almost all lymph nodes described. Lungs showed areas of atelectasis and small focal areas of bronchopneumonia. Cardiac changes seen were limited to moderate hypertrophy and restricted to the right ventricle. The gastrointestinal tract was almost devoid of pathology with the exception of moderate dilation and greater content volumes than normal. Livers and spleen of infected animals exhibited the greatest degree of enlargement and pathology. Livers of infected calves showed atrophic changes with significant increases in all cellular elements including the Kupffer cells. Periportal infiltrates were prominent as was the erythrophagocytic activity. The splenic changes were characterized by increases in the size and number of follicular units and a relative reduction of cell numbers in the

T-dependent areas around the splenic arterioles. Splenic imprints show a considerable degree of extramedullary hematopoiesis. Nephropathology was evident and described as membranoproliferative with focal lymphocytic accumulations at the cortico-medullary junction.

Anemia is considered by many a cardinal sign of bovine trypanosomiasis. Dargie et al. (1979) described the anemia as consisting of two phases. The first was an acute rapidly developing hemolytic anemia which correlated closely with the degree of parasitemia. The second phase was chronic and began when parasitemia levels fell off. Red cell levels remained low at this stage with red cell life spans averaging about a third of that of control animals. The anemia is reflected by a marked erythroid shift and mild hemosiderosis of the bone marrow (Valli et al., 1979a). The synchronous nature of maturing erythroid elements and marrow hypercellularity indicated an effective response. Holmes and Mamo (1975) using radioactive tracers excluded dyshemopoiesis as the cause of anemia. Similar findings have been reported by Valli et al., (1979a) and Dargie et al., (1979).

Pathogenesis of African Trypanosomiasis

Toxic Aspects

The clinical manifestations and morbid anatomy of human and animal trypanosomiases have been well documented over the years, yet much remains uncertain about the mechanisms that bring about these changes. The conception of a toxic basis for disease in trypanosomiasis appeared popular at the turn of the century and references to trypanosome-derived toxins are still cited in modern textbooks of parasitology

(Faust et al., 1975). For this reason, a short review of the literature regarding postulated toxins seems appropriate.

Early workers in trypanosomiasis research believed that toxins of the trypanosomes were responsible for the high fevers observed (Laveran and Pettit, 1911; Novy et al., 1917). Their hypothesis appeared to be reasonable at the time since high parasite numbers and fever coincided during the course of infection. This correlation has been well documented over the years but support of the hypothesis is lacking (Strong, 1944; Boreham, 1968; Goodwin, 1970). Renewed interest in this subject occurred with the description of phospholipase A activity in lysates of *Trypanosoma congolense* (Tizard and Holmes, 1976; Tizard et al., 1978). The production of free fatty acids by phospholipase action on phosphatidylcholine resulted in the *in vitro* lysis of erythrocytes and the authors suggested that this activity was linked to anemia, immunosuppression, thrombosis, and myocarditis. In addition, complement-activating substances have been demonstrated on the surface of trypanosomes of a variety of species (Musoke and Barbet, 1977, Nielson et al., 1977, 1978a). In spite of these documented *in vitro* phenomena, evidence that such toxic factors have a role in the *in vivo* pathogenesis of the disease is absent.

As a final note on the topic, vascular permeability changes have been induced in rabbits by the inoculation of specific protein fractions from *T. gambiense* homogenates (Seed, 1968). It was later demonstrated in a report by Boreham and Wright (1976a) that these changes were a result of excessive kinin generation brought about by a complex interaction involving Hageman factor and immune complexes. In light

of the increasing amount of *in vivo* evidence supporting the role of kinins in the pathogenesis of the disease, this subject will be reviewed in detail later under a separate topic.

Anemia

While anemia may be mild in patients with Gambian sleeping sickness, it is the hallmark of the Rhodesian form of trypanosomiasis. There is general agreement among workers that immune mechanisms are involved but to what degree is of question. Experiments performed on African patients by Woodruff et al., (1973) with chromium^{51} labelled autologous erythrocytes demonstrated shortened red cell survival rates with the accumulation of the isotope activity in the liver and spleen. In addition, these investigators detected the presence of complement and immunoglobulins on the surface of erythrocytes suggesting that hemolytic processes were contributing to the cause of the anemia. This suggestion is supported by reports of positive anti-globulin (Coombs) tests conducted by others on human patients with sleeping sickness (Zoutendyk and Gear, 1951; Barrett-Connor et al., 1973; Mackenzie and Boreham, 1974a).

Substantial evidence supporting an immunologic etiology of the anemia in trypanosomiasis has been gathered from experimental animal infections. While a number of factors may be involved there is considerable evidence that in certain phases of the disease process, the anemia is hemolytic. Jennings et al., (1974) found that in rodents infected with *T. brucei*, the circulating half-life of chromium^{51} labelled erythrocytes was reduced and that the label was excreted in the urine suggesting intravascular destruction of erythrocytes. Iron^{59} was rapidly removed from the serum and incorporated into circulating red cells without loss of iron from the body. These changes were accompanied

by marked splenic erythrophagocytosis and hemosiderosis. The anemia was characterized as a macrocytic normoblastic one with reticulocytosis, normoblastic hyperplasia of the bone marrow and extramedullary erythropoiesis in the liver and spleen. Jennings and coworkers (1974) concluded that these findings were indicative of an acquired hemolytic anemia. Although these investigators did not substantiate their conclusions with immunological studies, they are consistent with Woodruff and associates' (1973) investigations on human cases.

Further support of an immunologically mediated anemia can be found in a study of *T. brucei* infected mice where suppression of the immune response by corticosteroids reduced the degree of anemia (Balber, 1974). In addition, Coombs positive anemia has been reported in trypanosome infections of rabbits by Mackenzie and Boreham (1974a) and Dodd et al., (1978) and of cattle by Kobayashi et al., (1976). The latter investigators substantiated their findings by eluting trypanosomal antigens and immunoglobulins from the red cells of infected calves. By means of complement fixation and hemagglutination inhibition tests, the eluted antibodies of both IgM and IgG classes showed serologic reactivity with *T. congolense* antigen preparations. On the basis of their findings, Kobayashi and associates (1976) postulated that trypanosome antigen-antibody complexes deposited on erythrocytes resulted in the immune elimination of cells leading to the observed anemia. It was suggested and supported by previous *in vitro* studies (Woo and Kobayashi, 1975) that the antigens of *T. congolense* can bind directly to erythrocytes at 37°C. However, in that study, rabbit red cells were used. It is not yet definitively known whether trypanosome antigen and antibody are adsorbed onto erythrocyte surface separately or as a complex. Furthermore, the role of serum complement in the process of adsorption has not been elucidated.

If the anemia of trypanosomiasis is of an immune origin, activation of the complement system should be evident (Woodruff et al., 1973). Low serum C3 levels and split C3 products have been detected in the serum of humans infected with trypanosomes (Greenwood and Whittle, 1976b). Both C4 and factor B levels were reduced suggesting the activation of both alternative and classical pathways (Greenwood and Whittle, 1976b). A similar report on reduced serum complement levels in monkeys infected with *T. rhodesiense* was given by Nagle et al., (1974). Kobayashi and Tizard (1976) found that the anemia in cattle infected with *T. congolense* coincided with a drop in parasitemia, the appearance of complement-fixing antibodies, and a progressive decrease in serum C3 levels. Tabel et al., (1977) and Nielsen et al., (1978b) reported low complement levels in cattle infected with *T. vivax* and *T. congolense*, respectively. The observation of hypocomplementemia in bovine trypanosomiasis has since been confirmed by Ruranguvia et al., (1980).

Additional immunologic factors with serologic reactivity against various erythrocyte preparations have been associated with the anemia of trypanosomiasis. The first report of heterophil antibodies in human cases of Gambian sleeping sickness was provided by Henderson-Begg (1946) with subsequent reports by Klein and Mattern (1965), Houba and Allison (1976) and Honba et al. (1969). The latter investigators demonstrated that the anti-sheep red cell antibodies (Anti-SRBC) were mercaptoethanol-sensitive 19S IgM immunoglobulins that were distinct from the classic Forsmann antibodies. Kobayakawa et al. (1979) detected Anti-SRBC antibodies using tannic acid treated cells, a method similar to the trypsin treatment that Rickman and Cox (1979) employed to detect cold-active hemagglutinins to homologous cells in

experimental rodent trypanosomiasis. In experiments conducted with *T. gambiense*-infected rabbits, Mattern et al. (1980) demonstrated that purified anti-SRBC globulins did not possess anti-trypanosome specificity by absorption studies. Evidence supporting a pathogenic role of such antibodies in the progression of anemia appears to be absent.

Cardiac Lesions

In an attempt to study the pathogenesis of the cardiac lesions of human trypanosomiasis, Poltera (1980) investigated the progression of cardiac pathology in mice infected with *T. b. brucei*. The autopsy findings had a very striking resemblance to that which Poltera et al. (1976; 1977) and Poltera and Cox (1977) reported in their post-mortem investigations of cardiac pathology of human trypanosomiasis. By sequentially killing infected mice during the course of a ten-week infection, it was shown that as parasites infiltrated the various layers and valves of the heart, a cellular inflammatory response consisting of mononuclear cells occurred. The parasites were observed in the interstial spaces of cardiac tissue and in the lymphatic channels draining the heart. Immunofluorescent studies demonstrated that the removal of parasites through the lymphatic vessels was accompanied by positive tests for immunoglobulins and C3, the distribution of which was confined to that of the lymphatics. As infection progressed, granular deposits of immunoglobulin and C3 could be detected throughout all tissue layers especially in the subendocardium. It is of significance that at the time extensive inflammation was observed in the cardiac tissues, high titered anti-trypanosomal antibodies, depressed C3 levels and immune complexes were demonstrated in the serum of

infected animals. In advanced stages of the disease, the regional lymph nodes were depleted of lymphocytes and plasma cells and replaced by histocytes. The lymphatic channels and marginal sinuses were dilated and convoluted, with trypanosomes and edema fluid filling the lumina. Obstruction of the vessels was suggested to account for the extensive accumulation of trypanosomes in the draining channels of the endocardium. These reactions, which in mice are characteristically followed by fibrosis (Murray, 1974), could explain the occurrence of endomyocardial fibrosis reported in previous studies of human cases.

Traditionally, cardiac pathology has not been considered to result from bovine trypanosomiasis. However, Valli and Forsbert (1979) reported a slight to moderate myocarditis in *T. congolense*-infected calves accounting for this oversight by earlier investigators. As in the case of human trypanosomiasis, pathologic changes in the myocardium were described as a mononuclear reaction with hypertrophy and hyperplasia. In severely affected calves, fiber atrophy was apparent but without any indication of necrosis. Of interest was the observation that dilation of the myocardial blood vessels was accompanied by aggregates of erythrocytes, platelets, macrophages and trypanosomes. Because of the relatively recent documentation of cardiac pathology, studies of the pathogenesis of this aspect of bovine trypanosomiasis have not been reported.

Cerebral Lesions

The pathologic picture of the lesions within the central nervous system has been documented as a diffuse meningoencephalitis (Strong, 1944; Binford and Connor, 1976; Poltera et al., 1977). As with the cardiac manifestations of the disease, investigators have had to rely

on experimental models to unfold the events leading to cerebral pathology. Poltera (1980) showed that infiltration of the brain tissues by trypanosomes occurred in the fourth week during *T. b. brucei* infection in mice. Slight edema and cellular infiltration accompanied the parasites which were localized in the interstitium, with a relatively lower amount of trypanosomes accumulating in the meninges. This histologic pattern coincided with occurrence of immunologic reactions in the blood (increased anti-parasite antibodies, depressed C3 levels and increased levels of soluble immune complexes) and progressed in concert with the progression of the disease. Granular deposits of C3 and immunoglobulin were shown to be distributed in discrete focal areas of the cerebral region by immunofluorescent antibody tests. Electron microscopic analysis of tissues revealed accumulation of electron dense deposits around the basement membrane of blood vessels in the subependymal and choriod plexus regions of the brain supporting their suggestion that immune complexes participated in the pathogenesis of these lesions. In addition, Poltera (1980) described unpublished evidence implicating both immune complexes and autoantibodies directed against cerebral ganglion cells in the pathogenesis of lesions in man.

Renal Lesions

Renal pathology as a result of infection with African trypanosomes was first described by Sadun et al., (1973). Experimentally infected rhesus monkeys developed a proliferative glomerulonephritis characterized by hypercellularity of the glomerular tuft, thickening and duplication of the capillary basement membrane and infiltrations of the interstitium by mononuclear leukocytes. Additional evidence of renal pathology was supplied by electronmicroscopic analysis of renal tissue demonstrating swelling and simplification of the visceral

epithelial cells with loss of filtration slits. Other findings included synechial and crescent formation along with the accumulation of proteinaceous casts within the tubular elements. Similar nephropathologic changes have been associated with experimental trypanosome infections of monkeys, mice and rats by other investigators (Nagle et al., 1974; Lindsley et al., 1974; 1978; 1980; Murray, 1974; Kabil et al., 1979; Rickman and Cox, 1979).

Investigations on the pathogenesis of renal disease in African trypanosomiasis have suggested that the deposition of immune complexes along the glomerular capillary bed may be responsible for the subsequent appearance of renal pathology (Nagle et al., 1974; Murray, 1974; Lindsley et al., 1978; Kabil et al., 1979). Electron-microscopic analysis of renal tissues from *T. b. rhodesiense*-infected rhesus monkeys demonstrated that pathologic alterations in the visceral epithelial cell morphology were associated with the accumulation of electron-dense material in the subepithelial spaces of the glomerulus (Nagle et al., 1974). These investigators detected immunofluorescent deposits of IgM, C3, and properdin along the glomerular capillary bed suggesting that these electron-dense materials may be composed of antigen-antibody complexes. Similar immunohistochemical studies conducted by Lindsley et al., (1978) and Murray (1974) suggested the involvement of the IgG subclasses of immunoglobulins in the development of glomerular pathology. The pattern of fluorescence in all these studies have been consistently described as discrete granular deposits. In contrast to the linear deposition of immunoglobulin seen in nephrotoxic nephritis, this "lumpy" pattern is consistent with immune-complex mediated glomerulonephritis as described by Dixon (1971b) in a review on the pathogenesis of immunologically mediated nephritis.

Lambert and Houba (1974) presented evidence implicating trypanosome antigen-antibody complexes in the pathogenesis of glomerular pathology. Immunofluorescent tests on renal tissues revealed the presence of trypanosomal antigens in the glomerular tuft. Antibodies of the IgG and IgM classes were eluted from renal tissues and shown to have serologic specificity against trypanosome antigen preparations. With the additional demonstration of C3 in renal tissues, Lambert and Houba (1974) suggested the trypanosome antigen-antibody-complement complexes were responsible for the glomerular lesions. However, Lindsley et al., (1974; 1978) provided evidence implicating non-trypanosomal immune complexes in the development of nephritis. These investigators detected antibodies against single-stranded DNA and RNA in serum of trypanosome infected humans, monkeys, and rats at the onset of glomerular pathology and suggested that their participation in the pathogenesis cannot be excluded citing evidence that nucleic acid antibodies and antigens participated in the renal lesions of systemic lupus erythematous.

Concepts of Immunopathologic mechanisms

"The conception that the antibodies, which should protect against disease, are also responsible for the disease, sounds at first absurd. This has as its basis the fact that we are accustomed to see in disease only the harm done to the organism (host) and to see in the antibodies solely antitoxic substances. One forgets too easily that the disease represents only a stage in the development of immunity, and that the host often attains the advantage of immunity only by means of disease," (Von Pirquet and Schick, 1905).

Some two hundred years prior to the publication of the above in Die Serumkrankheit by Von Pirquit and Schick (1905), Jenner (1785) had clearly established the interrelationships of infection, disease and immunity. It is apparent from Jenner's observations on the protection afforded against smallpox by previous exposure to cowpox virus that the inflammatory reactions associated with the rejection of variola virus were a result of the acquired immunity obtained from vaccina infection. Jenner stated in a footnote concerning the nature of the rejection phenomena: "It is remarkable that variolous matter, when the system is disposed to reject it, should excite inflammation on the part to which it is applied more speedily than when it produces the smallpox. Indeed it becomes almost a criterion by which we can determine whether the infection will be received or not. It seems as if a change, which endures through life, had been produced in the action, or disposition to action, in the vessels of the skin, and it is remarkable too, that whether this change has been effected by the smallpox or the cowpox, that the disposition to sudden cuticular inflammation is the same on application of variolous matter." Jenner's subject, Mary Barge of Woodward, had contracted cowpox some 30 years previous and had been employed since as a nurse to smallpox patients without ever experiencing any illness relatable to smallpox. Although these classical investigations laid the foundation for modern immunology, the role of the immune response in the pathogenesis of many infectious diseases is still unappreciated by many scientists.

The role of antibody production in the pathogenesis of serum sickness was recognized by Von Pirquet and Schick (1905). These early investigators clearly demonstrated that the elaboration of precipi-

tating antibodies accompanied the clinical manifestations of serum sickness. Following the inoculation of antitoxin (prepared in horses) into naive subjects, a period of 7-14 days was required before subjects presented with the symptomotology of serum sickness: fever, rash, edema, regional lymphoadenopathy and albuminuria. At the site of inoculation, a papular eruption accompanied by erythema and edema appeared at the time when generalized symptoms became evident. Furthermore, Von Pirquet and Shick (1905) observed a quantitative relationship governing the presentation of the disease, that is, the degree of pathology which ensued was directly proportional to the amount of precipitin generated. It seems that Jenner would have appreciated these concepts since he had recognized that the events leading to rejection of variolous matter, which would be considered protective, was accompanied by a significant degree of inflammation (pathology).

Dixon and associates (Dixon, et al., 1958; Dixon, 1963; Dixon, 1971a) have extended the concepts of Von Pirquet and Schick (1905) explaining the pathogenesis of the inflammatory lesion of serum sickness and many other seemingly unrelated conditions such as systemic lupus erythematous, rheumatoid arthritis and glomerulonephritis. The essential prerequisites for the production of classic serum sickness lesions outlined by Dixon (1971a) are: (1) the antigen employed must be capable of persisting in the circulation until antibody is generated, such as native serum proteins, homologous or heterlogous, and (2) the amount of antigen used should be sufficient enough that when complexing occurs, antigen excess must be maintained. The work of Dixon et al. (1958) established that the inflammatory lesions of serum sickness occurred during the phase of immune elimination of antigen,

at a time when antigen-antibody complexes are present in the circulation. The demonstration of antigen and antibody in the lesions within the kidneys supported the hypothesis that the pathologic changes were the result of localization of antigen-antibody complexes at the site of the lesion. Lesions, similar to those of the serum sickness type, have to be induced within 3-4 days by intravenous administration of preformed antigen-antibody complexes solubilized in antigen excess (McCluskey and Benacerraf, 1959; McCluskey, et al., 1960).

A variety of inflammatory mediators have been implicated in the generation of the lesions produced by soluble immune complexes. Dixon et al. (1958) points out that serum complement levels decreased substantially at the time of immune complex deposition and subsequent tissue distruction. In a review on the subject, Dixon (1971a) documented evidence suggesting the involvement of several plasma constituents including fibrinolysin, anaphylatoxin, and vasoactive peptides of the kinin-variety. At the cellular level, Dixon (1971a) included mast cell degranulation, platelet aggregation, and degranulation of polymorphonuclear leukocytes as contributory elements since all have been documented to produce potent permeability and cytotoxic substances. The precise role of these factors and possibly others in the development of inflammatory lesions associated with immune complexes remains to be determined.

Role of Complement in Immunologic Injury

Since the first description provided by Bordet (1896) of a serum factor essential for the lysis of bacteria and red cells, biochemists have unraveled a complex series of reactions involving 18 distinct serum proteins known today as the complement system. Operationally the complement system may be subdivided into two pathways, the classical

and alternative (properdin). Activation of complement has been described as having two distinct biological consequences: (1) irreversible structural and functional alterations of biological membranes and cell death, and (2) activation of specialized cell functions (Müller-Eberhard, 1975). The involvement of the complement system in the pathogenesis of immunologically induced reactions of passive cutaneous anaphylaxis, and the vasculitis and glomerulonephritis of serum sickness has been reviewed by Ward (1971). The major biological activities are associated with complement components C3 and C5, which contain peptides of significance in the phenomena of anaphylaxis, immune adherance, phagocytosis and chemotaxis. Lepow et al. (1969) outlined the pathobiological properties of C3a and C5a in the generation of acute inflammatory reactions involving smooth muscle contraction, vascular permeability and the degranulation of histamine containing cells.

An additional consequence of complement activation that has not been appreciated by many scientists, is the generation of intermediates that can participate in the phenomenon known as conglutination. With the discovery of a peculiar substance in normal bovine serum, "colloide de boeuf," Bordet and Streng (1909) documented the agglutinating properties of bovine conglutinin. In the presence of antibody-sensitized cells, either bacteria or erythrocytes, which had been absorbed with complement, the addition of conglutinin resulted in powerful clumping instead of hemolysis. It was shown by Streng (1909a; 1909b) and Streng and Ryti (1923) that conglutinin was a nonantibody fraction of bovine globulin that reacted with antigen-antibody fixed complement but that it does not react with unactivated complement. Streng (1930) demonstrated that rabbits immunized with sensitized bacteria alexinated with

complement, produced antisera that reacted with fixed complement absorbed onto heterologous sensitized bacteria. Since Streng's "immune body" had anti-complement specificity, a property similar to bovine conglutinin, he suggested the name immunoconglutinin. Verifying Streng's work, Wartiovaara (1932) demonstrated powerful immunoconglutinins by intravenous immunization with washed bacteria. Ingram (1962a; 1962b) generated immunoconglutinins by injections of complex carbohydrates (agar, starch and mucin), protein derivatives (lactoalbumin hydrolysate, bovine albumin, peptone) and particulate substances with active surfaces (animal charcoal, kaolin, red gold sol).

The distinction of immunoconglutinin from conglutinin found in normal bovine serum was demonstrated by Lachmann (1962) by employing modern biochemical techniques. In contrast to the non-immunoglobulin nature of bovine conglutinin, immunoconglutinin is an antibody of the IgM class having been shown to be mercaptoethanol-sensitive, excluded from Sephadex G-200, sedimented in the 19S region of zone ultracentrifugation and showed antigenicity of IgM (Lachmann, 1962; 1967; Thoongsuwan, et al., 1978b). Coombs and Coombs (1953) were the first to suggest that immunoconglutinin possessed autoreactivity properties. Definitive proof of autoreactivity was provided by Bienenstock and Block (1966) demonstrating that immunoconglutinin reacted *in vitro* with soluble immune complexes absorbed with autologous complement. Evidence of *in vivo* autoreactivity was provided by Ingram (1962c) observing sharp decreases in both lytic complement and immunoconglutinin levels following intravenous challenge with soluble immune complexes. Coombs, Blomfield, and Roberts (1950) using conventional complement reagents,

showed that immunoconglutinin reacted serologically with sheep erythrocytes complexed with antibody, C1, C2, and C4(EAC142). Later Lachmann (1962) demonstrated reactivity with EAC1423 and with the use of purified human complement components showed reactivity with EAC1423b, EAC1423d, and EAC142a (Lachmann, 1966; 1967). These serologic specificities have been confirmed by Rice (1963) and Thoongsuwan et al. (1978b).

In a review compiled by Lachmann (1967) evidence was provided supporting the participation of immunoconglutinin in the augmentation of complement fixation, immune adherence, opsinization and the formation of anaphylatoxin in allergic reactions. Ingram (1969) advanced the conception of immunoconglutinin's properties demonstrating that it could activate complement and in the presence of sufficient immunoconglutinin, autologous complement could be exhausted. The role of immunoconglutinin in the non-specific resistance to bacterial, protozoan and viral infections has been well documented (Ingram, et al., 1959; Ingram, 1959a; 1959b; 1965a; 1965b; Coombs, et al., 1961; Thoongsuwan, et al., 1978a). Immunoconglutinin has been shown to be protective against the *in vitro* cytopathic effects of certain viruses by Joshi et al. (1965) and to enhance to phagocytic activity of peritoneal macrophages by Parrappally and Ingram (1973). It appears that immunoconglutinin is on one hand protective, however, as it was pointed out by Thoongsuwan et al. (1978a; 1978b) and Rickman and Cox (1979), immunoconglutinin may also potentiate the pathologic consequences of infection such as the anemia and glomerulonephritis. This paradoxical concept of immunoconglutinin's role in disease has as its basis the fact that "one

forgets too easily that the disease represents only a stage in the development of immunity," Von Pirquet and Schick (1905).

Interrelationships of Clotting, Kinin and Complement Systems

The role of the coagulation system in the generation of inflammatory and allergic responses has received considerable attention and has been reviewed (McKay, 1965; Ratnoff, 1969; Kaplan and Austen, 1975). Reactions leading to the activation of the clotting, fibrinolytic, complement and kinin cascades center around the activation of Hageman factor (Blood Clotting Factor XII) and hence, referred to as Hageman factor dependent pathways (Kaplan and Austen, 1975). Hageman factor is a plasma beta-globulin that circulates in blood in an inactive form being activated by contact with a wide variety of materials possessing a negative charge such as collagen, endotoxin, damaged endothelium, crystals and glass (Wuepper and Cochrane, 1972; Cochrane, et al., 1973). Robbins and Stetson (1959) originally documented evidence supporting the *in vitro* activation of Hageman factor by immune complexes; however, Cochrane et al. (1972), employing purified components, demonstrated that this was not the case. Boreham and Goodwin (1970) and Boreham and Wright (1976a) demonstrated that the *in vitro* and *in vivo* activation of Hageman factor by immune complexes was dependent on kallikrein generation. An additional pathway involving the interaction of immune complexes, complement and platelets, can generate a number of substances capable of activating the clotting cascade, a subject reviewed by Pfueller and Lüscher (1972).

Hageman factor acts in association with a cofactor to activate the fibrinolytic pathway by converting plasminogen to its active peptide

form, plasmin (Kaplan and Austen, 1975). The activity of plasmin is not restricted to the generation of fibrinogen/fibrin degradation products since it can convert C1s to its active esterase form initiating activation of the complement cascade and can convert kallikreinogen to its active form kallekrein which is a potent generator of kinins leading to increases in vascular permeability (Ratnoff, 1969). As an additional consequence of plasmin, native C3 can be cleaved into its biologically active components C3a and C3b, by-passing the requirement for properdin or immune complexes in the activation of complement (Ward, 1967). Ward (1967) also points out that these cleavage products of C3 possess chemotactic and permeability properties which may function in the pathogenesis of inflammatory reactions. The limited list of reactions described above are considered to be among the major pathways participating in the pathogenesis of inflammatory lesion; however, for a detailed treatise on the topic one is directed to Movat (1971).

Boreham and Wright (1976b) in a review of pharmacologically active substances occurring in parasitic infections suggested that the activation of Hageman factor accounted for the kinin generation and fibrinolytic phenomena they observed in experimental infections, including trypanosomiases and babesiosis. The activation of kallereins (kininogenases) results in the generation of three varieties of kinins, namely, bradykinin, kalliden and kinin III. The serum levels of kallekrein along with the kinins have been shown to be elevated during infections with many parasitic agents, protozoan and helminth (Boreham and Wright, 1976b; Boreham, 1970; 1979).

In another report, Boreham and Facer (1974a) demonstrated elevated plasmin and fibrin degradation products in rabbits infected with *T. b. brucei*. During their attempts to measure the levels of serum fibrin degradation products, it was found that a factor was causing the agglutination of fibrinogen coated test cells which interferred with the results of the assay. A subsequent report indicated that the infected rabbits generated an antibody directed against a component of the fibrin/fibrinogen system, hence termed Anti-F (Boreham and Facer, 1974b). Anti-F was shown to be IgM by Sephadex G-200 chromatography and was mercaptoethanol-sensitive. Absorption studies indicated that Anti-F reacted with determinants exposed on membrane bound fibrinogen, fibrin, and their degradation products. Reactivity with autologous fibrin and fibrinogen components indicated that Anti-F was an auto-antibody. Thoongsuwan et al. (1979) have shown that Anti-F was not unique to trypanosome-infected rabbits having been detected in rats with malaria and babesiosis.

In conclusion, it appears that the fibrinolytic, clotting, complement, and kinin systems are intimately involved in the pathobiology of many immunologically mediated phenomena. Not only are the factors generated by these pathways directly involved in the pathogenesis of lesions, but it appears that the intermediates produced may result in autoimmunization. Perhaps, this would explain the wide occurrence of immunoconglutinins and Anti-F in many infectious diseases. It is clear that further investigations directed towards determining the role of each of these systems in the pathogenesis of disease are needed. The knowledge acquired from such studies could contribute greatly to our understanding of immunopathologic mechanisms of disease.

REFERENCES

BALBER, A. E. 1974. *Trypanosoma brucei:* attenuation by corticosteriods of the anemia in infected mice. Exp. Parasit. 35:209-218.

BARRETT-CONNOR, E., R. J. UGORETZ, and A. I. BRAUDE. 1973. Disseminated intravascular coagulation in trypanosomiasis. Arch. Int. Med. 131: 574-579.

BIENENSTOCK, J., and K. J. BLOCH. 1966. Some characteristics of human immunoconglutinin. J. Immunol. 96:637-645.

BINFORD, C. H., and D. H. CONNOR. 1976. African trypanosomiasis. IN Pathology of Tropical and Extraordinary Diseases, C. H. Binford and D. H. Connor (eds.). Armed Forces Institute of Pathology, Washington, DC, pp. 340-389.

BORDET, J. 1896. Sur le mode d'action des serum preventifis. Ann. Inst. Pasteur 10:143.

BORDET, J., and O. STRENG. 1909. Les phénomenes d' absorbtion et la conglutinine du serum de boeuf. Zbl. Bakt. (Orig.) 49:206-276.

BOREHAM, P.F.L. 1968. Immune reactions and kinin formation in chronic trypanosomiasis. Br. J. Pharmaco. 32:493-504.

BOREHAM, P.F.L. 1970. Kinin release and the immune reaction in human trypanosomiasis caused by *Trypanosoma rhodesiense.* Trans. R. Soc. Trop. Med. Hyg. 64:394-400.

BOREHAM, P.F.L. 1979. The pathogenesis of African and American trypanosomiasis. IN Biochemistry and Physiology of Protozoa, 2nd edition, J. P. Krierer (ed.). Academic Press, Inc., New York, Volume 2, pp. 429-457.

BOREHAM, P.F.L., and C. A. FACER. 1974a. Fibrinogen and fibrinogen/fibrin degradation products in experimental African trypanosomiasis. Int. J. Parasitol. 4:143-151.

BOREHAM, P.F.L., and C. A. FACER. 1974b. Autoimmunity in trypanosome infections II. Anti-fibrin/fibrinogen (Anti-F) autoantibody in *Trypanosoma (Trypanozoon) brucei* infections in the rabbit. Int. J. Parasitol. 4:601-607.

BOREHAM, P.F.L., and L. G. GOODWIN. 1970. Kinins in experimental *Trypanosoma brucei* infections in the rabbit. Adv. Exp. Med. Biol. 8:539.

BOREHAM, P.F.L., and I. G. WRIGHT. 1976a. The release of pharmacologically active substances in parasitic infections. IN Progress in Medicinal Chemistry, G. P. Ellis and G. B. West (eds.). North Holland Publishing Company, Volume 13, pp. 159-204.

BOREHAM, P.F.L., and I. G. WRIGHT. 1976b. Hypotension in rabbits infected with *Trypanosoma brucei*. Br. J. Pharmaco. 58:137-139.

BRUCE, D. 1895. IN Preliminary Report on the Tsetse Fly Disease of Nagana in Zululand. Durban: Bennett & David.

BUXTON, P. A. 1955. The Natural History of Tsetse Flies. H. K. Lewis and Company, London, pp. 816.

COCHRANE, C. G., K. D. WUEPPER, B. S. AIKINO, S. D. REVAK, and H. L. SPIEGELBERG. 1972. The interaction of Hageman Factor and immune complexes. J. Clin. Invest. 51:2736-2745.

COCHRANE, C. G., S. D. REVAK, and K. D. WUEPPER. 1973. Activation of Hageman Factor in solid and fluid phases: a critical role of Kallikrein. J. Exp. Med. 138:1564-1583.

COOMBS, R.R.A., A. M. BLOMFIELD, and G. F. ROBERTS. 1950. The conglutination phenomenon. VII. A study of the interactions of complement components and immunoconglutinin in the process of conglutination. J. Hyg. Camb. 48:484-499.

COOMBS, R.R.A., and A. M. COOMBS. 1953. The conglutination phenomenon. IX. The production of immunoconglutinin in rabbits. J. Hyg. Camb. 51L509-531.

COOMBS, R.R.A., A. M. COOMBS, and D. G. INGRAM. 1961. The Serology of Conglutination and its Relation to Disease, Blackwell Scientific Publications, Oxford, pp. 268.

DARGIE, J. D., P. K. MURRAY, M. MURRAY, W.R.T. GRIMSHAW, and W.I.M. McINTYRE. 1979. Bovine trypanosomiasis: the red cell kinetics of N'dama and Zebu cattle infected with *Trypanosoma congolense*. Parasitology 78:271-286.

DIXON, F. J. 1963. The role of antigen-antibody complexes in disease. Harvey Lecture Series, 58:21-58.

DIXON, F. J. 1971a. Experimental serum sickness. IN Immunological Diseases by Max Samter (ed.), Volume I. Chapter 14:253-264. Little, Brown and Company, Boston.

DIXON, F. J. 1971b. The immunopathology of glomerulonephritis. IN Immunological Diseases by Max Samter (ed.), Volume II. Chapter 68: 1125-1133. Little, Brown and Company, Boston.

DIXON, F. J., J. J. VAZQUEZ, W. O. WEIGLE, and C. G. COCHRANE. 1958. Pathogenesis of serum sickness. AMA Arch. Path. 65:18-28.

DODD, B. E., G. C. JENKINS, P. J. LINCOLN, and P. McCRORIE. 1978. The advantage of a build-up antiglobulin technique for the detection of immunoglobulin on the red cells of rabbits infected with trypanosomes. Trans. R. Soc. Trop. Med. Hyg. 72:501-505.

DUKE, B.O.L. 1978. The relevance of parasitology to human welfare today - Medical aspects, IN The Relevance of Parasitology to Human Welfare Today, A.E.R. Taylor and R. Muller (eds.). Blackwell Scientific Publications, Oxford, pp. 1-13.

FAUST, E. C., P. C. BEAVER, and R. C. JUNG. 1975. Animal Agents and Vectors of Human Disease. Lea and Febiger, Philadelphia, pp. 479.

GOODWIN, L. G. 1970. The pathology of African trypanosomiasis. Trans. R. Soc. Trop. Med. Hyg. 64:797-812.

GREENWOOD, B. M., and H. C. WHITTLE. 1976a. Coagulation studies in Gambian trypanosomiasis. Amer. J. Trop. Med. Hyg. 25:390-394.

GREENWOOD, B. M., and H. C. WHITTLE. 1976b. Complement activation in patients with Gambian sleeping sickness. Clin. Exp. Immunol. 24:133-138.

GREENWOOD, B. M., and H. C. WHITTLE. 1980. The pathogenesis of sleeping sickness. Trans. R. Soc. Trop. Med. Hyg. 74:716-725.

GRIFFITHS, R. B. 1978. The relevance of parasitology to human welfare today - veterinary aspects. IN The Relevance of Parasitology to Human Welfare Today, A.E.R. Taylor and R. Muller (eds.). Blackwell Scientific Publications, Oxford, pp. 41-66.

HENDERSON-BEGG, A. 1946. Heterophile antibodies in trypanosomiasis. Trans. R. Soc. Trop. Med. Hyg. 40:331-339.

HOARE, C. A. 1972. The Trypanosomes of Mammals. Blackwell Scientific Publications, Oxford, pp. 749.

HOLMES, P. H., and E. MAMO. 1975. Factors influencing the development of trypanosomal anemia in Zebu cattle. Trans. R. Soc. Trop. Med. Hyg. 69:274.

HOUBA, V., and A. C. ALLISON. 1966. M-antiglobulins (rhematoid factor-like) globulins and other gammaglobulins in relation to tropical parasitic infections. Lancet i:848-850.

HOUBA, V., K. N. BROWN, and A. C. ALLISON. 1969. Heterophil antibodies, M-antiglobulins and immunoglobulins in experimental trypanosomiasis.

INGRAM, D. G. 1959a. The conglutination phenomenon. XIII. *In vivo* interactions of conglutinin and experimental bacterial infections. Immunology 2:322-333.

INGRAM, D. G. 1959a. The conglutination phenomenon. XIV. The resistance enhancing effect of conglutinin and immunoconglutinin in experimental bacterial infections. Immunology 2:334-345.

INGRAM, D. G. 1962a. The production of immunoconglutinin. III. Factors affecting the response to autostimulation in mice. Can. J. Micro. 8:335-344.

INGRAM, D. G. 1962b. The production of immunoconglutinin. IV. Factors affecting the response of rabbits to autostimulation. Can. J. Micro. 8:345-360.

INGRAM, D. G. 1962c. The production of immunoconglutinin. V. The stimulus for the production of immunoconglutinin by autostimulation. Can. J. Micro. 8:461-471.

INGRAM, D. G. 1965a. The production of immunoconglutinin. IX. In acute bacterial infections. Can. J. Micro. 11:151-160.

INGRAM, D. G. 1965b. The production of immunoconglutinin. X. In chronic bacterial infections. Can. J. Micro. 11:161-165.

INGRAM, D. G. 1969. The serological activities of immunoconglutinin. Can. J. Micro. 15:981-987.

INGRAM, D. G., H. BARBER, D. M. McCLEAN, M. A. SOLTYS, and R.R.A. COOMBS. 1959. The conglutination phenomenon. XII. Immunoconglutinin in experimental infections of laboratory animals. Immunology 2:268-282.

JARVINEN, J. A., and A. P. DALMASSO. 1976. Complement in experimental *Trypanosoma lewisi* infections of rats. Inf. Immun. 14:894-902.

JENNER, E. 1798. An inquiry into the causes and effects of the variolae vaccine, a disease discovered in some of the western countries of England, particularly Gloustershire and known by the name of the cowpox. Printed for the author by Sampson Low, Soho, London.

JENNINGS, F. W., P. K. MURRAY, M. MURRAY, and G. M. URGUHART. 1974. Anemia in trypanosomiasis: Studies in rats and mice infected with *Trypanosoma brucei*. Res. Vet. Sci. 16:70-76.

JOSHI, N. N., M. SAVAN, and D. G. INGRAM. 1965. The activity of immunoconglutinin in *in vitro* viral infections. Can. J. Micro. 11: 1023-1025.

KABIL, S. M., A. W. WOODRUFF, and L. E. PETTIT. 1979. The role of complement in the production of nephropathology in trypanosomiasis. Trans. R. Soc. Trop. Med. Hyg. 73:320.

KALINER, G. 1974. *Trypanosoma congolense*. II. Histopathologic findings in experimentally-infected cattle. Exp. Parasitol. 36: 20-26.

KAPLAN, A. P., and K. F. AUSTEN. 1975. Activation and control mechanisms of Hageman Factor-dependent pathways of coagulation, fibrinolysis, and kinin generation and their contribution to the inflammatory response. J. Allergy Clin. Immunol. 56:491-506.

KLEIN, F., and P. MATTERN. 1965. Rheumatoid factors in primary and reactive macroglobulinemia. Ann. Rheum. Dis. 24:458-464.

KLEIN, F., P. MATTERN, and H. J. KORNMAN-v.d. BOSCH. 1970. Experimental induction of rheumatoid factor-like substances in animal trypanosomiasis. Clin. Exp. Immunol. 7:851-863.

KOBAYAHAWA, T., J. LOUIS, S. IZUI, and P. H. LAMBERT. 1979. Autoimmune responses to DNA, red blood cells and thymocyte antigens in association with polyclonal antibody synthesis during experimental African trypanosomiasis. J. Immunol. 122:296-301.

KOBAYASHI, A., and I. R. TIZARD. 1976. The response to *Trypanosoma congolense* infection in calves: Determination of immunoglobulins IgG1, IgG2, IgM and C3 levels and the complement fixing antibody titers during the course of infection. Tropenmed. Parasitol. 27: 411-417.

KOBAYASHI, A., I. R. TIZARD, and P.T.K. WOO. 1976. Studies on the anemia in experimental trypanosomiasis. II. The pathogenesis of the anemia in calves infected with *Trypanosoma congolense*. Amer. J. Trop. Med. Hyg. 25:401-406.

LACHMANN, P. J. 1962. A comparison of some properties of bovine conglutinin with those of rabbit immunoconglutinin. Immunology 5:687-705.

LACHMANN, P. J. 1966. A sedimentation pattern technique for measuring conglutination: Its application to demonstrating immunoconglutinins to C4. Immunology 11:263-271.

LACHMANN, P. J. 1967. Conglutinin and immunoconglutinin. Advances Immunol. 6:479-486.

LACHMANN, P. J., and R.R.A. COOMBS. 1965. Complement, conglutinin and immunoconglutinin. IN Complement, G.E.W. Wolstenholme and J. Knight (eds.). Ciba Foundation Symposium. Little, Brown and Company, Boston, pp. 242-273.

LAMBERT, P. H., and V. HOUBA. 1974. IN Progress in Immunology II. Brent and Holborow (eds.). North Holland Publishing Company, Volume 5, pp. 57-64.

LAVERAN, A., and A. PETTIT. 1911. Au sujet de *Trypanosoma rhodesiense*. Bull. Soc. Pathol. Exot. 4:42-45.

LEPOW, I. H., W. DIAS da SILVA, and R. A. PATRICK. 1969. Biologically active cleavage products of components of complement. IN Cellular and Humoral Mechanisms in Anaphylaxis and Allergy. H. Z. Movat, (ed.). Karger, Bazel/New York, pp. 237-252.

LEVINE, N. D. 1973. IN Protozoan Parasites of Domestic Animals and Man. 2nd Edition. Burgess Publishing Company, Minneapolis, pp. 406.

LINDSLEY, H. B., S. KYSELA, and A. D. STEINBERG. 1974. Nucleic acid antibodies in African trypanosomiasis: Studies in Rhesus monkeys and man. J. Immunol. 113:1921-1927.

LINDSLEY, H. B., R. B. NAGLE, and D. J. STECHSCHULTE. 1978. Proliferative glomerulonephritis, hypocomplementemia and nucleic acid antibodies in rats infected with *Trypanosoma rhodesiense*. Amer. J. Trop. Med. Hyg. 27:864-872.

LINDSLEY, H. B., R. B. NAGLE, P. A. WERNER, and D. J. STECHSCHULTE. 1980. Variable severity of glomerulonephritis in inbred rats infected with *Trypanosoma rhodesiense*. Amer. J. Trop. Med. Hyg. 29:348-357.

MACKENZIE, A. R., and P.F.L. BOREHAM. 1974a. Autoimmunity in trypanosome infections. III. The antiglobulin (Coombs) test. Acta Tropica 31:360-368.

MACKENZIE, A. R., and P.F.L. BOREHAM. 1974b. Autoimmunity in trypanosome infections. IV. Natural infections in hartebeest, hyaena, and lion in the Serengeti National Park, Tanzania. Acta Tropica 31:369-372.

MATTERN, P., F. KLEIN, R. PAUTRIZEL, and Y.E.J.M. JONGEPIER-GEERDES. 1980. Anti-immunoglobulins and heterophil agglutinins in experimental trypanosomiasis. Infect. Immun. 128:812-817.

McCLUSKEY, R. T., and B. BENACERRAF. 1959. Localization of colloidal substances in vascular endothelium, a mechanism of tissue damage. II. Experimental serum sickness with acute glomerulonephritis induced passively in mice by antigen-antibody complexes in antigen excess. Amer. J. Path. 35:275-283.

McCLUSKEY, R. T., B. BENACERRAF, J. L. POTTER, and F. MILLER. 1960. The pathologic effects of intravenously administered soluble antigen-antibody complexes. I. Passive serum sickness in mice. J. Exp. Med. 111:181-193.

McKAY, K. D. 1965. IN Disseminated Intravascular Coagulation: An Intermediary Mechanism of Disease. Harper and Row, New York, pp. 493.

MORNET, P. 1954. Les trypanosomes pathogenes de l'A.O.F. considerations sur leur repartition, leur frequence, les taux d'infestation des animaux domestiques. Bull. Soc. Path. Exot. 47:709-716.

MOULTON, J. E., and A. E. SOLLOD. 1976. Clinical, serologic and pathologic changes in calves with experimentally induced *Trypanosoma brucei*. Amer. J. Vet. Res. 37:791-802.

MOVAT, H. Z. 1971. The acute inflammatory reaction. IN Inflammation, Immunity and Hypersensitivity, H. Z. Movat (ed.). Harper and Row, New York, pp. 1-129.

MÜLLER-EBERHARD, H. J. 1975. Complement. Ann. Rev. Biochem. 44: 697-724.

MURRAY, M. 1974. The pathology of African trypanosomiasis. IN Progress in Immunology II. Brent and Holborow (ed.). North Holland Publishing Company, Volume 5, pp. 181-192.

MURRAY, M., P. H. LAMBERT, and W. I. MORRISON. 1975. Renal lesions in experimental trypanosomiasis. Médecine et Maladies Infectieuses 12:638-641.

MURRAY, M., P. K. MURRAY, F. W. JENNINGS, E. W. FISCHER, and G. M. URQUHART. 1974. The pathology of *Trypanosoma brucei* infections in the rat. Res. Vet. Sci. 16:77-84.

MUSOKE, A. J., and A. F. BARBET. 1977. Activation of complement by variant-specific surface antigen of *Trypanosoma brucei*. Nature 270:438-440.

NAGLE, R. B., P. A. WARD, H. B. LINDSLEY, E. H. SADUN, A. J. JOHNSON, R. E. BERKAW, and P. K. HILDEBRANDT. 1974. Experimental infections with African trypanosomes. VI. Glomerulonephritis involving the alternative pathway of complement activation. Amer. J. Trop. Med. Hyg. 23:15-26.

NAYLOR, D. C. 1971. The hematology and histopathology of *Trypanosoma congolense* infections in cattle. I. Introduction and histopathology. Trop. Anim. Health Prod. 3:24-32.

NIELSON, K., and J. SHEPPARD. 1977. Activation of complement by trypanosomes. Experientia 33:769-770.

NIELSEN, K., J. SHEPPARD, I. R. TIZARD, and W. HOLMES. 1977. *Trypanosoma lewisi:* Characterization of complement-activating components. Exp. Parasitol. 43:153-160.

NIELSEN, K., J. SHEPPARD, I. R. TIZARD, and W. HOLMES. 1978b. Direct activation of complement by trypanosomes. J. Parasitol. 64:544-546.

NIELSEN, K., J. SHEPPARD, W. HOLMES, and I. R. TIZARD. 1978b. Experimental bovine trypanosomiasis: changes in serum immunoglobulins, complement and complement components in infected cattle. Immunology 35:817-826.

NOBLE, E. R., and G. A. NOBLE. 1976. Parasitology. Lea and Febiger, Philadelphia, pp. 566.

NOVY, F. G., P. H. DEKRUIF, and R. L. NOVY. 1917. J. Infect. Dis. 20:499-535.

PARAPPALLY, N. P., and D. G. INGRAM. 1973. Role of immunoconglutinin in resistance to infection: The *in vitro* promotion of phagocytosis by immunoconglutinin. Immunology 25:523-530.

PFUELLER, S. L., and E. F. LÜSCHER. 1972. The effects of immune complexes on blood platelets and their relationship to complement activation. Immunochem. 9:1151-1165.

POLTERA, A. A. 1980. Immunological and chemotherapeutic studies in experimental trypanosomiasis with special reference to the heart and brain. Trans. R. Soc. Trop. Med. Hyg. 74:706-716.

POLTERA, A. A., and J. N. COX. 1977. Pancarditis with vasculitis in endomyocardial fibrosis and in human African trypanosomiasis: A comparative histological study of four Ugandan cases. Virchows Archiv. Path. Anat. Hist. 375:53-70.

POLTERA, A. A., J. N. COX, and R. OWOR. 1976. Pancarditis involving the conducting system and all valves in human African trypanosomiasis. Br. Heart. J. 38:827-837.

POLTERA, A. A., R. OWOR, and J. N. COX. 1977. Pathologic aspects of human African trypanosomiasis in Uganda. A post-mortem survey of fourteen cases. Virchows Archiv. Path. Anat. Hist. 373:249-265.

RATNOFF, O. D. 1969. The interrelationships of clotting and immunologic mechanisms. IN Immunobiology, R. A. Good and D. N. Fischer (eds.). Sinauer Associates, Inc., Stanford, pp. 135-144.

RICE, C. E. 1953. A consideration of the components concerned in the conglutinating activities of certain complements. J. Immunol. 70: 497-502.

RICKMAN, W. J. 1978. Immunopathology of experimental African trypanosomiasis. Masters Thesis. Michigan State University, East Lansing, Michigan, pp. 66.

RICKMAN, W. J., and H. W. COX. 1979. Associations of autoantibodies with anemia, splenomegaly and glomerulonehpritis in experimental African trypanosomiasis. J. Parasitol. 65:65-73.

ROBBINS, J., and C. A. STETSON. 1959. An effect of antigen-antibody interaction of blood coagulation. J. Exp. Med. 109:1-8.

ROBBINS-BROWNE, R. M., and J. SCHNEIDER. 1977. Coagulation abnormalities in African trypanosomiasis. IN Medicine in a Tropical Environment, J.H.S. Gear (ed.). A. A. Balkema, Rotterdam, pp. 565-572.

ROBINS-BROWNE, R. H., J. SCHNEIDER, and J. METZ. 1975. Thrombocytopenia in trypanosomiasis. Amer. J. Trop. Med. Hyg. 24:226-231.

RURANGIRWA, F. R., H. TABEL, G. LOSOS, and I. R. TIZARD. 1980. Hemolytic complement and serum C3 levels in Zebu cattle infected with *Trypanosoma congolense* and *Trypanosoma vivax* and the effect of trypanocidal treatment. Infect. Immun. 27:832-836.

SADUN, E. H., A. J. JOHNSON, R. B. NAGLE, and R. E. DUXBURY. 1973. Experimental infecions with African trypanosomes. V. Preliminary parasitological, clinical, hematological, serological, and pathological observations in Rhesus monkeys infected with *Trypanosoma rhodesiense*. Amer. J. Trop. Med. Hyg. 22:323-330.

SEED, J. R. 1969. *Trypanosoma gambiense* and *Trypanosoma lewisi:* Increased vascular permeability and skin lesions in rabbits. Exp. Parasitol. 26:214-223.

STRENG, O. 1909a. Studien über das verhalten des rinder serums gegenüber den microbien. Versuch einer neuen serodiagnostichen method. Zbe. Bakt. (Orig.) 50:47-78.

STRENG, O. 1909b. Agglutinin oder konglutinin? Zgl. Bakt. (Orig.) 52:523-531.

STRENG, O. 1930. Immunokonglutinin-antikomplement. Acta. Path. Microbiol. Scand., Suppl. III. 20:411-429.

STRENG, O., and E. RYTI. 1923. Vergleichende untersuchunger über die agglutination und die konglutination der blutkorperchen and bakterium in neutrolsalzlösungen. Acta. Med. Duodecin. IV. No. 3:1-68.

STRONG, R. P. 1944. IN Stitt's Diagnosis, Prevention and Treatment of Tropical Diseases, 7th Edition. Blakiston Company, Philadelphia, pp. 183-193.

TABEL, H., G. L. LOSOS, and G. M. MAXIE. 1977. Hypocomplementemia in bovine trypanosomiasis. Proc. Can. Fed. Biol. Soc. 20:471.

THOONGSUWAN, S., and H. W. COX. 1978. Anemia, splenomegaly and glomerulonephritis associated with autoantibody in *Trypanosoma lewisi* infections. J. Parasitol. 64:669-673.

THOONGSUWAN, S., H. W. COX, and R. A. PATRICK. 1978a. Immunoconglutinin associated with nonspecific acquired resistance in malaria, babesiosis and other anemia-inducing infections. J. Parasitol. 64:1050-1056.

THOONGSUWAN, S., H. W. COX, and R. A. PATRICK. 1978b. Serologic specificity of immunoconglutinin associated with infectious anemia of rats and its role in nonspecific acquired resistance. J. Parasitol. 64:1060-1066.

THOONGSUWAN, S., H. W. COX, and W. J. RICKMAN. 1979. Antibody to fibrinogen/fibrin products (Anti-F) in malaria, babesiosis and trypanosomiasis of rodents. J. Parasitol. 65:426-429.

TIZARD, I. R., and W. L. HOLMES. 1976. Hemolysin activity associated with extracts of *Trypanosoma congolense*. Experientia 32:1533-1534.

TIZARD, I. R., A. MELLORS, W. L. HOLMES, and K. NIELSEN. 1978. The generation of phospholipase A and hemolytic fatty acids by autolysing suspensions of *Trypanosoma congolense*. Tropenmed. Parasitol. 29:127-133.

VALLI, V.E.O., C. M. FORSBERG, and G. A. ROBINSON. 1978a. The pathogenesis of *Trypanosoma congolense* infection in calves. I. Clinical observations and gross pathologic changes. Vet. Path. 15:608-620.

VALLI, V.E.O., C. M. FORSBERG, and B. J. McSHERRY. 1978b. The pathogenesis of *Trypanosoma congolense* infection in calves. II. Anemia and erythroid response. Vet. Path. 15:732-745.

VALLI, V.E.O., C. M. FORSBERG, and J. H. LUMSDEN. 1979a. The pathogenesis of *Trypanosoma congolense* infection in calves. III. Neutropenia and myeloid response. Vet. Path. 16:96-107.

VALLI, V.E.O., and C. M. FORSBERG. 1979b. The pathogenesis of *Trypanosoma congolense* infection in calves. IV. Quantitative histological changes. Vet. Path. 16:334-368.

VON PIRQUET, C. F., and B. SCHICK. 1905. IN Die Serumkrankheit. Franz Deutiche, Leipzig and Wien. Translated by B. Schick. 1951. The Williams and Wilkens Company, Baltimore, pp. 130.

WARD, P. A. 1967. A plasmin-split fragment of C3 as a new chemotactic factor. J. Exp. Med. 126:189-196.

WARD, P. A. 1971. The role of complement in inflammation and hypersensitivity. IN Inflammation, Immunity and Hypersensitivity, Henry Z. Movat (ed.). Harper and Row, New York, pp. 459-477.

WARTIOVAARA, T. W. 1932. Über die entwicklung der konglutinierenden eigenschaft bei der immunisierung. Acta. Soc. Med. 'Duodecim' Ser A. 14:1-143.

WEITZ, B. 1970. Infection and resistance. IN The African Trypanosomiases, H. W. Mulligan (ed.). London, pp. 97.

WELLDE, B. T., R. M. KOVATCH, D. A. CHUMO, and D. E. WYKOFF. 1978. *Trypanosoma congolense:* Thrombocytopenia in experimentally infected cattle. Exp. Parasitol. 45:26-33.

WOO, P.T.K., and A. KOBAYASHI. 1975. Studies on the anemia in experimental African trypanosomiasis. I. A preliminary communication on the mechanism of the anemia. Ann. Soc. Belge. Med. Trop. 55:37-45.

WOODRUFF, A. W. 1973. Mechanisms involved in anemia associated with infection and splenomegaly in the tropics. Trans. R. Soc. Trop. Med. Hyg. 67:313-328.

WOODRUFF, A. W., J. L. ZIEGLER, A. HATHAWAY, and T. GWATA. 1973. Anemia in African trypanosomiasis and 'Big Spleen Disease' in Uganda. Trans. R. Soc. Trop. Med. Hyg. 67:329-337.

WUEPPER, K. D., and C. G. COCHRANE. 1972. Plasma prekallikrein: Isolation, characterization and mechanism of activation. J. Exp. Med. 135:1-20.

YORKE, W. 1911. Autoagglutination of red blood cells in trypanosomiasis. Amer. Trop. Med. Parasitol. 4:529-552.

ZOUTENDYK, A., and J. GEAR. 1951. Autoantibodies in the pathogenesis of disease. A preliminary study of autosensitization of red cells in various diseases. So. Afr. Med. J. 25:665-668.

ARTICLE 1

IMMUNOLOGIC REACTIONS ASSOCIATED WITH ANEMIA, THROMBOCYTOPENIA AND COAGULOPATHY IN EXPERIMENTAL AFRICAN TRYPANOSOMIASIS

William J. Rickman

Department of Microbiology and Public Health

Michigan State University

East Lansing, Michigan 48824

(Published in the Journal of Parasitology)

(66:28-33, 1980)

IMMUNOLOGIC REACTIONS ASSOCIATED WITH ANEMIA, THROMBOCYTOPENIA AND COAGULOPATHY IN EXPERIMENTAL AFRICAN TRYPANOSOMIASIS*

William J. Rickman
Department of Microbiology and Public Health
Michigan State University
East Lansing, Michigan 48824

*This research was supported in part with funds from the College of Veterinary Medicine, Michigan State University, and in part by a Grant from the Michigan Heart Association. This publication is Journal Article No. 8930 from the Michigan Agricultural Experiment Station.

This communication is from a dissertation entitled, "Immunoconglutination in Experimental African Trypanosomiasis," submitted by the author in partial fulfillment of the requirements for the Ph.D. degree at Michigan State University.

ABSTRACT

Rats infected with *Trypanosoma brucei rhodesiense* developed anemia, thrombocytopenia and hypocomplementemia. Anemia, thrombocytopenia and sharp reductions in parasitemia were associated with elevated titers of cold-active hemagglutinin, antibody to fibrinogen/fibrin related products and immunoconglutinin. Depletion of lytic complement, prolonged partial thromboplastin times and presence of fibrin monomers in the blood occurred at the time anemia and significant elevations in precipitable immune complexes were observed. Terminally, consumption of immunologic factors coincided with accelerated partial thromboplastin times. At death convulsions and hemoptysis with labored breathing suggested that the animals died of respiratory failure and that disseminated intravascular coagulation may have occurred. It is suggested that microthrombiosis might have resulted from the immunologic interaction of complex coated blood cells with immunoconglutinin and contributed to the terminal disease signs.

INTRODUCTION

In a previous communication we associated anemia, splenomegaly and glomerulonephritis in rats infected with *Trypanosoma brucei rhodesiense* to the presence of antibody against fibrinogen/fibrin related products (Anti-F) and antibody to the third component of fixed complement or immunoconglutinin (IK). In addition to the above disease signs, *in vivo* and *in vitro* clotting abnormalities were observed and the rats appeared to die of shock with pulmonary involvement. Evidence was presented indicating that soluble immune complexes of Anti-F,

fibrinogen/fibrin related products (FRP), complement and IK may have had a causal role in anemia (Rickman and Cox, 1979).

In view of the multiplicity of immune complex interactions, we conducted further experiments to determine if the complement and coagulation systems are affected during acute *T. b. rhodesiense* infections of rats. We here report the associations of Anti-F, IK, FRP and lytic complement consumption with anemia, thrombocytopenia and coagulation abnormalities in rats with acute trypanosomiasis.

MATERIALS AND METHODS

Experimental animals and infections

The origin of rats and the *Trypanosoma brucei rhodesiense* used in these experiments has been reported. For these experiments each animal received an intraperitoneal injection of 1 ml of diluted blood containing 10^6 parasites as described (Rickman and Cox, 1979).

Experimental design

Seventy-two rats were infected for the experiment. Daily six animals were randomly selected for study. Trypanosome (TRYP), erythrocyte (RBC), and platelet (PLT) counts were performed on each rat as described, except that Rees-Ecker diluting fluid was used (Rickman and Cox, 1979; Rees and Ecker, 1923). Each of the rats was exsanguinated by cardiac puncture under ether anesthesia. Three of the rats were bled for serum and three for platelet-free plasma as recommended by Tsang and Damian (1977).

Serologic examinations

Cold-active hemagglutinin (CAH) and immunoconglutinin (IK) were detected by reported methods (Rickman and Cox, 1979). Antibody to

fibrinogen/fibrin related products (Anti-F) was quantitated by the methods of Thoongsuwan et al., (1979) using as antigen human fibrinogen, which was donated by Dr. John E. Mercer of the Blood Products Laboratory, Michigan Department of Health, 3500 North Logan Street, Lansing, Michigan.

Lytic complement titers (LCT) were determined by the ability of 50 µl of serially diluted unheated test serum to completely lyse an equal volume of a 0.5% suspension of sensitized sheep erythrocytes (SRBC). Four volumes of a 5.0% suspension of SRBC were sensitized by incubation at 37°C for 20 min with one volume of anti-serum prepared in rats by intraperitoneal injection of 10^8 washed SRBC 9 days prior to bleeding. Veronal buffered saline (pH 7.4) containing 102 mg/ml of $MgCl_2 \cdot 6\ H_2O$ and 17 mg/ml of $CaCl_2$ was used as diluent in serial dilutions and for washing SRBC. The test was incubated at 37°C for 30 min in Cooke microtiter plates. The endpoint of the lytic titer consisted of the highest dilution of test serum resulting in complete hemolysis after storage overnight at 4°C. Controls for the assay consisted of heated and unheated normal rat serum and heated test serum titered against both unsensitized and sensitized SRBC.

Soluble immune complexes were detected in experimental test serum by precipitation with polyethylene glycol 6,000 (PEG) by the method of Bout et al., (1977). The PEG values are expressed as the optical density at 280 nm.

Preparation of coagulation reagents

A fresh normal bovine brain was homogenized and extracted with acetone (Langell et al., 1955). The acetone-dried bovine brain powder (BBP) was then stored at -20°C. Thromboplastin was prepared from BBP according to the method described by Tocantins (1955). Activated

partial thromboplastin was prepared from BBP as described by Langell et al., (1955).

Coagulation Assays

Prothrombin times (PT) were determined by the one-stage method of Quick as described by Tocantins (1955). Activated partial thromboplastin times (APTT) were performed according to the method of Langell et al., (1955). Each plasma sample was tested in quadruplicate. Fibrin monomers were detected with serial dilutions of protamine sulfate (SDPS) by described methods (Niewiarowski and Gurewich, 1971).

Preparation of antigens and antibodies

Normal rat fibrinogen (RF) was purified from platelet-free plasma as described by Kazal et al., (1955) with several modifications. (1) The entire purification process was performed without glass contact. (2) Epsilon animo caproic acid (EACA) was incorporated into all buffers at a 4.0 mM concentration to avoid *in vitro* fibrinolysis. (3) Ammonium sulfate at a final concentration of 25% saturation was used instead of glycine for final purification (MacFarlane, 1963). (4) The final product was reconstituted in and dialyzed against 0.055 M sodium citrate containing 4.0 mM EACA until free of ammonium sulfate.

Monospecific antiserum to RF was prepared in a normal rabbit. One ml of purified RF (10 mg/ml) was mixed with an equal volume of a 0.5% suspension of polystyrene latex particles coated with antihuman fibrinogen degradation fragments D and E globulins obtained from Burroughs Wellcome Co., Research Triangle Park, North Carolina. The mixture was incubated at 22°C for 5 min and was washed five times in cold PBS. After the final washing the pellet was resuspended in 1 ml

of PBS and mixed with an equal volume of Freund's Complete Adjuvant (Difco Laboratories, Detroit, Michigan). The rabbit was given two intramuscular injections of 1 ml per thigh fourteen days apart. On the twenty-first day after the initial injection the rabbit was exsanguinated by cardiac puncture. The specificity of rabbit anti-RF was demonstrated by immunoelectrophoresis according to the method of Leid and Williams (1974).

Double immunodiffusion (DID) gel analysis of PEG precipitated immune complexes

Pools of experimental serum were precipitated with PEG and resuspended in PBS for DID analysis. The gels were prepared as described by Thoongsuwan and Cox (1973).

RESULTS

Relationships of hematologic changes and serum complement levels with the presence of CAH, IK and Anti-F

Mean values for TRYP, RBC and PLT counts are presented in Table 1. Parasites were first detected on day 2 by wet mount examination of the peripheral blood. The parasitemia peaked on day 5 and again on day 9 with marked reductions between peaks. This reduced parasitemia coincided with anemia and thrombocytopenia. Both RBC and PLT counts remained substantially reduced throughout the remainder of the experiment.

Mean titers for CAH, IK and Anti-F are also presented in Table 1. CAH was first detected on day 3 reaching its highest concentration on day 6. IK titers did not significantly increase over the normal

values until day 5 and peaked on day 6. Anti-F titers reached their highest value on day 5 of the infection. Titers of all three antibodies were reduced on day 9 when most of the rats died showing signs of respiratory failure.

The LCT titers were markedly reduced on day 4 and were undetectable on days 7 through 9 of the infection (Table 1).

Coagulation status of rats infected with *T. b. rhodesiense*

Mean values for PT and APTT are presented in Table 2. The PT values did not significantly change throughout the infection as judged by statistical analysis using the student's T test for paired observations. On days 4 and 5 the APTT values were significantly increased over normal control animals. This abnormality was associated with prolonged and excessive bleeding when the rats' tails were snipped for blood counts. On days 6, 8 and 9 the APTT values were significantly decreased. During the last two days of the infection the infected animals exhibited signs of shock. Convulsions and hemoptysis with labored breathing were common. Blood drawn just prior to death often clotted in spite of the use of adequate anticoagulants. For this reason additional animals had to be bled on days 8 and 9 to obtain suitable plasma samples. Early prolonged bleeding and terminal shock signs accompanied by hyperclottability have been consistently observed in subsequent experiments and in passage animals.

The SDPS Test results demonstrated that fibrin monomers were present in the plasma of infected animals on days 4 and 9.

Detection of soluble immune complexes in the serum of infected animals

Statistically significant elevations in PEG precipitable material were demonstrated during the course of the infection (Table 2). The

highest values were obtained on days 3 through 9 indicating the presence of soluble immune complexes in the serum of infected animals. Anti-RF reacted in DID with the pools of PEG precipitates on days 5 through 9 indicating that fibrinogen/fibrin related products were present.

DISCUSSION

In a previous communication we reported that a syndrome of anemia, splenomegaly and proliferative glomerulonephritis accompanied by CAH, Anti-F and IK was associated with acute *T. rhodesiense* infections of rats. Coating of erythrocytes and parasites with fibrin and complement suggested that immune complexes of FRP, Anti-F and complement may have coated the cells and parasites and that the conglutinating action of IK caused both to be sequestered and phagocytized in the spleen (Rickman and Cox, 1979).

We here report that thrombocytopenia, hypocomplementemia and coagulation abnormalities manifested as hypoclottability with prolonged bleeding may also be a part of the syndrome. Anemia, thrombocytopenia and reduced parasitemia were associated with elevated titers of each of the three autoantibodies, depletion of lytic complement, prolonged APTT and with the presence of fibrin monomers in the blood. Coagulopathy was also indicated by prolonged bleeding from the snipped tips of the tails.

Except for paleness of ears and eyes, the infected rats appeared to remain in good condition up to the time of death. Terminal morbid signs, which appeared on the ninth day, were sudden in onset and resembled acute respiratory failure. At that time trypanosome counts were elevated but erythrocyte and thrombocyte counts remained low.

Lytic complement remained absent and titers of CAH, Anti-F and IK were reduced. Fibrin monomers and PEG precipitable soluble immune complexes were present. Significant shortening of APTT on day 9 was indicative of hyperclottability as was also the apparent clotting of blood drawn into anticoagulant. The findings would suggest that the rats had died of pulmonary thrombosis or disseminated intravascular coagulation.

Prolonged bleeding, extended APTT, the presence of Anti-F and the presence of fibrin monomers early in the infection indicated activation of the intrinsic (Hageman Factor) pathway of coagulation. Alterations in this pathway have been reported in humans and cattle with acute trypanosomiasis (Robins-Browne and Schneider, 1977; Wellde et al., 1978). On the basis of thrombocytopenia and elevated fibrin degradation products, investigators have implicated coagulation disease as an additional consequence of acute trypanosome infections (Barrett-Conner et al., 1973; Boreham and Facer, 1974; Robins-Browne et al., 1977; Wellde et al., 1978). In our experiments the presence of fibrin monomers on days 4 through 7 along with abnormal APTT values indicated the occurrence of coagulopathy that resulted from secondary fibrinolysis (Niewiarowski and Gurewich, 1971).

While coagulation abnormalities occurred during the infection, the etiology is not clear. Robbins and Stetson (1959) indicated activation of the intrinsic pathway occurred in the presence of immune complexes, but Cochrane et al. (1972) reported that immune complexes could not activate purified Hageman Factor *in vitro*. On the other hand, indirect activation may result from the interaction of complement fixing immune complexes with cellular elements, e.g., platelets

(World Health Organization, 1977). The loss of platelets and complement at the time immune complexes were detected would indicate that complexes have a role in coagulation activation *in vivo*.

Hypocomplementemia in trypanosomiasis has been reported by several investigators (Nagle et al., 1974; Jarvinem and Dalmasso, 1976; Kobayashi and Tizard, 1976; Greenwood and Whittle, 1976). Several contributory mechanisms were suggested. One mechanism, nonspecific activation by variant-specific surface antigens of trypanosomes, has received considerable attention and was reviewed (Musoke and Barbet, 1977; Nielson et al., 1977; Nielson et al., 1978). Others have advocated activation by antigen-antibody complexes (Woodruff et al., 1973; Woo and Kobayashi, 1975; Kobayashi et al., 1976). In the present study, demonstrating complexes in the blood when lytic complement was depleted is supportative of the latter concept. Further, a significant correlation between the presence of PEG detectable immune complexes and the C1q binding potential of serum has been demonstrated (Bout et al., 1977). Finally, the fact that IK was stimulated was indicative of the presence of complement fixing immune complexes (Lachmann and Coombs, 1965). Our DID analysis of PEG precipitates indicated that FRP were present, possibly complexed with Anti-F. Thus complexes of FRP and Anti-F could have contributed to complement consumption.

The presence of fibrin monomers has important implications. The action of thrombin on fibrinogen results in the formation of fibrin monomers which combine with fibrinogen or late fibrinogen degradation products and remain soluble in circulation (Niewiarowski and Gurewich, 1971). In the present experiments the possibility that fibrin

monomers served as a soluble antigen to form immune complexes with Anti-F must be considered. Following the time monomers were conspicuous, titers of both Anti-F and IK were suddenly reduced. This finding could be interpreted as a result of the complexing of the monomers with Anti-F and the binding of the complexes to blood cells. Binding of various antigens and their respective antibody to erythrocytes and the subsequent fixation of complement has been demonstrated (Rickman and Cox, 1979; Woodruff et al., 1973; Kobayashi et al., 1976). The conglutination of the complex coated cells by IK could result in the formation of aggregates. It is possible that such aggregates, or conglutinates of cells served as thrombi or pseudothrombi to occlude the microvasculature of the lungs and result in the terminal disease signs observed in the present experiments.

Spontaneous clumping of blood cells from trypanosomal infections was observed and cold-active agglutinins for intact autologous, homologous and heterologous erythrocytes were demonstrated (Yorke, 1911). CAH agglutination of trypsinized homologous erythrocytes was demonstrated in the present experiments. In view of the fact that Yorke was dealing with intact erythrocytes it is possible that the autoagglutination he observed may have been a result of IK conglutination of immune complex coated erythrocytes.

LITERATURE CITED

1. BARRETT-CONNER, E., R. J. UGORETZ, and A. I. BRAUDE. 1973. Disseminated intravascular coagulation in trypanosomiasis. Arch. Int. Med. 131:574-579.

2. BOREHAM, P.F.L., and C. A. FACER. 1974. Fibrinogen and fibrinogen/fibrin degradation products in experimental African trypanosomiasis. Int. J. Parasitol. 4:143-151.

3. BOUT, D., F. SANTRO, Y. CARLIER, and A. CAPRON. 1977. Circulating immune complexes in schistosomiasis. Immunology 33:17-22.

4. COCHRANE, C. G., K. D. WUEPPER, B. S. AIKEN, S. D. REVAK, and SPIEGELBERG, H. L. 1972. Interaction of Hageman factor and immune complexes. J. Clin. Invest. 51:2736-2745.

5. GREENWOOD, B. M., and H. WHITTLE. 1976. Complement activation in patients with Gambian sleeping sickness. Clin. Exp. Immunol. 24:133-138.

6. JARVINEM, J. A., and A. P. DALMASSO. 1976. Complement in experimental *Trypanosoma lewisi* infections of rats. Infect. Immun. 14:894-902.

7. KAZAL, L. A., G. F. GRANNIS, and L. M. TOCANTINS. 1955. Preparation of fibrinogen by glycine preparation. In L. M. Tocantins and L. A. Kazal (eds.), Blood coagulation, hemorrhage and thrombosis. Grune and Stratton, Inc., New York, p. 232-239.

8. KOBAYASHI, A., and I. R. TIZARD. 1976. The response to *Trypanosoma congolense* infection in calves: Determination of immunoglobulins IgG1, IgG2, IgM and C3 levels and the complement fixing antibody titers during the course of infection. Tropenmed. Parasitol. 27:411-417.

9. _____, _____, and P.T.K. WOO. 1976. Studies on the anemia in experimental African trypanosomiasis. II. The pathogenesis of the anemia in calves infected with *Trypanosoma congolense*. Am. J. Trop. Med. Hyg. 25:401-406.

10. LACHMANN, P. J., and R.R.A. COOMBS. 1965. Complement, conglutinin and immunoconglutinin. In G.E.W. Wolstenholme and J. Knight (eds.), Complement. Little, Brown and Company, Boston, p. 242-273.

11. LANGELL, R. D., R. H. WAGONER, and K. M. BRINKHOUS. 1955. Determination of partial thromboplastin time (PTT). In L. M. Tocantins and L. A. Kazal (eds.), Blood Coagulation, Hemorrhage and Thrombosis. Grune and Stratton, Inc., New York, p. 103-107.

12. LEID, R. W., and J. F. WILLIAMS. 1974. The immunological response of the rat to infection with *Taenia taeniaformis*. II. Characterization of reaginic antibody and an allergen associated with the larval stage. Immunology 27:209-225.

13. MACFARLANE, A. S. 1963. *In vivo* behavior of ^{131}I-fibrinogen. J. Clin. Invest. 42:346-361.

14. MUSOKE, A. J., and A. F. BARBET. 1977. Activation of complement by variant-specific surface antigen of *Trypanosoma brucei*. Nature 270:438-440.

15. NAGLE, R. W., P. A. WARD, H. B. LINDSLEY, E. H. SADUN, A. J. JOHNSON, R. E. BERKAW, and P. K. HILDEBRANDT. 1974. Experimental infections with African trypanosomes. VI. Glomerulonephritis involving the alternative pathway of complement activation. Am. J. Trop. Med. Hyg. 23:15-26.

16. NIELSEN, K., J. SHEPPARD, I. TIZARD, and W. HOLMES. 1977. *Trypanosoma lewisi:* Characterization of complement-activating components. Exp. Parasitol. 43:153-160.

17. _____, _____, _____, _____. 1978. Direct activation of complement by trypanosomes. J. Parasitol. 64: 544-546.

18. NIEWIAROWSKI, S., and V. GUREWICH. 1971. Laboratory identification of intravascular coagulation. J. Lab. Clin. Med. 77:665-676.

19. REES, H. M., and E. E. ECKER. 1923. An improved method for counting blood platelets. JAMA 80:621-624.

20. RICKMAN, W. J., and H. W. COX. 1979. Associations of auto-antibodies with anemia, splenomegaly and glomerulonephritis in experimental African trypanosomiasis. J. Parasitol. 65: 65-73.

21. ROBBINS, J., and STETSON, C. A. 1959. An effect of antigen-antibody interaction of blood coagulation. J. Exp. Med. 109:1-8.

22. ROBINS-BROWNE, R. M., and J. SCHNEIDER. 1977. Coagulation abnormalities in African trypanosomiasis. In H.H.S. Gear (eds.), Medicine in a Tropical Environment. A. A. Balkema, Rotterdam, p. 565-572.

23. _____, _____, and J. METZ. 1975. Thrombocytopenia in trypanosomiasis. Am. J. Trop. Med. Hyg. 24:226-231.

24. THOONGSUWAN, S., and H. W. COX. 1973. Antigenic variants of the haemosporidian parasite, *Babesia rodhaini*, selected by *in vitro* treatment with immune globulin. Ann. Trop. Med. Parasitol. 67:373-385.

25. _____, _____, and W. J. RICKMAN. 1979. Antibody to fibrinogen/fibrin products (Anti-F) in malaria, babesiosis and trypanosomiasis of rodents. J. Parasitol. 65:426-429.

26. TOCANTINS, L. M. 1955. Estimation of prothrombin (one-stage method of Quick). In L. M. Tocantins and L. A. Kazal (eds.), Blood Coagulation, Hemorrhage and Thrombosis. Grune and Stratton, Inc., New York, p. 148-150.

27. TSANG, V.C.W., and R. T. DAMIAN. 1977. Demonstration and mode of action of an inhibitor for activated Hageman factor (Factor XIIa) of the intrinsic blood coagulation pathway from *Schistosoma mansoni*. Blood 49:619-633.

28. WELLDE, B. T., R. M. KOVATCH, D. A. CHUMO, and D. E. WYKOFF. 1978. *Trypanosoma congolense:* Thrombocytopenia in experimentally infected cattle. Exp. Parasitol. 45:26-33.

29. WOO, P.T.K., and A. KOBAYASHI. 1975. Studies on the anemia in experimental African trypanosomiasis. I. A preliminary communication on the mechanism of the anemia. Ann. Soc. Belge Med. Trop. 55:37-45.

30. WOODRUFF, A. W., J. L. ZIEGLER, A. HATHAWAY, and T. GWATA. 1973. Anaemia in African trypanosomiasis and "Big Spleen Disease" in Uganda. Trans. R. Soc. Trop. Med. Hyg. 67: 329-337.

31. WORLD HEALTH ORGANIZATION 1977. The role of immune complexes in disease. WHO Technical Report, Series Number 606.

32. YORKE, W. 1911. Autoagglutination of red blood cells in trypanosomiasis. Am. Trop. Med. Parasitol. 4:529-552.

Table 1. Mean ± standard error of trypanosome counts per mm^3 (TRYP x 10^5 ± S.E.), erythrocyte counts per mm^3 (RBC x 10^6 ± S.E.), platelet counts per mm^3 (PLT x 10^5 ± S.E.), mean lytic complement titer (LCT), and mean titers of cold-active hemagglutinin (CAH), immunoconglutinin (IK) and antibody to fibrinogen/fibrin related products (Anti-F) of rats autopsied daily during the course of *Trypanosoma brucei rhodesiense* infection.

Days Post-Infection	TRYP x 10^5 ± S.E.*	RBC x 10^6 ± S.E.*	PLT x 10^5 ± S.E.*	LCT**	CAH**	IK**	Anti-F**
0	0	6.92 ± 0.06	3.86 ± 0.21	128	0	10	8
1	0	6.84 ± 0.13	4.36 ± 0.20	128	0	10	8
2	(+)	7.21 ± 0.18	3.65 ± 0.21	128	0	10	16
3	0.28 ± 0.06	6.87 ± 0.18	3.95 ± 0.28	128	8	40	64
4	2.35 ± 0.44	5.96 ± 0.29+	2.17 ± 0.29+	8	256	40	64
5	5.28 ± 0.77	4.89 ± 0.38	0.65 ± 0.08	4	256	2560	256
6	3.81 ± 0.89	4.39 ± 0.29	0.51 ± 0.09	8	512	5120	128
7	1.91 ± 0.83	4.69 ± 0.07	0.95 ± 0.14	0	256	1280	256
8	0.93 ± 0.17	4.31 ± 0.13	1.34 ± 0.18	0	128	1280	256
9	5.86 ± 0.76	4.40 ± 0.19	1.12 ± 0.09	0	8	320	64
Post Infection Normal Control	0	7.26 ± 0.04	4.33 ± 0.23	128	0	10	8

* Six animals examined

** Three animals examined

\+ Significant at $P<0.02$. Students "t" test

Table 2. Mean ± standard error of prothrombin times in seconds (PT ± S.E.), activated partial thromboplastin times in seconds (APTT ± S.E.), polyethylene glycol precipitation of soluble immune complexes in optical density units at 280 nm (PEG ± S.E.) and results of serially diluted protamine sulfate test for fibrin monomers (SDPS Test) of rats autopsied daily as groups of three during the course of *Trypanosoma brucei rhodesiense* infection.

Days Post Infection	PT ± S.E.	APTT ± S.E.	PEG ± S.E.	SDPS Test
0	17.56 ± 0.13	27.26 ± 0.61	0.55 ± 0.01	0/3
1	18.03 ± 0.17	27.20 ± 0.58	0.54 ± 0.01	0/3
2	17.03 ± 0.37	27.83 ± 0.57	0.65 ± 0.05	0/3
3	17.43 ± 0.29	28.10 ± 1.80	0.72 ± 0.05*	0/3
4	17.20 ± 0.35	37.73 ± 1.19*	0.60 ± 0.03	1/3
5	17.60 ± 0.35	31.43 ± 1.06*	0.67 ± 0.05	3/3
6	17.96 ± 0.37	25.50 ± 0.79*	0.65 ± 0.03*	3/3
7	17.86 ± 0.43	28.23 ± 2.71	0.70 ± 0.01*	2/3
8	18.20 ± 0.49	23.40 ± 1.55*	0.88 ± 0.09*	1/3
9	18.73 ± 1.64	22.66 ± 0.36*	0.70 ± 0.05*	3/3
Post Infection Normal Control	17.50 ± 0.09	28.46 ± 0.28	0.54 ± 0.01	0/3

*Significant at $P<0.05$ "t" test.

ARTICLE 2

INTERACTIONS OF IMMUNOCONGLUTININ AND IMMUNE COMPLEXES IN COLD AUTOHEMAGGLUTINATION ASSOCIATED WITH AFRICAN TRYPANOSOMIASIS

William J. Rickman

Department of Microbiology and Public Health

Michigan State University

East Lansing, Michigan 48824

(Published in the Journal of Parasitology)

(67: IN PRESS, 1981)

INTERACTIONS OF IMMUNOCONGLUTININ AND IMMUNE COMPLEXES IN COLD AUTOHEMAGGLUTINATION ASSOCIATED WITH AFRICAN TRYPANOSOMIASIS

William J. Rickman
Department of Microbiology and Public Health
Michigan State University
East Lansing, Michigan 48824

*This research was supported in part with funds from the College of Veterinary Medicine, Michigan State University, in part from a Grant-in-Aid from Michigan Heart Association, and in part from Grant No. AI-17018-01 from National Institutes of Health. This publication is Journal Article No. 9635 from the Michigan Agricultural Experiment Station.

This communication is from a dissertation entitled, "Immunoconglutination in Experimental African Trypanosomiasis," submitted by the author in partial fulfillment of the requirements for the Ph.D. degree at Michigan State University.

ABSTRACT

Blood cells from rats infected with *Trypanosoma brucei rhodesiense* agglutinated when cooled in an ice bath and dissociated when warmed to 20°C. This autohemagglutination became evident at the time reduced erythrocyte and parasite counts became evident. Pools of plasma from rats showing the reaction agglutinated normal rat blood cells (NRBC) and sheep red blood cells (SRBC) at similar titers at 4°C and the agglutinated cells dissociated at 20°C. The active plasma contained elevated titers of cold-active hemagglutinin (CAH) detected with trypsinized erythrocytes, immunoconglutinin (IK) and antibody against fibrinogen products (Anti-F). The quantity of precipitable soluble immune complex (SIC) was elevated. Absorption of the plasma with NRBC and SRBC at 4°C reduced the autohemagglutination activity and reduced titers of CAH, IK, Anti-F and the quantity of SIC. Hemagglutination and each of the serologic activities, except CAH, were detected in eluates of the blood cells used for absorption. Specificity of CAH was shown to be for erythrocyte stroma, and since it was not found in the eluates, it appeared that this autoantibody had no role in the observed autohemagglutination activity. Heat inactivated plasma did not agglutinate SRBC at 37°C suggesting that heterophile antibody was not involved. Since IK, Anti-F and SIC were recovered from the cells used for absorption and the eluate from the cells agglutinated NRBC and SRBC at 4°C, it was indicated that those factors had interacted at 4°C to agglutinate the cells. It is suggested that SIC, including complexes of fibrinogen products and Anti-F, had fixed complement, became bound to the blood cells and that cells were conglutinated by IK. It is further suggested that this phenomenon of panautohemagglutination, which may be

associated with a variety of infectious diseases, is actually a cold-active immuno-conglutination.

INTRODUCTION

We reported that rats infected with *Trypanosoma brucei rhodesiense* developed a syndrome of anemia with thrombocytopenia, splenomegaly and glomerulonephritis accompanied by coagulation abnormalities. Onset of the signs, with a hypoclottable state, was associated with appearance in the blood of antibody against the third component of fixed complement or immunoconglutinin (IK), cold-active hemagglutinin detected with trypsinized blood cells, Anti-F, which is antibody against fibrinogen/fibrin products and by a reduction in titers of lytic complement. Terminal signs, which resembled those of pulmonary thrombosis or disseminated intravascular coagulation (DIC), were accompanied by consumption of Anti-F and IK and absence of lytic complement. Blood drawn to anticoagulant at or near the time of the terminal crisis appeared to clot as it cooled. It was therefore suspected that this apparent clotting might be relatable to the phenomenon of cold autohemagglutination described by Yorke (1911) in clinical trypanosomiasis (Rickman and Cox, 1979, 1980).

From the studies of Rickman and Cox (1979), it was perceived that complement fixing immune complexes became bound to blood cells and trypanosomes. Since the cells were coated with the antigen for IK, i.e. complement fixing immune complex, they could be conglutinated by IK. We studied blood drawn from rats on various days during the course of *T. b. rhodesiense* infection. Tests for autohemagglutination became positive at the time that anemia became evident. Plasma samples that

agglutinated homologous normal blood cells contained high titers of IK, coldactive hemagglutinin (CAH), Anti-F and immune complexes. Eluates from agglutinated cells, agglutinated normal rat erythrocytes at 2°C. Serologic study indicated the eluates contained Anti-F, IK and soluble immune complexes. Details of the studies and their results are presented.

MATERIALS AND METHODS

Experimental animals and infection.

Sprague-Dawley rats and the *T. b. rhodesiense* infection were from sources described (Rickman and Cox, 1979). Cardiac bleeding and terminal euthanasia was achieved with ether anesthesia. Standard infections consisted of 1×10^6 trypanosomes prepared as described and administered intraperitoneally (Rickman and Cox, 1979). Erythrocyte and trypanosome counts were performed as reported (Rickman and Cox, 1979).

Serologic procedures.

Cold-active hemagglutinin (CAH) was detected with trypsin-treated rat erythrocytes as described (Thoongsuwan and Cox, 1973). The specificity of CAH was tested on CAH absorbed from plasma of rats infected with *Babesia rodhaini* using one volume of trypsinized rat cells for 5 volumes of plasma and incubation overnight at 4°C. These cells were washed twice with 10 volumes of ice cold 0.01 M phosphate buffered saline (PBS), pH 8.4 and the cells, suspended in 10 volumes of PBS, were incubated for 1 hr at 37°C. The supernatant was recovered after centrifugation at 2,000 g for 10 min. This absorption and elution was repeated until CAH was no longer detected in the plasma (Soni and Cox, 1975). Globulins in eluted fluids were then precipitated at 50% saturation with ammonium sulfate and the precipitate was solubilized by dialysis against borate

buffered saline at 0.01 M and pH 7.4. This precipitation was repeated until the recovered globulin was free of hemoglobin discoloration. The globulin was dialysed against the buffer until sulfate ion was no longer detected with barium chloride solution. It was then conjugated with fluorescein isothiocyanate as described (Thoongsuwan et al., 1978a). The conjugate was reacted with blood films of intact normal rat blood cells, trypsin-treated normal rat blood cells and residue of normal rat cells disrupted by freezing and thawing. The preparations were examined for immunofluorescent activity as described (Thoongsuwan et al., 1978a).

Titers of IK were detected as described (Rickman and Cox, 1980). Polyethylene glycol (PEG) precipitable immune complexes were recovered after centrifugation. Concentration was expressed as the optical density at 280 nm of the solubilized pellet (Bout et al, 1977, Rickman and Cox, 1980). Antibody against fibrinogen/fibrin products (Anti-F) was detected as described (Thoongsuwan et al., 1979).

Autohemagglutination activity (AA) in blood of infected rats was detected by modifications of methods described by Yorke (1911). An 8 ml volume of blood was freshly drawn by cardiac puncture to 1 ml of 3.8% trisodium citrate solution. A 0.5 ml volume of the blood was added to an equal volume of 1% trisodium citrate in 0.85% NaCl solution. The suspension was placed in an ice bath for 1 to 4 hr and examined for agglutinated cells. Tubes showing agglutination were held at room temperature for 20 min and examined for evidence of dissociation.

Platlet and trypanosome poor plasma, harvested while warm from blood of rats exhibiting autohemagglutination activity, was pooled. Four aliquots of this pool, and a pool of normal rat plasma, were absorbed with erythrocytes of normal rats. Normal rat erythrocytes were prepared

from citrated whole blood by repeated washings with phosphate buffered saline, 0.01 M, pH 7.6 (PBS) at 100 g for 12 min at 22°C until free of platlets. The erythrocytes were then sedimented by centrifugation at 3,000 g for 10 min and washed three times with PBS. Aliquots of 2.0 ml of plasma were each mixed with a 0.1 ml volume of packed cells and were refrigerated overnight at 4°C. Plasma supernatant was recovered after centrifugation at 3,000 g at 2°C for 15 min. The sedimented cells were resuspended in 10 ml of ice cold PBS and sedimented by centrifugation as described. One drop of the PBS supernatant was tested for protein with 10% trichloroacetic acid, and if precipitate was detected, the cells were washed again, with cold buffer. The sedimented cells were suspended in 0.5 ml of PBS, incubated at 37°C for 30 min and the supernatant recovered after centrifugation at 1,000 g for 15 min at 22°C.

Two-fold serial dilutions of unabsorbed plasma, absorbed plasma and the supernatant, containing eluate from cells used for absorption, were tested for titers of cold hemagglutinating activity against an equal volume 1% normal rat erythrocyte suspension. The test was carried out in 13 mm x 100 mm glass test tubes with a final volume of 0.2 ml. Titers of cold hemagglutinating activity (AA) are expressed as the reciprocal of the greatest dilution exhibiting gross agglutination after minimal resuspension. Questionable reactions were examined microscopically on 4°C glass microscope slides without a coverslip. Care was taken to assure that all cell preparations remained at 2°-4°C while recordings were made. In addition, plasmas and eluates were examined for CAH, IK, Anti-F and PEG precipitable immune complexes.

This experiment was repeated with new pools of infected and normal rat plasmas. The contents of the pools were determined as described. Cold hemagglutination activity was tested with both normal rat blood cells

and normal sheep blood cells as described. Absorption procedures utilized the sheep cells in the same manner as described for rat cells. In addition to the tests described, two-fold serial dilutions unabsorbed and absorbed plasmas and eluate preparations were heat inactivated at 56°C and tested for agglutination activity against a 1% suspension of SRBC after incubation at 37°C for 30 min. These tests were again examined after standing overnight at 4°C.

EXPERIMENTAL RESULTS

The relationships of autohemagglutination activity and erythrocyte and trypanosome counts are shown, Table I. Blood cells from infected rats agglutinated in the cold at the time reductions in the counts of blood cells and trypanosomes became evident, i.e., day 7. The agglutinated cells dissociated when warmed to room temperature.

Results of the study of plasma of infected rats are presented, Table II. Before absorption the pooled plasma agglutinated normal rat cells at a titer of 32. The titer was reduced by absorption and eluates from the cells used had agglutinating activity. Titers of CAH, IK and Anti-F and the quantity of precipitable immune complex in the plasma were reduced by the absorption. These factors, except for CAH, were detected in the eluate. Only a trace of protein was detected with trichloroacetic acid in the cold buffer used to wash the agglutinated cells whereas the eluates gave strong reactions for protein.

Results of the absorption of the pooled plasma of infected rats with sheep blood cells are presented, Table III. These are essentially similar to those found in the previous experiment. Agglutinating activity in the plasma was as effective for sheep cells as it was for normal rat blood cells. Heat inactivated plasma or eluate did not agglutinate sheep cells

after incubation at 37°C. After overnight storage at 4°C the cold hemagglutination titers had been reduced as compared to unheated test samples.

CAH, absorbed from plasma of rats with acute babesiosis, and conjugated with fluorescein did not react with intact rat erythrocytes. Reaction with trypsinized cells was variable, and the reaction with stroma of freeze-thaw disrupted erythrocytes was intense.

DISCUSSION

The cold-autohemagglutination phenomenon observed by Yorke (1911) in human and animal cases of trypanosomiasis has been observed in rats with acute *T. b. rhodesiense* infection. Onset of the activity occurred at a time when anemia was evident and was most pronounced when parasite counts were reduced following the first parasitemia peak. Plasma drawn from the rats during this time agglutinated homologous normal blood cells after incubation at 4°C, and the agglutinates dissociated after warming.

Plasma that agglutinated normal cells contained titers of CAH, IK, Anti-F and PEG precipitatable immune complexes. These were reduced after the plasma was absorbed at 4°C with normal cells. These factors, except CAH, were detected in fluids eluted at 37°C from the absorption cells. This eluate fluid agglutinated homologous blood cells at a titer of 4. Inasmuch as the absorbing cells were washed with ice cold buffer until wash supernatant gave little or no protein reaction with 10% trichloroacetic acid solution, it appeared that the hemagglutinating factor(s) had been bound to the cells at 2°C and dissociated from the cells when warmed.

Similar results were obtained on another infected rat plasma pool using normal sheep cells for absorption and elution. The agglutinating activity of the plasma was as effective for sheep blood cells as it was for normal rat cells. Plasma that had been heat inactivated did not agglutinate sheep cells after incubation at 37°C. The reduced titers of heat inactivated plasma against sheep cells after overnight storage at 4°C indicated that the cold hemagglutination reaction observed may be enhanced by the presence of complement.

From the present experiments it appeared that CAH had specificity for erythrocyte stroma and had little or no reactivity with normal erythrocytes. Further, Thoongsuwan and Cox raised the antibody in rats with injections of autologous blood cells disrupted by freezing and thawing (unpublished results). This antibody was not detected in the eluate fluid of cells used to absorb plasma which agglutinated NRBC and SRBC at 4°C. It therefore seems improbable that CAH was involved in the cold hemagglutination here discussed.

We reported that blood cells of rats infected with *T. b. rhodesiense* became coated with fibrinogen, or its products and that the cells gave positive reactions for fixed C3. It was suggested that the coating represented, in part, immune complexes of fibrinogen/fibrin products and Anti-F that had fixed complement and became bound to the cells by means of receptors for fixed C3 (Rickman and Cox, 1979). Finding that Anti-F was eluted from our absorption cells tends to support the suggestion. Both the antigen and antibody moiety of the hypothesized complex have now been demonstrated as parts of the materials coating the cells.

We also suggested that anemia of *T. b. rhodesiense* infection was due in part to the conglutinating action of IK on cells coated with antigen, i.e., complement fixing immune complexes (Rickman and Cox, 1979). With the recovery of PEG precipitable immune complexes and IK in the eluates, this suggestion seems to be supported.

From these experiments it seems possible that the cold autohemagglutination reaction that Yorke (1911) associated with trypanosmiasis, and which we have confirmed, may not be an action of cold-active antibody against autologous blood cells. Participation of CAH seems to have been contraindicated by the experiments. Alternatively, we suggest that the agglutination was a result of the conglutination by IK of blood cells that had become coated with the antigen for IK, i.e., complement-fixing immune complexes. This concept is supported by the observation that plasma that was heated to destroy complement did not agglutinate sheep cells at 37°C. Reduction in agglutinating activity at 4°C of heat inactivated test samples indicates that the presence of complement enhances the agglutination reaction.

Though complexes of fibrinogen/fibrin products and Anti-F are associated, we would not imply that this antigen and antibody were the only causal factors. Soluble trypanosome antigen, such as variant-specific surface antigen(s), should have furnished a significant antigenemia (Diffley et al., 1980). It, with its antibody, could have fixed complement and became bound to both blood cells and trypanosomes. Other antigens generated or activated during infection, with their antibodies, could play a similar role. We suggest that immune complexes merely form the intermediate for the fixation of complement. The complement containing complexes could then bind to the rat erythrocyte membrane through the C3b, C4b or C5b receptors, similar to those

described on sheep erythrocytes by Law and associates (1980). The observed agglutination, which is enhanced by cold temperatures and complement, could be mediated by IK since its serologic specificity is for fixed C3b (Thoongsuwan et al., 1978b).

Stats and Wasserman (1943) point out that cold hemagglutination activity is associated with a number of infectious diseases as diverse as infectious mononucleosis and trypanosomiasis, and that the phenomenon was actually a panhemagglutination rather than autoagglutination. Immune globulins of the IgM class were implicated. Our present observations are in agreement. The agglutination activity was as effective for SRBC as it was for NRBC and the antibodies implicated, IK and Anti-F, were identified as IgM globulin (Boreham and Facer, 1974; Thoongsuwan et al., 1978b; Thoongsuwan et al., 1979).

Though Stats and Wasserman (1943) associate cold hemagglutination with diseases causing anemia, they do not discuss a causal association. A temporal association is clear in the present studies, and immune factors, IK and immune complexes, here related to cold hemagglutination, were implicated in anemia from various infections (Rickman and Cox, 1979; Thoongsuwan et al., 1978a, 1978b, 1979). This relationship is supported by Woodruff et al (1973) who related anemia and IK in a number of infectious diseases. It is possible that mechanisms causing anemia with splenomegaly and reduced parasitemia are the same as those responsible for cold hemagglutination.

From the study, we would suggest that the cold hemagglutination action here studied is a phenomenon of cold-active immuno-conglutination and that IK is the effector antibody.

LITERATURE CITED

BOREHAM, P.F.L. AND C. A. FACER. 1974. Autoimmunity in trypanosome infections. II. Anti-fibrin/fibrinogen (Anti-F) autoantibody in *Trypanosoma (Trypanozoon) brucei* infections of the rabbit. Int. J. Parasitol. 4:601-607.

BOUT, D., F. SANTRO, Y. CARLIER, AND A. CAPRON. 1977. Circulating immune complexes in schistosomiasis. Immunology 33:17-22.

DIFFLEY, P., J. E. STRICKLER, C. L. PATTON, AND B. H. WAKSMAN. 1980. Detection and quantification of variant specific antigen in the plasma of rats and mice infected with *Trypanosoma brucei*. J. Parasitol. 66:185-191.

LAW, S. K., N. A. LICHTENBERG, F. H. HOLCOMBE, AND R. P. LEVINE. 1980. Interaction between the labile binding sites of the fourth (C4) and fifth (C5) human complement protein and erythrocyte cell membranes. J. Immunol. 125:634-639.

RICKMAN, W. J., AND H. W. COX. 1979. Association of autoantibodies with anemia, splenomegaly, and glomerulonephritis in experimental African trypanosomiasis. J. Parasitol. 65:65-73.

_____, AND _____. 1980. Immunologic reactions associated with anemia, thrombocytopenia, and coagulopathy in experimental African trypanosomiasis. J. Parasitol. 66:28-33.

SONI, J. L., AND H. W. COX. 1975. Pathogenesis of acute avian malaria. II. Anemia mediated by a cold-active autohemagglutinin from the blood of chickens with acute *Plasmodium gallinacium* infection. Am. J. Trop. Med. Hyg. 24:206-213.

STATS, D., AND L. R. WASSERMAN. 1943. Cold Hemagglutination - an interpretive review. Medicine 22:363-424.

THOONGSUWAN, S., AND H. W. COX. 1973. Antigenic variants of the haemosporidian parasite, *Babesia rodhaini*, selected by in vitro treatment with immune globulin. Ann. Trop. Med. Parasitol. 67:373-385.

_____, _____, AND R. A. PATRICK. 1978a. Immunoconglutinin associated with nonspecific acquired resistance in malaria, babesiosis and other anemia-inducing infections. J. Parasitol. 64:1050-1056.

_____, _____, AND _____. 1978b. Serologic specificity of immunoconglutinin associated with infectious anemia of rats and its role in non--specific acquired resistance. J. Parasitol. 64:1060-1066.

_____, _____, AND W. J. RICKMAN. 1979. Antibody to fibrinogen/fibrin products (Anti-F) in malaria, babesiosis, and trypanosomiasis of rodents. J. Parasitol. 65:426-429.

WOODRUFF, A. W., J. L. ZIEGLER, A. HATHAWAY, AND T. GWATA. 1973. Anaemia in African trypanosomiasis and "Big Spleen Disease" in Uganda. Trans. R. Soc. Trop. Med. Hyg. 67:329-337.

YORKE, W. 1911. Autoagglutination of red blood cell in trypanosomiasis. Ann. Trop. Med. Parasitol. 4:529-552.

TABLE I. Mean trypanosome counts per mm^3 (TRYP x 10^5), erythrocyte counts per mm^3 (RBC x 10^6) and ratios of number of rats positive for agglutination of autologous erythrocytes to total number of rats tested (AA) after infection with *Trypanosoma brucei rhodesiense*.

Days Post infection	TRYP x 10^5	RBC x 10^6	AA
0	0	8.32	0/4
2	0	8.17	0/4
3	0.45	8.60	0/4
4	1.76	7.54	2/4
5	7.02	6.49	4/4
6	5.17	5.18	4/4
7	2.25	4.65	4/4
8	2.81	4.72	4/4
9	8.97	4.41	4/4
Post Infection Control	0	9.14	0/4

TABLE II. Mean titers for agglutination of homologous erythrocytes (AA), immunoconglutinin (IK), cold-active hemagglutinin (CAH), antibody to fibrinogen products (Anti-F) and polyethylene glycol precipitated soluble immune complexes in optical density units (PEG) in the plasma pool of normal rats and in the plasma pool of rats with acute *Trypanosoma b. rhodesiense* infections before and after absorption with normal rat erythrocytes at 2°C and in the eluates from the cells after incubation at 37°C.

	Normal Rat Plasma					Infected Rat Plasma				
Material Tested	AA	IK	CAH	Anti-F	PEG	AA	IK	CAH	Anti-F	PEG
Whole Plasma	0	20	0	0	.71	32	1280	64	256	1.11
Absorbed Plasma	0	10	0	0	.72	2	320	16	128	.92
37°C eluate from absorbing cells	0	0	0	0	.02	4	40	0	8	.12

Table III. Mean titers of autoagglutination of rat erythrocytes (AA), agglutination of sheep erythrocytes (4°C Anti-SRBC) and 37°C (37°C Anti-SRBC), immunoconglutinin (IK), cold-active hemagglutinin (CAH), antibody to fibrinogen products (Anti-F), and polyethylene glycol precipitation of soluble immune complexes in optical density units (PEG) in the plasma pools of normal rats and rats with acute *Trypanosoma b. rhodesiense* infections before and after absorption with normal sheep erythrocytes at 2°C and in the eluates from the cells after incubation at 37°C.

	Normal Rat Plasma							
		4°C*	37°C† Anti-SRBC					
Material Tested	AA	Anti-SRBC	Before overnight cold storage	After overnight cold storage	IK	CAH	Anti-F	PEG
Whole Plasma	0	0	0	0	10	0	0	.54
Absorbed Plasma	0	0	0	0	10	0	0	.47
37°C eluate from absorbing cells	0	0	0	0	0	0	0	.08

	Infected Rat Plasma							
		4°C*	37°C† Anti-SRBC					
Material Tested	AA	Anti-SRBC	Before overnight cold storage	After overnight cold storage	IK	CAH	Anti-F	PEG
Whole Plasma	16	12	0	6	320	16	256	.93
Absorbed Plasma	4.5	5	0	2	160	16	64	.69
37°C eluate from absorbing cells	2.5	4	0	0	15	0	24	.16

ARTICLE 3

TRYPANOSOME ANTIGEN-ANTIBODY COMPLEXES AND IMMUNOCONGLUTININ ASSOCIATED WITH ANEMIA AND REDUCED PARASITEMIA IN AFRICAN TRYPANOSOMIASIS

William J. Rickman

Department of Microbiology and Public Health

Michigan State University

East Lansing, Michigan 48824

(To be submitted to the Journal of Parasitology)

TRYPANOSOME ANTIGEN-ANTIBODY COMPLEXES AND IMMUNOCONGLUTININ ASSOCIATED WITH ANEMIA AND REDUCED PARASITEMIA IN AFRICAN TRYPANOSOMIASIS*

William J. Rickman

Department of Microbiology and Public Health

Michigan State University

East Lansing, Michigan 48824

*This research was supported in part with funds from the College of Veterinary Medicine, Michigan State University, in part from a Grant-in-Aid from Michigan Heart Association, and in part from Grant No. AI-17018-01 from National Institutes of Health. This publication is Journal Article No. from the Michigan Agricultural Experiment Station.

This communication is from a dissertation entitled, "Immunoconglutination in Experimental African Trypanosomiasis," submitted by the author in partial fulfillment of the requirements for the Ph.D. degree at Michigan State University.

ABSTRACT

Rats infected with *Trypanosoma brucei rhodesiense* developed parasitemia levels which peaked on days 5 and 9 of infection. Soluble antigens of *T. b. rhodesiense* were demonstrated in the plasma of infected animals. Antibody to parasite antigens were examined by indirect hemagglutination, microtiter enzyme-linked immunosorbent assay (ELISA) and single-tube kinetic-based ELISA tests. With the generation of anti-trypanosomal antibodies, soluble trypanosome antigens were no longer detectable in the circulation. Demonstration parasite antigens in precipitable immune complex fractions suggested that removal of circulating parasite antigen was a result of complexing with anti-trypanosomal antibodies. Complexing of trypanosome antigen and antibody was accompanied by high titers of immunoconglutinin and low serum complement levels. The occurrence of anemia at the time of complexing suggested a causal relationship. Passive transfer studies employing anti-parasite and immunoconglutinin globulins, alone or in combination, were conducted. An earlier anemia and reduced parasitemia was observed in animals receiving both globulin preparations when compared to control animals. The role of trypanosome antigen-antibody complexes in the development of anemia, parasitemia and nonspecific resistance in acute African trypanosomiasis are discussed.

INTRODUCTION

In a previous communication, we demonstrated that complement-fixing immune complexes were present on erythrocytes of rodents infected with *Trypanosoma brucei rhodesiense* (Rickman and Cox, 1979). It was

suggested that the interaction of immunoconglutinin (IK) with the complement moiety of the erythrocyte-bound immune complexes resulted in the formation of intravascular microthrombi contributing to the anemia and coagulopathies observed in the terminal stages of infection (Rickman and Cox, 1979; 1980). More recently, we demonstrated that the *in vitro* consumption of immune complexes and IK at cold temperatures by normal erythrocytes resulted in the formation of cellular aggregrates (Rickman et al., 1981). Since it had been shown previously that trypanosomes also became coated with complement-fixing complexes, it was suggested that IK could react with the parasites in a fashion similar to that described for erythrocytes contributing to the observed reductions in parasitemia levels (Rickman and Cox, 1979; 1980).

Our attempts to assess anti-trypanosome antibody responses during these investigations had been unsuccessful and were not reported. With the development of enzyme-linked immunosorbent assays (ELISA) and the recent availability of commercially produced conjugates with specificity against rat immunoglobulins, this investigation was designed to study the associations of parasite antigens, its antibody, complement and IK. The results of this work are presented in this report.

MATERIALS AND METHODS

Experimental animals and parasites

The source of Sprague-Dawley male rats (200-224 g), their care and the source and maintenance of *Trypanosoma brucei rhodesiense* have been described elsewhere (Rickman and Cox, 1979). For these experiments, all infections were established by a single intraperitoneal injection

of 1 ml of diluted blood containing 10^6 organisms of a serologically defined population of trypanosomes designated as P31.

Preparation of antigens and antibodies

A soluble trypanosome antigen was prepared from the blood of rats infected with the P31 population of trypanosomes. Parasites were isolated from other blood elements by DEAE-cellulose chromatography according to the method of Lanham and Godfrey (1970). The trypanosome-enriched fractions were pooled and washed three times in phosphate buffered saline (pH 7.6, 0.01 M; PBS). Trypanosome antigens of P31 were solubilized by repeated sonication for 3 min at 60-kilocycles. The sonicate was centrifuged at 5000 g for 1 hr at 4 C before storage in small aliquots. The final protein concentration was determined by the method of Kalb and Bernlohr (1977) and found to be 8.25 mg/ml.

Antisera to the soluble trypanosome antigen P31 (Anti-P31) was prepared by immunizing 12 rats with the P31 antigen incorporated into Freund's complete adjuvant (Difco Laboratories, Detroit, MI). Each animal received a 1 ml intramuscular injection of emulsified antigen per thigh 14 days apart. On the 21st day after the initial injection, the rats were exsanguinated by cardiac puncture. Since large amounts of IgG globulin were needed, an IgG-enriched fraction was prepared from the pooled immune sera by repeated ammonium sulfate precipitation at a final concentration of 33%. The final precipitate was minimally solubilized in PBS and dialyzed against several changes of PBS until sulfate ion was no longer detectable with 1% barium sulfate. The final protein concentration of the Anti-P31 globulin was 12 mg/ml (Kalb and Bernlohr, 1977). Immunoconglutinin (IK) was raised by autostimulation

of normal rats by injections of normal rat serum (NRS) absorbed on kaolin particles as recommended by Coombs et al. (1961). An IgM-enriched fraction of the pooled IK sera was prepared by first removing the IgG globulins by ammonium sulfate precipitation at a final concentration of 33%. The IgG globulins were sedimented by centrifugation at 7500 rpm for 20 min at 4 C and the resultant supernatant was brought to 50% saturation with additional saturated ammonium sulfate. Precipitation at 50% saturation was repeated until the recovered globulin was free of hemoglobin discoloration. The globulin was minimally solubilized in PBS and dialyzed against PBS as described above. The final protein concentration of the IK globulin was 19.2 mg/ml. The specificities of the Anti-P31 and IK globulins were confirmed by serologic and immunodiffusion analysis.

Serologic examinations

Immunoconglutin (IK) was titered in test samples by the test tube method of Coombs et al. (1961) except that rats were used the generate anti-sheep erythrocyte serum (Thoongsuwan et al., 1978a). Complement levels were determined by the lytic complement titer (LCT) assay performed in microtiter plates as described by Rickman and Cox (1980).

Antibodies to trypanosome antigen P31 were evaluated by three separate procedures. The first method employed was an indirect hemagglutination test (IHAT) developed by Woo and Kobayashi (1975) and adapted for detecting rat antibodies to *T. b. rhodesiense* by employing the soluble trypanosome antigen P31 described above. Sensitization of rat erythrocytes was accomplished without the use of a coupling agent.

Microtiter-enzyme-linked-immunosorbent-assay (m-ELISA) was also used for the detection of anti-trypanosomal antibodies employing the methods of Deedler et al. (1980) with the following modifications: (1) sensitization of polyvinyl flat-bottom microtiter plates (Dynatech Laboratories, Alexandria, VA) was accomplished by incubating 200 µl of a 1:50 dilution of solubilized trypanosome antigen P31 (165 µg/ml final protein concentration) in 0.1 M carbonate buffer (pH 9.6) per well for 18 h at 4 C, (2) the rabbit anti-rat globulin conjugated horseradish peroxidase (Miles Laboratories, Elkhart, IN) was diluted 1:500 in phosphate buffered saline (0.01 M, pH 7.6) containing 0.02% Tween 20 (Sigma Chemical Co., St. Louis, MO; PBS/T) and 100 µl of diluted conjugate was allowed to react for 1 h at 37 C. (3) Three washings with PBS/T were performed between each reaction step during the procedure. Titers of anti-trypanosomal antibodies were assessed visually 1 h after the addition of substrate.

For precise quantitative determination of anti-trypanosomal antibody levels, the single-tube kinetic-based ELISA (k-ELISA) developed by Tsang et al. (1980) was employed with the above consideration of sensitization, conjugate dilution and washing procedures. The modifications of the k-ELISA are as follows: (1) disposable polystyrene cuvettes with a 1-cm light-path and 4.5 ml maximum capacity (Fisher Scientific Co., Detroit, MI) were filled with 4.0 ml volumes of the various reaction mixtures, (2) substrate was prepared by methods of Deedler et al. (1980), (3) absorbance at 460 nm (A_{460}) was recorded with a Coleman spectrophotometer (Model 124) coupled to a 10-mV strip-chart recorder, and (4) peroxidase activity was computed from the slope of the readings obtained during the initial 5 min and expressed as ΔA_{460}/min.

Experimental plasmas were tested for circulating soluble parasite antigens by double-immunodiffusion (DID) analysis. Soluble immune complexes in experimental test samples were precipitated with polyethylene glycol 6000 (PEG) by the method of Bout et al. (1977). The precipitates were resuspended in PBS for DID analysis for the presence of parasite antigens employing the Anti-P31 described above. The gels were prepared by methods described by Thoongsuwan and Cox (1973).

Experimental design

In the first experiment, groups of four rats were randomly selected for study at intervals during the course of infection. Blood samples were obtained from the scissor-snipped tails of rats for erythrocyte (RBC) and trypanosome (TRYP) counts by hemacytometer methods described by Rickman and Cox (1979). While under either anesthesia, rats were bled by cardiac puncture for anticoagulated whole blood (1 part 3.8% trisodium citrate to 8 parts whole blood). Plasma was harvested from the blood samples by centrifugation at 5000 rpm for 15 min. Following the collection of anticoagulated whole blood, the rats were bled for an additional 4 to 6 ml of blood which was allowed to clot overnight at 4 C. Serum was harvested by centrifugation and both serum and plasma samples were stored at $^{-}$20 C until tested for serologic activity.

In a second experiment 60 rats were divided into 5 groups of 12 rats each. One group served as the control for erythrocyte counts, while the other four groups were inculated with 10^6 trypanosomes as described above. Two days after infection had been established the following intraperitoneal inoculations were administered. Group 1 (without infection) received 1 ml of IK globulin and 1 ml of Anti-P31 globulin.

Group 2 was given 1 ml of normal rat serum (NRS). Group 3 received 1 ml of IK globulin. Group 4 was injected with 1 ml of Anti-P31 globulin and Group 5 received 1 ml of IK globulin plus 1 ml of Anti-P31 globulin. Erythrocyte and trypanosome counts were performed daily by hemacytometer methods on 9 rats from each group (Rickman and Cox, 1979).

RESULTS

Comparative studies of IHAT, m-ELISA and k-ELISA assays for the detection of anti-trypanosomal antibodies

The IHAT was found unsuitable for titering anti-parasite antibodies since the trypanosome antigen preparation agglutinated the rat erythrocytes at sensitization dilutions ranging from 1:10 up to 1:80. Saline and NRS controls were positive at these dilutions, however, the reactions with acute plasmas and Anti-P31 appeared relatively stronger. With sensitization dilutions greater than 1:80, all serologic activity was lost.

Preliminary studies demonstrated a close relationship between the results of the m-ELISA and k-ELISA tests for parasite directed antibodies (Table 1). However, the quantitative nature of the k-ELISA appeared better suited for precise analysis of antibody levels. It was found that the rate of the reaction of bound conjugate with substrate was linear in all cases during the initial 5 min reaction. As shown in Table 2, the non-specific binding of NRS was irrespective of NRS concentration. In contrast, with the adjuvant stimulated Anti-P31 globulin, the rate of the reaction was directly proportional to the amount of Anti-P31 added.

Relationships of anemia and parasitemia with circulating trypanosome antigen, anti-trypanosomal antibody and IK levels

Mean values for TRYP and RBC levels are presented in Table 3. Trypanosomes first appeared in the peripheral blood of infected animals on the second day of the infection. Peak parasitemias occurred at 5 and 9 days post-infection. Anemia closely followed the first peak parasitemia and both RBC and TRYP counts decreased together thereafter except for the fact that parasite levels suddenly increased on the last day of infection. Reductions in RBC and TRYP numbers were accompanied by the concurrent presence of circulating soluble trypanosome antigens and anti-trypanosomal antibodies (Table 3). In addition, high titers of IK were observed at the time anemia and reduced parasitemia were evident. Anti-P31 reacted in DID with the PEG precipitates on days 5 through 9 indicating that trypansome antigens were present.

Lytic complement levels

While no changes in LCT's were observed in plasma samples during the course of the infection, marked reductions and depletion of lytic complement were observed with the blood samples allowed to clot overnight in the cold (Table 3). Reductions in LCT values occurred in those samples obtained on days 4 through 9 of the infection.

Passive transfer studies

The results of the effects of the administration of Anti-P31 and IK globulins on the anemia and parasitemia of *T. b. rhodesiense* infection are presented in Tables 4 and 5. Injections of both globulin appeared not to influence the erythrocyte counts of uninfected rats. Infected animals receiving the IK globulin developed anemia and parasitemia at a

rate which did not differ statistically from infected rats given NRS. Infected rats given Anti-P31 developed anemia at the same rate as the infected groups receiving NRS yet presented with slightly lower parasite levels. However, this lower parastemia was not found to be statistically significant. Infected rats receiving both IK and Anti-P31 globulins developed anemia earlier than all other infected animals. The erythrocyte counts of Group 5 were significantly lower on day 3 of infection when compared to the NRS group. The lower RBC counts in Group 5 were accompanied by statistically significant lower TRYP counts on days 3, 4, 5 and 7. All infected animals died by the eighth day of the infection except for one animal in Group 5 which survived until day 9.

DISCUSSION

This present study adds new information concerning the immunopathologic mechanisms we had previously implicated in the pathogenesis of experimental African Trypanosomiasis (Rickman and Cox, 1979; 1980; Rickman et al., 1981). The presence of circulating soluble trypanosome antigens early in infection (Day 2) was demonstrated by DID analysis. Following the appearance of antitrypanosomal antibodies on the fifth day of infection, soluble trypanosome antigens were no longer detected in the circulation. With the finding of trypanosome antigens in the PEG precipitable protein fractions, it is suggested that removal of antigen from the circulation had been a result of complexing with antibody. Since immune complexes are selectively precipitated at the concentrations of PEG employed in this study, this suggestion appears

to be supported. Additional support of complexing of parasite antigens and antibodies can be found in the elevated levels of IK demonstrated in the plasma of animals during the course of the infection.

In an earlier study, we presented evidence that suggested complexes of antibody to fibrinogen related products (Anti-F) and its antigen were contributory factors in the development of anemia and nephritis associated with acute Rhodesian trypanosomiasis (Rickman and Cox, 1979; 1980). The results presented here supports our previous suggestion that trypanosome antigen-antibody complexes may be similarly involved. Complexing of parasite antigens and antibodies occurred at the time anemia became evident and complexes, shown to contain parasite antigen, were detectable throughout the duration of anemia. In a recent report, we demonstrated that the *in vitro* adsorption of immune complexes onto the surface of rat erythrocytes was associated with the consumption of IK suggesting that a similar mechanism may be operative in the pathogenesis of anemia observed during infection (Rickman et al., 1981). It was suggested that complement containing immune complexes may have become bound to the erythocytes by means of the receptors for complement that Law et al. (1980) had demonstrated previously. Studies on the IHAT, suggests that an additional mechanism of binding may be operative. It appears that the binding of soluble trypanosome antigens to the red cell surface can be accomplished without the use of coupling agents. Kobayashi et al. (1976) reported similar findings demonstrating direct binding of *T. congolense* and *T. brucei* antigen extracts to bovine and rabbit erythrocytes, respectively. However, the presence of trypanosome antigen-antibody complexes in the antigen preparations of

Kobayashi et al. (1976) and P31 used here, has not been determined. Thus, whether the complexes containing trypanosome antigens become bound as a unit or separately remains to be determined.

In this and previous reports on *T. b. rhodesiense* infections in the rat, the onset of anemia has been consistently accompanied by a sharp reduction in circulating parasite numbers (Rickman and Cox, 1979; 1980; Rickman et al., 1981). Here we show that this reduction occurred at the time anti-trypanosomal antibodies were generated. It would appear that this antibody response may have brought about the reductions in parasitemia. However, infected animals administered the Anti-P31 globulin in the passive transfer experiment still developed high levels of parasitemia. Diffley, et al. (1980), who quantitated antigenemia in *T. b. rhodesiense* infections of rats, reported high levels of circulating antigens corresponding to each peak of parasitemia. In this respect, the high level of antigenemia occurring at peak parasitemia may block the effectiveness of anti-parasite response. It is suggested that the reductions in observed parasitemia may be the result of nonspecific binding of complexes, either of parasite or non-parasite origin, to the trypanosome and with the fixation of complement, IK could react with the parasites contributing to the observed reductions in parasitemia.

Support for this alternative hypothesis concerning the role of immune complexes in modulating parasitemia can be found in the results of the passive transfer study. Results of the erythrocyte and parasite counts of infected animals given IK alone indicated that this antibody was no more effective than NRS. While injections of Anti-P31 alone

resulted in minor reductions in parasitemia, when it was combined with injections of IK, early anemia and significantly reduced parasitemia were observed. The absence of anemia in uninfected rats receiving both Anti-P31 and IK indicated that these globulins lacked any anemia-inducing factors. This might be expected, since without infection (antigen), anemia-inducing complexes could not be formed with Anti-P31. Similarly, IK alone could not have an effect until complexes of parasite antigen and antibody had formed and fixed complement. Animals receiving both globulin fractions, (Anti-P31 and IK) had circulating Anti-P31 which could react with parasite antigens when generated early in infection. With the subsequent fixation of complement by parasite antigen-antibody complexes, the complexes could then bind to the erythrocytes and parasites. In the presence of sufficient IK supplied passively, these complex-coated cells could be removed from the circulation resulting in an earlier and more effective clearance as reflected by both lower erythrocyte and parasite counts.

The non-specific acquired resistance associated with IK has been demonstrated in malaria, babesiosis and viral infectious anemia by Thoongsuwan et al. (1978a, 1979b). In those studies, increased survival rates were demonstrated in mice receiving globulin fractions similar to those described here. In addition, non-specific acquired resistance could be stimulated by sublethal infections of one agent and shown to be protective against challenge with heterologous agents (Thoongsuwan et al., 1978a).

The role of immune complexes of parasite origin and IK implicated here in the development anemia and parasitemia may have additional significance. Our previous studies had shown that coagulation abnormalities,

hypocomplementemia, thrombocytopenia, glomerulonephritis and auto-agglutination phenomena were associated with the generation of immune complexes during *T. b. rhodesiense* infection. The participation of parasite derived complexes in the pathogenesis of these phenomena would seem likely. However, regardless of their origin, the complex itself appears to be the pathogenic factor.

LITERATURE CITED

1. BOUT, D., F. SANTRO, Y. CARLIER, and A. CAPRON. 1977. Circulating immune complexes in schistosomasis. Immunology 33:17-22.

2. COOMBS, R. R. A., A. COOMBS, and D. G. INGRAM. 1961. The serology of conglutination and its relation to disease. Blackwell Scientific Publications, Oxford.

3. DEELDER, A. M., D. KORNELIS, M. MAKBIN, H. N. NOORDPOOL, R. M. CODFRIED, J. P. ROTMANS, and B.F.J. OOSTBURG. 1980. Applicability of different antigen preparations in the enzyme-linked immunosorbent assay for schistosomiasis mansoni. Amer. J. Trop. Med. Hyg. 29:401-410.

4. DIFFLEY, P., J. E. STRICKLER, C. L. PATTON, and B. H. WAKSMAN. 1980. Detection and quantification of variant specific antigen in the plasma of rats and mice infected with *Trypanosoma brucei*. J. Parasitol. 66:185-191.

5. KALB, V. F., and R. W. BERNLOHR. 1977. A new spectrophotometric assay for protein cell extracts. Anal. Biochem. 82:362-371.

6. KOBAYASHI, A., I. R. TIZARD, and P.T.K. WOO. 1976. Studies on the anemia in experimental African trypanosomiasis. II. The pathogenesis of the anemia in calves infected with *Trypanosoma congolense*. Amer. J. Trop. Med. Hyg. 25:401-406.

7. LANHAM, S. M., and D. G. GODFREY. 1970. Isolation of salivarian trypanosomes from man and other mammals using DEAE-cellulose. Exp. Parasitol. 28:521-534.

8. LAW, S. K., N. A. LICHTENBERG, F. H. HOLCOMBE, and R. P. LEVINE. 1980. Interaction between the labile binding sites of the fourth (C4) and fifth (C5) human complement protein and erythrocyte cell membranes. J. Immunol. 125:634-639.

9. RICKMAN, W. J., and H. W. COX. 1979. Association of autoantibodies with anemia, splenomegaly, and glomerulonephritis in experimental African trypanosomiasis. J. Parasitol. 65:65-78.

10. RICKMAN, W. J., and H. W. COX, 1980. Immunologic reactions associated with anemia, thrombocytopenia, and coagulopathy in experimental African trypanosomiasis. J. Parasitol. 66:28-33.

11. RICKMAN, W. J., H. W. COX, and S. THOONGSUWAN. 1981. Interactions of immunoconglutinin and immune complexes in cold autohemagglutination associated with African trypanosomiasis. J. Parasitol. 67:(IN PRESS).

12. THOONGSUWAN, S., and H. W. COX. 1973. Antigenic variants of the haemosporidian parasite, *Babesia rodhaini*, selected by *in vitro* treatment with immune globulin. Ann. Trop. Med. Parasitol. 67:373-385.

13. THOONGSUWAN, S. , H. W. COX, and R. A. PATRICK. 1978a. Immunoconglutinin associated with nonspecific acquired resistance in malaria, babesiosis, and other anemia-inducing infections. J. Parasitol. 64:1050-1056.

14. THOONGSUWAN, S. , H. W. COX, and R. A. PATRICK. 1978b. Serologic specificity of immunoconglutinin associated with infectious anemia of rats and its role in nonspecific acquired resistance. J. Parasitol. 64:1060-1066.

15. TSANG, V.C.W., B. C. WILSON, and S. E. MADDISON. 1980. Kinetic studies of a quantitative single-tube enzyme-linked immunosorbent assay. Clin. Chem. 26:1255-1260.

16. WOO, P.T.K., and A. KOBAYASHI. 1975. Studies on the anemia in experimental African trypanosomiasis. I. A preliminary communication on the mechanism of the anemia. Ann. Soc. Belge. Med. Trop. 55:37-45.

Table 1. Comparative studies of m-ELISA and k-ELISA for anti-trypanosomal antibody levels. The mean k-ELISA values of samples for each titer level are presented.

Anti-trypanosomal m-ELISA titer	Anti-trypanosomal k-ELISA (ΔA_{460}/min) level
0	0.0092
8	0.0116
16	0.0142
32	0.0258
64	0.0280
128	0.0370
512	0.0402
1024	0.0474

Table 2. Comparison of mean k-ELISA values for different dilutions of normal rat serum (NRS) and antisera to soluble trypanosome antigen P31 (Anti-P31)

Type of serum added	ml of serum added[+]	k-ELSIA ΔA_{460}/min
Buffer control	0	0.0000
NRS	1	0.0102
NRS	2	0.0098
NRS	3	0.0083
NRS	4	0.0113
Anti-P31	1	0.0242
Anti-P31	2	0.0561
Anti-P31	3	ND[++]
Anti-P31	4	0.1384

Table 3. Mean trypanosome counts per mm^3 (TRYP x $10^5/mm^3$), mean erythrocyte counts per mm^3 (RBC x $10^6/mm^3$), results of immunodiffusion tests for soluble trypanosome antigens (TRYP AG), mean anti-trypanosomal antibody titer by m-ELISA (Anti-TRYP m-ELISA), mean anti-trypanosomal antibody level by k-ELISA (Anti-TRYP k-ELISA), mean plasma lytic complement titer (plasma LCT), mean serum lytic complement titer (serum LCT) and mean immunoconglutinin titer (IK) in samples collected from rats as groups of four during acute *T. b. rhodesiense* infection.

Days post-infection	TRYP x $10^5/mm^3$	RBC x $10^6/mm^3$	TRYP AG	Anti-TRYP m-ELISA	Anti-TRYP k-ELISA	Plasma LCT	Serum LCT	IK
0	0.00	8.38	0/4	0	0.0114	256	256	2
2	(+)	8.17	2/4	0	0.0105	256	256	4
3	0.45	8.60	0/4	0	0.0093	256	256	4
4	1.76	7.54	4/4	8	0.0190	128	32	32
5	7.02	6.49	4/4	32	0.0144	256	32	320
6	5.71	5.18	0/4	160	0.0359	256	4	2368
7	2.25	4.65	0/4	168	0.0328	256	8	704
8	2.81	4.72	0/4	128	0.0327	256	0	576
9	8.97	4.41	3/4	320	0.0387	128	8	1184
post control infection	0.00	9.14	0/4	0	0.0101	256	256	2

Table 4. Mean erythrocyte counts per mm^3 (RBC x $10^6/mm^3$) of uninfected rats injected with both antitrypanosomal globulin (Anti-P31) and immunoconglutinin globulin (IK), (controls) and rats infected with *Trypanosoma brucei rhodesiense* and injected with normal rat serum (NRS), the IK globulin, the Anti-P31 globulin and with both IK and Anti-P31 globulins.

	RBC x $10^6/mm^3$				
Days	Controls, IK + Anti-TRYP	NRS	IK	Anti-TRYP	IK + Anti-TRYP
0	8.32	8.21	8.18	8.25	8.16
1	8.17	8.24	8.16	8.41	8.06
2	7.91	7.96	7.93	8.05	8.10
3	8.00	7.73	7.76	7.71	7.11[5]
4	8.14	6.54	6.32	6.45	6.24
5	8.08	5.74	5.74	5.92	5.35
6	8.18	4.89	5.28	4.98	4.84
7	8.21	4.29[3]	4.57	4.45	4.29
8	8.12	D[1]	D	D	S[2]

[1] All animals dead.

[2] 11 animals dead, 1 survived until day 9.

[3] 1 animal dead.

[4] 2 animals dead.

[5] Significantly lower than NRS controls. $P \leq 0.05$ (student's t test).

Table 5. Mean trypanosome counts per mm^3 (TRYP x $10^5/mm^3$) of uninfected rats injected with both antitrypanosomal globulin (Anti-P31) and immunoconglutinin globulin (IK), (controls) and rats infected with *Trypanosoma brucei rhodesiense* and injected with normal rat serum (NRS), the IK globulin, the Anti-P31 globulin and with both IK and Anti-P31 globulins.

	TRYP x $10^5/mm^3$				
Days	Controls, IK + Anti-TRYP	NRS	IK	Anti-TRYP	IK + Anti-TRYP
0	-	0.00	0.00	0.00	0.00
1	-	0.00	0.00	0.00	0.00
2	-	(+)	(+)	(+)	(+)
3	-	2.34	1.97	2.01	1.48[5]
4	-	7.84	6.66	5.66	4.85[6]
5	-	7.89	6.42	5.02	3.38[6]
6	-	2.42	1.72	1.05	1.06
7	-	6.08[3]	7.33	6.76[4]	4.21[5]
8	-	D[1]	D	D	S[2]

[1] All animals dead.

[2] 11 animals dead, 1 survived until day 9.

[3] 1 animal dead.

[4] 2 animals dead.

[5] Significantly lower than NRS controls. $P \leq 0.05$ (student's t test).

[6] Significantly lower than NRS controls. $P \leq 0.01$ (student's t test).