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William James Rickman

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IMMUNOPATHOLOGY OF EXPERIMENTAL AFRICAN TRYPANOSOMIASIS

By

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ABSTRACT

IMMUNOPATHOLOGY OF EXPERIMENTAL
AFRICAN TRYPANOSOMIASIS

By

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Rats experimentally infected with *Trypanosoma brucei rhodesiense* developed anemia, splenomegaly and an acute proliferative glomerulonephritis which were accompanied by circulating cold-active hemagglutinin (CAH), immunoconglutinin (IK) and antibody to fibrinogen/fibrin related products (Anti-F). The infected rats died by the ninth day with signs of acute respiratory failure. Anemia, reduced parasitemia along with splenomegaly were observed when antibody titers peaked. Fluorescein isothiocyanate conjugated IK and Anti-F reacted with circulating erythrocytes and trypanosomes when anemia and reduced parasitemia were evident. These reactions suggested that the cells were coated with complement and fibrinogen related products. It is postulated that these substances were previously bound to the cells as complexes of antigen and antibody, thus contributing to their sequestration within the spleen. Evidence of pulmonary involvement and coagulation abnormalities suggested that death may have resulted from disseminated intravascular coagulation.

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INTRODUCTION

Yearly African trypanosomiasis has a devastating impact on domestic animal production and on the well-being of the human inhabitants of the African continent. The disease results from infections with a blood parasite of the Genus *Trypanosoma*, in particular, those of the *brucei*-complex. Since trypanosomiasis continues to be a serious hazard to health, there is a need for a thorough understanding of the pathogenesis of the disease.

Woodruff and associates (1973) documented this disease of humans as a syndrome of anemia with splenomegaly. These investigators implicated a pathologic role for antibody and complement coated erythrocytes in the etiology of the anemia associated with the infection. Sadun et al. (1973) expanded the definition of the syndrome to include glomerulonephritis utilizing rhesus monkeys infected with *Trypanosoma brucei rhodesiense*. Most data accumulated on infections with this agent have been limited to those collected from human and other primate sources.

This study was conducted to ascertain the suitability of a rodent model for investigating the immunopathology of African trypanosomiasis. Experiments were performed with rats infected with a human trypanosome, *Trypanosoma brucei rhodesiense*, to determine the pathophysiological sequence of the infection by observing the parasitological, hematological, serological and pathological aspects of the infection. The

associations of anemia, splenomegaly and glomerulonephritis with cold-active hemagglutinin, immunoconglutinin and antibody to fibrinogen/fibrin related products are reported in this thesis.

REVIEW OF LITERATURE

Hemoflagellate Parasites

Taxonomically hemoflagellate parasites belong to the Phylum *Protozoa* and fall within the Class *Zoomastigophorasida*. Hemoflagellates all belong to the Family *Trypanosomatidae*, which is represented by nine genera. Eight genera are parasitic with each requiring either one host (monoxenous) or two hosts (heteroxenous) for completion of their life cycles (Table 1). Only two of the genera are parasites of domestic animals and man, notably the genera *Leishmania* and *Trypanosoma* (Levine, 1973).

Many species of trypanosomes parasitize amphibians, fishes, reptiles, birds and mammals. In these cases leeches serve as intermediate hosts and biological vectors of trypanosomes parasitizing aquatic vertebrates. Arthropods generally serve as biological vectors of non-aquatic vertebrates (Faust et al., 1975). Transmission of trypanosomiasis may occur by mechanical means with the blood-sucking arthropods herein described through contamination of the proboscis with organisms from an interrupted blood meal.

In Cecil Hoare's (Hoare, 1972) most recent tabulation of trypanosome species, 125 were implicated as parasites of mammals with 12 of the 19 orders of mammals parasitized. Most hemoflagellates have adapted remarkably well to their host and are not lethal or even life-threatening. However, many species of the Genus *Trypanosoma* have been associated with disease and are therefore reviewed in Table 2.

Table 1. Genera of the Family *Trypanosomatidae* (adapted from Levine, 1973)

Genus	Type of Development	Host(s)
Blastocrithidia	monoxenous	Arthropods
Crithidia	monoxenous	Arthropods
Leptomonas	monoxenous	Invertebrates
Herpetomonas	monoxenous	Invertebrates
Rhynchoidomonas	monoxenous	Insects
Phytomonas	heteroxenous	Plants and hemipterous insects
Proleptomonas	coprozoic	Soil and feces
Leishmania	heteroxenous	Invertebrates and Vertebrates
Trypanosoma	heteroxenous	Invertebrates and Vertebrates

Table 2. Members of the Genus *Trypanosoma* as important agents of disease (adapted from Levine, 1973)

Subgenus	Species	Mammalian Host	Vector
Section Stercoraria			
Megatrypanum	*T. theilera*	Cattle	Hippoboscid Flies
	T. melophagium	Sheep	" "
	T. ingens	Cattle	" "
Herpetosoma	*T. lewisi*	Rats	Fleas
	T. duttoni	House mice	"
	T. rangeli	Man and dogs	"
Schizotrypanum	*T. cruzi*	Man,dogs,cats	Reduviid Bugs
Section Salavaria			
Duttonella	*T. vivax*	Ruminants, equids	Glossina Flies
	T. uniforme	Ruminants	" "
Nannomonas	*T. congolense*	Ruminants,pigs, equids	" "
	T. dimorphon	Ruminants,pigs, equids	" "
	T. simiae	Pigs	" "
Pycnomonas	*T. suis*	Pigs	" "
Trypanosoma	*T. brucei brucei*	All domestic mammals	" "
	T. b. rhodesiense	Man and domestic animals	" "
	T. b. gambiense	Man	" "
	T. evansi	Camels,bovids, equids	Tabanid Flies
	T. equinum	Equids,bovids	" "
	T. equiperdum	Equids	None (venereal)

The Genus *Trypanosoma* has been conveniently classified into two main sections, the *Stercoraria* and *Salivaria*. The *Stercoraria* develop in the posterior portion of the alimentary tract of the arthropod vector. Transmission to the vertebrate host occurs when fecal material containing infective forms is deposited on the host during a blood meal. The *Salivaria*, on the other hand, develop in the anterior portion of the alimentary tract of the arthropod with emergence of the infective form through the hypopharyngeal tube of the proboscis. The morphologic stages in the developmental cycles of both sections vary, depending upon the species of trypanosome (Hoare, 1972).

The *brucei*-Group of Trypanosomes (Subgenus *Trypanozoon*)

The developmental cycles of *Trypanosoma brucei brucei*, *T. b. gambiense*, and *T. b. rhodesiense*, are similar (Hoare, 1972). When trypanosomes enter the blood stream of the vertebrate host they divide by longitudinal binary fission producing the trypomastigote stage. This stage of the parasite is polymorphic, presenting with slender, intermediate and stumpy morphologic forms. The trypomastigotes average 29 microns in length, possess a posterior kinetoplast, undulating membrane and a single free flagellum. When trypomastigotes are ingested by the arthropod vector of the Genus *Glossina*, they localize in the posterior part of the midgut. At this site multiplication in the trypomastigote stage occurs. After ten days, forms appear which possess a kinetoplast located between the central nucleus and the posterior end of the organism. The undulating membrane of this form is less pronounced than that shown by the blood forms. By the fourteenth day in the insect, the midgut trypomastigotes migrate to the

proventriculus, esophagus and, finally, the salivary glands. Attachment of the trypanosomes' flagella to the walls of the salivary glands occurs about the twentieth day with subsequent transformation to the epimastigote stage. This stage differs slightly from the preceding trypomastigote in that the kinetoplast is located adjacent to the central nucleus. Additional multiplication of the epimastigote stage occurs at the salivary glands. The epimastigotes undergo further transformation, giving rise to the metacyclic trypmastigotes or the infective form. The entire developmental cycle from blood trypomastigote to infective metacyclic trypomastigote requires 15 to 35 days in the insect. The life cycle is completed when the tsetse fly obtains a blood meal from a suitable vertebrate host with the liberation of the infective metacyclic forms. Several thousand trypanosomes may be inoculated with a single bite of the fly. Most species of salivarian trypanosomes possess this typical characteristic developmental cycle (Hoare, 1972).

Trypanosoma brucei brucei (Plimmer and Bradford, 1899)

One of the most common and important parasites of domestic animals in tropical Africa is *Trypanosoma brucei brucei*. The disease caused by this trypanosome is known as nagana, a Zulu term meaning weak or feeble. In general, the disease is characterized by fever, anemia, edema, cachexia and paralysis with host dependent pathology. Horses, mules and donkeys appear to be most susceptible to this disease. Fatalities have been reported within three months after exposure to this organism (Levine, 1973). Levine (1973) has stated that because of this disease African agriculture has remained virtually wheel-less because cattle and horses cannot survive to pull wagons or plows.

Originally nagana, or "tsetse fly disease", in animals was thought to be caused by toxins of the fly. Its earliest description was provided in 1861 by Dr. Stanley Livingstone during his missionary travels in southern Africa. The causal relationship of trypanosomes to nagana was demonstrated by inoculating the blood of infected cattle into horses and dogs which subsequently developed an acute form of the disease (Bruce, 1895). In 1896 Sir David Bruce sent an infected dog to England, where the trypanosome biology was studied. Plimmer and Bradford (1899) described the morphology of the nagana parasite and named it *Trypanosoma brucei*. Kleine (1909) later showed that *Glossina palpalis* was able to transmit the infection to clean animals. This investigator further demonstrated that transmission occurred only after a developmental period of 18 to 20 days in the insect host, thereby indicating for the first time that trypanosomes underwent a necessary period of development in the insect. These discoveries laid the foundation for future investigations on the etiology of tsetse-borne trypanosomiasis of domesticated animals and humans.

Trypanosoma brucei gambiense (Dutton, 1902) and *Trypanosoma brucei rhodesiense* (Stephans and Fantham, 1910)

Sleeping sickness is a disease of humans that has been known in West Africa for centuries, with its earliest description given by John Adkins in 1734 (Goodwin, 1970). The elucidation of the causative agent, *T. gambiense*, occurred in 1902 when J. E. Dutton observed "wiggling bodies" in a European patient's blood with "trypanosoma fever." Up until 1903, all reported cases attributed to an infection with *T. gambiense* were clinically manifested as "trypanosoma fever"

and were thought to be a disease separate from that of sleeping sickness. Castellani (1903) found trypanosomes in the cerebrospinal fluid of patients in Uganda suffering from sleeping sickness, thereby establishing a connection between this disease entity and a trypanosome. Maxwell-Adams (1903) was the first to suggest that *T. gambiense* might be the causative agent of both "trypanosoma fever" and sleeping sickness. He hypothesized that early in the infection when parasitemia was pronounced the disease was manifested as "trypanosoma fever" and when the parasites invaded the central nervous system the clinical picture was one of sleeping sickness. This hypothesis was confirmed when Gray and Tullock (1907) followed 13 cases of "trypanosoma fever", all of which later developed into typical sleeping sickness.

Stephans and Fantham (1910) isolated a new trypanosome from a human case of trypanosomiasis that markedly differed in severity from the Gambian form of sleeping sickness previously described. They attributed this new disease to a new trypanosome, *Trypanosoma rhodesiense*. It was first thought that these new cases were a reflection of the spread of *T. gambiense* infection into areas outside the endemic foci of *T. gambiense*. However, transmission was by a different species of fly, *Glossina morsitans*. Laboratory studies utilizing this new trypanosome demonstrated a greater virulence and shorter duration of infection. Since 1910, the Rhodesian and Gambian forms of human trypanosomiasis have been well documented and the two species' identity established (Hoare, 1972).

The geographic distribution of the two types of human trypanosomiasis and their respective arthropod vectors support an evolutionary phylogenetic basis for these two host-parasite relationships. The

Gambian version of the disease is confined to tropical Africa between the Gambian and Congo Rivers, extending east to Lake Tanganyika (Hoare, 1972). The distribution of Gambian trypanosomiasis is discontinuous and corresponds to that of the vector (Levine, 1973). The primary vector, *Glossina palpalis*, is a riverine species feeding primarily on reptiles, in particular crocodiles (Hoare, 1931). The residents of these densely populated areas are frequently bitten by these flies as dependence upon the rivers for both travel and livelihood is common. Under these ecological conditions the disease can be transmitted from human to human without requiring a reservoir host, thus termed anthroponotic transmission.

The distribution of Rhodesian trypanosomiasis extends from the Zambezi River north to the upper Nile, overlapping the Gambian form only on the banks of Lakes Tanganyika and Victoria (Hoare, 1972). In this less populated region, game animals are plentiful and have been shown to serve as reservoir hosts for *T. rhodesiense* (Heisch et al., 1958; Goodwin, 1970). The tsetse fly responsible for disease transmission is *Glossina morsitans*. These flies feed primarily on game animals and rarely obtain blood meals from humans (Hoare, 1972). For decades the Rhodesian form of trypanosomiasis has been referred to as an occupational disease, affecting only those individuals who travel or hunt in the natural enzootic foci of the disease (Robertson and Baker, 1958). Unlike the Gambian form, the infection is maintained independent of man. Humans become infected only accidentally, a type of transmission termed anthropozoonotic.

It has been proposed that the human trypanosomes of Africa evolved from the *brucei*-complex of wild animals (Hoare, 1972). The Rhodesian

form of trypanosomiasis, manifested as a severely acute disease in humans, may be an ongoing evolutionary adaptation of *Trypanosoma brucei* to a human host. On the other hand, *Trypanosoma gambiense*, causing a less severe and more chronic disease, may represent a more adapted strain of *Trypanosoma brucei*. Morphologically these three parasites are indistinguishable and all exhibit considerable polymorphism. Any attempt to separate them on the basis of size, percentage of postnuclear and stumpy morphologic forms have resulted in inconsistent identification (Hoare, 1972). Presently, the classification of the *brucei* group of trypanosomes is most reliably achieved by knowing the source of the isolate, its virulence and pathogenicity in laboratory animals. Levine (1973) has stated that *T. brucei* can be separated from the other two morphologically similar parasites by its inability to infect humans, since all such attempts have been unsuccessful. In summary, this evolutionary relationship is reflected in the modern classification of the *brucei* group of trypanosomes. The Subgenus *Trypanozoon* or subgroup designation *brucei* is widely used to signify species (strain) differences (Hoare, 1972).

Pathogenesis of African Trypanosomiasis

Clinical Manifestations

The clinical course of African trypanosomiasis has been well documented and an excellent description by Dr. Richard P. Strong appears in *Stitt's Diagnosis, Prevention and Treatment of Tropical Diseases* (1944). Dr. Strong has divided this disease into three stages: (1) incubation, (2) febrile or glandular, and (3) cerebral. The time duration of each stage is irregular with respect to both

length as well as intensity. Inflammation at the site of the fly bite may occur within 48 to 72 hours. Within 10 to 12 days after the bite of an infected fly, trypanosomes can be found in the peripheral blood circulation. This period is designated as the incubation stage. Parasitemia becomes evident along with a fever fluctuating from normal to 104°F. Erythematous eruptions may occur in the epidermis with enlargement of the liver and spleen also observed. Early in the disease, involvement of the central nervous system can occur resulting in neuralgic pain and headaches. Enlargement of the lymph nodes, especially those of the posterior triangle of the neck, follows parasitemia. This condition has been referred to as Winterbottom's sign, a diagnostically important symptom. Aspirations from these nodes can reveal trypanosomes and serve to confirm the diagnosis of trypanosomiasis. The febrile stage may persist for months or years with considerable variation in severity. Generally this febrile stage is most severe in Europeans and less severe in Africans. The eventual progression of the disease includes a transient parasitemia with remittent fever with spontaneous recovery or, in about 13 percent of the cases, death.

Some cases of trypanosomiasis progress to the cerebral stage, and if trypanosomes are found in the cerebrospinal fluid, death is a common occurrence. The duration of the cerebral stage is quite variable with some patients surviving years while others succumb to complicating infections. Headaches, tremors, hysteria and mania can follow. Patients may become lethargic and apathetic in the last weeks of infection with walking being almost impossible. Further disease progression results in emaciation. The patient sleeps continuously,

the radial pulse becomes barely palpable, coma begins and death results.

The most striking lesions found at necropsy are observed in the lymphatic glands and in the central nervous system. The chief feature of the infection is a chronic lymphocytic inflammation of the lymphatic system resulting in an enlargement of the glands. Inflammation of the lymphatics of the brain often takes place. Trypanosomes can be found in the vessels but are distributed in an irregular manner.

The cerebrospinal fluid is increased and often turbid. There is usually congestion of the brain and spinal cord with occasional hemorrhages. The spleen is usually enlarged and the lungs may show pneumonic changes. A more detailed description of the pathology and morbid anatomy of human trypanosomiasis can be found in *The Pathology of Tropical Diseases* (Ash and Spitz, 1945).

Immunologic Mechanisms of Anemia and Splenomegaly

One of the most consistent observations of trypanosomiasis has been anemia. It has been reported in cattle, monkeys, rabbits, mice, rats and humans (Kobayashi et al., 1976; Murry, 1974; Sadun et al., 1973; Goodwin, 1970; Jennings et al., 1974; Woodruff et al., 1973). The clinical anemia is normochromic, normocytic accompanied by a marked reticulocytosis with a bone marrow examination revealing a significant erythrocytic hyperplasia (Sadun et al., 1973). There is also a clinically evident thrombocytopenia (Sadun et al., 1973; Greenwood and Whittle, 1976). In addition, Holmes and Mamo (1975) demonstrated with iron uptake studies that dyshemopoiesis played no part in the etiology of the anemia.

An immunologic mechanism of anemia in trypanosomiasis has been a popular concept since it was first suggested by Dr. Warrington Yorke (1911). He observed that fresh preparations of anticoagulated blood from infected animals appeared to clump at laboratory temperatures instead of forming rouleaux. Dr. Yorke's account of this phenomenon follows:

> These observations served to indicate that temperature played an important role in the development of the phenomena [of autoagglutination]. On this occasion three sets of tests were made: the first were placed in the incubator at 37°C, the second were left at laboratory temperature, the last were kept in the ice chest at 0°C. Even at the end of five minutes agglutination of red cells was noticeable in the tubes placed on ice, whilst in fifteen minutes were completely agglutinated. The reaction was also distinct in many tubes kept at laboratory temperature but it was neither so marked nor as quick as those subjected to lower temperatures. As before, no agglutination was observed in the tests which had been placed in the incubator.

This description appears to be the first observation that characterized a cold autoagglutinin in trypanosomiasis presently defined as cold-active hemagglutinin (CAH). Dr. Yorke investigated the phenomena utilizing the blood of monkeys, donkeys, goats, dogs, rabbits and rats infected with trypanosomes to confirm his earlier observation of a cold agglutinin. He further demonstrated that this phenomenon was reproducible with the serum of infected animals and homologous normal red cells. In addition, he observed that small quantities of cold agglutinins were present in the blood of many normal animals.

Other investigators of this era reported cold agglutinins in trypanosomiasis (Kanthack et al., 1899; Christy, 1904; Todd, 1910; Dubois, 1912; Marty, 1917). An important question, however, is whether anemia can be mediated by a cold hemagglutinin. Direct evidence of such a mechanism has only been reported in malarious chickens (Soni

and Cox, 1975a,b). Anemia was produced in normal chickens by injections of trypsinized autologous erythrocytes. Within three days serologically detectable titers of CAH were observed. These investigators absorbed CAH from malarious plasma with trypsinized human "O" blood cells and eluted the antibody from the cells with saline at 37°C. Injections of this eluate produced anemia in recipient normal chickens within 48 hours. CAH-mediated anemia has been suggested in many disease models. Among them are rodent malaria (Cox et al., 1966; Musoke and Cox, 1977; Musoke et al., 1977), *Haemobartonella* and *Eperythrozoon* infections in rodents (Cox and Calif-Iturri, 1976) and *Babesia* infections in rats (Thoongsuwan and Cox, 1973; Schroeder et al., 1966).

Woodruff et al. (1973) suggested that if an immunologic mechanism is responsible for anemia, complement coating of the erythrocytes is likely to occur. These investigators have compiled the most convincing evidence to date to indicate that the anemia of both kala azar and human trypanosomiasis is immunologic (Woodruff et al., 1972, 1973). Experiments performed on patients with leishmaniasis and trypanosomiasis using injections of chromium^{51} labeled autologous erythrocytes demonstrated a decreased erythrocyte survival time with accumulation of isotope activity predominantly in the spleen. Using antisera directed against human complement protein (Anti-C3), it was shown that complement coating of peripheral erythrocytes had occurred. These investigators suggested that such an event may reflect the previous presence of antigen-antibody-complement complexes on the red cells. Concurrent to complement coating of cells, immunoconglutinin titers were markedly elevated above normal ranges. Woodruff et al. (1973) suggested that

immunoconglutinin may assist in the immunologic sequestration of complement coated erythrocytes, thereby contributing to the splenomegaly observed in African trypanosomiasis.

Immunoconglutinin is an antibody directed against cleaved C3 (Lachmann, 1962; Lachmann and Coombs, 1965; Lachmann and Muller-Eberhard, 1968) and C4 (Lachmann, 1966) complement components of bound or fixed complement (not native C3 or C4) which are not related to the antigenic character of the infecting organism (Coombs et al., 1961). The actual role immunoconglutinin plays in anemia has not been demonstrated conclusively. Ingram et al. (1959) have suggested that immunoconglutinin enhances non-specific resistance to disease and the anti-complementary character of immunoconglutinin could have a role as an opsonin contributing to the pathogenesis of disease. Parappally and Ingram (1973) demonstrated that immunoconglutinin enhanced the phagocytic activity of mouse peritoneal macrophages against bacterial cells *in vitro*. The amplifying effect of immunoconglutinin on complement fixation *in vivo* was shown by Tedesco and co-workers (1972), suggesting that this antibody potentiated the ability of complement to bring about red cell destruction.

The presence of circulating immunoconglutinins has been reported by other investigators in *Trypanosoma lewisi* infections of rodents (Thoongsuwan, 1976) and *T. brucei* infections of rabbits (Ingram and Soltys, 1960). The association of immunoconglutinin with anemia is not restricted to trypanosomiasis having been reported in cattle with contagious bovine pleuropneumonia (Kakoma et al., 1973), *Streptococcus* and *Listeria* infections (Ingram et al., 1959), and *Babesia, Plasmodium* and viral infections (Thoongsuwan, 1976).

Nagle et al. (1974) suggested that hypocomplementemia observed in African trypanosomiasis may have resulted from an immunologic activation of the complement system. Reductions in complement C3 and C4 serum levels have been reported in infections with *Trypanosoma lewisi, T. rhodesiense* and *T. congolense* (Jarvinem and Dalmasso, 1976; Nagle et al., 1974; Kobayashi and Tizard, 1976). However, Kierszenbaum and Weinman (1977) demonstrated *in vitro* antibody-independent, complement-dependent lysis of epimastigotes of *T. cyclops*. Decomplementation with cobra venom factor resulted in an increased *in vivo* survival of *T. cruzi* trypomastigotes in chickens with *in vitro* studies suggesting that the lysis of these parasites occurred via the alternative pathway of complement activation (Kierszenbaum et al., 1976). Musoke and Barbet (1977), using purified parasite surface antigens of both *T. brucei* and *T. congolense* in the presence of purified human complement proteins, demonstrated *in vitro* decomplementation via both the classical and alternative pathways. In all cases these investigators have stated that complement activation occurred in the absence of antibody and/or immune complexes and therefore may have resulted from a non-immunologic activation of complement. However, whether or not non-immunologic activation of complement would contribute to anemia and splenomegaly has not been indicated.

Additional evidence for a proposed immunologic mechanism of anemia in trypanosomiasis can be supported by the consistent observation of positive anti-globulin (Coombs) tests. Coombs positive anemia has been reported in trypanosome infections of rabbits and humans by Mackenzie and Boreham (1974b), of humans by Woodruff et al. (1973)

and of cattle by Kobayashi et al. (1976). The latter investigators substantiated their findings by eluting antigens and antibodies from the erythrocytes of infected animals. The antibodies eluted were of the IgM, IgG_1 and IgG_2 classes. By means of complement fixation and hemagglutination inhibition tests, the eluates showed serologic reactivity with sonicated trypanosomes. These authors postulated that trypanosome antigen-antibody-complement complexes deposited on the surface of erythrocytes resulted in an immune elimination of the cells leading to clinical anemia. Many authors have suggested a pathologic role for immune complexes in the etiology of the anemia of infections with a variety of unrelated agents (Zuckerman, 1964; McGhee, 1964; Cox et al., 1966; Woodruff et al., 1966, 1972, 1973; Schroeder et al., 1966; Soni and Cox, 1974, 1975a,b; Musoke and Cox, 1977; Musoke et al., 1977).

In addition to cold-active hemagglutinin and immunoconglutinin, other autoantibodies (i.e., antibodies directed against autologous host antigens) have been reported in trypanosomiasis. Seed and Gam (1967) described an anti-liver antibody in rabbits infected with *T. gambiense*. Mansfield and Kreier (1972), using both complement fixation and precipitin tests, demonstrated antibodies to autologous kidney, liver, brain and heart antigens in rabbits with *T. congolense* infections. However, delayed hypersensitivity skin reactions and macrophage inhibition factor production could not be demonstrated using the same antigen preparations. Anti-liver, anti-fibrinogen and Wasserman antibodies have been documented in cattle and humans with trypanosome infections (Mackenzie et al., 1973). Absorption studies by Mackenzie and Boreham (1974) indicated that these

antibodies were not cross-reactive and were of the IgM class. These authors suggested that these antibodies may contribute to the elevated IgM levels observed in trypanosomiasis. No further studies have been reported concerning the possible role of these antibodies in the pathogenesis of anemia in trypanosome infections.

Immunologic Mechanisms of Glomerulonephritis

Glomerulonephritis was first recognized as a consequence of infections with African trypanosomes by Sadun and co-workers (1973). Their pathological studies revealed a marked hypercellularity of the mesangial cells within the glomerular tuft accompanied by sclerosis of the mesangium. Electron microscopic analysis demonstrated swelling and simplification of the visceral epithelial cells and apparent focal loss of filtration slits. Using special staining techniques, thickening and duplication of the capillary basement membrane, the presence of proteinaceous casts with polymorphonuclear leukocyte infiltration were seen. These morphologic changes in renal tissue have been consistently observed in experimental trypanosomiasis (Lambert and Houba, 1974; Murray, 1974; Murray et al, 1975; Nagle et al., 1974). Similar nephropathologic changes have been described in malarial, babesial and schistosomal infections (Ward and Kibuka-Musoke, 1969; Soni and Cox, 1974, 1975c; Musoke et al., 1977; Iturri and Cox, 1969; Mahmoud and Woodruff, 1972). The observed morphology of the renal lesions associated with these infections suggests that the pathologic mechanisms responsible may be similar in nature if not identical (Musoke et al., 1977).

Frank Dixon has contributed substantially to the understanding of the immunopathology of glomerulonephritis during the past two decades.

He has formulated two distinct pathologic mechanisms operative in human glomerulnephritis and proposes that they account for the pathology seen with most cases of this disease (Dixon, 1971b). The first mechanism (Type I) described involves the production of antibodies by the patient which are capable of reacting with the patient's own glomerular basement membrane. The immunologic events that occur in nephrotoxic nephritis have been well established (Dixon et al., 1958; Dixon, 1963; Unanue and Dixon, 1967; Vassalli and McCluskey, 1964). The second mechanism (Type II) involves the production of soluble non-glomerular antigen-antibody complexes which become deposited within the renal glomeruli.

The histological appearance of these two forms of nephritis are similar, varying only in the degree of intensity (Dixon, 1976lb). However, immunohistochemical techniques and electron microscopy have revealed distinct differences in the nature of the immune deposits (Dixon, 1963). The appearance of the lesions produced by the reaction of anti-glomerular antibodies and complement results in a smooth linear deposition of immunoglobulin and complement along the inner aspect of the basement membrane of the glomerular capillaries. In contrast, with Type II glomerulonephritis, non-glomerular antigens, antibody and complement accumulate in discrete, irregular, lumpy or granular deposits along the outer aspect of the capillary basement membrane.

Nagle and associates (1974) demonstrated using immunofluorescence that renal deposits of C3, properdin and IgM characteristic of Type II nephritis had occurred from *T. rhodesiense* infections. These observations were confirmed by electron microscopic examination as this

technique revealed irregular electron-dense deposits in the subepithelial spaces of the mesangial matrix. Similarly, Lambert and Houba (1974) demonstrated distinctly granular deposits of immunoglobulin (IgG and IgM) and C3 in the renal tissues of rodents infected with *T. brucei*. In more advanced cases of trypanosomiasis, electron microscopic studies showed extensive deposition of large, irregular electron-dense material between the glomerular basal lamina and the capillary endothelium, resulting in the occlusion of the capillary lumen (Lambert and Houba, 1974). These observations have been verified to occur in experimental trypanosomiasis by Murray (1974) and Murray et al. (1975).

Since von Piquet and Schick (1905) first observed that disease could result from a single injection of a foreign serum protein, serum sickness has been employed as a model for investigating the pathogenesis of many diseases. The immunologic sequence of events which occur in serum sickness result in the formation of soluble antigen-antibody complexes, thus producing pathologic renal lesions analogous to Type II nephritis (Dixon et al., 1958; Dixon, 1963; Weigle, 1961). By the use of iodine^{131} labeled bovine serum albumin, Dixon and co-workers (1958) documented the temporal relationships of antigen, antibody and complement in serum sickness. Following intravenous injection of a heterologous soluble antigen into an unimmunized host, there is a slow equilibration of antigen between intra- and extravascular components. A rapid elimination of antigen by antibody follows with the formation of small antigen-antibody complexes with a half-life of ten days. The initial accumulation of immune complexes in tissues occurs early during an excess antigen phase. The formation of immune complexes during excess antigen phase appears to be an essential

prerequisite for immune complexes deposition. The smaller complexes can persist in the circulation and, as more antibody becomes available, enlargement of the complexes occurs. Activation of complement results in the formation of larger complexes which are eliminated from the circulation. During the removal of these complexes serum complement levels are decreased and within a short period of time, acute focal inflammatory lesions appear in the kidneys (Dixon et al., 1958).

Considerable attention has been focused toward implicating antigenic variation of trypanosomes as a basis for antigen-antibody complex formation in African trypanosomiasis. Almost 70 years ago Ross and Thomson (1911) demonstrated that successive waves of antigen variation occurred in human sleeping sickness. Later, Russell (1396) proposed that the changes observed in antibody levels were due to the serologic variation in the infecting strain of trypanosomes. Gray (1962) presented further evidence of this observation by showing that host antibodies were developed against a series of immunologically distinct trypanosome variants. Each of these antibodies coincided with the disappearance of the homologous variant and the emergence of a new antigenic type. Murray and co-workers (1975) have suggested that large amounts of parasite antigen can result from the sequential release of surface trypanosome antigens during the course of infection, an event which favors immune complex formation. Such a concept would provide a means whereby persistent antigen excess similar to that observed with serum sickness would result in renal damage.

Lambert and Houba (1974) have presented evidence implicating trypanosome antigen-antibody complexes in the etiology of glomerulonephritis. Eluates from the kidneys of infected mice contained

antibodies which reacted specifically with trypanosome antigens of the initial inoculum. By direct immunofluorescence, trypanosomal antigens were observed in the renal glomeruli in a pattern analogous to that seen with Type II nephritis. The major parasite antigens present in the kidneys corresponded to those antigens of the initial population of trypanosomes used to establish the infection and not to the trypanosomes circulating in the blood at the time of sacrifice.

Further support of a Type II nephritis in trypanosomiasis can be obtained by Clq-binding studies. Circulating immune complexes can be demonstrated by the ability of complement (Clq) to bind with immune complexes in serum samples using a variety of assay systems (WHO Technical Report, 1977). Murray et al. (1975) have reported increased Clq-binding potential in the serum of mice infected with *T. brucei* suggesting serologic evidence of the involvement of immune complexes in the disease process. Nagle and co-workers (1974) associated depression of serum complement levels with the appearance of immune deposits in renal tissues during the course of *T. rhodesiense* infections. These investigators suggested that the alternative rather than the classical pathway of complement activation was involved in the observed immune complex deposition. This suggestion was supported by the depression of C3 but not C4 serum complement levels and the presence of properdin and C3 (not C4) in the immune deposits found in the renal glomeruli. Woodruff and associates (1973) have reported that serum immunoconglutinin titers are increased during infections with African trypanosomes. They suggested that the elevated immunoconglutinin levels may reflect the occurrence of complement binding to immune complexes, thus contributing to complex

deposition in the tissues. These observations bear a striking resemblance to the immunologic events of serum sickness nephritis and thus it appears that the mechanisms of nephritis in African trypanosomiasis may be very similar, if not identical to, those described for serum sickness.

Recently, the blood coagulation system has been recognized as a mediator of disease processes and has been reviewed (McKay, 1965; Kaplan and Austen, 1975). A pathogenic role for fibrin deposition has been reported in immunologically induced glomerulonephritis (Type I) by Vassali and McCluskey (1964). Evidence of fibrinolysis as reflected by elevated serum fibrin degradation products, has been demonstrated in trypanosome infections (Barrett-Conner et al., 1973; Boreham and Facer, 1974a; Greenwood and Whittle, 1976). Deposition of fibrin in renal tissues has been reported in African trypanosomiasis by Boreham and Facer (1972), Murray (1974) and Murray et al. (1975). Vassalli et al. (1973) induced intravascular fibrin formation in rabbits by intravenous injections of thrombin. This protocol resulted in renal lesions similar to those described in trypanosome infected animals. Electron microscopic study of renal tissues demonstrated that fibrin and fibrinoid material accumulated between endothelial cells and the basal lamina occluding the capillary lumen (Vassalli et al., 1973). The appearance of the material within mesangial cells of the glomeruli suggested that fibrin degradation could occur as a result of phagocytosis (Vassalli et al., 1973). These investigators have demonstrated the dependence of these abnormalities on the fibrinolytic system as injections of thrombin with epsilon-amino-caproic acid (EACA) abolished the pathologic deposition

of fibrin in the renal lesions. The latter, EACA, is a potent inhibitor of the activators of fibrinolysis (Soter et al., 1975).

In conclusion, a multiplicity of pathways leading to glomerulonephritis may involve the presence of circulating soluble immune complexes during persistent antigen excess. The role of complement activation, blood coagulation and fibrinolysis in trypanosomiasis needs further investigation directed toward determining the role of each in the pathogenesis of disease. The elucidation of the complex interrelationships of these biological systems in disease will contribute much in the future to the understanding of the immunopathologic basis of disease.

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ARTICLE

ASSOCIATIONS OF AUTOANTIBODIES WITH ANEMIA, SPLENOMEGALY AND GLOMERULONEPHRITIS IN EXPERIMENTAL AFRICAN TRYPANOSOMIASIS

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ABSTRACT

Rats experimentally infected with *Trypanosoma brucei rhodesiense* developed a syndrome characterized by anemia, splenomegaly and glomerulonephritis. Serologic evaluation revealed that the infection was accompanied by the presence of three autoantibodies: cold-active hemagglutinin, immunoconglutinin and antibody to fibrinogen/fibrin products. Fluorescein isothiocyanate conjugated antibody tests showed the presence of fixed complement and fibrinogen on both trypanosomes and erythrocytes. All infected rats died by the ninth day of the infection with five animals showing signs of pulmonary involvement and shock. From these observations it is suggested that autoantigens, autoantibodies and complement were causal in this syndrome.

INTRODUCTION

A syndrome of anemia, splenomegaly and glomerulonephritis accompanied by autoantibodies has been associated with infections of unrelated agents (Boreham and Facer, 1974b; Cox and Calaf-Iturri, 1976; Thoongsuwan, 1976). Identified autoantibodies are cold-active hemagglutinin (CAH), which is antibody to erythrocyte stroma; immunoconglutinin (IK), which is antibody to the third component of fixed complement; and Anti-F, which is antibody to fibrinogen/fibrin products (Soni and Cox, 1975a; Lachmann, 1967; Boreham and Facer, 1974b).

The consistent association of IK with the syndrome suggests that soluble immune complexes that had fixed complement were present in the blood and contributed to pathogenesis. This is supported by Mahmoud and Woodruff (1975), who associated immune complexes with

renal lesions in schistosomiasis. Allison et al. (1969) associated immune complexes with the nephrotic syndrome of malarious African children. Glomerular deposits of fibrin, IgM, IgG, IgA and complement were observed in kidneys of African children with quartan malarial nephritis (Ward and Kibuka-Musoke, 1969). Both antigen and antibody were detected in blood, in eluates of blood cells, in extracts of kidney tissues and in urinary wastes of malarious chickens by Soni and Cox (1975,b,c) and of malarious rats by Musoke et al. (1977). Both anemia and nephritis were induced in normal chickens by injections of parasite-free malarious plasma which contained antigen and antibody (Soni and Cox, 1974).

Thoongsuwan and Cox (1978) associated anemia, splenomegaly and glomerulonephritis with CAH in rats infected with *Trypanosoma lewisi*. Anemia with glomerulonephritis was observed with *Trypanosoma rhodesiense* infections of monkeys (Sadun et al., 1973). High titers of IK and Anti-F were detected in blood of rabbits with acute trypanosomiasis (Ingram and Soltys, 1960; Boreham and Facer, 1974b).

We have undertaken a study to determine the relationships of immune complexes and autoantibodies to pathogenesis of acute *T. brucei rhodesiense* infections. The associations of anemia, splenomegaly, glomerulonephritis, CAH, IK and Anti-F are demonstrated in this communication.

MATERIALS AND METHODS

Experimental Animals and Parasites

Sprague-Dawley male rats (200 to 224 grams) were purchased from Spartan Research Animals Inc., Haslett, Michigan. Care and experimental

procedures conformed to those promulgated by the National Institute for Laboratory Animal Resources of the National Research Council.

The strain of *Trypanosoma brucei rhodesiense* used in this experiment was obtained from LTC James C. Burke, MSC, Walter Reed Army Institute of Research, Walter Reed Army Medical Center, Washington, D.C., and kept in continuous rat passage. After the sixteenth passage, blood was drawn from an infected etherized rat by cardiac puncture and added 10 parts to 1 of heparinized saline (100 units sodium heparin/ml of 0.85% NaCl solution). The blood was diluted in normal saline (0.85% NaCl solution) to result in a final trypanosome concentration of 10^6 trypanosomes/ml and rats were given intraperitoneal (IP) injections of one milliliter each.

Experimental Design

Twenty-eight rats were infected and four rats were randomly selected for autopsy on days 0, 3, 5, 6, 7, 8 and 9. Four normal rats from the original group were sacrificed on day 10. Samples for blood counts were obtained by snipping the tips of their tails with scissors. Red blood cell counts (RBC) and trypanosome counts were made from blood collected in Sahli RBC pipettes and diluted with Hayem's diluting fluid. Counts were made microscopically using a Neubauer hemacytometer. The rats were then exsanguinated while under ether anesthesia by cardiac puncture. After standing overnight at 4°C the serum was collected and stored at -20°C. After bleeding all animals were then autopsied for histological purposes.

Splenomegaly was assessed by measuring spleen volumes by water displacement. Erythrophagocytosis was evaluated using spleen impression slides stained with Giemsa and counterstained with Wright's stain.

Kidney specimens of approximately 3 mm thickness were fixed in 10% buffered formalin. After processing, 5 μ paraffin sections were stained with hematoxylin-eosin according to methods described by Luna (1968).

The severity of kidney damage (SKD) was employed to evaluate a relative increase in the size of the glomerular tuft and to assess tubular swelling according to the method of Iturri and Cox (1969). Hyperplasia was assessed by the number of nuclei per glomerular tuft (NGT) by methods described by Kibuka-Musoke and Hutt (1967). Nuclear counts were based on an average for 25 randomly selected tufts. Photomicrographs of stained kidney sections and spleen impressions were made with a Zeiss Fluoroscope by tungsten filament illumination using Kodak High Speed Tungsten Ektachrome film at an exposure of 0.125 sec.

Serologic Examinations

Detection of cold-active hemagglutinin (CAH) was accomplished by titration of serum samples with trypsinized normal rat erythrocytes as described by Thoongsuwan and Cox (1973).

Immunoconglutinin (IK) from blood of experimental and control rats was titrated with horse complement-fixed sensitized sheep red blood cells (SRBC) as described by Coombs et al. (1961), except that rats were used to generate anti-SRBC serum. Rats were given a single IP injection of 1 ml of 2% suspension of washed SRBC and 9 days afterward the serum was recovered from blood drawn by cardiac puncture under ether anesthesia. The pooled serum was stored at -20°C. Optimal concentration of anti-SRBC giving least hemolysis and strong agglutination with IK positive serum was predetermined. Wells of Cooke

microtiter plates were charged with 0.025 ml of 2-fold diluted sample, 0.025 ml of 1:10 heat inactivated horse serum, 0.025 ml of 0.5% complement-fixed sensitized SRBC and 0.050 ml of saline in that order. Negative controls for each sample consisted of sensitized SRBC treated with heat inactivated horse serum. The plates were covered with adhesive plastic film and mechanically shaken for 1 min before incubation at 37°C for 30 min. Final readings of the conglutination titer were made after storage overnight at 4°C.

Antibody to fibrinogen/fibrin products (Anti-F) was detected by flocculation of sized bentonite particles absorbed with rat fibrinogen at a concentration of 10 µg/ml. Procedures for the test followed methods of Thoongsuwan and Cox (1973).

Preparation of Rat Fibrinogen

Rat fibrinogen was prepared by repeated ammonium sulfate precipitation of normal citrated rat plasma (1 part 3.8% sodium citrate and 8 parts whole blood) according to the method described by McFarlane (1963). The fibrinogen preparation was stored in small aliquots at -20°C in 0.01 M phosphate buffered saline (PBS), pH 7.5, at a concentration of 1 mg/ml of protein which was determined by the method of Lowry et al. (1951).

Preparation of Fluorescein Isothiocyanate Anti-F (FITC-Anti-F)

Four adult male rats were given 2 intramuscular injections of 1 ml per thigh of a mixture of equal amounts of Freund's complete adjuvant and human fibrinogen (1 mg/ml in PBS, pH 7.5) fourteen days apart. Human fibrinogen (65% clottable) was purchased from Miles Laboratories, Elkhart, Indiana, and adjuvant from Baltimore Biological

Laboratories, Cockeysville, Maryland. On the twenty-first day after the first injection the animals were bled by cardiac puncture. The pooled immune sera with an Anti-F titer of 1024 against rat fibrinogen was precipitated twice at 50% saturation with ammonium sulfate. The precipitate was restored to half the original volume in 0.1 M borate buffered saline (BBS), pH 7.5, and dialyzed against changes of BBS until free of ammonium sulfate. The immune globulin was adjusted to 20 mg/ml of protein as described for rat fibrinogen and conjugated with fluorescein isothiocyanate according to the method of Goldman (1968). To remove the non-specific reactivity, the FITC-Anti-F was absorbed with powdered animal charcoal for 1 hour at 4°C and then absorbed 30 min at 37°C with normal thrice-washed rat erythrocytes. To remove the IK activity detected in the immune globulin, the FITC-Anti-F was incubated with an equal volume of the complement-fixed sensitized SRBC for 30 min at 37°C. After ascertaining that the conjugate was free of IK, it was stored in small aliquots at -20°C.

Preparation of Fluorescein Isothiocyanate-IK (FITC-IK)

IK was raised by autostimulation of normal rats by injections of autologous serum absorbed on kaolin particles as recommended by Coombs et al. (1961). Serum recovered from the rats 21 days later had an IK titer of 5120. It was absorbed free of CAH by incubation with trypsin treated normal rat erythrocytes as recommended (Soni and Cox, 1974). Globulin recovered from the serum was conjugated with FITC as in preparation of FITC-Anti-F.

Fluorescent Antibody Tests and Microphotography

Blood smears from experimental and control animals were dried and fixed in absolute methanol for 3 min. Fluorescent antibodies diluted 1:4 in PBS, pH 7.5, were applied to the slides and incubated 30 min at 37 C, washed twice in PBS and coverslipped using 90% glycerol in PBS. Fluorescent antibody activity was studied with a Zeiss Fluoroscope equipped with a darkfield condenser and an Osram HBO 200 mercury lamp using excitor filter II BG 13 and barrier filter 50-00. Microphotographs were taken with Tri-X-Pan Kodak at an exposure of 90 sec.

EXPERIMENTAL RESULTS

Course of Anemia, Parasitemia and Splenomegaly

Following the inoculation of animals with 10^6 trypanosomes, no parasites were observed by peripheral blood examination until 48 hours after inoculation. Parasitemia peaked on day 6 of the infection, then decreased, only to peak again on day 9. By the fourth day of the infection anemia was evident and progressed until the animals died on day 9. Splenomegaly was grossly evident from day 5 through day 9, the period of rapid decline in erythrocyte counts. During this time spleen sizes had increased up to 7 times the normal size. The mean values for erythrocyte and parasite counts and spleen sizes are presented in Table 1.

Gross Observations

At no time during the course of the infection was hemoglobinuria observed. Other than the paleness of ears and eyes, the rats appeared

Table 1. Mean ± standard error of erythrocyte counts per mm^3 (RBC x 10^6 ± S.E.), trypanosome counts per mm^3 (TRYP x 10^5 ± S.E.), spleen volumes in ml (S.V. ± S.E.), severity of kidney disease (SKD ± S.E.), number of nuclei per glomerular tuft (NGT ± S.E.), and mean titers of cold-active hemagglutinin (CAH), immunoconglutinin (IK) and antibody to fibrinogen/fibrin (Anti-F) of rats autopsied as groups of four at intervals during the course of acute *Trypanosoma brucei rhodesiense* infection

Days post-infection	RBC x 10^6 ± S.E.	TRYP x 10^5 ± S.E.	S.V. ± S.E.	SKD ± S.E.	NGT ± S.E.	CAH	IK	Anti-F
0	8.77 ± 0.11	0	1.1 ± 0.05	29 ± 5.5	54 ± 1.8	0	20	0
3	8.20 ± 0.04	0.5 ± 0.05	1.8 ± 0.12	67 ± 7.6	61 ± 1.4	8	100	10
5	6.48 ± 0.13	7.0 ± 0.65	5.1 ± 0.31	190 ± 8.4	79 ± 2.5	20	2400	70
6	5.51 ± 0.16	8.5 ± 0.78	6.5 ± 0.24	191 ± 14.3	84 ± 4.6	576	7680	1120
7	4.60 ± 0.35	1.9 ± 0.22	7.3 ± 0.25	241 ± 21.3	87 ± 1.8	896	7680	7680
8	4.20 ± 0.22	1.8 ± 0.13	6.5 ± 0.24	311 ± 6.4	88 ± 1.3	448	6400	5760
9*	3.79 ± 0.42	8.2 ± 0.73	6.5 ± 0.24	299 ± 7.5	87 ± 1.5	597	427	1067
10**	8.62 ± 0.11	0	1.0 ± 0.05	23 ± 4.8	55 ± 1.7	0	15	0

*Only three rats survived to be autopsied.

**Four normal rats from original group autopsied after experiment.

to be normal and active until the ninth day, when 5 of the 8 remaining rats died. At death they experienced convulsions with signs of respiratory difficulties. During these seizures a serous exudate, often containing blood, oozed from the animal's nose, suggesting pulmonary involvement. For this reason only 3 animals lived to be examined on day 9. These morbid signs have since been consistently observed in passage animals.

An additional observation seen subsequently in passage animals is that blood taken from acute and terminal animals visually appeared to clot in the presence of heparin or sodium citrate concentrations which inhibit clot formation of normal rat blood.

Serologic Evaluation of Serum Samples

The presence of Anti-F and CAH was detected in all infected animals by day 3, while control animals were negative for both autoantibodies. High IK titers were detected in the infected animals by day 5, while uninfected controls had low IK titers ranging from 0 to 40. All three autoantibody titers were highest on day 6 through day 9, when anemia, splenomegaly and nephritis were clearly evident. Mean titers of IK, CAH and Anti-F of infected and control animals are also presented in Table 1.

Histopathologic Studies of Kidney Tissues

Data obtained from histologic examination of hematoxylin-eosin stained kidney sections of autopsied animals are shown in Table 1. The average SKD values from autopsied animals rose from 29 on day 0 to 311 on day 8. The rise in SKD values was accompanied by an increase in NGT values ranging from 54 on day 0 to 88 on day 8. A typical

normal glomerular tuft and tubular area are shown in Figures 1A and 1B, respectively.

The glomerular tufts of infected animals were hypercellular and often swollen to the extent that they completely filled Bowman's capsule when compared to that of normal animals (Figure 1C). The basement membrane of the glomerular tuft was thickened by day 9 (Figure 1D). On days 7 through 9 the epithelial cells of the tubules were swollen, and degeneration and necrosis were observed (Figures 1E and 1F). In general, the alterations observed can best be described as an acute proliferative glomerulonephritis.

Evaluation of Spleen Impressions

Evaluation of stained spleen impressions revealed an excessive amount of erythrophagocytosis by day 5 (Figures 2B and 2C), whereas exhaustive examination of normal spleen impressions revealed that this activity was rare in normal rats (Figure 2A). The phagocytic activity observed in the infected animals was only prominent during the onset of anemia and was difficult to find in the impression slides of animals examined on days 7 through 9.

Fluorescent Studies of Blood Smears

FITC-Anti-F conjugate reacted with smears of infected animals as early as day 3 (Figure 3B) as compared to that of controls (Figure 3A). Reactions with FITC-IK were similar and are shown in Figures 3D, 3E and 3F. It may be noted that the trypanosomes in these preparations reacted intensely on days 3 and 5. Since these conjugates react with fibrinogen and complement, respectively, it appeared that the organisms were coated with both fibrinogen and complement.

Figure 1. Changes observed in glomeruli and adjacent tubules in kidneys of rats autopsied during the course of *Trypanosoma brucei rhodesiense* infection.

A. Glomerulus and adjacent tubules on day 0 of infection. The normal sized glomerular tuft is surrounded by Bowman's space. The NGT count is 56 and SKD value is 0.

B. Normal tubular area on day 0 illustrating that the lumens of the tubules are patent.

C. Glomerulus and adjacent tubules on day 7 of the infection. The glomerular tuft is swollen and fills Bowman's space. The increased NGT count of 92 and estimated SKD value of 4 indicated acute nephritis.

D. The glomerulus of a rat autopsied on day 9 showing marked thickening of the basement membrane.

E. Tubular area of a kidney of a rat autopsied on day 7 with extensive swelling and hypercellularity. Note the edematous interstitial areas and casts within the lumens of the tubules.

F. Tubular area of a kidney on day 9 showing areas of degeneration and necrosis. (250 x)

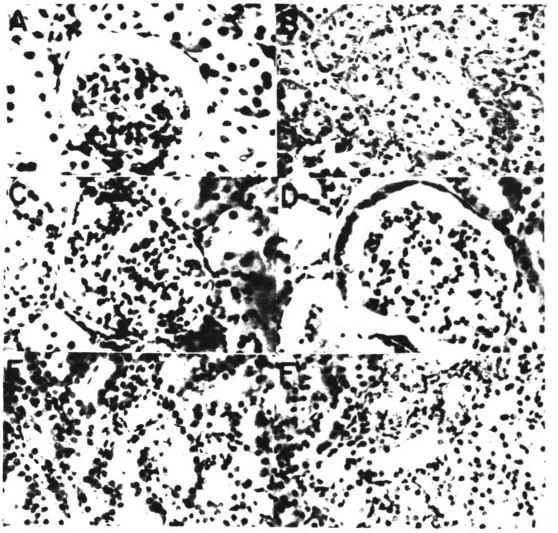

Figure 1

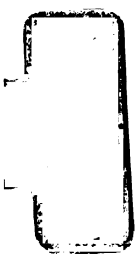

Figure 2. Giemsa stained spleen impressions counterstained with Wright's stain of rats autopsied during the course of *Trypanosoma brucei rhodesiense* infection. (A) Normal spleen cells of a rat autopsied on day 0 of infection. (B) and (C) Erythrophagocytic spleen cells of a rat autopsied on days 3 and 5, respectively. (1000 x)

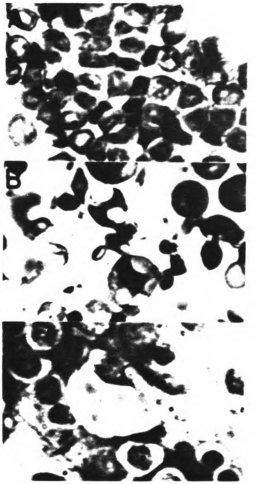

Figure 2

Figure 3. Fluorescein isothiocyanate conjugated antibody reactions of blood smears prepared from rats during the course of *Trypanosoma brucei rhodesiense* infection.

A. FITC-Anti-F test for reactivity with a blood smear prepared on day 0 of infection.

B. FITC-Anti-F reactivity with a blood smear prepared on day 3 of infection. Note the fluorescence of trypanosomes and material on erythrocyte surfaces.

C. FITC-Anti-F reactivity with a blood smear prepared on day 5 of infection.

D. FITC-IK test for reactivity with a blood smear prepared on day 0 of infection.

E. FITC-IK reactivity with a blood smear prepared on day 3 of infection. Note the fluorescence of both trypanosomes and material on erythrocyte surfaces.

F. FITC-IK reactivity with a blood smear prepared on day 5 of infection. Note the presence of two distinctly different fluorescent patterns of the trypanosomes.

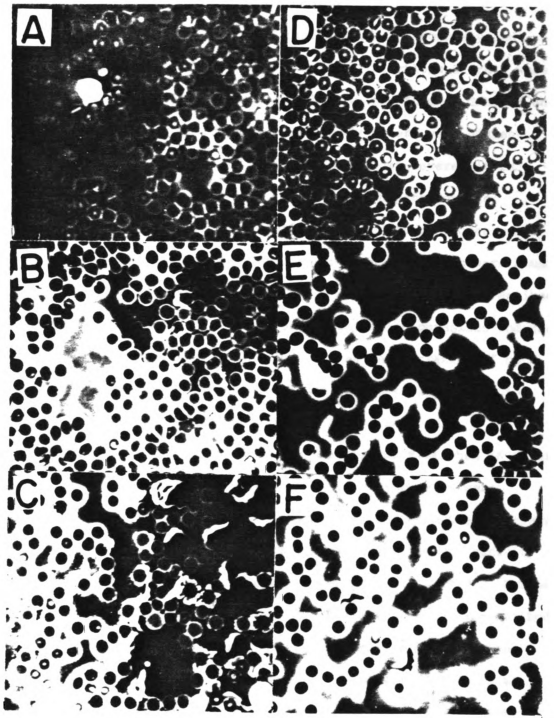

Figure 3

DISCUSSION

These observations indicated that anemia, splenomegaly and glomerulonephritis of *T. brucei rhodesiense* infections of rats were accompanied by circulating autoantibodies in the form of CAH, IK and Anti-F. Since it appeared that autoantibody titers were highest when anemia, splenomegaly and nephritis were most severe, it is possible that the autoantibodies have a role in pathogenesis.

Reactions of FITC-IK with erythrocytes and trypanosomes would suggest that complement fixing antigen-antibody complexes were bound to the cells and parasites. It is possible that the antigen-antibody-complement complexes did become bound to cells and parasites and that the conglutinating action of IK enhanced the sequestration of both cells and parasites by the spleen. Such action could account for reduced RBC counts, reduced parasitemia and splenomegaly.

This concept is supported by the observation that when IK titers of trypanosomiasis patients were high their erythrocytes were agglutinated by antibody to human complement, and when these patients were injected with ^{51}Cr labeled erythrocytes, the isotope activity was soon concentrated in the spleen (Woodruff et al., 1973). Further support was found in the demonstration that both antigen and antibody were present in plasma and that both could be eluted from saline washed blood cells of animals with malarial anemia (Musoke et al., 1977; Soni and Cox, 1975b). Further, antigen and antibody eluted from blood cells of malarious chickens produced anemia within 24 hr when injected into normal birds (Soni and Cox, 1975b).

Boreham and Facer (1974b) detected an antibody in blood of rabbits with experimental trypanosomiasis that reacted with fibrinogen,

fibrin and fibrinogen degradation products (FDP). In our experiments we detected an antibody against fibrinogen (Anti-F) in rats infected with *T. brucei rhodesiense*. High titers of Anti-F correlated with signs of anemia, splenomegaly and glomerulonephritis. FITC conjugates of Anti-F, stimulated in rats by injections of human fibrinogen, reacted with both the erythrocytes and trypanosomes. It thus appeared that they were coated with fibrinogen, fibrin and/or FDP. Since Anti-F was present in the blood of these animals, it is conceivable that these antigens had become bound to the cells as immune complexes, possibly by means of receptors for the third component of complement that had been fixed by the complex. The reaction of FITC conjugated IK with the cells and parasites would support this.

Histopathologic studies of kidney tissues of the infected rats demonstrated that glomerulonephritis with splenomegaly and autoantibodies accompanied the syndrome of anemia. The changes noted were swelling of the glomerular tuft, tubular epithelium and hypercellularity of the tuft. These changes were consistent with those observed in rats with acute babesiosis, in rats and chickens with acute malaria and in monkeys with acute trypanosomiasis (Iturri and Cox, 1969; Soni and Cox, 1974; Musoke et al., 1977; Nagle et al., 1974). Since antigen and antibody, which were identical to those eluted from blood cells, were extracted from kidneys and.were detected in the urinary wastes, it was suggested that soluble antigen-antibody complexes had a causal role in nephropathic changes (Soni and Cox, 1975c; Musoke et al., 1977). This suggestion was supported by reported observations on quartan malarial nephritis in African children (Ward and Kibuka-Musoke, 1969). In view of the present evidence that Anti-F

and its antigen were present in the blood of our infected rats and that complement had been fixed, it is possible that complexes of this antibody, fibrinogen products and complement had a role in the observed nephritis.

The incidental observation on the clotting of heparinized or citrated blood drawn by cardiac puncture from rats with acute trypanosomiasis has interested us. In these bleedings the blood was carefully drawn with a glass syringe containing heparinized saline at a ratio of 100 units of sodium heparin per 9 ml of blood, or 38 mg of sodium citrate per 9 ml. The fact that these ratios of anticoagulants have consistently prevented clotting of normal rat blood, and our observation on the terminal morbid signs of the infected rats, led us to suspect that clotting abnormalities might also be part of the disease syndrome of acute *T. brucei rhodesiense* infection of rats.

Coagulation abnormalities resembling disseminated intravascular coagulation (DIC) in infection have been reviewed (McKay, 1965). Boreham and Facer (1974a) reported elevated levels of FDP in blood of rabbits with acute trypanosomiasis, and Greenwood and Whittle (1976) reported similar elevations in Gambian trypanosomiasis. Punyagupta et al. (1974) described pulmonary insufficiency in *Plasmodium falciparum* malaria and conventional coagulation assays indicated DIC in their patients. Johnson et al. (1964) demonstrated that erythrocytes in lung tissue were coated with fibrin during irreversible hemorrhagic shock and considered that this might contribute to the formation of microthrombi. The reactions of FITC conjugated Anti-F with erythrocytes of infected rats here reported may indicate a similar mechanism of microthrombi formation.

The reactions of FITC conjugated Anti-F and IK with trypanosomes may have an important implication. The reactions did indicate that the parasites were coated with the antigens for these autoantibodies, and the interaction with IK indicated that complement had been fixed. Coatings of FDP, Anti-F and fixed complement could have led to conglutination and sequestration of the trypanosomes, thus contributing to the reduced parasitemia that was associated with higher titers of IK and Anti-F.

Elevated titers of CAH have been associated with anemia in rats infected with *Babesia rodhaini, Plasmodium berghei, P. chabaudi, Haemobartonella muris* and *Trypanosoma lewisi* (Schroeder et al., 1966; Cox et al., 1966; Cox and Calaf-Iturri, 1976; Musoke and Cox, 1977; Thoongsuwan and Cox, 1978). Injections of CAH isolated from blood of malarious chickens produced anemia in normal birds. In addition, anemia was also produced in normal chickens by injections of trypsinized autologous erythrocytes (Soni and Cox, 1974, 1975a). Such experiments in rats have not been reported.

From the evidence here cited and in view of modern concepts of the role of soluble antigen-antibody complexes in immunopathogenesis, it is considered that the autoantibodies presently related to acute *T. brucei rhodesiense* infections of rats might have formed complexes with their antigens to contribute to the observed anemia, splenomegaly and nephritis.

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