PRELIMINARY STUDIES ON THE DISEASE MECHANISM OF INFECTIOUS ANEMIA USING *HAEMOBARTONELIA MURIS* INFECTIONS IN RATS

Thesis for the Degree of M. S.
MICHIGAN STATE UNIVERSITY
Elizabeth F. Nelson
1970

ABSTRACT

PRELIMINARY STUDIES ON THE DISEASE MECHANISM OF INFECTIOUS ANEMIA USING HAEMOBARTONELLA MURIS INFECTIONS IN RATS

By

Elizabeth F. Nelson

The infectious anemias include a wide variety of different infections all characterized by such common characteristics as anemia, splenamegaly and obscure immunologic mechanisms. Studies of the malarias, babesiosis and anaplasmosis have shown that anemia was not always commensurated with parasitemia and was often more relatable to immunologic mechanisms.

Substances were found present in the sera of animals with acute malaria or acute babesiosis which produced an anemia when injected into normal animals, and these animals were resistant to subsequent challenge. There were also factors in the acute sera of animals with these infections that were not parasite specific and that would cross react serologically with the sera of animals recovered from other malarial or babesial infections. These factors were also shown to cross protect so that animals treated with the acute serum factors from one infection were resistant to challenge with other malarial or babesial infections.

Elizabeth F. Nelson

The fact that nonspecific serum substances occurred in a broad spectrum of different infectious anemias lead to the study of the serum of animals with infections of acute Haemobartonella muris. The sera of animals recovered from active H. muris infections were found to react serologically with serum substances from animals with acute infections of Plasmodium lophurae and Babesia rodhaini. However hemagglutinins for trypsinized red blood cells were not detected in the sera of rats with acute infections of H. muris, and anemia was not produced in normal rats treated with acute sera. The latter results were in contrast to those seen with the hemosporidian infections. The implications of these results were discussed and a theoretical discussion of its relationship to infectious anemia was also discussed.

PRELIMINARY STUDIES ON THE DISEASE MECHANISM OF INFECTIOUS ANEMIA USING *HAEMOBARTONELLA MURIS* INFECTIONS IN RATS

By

Elizabeth F. Nelson

A THESIS

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

MASTER OF SCIENCE

Department of Microbiology and Public Health

1970

ACKNOWLEDGEMENTS

The author gratefully acknowledges the guidance, advice and criticism of Dr. Herbert W. Cox during the course of this work.

Sincere appreciation is due Dr. Robert M. Corwin for his continuing encouragement, ready advice and assistance, and steadfast faith in me.

The writer also thanks Dr. Colin G. Ludford for his helpful advice and for reviewing the manuscript.

The technical assistance of Miss Carmen Vasques is gratefully acknowledged.

Thanks are also due to Miss Roberta Milar, Dale Goss and Harold McAllister from the Department of Microbiology and Public Health.

The author deeply appreciates the financial support furnished Dr. Herbert W. Cox by the Army Medical Research and Development Command which made it possible to conduct this investigation.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Anemia is a common symptom found in a wide variety of different erythrocytic infections. Early work with malaria demonstrated that the anemia was often not commensurate with the parasitemia (Maegraith, 1948). Recently it has been shown that the anemia is more relatable to immunologic mechanisms than to destruction of erythrocytes by parasites (Zuckerman, 1960; McGhee, 1960, 1964; Cox *et al.*, 1966). Similar results have been reported with babesiosis (Schroeder *et al.*, 1966) and with the rickettsial-like infection, *Anaplasma* (Ristic, 1961; Mann and Ristic, 1963 a,b).

Substances were found present in the sera of animals with acute malaria and acute babesiosis that produce a anemia when injected into normal animals, and these animals were resistant to subsequent challenge (Corwin and McGhee, 1966; Sibinovic *et al.*, 1967 a,b, 1969). Cox *et al.* (1968) have shown that the factors in the acute sera are not parasite specific and will cross react serologically with the sera of animals recovered from other malarial or babesial infections. These factors were also shown to cross protect so that animals treated with the acute serum factors from one infection were resistant to challenge with other malarial or babesial

infections (Cox, 1966; Cox and Milar, 1968; Corwin and Cox, 1969).

Thus it appears that there are substances free in the serum of animals with acute hemosporidian infections which act not only in producing the anemia but which are also immunogenic. Since these factors have been found in several different parasitic systems, it was of interest to determine whether the anemia and immunity found in other erythrocytic infections might also be mediated by similar mechanisms. The relationship of immunologic mechanisms to the anemia in Anaplasma suggests that similar mechanisms might be present in the infections of other rickettsial-like organisms, such as Haemobartonella. Thus it is the purpose of this paper to attempt to show the presence of immunologic mechanisms which may be active in producing the anemia found in Haemobartonella infections.

LITERATURE REVIEW

1. HAEMOBARTONELLA

Small microorganisms normally found closely associated with erythrocytes and producing anemia have been found in various vertebrate hosts. They have been called the "bartonellas," and the group is thought to be made up of three genera: Bartonalla, Haemobartonella and Eperythrozoon (Weinman, 1968).

These genera are related by several broad characteristics. They all appear to be situated on the surface of red blood cells, cause an acute anemia in their vertebrate host, are carried latently for long periods of time, and are transmitted by an arthropod vector. There are, however, important features which separate Bartonella from the other two genera.

Bartonella was first described in 1905 by Alberto Barton (1905). It was shown to cause two different clinical conditions. The initial symptom usually is an acute febrile anemia known as Oroya fever. Survivors usually develop benign skin eruptions known as verruga peruana. These two clinical forms, which may occur together or separately, constitute Carrión's disease. Relapses usually occur in the

verrucose form. The infection is limited to a narrow strip of the Andes in Peru, Columbia and Equador. In endemic areas there are many asymptomatic latent infections. Man and the arthropod vector, Phlebotomus spp., are the only spontaneous hosts for the organism (Schultz, 1968; Weinman, 1944, 1968).

Bartonella has been shown to be bacteria-like with a cell wall and lophotrichate flagella (Peters and Wigand, 1955). It is grown readily on culture media being rod-shaped in young cultures and mostly coccoid in older ones. It grows in tissues of the body other than the blood, the reticulo-endothelial system and vascular endothelium, and thus causes the cutaneous eruptions as well as anemia. The in vivo multiplication of the organism has not been shown to be directly controlled by the spleen. It is not affected by arsenicals, but by the more classical antibiotics such as penicillin. Peters and Wigand (1955), taking account of all these factors, suggested that Bartonella be classified among the bacteria. Breed, Murray and Smith (1957) in Bergey's Manual of Determinative Bacteriology place it in the order Rickettsiales due to the close association it has with the vascular endothelium and erythrocytes, and to analogies in size and arthropod transmission. However, Peters and Wigand (1955) failed to show a serologic relationship between Bartonella and the rickettsiae. This, in addition to the fact that Bartonella is flagellated and can be cultivated on artificial media, makes the classification tenuous.

Haemobartonella spp. and Eperythrozoon spp., in contrast, are natural parasites of erythrocytes. They have not been shown to multiply in other tissues and thus produce no cutaneous eruptions. They have a worldwide distribution, occurring naturally in a variety of vertebrate hosts, but are not known to occur in man. Disease, in most species, is rarely produced except in splenectomized animals. The notable exceptions are H. felis in cats, E. ovis in sheep and E. suis in swine which all cause a marked anemia in normal animals, although mortality is rare. The organisms are not easily cultured, are markedly influenced by arsenicals, and are transmitted by arthropods.

Breed, Murray and Smith (1957) place these organisms in the order Rickettsiales, family Bartonellaceae. However, Peters and Wigand (1955) believe that even though Bartonella can be classified with fair certainty, the classification of Haemobartonella and Eperythrozoon is still questionable. They are distinguishable from true protozoa by their small size and lack of cellular structure. They differ from mycoplasmas and L forms of bacteria in that they lack pleomorphism, their method of multiplication, and in their ability to be readily grown on culture media. The analogies with the rickettsiae are in size, transmission by insects, and association with blood elements. However, they differ in structural details from the rickettsiae and show no serologic relationship with them. They are quite different from viruses

because of their extracellular mode of life. Thus, there is no place in the present taxonomic system of the bacteria or viruses where these two organisms can be properly placed (Peters and Wigand, 1955; Tanaka et al., 1965).

Kreier and Ristic (1968) list 28 different species of Haemobartonella plus eight species of animals from whose blood bodies resembling Haemobartonella have been described. The species are distinguished mainly on the basis of host origin, subtle morphologic differences and susceptibility of other hosts to experimental infections. Probably some of these are misclassifications while others are misnomers or synonyms.

Haemobartonella muris is the type species of the genus (Weinman, 1944). It was first observed by Mayer and Zeiss (1920) in the blood of rats recovering from treated trypanosome infections. Mayer (1921) proposed the name Bartonella muris because of the close morphologic resemblance of the parasite to Bartonella bacilliformis. Later, Tyzzer and Weinman (1939) proposed a separate genus, Haemobartonella, because the organism did not multiply outside the blood and did not cause cutaneous eruptions, it rarely produced disease without removal of the spleen, and it was markedly affected by arsenotherapy.

Light microscopy of stained blood films shows the organisms as very pleomorphic, but predominantly rod-shaped often with rounded ends, although coccoid and dumbbell forms

are common. They may occur individually, in short chains, or in high infections in parallel groups. The coccoids have a diameter of 0.1 to 0.2 μ while the rod-forms measure 0.7 μ by 0.1 μ (Weinman, 1944). Romanowsky-type stains are preferred with these organisms which appear intensely red with Giemsa's stain and bluish with Wright's stain. There is no differentiation into cytoplasm and nuclear region. They are gram-negative. Enzyme treatments and histochemical staining indicate the presence of both RNA and DNA diffusely distributed in the organism (Peters and Wigand, 1955).

The many various attempts at cultivating the parasite have not proved satisfactory although a wide variety of different blood-containing media has been tried (Kreier and Ristic, 1968). However, Ford and Murray (1959) using serum-tryptone media have reported cultivation of a diplococcus from Sprague-Dawley rats known to be H. muris carriers. The isolated organism was infective for splenectomized rats, although the disease produced was not described. Conflicting results have also been reported on the ability to culture the organism in embryonated eggs. Laskowski et al. (1950) reported success, although the isolated organism was not infective when reinoculated into splenectomized rats. Wigand (1958) was unable to show growth.

There have also been conflicting reports on motility. Early reports described Brownian movement, a slow sinuous motion in the red blood cell, and a rapid motion in the

plasma (Weinman, 1944). Later work using phase contrast microscopy (Wigand and Peters, 1952; Tanaka et al., 1965) failed to demonstrate motility. Moore et al. (1965) using diffusion techniques reported the organism as nonmotile. Wigand and Peters (1950) failed to show flagella by light microscopy.

Because of the extremely small size of Haemobartonella muris, more detailed work was done with the electron microscope. Peters and Wigand (1955) showed the organisms to be coccoid in shape with the different pleomorphic forms being chains of the coccoid particles. They found no structural details within the cells and no cell wall resembling that of bacteria. Tanaka et al. (1965) used ultrathin sections to study the cells of the peripherial blood of rats suffering with H. muris infections. They found the organism to be round or slightly ellipsoidal in shape, ranging from 350 to 700 mμ in diameter. There was only a single limiting membrane showing unit membrane structure and no cell wall. Internally there were a number of diffusely distributed small granules, 10 to 15 mμ in size, and often a few filamentous structures of varying widths. No distinct nucleoid structure was seen. The bodies occurred singly, in pairs, and frequently in groups in depressions on the erythrocyte surface. Tangential sections showed the bodies arranged linearly in shallow canaliculi. About half the surface area of the parasite was in contact with the erythrocyte with the distance

between the limiting membranes of the organism and the erythrocyte being rather constant, from 10 to 15 mμ. The red blood cell-parasite combination was rather tenacious in that various enzymes and reagents normally reported effective in isolating tissue cells or in eluting virus did not release the parasite from the erythrocyte.

Tanaka et al. (1965) also demonstrated various morphologic forms which they considered to be stages of binary fission. They found elongate forms, dumbbell forms and pairs of round forms connected to each other. This morphologic evidence along with the linear arrangement of the parasites on the erythrocyte surface suggested to them binary fission.

Transmission of the disease was shown by Cannon and McClelland (1928) to be by the spined rat louse, Polyplax spinulosa. Early work demonstrated that contact transmission and infection by ingestion did not occur (Weinman, 1944). Crystal (1958, 1959) studied the transmission of the disease fairly extensively and found that neither ingested lice nor lice feces were infective. He did show that lice crushed onto the skin produced infection in susceptible rats. He found that a louse could transfer the infection by biting if a short period (4 hours) passed between bites, but lice starved for 24 hours could not infect by biting. An incubation period of 4 to 7 days was required after being fed on a carrier rat before the lice again became infective. These facts suggest that the louse may act as either a mechanical or as a biological carrier of H. muris.

The course of the infection depends on the state of the host. H. muris is a natural infection of the albino rat and is carried latently in many laboratory strains. Usually it remains latent unless activated by splenectomy.

Following splenectomy there is often a slight increase in erythrocyte numbers due to a relative anhydremia which is seen in normal as well as in carrier animals (Weinman, 1938). The incubation period is usually 3 to 5 days but can vary from one to 20 days or more following splenetomy. Shortly before symptoms occur, there is a precipitous decrease in erythrocytes with the animal's erythrocyte count dropping as much as 5 or 6 million per mm^3 in 24 hours. The parasites occur in the blood a few days before the appearance of the anemia, reaching maximal numbers just before the terminal drop in red cells (Weinman, 1944). During the crisis, the animals become quiet, lose their appetite and rapidly lose weight. They take on a characteristic huddled appearance, with roughened hair coat and a distinct pallor of feet, ears, nose and eyes. Hemoglobinuria frequently occurs. The symptoms last for a few days, and then 40 to 80% of the animals die (Griesemer, 1958). When recovery takes place, the anemia and parasites gradually disappear, and the blood picture returns to normal in one to two months (Weinman, 1944). Relapses may occur at intervals of several weeks.

Blood examination during crisis shows a very rapid sedimentation rate even after correction for anemia. The hematocrit may be below 10%, the erythrocyte count below one

million per mm^3, and the hemoglobin 3 or 4 g. per 100 ml. The mean corpuscular volume increases from a normal of about 61 μ^3 to 125 μ^3 or more (Griesemer, 1958). The anemia is of the macrocytic and hypochromic type (Weinman, 1938) with the numbers of leucocytes rapidly increasing, starting even before the onset of anemia, up to 40,000 per mm^3 or more. There is also a drastic rise in reticulocyte numbers, from the normal to less that 5% in adults to 40 to 60%. Diagnosis is made by demonstration of the organisms in the blood.

Autopsy reveals that the tissues are pale and have an icteric ringe. Fluid accumulates in the thoracic and abdominal cavity, and the mesenteries are congested. The liver increases in size, becomes soft and friable and appears yellow. Microscopically there are multiple, centrolobular necrotic foci, a diffuse change in fatty content of the hepatic cells and a mobilization of the Kupffer cells which contain numerous erythrocytes and iron pigment. The kidneys are pale and swollen unless hemoglobinuria occurs, in which case they are blackish-red. Microscopically there are scattered zones of glomerular necrosis, and in acute cases there is severe tubular damage with necrotic lesions and desquamation of the epithelium. The bladder is distended with dark purple hemoglobinuric urine, and the urine contains hyaline, granular and blood casts, desquamated epithelia, blood elements and large amounts of protein. The bone marrow is red, almost watery and has a low myeloerythroid ratio (Weinman, 1938, 1944; Griesemer, 1958).

The infection can also be transmitted by inoculation. Whole blood, washed erythrocytes, plasma, liver suspensions and the hemoglobinuric urine are infective. Infection can be produced by the common routes of inoculation but not orally. Wigand (1958) found that as few as two parasitized erythrocytes could produce the disease in splenectomized rats when the diluent was uninfected rat blood. He also found that comparable numbers of parasitized erythrocytes could infect intact rats. Kessler (1943 a,b) observed age to be an important factor in determining resistance to H. muris infection with rats of 12 days of age being most resistant. Those of 27 days were most susceptible in terms of short prepatent period and survival time, but older animals showed essentially the same course of infection. The resistance of the young rats was not due to passive transfer of some inhibitory or hormone-like substance produced in the spleen of the mother, for rats born of splenectomized mothers were as resistant as those born of normal ones. He also found the length of the prepatent period to be a direct function of the infective inoculum.

Weinman (1944) pointed out that the actual outcome of the infection seemed to be controlled by two principal factors 1) the presence of the spleen and 2) the presence of the parasite. Adult non-splenectomized Haemobartonella-free rats show a slight disease and transient anemia when inoculated with the parasite while splenectomized Haemobartonella-free rats when inoculated show the same acute disease as seen

in splenectomized carrier rats. However, adult non-splenectomized carrier rats show no haemobartonellosis on inoculation, although very large doses can lead to a transient parasitemia and anemia. Adult splenectomized infected rats are generally refractile for 15 weeks to 8 months after the acute disease. The animals then eliminate the infection, become susceptible again and suffer the acute disease on reinoculation. Thus immunity to disease is a state of premunition, rather than classic immunity. The spleen aids in limiting parasite multiplication and subsequent anemia, but allows the parasite to exist indefinitely in the host. The spleen's role, although usually decisive in the outcome of the infection, is still not well understood.

As little as 10% of the spleen will prevent relapses in carrier rats (Domenico, 1956), and a quarter left intact will prevent the development of the disease when the organisms are injected into normal rats (Perla and Marmorston-Gottesman, 1930).

Perla and Marmorston-Gottesman (1930) demonstrated that minute splenic autoplastic transplants made seven weeks prior to splenectomy protected a number of splenectomized rats against Haemobartonella infection. Removal of the transplants and histological studies showed destruction of the pulp in the unprotected rats and regeneration in the protected rats. An explanation for these data was that the reticular and endothelial cells of the pulp of the spleen elaborated some

secretory substance which prevents Haemobartonella infection. Later Perla and Marmorston-Gottesman (1932) prepared an aqueous lipid extract from ox spleen which seemed to have preventive action, but eventually they reported (1941) that it seemed to have lost its effectiveness in preventing infection.

Serological studies were undertaken to determine the role, if any, of serum substances in producing immunity. It was found that blood, plasma or serum of adult normal rats or of splenectomized rats which had survived the anemia had no curative or preventive effect. Also "immune sera" derived from guinea pigs or rabbits were not effective (Weinman, 1944). Wigand (1958) could not demonstrate neutralization of the organisms with serum from intact carrier rats, from recovered splenectomized rats or from recovered splenectomized hamsters. Wigand (1956) used antigens prepared by lysis of infected erythrocytes to perform complement fixation tests on sera from infected and noninfected Haemobartonella rats. He found antibodies in non-splenectomized Haemobartonella-free rats about the fifth day after inoculation, and they reached a maximum in about 3 to 5 weeks. Thereafter, they persisted for a considerable period of time. When the animals were treated with neoarsphenamine, the antibodies gradually decreased. There was a temporary fall in antibody titer in infected animals after splenectomy and during relapses. The titers increased again after the peak parasitemia had passed.

No positive Weil-Felix reactions have been obtained (Kreier and Ristic, 1968). Complement fixation was not found when serum from infected rats was tested with antigens of endemic or epidemic typhus, Rocky Mountain Spotted fever, psittacosis or vaccinia nor in Wasserman tests.

Parabiotic studies were done in an attempt to study further the role of humoral substances in immunity. It was found that removal of the spleen of one parabiont had no effect on the disease. However, subsequent removal of the second spleen lead to the typical acute disease in both animals (Flaum and Lauda, 1931; Scheff et al., 1956). Flaum and Lauda (1931) interpreted their results to mean that there was a protective humoral substance produced in the spleen, but because the blood of the splenectomized parabiont passes through the spleen of the partner, the spleen may have been acting as a filter. Demenico and Andreotti (1959) were interested in determining whether there was a humoral substance elaborated by the spleen which played a protective role in the disease. They found that a splenectomized rat could be protected when attached to a normal partner in a true parabiotic situation, i.e., where there was cross circulation as detected by the circulation of ^{51}Cr-labelled erythrocytes in both animals. However, the splenectomized rat became infected when there was not a true parabiotic situation, i.e., where there was union and fluid exchange but no cross circulation as determined by the lack of circulation of

^{51}Cr-labelled erythrocytes in both animals. They believed actual fluid exchange took place between the two false parabionts, for arsenical therapy given to the unsplenectomized member suppressed the infection in the splenectomized individual. The authors concluded that splenic protection was local and not mediated by humoral substances.

Anigstein and Pomerat (1945) induced typical haemobartonellosis in carrier rats with homologous antireticuloendothelial serum. The antiserum was prepared against the elements of the spleen and bone marrow. Later, Pomerat et al. (1947) were unable to induce Haemobartonella infection in carrier rats injected with antiserum against rat erythrocytes even though clinical anemia was produced. These data indicate a role for the reticuloendothelial system in control of haemobartonellosis. Thomas et al. (1949) described the gross histopathology of carrier rats that had received the anti-erythrocytic serum, but they did not discuss the effects of the antispleen serum.

Cortisone is known to suppress the inflammatory reaction and to diminish either phagocytosis or the rate at which the cells of the reticuloendothelial system dispose of ingested particles (Humphrey and White, 1963). Laskowski et al. (1954) intraperitoneally administered 7.5 mg of cortisone daily for a week prior to splenectomy and 5 days thereafter. They found that the disease developed in a typical manner. When the same amount was administered starting the day of splenectomy or

3.0 mg/kg doses were given for 3 days prior to the operation, there was no effect on the development of the disease.

Scheff et al. (1956) inoculated 5 mg doses of cortisone subcutaneously for various periods of time. The treatment was given to a group of infected rats for 24 days prior to splenectomy. During this period the infection remained latent, although there was a slight but constant rise in the number of erythrocytes which was interpreted to be due to bone marrow stimulation. The treatments were discontinued, and the animals splenectomized. No disease developed. However, rats treated only 10 days prior to splenectomy showed severe anemia and typical disease. The cortisone was also found to induce relapse with severe anemia and large numbers of organisms. Histologic studies showed that cortisone treatment administered over a long period of time caused atrophy of the splenic white pulp. The workers felt that the cortisone stimulated the hematopoietic potential of the bone marrow and suppressed that of the spleen. The bone marrow stimulation was then enough to maintain the host-parasite equilibrium when the spleen was removed, and the animal suffered no relapse after splenectomy. When the stimulation was not given long enough, the aggravation of the reticuloendothelial system was enough to cause the animal to relapse.

Compensatory changes were found when young rats (10 weeks of age) were splenectomized 10 weeks prior to infection (Kessler, 1943 b). These animals had a mortality rate of 54% as compared to 100% for controls receiving the

inoculation upon splenectomy. They believed that the compensation was due to changes in the reticuloendothelial system.

Elko and Cantrell (1968) showed enhanced removal of intravenously injected colloidal carbon in rats with active Haemobartonella infections. This was true whether the infection was given to intact rats or induced by splenectomy. Cantrell and Elko (1966) had previously shown that splenectomy alone does not effect carbon clearance significantly. The clearance in carrier rats was about the same as in normal noncarrier rats. Also carrier rats, when splenectomized and treated with oxophenarsine to suppress the Haemobartonella infection, did not show an increased phagocytic index. The authors found this data to indicate that sufficient numbers of the parasite can stimulate the phagocytic system, but since during latency phagocytosis is about normal, hyperphagocytosis is not the sole method of maintaining latency.

Thus a clear picture of the regulatory function of the spleen has still not been elucidated. Probably the spleen acts in conjunction with the bone marrow and other reticuloendothelial elements in the control of the disease, but the exact mechanism is still unknown.

The other outstanding feature of this infection is the profound anemia the organism induces. The mechanism of this anemia still has not been satisfactorily described.

The great erythrocytic regeneration with large numbers of reticulocytes seen during and after the acute phase of the disease has long led workers to search for a cause of erythrocyte destruction rather than a block in erythrocyte production. Weinman (1938) searched for serum hemolysins in the blood of rats suffering from hemoglobinuria and hemoglobinemia. He found none, but the hemoglobinuria suggested erythrolysis. Since he could not detect a serum hemolytic factor, he decided to observe the red cells themselves. He found marked in vitro lysis of erythrocytes from the acute phase of the infection, especially at body temperature (37°C). He concluded that the cause of the lysis was due to damage of the red cells themselves, for lysis occurred only when the parasite was present. Cells from normal animals and from carriers did not demonstrate the lysis. He also found that the blood of animals intensely parasitized showed considerable variance in the amount of hemoglobin liberated in vitro, and this was not completely correlated with the degree of parasitemia. He believed that this was due to the rapidity with which the animal developed immunity against the parasite. He observed the cells themselves and found deformations at the sites where parasites were located, suggesting weaknesses. The dehemoglobinized cells almost always contained parasites. This lead him to conclude that the parasite causes alterations in the erythrocyte surface.

Rudnick and Hollingsworth (1959) studied the survival of ^{51}Cr-labelled erythrocytes in H. muris infected rats. They observed the rate of red blood cell destruction to be markedly accelerated in splenectomized animals coincident with the appearance of the hemolytic anemia. They then took cells at various times during the infection, labelled them and placed them into intact rats. They found that cells taken after splenectomy but before the organisms were present had a normal life span. However, as the organisms began to appear, the life span shortened. When terramycin was given to a recipient rat to control the Haemobartonella infection, the cells were eliminated at the same rate as in normal rats. They interpreted this data as confirming Weinman's work that there was irreversible damage to the erythrocyte caused by direct action of the parasite on the red blood cell membrane. The mechanism of the action was not known.

Thompson et al. (1961) found that the survival of autologous blood in Haemobartonella carrier rats was similar to the survival in normal noncarrier rats. These results coincide with those of Elko and Cantrell (1968) menticned earlier in that some other means than phagocytosis must control the infection in the carrier state.

Elko and Cantrell (1968) also noted high hematocrit values in splenectomized rats on the day preceding the appearance of the anemia. Correlating the changes in hemoglobin concentration, red blood cell counts and packed cell volume,

they too found an increased mean red cell volume which was accompanied by a decrease in mean corpuscular hemoglobin concentration. They interpreted these findings to indicate a disturbance in the osmotic equilibrium of the cell which leads to its initial swelling and eventual rupture.

2. ANEMIA IN ERYTHROCYTIC INFECTIONS

Anemia is a common feature of erythrocytic infections. It can be seen in such diseases as malaria, babesiosis and anaplasmosis as well as in haemobartonellosis and bartonellosis. Early work with the hemosporidian infections led workers to believe the anemia was due to direct lysis of the infected cells by escaping parasites. However, the syndrome of blackwater fever, often noted with cases of Plasmodium falciparium, shows intravascular agglutination of erythrocytes followed by an extensive intravascular hemolysis of both parasitized and unparasitized blood cells (Oliver-Gonzales, 1944). This suggests that some other mechanism than parasitic lysis is destroying red cells. Christophers and Bentley (1908) found considerable phagocytosis of normal red blood cells in the spleen of patients with blackwater fever, and stated that the disease could not be directly attributed to the parasite but must be the result of some other mechanism related to the malarial infection.

The role of phagocytosis in malarial infections was extensively studied, and the work led to the hypothesis of

a cellular immune mechanism. It was observed that before the parasitic crisis, there was scavenging of parasitic and cellular debris by phagocytes of the reticuloendothelial system, and after the crisis there was active phagocytosis of debris and parasitized and normal erythrocytes. Recovery was attributed to the increased phagocytic effectiveness of the individual macrophages and to the increased number of phagocytes (Taliaferro and Mulligan, 1937; Maegraith, 1948); erythrophagocytosis was thought to be the main immune mechanism (Taliaferro and Mulligan, 1937; Coggeshall, 1943).

The principal site of erythrophagocytosis is the spleen. Motulsky et al. (1958 a,b) reviewed the role of the spleen in relationship to anemia and concluded that the spleen probably contributes to anemia by destroying more than a physiologically normal number of red cells. Under certain pathological conditions, the spleen becomes greatly enlarged, and there is a relative stasis of blood flowing through the organ. This circulatory delay leads to sequestration of an abnormal number of red cells in the spleen and allows defective cells to be taken up in great numbers. However, circulatory stasis has been shown to be harmful to erythrocytes, and the repeated passage of cells through the enlarged spleen could lead to cell damage and to eventual cellular destruction by the splenic phagocytes.

Using tissue culture techniques, Marby et al. (1956 a,b) demonstrated that heat-stable, specific, absorbable factors

and complement in normal serum could produce maximal phagocytosis by splenic macrophages of heterologous erythrocytes and homologous erythrocytes modified with immune serum, trypsin or virus (i.e., Newcastle disease virus). Homologous normal erythrocytes were never phagocytized more than slightly. These data suggest that normal serum can act as an opsonin to give maximal erythrophagocytosis of damaged red blood cells without any specific immune mechanisms.

Gorstein and Benacerraf (1960) found striking erythrophagocytosis in the macrophages of the spleen and liver of mice treated with a nonspecific reticuloendothelial system stimulant, zymosan. They thought that the anemia produced was due to the stimulation of phagocytosis alone, for the erythrocytes had normal survival times when injected into normal animals and no gamma globulin was detected on the red cell membranes. Nussenzweig (1967) found that stimulation of the reticuloendothelial system of mice with killed Corynebacterium parvum prior to infection with sporozoites of Plasmodium berghei produced total protection in a considerable number of animals and a delayed infection in all other pretreated animals.

Thus, many believe that the anemia and immunity seen in malaria is a direct consequence of cellular destruction by the cells of the reticuloentothelial system. The enhanced erythrophagocytosis of both parasitized and normal erythrocytes seen during infection is believed to be due to the hypersplenism induced by the infection (George et al., 1966).

The first indication that humoral substances may play a role in inducing anemia was presented by Oliver-Gonzales (1944). He studied the sera of humans with blackwater fever and found a cold agglutinin for human erythrocytes. He suggested that the autoagglutination seen in the disease may be due to an autoagglutinin which results from the immunization of the host with parasite substances related to human isoagglutinogens. Gear (1946) was the first to suggest a role for an autohemolysin in the pathogenisis of blackwater fever. He reviewed the clinical symptoms and correlated them with the observations of Foy et al. (1941, 1945) that cells from healthy donors hemolyze as rapidly as the cells of patients when transfused into a patient with hemolysing blackwater fever, and red cells from a patient with blackwater fever transfused into a healthy recipient, hemolyzed as rapidly as the cells remaining in the patient. This correlation suggested that the hemolytic syndrome of blackwater fever was related to the development of an autoimmune response to erythrocytes altered by the parasites and/or drugs. However, work since has not yet demonstrated a circulating hemolysin (Zuckerman, 1964).

It was not until 1960 that autoantibody was demonstrated in malarial infections. Zuckerman (1960) and Maegraith (1948) had noted that in both vertebrate and human malarias there was often a blood loss more extensive than could be attributed to direct parasitic rupture alone. To explain the phenomenon,

she chose as a working hypothesis that red cells may be sufficiently altered by the parasites to become antigenic and give rise to autoantibodies which of themselves can lead to red cell destruction. In testing this theory she found that rats with Plasmodium berghei had positive antiglobulin tests following the parasitic crisis. Later, however, positive tests were found in animals which had been repeatedly bled or made anemic with phenylhydrazine hydrochloride (Zuckerman and Spira, 1961; Zuckerman, 1963). Thus the antiglobulin test was positive in the absence of any infectious agent, and it occurred before the onset of immunity; with the P. berghei infection however, the tests were not positive until after the time of the onset of acquired immunity.

McGhee (1960, 1964), working with the duck malaria, Plasmodium lophurae, found that ducklings with light or quinine-suppressed infections had an aerythroblastic anemia which approached that seen in ducklings with fulminating blood infections. He suggested an autoimmune mechanism. McGhee and Corwin (1964) studied the bone marrow during the anemic crisis of quinine-suppressed infections and found that the macrophages actively engulged both infected and uninfected red cells.

Corwin and McGhee (1966) found that when plasma from ducklings acutely infected with P. lophurae was intravenously injected into uninfected ducklings, it resulted in anemia and led to the immunization of the ducklings against subsequent challenge. Cox (1966) found similar results with

globulins from acute Plasmodium knowlesi infections. When injected into normal rats, the globulins produced anemia, and later the rats were markedly resistant to challenge with P. berghei. The work of these authors suggests that there are substances free in the serum during the acute infection which act not only in producing an anemia but which also are immunogenic.

Spira and Zuckerman (1965 a,b) suggested that if the anemia is due to an immune mechanism, splenectomy should delay its onset but not abolish it, for the spleen is not only a site of erythrophagocytosis but an important site of antibody production. However, since the spleen is not the exclusive site of either of these functions, the other elements of the lymphoid-macrophage system take them over and the anemia ensues. The results were as predicted; there was a delayed onset in the anemia of splenectomized rats infected with P. berghei. Similar results were found with acute babesial infections in rats (Todorovic et al., 1967).

Cox et al. (1966) showed the anemia in rats infected with P. berghei to be accompanied by extensive phagocytosis of normal as well as infected erythrocytes in the spleen and bone marrow. Correlated with the anemia and erythrophagocytosis was the presence of agglutinins for trypsin-treated homologous erythrocytes. Sera of animals made anemic by repeated bleedings showed only occasional low titers. The authors suggested that the autohemagglutinins they detected

may act as opsonins enhancing the phagocytosis of both normal and parasitized erythrocytes.

Work with other hemosporidian infections has demonstrated similar types of phenomena. Schroeder et al. (1966) found with Babesia rodhaini that the severity of the anemia was not commensurate with the parasitemia, and examination of the spleen and bone marrow showed macrophages phagocytizing both normal and parasitized erythrocytes. Hemagglutinins for trypsin-treated autologous and homologous erythrocytes were associated with the anemia and the onset of erythrophagocytosis in the spleen and bone marrow. This suggested to the workers that since similar findings of autoimmune activity have been found in several different parasitic systems, the anemia of other erythrocytic infections might also be mediated by similar mechanisms.

Sibinovic et al. (1965) found an antigen in the sera of horses with acute infections of a mixture of Babesia caballi and Babesia equi which could be demonstrated in gel precipitation tests with sera of clinically recovered horses. They found similar antigens in the sera of dogs with acute Babesia canis and rats with acute B. rodhaini (Sibinovic et al., 1967 a,b). The antigen was present during the period of acute parasitemia and was not detectable after the parasites had disappeared from the periphaeral blood. Fractionation of the acute sera from each of these three infections produced two different antigens. Antigen A was associated with the

gamma globulins while Antigen B was associated with the beta serum fraction. Each of the antigens reacted with recovered sera from each of the three infections, but not with sera of uninfected animals. In the gel diffusion test, all the A fractions showed lines of identity, and the heterologous B fractions also showed lines of identity. However, no reactions were seen between homologous and heterologous A and B fractions.

Further study of these purified antigens (Sibinovic et al., 1967 b) showed that both fractions were immunogenic although fraction B, especially of B. canis origin, was far superior. A fairly marked and persistant anemia was associated with the injection of the dog origin B antigen and a crude concentrate of antigen of rat origin. The degree of anemia seemed to be correlated with the immunizing effectiveness of the antigens. Thus rats immunized with concentrates of serum antigen prepared from dogs or rats showed marked protection when challenged with B. rodhaini while rats treated with antigens of horse origin showed no anemia and only some protection. Immunofluorescence studies demonstrated that the antigens combined with the erythrocytes after the injection and caused the cells to sequester in the spleen in 24 hours. They were shown to react with the surface or cytoplasm of infected erythrocytes with greater avidity than they did with the parasite (Sibonovic et al., 1969).

Cox et al. (1968) found serologic cross reactions between the serum antigens of rats infected with P. berghei, of rats infected with B. rodhaini, and of ducks infected with P. lophurae and the sera of animals recovered from each of the three infections. Cox and Milar (1968) also demonstrated that mice recovered from Plasmodium chabaudi resisted challenge with B. rodhaini; rats recovered from B. rodhaini were resistant to P. berghei, and rats recovered from P. berghei were resistant to B. rodhaini challenge. Corwin and Cox (1969) used a duck as the host model and found that plasma from acutely infected Plasmodium "spartani" or P. lophurae ducks, from chickens with acute Plasmodium gallinaceum or serum from rats acutely infected with B. rodhaini all induced protection to challenge with P. lophurae. It was suggested that since substances in the serum of acute malarial and babesial infections were shown to cross react aerologically as well as to cross protect, the anemia and subsequent immunity of these infections might be mediated by identical or similar substances.

While these studies were being carried out with the hemosporidian infections, similar results were found with Anaplasma infections. Anaplasma marginale is a rickettsial-like organism infecting erythrocytes of cattle (Breed, Smith and Murray, 1957). The organisms are transferred by a large variety of ticks. The initial body of Anaplasma appears to be structurally identical to the Haemobartonella and

Eperythrozoon parasites except for the presence of a double membrane around the initial body (Ristic, 1960; Small and Ristic, 1968). Tanaka et al. (1965) suggested that this double membrane is probably enough to separate the two groups, but not too remotely.

It was shown that the severity of the macrocytic anemia in anaplasmosis was not necessarily related to the severity of the disease (Ristic, 1968). Thus, some other mechanism was looked for to account for the discrepancy.

Ristic (1961) demonstrated erythrocyte-fixed autohemagglutinins in bovine anaplasmosis by means of an autolytic test. The autohemagglutinin could be eluted from the erythrocytes and used to sensitize normal erythrocytes so they would hemolyze in the autolytic test. Maximum autoantibody titer coincided with the time of the anemic crisis, and this suggested a causal relationship. There was no indication that the autohemagglutinin might produce intravascular hemolysis.

Mann and Ristic (1963 a,b) demonstrated the presence of free serum hemagglutinins for trypsinized red cells during the acute and convalescent stages of Anaplasma infections in calves. They also found the erythrocyte-bound autohemagglutinin. There was no correlation between the results of the hemagglutination tests and the degree of parasitemia. The hemagglutinin was associated with the euglobulin fraction of the serum and was a beta 2-M globulin. It was found to be a nonspecific cold reacting antibody of the type associated with

autoimmune diseases in man (Dacie, 1962). The authors pointed out that Dimopoullos and Bedell (1962) had shown that in the acute stages of Anaplasma infections, biophysical changes occur in the erythrocyte membrane as a result of a decreased phospholipid concentration. They believed that the production of the hemagglutinins may be related to the marked physical and chemical changes which occur in invaded erythrocytes.

The relationship of the autoimmune process to anemia was studied by Schroeder and Ristic (1965 a,b). They confirmed the presence of the erythrocyte-bound and the free serum autohemagglutinins during the acute and convalescent phases of the infection. The hemolytic test readings of the erythrocyte-bound autohemagglutinin were transient, varied proportionally with the changes in the packed cell volume, and showed no correlation with the degree of parasitemia. The hemagglutinin for trypsinized cells appeared about the time of the initial parasitemia and persisted throughout the period of anemia.

The erythrocyte survival studies of Baker et al. (1961) indicated that there was apparently an indiscriminate removal of erythrocytes in anaplasmosis without regard to the presence of the parasite itself. These results suggest that the infection initiates some mechanism which then continues to operate independently of the infection. The presence of the hemagglutinin for trypsinized red blood cells suggests an autoimmune mechanism.

In contrast are the complement fixing antibodies and those detected by the capillary tube agglutination test (Ristic, 1962, 1968). These antibodies were present from the onset of parasitemia and on into the carrier stage of the infection. Thus there appears to be two immunological systems operating in anaplasmosis. The first is directed toward the Anaplasma antigens themselves and can be detected throughout the carrier stage. The second is triggered by the infection, and its intensity and duration is not necessarily correlated with that of the infection. It appears to be directed toward native erythrocyte components and to be at least partially responsible for the anemia.

Kreier et al. (1964), in studying the bone marrow during the course of Anaplasma infections, found no evidence of hemopoietic depression. On the contrary, they found that the erythroid elements became more prominent during the period of severe anemia. Erythrophagocytosis of both normal and Anaplasma infected erythrocytes was seen at the time of crisis, and in some cases, erythrophagocytosis occurred synchronously with the appearance of the agglutinin for trypsinized red blood cells. The data suggested to the authors that the anemia in animals with anaplasmosis was initiated by parasitic damage to erythrocytes and antierythrocyte autoantibodies.

To further elucidate the role of the free serum hemagglutinin, Schroeder and Ristic (1968) conducted experiments to determine whether the autoantibody might be acting as

opsonin and thus bringing about the erythrophagocytosis seen at crisis. They utilized an *in vitro* test in which sensitized erythrocytes were exposed to phagocytosis by macrophages of mouse peritoneal exudate. The degree of opsonic activity was determined by the per cent phagocytes containing one or more erythrocyte and the highest serum dilution used to sensitize the erythrocytes in which at least 3% of the phagocytes contained red blood cells. Maximal opsonization was seen during the period of maximal anemia. Opsonic activity coincided with the erythrophagocytic activity seen in the spleen and bone marrow and was correlated with the development and persistance of the anemia. The free serum hemagglutinins were not the opsonin, for the hemagglutinins could be selectively removed by absorption of the serum with trypsin-treated cells at 25°C. Thus, the hemagglutinins do not seem to sensitize erythrocytes to phagocytosis, and their role in the pathogenesis of anemia still remains unexplained.

Further evidence for the role of immune mechanisms was presented by Jones *et al*. (1968). They compared *A. marginale* infections in intact and splenectomized calves. No significant difference was seen between the duration of the prepatent period in either group; however, the onset of anemia was delayed in the splenectomized group. In the intact group there was a discrepancy between the numbers of erythrocytes destroyed and those infected, and it was not entirely abolished by splenectomy. The data is reminenscent of the work of

Spira and Zuckerman (1965 a,b) with P. berghei. It again suggests that the removal of an important site of antibody production as well as erythrophagocytosis delays the anemia because the autoimmune response cannot develop. That the anemia occurs, although it appears to be more relatable to the parasitemia, would indicate that other organs of the reticuloendothelial system are taking over the splenic functions.

Much of the data related to anemia in Anaplasma seems to support the idea that the infectious agent triggers immunologic reactions which contribute to the pathogensis of the disease. They are suggestive of an autoantibody-like reaction occurring during the infection which plays a role in producing the anemia.

Antigens of several types have been extracted from the blood and erythrocytes of Anaplasma infected animals (for a review, see Ristic, 1968). In an attempt to induce active immunity, preparations of whole blood, washed erythrocytes, a complement fixing Anaplasma antigen, the antigen used in the capillary tube agglutination test, the soluble erythrocytic antigen known as the "protamine sulfate antigen," free serum antigen known as "exoantigen," and killed Anaplasma vaccines were used. No clinically useful immune response was seen in mature cattle treated with any of these antigens. However, the animals treated with the killed Anaplasma vaccines had only 60 to 80% of the parasitemias seen in

non-vaccinated animals, although the anemias were often similar. The treatment of animals with these antigens resulted in the appearance of agglutinating, precipitating and complement fixing antibodies which persisted for approximately 3 days to 4 months (Ristic, 1968).

A review of the literature of some erythrocytic infections points to an underlying mechanism in the production of the anemia, i.e., that the anemia seen in these infections is related not only to direct parasitic destruction but also to erythrophagocytosis of infected and normal red blood cells. The mechanism which leads to an anemia not commensurate with the parasitemia seems to be mediated by an autoimmune-type response induced by the parasite (for reviews, see Zuckerman, 1964, 1966; Schroeder and Ristic, 1968). It appears that with the hemosporidian infections the anemia-inducing component present in the sera of acutely infected animals leads to a nonspecific type of immunity. It was of interest to determine if similar mechanisms might be found in the erythrocyte infection, H. muris. Its close relationship to Anaplasma suggests that the severe anemia seen in Haemobartonella infections might be mediated by similar factors. The alterations in the cell surface caused by the parasites as reported by Weinman (1938) could expose antigenic sites which induce antibodies reactive against all red blood cells, and these antibodies may play a role in inducing the anemia.

MATERIALS AND METHODS

Maintenance of Infections: *Haemobartonella muris* parasites were present as a latent, contaminating infection in male Wistar rats obtained from Harlan Industries, Cumberland, Indiana. Upon splenectomy, the infection became patent. The parasites were maintained in male Sprague-Dawley rats obtained from Spartan Research Animals, Inc., Haslett, Michigan, which were free of the *Haemobartonella* contaminant. At monthly intervals, the parasites were transferred by intraperitoneal inoculation of infected erythrocytes. Later, Wistar rats were received from Harlan Industries that were *Haemobartonella*-free, and these rats were used for experimentation.

The *Plasmodium lophurae* and *Babesia rodhaini* infections used were obtained from Dr. Paul E. Thompson, Parke, Davis and Co., Ann Arbor, Michigan. The *P. lophurae* infection was maintained by blood passage at weekly intervals in 4 to 6 week old white Pekin ducklings obtained from Ridgeway Hatcheries, La Rue, Ohio. The *B. rodhaini* infection was maintained by blood passage in white Swiss mice obtained from Spartan Research Animals, Inc.

Hematological Methods: Blood for preparing films and red blood cell counts was obtained by snipping the tip of the tail of the rat. Blood films were stained with either Wright's or Giemsa's stain. Red blood cell counts were made either with a hemocytometer or a Coulter Model B Counter. Blood to be counted with a hemocytometer was collected in red blood cell hemocytometer pipettes and diluted with Hayem's solution. Counts were made microscopically using a Spencer hemocytometer counting chamber. Blood to be counted with the Coulter counter was collected in 10 mm^3 amounts in Sahli pipettes and diluted in 0.85% saline solution.

Splenectomization: The rats were anesthetized by intraperitoneal injection of approximately 0.5 ml of Pentobarbital diluted in 0.5 ml of 0.85% saline. An incision was made in the left side of the animal below the costal margin of the rib cage. The splenic vessels were tied off with Supramid synthetic suture of medium strength, and the spleen removed by cutting distal to the ligature. The incision was sewn up using Supramid suture. The animals usually regained consciousness about an hour post operation and were allowed at least two weeks recovery before being used experimentally. If they carried the Haemobartonella infection, the rats became acutely anemic and died within about a week.

Preparation of the Antigen: Ducklings, 6 to 8 weeks of age, were injected intravenously with 2 to 3 ml of heavily

parasitized blood from P. lophurae-infected ducks. When parasitemia was near maximal levels, the ducks were exsanguinated by cardiac puncture. One ml of heparinized saline solution (100 units) was added to each 10 ml of duck blood collected.

Rats (Wistar) were inoculated intraperitoneally with 0.2 ml of heavily parasitized blood from rats infected with B. rodhaini. When parasitemia reached its peak, the rats were etherized and bled for plasma by cardiac puncture using one ml of heparinized saline per 10 ml of blood collected.

In both cases, the plasma and the cells were separated by centrifugation. The plasma was stored at -20°C. When the plasma was to be processed, it was thawed, and fibrin clots were removed by centrifugation at 2000 G and 4°C for 15 minutes.

The plasma from acute P. lophurae infections was filtered through an 8% granulated agar column (Difco, Detroit, Michigan). Ten ml samples of plasma were processed through the agar column (2.0 by 40.0 cm) and eluted in 10 ml samples with 0.85% saline. Those samples which contained protein as detected with 10% $BaSO_4$ were concentrated by centrifugation at 47,000 rpm (190,000 G) in the 50 rotor of a Spinco Model L centrifuge for 15 hours. The procedure resulted in the separation of a clear supernatant and a viscous or amber residue over a pellet (Sibinovic et al., 1967 a). The residue and pellets were resuspended in 1 ml of phosphate buffered saline (PBS, 1 molar phosphate in 0.85% NaCl, pH 7.2), and each tested for antigen activity by using it to

sensitize bentonite particles and testing it in the tube bentonite flocculation test against P. lophurae plasma from recovered ducks (Cox et al., 1968). The active samples were pooled and used as test antigen.

The acute babesial plasma, after the precipitated fibrin was removed, was salted with 18% Na_2SO_4 by placing the plasma in 8-mm dialysis tubing and dialysing against 18% Na_2SO_4 at 4°C for 2 hours. The material was removed from the dialysis tube and centrifuged at 2000 G for one hour to sediment the precipitated globulins. The supernatant fluid was discarded. The sediment was resuspended in a minimum volume of 0.85% saline and dialyzed in 20-mm tubing against 0.85% saline at 5°C for 48 hours. The material from the tubing was centrifuged as before and reconstituted in a minimum volume of saline. The globulins were stored at -20°C until needed.

Preparation of the Bentonite Stock Suspension: The tube bentonite flocculation (TBF) test was used for examination of sera and plasma from experimental and control animals. The stock suspension was prepared as described by Bozicevich et al. (1960). Crude bentonite was obtained from the American Colloid Company, Skokie, Illinois, under the trade name Volclay Bentonite, BC. The material was pulverized with a mortar and pestle, and 1.5 g of the powder weighed out and suspended in 300 ml of distilled water. The mixture was homogenized in a Waring Blender for 2 minutes, allowed to stand for 5 minutes and rehomogenized for another 2 minutes.

The blended mixture was then diluted to 1,500 ml with distilled water in a large graduate cylinder and stirred for about a minute. It was then allowed to stand at room temperature for an hour. The supernatant was poured into 100 ml centrifuge tubes and centrifuged for 15 minutes at 500 G in an International Swinging Bucket Centrifuge. The supernatant from this centrifugation was then recentrifuged at 750-800 G for 15 minutes. The sediment was resuspended in 300 ml of distilled water and homogenized for one minute. This mixture was the stock bentonite solution and was stored at 5°C until needed for testing.

Sensitization of Bentonite Particles: The stock bentonite suspension was sensitized by adding 10 ml of the stock solution to 5 ml of the antigen concentrate diluted 1:25 with PBS. The mixture was allowed to stand overnight at 5°C. One ml of 0.1% aqueous methylene blue was added to the sensitized mixture and shaken. The mixture was centrifuged at 800 G for 5 minutes, the supernatant removed, and the sediment resuspended in 10 ml PBS. After a second washing, the sediment was again suspended in 10 ml PBS, and 0.2 ml of 0.5% bovine serum albumin solution in distilled water was added. The suspension was stored at 5°C and was used that day for testing.

Tube Bentonite Flocculation Test: Sera to be tested were diluted in PBS in serial twofold dilutions starting at 1:20. The final test volume of each dilution was 0.1 ml. One tenth

ml of the bentonite-acute globulin suspension was added to each dilution. The mixtures were agitated on a rotary shaker at 120 oscillations per minute for 20 minutes. Each test was read within 10 minutes after shaking. A button-like aggregate of particles constituted a positive reaction. Control sera from ducks recovered from P. "spartani" infections (Corwin and Cox, 1969) gave a titer of 1:640 in the test while normal duck serum pool was invariably negative. Pooled sera of rats recovered from B. rodhaini titered 1:160 while pooled normal rat sera were invariably negative. A saline control was also used to demonstrate that nonspecific agglutination of the bentonite-acute globulin suspension did not occur.

Hemagglutination (HA) tests: HA tests with trypsin-treated normal rat erythrocytes were carried out on various recovered and acute Haemobartonella plasma. The technique used was developed by Morton and Pickles (1947) and modified by Mann and Ristic (1963 a). The trypsin solution was prepared by adding 0.25 g trypsin (Trypsin, 1:250, Difco, Detroit, Michigan) to 100 ml of 0.85% saline. If the solution was cloudy it was filtered through Whatman no. 2 filter paper. Erythrocytes were obtained by cardiac puncture from normal rats, and 2 ml of blood was mixed with 1 ml of heparinized saline. The plasma was removed after centrifuging the blood at 800 G for 10 minutes. The cells were then washed twice in 5.0 ml of saline solution. To 0.5 ml of the packed washed cells, 4.5 ml of the 0.25% trypsin solution

was added, and the mixture incubated for 20 min in a 37°C water bath. The treated cells were washed 3 times with 5.0 ml of saline centrifuging at 800 G for 10 minutes after mixing. A 2% suspension of the packed treated cells was prepared in 0.85% saline. The plasmas to be tested were diluted in saline in serial twofold dilutions to a final test volume of 0.2 ml. An equal quantity of the 2% red blood cell suspension was added to each dilution, and the test was incubated at room temperature for 4 hours and overnight at 5°C. A positive test was represented by the formation of a single clump which did not break up readily on mild agitation of the tube. A negative test was characterized by a uniform resuspension of the red blood cells in the saline following mild agitation. The aggregates that remained intact after agitation were considered 4+ and less tenacious aggregates were 3+, 2+, 1+. Because trypsinized erythrocytes tend to be excessively sensitive to agglutination, readings of 1+ were considered doubtful and thus negative.

RESULTS

Haemobartonella Infection in Haemobartonella-free Rats: A group of 15 Sprague-Dawley male rats were splenectomized and observed for possible exacerbation of H. muris infection for at least two weeks. After this period, when no sign of the infection was seen, these rats, along with 25 normal intact rats, were all bled by cardiac puncture after mild etherization for 5.0 ml of blood. Each individual sample was stored overnight at 5°C, and the serum separated from the clot after mild centrifugation. The samples were then stored at -20°C.

Eleven splenectomized and 10 intact rats were infected intraperitoneally with 0.1 ml of a 1% suspension of blood from H. muris carrier rats (stock Wistar rats). Ten intact rats were set aside for later infection with H. muris, and 9 rats were used as controls. Daily red blood cell counts and blood smears were taken from all rats to follow the course of the infection and anemia. By the ninth day after infection, the splenectomized rats all had high parasitemias and were very anemic and moribund (Figure 1). They were exsanguinated by cardiac puncture for plasma. The plasma was harvested and stored at -20°C. One ml of the concentrated infected red

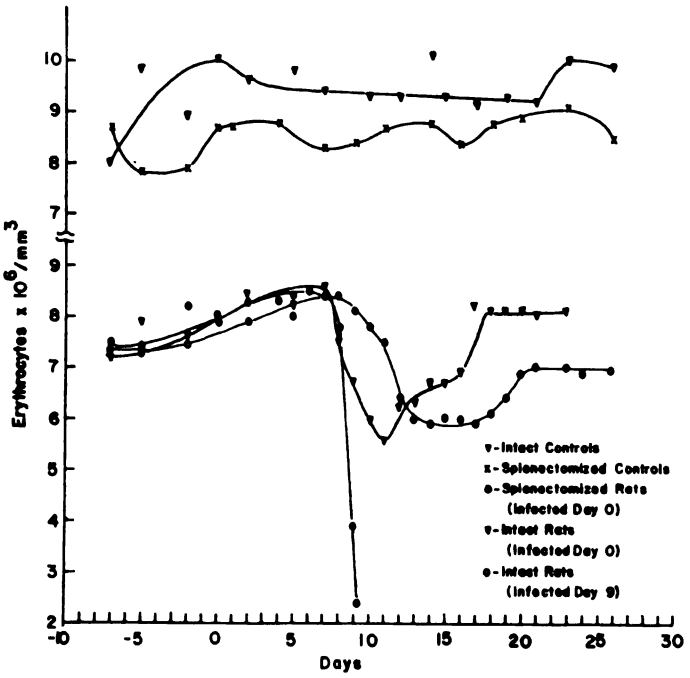

Figure 1. The course of Haemobartonella muris infections in splenectomized and intact rats.

cells was injected intraperitoneally into each of the group of 10 intact rats. Erythrocyte counts and blood smears were continued on these animals. When the intact rats had recovered as seen by the return of the erythrocyte counts to a normal level and the disappearance of detectable parasites from the blood, they were bled at weekly intervals for 2 or 3 weeks. The plasmas were stored at -20°C. Table 1 shows the results of HA tests carried out with the plasma from some of the acutely infected splenectomized rats and from some of the recovered intact rats. The results showed that neither the acute nor the recovered sera had activity for trypsinized red cells while the acute and recovered B. rodhaini plasmas had significant activity. The normal rat plasma pool was also negative.

The Presence of Antibodies to Acute Plasmodium lophurae Antigens in Intact Rats Recovered from Haemobartonella Infections: A group of 11 stock carrier Wistar rats was splenectomized, and the H. muris infection exacerbated in six days. When the rats became moribund, they were etherized and exsanguinated by cardiac puncture for red blood cells. The cells were gently washed once in 0.85% saline, and one ml of this cell concentrate was injected intraperitoneally into a group of 10 young intact carrier Wistar rats. Another group of 10 rats was set aside as controls. Per cent parasitemias and blood cell counts were done on alternate days until the animals recovered. One month after infection both

Table 1. Agglutinins for trypsinized erythrocytes in plasma from splenectomized rats acutely infected with *Haemobartonella muris* and from intact rats recovered from the infection one week. The tests were read after 4 hours incubation at 25°C and overnight at 5°C. The titers are expressed as reciprocals of the HA test titers.

Acute Plasma Rate Number	Titer after: 4 hours	24 hours
1	-	-
2	4	-
3	-	-
4	-	-
5	-	-
Recovered Plasma		
1	-	-
2	-	-
3	-	-
4	-	-
5	-	-
Acute *B. rodhaini* serum no. 1	8	32
Acute *B. rodhaini* serum no. 2	8	64
Recovered *B. rodhaini* serum	16	32
Normal rat plasma pool	-	-

groups were bled out by cardiac puncture. The plasma from each was stored at -20°C.

Each sample was tested for activity against bentonite sensitized with serum antigens of P. lophurae origin. Table 2 shows that the plasma of the animals immediately recovered from the infection did have activity toward the P. lophurae antigens with titers ranging from 1:640 to 1:80. Some of the control sera which were from animals which carried the infection latently also had low activity toward the antigens.

HA tests were also done with some of the sera of animals that had recovered from the infection. Table 3 illustrates that they were either negative or only slightly positive.

The Presence of Antibodies to Acute Plasmodium lophurae and Babesia rodhaini Antigens in Intact Rats Recovered from Haemobartonella Infections: A group of 20 stock carrier Wistar rats was etherized and bled by cardiac puncture for 5.0 ml of blood for preinfection serum. Ten of the prebled group were splenectomized and within 5 days were heavily parasitized, anemic and moribund. They were then etherized and bled by cardiac puncture for plasma. The plasma was harvested and stored at -20°C. One ml of the concentrated cells of each rat was injected intraperitoneally into a counterpart rat of the intact group. These rats were examined for per cent parasitemias and blood cell counts on alternate days until they had recovered from the infection as indicated

Table 2. Antibodies in the plasma of rats carrying Haemobartonella muris latently and of rats recovered from the infection one month previously to serum antigen from ducks with acute Plasmodium lophurae.

Experimental Rat Number Latent	Titer*	Experimental Rat Number Recovered	Titer
1	20	1	80
2	40	2	NT
3	20	3	320
4	0	4	640
5	0	5	320
6	20	6	640
7	0	7	160
8	0	8	80
9	0	9	80
10	0	10	160
Normal duck plasma pool			0
Recovered duck plasma**			640
Normal rat serum pool			0

* Reciprocals of TBF test titers

** Plasmodium sp. isolated from Canvasback duck, Plasmodium "spartani".

Table 3. Agglutinins for trypsinized erythrocytes in plasma from intact rats recovered from Haemobartonella muris superinfection. Each plasma was tested twice and the titers were read after 4 hours incubation at 25°C and overnight at 5°C. The titers are expressed as reciprocals of the HA test titers.

Recovered Plasma Rat Number	Titer after: 4 hours Test 1	4 hours Test 2	24 hours Test 1	24 hours Test 2
1	-	-	-	-
3	-	-	-	-
5	-	8	-	-
6	-	-	16	-
8	NT*	8	NT	4
Acute B. rodhaini serum no. 1	8	32	32	8
Acute B. rodhaini serum no. 2	8	32	64	32
Recovered B. rodhaini serum	16	8	32	8
Normal rat plasma pool	-	-	-	-

* Not tested

by the absence of detectable parasites in the blood and a return of the red blood cell counts to preinfection levels (Figure 2). Weekly blood samples were taken from each of the rats starting at recovery and were carried on for three more weeks. Five ml of blood were taken at each bleeding, and the plasma from each rat stored at -20°C. The experiment was terminated at the end of four weeks by exsanguinating each rat and harvesting the plasma.

Each serum sample was tested for activity in the TBF test using acute P. lophurae antigens and acute babesial antigens. Table 4 illustrates the presence of antibodies in the recovered intact Wistar rats to acute P. lophurae duck serum antigen. Activity was maximal the first week after recovery and by the third week had disappeared. Similar results were seen with the response to acute B. rodhaini rat serum antigen (Table 5). The ten preinfection samples from the rats were all negative in the test even though these animals were carrying the infection latently. The control positive P. "spartani" plasma exhibited its usual 1:640 titer.

Some of the plasma from the acutely infected splenectomized rats were tested for activity for trypsinized red blood cells. Table 6 shows there was no activity in most of the samples.

Anemia Induced by Acute Haemobartonella Serum: A group of 24 Sprague-Dawley, Haemobartonella-free rats was splenectomized and observed for at least two weeks. Each rat was

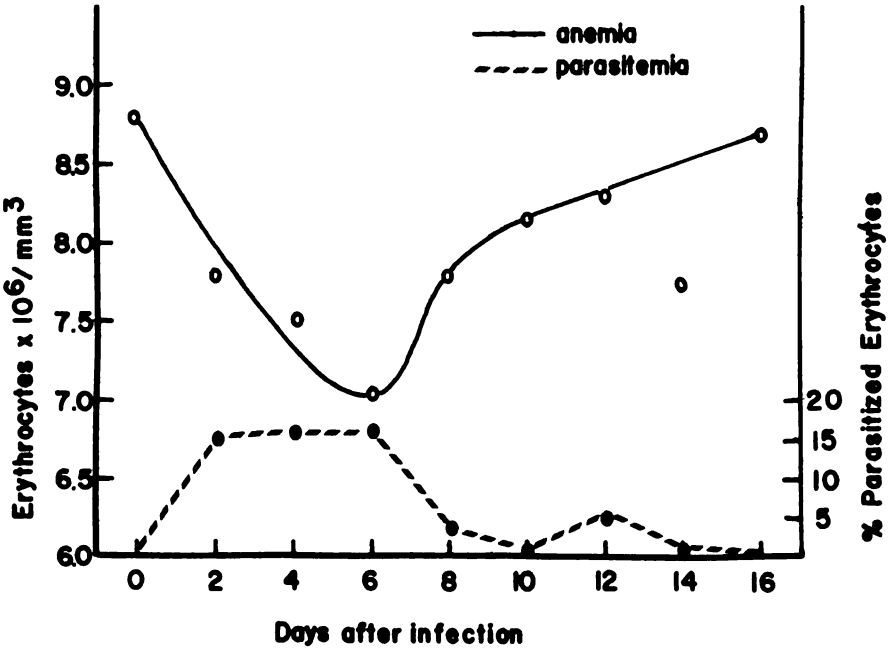

Figure 2. The course of a Haemobartonella muris superinfection in a group of carrier Wistar rats.

Table 4. Antibodies in rats before infection and recovered from Haemobartonella muris to serum antigen from ducks with acute Plasmodium lophurae.

Experimental Rat Number	Pre-infection	Recovered	Recovery 1st wk	Recovery 2nd wk	Recovery 3rd wk
1	0	80*	80	40	0
2	0	20	NT**	NT	NT
3	0	20	160	160	0
4	0	20	NT	NT	NT
5	0	20	160	40	0
6	0	40	80	40	0
7	0	20	40	80	0
8	0	20	80	40	0
9	0	20	160	80	0
10	0	20	NT	NT	NT
Mean	0	28	109	69	0
Rec'd Duck Plasma***	640	640	640	640	640
Normal Rat Serum Pool	0	0	0	0	0

*Titers expressed as reciprocals of TBF test titers.

**Not tested.

***Plasmodium sp. isolated from Canvasback duck, P. "spartani".

Table 5. Antibodies in rats before infection and recovered from Haemobartonella muris to serum antigen from rats with acute Babesia rodhaini.

Experimental Rat Number	Pre-infection	Recovered	Recovery 1st wk	Recovery 2nd wk	Recovery 3rd wk
1	0	20*	80	20	0
2	0	20	NT	NT	NT
3	0	20	20	20	0
4	0	20	NT	NT	NT
5	0	40	40	20	0
6	0	40	80	20	0
7	0	NT	20	40	0
8	0	20	20	20	0
9	0	NT	40	40	0
10	0	20	NT	NT	NT
Mean	0	25	45	24	0
Rec'd Duck Plasma**	640	640	640	640	640
Normal Rat Serum Pool	0	0	0	0	0

* Titers expressed as reciprocal of TBF test titers.

** Plasmodium sp. isolated from Canvasback duck, P. "spartani".

Table 6. Agglutinins for trypsinized erythrocytes in plasma from splenectomized rats acutely infected with *Haemobartonella muris*. The titers were read after 4 hours incubation at 25°C and overnight at 5°C. The titers are expressed as reciprocals of the HA test titers.

Acute Plasma Rat Number	Titer after: 4 hours	24 hours
6	-	-
7	-	-
10	4	-
Acute *B. rodhaini* serum no. 1	8	32
Acute *B. rodhaini* serum no. 2	8	64
Recovered *B. rodhaini* serum	16	32
Normal Rat Plasma Pool	-	-

infected with 0.1 ml of a 1% suspension of blood from H. muris passage rats. When they became anemic and moribund, they were etherized and bled by cardiac puncture for serum. A series of 24 normal Sprague-Dawley rats were also bled for normal serum. Each individual sample was allowed to stand overnight at 5°C, and the serum was drawn off after centrifugation. The acute sera and the normal sera were each pooled and stored at -20°C.

A group of 30 Wistar (Haemobartonella-free) rats was set aside and red blood cell counts were made at intervals one week prior to the experiment. The rats were then divided into three groups of ten. One group received five intraperitoneal injections of 1.0 ml apiece of the acute serum at hourly intervals and 1.0 ml of acute Haemobartonella blood thawed after storage in liquid nitrogen with the first acute serum injection. In the second group each rat received five intraperitoneal injections of 1.0 ml apiece of normal serum at hourly intervals and 1.0 ml of the acute Haemobartonella blood with the first inoculation. The final group of rats each received only the acute Haemobartonella blood. Red blood cell counts and per cent parasitemias were taken at approximate daily intervals.

Figure 3 shows that all the groups had a marked drop in red blood cell counts on day one. On day 2 the counts were approximately their preinfection levels, and by day 4 an anemia due to the infection itself had begun. Peak parasitemia

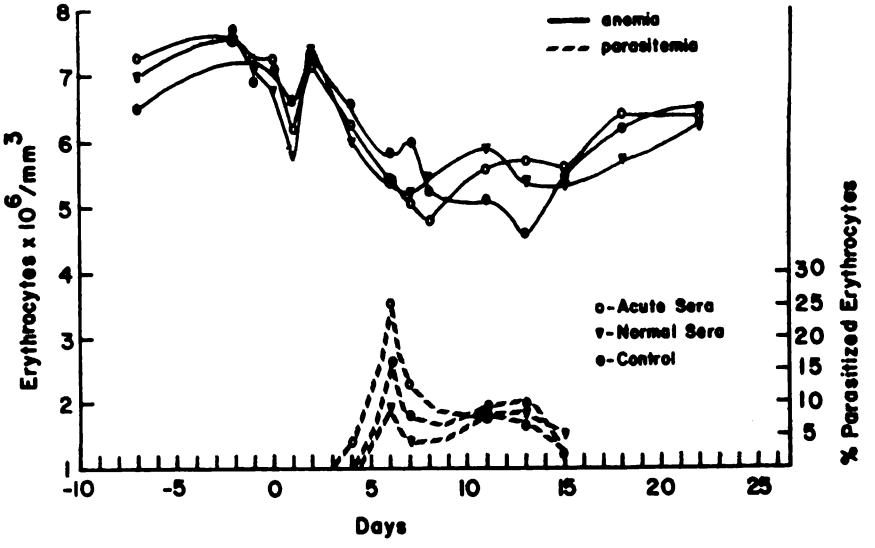

Figure 3. The effect of 5 hourly intraperitoneal injections of acute Haemobartonella muris serum into normal rats. Each animal was treated with acutely infected red blood cells.

was on day 6 in all three groups. The anemia was most pronounced between days 7 and 13 after which the blood picture started to return to normal. Because all three groups received the thawed acute Haemobartonella blood, it was thought that it might have produced the transient anemia seen in the three groups. There might also have been some hemodilution, for the two groups which received 6.0 ml of the blood and plasma showed a greater drop in blood cell counts than the control group that received only 1.0 ml of Haemobartonella blood.

To determine the role of the acute sera itself, a new experiment was set up. A second group of 24 Wistar rats was divided into 4 groups of 6 rats each. Each rat of the first group received 5.0 ml of the acute sera, each of the second group received 5.0 ml of normal sera, the third group each received 5.0 ml of saline and the fourth group received nothing. Injections were made intraperitoneally, and the inoculum was given all at once. Each rat was given daily intraperitoneal injections of 10 mg/kg of oxophenarsine hydrochloride (Mepharsen) to control the Haemobartonella infection (Thompson and Bayles, 1966). Erythrocyte counts were made daily. Figure 4 shows that the treatment did not effect the blood counts of any of the groups.

A final experiment was undertaken to determine the effect of the acute serum given intravenously. Another group of 24 young Wistar rats was divided as before. In this case

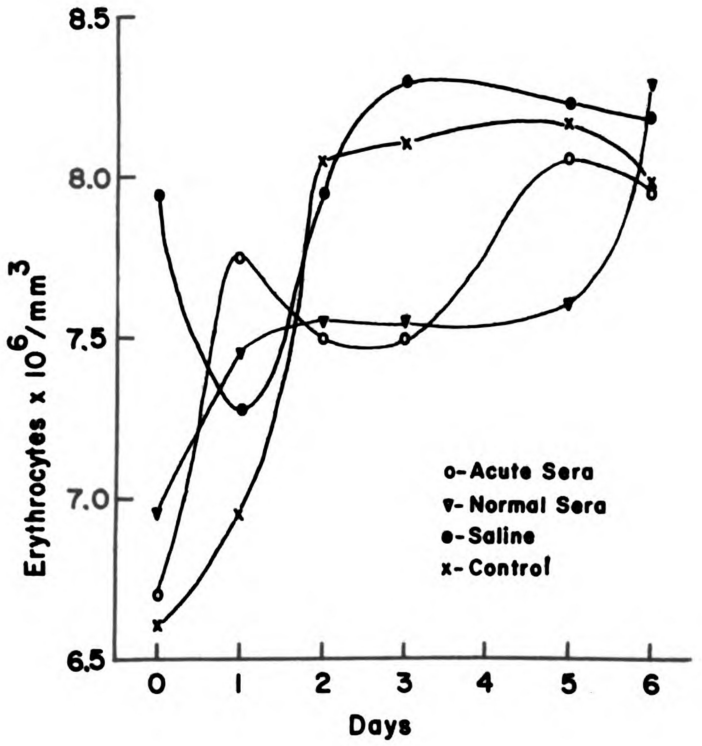

Figure 4. The effect of the intraperitoneal injection of acute Haemobartonella muris serum into normal rats. Each animal was treated with 10 mg/kg oxophenarsine daily.

injections were made intracardially. In the first group each rat received 2.0 ml of acute Haemobartonella sera; the second 2.0 ml of normal sera; the third 2.0 ml of 0.85% saline, and the final group received no treatment. Each animal again was treated daily with oxophenarsine. Erythrocyte counts were taken daily.

Figure 5 shows that all the groups had a decline in their erythrocyte counts shortly after the treatment and the first day. By the second day the counts were returning to their pretreatment levels. These results suggest a dilution phenomenon.

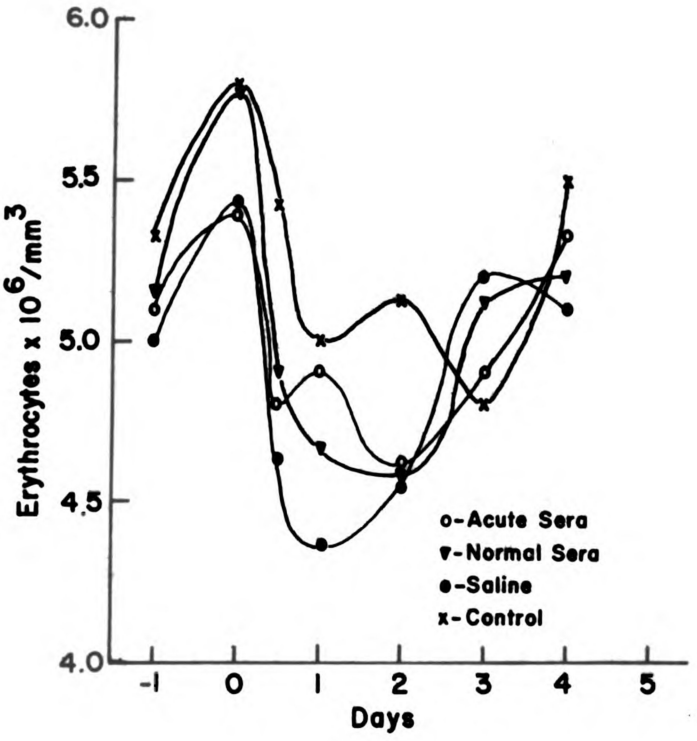

Figure 5. The effect of intravenous injection of acute Haemobartonella muris serum into young normal rats. Each animal was treated with 10 mg/kg oxophenarsine daily.

DISCUSSION

The data presented in this paper are not strongly indicative of an autoimmune reaction occurring in Haemobartonella infections. The observations of earlier workers on the course of the infection in normal and splenectomized rats (Weinman, 1944; Grieserman, 1958) were reconfirmed with our system. The splenectomized group showed a drop in erythrocyte numbers of 5.2 million per mm^3 within a 24 hour period starting the eighth day of the infection. In the intact group the blood cell loss was only 2.0 million per mm^3 over a 4 day period. The anemia began on the same day in both groups. The prepatent periods were also identical, although the number of parasites rose quickly in the splenectomized group. Spira and Zuckerman (1965 a,b) with P. berghei and Jones et al. (1968) with Anaplasma found a delay in the onset of the anemia in their splenectomized animals as compared to intact animals. They felt that the delay was suggestive of the role of the spleen in establishing an immune response toward erythrocytes. Such a delay was not seen with Haemobartonella which may mean that the same mechanism is not applicable or that direct parasitic destruction may be obscuring the presence of the immune response. It was of interest that

the group of animals given very large numbers of parasites demonstrated maximal anemia within 3 days of inoculation, and even though the anemia persisted at that level for a few more days, it did not become more severe after the expected time of the onset of the immune response. Thus the data from noninfected splenectomized and intact rats given H. muris infections tends to indicate that the anemia was directly related to the concentration of the parasites, and other mechanisms were not apparent.

Hemagglutinins for trypsinized red blood cells have been associated with the anemia seen during the acute stages of Anaplasma (Mann and Ristic, 1963 a,b), P. berghei (Cox et al., 1966), and B. rodhaini infections (Schroeder et al., 1966). However, they were not detected during the acute phase of Haemobartonella infections. The acute Haemobartonella plasmas tested were all taken from splenectomized animals while the acute plasmas tested in the hemosporidian and Anaplasma infections were taken from intact animals. Since the spleen is known to be the major immunogenic site of the body and its loss has been shown to be detrimental to the establishment of the immune response, it might be expected that hemagglutinins would not be found in the acute Haemobartonella plasmas. This may be especially true with Haemobartonella, for the infection in splenectomized animals becomes fatal before it is possible for the host to establish an immune response. With these thoughts in mind, hemagglutinins

were sought during the acute phase of the infection in intact rats, and preliminary data with 2 rats suggests that they are present in low titers. This aspect should be studied more thoroughly.

Recovered sera of various hemosporidian infections were shown to cross react serologically with substances in the globulins of the acute sera of other babesial or malarial infections (Cox et al., 1968) suggesting that similar substances are elaborated during the acute phases of these different diseases. It was thus of interest that substances were also found in the sera of rats recovered from Haemobartonella infections which cross reacted with globulins from ducks with acute P. lophurae and from rats with acute B. rodhaini infections. The antibodies were not present prior to infection or were present at very low titers even though the animals all carried the parasite latently. This suggests that the parasite itself is fairly innocuous and that the anemia is the necessary factor to elicit the formation of these serum substances.

Several groups of workers (Corwin and McGhee, 1966; Cox, 1966; Sibinovic et al., 1967 b; Cox and Milar, 1968; and Corwin and Cox, 1969) demonstrated the presence of anemia inducing factors in the acute sera of animals with hemosporidian infections. Recipients of the acute sera were later found to be resistant to challenge with homologous and heterologous infections. It was thought that substances

related to these factors were present in the acute sera of Haemobartonella rats because antibodies against them were detected in recovered rats. However, no anemia was produced in normal rats injected with acute Haemobartonella sera. This suggests that the acute sera itself plays no role in the production of the anemia. It may be that the lack of detectable hemagglutinins is relatable to the failure of the acute sera of induce anemia. Similar results were seen with antigens with B. caballi-B. equi infected horses. The acute antigenic globulin fractions did not induce anemia and were poorly immunogenic when injected into rats later challenged with B. rodhaini (Sibinovic et al., 1967 b). It would be of interest to determine whether animals exposed to acute Haemobartonella sera would be resistant to challenge with one of the hemosporidian infections.

Thus none of the data conclusively demonstrates the presence of an autoimmune response in Haemobartonella infections. However, the presence of substances in the recovered serum which serologically cross react with globulins from acute P. lophurae infections and from acute B. rodhaini infections suggests that a similar mechanism occurs in these different infections. The role these substances play in haemobartonellosis is still not known.

The fact that nonspecific serum substances occur in a broad spectrum of different infectious anemias has some important theoretical implications. They have been shown

to be present in all of the hemosporidian infections tested (Table 7) being nonspecific both serologically and immunogenically and being related antigenically neither to the parasite nor to the normal red blood cell but to each other. The first indication that similar substances may play a role in other erythrocytic infections came with the discovery of the role of autoimmune factors occurring during the anemic crisis of anaplasmosis (Mann and Ristic, 1963 a; Schroeder and Ristic, 1968). Now similar nonspecific acute factors have been demonstrated in Haemobartonella infections and also in Eperythrozoon infections (Cox, unpublished data).

The acute factors in all these various infections are antigenically similar but are not related to either the parasite or the erythrocyte. A similar type of reaction occurs with the tumor viruses. Several different tumor viruses were shown to produce malignancies with antigenic determinants which were neither directly related to the virus itself nor to the host but which were common to the several tumors (Good, 1968). Thus the various agents were causing cells to produce new antigenic sites which were not relatable to the host or to the virus but to each other. Habel (1961) suggested that the new antigen was produced by the normal cell after it had been transformed by the viral infection with the new antigen being only slightly different from the corresponding antigens in normal cells. A similar mechanism may be occurring in the erythrocytic infections, and the new antigens produced

Table 7. Immunopathologic reactions in infectious anemia (after Cox).

	INFECTION ANEMIA	SPLENOMEGALY	PHAGOCYTOSIS OF R.B.C.	NEPHRITIS	HEMAGGLUTININS	OPSONIN FOR R.B.C. or ANEMIC FACTOR	SERUM ANTIGEN	SERUM ANTIGEN IMMUNIZATION	HOST ANIMAL
A. marginale	+	+	+		+	+			Cattle
A. ovis	+	+		+					Sheep
E. coccoides	+	+					+		Mice
H. muris	+	+		+	?	?	+		Rats
D.I.A.*	+	+	+						Duck
P. lophurae	+	+	+			+	+	+	Duck
P. "spartani"	+	+				+	+	+	Duck
P. gallinacium	+	+				+	+	+	Hen
P. berghei	+	+	+	+	+	+	+	+	Mice-Rat
P. chabaudi	+	+							Mice
P. knowlesi	+	+	+			+	+	+	Monkey
P. falciparum	+	+		+	+		+		Man
B. rodhaini	+	+	+	+	+	+	+	+	Mice-Rat
B. canis	+	+	+			+	+	+	Dog
B. equi	+					-	+	-	Horse

*Duck Infectious Anemia (Ludford, 1969)

may lead to the induction of an autoimmune response to erythrocytes. Since all the erythrocytic infections attack a common cell, the red blood cell, it might be expected that similar antigenic sites are exposed under the influence of several different infectious processes.

Corwin and Cox (1969) felt that the acute factor could be related to a common parasite antigen but more probably to an ubiquitious tissue antigen analogous to the stromal T antigen described by Thomsen et al., (1930). The presence of common antigens in widely separate species of bacteria has long been known and used as the basis of some common diagnostic tests (Smith et al., 1964). An example is the Weil-Felix reaction where Proteus OX 19 shares a common antigen with Rickettsia prowaseki and R. rickettsii. Thus a parasitic factor could be common among the different erythrocytic infections being exposed when the parasite interacts with the red blood cell. However, this hypothesis becomes somewhat tenuous when similar substances are found in the infections of organisms very different from the hemosporidian, such as Haemobartonella.

The serum factor could be related to an erythrocytic antigen common among different species of animals. The stromal T antigen (Thomsen et al., 1930) is an example of such an antigen. This stromal antigen is exposed in vitro by treating erythrocytes with various bacteria or enzymes. The treated cells are then agglutinable by normal serum. It was

thought that the bacteria uncover an antigen which was common to erythrocytes of all species but which is not exposed under normal conditions (Raffel, 1961). The injection of enzymes obtained from Vibrio cholera was found to modify the animal's own red blood cells in vivo so that the agglutinin for the modified erythrocytes increased in titer (Humphrey and White, 1963). Thus it is possible that the action of the various infectious agents on the red blood cells leads to the exposure of analogous hidden antigenic sites or to the release of antigenic cell substances into the serum which are common among different hosts. Similar antigenic sites or substances may be exposed in normal, non-infected erythrocytes during splenic stasis (Motulsky et al., 1958 a,b), for splenomegally is an invariable symptom of these diseases (Table 7). Thus the parasites may initiate the anemia by exposing hidden antigenic sites which cause the production of antibodies that react not only with the infected cells but with normal cells slightly damaged by splenic stasis, and an autoimmune anemia ensues.

It is not known which of the various factors in the blood are active in eliciting the immunoserologic reactions produced by the acute serum. There is no real evidence that the anemia inducing factor is the same as that producing immunity. Work with a duck infectious anemia (Ludford, 1969) indicates that the amount of anemia produced is not directly relatable to the immunity produced in recipient animals. With Haemobartonella an antigenic factor was present in the acute

sera, but an anemia producing agent was not. This further suggests that they are not one and the same. Since the acute sera lacked hemagglutinins for trypsinized red blood cells, it could be that the hemagglutinins act in eliciting the anemia while the acute serologic factor plays a role in immunity. Similar results were seen with acute Anaplasma infections where free serum autohemagglutinins were present in the acute sera, but when injected into normal animals, the acute plasma did not elicit immunity (Ristic, 1968).

Studies on the nature of the serum antigen are scarce. Sibinovic et al. (1967 a) did work with the acute sera of babesial infections and found at least two antigens neither of which could be separated from the normal globulins of the blood. It appeared that the factor in the beta-globulin fraction was especially effective in inducing anemia and eventual immunity, but it was not certain whether the antigen was single or multiple, for the gel precipitation lines were somewhat blurred. Further work is needed in isolation and characterization of the acute serum factor(s).

As of now, it can be stated that there appear to be in the serum of animals with acute infectious anemias nonspecific antigenic factors which may play a role in producing the anemia and subsequent immunity seen in these infections. That the phenomenon exists seems to be well verified, but its exact role in the pathogenesis of infectious anemias still remains to be elucidated.

SUMMARY

The sera of animals recovered from active Haemobartonella infections were found to react serologically with serum substances from acute infections of P. lophurae and B. rodhaini. These data suggest that similar factors are present in the sera of animals with acute haemobartonellosis. Hemagglutinins for trypsinized red blood cells were not detected in acute plasmas, and anemia was not produced in normal rats when they were treated with acute Haemobartonella sera. These results are in contrast to those found in the hemosporidian infections, and the theoretical implications of the difference were discussed. The course of the infection in intact and splenectomized rats did not show a delay in the onset of the anemia in the splenectomized group. A delay has been shown in malaria, babesiosis and anaplasmosis and was interpreted to indicate the presence of autoimmune mechanisms mediated by the spleen. Thus the results of this report did not settle the question of the role of autoimmune mechanisms in the anemia of Haemobartonella infections.

BIBLIOGRAPHY

Anigstein, L. and Pomerat, C. M. 1954 Reticulo-endothelial immune serum (REIS). IV. Experimental production of anemia and bartonellosis in rats by inhibitory dosage. Texas Rept. Biol. Med., 3, 545-557.

Baker, N. F., Osebold, J. W. and Christensen, J. F. 1961 Erythrocyte survival in experimental anaplasmosis. Am. J. Vet. Res., 22, 590-596.

Barton, A. L. 1909 Descripción de elementos endo-globulares halladoes en los enfermos de fiebre verrucosa (Artículo preliminar). Cronica Med. (Lima), 26, 7.

Bozicevich, J., Nasou, J. P. and Kayhoe, D. E. 1960 Desoxyribonucleic acid (DNA)-bentonite flocculation test for lupus erythematosus. Proc. Soc. Exptl. Biol. Med., 103, 636-640.

Breed, R. S., Murray, E. D. G. and Smith, N. R. 1957 Bergey's Manual of Determinative Bacteriology. 7th edition, The Williams and Wilkins Company, Baltimore.

Cannon, P. R. and McClelland, P. H. 1928 Role of ectoparasites in bartonella infections of albino rats. Proc. Soc. Exptl. Biol. Med., 26, 157-158.

Cantrell, W. and Elko, E. E. 1966 Effect of splenectomy on phagocytic activation by Plasmodium berghei. J. Inf. Dis., 116, 429-438.

Christophers, S. R. and Bentley, C. 1908 Phagocytosis of red cells in blackwater fever. J. Am. Med. Assoc., 50, 1383.

Coggeshall, L. T. 1943 Immunity in malaria. Medicine, 22, 87-102.

Corwin, R. M. and Cox, H. W. 1969 The immunogenic activities of the nonspecific serum antigens of acute hemosporidian infections. Mil. Med., (Suppl) 134:1258-1263.

Corwin, R. M. and McGhee, R. B. 1966 Anemia in ducklings treated with malarious plasma. Exp. Parasitol., 18, 281-289.

Cox, H. W. 1966 A factor associated with anemia and immunity in Plasmodium knowlesi infections. Mil. Med., 131 (Suppl.), 1195-1200.

Cox, H. W. and Milar, R. 1968 Cross protection in immunization by Plasmodium and Babesia infections in rats and mice. Am. J. Trop. Med. Hyg., 17, 173-179.

Cox, H. W., Milar, R. and Patterson, S. 1968 Serologic cross reactions of serum antigens associated with acute Plasmodium and Babesia infections. Am. J. Trop. Med. Hyg., 17, 13-18.

Cox, H. W., Schroeder, W. F. and Ristic, M. 1966 Hemagglutination and erythrophagocytosis associated with the anemia of Plasmodium berghei infections of rats. J. Protozool., 13, 327-332.

Crystal, M. M. 1958 The mechanism of transmission of Haemobartonella muris (Mayer) of rats by the spined rat louse, Polyplax spinulosa (Burmeister). J. Parasitol., 44, 603-606.

Crystal, M. M. 1959 Extrinsic incubation period of Haemobartonella muris in the spined rat louse, Polyplax spinulosa. J. Bacteriol., 17, 1959.

Dacie, J. V. 1962 The haemolytic anemias. II. Autoimmune haemolytic anemias. J. & A. Churchill Limited, London.

Dimopoullos, G. T. and Bedell, D. M. 1962 Studies of bovine erythrocytes in anaplasmosis. II. Role of chemical and physical changes in the mechanism of anemia in splenectomized calves. Am. J. Vet. Res., 23, 813-820.

Doménico, A. D. 1956 Anemia hemolítica provocada por la esplenectomía en la rata blanca portadora de Bartonellas. Acta Physiol. Latinoam., 6, 60.

Doménico, A. D. and Andreotti, H. T. 1959 Mechanism of splenic protection against Bartonella muris in the parabiotic rat. Am. J. Physiol., 197, 795-798.

Elko, E. E. and Cantrell, W. 1968 Phagocytosis and anemia in rats infected with Haemobartonella muris. J. Inf. Dis., 118, 324-332.

Flaum, E. and Lauda, E. 1931 Zur Frage der immeren Sekretion der Milz. Ztschr. f. Exper. Med., 77, 410.

Ford, A. C. and Murray, T. J. 1959 Studies on Haemobartonella infection in the rat. Can. J. Microbiol., 5, 345-350.

Foy, H., Kondi, A. and Moumjidis, A. 1941 Transfusion of blackwater fever blood into a normal individual during haemolytic crisis. Trans. R. Soc. Trop. Med. Hyg., 35, 119-123.

Foy, H., Kondi, A., Rebelo, A. and Soliro, A. 1954 Survival of transfused red cells in blackwater fever circulation and of blackwater red cells in normal circulation. Trans. R. Soc. Trop. Med. Hyg., 38, 271-286.

Gear, J. 1946 Autonatigens and autoantibodies in the pathogenesis of disease with special reference to blackwater fever. Trans. R. Soc. Trop. Med. Hyg., 39, 301-314.

George, J. N., Stokes, E. F., Wicker, D. J. and Conrad, M. E. 1966 Studies of the mechanism of hemolysis in experimental malaria. Mil. Med., 131 (Suppl.), 1217-1224.

Good, R. A. and Finstad, J. 1968 The association of lymphoid malignancy and immunologic functions. In "Proceedings of the International Conference on Leukemia-Lymphoma." (C. J. D. Zarafonetis, ed.), pp. 175-197, Lea & Febiger, Philadelphia.

Gorstein, F. and Benacerraf, B. 1960 Hyperactivity of the reticulo-endothelial system and experimental anemia in mice. Am. J. Path., 37, 569-578.

Griesemer, R. A. 1958 Bartonellosis. J. Natl. Cancer Inst., 20, 949-956.

Habel, K. 1961 Resistance of polyoma virus immune animals to transplanted polyoma tumors. Proc. Soc. Exptl. Biol. Med., 106, 722-725.

Humphrey, J. H. and White, R. G. 1963 Immunology for Students of Medicine. F. A. Davis Company, Philadelphia.

Jones, E. W., Norman, B. B., Kliewer, I. O. and Brock, W. E. 1968 Anaplasma marginale infection in splenectomized calves. Am. J. Vet. Res., 29, 523-533.

Kessler, W. R. 1943 a Studies on experimental Bartonella muris anemia in the albino rat. I. Age and resistance. J. Inf. Dis., 73, 65-76.

Kessler, W. R. 1943 b Studies on experimental Bartonella muris anemia in the albino rat. II. Latent infection and resistance. J. Inf. Dis., 73, 77-84.

Kreier, J. P., Ristic, M. and Schroeder, W. F. 1964 Anaplasmosis. IV. The pathogenesis of anemia produced by infection with Anaplasma. Am. J. Vet. Res., 25, 343-352.

Kreier, J. P. and Ristic M. 1968 Haemobartonellosis, Eperythrozoonosis, Grahamellosis, and Ehrlichiosis. In "Infectious Blood Diseases of Man and Animals." (D. Weinman and M. Ristic, eds.), vol. II, Academic Press, New York.

Laskowski, L., Pinkerton, H. and Greiff, P. 1950 A non-pathogenic Bartonella-like organism developing in embryonated eggs after injection with Haemobartonella muris. J. Immunol., 64, 455.

Lawkowski, L., Stanton, M. F. and Pinkerton, H. 1954 Chemotherapeutic studies of alloxan dehydroascorbid acid and related compounds in murine haemobartonellosis. J. Inf. Dis., 95, 182-190.

Ludford, C. G. 1969 Personal communication.

Maegraith, B. G. 1948 Pathological processes in malaria and blackwater fever. Blackwell Scientific Publications, Oxford, England.

Mann, D. K. and Ristic, M. 1963 a Anaplasmosis. XIII. Studies concerning the nature of autoimmunity. Am. J. Vet. Res., 24, 703-708.

Mann, D. K. and Ristic, M. 1963 b Anaplasmosis. XIV. The isolation and characterization of an autohemagglutinin. Am. J. Vet. Res., 24, 709-712.

Marby, D. S., Bass, J. A., Dodd, M. C., Wallace, J. H. and Wright, C-S. 1956 a Differential phagocytosis of normal, trypsinized and virus-treated human and rabbit erythrocytes by macrophages in tissue culture. J. Immunol., 76, 54-61.

Marby, D. S., Wallace, J. H., Dodd, M. C. and Wright, C-S. 1956 b Opsonic factors in normal and immune sera in the differential phagocytosis of normal, trypsinized and virus-treated human and rabbit erythrocytes by macrophages in tissue culture. J. Immunol., 76, 62-68.

Mayer, M. 1921 Ueber einige bakterienähnliche Parasiten der Erythrocyten bei Menschen und Tieren. Arch. f. Schiffs- u. Tropen-Hyg., 25, 150.

Mayer, M. and Zeiss, H. 1920 Versuche mit einem neuen Trypanosomenheilmittel "Bayer 205" bei menschen- und tierpathogenen Trypanosomen. Arch. f. Schiffs- u. Tropen-Hyg., 24, 257.

McGhee, R. B. 1960 An autoimmune reaction produced in ducklings in response to injections of duck embryo blood infected with Plasmodium lophurae. J. Inf. Dis., 107, 410-418.

McGhee, R. B. 1964 Autoimmunity in malaria. Am. J. Trop. Med. Hyg., 13, 219-224.

McGhee, R. B. and Corwin, R. M. 1964 Bone marrow dyscrasis in malarious ducklings. J. Parasitol. Suppl., 50, 49-50.

Moore, D. H., Arison, R. N., Tanaka, H., Hall, W. T. and Chanowitz, M. 1965 Identity of the filterable hemolytic anemia agent of Sacks with Haemobartonella muris. J. Bacteriol., 90, 1669-1674.

Morton, J. A. and Pickles, M. M. 1947 Use of trypsin in the detection of incomplete anti-Rh antibodies. Nature, 159, 779-880.

Motulsky, A. G., Casserd, F., Giblett, E. R., Brown, G. O. and Finch, C. A. 1958 a Anemia and the spleen. New Engl. J. Med., 259, 1164-1169.

Motulsky, A. G., Casserd, F., Giblett, E. R., Brown, G. O. and Finch, C. A. 1958 b Anemia and the spleen (Concluded). New Engl. J. Med., 259, 1215-1219.

Nussenzweig, R. S. 1967 Increased nonspecific resistance to malaria produced by administration of killed Corynebacterium parvum. Exptl. Parasitol., 21, 224-231.

Oliver-Gonzáles, J. 1944 Blood agglutinins in blackwater fever. Proc. Soc. Exptl. Med., Biol., 257, 25-26.

Perla, D. and Marmorston-Gottesman, J. 1930 Studies on Bartonella muris anemia of albino rats. III. The protective effect of autoplastic splenic transplants on the Bartonella muris anemia of splenectomized rats. J. Exptl. Med., 52, 131-143.

Perla, D. and Marmorston-Gottesman, J. 1932 Studies on Bartonella muris anemia. VI. A lipoid extract of the spleen that prevents Bartonella muris in splenectomized albino rats. J. Exptl. Med., 56, 777-782.

Perla D. and Marmorston, J. 1941 Natural resistance and clinical medicine. Little, Brown and Company, Boston.

Peters, D. and Wigand, R. 1955 Bartonellaceae. A review. Bacteriol. Rev., 19, 150-155.

Pomerat, C. M., Frieden, E. H. and Yeager, E. 1947 Reticuloo-endothelial immune serum (REIS). V. An experimental anemia in Bartonella infected rats produced by anti-blood immune serum. J. Inf. Dis., 80, 154-163.

Raffel, S. 1961 Immunity. 2nd Ed. Appleton-Century-Crofts, Inc., New York.

Ristic, M. 1960 Structural characterization of Anaplasma marginale in acute and carrier infections. J. Am. Vet. Med. Assoc., 136, 417-428.

Ristic, M. 1961 Studies in anaplasmosis. III. An autoantibody and symptomatic macrocytic anemia. Am. J. Vet. Res., 22, 871-876.

Ristic, M. 1962 A capillary tube agglutination test of anaplasmosis. A preliminary report. J. Am. Vet. Med. Assoc., 141, 588-594.

Ristic, M. 1968 Anaplasmosis In "Infectious Blood Diseases of Man and Animals." (D. Weinman and M. Ristic, eds.), vol. II, pp. 473-542, Academic Press, New York.

Rudnick, P. and Hollingsworth, J. W. 1959 Lifespan of rat erythrocytes parasitized by Bartonella muris. J. Inf. Dis., 104, 24-27.

Scheff, G. J., Scheff, I. M. and Eiseman, G. 1956 Concerning the mechanism of Bartonella anemia in splenectomized rats. J. Inf. Dis., 98, 113-120.

Schroeder, W. F. and Ristic, M. 1965 a Anaplasmosis. XVII. The relation of autoimmune processes to anemia. Am. J. Vet. Res., 26, 239-245.

Schroeder, W. R. and Ristic, M. 1965 b Anaplasmosis. XVIII. An analysis of autoantigens in infected and normal bovine erythrocytes. Am. J. Vet. Res., 26, 679-682.

Schroeder, W. F. and Ristic, M. 1968 a Blood serum factors associated with erythrophagocytosis in calves with anaplasmosis. Am. J. Vet. Res., 29, 1993-1995.

Schroeder, W. F. and Ristic, M. 1968 b Autoimmune response and pathogenesis of blood parasite diseases. In "Infectious Blood Diseases of Man and Animals." (D. Weinman and M. Ristic, eds.), vol. I, pp. 63-77, Academic Press, New York.

Schroeder, W. F., Cox, H. W. and Ristic, M. 1966 Anaemia, parasitaemia and erythrophagocytosis and haemagglutinins in Babesis rodhaini infections. Ann. Trop. Med. Parasitol., 60, 31-38.

Schultz, M. G. 1968 A History of Bartonellosis (Carrión's Disease). Am. J. Trop. Med. Hyg., 17, 503-515.

Sibinovic, K. H., Ristic, M., Sibinovic, S. and Phillips, T. N. 1965 Equine babesiosis: Isolation and serologic characterization of a blood serum antigen from acutely infected horses. Am. J. Vet. Res., 26, 147-153.

Sibinovic, K. H., MacLeod, R., Ristic, M., Sibinovic, S. and Cox, H. W. 1967 a A study of some of the physical, chemical and serologic properties of antigens from sera of horses, dogs, and rats with acute babesiosis. J. Parasitol., 53, 919-923.

Sibinovic, K. H., Sibinovic, S., Ristic, M. and Cox, H. W. 1967 b Immunogenic properties of babesial serum antigens. J. Parasitol., 53, 1121-1129.

Sibinovic, K. H., Milar, R., Ristic, M. and Cox, H. W. 1969 In vitro and in vivo studies of the effects of serum antigens of acute babesiosis and their antibodies on parasitized and normal erythrocytes. Ann. Trop. Med. Parasitol. In press.

Small, E. and Ristic, M. 1967 Morphologic features of Haemobartonella felis. Am. J. Vet. Res., 28, 845-856.

Smith, D. T., Conant, N. F. and Overman, J. R. 1964 Zinsser Microbiology. 13th edition, Appleton-Century-Crofts, Inc., New York.

Spira, D. and Zuckerman, A. 1965 a The effect of splenectomy on anemia in the malarious rat. Prog. in Protozool., IInd Internat. Cong. Protozool., London, p. 169.

Spira, D. and Zuckerman, A. 1965 b Blood loss and replacement in plasmodial infections. VI. Plasmodium berghei in splenectomized rats. J. Inf. Dis., 115, 337-344.

Taliaferro, W. H. and Mulligan, H. W. 1937 The histopathology of malaria with special reference to the function and origin of the macrophages in defense. Indian Med. Res. Memoir, 29, 1-138.

Tanaka, H., Hall, W. T., Sheffield, J. B. and Moore, D. H. 1965 Fine structure of Haemobartonella muris as compared with Eperythrozoon coccoides and Mycoplasma pulmonis. J. Bacteriol., 90, 1735-1749.

Thomas, T. B., Pomerat, C. M. and Frieden, E. H. 1949 Cellular reactions in Haemobartonella infected rats with anemia produced by anti-blood immune serum. J. Inf. Dis., 84, 169-186.

Thompson, J. S., Gurney, C. W., Hanel, A., Ford, E. and Hofstra, D. 1961 Survival of transfused blood in rats. Am. J. Physiol., 200, 327-331.

Thompson, P. E. and Bayles, A. 1966 Eradication of Eperythrozoon coccoides with oxophenarsine in normal and drug-resistant lines of Plasmodium berghei in mice. 52, 674-678.

Thomsen, O., Friedenreich, V. and Worsaae, E. 1930 Uber die Mogucheit Der Existenz Zweier neuer blutgruppen; auch ein Beitrag zur beleuchtung sogenanneter untergruppen. Acta path. et microbiol. Sacndinav., 7, 157-190.

Todorovic, R., Ferris, D. and Ristic, M. 1967 Roles of the spleen in acute plasmodial and babesial infections in rats. Exp. Parasitol., 21, 354-372.

Tyzzer, E. E., and Weinman, D. 1939 Haemobartonella, N. G. (Bartonella Olin Pro Parte), H. microti N. Sp., of the field vole, Microtus pennsylvanicus. Am. J. Hyg., 30 141-156.

Weinman, D. 1938 On the cause of anemia in the Bartonella infection of rats. J. Inf. Dis., 63, 1-9.

Weinman, D. 1944 Infectious anemias due to Bartonella and related red cell parasites. Trans. Am. Phil. Soc., 33, 243-287.

Weinman, D. 1968 Bartonellosis In "Infectious Blood Diseases of Man and Animals." (D. Weinman and Ristic, M., eds.), vol. II, pp. 3-24, Academic Press, New York.

Wigand, R. 1956 A complement-fixation reaction with Haemobartonella muris and Eperythrozoon coccoides. Nature, 178, 1288-1289.

Wigand, R. 1958 Morphologische Biologische und Serologische Eigenschaften der Bartonellen. Thieme, Stuttgart.

Wigand, R. and Peters, D. 1950 Z. Tropenmed. Parasitol., 2, 206. Cited by Kreier, J. P. and Ristic, M., 1968.

Wigand, R. and Peters, D. 1952 Z. Tropenmed. Parasitol., 3, 431. Cited by Kreier, J. P. and Ristic, M., 1968.

Zuckerman, A. 1960 Autoantibody in rats with Plasmodium berghei. Nature, 185, 189-190.

Zuckerman, A. 1963 Immunity in malaria with particular reference to red cell destruction. In "Immunity to Protozoa. A Symposium." (Garnham, P. CC., Pierce, A. E. and Roitt, F. A., eds.), pp. 78-88, F. A. Davis Company, Philadelphia.

Zuckerman, A. 1964 Autoimmunization and other types of indirect damage to host cells as factors in certain protozoan diseases. Exp. Parasitol., 15, 138-183.

Zuckerman, A. 1966 Recent studies on factors involved in malarial anemia. Mil. Med., 131 (Suppl.), 1201-1216.

Zuckerman, A. and Spira, D. 1961 Blood loss and replacement in plasmodial infections. V. Positive antiglobulin tests in rat anemias due to the rodent malarias Plasmodium berghei and Plasmodium vinckei, to cardiac bleeding, and to treatment with phenylhydrazine hydrochloride. J. Inf. Dis., 108, 339-348.