PATHOGENESIS OF ACUTE AVIAN MALARIA

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ABSTRACT

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Factors in the plasma of chickens with acute *Plasmodium gallinaceum* infection were found to be responsible for both anemia and acute glomerulonephritis. Intravenous injection of plasma of malarious birds into normal chickens produced a 35% reduction in the red blood cell counts within 48 hours which persisted for 8 to 9 days. A similar anemia resulted from the injection of material eluted from cells of malarious blood. Anemia was also induced by injections of a cold-active agglutinin absorbed from the plasma of malarious chickens with trypsinized human type "O" erythrocytes and dissociated from the cells by incubation at 37 C. The injection of the agglutinin also produced an anaphylactic-like shock in the recipients, but no deaths. When the plasma that had been absorbed free of agglutinin was injected into normal chickens it too produced anemia. Thus there appeared to be 2 substances in malarious blood that caused anemia.

Acute glomerulonephritis was produced in normal chickens by the injection of plasma from malarious birds. Except that it appeared much earlier, this nephritis did not differ from that seen in chickens with acute *P. gallinaceum* infection. Since the plasma did not cause nephritis in chickens that had recovered from acute *P. gallinaceum* infection, it

was considered that the inducing substances were immunologic rather than host permeability factors.

Study of the blood of malarious chickens revealed that the plasma contained the cold-active hemagglutinin, globulin associated antigen (serum antigen), antibody to the antigen, and antibody to extracted parasite antigen. Material leached from cells of malarious blood contained predominantly serum antigen and antibody that did not differ from that found in the plasma. The parasites liberated from washed disrupted erythrocytes revealed the presence of one or more antigens that were not differentiated one from the other. These antigens did not react with antibody to the serum antigen but did react with the antibody to parasite antigen in plasma of malarious birds.

Study of the cold-active agglutinin revealed that it was associated with the beta globulin fraction of the plasma, was inactivated by 2-mercaptoethanol cleavage, and reacted with red blood cells, and antiserum to whole chicken globulin. Cells of chickens that had been injected with the cold agglutinin reacted with fluorescein isothiocyanate conjugated anti-chicken globulin, but did not react with conjugates of antibody to serum antigen or antibody to parasite antigen.

Both serum antigen and antibody to serum antigen were extracted from the kidneys of malarious chickens and both were identified and partially separated from the various serum proteins that were found in the urinary droppings of malarious chickens. As birds recovered from acute infection, only the antibody and low molecular weight protein continued to be excreted.

When kidney sections from malarious chickens were reacted with conjugates of antibody to serum antigen, antibody to parasite antigen, and anti-IgG of chickens, immunofluorescent reactions were seen with

each; however, the reactions of the glomeruli with the first 2 conjugates was stronger, more diffuse and granular than was the reaction with anti-parasite antigen. Kidney sections of chickens with nephritis induced by plasma injections reacted with conjugates of antibody to serum antigen and with anti-IgG of chickens but did not react with antibody to parasite antigens.

Since globulin associated serum antigen and its antibody were implicated in both anemia and nephritis, further study was made. Serum antigen from rats with acute *Babesia rodhaini* was used to immunize chickens. The globulins from these chickens reacted in serologic tests with the plasma of chickens with acute *P. gallinaceum* infection, with the antigens found in their kidneys, and with the antigen extracted from droppings of malarious chickens. Conjugates of the antibody reacted with the glomeruli of malarious chickens, and those of chickens with plasma induced nephritis, in the same manner as did antibody to serum antigen from malarious chickens. Antibody to serum antigen of chicken origin reacted in serologic tests with serum antigen in the plasma of rats with acute *B. rodhaini* infection. Thus it was further indicated that serum antigen was not a part of *P. gallinaceum* parasites. It appears to be an antigen that is in some way elaborated during acute red blood cell infections with parasites of other genera, as well as those of genus *Plasmodium*.

This work has incriminated two factors, the cold-active hemagglutinin, and complexes of serum antigen and antibody in the anemia of acute malarial infections of chickens. The nephritis of acute malaria of chickens appears to be primarily an immune complex disease involving serum antigen.

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By

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Dedicated

to

my wife Mrs. Gayatri Soni

and

my family

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INTRODUCTION

Anemia and changes in the vascular system that were manifested as glomerulonephritis have long been a recognized part of malarial disease. Earlier investigators had indicated that anemia had been a result of disruption of the erythrocytes by emerging parasites and that the vascular changes associated with nephritis might have been caused by such mechanisms as anoxia resulting from blood loss, toxic substances of the parasite, or blockage of blood vessels by parasites released from blood cells by hemolysis (Maegraith, 1948). More recent studies have furnished sufficient evidence to warrant the presumption that anemia and nephritis of acute malaria, and other red blood cell infections, were mediated by immune mechanisms. Various investigators have furnished evidence allowing the suggestion that malarial anemia and nephritis might have been mediated by auto-antibody (Zuckerman, 1960; McGhee, 1960; 1964; Cox *et al.*, 1966; Iturri and Cox, 1969). Others have suggested that both anemia and nephritis of acute malaria were mediated by antigen-antibody complexes, which in combination with complement might act as a hemolysin, or as a vascular permeability factor to produce nephritis (Dixon, 1966; 1972; Ward and Conran, 1966; 1969). These suggestions have been based in large part on the findings of agglutinins for trypsinized erythrocytes, or a positive Coomb's test, associated with malarial anemia, and on the detection of immune gamma globulin (IgG) in nephrotic lesions of the kidney, with fluorescein isothiocyanate conjugated anti-IgG.

The definitive experiments, which would in essence fulfill Koch's postulates for the definition of the etiologic agent of an infectious disease, have not been performed. Neither malarial anemia nor nephritis had been induced in experimental animals in absence of infection by injecting them with the immune substances found associated with acute malarial disease. Neither have the antigens, which would be the essential ingredient for immunological diseases, either autoimmune or immune complex, been identified and their roles in disease indicated.

It is therefore believed that the evidence that immune mechanisms are the mediators of malarial nephritis and anemia is at best presumptive. There is a real need for definitive experiments that prove a causal relationship of these immune activities in absence of infection.

In the research for this thesis, using as a model *Plasmodium gallinaceum* infections of White Leghorn cockerels, an effort has been made to implicate immune activity associated with acute infection in both anemia and nephritis, and to indicate the antigens responsible for immunopathogenesis. The review of the background literature, the methodology, and the results of this research are presented.

REVIEW OF LITERATURE

Hemosporidian Parasites

Taxonomically hemosporidian parasites belong in the Phylum *Protozoa* and fall within the Class *Sporozoa*, Order *Hemosporidia*. The families of the Class which are important from a pathological point of view are the *Plasmodiidae*, *Hemoproteidae*, *Theileriidae*, and *Babesiidae* and the species which have most significance as causes of disease in man and domestic animals are members of the genera *Plasmodium*, *Hemoproteus*, *Leucocytozoon*, *Theileria*, and *Babesia* (Garnham, 1966).

These parasites typically have an intraerythrocytic stage in the definitive host and their life cycle is characterized by 2 or more reproductive phases: sporogony, a sexual life cycle, which takes place in the body of blood sucking arthropods, and schizogony, an asexual life cycle, which occurs in erythrocytes of the vertebrate host.

Plasmodium gallinaceum Brumpt 1935

Discovery and natural transmission. *P. gallinaceum* was probably seen for the first time by Brousais in 1910 at Nhatrang, Indo-China, in the blood of a native fowl. However, it was Brumpt (1935) who first described the parasite under the name of *Plasmodium gallinaceum*.

Omer *et al.* (1962) and Niles *et al.* (1965) found *Aedes aegypti* and *Mansonia crassipes*, respectively, to be the natural vectors of *P. gallinaceum*. Experimental infections of mosquitoes of the genera *Aedes*, *Aermigeres*, *Culex*, *Culiseta* and *Mansonia* have been demonstrated (Brumpt,

1949), but *A. aegypti* is preferred for laboratory transmission since 100% of fed mosquitoes may become infected, they are easily reared in the laboratory, and will readily feed on many avian species. *P. gallinaceum* was readily adopted to domestic chickens and has since been a popular model for the study of plasmodial infections in both vertebrate and mosquito hosts.

Developmental aspects in the avian host. Coulston, Cantrell and Huff (1945) discovered the exoerythrocytic schizogony of plasmodial parasites using *P. gallinaceum*. Multiplication took place first in the macrophages of the skin after introduction of sporozoites. The products of fission were called cryptozoites. Subsequent fission produces metacryptozoic stages. A morphologically distinct stage called phanerozoites then developed which was capable of widespread invasion of the cells of the lymphoid-macrophage system, and also the endothelial cells lining the sinusoids and capillaries of internal organs. After two generations, the progeny were capable of invading erythrocytes.

Huff (1952) recorded the frequency of metacryptozoite invasion of different organs in experimentally infected birds in the following order: spleen, lungs, heart, kidney, liver, brain, intestine, thymus, and testes. Bray (1957) reported similar findings but also observed parasites in cells of the pancreas and bone marrow.

Developmental and morphologic studies have been made on stages of *P. gallinaceum* growing under several artificial situations. McGhee (1949) found that sporozoites of *P. gallinaceum* will develop in the lymphoid-macrophage system of 12- to 16-day-old chick embryos with cryptozoic and metacryptozoic stages similar in duration and morphology to those observed in hatched birds. Sporozoites will infect tissue cultures of embryonic spleen cells where development takes place in the

macrophages, and Dobin *et al.* (1949) found as many as 12 parasites in a single cell.

Studies of the nucleus and cytoplasm of erythrocytic schizonts in tissue culture have been reported by Huff *et al.* (1960). Three developmental forms of cytoplasmic division were observed which ultimately led to cytomere formation and release of merozoites. Little diminution in virulence or of the ability of the parasite to invade the erythrocytes was found after the exoerythrocytic stage of *P. gallinaceum* had been maintained in tissue culture continuously for 4 years (Meyer and Musacchio, 1963).

A fairly synchronous 36 hour schizogony cycle has been shown to occur in blood (Giovannola, 1938). At the height of infection an intense parasitemia occurs, at which time a single erythrocyte may contain 2 or more trophozoites. At this stage the blood may contain many immature erythrocytes. The increase in parasitemia continues until there are more parasites than corpuscles. The acute phase lasts for about 9 days, when it is terminated by a crisis.

Barretto and deFretes (1945) studied the correlation between age and weight, and the mortality rate in *P. gallinaceum* infected chickens. They found a 100% mortality in young chicks weighing less than 250 grams; 87% in chicks of 300 to 350 grams; and 45% in those weighing 1000 grams. The disease generally followed a milder course in adult birds. The proportion of gametocytes to asexual parasites was always low (1:100 to 2:100), but usually higher in the more acute attacks of young chicks.

Ultrastructural studies related to *Plasmodium gallinaceum*. Rudzinska and Trager (1957) share major credit for their pioneer work concerning the fine structure of erythrocytic stages of malarial parasites. The

literature on ultrastructure has since become voluminous and only a few pertinent references are cited.

Various organellae including a large nucleus, mitochondria with microtubular cristae, well developed endoplasmic reticula, food vacuoles with osmophilic malarial pigment particles, and double plasma membranes were originally demonstrated and described by Rudzinska and Trager (1957). Since then various new structures have been detected such as the "conoid" and a system of peripheral fibrils, microtubules and convoluted tubules (Garnham *et al.*, 1960; Garnham, 1961; Garnham *et al.*, 1961; Garnham *et al.*, 1963).

In the erythrocytic stages of *Plasmodium lophurae* parasites the observation of random invaginations of the parasite led Rudzinska and Trager (1965) to suggest that they fed by intracellular phagotrophy. Ristic and Kreier (1964) observed a similar phenomenon in their studies of ultrathin sections of erythrocytic stages of *P. gallinaceum*. However, in both erythrocytic and exoerythrocytic stages, a specialized structure or "cytostome" has also been described on the surface of *P. gallinaceum* (Meyer and Musacchio, 1965; Aikawa, 1966; Aikawa *et al.*, 1966). Further studies have detected differences in the size of the cytostome and consequently of food vacuoles in different species, but Aikawa *et al.* (1966) have suggested that the process of feeding is the same in both avian and primate malarial parasites.

Garnham *et al.* (1960) carried out electron microscopic studies on exflagellating gametocytes and described 3 zones. The outermost envelope represented degenerating erythrocytic material. Next was a dense region, corresponding to the cytoplasm of the parasite which contained the golgi apparatus, endoplasmic reticulum, mitochondria, pigment, and the developing gamete, and finally there was a centrally located nucleus undergoing endomitotic division.

Histochemical studies have been made on different species of malarial parasites, but few on *P. gallinaceum*, and the organism remains poorly characterized in this respect. An electron microscopic cytochemical study of glucose-6-phosphate-dehydrogenase (G-6-PD) activity in erythrocytes of malarious mice, monkeys, and chickens reported by Theakston and Fletcher (1971) suggested that mammalian species of parasites have a predilection for erythrocytes possessing detectable amounts of G-6-PD activity. In *P. gallinaceum* infected chicken erythrocytes the distinction was much less obvious. No G-6-PD activity was found in parasites; however, only a small proportion of chicken erythrocytes showed activity. The study suggested that G-6-PD utilized by parasites was of erythrocytic origin.

Histochemical tests using both light and electron microscopy have been made for the presence of 3 acid hydrolases in plasmodia: acid phosphatase, β-glucuronidase and aryl sulphatase were all found to be located in lysosomes of some parasites (Scorza *et al.*, 1972). All of the enzymes were detected in normal erythrocytes of each of the hosts; however, *P. gallinaceum* parasites showed only aryl suphatase activity.

Pathology of *P. gallinaceum* infection. Most of the pathological aspects of natural *P. gallinaceum* infection have been reviewed by Lund and Farr (1965) and by Garnham (1966). The parasite is said to exert its pathogenic effects in 2 ways; firstly by invasion of blood cells and secondly by the development of exoerythrocytic stages in the brain. Crawford (1945) described the disease as running a very acute course in which birds lie in a corner with their faces and combs congested, then rapidly become pale, weak, and diarrheic, and die within 7 days. Quinine treated birds survived longer but eventually died due to posterior paralysis. Intense parasitemia and profound anemia with an

erythrocyte count below 1 million cells per cubic millimeter was frequently found. The anemia was also attributed to invasion of erythropoietic stem cells. A variety of pathological changes occur in the acute stage of the disease but marked degenerative changes were seen particularly in the adrenal glands, kidney, heart, and spleen along with centrilobular necrosis of the liver. Death in cases of avian malaria is generally considered to be due to cerebral lesions, resulting from capillary blockage by schizonts from lysed red cells.

Taliaferro and Taliaferro (1955) made a correlative histopathological study with special reference to the phagocytic response in avian malaria. Splenomegaly with black coloration due to malarial pigment developed with damage to the malpighian bodies of the spleen and lymphoid tissues very evident in advancing cases. An extensive hyperplasia of lymphoid tissues occurred during recovery which they ascribed to transformation of many cells to the macrophage form.

Two sporadic outbreaks in domestic fowl have been reported from India (Rao *et al.*, 1951; Das *et al.*, 1952). Parasites of one outbreak were studied experimentally and a detailed account given of the clinical signs, lesions, and immunity.

Jungle fowl are relatively resistant, but outbreaks of disease may occur in domestic or newly introduced breeds of poultry in endemic areas (Levine, 1967).

Immunological Studies

Avian Immunology

Infectious diseases in chickens are evidently affected by immunological events involving both humoral and cellular responses and some comments on the immunological system of avians are therefore pertinent.

The use of birds in experimental immunology has gained popularity since the recognition of the role of the lymphoid tissues of the bursa of Fabricius in the immune response (Pierce *et al.*, 1966; Thorbecke *et al.*, 1968; Forget *et al.*, 1970; Good, 1972). Lymphoid cells proliferating within the bursal follicles first synthesize IgM and afterwards IgG producing cells appear. Many experiments have now shown that this change in immunoglobulin synthesis can occur only within the bursa of Fabricius. Toivanen *et al.* (1972) found that bursal lymphocytes given in sufficient number to inbred chickens will completely reconstitute the morphological characteristics and functional capacities of bursectomized chickens, or of chickens made agammaglobulinemic by cyclophosphamide treatment in the neonatal period. On the other hand, cellular immune events mediating delayed hypersensitivity and graft rejection have been shown to be dependent upon the regulatory effect of the thymus lymphocyte system (Brown, 1969; Good, 1972).

Primary immune responses in chickens are associated with the production of IgM, followed by IgG. Thus 6-day antiserum to bovine serum albumin contained mainly IgM antibody and synthesis of IgG followed later (Benedict, 1967). Chickens inoculated with a single dose of hapten-conjugated chicken protein produce antihapten IgG and an antibody identified as IgA (Dreesman *et al.*, 1965). Three antigenically and physicochemically distinct immunoglobulins have therefore been reported to be present in chicken serum. However, the nature of secretory immunoglobulins in chickens has been a matter of dispute. Leslie *et al.* (1971) described a major secretory immunoglobulin from chickens with a sedimentation value of 7 Svedberg units (S) which differed from IgG and designated it "IgY." The 10.8 S moiety isolated from seminal fluid of chickens was described as a dimer of IgY with or without

secretory component. More recently Bienstock *et al.* (1973) described a different immunoglobulin which they regard as the chief secretory component and showed that it was 11.9 to 16.2 S IgA. Other immunoglobulins, particularly those mediating immediate hypersensitivity, have yet to be characterized.

Participation of humoral and cellular responses in human malarial infection has been discussed and reviewed and IgM, IgG, and IgA have been shown to possess specific antibody activity; however, protective immunity was ascribed to IgG only (Turner and Voller, 1966; Rowe *et al.*, 1968; Brown, 1969). So far there is little information in relation to the specific immunoglobulins in the immune response of the chicken to *P. gallinaceum*. In the following review an attempt will be made to provide a comparative account of the antigens and antibodies which participate in immune reactions and their consequences both in terms of detection and the pathogenesis of the disease which is seen in malarious birds.

Parasite Antigens

The chemical composition of *Plasmodium* parasites has been extensively studied, but characterization of antigens has been a more recent effort. Antigenic analysis has been undertaken employing various physicochemical and immunological techniques.

A number of antigens are thought to be shared by different species. Indirect evidence suggestion of antigen-sharing by various plasmodial species has been provided by cross reactions in fluorescent antibody tests and by passive hemagglutination tests (Voller, 1964; Bray, 1965; Collins *et al.*, 1966), but each species also has its species specific antigens (Zuckerman and Spira, 1965). Spira and Zuckerman (1966)

obtained 12 to 16 protein bands in disc electrophoresis of cell free extracts of most of the simian and avian malaria parasites, and the major components were shown to be shared by different species. However, the antigenic nature of these bands was not indicated.

Some of the difficulties encountered in the analysis of parasite antigens are derived from the problem of separation of the organism from host blood cells. Leukocytes have been found to interfere with the efficient extraction and, accordingly, Sherman and Hull (1960), Diggs (1966), and Sodeman and Meuwissen (1966) found it necessary to remove the buffy coat during repeated washings of infected cells. Spira and Zuckerman (1966) eliminated leukocytes and thrombocytes from their preparation by employing 3 to 6% dextran solution in which the parasitized red cells sedimented rapidly. Leukocytes may also be removed by passing whole parasitized blood through a column of packed filter paper (Aikawa and Cook, 1972).

In order to improve the purity of parasite antigens, attempts have been made to extract parasitic material from a population of almost pure infected cells. Separation of parasitized and non-parasitized cells has been achieved by using sucrose density gradients or density gradient mixtures of di-N-butyl and methyl-phthalate solutions. Parasite bearing cells were found in the low density area (Williamson and Cover, 1966; F. E. G. Cox, 1970; Saunders, 1970; Miller and Chien, 1970).

Various methods have been used for releasing plasmodial parasites from infected cells, including saponin lysis, disruption by French pressure cells, enzymic digestion, and immune lysis (Bangham and Horne, 1962; D'Antonio *et al.*, 1966; 1970; Bahr, 1966; Turner and McGregor, 1969; Killby and Silverman, 1969). These methods were subjected to evaluation by ultrastructural study of the released parasites by Aikawa

and Cook (1972) and their results suggested that saponin release of malarial parasites was highly satisfactory. Different methods for the extraction of antigens from released parasites have been used. Sonic disruption, freezing and thawing, the Hughes press, and homogenization in tissue grinders followed by extraction in veronal acetate buffer, have all been used with varying success (Diggs, 1966; Zuckerman, 1966; Turner and McGregor, 1969).

In general extracts prepared in these ways from different *Plasmodium sp.* have been shown to be extremely complex mixtures of protein, lipoprotein, carbohydrate, and lipids. Antigenic activity has been most often associated with proteinaceous moieties (Zuckerman, 1966). Immunological analysis and fractionation of parasite constituents of *Plasmodium berghei* were carried out by Chavin (1966) and 5 rivenol precipitated proteins were found to be antigenic when tested against serum of rats immunized with *P. berghei* infection.

Serological Cross Reactions of Plasmodial Antigens

The occurrence of cross reactions between antigens within the genus *Plasmodium* has already been referred to (Voller, 1964; Bray, 1965; Collins *et al.*, 1966). Kielman *et al.* (1970a,b) were able to use *P. gallinaceum* infected erythrocytes as antigen for the immunofluorescent diagnosis of human malaria infection. No difference in antibody titers was observed using *P. gallinaceum, Plasmodium falciparum, Plasmodium cyanomolgi* or *P. berghei* as antigen for the indirect immunofluorescence test. High titers were seen in positive cases whereas in normal serum samples no titers greater than 1:10 were observed.

Species of *Plasmodium* and *Babesia* have been shown to share antigenic substances. Serological cross reactions have been reported with sera of

recovered animals and serum of animals acutely infected with *Plasmodium* and *Babesia* (Cox *et al.*, 1968). This observation has been confirmed and the indirect fluorescent antibody test used to study the relationship betweeen antigens of *Plasmodium vinckei*, *Plasmodium chabaudi*, and *P. berghei* and to those of *Babesia rodhaini* and *Babesia microti*. Cross reactivity of antigens of *Plasmodium falciparum*, and *Plasmodium vivax* with *Babesia argentina* (Cox and Turner, 1970) was also found (Ludford *et al.*, 1972; Kagan *et al.*, 1972). Additionally, a considerable degree of protective cross immunity has been shown to occur between malaria parasites and piroplasms in rats and mice (Cox and Milar, 1968; Cox, 1972a,b).

Antigens in Plasma

A variety of immunologic activities is detectable in plasma during malarial infections. Blood of both experimental and naturally infected animals has been shown to contain circulating antigenic substances which have been designated as "serum antigens" (SA) or serum soluble antigens (SSA). SA have been reported in monkeys with *Plasmodium knowlesi* infection (Eaton, 1939; Cox, 1966), in ducks with *P. lophurae* (Torry and Kahn, 1949) and in chickens with *P. gallinaceum* (Todorovic *et al.*, 1968; Smith *et al.*, 1969; Lykins *et al.*, 1971). In human malaria infection SA have been reported by Turner *et al.* (1971), McGregor *et al.* (1968) and Wilson *et al.* (1969). SA were associated with *P. berghei* malaria by Cox *et al.* (1968), Wilson and Voller (1970), and Seitz (1972).

SA have been reported in other hemosporidian infections, particularly in babesiosis in cattle (Mahoney, 1967), dogs, horses and rats (Sibinovic *et al.*, 1967a,b; 1969; Ristic *et al.*, 1971).

SA generally appear during the acute phase of malaria infection and persistence is variable (Turner and McGregor, 1969; McGregor, 1972). In

P. gallinaceum infected chickens SA first appeared at the peak of parasitemia and persisted in detectable quantities during the declining phase of parasitemia over about 2 weeks (Todorovic *et al.*, 1968).

In treated human patients with *P. falciparum*, relapse or recurrence of parasitemia is followed by reappearance of SA but the specificity of the SA produced differs according to the time at which the sample is taken. Reappearance within a month leads to production of SA identical to those seen in the acute phase of early infection. However, SA of different specificities appear during parasitemia after one month has passed (McGregor *et al.*, 1968; McGregor, 1972).

The influence of treatment on persistence of SA has been discussed at length by McGregor (1972). He reported that such antigens tend to disappear quickly in patients responding effectively to antimalarial therapy. However, the higher the titer at the time of treatment the longer SA persisted in the circulation. The intensity of parasitemia and the onset of production of antibody to SA were also found to affect persistence (McGregor, 1972).

A considerable amount of quantitative and qualitative data has been accumulated on the nature of SA and it appears that many different substances are involved. In early work SA with different precipitation characteristics were described. Thus a SA which precipitated at pH 3.2 was reportedly present in greater concentration and was more stable than a second antigen which precipitated at pH 5.6 according to Torry and Kahn (1946). Acid precipitation at a pH lower than 5.5 led to loss of antigenic activity (Eaton, 1939). In more recent work the SA present in most human malarial infections have been found to be associated with the macroglobulin peak when subjected to Sephadex G-200 gel filtration (Turner, 1967; Turner and McGregor, 1968; Smith *et al.*, 1969; 1970) and

the molecular weights have been variously estimated within the broad range from 300,000 to 900,000 (Turner, 1967; McGregor *et al.*, 1968). For example, on the basis of gel filtration and thin layer Sephadex G-200 chromatography, Wilson *et al.* (1969) determined that the SA in *P. falciparum* designated 'S' (heat stable) had a molecular weight of 400,000.

The physicochemical characteristics of several different SA in *P. gallinaceum* have been used to classify these antigens as follows: SA1 is a protein with a molecular weight of 500,000 to 1,000,000; SA2 is a lipoprotein with a molecular weight of 150,000 to 250,000; and SA3 is a protein of less than 70,000 mw. Other criteria such as the diffusion rate in agar gel, sodium sulphate precipitation, anion exchange chromatography and dextran sulphate precipitation were used to separate and characterize SA in *P. gallinaceum* infection in the chicken. SA1 was precipitable with 10% sodium sulphate, and eluted from DEAE in 0.02 M and 0.1 M phosphate buffer fractions. SA2 was precipitated with dextran sulphate only (Lykins *et al.*, 1971).

Using sucrose density gradient centrifugation, two fractions A and B have been obtained and characterized from SA found in globulin of horses, dogs and rats with acute babesiosis. Fraction A was located in the 25% sucrose zone and had a sedimentation coefficient of 8S. Fraction B was found in the 40% sucrose zone and had an S value of 20 to 23. Physicochemical studies revealed them to be very complex structures containing peptides, lipids, phosphatides and polysaccharides, all of which were reported to contribute to antigenicity (Sibinovic *et al.*, 1967a).

Heat susceptibility has also been used to classify serum antigens into S (stable), L (labile) and R (resistant) antigens both in man and

in owl monkeys (Wilson *et al.*, 1969; Wilson and Voller, 1970). In 50 serum antigen samples at least 18 different S antigens have been reported. S antigens in *P. falciparum* infection differ in physico-chemical characteristics from those which occur in *Babesia* infections and those in *P. gallinaceum* infection in chickens (Wilson *et al.*, 1969).

Serum antigens of malarial infections have been discussed by Smith *et al.* (1972) and the relationships of SA classified, following the different criteria listed above.

Origin of Serum Antigens

The origin of serum antigens is still a controversial issue. On the basis of their observations on the appearance of SA in *P. gallinaceum* infection at the peak of parasitemia and their persistence even during the decline of parasitemia, when many erythrocytes are destroyed, Todorovic *et al.* (1968) suggested that the origin of some SA could be the parasitized erythrocytes themselves. On the other hand, Wilson *et al.* (1969) have hypothesized that SA are derived essentially from the parasite and suggest that either antigenicity is changed or a considerable degree of antigenic heterogeneity is maintained within the plasmodial population, which could account for the many SA detected. However, the possibility that their 'S' antigen was elaborated by infected erythrocytes was not ruled out (Wilson *et al.*, 1969).

It has been suggested by Turner and McGregor (1969a) that the alpha SA antigen is a soluble product of infected cells whereas the beta SA may be associated with the parasite itself. However, SA have also been referred to as exo-antigens (Weitz, 1960; Fife, 1971) and they have also been suggested to be secretions of excretions of the parasite (Fife, 1971). In view of these many conflicting opinions, it

is not possible at this time to offer a definitive statement of the origin of these substances.

The Role of Serum Antigens in the Pathogenesis of Disease

At the present time it is not clear whether serum antigens and their antibodies have an important role in the pathogenesis of hemosporidian disease. It is generally conceded that anemia cannot be completely attributed to the destruction of erythrocytes by the parasites, and there is evidence relating SA to this phenomenon. Serum antigens of acute malaria and babesiosis produced anemia when injected into normal animals (Cox, 1966; Sibinovic *et al.*, 1969). Adsorption of SA on the surface of red blood cells has also been reported by Weitz (1960), Zuckerman (1964a) and Sibinovic *et al.* (1969). In discussing the immunopathological mechanism of anemia in malarial infection Dixon (1966) suggested that antigen-antibody complexes coating receptors on the surface of RBCs are indeed considered to occur and, in association with complement fixation, lead to opsonization or outright lysis of erythrocytes. Such a mechanism involving serum antigen could have a role in anemia.

Soluble antigens were suspected of being associated with glomerular disease through the deposition of soluble immune complexes (Dixon, 1966; Cohen *et al.*, 1969). Presumptive evidence of immune complex deposits in malarial nephritis was obtained from renal biopsy specimens by immunofluorescent staining with anti-IgG (Ward and Conran, 1966; 1969; Soothill and Hendrickse, 1967; Allison *et al.*, 1969).

Immunizing Properties of Serum Antigens

Although the results have not been uniformly consistent there has accumulated over the past several decades considerable evidence to

suggest that protection against challenge may result from immunization with serum antigens. In his classical early work Eaton (1939) observed that soluble antigen from the serum of monkeys heavily parasitized with *P. knowlesi* when injected into normal monkeys could produce complement fixing antibodies similar to those seen in malaria infection. No protection seemed to have been produced against *P. knowlesi* challenge. However, ducklings were found to be immunized against challenge after inoculation with plasma from ducklings acutely infected with *P. lophurae* and serum of rats acutely infected with *P. rodhaini*, and more than half of the immunized ducks showed significantly lower parasitemia than that which developed in control birds (Corwin and Cox, 1969). Similarly, rats immunized with serum antigens from *P. knowlesi* infected monkeys also became resistant to challenge, in this case with *P. berghei* (Cox, 1966); and plasma obtained from chickens infected with *P. gallinaceum* has been used successfully to immunize chickens and protect them against homologous challenge (Todorovic *et al.*, 1967).

The phenomenon has been shown to occur with both homologous and heterologous hemosporidian infections, for example, plasma taken from rats or dogs with acute babesiosis was effective in immunizing rats against *B. rodhaini* and dogs against *B. canis* (Sibinovic *et al.*, 1967a,b). Immunization has been achieved using serum antigens of *Plasmodium* or *Babesia* species and challenge of the animals with another species (Cox, 1966; Sibinovic *et al.*, 1967a; Corwin and Cox, 1969). Additionally immunization by infection with *Plasmodium* and *Babesia* species confers resistance to challenge with heterologous species and genera of parasites (Cox and Miler, 1968; F. E. G. Cox, 1968). Immunogenicity of serum antigens has therefore been shown to lack both parasite and host specificity (Corwin and Cox, 1969).

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Cold-Active Agglutinin for Trypsinized Erythrocytes

A cold-active agglutinin was detected in the sera of persons suffering from black water fever (Oliver-Gonzalez, 1944). He suggested that it might contribute to the development of the disease.

An association of cold agglutinin for trypsinized red cells and anemia and erythrophagocytosis in *P. berghei* and *B. rodhaini* infections in rats was reported by Cox *et al.* (1966) and Schroeder *et al.* (1966). They suggested that the agglutinin might have been stimulated by the "T" stroma antigen of Hubner-Thomson-Fredenrich which were exposed by enzyme treatment as was suggested by Springer (1963). Morton and Pickles (1947) suggested that such agglutinin was incomplete antibody to erythrocyte antigens. Iturri and Cox (1969) reported that an anteration in kidney vascular endothelium was associated with this agglutinin and suggested that it might have a causal relationship.

Some physicochemical characteristics of cold agglutinins in infections of *P. berghei* in rats and *P. lophurae* infected chickens have been reported by Kreier *et al.* (1966) and Barrett *et al.* (1970). In both instances hemagglutinins were found to be more active at 4 C, occurred in the macroglobulin fraction of serum and were eluted from erythrocytes at 37 C. The agglutinins were susceptible to reductive cleavage by 2-mercaptoethanol. Kano *et al.* (1968) observed an increase in IgA levels in the serum of malaria infected humans and suggested that this may represent cold agglutinin. These authors all speculated that hemagglutinins were produced as a consequence of excessive erythrocyte destruction or to exposure of normally hidden antigenic sites on these cells. Further reference to the role of cold agglutinins is made below in the discussion of immune mechanisms in anemia in malaria.

Immunological Mechanisms in Malarial Disease

Anemia in Malaria

There are several disease entities in which the extent of the anemia is not related directly to the number of infective agents in the circulation and immunologic mechanisms have been suspected to be responsible (Zuckerman, 1964a; McGhee, 1964; Cox *et al.*, 1966; Schroeder *et al.*, 1966). In recent years a separate classification of immunologically mediated anemias has been developed in which autoimmune mechanisms associated with incomplete warm and cold agglutinins have been implicated (Davidson and Neelson, 1969).

In human malarial infections it has long been known that the severity of anemia is not always correlated with the degree of parasitemia and this observation has been extended to experimental models (Zuckerman, 1966). Several complex mechanisms hypothesized for the production of anemia by immunologic means have been discussed and reviewed by Brown (1969), including the liberation of antigenically altered red cell components from infected red cells, the combination of parasite products with the erythrocytes, and the production of parasite antigens closely resembling host components. These may be further compounded by the development of rheumatoid factor or immunoconglutinin (Houba and Allison, 1966). Dixon (1966) considered that the most likely explanation of the anemia was that antigen and antibody complexes, unrelated to the erythrocytes, could be adsorbed onto the surface of normal red cells which might then be lysed by a complement dependent system or phagocytosed. Evidence for these various hypotheses is derived from many different observations. Enhanced phagocytosis of non-infected cells has been observed by many workers and this phenomenon

is particularly marked at, or just following, the anemic crisis, but may persist for some time after the bulk of the parasites have been removed from the circulation (Zuckerman, 1964a; 1966; McGhee, 1965; Cox *et al.*, 1966).

Serum antigens circulating in the plasma have been found to be associated with anemia (Cox, 1966; Todorovic *et al.*, 1966a; McGregor *et al.*, 1968). Cold active agglutinins for trypsinized erythrocytes have been demonstrated with various hemosporidian infections (Cox *et al.*, 1966; Schroeder *et al.*, 1966; Kreier *et al.*, 1966; Iturri and Cox, 1969; Barrett *et al.*, 1970). Although the role of hemagglutinins is not clear, when these antibodies are present they are better related to the onset of anemia than is parasitemia. Further, it has been suggested that these hemagglutinins react with autoantigens that are on the surface of erythrocytes which are exposed during infection or by enzyme treatment (Schroeder *et al.*, 1965a).

In *P. gallinaceum* infection in the chicken, Gautam *et al.* (1970) showed that immune globulin becomes coated onto infected erythrocytes. This immune globulin agglutinated trypsinized erythrocytes from uninfected animals, whereas non-trypsinized erythrocytes were not agglutinated. It was suggested by these authors that the mechanism of erythrocyte destruction in malaria directly depends on the presence of parasites or some of their products which are absorbed from the blood stream onto erythrocytes. On the other hand, splenomegaly and anti-erythrocyte antibodies have been shown by Swann and Kreier (1973) to be produced as a result of erythrocyte destruction and were felt to be responsible for removal of damaged erythrocytes rather than causal in the anemia itself in *P. gallinaceum* infection.

Malarial Nephropathy

The association of nephritis with malaria is historically well established and was reported in chronic quartan malaria during the 19th century (Atkinson, 1884; Thayer, 1899; Bignami, 1900). Nephritis as a complication of malarial infection has been consistently observed since that time (James, 1910; 1912; Clarke, 1912; Deeks, 1916; Goldie, 1930; Giglioli, 1930; Jansco and D'Angel, 1931). Nephrotic lesions are observed in many instances of infectious anemia in domestic animals (Banks *et al.*, 1972). Malarial nephritis has not been reported in avian species.

Although advanced glomerular changes were associated primarily with chronic or recurrent cases of *Plasmodium malariae* infection, Giglioli (1932) on the basis of histopathological findings in 5 fatal cases of malarial nephritis, suggested that renal lesions in acute *P. falciparum* and occasionally *P. malariae* infection may spontaneously cure, but may sometimes cause chronic or even fatal kidney disease. Later, Maegraith and Findlay (1944) observed in their pathological studies that in the malarious kidney the lumina of the tubules were frequently filled with "casts" which appeared to range from desquamated epithelial cells and red blood cells to reddish-brown granules, the composition of which was reported to be uncertain. The casts were most abundant in the distal convoluted tubules, the ascending loop of Henle and in the collecting tubules. Spitz (1946) also reported hypercellularity and swelling of the glomerular tuft and established that tubular changes were restricted in occurrence to the disease entity known as malignant tertian malaria. Kibuka-Musoke and Hutt (1967) categorized several basic histological groups in 77 cases of malarial nephrotic syndrome which they studied. A majority of the cases (55) fell under the description of proliferative

glomerulonephritis which was further subdivided into 5 groups consisting of diffuse, lobular, focal, chronic, and minimal or "no change" types. In a smaller population of cases "membranous glomerulonephritis" was used to denote thickening of the basement membrane of glomeruli without proliferation of the glomerular tuft or capsule. Similar pathological observations have been reported by Burger *et al.* (1967) in renal biopsy findings in persons suffering from *P. falciparum* malaria. The glomeruli in these cases were found to be hypercellular, avascular and usually filled the capsular space. Adhesion of the glomerular tuft to Bowman's capsule was frequent. In addition, scattered glomeruli exhibited thickening and splitting of glomerular basement membranes when treated with periodic acid-Schiff stain (Soothill and Hendrickse, 1967).

The relationship of clinical nephritis to the immunological events in serum sickness was shown by von Pirquet (1911), and subsequent studies have led to the suggestion that immunological renal injuries can be produced by two distinct means: (1) antibody against antigen fixed in the kidneys (e.g., against glomerular basement membrane), (2) circulating antigen and antibody complexes (Weigle, 1961; Unanue and Dixon, 1967; Dixon, 1968; Carpenter, 1970).

These two types of nephritis differ in the deposition pattern of immune substances in the kidneys. In the latter type of nephritis antigens of streptococcal, viral, staphylococcal, malarial, and D-penicillin have been implicated (Dixon, 1966; 1968; 1972; Koffler *et al.*, 1967; Lambert and Dixon, 1968; Haslett *et al.*, 1968; Jaffe *et al.*, 1968; West *et al.*, 1968; Stickler *et al.*, 1968; Stollerman and Pearce, 1968; Ward and Kibuka-Musoke, 1969; Banks *et al.*, 1972; Banks and Hanson, 1972).

The ultrastructure of such immune deposits in renal glomeruli has been reviewed by Churg and Grishman (1972). Deposits differing in composition, location and ultrastructural characteristics have been described. In malarial nephritis electron microscopic observations showed fusion of foot processes of epithelial cells and thickening of the basement membrane. There is generally an increase in the subendothelial zone as well as occasional aggregation of electron dense and cytoplasmic material within the basement membrane itself (Allison *et al.*, 1969; Houba *et al.*, 1971). The presence of small lacunae scattered throughout the basement membrane is considered diagnostic of quartan malarial nephrotic syndrome by Hendrickse *et al.* (1972). On the basis of their ultrastructural studies, Boonpucknavig *et al.* (1973) concluded that glomerular lesions in *P. berghei* infection were also induced by immune complexes.

Similar electron microscopic observations have been made in ultrastructural studies of immune complex nephritis due to other causes in dogs (Murray *et al.*, 1971; Halliwell and Blakemore, 1972; Kurtz *et al.*, 1972), in cats (Slauson *et al.*, 1971), in glomerulitis of horses (Banks *et al.*, 1972), and in experimental renal disease induced by DNA-anti-DNA immune complexes in rabbits (Natali and Tan, 1972).

The fluorescent antibody technique has been extensively used to demonstrate the deposition of immune complexes in nephritis. The specific pattern of these complexes in glomeruli is in the form of fine, granular or lumpy discontinuous deposits along the glomerular capillary walls (Dixon *et al.*, 1958; Dixon, 1963; Kniker and Cochrane, 1965). This appearance differs markedly from the linear deposits of nephrotoxic antibodies along the capillaries in nephrotoxic antiserum nephritis described by Unanue and Dixon (1967). In recent studies employing

fluorescein conjugated anti-IgG, IgM and β_1C, evidence has been obtained suggesting that immunopathologic mechanisms in human malaria are mediated by formation and localization of malarial antigen-antibody complexes (Ward and Conran, 1966; 1969; Dixon, 1968; Allison *et al.*, 1969; Ward and Kibuka-Musoke, 1969; Adeniyi *et al.*, 1972; Hendrickse *et al.*, 1972). IgG and β_1C cryoglobulins have been incriminated as etiological agents in acute glomerulonephritis, on the basis of granular deposits demonstrable by immunofluorescence (Grupe, 1968). The characteristic deposition patterns seen in FA studies have also been used to confirm immune complex glomerulonephritis due to causes other than malaria (Slauson *et al.*, 1971; Murray *et al.*, 1971; Porter and Porter, 1971; Oldstone and Dixon, 1971; Halliwell and Blakemore, 1972; Kurtz *et al.*, 1972; Banks *et al.*, 1972; Natalli and Tan, 1972).

The pathogenic mechanisms of nephritis in autologous and heterologous phases have been described at length by Unanue and Dixon (1967), and Dixon (1972). A latent period was observed between the time at which antigen became demonstrable in the glomeruli and the appearance of histological changes. During this latent period a continuous immunological interaction in the glomerular capillaries occurred in which host antibodies and complement were associating and dissociating from the most permanent of the "planted" antigens (Unanue and Dixon, 1967). Small complexes, found when there was antigen excess, tended to remain in the circulation, whereas larger complexes, found at equivalence or in antibody excess, were rapidly removed by phagocytes. Intermediate sized complexes, which were found in moderate antigen excess, may remain soluble but large enough to react with complement. These were considered more likely to be trapped in the vessel wall where focal inflammation may be consequently induced (Dixon, 1968).

Cochrane and Dixon (1968) considered the likely sequence of action for the pathogenic activity of immune complex nephritis to be as follows: (a) formation of an antigen-antibody complex in the circulation; (b) platelet clumping by the complexes with release of vasoactive amines, (c) increased vascular permeability, (d) trapping of large soluble complexes in the basement membrane and (e) complement fixation and polymorphonuclear leukocyte accumulation.

Proteolytic enzymes and basic protein complexes are released which "chew up" the basement membrane according to Dixon (1972). However, the intensity and severity of lesions may also be dependent upon the metabolic state of the glomerulus, the phagocytic mesangial cells, and the degree of activity of the clotting system (Carpenter, 1970).

In spite of the growing mass of evidence concerning the immunologic basis of malarial nephritis, few people have attempted to elute antigens from affected kidneys. Antibodies were successfully eluted using 0.1 M citric acid pH 2.5 (Allison *et al.*, 1969), citric acid or glycine buffer of pH 2.5 (Houba *et al.*, 1971), by 1 M propionic acid (Gallo, 1970), or 0.02 M citrate buffer (Banks *et al.*, 1972).

In conclusion, there is sufficient indirect evidence to warrant the presumption that anemia and nephritis of acute malaria are mediated by immune mechanisms. However, the definitive experiments in which the disease signs were mediated by immune substances in absence of infection have not been performed. Neither has a comprehensive study of the antigens, which are the essential ingredients of immunologic disease, been made. Until it has been shown that a specific antigen and its antibody can mediate a lesion and that they are present in the lesion, the evidence for immune mechanisms in malarial disease is seriously deficient. The objectives of this research are to produce malarial

disease, anemia and nephritis, without infection, and to identify the antigens and antibodies responsible.

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Article 1

PATHOGENESIS OF ACUTE AVIAN MALARIA

I. IMMUNOLOGIC REACTIONS ASSOCIATED WITH ANEMIA, SPLENOMEGALY, AND NEPHRITIS OF ACUTE *PLASMODIUM GALLINACEUM* INFECTIONS OF CHICKENS

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ABSTRACT

Anemia, splenomegaly and glomerulonephritis in acute *Plasmodium gallinaceum* infections of chickens were associated with cold-active hemagglutinin for trypsinized human "O" erythrocytes, serum antigen, and the concurrent presence of antibody to serum antigen, as well as with the parasitemia of acute infection. Anemia with splenic enlargement was produced in normal chickens within 24 hours by intravenous injection of plasma from malarious chickens that contained high titers of hemagglutinin, serum antigen, and antibody. Similar anemia and splenic engorgement resulted in normal birds from injection of eluates from the washed cells of malarious chickens. The malarious plasma produced an acute glomerulonephritis within 24 hours similar to that observed on the 5th day of infection. The concurrent presence of the hemagglutinin, serum antigen, and its antibody in the injected plasma, and the failure of the plasma to produce nephritis in immunized chickens, suggested that immune substances might be causal in acute malarial anemia, and nephritis.

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INTRODUCTION

Anemia with splenomegaly, intravascular hemolysis and hemoglobinuria are well known disease signs resulting from infection with red blood cell parasites such as *Plasmodium* or *Babesia* species. Renal complications have also been associated with acute malarial and babesial infections, especially acute malignant tertian malaria and acute canine babesiosis (16,17,26). In earlier reports blood loss was attributed to red cell disruption by the emerging parasites, and mechanisms such as anoxia following blood loss, parasite toxins, and blood-parasite thrombi occlusion of the convoluted tubules have been suggested as causal in the renal disease (16,17).

Anemia of acute malaria and babesiosis has been associated with autoimmune-like mechanisms. Acute blood loss and splenomegaly with phagocytosis of uninfected erythrocytes were correlated with the presence of agglutinins for trypsinized erythrocytes (6,22). The induction of anemia in normal animals by injection of globulin from animals with acute malaria or babesiosis further indicated immune mechanisms in blood loss. The injected globulins apparently became fixed to the erythrocytes causing them to be sequestered in the spleen (5,12,24).

The correlation of the severity of kidney damage with the titer of agglutinin for trypsinized erythrocytes prompted the suggestion that autoimmune mechanisms might be causal in the acute glomerulonephritis observed in acute *Babesia rodhaini* infections of rats (13). However, Ward and Conran (30,31) suggested that antigens of the parasites were the substances principally responsible for stimulating the antibodies involved in nephritis of acute malaria.

Weigle (33) and Dixon (8) emphasized that soluble immune complexes may cause increased vascular permeability, generalized anaphylaxis, acute vasculitis and glomerulonephritis. It was also emphasized that immune complexes could combine with red blood cells and act as opsonins to cause phagocytosis or, in proper combination, fix complement and cause hemolysis (10). Investigations of malarial nephritis indicated that immune complexes might be present in kidney lesions (30,31,32).

In addition to cold-active hemagglutinin, serum antigens have been detected in serum from animals with acute red blood cell infections (3,5,7,23,25). Further, both serum antigen and its antibody may be concurrently present in the blood of moribund animals (28). It seemed possible that this antigen and its antibody could form complexes that could combine with uninfected red cells causing them to be destroyed, or could react with vascular endothelium to increase vascular permeability.

We have undertaken a study that attempts to define the relationship of the immunologic activities, found in blood during acute red blood cell infection, to anemia and nephritis. The present communication reports results of experiments which indicated that immunologic activity was related to anemia and nephritis in acute *Plasmodium gallinaceum* infections of chickens.

MATERIALS AND METHODS

P. gallinaceum was obtained from Dr. Julius P. Kreier, Faculty of Microbiology and Cellular Biology, The Ohio State University, Columbus, Ohio, and has been maintained by blood passage in White Leghorn cockerels. The chickens were obtained from Rainbow Trails Hatcheries, St. Louis, Michigan, as day-old chicks and were reared in

a brooder to the age of 4 to 6 weeks prior to use. Inocula for passage and for experiments were made with blood of infected roosters obtained near the time of peak parasitemia in order to maintain maximum virulence of the strain. Ten parts of blood, obtained from veins of the legs or wings, were added to 1 part heparinized 0.78% NaCl solution (100 units heparin per ml saline). After centrifugation at 800 g for 10 minutes the plasma was removed and the cells were resuspended in 0.78% saline. Inocula were administered intravenously (IV) after determining the number of parasitized erythrocytes (PE) per inoculum by described methods (3).

Blood for Wright's stained smears or for RBC counts was obtained by pricking the skin over the leg or foot veins. RBC counts were made with Sahli dilution pipettes and a microscope hemacytometer. Blood for experimental study was obtained by cardiac puncture under ether anesthesia, and was mixed with heparinized saline. Plasma was recovered after centrifugation and stored at -18 C. Prior to use it was clarified by centrifugation in a refrigerated centrifuge at 2000 g for 30 minutes.

Blood cells from infected chickens were washed twice with 0.78% NaCl solution. A volume of 1.2% NaCl solution equal to the packed cell volume was added with mixing and the mixture was allowed to stand overnight at 4 C. The supernatant was recovered after centrifugation and was dialyzed overnight at 4 C in 0.78% NaCl solution to reduce the salt concentration. The volume of the supernatant was reduced to 1/10 of the original by ultrafiltration in dialysis tubing under vacuum at 0 C. The concentrated cell eluate was then dialyzed against 0.78% saline overnight at 4 C and was then stored at -18 C until used.

Serologic tests were performed with clarified plasma samples. Titers of hemagglutinin (HA) for trypsinized erythrocytes were determined by methods modified from those of Cox *et al.* (7), using trypsin-treated human type "O" erythrocytes (1). Serum antigen (SA) and antibody to serum antigen (ABSA) were assayed by means of a tube bentonite flocculation (TBF) test described by Cox *et al.* (7), and modified by Thoongsuwan and Cox (28). To detect antigen, bentonite was treated with globulin of rats recovered from babesiosis while globulin of rats with acute *Babesia rodhaini* was used to measure antibody.

Chickens used for postmortem study were exsanguinated under ether anesthesia and autopsied. The spleen was removed and its volume determined by water displacement. A kidney tissue sample 5 mm in thickness was taken from the cortex area and was preserved in Bouin's fixative for 6 hours. The samples were washed, processed and mounted in Paraplast. Sections were cut at 5-6 μ and stained with either Hematoxylin, Giemsa's or Mallory's stain following recommended procedures (15).

The severity of kidney damage (SKD) was determined quantitatively by microscopic study of 100 Nephrons (glomerulus and adjacent convoluted tubules) scoring severity from 0 to 4 and totaling the scores as described (13).

Clarified plasma and the eluate from blood cells of malarious chickens were tested for anemia inducing activity by IV injection into normal birds using plasma and cell eluate from normal chickens as controls. Blood counts were made before injection and daily thereafter until the birds were discarded.

Normal chickens and those that had recovered from *P. gallinaceum* infection were injected IV with malarious or normal plasma and 24 hours

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later were exsanguinated and the kidney removed to be processed for histopathologic study as described for malarious chickens.

EXPERIMENTAL RESULTS

Immunopathologic studies of acute avian malaria. Twenty-one 8-week-old cockerels were infected with 10^8 *P. gallinaceum* infected erythrocytes. Three uninfected birds were autopsied and examined on day zero and three of the infected birds were sacrificed at daily intervals through the 5th day. Infections were patent on the day after inoculation and the percentage of parasitized erythrocytes rose rapidly to 70% by the 4th day. On the 5th day there was a drop in parasitemia which was followed by death of all birds before the 6th day. The %PE, RBC counts, score of kidney damage (SKD), spleen volume, and the titer of SA, ABSA and HA are presented in Table 1.

A second experiment was carried out using a smaller inoculum of 10^6 PE. Parasitemia was not detected in birds of this experiment until the 3rd day and did not reach a peak until the 9th day. Thereafter, there was a decline in parasitemia until the last of the birds were sacrificed on the 16th day. Data on parasitemia red cell counts, splenic enlargement, SKD, HA, SA and ABSA are presented in Table 2.

Gross observations of infected chickens showed that in birds given the larger inoculum of parasites, cyanosis of the comb and wattles was detected on the 3rd day and the birds had elevated temperatures. During the 4th or 5th day they would suddenly develop subnormal temperatures and became moribund. Combs and wattles would become deeply cyanotic. A greenish diarrheal discharge was also evident. The subnormal temperatures were followed by death within 24 hours. Just prior to death, combs and wattles appeared blanched.

In birds infected with 10^8 PE massive blood loss was evident on the 5th day, but not until the 9th day in those infected with 10^6. Anemia appeared to be better correlated with high titers of ABSA than to splenic enlargement or the HA titer.

Nephropathic changes were detected early in both experiments but scores were low. At this time, tests for both HA and SA were positive. Marked increases in SKD values were associated with marked increases in titers of ABSA. High SKD scores were indicated by swelling of the endothelial cells of the capillary of the glomerulus which appeared to obliterate the lumen of the capillary and cause the capillary loop to completely fill Bowman's space. Increases in the number of mesangial cell nuclei indicated a degree of hypercellularity of the tuft. There was also extensive swelling of the epithelial cells of the adjacent convoluted tubules that appeared to close the lumen, but without a marked increase in the number of nuclei present. It was also noted that blood vessels adjacent to the nephron were not patent and were void of blood cells. A nephron appearing in this condition was given an SKD value of 4. Study of the tubular elements reveals the presence of hyaline casts and edema of the basement membrane (Figure 1, A, B and C).

Anemia from inoculations of plasma of malarious chickens. Normal chickens were injected IV with plasma of malarious chickens that had been tested and found positive for HA, SA and ABSA. Prior to injection red cell counts were made on each bird. The results of counts made on normal birds injected with 2 ml of malarious plasma and those given normal plasma are shown (Table 3). The counts indicated that 24 hours after injection the birds given malarious plasma had 31% fewer RBC than

birds given equal amounts of normal plasma. The counts were reduced further to 41% two days after injection. Recovery from the blood loss did not become apparent until after the 6th day.

Blood smears prepared from birds given the malarious plasma were free of parasites on each day of observation. On the 11th day, 1 ml samples of blood were taken from each chicken, pooled, and injected into a normal chicken. Blood smears taken each day from this bird for 10 days showed no parasites.

Anemia from substances eluted from blood cells of malarious chickens. Normal chickens inoculated IV with 2 ml of the eluate of cells from malarious blood, and from cells of normal chickens, were subjected to red blood cell counts daily (Table 4). The eluted supernatant from normal chicken erythrocytes had no effect on red cell counts of normal chickens while reduction in counts which persisted for 5 days were observed in the birds injected with the solution eluted from erythrocytes of malarious birds.

Nephritis induced with plasma of chickens with acute *P. gallinaceum*. A group of normal cockerels and another group that had recovered from *P. gallinaceum* infection were each divided into two groups. One group of normal and a group of the recovered birds were injected with 2 ml of plasma from malarious birds that contained HA, ABSA, and SA. The other two groups were injected with the same amount of normal chicken plasma. All of the birds were sacrificed 24 hours after injection and kidney samples were taken for histologic study. The SKD values estimated for each kidney are recorded (Table 5). The SKD estimates for the chickens injected with the plasma of malarious birds were as severe 24 hours after injection (average of 304) as was

observed on the 5th day (average of 274) in chickens infected with 10^8 *P. gallinaceum* infected erythrocytes, or on the 9th day (average 300) in birds inoculated with 10^6 infected cells (Tables 1 and 2).

Histopathologic changes seen in kidneys of chickens 24 hours after injection of normal plasma or plasma from malarious chickens are shown (Figure 2, A, B, C and D). The nephron seen in Figure 2A shows cellular hydration of endothelial cells of the glomerular tuft and of the epithelial cells of the adjacent convoluted tubules similar to that shown in Figure 1B. The presence of casts and changes in tubular elements were noted (Figure 2C). Cellular hydration of tuft endothelium and tubular epithelium was not evident after injection of malarious plasma into chickens that had recovered from acute *P. gallinaceum* infection (Figure 2D).

DISCUSSION

It has been shown that along with severe anemia, there is an acute glomerulonephritis associated with acute *P. gallinaceum* infections of chickens. Nephritis became evident soon after the development of patent parasitemia and increased markedly in severity with the appearance of hemagglutinin for trypsinized erythrocytes, and antibody to serum antigen in blood of the malarious birds. The sudden increase in severity also preceded the anemia crisis and the profoundly moribund condition usually noted just prior to the death of the birds.

The most severe form of the nephritis was manifested as cellular hydration, or swelling of the endothelial cells of the glomerular tuft. Capillary endothelium was swollen to the extent that the lumen of the capillary was obliterated and the tuft completely filled Bowman's capsule. The absence of blood cells in the capillary and in blood

vessels adjacent to the glomerulus suggested that blood supply to the kidney might have been limited. Swelling of the epithelial cells of the proximal convoluted tubules was also extensive and it appeared that the lumen of the tubules were closed. The extent to which the blood vessels and tubules appeared to be shut down and the number of nephrons involved suggested that the kidneys of the birds might have been non-functional during that stage of the disease. Evidence of extensive extravasation of serous substance was indicated by the number of hyaline casts seen in the lumen of the distal tubules. The histologic appearance and the rapid onset of the changes are reminiscent of rapid changes observed in hydrocarbon-induced "toxic" nephritis described by Smith and Jones (27).

In birds that survived acute malaria the changes observed in moribund chickens were not evident. Nephropathic changes in these birds would be best described as subacute or chronic as indicated by some semilunar crescenting and fibrinous adherence of the tuft to Bowman's membrane. While the glomerular side of the nephron later appeared nearly normal, desquamated epithelium in the tubules remained evident for some time.

This "toxic" nephritis syndrome was observed in normal chickens 24 hours after intravenous injection of plasma from malarious birds, but was not produced in chickens that had recovered from acute *P. gallinaceum* infection. The absence of plasma induced nephritis in immune birds led us to suspect that immune mechanisms were responsible for malarial nephritis.

The nephritis seen in malarious birds was similar in appearance to that reported for rats with acute *B. rodhaini* infection. Since, in the rats, severity was correlated with the titers of agglutinin for

trypsinized erythrocytes, it was suggested that autoantibodies might have been involved, and that the nephritis might have been similar to that observed by Masugi (18) which was induced with antiserum to kidney tissues (13). However, it is now known that serum of rats with severe babesiosis may contain antibody to serum antigen and serum antigen as well as the hemagglutinin (28). Thus, the nephritis of acute babesiosis could have resulted from immune complexes of serum antigen and antibody.

Blood loss within 24 hours following the injection of plasma from malarious chickens indicated the presence of anemia inducing substances in the plasma. The observation of blood loss following the injection of substances eluted from blood cells of malarious birds suggested that the anemia factor(s) in plasma had combined with erythrocytes.

Blood loss following injection of plasma of malarious animals in these experiments confirmed earlier observations. Cox (5) reported a sustained reduction in red cell counts of rats that had been inoculated with globulin of monkeys with acute *P. knowlesi* malaria. Corwin *et al.* (4) observed blood loss in ducklings injected with plasma of malarious ducks. Serum globulin of both rats and dogs with acute babesiosis caused blood loss in normal rats following injection. That globulin had combined with erythrocytes was indicated by reactions of the blood cells of the recipient rats with fluorescent antibody to serum antigen. Since the reacting cells disappeared from the blood and the spleens of the rats sacrificed for autopsy 24 hours after injection were enlarged, it was suggested that the cells had been sequestered in this organ. The presence of large numbers of hyperchromic macrocytic erythrocytes in the blood of recipient rats 48 hours after injection indicated that the sequestered cells had been

destroyed. Since this phenomenon was not evident after injection of normal globulin, blood loss was attributed to substances that had been elaborated during acute babesial infection (24). In this earlier work the only substance identified in the injected globulin was serum antigen. We now know that serum of rats with acute *B. rodhaini* infection may contain antibody to serum antigen as well, and it is now suspected that Sibinovic *et al.* (24) may have been staining the antigen side of a complex with the conjugated antibody.

It is possible that both anemia and acute nephritis might have been in part mediated by the same immune substances. Since both serum antigen and antibody were detected concurrently in plasma of moribund birds it is possible that these ingredients could have been causal. As was suggested by Dixon (10), we suspect that concentrations of antigen and antibody might be obtained that would be optimal for binding erythrocytes, causing them to be sequestered in the spleen, or for binding complement to cause intravascular hemolysis. As suggested by Ward (29) complexes of antigen, antibody and complement may alter vascular permeability, and thus in part contribute to nephritis. The failure of malarious plasma to induce nephritis in immune recovered birds encourages us to favor the idea that immune complexes, rather than blood permeability factors, might have been causal. However, permeability factors have been demonstrated in blood of malarious animals (19,20,21). These too must be given due consideration in determining the pathogenic mechanisms of vascular-renal disease associated with acute malaria.

We do not wish to imply that the observed nephritis was a major cause of mortality. While it did appear that the kidneys were nonfunctional, this condition apparently existed for only a short time

and histopathologic study of kidneys of recovered birds did not reveal that major damage had been done. It is probable that the capillary beds of all major organs of the body were affected in a similar manner and that malfunction of the brain or the lungs could have contributed to death.

Finally, we would not wish to incriminate serum antigen and antibody as solely responsible for morbidity and mortality in acute malaria. In addition to the parasites, cold-active hemagglutinin was present, and it is possible that there are other soluble antigens present in the blood of malarious animals that might stimulate antibodies. Ultimate definition of the causal substances depends upon isolation of each immunologically active substance, purification of antigens, the detection of early appearing antibody and detecting the antigen and antibody in extravascular lesions with fluorescein conjugated monospecific antisera. Attempts to achieve these definitive studies are in progress.

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Table 1. Average of percentage of parasitized erythrocytes (%PE), red blood cell counts (RBC x 10^6), spleen volume in ml., severity of kidney damage (SKD), the titers of hemagglutinin for trypsinized erythrocytes (HA), serum antigen (SA) and antibody to serum antigen (ABSA) in chickens infected with 10^8 *Plasmodium gallinaceum* infected erythrocytes.

Days of Infection	Ave. % P.E.	Ave. RBC x 10^6	Ave. Spleen Vol. ml.	Ave. SKD	Ave. HA	Ave. SA	Ave. ABSA
0	0	2.45	1.8	39.3	0	0	0
1	2.75	2.16	3.9	130.7	0	8	0
2	15.75	1.92	5.6	209.0	16	64	16
3	44.50	1.93	7.2	202.7	32	128	128
4	69.75	1.85	4.6	261.0	128	32	256
5	60.00	1.01	5.3	274.0	16	8	256

Table 2. Averages of percentage of parasitized erythrocytes (%PE), red blood cell counts (RBC x 10^6), spleen volume in ml., severity of kidney damage (SKD), the titers of hemagglutinin for trypsinized erythrocytes (HA), serum antigen (SA) and antibody to serum antigen (ABSA) in chickens infected with 10^6 *Plasmodium gallinaceum* infected erythrocytes.

Days of Infection	% P.E.	RBC x 10^6	Spleen Vol. ml.	SKD	HA	SA	ABSA
0	0	2.68	1.5	36	0	0	0
3	0	2.60	2.0	165	16	16	0
5	1.0	2.25	1.67	161	8	128	8
7	61.0	1.61	1.60	257	16	1024	256
9	61.3	1.29	2.67	300	8	64	128
11	76.3	0.92	6.70	302	64	128	256
13	47.3	1.03	5.80	327	16	128	1024
16	0	1.49	4.80	294	8	0	128

Table 3. Average red blood cell counts (RBC x 10^6) on normal chickens injected with the plasma of chickens with acute *Plasmodium gallinaceum* infection (Exptl.) and normal chickens injected with plasma of normal birds (Control).

Days after Injection	Average RBC x 10^6 Control	Exptl.	% RBC lost	D.F.	t.	p.
0	2.71 ± 0.14	2.67 ± 0.17	-1	6	0.30	N.S.
1	2.67 ± 0.11	1.68 ± 0.12	31	6	11.49	<0.001
2	2.66 ± 0.17	1.58 ± 0.18	41	6	8.56	<0.001
3	2.69 ± 0.15	1.83 ± 0.05	27	6	10.28	<0.001
4	2.78 ± 0.10	1.79 ± 0.07	36	6	14.97	<0.001
5	2.73 ± 0.17	1.74 ± 0.16	36	6	8.31	<0.001
6	2.63 ± 0.12	1.87 ± 0.20	28	6	6.31	<0.001
7	2.60 ± 0.13	1.91 ± 0.25	26	6	4.76	<0.005
8	2.67 ± 0.04	2.02 ± 0.09	24	6	12.00	<0.001
9	2.71 ± 0.11	2.28 ± 0.25	16	6	3.03	<0.025
10	2.62 ± 0.06	2.30 ± 0.25	12	6	2.42	N.S.

Table 4. Red blood cell counts (RBC x 10^6) of normal chickens injected with "eluate" from *P. gallinaceum* infected erythrocytes and normal chickens injected with "normal" red cell eluate.

Days after Injection	Average RBC x 10^6 Control	Exptl.	% RBC lost	D.F.	t.	p.
0	2.89 ± 0.27	2.94 ± 0.19	-1	8	0.35	N.S.
1	3.21 ± 0.50	2.23 ± 0.10	30	8	4.22	<0.005
2	3.09 ± 0.29	2.17 ± 0.07	30	8	6.70	<0.001
3	3.23 ± 0.40	2.34 ± 0.13	30	8	4.74	<0.005
4	3.03 ± 0.25	2.67 ± 0.23	13	8	2.38	<0.050
5	2.83 ± 0.30	2.06 ± 0.22	25	7	4.18	<0.005
7	2.82 ± 0.35	2.15 ± 0.43	23	8	2.64	<0.050
9	2.97 ± 0.18	2.44 ± 0.62	9	8	1.81	N.S.

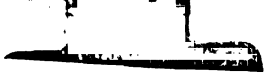

Table 5. Severity of kidney disease (SKD) in normal chickens and in chickens recovered from *Plasmodium gallinaceum* 24 hours after intravenous injection of plasma of chickens with acute malaria.

Normal Chicken Malarious Plasma		Normal Chicken Normal Plasma		Recovered Chicken Malarious Plasma		Recovered Chicken Normal Plasma	
Chicken No.	SKD	Chicken No.	SKD	Chicken No.	SKD	Chicken No.	SKD
2391	355	2393	81	1370	104	1368	101
2392	310	2396	168	1342	131	1372	110
2394	370	2386	74	1377	150	1347	132
2395	294	2400	106	1366	128	1364	142
2397	245			1343	149		
2398	316			1348	94		
2385	256			1344	154		
2399	287			1365	129		
Average SKD							
304		107		130		121	

Figure 1. Nephritis in acute *Plasmodium gallinaceum* infections of chickens.

A. Nephron (glomerulus and adjacent proximal convoluted tubules) of normal chicken kidney. Note the size relationship of the glomerular tuft and Bowman's space. Adjacent capillaries are patent and erythrocytes are clearly visible within the lumens. The lumen of the adjacent convoluted tubules is patent and the tubular epithelium is intact. In estimating severity of kidney damage (SKD) this nephron would be evaluated as zero (H and E, 250x).

B. Nephron of a kidney taken from a malarious chicken at the peak of the parasitemia-anemia crises. Note that capillaries adjacent to the glomerulus are not obvious, the tuft completely occupies Bowman's space, there is an increase in the number of mesangial cell nuclei, and the lumen of most of the adjacent convoluted tubules is obliterated. A nephron in this condition was given an SKD evaluation of 4 plus (H and E stain, 400x).

C. Section from the proximal convoluted tubules of a kidney taken from a malarious chicken at the peak of the parasitemia-anemia crises. Note that the tubule (top right) contains a hyaline cast. The tubule in the center contains a cast consisting of cellular debris. The basement membrane of several of the tubules has separated (blistered) from the tubular epithelium. Interstitial edema is also evident (Giemsa stain, 250x).

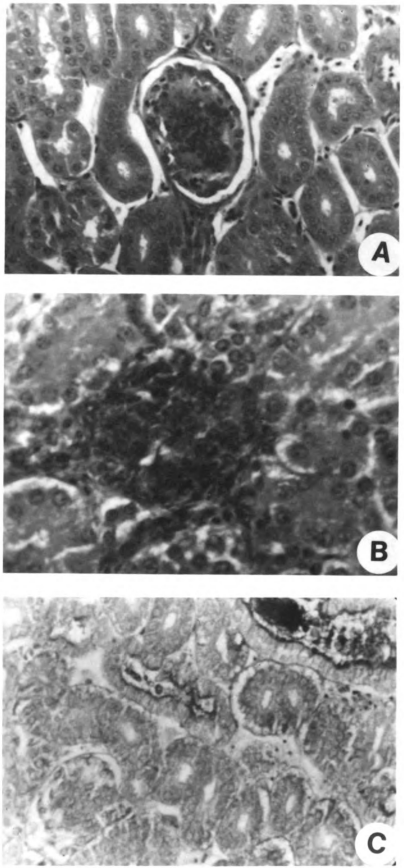

Figure 1

Figure 2. Nephritis in chickens induced by injection of plasma of chickens with acute *Plasmodium gallinaceum* infection.

A. A nephron (glomerulus and adjacent proximal convoluted tubules of a kidney taken from a chicken 24 hours after injection of malarious plasma. Note that the glomerular tuft completely occupies Bowman's space; however, hypercellularity is not evident. The tubule at the top left appears necrotic. Epithelium of other tubules is swollen and the lumen of the two lower tubules appears to contain casts. A nephron in this condition was given a severity of kidney damage (SKD) evaluation of 4 plus (Mallory's stain, 250x).

B. Nephron of a kidney taken from a chicken 24 hours after injection of normal chicken plasma. While the nephron appears slightly edematous, it does not differ remarkably from the nephron shown in Figure 1A. The SKD was estimated at 1 plus (H and E stain, 250x).

C. Section from area of distal convoluted tubules of a kidney taken from a chicken 24 hours after injection of malarious plasma. Note that 3 tubules at the center are filled with hyaline casts. The epithelium of other tubules is swollen (Giemsa stain, 250x).

D. Nephron of kidney from a chicken that had recovered from acute *P. gallinaceum* infection, 24 hours after injection of malarious plasma. The semilunar crescenting, the fibrinous adherence of the tuft to Bowman's membrane, and the evidence of desquamation of tubular epithelium are characteristic of the nephron of recovered chickens (H and E stain, 250x).

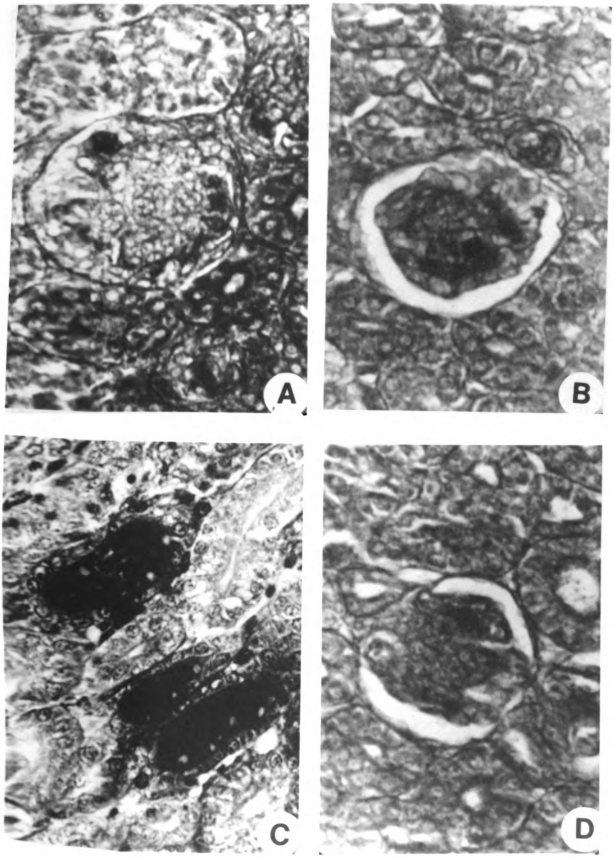

Figure 2

Article 2

PATHOGENESIS OF ACUTE AVIAN MALARIA

II. A STUDY OF ANTIGENS AND ANTIBODIES ASSOCIATED WITH ANEMIA OF ACUTE *PLASMODIUM GALLINACEUM* INFECTION OF CHICKENS

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ABSTRACT

The anemia of acute *Plasmodium gallinaceum* malaria of chickens had been found to be in part mediated by substances in the plasma of malarious chickens. Injections of malarious plasma into normal chickens produced a 30% reduction in red blood cell counts within 48 hours which persisted for more than a week. Injections of hypertonic salt solution eluates from cells of malarious blood produced a similar anemia. Serologic study of plasma implicated two mechanisms, a cold-active IgM class of hemagglutinin and a soluble globulin associated serum antigen and its antibody. The serum antigen was shown to be serologically unrelated to parasite antigen and similar, if not identical, to serum antigen elaborated during red blood cell infections with parasites other than those of the genus *Plasmodium*, e.g., *Babesia*. Study of cell eluates from chickens made anemic by plasma injections revealed the presence of both serum antigen and its antibody. Immunofluorescent study revealed that

the blood cells of these birds reacted strongly with conjugates of antibody prepared in chickens to serum antigen from rats with acute babesiosis, and from chickens with acute *P. gallinaceum* malaria. The cells did not react with conjugates of antibody to *P. gallinaceum* parasite antigen. Strong reactions with anti-chicken 7S globulin suggested that antibody was also present on the surface of the blood cells. Immunofluorescent activity was substantially reduced three days after injection when anemia was maximal. These experiments lead to the conclusion that the anemia of acute *P. gallinaceum* malaria of chickens is in part an immune complex disease, and that serum antigen, which was serologically unrelated to parasite antigen, was a principal causal ingredient.

INTRODUCTION

Immune mechanisms have been implicated in the anemia of acute haemosporidian infections. The association of cold active hemagglutinin for trypsinized erythrocytes with anemia, splenomegaly, and phagocytosis of erythrocytes by splenic macrophages in acute malaria and babesiosis of rodents led to the suggestion that autoimmune-like mechanisms might have been in part responsible (1,2). Anemia following the injection of globulins from animals with acute malaria or babesiosis further indicated that there were anemia inducing substances in the blood of such animals (3,4). This observation was confirmed by injecting normal chickens with plasma of chickens with acute *Plasmodium gallinaceum* infection. A 30% reduction in the red cell counts was achieved within 24 hours and the birds did not recover from this anemia until after 8 days. It was further indicated that anemia inducing substances in the plasma had combined with red cells, by producing a

similar anemia with material leached from blood cells of malarious chickens (5).

Cold-active hemagglutinin was associated with acute avian malaria and was identified as cold-active IgM (6). These observations were confirmed by Soni and Cox (5). It was also found that the hemagglutinin recovered from the plasma of chickens with acute *P. gallinaceum* infection by absorption with, and elution from, trypsinized erythrocytes, caused anaphylactic-like shock when injected into normal chickens. Plasma samples collected 24 hours later showed signs of hemolysis and the red cell counts of the birds were reduced by 25%. When plasma that had been absorbed free of hemagglutinin was injected into normal chickens, it too caused a 25% drop in red cell counts. Thus it was indicated that there was more than one anemia inducing factor in the plasma of chickens with acute *P. gallinaceum* malaria (7).

In earlier work showing that serum from animals with acute malaria and babesiosis contained anemia inducing substances, the only identified component injected had been an immunogen termed serum antigen. With fluorescein conjugated antibody to serum antigen, it was shown that the antigen had combined with the red cells of the injected animals and had caused them to be removed from the circulation (3,4). The means whereby serum antigen could combine with erythrocytes was not indicated. An insight was furnished by the observation that the anemia of acute *Babesia rodhaini* infections of mice was better correlated with the concurrent presence of serum antigen, and antibody to serum antigen, than it was to parasitemia or to the titers of hemagglutinin for trypsinized erythrocytes (8).

Thus a mechanism suggested by Dixon (9) that involved the binding of soluble antigen-antibody complexes to erythrocytes to act as opsonins was indicated as a possible anemia inducing factor. The antigens and antibodies in the blood of chickens with acute *P. gallinaceum* infection have been identified and those that react *in vivo* with normal erythrocytes have been indicated. The details of this study are presented in this communication.

MATERIALS AND METHODS

Animals and Infections: The source of the *P. gallinaceum* infection, the chickens used, methods for maintenance, experimental infections, and the parameters for evaluating infection and pathogenesis have been described elsewhere (5).

Chickens infected with *P. gallinaceum* were exsanguinated at the peak of parasitemia and the blood was mixed 10:1 with heparinized saline (100 units Sodium Heparin to 1.0 ml of 0.78% NaCl solution) as described (5). After centrifugation at 800 g for 10 minutes the supernatant plasma was removed and stored at -18 C. The sedimented cells were resuspended and washed twice with saline (0.78% NaCl solution) by centrifugation at 800 g and resuspension in saline. The washed cells were then leached with hypertonic NaCl (1.2%) solution (5). This leached material was stored at -18 C.

Preparation of Parasite Antigen: Washed cells of malarious chicken blood were treated to liberate parasites by methods modified from those of Sherman and Hull (10) (see Flow Chart). After concentration and tests for antigen activity, the soluble parasite antigen (PA) was stored at -18 C.

The concentrated PA was further purified by column chromatographic methods using Sephadex G-200 as described below. Fractions from the column were pooled as shown (Figure 2). After concentration by polyethylene glycol treatment, the pools were tested for antigen in double diffusion in gel tests using plasma of recovered chickens. Active fraction pools were stored at -18 C.

Preparation of Serum Antigens: Serum antigen was detected in plasma of malarious chickens by means of a tube bentonite flocculation (TBF) test using bentonite suspension treated with globulin of rats recovered from *B. rodhaini* as described by Thoongsuwan and Cox (8). This test was later modified to use bentonite treated with globulin of chickens that had been immunized with serum antigen of rats with acute *B. rodhaini* infection (ABr).

Plasma with serum antigen was clarified by centrifugation at 2000 g for 30 minutes at 5 C. The plasma was then subjected to column chromatography using Sephadex G-200 as described below. Fraction pools obtained as shown in Figure 2 were concentrated by ultrafiltration in dialysis tubing under negative pressure, or by polyethylene glycol dehydration. After tests for activity against ABr, the active fractions were stored at -18 C.

Attempts were made to further separate serum antigen fractions by precipitation with sodium sulphate and dextran sulphate following methods described by Lykins *et al.* (11).

Preparation of Leach Antigen: Antigens were eluted from the washed cells of malarious chicken blood with hypertonic 1.2% NaCl solution as described (5). These preparations were then subjected to column chromatography using Sephadex G-100 as described below. Fraction pools

as shown in Figure 2 were tested for antigen activity in gel tests using ABr and plasma of chickens recovered from *P. gallinaceum* infection. Fractions with antigen activity were stored at -18 C.

Column Chromatography Methods: Molecular sieving methods used were Sephadex G-200 or G-100, prepared in borate buffered NaCl solution, pH 8.2, with an ionic strength of 0.16 (BBS) as described by Benedict (12). The column size was 100 x 2.5 cm with a packed column size of 90 x 2.5 cm. The flow rate was adjusted to 15 ml per hour and the collector was set for 3.0 ml. All molecular sieving was done at a constant room temperature of 22 C. Four ml volumes, containing 160 mg of protein, were equilibrated by dialysis overnight against BBS. The sample was then mixed with 0.16 gm of sucrose and applied to the column. Fractions were tested for absorbance of light at 280 nm and appropriate fractions were pooled (Figure 2). Each pool was dialyzed in 1:100 BBS overnight, and then concentrated by lyophilization, ultrafiltration, or dehydration with polyethylene glycol.

Double Diffusion in Gel Test Methods: Modifications of the gel diffusion test methods of Ouchterlony (13) as described by Lykins *et al.* (11) were employed, using as a base 1% Colab Ion Agar No. 2. The agar gel was made in 1.5 M NaCl to test for serum antigen, and in Veronal Acetate buffer at pH 8.6, ionic strength of 0.1, for other antigens. Sodium azide to give a concentration of 0.02% was added to the gel to prevent contaminant growth. All gel slides were incubated at 22 C for as long as 5 days and multiple feedings of wells was done as needed. Precipitin lines were stained by methods of Uriel (14). Photographic records were made of reactions in gel by photographing either the fresh wet preparations, or stained dried slides.

Immunoelectrophoresis (IEP) Methods: IEP was accomplished with a Gelman Instrument Co. apparatus and Gelman's high resolution Tris-Barbitol-Sodium Barbitol buffer, pH 8.8, with an ionic strength of 0.05. Slides were prepared from 1% Colab Ion Agar No. 2 in Gelman's buffer. Test slides were exposed to 18 ma of current for 65 minutes. After electrophoresis, the slides were reacted against anti-chicken serum or anti-chicken globulin for 48 to 72 hours at 22 C. The slides were washed and stained as described by Uriel (14).

Disc Electrophoresis (DEP) Methods: The physicochemical nature of blood fractions showing antigen activity was determined by disc electrophoresis using modifications of methods described by Davis (15). The running gel consisted of 7% cyanogum in Tris-HCl buffer pH 8.6 which was polymerized by addition of TEMED (N,N,N',N'-Tetramethylethylene) and Ammonium persulphate (AP). The spacer gel consisted of 4% cyanogum in Tris-HCl buffer, pH 8.2, polymerized with TEMED and AP. Samples to be run were made up to a 4% sucrose solution and 20 µl were applied to the spacer gel, along with a drop of Bromophenol blue to serve as tracking dye. The current was applied at 5 ma per tube until the migration was complete. Discs were stained differentially for protein, lipids, carbohydrates, and for deoxyribonucleic acid as described by Turner and McGregor (16).

Tube Bentonite Flocculation (TBF) Test for Antigens and Antibodies: Suspensions of sized bentonite particles have been used to titrate antigens by treating them with antibody and for titrating antibody by treating the bentonite with antigen (5,8). The methods for preparing stock bentonite suspension, treating the bentonite with antigens or antibodies, and accomplishing the TBF test have been described

(5, 8,17). In the present work, the TBF test was used to titrate antigens present in material leached from blood cells, antigens prepared from parasites, and serum antigen. One tenth ml of globulin from immunized chickens, diluted 1:20 with phosphate buffered 0.85% NaCl solution, pH 7.2 (PBS), was used to sensitize 10 ml of bentonite suspension.

Preparation of Antiserum: ABr was prepared in chickens by immunizing them with serum antigen from rats with acute *B. rodhaini* infection. Each chicken was given 0.5 ml of plasma mixed with 0.5 ml of complete Freund's adjuvant injected at multiple sites, followed by similar injections one week later. After one week, a test bleeding of the chickens was made and they were given a 3rd injection of the antigen with adjuvant. Two weeks later the birds were exsanguinated and the plasma recovered. Plasma from these birds was tested for antibody in gel diffusion tests against serum antigen bearing plasma of chickens with acute *P. gallinaceum* infections. After clarification, the plasma was stored at -18 C.

Antisera to each of the purified antigens from the blood of malarious chickens was prepared in chickens by immunizing them, each with 1.0 mg of antigen in 0.5 ml of saline mixed with an equal volume of complete Freund's adjuvant as described above. These birds were exsanguinated 2 weeks after the 3rd injection and the plasma tested against the respective antigen in gel diffusion tests. They were also tested for specificity against the homologous and heterologous antigens in gel and TBF tests.

Antisera to Normal Chicken Serum and to Normal Chicken Globulin: Blood from normal chickens was withdrawn and allowed to stand overnight at 5 C. The next day it was centrifuged at 2000 g for 30 minutes and

the supernatant serum was recovered and stored at -18 C. A portion of the serum was precipitated with an equal volume of saturated ammonium sulphate and the recovered globulin was dialyzed free of sulphate as described (5). One mg of serum, or 1.0 mg of globulin, in 0.5 ml of 0.78% NaCl solution was mixed with an equal volume of complete Freund's adjuvant and each was given by multiple site intramuscular injection to rabbits. The rabbits were given a second injection a week later and after another week a trial bleeding was made. Sera from these rabbits reacted strongly in gel tests with chicken serum, or with whole globulin. The rabbits were given an additional injection and were exsanguinated the following week.

Antiserum to chicken 7S globulin was obtained from Nutritional Biochemical Corporation, Cleveland, Ohio.

Fluorescein Isothiocyanate (FITC) Conjugation of Antisera: Antisera to be used for fluorescent antibody testing were conjugated with FITC. Globulins obtained from sera or clarified plasma, by 50% ammonium sulphate precipitation, were dialyzed against BBS for 2 days with a change of buffer after 24 hours, to remove ammonium sulphate. The protein concentration of the globulins was determined as described by Lowery *et al.* (18) and was adjusted to a concentration of 20 mg per ml by addition of .85% NaCl solution. FITC conjugation was achieved by methods described by Cherry *et al.* (19). The conjugate was then tested in gel against antigen to ensure activity before it was stored in small aliquots at -70 C.

Fluorescent Antibody Tests and Microphotography: Blood slides from malarious chickens, chickens injected with plasma of malarious chickens, and control birds were prepared and stored at -70 C as recommended by

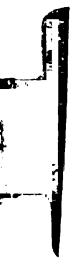
Sulzer and Wilson (20). They were gradually equilibrated to room temperature by holding the slides at -20 C for 30 minutes, at 4 C for 30 minutes and then at room temperature before the slides were fixed in Acetone for 10 minutes. The slides were rinsed in PBS, pH 7.0. Direct staining was accomplished as described by Ward and Conran (21).

Fluorescence was determined with a Carl Zeiss Fluorescope equipped with an Osram HBC 200 Mercury lamp using excitor filter II BG 13 and barrier filter 50/44. Microphotographs were taken with TRI-X-Pan Kodak film at an exposure time of 30 and 60 seconds.

EXPERIMENTS AND RESULTS

Tests of Components of Blood from Malarious Chickens for Antigens: The plasma, the material leached from the washed blood cells with hypertonic NaCl solution, and the material extracted from liberated parasites each reacted with plasma of chickens that had recovered from acute *P. gallinaceum* infection (Figure 1-1). The plasma, here designated as serum antigen (SA), the leach antigen (LA), reacted with globulin of chickens immunized with plasma of rats with acute *B. rodhaini* (ABr). However, the parasite antigen (PA) did not react with ABr (Figure 1-2).

In further tests the leached material reacted in gel tests with plasma of recovered chickens, ABr and antibody to serum antigen from chickens (ASA) but did not react strongly with antibody to parasite antigen (APA (Figure 1-3). The reaction with serum antigen from rats with acute *B. rodhaini* (BrA) indicated the presence of antibody to serum antigen, as did also the reaction with serum antigen from malarious chickens (SA2), and the reaction with anti-chicken 7S globulin (Figures 1-4, 1-5, 1-6).



In immunoelectrophoresis, tests of LA with anti-chicken serum, the presence of 19S and 7S globulins, along with albumin, was indicated. Immunoelectrophoresis of leach from normal blood cells, tested with anti-chicken globulin, revealed only traces of protein (Figure 1-7). However, disc electrophoresis of both leach preparations revealed the presence of a variety of bands taking protein stain. One band in the 19S globulin range distinguished the leach from malarious blood cells from that of the normal cells (Figure 1-8).

After column chromatography of each of the crude components from malarious blood, fraction pools as indicated in Figure 2 were made and tested in gel against plasma of recovered chickens. Pools 1, 2 and 3 of the fractionated SA, Pools 2 and 3 of leached material, and all pools collected from the column for parasite extract reacted. Thus it appears that antigen(s) present in plasma was restricted to the 19S and 7S protein peaks. Antigen activity in pool 2 of the leached material was eluted with the globulin class proteins. However, the activity in pool 3 was associated with proteins of lower molecular weight. The results from the tests of column fraction pools of parasite antigen are unclear. Antigen activity was found in all pools, including the 5th, which did not show absorbance at 280 nm.

An attempt was made to further purify the active SA fractions by precipitating each with Dextran Sulphate. The supernatant was then precipitated with 10% sodium sulphate. In each fraction pool, the material precipitated with the sodium sulphate (SA-1) and Dextran Sulphate (SA-2) reacted with both recovered chicken globulin and with ABr. SA-2, but not SA-1, reacted in gel tests with antibody to parasite antigen.

Antibody prepared in chickens to the various column fraction pools was used to test for monospecific antigen and antibody in both gel and TBF tests. The tests indicated that the only pure antigen and antibody obtained was SA-1 and its antibody. While the leached pools contained serum antigen, it was also evident that they contained parasite antigen. Cross reactions with parasite antigen pools and their various antibodies did not further clarify the status of parasite antigen, except that they did not react with anti-SA-1.

Antibodies Found in Blood During the Course of Acute Avian Malaria: Chickens 6-8 weeks old were inoculated intravenously with 10^8 *P. gallinaceum* parasitized erythrocytes and control birds were given an equivalent injection of normal chicken cells. Birds of both groups were examined daily for RBC counts, the percent of erythrocytes parasitized (%PE) was determined, and blood plasma samples were taken for serologic study. These were tested for antibody to SA, LA, and PA using the TBF test with bentonite sensitized with SA-1, LA-2, and PA-4. The results of these studies are shown in Figure 4. Parasitemia was first patent on day 2 and reached its peak on day 8. The RBC counts did not decrease sharply until after the 5th day of infection and between the 5th and 8th days fell from approximately 2.25×10^6 to 0.8×10^6. None of the plasma samples collected on days 1 and 2 reacted with antigen sensitized bentonite. However, those collected thereafter reacted with all three antigen preparations, reaching their highest titers between the 5th and 8th days. In general, the appearance of antibodies and high parasitemia were correlated with anemia.

Immunologic Study of Anemia Inducing Factors in Plasma from Chickens with Acute *P. gallinaceum* Infections: For these experiments, 4 chickens were given 4 ml of plasma from chickens with acute *P. gallinaceum* infection by intravenous injection. Four other birds were inoculated in the same manner with 4 ml of plasma from normal chickens. In both cases the protein content of the inocula was estimated to be approximately 164 mg. Red blood cell counts were made prior to injection, and subsequently at 3, 6, 12, 24, 48, 72, 96 and 120 hours after inoculation. The results of these counts are presented in Table 1. The cell counts were reduced by 21% after 3 hours and to 34% at 24. Recovery from the effects of the injections was not evident until after 72 hours. Statistical analysis indicated that the differences in the cell counts were highly significant.

Another group of 4 experimental and 4 control chickens were given plasma injections as described above. One experimental and one control bird were sacrificed by exsanguination at 6, 12, 18 and 24 hours after injection. After centrifugation, the plasma was recovered and the cells were divided into equal portions. To one portion, 0.78% NaCl solution was added to suspend the cells to original volume. After gentle agitation of the suspension, the supernatant was removed and held for study. These cells and the portion of unwashed cells were subjected to leaching. The plasma, the cell washings, and the material leached from both cell portions were then tested for antibody using the TBF test with bentonite sensitized with serum antigen from rats with acute *B. rodhaini*, and with globulin of chickens with acute *P. gallinaceum*, infection. The preparations were also tested for serum antigen with bentonite treated with ABr, and with globulin of recovered chickens. Results of the tests are presented in Table 2. Titers of the antigen and antibodies did not

differ, regardless of the origin of serum antigen, or antibody to serum antigen, used to sensitize the bentonite. Serum antigen and its antibody were present in the plasma of the recipient chickens and persisted throughout the 24 hours of the experiment. The antigen and antibody were also present in the wash solution, and in the material leached from both washed and unwashed cells. It was also noted that antigen titers tended to be higher than those of antibody throughout the experiment. Tests of materials from chickens injected with normal plasma were negative.

Blood smears were prepared from chickens after injection of plasma from malarious birds and were incubated with FITC conjugated with ABr, anti-SA-1, anti-PA, and anti-chicken 7S globulin. In slides prepared up to 24 hours after injection, fluorescence was observed about the periphery of numerous cells. Immunofluorescence was observed with ABr, anti-SA-1, and anti-7S, but not with anti-PA conjugate. Blood cells from chickens injected with normal plasma did not react (Figure 5).

DISCUSSION

This study confirmed the observation that plasma of chickens with acute *P. gallinaceum* infection contained both antigen and antibody, and that injection of such plasma into normal chickens caused anemia. It also confirmed the observation that material leached from washed blood cells of malarious birds produced anemia in a similar manner (5).

Serologic study of material leached from the washed cells of malarious chickens, and from washed blood cells of chickens made anemic with plasma injections, indicated that both antigen and antibody were present. That antigen and antibody had combined with the cells *in vivo* was indicated by fluorescent antibody staining of blood cells withdrawn

from the recipient birds, with conjugated antibody to the antigen and with conjugated antibody to chicken 7S globulin. Since the number of cells showing fluorescence was great soon after injection of malarious plasma and was considerably diminished after 3 days, it is suggested that reacting cells had been removed from the circulation.

From the data it is suggested that malarial anemia may in part be mediated by complexes of soluble antigens and antibodies as was suggested by Dixon (9).

To identify the antigens and antibodies responsible for anemia, an attempt was made to detect all of the antigenic substances that were present in the blood of malarious chickens. In addition to the plasma, and the material leached from the washed cells, antigens were extracted from parasites (PA) that had been liberated from infected erythrocytes.

Column chromatographic and precipitation treatment of plasma from malarious chickens revealed the presence of two fractions with antigenic activity which were termed serum antigen, SA-1 and SA-2. Both migrated through Sephadex G-200 along with the 19S and 7S globulin fractions. The SA-1 fraction reacted in serologic tests only with plasma of chickens recovered from malaria, anti-SA-1, and with the plasma of chickens immunized with plasma of rats with acute *B. rodhaini* infection (ABr). SA-2 also reacted with these antibodies, but also reacted with antisera to PA.

Antigen in the material leached from cells of malarious birds was partly associated with globulin fractions, but was also associated with lower molecular weight proteins present in the leach. Two column fraction pools with antigen activity were obtained, LA-2 and LA-3, both of which reacted with plasma from recovered birds and ABr. LA-3 also reacted with antibody to PA, indicating that a mixture of antigens was present.

Attempts to obtain pure antigens from extracted parasite material were not successful. All fractions collected after column chromatography reacted with plasma of recovered chickens; however, none of them reacted with ABr or with anti-SA-1. Gel tests using various PA fractions, or their antibodies, did not cross react with SA-1 or anti-SA-1. It was therefore indicated that there were two distinct classes of antigens present in blood of the chickens: the PA class that was associated with parasites, and the SA class which was without parasite species specificity.

Plasma samples taken daily during the course of *P. gallinaceum* infection were tested for antibody using SA-1, LA-2, and PA. Antibody to each of the antigens was detected on the 4th day and persisted throughout the course of the experiment. However, except for the evidence for the presence of PA in the SA-2 preparation, the only antigen present in the plasma from malarious blood was the globulin associated serum antigen.

While antibody to PA could have reacted with infected cells to cause them to be sequestered, it is unlikely that it had a role in anemia from the injection of plasma of malarious birds, since antigen for complex formation was not found in great concentration. It is therefore suggested that in these experiments, globulin associated serum antigen and its antibody were the principal reactants detected in plasma, and in eluates from blood cells of plasma injected chickens, that could have caused immune complex anemia.

In other work attempting to identify the mediators of anemia in experimental malarial infections, Musoke (22) found that serum antigen and its antibody could not have been implicated in anemia from *Plasmodium chabaudi* infections of rats. Plasma of rats made anemic by

P. chabaudi infection contained a soluble antigen that was species-specific for *P. chabaudi* and antibody to this antigen was also detected. Both antigen and antibody were eluted from the washed blood cells of anemic rats. It was suggested that immune complexes of this antigen and antibody might have been instrumental in anemia. It therefore appears that immune complexes, involving antigens other than serum antigen, may have a role in malarial anemia.

Another immune mechanism associated with anemia of acute malaria has been investigated. A cold-active hemagglutinin for trypsinized erythrocytes was shown to be associated with the anemia of both acute malaria and babesiosis of rats (1,2,22).

Barrett *et al.* (6) detected cold-active agglutinin in the blood of chickens with acute *P. lophurae* infection using trypsinized human type "O" erythrocytes. It was felt that this work should be further investigated since McGhee and Loftis (23) and Ludford *et al.* (24) had indicated that *P. lophurae* at their respective laboratories was contaminated with an anemia inducing agent, duck infectious anemia (DIA) virus. Ludford *et al.* (25) found that *P. lophurae* strains maintained at four laboratories were contaminated, and suggested that DIA virus might have been an unrecognized companion of *P. lophurae* for many years. The strain of *P. gallinaceum* used at this laboratory was tested extensively for DIA virus contamination and was found to be clean.

Cold-active hemagglutinin was detected in the blood of chickens with *P. gallinaceum* anemia with trypsinized human type "O" erythrocytes as reported by Barrett *et al.* (6) and was generally associated with the anemia of infection (5).

Injection of hemagglutinin absorbed from malarious plasma into normal chickens produced both an anaphylactic-like reaction and anemia.

Plasma from which hemagglutinin had been absorbed also produced anemia. Since the hemagglutinin did not react with other antigens, or antibodies from blood of malarious chickens, it was considered to be an auto antibody-like IgM (7).

The presence, or absence, of a serum antigen that was common to acute babesial and malarial infections, and the role of such antigens in immunity, has become a controversy (26). Serum antigen was found in the blood of monkeys with acute *Plasmodium knowlesi* malaria by Eaton (27). The presence of such an antigen was confirmed by Cox (3), who also reported that the antigen from malarious monkeys conferred immunity in rats challenged with *P. berghei*.

Serum antigen from the blood of horses, dogs, and rats with acute babesiosis each reacted in serologic tests with sera from recovered horses, dogs, and rats. The antigens from rats and dogs each conferred resistance to challenge with both heterologous and homologous *Babesia* species (4,28,29). Cox *et al.* (30) demonstrated with serological methods that the serum antigens associated with acute malaria and babesiosis were similar if not identical. This observation was confirmed independently by Ludford *et al.* (31). Cross protective immunization with serum antigen was demonstrated with serum antigen from rats with babesiosis against malaria in ducks (32). Recovery from acute malaria conferred resistance to babesiosis, and recovery from babesiosis conferred resistance to malaria (33). This cross immunization between heterologous species and genera of haemosporidian parasites has been repeatedly confirmed (34).

We detected serum antigen in the blood of rats with acute *B. rodhaini* infection and used this antigen to immunize chickens. Antibody from these chickens reacted in serologic tests with the plasma of

chickens with acute *P. gallinaceum* infection. The purified SA-1 fraction from plasma of these chickens stimulated antibody in chickens that reacted in serologic tests with the plasma of rats with acute *B. rodhaini*. These findings should satisfy objections raised by Smith *et al.* (26) concerning the existence or serologic cross-reactivity of globulin associated serum antigens.

The origin of serum antigen and the means whereby it functions in acquired resistance have not been satisfactorily demonstrated. Sibinovic *et al.* (28) found the immunogenic component to be associated with macroglobulin and that its sedimentation coefficient was approximately 22S. Amino acid analysis and enzyme studies did not differentiate it from host globulin. The results of the present experiments suggest that the serum antigen from chickens with acute *P. gallinaceum* infection is a globulin associated antigen and may be similar to the one described by Sibinovic *et al.* (28).

It is difficult to visualize how an antigen that is unrelated to a parasite could stimulate acquired resistance to infection. However, it seems clear that this antigen and its antibody reacted with erythrocytes and caused a dramatic and rapid reduction in circulating red blood cells.

It is suggested that complexes of the antigen and antibody had acted as opsonin to cause the cells to be sequestered in the spleen, or in other cases caused complement fixation and lysis of the cells. Since this mechanism would involve infected cells, it could be speculated that in a serum antigen immunized animal, parasitized as well as normal red cells might be removed from the circulation mor rapidly than they would have been in nonimmune controls. Such a mechanism might contribute in this nonspecific manner to recovery from acute malaria.

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TABLE 1

Red blood cell counts (RBC x 10^6) of normal chickens after injection of plasma from chickens with acute *Plasmodium gallinaceum* infection (Exptl.) and plasma of normal chickens (Control) and the percent of RBC lost (% RBC lost).

Particulars	Exptl.	Control			
Number of chickens	4	4			
Time after Injections	Ave. RBC x 10^6	Ave. RBC x 10^6	% RBC lost	t value	P
0 hours	3.08 ± 0.27	2.97 ± 0.59	-3.7	0.26	N.S.
3 hours	2.32 ± 0.09	2.93 ± 0.42	20.8	2.81	<0.050
6 hours	1.95 ± 0.24	2.81 ± 0.30	30.6	4.43	<0.005
12 hours	1.88 ± 0.15	2.81 ± 0.26	33.1	6.06	<0.001
24 hours	1.84 ± 0.17	2.80 ± 0.30	34.3	5.43	<0.005
48 hours	1.88 ± 0.54	2.86 ± 0.17	34.2	3.42	<0.025
72 hours	2.00 ± 0.16	2.83 ± 0.09	29.33	8.57	<0.001
96 hours	1.91 ± 0.23	2.84 ± 0.10	32.7	7.19	<0.001
120 hours	2.11 ± 0.50	2.73 ± 0.13	22.7	2.61	<0.050

TABLE 2

Titers of serum antigen (SA) and antibody to serum antigen (ABSA) in plasma (I), saline washings of blood cells (II), hypertonic saline leach of unwashed cells (III), and leach from washed cells (IV) from the blood of chickens sacrificed at 6, 12, 18 and 24 hours after the injection of 4 ml of plasma from chickens with acute *Plasmodium gallinaceum* malaria, and in chickens injected with plasma of normal chickens.* Tested with the tube bentonite flocculation test using bentonite treated with SA from rats with acute babesiosis (R) and from chickens with acute malaria (C) to detected ABSA, and ABSA of both rat and chicken origin to detect SA.

Titers of SA and ABSA

		6 Hrs.		12 Hrs.		18 Hrs.		24 Hrs.	
		SA	ABSA	SA	ABSA	SA	ABSA	SA	ABSA
I	C	64	32	32	0	64	32	64	32
	R	64	64	32	16	64	32	64	64
II	C	64	64	64	32	32	32	32	16
	R	16	8	32	16	32	16	16	8
III	C	32	32	64	16	32	16	32	16
	R	16	16	16	16	32	16	32	16
IV	C	32	32	32	16	16	8	16	8
	R	32	16	64	16	32	16	16	8

*All tests made on blood from chickens injected with normal plasma were negative.

Figure 1. The serologic relationships of antigens found in the blood of chickens with acute *Plasmodium gallinaceum* infection, and the physicochemical properties of material leached from cells of malarious blood.

1-1. The reactions in double diffusion in gel tests of malarious plasma (SA), material leached from cells of malarious blood (LA), and the material extracted from *P. gallinaceum* parasites (PA) with the plasma of chickens recovered from *P. gallinaceum* infection (R).

1-2. The reactions of LA, SA, and PA with plasma of chickens that had been immunized with plasma of rats with acute *Babesia rodhaini* infection (ABr). Note the line of identity between LA and SA and the failure of PA to react with this antibody.

1-3. The reactions of LA with ABr, R, antibody to serum antigen from chickens with acute *P. gallinaceum* infection (ASA), and antibody to *P. gallinaceum* parasite antigen (APA). Note the failure of APA to react with LA.

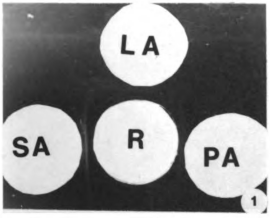
1-4. The reaction of LA with the plasma of rats with acute *B. rodhaini* infection (BrA) and the failure of normal rat serum (NRS) to react.

1-5. The reaction of LA with purified serum antigen from chickens with acute *P. gallinaceum* infection (SA2) and failure of equivalent plasma fraction of normal (N) chickens to react.

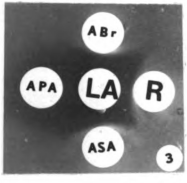
1-6. Tests of LA and material leached from cells of normal chicken blood (N) with antibody to chicken 7S globulin.

1-7. Immunoelectrophoretic reaction of LA and normal chicken serum with antibody to whole chicken serum (Top), and material leached from cells of normal chickens with antibody to chicken globulin (Lower).

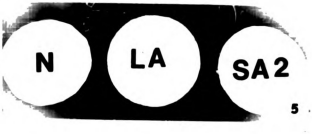
1-8. Disc electrophoresis of LA and material leached from cells of normal chicken blood (N) stained for protein. Arrow indicates the presence of a high molecular weight protein in leach from infected cells (I) which was not seen in the leach from normal cells (N).


LA
SA
R
PA
1

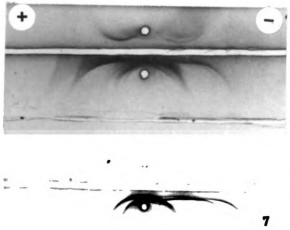

LA
PA
ABr
SA
2


ABr
APA
LA
R
ASA
3


NRS
LA
BrA
4


N
LA
SA2
5


LA
ATs
N
6


+
−
7


I
↓
N
8

Figure 1

Figure 2. Elution profile after column chromatography in Sephadex, of plasma of malarious chickens, material leached from cells of malarious chicken blood with hypertonic (1.2%) NaCl solution, and extract from *Plasmodium gallinaceum* parasites. The columns were 2.5 x 100 cm, the packed column size 2.5 x 90 cm, and columns were charged with 160 mg protein. Elution was with borate buffered saline, pH 8.2, I = 0.16, the flow rate was 15 ml per hour, and the fraction volume 3 ml/tube. All columns were run at 22 C.

Pools of column fractions were made as shown. In tests for antigenic activity with plasma of chickens recovered from *P. gallinaceum*, pools 1, 2 and 3 of plasma protein, pools 2 and 3 of the leached material and all pools of the parasite extract reacted.

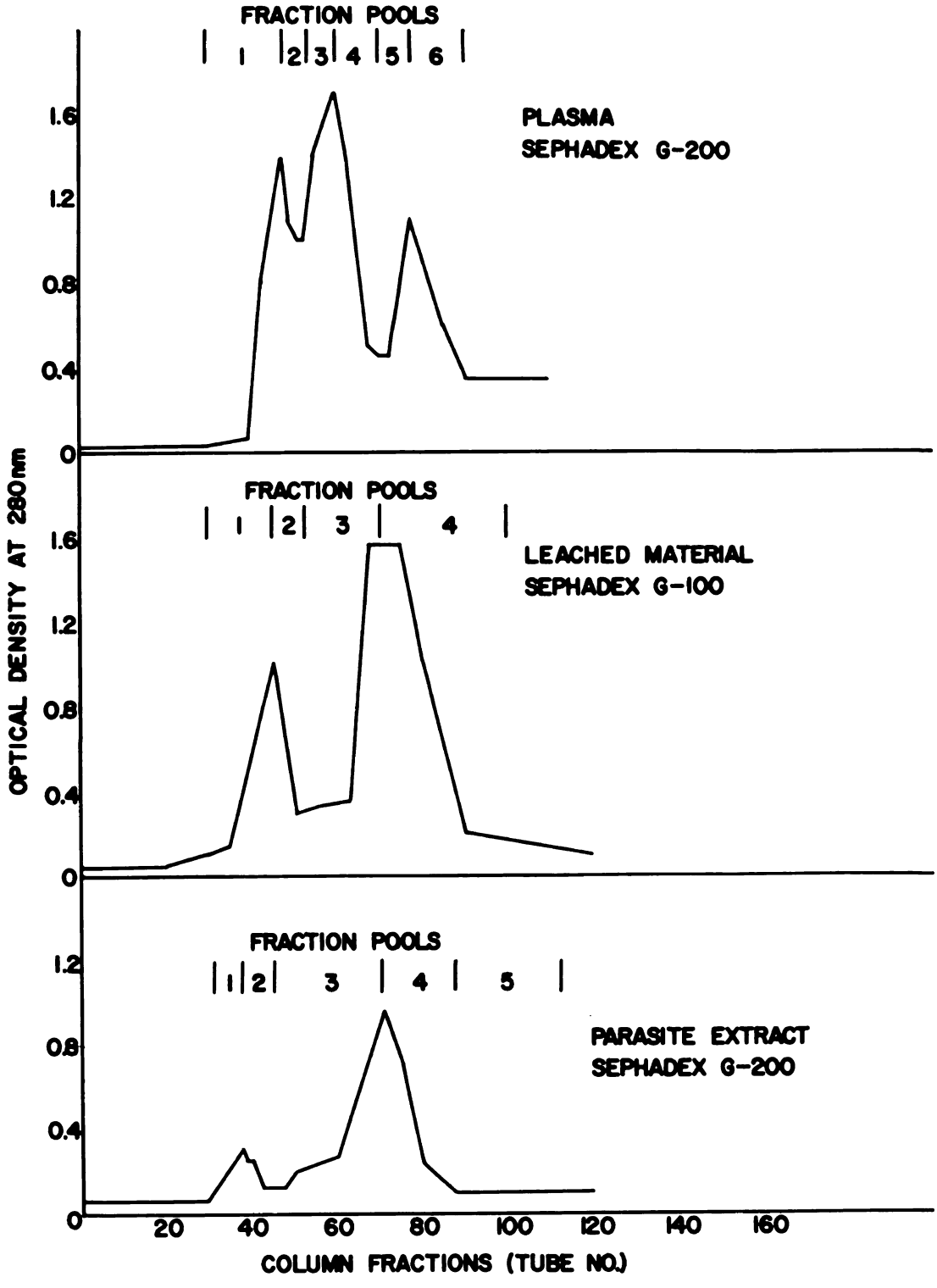

FRACTION POOLS
1
2
3
4
5
6
PLASMA
SEPHADEX G-200
FRACTION POOLS
1
2
3
4
LEACHED MATERIAL
SEPHADEX G-100
FRACTION POOLS
1
2
3
4
5
PARASITE EXTRACT
SEPHADEX G-200
OPTICAL DENSITY AT 280nm
0
0.4
0.8
1.2
1.6
20
40
60
80
100
120
140
160
COLUMN FRACTIONS (TUBE NO.)

Figure 2

Figure 3. The relationships of parasitemia (average % parasitized erythrocytes), anemia (average RBC counts x 10^6) and average antibody titers against serum antigen, leached antigen, and parasite antigen in the blood of chickens throughout the course of *Plasmodium gallinaceum* infection. Titers were determined with the tube bentonite flocculation test. Serum antigen from rats with acute *Babesia rodhaini* infection, as well as serum antigen from malarious chicken plasma, was used to test for antibody to serum antigen.

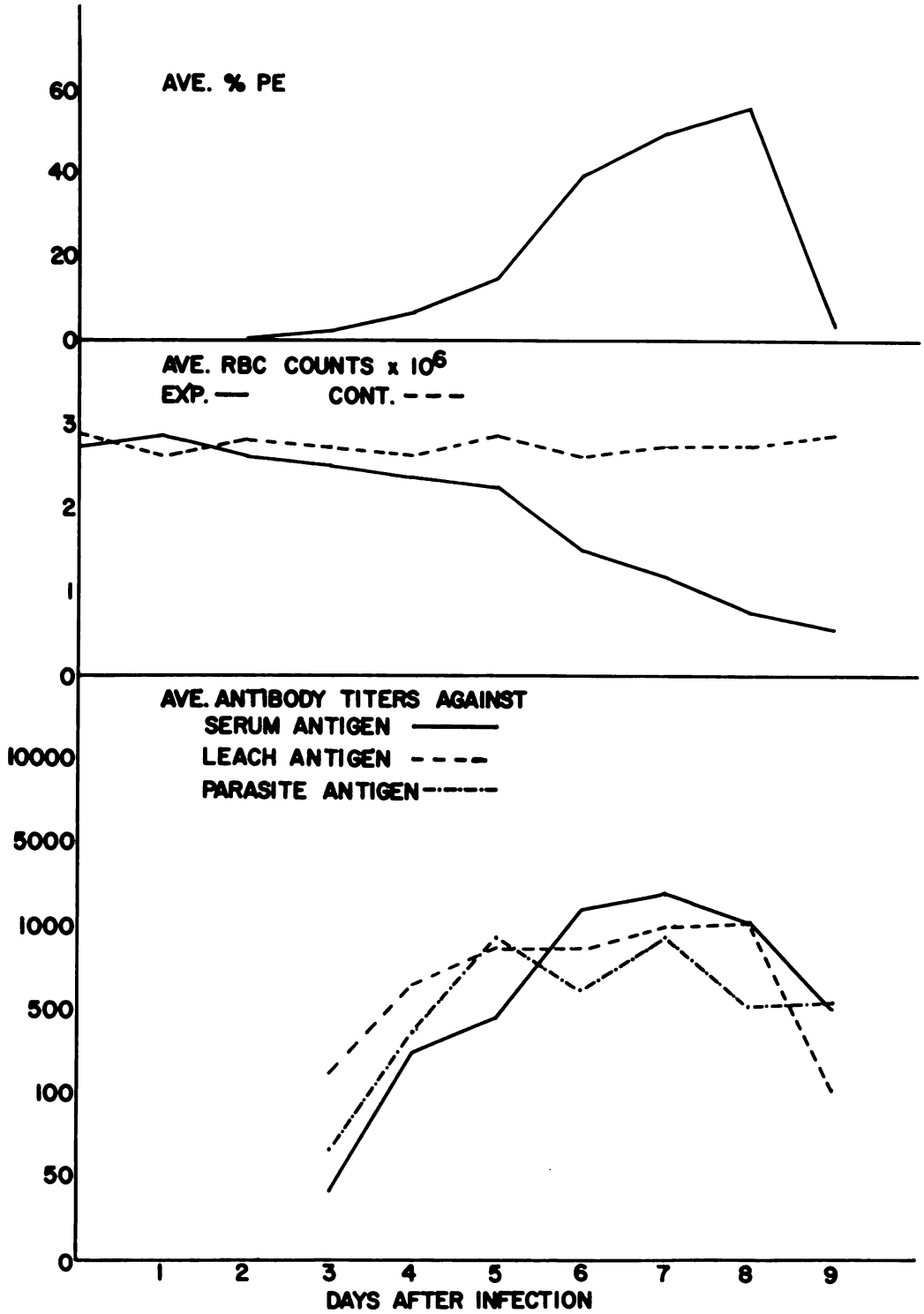

AVE. % PE
60
40
20
0
AVE. RBC COUNTS x 10^6
EXP. —
CONT. - - -
3
2
1
0
AVE. ANTIBODY TITERS AGAINST
SERUM ANTIGEN
LEACH ANTIGEN
PARASITE ANTIGEN
10000
5000
1000
500
100
50
0
1
2
3
4
5
6
7
8
9
DAYS AFTER INFECTION

Figure 3

Figure 4. Reactions of the blood cells of normal chickens that had been injected intravenously with the plasma of chickens with acute *Plasmodium gallinaceum* infections, with fluorescein isothiocyanate conjugates of antibody to serum antigen of rats with acute *Babesia rodhaini* infection (ABr) prepared in chickens, antibody to serum antigen from malarious chickens prepared in chickens (ASA), antibody to chicken 7S globulin (IgG) (commercial, prepared in rabbit), and antibody to *P. gallinaceum* parasite antigen (APA) prepared in chickens.

A. Reaction between blood cells and ABr 24 hours after injection of plasma from malarious chickens. Cells from chickens injected with normal plasma did not react.

B. Reactions seen with ABr conjugate 3 days after injection of malarious plasma. Note that cells showing immunofluorescent activity were reduced in number and appeared to be clumped.

C. Reaction of blood cells with ASA 24 hours after the chickens had been injected with malarious plasma.

D. Reaction of blood cells with anti-7S globulin 24 hours after the chickens had been injected with malarious plasma.

The APA conjugate did not react with cells from chickens injected with malarious plasma.

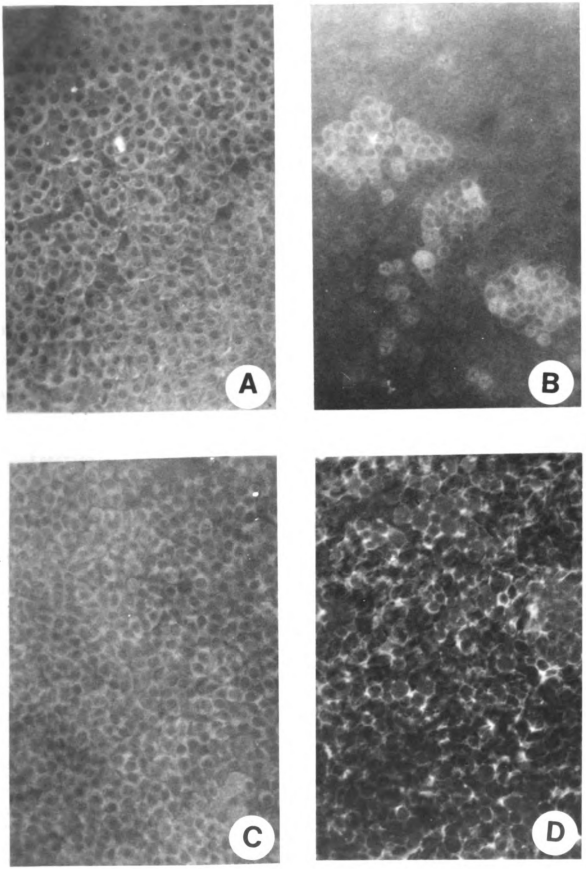


Figure 4

Procedural steps for separating *Plasmodium gallinaceum* parasites from erythrocytes and preparing parasite antigen (PA). Modified from methods of Sherman and Hull (10).

1. Draw blood from heavily infected chickens and add 1 ml of heparinized saline (100 I. U. sodium heparin/ml of 0.78% NaCl solution) per 10 ml of blood. Mix. Centrifuge at 800 g for 5 minutes. Discard plasma.
2. Wash cells 3 times in modified Trager's buffer (MTB) QS to original blood volume, with centrifugation at 800 g for 5 minutes. Remove buffy coat after each washing.
3. Add 5 volumes of 1% saponin per volume of packed cells, mix and incubate at 39 C for 15 minutes.
4. Centrifuge at 2000 g for 15 minutes at 4 C and discard the supernatant. Wash the sediment 3 times with 5 volumes of MTB and centrifugation at 2000 g for 15 minutes at 4 C.
5. Mix sediment with 5 volumes of 1.0 M NaCl solution and incubate at 39 C for 30 minutes. Centrifuge at 2000 g for 15 minutes at 4 C. Discard supernatant.
6. Wash sediment 2 times with 5 volumes of MTB and centrifugation as in 5.
7. Mix sediment in 10 ml of MTB and freeze-thaw 5 times by alternate acetone-dry ice freezing and thawing at 37 C.
8. Centrifuge at 3000 g for 30 minutes at 4 C. Discard sediment. Concentrate supernatant to 25% of original volume by dehydration in dialysis tubing with polyethylene glycol flakes at 4 C.
9. Test for antigenic activity with globulin of chickens recovered from *P. gallinaceum* infection in double diffusion in gel tests.

Article 3

PATHOGENESIS OF ACUTE AVIAN MALARIA

III. IMMUNOLOGIC MEDIATORS OF NEPHRITIS IN ACUTE *PLASMODIUM GALLINACEUM* INFECTIONS OF CHICKENS

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(To be submitted to The American Journal of Tropical Medicine & Hygiene)

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ABSTRACT

Nephritis associated with acute malaria has been suspected of being an immunologic disease; however, the responsible antigens have not been implicated. To get this basic information a study was made of the kidneys and kidney functions of chickens with acute *Plasmodium gallinaceum* malaria. A study of the urinary wastes of malarious chickens revealed that serum proteins were extravasated into the kidney to pass in the urine. The leakage did not begin until globulin associated serum antigen and its antibody were both detected in blood. This antigen and its antibody were detected in the urine wastes for as long as antigen was present in the blood. Afterwards, the presence of 7S globulin indicated that protein leakage continued. A study of the materials extracted from kidney tissues indicated the presence of serum proteins that were not detected in tissues from normal chickens. Serum antigen and its

antibody were also present in these proteins. Frozen kidney sections from malarious chickens were reacted with fluorescein isothiocyanate conjugated antibody prepared in chickens against serum antigen from rats with acute *Babesia rodhaini* infection (ABr), purified serum antigen from malarious chickens (ASA), purified *P. gallinaceum* parasite antigen (APA), and antibody to chicken 7S globulin prepared in rabbits (A7S). With the ABr, ASA, and A7S a diffuse, granular "lumpy-bumpy" type of immunofluorescence that has been described as characteristic for immune complex nephritis was observed. Immunofluorescence with APA conjugate differed in that the activity was localized to the glomerular tuft. In the course of infection immunofluorescent activity was not observed until both serum antigen and its antibody were detected in the blood, and activity with ABR, ASA and APA ceased on the day serum antigen and parasitemia were no longer detected in the blood. Fluorescence with A7S continued undiminished well into the time the birds appeared to have recovered from malaria. When sections from kidneys of birds with nephritis induced by injections of malarious plasma were studied, immunofluorescent activity of the diffuse granular "lumpy-bumpy" type was observed with ABr, ASA and A7S. No reaction was observed in tests with APA. It was therefore concluded that parasite antigen played no role in the nephritis induced by malarious plasma. Globulin associated serum antigen, which was serologically unrelated to *P. gallinaceum* parasite antigen, and which is associated with infections with parasites other than those of the genus *Plasmodium*, has been strongly implicated as a causal agent in the acute nephritis associated with *P. gallinaceum* malaria of chickens.

INTRODUCTION

Glomerulonephritis associated with malaria is best known as the chronic quartan malarial nephritis syndrome associated with *Plasmodium malariae* infection in man. This disease is chronic and progressive in nature, and eventually leads to renal failure with disease signs consistent with those described by Bright.[1] A nephritis with sudden onset, which often resulted in renal shutdown, was less well recognized, and has in recent years been found to be associated with acute severe *Plasmodium falciparum* infections, especially those that originated in South East Asia.

In earlier studies it was suggested that factors such as anoxia from acute anemia, or vascular failure due to the occlusion of the blood vessels of the kidney,were the mediators of the disease.[2,3,4]

However, recent investigators have suggested that immunologic mechanisms might have been involved. This suggestion has been based on the observation that immune substances in the form of IgG, IgM, and B_1C globulin have been seen as deposits in kidney sections stained with fluorescein isothiocyanate (FITC) conjugated anti-globulin.[5,6,7] Ultra structure studies revealed alterations, such as fusion of the foot processes of podocytes, and electron dense deposits in the cytoplasm and basement membrane of these cells, which were considered as suggestive of immune complex nephritides.[7,8,9] In such studies, there have been only a few attempts to identify antigens that had stimulated the immune globulins involved and to determine that these antigens were part of immune complexes associated with lesions.[10,11]

In studies of the pathogenesis of acute avian malaria, both anemia and nephritis of acute *Plasmodium gallinaceum* infections of chickens were associated with the appearance of cold-active hemagglutinin for

trypsinized erythrocytes, and the concurrent presence of serum antigen and antibody in the blood of the malarious chickens. Both anemia and acute nephritis were induced in normal chickens by injection of plasma which contained these immunologically active substances.[12] In subsequent work it was shown that both serum antigen and antibody could be eluted from the washed blood cells of birds that had been injected with the plasma of malarious chickens. Since these were the only antigens and antibodies found reacting with the cells, it was suggested that immune complexes involving serum antigen and its antibody were in part responsible for the anemia of acute malaria of chickens.[13]

In the present studies the nephritides of acute avian malaria have been investigated to determine whether or not immune complexes might have been causal and to attempt to identify the antigens and antibodies responsible. The results of these studies are presented in this communication.

MATERIALS AND METHODS

Animals and Experimental Infections: The source and methods of maintenance of the white leghorn cockerel chickens, the *P. gallinaceum* strain, and the *B. rodhaini* strain used for this work have been described.[12] Chickens for experimental studies were all infected by intravenous inoculation of 10^8 or 10^6 parasitized erythrocytes, standardized by methods described.[14] Birds that furnished plasma for the experiments were exsanguinated by cardiac bleeding under anesthesia as described.[12] Chickens that were to be subjected to postmortem study were exsanguinated as described. Data on the parasitemia, expressed as the percentage of parasitized erythrocytes (% PE) and anemia expressed as red blood cell counts (RBC X 10^6), was obtained by described methods.[12]

Kidneys of experimental and control chickens were taken at autopsy. Portions to be used for immunopathologic studies were taken from the cortex area and cut into pieces 3-5 mm square which were quick frozen in acetone-dry ice mixture and stored at -70 C until needed for study. Kidney tissue from which antigen and antibody were to be extracted was minced into very small pieces and processed immediately.

Reagent Antigens and Antibodies and Serologic Tests: Antigens for these experiments consisted of serum antigen from the globulin of rats with acute *B. rodhaini* infection (BrA), purified serum antigen (SA-1 and SA-2) from the plasma of malarious chickens, antigen leached from blood cells (LA-3), and parasite antigen (PA) prepared as described.[13] Antisera were prepared against each of these antigens in chickens.[13] Portions of the plasma from immunized chickens were precipitated and the globulin conjugated with fluorescein isothiocyanate (FITC) as described.[13] Antibody to serum antigen from rats with acute *B. rodhaini* infection was referred to as ABr, antibody to serum antigen from malarious chickens as ASA, and antibody to parasite antigen as APA. FITC conjugated anti-chicken 7S globulin was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio.

Double diffusion in gel and the tube bentonite flocculation tests were used to detect and titer the antigens and antibodies present in materials from malarious chickens following described methods.[12,13]

Study of Protein Secreted in Droppings of Chickens with Acute *P. gallinaceum* Infection: Physical evidence of kidney disease was sought for by studying droppings from malarious birds. The droppings, which contained the urinary secretions, were obtained at daily intervals from infected and control chickens. Fecal material was in part avoided

by collecting the excrement that was predominantly white, or greenish, from malarious birds. Approximately 10 gm of the droppings from experimental and control chickens were each homogenized in a Waring blender with 50 ml of veronal acetate buffer, pH 8.6, ionic strength of 0.1, for 1 minute at medium speed. The homogenate was then centrifuged at 800 g for 5 minutes, the supernatant fluids recovered, and allowed to stand overnight at 4 C. The supernatant was further clarified by centrifugation at 2000 g for 15 minutes at 4 C and each preparation was concentrated to a volume of 4 ml by ultrafiltration at 4 C. These preparations were stored at -18 C until needed for study. Because of discoloration and turbidity, total protein concentrations were not determined on the droppings extracts. They were tested for antigen and antibody in double diffusion in gel tests, and subjected to immunoelectrophoresis and disc electrophoresis study by methods described.[13] The droppings extracts were subjected to column chromatography with Sephadex G-100 following described methods.[13]

Studies of Kidney Tissues of Chickens with Acute *P. gallinaceum* Infection for Antigens and Antibodies: Kidney pieces taken from malarious chickens at the peak of infection and from control birds were washed 4 times with cold phosphate buffered 0.85% NaCl saline (PBS) pH 7.2. Approximately 5 volumes of cold PBS were added to the minced tissues and the mixture homogenized in a Waring blender for 2 minutes at medium speed. The homogenate was then centrifuged at 2000 g for 30 minutes at 4 C. The clear supernatant was collected and dehydrated to 1/5th of original volume by ultra filtration, or by dehydration in dialysis tubing with polyethylene glycol treatment, at 4 C. This preparation was tested for antigens.

The sediment of homogenized kidney tissue was resuspended and washed with centrifugation in 5 volumes of PBS. After the 7th washing the pellet was resuspended in 5 volumes of 0.1 N citric acid solution, pH 2.5, and placed on a mechanical stirrer for 16 hours at 4 C. The mixture was then centrifuged at 2000 g for 15 minutes at 4 C and the supernatant recovered. This supernatant was neutralized to pH 7.0 with 0.1 N NaOH, dialyzed overnight against PBS at 4 C, and dehydrated to a volume of 4.0 ml by polyethylene glycol treatment at 4 C. These procedures were adapted from those described by Gallo[15] and Houba *et al.*[16] This preparation was tested for antigen and antibody in double diffusion in gel, and in the TBF tests. It was also subjected to study after immunoelectrophoresis and disc electrophoresis as described by Soni and Cox.[13]

Detection and Identification of Immune Complexes Associated with Nephritis in Acute *P. gallinaceum* Infections of Chickens: Frozen kidney tissue samples from malarious chickens, chickens that had been injected with plasma of malarious chickens, and from control birds were oriented in Lipshaw M-1 embedding matrix and frozen to -20 C. Sections were cut at 4 microns with a Lipshaw Cryotome, mounted onto microscope slides precooled to -20 C, and fixed for 10 minutes in acetone. The slides were then washed in PBS, pH 7.0, for 10 minutes and FITC conjugated antiserum, diluted to 1:20 or 1:40, was added. The preparations were incubated at 37 C for 30 minutes. They were then washed in PBS, pH 7.0, for 10 minutes and a No. 1 cover glass was mounted with 90% glycerine in PBS pH 7.0. The sections were examined with a Zeiss Fluorescope illuminated with an Osram HBO 200 super high pressure mercury lamp using Excitor filter II and a Barrier filter 50/44. Microphotographs were taken with Kodak Tri-X-Pan film with an exposure time of 30 to 60 seconds.

EXPERIMENTS AND RESULTS

Antigen Extracted from the Kidney Tissues of Chickens with Acute *P. gallinaceum* Infection: When the material extracted from the homogenized kidney tissues of malarious chickens was tested in double diffusion in gel tests with the plasma of recovered chickens, a single line of precipitation was observed, indicating that there was at least one antigenic component present in the extract (Figure 1A). Tests of the extract with ABr indicated that it contained serum antigen. Tests with APA were negative. No reactions were seen in any of the tests with material extracted from kidneys of normal chickens.

Immunoelectrophoresis reaction with antiserum to normal chicken serum indicated that the serum proteins of the extract were IgM and IgG (Figure 1B). In disc electrophoresis at least 23 protein bands were observed in the extract from malarious chickens while none were observed in tests of samples from control birds (Figure 1C). Stains for carbohydrate, lipid, and DNA were negative.

Antibody Extracted from the Kidney Tissues of Chickens with Acute *P. gallinaceum* Infection: The material extracted with citric acid reacted in double diffusion in gel tests with serum antigens SA-1 and SA-2. A faint line of reaction was also seen with material leached from cells of malarious blood (LA-3), but no reaction was observed in a test with parasite antigen preparation (Figure 2A). The finding that antibody to serum antigen, but not to parasite antigen, was present in the extracted material was also confirmed with the tube bentonite flocculation test using bentonite sensitized with parasite antigen and serum antigen. Tests with parasite antigen were negative and antibody to serum antigen was consistently detected.

The citric acid extracted material exhibited two lines of precipitation in tests with anti-chicken globulin and anti-7S globulin, which suggested that there was more than one class of globulin present (Figure 2B and 2C). Material extracted from kidneys of normal chickens did not exhibit these reactions.

Immunoelectrophoresis with anti-normal chicken globulin indicated that the protein migrated in a manner similar to IgG, and that the extract from normal kidney did not contain this protein (Figure 2E). In disc electrophoresis the material exhibited 10 protein bands, indicating that protein other than IgG had been extravasated into the tissue. Again, protein was not detected in the extracts of normal kidney (Figure 2F).

To further substantiate that the material extracted from the kidneys of malarious chickens was antibody, absorption was made of the extract with equal amounts of SA-1, SA-2, and LA-3. The absorbed extract gave no reactions when retested with the antigens to which it had previously reacted (Figure 2G and 2H).

Study of Extracts of Droppings of Chickens with Acute *P. gallinaceum* Infection for Immunologic Activity: Extracts of droppings of malarious chickens did not lend themselves to quantitative study of protein secretion. However, in disc electrophoresis as many as 16 bands taking protein stain were detected in the test of the extracts from malarious birds while none were evident in tests of material from control chickens (Figure 3D).

In immunoelectrophoresis with anti-chicken serum, reaction bands suggestive of IgG and albumin also indicated that a diversity of serum proteins were passing in the droppings (Figure 3C).

In double diffusion in gel tests the droppings extract reacted with plasma of chickens with acute *P. gallinaceum* infection, A. Reaction with ABr, B indicated that serum antigen was secreted (Figure 3A). Gel tests for parasite antigen and antibody were negative. Tests with anti-7S indicated that IgG had been secreted (Figure 3B).

After molecular sieving through Sephadex G-100, protein bearing fractions were collected as shown in Figure 4. Two peaks of concentration, one similar to globulin and the other to low molecular weight protein, were consistently found in the extracts from malarious chicken droppings. Antigen and antibody activities both were confined to the globulin peak.

The Interrelationships of Serum Antigen and Antibody in Plasma, and in Droppings, to the Course of Acute *P. gallinaceum* Infection: A group of chickens were given a light infection of 10^6 parasitized erythrocytes. Slides for estimating parasitemia, blood for RBC counts and droppings for extraction were collected daily. Plasma for serologic tests was collected at 2 day intervals. Both plasma and droppings extracts were tested for serum antigen and antibody using the TBF test. Table 1 shows the results of this experiment. Parasitemia was first detected on the 3rd day, reached a peak on the 6th, and fell rapidly thereafter. Reductions in red cell counts were observed on the 2nd day and were lowest on the 6th. Thereafter, the counts increased at about the rate they had fallen and were normal again the 14th day. Serum antigen was detected in plasma samples on the 2nd day but was not found in droppings extracts until the 3rd, when both antigen and antibody were present. The titers of serum antigen fell precipitously after the 8th day and were no longer present in either plasma or droppings after the 10th.

Antibody was detected in both plasma and droppings from the 4th day throughout the experiment.

Fluorescent Antibody Studies of Kidney Sections from Chickens with Acute *P. gallinaceum* Infection: Sections of frozen kidney tissues from malarious chickens were reacted with FITC conjugated ABr, ASA and APA. The presence of extravasated immune globulin was detected with conjugated A7S. Examples of the reactions obtained are shown in Figure 5.

Immunofluorescent staining of kidney sections from malarious birds was obtained with each of the conjugates. The staining with the ABr and ASA conjugates showed diffuse "lumpy-bumpy" type of immunofluorescent reactions. Similar types of reaction patterns were observed with the A7S conjugate. Fluorescence with the APA conjugate was spotted, less diffuse, and appeared to be confined to the capillary loop of the glomerular tuft.

The Relationships of Parasitemia, Anemia, Serum Antigen, and Antibody to Serum Antigen in Blood to Immunofluorescent Reactions of Kidney Tissues in Chickens Infected with *P. gallinaceum*: Parasitemia, red blood cell counts, tests for serum antigen and antibody were made daily on chickens brought to autopsy after *P. gallinaceum* infection. Frozen kidney sections were tested with FITC conjugates of ABr, ASA, APA, and A7S, and evaluated as 0, 1+, 2+, 3+, or 4+ on the basis of intensity of immunofluorescent activity. The results of the experiment are presented (Table 2).

Parasitemia did not become evident until the 5th day of infection; however, red blood cell counts declined slightly. Titers of SA and ABSA were not detected in plasma until the 7th day; however, kidney sections showed slight reactions with each of the conjugates on day 6 and were

near maximal on day 7. Thereafter each conjugate reacted strongly with kidney sections until day 15, when reactions with ABr, ASA and APA became negative. These changes were accompanied by the disappearance of parasites and SA from the blood. Blood titers of ABSA, and reactions of the tissue sections with A7S, remained strong throughout the experiment.

Immunofluorescent Reactions of Kidneys of Normal Chickens after Injection of Plasma from Chickens with Acute *P. gallinaceum* Malaria: Immunofluorescent studies were made of the kidneys of normal chickens that had been injected intravenously with plasma of malarious chickens bearing serum antigen and antibody. Immunofluorescent reactions with ABr, ASA and A7S, which were similar to those seen in kidney sections from malarious birds, were evident in chickens brought to autopsy at 24 and 48 hours after injection. Again the diffuse "lumpy-bumpy" pattern of the fluorescence indicated that the reacting antigens and globulins were in extravascular tissues. No reactions were obtained in tests with APA conjugates in this experiment (Figure 6).

DISCUSSION

Earlier work indicated that there was an acute glomerulonephritis associated with acute *P. gallinaceum* infections of chickens. The severity of the nephritis was associated with the concurrent presence of serum antigen and antibody in the blood of the malarious birds. When plasma containing the antigen and antibody was injected intravenously into normal chickens, nephritis similar to that seen in malarious chickens was detected 24 hours after injection. Since these changes were not produced by injection of chickens that had recovered from *P. gallinaceum* infection, it was considered that they had been mediated by immunologic mechanisms, rather than by blood permeability factors.[12] In the present

work additional evidence was furnished indicating that kidney malfunction was a part of the disease syndrome. Serum proteins were found in the urinary part of droppings of malarious chickens soon after parasitemia became patent and continued to be present at the time the birds were well recovered from the parasitemia and anemia of acute infection. Serum protein, which was not evident in extracts of normal chicken kidneys, further indicated that extravasation of blood substances into the kidney tissues had occurred.

Immunochemical studies of the protein found in the droppings indicated that the larger portion was low molecular weight material. There was, however, a substantial quantity of high molecular weight protein that was eluted from the Sephadex column in the range of serum globulin. Immunoelectrophoresis and disc electrophoresis studies of the extracts confirmed the presence of globulin and albumin. In serologic tests, the globulin bearing samples reacted with plasma of recovered chickens which indicated the presence of antigen. Tests with plasma of acutely malarious chickens indicated the presence of antibody. Samples bearing low molecular weight protein failed to react. Further study of the droppings extracts revealed that they reacted in gel with antibody to serum antigen from rats with acute *B. rodhaini* infection. Reactions with purified parasite antigen, or antibody to parasite antigen, were not observed. Thus the only antigen and antibody detected in the urinary secretions of the malarious chicken was the globulin associated serum antigen and its antibody.

In an experiment, the relationships of parasitemia and anemia to the presence of serum antigen and antibody in plasma and in urinary wastes were studied. There appeared to be a relationship of the presence of antibody to a rapid decline in red blood cell counts and the

extravasation of protein, as was indicated by the appearance of antigen and antibody in the droppings. Serum antigen was detected in plasma on the 2nd day when parasitemia was very low. It was first detected in droppings on the 3rd day when both antigen and antibody were present. From then until the 7th day, titers of antigen were higher than those of antibody in both the plasma and droppings, suggesting that there was an excess of the antigen. This was associated with the rapid fall in red blood cell counts and increases in the titers of antigen and antibody secreted in the urine. From the 8th day antibody titers were higher in both plasma and droppings, and antigen was not detected after the 10th day. This change was accompanied by a rapid improvement in the red blood cell counts and a remarkable improvement in the appearance of the chickens, in spite of the persistence of fairly high parasitemia. Changes in protein output in the urine were not evident since the titers of antibody to serum antigen in the droppings extracts approximated those found in plasma.

Study of material extracted from the kidneys of malarious chickens revealed the presence of serum proteins that were not present in extracts of kidneys from normal chickens. Immunoelectrophoresis and disc electrophoresis again indicated that a variety of protein, ranging from globulin to albumin, had been extravasated into the tissue. Serologic tests of the extracts indicated the presence of globulin associated serum antigen and its antibody. Tests with parasite antigen and its antibody did not show reactions.

In the immunofluorescent study of kidney sections from chickens with acute *P. gallinaceum* malaria, FITC conjugates of antibody to serum antigen from rats with acute *B. rodhaini* infection, and antibody to serum antigen from malarious chickens, both of which were prepared in

chickens, reacted strongly, giving a picture of diffuse granular type of fluorescence which was described by Dixon[17] as "lumpy-bumpy" and associated with immune complex glomerulonephritis. Similar "lumpy-bumpy" fluorescence was seen in sections reacted with conjugates of anti-chicken 7S globulin. Sections from chickens with acute *P. gallinaceum* malaria also reacted with FITC conjugates of antibody to parasite antigen that had been repared in chickens. In these tests the reacting areas showed strong fluorescence, but diffuse immunofluorescence, as was seen with antibody to serum antigen and with anti-7S, was not evident. That is, it appeared that the parasite antigen had not diffused into the tissues or the glomerular tuft as extensively as had serum antigen.

Sections from kidneys of uninfected chickens with nephritis induced by injections of plasma from malarious chickens also reacted with conjugates of antibodies to serum antigens of babesial and plasmodial origin, and with anti-7S conjugates, just as described above, except that the diffuseness of immunofluorescence was less than that observed in sections from acutely malarious chickens. Tests with conjugates of antibody to parasite antigen did not give immunofluorescent reactions with sections taken from the kidneys of these chickens.

In an experiment showing the relationships of parasitemia, anemia, serum antigen and antibody in blood, to immunofluorescent activity in kidney sections prepared from chickens sacrificed daily after infection, the sections were tested with conjugates of antibodies to serum antigens of babesial and malarious origins, anti-parasite antigen and with anti-chicken 7S globulin. The beginning of immunofluorescent activity with each of the conjugates was associated with the concurrent presence of serum antigen and antibody in blood of the chickens. Intensity of the

reactions, graded from zero to 4 plus, was approximately equal for each conjugate from the 6th through the 13th day. Reactivity with the antibodies to the serum antigens and parasite antigen all ceased on the same day. The reversion of these reactions to negative tests was associated with the disappearance of serum antigen from the blood and the recovery from parasitemia. Reaction with tbe anti-7S conjugate continued to be strong for the remaining observations of the experiment, and were associated with the presence of antibody to serum antigen in the blood of the animals. Thus it was indicated that serum globulins were extravasated into the kidney tissues for some time after recovery from acute malaria, just as was indicated by the presence of antibody to serum antigen in the droppings of recovered chickens.

In supposing that immune complex nephritis would be dependent upon the presence of both soluble antigen and antibody in the blood, and that antigen excesses are usually associated with the disease, it seems that the relationships of the globulin associated serum antigen and its antibody in the blood, in the kidney tissues, and in the urinary wastes of the malarious birds fulfill these requirements.

In the study of the antigens present in the blood of chickens with acute *P. gallinaceum* malaria, there was but little evidence that soluble parasite antigen was present free in the plasma, even though a high titer of antibody to parasite antigen was evident, along with a high titer of antibody to serum antigen. On the other hand, a high titer of serum antigen, which was clearly shown to be serologically unrelated to parasite antigen, was a constant finding.[13] Thus the soluble serum antigen, and its antibody, were the obviously available ingredients in the plasma for forming immune complexes in these experiments.

The reactions of antibody to parasite antigen with the kidney sections from malarious chickens must bear the burden of being the best evidence found that parasite antigen and its antibody had a role in the nephritis of acute *P. gallinaceum* malaria of chickens. Since this antigen and antibody were not evident in the nephritis induced in normal chickens by injections of malarious plasma, it was further indicated that serum antigen and its antibody were important mediators of nephritis in these experiments.

The results of these experiments lead to the suggestion that complexes of a globulin associated serum antigen which was serologically unrelated to *P. gallinaceum* parasite antigen, and which is found in the blood of animals with acute red blood cell infections of parasites other than those of the genus *Plasmodium*, was along with its antibody in part the mediator in glomerulonephritis in chickens with acute *P. gallinaceum* malaria. However, it is not implied that the immune complexes alone were the causal agents. It is probable that the β1C (C3) fraction of complement, activated by the complex, had a major role in altering cell membranes to allow cellular hydration and in altered vascular permeability as was suggested by Ward and Conran.[10,11]

The results of these experiments do not indicate that serum antigen and antibody are the sole mediators of nephritis in acute malaria. Glomerulonephritis was found to be associated with acute *Plasmodium chabaudi* malaria of rats, where it was conclusively shown that serum antigen was not elaborated during acute infection. However, in these rats it was indicated that soluble complexes of parasite specific antigen, and its antibody, were present in blood, in the kidney tissues, and were secreted in the urine.[18] Thus, while the antigens and antibodies involved differed in the two infections, the mechanisms implicated

in acute *P. chabaudi* infection of rats, and acute *P. gallinaceum* infection of chickens, seem essentially the same. That is, both diseases appear to have been mediated by immune complexes.

The role of cold-active hemagglutinin for trypsinized erythrocytes in acute glomerulnephritis has not been evaluated. In a report of nephritis associated with acute *B. rodhaini* infections of rats, it was suggested that the hemagglutinin might have been in part responsible, since the titers were correlated with the severity of kidney damage.[19] This agglutinin was also found in acute *P. gallinaceum* infections of chickens.[12] The role of the agglutinin in anemia has been studied and will be discussed in a subsequent communication.

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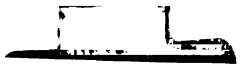


TABLE 1

The average percent of parasitized erythrocytes (PE), the red blood cell counts (RBC X 10^6), the average of the titers of serum antigen (SA) and antibody to serum antigen (ABSA) in plasma, and fecal droppings from chickens with acute *Plasmodium gallinaceum* malaria.

Days Post Infection	P.E.	RBC X 10^6	Plasma SA	Plasma ABSA	Fecal SA	Fecal ABSA
1	0	2.67	0	0	0	0
2	+	2.15	32	0	0	0
3	3.66	2.02	N*	N*	32	12
4	25.9	1.49	192	48	64	16
5	42	0.94	N*	N*	96	20
6	81.2	0.58	288	178	96	20
7	47	0.78	N*	N*	96	24
8	22	1.39	298.6	512	96	136
9	16.5	1.61	N*	N*	80	192
10	13	1.90	20	288	16	256
11	3	2.37	N*	N*	0	128
12	2	2.42	0	160	0	128
13	1	2.65	N*	N*	0	128
14	-	2.79	0	48	0	32

*No test. Plasma samples were taken on alternate days.

TABLE 2

Parasitemia (av. % PE) and anemia (av. RBC X 10^6) in chickens infected with *Plasmodium gallinaceum*, the titer of serum antigen (SA) and antibody to SA (ABSA) in plasma, the intensity of fluorescent activity in fluorescent antibody tests (FAT) with conjugated antibody to serum antigen from rats with acute *Babesia rodhaini* infection (ABr), antibody to serum antigen from malarious chickens (ASA), antibody to *P. gallinaceum* parasite antigen (APA), and anti-chicken 7S globulin, in frozen kidney sections from a chicken brought to autopsy at daily intervals after infection.

Days Post Infection	Av. % P.E.	Av. RBC 10^6	SA	ABSA	Results of FAT			
					ABr	ASA	APA	A7S
1	0	3.02	0	0	0	0	0	0
2	0	2.73	0	0	0	0	0	0
3	0	2.72	NT*	NT	0	0	0	0
4	+**	2.57	0	0	0	0	0	0
5	0.31	2.55	0	0	0	0	0	0
6	2.3	2.33	0	0	+	+	+	+
7	12.2	2.26	128	128	3	3	3	2
8	27.7	2.20	512	256	4	4	4	3
9	24.5	2.01	NT	NT	NT	NT	NT	NT
10	28.9	1.32	1024	512	4	4	4	4
11	28.4	1.16	512	128	3	3	3	2
12	18.4	1.01	64	256	2	2	2	3
13	7.2	1.13	32	256	2	2	2	3
14	3.0	1.25	NT	NT	NT	NT	NT	NT

TABLE 2 (CONT'D.)

Days Post Infection	Av. % P.E.	Av. RBC 10^6	SA	ABSA	Results of FAT			
					ABr	ASA	APA	A7S
15	0.75	1.33	0	128	0	0	0	2
16	+	1.35	NT	NT	NT	NT	NT	NT
17	+	1.70	0	64	0	0	0	2
18	0	1.95	NT	NT	NT	NT	NT	NT
19	0	1.69	0	512	0	0	0	4
20	0	NT	NT	NT	NT	NT	NT	NT
21	0	1.85	0	256	0	0	0	3

*Not tested.

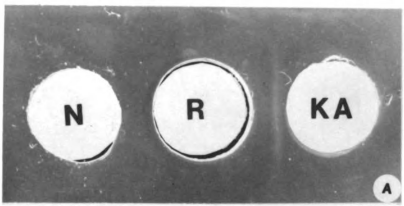
**Parasites detected, too few to count.

Figure 1. Reactions of antigen extracted from the kidneys (KA) of chickens with acute *Plasmodium gallinaceum* infection.

1-A. Reaction in double diffusion in gel test with plasma of chickens recovered from *P. gallinaceum* malaria.

1-B. Reaction of KA, and normal control, after immunoelectrophoresis with anti-normal chicken serum (Top), compared to the immunoelectrophorogram of normal chicken serum (Bottom).

1-C. Disc electrophoresis of KA (Left) compared to normal chicken globulin (Center) and extract of normal kidney (Right) after staining for protein.



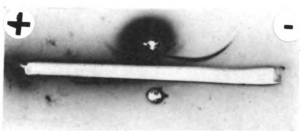


Figure 1

Figure 2. Tests of antibody extracted from the kidneys (KAb) of chickens with acute *Plasmodium gallinaceum* infection.

2-A. Reactions of KAb in double diffusion in gel tests showing positive reactions with serum antigen from malarious chickens (SA1 and SA2) but no reactions with parasite antigen (PA). A faint precipitin line not seen in the photograph was seen in the test with material leached from the cells of malarious blood (LA3).

2-B. Reaction of KAb with anti-normal chicken globulin (ACG). No reaction was seen with extract from normal kidney (N).

2-C. Reaction of KAb with anti-chicken 7S globulin and absence of reaction with N.

2-D. Reaction of KAb with plasma of recovered chickens (R) indicating that antigen was associated with KAb.

2-E. Immunoelectrophoresis with anti-chicken globulin of KAb (Top) and extract of normal kidneys (Bottom) compared to whole chicken globulin.

2-F. Disc electrophoresis of KAb compared to extract of normal kidney after staining for protein.

2-G. Reaction of KAb with SA1, SA2, and LA3 before absorption. A reaction, not seen in the photograph, was observed with LA3 in the original slide.

2-H. Absence of reaction with SA1, SA2, and LA3 of the absorbed supernatant (AS) from KAb, after incubation with SA1, SA2, and LA3.

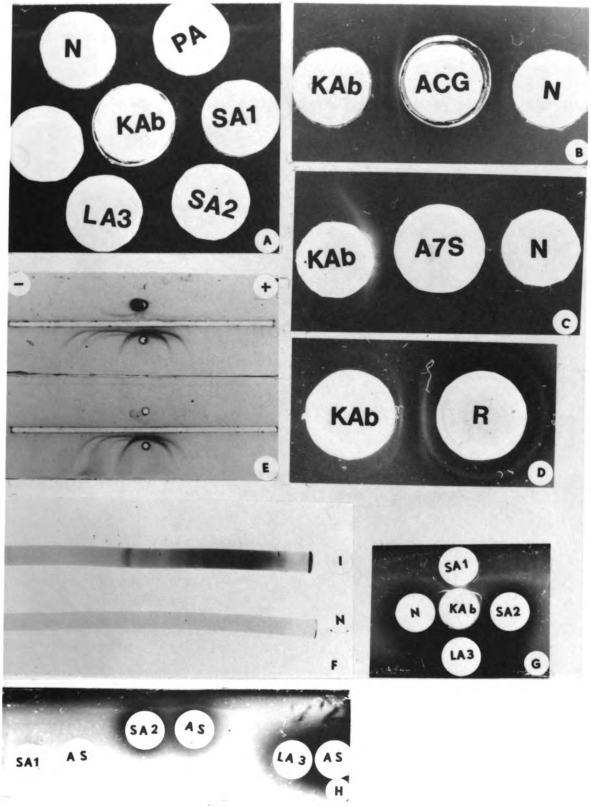

N
PA
KAb
SA1
LA3
SA2
A
KAb
ACG
N
B
KAb
A7S
N
C
KAb
R
D
E
I
N
F
SA1
N
KAb
SA2
LA3
G
SA1
AS
SA2
AS
LA3
AS
H

Figure 2

Figure 3. Reactions of extracts of droppings from chickens with *Plasmodium gallinaceum* malaria.

3-A. Reaction of extracts of droppings on day 4 with antibody to serum antigen from rats with acute *Babesia rodhaini* infection (B) and below with plasma of malarious chickens (A) compared to control extract (N).

3-B. Reactions of droppings extract from day 8 with anti-chicken 7S globulin (A7S) compared to N.

3-C. Immunoelectrophoresis with anti-chicken globulin of droppings extracts from malarious chickens (Top) and extracts from droppings of normal chickens (Bottom), compared to normal chicken globulin.

3-D. Disc electrophoresis of droppings extracts from malarious chickens (Left) and normal chickens (Right) after staining for protein.

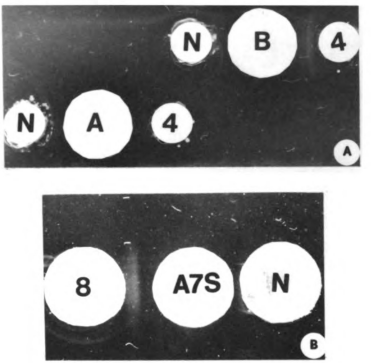


Figure 3

Figure 4. Elution profile of protein eluted from extracts of droppings from chickens with acute *Plasmodium gallinaceum* malaria, after column chromatography with Sephadex G-100. Column size: 2.5 x 100 cm. Packed column: 2.5 x 90 cm. Charge: 4 ml of extract. Elution with: Borate buffered saline, pH 8.2, I = 0.16. Flow rate: 15 ml/hour. Fraction volume: 3 ml.

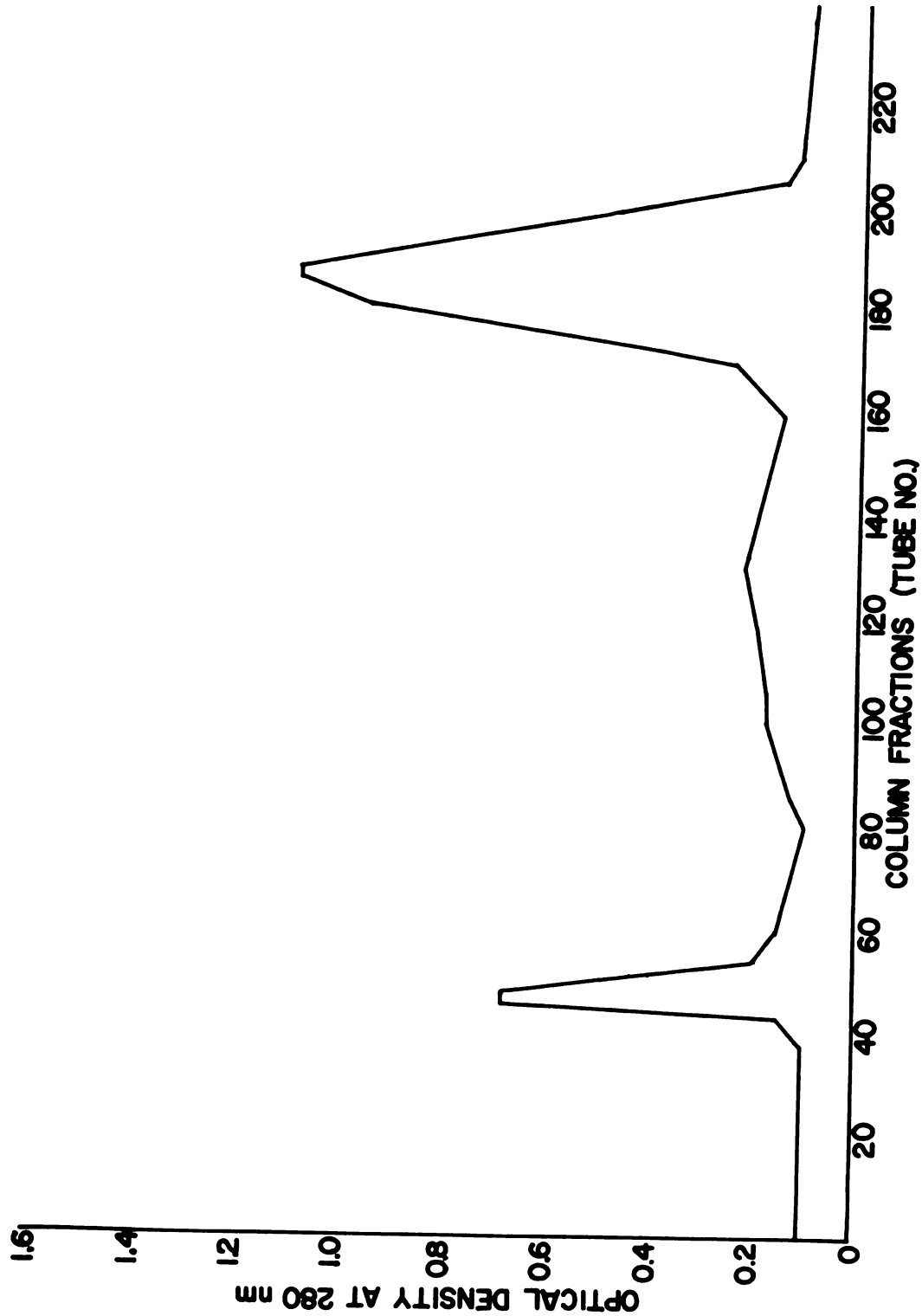

OPTICAL DENSITY AT 280 nm
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
20
40
60
80
100
120
140
160
180
200
220
COLUMN FRACTIONS (TUBE NO.)

Figure 4

Figure 5. Reactions of kidney sections from chickens with acute *Plasmodium gallinaceum* malaria, with fluorescein isothiocyanate conjugated antibody to serum antigen from rats with acute *Babesia rodhaini* infection (A), antibody to serum antigen from chickens with acute *P. gallinaceum* malaria (B), antibody to *P. gallinaceum* parasite antigen (C), and anti-chicken 7S globulin (D) (400 X).

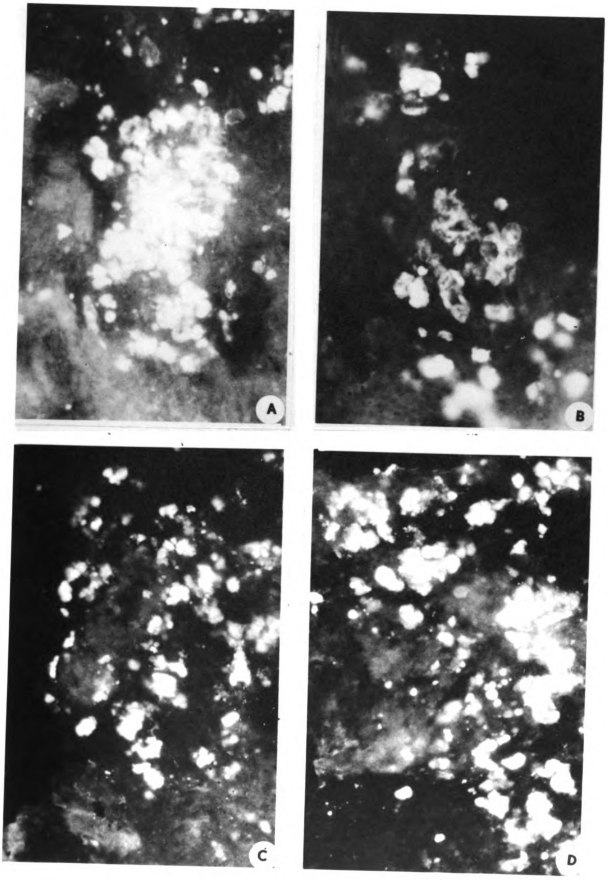


Figure 5

Figure 6. The reactions of kidney sections from chickens taken 48 hours after injection of plasma from chickens with acute *Plasmodium gallinaceum* malaria, with fluorescein isothiocyanate conjugated antibody to serum antigen from rats with acute *Babesia rodhaini* infection (A), antibody to serum antigen from chickens with acute *P. gallinaceum* malaria (B), anti-chicken 7S globulin (C), and antibody to *P. gallinaceum* parasite antigen (D) (400 X).

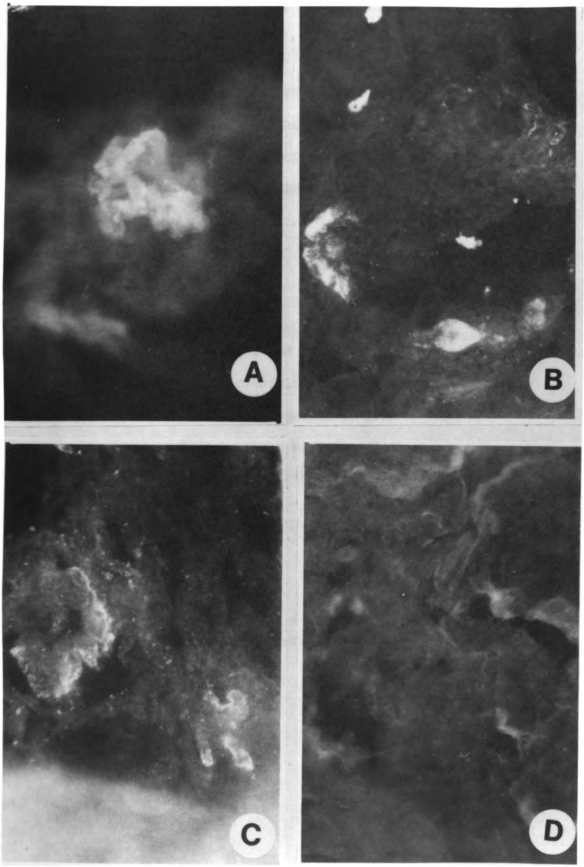


Figure 6

Article 4

PATHOGENESIS OF ACUTE AVIAN MALARIA

IV. ANEMIA MEDIATED BY THE COLD-ACTIVE AGGLUTININ FOR TRYPSINIZED ERYTHROCYTES FROM THE BLOOD OF CHICKENS WITH ACUTE *PLASMODIUM GALLINACEUM* INFECTION

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ABSTRACT

A cold-active agglutinin was absorbed from the plasma of chickens with acute *Plasmodium gallinaceum* malaria with trypsinized type "O" human erythrocytes. It was first detected at the beginning of parasitemia, reached its highest titer just prior to the parasitemia-anemia crises, and thereafter was detected only at low titer. Reinfection of the chickens did not produce a marked elevation of the titers. The agglutinin was found to be associated with the 19S and 7S globulin fractions of malarious chicken blood, but cleavage with 2-mercaptoethanol indicated that it was primarily of the IgM class of antibody. In serologic tests the agglutinin reacted only with trypsinized erythrocytes, anti-chicken globulin and with anti-chicken 7S globulin. It did not react with any of the antigens or antibodies detected in the blood of malarious chickens. Neither were titers of antigen and antibody in malarious plasma other than the agglutinin altered by the absorption. When the absorbed agglutinin was injected intravenously into normal

chickens it produced an anaphylactic-like shock and caused a 25% reduction in red blood cell counts within 48 hours. Plasma samples collected during this interval showed signs of hemolysis. Immunofluorescent study of blood cells removed from the injected birds showed reactions with conjugated anti-chicken globulin, but did not show reactions with conjugates of antibody to any of the antigens found in blood of malarious chickens. The absence of fluorescent activity 3 days after injection suggested that erythrocytes that had reacted with the agglutinin had been removed from the circulation. Anemia following the injection of malarious plasma that had been absorbed free of agglutinin indicated that anemia inducing factors other than the agglutinin were also present. While the antigenic determinants for the agglutinin were not indicated, the data suggest that the agglutinin is an autoantibody, and clearly indicates that it may have a causal role in malarial anemia.

INTRODUCTION

Anemia that was incommensurate with parasitemia has come to be a well known part of disease in red blood cell infections. The association of a positive Coomb's test with anemia of *Plasmodium berghei* infections of rats led to the suggestion that the anemia might have been mediated by autoimmune substances (19). Similar suggestions were made for the anemia associated with acute *Plasmodium lophurae* infections of ducklings and for *Anaplasma marginale* infections of cattle (9,10,13).

The association of cold-active agglutinin with anemia, splenomegaly, and erythrophagocytosis in *A. marginale*, *P. berghei* and *Babesia rodhaini* infections led to the suggestions that in each of these cases anemia may have been in part due to this autoantibody-like substance (3,14,15). Barrett *et al.* (1) demonstrated cold-active agglutinin

associated with anemia of *Plasmodium lophurae* infection in chickens using trypsinized human type "O" erythrocytes. They associated the agglutinin with 19S globulin and found its activity to be destroyed by 2-mercaptoethanol treatment. The titers of the agglutinin did not diminish after recovery from malaria and subsequent injections of erythrocyte from normal chickens resulted in a decrease in the titers.

The association of cold-active agglutinin for trypsinized human "O" erythrocytes with acute malaria was confirmed using acute *Plasmodium gallinaceum* infections of chickens. However, it was found that the agglutinin titers tended to fall quickly after recovery from parasitemia (16). Further, questions concerning the persistence of high titers of agglutinin were raised since it was found that *P. lophurae* at this laboratory and at four others was contaminated with an anemia inducing virus agent which caused a disease in ducks that mimicked acute malaria (7,8). The virus of duck infectious anemia (DIA) could be transmitted as aerosol from infected to clean birds, including chickens (8). Before starting the present work our strain of *P. gallinaceum* was extensively screened for DIA virus, all work with DIA was discontinued, and our animal rooms were sanitized.

Anemia of acute *P. gallinaceum* infection in chickens was found to be due in part to immune complexes of soluble antigens and antibodies found in the blood of the birds during acute infection (17). The role of the cold-active agglutinin has also been studied. The results of the studies are reported in the present communication.

MATERIALS AND METHODS

Experimental animals and infections. Mature White Leghorn cockerels for the experiments were obtained from Rainbow Trails Hatchery, St.

Louis, Michigan, as day-old chicks and reared in departmental animal facilities. The *P. gallinaceum* infection was obtained from Dr. Julius P. Kreier, Department of Microbiology, Ohio State University, Columbus, Ohio. After extensive testing for DIA virus contamination, it was stored in liquid nitrogen, or maintained by blood passage in chickens. For this research it was considered essential that the parasites be maintained at maximal virulence. Therefore severity of anemia and moribund condition of the birds were the criteria used in selecting infected birds to be used for passage. The methods of inoculation, evaluating anemia, and parasitemia have been described (16).

Detection of cold-active agglutinin for trypsinized erythrocytes. The agglutinin was detected and titrated in plasma from malarious chickens using trypsinized human type "O" erythrocytes prepared by methods modified from those of Cox *et al.* (3) and have been described (16).

Absorption and elution of agglutinin. Chickens infected with 10^8 parasitized erythrocytes that had been washed with 0.78% NaCl solution were exsanguinated at the peak of parasitemia and anemia, and the blood was added 10 parts to 1 part heparinized saline (100 I.U. Sodium heparin/ml of 0.78% NaCl solution). After centrifugation at 800 g for 15 minutes, the plasma was recovered and stored at -18 C until tested. For the test frozen plasma was thawed and clarified by centrifugation at 2000 g for 30 minutes at 4 C and 10 ml of plasma was added to 5 ml of packed trypsinized human type "O" cells. After mixing, the suspension was held at 4 C for 4 hours. The plasma was removed after centrifugation and the cells were washed 4 times with cold 0.85% NaCl solution. The packed cells were then suspended in an equal volume of the salt solution

and incubated at 37 C for 30 minutes. After centrifugation at 1500 g for 10 minutes, the supernatant was recovered, placed in dialysis tubing and dehydrated to 1/5th volume by polyethylene glycol treatment at 4 C. This material was then tested for agglutinin with trypsinized human type "O" erythrocytes, and was stored at -18 C.

In vivo tests of cold-active agglutinin for biological activity. Four chickens were injected intravenously with the agglutinin that had been standardized to contain 4.0 mg of protein per inoculum. Four other chickens were inoculated with eluates prepared from trypsinized human "O" red cells used to absorb plasma of normal chickens. The absorbed plasma was tested for anemia inducing factors by injection of 4 ml intravenously into each of 4 other normal chickens. Anemia following the injections was determined by daily red blood cell counts over a 10 day period as described (16).

Slides from the blood of chickens injected with agglutinin were prepared and tested for reactivity with fluorescein isothiocyanate (FITC) conjugates of anti-chicken whole globulin, anti-serum antigen from rats with acute *B. rodhaini* infection (ABr), and anti-*P. gallinaceum* parasite antigen (APA) prepared as described (17).

Immunochemical properties of cold-active agglutinin. Four ml (160 mg) of globulin salted from the plasma of malarious chickens at 4 C with 50% saturated ammonium sulphate solution was subjected to study by column chromatography using Sephadex G-200 as described (17). Each sample from the column was tested for agglutinin activity with trypsinized human type "O" cells as described (16).

The agglutinin was subjected to 0.1 M 2-mercaptoethanol (2-ME) treatment as described by Chan and Deutsch (2). Reductive cleavage

was determined by testing the treated material with trypsinized "O" erythrocytes.

Absorbed agglutinin and 2-ME cleaved agglutinin were studied by immunoelectrophoresis and reacted with anti-normal chicken globulin following described methods (17). Disc electrophoresis study of the agglutinin was made following described methods (17).

The agglutinin, the 2-ME cleaved agglutinin and the absorbed plasma were each tested for reactivity with serum antigen and antibody to serum antigen of rat babesiosis origin, using the tube bentonite flocculation (TBF) test as described by Thoongsuwan and Cox (18) and Soni and Cox (16). The agglutinin was also tested in double diffusion in gel tests following described methods (6,17). Reactions were tested for with: anti-normal chicken globulin, anti-chicken 7S globulin, plasma of chickens recovered from *P. gallinaceum* infection, plasma of chickens with acute *P. gallinaceum* infection, anti-*P. gallinaceum* parasite antigen, *P. gallinaceum* parasite antigen, and purified serum antigen from chickens with acute *P. gallinaceum* infection.

RESULTS

Absorption and recovery of cold-active agglutinin from plasma of chickens with acute *P. gallinaceum* infection. The titer of cold-active agglutinin for trypsinized human type "O" erythrocytes, before and after absorption, in the plasma from 5 malarious chickens, and the titer of the agglutinin eluted from the cells, are shown in Table 1. The average agglutinin titer of 122 for the plasma was reduced to zero after absorption. The material eluted from the cells had a titer of 51. The average titer for the 4 chickens hyperimmunized by repeated *P. gallinaceum* infection was 64. Cleavage with 2-ME destroyed agglutinin activity

in plasma from malarious chickens, as well as the low titers of agglutinin found in plasma of normal chickens and the residual titers found in plasma of birds that had been hyperimmunized by repeated *P. gallinaceum* infection.

In vivo effects of cold-active agglutinin on normal chickens. The red blood cell counts on chickens injected intravenously with eluted agglutinin are shown in Table 2. Injection of the agglutinins produced a drop of 14.6% in the number of circulating erythrocytes within 24 hours. The maximum reduction of nearly 25% was attained on day 2. Recovery from blood loss was evident after the 7th day. Immediately after injection of the agglutinin the recipient chickens went into anaphylactic-like shock. Breathing was labored, their combs became cyanotic and the birds were near collapse. About 2 hours were required before they recovered. Plasma taken at 24 and 48 hours after injection showed distinct hemolysis.

Injection of the absorbed plasma produced a drop of 30% in the red cell counts 3 days after injection (Table 3). Blood cell counts did not revert to normal levels until 9 days after injection.

Immunochemical studies of the cold-active agglutinin. Protein concentration and the titers of the agglutinin in fractions from globulin salted from malarious chickens with 50% saturated ammonium sulphate solution after Sephadex G-200 chromatography are shown (Figure 1). Protein bearing column sample numbers 53 through 76 were positive when tested with trypsinized cells, the highest titers being in samples 59 through 65. The remainder of the samples did not exhibit agglutinin activity. The elution pattern indicated that agglutinin was located primarily in the 19S peak but was also present in 7S fractions.

In double diffusion in gel tests the cold-active agglutinin (CA) reacted with anti-chicken whole globulin (ACG) and with anti-chicken 7S globulin (A7S) (Figure 2-1). Tests with anti-*P. gallinaceum* parasite antigen, parasite antigen, purified serum antigen from malarious chickens and antibody to serum antigen, were all negative.

In immunoelectrophoresis of column fraction pools, pool 1, consisting of fractions 50-61, gave a precipitin line consistent with pure 19S globulin while pool 2, consisting of fractions 62-70, showed a mixture of 19S and 7S (Figure 2-2).

The reactions of absorbed agglutinin before and after 2-ME cleavage with anti-chicken globulin in immunoelectrophoresis are shown in Figure 2-3. In the test shown (1) the agglutinin (top well) shows the presence of both 19S and 7S globulin. The reaction of material absorbed from normal plasma (N) showed similar but less distinct lines. The 2-ME cleaved agglutinin (ME) in the lower well did not show any of the lines seen with the uncleaved agglutinin (top well).

In disc electrophoresis the agglutinin exhibited 7 bands that took protein stain. A preponderance of 19S globulin was indicated by the presence of a heavy band taking protein stain which remained primarily in the spacer gel (Figure 2-4).

Reactions of erythrocytes of chickens injected with cold-active agglutinin with fluorescein isothiocyanate (FITC) conjugated antibodies. Slides prepared from the blood of chickens after intravenous injection of cold-active agglutinin were tested with FITC conjugated antibodies to antigenic substances found in the blood of chickens with acute *P. gallinaceum* infection, with conjugate of anti-chicken whole globulin, and with anti-chicken 7S globulin. Immunofluorescent activity with

anti-whole globulin was observed about the periphery of erythrocytes taken from the injected birds 24 hours after injection. The number of cells showing fluorescent activity was substantially reduced at 48 hours and was no longer detected after 3 days. Blood from chickens injected with control material showed no fluorescent activity. FITC conjugated antibody to serum antigen and to parasite antigen did not show fluorescence (Fig. 2-5).

Results of tests for agglutinin, serum antigen and antibody to serum antigen in plasma of malarious chickens, before and after absorption, are presented (Table 4). Absorption with trypsinized cells removed all of the agglutinin from the plasma without affecting the titers of serum antigen and antibody. Agglutinin but no serum antigen or antibody was detected in the eluate from the cells used for absorption.

DISCUSSION

These experiments have confirmed observations of Cox *et al.* (3) that the anemia of acute malarial infections is associated with the presence of cold-active agglutinin for trypsinized erythrocytes. They also confirm the observations of Barrett *et al.* (1) and Soni and Cox (16) that this, or a similar, agglutinin can be detected and titrated in the blood of chickens with acute malaria using human type "O" trypsinized erythrocytes.

The observation of Barrett *et al.* (1) that the agglutinin detected with trypsinized cells, after Sephadex G-200 column chromatography of globulin of malarious chickens, was exclusively 19S globulin was not confirmed. A portion of the protein eluted from our column in the area of 7S globulin contained agglutinin activity. Our immunoelectrophoresis

study also indicated the presence of 7S globulin in the eluted material. Neither did we find that reinoculation of chickens recovered from acute malaria with infected erythrocytes greatly increased the titer of the agglutinin as was reported by Barrett *et al.* (1).

While it is possible that *P. lophurae* infections of chickens differ from those of *P. gallinaceum*, it is pointed out that *P. lophurae* used in the past at this laboratory, and at others, was contaminated with DIA virus and that acute DIA closely mimicks acute malarial infection, even to the extent that ducks recovered from acute DIA were resistant to plasmodial challenge (7,8). In unpublished work from this laboratory it was found that agglutinin to trypsinized erythrocytes appeared in the blood of ducks with acute DIA and that the titers of the agglutinin persisted long after apparent recovery. We do not doubt that the agglutinin was raised during acute *P. lophurae* infection, but a possible role of acute and chronic DIA virus infection must be considered in attempts to draw conclusions about experiments performed with *P. lophurae*.

It was found that intravenous injections of the agglutinin produced anemia in normal chickens. Red blood cell counts of the experimental birds were reduced by nearly 15% over those of controls after 24 hours and to nearly 25% on day 2. Recovery from the anemia was not complete until the 9th day after injection. The birds injected with the agglutinin also suffered an anaphylactic-like shock characterized by extreme prostration, blanched or cyanotic boms, and labored breath. However, none of the birds died and most had recovered after 2 hours. That the shock could have been related to the agglutinin was further indicated by hemolysis of all plasma samples taken from these birds on days 1 and 2. No signs of shock were seen in the chickens injected with control material and all plasma samples were clear.

Fluorescent antibody studies of blood slides obtained from the chickens using a FITC conjugate of anti-chicken globulin indicated that the agglutinin had reacted with the erythrocytes of the recipient chickens. The marked reduction in immunofluorescent activity after the 3rd day suggested that the reacted cells had been removed from the circulation.

Destruction of the hemagglutinating activity after treatment with 2-ME indicated that the agglutinin itself was a 19S immune globulin as suggested by Barrett *et al.* (1). It is therefore probable that in column chromatography study the hemagglutination activity present in samples eluted from the column along with the 7S globulin was 19S contaminant. This information, along with the fact that it was readily dissociated from the trypsinized cells, suggests that the agglutinin was in fact cold-active IgM.

Aside from the serologic and *in vivo* reactions with erythrocytes and with anti-chicken globulin, the agglutinin did not react in serologic tests with any of the antigens or antibodies found to be associated with *P. gallinaceum* infections of chickens. Further, the blood cells from chickens injected with agglutinin did not react with FITC conjugates of any of the antibodies prepared against antigens found in the blood of malarious chickens. The cells reacted only with anti-chicken globulin conjugate.

The information from these experiments indicates this agglutinin is antibody to red blood cell substances that are common to erythrocytes of heterologous species of animals, and that it has a role in the pathogenesis of acute *P. gallinaceum* infections of chickens. While the antigenic determinants for the agglutinin have not been indicated, the present work furnishes further indirect evidence that it might be an autoantibody as was suggested by Cox *et al.* (3).

Plasma that had been absorbed free of agglutinin still contained anemia inducing substances. Such plasma when injected into normal chickens produced a 30% reduction in red blood cell counts within 3 days. This substantiates the evidence that immune complexes of serum antigen and antibody might have a role in anemia (17).

Dacie (5) has pointed out that the association of cold-active agglutinin for trypsinized erythrocytes with congenital and idiopathic anemias in man is a common finding and that the agglutinin is generally considered to be an autoantibody that is causal in anemia. This, or a similar type of agglutinin, has now been associated with anemia in a diversity of animals infected with agents of equal diversity. Anemia associated with the agglutinins was found in acute anaplasmosis of cattle, acute *B. rodhaini* infection of rats, *P. berghei* infection of rats, *P. chabaudi* infection of rats and mice, acute equine infectious anemia virus infection, and acute *Haemobartonella muris* and *Eperythrozoon coccides* infections of rats and mice, respectively (3,4,11,12,14,15). To our knowledge, the present study is the only one in which a causal relationship of the agglutinin to anemia has been indicated.

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Table 1. Average titers of cold-active agglutinin for trypsinized human type "O" erythrocytes in the plasma of 5 chickens with acute *Plasmodium gallinaceum* infection, 3 normal chickens and 4 hyperimmunized chickens, before and after absorption with the trypsinized cells at 4 C, the titers of the agglutinin in saline used to elute cells at 37 C, and tests of each material for agglutinin activity after 2-mercaptoethanol treatment (2-ME).

	Titers Before and After 2-ME					
	Infected		Normal		Hyperimmune	
Material Tested	Before	After	Before	After	Before	After
Whole plasma	122	0	8	0	64	0
Absorbed plasma	0	0	0	0	0	0
1st 37 C eluate from absorbing cells	51	0	4	0	16	0
2nd 37 C eluate from absorbing cells	11	0	0	0	0	0
3rd 37 C eluate from absorbing cells	0	0	0	0	0	0

Table 2. Average red blood cell counts (RBC x 10^6) on 4 chickens (Exptl.) injected with cold agglutinin absorbed from plasma of chickens with acute *Plasmodium gallinaceum* infection with trypsinized human type "O" erythrocytes, and on 4 chickens injected with control materials from normal chicken plasma (Control).

Days Post Injection	Av. TBC Count x 10^6/cmm		% RBC Loss	t value	P
	Exptl.	Control			
0	3.23 ± 0.50	3.21 ± 0.42	-0.6	0.06	N.S.
1	2.51 ± 0.54	2.94 ± 0.31	14.6	1.45	N.S.
2	2.46 ± 0.46	3.27 ± 0.30	24.8	2.90	<0.025
3	2.49 ± 0.26	3.27 ± 0.18	23.8	4.79	<0.005
4	2.54 ± 0.57	3.25 ± 0.23	21.8	2.28	N.S.
5	2.67 ± 0.73	3.13 ± 0.14	14.6	1.22	N.S.
6	2.70 ± 0.75	3.14 ± 0.14	14.0	1.14	N.S.
7	2.83 ± 0.60	3.22 ± 0.16	12.1	1.26	N.S.
8	3.07 ± 0.32	3.24 ± 0.19	5.2	1.27	N.S.
9	3.23 ± 0.36	3.28 ± 0.21	1.0	0.24	N.S.

Table 3. Average red blood cell counts (RBC x 10^6) on 4 chickens injected with plasma of chickens with acute *Plasmodium gallinaceum* from which cold-active agglutinin had been absorbed with trypsinized human type "O" erythrocytes (Exptl.), and on 4 chickens injected with normal plasma treated with the trypsinized cells (Control).

Days Post Injection	Av. RBC Count x 10^6/cmm		% RBC Loss	t value	P
	Control	Exptl.			
0	3.15 ± 0.14	3.19 ± 0.20	-1.0	0.02	N.S.
1	3.24 ± 0.07	2.77 ± 0.03	14.5	10.90	<0.001
2	3.21 ± 0.07	2.67 ± 0.22	16.8	4.37	<0.005
3	3.20 ± 0.11	2.24 ± 0.60	30.0	3.14	<0.025
4	3.26 ± 0.12	2.35 ± 0.34	27.7	5.01	<0.005
5	3.18 ± 0.01	2.42 ± 0.21	23.9	6.95	<0.001
6	3.22 ± 0.09	2.57 ± 0.13	20.2	9.37	<0.001
7	3.19 ± 0.03	2.70 ± 0.08	15.3	10.86	<0.001
8	3.20 ± 0.04	2.85 ± 0.09	10.9	6.86	<0.001
9	3.21 ± 0.05	2.92 ± 0.16	9.3	3.36	<0.025
10	3.26 ± 0.04	3.07 ± 0.15	5.8	2.28	N.S.

Table 4. The titers of cold-active agglutinin (CA), serum antigen (SA) and antibody to serum antigen (ABSA) in plasma from chickens with acute *Plasmodium gallinaceum* infection, before and after absorption with trypsinized human type "O" erythrocytes.

Chicken Numbers		1451	1446	820	1351	831	Cont. 1.	Cont. 2.	Cont. 3.
Pre-Absorption	CA	128	64	32	256	128	8	8	8
	SA	512	128	256	128	512	0	0	0
	ABSA	512	64	128	64	128	0	0	0
After Absorption	CA	0	0	0	0	0	0	0	0
	SA	512	128	256	128	256	0	0	0
	ABSA	512	64	128	64	128	0	0	0
Eluates from Trypsinized Erythrocytes	CA	32	32	32	128	32	4	4	4
	SA	0	0	0	0	0	0	0	0
	ABSA	0	0	0	0	0	0	0	0

Figure 1. The titers of cold-active agglutinin for trypsinized human type "O" erythrocytes in column fraction samples after column chromatography with Sephadex G-200 of globulin from chickens with acute *Plasmodium gallinaceum* infection.

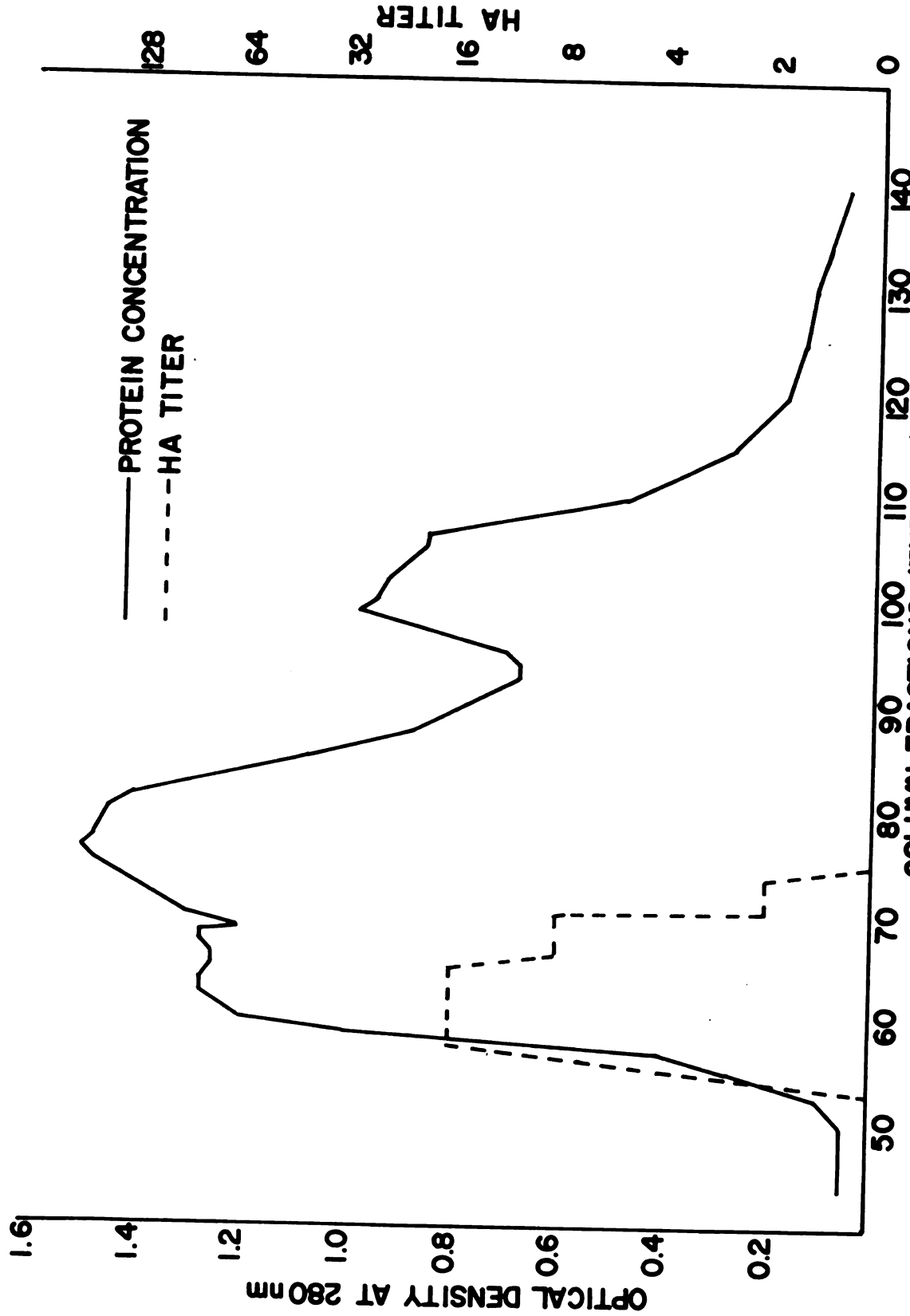

PROTEIN CONCENTRATION
HA TITER
OPTICAL DENSITY AT 280nm
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
HA TITER
128
64
32
16
8
4
2
0
COLUMN FRACTIONS (TUBE NO.)
50
60
70
80
90
100
110
120
130
140

Figure 1

Figure 2. The serologic and physicochemical properties of cold-active agglutinin absorbed from the plasma of chickens with acute *Plasmodium gallinaceum* malaria at 4 C with trypsinized human type "O" erythrocytes and eluted from the cells at 37 C.

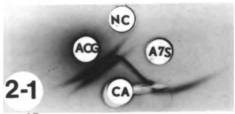
2.1. Reactions of cold-active agglutinin (CA) with anti-chicken globulin (ACG) showing 2 precipitin lines and a single line with anti-chicken 7S globulin (A7S). No reaction was observed with absorbed eluate from normal chicken plasma (NC).

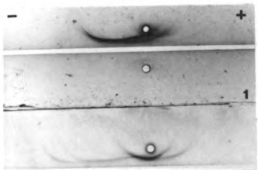
2.2. Immunoelectrophoresis with anti-chicken globulin of Sephadex G-200 column fractions 50-61 (1) and 62-70 (2) from globulin of malarious chickens, both of which contained cold agglutinin activity. Pool 1, lower well, migrated as nearly pure 19S globulin. Pool 2, lower well, exhibited migration patterns consistent with 19S and 7S globulins.

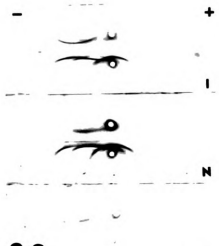
2.3. Immunoelectrophoresis with anti-chicken globulin of cold agglutinin eluted from trypsinized erythrocytes after absorption of malarious plasma at 4 C and elution at 37 C. Eluate from malarious plasma (I), top well, indicated the presence of protein migrating as 19S and 7S globulin. Eluate from normal plasma (N), top well, also gave evidence of containing serum globulin. The eluate from malarious plasma after cleavage with 2 mercaptoethanol (ME), lower well, did not give evidence of globulins.

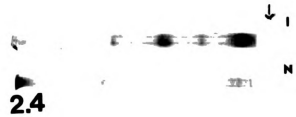
2.4. Disc electrophoresis stained for protein of eluate absorbed from malarious plasma (I) and eluate absorbed from normal chicken plasma (N). The arrow indicates a band in the range of 19S globulin that was not seen in the control material.

2.5. Immunofluorescent study of erythrocytes from chickens after injection of cold agglutinin. Reactions of conjugated anti-chicken globulin with cells from an agglutinin injected bird (I) and a bird injected with eluate absorbed from normal chicken plasma (C) are shown. Cells did not react with conjugated antibody to any of the antigens found in malarious blood.









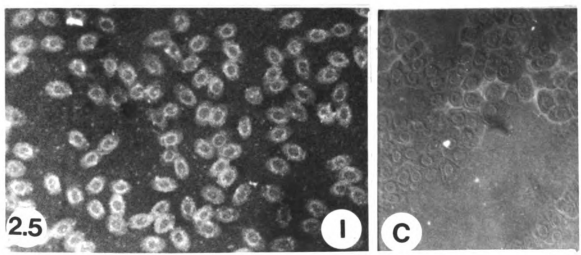


Figure 2