ANEMIA AND MECHANISM OF
IN DUCKS WITH ACUTE LEUCOCYTOZOON DISEASE

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ABSTRACT

ANEMIA AND MECHANISM OF RED CELL DESTRUCTION
IN DUCKS WITH ACUTE LEUCOCYTOZOON DISEASE

by Richard M. Kocan

A study of the anemia which accompanies infection by Leucocytozoon simondi in Pekin ducks revealed that there was a far greater loss of erythrocytes than could be accounted for as a result of direct physical rupture by the parasite. Erythrocyte loss began at the same time the first parasites appeared in the blood and was severest just prior to maximum parasitemia. Blood replacement and parasite loss occurred simultaneously. Examination of the spleen and bone marrow revealed that erythrophagocytosis was not the cause of anemia as reported for infections by Plasmodium, Babesia and Anaplasma. It was possible, however, to demonstrate an anti-erythrocyte (A-E) factor in the serum of acutely infected ducks which agglutinated and hemolysed normal untreated duck erythrocytes as well as infected cells. This A-E factor appeared when the first red cell loss was detected and reached its maximum titer just prior to the greatest red cell loss. Titers of the A-E factor were determined using normal uninfected erythrocytes at temperatures between 4 and 42 C. Cells agglutinated below 25 C and hemolysed at 37 and 42 C. These results indicated that the A-E factor could be responsible for loss of cells other than those which were infected and thus produce an excess loss of red cells.

Attempts to implicate the A-E factor as an autoantibody were all negative. The A-E factor was present in the gamma fraction of acute serum but no anamnestic response could be detected when recovered ducks

were reinfected. Anemia was never as severe in reinfections as in primary infections. The A-E factor also never reached as high a titer and was removed from the circulation very rapidly in reinfected ducks. The studies on characterization of the A-E factor were possible through the development of a technique of freeze-preservation in liquid nitrogen. This made it possible to give equal doses of sporozoites in both the primary infection and upon reinfection.

Acute serum was also shown to agglutinate red cells of numerous other species of mammals and birds. Further, acute serum was observed to inhibit heterogenetic antigen-antibody reactions between normal duck serum and cells which it normally agglutinates and/or hemolyses. These included human A, B and O Rh+ erythrocytes. Whether or not the A-E factor and the inhibitory factor are the same is uncertain at this point but preliminary evidence indicates that they are not.

It is concluded that red cell loss in ducks with acute Leucocytozoon disease results from intravascular hemolysis rather than erythrophagocytosis. The A-E factor responsible for hemolysis is more likely a parasite product than autoantibody.

# ANEMIA AND MECHANISM OF RED CELL DESTRUCTION IN DUCKS WITH ACUTE LEUCOCYTOZOON DISEASE

By

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# INTRODUCTION

Anemia is frequently described as one of the symptoms of an infection by any of the hemosporidia. It is not surprising that this should be the case since all hemosporidia have stages of their life cycle which are invaders of the circulatory system and in most cases of the erythrocytes. The method of reproduction of most of these organisms in the vertebrate host is one of asexual reproduction which is referred to as schizogony. This reproductive process produces many merozoites or spores from a single parasite within a host cell. Liberation of these merozoites results in the destruction of the host cell leading to the common belief that this was the means of red cell destruction. It has become evident in recent years that this explanation falls short of accounting for all of the cells lost during infection, and in addition the timing of the release of merozoites with rupture of the host cell does not coincide with the period of blood loss.

Several hypotheses have been presented to explain the excessive loss of red cells. These include non-specific phagocytosis of both infected and uninfected red cells by the spleen, and the production of an autoantibody. The most popular is the autoantibody hypothesis since it can explain both increased erythrophagocytosis and non-specific hemolysis.

The intention of the research being presented here is to describe the anemia which accompanies infection by Leucocytozoon simondi (Mathis and Leger, 1910) and to elucidate some of the factors which may contribute to an understanding of the mechanism of erythrocyte destruction.

## HISTORY

The loss of erythrocytes, one of the most outstanding features of a malarial infection, was not always recognized as a symptom of the disease. As early as 1901, Marchiafava and Bignami indicated that blackwater fever could occur under varied circumstances but indicated that the etiological significance of infection by Plasmodium could not be overlooked. Stephens (1913) showed a statistical relationship between the number of cases of blackwater fever and the incidence of malaria while working in the Canal Zone. At this time the immediate relationship between the organism and loss of red cells was unknown. It was evident, however, that the highest incidence of blackwater fever occurred among those persons who were newcomers to the endemic area (Ross, 1932), and in contradistinction, Negroes and other natives of the area showed a low incidence of blackwater fever which corresponded closely to their immunity to Plasmodium.

Once it was established that anemia and malaria were in some way associated, a search for the mechanism of blood loss began. The most obvious mechanism was that of direct rupture of the infected erythrocytes by the escaping parasites; but this hypothesis broke down when comparisons of parasite density and the number of erythrocytes lost showed a large discrepancy (Deeks and James, 1911; Kitchen, 1941; Zuckerman, 1945, 1960; McGhee, 1960; Terzian, 1941). The importance of destruction of normal cells was stressed by Draper (1960) who considered the loss of uninfected red cells as important to malarial anemia as the loss of parasitized cells. Blacklock and Macdonald (1928) proposed that the cause of hemolysis in blackwater fever was a hyperlactacidemia resulting from deficient oxygenation, but Ross (1932) was

unable to demonstrate excess lactic acid in the blood of blackwater fever patients. At about the same time the above authors proposed their theory for hemolysis, Cort (1929) stated that the available evidence indicated that perhaps certain strains of malaria "...elaborated a potent hemolysin."

Correlation between the physiological activities of the spleen and anemia was made by Manson-Bahr (1931) when he noted that the extent of spleenomegaly decreased as hemolysis diminished. This discovery probably led to the present concept of anemia resulting from an immune response. Since the spleen contains both phagocytic cells and globulin producing cells, these two factors began to receive more attention in relation to their role in erythrocyte destruction.

Zuckerman (1945) was one of the first investigators to demonstrate that the serum of ducks infected with Plasmodium lophurae and chickens infected with P. gallinaceum was capable of increasing the rate of phagocytosis of normal and infected erythrocytes in vitro. Since that time the organisms in which autoimmunity has been bruited include leishmania, trypanosomes, piroplasms, and plasmodia (Zuckerman, 1964) and more recently Leucocytozoon (Kocan and Clark, 1966).

The discovery of normal cells being phagocytized along with infected cells in vivo (Taliaferro and Mulligan, 1937; Clark and Tomlinson, 1949) stimulated investigators to look for a factor which might be responsible for the disproportionate loss of erythrocytes in malaria infections. Zuckerman (1945) further noted that the opsonin she found in malarious serum could be absorbed with normal erythrocytes. In agreement with this discovery was the work of Coffin (1951a), in which he showed that the protection offered normal birds against lophurae-malaria is lost when hyperimmune serum is first absorbed with normal duck erythrocytes,

and (1951b) that chickens are resistant to infection by P. gallinaceum when first immunized with normal chicken red cells. Schwink (1954) showed that this immunity is transferable and he was able to absorb the agent with normal chicken cells.

Trowell and Vaizy (1956) treated blackwater fever patients with prednisone, a corticosteroid, and found that the compound significantly reduced the severity of the anemia. This suggested that perhaps there was an immune mechanism involved in red cell loss since corticosteroids often depress immunologic activity. Further evidence for an extraparasitic factor in hemosporidian anemia was discovered by Fogel et al. (1966). They found that avian cells taken during an acute malarial infection showed a greater osmotic fragility than did cells from uninfected birds. This indicates that although the cells are intact their membranes or osmoregulatory mechanism has been disrupted during the infection. In contrast to this Maegraith et al. (1957) pointed out that red cell osmotic fragility in Babesia canis infections did not increase but that the sedimentation rate did. An increased sedimentation rate is often associated with an alteration in the surface charge of red cells which can come about as a result of their being coated with antibody.

Although the above evidence suggests a possible autoimmune mechanism for the disproportionate erythrocyte loss, one must look at several other circumstances which may superficially appear to be autoimmunity but in reality may be a quite different situation.

In the strictest sense autoimmunity is defined (Brent and Medawar, 1959) as the production of antibody by an organism which reacts against one of its own unmodified antigens, or against a foreign antigen whose

antibody cross reacts with native self antigen. Zuckerman (1964) suggests four models any of which can superficially appear to be the result of autoantibody: Model 1. The infectious agent might share an antigen with the host cell. In this way antiparasitic antibody would react with the heterogenetic antigen. This, however, would conflict with Burnet's (1959) clonal selection theory unless one presupposes that the heterogenetic antigen had been unexposed prior to infection. Model 2. The target host cell might be coated with parasite antigen which when combined with antibody injures the cell. Model 3. The target host cell might have adsorbed parasite antibody onto its surface and thereby become sensitized. When the parasite antigen combines with the antibody, injury to the cell results. Model 4. The molecular structure of the target host cell might be so modified by the parasite or its products that it becomes autoantigenic.

The idea of an infectious agent being responsible for autoimmune-like symptoms is not new. Rheumatic fever (Kabat et al., 1947; Cavelti, 1955) and glomerulonephritis (Cavelti, 1955) are among the diseases which are suspected of resulting from a combination of antibody with streptococcal products which are fixed to various tissues. Paroxysmal cold hemoglobinuria is a hemolytic disease often recognized as a sequel to syphilis and possibly resulting from a reaction of spirochaete antigen and host antibody (Jordan et al., 1952; Dameshek, 1955). Cavelti (1955) supports the autoimmune mechanism behind the pathology of these infectious agents since he can experimentally mimic the symptoms by the injection of self antigens treated with microbial products.

The precise mechanism by which excessive numbers of erythrocytes are lost in hemosporidian infections has received much attention recently

(Cox, 1964, 1966; Cox et al., 1966; Schroeder et al., 1966; Corwin and McGhee, 1966; McGhee, 1964; Zuckerman, 1964).

In an attempt to prove or disprove an autoimmune mechanism as the cause of anemia in Plasmodium infections Zuckerman (1960) demonstrated that rat erythrocytes were Coomb's positive throughout the period of anemia resulting from infection with P. berghei. A continuation of this work (Zuckerman and Spira, 1961) showed that rats made anemic by cardiac bleeding or injections of phenylhydrazine hydrochloride also had Coomb's positive red cells during the ensuing anemia. This resulted from the adsorbtion of a serum globulin onto the reticulocytes which were produced in response to the anoxia which resulted from blood loss (Jandl, 1960). The clumps of agglutinated erythrocytes observed by Zuckerman consisted primarily of reticulocytes which supported the false antiglobulin test. In spite of this problem of non-specific agglutination she still contends that an antibody may be involved. This view is based primarily on the fact that normal erythrocytes can absorb opsonizing antibody from hyperimmune chicken serum and that the unexplained increment of blood loss occurs chiefly after parasite crisis when parasitemias are waning or even latent. This last phenomenon is verified by Cox et al. (1966) and Charmot et al. (1963). Spira (1958 - in Zuckerman, 1963) described still more evidence favoring an autoimmune etiology for severe anemia. In this work it was demonstrated that splenectomy of rats infected with P. berghei delayed but did not abolish anemia. This could occur if other parts of the reticuloendothelial system function to compensate for the loss of the spleen. In a later study, Zuckerman (1966) reported that the maximum uptake of erythrocytes by phagocytes occurs at the peak of parasitemia and that uninfected cells are

more frequently taken up than infected cells even when parasitemia reaches 60%. She believes that it is unlikely that the antibody present in P. berghei infections is a hemolysin since serum collected at the peak of the anemic bout contained no bilirubin (Zuckerman, 1966). She also noted that no spontaneous agglutination occurred, thus the antibody must be monovalent.

Cox et al. (1966) also gave evidence in support of erythrophagocytosis as the primary cause of anemia in rats infected with P. berghei. They observed, as did Zuckerman (1966) that anemia was greatest during the acute and early stages of recovery and that uninfected cells were much more frequently phagocytized than infected cells. In addition, they demonstrated an agglutinin for trypsinized rat erythrocytes which corresponded in time very closely with the occurrence of the anemic condition. There was no indication by the authors, however, that their agglutinin was capable of reacting with normal untreated erythrocytes. The demonstration of this reaction would be even stronger evidence that an agglutinating antibody was responsible for the observed anemia.

Motulsky et al. (1958a, b) in discussing hypersplenism, noted that the spleen often enlarges during certain pathologic conditions and a relative stasis of blood occurs with an accompanying removal of more than the normal "physiological" number of erythrocytes. Hypersplenism is often associated with hemosporidian infections and may well be the cause of excessive red cell destruction.

Cox (1966) attempted to isolate the factor which was responsible for anemia from the plasma of monkeys infected with P. knowlesi. He obtained two serum fractions which he designated $F_1$ and $F_2$. The $F_1$ fraction was precipitated from acute monkey plasma with 18% $Na_2SO_4$ and

the $F_2$ fraction was precipitated with 33% $Na_2SO_4$. Using these two serum fractions to immunize rats he found that those injected with the $F_2$ fraction developed moderately severe anemia while those given the $F_1$ fraction showed no signs of blood loss. When the two groups of rats were subsequently challenged with P. berghei infected mouse cells, both groups showed excessive anemia but were also markedly resistant to the challenge infections. During the course of these same experiments he also observed that the sera from animals recovered from P. berghei, P. falciparum and P. gallinaceum infections would form precipitin bands when allowed to diffuse against serum from animals acutely infected with P. knowlesi. He suggested that this indicated the presence of similar antigens in each of these organisms.

Sibinovic (1966) found a beta globulin which was active in causing anemia in dogs infected with Babesia canis and Cox (1966) states that his $F_2$ fraction of monkey serum is similar to this beta globulin. The relationship may be present but Cox probably did not have a beta globulin since 33% $Na_2SO_4$ usually does not precipitate beta globulin.

Schroeder et al. (1966) studying infections of Babesia rodhaini in rats and Kreier et al. (1964) and Schroeder and Ristic (1965) studying Anaplasmosis in bovines found essentially the same thing as that found in Plasmodium infections. Maximal anemia coincided with or followed the peak parasitemia and was in excess of that expected from the level of parasitemia. In Babesia infections there was also an excess of uninfected erythrocytes phagocytized. There were also hemagglutinins for trypsinized erythrocytes present coincidentally with the period of anemia.

Kreier et al. (1966) found that the agglutinin in the serum of rats infected with P. berghei would elute from trypsinized rat cells if they

were incubated at 37 C after the initial agglutination reaction. This cold agglutinin, as they call it, might be the same agglutinin observed by Cox et al. (1966) and Schroeder et al. (1966) since they conducted their tests at "room temperature."

Schroeder and Ristic (1965) reported that calves infected with Anaplasma showed a pattern of anemia and parasitemia similar to that reported for Plasmodium and Babesia. They demonstrated a hemolysin and hemagglutinin for trypsinized erythrocytes which occurred at the time of or following peak parasitemia and maximum blood loss. When they challenged a recovered calf with Anaplasma no parasites could be demonstrated, but a mild anemia occurred and was accompanied by a low titer agglutinin and hemolysin. In the interpretation of their results they emphasize that their "autoantibody" decreases rapidly as the parasitemia wanes.

The greatest problem in proving the presence of an autoantibody against erythrocytes is the demonstration of that antibody free in the plasma. Cox (1966) and McGhee (1965) have been the only ones thus far to approach such a demonstration. Corwin and McGhee (1966) have reported a circulating factor in the malarious plasma of ducks. They demonstrated a loss of over $1 \times 10^6$ erythrocytes on the 13th day following the last of three treatments with 0.2 ml of malarious plasma. The delay of the blood loss suggests that this would be a situation similar to Zuckerman's Model 3 rather than an autoantibody. The injected plasma may have contained parasite antigen which adsorbed to the surface of the erythrocytes, and when antibody was elaborated the sensitized cells were destroyed. These investigators did make one curious discovery in relation to cell destruction - it appeared to make little difference how much plasma was injected (0.2 or 2.0 ml) as to how many cells were lost. In both cases

they indicated that red blood cell counts fell to just under 1 x $10^6$ erythrocytes per $mm^3$ of blood.

There is evidence both for and against the coating of host cells with parasite antigen. Tobie and Coatney (1961) working with human volunteers found the stroma of normal cells from malarious patients to be fluorescent while cells from uninfected persons did not show such fluorescence. Their interpretation was that the red cells of infected patients were coated with parasite antigen. Davis (1948) and Eaton (1939) reported that a complement fixing antibody for soluble antigens in knowlesi infected monkey serum increased in conjunction with parasitemia. This may be the $F_2$ factor described by Cox (1966). On the other hand Zuckerman (1963) points out that anemia occurs during and after the parasite crisis and often into the period of latency, a time when soluble parasite antigen is not likely to be present.

It appears that several processes may be involved in the excessive blood loss in hemosporidian infections: 1. Opsonizing antibody possibly coats normal as well as infected cells; 2. A "hemolysin" which lyses cells in vivo may be produced; 3. An "agglutinin" for trypsinized erythrocytes could occur and 4. Some nonspecific erythrocyte removal occurs due to hypersplenism as proposed by George et al. (1966) and Gorstein and Benacerraf (1960).

The most convincing proof of autoantibody would be the detection of antibody globulin combined with normal untreated cells. This approach was attempted by George et al. (1966) and they found no evidence for such antibody in rats infected with P. berghei. Direct and indirect Coomb's tests proved negative for gamma globulin and no complete agglutinins could be detected at 4 or 37 C. They also found that infected

rats lost $^{51}Cr$ tagged erythrocytes far more rapidly than did uninfected rats but could not detect the same rapid loss of tagged normal cells in rats injected with acute or recovered malarious sera. As a result of these findings they concluded that anemia in P. berghei infections was the result of hypersplenism without an autoimmune basis. Assuming that their results are valid, some doubt is cast on an autoimmune mechanism for anemia in P. berghei infections.

A hemosporidian which has not been investigated in terms of the excessive anemia resulting from infection by it is Leucocytozoon. The anemia accompanying an infection by this organism was first described by Fallis et al. (1951) but they gave no indication of the relationship between parasite density and erythrocyte loss. It is difficult to compare their variations of anemia with that of parasitemia since they exposed their ducks to infection for one to seven days. This would undoubtedly result in several infections by the same organism superimposed on each other giving a distorted picture of the onset, severity and termination of any accompanying anemic bout.

Newberne (1957) described marked damage to the liver and enlargement of the spleen from congestion and macrophage proliferation. These changes were attributed to the massive red cell destruction and anemia often accompanying infection by Leucocytozoon. Huff (1963) suggested that the mechanism for this anemia may be similar to that which causes anemia in Plasmodium infections.

Using short term exposures to sporozoites Kocan and Clark (1966) described the pattern of anemia as it relates to the development of parasitemia. It was evident from their data that the loss of erythrocytes is far in excess of that which might be expected from direct

rupture by the parasite. This is identical with the situation described above for Plasmodium infections. There was, however, a considerable difference in the time of maximum anemia in relation to parasitemia when compared to that reported for Plasmodium and Babesia. Ducks infected with L. simondi started to lose erythrocytes on the same day that parasitemia became evident on thin blood smears. The maximum loss of cells usually preceded the maximum parasite density by 1 to 5 days, often beginning to return to normal prior to the parasite crisis. This would indicate that red cell loss is not directly related to the presence of parasites in the circulating blood.

## MATERIALS AND METHODS

### I. CHARACTERIZATION OF ANEMIA

White Pekin ducklings ranging in age from one to seven weeks were used in all experiments. These were obtained from a local hatchery as day old birds.

Leucocytozoon infections were induced by means of sporozoites either by exposure of the ducklings to the bites of infected black flies or by intravenous injection of ground up infected black flies. During seasons when black flies were not available infections were initiated by means of sporozoites which had been collected during the summer and frozen in liquid nitrogen. This procedure has been shown to delay the initial development of the parasite in the duck but does not otherwise alter the pattern of infection (Kocan et al., 1967). Therefore the data obtained from birds infected with frozen sporozoites were comparable to those from natural infections if the 2 to 3 day delay in development was taken into account.

Erythrocytes were quantitated either by direct red cell counts or by packed cell volume (hematocrit). The red cell counts were made on a hemocytometer with blood diluted in a Unopette disposable pipette. When available a Coulter Counter was used for red cell counts since it is more accurate and facilitates more rapid processing of samples. Values for erythrocyte density were expressed as erythrocytes per $mm^3$ of whole blood. Packed cell volumes were determined by the use of micro-hematocrit capillary tubes which were centrifuged in a Model CL International Clinical centrifuge with a capillary tube head at maximum speed for 10 minutes. The values obtained in this way were expressed as per cent

packed erythrocytes. Although the packed cell volume is not directly convertible to erythrocyte density it is an excellent check on the accuracy of the red cell counts since there is minimal error with this technique. Provided there is no prolonged microcytic or macrocytic anemia the curves of the two values should parallel each other.

In order to correlate erythrocyte loss with parasite density it was necessary to determine the number of parasites per volume of blood. Using the equation $\frac{\text{gametocytes}}{\text{mm}^3 \text{ blood}} = \frac{\text{gametocytes per } 10^3 \text{ rbc x rbc per mm}^3}{1000}$, it was possible to express the density of erythrocytes and parasites in the same units.

Samples of blood were removed from the tibial vein of the ducks either daily or on alternate days by means of a small puncture. Excessive blood loss was avoided to reduce experimental altering of the natural course of anemia. A series of birds was also bled by cardiac puncture to determine how much blood must be removed to produce measurable anemia. Replacement of the plasma followed the removal of blood so that only cell loss and replacement were being measured.

In order to determine how the lost cells were being removed ducks were killed daily from days six through eleven of the infection and spleen and bone marrow presses were made. These were stained with Wright's stain and examined under oil immersion (X 970) in order to determine the extent of erythrophagocytosis. Histologic sections were also made from the spleens of these birds in order to follow the development of megaloschizonts. These were cut at 10 u and stained with eosin and hemotoxlin.

## II. CHARACTERIZATION OF THE ANTI-ERYTHROCYTE FACTOR

### A. In Vivo

Anti-erythrocyte titers of serum from infected birds were determined on days 3, 5 and 7 through 25 of the primary infection. Serum was obtained by drawing 1.5 ml of blood from the jugular vein on each collection date. This was used for rbc counts and hematocrits before being centrifuged to remove all cellular elements. These sera were incubated in phosphate buffered saline with the duck's own cells and with a 1% suspension of cells from uninfected ducks which were bled daily and acted as controls. The serum-cell suspension was kept at 4 C for 90 minutes. The suspension was shaken frequently for the first 30 minutes and allowed to settle undisturbed for the next hour. At the end of this time the tubes were read by examining the bottom of the tubes for the settling pattern. A small compact button of cells was considered negative and a diffuse pattern which covered the entire bottom of the tube was read as ++++. Titers of +, ++ and +++ were based on intermediate settling patterns. Those ducks which survived the primary infection were reinfected with the same number of sporozoites within two weeks after recovery and their serum titers determined in the same manner as before.

To test the activity of the anti-erythrocyte factor in living uninfected ducks the acute serum was injected intravenously into one week old ducklings. This was done by first removing approximately ½ of the total blood volume of the duckling in a heparinated syringe and removing the plasma by centrifugation. The sedimented cells were then resuspended in acute serum and immediately reinjected into the

jugular vein. Controls were treated identically but normal serum from different ducks was used in place of the acute serum.

To determine whether a stage of the parasite was responsible for the production or stimulation of the anti-erythrocyte factor, young ducks were inoculated with semipurified gametocytes and purified megaloschizonts in Freund's Adjuvant. This was done by mixing the parasite antigen, made soluble by grinding with alumina, with adjuvant and injecting the mixture intramuscularly on two occasions one week apart. Ten days after the second injection the birds were bled for serum and this was tested against the bird's own red cells to determine if autoagglutinins were present. During the period of immunization, daily blood counts and hematocrits were made to determine if any cells were lost as a result of the treatment.

The two parasite stages were purified by the following procedures: Whole infected blood was collected into a heparinated tube and centrifuged until all of the cellular elements were sedimented. Above the red cells there appeared a thick buffy coat which was made up primarily of gametocytes and leucocytes. This was removed with a pipette and resuspended in saline and centrifuged again. Most of the trapped erythrocytes were removed during the second centrifugation but some still remained in the preparation. These plus the leucocytes which are always present in the buffy coat constitute what is designated as a semipurified antigen (Figure 1A).

The megaloschizonts were purified by mincing the spleens of infected ducks on the ninth and/or tenth days of infection. The minced spleen was then poured through four thicknesses of gauze and the filtrate allowed to settle in a conical glass centrifuge tube. The

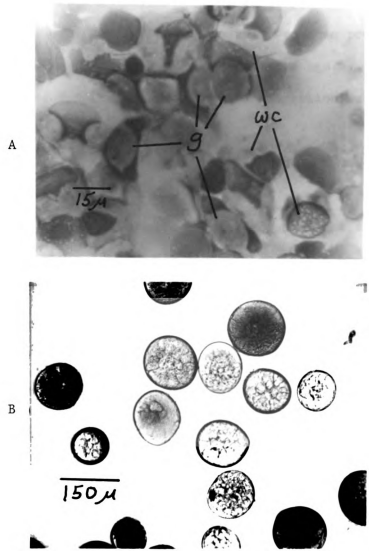


Figure 1. Purified parasite stages used for immunization.
A. Gametocytes (g) obtained by centrifugation and washing of infected blood. Note the presence of some white cell (wc) contaminants of host origin.
B. Megaloschizonts obtained on 9th-10th day of infection by mincing spleens. No host material was observed in this preparation when examined microscopically.

megaloschizonts are much more dense than spleen cells or blood cells and settled out first. The supernatant was then poured off and the parasites resuspended and allowed to settle again. This was repeated until a microscopically pure parasite suspension was obtained (Figure 1B).

B. In Vitro

Blood for serum samples was collected on the same time schedule given in A above. These samples were collected by means of cardiac puncture from moribund birds or via the jugular vein from those not apparently dying. Each sample was collected in a heparinated syringe and centrifuged immediately to obtain a cell free plasma. Hematocrits and red cell counts were made prior to centrifugation. As far as possible tests were run using freshly collected plasma but when not used immediately it was frozen at -20 C.

Except for titers all tests were carried out using a ½ dilution of plasma or serum in buffered saline at a pH of 7.0 with cells from uninfected ducks serving as indicator cells. Titers were determined in two fold dilutions in the same buffered saline.

To determine what fraction(s) of the serum contained the active component(s), $(NH_4)_2SO_4$ was used to salt out the globulins. Since autoimmunity was a possiblity the gamma fraction was looked at first. To isolate this fraction a measured volume of whole serum was mixed with one half of its volume of saturated ammonium sulfate solution. This gave a final dilution of 33% which should salt out gamma globulin. The mixture was stirred with a magnetic stirrer for one hour after which the precipitated material was removed by centrifugation. The pellet was

then reconstituted to the original volume of whole serum with borate buffered saline (pH-8.4) and again mixed with ammonium sulfate as described above. This procedure was repeated twice more and the final product was a pure white precipitate of gamma globulin as determined by cellulose acetate strip electrophoresis. The technique is exactly as described by Campbell et al. (1963).

# RESULTS

## I. COURSE OF ANEMIA IN INFECTED DUCKS

The first erythrocyte loss associated with Leucocytozoon infection began on or immediately following the first appearance of blood stages of the parasite. This occurred within 24 hours of the 7th day post infection in over 90% of the ducks infected with fresh sporozoites and the 10th day in those infected with frozen sporozoites. There was a continuous abnormal loss of erythrocytes until the severest anemia occurred 1 to 5 days prior to the maximum gametocyte density. Those ducks which succumbed to the infection exhibited a chronic anemia for several days prior to death. Ducks which survived the infection began to replace lost erythrocytes immediately after the maximum anemia was observed. Recovery from anemia and parasitemia proceeded coincidentally until the fourth week when the infection became latent.

Hematocrit (packed cell volume) values closely paralleled erythrocyte counts indicating that there was no chronic macrocytic or microcytic anemia. Polychromatic erythrocytes, as described by Lucas and Jamroz (1961), began to increase shortly after the beginning of erythrocyte loss and reached a maximum level of 35 to 40% one to two days after maximum red cell loss. The polychromatophils then gradually decreased until a normal value of 2 to 3% was reached just prior to when the red cell counts returned to normal.

Microscopic examination of slide presses made from spleen and bone marrow of infected ducks showed no signs of erythrophagocytosis. It was possible, however, to see merozoites (1 u to 2 u) within the phagocytic spleen and marrow cells (Table I). This indicated that perhaps

TABLE I. Spleen and bone marrow smears of 13 ducks infected with fresh sporozoites.

| | Days Post Infection | | | | | | |
|---|---|---|---|---|---|---|---|
| | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
| Merozoites | - | - | + - | ++ | ++ | ++++ | + |
| Gametocytes | - | - | + - | ++ | ++ | ++++ | + |
| Erythro-phagocytosis | - | - | - - | -- | -- | ---- | - |
| No. of ducks examined | 1 | 1 | 2 | 2 | 2 | 4 | 1 |

some process other than phagocytosis was responsible for erythrocyte loss.

Hemoglobin was often noted in the serum of infected ducks but could not be correlated with the severity of anemia. There was, however, a greater number of birds with free hemoglobin among those which succumbed to the infection than among those which survived even though the latter often had a more severe blood loss. In general, the younger the duck at the time of infection the more frequent the appearance of intravascular hemolysis and the more likely it was to succumb. This was true even when the red cell loss per $mm^3$ of blood was equivalent in the different age groups. The observance of free hemoglobin in the plasma suggested that blood loss may have been due to intravascular hemolysis.

In the birds studied, erythrocyte loss always preceded the maximum gametocyte density by 1 to 5 days. There was also a tremendous number of erythrocytes lost in comparison to the maximum number of gametocytes (Table II). Although maximum erythrocyte loss precedes maximum gametocyte density, it follows the peak density of round gametocytes which always precedes elongate forms in their appearance. The relationship between these two forms is still in doubt.

TABLE II. Comparison of gametocyte density and erythrocyte loss and their relative time of occurrence during acute infection.

Natural Infections

| | Erythrocytes | | Gametocytes | |
|---|---|---|---|---|
| Bird # | Maximum Loss x $10^3$ | Day | Maximum Density x $10^3$ | Day |
| 1 | 600 | 11 | 15 | 14 |
| 2 | 200 | 13 | 15 | 13 |
| 3 | 500 | 13 | 23 | 17 |
| 4 | 700 | 13 | 37 | 13 |
| 5 | 50 | 12 | 29 | 15 |
| 6 | 800 | 12 | 27 | 15 |
| 7 | 50 | 12 | 11 | 16 |
| 8 | 300 | 11 | 25 | 14 |
| 9 | 650 | 11 | 11 | 13 |
| 10 | 810 | 7 | 10 | 13 |
| 11 | 390 | 12 | 24 | 13 |
| 12 | 1800 | 12 | 18 | 10 |
| 13 | 1000 | 11 | 49 | 12 |
| | | $\bar{x}$ = 11.5 | | $\bar{x}$ = 13.7 |

Infected with Frozen Sporozoites

| | | | | |
|---|---|---|---|---|
| 1 | 2000 | 11 | 11 | 18 |
| 2 | 1600 | 15 | 37 | 17 |
| 3 | 1240 | 15 | 34 | 17 |
| 4 | 1370 | 15 | 23 | 17 |
| 5 | 1780 | 15 | 6 | 18 |
| | | $\bar{x}$ = 15.0 | | $\bar{x}$ = 17.4 |

A comparison of the onset of anemia with the development of asexually reproductive stages shows that the hepatic schizonts (Figure 2) have already made their appearance prior to the first blood loss while the megaloschizonts first begin to appear coincidentally with blood

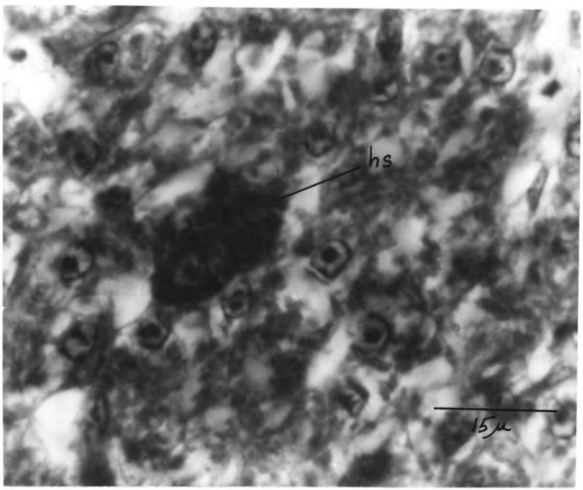


Figure 2. Histologic section of liver containing a hepatic schizont (hs) on the 4th day of a L. simondi infection initiated with fresh sporozoites. Erythrocyte loss does not begin until the 6th or 7th day post infection when this stage is no longer present.

loss and disappear just prior to maximum anemia (Figure 3).

## II. CHARACTERISTICS OF THE ANTI-ERYTHROCYTE FACTOR

### A. In Vivo

#### Experiment A-1

Since erythrophagocytosis could not be detected in any infected birds the serum was examined as a possible source of anti-erythrocyte (A-E) activity. Three two-week old ducklings were transfused with acute serum equal to approximately 1/4 of their total plasma volume. Three additional ducklings were transfused with normal serum and were used as controls. Figure 4 shows the red cell counts of these six ducklings. The three receiving acute serum lost approximately $4 \times 10^5$ erythrocytes per $mm^3$ of blood as compared to no change in the controls.

#### Experiment A-2

To determine the pattern of A-E activity during an infection, serum was collected from ten two-week old ducklings ($500 \pm 40$ grams) throughout the primary infection. Titrations were made at two fold dilutions against normal homologous erythrocytes and at a 1/4 dilution against erythrocytes from the same bird from which the serum was collected (autologous cells). When these results were compared with the course of anemia it was observed that the highest titer in both homologous and autologous cell test systems occurred one to two days prior to the maximum red cell loss. The first appearance of the A-E factor was at about the same time as the first erythrocyte loss and as the titer decreased the red cell counts began to increase toward a normal level (Figure 5).

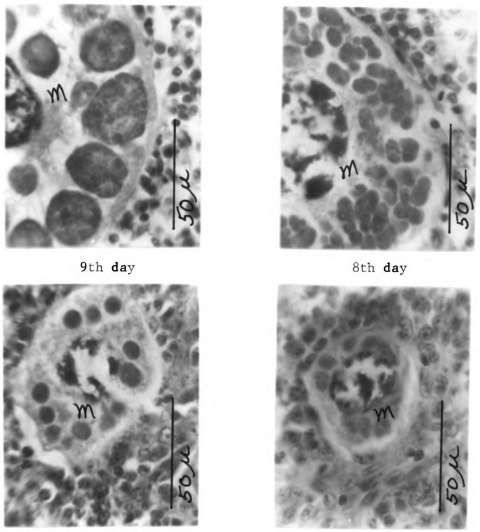


Figure 3. Histologic sections of infected duck spleen showing development of the megaloschizont (m) from the 6th to 9th day. Between the 10th and 11th day post infection (fresh sporozoites) nearly all of these schizonts are lost. Blood loss begins on the 6th day and is severest on the 11th to 12th day post infection.

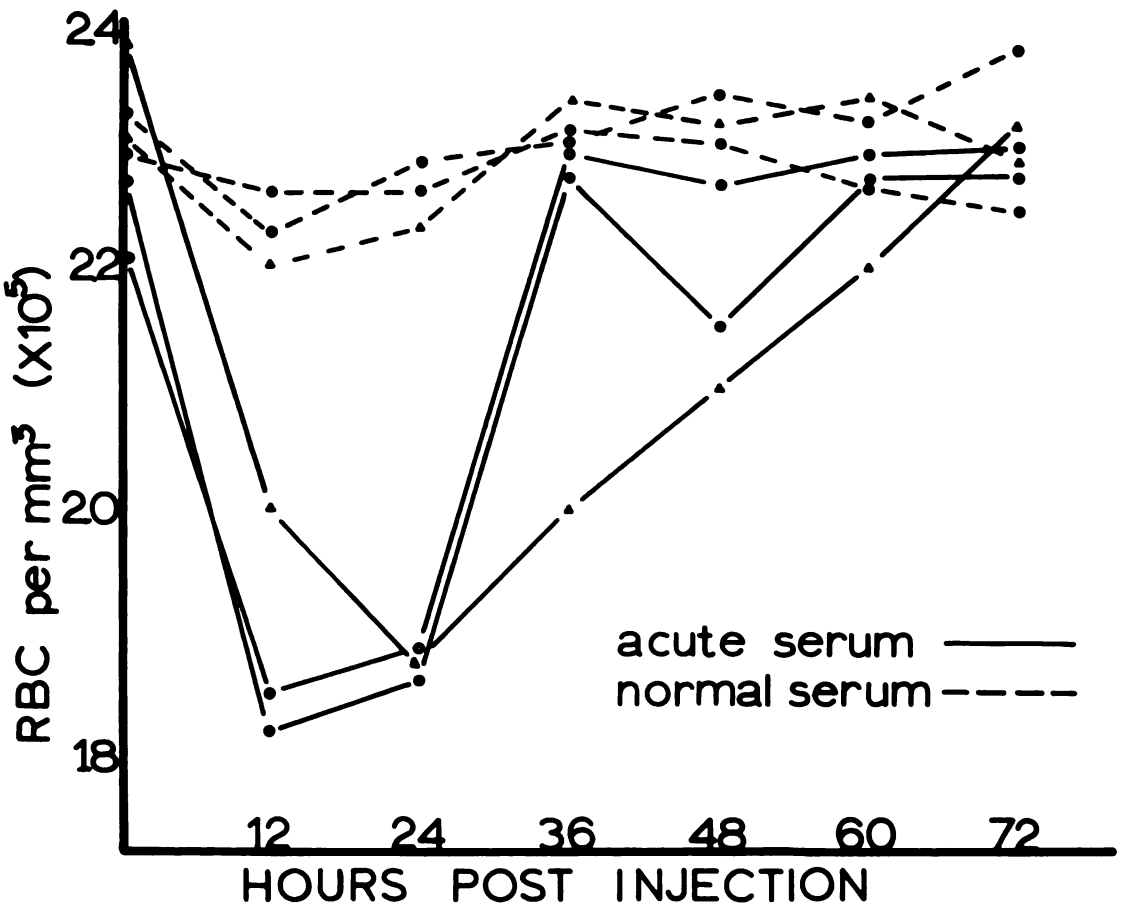


Figure 4. Massive plasma transfusions from acutely infected ducks to three normal ducklings produced a mean red cell loss of 400,000 cells per $mm^3$ while identical transfusions of normal plasma had no effect. The pattern of red cell loss and replacement is similar to that observed when cells are removed by syringe in one large aliquot.

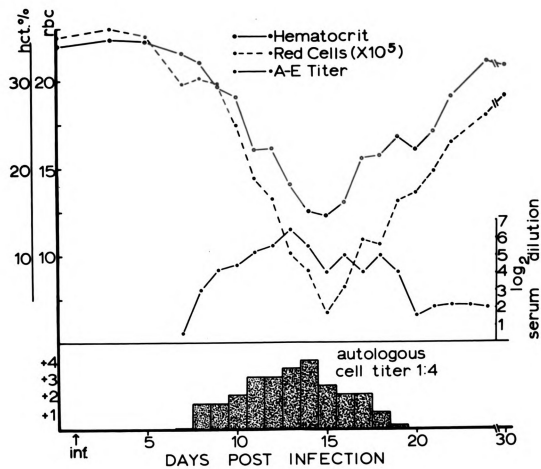


Figure 5. A comparison of erythrocyte loss per $mm^3$ of blood and anti-erythrocyte agglutination titer in two-week old (500 gram) ducklings shows that the peak titer occurs just prior to the maximum red cell loss. The rise in A-E titer occurs simultaneously with red cell loss, and recovery from anemia accompanies a drop in A-E titer. The parallel curves for hematocrit and red cell count indicate that no chronic macrocytic or microcytic anemia occurs during infection by L. simondi. In addition to paralleling the heterologous cell titer, the autologous cells were occasionally observed to spontaneously agglutinate in buffered saline.

The use of autologous cells was discontinued when it was noted that washed erythrocytes from infected birds would occasionally settle in a diffuse pattern when suspended in buffered saline alone. It was suspected that this might influence the titer and would therefore be inadequate as a means of quantitating the hemagglutinin.

## Experiment A-3

A second group was tested in the same manner as above in order to determine what effect age and size had on the course of anemia. This group consisted of six four-week old ducklings with a mean weight of $1000 \pm 55$ grams. To determine if the daily withdrawal of blood influenced the course of anemia, two three-week old ducklings were used as controls. These were not infected but were bled daily and tested for agglutinins along with the infected birds. Both age groups received identical aliquots of the sporozoite suspension. These sporozoites had been frozen in liquid nitrogen. The anemias recorded from the two groups are compared in Figures 5 and 6. It is obvious that the mean blood loss per $mm^3$ is identical in both groups while the A-E titer is lower in the older and larger group. This group also showed a more rapid decline in A-E titer than did the younger group. Six ducks in the younger group died between the 12th and 15th day post infection while there was complete recovery in all of the ducks in the older group. There was no alteration in blood values or agglutinins in the two uninfected controls. This is in agreement with results obtained from bleeding experiments which showed that a loss of up to 20% of the red cells produced no detectable change in blood count.

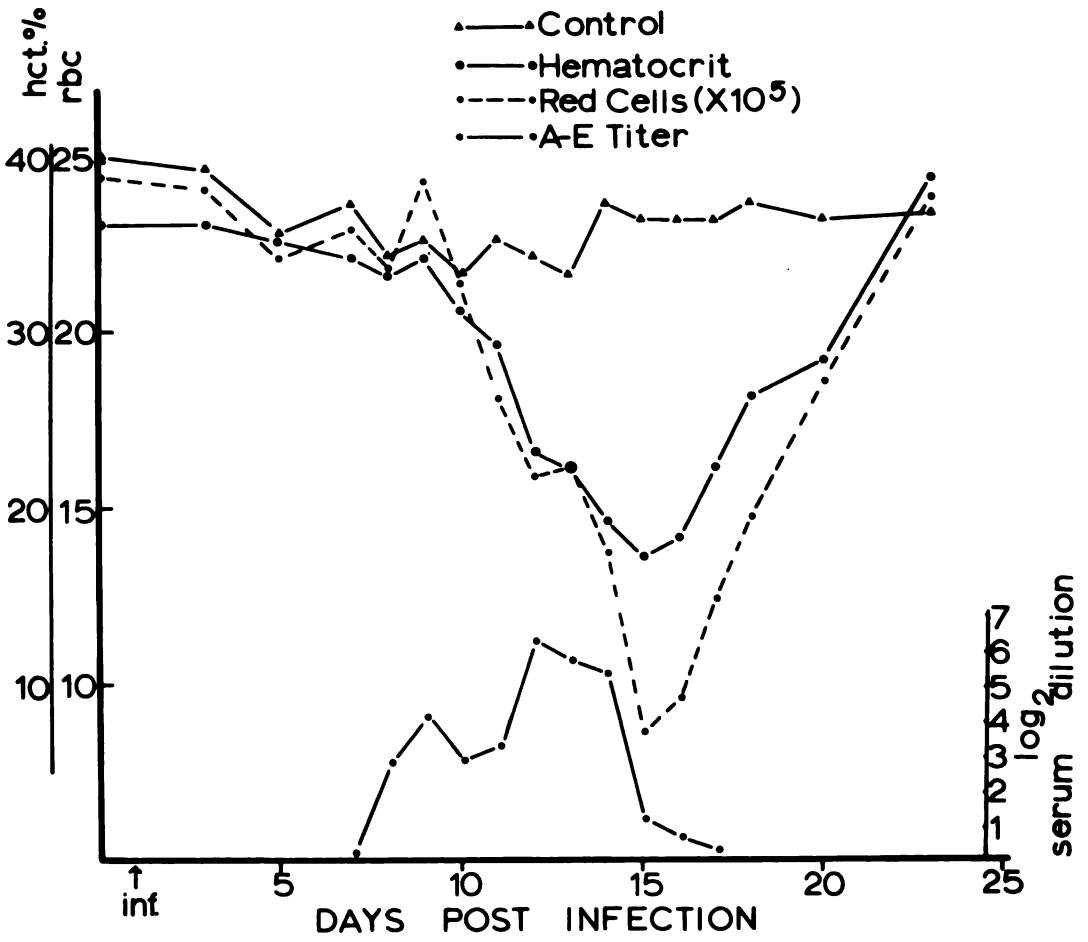


Figure 6. Comparison of anti-erythrocyte agglutination titer with red cell loss per $mm^3$ of blood in four-week old (1000 gram) ducklings shows a coincidental rise in titer and loss of red cells with the peak titer occurring just prior to maximum red cell loss. The drop in titer occurred more rapidly in the older ducks than in the younger. The erythrocyte counts for the uninfected controls indicate that daily withdrawal of blood at the level used in these experiments does not produce a detectable anemia. There was also no A-E titer produced as a result of blood removal.

## Experiment A-4

The four survivors from the younger group described above were reinfected with an identical dose of sporozoites one week after they had shown a latent primary infection. These birds were 2000 grams at the time of reinfection, four times their weight when first infected. This experiment was carried out to determine if the A-E factor responded anamnestically to a second challenge as one might expect it to if it were an autoantibody. Figure 7 compares the blood loss and A-E titers of these four birds during the primary and reinfection. It is obvious that the blood loss, if any, is greatly reduced during the reinfection. The A-E titer parallels that of the primary infection from days 7 to 12 post infection but then suddenly drops to zero. No evidence for an anamnestic response could be found at any stage of the reinfection.

## Experiment A-5

Ducklings were inoculated with three different materials in an attempt to artificially produce anemia or an A-E titer in uninfected ducks.

Gametocytes. A mixture of round and elongate gametocytes was harvested from an infected duck and ground with alumina to solubilize the antigens. This was then mixed with Freund's Adjuvant and used to immunize two three-week old ducklings with two intramuscular injections given one week apart. During the course of immunization and for ten days following the last injection, blood counts and hematocrits were made to determine if any erythrocytes were lost. Serum and erythrocytes were collected and titered against each other at the end of the ten days following the last injection. No blood loss or A-E activity could be detected.

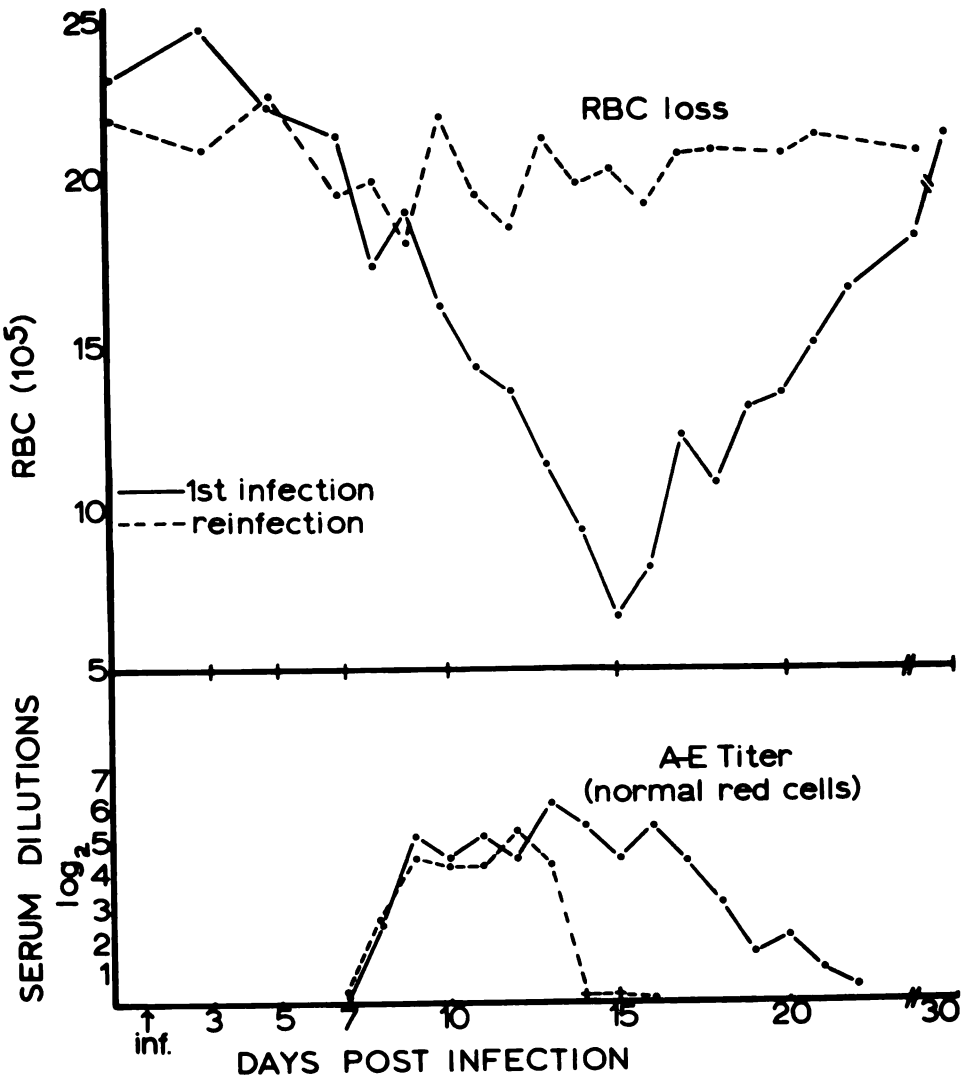


Figure 7. In order to determine if an anamnestic response occurs upon reinfection by L. simondi, the A-E titers and red cell counts of three ducklings were compared for the primary infection and reinfection. Red cell loss on reinfection was very slight. The A-E titer paralleled that of the primary infection for four to five days followed by a precipitous drop in titer. No evidence for an anamnestic response could be found.

Megaloschizonts. Megaloschizonts were collected on the two days prior to their disappearance and treated in the same manner as the gametocytes. The two birds in this experiment also showed no change in blood count or hematocrit and no A-E titer could be detected after immunization.

Acute Serum. Pooled acute serum was injected intravenously (0.1 ml) into six ducklings on alternate days for two weeks. During this period and for ten days following the last injection no change in blood count or hematocrit was observed. There was also no observable A-E titer.

### Experiment A-6

To test for the presence of organisms other than L. simondi which might be responsible for the observed anemia ducklings were injected intraperitoneally with host tissues collected at various phases of parasite development. These included: 1 - blood collected prior to parasitemia or anemia, blood collected during the acute phase of the infection, and from recovered ducks; 2 - spleen and liver removed just prior to maximum blood loss; 3 - spleen and bone marrow collected immediately prior to the first blood loss. None of the injected ducklings exhibited symptoms of the disease such as lower hematocrits or a drop in red cell count. Round gametocytes were observed in very low numbers in those ducklings given blood, liver or marrow from prior to blood loss or parasitemia.

## B. In Vitro

### Experiment B-1

To determine what component(s) of the serum contained the A-E factor(s) ammonium sulfate precipitation of the components was carried out.

Gamma globulin was the only fraction which showed A-E activity. There was, however, a drastic reduction in the amount of activity in the gamma fraction as compared to whole serum even when a 4x concentration was used. The remainder of the serum was also tested up to a 4x concentration but showed no activity.

To check if some normal component of serum removed by precipitation might be needed for full activity a test was run using gamma globulin plus normal serum. The results are recorded in Table III. Apparently some activity was lost as a result of manipulating the protein since restoration was not possible with normal serum.

TABLE III. Fraction of acute serum containing A-E activity.

| | (1:4 dilution) |
|---|---|
| Normal serum | - |
| Acute serum | ++++ |
| Gamma free acute serum | - |
| Acute gamma globulin | + |
| Acute gamma globulin + normal serum | + |

## Experiment B-2

The activity of the acute serum was tested at a series of physiological temperatures to determine its effective temperature range. Acute serum and normal erythrocytes were incubated at 4, 10, 15, 22, 37 and 42 C and checked for agglutination. The cells were resuspended frequently for the first half hour then allowed to settle for one hour and read for agglutination. All tubes below 37 C showed the same degree of

agglutination. The tubes at 37 and 42 C, however, showed some hemolysis if left over one hour. If resuspended and allowed to settle after the one hour incubation these higher temperature tubes showed a reduction in the agglutination pattern due to the hemolysis of most of the cells. When serum with a high titer was tested it was noted that there was often no agglutination at the higher temperatures but hemolysis did occur. It was assumed that the cells were hemolysing before lattice formation or whatever process was responsible for agglutination could occur. The results of temperature range experiments given in Table IV show that all temperatures up to that of the host are favorable for A-E activity.

TABLE IV. Temperature range of A-E activity (1:4 dilution).

| 4 C | 10 C | 15 C | 22 C | 37 C | 42 C |
|---|---|---|---|---|---|
| ++++ | ++++ | ++++ | ++++ | Hemolysis | Hemolysis |

Experiment B-3

Since cells incubated at higher temperatures were lysed by acute serum, titers were determined for the strongest hemolytic sera. This serum was collected from moribund ducks on the tenth day of the infection and had the appearance of chocolate milk. The cells and serum were incubated for three hours at 37 C with frequent resuspension of the cells. At the end of this period a slide was made of the cells from each dilution. Figure 8 shows the results of this test in one duck. It is apparent that a 1/64 dilution is still capable of hemolysing 50% of the normal erythrocytes in the tube while a 1/128 dilution causes slight

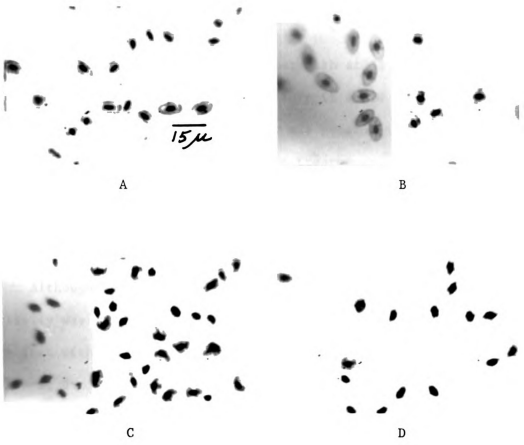


Figure 8. Stained erythrocytes after 10 hours incubation at 37 C with various dilutions of the anti-erythrocyte factor. A - normal serum; B - 1/128 acute serum; C - 1/64 acute serum; D - 1/32 acute serum. The 50% end point for this serum is the 1/64 dilution since one half of the cells have been destroyed. There was some cytoplasmic derangement in the 1/128 dilution. The control was allowed to incubate for 24 hours but still showed no signs of red cell alteration.

derangement of the red cell cytoplasm. The control cells were allowed to incubate up to 24 hours but showed no signs of hemolysis.

## Experiment B-4

Acute serum was placed in a water bath at 56, 60 and 65 C for 30 minutes to determine at what temperature it would be deactivated. Full hemolytic and agglutinating activity was present in the 56 C sample but it was lost in the higher temperature treatments. Apparently complement is not required for these reactions. At 60 C and above it was noted that a precipitate began to form and it was assumed that the serum proteins were being heat denatured.

Although 56 C did not deactivate the A-E factor it was noted that activity was lost after the serum or plasma had been stored. Activity was lost within a few hours at room temperature and within 24 to 48 hours at 0 to 4 C. Freezing at -20 C preserved the activity for several months but even this was lost with prolonged storage. No experiments were carried out to determine the exact half-life of the A-E factor at various temperatures. It was observed, however, that when stored in an unfrozen state a precipitate formed in the acute serum but not in normal serum. This precipitate may have contained the A-E factor and was therefore responsible for the loss of activity.

## Experiment B-5

Serum from days 7 through 11 was pooled from ten ducks and tested at a ½ dilution for activity against the erythrocytes of other avian and mammalian species. Table V gives the results of these tests. Controls consisted of serum pooled from six uninfected ducks and determined at

the same dilution as the acute serum.

TABLE V. Specificity of the A-E factor (1:4 dilution).

| Source of Erythrocytes | Acute Serum | Normal Serum |
|---|---|---|
| Chicken (W. L.) | ++++ | + |
| Cow (Guernsey) | ++++ | - |
| Mink | ++ | - |
| Dog (Beagle) | ++ | - |
| Canada Goose | +++ | - |
| Bob White Quail | +++ | + |
| Khaki Duck | ++++ | - |
| Human A+ | - | ++++ |
| Human B+ | - | ++++ |
| Human O+ | - | ++++ |

All of the species tested, with the exception of human cells, gave a positive agglutination reaction. Several species exhibited slight heterogenetic reactions with normal serum but all of these increased greatly when incubated with acute serum.

The lack of agglutination observed with acute serum and human erythrocytes was tested to determine if the inhibition occurred at the cell surface or in the non-cellular blood components. The cells which had incubated with acute serum were washed three times in buffered saline and divided into two aliquots of each blood group. One aliquot was allowed to settle out in buffered saline while the other settled in 1/4 normal serum and saline. Table VI gives the results of this test.

TABLE VI. Site of agglutination inhibition of acute serum for human A, B and O Rh+ cells.

| Blood group | Cells + acute serum | Cells washed after treatment with acute serum + normal serum |
|---|---|---|
| A+ | - | ++++ |
| B+ | - | ++++ |
| O+ | - | ++++ |

It appears that there was either no permanent alteration of the erythrocyte surface since the normal serum was still able to agglutinate the cells which had been washed free of acute serum or that the inhibition resulted from a reaction between acute and normal serum.

A second test was set up in which 0.05 ml of acute serum was added to a series of normal serum dilutions and 1% human erythrocytes to determine if acute serum could directly inhibit the agglutination reaction. Table VII shows the results of this test.

TABLE VII. Ability of acute serum to inhibit activity of normal serum.

| Components | ml of each component | | | | | |
|---|---|---|---|---|---|---|
| Acute serum | 0.0 | 0.0 | 0.0 | 0.05 | 0.05 | 0.05 |
| Saline | 0.80 | 0.925 | 0.988 | 0.75 | 0.88 | 0.938 |
| Normal serum | 0.25 | 0.125 | 0.062 | 0.25 | 0.125 | 0.062 |
| Normal cell suspension | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| Agglutination titer | ++++ | +++ | ++ | + | - | - |
| Total ml | 1.15 | 1.15 | 1.15 | 1.15 | 1.15 | 1.15 |

It is apparent that the 0.05 ml of acute serum had an inhibitory effect on the agglutinating activity of normal serum which is not the result of dilution.

## DISCUSSION

Contrary to descriptions of anemia in animals infected with Plasmodium, Babesia, or Anaplasma the onset and severest blood loss in ducks infected with Leucocytozoon occurs prior to the peak parasitemia. The significance of this observation is difficult to assess at this time because the infections of L. simondi were initiated with sporozoites while the others were initiated with merozoites in blood. If erythrocyte destruction in L. simondi infections results from the presence of some prepatent tissue stage, this would be masked in blood induced infections because of the presence and development of the blood stages simultaneously with the tissue stages. In sporozoite induced infections these two factors would be separated - tissue stages only occurring during the prepatent period and blood stages occurring later. Until studies on the course of anemia in Plasmodium infections initiated with sporozoites have been carried out, this discrepancy will have to remain unexplained. Another factor which makes comparison of Leucocytozoon with Plasmodium and Babesia infections difficult is the presence of reproducing stages in the peripheral blood in the two latter types of infection. No asexual parasite reproduction has been observed in the peripheral circulation during infections of Leucocytozoon.

The replacement of lost cells in infected hosts was probably the result of increased erythropoiesis. The increase in polychromatic erythrocytes during the period of anemia indicated that there was not an inhibition of erythropoiesis as suggested by McGhee (1965). Cox et al. (1966) and Zuckerman (1963) did observe reticulocytes in their infected animals, and their values correspond to those obtained for L. simondi. It seems unlikely that any animal would survive an infection which

produced both anemia and inhibition of erythropoiesis. What is probably occurring in L. simondi infections is an increase of erythropoietic activity in response to tissue anoxia. During the acute phase of the infection an anti-erythrocyte (A-E) factor and erythropoiesis are acting antagonistically, and survival of the host depends in part on whether erythrocyte replacement can compensate for loss until the titer of the A-E factor is reduced.

Although there is no agreement on the mechanism of blood loss in hemosporidian infections investigators in this field agree that there is a loss of cells incommensurate with the parasite density. The report by Kocan and Clark (1966) and the results presented here show that L. simondi infections also fit this pattern. There is no doubt that a tremendous number of erythrocytes are lost in excess of the parasite density and that the maximum values do not correspond in time. There may be a correlation between the tissue schizonts and blood loss, but thus far there has been no accurate comprehensive work done on the pattern and timing of development of these stages.

A large body of evidence has been presented incriminating erythrophagocytosis as the major cause of blood loss in hemosporidian infections. I was unable to detect any increase of erythrophagocytosis during L. simondi infections. Since thirteen birds were examined from the first signs of blood loss until they died with an extremely low blood count, it is unlikely that this phenomenon was overlooked. The presence of parasites in phagocytic cells indicates that phagocytosis is not being inhibited by Leucocytozoon. It is possible that the A-E factor is able to act as an opsonin, but as noted in the experiments concerned with the effects of temperature on A-E activity it was found that the

A-E factor acts to hemolyse red cells in vitro at the body temperature of the duck. In vitro tests at lower temperatures would have to be carried out to determine whether or not the A-E factor possesses opsonizing activity, but this would not give a true picture of what is occurring in vivo.

The presence of free hemoglobin in the plasma of many of the infected ducks indicates that intravascular hemolysis may be the cause of erythrocyte destruction. The observation that some birds did not have free hemoglobin in their plasma, even though they had an anemia comparable to those which did, is probably a manifestation of the bird's ability to reclaim the hemoglobin.

There is no doubt that some type of an anti-erythrocyte factor is present in the serum of acutely infected ducks. The drop in blood count of the ducklings transfused with acute serum is strong support of the A-E factor's ability to destroy erythrocytes in vivo. This experiment should be verified with other more sophisticated techniques. The withdrawl of large volumes of blood and the massive replacement with foreign serum produces major physiological changes in the circulatory system which can not be controlled. The use of $^{51}Cr$ or $^{59}Fe$ tagged cells would have a far less drastic effect on the experimental animal's circulatory system than massive transfusion.

The A-E factor is first detected in the serum at the same time anemia begins. It may be present prior to that time but in such low quantities that it is undetectable or it may all be bound to erythrocytes. The increase in titer which parallels the loss of red cells and the gradual decrease in titer during recovery definitely indicates that the A-E factor is a prime suspect as the major contributor to blood loss. The peak

titer just prior to maximum blood loss and its decline immediately thereafter may indicate that it is being released by one of the parasite stages during its growth and development and stops being produced once maturity or loss of that stage occurs. The presence of an erythrocyte destroying factor prior to the parasite crisis is contrary to the results of all other investigators who have studied anemia in other hemosporidian infections. In all cases where a serum component has been shown to affect red blood cells, the activity has been in convalescent rather than acute serum - a fact which has been one of the major arguments in support of the autoimmune mechanism of blood loss.

Until now a serum component which can directly affect normal untreated erythrocytes has not been demonstrated. Since for most hemosporidian infections the excess blood loss has been attributed to the destruction of non-parasitized cells as well as parasitized cells it is imperative that a reaction against normal cells be demonstrated. Zuckerman (1945) did demonstrate opsonizing activity of malarious plasma against non-parasitized cells supporting the erythrophagocytosis mechanism of blood loss. All other evidence of activity against non-parasitized cells has been with the use of trypsinized or tanned erythrocytes. Since these treatments cause the cells to adsorb protein, there is no way of knowing what has been adsorbed to the cell surface. At least in the work which has been reported there has been no attempt to identify the adsorbed material. Existing data do not provide a basis for calling agglutination of treated cells the result of an antigen-antibody reaction. With a treated cell system, lattice formation can occur by several cells adsorbing to the same protein molecule. This could be normal gamma globulin, parasite antigen, or a combination of the two. There is no doubt that normal

erythrocytes are affected by serum from acute Leucocytozoon infections, but there is no evidence that this factor is present in recovered duck serum.

Comparison of the data obtained from the various hemosporidian infections strongly suggests that the mechanism of anemia is not the same in all cases. As the situation stands presently, there is evidence for a hemolytic autoantibody, an opsonizing autoantibody, direct physical rupture of infected cells by the parasite, and parasite A-E factor. The A-E factor is believed to be the case for L. simondi infections.

From the available evidence it is impossible to determine what stage(s) of the parasite is responsible for the anemia. It is possible that the parasite is not directly responsible for red cell destruction but that it stimulates some host cells to release the A-E factor. Two possible host products which could be released as a result of infection are autoantibody or tissue hemolysins. Autoantibody would be produced in response to some parasite antigen or against altered or exposed host antigen. Tissue hemolysins could result from cells damaged by the parasite through direct invasion or some metabolite of the parasite.

Location of the A-E factor in the gamma fraction of the acute serum lent support to the autoantibody mechanism. Other evidence, however, does not support this theory. First, the ability of deactivated serum to hemolyse red cells without the aid of complement does not agree with the known antigen-antibody explanation of hemolysis. Also, the relative instability of the A-E factor upon standing is not common for antibody; although this property does not lend strong support against antibody. Second, the drop in A-E titer which paralleled recovery from parasitemia is not reminiscent of antibody production. In general one would expect

that an autoantibody titer would be at its highest level during the recovery phase when anti-parasite antibody is at its highest level. This line of reasoning is logical. If the autoantibody is produced in response to the parasite antigen it should parallel anti-parasite antibody which is also produced in response to parasite antigen. If the autoantibody resulted from alteration or exposure of host antigen, it would still be produced sometime after the parasite has had an opportunity to alter the host cells or antigens. Other evidence in opposition to an autoantibody mechanism is the lack of an anamnestic response upon reinfection. Most suspected autoantibody diseases become more severe upon re-exposure to the antigen. The evidence presented above shows that the A-E factor did not appear any earlier in the re-exposed birds than it did in the primary infections, and it never reached as high a titer. In addition, when the A-E titer in the primary infections was at its highest, the reinfected group showed a precipitous drop in titer. There was also little if any blood loss upon reinfection which is the opposite of what would be expected if autoantibody were responsible. In the classical anamnestic response antibody appears earlier than on the first exposure, reaches a higher titer, and can be detected for a longer period after elimination of the antigen. Results reported by Kocan (1965) show that recovered ducks reinfected with a larger number of sporozoites than they received on the primary infection developed severe anemia and died. Comparing these two sets of results it is clear that autoantibody is not likely the cause of anemia in these infections. The evidence better supports a mechanism of parasite products or tissue hemolysins being the A-E factor.

If one assumes that a constant amount of A-E factor is released by

a given number of parasites or a given number of infected cells, which would also depend on the parasite density, the results given above could be readily interpreted. The lower titer observed in the older birds could be the result of dilution of the A-E factor. Since the older group was twice the size of the younger group it would be ideal to have a titer one half that of the younger group. It is felt, however, that since unbound rather than total A-E factor is being measured a simple dilution might not be the case. Some sort of an equilibrium between erythrocyte receptor sites and the A-E factor might be in operation. Until the nature of the binding reaction is revealed there is no way of determining what per cent of the A-E factor is in the unbound state.

A second factor in support of a parasite product is the rapid drop in titer which occurs as the birds recover. This is reminiscent of antigen removal in an animal which has developed antibody to an antigen which is still circulating. An example would be serum sickness. Even stronger support is the precipitous drop in A-E titer in the reinfected birds. If the A-E factor were antigenic, the first infection would immunize the bird and the second would result in its more rapid removal. The anemia from larger numbers of sporozoites in reinfected birds reported by Kocan (1965) could be the result of the A-E factor being present in such large quantities that it overwhelms the antibody system developed against it. If the A-E factor were a weak antigen, this situation could be readily visualized. It is also possible that the birds are destroying stages of the parasite before they are able to produce the A-E factor resulting in a precipitous drop in titer as the parasite is killed.

The scheme described above is better suited to a parasite product than to a tissue hemolysin. The latter would be difficult to explain

in terms of the antigenic explanation for the rapid removal in reinfected birds.

The immunization experiments did not support nor refute either of the mechanisms previously described. The negative results following immunization with the three different parasite products indicates that they are not capable of stimulating anti-erythrocyte antibody by the procedure or in the quantity used. It is possible that one of these products would be a better antigen under different conditions of immunization, or in the living state they may produce some substance which is antigenically active and results in the production of anti-erythrocyte antibody. It is also possible that the hepatic schizonts, which were not tested, are responsible for the blood loss. Before these can be tested a method for purifying them must be developed, otherwise the problem of large amounts of host antigen being present in the preparation will complicate the results.

In any situation where natural transmission is used, or where material other than a pure culture of parasites is used to initiate an infection, there is the possibility of infection by some organism other than the one being studied. The routine tests for determining the presence of hemagglutinating organisms were all negative. The myxoviruses can be ruled out since they cause hemagglutination at 37 C, whereas the A-E factor causes lysis at this temperature. Myxoviruses also destroy their receptors and elute while the A-E factor remains attached. The basophilic viruses are also known to cause hemagglutination but the activity is destroyed at 56 C for 30 minutes and they elute at 4 C. The hemagglutinating arboviruses are active between pH 6.4 and 6.8 while that observed here occurred between 7.0 and 7.2 In all cases cited

above, recovery produces a strong hemagglutinin inhibiting antibody. In the recovered birds studied free active A-E factor was always produced; thereby ruling out any strong inhibiting antibody.

Since the A-E factor was at first considered to be autoantibody it was thought that testing the acute serum against erythrocytes of other species might give some evidence as to what antigen the A-E factor affected. From the results it is apparent that there is very little species specific activity. This indicates that there are similar receptor cites on the erythrocytes of all of those species tested and shown to react positively. The loss of normal hemagglutinating activity to human cells by the acute serum was a complete reversal of what might be expected from the previous discussion and eliminated these antigens as possible receptors. The experiments to determine the location of inhibition indicated that either the inhibitor acted at the cell surface but was so loosely bound that it could be washed free or that the inhibition resulted from an alteration of some component of normal serum. Since this inhibitory activity remains active after the A-E factor activity is lost it is felt that they are two different components of the acute serum. It is possible, however, that the same molecule is responsible for both reactions but that it has two active sites, one more stable than the other. Results reported by Becker et al. (1951), Becker et al. (1952) and Becker and Schwink (1953) indicate that they found a similar component in duck serum. They called their substance the "sparing" factor and reported that it was stable at 56 C. It had the ability to inhibit the hemagglutinating activity of normal chicken plasma on duck erythrocytes. This factor was present in normal as well as infected serum. They did not test it against other hemagglutinating systems so it is

difficult to tell if it is the same factor reported here. Since it is present in normal as well as infected serum it may not be the same substance as the inhibiting factor discussed here.

In view of the results obtained while studying anemia in ducks with acute Leucocytozoon disease, the following conclusions have been made: The anemia which accompanies Leucocytozoon infections is not the result of direct physical rupture of the red cells by the parasites nor from erythrophagocytosis as reported for other types of infection. There is also no sound evidence in support of an autoantibody mechanism. The evidence does support a parasite product as the cause of hemolysis. This is released into the circulation during the acute phase of the disease and can be detected in an unbound form in serum collected during this period. The A-E factor, as I have called it, is heat stable at 56 C but does lose activity with time. Very little specificity from species to species is present.

SUMMARY

Erythrocyte loss in ducklings which accompanies infection by Leucocytozoon simondi (Mathis and Leger, 1910) was found to be far in excess of that which would be expected from direct physical destruction by the parasite. Cell destruction began coincidentally with the appearance of parasites in the blood and reached its severest point one to five days prior to maximum gametocyte density. Loss of circulating parasites and red cell replacement occurred simultaneously.

No evidence could be found to incriminate erythrophagocytosis as the mechanism of erythrocyte destruction. It was possible, however, to demonstrate a humoral anti-erythrocyte (A-E) factor in the serum of acutely infected ducks which agglutinated and/or hemolysed normal untreated erythrocytes. This factor is located in the gamma portion of the acute serum and is active from 4 to 42 C. Attempts to demonstrate an anamnestic response by the A-E factor in reinfected ducks were all negative. It was concluded that the A-E factor was probably not autoantibody.

Incubation of acute serum with red cells of other avian and mammalian species showed that the A-E factor was not species specific. It was also found that agglutination of heterogenetic cells by normal duck serum was inhibited by acute serum. Tests indicate that the inhibitor is either very loosely bound to the erythrocyte surface and can be washed off or the inhibition occurs between humoral factors in normal and acute serum. Evidence suggests that the A-E factor and inhibitory factor are not the same.

In order to obtain year round infections it was necessary to develop a method of preserving L. simondi sporozoites. This was accomplished by freeze-preservation in liquid nitrogen. With this technique sporozoites were kept viable and infective for at least eight months.

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