Characteristics of the Hemagglutinin Produced During *Plasmodium lophurae* Malaria in Chickens

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During the course of *Plasmodium lophurae* infections in normal chickens, there was a sharp increase in the titer of a hemagglutinin which reacted better with trypsinized than with normal erythrocytes. This hemagglutinin was a typical "cold hemagglutinin" in that it was much more active at 4°C than at 37°C, was found in the macroglobulin fraction of the serum, was eluted from erythrocytes at 37°C, and was easily destroyed by 2-mercaptoethanol reduction. The injection of allelo-genic erythrocytes into chickens prior to their exposure to *P. lophurae* resulted in a coexistent increase in cold hemagglutinin titers and enhanced resistance to parasitemia. Since this antibody was more active against altered erythrocytes than against normal erythrocytes, it may moderate resistance to malarial infections by specifically stimulating phagocytosis of parasitized erythrocytes.

Blood-induced malarial infections must by necessity be accompanied by the introduction of erythrocytic antigens into the experimental animal. The resulting immune response may be directed towards the antigens of the parasite and the erythrocytic antigens of the donor animal. The development or presence of such hemagglutinins may afford some protection against the subsequent infection by encouraging the removal of parasitized red blood cells.

Coffin (4) and Schwink (13) demonstrated a nonspecific resistance to avian malaria following immunization with normal allelo-genic erythrocytes, although neither of these investigators attempted a detailed examination of the hemagglutinins produced. Coffin (4) observed that the agglutinin(s) could be absorbed from the serum of superinfected ducks by either parasitized or noninfected duck red cell stroma. Intact allelo-genic duck erythrocytes were inconsistent in their ability to remove the hemagglutinins. Conversely, Schwink (13) reported that the hemagglutinins in hyperimmune chicken serum could be removed by absorption with intact, allelo-genic erythrocytes. Both investigators observed that the agglutinins functioned at room temperature, but since the incubation times were not recorded it cannot be positively stated whether or not they were measuring "cold" hemagglutinins. Hemagglutinins developed in other forms of avian malaria have been studied only with respect to their effect on immunity (10, 17).

Because detailed serological and immunochmical descriptions of the hemagglutinins produced during *Plasmodium lophurae* malaria are lacking, we are reporting our study of the hemagglutinin response of normal and erythrocyte-preimmunized chickens during their *P. lophurae* infection and are presenting a partial characterization of these hemagglutinins. A preliminary report of portions of the data has appeared elsewhere (Rigney, Barrett, and Breitenbach, Bacteriol. Proc., p. 101, 1969).

MATERIALS AND METHODS

Experimental infections. White Leghorn cockerels 4 to 6 weeks of age were used in all experiments. Each experimental group of chickens was composed of birds from the same hatch and housed under identical conditions. *P. lophurae* strain 12a, an avian malarial parasite, was used throughout this investigation. This parasite was maintained by twice weekly blood transfers in chickens.

Blood-induced infections were accomplished by the administration of a volume of heparinized, infected, whole blood calculated to contain 10⁹ malarial parasites per kg of body weight. Parasitemia levels were calculated by the method of Gingrich (6).

Hemagglutination tests. Agglutination studies were performed with freshly drawn erythrocytes from various animal sources (Table 1). In addition, aged chicken erythrocytes and trypsinized human erythrocytes were employed as test cells. Trypsinized human erythrocytes were washed with saline and used as controls.

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erythrocytes (O, Rh-positive) were prepared by the method of Mann and Ristic (9). The agglutination tests were conducted as described by Salk (12), and the titers were determined after 1.5 hr of incubation.

Characterization of the hemagglutinin. All physical and chemical treatments used in characterizing the hemagglutinin were performed on serum containing demonstrable agglutinins, which was obtained from chickens acutely ill with *P. lophurae* infections. Hemagglutination tests were performed on each sample of untreated serum before and after the treatment. All except the initial experiments were performed with trypsinized, O, Rh-positive erythrocytes at 4 C.

The reduction cleavage of macromolecules was accompanied by treatment with 0.1 M 2-mercaptoethanol followed by dialysis against 0.2 M iodoacetamide (3).

Adsorption and elution of antibody. Agglutinins were adsorbed from untreated sera of acutely ill chickens by treatment with trypsinized, O, Rh-positive human erythrocytes at 4 C for 4 hr. The erythrocytes were then washed four times in cold saline and resuspended in a volume of saline equal to the original volume of serum. This saline-erythrocyte suspension was incubated at 37 C for 30 min. The eluted agglutinin was then separated from the erythrocytes by centrifugation, dialyzed, lyophilized, and stored at −20 C until used.

Electrophoresis. A Beckman model R-101 Microzone electrophoresis system was used to separate the protein components in sera and the eluted antibody.

Sephadex gel filtration. Globulins were precipitated from the acute sera by one-half saturation with ammonium sulfate. The globulins were redissolved in a minimal amount of 0.1 M phosphate-buffered saline (pH 7.4) and were dialyzed overnight against this buffer. Separation of the globulins in this mixture was accomplished by gel filtration through a Sephadex G-200 column (4 by 70 cm) and elution with 0.1 M phosphate-buffered saline (pH 7.4). Fractions of the eluate were scanned at 280 nm to estimate protein content. In addition, each fraction was assayed for hemagglutinin activity.

Nonspecific resistance. Two groups of 4.5-week-old chicks were used. The experimental group of 11 chicks received two intravenous injections of 1.0 ml of a 50% suspension of washed normal chicken erythrocytes. One injection was administered 6 days before infection and the second was given 1 day prior to infection. The control group of nine chicks received the same volume of 0.85% saline on these days. The following day, all of the chicks were infected with *P. lophurae*. For 10 days after infection, thin blood smears were taken from each chick in the morning hours of each day for parasitemia determinations. In addition, whole blood was taken from six birds in each group to provide sera for the hemagglutination studies.

RESULTS

Normal White Leghorn chickens possessed low levels of heteroagglutinins for erythrocytes of several species, including sheep, rabbit, guinea pig, and man, but not for allogeneic cells (Table 1). Upon recovery from *P. lophurae* malaria, the hemagglutinin titers of these chicken sera were generally increased, and an agglutinin which was active against aged allogeneic chicken cells was also detectable. When the agglutinin for human erythrocytes was removed by absorption, the agglutinin for chicken erythrocytes was also removed.

At 4 C, the hemagglutinin titer of normal chickens for the trypsinized human erythrocytes averaged only 1:8 (Table 2). The titer increased sharply during the acute phase of the infection (days 2 and 3 of the illness), usually reaching 1:1,024. The hemagglutinin titers customarily decayed during the recovery phase. Hyperimmune chickens, which were rechallenged several times with *P. lophurae* (blood-borne), developed and maintained elevated hemagglutinin titers which ranged as high as 1:4,096. Although similar fluctuations in the hemagglutinating capacity of the sera were also demonstrable when the tests were performed at 25 and 37 C (Table 2), the magnitude of the titers at 4 C suggested that a typical cold hemagglutinin was responsible for these results. This was supported by the observa-

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**Table 1. Hemagglutinating activity of normal and Plasmodium lophurae-immune chicken serum**

<table>
<thead>
<tr>
<th>Erythrocyte species</th>
<th>Normal serum</th>
<th>Untreated immune serum</th>
<th>Human O, Rh+ absorbed immune serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheep</td>
<td>4</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Rabbit</td>
<td>64</td>
<td>256</td>
<td>256</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Chicken, fresh</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Chicken, aged</td>
<td>0</td>
<td>32</td>
<td>0</td>
</tr>
<tr>
<td>Human O, Rh+</td>
<td>4</td>
<td>32</td>
<td>0</td>
</tr>
<tr>
<td>Human O, Rh+,</td>
<td>8</td>
<td>256</td>
<td>0</td>
</tr>
</tbody>
</table>

**Table 2. Identification of a cold hemagglutinin for human erythrocytes associated with Plasmodium lophurae infection**

<table>
<thead>
<tr>
<th>Sources of chicken serum</th>
<th>Reciprocal of antibody titera</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 C</td>
</tr>
<tr>
<td>Normal</td>
<td>8</td>
</tr>
<tr>
<td>Acutely ill, <em>P. lophurae</em></td>
<td>1,024</td>
</tr>
<tr>
<td>Recovered, <em>P. lophurae</em></td>
<td>256</td>
</tr>
<tr>
<td>Hyperimmune, <em>P. lophurae</em></td>
<td>2,048</td>
</tr>
</tbody>
</table>

a Six or more animals in each group.
tion that after mercaptoethanol reduction the titers of normal and recovered chicken sera were completely eliminated, and titers of sera from acutely ill chickens were active only when used undiluted. Hyperimmune sera still retained some activity.

Further evidence that the hemagglutinin was of the cold variety was demonstrated by its ready elution at 37°C from erythrocytes which had been agglutinated at 4°C.

**Electrophoresis of the eluted hemagglutinin.** Figure 1 contains reproductions of the electrophoretic patterns obtained with typical acute and hyperimmune serum. The bottom pattern was obtained by electrophoresis of the protein eluted from erythrocytes which had been agglutinated by the acute sera. It can be seen that the eluted protein migrated with the beta-2-globulin of the acute and hyperimmune samples.

**Gel filtration.** Fractionation of the ammonium sulfate-precipitated globulins from plasma of acutely infected chickens was accomplished by gel filtration on a Sephadex G-200 column. Two definite absorption peaks were obtained by this procedure (Fig. 2). The first emerged from the column immediately after the void volume, and another peak began at tube 110. This second peak trailed for a considerable distance until all protein was washed from the column. All hemagglutinin activity was found in the first peak.

**Nonspecific resistance to *P. lophurae*.** The effects of prior inoculation of normal allogeneic chicken erythrocytes on the course of *P. lophurae* infection are presented in Table 3. Animals in the control and experimental (erythrocyte-inoculated) groups exhibited parasitemia on day 1, the level of parasitemia being higher in birds in the control group (5.2%) than in those of the experimental group (1.1%). Birds in the control group attained a peak parasitemia of 29.4% on day 3, whereas the chicks in the experimental group permitted a low level of parasitemia to develop (5.3 to 6.7% for days 2, 3, and 4, day 4 being the peak day). The parasite was essentially eliminated from the peripheral circulation of chicks in both groups by day 5, although four birds in the control group had died. The differences in parasitemias cited for days 1, 2, and 3 were statistically significant at the 0.05-level.

The prior injection of normal allogeneic chicken erythrocytes resulted in the production of a geometric mean hemagglutination titer of 1:853 (Table 3). After the administration of the infecting dose of *P. lophurae* on day 0, the titer

![Fig. 1. Electrophoretic comparison of (a) acute infection serum, (b) hyperimmune serum, and (c) eluate from agglutinated erythrocytes. Electrophoresis was performed at pH 8.6, ionic strength 0.1, barbital buffer on cellulose acetate.](image1)

![Fig. 2. Separation of serum from acutely infected chickens by Sephadex G-200 gel filtration. Conditions: 750 mg of protein, phosphate-buffered saline, 0.1 M, pH 7.4.](image2)
TABLE 3. Parasitemia and hemagglutinin levels in normal and erythrocyte-immunized chickens

<table>
<thead>
<tr>
<th>Day</th>
<th>Control group</th>
<th>Exptl group</th>
<th>Control group</th>
<th>Exptl group</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.2</td>
<td>1.1*</td>
<td>6.7</td>
<td>853.0*</td>
</tr>
<tr>
<td>2</td>
<td>18.6</td>
<td>5.3*</td>
<td>53.3</td>
<td>138.7*</td>
</tr>
<tr>
<td>3</td>
<td>29.4</td>
<td>6.5*</td>
<td>138.7*</td>
<td>28.0</td>
</tr>
<tr>
<td>4</td>
<td>5.0</td>
<td>6.7</td>
<td>409.6*</td>
<td>27.2</td>
</tr>
<tr>
<td>6</td>
<td>0.0</td>
<td>0.8</td>
<td>89.6</td>
<td>108.8</td>
</tr>
<tr>
<td>8</td>
<td>ND*</td>
<td>ND</td>
<td>153.6</td>
<td>460.8</td>
</tr>
</tbody>
</table>

a Statistically significant lower parasitemia, probability < 0.05.

b Statistically significant higher hemagglutinin titer, probability < 0.05.

c Not done.

decreased to a low level of 1:28 on day 3. The hemagglutinin titer increased during the recovery phase to a value of 1:461 on day 8. The control group showed a gradual rise in hemagglutinin which peaked on day 4 and fluctuated at a lower level thereafter.

**DISCUSSION**

Hemagglutinins have been previously noted in fowl malaria due to *P. circumflexum* (10), *P. gallinaceum* (4, 17), and *P. lophurae* (4, 7, 13, 14). Only in the last instance, in the studies by Coffin (4) and Schwink (13), has the hemagglutinin been partially characterized on a serological basis. The results presented here more fully described the *P. lophurae*-induced agglutinin in serological and immunological terms.

As expected, "natural" hemagglutinins for heterologous erythrocytes were noted in normal chicken serum. During the malaria infection, however, a hemagglutinin developed which was active upon aged chicken erythrocytes and upon normal and trypsinized human O, Rh-positive erythrocytes. The agglutinin for these three cell types was removed by adsorption with normal human O, Rh-positive cells. On this basis, the use of the enzyme-modified human erythrocytes in later experiments was justified. The higher titer of the agglutinin for these cells was a further advantage.

The thermal requirements of the hemagglutinin presently described indicated that it was a typical cold agglutinin (5, 11). It exhibited its highest titers at 4 C and eluted readily from erythrocytes at 37 C. Moreover, the agglutinin was eliminated early from a Sephadex G-200 column, migrated in the beta-2-globulin region of the serum electrophoretic profile, and lost it serological activity upon reduction with 2-mercaptoethanol. Its appearance early in the course of the disease is a further characteristic of 19S macroglobulins.

Elevated levels of this hemagglutinin were attained in birds which had received prior injections of normal chicken erythrocytes or in birds during the course of blood-induced infections with *P. lophurae*. Preimmunized chicks maintained high levels of the agglutinin only during the period of low parasitemia; agglutinin titers diminished as the parasitemia level increased, probably owing to utilization of the globulin as the parasites were removed. After the removal of the parasite from the circulation, the hemagglutinin titer rebounded and then stabilized.

A serum factor of *P. berghei*-infected rats which agglutinated trypsinized autologous and homologous erythrocytes has been described as a mercaptoethanol-sensitive cold agglutinin (8). In human malaria, increases in 19S macroglobulins (1, 15) have also been recorded. In other protozoan diseases, trypanosomiasis (16), leishmaniasis (16), and anaplasmosis (9), macroglobulin increases with coexistent increases in hemagglutinin have been observed.

The cause for the development of hemagglutinins during malarial infections is conjectural (18). It has been suggested that plasmodia share antigens with erythrocytes and that autologous erythrocytes are antigenically altered by the parasite to become neoantigens. Regardless of the mechanism for their development, these hemagglutinins have been reported to contribute to nonspecific immunity against malaria (2, 18).

It has been suggested that these hemagglutinins may function as opsonins which, since they are more active against altered than normal erythrocytes, promote the specific destruction of parasitized red blood cells (2, 18). The antibody described herein was not active upon normal allogeneic cells and hence would not appear to moderate the course of the disease by masking erythrocytes against plasmodial invasion, although an effect due to other mechanisms, such as nonspecific stimulation of the reticuloendothelial system, may have occurred (2).

**ACKNOWLEDGMENT**

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**LITERATURE CITED**