Innate and Acquired Agglutinins in Ducks to the Malaria Parasite *Plasmodium lophurae*. (18336)

WILLIAM TRAGER,* LESLIE A. STAUBER,† AND SHULAMITE BEN HAREL.‡

From the Laboratories of the Rockefeller Institute for Medical Research and the Zoology Department, Rutgers University.

A large proportion of mature female ducks and a small proportion of mature male ducks are relatively resistant to infection by *Plasmodium lophurae*(1,2). It has been shown that the natural or innate resistance of these adult ducks depends at least in part on the presence in their plasma of materials which exert an anti-plasmodial effect when the plasma is injected into young ducklings infected with *Plasmodium lophurae*(2). When preliminary observations revealed that the plasma of uninfected ducks agglutinated free-parasite suspensions of *P. lophurae*(3) and that the degree of agglutination differed with the individual birds, it seemed possible that the agglutinating power of a plasma might be correlated with its protective power when injected into infected ducklings, and with the innate resistance of the duck from which it was obtained. Detailed experiments have not substantiated this possibility, but have indicated a much more complex situation involving the interplay of innate and acquired agglutinins and of unknown factors.

Materials and methods. All the ducks were obtained as day-old ducklings and were fed commercial duck-pellet feeds. They were reared indoors until they were about 3 months old, when they were placed in an outdoor pen until such time as they were to be used in an experiment. The strain (12A) of *P. lophurae* was maintained by the weekly passage of infected blood in ducklings 2 to 3 weeks old. Experimental ducks received intravenously a dosage of blood (taken from a donor on the sixth day of its infection) sufficient to introduce about 25 million parasites per 100 g of body weight. Infections were followed in the usual manner by means of blood films stained with Giemsa's stain. In the experiments with mature or 3- to 4-month-old ducks, a blood sample was taken for agglutinin assay shortly before the ducks were inoculated with the parasites. Samples were taken again on the fourth or fifth day and on the seventh day of the infection. In one experiment with 1-month-old ducklings a portion of the animals was saved by treatment with quinine and additional samples for agglutinin assay were taken over a period which included relapses in a few of the survivors. The details of this experiment will be considered subsequently with the results which were obtained. Ducks were bled from the right jugular vein with heparin solution at the rate of 0.1 ml per 1 ml of blood as an anticoagulant. The heparin solution contained 27 mg of Connaught heparin in 100 ml of 0.85% sodium chloride solution. A 3 ml sample of blood provided enough plasma for a considerable number of agglutination tests, and the sample was limited to this amount whenever frequent tests were to be made on the same duck. When duck plasma was heated for one-half hour at 55° to 56°C a fine precipitate usually formed. In order to use such heated plasma in the agglutination test it was necessary to remove the precipitate by centrifugation. The antigen for the agglutination tests consisted of dense suspensions of *P. lophurae* largely freed from erythrocyte material by the method of Stauber and Walker(3). The suspensions were stored at 4°C. A single preparation was used throughout the course of an experiment. For use, a small amount of material was diluted with enough buffered saline (pH 7.4) to give an opacity corresponding to that of a 1:10 dilution of full-strength material(3).

* Rockefeller Institute, N. Y.

† Rutgers University, New Brunswick.

‡ Visiting Investigator, Rockefeller Institute, 1949-1950.

1. Trager, W., *J. Parasitol.*, 1948, v34, 389.

2. Trager, W., and McGhee, R. B., *J. Exp. Med.*, 1950, v91, 365.

3. Stauber, L. A., and Walker, H. A., PROC. SOC. EXP. BIOL. AND MED., 1946, v63, 223.

The agglutination tests were prepared and read essentially after the manner of Stauber, Richardson and Walker(4). Appropriate dilutions of each plasma to be tested were made in buffered saline. Two drops of plasma or plasma dilution and one drop of dilute antigen suspension were mixed on a clean glass slide by a rotary movement of the slide. The mixture was then incubated at 37°C in a moist chamber for 10 minutes, stirred again and examined under the microscope with a 16 mm objective. Frequent controls were prepared consisting of buffered saline and antigen. These never showed agglutination, but presented a uniform dispersion of fine particles (the parasites) with occasional gross, somewhat flaky, masses which were present in the antigen suspension and could not be confused with a positive agglutination reaction. In the strongest agglutination reaction, which was read as 4+, large clumped masses were present with very little finely dispersed material. Reactions with progressively smaller clumps and more unclumped material were read as 3+ to +. A ± reaction was one which showed very small but still perfectly definite agglutinated clumps in part of the preparation and much unagglutinated material in the rest of it. If mixtures giving any degree of positive reaction after 10 minutes' incubation were allowed to stand several additional minutes at room temperatures, many of them showed a further intensification of the reaction, but mixtures which were negative at ten minutes did not become positive upon longer standing.

Results and discussion. The agglutination reaction. Plasma from ducks which had not been infected with *P. lophurae* always gave the strongest reaction in the preparation with undiluted plasma. The reaction of different plasmas varied from – to 4+. It was usually 2+ to 3+ in ducks 3 months of age or older and + to 2+ in ducklings one month old or younger. The reaction disappeared rapidly with dilution of the plasma. The plasma of most young ducklings showed a negative reaction at 1:2 dilution (1 part of plasma to 1 part of buffered saline). Among the older ducks even plasma which gave a 4+ reaction in the undiluted state rarely gave any positive reaction at a dilution beyond 1:4. A zone phenomenon was not observed. The agglutinating activity of plasmas from uninfected ducks or ducklings was usually completely removed by heating for one-half hour at 55-56°C followed by centrifugation to remove the fine precipitate which formed. The agglutinating activity also tended to decrease with prolonged storage of the plasma in the refrigerator. The addition of guinea pig serum to a heated plasma which had been active in the unheated state did not restore its activity. Plasmas from ducks undergoing an infection with *P. lophurae* varied in their agglutinin titer even more than plasmas from uninfected ducks. With unheated plasma there might be any one of the following patterns: (1) no positive reaction at any dilution, a type of result observed chiefly with very young ducklings; (2) a strong positive reaction with undiluted plasma which rapidly disappeared upon dilution, as with the plasma of uninfected ducks; (3) a negative reaction with undiluted plasma and with plasma diluted 1:2, followed by a positive reaction at higher dilutions, sometimes up to over 1:1000; (4) a positive reaction with undiluted plasma followed by a zone of weak or negative reactions and then by positive reactions up to a high dilution. The 3 latter types of reactions are illustrated in Table I.

Plasmas obtained from infected ducks and subjected to heating for one-half hour at 55-56°C followed by centrifugation usually gave a negative reaction in the undiluted state. If the unheated plasma gave a reaction pattern of type 1 above, then the heated plasma was negative at higher dilutions as well as in the undiluted state. If the unheated plasma gave a pattern of type 2 the heated plasma might be either entirely negative as in uninfected ducks (Table I, duck 776), or it might be positive in the lower dilutions (Table I, duck 973). If the unheated plasma gave a pattern of type 3 or 4 then the heated plasma usually showed a positive agglutination at 1:2 followed by equally strong or even

4. Stauber, L. A., Richardson, A. P., and Walker, H. A., in press.

TABLE I. Results of Agglutination Tests with Plasma Taken from Ducks on the 7th Day of Infection with *P. lophurae*.

Duck No.	Degree of agglutination at the indicated dilutions of plasma.* (U = Unheated plasma; H = plasma heated ½ hr at 55-56°C and cleared by centrifuging).																	
	Undiluted		1:2		1:4		1:8		1:16		1:32		1:64		1:128		1:256	
	U	H	U	H	U	H	U	H	U	H	U	H	U	H	U	H	U	H
776	3+	—	2+	—	±	—	—	—	—	—	—	—						
778	—	—	—	±	±	+	±	+	±	+	+	+	+	+	±	+	±	±
780	2+	—	2+	—	±	±	+	±	+	±	±	+						
973	3+	—	2+	±	±	+	—	±	—	—	—	—						
974	+	—	±	—	—	±	+	+	±	+	—	+						
975	3+	—	2+	±	±	+	+	2+	+	2+	+	2+	+	2+	+	+	±	±
987	2+	—	+	±	±	±	—	+	—	+	—	±						
988	3+	—	+	±	+	±	±	2+	±	+	+	+						

* Blank space indicates no test made.

stronger reactions at higher dilutions and going out to as great a dilution as that of the unheated plasma (Table I, ducks 778, 975). Sometimes the reaction of heated plasma at the higher dilutions was stronger than that of the same plasma unheated. Heat stable agglutinins were not observed in the plasmas of a series of ducklings inoculated when they were one month old. The positive agglutinations seen with heated plasma differed in character from those with unheated plasma. Heated plasmas have never at any dilution tested given more than a 2+ reaction, and usually they gave a + or ± reaction. This was true even of plasmas which still gave a ± reaction at a dilution of 1:1024. Positive unheated plasmas differed from each other chiefly in the degree of agglutination shown by the undiluted plasma, which ranged from the formation of very small clumps to the formation of very large ones easily seen macroscopically. Positive heated plasmas on the other hand generally formed only small clumps throughout their positive range, and differed from each other chiefly in the extent to which they could be diluted and still show agglutination.

These results may be interpreted in terms of innate heat-labile agglutinins and acquired heat-stable agglutinins. The latter are probably of the nature of the usual antibodies which develop in response to antigenic stimuli. Ducks are generally poor antibody producers (5). Nevertheless the plasma of some mature and 3- to 4-month-old ducks which had been infected with *P. lophurae* showed heat-stable agglutinins for free parasite preparations of this species even at a dilution of 1:1024. Agglutinating antibodies to such preparations of *P. lophurae* have been produced in rabbits by injecting them with infected duck blood(4). The innate agglutinins are possibly similar to the heat-labile components of normal serum which have been observed to react against other infectious agents such as Toxoplasma (6) and some of the viruses(7).

The changes in the heat-labile and heat-stable agglutinins during the course of infection in mature and 3- to 4-month-old ducks. The results of a typical experiment are presented in Table II. In this experiment and 2 others like it totals of 8 mature females, 8 mature males and 8 3- to 4-month-old ducks were used. Of these, 7 females, 1 male and 3 immature ducks developed infections with a peak parasitemia of less than 100 per 1000 red cells, confirming again the relatively greater resistance of the mature females(1,2). There was no correlation between the strength of the agglutination reaction exhibited by the plasma before inoculation and the subsequent course of the infection. Except in some very light infections (Table II, duck 990) the reaction given by the unheated, undiluted plasma decreased as the infection progressed. Ducks which showed this change to a marked

5. Wolfe, H. R., and Dilks, E., *J. Immunol.*, 1949, v61, 251.

6. Sabin, A. B., and Feldman, H. A., *Science*, 1948, v108, 660.

7. Ginsberg, H. S., and Horsfall, F. L., Jr., *J. Exp. Med.*, 1949, v90, 475.

TABLE II. Course of the Parasitemia and Changes in the Heat-Labile (U) and Heat-Stable (H) Agglutinins in Ducks Infected with *P. lophurae*.

Duck			Parasites per 1000 red cells on days after inoculation						Degree of agglutination (— to 4+) given by undiluted, unheated plasma (U), and highest dilution of heated plasma giving a positive agglutination (H) on days after inoculation					
									0		5		7	
No.	Sex	Age, mo.	4	5	6	7	8	9	(U)	(H)	(U)	(H)	(U)	(H)
744	♀	7	30	11	0.1	0	0	0	+	0	2+	1:64*	+	1:64*
747	♂	7	122	468	784	1592	D		3+	0	+	1:4	—	1:2
751	♀	7	26	66	368	546	1125	1340	2+	0	+	1:8	—	1:4
781	♀	3	160	380	212	484	434	252	3+	0	+	1:64*	—	1:64*
784	♂	3	44	74	66	2	4	5	2+	0	3+	1:64*	+	1:64*
785	♀	3	116	586	780	1535	D		3+	0	2+	0	+	1:4
957	♂	13	76	201	112	344	310	406	3+	0	—	1:64*	±	1:64*
990	♀	12	4	2	0.2	0	0	0	3+	1:4	3+	1:32	3+	1:64*

D denotes death. * Highest dilution tested.

degree and which, in addition, developed only a low titer of heat-stable agglutinins, also showed the most severe infections (Table II, ducks 747, 751, 785). A few of the resistant ducks maintained a high level of heat-labile agglutinins and developed no heat-stable agglutinins, but most of them also acquired the heat-stable agglutinins. In moderately susceptible ducks, the heat-labile agglutinins decreased and heat-stable agglutinins appeared in relatively high titer (Table II, ducks 781, 957). The decrease in heat-labile agglutinins which occurs in the more susceptible older ducks and in all young ducklings could as well be a result rather than a cause of the concomitant high parasitemia. It is noteworthy that complement similarly undergoes a marked decrease in titer during the course of infection of chicks and ducklings with *P. lophurae*(8,9).

Changes in the heat-labile agglutinins of ducklings during infection with P. lophurae *and during latency and relapse.* A number of observations with young ducklings indicated that their heat-labile agglutinins disappeared completely at the peak of infection and that they did not develop heat-stable agglutinins. The following more detailed experiment confirmed these observations and provided some additional information concerning a possible relation of the agglutinins to relapse. A uniform batch of 24 ducklings was used. When these were 25 days old a blood sample was taken from each for determination of the agglutinins. Two days later 19 of the ducklings were inoculated with *P. lophurae*. Blood films from these ducklings were prepared daily from the second to the 8th day after inoculation, and at 3- to 4-day or sometimes shorter intervals thereafter. Twelve of the inoculated ducklings were treated with quinine on the 5th, 6th and 7th days of their infection. Each treated duckling received by stomach tube enough of a 5% solution of quinine dihydrochloride to supply it with 10 mg of the drug per 100 g of body weight. Blood samples for agglutinin determinations were taken from all the ducklings (including the 5 uninfected ones) on days 2, 4 and 7 after the day of inoculation, and from the survivors on days 19, 28, 40, 49 and 56.

Although all the plasmas were tested at several dilutions for heat-stable agglutinins, none were observed. Positive reactions with unheated plasma were rare at dilutions beyond 1:2. Hence it will be sufficient to consider only the degree of agglutination with the unheated, undiluted plasma. Among the 5 uninfected ducks, 2 gave a + and 3 a 2+ reaction in the first sample taken at 25 days of age. Most of the later samples were + or 2+. In the last samples taken when the ducklings were 83 days old, 3 showed a 2+ and 2 a 3+ reaction. Among the 7 ducks which were infected and not treated with

8. Trager, W., *J. Exp. Med.*, 1947, v85, 663.
9. McGhee, R. B., unpublished results.

TABLE III. (Changes in Heat-Labile Agglutinins of Ducklings During Infection with *P. lophurae* and During Latency and Relapse. Ducklings inoculated when 27 days old. On the 5th to 7th days of infection each duckling received by stomach tube quinine dihydrochloride (as a 5% sol.) 10 mg per 100 g of body wt. See text for results with uninfected control ducklings and with infected ducklings not treated with quinine.

No.	Parasites per 1000 red cells on days after inoculation													Degree of agglutination by undiluted plasma on days after inoculation								
	6	7	8	11	14	19	25	28	32	37	40	42	46	49	56	−2	2	4	7	19	28	40	49	56
129	240	260	225	3	0.5	0	0	0.2	0.6	45	436	11801D				3+	3+	3+	+	4+	2+	±		
130	498	370	332	27	0	0.5	0.1	0.1	0.8	0	0	0	0	0	0	±	2+	2+	—	3+	3+	3+	3+	3+
135	254	196	168	29	7	1220D										++	3+	3+	++	—	—			
136	440	478	486	12	0	0	0	0	0	0.1	0.1	0	0	0	0.1	+	3+	2+	++	3+	2+	3+	3+	3+
137	404	388	186	0	0.5	4	0	0	0	69	1055	D				±	2+	+	+	—	—	—		
138	220	124	5	0	0	0.1	0	0	0.2	0.3	1	2	12	0.7	0.1	±	2+	3+	2+	+	+	±	2+	2+

D denotes death on or just after indicated day. See text.

quinine, four had a +, two a 2+ and one a 3+ reaction in the initial samples taken 2 days before inoculation. Two days after inoculation all 7 had a 2+ reaction and this was essentially the case 4 days after inoculation, but on the 7th day all were entirely negative. The ducks died on the 8th day. Among the 12 infected ducks treated with quinine 4 died on the 9th day and 2 others a few days later, in spite of some reduction in parasitemia effected by the quinine. The course of events in the 6 ducks which survived to be sampled on the 19th day after inoculation is shown in Table III. All of these ducks showed a drop in the agglutination reaction on the 7th day. In 2 (ducks 130, 136) this drop was followed by an increase which persisted in subsequent samples, and few or no parasites were found in the blood films. A 3rd duck (129) exhibited an increased reaction on the 19th day followed by a decrease to ± on the 40th day. The decrease was accompanied by a severe relapse which ended in death on the 43rd day. In duck 138, a slowly decreasing reaction was accompanied by a slowly increasing but low parasitemia which reached a peak around the 46th day and then fell off, while the agglutinin reaction rose to 2+. Duck 135 with a negative reaction on the 19th day was then at the peak of a relapse and died on the following day. The 6th survivor (137) had a negative reaction on the 19th day and also in the 2 subsequent samples. It developed a severe relapse and died on the 42nd day. The 28-day sample of blood from this bird contained more than half as great a volume of white blood cells as of red cells. These white cells appeared to be hemocytoblasts and monocytes. They were present in smaller numbers at 32 days, just before the malarial relapse began. Subsequent samples, taken during the relapse, presented only the usual blood picture.

Summary. Erythrocyte-free suspensions of *Plasmodium lophurae* were agglutinated to a variable degree by the fresh plasma of ducks which had not been infected by this parasite. The agglutinating activity of the plasma was in general higher in older ducks than in young

ducklings. It was rarely evident beyond a 1:4 dilution of the plasma, and was fully removed by heating the plasma for one-half hour at 55-56°C followed by centrifugation to remove the precipitate which formed. In infected ducks 3 months of age or older these heat-labile agglutinins generally decreased as the infection progressed, except in individuals which exhibited a strong innate resistance to the parasites. The heat-labile agglutinins disappeared completely at the peak of a severe infection. Heat-stable agglutinins appeared in the plasma of most of the infected birds. These, unlike the heat-labile agglutinins, were not demonstrable in undiluted plasma but often were active in plasma diluted as far as 1:1000. In ducklings infected when they were one month old or younger, heat-stable agglutinins did not appear. As in severely infected older ducks, the heat-labile agglutinins vanished at the peak of the infection. If such ducklings were saved from death by treatment with quinine the heat-labile agglutinins reappeared to a varying degree. Relapses, like initial infections, were accompanied by a lack of heat-labile agglutinins.

Received October 18, 1950. P.S.E.B.M., 1950, v75.

Experimental Studies on Pathogenesis of Hemoglobinuric Nephrosis. (18337)

VICTOR PARSONNET, J. STUART FISHLER, AND WILLIAM THALHIMER.
From the Division of Pathology, Grasslands Hospital, and the Department of Laboratories and Research of Westchester County, Valhalla, N. Y.

The exact mechanism of the production of the renal lesion following the transfusion of incompatible blood is as yet unclear. It has become apparent that no one factor is responsible, but probably some combination of hemoglobin excretion, the pH of the urine, shock (renal anoxia), and the incompatible reaction *per se* is involved. Many factors have been investigated in the production of hemoglobinuric nephrosis. Among these factors are the "toxicity" of hemoglobin(1-11), the chemical form of the excreted hemglobin (7,12-14), the pH of the urine(1,6,13,14), dehydration(8,9,11,14,15), renal anoxia(16, 17), hemorrhagic shock(2,5), subcortical renal vascular shunt(18), and the incompatible transfusion reaction *per se*(19-21). It has been suggested(22,23,25) that hemo-

1. De Navasquez, S., *J. Path. and Bact.*, 1940, v51, 413.
2. Lamson, P. D., Robbins, B. H., and Grieg, M. E., *J. Pharmacol. and Exp. Therap.*, 1945, v83, 225.
3. Ottenberg, R., and Fox, C. L., Jr., *Am. J. Physiol.*, 1938, v123, 516.
4. Bayliss, W. M., *Brit. J. Exp. Path.*, 1920, v1, 1.
5. Hamilton, P. B., Hiller, A., and Van Slyke, D. D., *J. Exp. Med.*, 1947, v86, 477.
6. Degowin, E., Warner, E. D., and Randall, W. L., *Arch. Int. Med.*, 1938, v61, 609.
7. Harrison, H. E., Bunting, H., Ordway, N. K., and Albrink, W. S., *J. Exp. Med.*, 1947, v86, 339.
8. Yorke, W., and Nauss, R. W., *Ann. Trop. Med.*, 1911, v5, 287.
9. Maluf, N. S. R., *Ann. Surg.*, 1949, v180, 49.
10. Ayer, G. D., and Gauld, A. G., *Arch. Path.*, 1942, v33, 513.
11. Lalich, J. J., *J. Exp. Med.*, 1948, v87, 157.
12. Anderson, W. A. D., Morrison, D. B., and Williams, E. F., *Arch. Path.*, 1942, v33, 589.
13. Bing, R. J., *Bull. Johns Hopkins Hosp.*, 1944, v74, 161.
14. Baker, S. L., and Dodds, E. C., *Brit. J. Exp. Path.*, 1925, v6, 247.
15. Lalich, J. J., *J. Exp. Med.*, 1947, v86, 153.
16. Koletsky, S., and Gustafson, G. E., *J. Clin. Invest.*, 1947, v26, 1072.
17. Van Slyke, D. D., *Ann. Int. Med.*, 1948, v28, 701.
18. Trueta, J., Barclay, A. E., Daniel, P. M., Franklin, K. J., and Prichard, M. L., Blackwell Scientific Publications, Oxford, England, 1947.
19. Ottenberg, R., Kaliski, D. J., and Friedman, S. S., *J. Med. Res.*, 1913, v28, 141.