THE AGGLUTINATION OF PLASMODIUM KNOWLESI BY IMMUNE SERUM

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PLATES 37 AND 38

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The demonstration of passive immunity in experimental monkey malaria by Coggeshall and Kumm (1, 2) indicates that immune substances are present in the sera of animals with chronic malarial infections and of animals that have been hyperimmunized by superinfection. The nature and mode of action of the protective substances is at present unknown. The existence of precipitins and complement-fixing antibodies in human malaria has been reported, but up to the present time the specific sensitization and agglutination of malarial parasites by immune serum has not been demonstrated. This paper will describe and discuss the significance of a specific agglutination of *Plasmodium knowlesi* by immune serum from monkeys. The immune body concerned in this agglutination apparently has a general similarity to antibacterial and other antibodies.

Using antigens prepared from parasitized red blood cells or from malarial spleens, Thomson (3), Kingsbury (4), and others have obtained fixation of complement by sera from cases of benign and malignant tertian malaria. Generally the reactions were rather weak and pseudopositive reactions with syphilitic sera were observed. Precipitin tests for malaria have been described by Pewny (5), Taliaferro, Taliaferro, and Fisher (6), and Row (7). Taliaferro obtained a large number of positive reactions with malarial sera using an antigen prepared from placentas infected with *Plasmodium falciparum*, but this author was unable to repeat the results later with different antigens and sera (8). Of a different nature is the non-specific seroflocculation reaction in malaria described by Henry (9). The character of the antigen (melanin) and of the reactive substances in the malarial sera excludes the possibility that the Henry reaction is a true precipitation of antigen by antibody (10).

In avian malaria the serum of the bird acquires the ability to reduce the electric

charge of both parasitized and unparasitized red cells. Brown (11) has reported that the reduction in the charge on the red cells is related to the degree of resistance to the infection. This implies a non-specific sensitization of all the red cells, but no agglutination was observed. Malamos (12) observed in the blood of a monkey infected with *P. knowlesi* and treated with atebrin an agglomeration of red cells which affected, during the early stages of the disease, only the parasitized cells. Later an agglomeration of all of the red cells occurred and the animal died, despite the fact that the parasite count had been greatly reduced. The phenomenon was observed in only one of 200 animals.

Agglutination of leishmania and trypanosomes by sera from animals or human beings chronically infected with these organisms has been demonstrated repeatedly. In certain instances auto-agglutination of red cells by these sera was also observed. Leishmania and trypanosomes are agglutinated by normal sera in dilutions lower than the dilutions of immune sera required for agglutination. A summary of the work on serological reactions in various parasitic infections will be found in the book by Taliaferro (8).

Materials

Antigen.—The blood of *rhesus* monkeys dying from acute infection with *P. knowlesi* (1) was collected in citrate by bleeding the animals from the heart. In most cases the parasite count of these animals is between 2,000 and 5,000 per 10,000 red cells. Blood with a lower parasite count is generally not suitable for the preparation of antigen because of the difficulty of obtaining a sufficient proportion of mature parasites. The serum is separated from the cells by centrifugation. The blood is resuspended in saline and centrifuged at low speed, or the cells are allowed to settle by gravity in a cylinder. An upper brown layer containing parasitized cells and leucocytes may then be separated from the unparasitized cells which settle more rapidly. With blood which has stood in the ice box for more than a few hours or with old antigens it is advisable to filter the suspension through cotton and use the settling method to remove pieces of clot or clumps of cells.

The cells to be used for antigen are suspended in saline so that an opacity approximately equivalent to that of a 0.5 per cent suspension of red cells is obtained. The resulting suspension should contain 50 per cent or over of cells with mature malarial parasites. It should be free of clumps as determined by macroscopic and microscopic examination, and it should not contain a large excess of white cells. The leucocytes tend to agglutinate spontaneously and this may be confused with agglutination of the parasitized cells. The antigen suspension should be definitely brown. If the color is predominantly red, too many normal cells and immature parasites are present.

Sera.—Animals were bled from the femoral vein and the serum obtained in the usual manner. Except in the experiment with heated and unheated serum, all sera used for agglutination were heated at 56°C. for one-half hour. Sera were collected from twenty normal monkeys, from five monkeys during the acute stages

of the infection, from ten monkeys a few weeks after the acute infection had subsided, from five monkeys with chronic malaria, and from ten superinfected monkeys. The sera from chronically infected and superinfected monkeys were the same as those used in the experiments of Coggeshall and Kumm (2). The animals were superinfected by nine or more injections of 5 cc. each of heavily parasitized blood given at intervals of 7 to 10 days. Sera from five monkeys having chronic infections with *P. inui* of various durations were also tested for agglutination of *P. knowlesi*.

Method of Performing the Agglutination Test

Macroscopic Agglutination.—Diluted or undiluted serum in amounts of 0.2 cc. is pipetted into small tubes 0.8 cm. in diameter and 9.0 cm. long. The suspension of parasitized cells (0.3 cc.) is then mixed with the serum and the tubes allowed to stand at room temperature for 2 hours. Occasional gentle agitation during the first 15 minutes facilitates the agglutination. The agglutinated parasitized cells appear as small granules at the bottom of the tube. In the negative controls the red cells should settle compactly to the bottom of the tube and no granules should be visible. With long standing the granules of agglutinated cells may be obscured by unagglutinated cells which have settled around them. After examination of the bottoms of the tubes for granules the cells are resuspended by gentle shaking and the presence or absence of visible granules or floccules in suspension is noted.

Microscopic Agglutination.—A drop of the mixture of parasitized cells and serum is removed from the tube and examined on a microscope slide under a cover slip with the high power objective. In a positive test clumps of parasitized cells containing pigment granules are visible but there should be no clumping of unparasitized cells. If the reaction is non-specific clumps of both parasitized and unparasitized cells will be seen. Normal serum should give no microscopically visible agglutination.

The hanging drop technique may also be used for performing the microscopic agglutination test. A drop of diluted or undiluted serum is mixed with a drop of the suspension of parasites on a cover slip, then inverted over a hollow ground slide and the preparation sealed with paraffin oil. With strongly positive sera agglutination of the parasitized cells is visible in 15 to 30 minutes.

The macroscopic method just described will detect agglutinins in all but a few of the weakest sera when a good antigen is obtainable. Unless the antigen contains over 50 per cent of mature parasites it may be difficult to see the agglutinated clumps with the naked eye because they are obscured by unparasitized cells which do not agglutinate. With the microscopic technique agglutination may be seen in a suspension containing as little as 5 to 10 per cent of parasitized cells. Nonspecific agglutination of red cells and spontaneous clumping of red or white cells can best be distinguished from agglutination of the parasitized cells by examination under the microscope.

Agglutination Titers of the Sera of Immune Monkeys

The sera of twelve monkeys with chronic infections of various durations were tested for agglutination at dilutions up to 1:4,096. Eight of these monkeys had been superinfected repeatedly and their sera, in

TABLE I

Agglutination of Plasmodium knowlesi by Immune Serum from Chronically Infected and Superinfected Monkeys. Titer of Agglutinins

Monkey No.	Infection and duration		Dilution of serum					
			1:4	1:16	1:64	1:256	1:1,024	1:4,096
		mos.						
B2	Chronic,	5	++	++	+	+	±	−
B9	"	2	++	+	+	−		
3-9	"	5	++	+	±	−		
4-0	"	5	+	±	−	−		
5-4	"	2	±	−	−	−		
9	Superinfected,	9	+++	++	++	+	±	−
1-2	"	8	+++	++	+	±	−	−
8	"	11	++	+	+	+	±	−
3	"	9	+++	++	++	+	±	−
4	"	10	++	+	+	±	−	−
7	"	10	++	+	+	−	−	−
2	"	13	++	+	+	−	−	−

Controls: 20 normal sera tested undiluted against the antigen used in these tests gave no agglutination

+++, strong macroscopic agglutination, large clumps of parasitized cells visible in suspension.

++, moderate macroscopic agglutination, clumps definitely visible in suspension.

+, small clumps barely visible in suspension, but definitely visible as granular sediment in the bottom of the tube and under the microscope.

±, agglutination detectable only as slight granular sediment in the bottom of the tube and as small clumps of parasitized cells under the microscope.

−, no agglutination detectable microscopically.

general, possessed stronger protective properties than the sera of the five animals which had not been superinfected (1, 2). The results presented in Table I indicate that the sera of the group of superinfected monkeys agglutinated the parasites at higher dilutions than the sera

of the group of chronic monkeys which had not been superinfected. Four of the sera, three being from superinfected animals, gave definite microscopic agglutination at a dilution of 1:1,024. All the sera from superinfected animals and two of the five sera from chronic infections gave macroscopic agglutination at a dilution of 1:64. In the second column of the table the duration of the infection in months is given. It will be noted that the serum of monkey B2, which had an infection of 5 months' duration without superinfection, gave as high an agglutination titer as the sera of the superinfected animals. Four other monkeys in this group had infections of shorter duration than the superinfected animals and their sera agglutinated at considerably lower titers. Further work with a larger series of monkeys will be necessary before the relationship of superinfections and duration of infection to the strength of agglutination can be definitely determined.

Development of Agglutinins in the Sera of Monkeys during Recovery from Acute Infection

Agglutination of *P. knowlesi* by the sera of normal monkeys taken before infection has not been detected. The sera of five monkeys taken at the height of the acute infection when the parasite count was over 1,000 per 10,000 red cells also gave no agglutination. The blood of these animals had contained demonstrable parasites for 5 to 7 days. After the fall in the parasite count which follows treatment with quinine or immune serum, the sera of most of the monkeys so far tested have shown agglutination.

Table II shows the rise in the titer of agglutinins in the serum of a monkey treated with quinine. At 15 days, after recovery from the acute infection, weak microscopic agglutination was detected. 2 weeks later the agglutination was stronger and during a series of relapses in the next 2 months the titer of agglutinins definitely increased. After 3 months the animal no longer had relapses with high parasite counts and the agglutination titer had reached a relatively high level.

The sera of eight monkeys which had been treated by daily injections of 2 cc. of immune sera for 10 days after the injection of parasites were also tested for agglutination. In this case the presence of passive immunity makes the detection of agglutinins in the early stages of the disease less significant because the agglutination may be due to the

injected immune serum. The results presented in Table III show that five of the eight sera agglutinated the parasites 23 and 33 days after infection. At 44 and 57 days all of the sera gave positive

TABLE II

Development of Agglutinins during Recovery from Acute Infection with Plasmodium knowlesi by a Monkey Treated with Quinine

Time after infection	Dilution of serum				
	Undiluted	1:4	1:16	1:64	1:256
days					
0 (quinine)	−	−	−	−	−
15	±	−	−	−	−
29	+	±	−	−	−
45	++	++	±	−	−
55	++	++	±	−	−
67	++	+	−	−	−
77	++	+	−	−	−
88	++	++	+	±	−
101	++	++	+	±	−

TABLE III

Agglutination of Plasmodium knowlesi by the Sera of Monkeys Recovering from Acute Infection after Treatment with Immune Serum

Monkey No.	Serum	Days after infection			
		23	33	44	57
5-6	Undiluted	−	−	+	
5-8	"	±	±	+	±
5-9	"	+	+	±	+
6-0	"	−	−	±	±
6-1	"	−	−	+	+
6-2	"	+	+	+	+
6-5	"	+	+	±	±
6-6	"	±	+	±	±

agglutination. In addition to these, the sera of two monkeys which recovered from the disease without treatment agglutinated parasites after infections of 18 and 44 days, respectively. These animals repre-

sent an exception to the general rule that untreated infection with *P. knowlesi* is fatal to *rhesus* monkeys.

Tests for Cross-Agglutination of Plasmodium knowlesi by the Sera of Monkeys Chronically Infected with Plasmodium inui

In order to determine whether a chronic infection with another species of malarial parasite causes the production of agglutinins for *P. knowlesi*, sera from monkeys with chronic infections with *P. inui* were tested against the *P. knowlesi* antigen. The duration of infection in the five monkeys tested was 2, 3, 8, 11, and 13 months, respectively. None of the sera gave any agglutination detectable by microscopic examination. Experiments using an antigen prepared from *P. inui* have not been done because the low parasite count in monkeys infected with this species makes the preparation of a satisfactory antigen difficult.

Individual Variations in the Sensitivity of Antigens

Because of the lability of the antigen used in the agglutination tests described in the previous sections of this paper, it has not yet been possible to prepare standardized antigens of uniform sensitivity. Differences in the sensitivity of various preparations of antigen may be attributed to aging, partial clotting of the blood, percentage of parasitized cells, stage of development of the parasites, and possibly individual differences in the cells of different *rhesus* monkeys. With sera which give weak positive reactions these variations may be great enough to determine whether the reaction is positive or negative, but with definitely positive immune sera all the antigens tested have given positive results. In Table IV are presented the results of agglutination of eight separate antigens by ten immune sera. Weak sera, such as Nos. 2, 7, 1-4, and 3-9, give moderate agglutination with all the antigens tested, while relatively stronger sera give corresponding uniformly strong reactions. Antigens D and G may be considered relatively insensitive while antigens C, E, and F are quite sensitive and give strong agglutination with most of the sera. This table also shows that the agglutination of the parasitized cells is apparently independent of the possible existence of blood groups or other individual differences in the blood of monkeys.

Occasional Agglutination of Normal Red Cells by Normal and Immune Sera

During the experiments on the agglutination of the parasitized red cells the positive sera were examined repeatedly for agglutination of normal red cells. This was done both by mixing the sera with the cells of normal monkeys and examining for macroscopic agglutination,

TABLE IV

Agglutination of Separate Preparations of Antigen from Eight Monkeys Dying of Acute Infection with Plasmodium knowlesi

Serum No.	Antigens							
	A	B	C	D	E	F	G	H
Immune sera undiluted								
2	x	x	x	+	++	++	x	x
3	+++	+++	x	++	+++	++	++	++
4	+++	x	+++	++	++	+++	+	x
5	++	++	++++	x	x	x	x	+++
7	+	x	x	x	++	++	x	+
8	++	x	+++	x	++	++	++	x
9	+++	++	x	x	+++	+++	++	++
1-2	++	+++	x	x	++	+++	+	++
1-4	+	x	++	x	x	x	x	x
3-9	x	x	x	+	+	++	x	++
Normal sera undiluted								
N1	−	x	−	x	−	−	−	−
N2	−	−	−	x	x	x	x	x
N3	x	−	x	−	−	x	x	x
N4	x	−	x	x	−	−	x	x

x, not done.

and by examining mixtures of parasitized blood and immune serum under the microscope for agglutination of unparasitized cells. One of the immune sera not included in the preceding tables regularly gave macroscopic agglutination of normal cells from three of four other monkeys. A few other sera, both normal and immune, occasionally gave slight clumping of normal red cells visible under the microscope. This suggests the possible existence of iso-agglutinins

in the blood of *rhesus* monkeys although these have not been found by other investigators (13, 14).

Characteristics of the Material Which Agglutinates

The suspension of parasitized cells used in the agglutination test rapidly deteriorates on standing in the ice box. Spontaneous hemolysis which affects first the parasitized cells occurs after 24 hours. The parasites liberated by hemolysis tend to coalesce into a mass which is not readily dispersed. Attempts to preserve the suspensions in solutions of 25 per cent glycerol, 12 per cent dextrose, or 1 per cent formalin have been unsuccessful. Formalin fixes the parasitized cells but destroys their ability to agglutinate after 24 hours in the ice box. When kept in 25 per cent normal monkey serum the parasitized cells remain agglutinable for as long as 6 days but the sensitivity of the antigen is considerably less than that of a fresh suspension.

The possibility that the agglutination is due to a soluble and readily diffusible antigen on the surface of the red cells was considered. If this were the case, washing should remove the antigen and make the parasitized cells inagglutinable. However, washing the parasitized cells four times with saline slightly diminishes but does not abolish their agglutinating ability.

As may be seen in Figs. 1 and 2, the cells containing the small rings do not agglutinate as readily as the red cells with mature parasites. This indicates that the antigen does not become accessible to the antibody until the parasites have grown to such a size that they fill most of the red cell. In the clumps of agglutinated cells a clear margin around each parasite is generally visible (Fig. 2). This shows that the membrane of the red cell has not been broken. Possibly the growth of the parasite inside the red cell finally damages the cell membrane to such an extent that it becomes permeable to antigen, antibody, or both. The increased fragility of the parasitized red cells, as compared with the unparasitized cells in the same sample of blood, lends support to this view.

Parasites liberated from the red cells by spontaneous hemolysis agglutinate in much the same way that they do when inside of the red cell.

A suspension of "free" parasites was obtained from a sample of heavily parasitized citrated whole blood which had stood overnight in the ice box. Many of the cells containing mature parasites had gone to pieces but there was very little hemolysis of the unparasitized cells. By centrifuging, the free parasites were readily separated in an upper layer, washed once in saline, and resuspended in normal serum. In a smear of this suspension stained by Giemsa it was seen that some of the mature segmenters stained a deep blue, a normal staining reaction of live malarial parasites, but many of the parasites were stained a lighter shade of lavender (Fig. 3). These latter were probably damaged or dead parasites.

The suspension of free parasites was completely agglutinated to medium sized clumps by immune sera but this antigen was not more sensitive than an antigen prepared from unhemolyzed parasitized

TABLE V

Effect of Heat on the Agglutinins in Immune Monkey Serum

Serum No.	Temperature and time of heating	Dilution of serum and agglutination of *P. knowlesi*				
		1:4	1:16	1:64	1:256	1:1,024
B2	Not heated	++	++	+	±	−
"	56°C. for 60 min.	++	+	+	±	−
"	65°C. for 30 min.	+	+	±	−	−
"	75°C. for 30 min.	±	−	−	−	−
3	Not heated	++	++	+	+	±
"	56°C. for 60 min.	++	++	+	+	±
"	65°C. for 30 min.	+	+	±	±	−
"	75°C. for 30 min.	−	−	−	−	−

cells. On staining the agglutinated free parasites it was seen that both the lavender and the blue staining parasites had agglutinated (Fig. 4). This indicates that it is not essential for the parasites to be alive in order to agglutinate. The material which agglutinates apparently has a staining reaction similar to the nuclear material of the parasites.

Effect of Heat on the Agglutinin for Plasmodium knowlesi

The immune body concerned in the agglutination of *P. knowlesi* resembles other antibodies in its behavior to heat. The results presented in Table V show that the agglutinin is not appreciably affected by heating at a temperature of 56°C. for 1 hour, is partly

inactivated at 65° for 30 minutes, and is almost completely inactivated by a temperature of 75° for 30 minutes.

DISCUSSION

The specific agglutination of *Plasmodium knowlesi* by immune serum suggests that a specific sensitization of the parasites may occur *in vivo* and this renders them more susceptible to phagocytosis by macrophages of the spleen and by other phagocytic cells. The identity of sensitizing and agglutinating antibodies for bacteria has been clearly demonstrated. Since some of the immune sera agglutinate the parasites at dilutions as high as 1:1,000 it is probable that as little as 1 cc. of these sera injected into an infected animal could produce a sensitization of the parasites in the blood stream. The appearance of agglutinins in the sera of monkeys after the recovery from the acute phase of the infection with *Plasmodium knowlesi* also suggests that the relative immunity of these animals is associated with the presence of sensitizing antibodies in the blood stream. The data presented in this paper are not considered to be extensive enough to demonstrate conclusively a relationship between agglutinins and passive and active immunity to malaria but further investigations along these lines will be carried out.

Findlay and Brown (15) have shown that during an attack of avian malaria the degree of non-specific sensitization of parasitized and unparasitized red cells by serum *in vitro* (as measured by the amount of reduction of the electric charge on the red cells) is correlated with the size of the spleen and with the rate of phagocytosis of infected red cells by the macrophages of the spleen. This non-specific sensitization, which is apparently due to an increase in the euglobulin fraction of the serum (11), is not to be confused with the specific sensitization and agglutination observed in monkey malaria. Since agglutination of normal red cells by the serum of one monkey with chronic malaria has been observed, it is possible that non-specific factors do play a part in the agglutination and phagocytosis of parasitized cells, but in monkey malaria these non-specific effects are not as prominent as the specific agglutination.

That specific factors do exist in other forms of malaria, including that of birds, is indicated by the fact that tolerance of one species

of malarial parasite does not confer protection against another species. As Findlay and Brown have pointed out, if the serum plays a part in this specific protective mechanism, the reaction between parasite and serum as a surface phenomenon can probably occur only during the short extracellular stage of the parasite. This is borne out by the observation that the red cells containing the immature ring forms of *P. knowlesi* do not agglutinate. On the other hand, the segmenters which are undoubtedly intracellular do agglutinate despite the apparent intervention between the parasite and the antibody of the membrane and cytoplasm of the red cell. Increased permeability of the membrane has been offered as a possible explanation for this. In view of this observation it seems likely that the parasites may be susceptible to the immune effects of the serum over a longer period of their life cycle than was assumed by Findlay and Brown.

At the present stage of the work the value of the agglutination test as a diagnostic measure in malaria is diminished by the difficulties of preparing a suitable antigen. The sera of a number of human cases of paresis which had been treated by infection with monkey malaria were tested against an antigen prepared from the cells of an infected monkey. In no case was it possible to differentiate between non-specific agglutination of all the red cells, due to hetero-agglutinins in the human sera, and specific agglutination of the parasitized cells. Suitable material for the preparation of antigen from human blood infected with tertian, sub-tertian, or quartan malaria has not been available.

SUMMARY

A specific agglutination of *Plasmodium knowlesi* detectable both by macroscopic and by microscopic methods is described.

Agglutinins for *Plasmodium knowlesi* appear in the sera of monkeys between 15 and 45 days after the onset of the infection and become progressively stronger as the malarial infection gradually subsides.

Agglutinins persist in the sera of chronically infected animals for a year or longer. The sera of animals which have been repeatedly superinfected agglutinate parasites at dilutions as high as 1:1,000.

Sera from normal monkeys, from monkeys acutely ill with malaria, and from monkeys chronically infected with a different species of

malarial parasite (*Plasmodium inui*) do not agglutinate *Plasmodium knowlesi*.

Immune serum agglutinates mature intracellular or extracellular parasites but does not agglutinate unparasitized cells or cells containing immature parasites.

The relation of these observations to the mechanism of active and passive immunity in monkey malaria is discussed.

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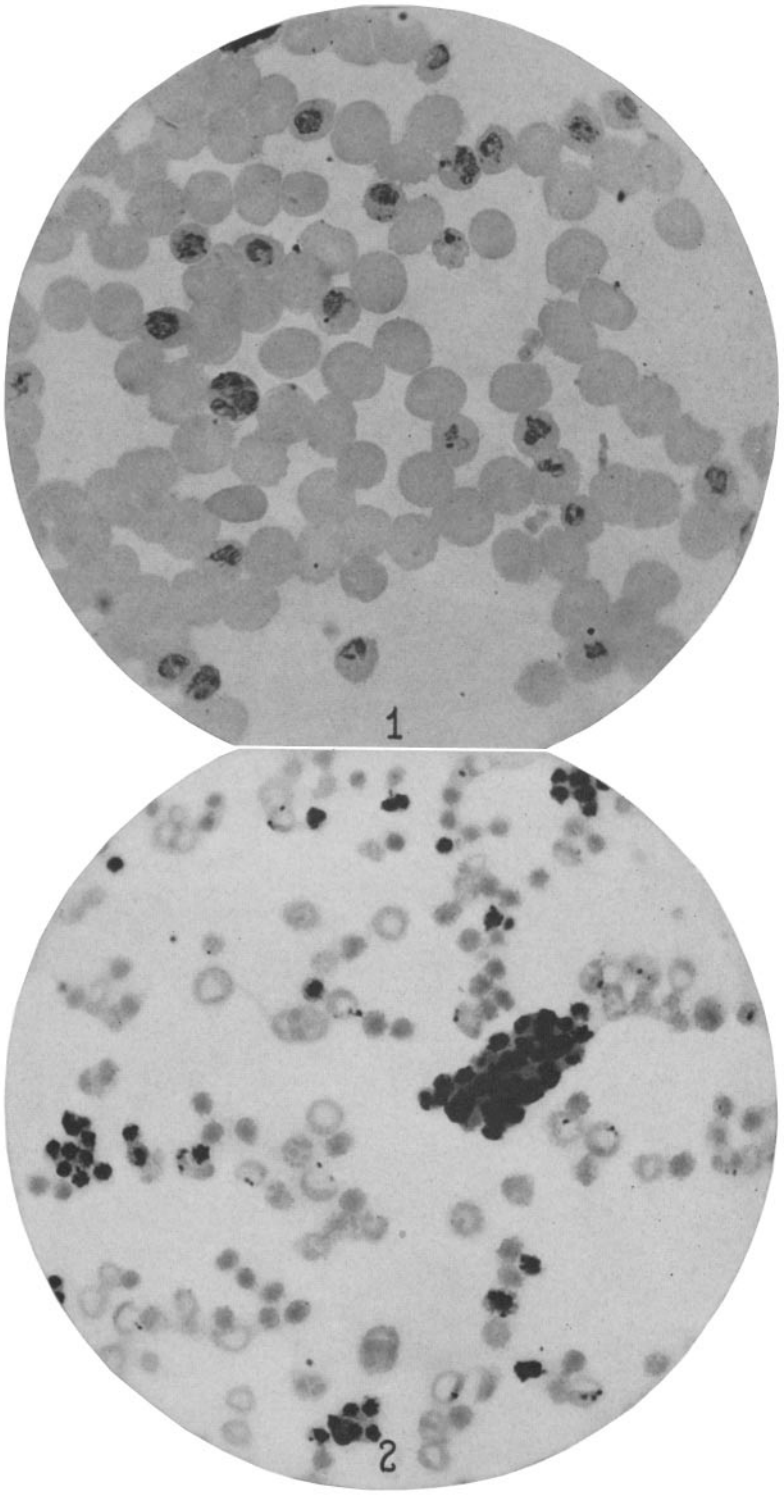
EXPLANATION OF PLATES

PLATE 37

FIG. 1. Red cells containing *P. knowlesi* at various stages of development, showing absence of agglutination in normal monkey serum. × 820.

FIG. 2. Agglutination by immune serum of red cells containing mature forms of *P. knowlesi*. In the clumps of parasitized cells a lightly stained zone around each parasite represents the remaining cytoplasm and membrane of the red cell. Cells containing immature parasites, visible in the photograph as small dots, are not agglutinated. The clumps of parasitized cells include no unparasitized red cells. × 820.



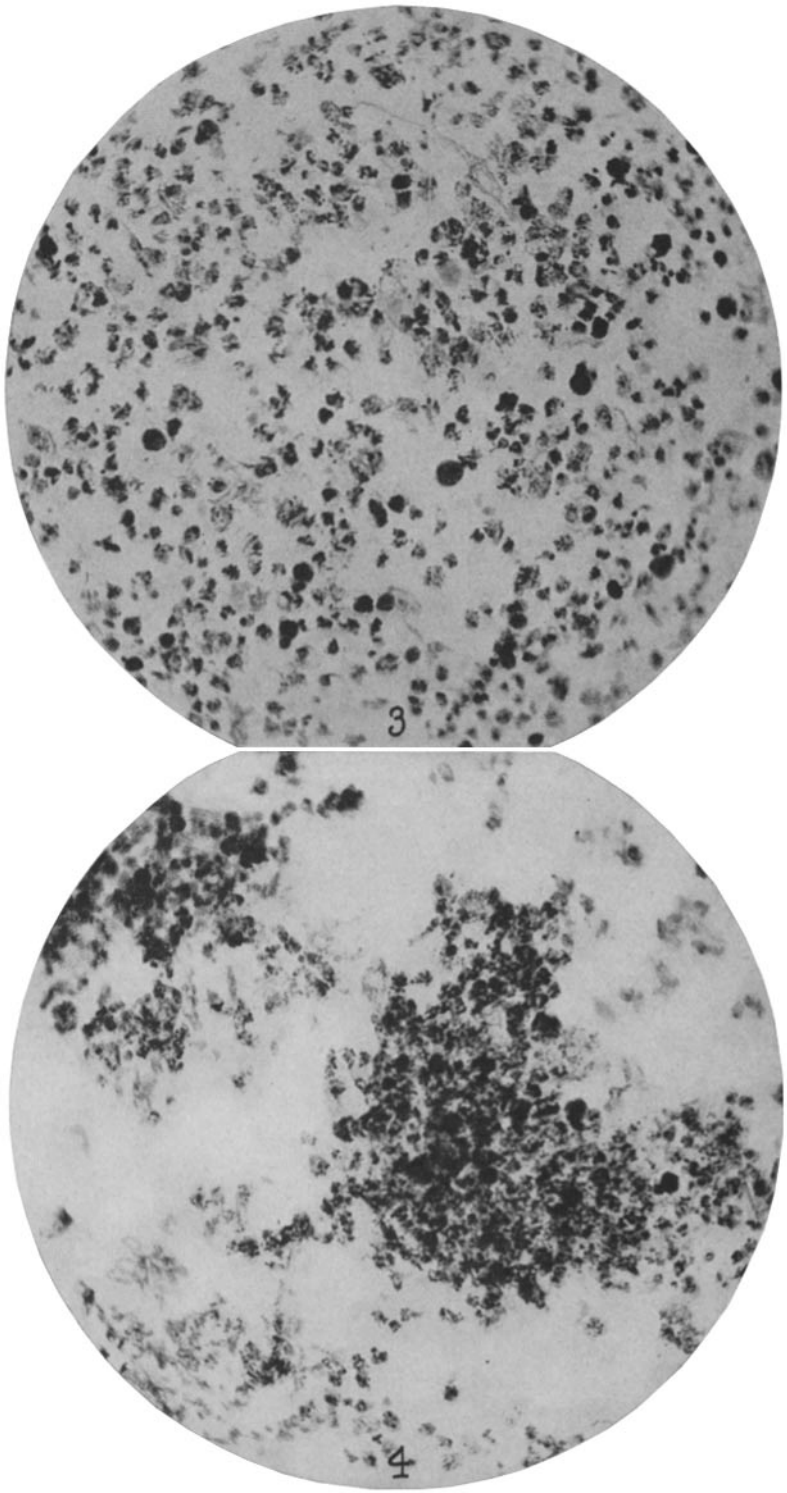
Photographed by Joseph B. Haulenbeek

(Eaton: Agglutination of *Plasmodium knowlesi*)

PLATE 38

FIG. 3. Free parasites resulting from spontaneous hemolysis of parasitized cells 24 hours after drawing the blood. Parasites suspended in normal serum. The large, round, darkly stained (blue) forms are mature segmenters. Lighter and irregularly stained (lavender) forms are probably damaged or dead parasites containing pigment granules. The suspension contains very few intact red cells. × 820.

FIG. 4. Agglutination of free parasites by immune serum. The clumps contain both light and dark staining forms. Practically all of the material has agglutinated. × 820.



Photographed by Joseph B. Haulenbeek

(Eaton: Agglutination of *Plasmodium knowlesi*)