

COMPLEMENT FIXATION IN HUMAN MALARIA WITH AN
ANTIGEN PREPARED FROM THE MONKEY PARASITE
PLASMODIUM KNOWLESI

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The development of specific complement-fixing antibodies in the sera of monkeys infected with the malarial parasite *Plasmodium knowlesi* has been reported in a previous paper (1). Attempts to obtain a similar specific complement fixation reaction with sera from human beings with *Plasmodium vivax* and *Plasmodium falciparum* infections have, in the past, led to inconclusive results because no sensitive, specific, and easily standardized antigen was available. The intense infection produced in *rhesus* monkeys by *P. knowlesi* provides an abundant source of parasites for the preparation of an antigen which gives a definite and specific reaction with malarial sera from monkeys. This paper will describe the use of this *P. knowlesi* antigen in complement fixation tests with sera from human beings infected with *P. knowlesi* and also with the sera of those infected with *P. vivax* and *P. falciparum*. Because most of the human sera tested came from patients being treated for paresis by malarial therapy, it was necessary to eliminate certain non-specific reactions. This necessitated the preparation of antigens of the greatest possible specificity and the performance of many control tests with luetic and normal sera from persons with no malaria, and tests with antigens prepared from normal monkey cells. After the selection of a suitable antigen the intensity and duration of the complement fixation reaction due to malaria was studied.

The literature concerning various serological reactions in malaria and other protozoal infections of man and animals has been reviewed in previous papers (1, 2). In relation to the present studies the work of Kingsbury (3) on comple-

ment fixation in human malaria is of particular interest. This author used antigens prepared from the blood and internal organs of human beings with heavy *P. vivax* and *P. falciparum* infections. The most sensitive and specific antigen was obtained by laking washed parasitized blood corpuscles. With the antigens prepared from *P. falciparum* 48 per cent of the *falciparum* sera and 50 per cent of the *vivax* sera gave positive reactions. With *P. vivax* antigen 31 per cent of the *falciparum* sera and 67 per cent of the *vivax* sera gave positive reactions. These results indicate that the complement-fixing antibody is not species-specific. Kingsbury reported non-specific reactions due to differences in blood group of the antigen and the serum, and a considerable proportion of doubtful or positive reactions between luetic sera and all the antigens, especially those made from spleen, cerebral cortex, and liver. The question of serological reactions between malarial antigens and luetic sera has been reinvestigated during the course of the present work. There are many reports of positive reactions between Wassermann or Kahn antigens and non-luetic malarial sera. As many as 50 per cent of sera tested at the height of the acute malarial infection have given positive Wassermann or Kahn reactions when these same sera tested before the inoculation with malaria were negative (4). Other investigators have denied that malaria *per se* will cause a positive Wassermann (8, 9).

Materials and Methods

Antigens.—Tests with four different malarial antigens will be described in this paper. Two of these were prepared from the blood and two from the spleens of monkeys dying of acute infection with *P. knowlesi*. The parasitized blood cells were concentrated, frozen, dried, and preserved in sealed tubes according to the methods described in a previous paper (1). The spleens were dried in a similar manner. Details of preparing the antigen solutions follow.

Antigen 1 was prepared from dried parasitized blood cells by rehydrating the dried equivalent of 1 cc. of packed cells with 10 cc. of saline. The resulting suspension was then frozen and thawed four times, centrifuged, and the supernatant used as antigen.

Antigen 2 was prepared as previously described (1) from dried parasitized cells by grinding in a ball mill and extracting with saline. The proportion of dried cells to saline was the same as for antigen 1.

Antigen 3 was prepared from dried spleen in the same way as antigen 2, using 1 gm. of dried spleen to each 10 cc. of saline.

Antigen 4 was prepared from dried spleen by rehydrating with saline in the proportion of 10 cc. to each gram of dried material and freezing and thawing as with antigen 1.

In addition to these malarial antigens, an antigen was prepared from normal monkey red cells in the same way as antigen 1. This antigen was used for a normal control in the tests with malarial sera. It is designated as antigen N.

Antigen 1 was not anticomplementary undiluted and was used in the tests at a

dilution of 1:4. Antigen 2 was slightly anticomplementary undiluted and was used at a dilution of 1:10. Antigen 3 was definitely anticomplementary undiluted and was used at a dilution of 1:10. Antigen 4 was anticomplementary at a dilution of 1:2 and was used at a dilution of 1:12. Antigen N was not anticomplementary undiluted. None of these antigens showed any hemolytic properties when tested with sensitized sheep cells.

Sera.—Practically all of the tests described in this paper were made with sera from patients with paretic malaria who were being treated by induced malarial infections with *P. knowlesi*, *P. vivax*, or *P. falciparum*. Part of the sera were collected by us at the Manhattan State Hospital, and part were furnished by Dr. Mark F. Boyd of the Station for Malaria Research at Tallahassee, Florida. Nine non-luetic malarial sera were also sent by Dr. Boyd. Specimens were taken during the clinical attack and at various times up to 6 or more months afterward. From twelve patients series of samples were taken at intervals before, during, and after the malarial attack. Many of the patients, especially those with *vivax* and *falciparum* malaria, were treated with quinine after the first course of malarial paroxysms. Luetic sera from patients presumably free of malaria were collected at various clinics in New York City.

Method of Performing the Complement Fixation Test.—The tests with human sera were made by the same procedure as that already described in detail for monkey sera (1) except that 2 units of complement were used instead of 2½ units as for the monkey sera. This makes the test with human sera somewhat more sensitive. Except where it was desired to know the titer, the sera were not run at various dilutions but only in amounts of 0.1 cc., and the results were recorded as ±, +, ++, +++, and +++++, according to amount of unhemolyzed cells remaining.

Standardization of Antigens by Serological Measurements of the Relative Amounts of Specific and Non-Specific Material.—In complement fixation tests with malarial sera requisites of a suitable antigen are: (a) low anticomplementary activity, (b) relatively low content of non-specific material, and (c) relatively high content of specific malarial antigen. For the purpose of measuring the relative amounts of specific (malarial) and non-specific (monkey) antigens in preparations of parasitized blood and parasitized spleen, a method of serological titration was devised. The specific antigen was titrated against hyperimmune monkey serum. In this reaction non-specific factors may be considered negligible. The amount of reactive monkey protein was measured by titration against the serum of a rabbit immunized with normal monkey erythrocytes. The method and results are illustrated in Table I where two antigens, one from blood and one from spleen, are compared.

With the anti-monkey (rabbit) serum, antigen 4 gives fixation of complement at a dilution which is at least four times the highest dilution of antigen 1 which gives a positive reaction. With the antimalarial (monkey) serum, antigen 4 is slightly less reactive than antigen 1.

This indicates that antigen 4 contains more non-specific material (monkey

protein) per reacting unit of malarial antigen than does antigen 1. Also, antigen 4 is more anticomplementary than antigen 1. Although it is possible that these reactions of complement fixation with monkey and rabbit sera are not entirely

TABLE I
Measurements of Relative Amounts of Specific (Malarial) and Non-Specific (Monkey) Antigens in Parasitized Blood, Parasitized Spleen, and Normal Blood

Serum	Antigen No.	Dilution of antigen							
		1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256
Anti-monkey (rabbit)	1:8 1 (blood)	++++	++++	++++	++++	+++	+	-	-
"	" 1:8 4 (spleen)	++++	++++	++++	++++	++++	++++	++++	++++
"	" 1:8 N(blood)	++++	++++	++++	++++	++++	+++	++	-
Antimalarial (monkey)	1:10 1 (blood)	++++	++++	++++	++++	++	-	-	-
"	" 1:10 4 (spleen)	++++	++++	++++	+++	±	-	-	-
"	" 1:10 N(blood)	-	-	-	-	-	-	-	-
None (saline)	1 (blood)	-	-	-	-	-	-	-	-
"	" 4 (spleen)	++++	±	-	-	-	-	-	-
"	" N(blood)	-	-	-	-	-	-	-	-

TABLE II
Tests for Complement Fixation by Malarial Antigens with Luetic and Normal Sera

Antigen No.	Sera		Total number of sera tested	Reactions, per cent of total			
	Source	Wassermann		++ to ++++	+	±	-
1 (blood)	Lues	Positive	39	3	5	15	77
"	"	Negative	13	8	8	8	76
"	Normal	"	37	0	8	11	81
2 (blood)	Lues	Positive	31	0	13	10	77
"	"	Negative	13	0	8	15	77
3 (spleen)	"	Positive	29	35	13	17	35
"	"	Negative	13	16	8	16	60
"	Normal	"	53	8	6	8	79
4 (spleen)	Lues	Positive	33	6	9	15	70
"	"	Negative	13	8	16	8	68

comparable to reactions with human serum, this method appears to be useful as a preliminary test for selecting a suitable antigen, and it eliminates the time-consuming process of testing many antigens against many human sera. Furthermore, the results with this method are confirmed by the tests with human sera shown later in Table II and Fig. 1.

In Table I it may be seen that antigen N prepared from normal monkey erythrocytes reacts to a slightly higher titer with the anti-monkey serum than did antigen 1 which was prepared in the same way from parasitized cells. In the complement fixation tests with human sera both antigens were used at a dilution of 1:4. The sensitivity of antigen N to substances reactive with the monkey cells should, on the basis of the results with rabbit serum, be slightly greater than the sensitivity of antigen 1. In this way false positive reactions due to constituents of the red cells can be detected.

The standardization of malarial antigens and of normal control antigens can be accomplished with reasonable accuracy when the methods just described are used in conjunction with tests against malarial and non-malarial human sera. When a new preparation of antigen is made, this is compared with the old antigen by parallel tests, and the concentration is then adjusted so that the new antigen gives reactions of similar sensitivity and specificity.

Tests of Malarial Antigens for Non-Specific Complement Fixation with Luetic and Normal Human Sera

The four malarial antigens, prepared as described in the section on materials and methods, were tested with normal human sera and with Wassermann-positive and Wassermann-negative luetic sera. For the most part, each individual serum was tested with each of the four antigens. However, in some cases there was only enough serum to do tests with two or three of the antigens. The results of these complement fixation tests are summarized in Table II.

Antigens 1 and 2, prepared from parasitized blood cells, gave less frequent and weaker positive reactions than antigens 3 and 4, prepared from malarial spleens. Only two sera gave reactions stronger than + with antigen 1, and none of the sera tested gave reactions of ++ or greater with antigen 2. With antigens 1 and 2 approximately the same percentage of + and ± reactions occurred with luetic as with normal sera. With antigen 3 a larger number of positive reactions was obtained with Wassermann-positive than with Wassermann-negative luetic sera, and the percentage of positive reactions with normal sera was considerably lower, being about the same as with antigens 1 and 2. These results indicate that antigen 3 contained considerable amounts of substances which fix complement in parallel with the Wassermann reaction. Antigen 4, also prepared from spleen, did not give a greater percentage of positive reactions with Wassermann-positive than with Wassermann-negative luetic

sera, but the proportion of positive reactions with these sera was slightly higher than was given by antigens 1 and 2 from blood.

It will be seen that antigen 3 cannot be considered reliable for tests with malarial sera which come from patients suffering from paresis, because of its reactivity with non-malarial luetic sera. The other three antigens gave less cross reaction with luetic sera, and with these antigens a reaction of ++ with serum in amounts of 0.1 cc. or less could be considered significant.

Serological Reactions of Normal Monkey Red Cells with Normal, Luetic, and Malarial Human Sera

Several investigators have recorded an agglutination of the red cells of *rhesus* monkeys by some human sera. This is apparently due to hetero-agglutinins not related to the human blood groups or to the Forssman antigen (5). In the present work, some of the patients with *P. knowlesi* infection had been inoculated with the infected blood of *rhesus* monkeys, and their sera showed definite agglutination of monkey cells. The presence of normal hetero-agglutinins in human sera apparently accounted for several other examples of strong agglutination occurring after 2 hours at 37°C. and numerous instances of weaker agglutination of monkey cells which occurred only after 24 hours in the ice box. It was, of course, necessary to determine to what extent these reactions affected the complement fixation with monkey antigens and human sera in the detection of malarial antibodies.

The results of complement fixation tests with the antigen prepared from normal monkey erythrocytes and with human sera from normal individuals and patients with syphilis and malaria are presented in Table III. The antigen was prepared and standardized for sensitivity by titration against anti-monkey rabbit serum as described in previous sections, and was used at a dilution of 1:4. Of the total of 150 sera tested, only one gave a reaction greater than + in an amount of 0.1 cc. with the normal monkey antigen. In the incidence of positive (+) and doubtful (±) reactions there was no important difference between luetic sera giving a positive Wassermann, those giving a negative Wassermann, and the normal sera. In the three series of malarial sera the percentages of positive and doubtful reactions were considerably higher than with the non-malarial sera. This suggests that malarial infection stimulates the production of hetero-antibodies which react with normal monkey cells, and in two or three cases the apparent development of such antibodies at low titer during the course of the malarial paroxysms was observed in patients who had not received monkey blood. These observations demonstrate the importance of using, in complement fixation tests for malaria, a control antigen made from normal cells in the same way and used at the same effective dilution as the malarial antigen.

On twenty-one non-malarial sera and twenty-eight malarial sera, agglutina-

tion and complement fixation tests with normal monkey cells were run in parallel. Of the total of forty-nine sera, twenty-eight (fourteen in each group) gave negative reactions in both tests. With five malarial sera and five non-malarial sera, the agglutination test was positive and the complement fixation test negative, and with nine malarial and one non-malarial sera both tests were positive. One serum gave weak complement fixation but no agglutination. These results indicate that with a fair proportion of sera the hetero-antibodies that cause agglutination of normal monkey cells react weakly or not at all in the complement fixation test.

TABLE III
Tests for Complement Fixation by an Antigen Prepared from Normal Monkey Red Cells (Antigen N) with Normal, Luetic, and Malarial Human Sera

Source of sera	Total number of sera tested	Reactions, per cent of total			
		++ to ++++	+	±	-
Normal.....	37	0	6	6	88
Lues (Wassermann-positive).....	39	0	3	10	87
Lues (Wassermann-negative).....	13	0	8	0	92
<i>P. knowlesi</i> infection and lues.....	25	0	12	24	64
<i>P. vivax</i> infection and lues.....	22	0	14	23	63
<i>P. falciparum</i> infection and lues.....	14	7	7	29	57

Detection of Pseudopositive Reactions with "Normal" Antigen

According to the results which are presented in Table III, strong complement fixation reactions due to the antigens in normal monkey cells are rare. Consequently, strong reactions with a malarial antigen prepared from parasitized red blood cells may be considered significant. When the reaction with malarial antigen is weak or doubtful, the control with normal antigen is of value in eliminating a certain number of false positives.

In Table IV are presented results showing the relative intensity of false positive reactions using normal and luetic sera with normal monkey blood and the four previously described malarial antigens. Only those sera which gave a definitely positive reaction with more than one antigen are included in the table. In general, pseudopositive reactions were more frequent and stronger with the antigens prepared from spleen than with the antigens prepared from blood. Three sera, Gz, Ml, and Rg, were exceptional in giving positive reactions

with the blood antigens and negative reactions with the spleen antigens. Of the fourteen sera which gave positive or doubtful reactions with the malarial antigens prepared from parasitized red cells, six also gave reactions of similar intensity with the antigen N, prepared from normal red cells. Two sera, Gs and Gz, gave strong pseudo-positive reactions with the antigens from malarial blood but none with

TABLE IV
Relative Intensity of False Positive Reactions with Various Malarial Antigens and Normal and Luetic Sera

Human serum	Antigen N (blood)	Malarial antigens				Wassermann
		1 (blood)	2 (blood)	3 (spleen)	4 (spleen)	
Bi	—	—	—	+	++	++++
Fl	—	+	±	++		+
Gs	—	++	—	++	++	++++
Ha	±	±	—	++	±	++++
Lk	—	±	—	++	—	++++
Lm	—	—	—	++	+	+
Lz	—	—	±	++++	++++	++++
Pc	—	—	—	++	±	++
Ss	±	±	+	+++		++++
Do	—	—	—	+	+	—
Gz	—	+++	+	—	—	—
Ml	+	+	—	—	—	—
Pr	±	±	±	++	+++	—
Bs	—	±		+++	+	Normal serum
Gr	—	—		+	++	“ “
Kl	—	+		++	++	“ “
Lr	+	+		+	+	“ “
Rg	+	+		—	—	“ “
Zl	—	±		++++	+	“ “

the antigen from normal blood. Nine sera gave strong false positive reactions with one or both of the spleen antigens but no reaction at all with antigen N. From these results it is evident that the control tests run with antigen N are of value in eliminating about half of the false positive reactions when parasitized blood is used for the test, but such controls are of little value in eliminating the more frequent false positive reactions with spleen antigen.

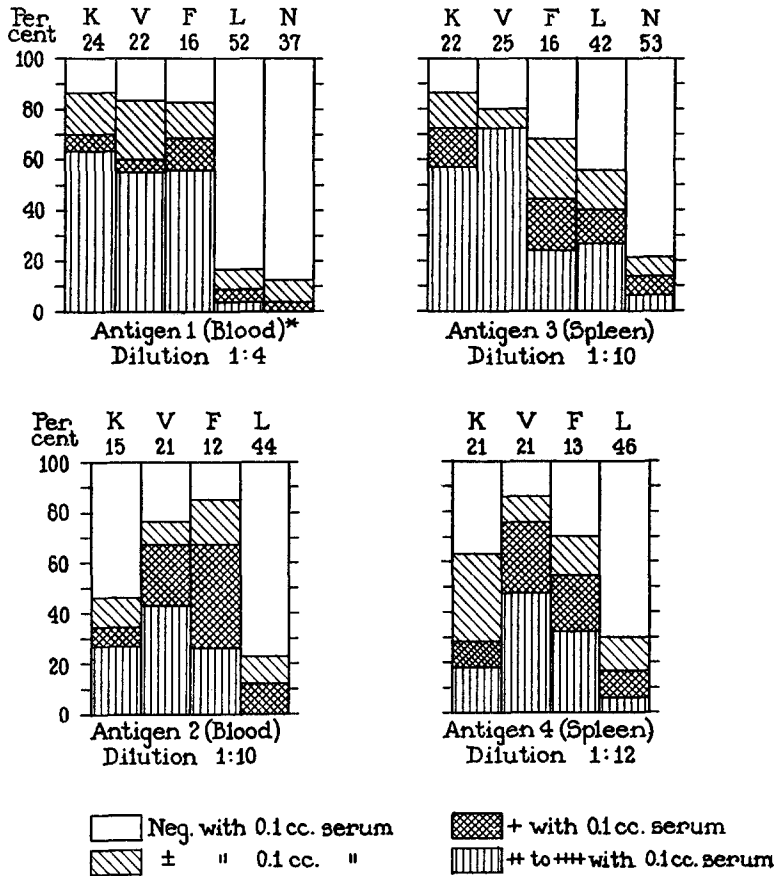
Complement Fixation with P. knowlesi Antigens and Sera from Patients Infected with P. knowlesi, P. vivax, and P. falciparum; Sensitivity and Specificity of the Antigens

Complement fixation tests with sera from paretic patients receiving malaria therapy were done with four antigens prepared from the blood or the spleens of monkeys dying of *P. knowlesi* infection. The results are summarized in Fig. 1. Control tests with antigen N, prepared from normal monkey erythrocytes, were run in parallel with the tests of the malarial sera with antigen 1. Several sera gave weak positive reactions of equal intensity with antigen 1 and antigen N. These were recorded as negative. When the reaction with antigen 1 was + or ++ and that with antigen N \pm , the result was recorded as doubtful. Stronger reactions with antigen 1 were recorded as ++ to ++++, depending upon whether the reaction with antigen N was negative, \pm , or +. Similar controls with antigen N were run with the luetic and normal sera from patients with no malaria, and in the corrected results shown in Fig. 1 about one-third of the pseudopositive reactions have been eliminated.¹ Antigens 2, 3, and 4 were tested with the same sera as antigen 1, but no control tests with normal antigen were done. The results with these antigens have, therefore, been recorded as positive or negative, regardless of what reaction the sera gave with antigen N.

From the results shown in Fig. 1, it is obvious that antigens prepared from *P. knowlesi* fix complement not only with homologous antisera but also with sera from *P. vivax* and *P. falciparum* infections. In fact, antigens 2 and 4 gave a higher percentage of fixation with *vivax* and *falciparum* sera than with *knowlesi* sera. Antigens 1, 2, and 4 gave significantly higher percentages of positive reactions with the three kinds of malarial sera than with sera from normal individuals or from those with syphilis alone. Antigen 3 gave a relatively high percentage of strongly positive reactions with *knowlesi* and *vivax* sera, but there were also many cross reactions with the luetic and normal sera. Although antigens 2 and 4 gave relatively few positive reactions with non-malarial luetic sera, the positive reactions with

¹ The results for antigen 1 recorded in Table II include all of the positive reactions with this antigen, irrespective of the reaction of these sera with antigen N.

malarial sera were generally weaker than those of antigens 1 and 3. With the malarial sera taken as a group, antigen 1 was superior both in specificity and sensitivity to the other three antigens, and with the homologous anti-*knowlesi* sera this antigen gave the highest per-



* Percentages corrected for false positive reactions with N monkey cells.

FIG. 1. Specificity and sensitivity of four malarial antigens in complement fixation test with human sera. Letters and numbers at heads of columns indicate kind and number of sera tested. K, sera from *P. knowlesi* infection and lues; V, sera from *P. vivax* infection and lues; F, sera from *P. falciparum* infection and lues; L, luetic sera, no malaria; N, normal sera.

centage of strongly positive reactions. This superiority of antigen 1 was also evident before the results were corrected for reaction with normal monkey cells (see Table II).

Sera from patients without syphilis but infected with *P. vivax*, *P. falciparum*, or both, were tested with antigens 1 and 3. Of the six sera tested with antigen 1, ++ to ++++ reactions were obtained with four sera, one was negative, and one gave doubtful reactions with both antigen 1 and antigen N. Of the nine sera tested with antigen 3, reactions with three sera were ++ to ++++, with two were +,

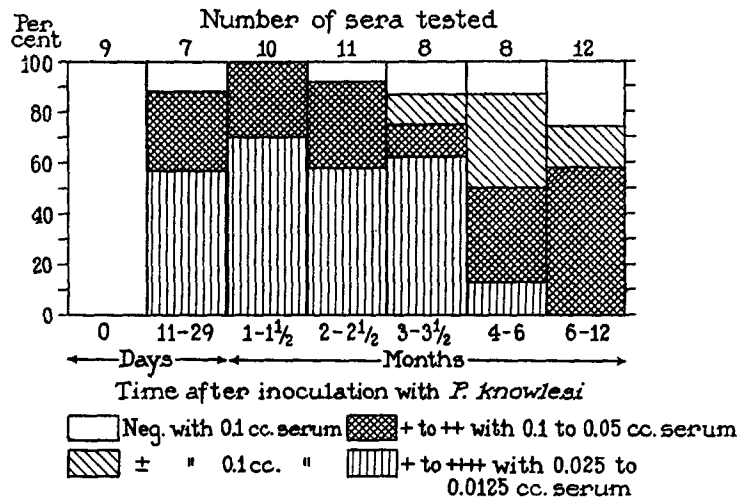


FIG. 2. Complement fixation reactions with sera collected at various intervals after infection with *P. knowlesi*.

and four sera were negative. This series is too small to draw any conclusions except that definite complement fixation may be obtained with sera from patients having malaria alone.

Duration of Positive Complement Fixation after Infection with Plasmodium knowlesi

The results of complement fixation tests with the sera of the twenty-four patients with *P. knowlesi* infection tested with antigen 1, as shown in Fig. 1, and, in addition to these, sera taken from six other patients at various intervals after infection, have been arranged according to time in Fig. 2. Nine sera tested at the time of inoculation

gave completely negative results. The percentage of positive reactions reaches a maximum about 1 to 1½ months after inoculation when all of the ten sera tested were positive, and seven of these gave strong reactions in amounts less than 0.05 cc. After 4 months the number of strongly positive reactions diminishes rapidly and most of

TABLE V
Tests for Plasmodium knowlesi Infection by Inoculation of Monkeys with Blood and by Complement Fixation with Serum

Patient	Time after infection <i>mos.</i>	Result of inoculating monkey	Complement fixation test
Bf	1	+	++
Gt	2.5	0	-
Wi	2.5	0	++++
Lx	2.5	0	+++
Jn	2.5	0	+++
Dl	2.5	0	+
Rl	3	0	++++
Pc	3.5	0	++++
Fz	4	+	+++
Ws	4	0	±
Sn	4.5	0	±
Pj	7	0	-
Pc	7	0	++
Lo	7	0	±
Fo	8	0	-
Wk	9	0	+
Sr	9	0	-
Lo	11	0	-
Ce	11	0	+
Pn	12	0	+++

+ = fatal infection with *P. knowlesi*.

0 = no infection.

the results are moderately positive or doubtful. No strong reactions with less than 0.05 cc. of serum were obtained after 6 months.

Studies on the duration of *P. knowlesi* infections in man (6) have revealed that blood smears, after being positive for about 1 week, usually become negative 12 to 31 days after inoculation and remain negative after this. Parasitological and clinical relapses have been reported (7). For some time after the blood smear has become negative, inoculation of 5 cc. of blood into a monkey will produce an acute

infection with *P. knowlesi*. This indicates that the infection in man passes into a latent phase, detectable only by subinoculation into monkeys, which may last as long as 5 months. Twenty samples of blood were taken from patients at various times after acute infection and tested simultaneously by monkey inoculation and by complement fixation. The results are shown in Table V.² Only two of the bloods were positive both by monkey inoculation and by complement fixation. Of the eighteen which were negative by monkey inoculation, five were also negative by complement fixation, three gave doubtful reactions, and ten were positive to various degrees. Two patients, Pc and Lo, were tested at two different times after infection.

Although the results of Milam and Coggeshall (6) with monkey inoculations were obtained on a different series of patients, a summary of these results in comparison with the results of the present work on complement fixation is of some interest. Of twenty-seven patients tested at 1 to 1½ months for *P. knowlesi* infection by subinoculation of their blood into monkeys, 60 per cent were positive. This compares with 100 per cent positive reactions in the complement fixation test at 1 to 1½ months (Fig. 2). At 2 to 2½ months thirteen patients were tested by monkey inoculation and 23 per cent were positive, compared with 90 per cent positive in the series tested by complement fixation. Similarly, at 3 to 3½ months 17 per cent of twelve patients were positive by monkey inoculation and 74 per cent by complement fixation; and at 4 to 12 months 10 per cent of ten patients were positive by monkey inoculation, compared with about 50 per cent positive by complement fixation. Although the data presented here are incomplete, they indicate that the complement fixation reaction may remain positive for some time after the presence of infection can no longer be detected by inoculation of the blood into monkeys.

Changes in the Titer of Complement-Fixing Antibodies during Infection with Plasmodium knowlesi or Plasmodium vivax

The changes in antibody titer in the sera of six patients during the course of acute infection with *P. knowlesi* are shown in Fig. 3. The

²The series of complement fixation tests represents part of those shown in Fig. 2.

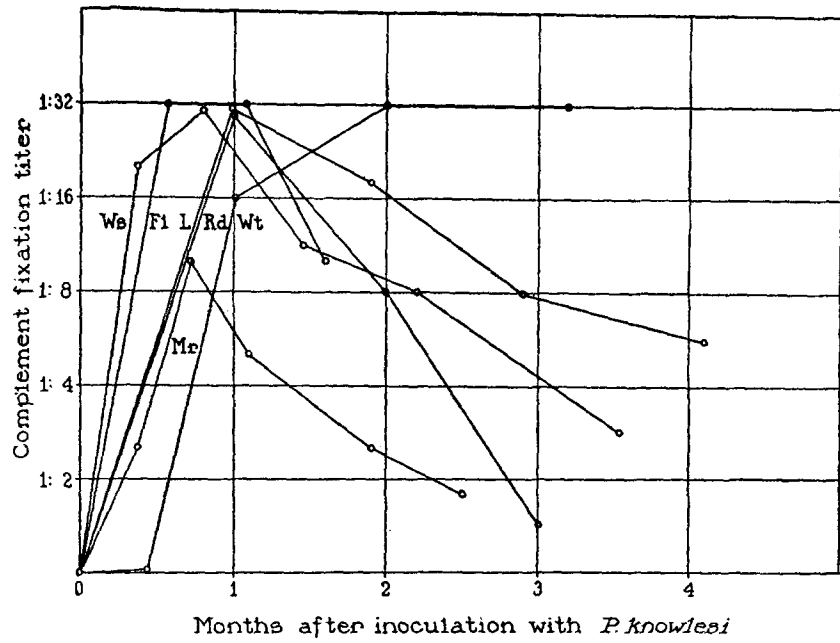


FIG. 3. Changes in complement fixation titer of six human sera before, during, and after acute infection with *P. knowlesi*.

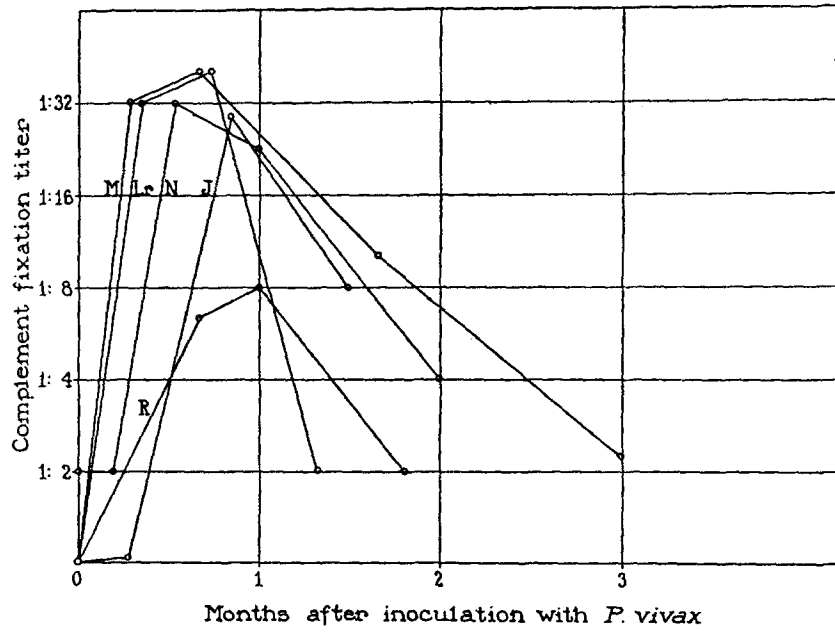


FIG. 4. Changes in complement fixation titer of five human sera before and during infection with *P. vivax*.

complement fixation tests were done with antigen 1, all being set up simultaneously. Controls with antigen N were run on each of the samples of sera. In some cases weakly positive or doubtful reactions were obtained with the normal blood antigen when the titer of complement-fixing antibodies for the malarial antigen was at a maximum. Corrections were made as described in a previous section of this paper. In no case did the titer against the normal antigen go above 1:2.

The titer of complement-fixing antibodies reached a maximum in four of the sera between 12 and 30 days after inoculation. In one patient the reaction was negative 15 days after inoculation, and the titer did not reach a maximum until 2 months had elapsed. It will be noted that the titer tends to fall off rather rapidly in most cases after subsidence of the acute infection. This differs from the results with monkeys (1) in which the infection passes into a prolonged chronic phase with frequent relapses and the complement fixation titer tends to remain at a relatively constant level.

Similar results were obtained with the sera of five patients infected with *P. vivax* in complement fixation tests with the *P. knowlesi* antigen (antigen 1). From the results shown in Fig. 4 it may be seen that the rise in titer is comparable to that resulting from *P. knowlesi* infection, but the subsequent fall in titer appears to be somewhat more rapid after the acute infection with *P. vivax*. This may be due in part to the fact that the patients receiving *P. vivax* were treated with quinine after ten or more paroxysms, while those with *P. knowlesi* usually recovered without quinine.

Effect of Absorbing Human Sera with Normal Monkey Erythrocytes on the Complement Fixation Test for Malaria

If the fixation of complement by human malarial sera and antigens from parasitized monkey cells were due to some constituent of the red cells, and if the control tests with antigen N failed to reveal the antibodies concerned, then, possibly, absorption of the malarial sera with normal monkey erythrocytes might remove the reacting substances. Absorptions were done by the usual method on fifteen sera from patients with *P. knowlesi* or *P. vivax* infection, using 0.5 cc. of packed cells to 1.0 cc. of serum diluted 1:2. The absorption was

repeated once. In some cases the monkey erythrocytes were agglutinated by the sera during the first absorption, but in no case during the second. The absorbed and unabsorbed sera were then tested with malarial antigens from blood and spleen.

Usually the absorption had no effect on the complement fixation with malarial antigen, the titers being undiminished. Examples in

TABLE VI
Effect of Absorption with Normal Monkey Erythrocytes on Complement Fixation with Human Malarial Sera and Plasmodium knowlesi Antigens

Patient	Infecting parasite	Antigen No.	Treatment of sera	Dilutions of serum		
				1:2	1:4	1:8
Fz	<i>P. knowlesi</i>	1 (blood)	Unabsorbed	++	+	±
"	"	" "	Absorbed	++	±	-
Wt	"	" "	Unabsorbed	++++	++++	+
"	"	" "	Absorbed	++++	++	±
"	"	4 (spleen)	Unabsorbed	+	-	-
"	"	" "	Absorbed	±	-	-
Lc	"	1 (blood)	Unabsorbed	++++	+++	±
"	"	" "	Absorbed	++++	+++	-
"	"	4 (spleen)	Unabsorbed	+	-	-
"	"	" "	Absorbed	-	-	-
Ir	<i>P. vivax</i>	1 (blood)	Unabsorbed	++++	+++	+
"	"	" "	Absorbed	+++	++	-
"	"	4 (spleen)	Unabsorbed	++++	+++	-
"	"	" "	Absorbed	++++	+	-
Mz	"	1 (blood)	Unabsorbed	+	±	-
"	"	" "	Absorbed	+	-	-
"	"	4 (spleen)	Unabsorbed	±	-	-
"	"	" "	Absorbed	±	-	-
Al	"	1 (blood)	Unabsorbed	++	+	-
"	"	" "	Absorbed	++	±	-
"	"	4 (spleen)	Unabsorbed	+++	++	±
"	"	" "	Absorbed	+++	++	-

which slight effects were noted are presented in Table VI. It will be seen that even with sera giving weak complement fixation, such as Fz, Mz, and Al, the effect of absorption with normal monkey cells was negligible. With one serum, Lc, the reaction with spleen antigen was changed from positive to negative by absorption. With one *P. vivax* serum, Ir, there was a moderate reduction in titer.

DISCUSSION

In the complement fixation test a positive reaction between any serum and a complex material prepared from blood or spleen is a summation of the reactions between several antigens and their corresponding antibodies. In this work we have endeavored to analyze all the possible reactions which occur when an antigen, prepared from the red cells or spleen of a monkey infected with *P. knowlesi*, reacts with the serum of a human being with malaria and syphilis.

With luetic sera from patients having no malaria, three of the four malarial antigens tested gave no higher percentage of positive reactions with Wassermann-positive than with Wassermann-negative sera, and antigen 1, prepared from parasitized red cells, was not more reactive with luetic sera than with normal sera (Table II). When the reactions of normal and luetic sera with antigens prepared from parasitized red cells (antigen 1) and normal red cells (antigen N) are compared, it is found that the malarial antigen gives a slightly higher percentage of positive reactions with both normal and luetic sera, but the differences in reactivity of antigen 1 and antigen N with these sera are not great enough to be very significant. Furthermore, the studies of Kingsbury (3) and our own investigations (1) have shown that the malarial antigen cannot be prepared in the same way as the Wassermann antigen, namely, by alcoholic extraction of organs. The malarial antigen appears to be a water-soluble protein, whereas the Wassermann antigen is probably a lipoid. Thus, the malarial antigen has been shown to be distinct both chemically and serologically from the Wassermann antigen. Certain preparations may contain both the Wassermann antigen and the malarial antigen. For example, antigen 3, prepared from malarial spleen, gave a much higher percentage of positive complement fixation with Wassermann-positive sera than with Wassermann-negative or normal sera.

Certain human sera contain hetero-agglutinins for normal monkey cells, and these antibodies also give a weak complement fixation reaction with extracts of frozen and thawed monkey erythrocytes. The results shown in Table III suggest that these hetero-antibodies are increased during malarial infection, but the increase in hetero-antibodies is not nearly so great as the increase in immune bodies which fix complement with the malarial antigen. Absorption of

malarial sera with normal monkey erythrocytes reduces the complement fixation titer against malarial antigen from parasitized blood only slightly or not at all. This indicates that most of the fixation of complement obtained with malarial sera is due to malarial antigen-antibody reactions and not to reactions of hetero-antibodies with constituents of the erythrocytes.

The discovery that antigen prepared from *P. knowlesi*, a natural parasite of certain Java monkeys, fixes complement with sera from human beings infected with *P. vivax* and *P. falciparum* is analogous to the results of others (3, 10) who found that antigens from *P. vivax* gave cross reactions with *falciparum* antisera and *vice versa*. The protective and agglutinative antibodies against *P. knowlesi* seem to be species-specific (2, 11). There is also considerable evidence that general resistance to superinfection with malarial parasites in birds (12), monkeys (13), and man (14) is species-specific or even strain-specific. In contrast, the complement fixation reaction for malaria is group-specific. The possibility that this cross reaction may extend to other protozoal infections of man and animals has not yet been investigated.

With *P. knowlesi* infections in man, the duration of positive complement fixation seems to be greater than the duration of the active infection as determined by blood smears or by subinoculation of the blood into *rhesus* monkeys. In man, immunity to superinfection with *P. knowlesi* persists for a year or more, which may or may not be due to continued presence of parasites in the body. The infection in man, however, rapidly passes into a latent phase with corresponding diminution in the titer of complement-fixing antibodies, while in monkeys the repeated relapses stimulate the production of complement-fixing antibodies so that the titer is maintained. In natural *P. vivax* or *P. falciparum* infections in man the disease runs a course similar to that of *P. knowlesi* in monkeys. Most of the cases of *P. vivax* or *P. falciparum* infections studied by complement fixation in the course of the present work were treated with quinine so that relapses did not occur. It is possible, however, that relapses in human malaria produce a rise in the complement fixation titer similar to those observed in monkey malaria. The value of the complement fixation test as a diagnostic aid in malaria can be determined only after an extensive

study of the reactions of sera from human beings with known malaria in places where the disease is endemic.

SUMMARY

In the studies of complement fixation described in this paper, the antigens were prepared from (a) normal monkey red cells, (b) parasitized red cells of monkeys dying with *Plasmodium knowlesi* infection, (c) the spleens of monkeys dying with *Plasmodium knowlesi* infection; the sera came from (a) normal human beings, (b) patients with syphilis, (c) patients with paresis who were receiving malaria therapy with *Plasmodium knowlesi*, *Plasmodium vivax*, or *Plasmodium falciparum*, and (d) patients with malaria alone.

The malarial antigens gave negative complement fixation reactions with 70 to 80 per cent of the luetic and normal sera and weak or doubtful reactions with the remaining 20 to 30 per cent. With the exception of one antigen prepared from spleen, there was no evidence that the malarial antigens were more reactive with Wassermann-positive than with Wassermann-negative sera.

Some human sera give weak complement fixation with antigens prepared from normal monkey erythrocytes, and the percentage of these positive reactions is slightly higher with malarial sera than with normal or luetic sera.

The most sensitive and specific malarial antigen was prepared from dried parasitized red cells by extraction with saline, freezing, and thawing. This *P. knowlesi* antigen gives strong complement fixation with malarial sera from human beings infected with *P. knowlesi*, *P. vivax*, or *P. falciparum*.

The titer of complement-fixing antibodies reaches a maximum about 1 month after the beginning of the acute infection. At this time all of the *P. knowlesi* sera tested were positive. After 4 months the reaction diminishes rapidly in titer but may remain positive for 12 months or longer. With *P. knowlesi* infections in man, the complement fixation reaction remains positive for some time after the infection has apparently disappeared as judged by daily smears and inoculation of monkeys with the blood.

The complement fixation reaction in malaria is group-specific rather than species-specific. Sera from patients infected with *P. vivax* or

P. falciparum react in the same way with the *P. knowlesi* antigen as the homologous sera.

Absorption of malarial human sera with normal monkey erythrocytes does not remove the immune bodies which fix complement with malarial antigens.

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