COMPLEMENT FIXATION IN HUMAN MALARIA USING AN ANTIGEN PREPARED FROM THE CHICKEN PARASITE *PLASMODIUM GALLINACEUM*

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The literature concerning various serological reactions in malaria in man has been well reviewed (1). According to this review, attempts to obtain a specific complement fixation reaction with sera from human beings infected by *Plasmodium vivax* or by *Plasmodium falciparum* have led in the past to inconclusive results because no sensitive, specific, and easily standardized antigen was available. Due to the difficulty in obtaining sufficient quantities of heavily infected human blood from which to prepare an antigen, these investigators developed an antigen from *Plasmodium knowlesi* which occurs naturally in the malaria of monkeys and can be used to induce malaria in man. A complement fixation test using such an antigen was found to give a group reaction with sera from human beings infected with *Plasmodium vivax* and *Plasmodium falciparum.*

When the onset of war made procurement of monkeys impracticable, two workers (2) prepared an antigen from the chicken malarial parasite *Plasmodium gallinaceum.* One of them (3) demonstrated the group specificity of this antigen, using the sera of soldiers with malaria at Percy Jones General Hospital. Further studies appeared desirable since there are no certain clinical criteria indicative of a complete cure in malaria and a test that could detect latent malaria would obviously serve a very useful purpose.

The present report deals with the serological investigations conducted at the Harmon General Hospital. The antigen prepared from *Plasmodium gallinaceum* was supplied originally by the aforementioned workers and subsequently by the Army Medical School. The specificity and sensitivity of this complement fixation test was studied on 11,367 sera. These sera were obtained from 1000 normal healthy soldiers, from 95 soldiers with febrile illnesses other than malaria, from 481 men with syphilis, and from 505 soldiers who gave a history of malarial infection while in the South Pacific. During the period of study of the last-named group, there were 434 recurrent attacks of malaria due to *Plasmodium vivax.*

MATERIALS AND METHODS

Sera. One thousand sera for controls were obtained from normal healthy soldiers. None of these men had been overseas or had a history of malaria. Four hundred and seventy-five sera were obtained from 95 soldiers who likewise had never been overseas or had malaria but who were suffering from a febrile illness in which the body temperature was not less than 100° F. at the time of drawing the initial blood sample. From each of these 95 patients, sera were taken for 5 consecutive days. Sera were taken from 481 men with syphilis who had never been overseas. These men were negro soldiers from various parts of the country and it is possible that some from the South may have had malaria contracted in the past. From the 505 patients with a history of malaria contracted in the South Pacific, sera were obtained at 5-day intervals and, when a recurrent attack of malaria developed, sera were taken on the day of onset and on each of the next 4 days. The taking of sera at 5-day intervals was then resumed.

Smears. A thick or thin blood smear was made from capillary blood each time that serum was obtained by venepuncture. Staining was done by the Giemsa technique. The species of plasmodium was determined in each instance with special reference to the possibility of mixed infections.

Treatment. Acute malarial attacks were treated with atabrine, 2.8 grams in 7 days, for 50 per cent of the attacks; atabrine, 2.8 grams in 7 days followed by atabrine 0.1 gram daily for 60 days, in an additional 31 per cent; quinine, 16 grams in 7 days, in 3 per cent of attacks; quinine, 6.0 grams in 2 days followed by atabrine 1.5 grams in 5 days followed by plasmochin 0.15 gram in 5 days, in 3 per cent; and various other combinations of these drugs in 5 per cent of attacks. The details of treatment for the remaining 8 per cent are not known because the attacks took place while the patients were absent from the hospital.

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Complement fixation technique. Patients were bled from an antecubital vein with sterile equipment and the blood placed in sterile tubes. Serum was removed promptly from specimens of blood and kept sterile so that retesting could be carried out at future dates. All stored sera were frozen and kept in this state. For use in the test, the sera were inactivated for 30 minutes at 56° C.

The antigen consisted of lyophilized chicken red blood corpuscles infected with *Plasmodium gallinaceum.* Using the titer stated on the container, the volume was always restored according to directions.

Complement was obtained from the Army Medical School to which it had been furnished by a commercial firm in a lyophilized form, packaged *in vacuo.* The complement was always restored to the volume directed on the ampule. Before use, each lot of complement was titrated to determine its potency by the method given below.

The amboceptor used was obtained from the Army Medical School and its unit was determined by titration.

Fresh sheep red blood corpuscles were obtained as needed. Twenty ml. of citrated blood were obtained twice a week and washed as directed for use in the Kolmer test. The corpuscles were originally used in a 2.5 per cent suspension, but later when the test was revised, a 2 per cent suspension was used. The accuracy of the suspension was tested by centrifuging 10 ml. of the suspension in a graduated centrifuge tube and noting the volume of the packed cells. The suspension was never more than 2 days old at the time of use.

The salt solution originally used was made up to 0.85 per cent and unbuffered. Later the salt solution was buffered with 0.005 M phosphate (pH 7.2 to 7.4).

For a short period of time, the tests were set up according to Coggeshall and Eaton's technique as modified by Captain A. W. Frisch, MC, of Percy Jones General Hospital. Later the test was altered to conform to the Army Medical School procedure (4).

Frisch's technique was as follows:

1. Titration of amboceptor

A. Preliminary titration: Titrations were carried out in the presence of an excess of complement (*i.e.* 0.2 ml. of a 1:10 dilution). For titration purposes, the following dilutions of amboceptor were prepared: 1:100; 1:200; 1:400; 1:800; 1:1600; 1:3200; 1:6400.

To each tube containing 0.5 ml. of diluted amboceptor, 0.2 ml. of 1:10 complement and 0.2 ml. of saline were added. Then to each tube was added 0.25 ml. of 2.5 per cent sheep red corpuscles. The tubes were shaken and incubated in the water bath at 37° C. for 30 minutes and then read. The highest dilution of amboceptor showing complete hemolysis was selected for the final titration.

B. Final titration: Using the dilution of amboceptor, as determined above, the following graded amounts of amboceptor were added to a series of 7 tubes.

Tube	1	2	3	4	5	6	7
Amboceptor—ml.	0.50	0.40	0.30	0.20	0.10	0.05	0.025
Saline—ml.	0.20	0.30	0.40	0.50	0.60	0.65	0.675
Complement 1 : 10 ml.	0.20	0.20	0.20	0.20	0.20	0.20	0.20
2.5 per cent sheep R.B.C.—ml.	0.25	0.25	0.25	0.25	0.25	0.25	0.25

The tubes were then incubated in the water bath for 30 minutes at 37° C. and read. The smallest amount of amboceptor giving complete hemolysis was taken as 1 unit. In the final test, there were 2 units contained in a volume of 0.25 ml. The amboceptor dilution containing 2 units remained constant for each lot, provided that the red blood corpuscles were prepared properly.

2. Complement titrations

Complement was titrated each day that the tests were run. A series of tubes was set up containing 1:10 complement as follows:

Tube	1	2	3	4	5	6
Complement—ml.	0.20	0.15	0.10	0.075	0.050	0.025
Saline—ml.	0.50	0.55	0.60	0.625	0.650	0.675
Amboceptor (2 units)—ml.	0.25	0.25	0.25	0.25	0.25	0.25
Sheep cells (2.5 per cent)—ml.	0.25	0.25	0.25	0.25	0.25	0.25

The tubes were incubated for 30 minutes at 37° C. and read. The exact unit of complement was taken as the amount showing complete or almost complete hemolysis. For the final test two units of complement were used.

3. Preparation of antigen

The lyophilized antigen was restored to original volume with distilled water and then diluted 1:10 with saline. The antigen was shaken thoroughly and then allowed to stand to get rid of the larger particles. The turbid supernatant fluid was pipetted off and serial dilutions of the antigen from 1:10 through 1:320 were prepared.

A series of two rows of tubes, A and B, containing the following were set up:

Tube	1A	2A	3A	4A	5A	6A
Antigen 0.25 ml.	1:10	1:20	1:40	1:80	1:160	1:320
Complement units	2	2	2	2	2	2
Known pos. serum—ml.	0.2	0.2	0.2	0.2	0.2	0.2

Tube	1B	2B	3B	4B	5B	6B
Antigen 0.25 ml.	1:10	1:20	1:40	1:80	1:160	1:320
Complement units	2	2	2	2	2	2
Saline—ml.	0.2	0.2	0.2	0.2	0.2	0.2

The tubes were incubated in the water bath at 37° C. for 1 hour, after which 0.5 ml. of sensitized sheep red blood corpuscles were added. The tubes were then replaced in the water bath at 37° C. until the majority of tubes in row "B" showed complete hemolysis. The dilution of antigen showing complete fixation and no anticomplementary activity was selected as the end-point. It was found that the antigen unit invariably coincided with that given on the container.

The qualitative test proper: To each of two tubes the following were added:

	Tube 1A	Tube 1B
Serum, ml.	0.2	0.2
Complement units	2	2
Antigen, ml.	0.25	
Saline, ml.		0.25

In addition to the above, there were prepared at the same time two control tubes, one containing a known positive serum set up as above and an antigen control containing 0.25 ml. antigen, 2 units of complement, and 0.2 ml. of saline. The tests and controls were incubated at 37° C. for 1 hour and then 0.5 ml. of corpuscles sensitized with amboceptor were added to each tube. The tubes were incubated at 37° C. until the antigen controls were clear. The results were recorded in terms of plus signs, ranging from 4+ to 0—4+ indicating no hemolysis and each increment 25 per cent more hemolysis until complete hemolysis occurred. If no hemolysis occurred in either control tube or the test tube, the result was recorded as anticomplementary.

Quantitative titrations were performed on all sera that had given a 4+ reaction. In addition to repeating the test on these sera, the following dilutions were prepared: 1:5, 1:10, 1:15, 1:20, 1:30, 1:40, 1:60, 1:80, 1:120, and 1:160. Normal, undiluted sera and sera diluted 1:5 and 1:10, were included in these titrations as controls. After the dilutions had been prepared the tests were performed in the same manner as above.

After approximately 3000 tests had been run, a modification in technique was introduced. With this procedure, the 0.85 per cent NaCl was buffered with 0.005 M phosphate (pH 7.2 to 7.4). The reagents were prepared in the same manner but the volume of each was adjusted so that it was contained in 0.2 ml. and the total volume for each test was 1 ml.

After the reagents were added, the tests were shaken and placed in the refrigerator at 5° C. overnight. The next morning there was added to each tube 0.2 ml. of 2 per cent sheep's corpuscle suspension sensitized with 2 units of amboceptor, making a total volume of 0.4 ml. The tubes were incubated at 37° C. for 30 minutes and then read. The activity of the hemolytic system was tested by refrigeration of the controls overnight followed by addition of the indicator series the following morning.

More than 8000 tests were performed using the ice-box fixation method. At one time, because of the larger number of negative tests, it was thought that even though the complement unit was determined by titration, an excess was possibly being used. Therefore, duplicate tests were set up on the same sera using the complement unit as determined by titration for one series and a two-thirds unit of complement in the other series. This gave a larger number of positive results but also increased the number of anticomplementary results. Recently, we had the opportunity of comparing a large number of tests in duplicate using the old method and one recently introduced by Major C. H. Rein and Captain S. Bukantz (5). The method of Rein and Bukantz has a distinct advantage over the one now in use in that it lends itself readily to standardization. However, in comparing the results of the latter method with the one employed in this laboratory, it was found that there was no significant difference either in sensitivity or specificity in approximately 1500 tests run in duplicate.

RESULTS

A summary of the results of 11,367 complement fixation tests are shown in Table I. Single specimens of sera from 1000 normal healthy soldiers showed 99 per cent to be negative. Tests on 475 sera from 95 soldiers with febrile illnesses other than malaria showed 96 per cent to be negative. Of 481 sera from syphilitic men, 93 per cent were negative. Of 9411 sera from 505 patients with a history of malaria contracted in the South Pacific area, tested during the entire period in which they were under observation in the Harmon General Hospital, 67 per cent were negative, 30 per cent positive, and 3 per cent anticomplementary. A detailed analysis of the material recorded in Table I is presented in the following figures and tables. The specificity and sensitivity of the complement fixation test and its practical application to the problem of malaria is then discussed.

TABLE I

Results of complement fixation tests performed on sera of various controls and patients with history of malaria

Source of	No. pts.	No. tests	Negative		Positive		Anticomplementary	
			number	*per cent*	*number*	*per cent*	*number*	*per cent*
I. Control tests								
1. Healthy soldiers	1000	1000	989	99	9	Under 1	2	Under 1
2. Patients with febrile diseases other than malaria	95	475	455	96	14	3	6	1
3. Syphilitic men	481	481	449	93	24	5	8	2
II. Soldiers with history of malaria *	505	9411	6318	67	2777	30	316	3
Total tests		11367						

* All gave a history of malaria contracted in the South Pacific area but not all developed attacks while in Harmon General Hospital.

Serological controls. Sera were obtained from 4 groups of normal healthy soldiers for the control series. None of these men had been out of the United States and none gave a history of

having had malaria. The first and second groups were soldiers from the detachment of an affiliated hospital unit and their sera were taken during field training. The third and fourth groups were infantrymen from whom the blood was drawn just after completion of a final type physical examination. For each member of a given group, the blood was taken on the same day and all tests were run on the day following procurement of sera. Ninety-nine per cent of all the sera were negative. Additional sera were not obtained from the 9 men with positive complement fixation tests or from the 2 men with anticomplementary results.

As a further control, the results of the complement fixation test were determined in soldiers with febrile illnesses other than malaria. Ninety-five patients with a temperature of not less than 100° F. at the time of taking of the first serum also had specimens taken on 4 additional consecutive days. None of these patients had ever been overseas or had ever had malaria. The illnesses from which they suffered at the time of the tests were common respiratory infections, pneumonia, pleurisy, measles, and acute rheumatic fever. Table II shows that 96 per cent of the 475 sera tested were negative. The positive and anticomplementary results, totaling 20 tests, occurred in 14 patients.

The final series of control sera were from 481 syphilitic men. All of these soldiers had proven syphilis and were under treatment. At the time of testing, Wassermann, Kahn, and malaria complement fixation tests were performed on each serum. The results showed that 318 Wassermann and 359 Kahn tests were positive. Twenty-four malaria complement fixation tests were positive and 8 were anticomplementary. Out of 481 sera from the total group of syphilitics (none of whom had neurosyphilis), 93 per cent of the malaria complement fixation tests were negative, 5 per cent positive, and 2 per cent anticomplementary.

Titre of complement-fixing antibodies. As an indication of the serological sensitivity of the complement fixation test, 1335 strongly positive sera (4+) from malarial patients were further tested in 10 dilutions. The dilutions ranged from 1:5 through 1:160. The results are presented in Figure 1 and indicate that 40 per cent of all tests were positive in a 1:5 dilution, 29 per cent in a 1:10 dilution, 22 per cent in a 1:15 dilution, 13 per cent in a 1:20 dilution, 4 per cent in a 1:30 dilution, 3 per cent in a 1:40 dilution, 2 per cent in a 1:60 dilution, and 1 per cent from 1:80 to 1:160 dilution. Ninety-four per cent did not titre beyond a 1:30 dilution.

Relation of blood smear and complement fixation during 234 recurrent attacks. For the purpose of this analysis, no one was considered clinically to have a relapse unless a positive blood smear and temperature of at least 100° F. were obtained before treatment was instituted. Figure 2 shows that on the first day of 234 recurrent attacks, the percentage of positive smears was 100. By the second day, with treatment, it had dropped to 63 per cent, by the third day to 10 per cent, and by the fourth and fifth days to 1

TABLE II

Complement fixation in 95 patients with febrile illnesses other than malaria (475 tests)

Diagnosis	Number of patients	Serological results during 5 successive days														
		Negative					Positive					Anticomplementary				
		1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Common respiratory diseases	54	53	52	49	53	54	1	1	5	0	0	0	1	0	1	0
Measles	27	27	27	27	27	27	0	0	0	0	0	0	0	0	0	0
Pneumonia or pleurisy	13	11	11	13	11	12	2	2	0	2	1	0	0	0	0	0
Rheumatic fever	1	0	0	0	0	1	0	0	0	0	0	1	1	1	1	0
Total by days		91	90	89	91	94	3	3	5	2	1	1	2	1	2	0
Grand total	95	455					14					6				

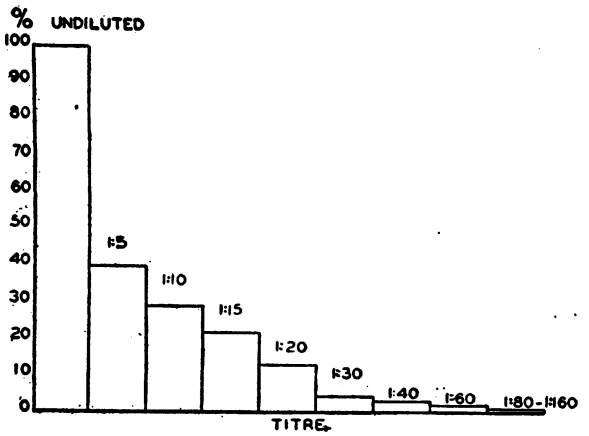

FIG. 1. DILUTION TESTS ON 1335 STRONGLY POSITIVE SERA (4+)

per cent. During the same period of time, the percentage of negative complement fixation tests was recorded (Figure 2) rather than the percentage of positive tests in order to account for the anticomplementary results. It is apparent that, from the first day through the fifth, negative tests were obtained between 41 and 46 per cent of the time. On any one of these 5 days, not over 58 per cent of the tests were positive. Table III shows the daily complement fixation results represented in Figure 2.

Complement fixation during the recurrent attack. Sera were drawn for 5 consecutive days during recurrent attacks beginning at the time a smear was found to be positive. Table IV shows the complement fixation results in 238 attacks

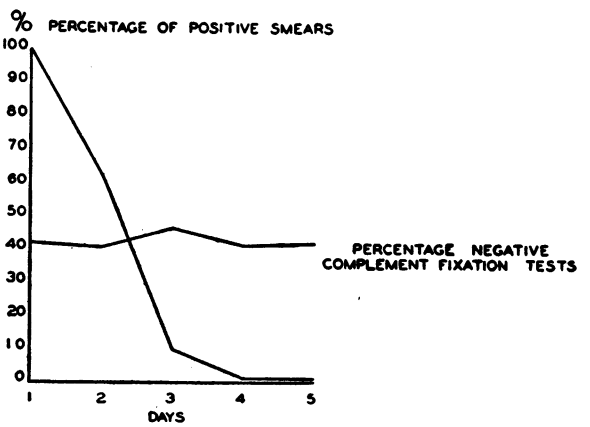

FIG. 2. COMPLEMENT FIXATION AND BLOOD SMEARS FOR 5 CONSECUTIVE DAYS BEGINNING WITH THE FIRST DAY OF A RECURRENT ATTACK (234 ATTACKS)

TABLE III

Daily complement fixation results represented in Figure 2

Days	Results of complement fixation tests						Negative
	0	1+	2+	3+	4+	AC	
							per cent
1	100	5	19	22	77	11	43
2	96	6	15	19	90	8	41
3	107	6	16	16	71	18	46
4	97	3	17	27	80	10	41
5	100	6	18	20	77	13	43

in which no anticomplementary results were obtained. The results are recorded as negative or positive for each day for each individual attack. The positive results include the entire range from 1+ to 4+. From these data it can be seen that there were 28 different types of serological responses. For example, in 54 attacks not a single positive test was found and in 42 attacks not a single negative test was found. In all the other attacks, there were variations in the days on which either positive or negative results were obtained. These results indicate that a positive

TABLE IV

Complement fixation results for 5 consecutive days in 238 recurrent attacks of malaria

Number in group	Days				
	1	2	3	4	5
54	−	−	−	−	−
4	−	−	−	+	−
1	−	−	+	−	−
9	−	+	−	−	−
5	−	+	+	−	−
3	−	+	+	+	−
1	−	−	+	−	+
2	−	+	−	−	+
1	−	+	+	−	+
1	−	+	−	+	+
10	−	−	−	−	+
8	−	−	−	+	+
8	−	−	+	+	+
13	−	+	+	+	+
3	+	−	+	−	−
3	+	−	−	+	−
1	+	−	+	+	−
12	+	−	−	−	−
8	+	+	−	−	−
4	+	+	+	−	−
12	+	+	+	+	−
3	+	−	−	−	+
3	+	+	−	−	+
5	+	+	+	−	+
5	+	−	−	+	+
8	+	+	−	+	+
9	+	−	+	+	+
42	+	+	+	+	+

TABLE V

Comparison of serological response in 2 successive attacks in 30 patients

Number	First attack Days					Second attack Days					Previous attacks	Interval in weeks
	1	2	3	4	5	1	2	3	4	5		
1	−	−	−	−	−	−	−	−	−	−	2	24
2	−	−	−	−	−	−	−	−	−	−	13	17
3	−	−	−	−	−	−	−	−	−	−	3	14
4	−	−	−	−	−	−	−	−	−	−	6	13
5	−	−	−	−	−	−	−	−	−	+	15	9
6	−	+	−	−	−	+	−	−	−	−	8	20
7	−	+	−	−	+	−	−	−	−	−	2	14
8	−	−	−	−	+	+	−	+	−	−	5	12
9	−	−	+	+	+	−	−	−	−	−	7	23
10	−	+	+	+	+	−	−	−	−	−	2	13
11	−	+	+	+	+	−	−	−	+	+	6	12
12	−	+	+	+	+	+	+	+	+	+	7	4
13	+	−	−	−	−	+	−	+	+	+	8	13
14	+	+	−	−	−	−	−	−	+	−	4	12
15	+	+	−	−	−	−	+	−	−	−	3	13
16	+	+	−	−	−	+	+	+	+	+	8	12
17	+	+	−	−	−	+	+	+	+	+	8	9
18	+	+	+	−	−	+	+	+	+	+	12	11
19	+	+	+	+	−	+	+	−	+	+	4	15
20	+	+	−	+	+	+	−	+	+	+	8	12
21	+	+	−	+	+	+	+	+	+	+	8	7
22	+	−	+	+	+	−	−	−	−	−	9	4
23	+	−	+	+	+	+	+	−	−	+	4	13
24	+	+	+	+	+	−	−	−	−	−	7	15
25	+	+	+	+	+	−	+	−	−	−	8	13
26	+	+	+	+	+	−	−	−	−	+	5	5
27	+	+	+	+	+	+	+	−	−	−	1	5
28	+	+	+	+	+	+	+	+	+	−	5	22
29	+	+	+	+	+	+	−	−	−	+	4	10
30	+	+	+	+	+	+	+	+	+	+	11	16

test might not appear at all during this 5-day period or might appear on any one of the 5 days and might be followed on the next day by a negative test and even later by a positive test. Thus, there was no constant serological response characterizing the attacks as a whole.

Comparison of the serological response in 2 successive attacks in 30 patients is shown in Table V. The number of attacks prior to the first one for which data are presented is given as well as weeks between the 2 successive attacks. There was no correlation between the number of previous attacks and complement fixation results during the attacks. The group of 42 men with positive tests on 5 successive days had an average of 7.0 previous attacks (range 1 to 15), while the group of 54 men with 5 negative results had an average of 7.3 attacks (range 1 to 17). Furthermore, the type of response in each attack was not usually the same.

Relation of complement fixation and blood smears during recurrent attacks. In Table VI, data are presented relating the results of complement fixation tests to the malaria smears obtained on each of 5 days in 200 malarial attacks in which treatment with atabrine was started after a positive smear and a temperature of 100° F. were attained. As previously indicated (Figure 2), the percentage of positive smears dropped sharply from 100 per cent before treatment to 60 per cent on the second day, 10 per cent on the third day, and 1 per cent on the 4th and 5th days of the attack. Since the percentage of positive complement fixation tests remained approximately 50 per cent on any day, it is seen that in the third, fourth, and fifth days of the attack, the complement fixation test was positive in 77, 101, and 101 patients, respectively, from whom negative smears were obtained. This might be interpreted as indicating that in those patients in whom treatment had been initiated without obtaining a smear, the complement fixation test might have been used for diagnostic purposes. Actually, in well over 400 recurrences, positive smears were obtained without difficulty during the initial paroxysm of the recurrence which brought the patient under observation; occasionally a positive smear was obtained during a period of prodromal symptoms, occasionally not until the day after the initial paroxysm, or during a second paroxysm. In only 3 patients, however, with symptoms suggesting a malarial recurrence, did smears remain persistently negative.

TABLE VI

Relation of complement fixation and blood smears during 5 days in 200 recurrent attacks of malaria

Day of relapse	Positive smears Positive C.F.		Positive smears Negative C.F.		Negative smears Positive C.F.		Negative smears Negative C.F.	
	number	*per cent*	*number*	*per cent*	*number*	*per cent*	*number*	*per cent*
First	105	53	95	48	0	0	0	0
Second	51	26	67	34	53	27	29	15
Third	8	4	12	6	77	39	103	52
Fourth	2	1	0	0	101	51	97	49
Fifth	2	1	0	0	101	51	97	49

Complement fixation in intervals between recurrent attacks of malaria. After determining the specificity and sensitivity of the complement fixation test, the most important phase of the entire problem was approached. This consisted in de-

termining whether the antibodies detectable by this procedure were consistently present between attacks. Should there be persistent antibodies as long as latent or subclinical malaria existed, the test would be of immense practical value in deciding in the individual case whether or not a cure had been established. The data in Tables VII, VIII, and IX answer this question.

TABLE VII

Complement fixation during 100 intervals between observed attacks

Complement fixation	Number tests
Negative	574
1+	32
2+	44
3+	60
4+	151
Anticomplementary	30
Percentage negative: 64	Total 891

Table VII shows the results of complement fixation tests in 100 intervals between 2 or 3 consecutive attacks, observed in 76 patients. The interval chosen was that period of time between the sixth day from the beginning of an attack (since the first 5 days as shown before are arbitrarily considered as the attack) and the last day preceding the next attack. The shortest interval was 4 weeks and the longest 24 weeks with the majority about 8 weeks. Out of a total of 891 tests, 64 per cent were negative and 33 per cent were positive. In other words, only one third of the tests were positive in patients who subsequently developed malaria. It is obvious that the results of initial casual testing during intervals free from clinical activity cannot be used for predicting subsequent recurrences.

Complement fixation directly before and after recurrent attacks. Complement fixation before and after 300 attacks, without reference to consecutive attacks, is shown in Table VIII. Of 171 tests performed 10 days before an attack, 67 per cent were negative. Five days after an attack (10 days from the initial day of the attack) in 223 tests, 48 per cent were negative; ten days after, in 242 tests, 52 per cent were negative; 15 days after, in 224 tests, 59 per cent were negative, and 20 days after, in 215 tests, 66 per cent were negative. It is to be recalled from Figure 2 that during the 5 days of the attack period, 41 per cent to 46 per cent were negative, and from

TABLE VIII

Complement fixation in sera from malarial patients taken 5 and 10 days, respectively, before a recurrent attack and 5, 10, 15, and 20 days after the fifth day of 300 recurrent attacks

Complement fixation	0	1+	2+	3+	4+	AC	Percentage negative
10 days before attack	114	7	9	10	26	5	67
5 days before attack	124	5	8	13	36	6	65
During attack							41 to 46
5 days after attack	108	8	5	16	68	18	48
10 days after attack	125	9	12	14	70	12	52
15 days after attack	133	10	12	25	40	4	59
20 days after attack	142	9	15	11	33	5	66

Table VII, that for the entire interval between 100 attacks, 64 per cent were negative. This shows that the number of positive tests reached a maximum of 58 per cent during an attack and that within 25 days from the onset of an attack there was a drop to 33 per cent positive tests. By chance, 22 tests were made on the day before an attack and 26 tests, 2 days before. Only 14 and 23 per cent, respectively, were positive as compared with 34 per cent 5 days prior to an attack. This finding is not contrary to the theory according to which the test may become negative just before the paroxysm because of absorption of antibodies by the increasing number of parasites. The small number of tests does not permit use of the data either in support or in contradiction of this suggestion.

Complement fixation during a period of 6 months. Table IX represents a continuous serological study covering 6 months' observation at Harmon General Hospital of 121 soldiers known to have had malaria in the South Pacific. Each of these men had a sick furlough of 3 weeks in this 6-month period. The interest in this table lies in the results of complement fixation tests

TABLE IX

Complement fixation results on sera from 121 malarial patients taken at 5-day intervals for 6 months except during the periods of recurrent attacks (5 days)

Number patients	Number of attacks in 6 months	Serology 0	1+	2+	3+	4+	AC	Percentage negative
1	4	14		2		3	1	70
17	3	210	11	17	25	64	23	60
42	2	580	41	45	56	164	15	64
35	1	602	32	32	39	123	11	72
26	No attack	539	13	24	22	63	5	81

performed at 5-day intervals, exclusive of the periods of recurrent attacks (5 days). The purpose of the compilation is to demonstrate persistence or disappearance of positive complement fixation in patients with recurrent attacks and in those in whom subsequent attacks did not occur during this period of observation and serological investigation. One patient had 4 attacks but during the symptom-free intervals had 70 per cent negative tests. Seventeen patients had 3 attacks with 60 per cent negative tests in the intervals. Forty-two patients with 2 attacks had 64 per cent negative tests. Thirty-five patients with 1 attack had 72 per cent negative tests. Twenty-six patients with no attacks while in the hospital had 81 per cent negative tests. During their period of observation, these 26 patients had no definite clinical attacks of malaria or parasitemia. Of these, 1 had an attack of unobserved illness, which was probably malaria, 5 at no time complained of symptoms suggesting malaria, and 20 complained irregularly of varying aches or malaise which might have represented subclinical activity. Since, for the most part, these men resided in rehabilitation barracks where routine taking of temperature was purposely omitted, data were not collected which would permit a precise estimate of the relation of occasional rises in complement fixation (19 per cent positive tests) to subclinical activity. If the tests of the first 3 months of the 6-month period are separated from those of the last 3 months, the percentage of negative tests for the latter period is found to be 86 per cent.

DISCUSSION

As long ago as 1907 DeBlassi (6) claimed to have secured positive specific reactions in human malaria with an antigen prepared from *Plasmodium vivax*. The idea of utilizing a group reaction was introduced by Coggeshall and Eaton who demonstrated that an antigen from *Plasmodium knowlesi* could be used in studies of human malaria. Subsequently Kligler and Yoeli (7) using an antigen from *Plasmodium gallinaceum* found that it was nearly as effective as that attained with *Plasmodium knowlesi*.

In our investigations, comparison of the number of positive tests in control sera with those of patients known to have had malaria showed that a group reaction could be obtained in sera from patients infected by *Plasmodium vivax* when an antigen prepared from *Plasmodium gallinaceum* was used. False positive tests reached as high a figure as 5 per cent in the group of syphilitic men studied, whereas it was under 1 per cent in normal healthy men. When the results on sera from the syphilitics are grouped with our other control data, the specificity of this test for malaria appears to be somewhat less than that of the various complement fixation tests for syphilis (8), although obviously the procedures are not strictly comparable.

The sensitivity of the malaria complement fixation test was determined by dilution tests on strongly positive sera. At a dilution of 1:5, only 40 per cent of the sera were still positive, and at a dilution of 1:30, only 4 per cent were positive. It is impossible to compare these dilution tests accurately with complement fixation tests for syphilis because of differences in the character of the diseases and in the types of antigens. Nevertheless, it is of some interest that in dilution tests performed on 275 of the positive Wassermann tests in our control series of 481 syphilitic men, 55 per cent were positive at a dilution of 1:60, 33 per cent at 1:120, and 3 per cent at 1:600.

The results of the malaria complement fixation test during the 5-day period of the attacks were variable. It was possible for an individual to have negative tests throughout or in contrast to have positive tests on each of the 5 days. The number of previous attacks had no relationship to the subsequent serologic findings during a future attack. Comparison of 2 successive attacks showed that the results in each were frequently quite different and that the interval between attacks had no relation to the complement fixation results during the attack.

In the interval between attacks, the test could not be used to predict that a recurrent attack would eventually appear or that a patient was cured. In a group of 300 attacks, it was found that the maximum number of positive tests occurred during the attack and that it decreased from 58 per cent to 33 per cent within 20 days. Five days prior to an attack, the percentage of positive tests was 34. In a very small series of

22 tests on the day before an attack and in 26 tests two days before an attack, only 14 and 23 per cent, respectively, were positive.

Over a 6-month period, the number of prior attacks and the total length of infection had no appreciable effect on the number of positive tests.

Whether the antibodies of immunity are those detected by this test is not known. The decline in percentage of positive tests following the attacks may conceivably be related to a fall or disappearance of sufficient available antibodies. This might then reflect the clinical evidence of lack of immunity as indicated by subsequent relapses. Such an impression would suggest that the *plasmodia* reside in reservoirs during the intervals between attacks at which time the circulating antibodies decline quantitatively, although some may be present at all times. However, it is also possible that the antibodies utilized in this test have no relation to the problem of immunity in this disease.

In syphilis, a positive complement fixation test is usually obtained while the patient is still actively infected and this may cover a period of years but in the type of malaria studied in this investigation, an individual removed from an endemic area may still be actively infected as shown by subsequent relapses and yet intermittently have negative tests.

Successful application of a complement fixation test in recurrent malaria depends both upon the specific properties of the antigen employed and the patient's antibody response to the disease. Factors which may have influenced the antibody response of these patients during the period in which these tests were performed were: (*1*) previous residence in endemic zones under more or less continuous suppressive treatment; (*2*) previous occurrence in most of the patients of multiple attacks of malaria due to infection with *Plasmodium vivax* and/or *Plasmodium falciparum,* with possible difference in strains within these species; (*3*) variation in length of time from original infection to the time of serological examination, with tendency toward a lower rate of attack with the passage of time; and (*4*) prompt initiation of therapy during the attacks under study with resultant disappearance of trophozoites from the blood.

SUMMARY

1. Complement fixation tests using an antigen prepared from the chicken parasite *Plasmodium gallinaceum* were performed on 11,367 sera.
2. Control sera obtained from 1576 men included:
   (*1*) Sera from 1000 healthy soldiers, who had never had malaria. These were negative in 99 per cent of the tests.
   (*2*) Sera obtained on 5 successive days from 95 soldiers with febrile illnesses other than malaria. These were negative in 96 per cent of the tests.
   (*3*) Sera from 481 syphilitic men without a history of malaria. These were negative in 93 per cent of the tests.
3. Sera were obtained from 505 soldiers evacuated from the South Pacific and known to have had malaria while in that area. The results on these sera showed that:
   (*1*) From this group, 9411 sera were positive in 30 per cent of the tests.
   (*2*) There was a maximum of 58 per cent positive tests on any one of 5 successive days during 234 recurrent attacks.
   (*3*) Complement fixation tests before and after 300 recurrent attacks showed that:
      a. Five days before the attacks, 33 per cent of the tests were positive; 5 days after the attacks, 47 per cent were positive; and 20 days after the attacks, 33 per cent were positive.
   (*4*) In 121 patients followed for 6 months at 5-day intervals, 95 had recurrent attacks during the period of study, whereas, the remaining 26 had no attacks during that period. The sera from all of these men, exclusive of the period of attacks, showed that only 36 per cent of the tests were positive.
4. These results indicate that the complement fixation test using an antigen prepared from *Plasmodium gallinaceum* gives a group reaction of undetermined sensitivity for the sera of human beings infected by *Plasmodium vivax,* but is of no practical value in detecting latent malaria or indicating when a patient is cured.

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