

THE COMPLEMENT FIXATION REACTION IN MONKEY MALARIA

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A specific complement fixation reaction for malarial infections has given negative results in the hands of several workers (1-3), while with others (4-8) positive findings have been obtained. In some instances failure to obtain specific reactions seems to have been due largely to faulty technique. At the present time there is no accepted complement fixation test which is adaptable for routine use in the investigation of malarial infections. The chief difficulty encountered has been the lack of any means whereby it would be possible to obtain a standardized specific antigen. Thomson (5), in an attempt to overcome this difficulty, used a modification of Bass and Johns' (9) method of cultivation in order to obtain an antigen with a larger proportion of parasitic material. He obtained rather weak complement fixation with this material. Kingsbury (8) utilized different internal organs and heart's blood of human beings with heavy *Plasmodium falciparum* infections in an attempt to obtain an antigen of low anticomplementary power but with high specificity and sensitivity. Saline emulsions of heavily parasitized heart's blood proved to be most effective, yet the sera from 25 known cases of *falciparum* malaria fixed complement in only 48 per cent of the instances. Manson-Bahr (10) felt that a very specific antigen could possibly be obtained from the oocysts on the stomachs of infected mosquitoes. He was unable to demonstrate any complement-fixing activity with the alcoholic extract of emulsified stomachs, but the material he had to work with was limited.

No noteworthy studies dealing with the complement fixation in malaria have appeared following the above reported contributions. However, in view of the promising results obtained by some of the workers, in spite of the lack of satisfactory source of antigenic material, it seemed likely that the intense infections of *Plasmodium knowlesi* in *rhesus* monkeys would provide an abundant source of parasites for the preparation of a suitable antigen. The present study deals with the procedures employed in the preparation of various antigens and the method of performing the test. The results with sera of monkeys

tested during the acute and chronic stages of *P. knowlesi* infections indicate that specific antibodies appear in the early stages of the disease and persist during the course of the subsequent chronic infection. The variations in titer may be correlated with the presence or absence of circulating parasites.

Materials and Methods

Antigen.—The material used for antigen in the complement fixation test was prepared from the spleen or the blood of monkeys dying of infection with *P. knowlesi*. Three types of antigen have been used.

1. The infected spleens were chopped up, frozen, and dried in the frozen state in a vacuum desiccator. The dried material was then ground in a ball mill at -70°C . according to the technique described by Mudd *et al.* (11). The ground material was extracted overnight in the refrigerator with 10 cc. of saline for each gram, and the insoluble residue centrifuged down. The supernatant was used for antigen.

2. Blood containing 20 to 50 per cent of parasitized red cells was collected in 2 per cent sodium citrate solution, centrifuged to separate the serum, and washed twice with saline. The packed red cells and parasites suspended in an equal volume of saline were then frozen, dried, and ground by the same procedure as that described for the spleens. The antigen was prepared by extraction with saline.

3. The parasitized blood, washed as described above, was mixed with three volumes of distilled water, toluene was added for a preservative, and the material was allowed to autolyze in the incubator for 48 hours. The insoluble residue was centrifuged down and the supernatant used for antigen after adding enough salt solution to bring it to isotonicity. Antigen was also prepared by adding three volumes of distilled water to parasitized blood which had undergone autolysis in the refrigerator for several weeks, and then centrifuging.

The antigens prepared from blood or spleens by the methods just described do not greatly differ in the complement fixation reaction with immune sera. The antigens prepared from blood are slightly more sensitive than those prepared from spleen and have been used in all but two or three of the series of tests described in this paper. Several other methods for preparing antigen have been tried. The material extracted from infected spleen by acetone, alcohol, or ether does not give a specific fixation of complement with immune serum. The material extracted from spleens or blood with acid or alkaline buffers is not a better antigen for complement fixation than that extracted by saline or distilled water. Attempts to separate the antigen from inactive protein by tryptic digestion were unsuccessful because the antigen was rapidly destroyed.

The antigens were titrated for anticomplementary and hemolytic properties. In the test a dilution of antigen was used which was at least 4 times the dilution showing slight anticomplementary effects, and 8 times the dilution showing

marked anticomplementary action. In most cases this was a dilution of the original antigen solution of 1:10 to 1:16. The antigens did not have any marked hemolytic activity.

Sera.—Monkeys were bled at regular intervals of 7 to 10 days, and the serum was inactivated and stored in the ice box. At intervals of 6 to 8 weeks complement fixation tests were done on the series of specimens collected at different dates from each animal. Successive samples of serum were collected from seven monkeys beginning shortly after the recovery from the acute phase of the malarial infection, from three monkeys during the chronic infection with frequent relapses, and from ten superinfected monkeys (12) with long standing infections and infrequent relapses.

Method of Performing the Complement Fixation Test.—The hemolytic system consisted of 5 per cent sheep cells and anti-sheep rabbit serum. The unit of amboceptor was taken as the smallest amount which produced complete hemolysis in the presence of an excess of complement (fresh or frozen and dried guinea pig serum). The complement was titrated on each day before the tests were set up, using 2 units of amboceptor. Complement having a unit greater than 0.15 cc. of a dilution of 1:10 was discarded. The complement fixation test was set up as follows:

0.2 cc. serum undiluted, 1:2, 1:4, 1:8, etc.

2½ units of complement (0.35 cc. or less of 1:10 dilution).

0.25 cc. antigen diluted as described.

Controls:

1. Normal serum with antigen and complement.
2. Immune serum undiluted, 1:2, 1:4, 1:8, etc., with complement and 0.25 cc. saline in place of antigen (serum control).
3. Antigen at dilution used in test with complement and 0.2 cc. saline in place of serum (antigen control).

Incubate for 1 hour at 37°C. in the water bath and add 0.5 cc. of a mixture of equal volumes of 5 per cent sheep red cells and amboceptor diluted so that 0.25 cc. contains 2 units.

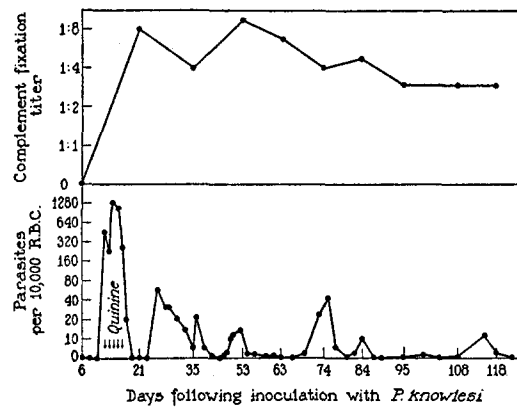
After adding the hemolytic system the tests are read as soon as the controls have cleared, usually between 15 and 30 minutes. Since monkey sera, undiluted or diluted 1:2, are frequently anticomplementary, the serum controls may not clear completely. In this case the test is read after 30 minutes if the controls with antigen and normal serum show complete hemolysis.

Because of the anticomplementary properties of monkey serum, it is necessary to sacrifice some sensitivity in the test by using 2½ units of complement instead of 2 or 1½ units. The excess of complement may cause weak reactions to fade quickly so that it is necessary to follow the progress of hemolysis rather closely. On the other hand, reactions with strongly positive sera do not change appreciably after long standing.

The titers of the sera in the tests are recorded as the highest dilution of serum which gave a fixation of complement demonstrable by the presence of unhemolyzed

red cells. In the graphic representation of the titers used in this paper, the location of the points on the graph was sometimes determined by interpolation. For example, if a serum gave a +++ reaction at 1:8 and a ± at 1:16, the point was placed midway between 1:8 and 1:16 on the graph. If the reaction was + at 1:8, the point was placed exactly at 1:8, but if the reaction was ++ at 1:8 and - at 1:16, the point was placed slightly above 1:8. When the sera were definitely anticomplementary, it was also necessary to make allowance for this. Thus when a serum gave a reaction equivalent to ++ or greater at 1:2 in the serum control without antigen, the titer was considered to be half of the figure indicated by the test with antigen. When the sera were anticomplementary at 1:4, the test was considered unsatisfactory and the results have not been used.

Parasite Counts.—The intensity of the infection and the occurrence of relapses were determined by making parasite counts in blood smears from the infected



TEXT-FIG. 1. Monkey B9.

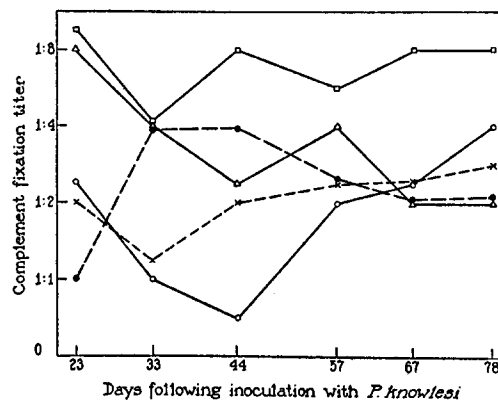
monkeys at intervals of 1 to 3 days. These are recorded as the number of parasitized cells per 10,000 red blood corpuscles.

Appearance of Complement-Fixing Antibodies in the Sera of Monkeys after Recovery from Acute Infection

The changes in parasite count and the complement fixation titer of the serum of monkey B9 treated with quinine are shown in Text-fig. 1. At 21 days, when the animal had recovered from the acute infection, the complement fixation titer was relatively high. Between 21 and 35 days the titer fell despite the constant presence of parasites in the blood. At 53 days the titer had risen again after a period of low grade infection followed by a relapse. In the next 2 months the

titer gradually fell despite the occurrence of three definite relapses with intervening low grade infection.

The titers of the sera of seven monkeys which had been treated by daily injections of 2 cc. of immune serum for 10 days after the injection of parasites were also followed in a similar manner. The results for five of the monkeys are shown in Text-fig. 2. Two monkeys in this group had traces of complement-fixing antibodies between the 23rd and 78th days after infection, but since their sera were quite anticomplementary, the results are not included in the graph. Four of the curves are similar in form to that shown in Text-fig. 1. At 23 days the titer was high, and this was followed by a decline to the 33rd day.



TEXT-FIG. 2. ●, monkey 5-8; ○, monkey 5-9; ×, monkey 6-0; □, monkey 6-4; △, monkey 6-5.

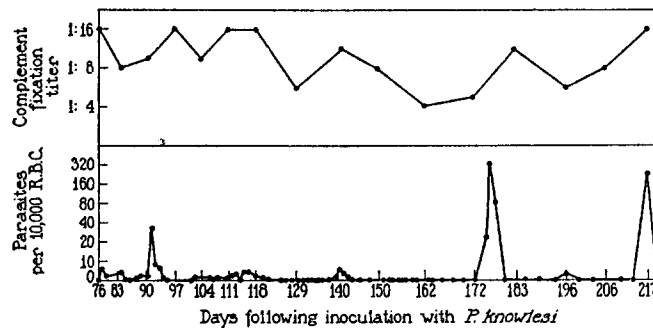
It is possible that part of the complement-fixing antibodies found in these sera on the 23rd day came from the injected immune serum, the last dose of which had been given 13 days previously. One of the sera (No. 5-8 from a monkey which also received immune serum) showed a low titer on the 23rd day, followed by a rise.

The secondary rise in titer and the subsequent gradual leveling off seen in the curve for the serum of the monkey treated with quinine may also be seen in four of the curves in Text-fig. 2 in the corresponding stages of the infection. The somewhat different response in monkey 5-8 may have been due to the fact that parasites did not appear in the blood of this animal until the 13th day after infection,

while the others became positive for parasites between the 4th day and the 8th day. The two monkeys which developed only traces of complement-fixing antibodies had infections which were as heavy and prolonged as those of the other animals.

Changes in the Complement Fixation Titer during the Chronic Relapsing Phase of Plasmodium knowlesi Infection

As indicated in the preceding section, the complement fixation titer of the sera of monkeys infected with *P. knowlesi* reaches a relatively constant level 2 to 3 months after the animals have recovered from the acute infection. This level is apparently maintained by the occurrence of repeated relapses with the appearance of a considerable



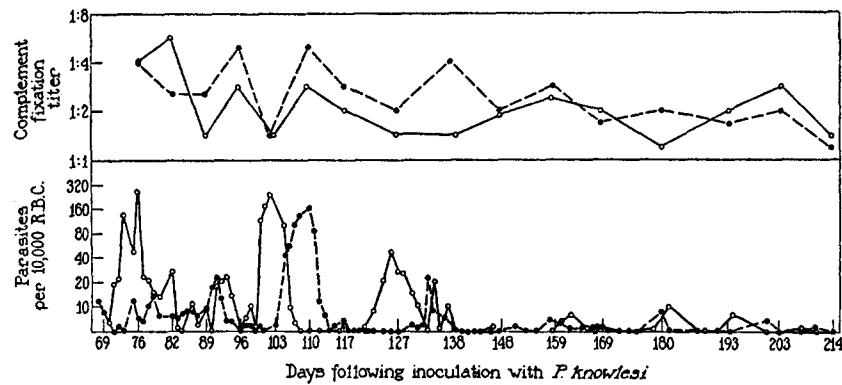
TEXT-FIG. 3. Monkey B2.

number of parasites in the blood. Different monkeys show wide individual variations in the level at which the complement-fixing antibodies are maintained.

During the chronic stage of the infection the rise and fall of the complement fixation titer of the serum appears to be related to the occurrence of relapses. This is illustrated in Text-figs. 3 and 4. Monkey B2 (Text-fig. 3) during the period of observation had three major relapses, on the 91st, 177th, and 217th days, respectively, and three minor relapses, on the 104th to 118th days, the 140th, and the 196th days, respectively. A fall in the curve for complement fixation titer preceded each of the six relapses, and a rise in the curve accompanied or closely followed each relapse. Since the points for the complement fixation titer are placed on the graph at intervals of 7

to 12 days, while the points for the parasite count are placed at intervals of 1 to 3 days, an exact conformity of all parts of the curves cannot be expected. For this same reason it is possible that some of the higher peaks on the complement fixation curve may have been missed.

Similar results are shown in Text-fig. 4, but here certain exceptions may be noted. Between the 76th and 89th days the sera of the two monkeys showed a fall in complement fixation titer which was accompanied by the almost constant presence of parasites in the blood. Between the 89th and 96th days peaks in the parasite count were followed by peaks in the curves for complement fixation. In monkey 3-9 on the 103rd day a fall in the curve followed a rise in the parasite



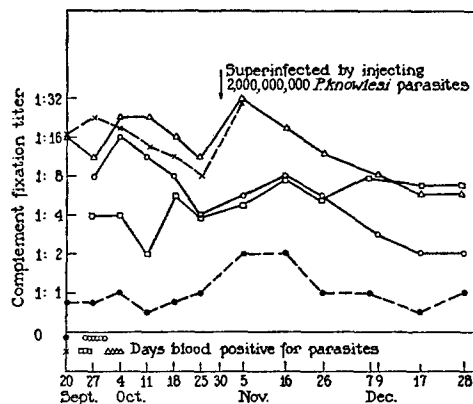
TEXT-FIG. 4. ●, monkey 4-0; ○, monkey 3-9.

count, but with the disappearance of parasites from the blood on the 110th day the complement fixation titer had risen again. In this same monkey there was no response to a major relapse on the 126th day, but later a rise in the titer followed several smaller relapses. The curves for monkey 4-0 show a relation between parasite count and rise and fall of complement fixation titer similar to that for monkey B2 (Text-fig. 3).

Effect of Superinfection on the Titer of Complement-Fixing Antibodies

A group of ten monkeys with chronic malarial infections ranging in duration from 8 to 13 months were tested at regular intervals over a period of 2 months for complement-fixing antibodies. Each of the

group of monkeys in Text-fig. 5 had a relapse some time during the period of 2 weeks from September 20 to October 4, and in each case the relapse was followed by a rise in the complement fixation titer, except in monkey 4 in which the rise was preceded by a slight fall in titer. In these animals the relapses are seldom severe. Frequently only one to five parasites per 10,000 red corpuscles are found over a period of a few days. This seems to be sufficient to cause as great a rise in the complement fixation titer as a more severe relapse in a monkey having chronic malaria of shorter duration (compare Text-figs. 4 and 5).

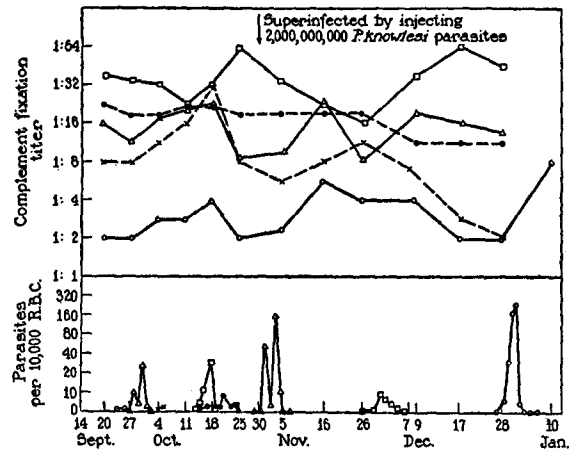


TEXT-FIG. 5. ●, monkey 2; ○, monkey 3; ×, monkey 1-4; □, monkey 4; △, monkey 5.

On October 30 each of the monkeys in the group was superinfected by the injection of blood containing approximately 2 billion parasites into the peritoneal cavity. All five animals had responded by November 5 with an increase in complement fixation titer. This rise in titer persisted in monkeys 2, 3, and 4 for 2 weeks and in No. 5 for only 1 week. Monkey 1-4 died as a result of bleeding. Following the rise after the superinfection, the curves tend to fall to constant levels.

The results with the other five monkeys in this group are shown in Text-fig. 6. Three of these monkeys each had one or more relapses of moderate severity. Monkey 7 had relapses on the 18th of October and 30th of November, each of which was followed by a sustained rise in the complement fixation titer, but this monkey failed to respond

with increased titer to the superinfection which was given shortly after a relapse. Monkey 1-2 showed no rise in titer as a result of a relapse on October 23 and superinfection a week later. In this monkey the level of complement-fixing antibodies remained relatively constant over the period of 2 months. Monkey 6 had a relapse on October 1 and another on the 1st to 5th of November immediately after superinfection. Each of these relapses was followed by a rise in the complement fixation titer. This monkey also showed a secondary rise on December 7 which was not accompanied by demonstrable parasites in the blood. Monkeys 8 and 9 showed rises in comple-



TEXT-FIG. 6. ●, monkey 1-2; ○, monkey 8; × monkey 9; □, monkey 7; △, monkey 6.

ment fixation titer following mild relapses and after superinfection, thus resembling in behavior the five monkeys in Text-fig. 5. Monkey 8 also had a severe relapse on December 30 which was followed by a sharp rise in the curve. In all of these curves there is a definite indication that the complement fixation titer tends to fall off gradually when the blood remains free of parasites for a long period of time.

DISCUSSION

In general, the best antigens were obtained from blood containing a high proportion of mature parasites, or from spleens taken from

monkeys with intense infections. In order to extract the antigen in a soluble form, it was apparently necessary to break down the parasites as completely as possible. It was found that freezing, drying, and grinding the parasitic material or permitting it to autolyze was sufficient to liberate a soluble antigen extractable with normal saline solution. Since the antigen was not extractable by lipoid solvents and was destroyed by tryptic digestion, the active principle behaves as a protein.

Monkeys treated in the acute stage of the infection with quinine or immune serum showed a similar response in the production of complement-fixing antibodies. The titer rises promptly after an acute attack and then proceeds to drop. There is a secondary rise which tends to level off, and subsequently the titer, whether high or low, seems to remain at a rather uniform level for any particular animal. During the chronic stage of the infection when major and minor parasitic relapses appear at irregular intervals, the titer is low immediately preceding a relapse and is elevated after the relapse has terminated. The severity of the relapse appeared to have no correlation with the complement-fixing titer. The continued presence of large numbers of parasites in the blood as the result of repeated relapses apparently may produce an effect on the titer opposite from that observed when relapses appear at longer intervals of time. The fall in complement fixation titer observed in those monkeys with frequent relapses is probably due to the removal of circulating antibody or to the exhaustion of the mechanism responsible for antibody production.

There is a wide variation among different monkeys in the degree of production of complement-fixing antibodies. Some animals maintain the titer at relatively high levels; others produce practically no antibodies detectable by complement fixation. Similarly, some animals show a prompt and extensive rise after a relapse or superinfection while others show a delayed response or none at all. The sera used in this study were taken from monkeys which had previously served as the source of serum in the demonstration of protective antibodies (13). It was noted in some animals that the complement fixation titer was low and the titer of the protective antibodies was high; also the sera of others having high complement fixation titers showed

little protective effects. However, in the individual animals any factor which influences the level of protective antibodies may also influence the corresponding level of the complement-fixing antibodies. This assumption has been experimentally demonstrated in monkeys which have been tested for complement-fixing and protective antibodies immediately before and after relapses and will be described in more detail in a later report. By analogy there is also an apparent relationship between these two antibodies, as a relapse is followed by an increase in the titer of complement-fixing antibodies; and as the animal is able to overcome the relapse spontaneously, there must be an increase in the concentration of protective antibodies. There is no evidence to show that complement-fixing antibodies and protective antibodies are identical.

SUMMARY

1. A specific complement fixation reaction test for *Plasmodium knowlesi* malaria in *rhesus* monkeys is reported with details involved in the preparation of the antigen and procedures employed in setting up the test.

2. It was found that specific complement-fixing antibodies appeared early in the course of the experimental disease and persisted during the course of the chronic infection.

3. The first appearance of complement-fixing antibodies was generally followed by a temporary fall in titer. During the first 2 months of infection there was no apparent relationship between the number of circulating parasites and the changes in complement fixation titer.

4. During the stage of chronic infection there was a fall in the titer of complement-fixing antibodies preceding each parasitic relapse, and after the relapse had terminated, there was an elevation of the complement-fixing titer.

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