

EFFECT OF REPEATED SUPERINFECTION UPON THE  
POTENCY OF IMMUNE SERUM OF MONKEYS  
HARBORING CHRONIC INFECTIONS OF  
PLASMODIUM KNOWLESI

BY LOWELL T. COGGESHALL, M.D., AND HENRY W. KUMM, M.D.

(From the Laboratories of the International Health Division, The Rockefeller  
Foundation, New York)

(Received for publication, April 13, 1938)

In a previous communication (1) it was reported that pooled serum obtained from monkeys with chronic infection of *Plasmodium knowlesi* contained protective antibodies as evidenced by the ability to protect normal animals from death due to infection produced by the injection of homologous parasites. The fact that large amounts of immune serum are necessary to produce an inhibitory effect upon the course of the experimental disease was taken as an indication that the antibodies were present in the serum of such animals in a rather low concentration. In addition, it was observed that the potency of the immune serum decreased progressively after repeated bleedings of the same donor animals. The purpose of this paper is to report attempts made to increase the potency of the immune serum in monkeys with chronic infections resulting from single or multiple injections of massive doses of homologous parasites.

*Materials and Methods*

Fifteen *rhesus* monkeys whose original acute attacks of *P. knowlesi* malaria had assumed a chronic form for a period of at least 8 months were used as source animals for the immune serum. These animals had been bled repeatedly for immune serum in earlier experiments (1). It was noticed, however, that after each bleeding there was an apparent diminution in the protective property of their serum. After about a month's rest from previous bleeding these animals were given a series of intravenous injections of massive doses of parasites at various time intervals. The parasites used for superinfection were obtained from a number of monkeys which were suffering from acute attacks of *P. knowlesi* infection, and they were bled shortly before death when their parasite count in the blood

was at its maximum density. The immune serum obtained from the superinfected animals was either pooled or kept separately as required for the particular experiment. For protection tests normal *rhesus* monkeys of approximately equal age and weight were used. The number of parasites used for the inoculum in each protection test was approximately 1,500,000, and the amount of infected blood necessary to contain this number was computed beforehand by making a simultaneous red blood cell and parasite count upon the source monkey. In the first few protective tests immune serum and parasites were injected separately into the abdominal cavity of the monkeys. In one experiment the two components were mixed and incubated at 37°C. for 30 minutes before injection. Parasite counts were made daily on all animals under observation by means of thin blood smears.

#### EXPERIMENTAL

*Experiment 1.*—The *rhesus* monkeys harboring chronic infections of varying durations were divided into 2 groups. Group 1 consisted of 8 monkeys, Nos. 1 to 8 inclusive, and group 2 of 7, Nos. 9 to 15 inclusive. The animals in group 1 were given a course of hyperimmunization which consisted of 7 injections of massive doses of parasites. These injections were given intravenously at approximately 7 day intervals, and the doses varied from 5 to 7 billion parasites. The monkeys in group 2 were allowed to continue their chronic course without intervention and were intended to serve as controls. 6 days after the last injection of parasites into group 1, the animals of both groups were bled and their serum collected into 2 respective pools. The serum collected from monkeys in group 1 was designated as pool 1, and that from group 2, pool 2. The following comparative protection experiment was carried out with these 2 pools.

As shown in Table I and Charts 1 and 2, 10 normal *rhesus* monkeys, Nos. 33 to 42 inclusive, were each inoculated with 1,500,000 *P. knowlesi* parasites. Immediately following the injection of the parasites and daily thereafter, 3 of the monkeys, Nos. 33, 34, and 35, were given intraperitoneally hyperimmune serum of pool 1 in amounts of 10 cc., 5 cc., and 2.5 cc. respectively, while a fourth monkey, No. 36, received 1 cc. of the same serum at similar intervals intravenously. Another group of 4 monkeys, Nos. 37, 38, 39, and 40, were each given serum of pool 2 in corresponding amounts and at the same intervals as those which received pool 1. Two additional control monkeys, Nos. 41 and 42, were given no serum.

As seen from Table I, the use of the immune serum in pool 1, which came from the superinfected group of monkeys, was followed by a very marked protective action. Monkey 33, which received 10 cc. of this serum daily for 10 days, never showed any detectable parasites in the peripheral blood. As a further proof for the protective action of this serum, blood from this monkey was subinoculated into normal

monkeys on six separate occasions at approximately weekly intervals during and following the protection test, and none of these developed malaria. Monkey 34 received 5 cc. of serum daily for 10 days, and parasites were first detected in its blood smear only on the 20th day after the original inoculation and reached a peak of 13 per 10,000 red blood cells on the 28th day. Monkey 35, which received 2.5 cc. of the same serum for 10 days, first showed parasites on the 13th day following inoculation; they reached a peak of 55 per 10,000 red blood cells on the 19th day, and then subsided into a chronic infection. Monkey

TABLE I

*Experiment 1*

Ten normal monkeys each inoculated with 1,500,000 parasites and immune serum of pools 1 and 2 given in doses and at intervals as follows:

Monkey No.	Injection of immune serum		Results
	Pool	Dose and route	
33	1	10 cc. i. p. daily for 10 days	Survived
34	1	5 cc. i. p. daily for 10 days	Survived
35	1	2.5 cc. i. p. daily for 10 days	Survived
36	1	1 cc. i. v. daily for 10 days	Died on 10th day
37	2	10 cc. i. p. daily for 10 days	Died on 14th day
38	2	5 cc. i. p. daily for 10 days	Died on 12th day
39	2	2.5 cc. i. p. daily for 9 days	Died on 10th day
40	2	1 cc. i. v. daily for 7 days	Died on 8th day
41		Control, no serum	Died on 7th day
42		Control, no serum	Died on 9th day

i. p., intraperitoneally.

i. v., intravenously.

36, which received only 1 cc. of serum intravenously for 10 consecutive days, died on the 10th day.

While serum of pool 1 showed definite protective properties, that of pool 2 showed only a slight inhibitory effect upon the course of the experimental disease in monkeys in which it was tested. As shown in Table I, monkey 37, which received the greatest amount of this serum, died on the 14th day following inoculation, and monkey 38, which had been given 5 cc. of serum for 10 days, died on the 12th day. Monkeys 39 and 40, which received 2.5 cc. and 1 cc. respectively, died

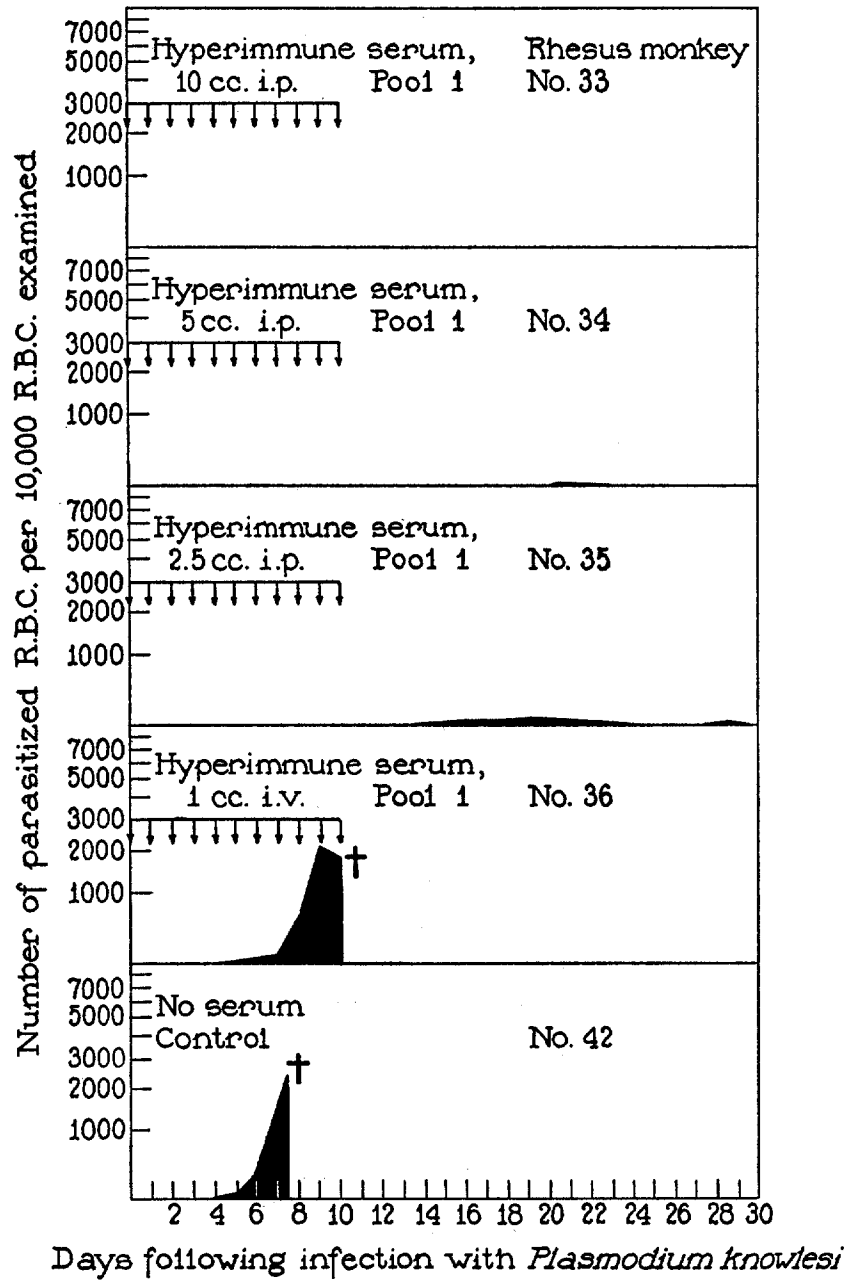


CHART 1

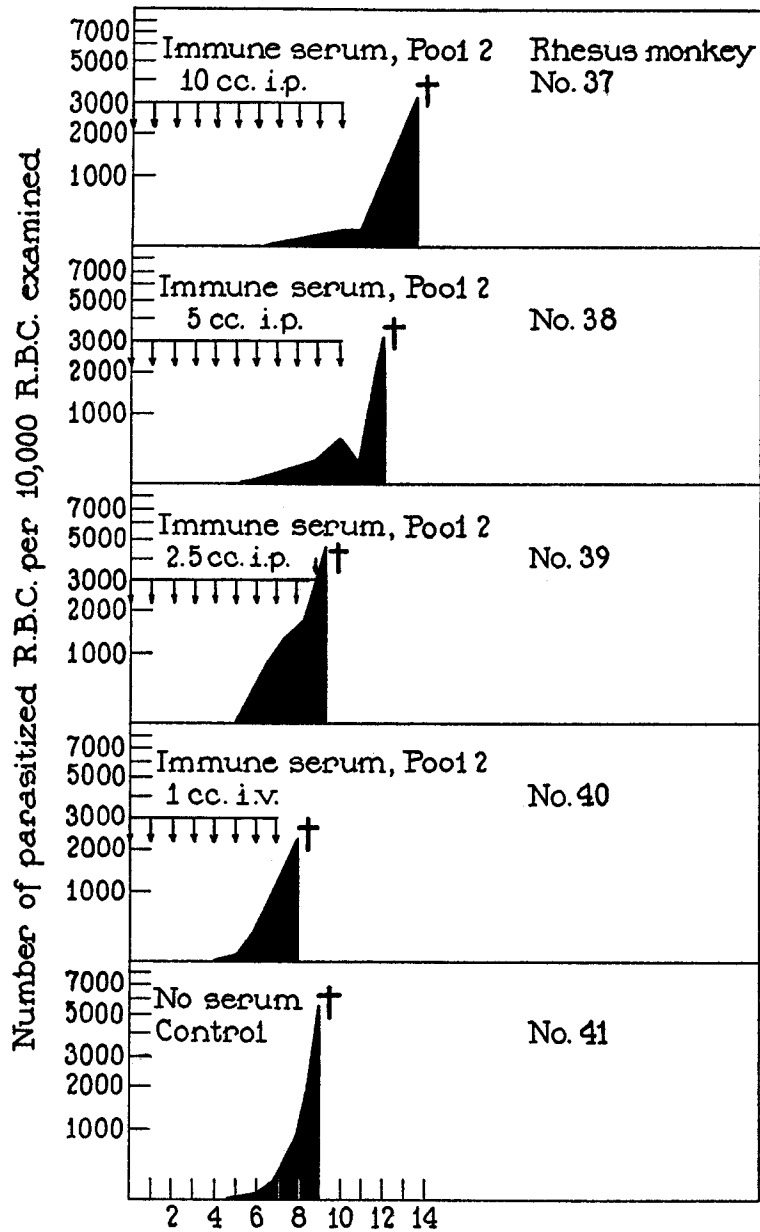


CHART 2

practically at the same time as the control monkeys, Nos. 41 and 42, which received no serum.

The results of this experiment indicate that the potency of the serum obtained from monkeys with chronic infections can be markedly increased by the repeated injections of large numbers of parasites.

*Experiment 2.*—The experiment above was repeated as follows: 4 monkeys of group 2, which harbored chronic infection and served as the source of serum for pool 2 used in the preceding experiment, were subjected to a prolonged course of hyperimmunization. At 6 day intervals they were given a total of 9 injections of parasites in doses varying from 2 to 13 billion. The total time required for this course of hyperimmunization was 2 months. On the 6th day after the last

TABLE II

*Experiment 2*

Five normal monkeys each inoculated with 1,213,000 parasites and immune serum of pool 3 given in doses and at intervals as follows:

Monkey No.	Injection of immune serum, dose, and route	Results
43	5 cc. i. p. daily for 10 days	Died on 12th day
44	2.5 cc. i. p. daily for 10 days	Died on 12th day
45	1 cc. i. p. daily for 10 days	Died on 12th day
46	1 cc. i. v. daily for 10 days	Died on 11th day
47	Control, no serum given	Died on 9th day

injection they were bled and the serum pooled, which was designated as pool 3. A protection test was set up with this pool as follows:

Five normal monkeys, Nos. 43 to 47 inclusive, were each inoculated intravenously with 1,312,000 parasites. Immediately after the injection of parasites and daily thereafter for a period of 10 days, monkeys 43, 44, and 45 were given 5 cc., 2.5 cc., and 1 cc. respectively, of serum from pool 3 intraperitoneally, and monkey 46 was given 1 cc. of same serum and at similar intervals intravenously. Monkey 47 received no serum and served as control. The results of this experiment are shown in Table II. It will be noted that this pool of serum showed practically no protective property as there was only a slight delay in the death of the animals which received daily doses of immune serum as compared with the control animal. Because of the inadequate amount of serum available, 10 cc. doses were not used in this experiment.

*Experiment 3.*—Seven monkeys with chronic infection, Nos. 2 to 8 inclusive, which in Experiment 1 had been included in group 1 and given a series of injections of massive doses of parasites, were now subjected to a second course of hyper-

immunization. Following a rest period of 1 month after the first course of hyperimmunization and bleeding, these monkeys were given 9 additional injections at 6 day intervals in doses varying from 2 to 13 billion parasites. On the 6th day after the last injection they were bled and the protective property of the serum of each tested separately as follows:

Eight normal monkeys, Nos. 48 to 55 inclusive, were each inoculated with 1,350,000 parasites. Seven of these were given serum from an equal number of hyperimmunized monkeys, while the eighth animal served as control. As shown in Table III, the serum of each hyperimmunized monkey was tested separately in a single animal; it was given intraperitoneally in 1 or 2 cc. amounts, beginning immediately after the injection of the parasites, and daily thereafter for 10 days, or until the death of the test animal.

TABLE III

*Experiment 3*

Eight normal monkeys each inoculated with 1,350,000 parasites. Immune serum from individual monkeys given intraperitoneally in doses and at intervals as follows:

Monkey No.	Source and dose of immune serum injected	Results
48	Serum from monkey 8 given 2 cc. daily for 10 days	Survived
49	Serum from monkey 4 given 2 cc. daily for 10 days	Died on 11th day
50	Serum from monkey 5 given 2 cc. daily for 9 days	Died on 9th day
51	Serum from monkey 6 given 2 cc. daily for 9 days	Died on 9th day
52	Serum from monkey 7 given 2 cc. daily for 8 days	Died on 8th day
53	Serum from monkey 3 given 1 cc. daily for 8 days	Died on 8th day
54	Serum from monkey 2 given 1 cc. daily for 8 days	Died on 8th day
55	Control, no serum given	Died on 8th day

The results are summarized in Table III. It will be noted that with a single exception all test monkeys died of malaria, showing that the serum of the hyperimmunized monkeys had no inhibitory effect upon the experimental disease. One animal, monkey 48 which received serum from hyperimmunized monkey 8, suffered a mild attack of malaria and survived. This monkey died a month later of generalized tuberculosis. The negative results of this experiment indicated that the monkeys had been excessively superinfected.

*Experiment 4.*—The results above indicate that immune serum of relatively high potency was obtained from monkeys after a series of 7 injections of massive doses of parasites in Experiment 1. On the other hand, the serum of the same animals, after a more intensive course of hyperimmunization consisting of 9

injections given at similar intervals and in somewhat larger doses, had practically no protective property. It was now considered of interest to determine the effect of a single injection of parasites upon the antibody content of the serum of the same animals. Accordingly, a group of 10 monkeys harboring chronic infection were chosen as the source of immune serum. Seven of these, Nos. 2 to 8 inclusive, had already been subjected to two courses of hyperimmunization as described in Experiments 1 and 5, while three, Nos. 9, 12, and 14, had received a single course of 9 injections in Experiment 2. These 10 animals were allowed a rest of 1 month after their last hyperimmunization and bleeding and were then

TABLE IV  
*Experiment 4*

Eleven normal monkeys each inoculated with 1,500,000 parasites. Immune serum from individual monkeys given intraperitoneally in doses and at intervals as follows:

Monkey No.	Source and dose of immune serum injected	Results
56	Serum from monkey 14 given 2 cc. daily for 10 days	Survived
57	Serum from monkey 4 given 2 cc. daily for 10 days	Died on 16th day
58	Serum from monkey 2 given 2 cc. daily for 10 days	Survived
59	Serum from monkey 5 given 2 cc. daily for 10 days	Survived
60	Serum from monkey 12 given 2 cc. daily for 10 days	Survived
61	Serum from monkey 8 given 2 cc. daily for 10 days	Survived
62	Serum from monkey 3 given 2 cc. daily for 10 days	Survived
63	Serum from monkey 6 given 2 cc. daily for 8 days	Died on 8th day
64	Serum from monkey 9 given 2 cc. daily for 10 days	Survived
65	Serum from monkey 7 given 2 cc. daily for 10 days	Survived
66	Control, no serum given	Died on 10th day

given a single injection of 7 billion parasites. One week later all were bled and the sera tested separately for protective antibodies as follows:

To 2 cc. of serum from each hyperimmune monkey were added 1,500,000 parasites and the mixtures incubated for 30 minutes at 37°C., after which each mixture was injected intraperitoneally into a separate normal monkey, while a control monkey received the same number of parasites incubated with 2 cc. of normal monkey serum. As shown in Table IV, the 10 test animals, Nos. 56 to 65 inclusive, each received 2 cc. of serum from a separate hyperimmune monkey daily for a period of 10 days following the injection of parasites. This also includes the amount mixed and incubated with the parasites at the time of inoculation. No subsequent injections of normal serum were given to the control monkey, No. 66.

The results are summarized in Table IV, and it will be seen that only 2 of the test animals died while 8 survived. The serum of mon-



keys 4 and 6 exhibited hardly any protective properties, although it must be pointed out that both of these monkeys suffered a relapse with a considerable number of parasites in the circulating blood at the time they were bled. In a later experiment the residue of serum from monkeys 3 and 12 was tested in a similar manner and against a similar number of parasites, except that the parasites and serum were injected separately. The test animal survived, indicating that the incubation of parasites and serum together had no influence upon the results of the above experiment.

#### DISCUSSION

A fundamental point in the study of immunity in malaria is the response of an animal with a chronic infection to superinfection. In the literature in this field are numerous reports which show that the superinfection of any animal with a chronic malaria infection by the injection of the homologous parasites results in a very rapid removal of the organisms from the blood stream (2-4). Information regarding the humoral immunity response to the injection of massive doses of homologous parasites is totally lacking. However, with numerous other infectious diseases it has been shown that the antibody titer of an animal can be elevated by hyperimmunization.

The results obtained in this experimental study indicate that the protective properties of immune serum of monkeys with chronic infections could either be increased or decreased by superinfection. An analysis of the outcome of the individual experiments deserves certain comment. In Experiment 1 it was noted, in the two comparable groups of immune monkeys, that those hyperimmunized had a much more potent protective serum than those which had been allowed to rest without superinfection. Of special interest was monkey 33 (Table I) in this experiment which was completely resistant to infection after receiving serum from the superinfected group. 6 weeks after the original inoculation this monkey, No. 33, was re-inoculated with exactly the same number of parasites as previously used. The second inoculation resulted in infection and death on the 19th day, which suggested that the animal had acquired no active immunity from the original inoculation but had retained enough passively acquired immunity to prolong the time of death.

The results of Experiment 2 and Experiment 3 show that after 9

superinfections the pooled sera of the four monkeys superinfected for the first time were no more potent than had been observed in Experiment 1 and that the sera from the 7 monkeys receiving the second course of superinfection and tested separately protected in only one instance. The survival of monkey 48 in this experiment cannot be attributed solely to the action by immune serum as it died of generalized tuberculosis 1 month after inoculation. It has been repeatedly observed that tuberculosis has an inhibitory effect on the course of *P. knowlesi* infection in monkeys.

The negative results of this experiment were unexpected. In view of the fact that the serum of the monkeys with chronic infection after the first course of hyperimmunization had shown a relatively high protective property, it had been expected that after the second course this property would be as high or even considerably higher. The only explanation that occurred to us as plausible for the failure was that repeated flooding of the animals with parasites apparently had exhausted most of the specific antibodies. This view is supported by the fact that, after the last injection of parasites and at the time of bleeding, most of the animals had a parasitic relapse, an occurrence which was absent during the course of hyperimmunization.

Experiment 4 showed that after a single superinfection all but 2 animals of those which had been used throughout as a source of immune serum showed protective antibodies. The blood of these 2 animals showed parasites at the time they were bled for the protection test, while the remaining 8 showed no parasites in their peripheral blood following the single superinfection.

We feel that the results of the present experimental work indicate that it is possible to influence the immune state of a monkey with chronic *P. knowlesi* infection by superinfection. The exact degree of alteration is difficult to state because at the present time there are no serological tests which will aid in the determination of protective antibody concentration other than the unwieldy and expensive monkey protection test.

#### SUMMARY

Protection tests have been utilized to determine the effect of superinfection upon the potency of immune serum of monkeys with chronic

*Plasmodium knowlesi* infections. The results of these tests showed that:

1. In 2 groups of monkeys with comparable *P. knowlesi* infections the immune serum of 8 monkeys which had been superinfected on 7 separate occasions over a period of 2 months was much more potent than the immune serum of a group of 7 monkeys which were allowed to continue their chronic course of infection without superinfection.

2. After a series of 9 more intense superinfections the serum from the same 2 groups of monkeys contained no demonstrable protective antibodies.

3. The serum from 8 of the 10 monkeys in the original 2 groups showed a relatively high concentration of protective antibodies following a month's rest and a single superinfection.

4. The results of the experiments indicate that it is possible to increase the potency of immune serum by superinfection, but it is also possible to obtain a decrease in the protective property of the serum by too severe superinfections.

#### BIBLIOGRAPHY

1. Coggeshall, L. T., and Kumm, H. W., *J. Exp. Med.*, 1937, **66**, 177.
2. Taliaferro, W. H., and Taliaferro, L. G., *J. Prevent. Med.*, 1929, **3**, 197.
3. Mulligan, H. W., and Sinton, J. A., *Rec. Malaria Survey India*, 1933, **3**, 809.
4. Nauck, E. G., and Malamos, B., *Z. Immunitätsforsch.*, 1935, **84**, 337.