THE QUANTITATIVE RELATIONSHIP BETWEEN IMMUNE SERUM AND INFECTIVE DOSE OF PARASITES AS DEMONSTRATED BY THE PROTECTION TEST IN MONKEY MALARIA

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In demonstrating the existence of protective antibodies in the serum of *rhesus* monkeys with chronic *Plasmodium knowlesi* infections (1) it was noted that a relatively large amount of immune serum was necessary to produce passive protection and that the use of an excessive number of parasites for the initiation of infection obscured the protective action of the serum. On the basis of these results it seemed important to determine whether a quantitative relationship exists between the amount of immune serum and the dosage of parasites.

The present study is concerned with the determination of the minimal infective dose of *P. knowlesi* in normal *rhesus* monkeys and the effect of varying the number of parasites and the amounts of immune serum used in protection tests. It has been repeatedly demonstrated in many of the infectious diseases that it is impossible to obtain a protective effect from a very potent immune serum if an excess of the infecting agent is used. In malaria, information on this subject is very meagre. Findlay (2) working with malaria in canaries has shown that an increase in the length of incubation time occurred when immune serum and parasites were injected into normal canaries as compared to normal canary serum and parasites. He also noted a progressive increase in incubation time with dilutions of parasites from 1:1 to 1:64.

Materials and Methods

Rhesus monkeys were used exclusively in this work, and all inoculations were made with a strain of *P. knowlesi* which had been under observation in this laboratory since 1933. In order to determine the minimal infective dose, a monkey

with a low grade chronic infection and a parasite count between 10 and 100 per 10,000 red cells was selected. Blood was collected in sodium citrate from the donor animal shortly after sporulation so that all parasites were present in the red cells as young rings. This was done to obviate an increase in the number of parasites in an inoculum due to rupture of segmenting forms. The number of parasites per cubic centimeter of blood was calculated from the red cell count and the parasite count. The citrated blood, immediately after being collected, was diluted in a medium containing equal parts of whole blood from a normal monkey and 2 per cent citrate solution. Results were unsatisfactory when only normal saline solution was used to dilute the infected blood since parasites were destroyed in the higher dilutions. The dilutions used in this study contained 100,000, 10,000, 1,000, 100, 10, and 1 parasites per cc. and 1 parasite per 10 cc. All inoculations were given by injecting 1.0 cc. of each dilution intraperitoneally.

In the experiments designed to show the quantitative relationship between the amount of immune serum and the number of parasites, dilutions of infected blood were made in the same way as in the experiments to determine the minimal infecting dose. The immune serum to be used was obtained from monkeys with long standing chronic infections and pooled so that there was a sufficient amount available for all desired tests. In the first protection experiment the amount of immune serum was kept constant and the number of parasites in the inoculum varied between 1,000,000 and 100. 2 cc. of serum and 1 cc. of each of the dilutions of parasites were incubated together at 37°C. for half an hour before injection, and 2 cc. of serum were administered daily for 9 successive days thereafter.

In the next experiment the same pool of immune serum was used in varying amounts from 2.0 cc. to 0.125 cc., but the number of parasites in the inoculum was kept constant. The monkeys with chronic infections which served as the source of immune serum were bled the second time after a 6 weeks' interval. The pool of serum obtained from this bleeding was set up in one experiment in which there were variations both in the number of parasites and the amount of immune serum.

EXPERIMENTAL

Experiment 1. Determination of the Minimal Infective Dose.—Eight normal monkeys, Nos. 1 to 8 inclusive, were used in this experiment. As shown in Table I, monkey 1 was inoculated with 100,000 *P. knowlesi* parasites, monkey 2 was inoculated with 10,000 parasites, while duplicate monkeys were inoculated with 1,000, 100, and 10 parasites. The results of these inoculations were infection and death in all 8 monkeys. Monkey 1, which received the largest dosage, was the first to show circulating parasites on the 5th day and died on the 9th day. There was a gradual increase in incubation time and the time of death as the number of parasites in the inoculum was decreased. Monkeys 7 and 8, which received the final dilution containing 10 parasites, first showed circulating parasites on the 9th and 13th days and died on the 13th and 17th days respectively.

Since all of the monkeys became infected and died, it was decided to repeat the experiment but to start with 1,000 parasites and dilute

so that the least number was 0.1 parasite (or 1 cc. of a dilution containing 1 parasite per 10 cc.), a theoretical number where infection should not occur.

Nine monkeys were used in the repeat experiment, and, as shown in Table II, monkey 9 was inoculated with 1,000 parasites, monkey 10 was inoculated with

TABLE I

Determination of Minimal Infective Dose of Plasmodium knowlesi Injected Intraperitoneally into Rhesus Monkeys

Monkey No.	Number of parasites injected	Day parasites appeared in blood stream	Results
1	100,000	5th	Died on 9th day
2	10,000	6th	Died on 10th day
3	1,000	7th	Died on 12th day
4	1,000	7th	Died on 11th day
5	100	7th	Died on 12th day
6	100	8th	Died on 13th day
7	10	9th	Died on 13th day
8	10	13th	Died on 17th day

TABLE II

Determination of Minimal Infective Dose of Plasmodium knowlesi Injected Intraperitoneally into Rhesus Monkeys

Monkey No.	Number of parasites injected	Day parasites appeared in blood stream	Results
9	1,000	6th	Died on 12th day
10	100	7th	Died on 14th day
11	10	8th	Died on 11th day
12	10	9th	Died on 14th day
13	1	11th	Died on 18th day
14	1	—	No infection
15	0.1*	—	No infection
16	0.1*	—	No infection
17	0.1*	—	No infection

* 1 cc. of a dilution containing 1 parasite per 10 cc.

100 parasites, monkeys 11 and 12 were each inoculated with 10 parasites, monkeys 13 and 14 were each inoculated with 1 parasite, while monkeys 15, 16, and 17 were each inoculated with 1 cc. of a dilution containing 1 parasite per 10 cc. The results of this experiment showed that the monkeys inoculated with 1,000, 100, and 10 parasites and monkey 13, which received 1 parasite, all became infected and died of acute malaria. Monkey 14, which received an inoculum

diluted so as to contain 1 parasite, and monkeys 15, 16, and 17, injected with 0.1 parasite, failed to develop infections.

The characteristic feature of the above experiments was the progressive increase in the incubation period as the monkeys received a decreasing number of parasites. However, following the detection of microscopically visible parasites, the severity of the disease course and the length of time until death (usually 4 to 5 days) were approximately the same in all animals that became infected.

Experiment 2. Effect of Variation in the Number of Parasites with a Constant Amount of Immune Serum.—Six normal monkeys, Nos. 18 to 23 inclusive, were used in this experiment. Five separate 2 cc. samples of the pooled immune serum were incubated with 1,000,000, 100,000, 10,000, 1,000, and 100 *P. knowlesi* parasites for half an hour at 37°C. Each of these mixtures was then injected intraperitoneally into individual monkeys while a control monkey received 1,000,000 parasites incubated with 2 cc. of normal monkey serum. The immune serum was injected daily in 2 cc. amounts for 9 consecutive days in all except the control animal.

In the results of this protection test, as shown in Table III, 2 monkeys, Nos. 18 and 19 which received the greatest number of parasites, became infected and survived. Monkey 18 had a more severe disease course than monkey 19 which received 10 times fewer parasites. Monkeys 20, 21, and 22, injected with 10,000, 1,000, and 100 parasites respectively, failed to become infected, while control monkey 23 died on the 8th day following inoculation. Since monkeys 20, 21, and 22 failed to become infected, it was considered necessary to show that they were not refractory to infection. These animals were reinoculated 1 month after their original inoculation, each with the same number of parasites that had been used previously. They all became infected and died during the acute attack of malaria.

From this experiment 10,000 was selected as the test dose of parasites that should result in survival with mild infection or no infection when used with 2 cc. amounts of the same pool of immune serum. By decreasing the amount of serum given daily after injecting 10,000 parasites it should be possible to reach an end point where no protection would occur. Accordingly, the following experiment was set up.

Experiment 3. Effect of Variation in Amount of Immune Serum with a Constant Number of Parasites.—The immune serum used in this experiment was taken

from the same pool used in the preceding test. The serum was divided into amounts of 2.0, 1.0, 0.5, 0.25, and 0.125 cc., and each sample was incubated at 37°C. for half an hour with 10,000 parasites. Five monkeys, Nos. 24 to 28 inclusive, were inoculated intraperitoneally with the individual mixtures of immune serum and parasites, while a control monkey, No. 29, was injected with

TABLE III

Protection Experiment with Constant Amount of Immune Serum and Varying Number of Plasmodium knowlesi Parasites in Inoculum

Monkey No.	Immune serum injections, amount and time	Number of parasites injected	Day parasites appeared in blood stream	Results
18	2 cc. daily for 10 days	1,000,000	13th	Survived, severe infection
19	2 cc. daily for 10 days	100,000	11th	Survived, mild infection
20	2 cc. daily for 10 days	10,000	—	No infection
21	2 cc. daily for 10 days	1,000	—	No infection
22	2 cc. daily for 10 days	100	—	No infection
23	Control, no serum given	1,000,000	4th	Died on 8th day

TABLE IV

Protection Experiment with 10,000 Plasmodium knowlesi Parasites and Varying Amounts of Immune Serum
(Same Serum as Used in Table III)

Monkey No.	Immune serum injections, amount and time	Number of parasites injected	Day parasites appeared in blood stream	Results
24	2 cc. daily for 10 days	10,000	—	No infection
25	1 cc. daily for 10 days	10,000	12th	Survived
26	0.5 cc. daily for 10 days	10,000	8th	Died on 17th day
27	0.25 cc. daily for 10 days	10,000	8th	Died on 13th day
28	0.125 cc. daily for 10 days	10,000	11th	Severe infection, survived
29	Control, no serum given	10,000	5th	Died on 8th day

10,000 parasites incubated with 2 cc. normal monkey serum. The immune serum was then given daily for 9 days to the 5 monkeys in the same amount as had been used in the incubation with parasites.

As shown in Table IV, monkey 24, which received the most immune serum (a total of 20 cc.), did not become infected. Monkey 25, which

received 10 cc. of serum, became infected but survived. Monkey 26, which received 5 cc. of serum, died on the 17th day following inoculation. With this animal there was a lengthening of the incubation period and the duration of the acute infection, indicating a slight protective action of the serum. Monkey 27, which received only 2.5 cc. of serum, died on the 13th day. Monkey 28, which received 1.25 cc. of serum during the course of 10 days, had a severe infection but survived. Control monkey 29 died on the 8th day.

From this and the preceding experiment it is evident that the occurrence of infection and the survival or death of the animal in protection tests depend both upon the amount of immune serum and upon the number of parasites in the inoculum. A greater degree of protection was demonstrated either by increasing the amount of immune serum or by decreasing the number of parasites.

Experiment 4. Protection Test Varying Both the Amount of Immune Serum and the Number of Plasmodium knowlesi Parasites.—The immune serum for this experiment was obtained by bleeding the same monkeys which furnished the serum for Experiments 2 and 3. A 6 weeks' interval had elapsed between the two bleedings. There was sufficient quantity of pooled serum to test the effect of variations in the amounts of immune serum and numbers of parasites in the same experiment. As seen in Table V, 5 monkeys in each of 3 groups received immune serum in amounts of 2, 1, 0.5, 0.25, and 0.125 cc. respectively. In the first group 1,000 parasites were incubated separately with the 5 different amounts of immune serum, and each mixture was injected into individual monkeys, Nos. 30 to 34 inclusive. Similarly, monkeys of the second group, Nos. 35 to 39 inclusive, each received 100 parasites, and those of the third group, Nos. 40 to 44 inclusive, 10 parasites. The immune serum was then given daily for 9 days in the amounts indicated, making a total of 20, 10, 5, 2.5, and 1.25 cc. for the respective monkeys. There were 4 control monkeys and each received an injection of parasites which had been incubated with 2 cc. of normal monkey serum for half an hour. Monkey 45 was injected with 1,000 parasites, monkey 46 with 100 parasites, and monkeys 47 and 48 with 10 parasites each.

The results of this experiment showed that among the first group of 5 monkeys, Nos. 30 to 34 inclusive, which were inoculated with 1,000 parasites, only 1 monkey survived. This monkey, No. 30, received the greatest amount of immune serum, a total of 20 cc., and failed to become infected. The remaining 4 monkeys, Nos. 31 to 34, died on the 24th, 31st, 18th, and 16th days respectively although No. 32, according to the parasite count, had apparently recovered but relapsed

and died. Control monkey 45 died on the 12th day with an overwhelming infection.

There was one survival, monkey 36, in the second group of 5 monkeys which were inoculated with 100 parasites. Monkey 35 appeared to be recovering from the acute attack of malaria as evidenced by

TABLE V

Protection Experiment with Varying Amounts of Immune Serum and Varying Number of Plasmodium knowlesi Parasites in Inoculum

Monkey No.	Immune serum injections, amount and time	Number of parasites injected	Day parasites appeared in blood stream	Results
30	2 cc. daily for 10 days	1,000	—	No infection
31	1 cc. daily for 10 days	1,000	18th	Died on 24th day
32	0.5 cc. daily for 10 days	1,000	13th	Died on 31st day
33	0.25 cc. daily for 10 days	1,000	10th	Died on 18th day
34	0.125 cc. daily for 10 days	1,000	9th	Died on 16th day
45	Control, no serum given	1,000	6th	Died on 12th day
35	2 cc. daily for 10 days	100	14th	Died on 25th day
36	1 cc. daily for 10 days	100	10th	Survived
37	0.5 cc. daily for 6 days	100	—	Died on 6th day*
38	0.25 cc. daily for 10 days	100	12th	Died on 19th day
39	0.125 cc. daily for 10 days	100	9th	Died on 15th day
46	Control, no serum given	100	7th	Died on 14th day
40	2 cc. daily for 10 days	10	—	No infection
41	1 cc. daily for 10 days	10	—	No infection
42	0.5 cc. daily for 10 days	10	—	No infection
43	0.25 cc. daily for 10 days	10	—	No infection
44	0.125 cc. daily for 10 days	10	10th	Died on 16th day
47	Control, no serum given	10	8th	Died on 11th day
48	Control, no serum given	10	8th	Died on 13th day

* Intercurrent infection.

decreasing numbers in the parasite count but relapsed and died on the 25th day. Monkey 36 became infected and recovered. Monkey 37 died with an intercurrent infection before the appearance of any circulating parasites. Monkeys 38 and 39 died on the 19th and 15th days after inoculation, while control monkey 46 succumbed on the 14th day.

In the last group of 5 monkeys, which were inoculated with 10 parasites, there was only one death. Monkey 44, which received 10 daily injections of 0.125 cc. of immune serum, died on the 16th day. The remaining 4 monkeys, Nos. 40 to 43 inclusive, never became infected, while the 2 controls, Nos. 47 and 48, died on the 11th and 13th days respectively.

The outcome of this experiment indicated that the protective effect of corresponding amounts of serum was more marked when the inoculum contained only 10 parasites than when it contained 1,000 parasites. It was also possible to demonstrate in the same experiment that the use of a greater amount of immune serum against a constant number of parasites resulted in a more marked protective effect.

DISCUSSION

The results of this study indicate that *rhesus* monkeys can be fatally infected with *P. knowlesi* by the injection of less than 10 parasites and probably by 1 or 2. This implies a marked lack of natural resistance in normal *rhesus* monkeys to infection with *P. knowlesi*. Increasing the number of parasites in the inoculum shortens the time required for the organisms to multiply to the extent that they become detectable in blood smears. In untreated monkeys the period from the first positive blood examination to the time of death is approximately the same whether 1 parasite or 100,000 parasites have been injected. A small dose of parasites does not result in a milder disease course by prolonging the incubation time and allowing the host more opportunity to develop active immunity.

It appears from the protection experiments described in this paper that the smaller the number of parasites in the inoculum, the smaller the amount of immune serum required to save the life of the animal. This is known to be true only when the immune serum is incubated with the parasites before injection and is given daily during the incubation period and the first stages of the infection. Once the blood becomes positive for parasites, it is more difficult to protect the animal with immune serum (1). It is probable that if the immune serum were given daily after the first appearance of parasites in the blood, the amount required for protection would be independent of the

number of parasites originally injected. No experiments of this sort have yet been done.

It is possible that incubation of the parasites with immune serum may have reduced the number of infective parasites in the inoculum. Hence the protective action of the serum may be a result of combined *in vitro* and *in vivo* effects. In Table V, for example, monkeys 40, 41, 42, and 43 received 10 parasites and developed no infection, while monkey 44 became infected and died at approximately the same time as the controls. The immune serum in amounts of 0.25 cc. or more may have been sufficient to make the 10 parasites in the inoculum non-infective. On the other hand, monkey 35, which received 100 parasites and 2 cc. of immune serum, became infected while monkey 30, which received the same amount of immune serum and an inoculum 10 times as large (1,000 parasites), did not become infected. This difference must be ascribed to a greater *in vivo* protective effect of the serum in monkey 30 than in monkey 35 due to individual variations. Other examples where the *in vitro* and *in vivo* effects of the serum have apparently combined to prevent infection can be seen in monkeys 20, 21, and 22 in Table III.

In Experiments 2 and 3 in which the amount of serum was kept constant and the number of parasites varied, or *vice versa*, consistent results usually were obtained. An exception was noted in monkey 28 (Table IV) which received 0.125 cc. of immune serum and recovered despite a severe infection, while monkeys 26 and 27 died although they received more immune serum and the same dose of parasites. Another exception is seen in Table V, monkeys 35 and 36. This indicates that individual variations in monkeys may sometimes adversely affect the expected results.

SUMMARY

The minimal infective dose of *Plasmodium knowlesi* for *rhesus* monkeys was found in this study to be between 1 and 10 parasites when injected intraperitoneally. As the dose of parasites is increased, the length of time prior to the appearance of circulating parasites is decreased. However, the severity of the infection once it is established is independent of the initial dose of parasites.

In passive protection experiments a quantitative relationship was

demonstrated between the number of parasites in the inoculum and the effective amount of immune serum given at the time of infection and in equal doses daily for 9 days thereafter. The smaller the inoculum, the less the quantity of immune serum required to prevent the death of the animal.

When relatively large amounts of immune sera and small numbers of parasites were used in the protection experiment, infection was prevented.

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