IMMUNE RESPONSE OF RABBITS TO INJECTION OF PLASMODIUM KNOWLESI

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The experiments reported in this paper were devised to determine whether or not the protective, agglutinating, and complement-fixing antibodies found in the blood of monkeys with chronic *Plasmodium knowlesi* infection (1–3) were also produced in the non-susceptible rabbit by injection of living or killed malarial parasites.

Apparently very little work has been done on antibody production by injecting protozoan parasites into non-susceptible animals. Intravenous injection of living cultures of organisms of the leishmania group into rabbits gives rise to complement-fixing and agglutinating antibodies (4, 5). Similar experiments with malarial parasites are complicated by the fact that the parasites cannot be completely separated from the red cells of the monkey or other animal in whose blood they multiply. Thus antibodies, not only to the parasites but also to certain constituents of the blood of the alien species, are produced by rabbits immunized in this way.[1]

Materials and Methods

Parasitized blood was obtained by bleeding from the heart monkeys dying of infection with *P. knowlesi*. When small quantities of infected blood were required, animals with chronic or acute infection were bled from the femoral vein. The blood was collected in 2 per cent sodium citrate solution.

Immunization of Rabbits and Preparation of Immune Sera.—Sixteen rabbits were immunized with various antigens prepared from parasitized blood. The animals were given two or three intraperitoneal injections, each equivalent to about 5 cc. of blood, and were bled from the ear 14 days later. After a period of 2 to 4 weeks the injections were repeated and the animals bled again after 2 weeks. Three rabbits died after intravenous injection of very small doses of parasitized cells.

Rabbit 1 received six injections of the stroma from washed and laked parasitized cells over a period of 2 months. Rabbits 3 and 5 each received seven injections of similar

[1] In this paper the expressions "immunize" and "immune serum" are used in their broadest sense to signify production of antibodies to an injected antigen and to designate a serum containing such antibodies. They are not intended necessarily to imply resistance to infection.

material over a period of 6 months. Rabbit 4 received three injections of washed whole parasitized cells over a period of 3 weeks. Rabbits 2, 6, 7, and 8 received thirteen to seventeen injections of washed parasitized cells over periods of 7 to 8 months, and rabbits 10, 11, and 12 received ten or eleven injections of similar material over periods of 4 to 6 months. After a rest period of 2 months rabbits 2, 7, and 8 were inoculated five times with whole citrated parasitized blood (cells plus serum) over a period of 4 months, and rabbits 10, 11, and 12 received similar injections of washed parasitized cells.

The remaining four rabbits were immunized with material in which the parasites were demonstrated to be dead by injection into *rhesus* monkeys. All were given nine injections over a period of 3 months. Rabbit 13 received parasites killed with 1 per cent formalin; rabbit 14, parasites heated to 56°C. for 1 hour; rabbit 15, parasites which were killed by drying in the frozen state; and rabbit 16, frozen and thawed stroma from laked parasitized cells. Rabbit 17 was immunized with the blood of normal *rhesus* monkeys.

Preparation of Absorbed Sera.—The immune rabbit sera prepared as described above agglutinated normal monkey erythrocytes to titers varying from 1:8 to 1:1,000. Because these agglutinins would mask any malarial antibodies that might be detected by complement fixation, or protection tests, it was necessary to remove them by absorption with normal red cells. The sera were inactivated by heating at 56°C. for 30 minutes. Before absorption the sera were diluted with an equal volume of saline. A volume of packed, washed, red cells equal to that of the undiluted serum was added, and after standing for 30 minutes at room temperature, with thorough mixing, the tubes were kept overnight in the ice box. The cells were centrifuged down and the serum removed. The procedure was repeated once or twice until agglutinins were no longer detectable in the absorbed sera. With some sera absorption with parasitized cells was carried out in a similar manner.

Complement Fixation, Parasiticidal, and Protection Tests.—The complement fixation tests with rabbit sera were performed by the method described for monkey sera in a previous communication (3). Saline extracts of frozen and thawed parasitized cells were used for antigen. Control tests were done with antigen prepared in a similar manner from normal red cells.

In the parasiticidal tests 1 cc. of blood containing a measured number of parasites was added to 1 cc. of rabbit serum and the mixture incubated at 37°C. for 30 minutes. The cells and serum were then injected intraperitoneally into monkeys. In performing these tests every precaution had to be taken to bring all of the cells into contact with the serum because only a few parasites sticking to the sides of the tube, if taken up into the syringe and injected, might be sufficient to cause infection. The fresh citrated parasitized blood was diluted in a mixture of equal parts of whole blood and sodium citrate solution as described previously (6). In this way dilutions containing approximately 10,000, 100,000, and 1,000,000 parasites per cc. were obtained.

The protection tests with rabbit sera on *rhesus* monkeys were done by methods similar to those described by Coggeshall and Kumm (1). A more detailed description of these procedures will be given in a following section of this paper.

Demonstration of Malarial Complement-Fixing Antibodies in Immune Rabbit Serum

All of the rabbits which had received injections of material obtained from parasitized blood developed antibodies which fixed complement, both with

antigen prepared from parasitized cells and with antigen prepared from normal monkey cells. Immune sera absorbed with normal monkey cells fixed complement with malarial antigen but not with antigen from normal erythrocytes. Absorption of the sera with parasitized cells removed complement-fixing antibodies for both malarial parasites and normal red cells. Because absorption of the sera with normal or parasitized cells frequently

TABLE I

Demonstration of Malarial Complement-Fixing Antibodies in the Serum of Rabbits after Injection of Living or Dead Plasmodium knowlesi

Serum No.*	Absorption	Antigen-malarial blood 1:30				Antigen-normal blood 1:20				No antigen	
		Serum diluted				Serum diluted				Serum	
		1:4	1:8	1:16	1:32	1:4	1:8	1:16	1:32	1:4	1:8
7	−	++++	++++	+++	±	+++	−	−	−	−	−
7	N	++++	++++	+	−	±	−	−	−	−	−
8	−	++++	++++	++++	+++	++++	++++	+++	−	−	−
8	N	++++	++++	+++	−	+++	−	−	−	+++	−
13	−	+++	++	−	−	±	−	−	−	−	−
13	N	+++	++	−	−	±	−	−	−	±	−
15	−	++++	++++	++++	++	+++	+++	±	−	−	−
15	N	++++	++++	+++	+	+	−	−	−	+	−
15	P	+++	−	−	−	+++	−	−	−	+++	−
14	−	++++	++++	+	−	+	−	−	−	−	−
14	N	++++	+++	++	−	±	−	−	−	±	−
14	P	++	−	−	−	±	−	−	−	±	−
11	−	++++	++++	+++	+	++++	±	−	−	−	−
11	N	++++	++++	++	−	+	−	−	−	+	−
11	P	+	−	−	−	+	−	−	−	+	−
16	−	++++	++++	+++	±	++++	++	−	−	−	−
16	N	+++	++++	++++	+	+	−	−	−	−	−
16	P	+++	±	−	−	+++	±	−	−	+++	±

− = not absorbed.

N = absorbed with normal monkey erythrocytes.

P = absorbed with parasitized monkey erythrocytes.

* Production of immune serum is described in section on Materials and Methods.

made them somewhat anticomplementary, it was difficult to obtain clear cut results with some of the samples.

Some of the more definite results of these tests are presented in Table I. It will be seen that in most cases absorption of the serum with normal monkey erythrocytes slightly reduced the complement fixation titer against malarial antigen which contains material both from parasites and from red cells. On the other hand, this absorption abolished the fixation of complement with normal blood antigen. Normal rabbit serum gave no

fixation of complement with antigens from parasitized or normal red cells. Attempts to demonstrate precipitating or agglutinating antibodies for malarial parasites in the absorbed rabbit sera were unsuccessful.

Parasiticidal Action in Vitro of Immune Rabbit Serum

As was mentioned in a previous section, the immune sera prepared by injecting parasitized cells into rabbits were capable of agglutinating monkey erythrocytes to a high titer. It was found that when blood containing a

TABLE II

Parasiticidal Effects of Antisera Prepared by Immunizing Rabbits with Normal and Parasitized Monkey Erythrocytes

Monkey No.	Serum No.	Rabbit immunized with	Absorption of serum with normal erythrocytes	Infection from parasites plus serum*
				days
1	1	Parasitized cells	Unabsorbed	0
2	4	" "	"	0
3	1	" "	Absorbed	7
4	1	" "	Unabsorbed	0
5	11	" "	Absorbed	7
6	11	" "	Unabsorbed	0
7	25	" "	Absorbed	5
8	17	Normal cells	—	0
9	17	" "	—	0
10	17	" "	—	10
11	N-1	Normal rabbit serum		5
12	N-2	" " "		7

* 1 cc. of rabbit serum and 1 cc. of a dilution of infected blood containing approximately 10,000 parasites mixed and incubated for ½ hour at 37°C., then injected intraperitoneally into the monkey. Time, in days after inoculation, of first positive blood smear. 0 = no infection.

small proportion of parasitized cells was mixed with the immune serum and incubated, the resulting agglutination of the red cells was accompanied by an effect which made the blood non-infective when it was injected into the peritoneal cavity of susceptible *rhesus* monkeys. When it was observed that the same samples of serum absorbed with normal monkey red cells failed to reduce the infectivity of similar doses of parasites, it became evident that this parasiticidal effect was not due to the malarial antibody itself. Furthermore, serum obtained from a rabbit immunized only with normal monkey erythrocytes also showed parasiticidal action.

The results of these experiments are summarized in Table II.

Monkeys 1, 2, 4, and 6 were inoculated intraperitoneally with an incubated mixture of 10,000 parasites and serum from rabbits which had been immunized with parasitized cells. These monkeys survived without infection. Monkeys 3, 5, and 7, receiving similar mixtures of parasitized cells and serum absorbed with normal monkey erythrocytes, first had demonstrable parasites in their blood 5 to 7 days after inoculation and later died of acute malaria. Monkeys 8, 9, 10 were inoculated with parasites and serum from a rabbit immunized with normal monkey cells. Two of these animals survived without infection. Parasites appeared in the blood of the third animal 10 days after inoculation. Since the usual incubation period for an infecting dose of 10,000 parasites is 6 or 7 days, the prolonged incubation period in this case indicated that a considerable proportion of the parasites in the inoculum were killed. Two monkeys receiving normal rabbit serum and parasites became infected and died within the usual length of time.

TABLE III

Relation of Number of Parasites to Parasiticidal Effect of Immune Rabbit Serum

Dosage of parasites with 1 cc. of immune serum	Number of monkeys tested	Number of tests	Number of infections	Per cent of infections
1,000	2	2	0	0
10,000	4	4	0	0
100,000	12	40	4	10
1,000,000	7	10	4	40
5,000,000 to 10,000,000*	3	4	2	50

* With the largest doses of parasites guinea pig complement was added to produce complete lysis of the red cells. No complement was added to the mixtures containing 1,000,000 parasites or less.

There appears to be a definite limit to the number of parasites that can be killed by incubation for ½ hour with 1 cc. of immune rabbit serum. Sixty tests were done with different doses of parasites incubated with serum 11. The results are presented in Table III.

The second column of the table shows the number of monkeys that were tested with each dose of parasites. A total of seventeen monkeys were used, but a number of these were tested successively with 10,000, 100,000, and 1,000,000 parasites so that duplications occur in the table. Although several of the animals received successive doses of parasites and rabbit serum, this did not appear to make them immune since all of these animals eventually were infected. With doses of 1,000 or 10,000 parasites and rabbit serum, no infection occurred. With a dose of 100,000 parasites infection resulted in four out of the forty trials, giving an incidence of 10 per cent. With 1,000,000 parasites or more, infections occurred in 40 to 50 per cent of the tests.

The possible effect of complement *in vitro* on the parasiticidal action of the antibody to the monkey erythrocytes was investigated in two ways.

With the largest doses of parasites enough fresh guinea pig serum was added, together with the immune rabbit serum, to produce complete lysis of the red cells. As will be seen from the last line of Table III, this failed to bring about killing of all of the parasites in the mixtures. The possible rôle of complement in the fresh monkey blood used to dilute the parasites was also considered. In one experiment (not shown in Table III) dilution of the parasitized blood was done in a mixture of inactivated monkey serum and washed monkey erythrocytes. The resulting dilution containing 10,000 parasites per cc. was then set up in two tubes with immune rabbit serum and normal rabbit serum. The monkey inoculated with parasites and immune rabbit serum did not become infected, while the control monkey which received parasites and normal rabbit serum became infected after 6 days and died on the 11th day.

Most of the experiments on the parasiticidal action of immune rabbit serum were done with blood containing young ring forms of *P. knowlesi*. However, in certain experiments a considerable proportion of mature forms and segmenters were also present, and it appeared that these were as readily killed as the immature rings.

Attempts to Demonstrate Protective Antibodies in the Serum of Rabbits Immunized with Parasitized Red Cells from Monkeys

Immune rabbit sera, when injected intravenously into normal monkeys in moderately large doses, are capable of causing severe anemia, apparently as a result of lysis or destruction of erythrocytes *in vivo*. This effect is attributable to antibodies to monkey cells in the immune rabbit sera, and it was therefore considered desirable to remove these antibodies by absorption with normal erythrocytes before protection experiments were attempted.

The passive protection tests were done by methods similar to those described by Coggeshall and Kumm except that the parasites and rabbit serum were not incubated together before injection. Doses of 1,000 to 10,000 parasites were injected intraperitoneally into *rhesus* monkeys, and rabbit serum was then given daily, either intraperitoneally or intravenously, beginning 24 to 48 hours after the parasites. The results of these experiments were, for the most part, negative. They are summarized in Table IV.

Experiment 1.—Monkeys 13 and 14 received 2 cc. of serum daily beginning the 1st day after inoculation. Monkey 13 became positive on the 6th day, while monkey 14 first had demonstrable parasites on the 14th day. Monkey 15 received no serum until the 6th day when two parasites were seen in the blood smear after a long search. A total of 8 cc. of serum was then given over a period of 3 days, and the blood smear remained

negative until the 16th day. The control animals receiving normal rabbit serum became positive on the 6th day, and both had a protracted infection with high parasite counts. Monkey 16 died, while monkey 17 survived.

Experiment 2.—In this experiment all of the animals received 2 to 3 cc. of serum daily beginning the 1st day after inoculation. Parasites appeared in the blood of all of the

TABLE IV

Passive Protection Experiments with Rabbit Serum

Experiment No.	Monkey No.	Number of parasites injected	Rabbit serum No.*	Total dose of serum	Duration of life after inoculation
				cc.	*days*
1	13	10,000	1 + 4†	15	15
	14	10,000	1 + 4	15	19
	15	10,000	1 + 4	12	22
	16	10,000	Normal	15	18
	17	10,000	"	8.5	S
2	18	10,000	3 + 5	27	16
	19	10,000	2, 6, 7, 8	27	13
	20	10,000	11, 12	22	13
	21	10,000	Normal	27	12
	22	10,000	—	0	11
3	23	1,000	2	22	13
	24	1,000	10	31	8
	25	1,000	13 + 14	31	S
	26	1,000	15 + 16	31	13
	27	1,000	Normal	31	14
	28	1,000	—	0	14
4	29	10,000	2, 7, 8	38	10
	30	10,000	10, 11, 12	45	S
	31	10,000	13	47	S
	32	10,000	14	52	13
	33	10,000	Normal	46	16
	34	10,000	—	0	14

S = monkey survived.

* See section on Materials and Methods for immunization procedure.

† This serum was unabsorbed. All other sera were absorbed with normal monkey erythrocytes.

animals between the 5th and 6th day. Serum was discontinued on the 11th day. Monkey 18 had high parasite counts on the 9th and 10th days. The count then fell and remained low for 2 days, after which it rose again and the animal died. The course of the disease in the other animals was not essentially different from that in the control monkey receiving no serum.

Experiment 3.—Serum in amounts of 2 to 3 cc. was given daily for the first 5 days.

Then on the next 2 days when the parasite counts were increasing the dose of serum was raised to 6 to 8 cc. One monkey, No. 24, died with a low parasite count as a result, apparently, of the toxic effects of the serum. No. 25 survived after a moderately severe infection. In the other animals no effect of the serum on the course of the disease was observed.

Experiment 4.—During the first 5 days after inoculation 1 cc. of serum was given to each of the monkeys daily. When parasites began to appear in considerable numbers in the blood, the daily dosage of serum was increased to about 10 cc. and this was continued for 5 to 6 days. Monkey 30 survived after a very severe infection, and monkey 31 had a moderate degree of infection and survived but died after a month with tuberculosis. The increased dose of serum had no effect on the infection in the other animals.

Another experiment in which serum was withheld until the appearance of parasites in the blood was completely negative.

DISCUSSION

The appearance of malarial complement-fixing antibodies in the serum of rabbits receiving injections of living or dead parasites apparently occurs in response to an antigenic stimulus similar to or identical with that which gives rise to complement-fixing antibodies in monkeys. In a subsequent paper the production of complement-fixing antibodies in monkeys by injection of parasites killed in various ways will be described.

The parasiticidal action on *P. knowlesi* of antibodies to the host cell (erythrocyte) appears to be open to several interpretations in the absence of a more detailed investigation. First, the parasites may be killed *in vitro* by the antibody to the red cells, by the action of complement on the sensitized cells, or by some parasiticidal substance which occurs normally in the serum of rabbits and is brought into action after lysis of the cells by antibody and complement. Second, sensitized red cells containing parasites may be more subject to phagocytosis in the peritoneum of the monkey than are parasitized red cells not coated with antibody. Third, the coating of the red cell with immune globulin and the resulting agglutination may interfere with the reproductive processes of the parasite, either by preventing the normal emergence of the merozoites or by bringing about changes in the cell which are unfavorable to the continued life of the parasite. The definite quantitative limitation to the number of parasites that can be killed by a given sample of immune serum would make it seem probable that some factor in the parasiticidal mechanism is used up when the dose of parasites is increased to 10,000,000 per cc. or over. In this connection it must be pointed out that dilutions of the parasitized cells were made in whole citrated blood so that the number of red cells present was constant and, therefore, the amount of coating of all cells by homologous antibody

was approximately the same in all experiments irrespective of the number of parasites present.

In the first protection experiment (Table IV) it appeared that the absorbed rabbit serum tended to keep the blood of monkeys 14 and 15 free of parasites for a week to 10 days longer than the control animals, but we have been unable to repeat this observation. Of the three monkeys which survived after treatment with rabbit serum, two received serum from a rabbit immunized with formalinized parasites, and one from a rabbit immunized with living parasites. From this it would appear that sera from rabbits immunized with living parasites do not possess any greater protective properties than do the sera from rabbits inoculated with killed parasites. Survival of monkeys infected with *P. knowlesi* and treated with normal rabbit serum has been observed in two cases, and in a third the life of the animal was prolonged. It has been reported that susceptible animals may be passively protected against certain trypanosome infections by normal serum from resistant animals (7). The results of the present work taken as a whole indicate that it is difficult, if not impossible, to produce a significant increase in protective antibodies against *P. knowlesi* by injecting living or dead parasites into rabbits.

SUMMARY

Specific complement-fixing antibodies are produced in the serum of rabbits in response to injections of living or dead *Plasmodium knowlesi*.

Sera from rabbits receiving injections of either parasitized or normal monkey erythrocytes are parasiticidal *in vitro* for *P. knowlesi*. Because absorption of parasiticidal rabbit sera with normal monkey erythrocytes abolishes the parasiticidal effect, it is concluded that the effect is largely due to an antibody to the red cells. Normal rabbit serum is not parasiticidal.

Experiments on passive protection in monkey malaria with serum from rabbits which have received intraperitoneal injections of living or dead *P. knowlesi* yield no conclusive evidence that protective antibodies are formed.

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