PRODUCTION IN MONKEYS OF COMPLEMENT-FIXING ANTIBODIES WITHOUT ACTIVE IMMUNITY BY INJECTION OF KILLED PLASMODIUM KNOWLESI

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Attempts to immunize susceptible animals with non-living material derived from protozoan parasites have yielded conflicting and sometimes inconclusive results. Wenyon (1) states that injection of killed trypanosomes into experimental animals may stimulate the production of complement-fixing and agglutinating antibodies, but that the existence of active immunity to infection has not been conclusively demonstrated. Schilling (2), Braun and Teichmann (3), and Ponselle (4) were able to produce active immunity to infection and to demonstrate protective antibodies in the serum of rats, mice, and guinea pigs after injection of killed preparations of *Trypanosoma brucei* and *T. equiperdum*. A more detailed review of this work will be found in the book by Taliaferro (5). The Sergents (6) reported that 29 per cent of a group of canaries were resistant to infection with *Plasmodium praecox* after the birds had been inoculated with sporozoites which were presumably killed by storage for 24 to 48 hours after being removed from the mosquitoes. Only 0.72 per cent of a group of normal canaries were resistant to infection with the parasite.

It is possible that in some of the experiments just mentioned the immunity resulted from an inapparent infection with living organisms. In the case of *P. knowlesi* infection in *rhesus* monkeys it appears that between one and five parasites are capable of producing infection, and that once the infection is established, it is almost invariably fatal unless treated (7). For this reason the production of active immunity in *rhesus* monkeys by a sublethal dose of *P. knowlesi* seems to be impossible.

The experiments described in this paper were performed for the purpose of finding out whether or not complement-fixing, agglutinating, and protective antibodies, and active immunity could be produced in *rhesus* monkeys by intraperitoneal or intravenous injection of non-living antigens prepared from *P. knowlesi*.

Materials and Methods

Parasitized blood was obtained by the methods described in a previous paper (8). Five different methods were used to kill the parasites for the preparation of the vaccines.

1. The washed parasites were suspended in saline, and 1 per cent of formaldehyde

was added. The pH was adjusted to 7.4 with phosphate buffer and the preparation stored in the ice box.

2. A suspension of washed parasites in saline was heated at 56°C. for 30 minutes and then stored in the ice box.

3. The washed parasitized cells were dried in the frozen state in pyrex glass tubes in a vacuum desiccator. The tubes were then sealed and stored in the ice box. Just before making the injections the dried material was rehydrated with saline and, if to be given intravenously, it was filtered through cotton. In some experiments whole blood of monkeys dying from infection with *P. knowlesi* was dried in the frozen state, and this was rehydrated with normal monkey serum.

4. The washed parasitized cells were laked with distilled water and the stroma and parasites centrifuged down. This concentrated material was then frozen and thawed four times and finally stored in the frozen state in the freezing compartment of the ice box.

5. Parasites were killed by incubation with the serum of a rabbit immunized with *P. knowlesi* according to the method described in the preceding paper (9).

Antigens prepared by the first four methods were injected intravenously or intraperitoneally in doses of 2 to 5 cc., containing the equivalent of approximately 500 million to 2 billion parasites. No infections were observed in twelve monkeys inoculated repeatedly with these vaccines. As indicated in the previous paper, infections were relatively frequent with vaccines prepared by the fifth method. With these preparations only small doses of 100,000 to 1,000,000 parasites could be injected at one time.

Three to four injections were given at intervals of 3 to 7 days, and after a period of 2 weeks the animals were bled from the femoral vein to obtain serum for complement fixation and agglutination tests. The injections and bleeding were then repeated as before. Complement fixation and agglutination tests were done according to the methods described in previous papers (8, 10).

EXPERIMENTAL

The results of injecting into monkeys parasites killed by the first four methods described in the previous section are presented in Table I.

Monkeys 35, 36, and 37, which received formalinized parasites, developed low titers of complement-fixing antibodies after 1 to 1½ months. After further injections these antibodies tended to fall off or disappear completely. The animals receiving heated or dried parasites, Nos. 38 to 44 inclusive, developed complement-fixing antibodies ranging in titer from 1:16 to 1:64. These appeared about 1 month after the first injections and gradually increased to a maximum at 2 to 3 months. Monkeys 45 and 46 received injections of frozen and thawed parasites. The development of complement-fixing antibodies by these animals was delayed, and the titers were low. There was no significant difference between the effects of intraperitoneal and intravenous injection.

Agglutination tests were done repeatedly with sera taken at various dates from all the monkeys in Table I. In no case were agglutinins demonstrated (8). At the end of the course of injections the animals were tested for active immunity by infecting them with live parasites. All of the animals

TABLE I

Production of Complement-Fixing Antibodies in Monkeys by Injection of Killed Parasites

Monkey No.	Method of killing parasites	Route of injection	Number of injections	Period	Complement-fixing antibodies	
					Maximum titer	Time to maximum
				mos.		*mos.*
35	Formalin	Intraperitoneal	9	3	1:6	1
36	"	"	9	3½	1:4	1½
37	"	Intravenous	10	2	1:2	1
38	Heat	Intraperitoneal	9	3	1:32	2½
39	"	"	9	3½	1:16	2
40	"	Intravenous	10	2	1:64	2
41	Dried	Intraperitoneal	9	3	1:64	2½
42*	"	Intravenous	16	5	1:32	2
43*	"	"	16	5	1:32	2
44*	"	"	18	7½	1:32	3
45	Frozen and thawed	Intraperitoneal	9	3	1:6	3
46	" " "	"	9	3½	1:4	3

* Monkeys 42, 43, and 44 received dried whole parasitized blood rehydrated with serum.

TABLE II

Attempts to Produce Immunity by Injection of Parasites Killed with Immune Rabbit Serum

Monkey No.	Number of injections and parasites	Period of immunization	Result of test for active immunity
		mos.	
47*	1 × 10,000 1 × 100,000	1	Survived after severe infection†
48	6 × 100,000	1½	" " " "
49	7 × 100,000 1 × 1,000,000	3	Died 28 days†
50	6 × 100,000 2 × 1,000,000	5	Died 20 days
51*	1 × 100,000	½	Survived after moderate infection†
52*	4 × 100,000	3	Died 13 days
53*	1 × 100,000	½	Died 18 days
54*	3 × 100,000 2 × 1,000,000	2	Died 10 days
55*	3 × 100,000 2 × 1,000,000	2	Died 14 days
56*	1 × 100,000	½	Died 15 days

* Animals became infected after receiving an injection of parasites and rabbit serum in which all parasites had not been killed (not shown in second column of table). Others were tested for immunity by injecting 10,000 living parasites.

† These animals later died of tuberculosis.

died with acute malaria, the only exception being monkey 35. This animal survived after a severe infection and died several months later with tuberculosis.

Effect of Inoculating Monkeys Repeatedly with Small Doses of Parasites and Immune Rabbit Serum

Ten monkeys were given intraperitoneal injections of a mixture of 1 cc. of serum from a rabbit immunized with *P. knowlesi* and 1 cc. of a dilution of parasites in whole citrated monkey blood which had been incubated for 30 minutes at 37°C. The injections were repeated at intervals of 1 to 2 weeks. The sera of these monkeys were tested for complement-fixing and agglutinating antibodies, but no significant reactions in either test were found at any time during the course of injections.

In these experiments an attempt was made to build up an immunity gradually by increasing the dose of parasites first to 1,000,000, then to 10,000,000. As a result of this procedure seven of the ten monkeys became infected before the course of immunization was completed. The other three were tested for immunity after six to eight injections by infecting them with 10,000 living parasites. The results are presented in Table II.

Three of the ten monkeys, Nos. 47, 48, and 51, survived infection with *P. knowlesi.* No. 47 had received only two injections and No. 51 only one injection, and both these animals later died of tuberculosis. No. 48 received six injections, survived the test for immunity, and has remained well. One other animal, No. 49, showed a slightly increased resistance as indicated by survival for 28 days after infection with 10,000 parasites. This animal appeared to be recovering but later died with a high parasite count and tuberculous peritonitis. The remaining six monkeys, Nos. 50, 52, 53, 54, 55, and 56, showed no significant resistance to malarial infection.

DISCUSSION

The relative ease with which complement-fixing antibodies against *P. knowlesi* can be produced in rabbits (9) and in monkeys by the injection of parasites killed by heat or drying, contrasted with the failure to produce significant amounts of protective antibodies in rabbits and active immunity in monkeys, suggests at once that separate immunity mechanisms are involved. The malarial complement-fixing antigen behaves like other antigenic substances when injected into animals in that the production of antibodies is stimulated quite promptly, but the appearance of complement-fixing antibodies is not accompanied by the production of agglutinating antibodies or protective antibodies. Furthermore, the complement-fixing antibody appears to be group specific, since reactions may be obtained with *P. knowlesi* antigen and sera from human beings infected with *P. vivax* or *P. falciparum*, while active immunity to malaria appears to be strictly species specific (11).

The antigenic component of the parasite concerned in agglutination appears to be different from that taking part in complement fixation. The lability of the agglutinating antigen has been indicated by studies previously reported (8) in which it was found that only fresh preparations of parasites gave definite agglutination with monkey serum. Parasites which have been dried and rehydrated do not agglutinate or precipitate with immune serum but do give good fixation of complement. The soluble complement-fixing antigen found in the serum of monkeys infected with *P. knowlesi* (12) also appears to be distinct from the antigen concerned in agglutination. These observations are supported by the finding reported in the present paper. The injection of parasites killed by drying stimulates the production of complement-fixing antibodies to a high titer but does not produce agglutinins.

In the experiments on active immunization of monkeys injection of parasites killed by heat, freezing and thawing, formalin, or drying apparently produced no resistance whatever to infection with living parasites. The results with the first five monkeys inoculated with parasites and immune rabbit serum seemed promising, but three of these animals had tuberculosis, and this apparently increased the resistance of monkeys to malaria. Further experiments with monkeys having no tuberculosis failed to confirm the earlier results. The failure of these animals to produce complement-fixing antibodies was probably due to the relatively small doses of parasites given.

The antigen concerned in the production of protective antibodies may be so labile both *in vitro* and *in vivo* that it is destroyed, under most conditions, when the parasite is killed. Lability of the antigen to heat, formalin, drying, and freezing and thawing would account for the failure to produce protective antibodies in monkeys with killed parasites. In the experiments with rabbits injection of live parasites which should contain intact antigen produces no protective antibodies. In order to explain this it would be necessary to postulate the destruction of the antigen at the same time as the parasite in the body of the rabbit.

Quantities of antigen concerned in immunity by infection and in artificial immunization must also be considered. In an infected monkey having a parasite count of 500 per 10,000 red cells there are approximately 25 billion circulating parasites, and at least this number is reproduced every 24 hours. Thus the amount of antigen produced in the body of an infected monkey over a period of a week may be conservatively estimated at several hundred times the amount contained in several doses of 1 billion killed parasites. If part of the antigen were destroyed in killing the parasites *in vitro*, the

differences in the amount of antigen available in infection and in artificial immunization would be even greater. In the experiments described in this paper the injection of killed parasites was continued over a period of several months in the hope that by repeated small stimuli the quantitative differences just discussed might be overcome.

Taliaferro (5) and others have called attention to cellular factors in immunity to malaria. Of these the great increase in phagocytic activity in the spleen seems to be the most important. This form of resistance is presumably brought about only by infection. However, the work of Coggeshall and Kumm (13) emphasized the importance of circulating protective antibodies in immunity of monkeys to infection with *P. knowlesi*. So far these protective antibodies have been produced in significant amounts only by infection, and their production is not brought about by injecting killed parasites into susceptible monkeys or living parasites into non-susceptible rabbits according to the methods described in this and the preceding paper.

SUMMARY

Injection into *rhesus* monkeys of *Plasmodium knowlesi* killed by heat, formalin, drying, or freezing and thawing stimulates the production of complement-fixing antibodies, but no demonstrable agglutinating or protective antibodies are formed.

Possible differences in the immunity mechanisms concerned in active infection and in artificial immunization are discussed.

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