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Antibody-Mediated Elimination of Malaria Parasites (*Plasmodium berghei*) In Vivo[1]

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An infective preparation of extracellular blood forms (FP) of *Plasmodium berghei* was used to study some aspects of the interaction between protective antibodies and malaria parasites. FP but not infected erythrocytes (IRBC) were shown by the fluorescent antibody technique to be coated by antibodies after in vitro incubation with immune serum. Preincubation of both FP and IRBC with immune serum followed by their washing did not result in enhanced elimination of the parasites in vivo. However, FP preincubated with immune serum and subsequently washed were eliminated more efficiently than FP preincubated with normal serum if the preparations were injected with some immune serum. Such an increase in the efficiency of elimination was not detected with similarly pretreated IRBC. It is thus probable that protective antibodies acted in vivo against extracellular parasites rather than against parasites in erythrocytes. The interaction between parasites and antibodies may be of a highly reversible nature, and washing of the in vitro-treated parasites may cause elution of antibody from the sensitized parasites so that the amount of antibody on the parasite falls below the critical level required for in vivo elimination.

The ability of antibodies to protect various animals including humans against malaria parasites has been repeatedly demonstrated by passive transfer experiments (1, 8, 10, 13, 18, 26) As in all of these studies, protective antibody activity was determined against a preexisting infection or against an inoculum of infected erythrocytes (IRBC), and it was impossible to determine whether IRBC or extracellular parasites were the target of the protective antibodies. That protective antibodies may exert their activity on free malaria parasites (FP) rather than on IRBC was suggested by Cohen et al. (10), on the basis of the observation that after hyperimmune serum was transferred from adult Gambians to children infected with *Plasmodium falciparum* a substantial drop in numbers of circulating parasites did not occur until after the parasites had undergone schizogony. An experimental evaluation of the hypothesis that antibody acts more efficiently against FP than against IRBC was recently made by Cohen and Butcher (9). These authors demonstrated that addition of relatively large amounts of hyperimmune serum to short-term in vitro cultures of *Plasmodium knowlesi*, a synchronous parasite of monkeys, did not interfere with the intraerythrocytic development of the parasites (as measured by uptake of [^{3}H]leucine) but did completely arrest development after schizogony. These authors suggested that the inhibitory antibody acts on merozoites as antiviral neutralizing antibodies act on viruses and that the antibody interferes with merozoite penetration into erythrocytes. A more recent study by Miller et al. (17) has shown that addition of hyperimmune serum to cultures of *P. knowlesi* schizonts causes aggregation of the emerging merozoites and that although attachment of the parasites to erythrocytes may be achieved, penetration is not completed. These authors suggested that the interaction of antibody with merozoites will not prevent penetration unless the merozoites are agglutinated. The in vivo situation in this respect is less clearly understood. Although mechanisms similar to those operating in vitro may also operate in vivo, additional factors may determine the fate of circulating FP and possibly of IRBC in the presence of antibody.

The question of whether protective antibodies act against FP or against IRBC could be approached directly by the use of preparations of FP and IRBC in comparative protection studies. The convenient and relatively inexpensive *Plasmodium berghei*-rodent system, combined with the sonic oscillation procedure (19, 20), provides a suitable preparation of infective FP, essentially devoid of IRBC for such studies. In

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the present work we used such preparations of free *P. berghei* parasites to investigate in a comparative way antibody-mediated in vivo elimination of FP and IRBC to obtain information on the nature of parasite-antibody interactions.

MATERIALS AND METHODS

Parasites. *P. berghei berghei* strain was obtained from M. Aikawa (Case Western Reserve University, Cleveland, Ohio). This strain, which originated from the Walter Reed Army Institute of Research, was maintained in our laboratory by serial passage in mice.

Harvesting of FP. FP were harvested from the blood of infected mature rats after parasitemia reached about 50%. To obtain such a high parasitemia in rats, it was necessary to pretreat them with phenylhydrazine in order to increase their reticulocyte counts and thus make them more susceptible to *P. berghei* infection. To do this, the rats were given two injections of 30 mg of phenylhydrazine per kg of body weight. The injections were given 48 h apart, and 48 h later the rats were infected by the intravenous injection of heavily infected mouse blood. Parasitemia reached 50% 3 to 4 days later. FP were isolated from the infected blood by a modified continuous-flow sonication procedure (19, 20). The only changes in the published procedure involved the centrifugation stage to separate FP from erythrocytes after sonication. The sonicated material was centrifuged at 1,450 × *g* for 10 min; the supernatant fluid containing predominantly small parasites and no unlysed erythrocytes was collected, and the parasites were washed twice in Alsever solution by centrifugation at 6,000 × *g* for 10 min.

Animals. Male inbred CDF rats (Dunning Fischer 344 descendants, Charles River Breeding Laboratories) were used throughout the experiments. Mature rats were used as a source for serum and parasites; 4-week-old rats were used for protection tests.

Sera. Normal rat serum was harvested from CDF rats and stored at −20 C. Immune serum was raised in rats as follows. Rats were infected by injecting them with 2×10^6 IRBC of rat origin and were reinjected 30 days later with a similar dose of IRBC. The rats were bled 30 days after the second injection, and serum was separated, pooled, and stored at −20 C. Fluorescein-labeled rabbit immunoglobulin G raised against rat gamma globulin was obtained from Microbiological Associates.

Protection test procedure. One volume (usually 0.5 ml) of a suspension containing 5×10^6 FP or 5×10^3 IRBC per 0.1 ml was mixed with an equal volume of serum, and 0.2 ml of the mixture was injected intravenously into 4-week-old rats. Blood smears were taken daily and stained with Giemsa, and numbers of IRBC per 10,000 erythrocytes were determined.

Pretreatment of parasites with serum. One volume (usually 0.5 ml) of a suspension containing 5×10^6 FP or 5×10^3 IRBC per 0.1 ml was mixed with an equal volume of serum and incubated in a water bath at 37 C for 30 min. After incubation, the suspension was centrifuged in the cold at 5,000 rpm for 10 min, the supernatant was removed, and the parasite pellet was suspended in 3 ml of cold Alsever solution, centrifuged as before, and resuspended to the original volume in Alsever solution.

Fluorescent antibody test. Fresh unfixed FP, IRBC, or normal erythrocytes were used after being washed three times in Alsever solution. Five × 10^6 cells in a volume of 0.1 ml were mixed with immune rat serum or normal rat serum and incubated for 15 or 30 min in a water bath at 37 C or at room temperature for 5 min. After three repeated washes with 3-ml volumes of Alsever solution, the parasites were resuspended to a volume of 0.1 ml and incubated for 30 min at 37 C with 0.05 ml of fluorescent anti-rat gamma globulin (diluted 1:2 in Alsever solution). This step was followed by three washes in Alsever solution and resuspension to a volume of 0.1 ml. A drop of each sample was examined under the fluorescence microscope.

RESULTS

A preliminary study was done by the fluorescent antibody technique on fresh unfixed cells to determine whether antiplasmodial antibodies bind to FP and/or IRBC (Table 1). FP pretreated with serum from recovered rats and washed extensively thereafter were strongly stained by fluorescent rabbit immunoglobulin G raised against rat gamma globulin, whereas infected and normal erythrocytes were not. FP in the preparation of IRBC were also stained. These results indicate that parasites were protected from antibody activity when within erythrocytes. However, once free from the protective envelope of the erythrocyte, the parasites became coated with antibody, even if they were separated from the antibody by washing after as short an interval as 5 min. Some fluorescence was also detected on FP treated with normal rat serum, which indicates that some

TABLE 1. *Interaction of serum from rats immune to P. berghei infection with malaria parasites*[a]

Antigen	Serum[b]	Results[c]
FP	IS**	++++
	NS***	±
IRBC (50% parasitemia)	IS	−[d]
	NS	−
Normal erythrocytes	IS	−
	NS	−

[a] Comparative study with FP and IRBC by the indirect fluorescent antibody technique using unfixed cells.

[b] IS, Serum from immune rats; NS, normal rat serum.

[c] Results were identical with incubation for either 15 or 30 min at 37 C or for 5 min at room temperature.

[d] Free parasites that were present in preparations of IRBC fluoresced.

nonspecific binding of gamma globulin to FP may occur.

In another experiment (data not shown), we demonstrated that the infectivity of both FP and IRBC could be inhibited to various degrees by sera from animals immune to *P. berghei* infection. These experiments were essentially passive transfer experiments and therefore could not give us a definitive answer to the question of whether protective antibodies act on FP or IRBC or both. Since it was found that FP but not IRBC are coated with antibodies after incubation with immune serum, we performed comparative experiments to determine whether in vitro pretreatment of FP and IRBC with immune serum, followed by their washing to remove unattached antibody, would reduce or destroy the parasites' infectivity. Two separate samples of FP were pretreated with immune serum and washed as described in Materials and Methods. One of the samples was resuspended in the original supernatant, which contained immune serum, and the other was resuspended in Alsever solution. The suspensions were then injected intravenously into rats (5×10^6 FP/0.2 ml per rat). A control group of rats received injections of FP pretreated with normal serum and then washed and resuspended in Alsever solution. Other rats received injections of IRBC (5,000/0.2 ml per rat) which were similarly pretreated with immune or normal serum and which underwent similar washing and resuspension. The initial stages of parasitemia in these rats is summarized in Tables 2 and 3. FP pretreated with immune serum under conditions allowing attachment of antibodies to the parasites and then washed and resuspended in immune serum were efficiently eliminated in vivo. However, if resuspension was made with Alsever solution, the initial stages of parasitemia were essentially similar to those of the control group, which received parasites pretreated with normal serum and resuspended in Alsever solution. IRBC that were pretreated with immune serum and then washed and suspended in buffer also initiated infection. When resuspended in immune serum, infection by IRBC was inhibited. In this study, therefore, antibody-coated FP did not behave differently from IRBC that were devoid of antibody coating, and the question of which of the two was the direct target for protective antibodies remained unsolved. These results agree with those made by Jerusalem et al. (16), that extracellular parasites coated with antibodies do not lose their infectivity, and could be interpreted to mean that the antibodies which coat FP have nothing to do with protection. An alternate explanation of these results would be that protective antibodies may be removed easily by washing. It is also possible that washing does not remove all protective antibodies but just reduces their concentration on the surface of the parasite below the critical level required for protection. We have done experiments designed to determine whether some protective anti-

TABLE 2. *Early stages of patent infection in rats given injections of free parasites pretreated in vitro with immune serum (IS) or normal serum (NS) and then washed and resuspended in immune serum or buffer*

Group	Serum used for pretreatment	Serum used for resuspension after washing	Early stages of patency at given day after inoculation[a]				
			5	6	7	8	9
A	IS	IS	0	0	0	0	0
B	IS	Buffer	4	74	2,046	2,266	3,100
C	NS	Buffer	7	64	1,199	2,662	3,366

[a] Figures represent the sum of parasitized erythrocytes per 10,000 erythrocytes counted for each of four rats in a group.

TABLE 3. *Early stages of patent infection in rats given injections of infected erythrocytes pretreated in vitro with immune serum (IS) or normal serum (NS) and then washed and resuspended in immune serum or buffer*

Group	Serum used for pretreatment	Serum used for resuspension after after washing	Early stages of patency at given day after inoculation[a]				
			3	4	5	6	7
A	IS	IS	0	0	0	0	0
B	IS	Buffer	7	89	844	4,016	3,620
C	NS	Buffer	9	110	1,390	3,982	4,048

[a] Figures represent the sum of parasitized erythrocytes per 10,000 erythrocytes counted for each of four rats in a group.

bodies are still associated with the pretreated parasites after their washing. Samples of FP were pretreated with immune serum, washed as before, and then suspended in Alsever solution to their original volume. Aliquots of the suspensions of antibody-treated FP were mixed with equal-sized aliquots of serial twofold dilutions of immune serum of the same batch as used for the in vitro pretreatment, and the mixtures were injected into rats (5×10^6 FP/0.2 ml per rat). Control rats received injections of FP similarly pretreated with serum before washing but with normal serum rather than with immune serum, and these FP were mixed with serial dilutions of the same immune serum. Parasitemia rose more slowly in rats injected with FP pretreated with immune serum and then washed and suspended in immune serum than in rats injected with FP pretreated with normal serum and then washed and suspended in immune serum (Table 4). This effect was particularly evident when the suspending immune serum was diluted up to 1:4. Furthermore, fewer rats showed patent infection after being injected with FP pretreated with immune serum than with FP pretreated with normal serum (Table 5). Thus, although immune serum diluted 1:8 completely inhibited parasitemia in 50% of rats, when injected with FP pretreated with immune serum it took more than twice that amount of immune serum to completely inhibit parasitemia in 50% of the rats when FP pretreated with normal serum were used. These results indicate that protective antibodies were still associated with the surface of the parasites after the pretreatment with immune serum and subsequent washings and that these antibodies contributed to the overall protective activity afforded by the immune serum injected along with the pretreated parasites. These results may also be interpreted to mean that a critical amount of antibody is required to prevent a parasite from initiating infection and that antibody on the parasite contributes to that amount.

A similar type of experiment was done with IRBC rather than FP (Table 6). The initial stages of parasitemia were essentially similar in rats receiving injections of pretreated IRBC together with the corresponding dilution of immune serum, regardless of whether pretreatment was done with immune serum or with normal serum. These results comply with the finding that IRBC are not coated with antibodies after treatment with immune serum (Table 1) and suggest, when compared with the results obtained with FP (Tables 4 and 5), that antibodies do not directly affect the elimination of IRBC during the initial stages of infection in animals being studied in passive transfer experiments. These experiments have been repeated with similar results.

In the experiments described so far, we used immune serum that was raised by infecting rats with *P. berghei* and reinoculating them after their recovery from infection. To find out whether reinoculation might have affected the binding character of the antibody, we harvested serum from rats 6 weeks after their recovery from *P. berghei* infection, without reinoculation, and used it in an experiment similar to that described in Table 4. The results of the experiment with serum from recovered animals are presented in Table 7. The protective activity of this serum was relatively weak since even undiluted serum allowed parasites to cause

TABLE 4. *Early stages of patent infection in rats inoculated with free parasites (FP) pretreated with immune serum (FPI) or with normal serum (FPN) and then washed and mixed with doubling dilutions of immune serum*

Parasite prepn	Immune serum dilution	Early stages of patency at given day after inoculation[a]						
		5	6	7	8	9	10	11
FPI	1:1	0	0	0	0	0	0	0
	1:2	0	0	0	0	0	0	8
	1:4	0	0	0	0	8	35	704
	1:8	0	0	2	10	77	913	1,960
	1:16	0	0	6	86	782	2,628	5,760
FPN	1:1	0	0	0	0	0	0	0
	1:2	0	0	0	0	2	10	100
	1:4	0	1	5	37	597	770	2,550
	1:8	0	1	4	22	395	1,617	9,400
	1:16	0	3	22	246	1,046	1,751	4,444

[a] Figures represent the sum of parasitized erythrocytes per 10,000 erythrocytes counted for each of four rats in a group.

patent infection; however, a protective effect of the immune serum was still evident. Pretreatment of parasites with this immune serum had only a marginal effect on the efficiency of elimination of the parasites after their injection with dilutions of immune serum. This suggests that washing removed a more substantial proportion of the protective antibodies from the parasites pretreated with this serum than from those pretreated with serum raised by infection and reinoculation (Tables 4 and 5).

TABLE 5. *Percent infectivity of free parasites (FP) pretreated with immune serum (FPI) or with normal serum (FPN) and then washed, mixed with dilutions of immune serum, and injected into rats*

Parasites	% Infected rats among rats given parasites in given serum dilution[a]				
	1:1	1:2	1:4	1:8	1:16
FPI	0	18	30	50	86
FPN	0	29	57	88	100

[a] Figures represent infectivity percentages obtained from groups of four rats analyzed by the method of Reed and Muench.

DISCUSSION

Protective antibody against *P. berghei* has been demonstrated by passive transfer experiments with both IRBC (1, 13, 26) and FP (Hamburger and Kreier, unpublished data). However, only FP have been shown to be coated by antibodies after incubation with immune sera, whereas IRBC are not (Table 1). Nevertheless, pretreatment of FP with immune serum does not increase the rate of their in vivo elimination if the parasites are washed before injection (Table 2). In this respect FP behave

TABLE 6. *Early stages of patent infection in rats inoculated with infected erythrocytes (IRBC) pretreated with immune serum (IRBCI) or with normal serum (IRBCN) and then washed and mixed with doubling dilutions of immune serum*

Parasite prepn	Immune serum dilution	Early stages of patency at given day after inoculation[a]					
		4	5	6	7	8	9
IRBCI	1:1	0	0	2	28	320	2,350
	1:2	0	3	40	274	2,585	2,019
	1:4	0	14	119	989	2,409	3,278
	1:8	3	41	237	2,453	3,014	3,389
	1:16	5	49	360	3,630	3,212	2,916
IRBCN	1:1	0	0	3	34	280	2,570
	1:2	0	1	36	348	2,211	2,079
	1:4	0	9	114	1,177	2,696	2,992
	1:8	2	39	261	2,637	3,719	3,960
	1:16	7	57	438	3,167	3,377	3,069

[a] Figures represent the sum of parasitized erythrocytes per 10,000 erythrocytes counted for each of four rats in a group.

TABLE 7. *Early stages of patent infection in rats inoculated with free parasites (FP) pretreated with a "weak" immune serum (FPI) or with normal serum (FPN), and then washed and mixed with doubling dilutions of immune serum*

Parasite prepn	Immune serum dilution	Early stages of patency at given day after inoculation[a]					
		5	6	7	8	9	10
FPI	1:1	0	0	2	64	368	2,024
	1:2	0	1	15	202	1,629	6,101
	1:4	0	9	106	1,600	4,268	8,591
	1:8	1	16	235	2,431	4,884	7,271
FPN	1:1	0	0	2	25	189	1,489
	1:2	0	1	20	167	1,089	5,727
	1:4	2	19	203	1,557	3,295	6,622
	1:8	2	24	480	3,229	6,479	5,423

[a] Figures represent the sum of parasitized erythrocytes per 10,000 erythrocytes counted for each of four rats in a group.

like IRBC subjected to similar pretreatment and washing conditions (Table 3). These observations with pretreated FP and IRBC confirm previous observations (6, 14, 16). It may seem, therefore, that antibodies which coat FP have no protective significance and may even have the capacity to enhance parasitemia as suggested by Jerusalem et al. (16). We found, however, that FP pretreated with immune serum and washed were eliminated in vivo more efficiently than were FP pretreated with normal serum if the preparation were injected with small amounts of immune serum (Tables 4 and 5). Immune serum pretreatment of IRBC, however, did not affect them at all (Table 6). It should be emphasized that in our experiments we chose the early stage of parasitemia for determining the activity of passively transferred antibodies since a correlation was found to exist between the reduction in potency of inocula of *P. berghei* and the delay in the rise of parasitemia (24). Later stages of the infection probably represent a more complex situation in which acquired immunity of the host may play a major role.

Our findings indicate that protective antibodies exert their activity on FP, thus supporting the suggestion of Cohen and Butcher (9), which was based on the observation that antibodies inhibit the in vitro development of *P. knowlesi* at stages following schizogony. They may also indicate that a critical amount of protective antibodies must be maintained in association with the parasites in order to allow their efficient elimination and that washing of the free parasites after their incubation with protective antiserum leaves only a subcritical amount of protective antibodies on the surface of the parasites. It still remains possible that a substantial proportion of antibodies that coat FP are not protective.

The relative ease by which protective antibodies seem to be removed by washing (Table 2) and the apparent requirement for continuous presence of unbound antibodies (Table 4) suggest that the association of protective antibodies with the parasites is of a highly reversible nature. This may be attributed to low avidity of the protective antibodies or to shedding of antigen-antibody complexes from the parasite's surface. The possibility that protective antibodies do not affect the parasites irreversibly and that protective antibody activity depends on additional conditions in the host emerges from the finding that similar immune sera had different protective activity in different animals, ranging from no apparent protection in mice to various degrees of protection in two different strains of rats (Hamburger and Kreier, unpublished data). Also, since complement activity does not appear to be required for protective antibody activity in vitro (9) and in vivo (15), it seems unlikely that activation of complement by parasite-antibody complexes leads to killing of parasites, nor is it likely that phagocytosis of parasites by macrophages is substantially enhanced by complement.

In relating to the possibility that the protective antibodies may have low avidity, it should be mentioned that the bulk of the protective activity against *P. berghei* lies in the immunoglobulin G fraction, whereas multivalent antibody moleucles, which may increase the overall avidity of protective sera, do not seem to play a major role in protection (13, 18; A. Zuckerman, J. Golenser, and D. T. Spira, Pre-Congress Bulletin, Int. Congr. Trop. Med. Malariol., 9th, Athens, Greece, 1973).

The ability of the merozoite to invade an erythrocyte relative to the efficiency of the reticuloendothelial system in ingesting the merozoites probably determines the fate of the malaria parasite, since the presence of circulating merozoites can be considered an event of short duration, culminating either in successful penetration or in elimination by phagocytes. It is known that manipulations that decrease total reticuloendothelial system activity, such as splenectomy, and those that increase the number of reticulocytes will also increase the susceptibility of rodents to *P. berghei* infection (25). In a situation where phagocytes, on the one hand, and erythrocytes, on the other, may compete with each other in taking up FP, it is not imperative that protective antibodies irreversibly neutralize the parasites in order to affect their elimination, since even an unstable state of neutralization may delay penetration and thus increase the chances for elimination. It has been proposed by Cohen and Butcher (9) that antibodies which inhibit in vitro development of *P. knowlesi* neutralize merozoites as antiviral antibodies neutralize viruses. Miller et al. (17), on the other hand, proposed that protective antibodies act as agglutinins and that agglutinated merozoites are incapable of penetration. A highly dissociable protective antibody may indeed be analogous to some virus-neutralizing antibody systems (11). It is not inconceivable, however, that both neutralization and agglutination of parasites by delaying penetration into erythrocytes may contribute in some degree to the elimination of the parasites by the phagocytes.

Antibody-mediated antigenic variation of malaria parasites has been described in rodent (2, 12), monkey (3, 5, 23), and human malaria (22) and is considered a major mechanism by

which malaria parasites survive in an immunologically hostile environment (4). Furthermore, it has been shown that antibodies which inhibit the in vitro growth of *P. knowlesi* are predominantly variant specific (7). In view of our findings, it appears possible that an easily reversible parasite-protective antibody interaction may also contribute to the ability of malaria parasites to evade the immune response.

Our results showed that a "weak" antiserum harvested after recovery and without reinoculation had a lesser capacity to remain associated with the parasites after pretreatment and washing than a serum produced by reinfection (Table 7). It appears possible, therefore, that repeated hyperimmunizations with plasmodia may induce antibodies of progressively higher affinity. Butcher and Cohen (7) have recently demonstrated that repeated reinfections of monkeys with the same variant of *P. knowlesi* induces the production of inhibitory antibodies against other variants. They postulated that this nonhomologous protection might be attributed to low-affinity cross-reacting antibodies or to antibodies produced by repeated immunization to common antigenic determinants present in small amounts in the parasites. The reversible nature of antibody-parasite interaction suggests that antibodies of low affinity may be involved in this type of nonhomologous protection.

The model used in our study provides a convenient and relatively inexpensive tool for studying the interactions between antiplasmodial antibodies and their immediate target, the free malaria parasites. It allows the combination between in vitro manipulation of free parasites and a subsequent assessment of infectivity in vivo. To our knowledge, no such system has yet been reported in the context of malaria parasites. A similar preparation of free parasites was recently used for a cytochemical study of the parasite's surface (21). Combined cytochemical immunological manipulations of free parasites may provide information about the molecular nature of the antigens on the surface of the parasites.

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