Plasmodium berghei Malaria: Effects of Acute-Phase Serum and Erythrocyte-Bound Immunoglobulins on Erythrophagocytosis by Rat Peritoneal Macrophages

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Acute-phase serum (APS) collected from Plasmodium berghei-infected rats inhibited phagocytosis of trypsinized rat erythrocytes and of erythrocytes from P. berghei-infected rats. Macrophages (MΦ) incubated with APS or heat-aggregated acute-phase serum (HAAPS) for 6 h, followed by 18 h incubation in serum-free medium, exhibited significantly higher levels of phagocytosis than MΦ similarly cultured but with normal rat serum. When APS was present at the time of assay, it inhibited erythrophagocytosis by MΦ which had been in culture for 0 or 24 h. MΦ activation by HAAPS was inhibited by 2-deoxy-D-glucose, which suggests that activation by HAAPS is Fc-receptor mediated. Adsorption of APS with staphylococcal protein A abrogated the ability of APS to inhibit phagocytosis and of HAAPS to effect MΦ activation, suggesting that immune complexes are involved in both processes. Surface-bound immunoglobulins eluted from erythrocytes of P. berghei-infected rats promoted phagocytosis of trypsinized erythrocytes by HAAPS-activated MΦ but not by resting MΦ. These results indicate that the immunoglobulins which bind to infected or damaged erythrocytes during malaria infections promote erythrophagocytosis by activated MΦ and that the immune complexes in serum from rats with acute malaria may inhibit erythrophagocytosis early in the infection but may, over time, induce changes in the MΦ which later facilitate erythrophagocytosis.

Malaria, caused by parasites of the genus Plasmodium, is one of the most important diseases in the world today. Wernsdorfer (36) estimated that malaria causes the deaths of one million infants and children annually. Despite the importance of malaria, the immunological mechanisms that enable the host to control plasmodial infection have not been clearly identified. Immune mechanisms directed against infected erythrocytes have been proposed (38), as have mechanisms directed against the merozoite when it passes from one erythrocyte to another (2, 5, 6, 8, 20). It is likely that both humoral and cellular responses are important in controlling infection (16, 22, 23, 26).

Both immunoglobulin G (IgG) and IgM antibodies are bound to erythrocytes (RBCs) of individuals with malaria (12, 18, 19, 34, 38, 39). Some of the IgM antibodies on the RBCs are cold reactive types, specific for lipids exposed by proteolytic degradation of the RBC membranes. They can be eluted by incubation at 37°C (7, 15, 18, 27). Attempts to demonstrate that antibody eluted from RBCs of infected rats enhances erythrophagocytosis by resting macrophages either in vitro (5, 9) or in vivo (17, 28) have failed.

Ingestion of immune complexes or heat-aggregated immunoglobulins triggers activation of macrophages (MΦ) (24). Immune complexes are present in the plasma of malarious mice (13, 33), humans (16), and rats (3). This paper describes experiments which provide indirect evidence that immune complexes may initially protect plasmodia from phagocytosis by blocking Fc-receptors, but that later in the course of the disease immune complex-mediated activation of MΦ may facilitate phagocytosis of opsonized RBCs. Phagocytosis of RBCs opsonized with antibodies by MΦ activated by ingestion of immune complexes may thus in part account for the anemia and drop in parasitemia that occur at the “crisis” period of plasmodial infection.

MATERIALS AND METHODS

Animals. Inbred Fisher 344 rats (CDF; Charles River Breeding Laboratories, Inc., Wilmington, Mass.) weighing from 150 to 200 g were used as the source of rat serum, RBCs, and peritoneal MΦ. Swiss albino mice were used to maintain P. berghei.

Parasites. P. berghei berghei (strain from Walter Reed Army Institute of Research) was stored in liquid nitrogen. Frozen stock parasites were thawed and used to infect Swiss albino mice as needed. The strain from the frozen stock was passed in mice no more than twice during these studies. Rats were infected with 10⁷ fresh, infected mouse RBCs by tail vein injection.

Serum collection, storage, and preparation. Rats were anesthetized with ether, and blood was collected by cardiac puncture. All sera were pooled, filter sterilized, and stored at −20°C. Acute-phase serum (APS) is defined here as serum which was collected and pooled 14 days postinoculation when infected rats had parasitemias ranging from 28 to 33%. Heat-aggregated normal rat serum (HANRS) and heat-aggregated acute-phase serum (HAAPS) were prepared by heating the sera at 63°C in a water bath for 30 min just before use.

MΦ harvest, staining, and counting. Normal rats were exsanguinated while anesthetized. The MΦ were obtained by filling the peritoneal cavity with 30 ml of sterile RPMI-1640 (GIBCO Laboratories, Grand Island, N.Y.) which had been supplemented with 25 mM HEPES (N-2-hydroxethylpiperazine-N'-2-ethanesulfonic acid; Sigma Chemical Co., St. Louis, Mo.), 25 U of penicillin per ml, 25 μg of streptomycin per ml, and sodium bicarbonate to give a pH of 7.0. Cells were collected through the peritoneal wall into a
sterile syringe and washed, and $2 \times 10^6$ viable cells were plated onto 35-mm plastic tissue culture dishes. The cells were incubated for 1 h at 37°C in a humidified, 7% CO$_2$ chamber; then nonadherent cells were removed by vigorous washing with sterile phosphate-buffered saline (PBS; pH 7.2). The adherent cells, which were >98% esterase positive, were used immediately as normal or resting MΦ unless stated otherwise. All phagocytosis assays were conducted at 37°C for 30 min in a humidified chamber with 7% CO$_2$ in air. All treatments were run in triplicate for each study, and experiments were repeated three times.

After each assay, plates were washed vigorously with PBS to remove RBCs which were not internalized, methanol fixed, and stained with Giemsa. Between 300 and 400 MΦ were counted for each plate, the number of MΦ containing one or more RBCs was determined microscopically, and the results are reported as the percent of phagocytic MΦ $\pm$ standard deviation (SD). Statistical analyses were made using the Student t-test. The use of a hypotonic solution to lyse bound RBCs was precluded, because this treatment sometimes caused mast cell degranulation and lysis of the MΦ monolayers.

**RBC harvest and preparation.** Normal or infected rats were anesthetized, then exsanguinated by cardiac puncture into an equal volume of cold Alsever’s solution. The RBCs were washed in cold PBS and then resuspended in RPMI-1640 to a concentration of $10^6$ per ml. A two-ml amount of the RBC suspensions was added to the MΦ monolayers to provide an RBC MΦ ratio of 100:1 in all experiments. Fresh RBC suspensions were prepared for each assay. Infected RBCs (IRBCs) were collected from rats having a 25 to 35% parasitemia, which was determined by counting 10,000 Giemsa-stained RBCs on a thin smear.

Uninfected RBCs (NRBCs) were trypsinized (TRBCs) by suspending 0.2 ml of packed, cells in 4 ml of a 0.25% trypsin solution in PBS. The suspension was placed in a water bath at 37°C for 15 min with occasional mixing. The TRBCs were then washed thoroughly before use.

**Effects of APS on phagocytosis by MΦ after various culture times.** MΦ were harvested and washed and described, except that 10% heat-inactivated fetal calf serum was added to the culture medium. MΦ monolayers that were assayed immediately were described as normal, resting, or zero-hour cultures. These cultures were used for baseline measures of the percent of normal MΦ that were phagocytic after various treatments.

MΦ cultured 24 h were incubated with 3 ml of RPMI-1640 containing 10% fetal calf serum. Monolayers were washed just before each assay, and MΦ were incubated with NRBCs, IRBCs or TRBCs prepared as previously described. Rat RBC suspensions either contained no serum or were supplemented with 10% heat-inactivated normal rat serum (NRS) or 10% heat-inactivated APS.

**MΦ activation using serum from acutely infected rats.** Based on the observations that heat-aggregated IgG or immune complexes can induce MΦ activation within 6 h (24), studies were conducted to determine whether MΦ exposed to HAAPS, APS, NRS or HANRS for 6 h would become activated. Freshly prepared MΦ monolayers were incubated at 37°C with 7% CO$_2$ for 6 h in 2 ml of RPMI-1640 supplemented with 10% NRS, APS, HANRS, or with HAAPS. After 6 h, the monolayers were washed with PBS and incubated in serum-free RPMI-1640 for an additional 18 h, to permit sufficient time for the MΦ to ingest bound aggregates and to regenerate Fc receptors (25). Monolayers were then washed, and suspensions of NRBCs, IRBCs, or TRBCs prepared as described earlier were placed on the monolayers, incubated, methanol-fixed, stained, and counted.

**Inhibition of MΦ activation with 2-DG.** RPMI-1640 containing 50 mM 2-deoxy-d-glucose (2-DG) was used to determine whether HAAPS activation of MΦ was mediated through Fc receptors, since it has been reported that 2-DG inhibits the phagocytosis of IgG aggregates or of IgG-antigen complexes which bind to Fc receptors (21). Fresh MΦ monolayers were incubated for 45 min in RPMI-1640 containing 2-DG before the addition of serum-supplemented medium. Medium containing 10% HAAPS and 50 mM 2-DG was then placed on the MΦ monolayers. Cells were incubated for 6 h, washed, and then incubated for 18 h in serum-free RPMI-1640. Monolayers treated with 2-DG but without HAAPS for the same time were checked for viability at 24 h by trypan blue dye exclusion. Positive controls without 2-DG were also run.

**Titration of RBC surface IgG and IgM by passive hemagglutination.** IRBCs or NRBCs, pooled from two to five animals, were collected in cold Alsever’s solution (50% [vol/vol]) and held at 4°C for 1 h to promote binding of cold hemagglutinins to the RBCs. Cells were then washed three times in cold PBS and resuspended to a 50% concentration. One half of each suspension was held at 4°C (nonluted RBCs) and the other half was held at 37°C (eluted RBCs) for 40 min. Cells were then centrifuged and supernatants were held for further analysis. Eluted and noneluted RBCs were suspended in cold PBS at a 0.5% concentration. Hemacytometer counts were used to adjust the cells to an equal cell concentration. All suspensions were held at 4°C before use.

Doubling dilutions of goat anti-rat IgG (Kirkegard and Perry Laboratories, Gaithersburg, Md.) were made with cold PBS, as were dilutions of rabbit anti-rat IgM (Miles Laboratories, Inc., Elkhart, Ind.). The one-half dilutions contained 25 μg of anti-rat IgG antibody or 1.5 mg of protein in the anti-IgM preparation (the antibody concentration was not specified). Tests were run in duplicate with 0.1 ml of the antisera dilutions and 0.1 ml of the eluted or non-eluted IRBCs or NRBCs. Sets of tubes were incubated at 4°C and 37°C, and titers were recorded at 4 h. The titer represents the highest dilution of antisera causing erythrocyte agglutination.

**Eluate preparation and storage.** Studies were conducted to determine whether immunooglobulins eluted from IRBCs could facilitate phagocytosis of TRBCs by HAAPS-activated MΦ. The term eluate refers to the supernatant that was removed from IRBCs which had been held at 37°C for 40 min. Procedures used were those described by Kreier et al. (18). To compensate for some loss of eluted immunoglobulins during handling, the eluate was concentrated to one half the original volume of blood collected, using XM100A Diaflo ultrafiltration membranes (Amicon Corp., Lexington, Mass.) to retain and concentrate molecules with molecular weights greater than 100,000. Samples were prepared on ice and then the concentrated eluate was filter sterilized and frozen at $-20°C$ until used. Dilutions of eluate were prepared in RPMI-1640 and comprised 10% of the final 2-ml volume of TRBC suspensions used in the assays. Presence of both rat IgG and IgM in the eluate was verified by immunoelectrophoresis.

**Adsorption of immune complexes from APS and HAAPS with protein A.** Protein A-binding staphylococci (The Enzyme Center, Walden, Mass.) were washed repeatedly in sterile PBS and then were pelleted by centrifugation. Pellets were resuspended in APS or HAAPS twice, 10 min each time, at room temperature. Adsorption times were selected to
10 min, since immune complexes or antibody aggregates should bind protein A in this time, while most monomeric IgG binds more slowly (14). Adsorbed serum samples were centrifuged to pellet the staphylococci to which the complexes were bound; then all samples were filter-sterilized and assayed immediately. Mφ cultured 24 h in medium containing 10% fetal calf serum were used to determine whether adsorption of APS with protein A would abrogate inhibition of phagocytosis.

RESULTS

Effects of NRS and APS on phagocytosis of RBCs by rat peritoneal Mφ cultured for various times. There was a slight increase in phagocytosis of NRBCs with increasing time of culture of Mφ in the absence of NRS. The level of phagocytosis of IRBCs and of TRBCs was consistently greater than that of NRBCs. The level of phagocytosis in each case was increased by cultivation of the Mφ for 24 h. The addition of APS to the cultures at the time of assay for phagocytic capacity at 24 h significantly inhibited phagocytosis of IRBCs and of TRBCs, as compared to the percent of phagocytic Mφ observed when NRS was used (P < 0.05). The inhibitory effect of APS was concentration dependent. At the low concentrations of APS, a greater proportion of Mφ incubated for 24 h phagocytized IRBCs and TRBCs than did Mφ at 0 h (Fig. 1), which indicates that low levels of culture-induced activation occur. Adsorption of APS with protein A abrogated the inhibition (data not shown).

Effect of culture for 6 h in the presence of APS and HAAPS on erythrophagocytic capacity of rat peritoneal Mφ after an additional 18 h cultivation. Cultivation for 6 h in APS or HAAPS, followed by 18 h cultivation in serum-free RPMI-1640, increased phagocytic capacity of Mφ over that of Mφ treated similarly but with NRS or HANRS in the initial 6 h incubation. The increases were apparent with all types of RBCs tested, but phagocytosis of IRBCs and TRBCs was always greater than phagocytosis of normal rat RBCs (Fig. 2). The percent of Mφ incubated with HAAPS which phagocytosed IRBCs or TRBCs was significantly higher (P < 0.05) than with all other treatments.

Relationship of RBC-bound immunoglobulins to parasitemia in rats infected with P. berghei. Both IgG and IgM were detected on surfaces of pooled, washed RBCs from infected rats by a passive hemagglutination test (Table 1). No RBC-bound IgG was detected on days 3 or 6 postinfection, even in animals having 10 to 13% parasitemias. Erythrocytes from rats which had a 55 ± 5% parasitemia 9 days postinfection had surface IgG and IgM titers of 32 and 512, respectively. Those rats which did not reach a 51 ± 5% parasitemia until 21 days postinfection had a higher IgG titer and a lower IgM titer than rats tested 9 days postinfection. The IgG titer in animals having a 31 ± 5% parasitemia at 15 days postinfection was 16, and rats of this same group had an IgM titer of 8. Pooled RBCs from rats which had 0% parasitemia by 23 days postinfection had no detectable surface IgG or IgM. In no case was hemagglutination observed at 37°C.

Effect of immunoglobulins eluted from the surfaces of RBCs of P. berghei-infected rats on phagocytosis of TRBCs by HAAPS-activated Mφ. RBCs from infected rats having high titers of RBC-bound IgG and IgM were held at 37°C to elute bound globulins. The eluates were concentrated and then added to suspensions of TRBCs. The mixture was then added to Mφ cultures. Activated Mφ in the cultures ingested few RBCs when held at 4°C under any conditions (data not shown). When assayed at 37°C, however, the number of Mφ ingesting TRBCs in the absence of eluate was significantly lower than the proportion of Mφ ingesting TRBCs that were supplemented with 10% eluate (Fig. 3). Addition of 5% eluate also enhanced phagocytosis. Enhancement of phagocytosis was most pronounced when the eluates were concentrated to 20% of the original volume.

![FIG. 1. Effects of NRS and APS on erythrophagocytosis by Mφ cultured 0 or 24 h before assay. Percent Mφ ingesting NRBCs, 1; IRBCs, 2; or TRBCs, 3. Bars represent the mean number of phagocytic Mφ ± SD.](http://iai.asm.org/)

![FIG. 2. Effect of culture for 6 h in the presence of NRS, APS, HANRS, or HAAPS on erythrophagocytosis by rat peritoneal Mφ after an additional 18-h cultivation in serum-free medium. Percent Mφ ingesting NRBCs, 1; IRBCs, 2; or TRBCs, 3. Bars represent the mean number of phagocytic Mφ ± SD.](http://iai.asm.org/)
phagocytosis above control levels by both 10 and 5% eluate was significant ($P < 0.05$). Enhancement was abrogated by diluting eluate to 2.5% or less. Eluate did not enhance phagocytosis by resting Mϕ at any of the concentrations tested (data not shown).

**Effect of 2-dG on activation of Mϕ by cultivation with HAAPS.** Mϕ that were cultured for 6 h in medium containing 50 mM 2-dG and 10% HAAPS, followed by 18 h culture in RPMI-1640 were not activated. This was indicated by the significantly lower phagocytic capacity of these Mϕ ($P < 0.05$) for NRBCs, TRBCs, and IRBCs than of Mϕ similarly incubated but without 2-dG (Fig. 4). Cultures incubated with and without 2-dG contained similar numbers of viable Mϕ as determined by microscopic examination and trypan blue exclusion.

**Effect of absorption of HAAPS with protein A on activation of Mϕ by cultivation with HAAPS.** Mϕ that were cultured for 6 h in medium containing HAAPS which had been adsorbed with protein A, followed by 18 h of culture in RPMI-1640, were less activated than Mϕ similarly treated but with unasorbed HAAPS. This is indicated by the observation that a significantly higher percentage ($P < 0.01$) of Mϕ incubated with nonadsorbed HAAPS than with adsorbed HAAPS engulfed NRBCs, TRBCs, or IRBCs (Fig. 5). Adsorption of complexes was perhaps not complete because phagocytes cultured with adsorbed HAAPS were more active than control Mϕ incubated with HANRS.

**DISCUSSION**

Reports of parasitized and nonparasitized RBCs in Mϕ of birds, monkeys, mice, and rats suffering from acute malaria have long suggested the importance of erythrophagocytosis in clearance of parasitemia (4, 35, 40). Massive erythrophagocytosis generally has a sudden onset and is associated with a precipitous decline in parasitemia and rapidly developing anemia. The stage of the disease in which these latter events occur is called the crisis (reviewed in reference 16; 29). It has generally been assumed that an immune response is responsible for the crisis, but there is no consensus among malarologists on the mechanism producing it (1).

This study and many others (12, 18, 19) have shown that as parasitemia develops, much of the erythron acquires a coat of immunoglobulin. As parasitemia develops, first antigens (31, 32) and then later immune complexes (3, 10, 13) appear in the plasma.

Immune complexes from malaria-infected mice block erythrophagocytosis in vitro (33, 34). In this study, we not only have confirmed that immune complexes inhibit phagocytosis of opsonized RBCs, but also we have shown that the complexes induced activation of resting Mϕ, thereby enhancing their capacity to phagocytize enzyme-damaged RBCs coated with antibodies eluted from RBCs of rats with malaria. The development of agglutinins during the acute anemia phase of malarial infection which can bind TRBCs may be related to exposure of RBC antigens which are also exposed as a result of proteolytic modification of RBCs during a natural infection (18). These may be autoantibodies, some of which have previously been shown to be IgM types (7, 18). Jayawardena et al. (11) reported that mice
incapable of producing IgM autoantibodies to enzyme-damaged RBCs developed more severe parasitemias after *Plasmodium yoelii* infection than did mice that were capable of producing such autoantibodies. Both types of mice finally controlled their infections when they developed IgG antibodies specific for *P. yoelii*. Green and Packer (8) recently reported that rheumatoid factors enhance agglutination of *Plasmodium falciparum* schizonts in vitro. It is possible that autoantibodies against infected or damaged RBCs act in a similar manner to facilitate erythropagocytosis. Both IgG and IgM were present in the eluates that were found to enhance erythropagocytosis by HAAPS-activated Mφ in this study. Significantly higher levels of phagocytosis of IRBCs and TRBCs than NRBCs may also indicate that the specificity of these immunoglobulins is for altered antigens on the RBC membrane. Such alterations may occur due to deposition of parasite antigens on membrane surfaces or to mechanical or proteolytic damage to RBCs during malarial infection.

The observation that HAAPS caused higher levels of activation than did APS is consistent with the observation of Pestel et al. (24) that IgG aggregates or immune complexes can induce Mφ activation and that the degree of activation increases when larger heat-aggregated globulins are present. It has also been shown that chemically cross-linked IgG oligomers have higher affinity for Fc receptors than does monomeric IgG, and that affinity increases with increasing IgG polymerization (30). Aggregation of immune complexes on the Mφ surface appears to be necessary for the efficient endocytosis and clearance of immune complexes in vivo (for review, see the study by R. G. Q. Leslie [18a]). Heat-induced aggregates may thus have higher affinity for Fc receptors and may also cross-link Mφ surface receptors to promote efficient clearance of the complexes.

These various observations are compatible with the hypothesis that crisis occurs when Mφ become activated by ingestion of immune complexes or aggregated immunoglobulins and acquire the ability to phagocytose RBCs coated with autoantibodies. In this scenario the defense of the parasite, based at least in part on a “smoke screen” (37) of soluble antigens and blocking immune complexes, is dissolved when Mφ become activated after ingesting immune complexes. The host response is thus modified by contact with components of the defense of the parasite, such as immune complexes, in such a way as to negate their smoke screen effect. It is important to note that this anti-erythrocyte-based system is only one part of the host defense. The study of Jayawardena et al. (11), for example, shows clearly the importance of the more direct antiparasite system in host defense. Antiparasite-IgG was required to control infections in mice that lacked the IgM autoantibody-based antiparasite system; and mice which produced both IgM and IgG experienced milder infections than did mice which lacked the ability to produce IgM.

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LITERATURE CITED


