IMMUNE PHAGOCYTOSIS IN MURINE MALARIA*

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Hosts infected by plasmodia show a highly complex and not always effective immune response. The high prevalence of this protozoan infection among human populations, the seriousness of the disease, and the search for protective vaccines have prompted numerous studies on the nature of the immunity to malaria, which appears to involve antibodies (1), complement (2), B cells, and T cells (3, 4).

The large increase in the number of spleen macrophages and the finding of parasitized and nonparasitized erythrocytes in phagocytic vacuoles of macrophages of monkeys and birds have been considered suggestive evidence of a role for mononuclear phagocytes in the clearance of parasites (5, 6). Similar observations have been made in spleens of rats infected with *Plasmodium berghei* (7). Previous attempts to reproduce these observations in vitro with rat peritoneal macrophages (8) and human monocytes (9) have been unsuccessful.

Recently, Lustig et al. (10) demonstrated that during *P. berghei* infection of mice, in which reticulocytes are preferentially invaded (11), parasitized and nonparasitized reticulocytes are coated with immunoglobulins (Ig) of the IgG and sometimes IgM classes. These Ig are bound mainly at 4°C, but some remain associated with the erythrocytes at 37°C. The amount of Ig increases with time after infection and parallels parasitemia. These observations suggested that opsonization by Ig followed by phagocytosis was a likely mechanism for the clearance of parasitized erythrocytes.

The present report shows that erythrocytes of malaria-infected mice can be ingested in vitro by macrophages obtained from the spleens of *P. berghei*-infected mice and by thioglycolate-stimulated peritoneal macrophages. This ingestion appears to be mediated by the Ig present on the parasitized erythrocytes. However, later in the course of infection, phagocytosis is inhibited by serum factors, possibly immune complexes. We also observed that malarial infection stimulates splenic macrophages, as detected by enhanced spreading and phagocytic ability.

An unexpected finding was that, in the course of the disease in mice, high levels of anti-Forssman antibodies are produced.

Materials and Methods

Reagents. Phosphate-buffered saline (PBS)[1] without CA^{++} and Mg^{++}, Dulbecco's Modified

* Supported by U. S. Public Health Service grants AI15235, CA23056, and the Malaria Component of the United Nations Development Programme/World Bank/World Health Organization Special Programme for Research and Training in Tropical Diseases.

‡ Recipient of Career Development Award CA00222 from the National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare.

[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; DMEM, Dulbecco's Modified Eagle Medium; EDTA, ethylenediaminetetraacetic acid; E, sheep erythrocytes; EIgG, E sensitized with IgG; EIgM, E sensitized with IgM; EIgMC, E sensitized with IgM plus complement; PBS, phosphate-buffered saline.

 J. Exp. Med. © The Rockefeller University Press · 0022-1007/79/06/1288/11 $1.00
Volume 149 June 1979 1288–1298

Eagle Medium (DMEM), and penicillin-streptomycin solution were obtained from Grand Island Biological Co., Grand Island, New York. Ethylenediaminetetraacetic acid (EDTA) and glucose were purchased from Fisher Scientific , Pittsburgh, Pa. Fetal calf serum, lot 29101068, was obtained from Flow Laboratories, Inc., Rockville, Md. Brewer thioglycolate medium (Difco Laboratories, Detroit, Mich.) was obtained from Beckman Instruments, Inc., Science Essentials Co., Mountainside, N. J. Trypsin and soybean trypsin inhibitor were from Worthington Biochemical Corp., Freehold, N. J. Purified protein A was from Pharmacia Fine Chemicals, Piscataway, N. J. Glutaraldehyde was obtained from Sigma Chemical Co., St. Louis, Mo., and sodium heparin was from Abbott Laboratories, Chicago, Ill. Bovine serum albumin (BSA) was from Miles Laboratories Inc., Elkhart, Ind.

Malaria Parasites. The NK65 strain of *P. berghei* was used. It is maintained by passage in hamsters with malaria-infected blood, alternating with mosquito-induced infections (12). SW mice were inoculated intravenously with 10^4 infected erythrocytes. Parasitemias of infected animals were determined by microscopic examination of Giemsa-stained smears.

Animals. Female SW mice (Taconic Farms, Inc., Germantown, N. Y.), 4–7 wk old, were used.

Macrophages. All cell preparations were prepared under sterile conditions, essentially according to Edelson and Cohn (13). Mice were sacrificed, the peritoneal cavity was washed with PBS, and the spleens were removed. Spleens were teased in PBS, pH 7.4, containing 0.01 M EDTA and 1% glucose. The spleen cell suspension was washed once by centrifugation, resuspended to 5×10^6 cells/ml in DMEM containing 20% fetal calf serum, 100 U/ml penicillin, and 100 mg/ml streptomycin. 0.1 ml of cells was pipetted onto 13-mm round glass cover slips (Clay Adams, Div. Becton, Dickinson & Co., Parsippany, N. J.) incubated at 37°C in 5% CO_2, 100% humidity for 45 min, and rinsed in DMEM. Macrophages were defined as adherent cells that ingested sheep erythrocytes sensitized with rabbit IgG. Lymphocytes, platelets, erythrocytic cells, and dendritic cells (14) were also observed on the cover slips. About 40% of the adherent cell population of the spleen from normal mice was constituted by macrophages. In the spleen cultures of malaria-infected animals, this percentage increased to ≅67% on day 7 and to 75% on days 14 and 21. These macrophages often contained malaria pigment (11).

Peritoneal macrophages were cultured as described for spleen cells except that 10% fetal calf serum was used. In some experiments, mice were injected intraperitoneally with 1 ml Brewer thioglycolate medium 4 d before harvest.

In trypsinization experiments 0.1 ml trypsin of a 100-μg/ml solution, was added to the macrophage monolayers for 15 min at 37°C and washed with medium containing 100 μg/ml soybean trypsin inhibitor for 15 min at 37°C.

In some experiments, mouse sera were first incubated for 30 min with a 100% suspension of formalin-fixed *Staphylococcus aureus* as a source of protein A (15) which binds to the Fc fragment of mouse IgG (16). The bacteria were removed by centrifugation. These, or untreated sera, were pipetted onto macrophage monolayers, and the cells were incubated for 40 min at 37°C and washed.

Parasitized Erythrocytes. These were obtained from mice 3 wk after infection. The blood was collected in heparin and washed three times with cold PBS.

Mouse Reticulocytes. These were induced with phenylhydrazine as described (10).

Preparation of Sensitized Sheep Erythrocytes. Sheep erythrocytes (E) stored in Alsever's solution were obtained from the City of New York, Department of Health, Bureau of Laboratories, Otisville Branch, and were always <2 wk old when used. E sensitized with IgG (EIgG), IgM (EIgM), and IgM plus complement (EIgMC) were prepared as in Bianco (17). IgG and IgM anti-E were obtained from Cordis Laboratories Inc., Miami, Fla. The IgM was further purified by incubation with protein A-Sepharose CL4B (Pharmacia Fine Chemicals). Serum from SW mice, diluted in Veronal buffer, was used as a source of complement (17).

In some experiments, 0.5% E were incubated for 40 min at 37°C with an equal volume of serum from *P. berghei*-infected animals, taken at various intervals after infection, or from age-matched controls. The cells were then centrifuged, washed twice at 750 *g*, and resuspended to 0.5%.

Spreading. Spleen and peritoneal cells were cultured for 1 h at 37°C, 5% CO_2 in medium with 10 or 20% fetal calf serum. They were washed and fixed with 1.25% glutaraldehyde in

PBS. Macrophages were considered spread when they were elongated or showed a large membrane apron. Macrophage diameters were measured with an ocular micrometer, and compared with a stage micrometer (18).

Phagocytic Assay. 0.1 ml of 0.5% erythrocytes suspended in DMEM was added to macrophage monolayers incubated for 45 min. The cover slips were quickly rinsed in PBS diluted 1:5 with distilled H_2O to lyse noningested erythrocytes. After glutaraldehyde fixation, 200 macrophages on duplicate cover slips were counted under phage contrast optics, and the results expressed as the mean number of erythrocytes ingested by 100 macrophages (17).

Indirect Hemagglutination. 0.5% E were incubated at 37°C for 40 min with an equal volume of serum from normal or *P. berghei*-infected mice. The E were washed twice in PBS and resuspended in PBS containing 0.5% BSA. Agglutination assays were performed with doubling dilutions of rabbit anti-mouse IgG (Meloy Laboratories Inc., Springfield, Va.) in the same diluent.

Results

Ingestion of Parasitized Erythrocytes during Malarial Infection. Cultures of splenic macrophages, obtained at weekly intervals after the inoculation of 10,000 parasitized erythrocytes, were incubated with mouse erythrocyte suspensions containing 50–60% parasitized cells. Controls consisted of the same macrophages incubated either with erythrocytes from normal mice or phenylhydrazine-treated mice. Phenylhydrazine-treated animals were included because the drug induces high levels of reticulocytes, comparable to reticulocyte levels of mice with *P. berghei* infections (11).

Splenic macrophages from normal animals (day 0) showed little ingestion of any of the target cells. In contrast, splenic macrophages obtained from malaria-infected mice ingested larger numbers of erythrocytes from parasitized mice but not the normal erythrocytes or reticulocytes. Significantly lower levels of ingestion were observed on days 15 and 21. Stimulated peritoneal macrophages from thioglycolate-inoculated mice also preferentially ingested erythrocytes from parasitized mice, indicating that the stimulus for ingestion is not necessarily specific (Fig. 1). In addition, after staining the cover slips, we observed that the thioglycolate-induced macrophages contained ≅60–70% parasitized erythrocytes and 30–40% nonparasitized erythrocytes from infected animals, as well as some free parasites.

The recognition of the parasitized erythrocytes seemed to be mediated by Ig because when they were pretreated with protein A, ingestion was reduced by 75%. Controls showed that protein A also prevented the ingestion of E coated with rabbit IgG antibodies. Protein A treatment of the macrophages had no effect on ingestion of sensitized erythrocytes (Table I).

Macrophage Activation by Malarial Infection. The experiments above suggested that spleen macrophages of the malaria-infected mice were activated during infection. To examine this possibility, we studied rapid spreading and ingestion of EIgMC and EIgG as assays for activation.

The degree of spreading of splenic macrophages during the course of *P. berghei* infection is shown in Table II. By day 7, 45.3% of splenic macrophages were spread compared with 21.0% of macrophages from spleens of noninfected controls. The percentage of splenic macrophages that were spread remained high throughout the infection. In contrast, peritoneal macrophages of infected animals were not spread.

A more quantitative evaluation of the spreading reaction was obtained by measuring the longest diameter of the cultivated cells. The size of splenic macrophages increased dramatically during the course of malarial infection (Table II). The

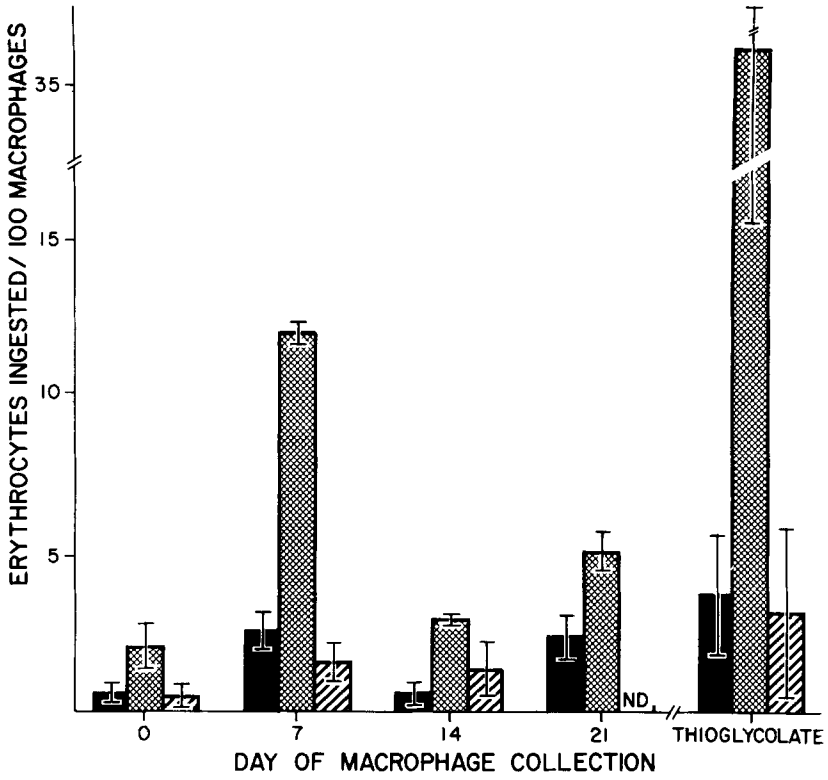

FIG. 1. Phagocytosis of malaria-parasitized erythrocytes. Macrophages obtained from spleens of normal (day 0), or *P. berghei*-infected mice (on days 7, 15, and 21 of infection) or thioglycolate-treated animals, were overlayered with erythrocytes from normal, heavily infected, or phenylhydrazine-treated mice. Phagocytosis was determined after a 45-min incubation by counting erythrocytes ingested by 200 macrophages on duplicate cover slips. Data represent the mean ingestion/100 macrophages of groups of three to five mice ± SE. Erythrocytes from: ■, normal; ■, *P. berghei* infected; ▨, phenylhydrazine treated mice.

TABLE I

Effect of Protein A on the Ingestion of Malaria-Infected Reticulocytes by Peritoneal Macrophages *

Erythrocytes	Erythrocyte pretreatment‡		
	Medium	Protein A	Reduction
			%
Parasitized erythrocytes§	24‖	6	75
	(20–28)	(4–7)	(67–86)
EIgG	357	18	95

* Peritoneal macrophages were obtained from mice injected 4 d earlier with thioglycolate for ingestion of parasitized erythrocytes. Normal macrophages were used for ingestion of EIgG.

‡ 2×10^8 parasitized erythrocytes or EIgG were incubated with 1 mg/ml protein A of *S. aureus* for 30 min in an ice bath and washed. Treatment of the macrophages with protein A had no effect.

§ Mouse blood containing 50–60% *P. berghei*-infected erythrocytes.

‖ 200 macrophages on duplicate cover slips were counted for each determination. Data are expressed as the mean ingested erythrocytes/100 macrophages. Numbers in parentheses refer to the range of values observed.

TABLE II

Spreading of Spleen Macrophages of P. berghei-Infected Mice‡*

Macrophages	Day of infection	Macrophages spread§	Mean length‖
		%	μm
P. berghei-infected mice			
Spleen	0	21.0 ± 3.6 (5)	12.5 ± 2.2
	3	11.4 ± 3.0 (4)	13.0 ± 4.5
	7	45.3 ± 6.3 (3)	22.9 ± 6.0
	14	45.4 ± 7.8 (5)	32.9 ± 11.8
	21	52.2 ± 2.2 (5)	39.1 ± 8.9
Peritoneal cavity	7–21	1.1 ± 0.3 (6)	11.2 ± 1.0
Controls			
Peritoneal cavity of normal mice		6.9 ± 4.0 (4)	16.2 ± 4.7
Peritoneal cavity of thioglycolate-injected mice (i.p.)		90.3 ± 1.5 (3)	45.5 ± 8.7

* Macrophages were allowed to spread for 1 h in DMEM containing 10 or 20% fetal calf serum.
‡ SW mice were inoculated with 10,000 infected erythrocytes.
§ Data represent the mean ± SE. Numbers in parentheses are the number of animals per group.
‖ Macrophage length was measured with an ocular micrometer. Data represent the mean ± SD.

macrophages were large, well spread, showed intense membrane ruffling, and contained large amounts of malaria pigment.

Another parameter of macrophage function studied was the ingestion of sensitized erythrocytes shown in Fig. 2. Increased phagocytic ability was observed throughout the infection. Ingestion of unsensitized E, which is unusual even for activated macrophages, was observed 1 wk after infection. Maximum ingestion of E occurred at 14 d and then diminished. The pattern of ingestion of EIgM was similar to that of unsensitized E. When fresh E were used (1 d after bleeding), significant phagocytosis was still obtained, although it was lower than the ingestion of older cells (not shown). It was recently reported that cultivated spleen macrophages ingest large numbers of E (19).

Ability to ingest EIgMC was evident 1 wk after the inoculation of *P. berghei*-parasitized erythrocytes (Fig. 2). Phagocytosis of the C3-coated particles was significantly higher than that of E or EIgM on day 7 ($0.005 > P > 0.001$). Splenic macrophages of normal mice (day 0) ingested minimal numbers of E, EIgM, and EIgMC.

Stimulation of splenic macrophages was also apparent from the fivefold enhancement of phagocytosis of IgG-coated erythrocytes by day 7 of the disease (Fig. 2). This enhancement was apparent throughout the infection, in contrast to the reduced ingestion of E, EIgM, and EIgMC at later time-points.

Peritoneal cells of malaria-infected SW mice were not stimulated above controls (not shown), indicating that the macrophage alteration may be restricted to certain organs.

Presence of Phagocytic Inhibitors in the Serum of Mice Infected with P. berghei. The diminished ingestion of EIgMC (Fig. 2) and of parasitized erythrocytes (Fig. 1) by macrophages from infected animals, seen on days 15 and 21 of the infection, could be due to the presence of disease-related immune complexes exerting a competitive effect at the level of the phagocytic cell membrane. Two indirect observations offer support for this interpretation: (*a*) macrophages from normal or malaria-infected mice incu-

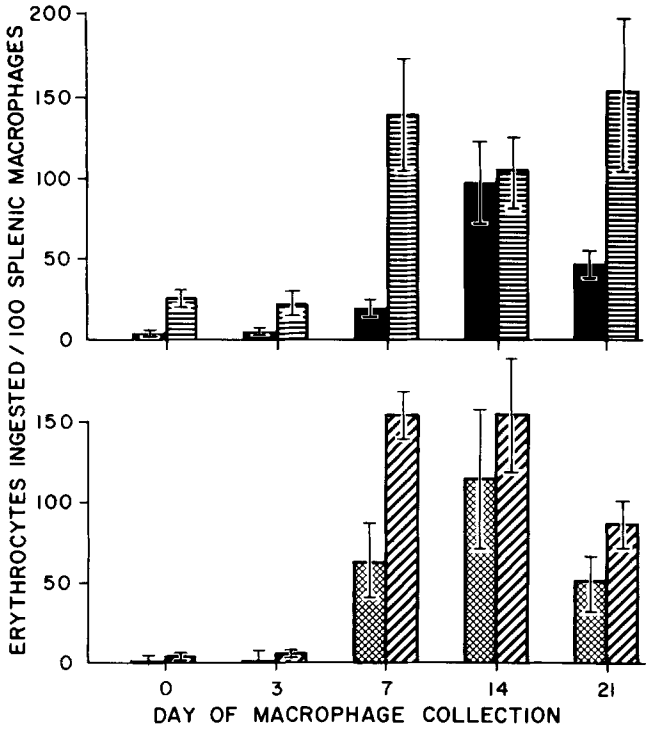

FIG. 2. Phagocytosis of E, EIgG, EIgM, and EIgMC by spleen macrophages obtained from *P. berghei*-infected mice. Macrophages were obtained from normal (day 0) or infected mice on days 3, 7, 14, and 21 of infection. They were incubated with the various erythrocyte preparations for 45 min. Ingestion was determined by counting 200 macrophages on duplicate cover slips. Data are expressed as the mean number of erythrocytes ingested/100 macrophages ± SE. Groups of three to nine animals were used per point. ■, E; ▤, EIgG; ▩, EIgM; ▨, EIgMC.

bated with sera from *P. berghei*-infected mice were strongly inhibited in their ability to phagocytize EIgG. Inhibition was correlated with the time following infection (Table III). (*b*) Pretreatment of the sera with the IgG-binding protein A from *S. aureus* removed the inhibitory component (Table III).

Evidence for the Presence of Antibodies to E in the Serum of Malaria-Infected Mice. A possible explanation of the ingestion of unsensitized E by macrophages of malaria-infected mice could be the presence on the macrophages of antibodies to E (cytophilic antibodies). In fact, indirect agglutination titers of sera obtained on days 14 and 21 after infection were consistently 1:1,024 or higher, whereas sera from uninfected mice were negative. Also, E preincubated with sera from infected animals were ingested in significant numbers by both normal mouse peritoneal macrophages and by thioglycolate-induced macrophages (Table IV). In the various experiments performed, maximum ingestion was observed with sera obtained between days 7 and 21 after inoculation with *P. berghei*.

Trypsin treatment of spleen macrophages, obtained from infected animals, abolished the ingestion of unsensitized E, indicating that the materials responsible for ingestion are associated with the plasma membrane and have a protein nature.

The antierythrocytic response of malaria-infected mice seems to be quite specific

TABLE III

Effect of Serum from P. berghei-Infected Animals on IgG-Mediated Phagocytosis*

Treatment of macrophages with serum from infected mice		Percent ingestion of EIgG as compared with controls‡	
Day of serum collection	Serum pretreatment	Normal peritoneal macrophages	Spleen macrophages from *P. berghei*-infected animal§
0	—	100	100
5	—	63	NT‖
10	—	23	NT
21–25	—	10	55
0	*S. aureus*¶	105	123
21–25	*S. aureus*	125	112

* Macrophages were incubated for 40 min at 37°C with 0.1 ml of serum from either normal (day 0) or infected animals (5, 10, 21–25 d after inoculation with 10,000 *P. berghei*-infected erythrocytes) and washed.

‡ Control values of EIgG ingested/100 macrophages were 62–220, normal macrophages; and 86, spleen macrophages.

§ SW mouse, 14 d after inoculation of 10,000 parasitized erythrocytes.

‖ Not tested.

¶ Sera treated with *S. aureus* as in Materials and Methods.

for E as no other erythrocytes tested could be opsonized by sera of these animals (Table V). This specificity was unexpected because of previous reports suggesting that malaria produces a polyclonal B-cell response (20, 21). These antibodies are directed to the Forssman antigen, because they can be completely adsorbed by guinea pig kidney cells as well as E (22).

Discussion

The present report demonstrates that splenic macrophages from malaria-infected mice are able to phagocytize *P. berghei*-parasitized erythrocytes in vitro. This ingestion appears to be mediated by disease-associated Ig which bind to the surface of parasitized cells (10). Whereas this phagocytosis may exert a protective effect during the course of malaria, it may also contribute to the anemia associated with the infection because both parasitized and nonparasitized erythrocytes were seen within macrophages.

It was of interest that spleen, but not peritoneal, macrophages were stimulated in the malaria-infected animals, indicating that the activation is localized, perhaps to a few organs. Peritoneal macrophages from thioglycolate-treated animals, however, also ingested parasitized cells, indicating that macrophage stimulation, even if nonspecific, is effective. The need for macrophage stimulation is explained by the relatively small numbers (3,000–6,000) of Ig molecules (10) and possible complement components (23) associated with the infected cells. It is possible that previous investigators failed to observe phagocytosis of parasitized erythrocytes in vitro because the macrophages studied were not stimulated (8, 9). In addition, the Ig on the parasitized erythrocytes was found to elute at room temperature or higher (10), so that preparation of the parasitized erythrocytes in the cold was essential.

In addition to the enhanced ability to ingest parasitized erythrocytes, spleen macrophages of malaria-infected mice showed increased spreading and modified

TABLE IV

Opsonic Activity of Serum from P. berghei-Infected Mice for E*

Peritoneal macrophages	Day of serum collection	E ingested/100 macrophages
Normal	4	3.6‡
	7	1.8
	14	203.6
	21	183.8
Thioglycolate-injected mice	4	72.5
	7	48.7
	14	554.0
	21	1,092.8

* Serum obtained from SW mice inoculated with 10,000 *P. berghei*-infected erythrocytes on the days indicated. It was heat inactivated at 56°C for 30 min before the experiment. Sera were incubated with equal volumes of 0.5% E, washed, and pipetted onto the macrophage monolayers.

‡ Controls with serum from age-matched uninfected mice induced ingestion of 0.7–1.3 erythrocytes by normal macrophages and 51.9–64.9 erythrocytes by thioglycolate-induced macrophages.

TABLE V

Opsonization of Erythrocytes of Different Species by Serum from P. berghei-Infected Mice

Erythrocytes	Ingestion* after incubation with	
	Serum from *P. berghei*-infected mice‡	Control serum
Sheep	263	12
Guinea pig	8	13
Rabbit	11	12
Rat	0	1
Human, type 0	16	2

* Macrophages obtained from mice injected intraperitoneally with thioglycolate. Phagocytic assay performed as in Materials and Methods.

‡ Pooled sera were obtained from mice infected with 10,000 *P. berghei*-infected erythrocytes 14 and 21 d earlier or age-matched control mice. All sera were heat inactivated at 56°C for 30 min.

§ Data represent the mean number of erythrocytes ingested/100 macrophages.

phagocytic properties, characteristic of activated macrophages (24). The exact mechanism of macrophage stimulation during malarial infection is unknown. However, T lymphocytes respond to malarial antigens in vitro (25), and could play the same role attributed to T cells in bacterial (26) and other parasitic infections (27, 28) in macrophage activation.

A large increase in circulating antibodies to E was also observed in the serum of infected mice. Although this may have been caused by a polyclonal stimulation during infection (20, 21), we could not demonstrate a substantial humoral response against rat, guinea pig, rabbit, or human type 0 erythrocytes. Interestingly, this antibody was specific to a Forssman antigen and could not be adsorbed by parasitized reticulocytes (data not shown). It is possible that the malaria parasite itself expresses a Forssman specificity at some point during its development. The suppressed immune

response to E antigens during malarial infection may be mediated by these anti-Forssman antibodies (29) because the response to several other antigens such as human serum albumin, and bacteriophage ϕx-174 remained unaffected (reviewed in reference 30).

The large increase in ingestion of EIgMC during *P. berghei* infection may have been facilitated by the presence of antibodies to E as synergy between the Fc and C3 receptors is known to occur (31, 32). Late in infection, phagocytosis of parasitized erythrocytes and C3-coated E was diminished. This may have been caused by immune complexes, often detected in both rodent (33, 34) and primate (35) malarias.

Another element that may affect the rate of clearance of parasitized erythrocytes is the body temperature of the host, because most of the reticulocyte-bound Ig were bound in the cold and smaller amounts were bound at 37°C. Assuming that the in vitro observations are reproduced in vivo, the higher the temperature of the host, the lower the number of IgG molecules bound, rendering less likely the recognition and ingestion of parasitized erythrocytes. Consequently, elevated body temperature, a constant feature of malarial infection, may contribute to the parasitemia.

The course of the disease as related to the clearance of malaria parasites depends, therefore, on the interaction of several elements of the immune system in addition to the levels of specific antimerozoite antibodies (36, 37); the amount of cold agglutinins bound to reticulocytes, the presence of circulating immune complexes, the degree of macrophage stimulation, and possibly, on the variations of body temperature of the host.

Summary

Spleen macrophages from *Plasmodium berghei*-infected mice are more efficient in the ingestion of parasitized reticulocytes than spleen macrophages obtained from normal animals. Other indications of spleen macrophage activation detected during malarial infection are enhanced macrophage spreading and increased phagocytosis of opsonized and nonopsonized sheep erythrocytes (E). Peritoneal macrophages are not activated to a significant degree.

The appearance of antibodies directed against Forssman antigen, but not to other erythrocyte antigens, is also a feature of this infection and explains the ingestion of unsensitized E by spleen macrophages of the diseased animals.

The recognition and ingestion of parasitized reticulocytes by infected mice is mediated by cold-agglutinin type immunoglobulins that appear during *P. berghei* infection and can be blocked by the Fc-binding protein A from *Staphylococcus aureus*. In advanced stages of the disease, the serum of infected animals inhibits phagocytosis, probably because of the high level of circulating immune complexes.

Thus, the clearance of malaria parasites is regulated by several elements of the immune system, in addition to levels of specific antimerozoite antibodies, including the amount of antibodies bound to reticulocytes, the presence of circulating immune complexes, and the degree of macrophage stimulation.

The authors are grateful to Ms. Charla Danforth, Mr. Paul Gil, and Ms. Gail Topper for technical assistance, and to Ms. Sharon Hecht-Ponger and Beatrice Robles for manuscript preparation. We would also like to thank Drs. Harry Danforth, Victor Nussenzweig, and Miercio E. A. Pereira for helpful discussions and review of the manuscript.

Received for publication 12 February 1979.

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