INFECTION AND IMMUNITY, June 1978, p. 827-835
0019-9567/78/0020-0827$02.00/0

Vol. 20, No. 3
Printed in U.S.A.

Role of the Surface Coat in In Vitro Attachment and Phagocytosis of *Plasmodium berghei* by Peritoneal Macrophages†

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Received for publication 5 January 1978

Evidence is presented to indicate that *Plasmodium berghei* merozoites, but not trophozoites, have an antiphagocytic capsule. The capsule appears to form around the developing merozoites of the schizont in the parasitophorous vacuole. Serum from animals immune to *P. berghei* reacts with this capsule. After reaction with immune serum, the antiphagocytic action of the capsule is lost. By the process of binding serum protein, the capsule becomes electron dense and can be readily visualized as the surface coat by electron microscopy. At physiological temperatures, phagocytosis by macrophages rapidly follows adhesion of antibody-coated parasites. Both tight and loose phagosomes are formed.

The ability of a host to overcome malaria infection depends partially on the phagocytic system. The frequency and effectiveness of parasite-erythrocyte contact as compared to the frequency and effectiveness of parasite-macrophage contact probably determines the outcome of the infection (11). The experimental demonstration of the phagocytic process has been difficult. This has been because free parasites were not available for study and when they were available, the experimental results obtained in phagocytic studies have been inconsistent. There has been, for example, much phagocytosis with normal serum in some experiments and only a quantitative additional effect with immune serum (3).

In the last few years some of the technical problems inhibiting the study of the interaction of parasites, antiserum, and phagocytes have been overcome. Specialized adaptations of culture techniques have permitted the harvest of mature merozoites of *Plasmodium knowlesi* (19), and improvements in culture (25) may soon permit the harvest of merozoites of *P. falciparium* as well, while a continuous-flow sonic oscillation technique (15, 16) yields free parasites in any of the forms parasitizing erythrocytes (13).

In this study we used a mixed population of trophozoites and merozoites freed from rat erythrocytes by sonification to study the role of immunoglobulin and stage of development of the parasites in attachment and phagocytosis of parasites by peritoneal macrophages in vitro.

† Army Malaria Project paper no. 1495.

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(This work is part of a thesis submitted by Carolyn Brooks as partial fulfillment of the requirements for a doctorate at the Ohio State University).

MATERIALS AND METHODS

Animals. Rats utilized for serum and parasite and macrophage harvests were inbred Fisher 344 (CD*F) males obtained from Charles River Breeding Laboratories, Wilmington, Mass. They were housed in the animal facility of the Department of Microbiology, The Ohio State University. Food and water were given ad libitum.

Parasites. The strain of *P. berghei* used originated from the Walter Reed Army Institute of Research and was maintained in our laboratory by serial passage in rats and mice or by storage in Alsever solution plus 10% (vol/vol) glycerol in liquid nitrogen (−193°C).

Harvesting of free parasites. Mature rats were used for harvesting parasites. To insure high parasitemias in these animals, it was necessary to increase the reticulocyte count by pretreatment with phenylhydrazine (11). When the parasitemia reached at least 50% and contained a high proportion of schizonts, the blood was harvested into cold Alsever solution by cardiac puncture. The blood was centrifuged at 650 × *g* for 10 min at 5°C. The supernatant fluid was removed by aspiration, and the cells were resuspended and washed twice more in cold Alsever solution. Thereafter, leukocytes were removed by filtration of a 10% suspension of washed erythrocytes through columns of packed powdered Whatman no. 1 filter paper over a plug of glass wool. The filtered erythrocytes were disrupted by treatment in a continuous-flow sonification system by methods previously described (15, 16). The sonificated material was centrifuged at 650 × *g* to remove the unlysed erythrocytes. The supernatant was then centrifuged at 1,450 × *g* for 10 min and the parasites collected were washed by an

additional centrifugation in Alsever solution.

Hyperimmune serum. Rats recovered from *P. berghei* received monthly reinoculations of infected erythrocytes from other inbred rats of the same strain. Sera collected 2 weeks after the last injection were separated, pooled, and stored at −20°C.

Macrophage collection. Rats were anesthetized with ether and then bled by cardiac puncture for normal serum. To obtain macrophages, the skin over the viscera was removed, the peritoneum was snipped, and the cavity was filled with sterile medium 199 (Microbiological Associates). The filled abdominal cavity was massaged, and the fluid was withdrawn into sterile pipettes and placed into sterile siliconized culture tubes. The macrophages were collected by centrifugation.

Parasite surface coat formation. For study of surface coat formation by electron microscopy, ultrasonically freed parasites were prepared by incubation at 37°C or in an ice bath in 5% hyperimmune or 5% normal serum or Alsever solution for 5 or 15 min.

Attachment studies. Attachment studies were carried out by light or electron microscopy. For both light and electron microscopic studies, parasites were exposed to rat origin peritoneal macrophages in suspensions in test tubes. For light microscopy, thin film preparations were made and stained by the Giemsa method. The techniques for staining were similar to those previously described (12).

Preliminary attachment studies revealed that 10 min of incubation at 4°C permitted attachment of parasites to macrophages in our system. After 10 min of incubation, the suspensions were removed and the pelleted cells were fixed and prepared for either light or electron microscopy.

The various combinations of macrophages, parasites, and normal or immune sera which were used in attachment and phagocytosis studies are listed in Tables 2 and 3. Parasitized erythrocytes were also studied in the same manner.

Phagocytosis. After 10 min of incubation in the cold to allow attachment, portions of macrophages and parasites prepared as previously described were placed in a 37°C water bath for 5- to 15-min intervals. At the appropriate times, the tubes were spun in a refrigerated centrifuge and the pellets were fixed for electron microscopy.

Electron microscopy. Parasite and macrophage preparations were fixed in 1.25% glutaraldehyde, 0.05 M phosphate buffer, pH 7.3, and 0.116 M sucrose. After washing in 0.05 M phosphate buffer, the preparations were postfixed in 1% osmium tetroxide for 1 h, washed, dehydrated in ethanol and propylene oxide, and embedded in an Epon 812 mixture. Sections were cut on a Sorvall Porter-Blum ultramicrotome equipped with a glass knife. The sections were mounted on uncoated 300-mesh copper grids, and stained with uranyl and lead citrate. The sections were examined on a 9S-2 Zeiss microscope.

RESULTS

Surface coat formation. Most of the merozoites present in sonically freed parasite preparations developed a thick electron-dense surface coat upon incubation for 15 min in immune serum (Fig. 1). Trophozoites present in the same preparations were often agglutinated by the immune serum, but did not develop a similar thick electron-dense surface coat (Fig. 2). Incubation in normal serum caused formation of a thin surface coat on most merozoites, but had no visible effect on trophozoites. Schizonts freed from erythrocytes also developed a thick surface coat upon incubation in immune serum (Fig. 3). Many of the merozoites incubated in immune serum for 5 min had an electron-lucid area (Fig. 4) between the electron-dense surface coat and the plasmalemma, whereas the surface coat surrounding the merozoites incubated in immune serum for 15 min was in direct contact with the plasmalemma.

Effect of addition of immune serum to macrophage-parasite mixtures. In macrophage-parasite preparations held at 5°C, more macrophages bound parasites in the presence of immune serum than in the presence of normal serum or in the absence of serum. The average number of parasites on the macrophages was also greater in the presence of immune serum than in the presence of normal serum or in the absence of serum. These results were similar

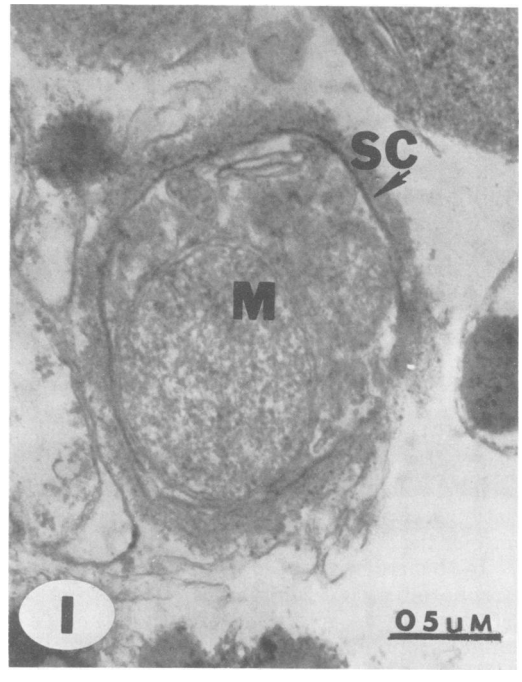

FIG. 1. *Thin-sectioned merozoite (M) that has been incubated in medium containing 5% hyperimmune serum for 15 min at 37°C. The parasite developed a very thick surface coat (SC).*

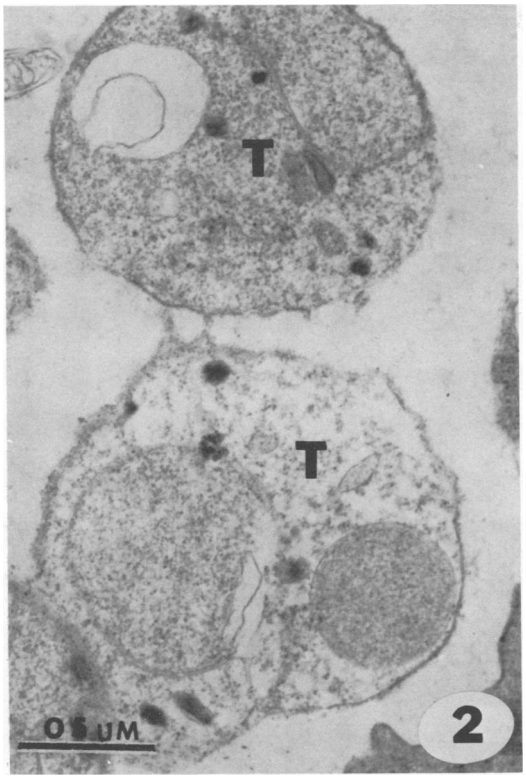

FIG. 2. *Trophozoites (T), which were incubated in 5% hyperimmune serum, with small amounts of material on their surfaces but no surface coat similar to the merozoites. The trophozoites were agglutinated by the immune serum.*

whether the parasites and serum were added to macrophage suspensions which were subsequently fixed, Giemsa stained, and examined by light microscopy or were prepared for examination by thin-section electron microscopy (Table 1).

Relative roles of cytophilic and opsonic components in immune serum on parasite attachment to macrophages. In macrophage preparations held at 5°C, more macrophages bound parasites which had been pretreated with immune serum than those pretreated with normal serum or not pretreated. The average number of antibody-pretreated parasites bound per macrophage was also greater than was the average number of normal serum-pretreated or untreated parasites. Pretreatment of the macrophages with immune serum enhanced their capacity to bind untreated parasites, but to a lesser degree than did pretreatment of parasites. Pretreatment of both macrophages and parasites with immune serum enhanced the degree of attachment of parasites to macrophages more than did treatment of parasites or macrophages alone (Table 2).

Effect of immune serum treatment upon the development stage of free parasites bound to macrophages. It was not possible to differentiate between merozoites and small free trophozoites by light microscopic examination of Giemsa-stained preparations of sonically freed parasites. This differentiation was, however, made by thin-section electron microscopy. When the parasites attached to macrophages were classified according to whether they were trophozoites or merozoites, it became apparent that practically all of the parasites which attached to macrophages in the presence of normal serum or the absence of serum were trophozoites (96–100%), whereas in the presence of immune serum, substantial numbers of merozoites (12 to 29%) also adhered to macrophages (Table 2).

Relative roles of cytophilic and opsonic components in immune serum on parasite ingestion by macrophages. Cytophilic antibody which facilitates attachment of parasites to macrophages may not facilitate their internalization. In fact, unopsonized parasites attached to macrophages by cytophilic antibody may come off upon prolonged incubation at 37°C (Table 3, lines 1 and 2). Attached parasites which have been opsonized, i.e., coated with antibody, on the other hand, are internalized within 5 min when the temperature of the cultures is raised to 37°C from 5°C (Table 3, lines 3, 4, 5, 6).

Process of phagocytosis of attached parasites. Parasites are taken into the cytoplasm of macrophages which initially project thin pseudopods around the parasite (Fig. 5). The pseudopods begin ingestion with such avidity that parasites may be distorted in the process. The cytoplasm around the forming phagosome is usually devoid of organelles. Parasite-containing vacuoles are found at random locations in the cytoplasm of macrophages; some lie in the area of the nucleus and others are situated peripherally, near the cell membrane. The phagosomes could be seen in two forms, the tight phagosome (Fig. 6) and the loose phagosome (Fig. 7). The tight phagosomes are those in which the phagosomal membrane is directly opposed to the cell membrane of the engulfed organism. The loose type has a wide space between the membrane of the phagosome and perimeter of the organism. Generally, plasmodia observed in loose phagosomes are relatively unaltered. In some loose phagosomes the space was occupied by flocculent material of moderate density; in others the space appeared completely electron lucent. Some of the vacuoles containing parasites were lined by electron-dense material.

Phagocytosis of erythrocytes. In vitro

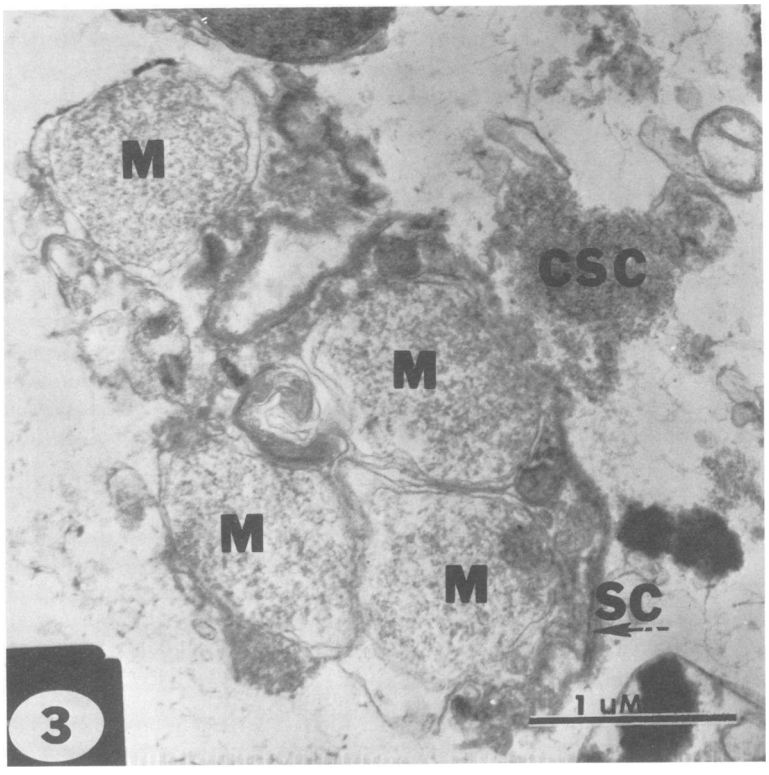

FIG. 3. *The matrix of the parasitophorus vacuole of this free schizont still partially surrounds the developing merozoites (M). The portion of the matrix which adheres to the parasites has reacted with the immune serum in which the schizont was incubated and formed a thick surface coat (SC). A clump which may be surface coat material appears to be partially shed from the schizont (CSC).*

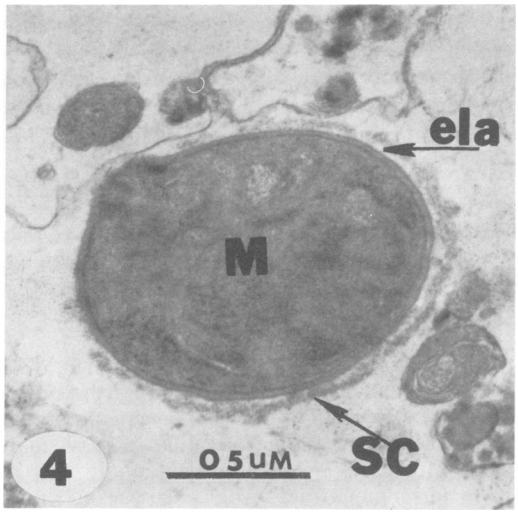

FIG. 4. *An electron-lucid area (ela) is present between the dense surface coat (SC) and the plasma membrane on this merozoite, which was incubated for 5 min in 5% hyperimmune serum at 37°C.*

phagocytosis of erythrocytes, either infected or noninfected, was a very rare event. The rareness of erythrophagocytosis was not affected by any serum treatment used.

DISCUSSION

The presence of a surface coat on merozoites of plasmodia was first reported by Ladda et al. (14). The existence of this surface coat on merozoites which have been exposed to immune serum and even to normal serum has been amply confirmed (1, 18). It has usually been assumed that this coat would be absent from trophozoites, but its absence has not been demonstrated because previous studies were done on parasite preparations which did not contain free trophozoites. The function of this surface coat has not been determined, nor has its origin.

Mason et al. (17) have suggested that the surface coat is of parasitic origin and that it is secreted after the merozoites are released. They showed a schizont inside a parasitophorous vacuole which they considered to have no surface

TABLE 1. *Effect of added immune (IS) or normal serum (NS) upon attachment of free P. berghei parasites to peritoneal macrophages in cultures incubated at 5°C*

Serum	Type of microscopy	No. of macrophages examined	No. of parasites on macrophages		No. of macrophages with parasites attached	
			Total	Per macrophage	Total	%
IS	Light	822	1,260	1.53	658[a]	80
	Electron	110	204	1.85	92[a]	84
NS	Light	723	745	1.03[a]	412[a]	58
	Electron	84	109	1.30	56[a]	67
None	Light	562	382	0.68	242	43
	Electron	84	60	0.71	43	51

[a] Average number of macrophages binding parasites and number of parasites bound per macrophage is significantly greater ($P < 1\%$) in these immune serum treatment groups than in the corresponding normal serum groups (Z test for equality of two proportions; 9).

TABLE 2. *Effect of various immune serum (IS) or normal serum (NS) treatments of parasites and macrophages on the percentage of macrophages showing attached free parasites, average number of attached free parasites per macrophage, and development stage of free parasites which attach to macrophages, as determined by thin-section electron microscopy*

Treatment	No. of macrophages examined	% Macrophages with attached parasites	Attached parasites		Attached merozoites		Developmental stage of attached parasites (%)	
			Total	Per macrophage	Total	Per macrophage	Trophozoites	Merozoites
IS added to parasite-macrophage culture	110	84	212	1.93	62[a]	0.56	71	29
NS added to parasite-macrophage culture	84	66	106	1.27	0	0	100	0
Macrophages pretreated with IS	90	72	147	1.63	18[a]	0.20	88	12
Macrophages pretreated with NS	83	58	108	1.30	0	0	100	0
Parasites pretreated with IS	90	93	216	2.40	41[a]	0.46	81	19
Parasites pretreated with NS	90	57	81	0.90	2	0.03	98	2
Parasites and macrophages pretreated with IS	100	96	242	2.42	51[a]	0.51	79	21
Parasites and macrophages pretreated with NS	72	60	67	0.93	3	0.04	96	4
All IS groups	390	86	817	2.09	172[a]	0.46	80	21
All NS groups	329	60	362	1.10	5	0.02	99	1
No serum in culture	87	47	69	0.79	0	0	100	0

[a] Number of merozoites attached to macrophages in the presence of immune serum is significantly greater ($P < 1\%$) than the number of merozoites attached to macrophages in the presence of normal serum in all treatment groups (Z test for equality of two proportions; 9).

coat, although it obviously was in a relatively low density matrix which appeared to serve as a spacer between the component parts of the schizont and the membrane of the parasitophorous vacuole. Figure 3 shows a schizont similar to the one shown by Mason et al. (17). This schizont, however, has been ripped from its vacuole by sonification and exposed to immune serum. It has a thick coat which occupies a part of the area filled by the matrix in the parasitophorous vacuole. A mass (CSC) which has the appearance of surface coat material can be seen partially detached from the schizont (Fig. 3). The nature of this material cannot be proven by techniques currently available, and, thus, alternative interpretations of its nature are possible.

The presence of an electron-lucid area between the surface coat and the plasmalemma in

TABLE 3. *Effects of various immune (IS) and normal (NS) serum treatments upon the percentages of macrophages showing phagocytosis of free parasites after 5 or 15 min of incubation at 37°C after 10 min of incubation at 5°C to permit parasite attachment*

Treatment	No. of macrophages examined	% Macrophages with no attached or phagocytozed parasites		% Macrophages with attached parasites only		% Macrophages with ingested parasites	
		5 min	15 min	5 min	15 min	5 min	15 min
Macrophages pretreated with IS	102	26	50	44	18	29	31
Macrophages pretreated with NS	92	53	60	17	8	31	32
Parasites pretreated with IS	109	7	10	5	0	88	90
Parasites pretreated with NS	88	31	30	32	21	38	49
Parasites and macrophages pretreated with IS	84	4	3	7	19	89	91
Parasites and macrophages pretreated with NS	91	33	37	6	12	48	51
No serum in culture	296	55	62	21	13	25	29

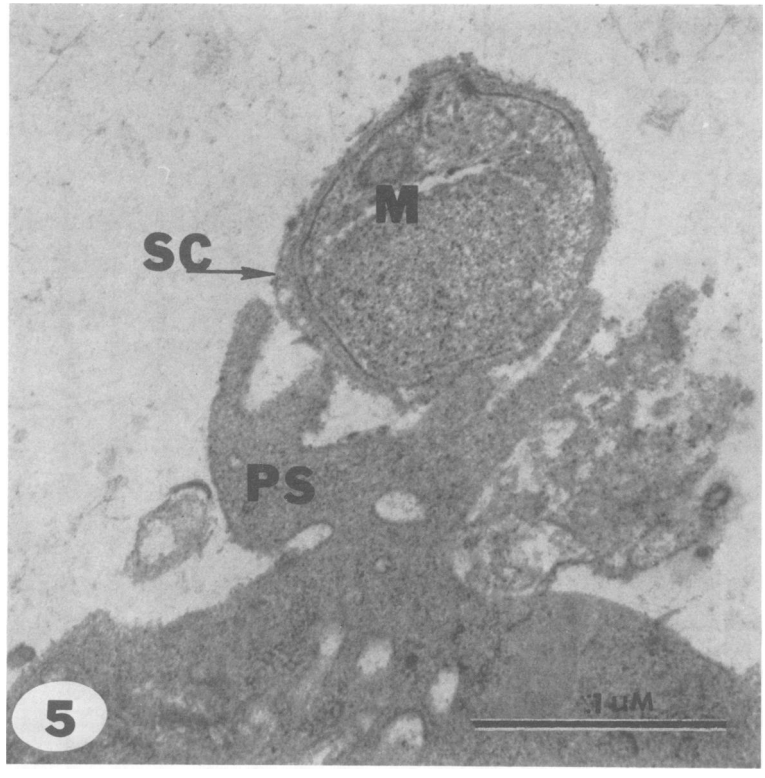

FIG. 5. *Both the parasite and macrophage were sensitized in 5% hyperimmune serum. The pseudopodia (PS) of the macrophage have begun to ingest the merozoite (M). The merozoite has a distinct surface coat (SC) which is also being ingested.*

parasites incubated for a short time in immune serum (Fig. 4) but its absence from parasites subjected to longer incubation (Fig. 1) is best explained by postulating a slow penetration of antibody into a loose gel surrounding the parasites rather than by postulating a secretion of capsule during incubation.

It is well known to bacteriologists that rough

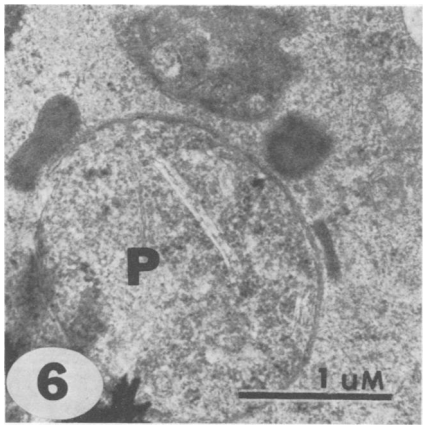

FIG. 6. *Tight phagosome in which the phagosomal membrane is directly apposed to the cell membrane of the engulfed organism (P).*

(i.e., nonencapsulated) strains of pathogenic bacteria are avirulent and easily phagocytized, whereas smooth (i.e, encapsulated) strains are virulent. The capsule of virulent pneumococci swells on exposure to immune serum (the Quellung reaction) (5). The pneumococcal capsule is electron lucid, but becomes electron dense when it imbibes serum protein, particularly specific immunoglobulin (21). The surface coat of the plasmodial merozoite which becomes visible after incubation of the parasite in serum could be an electron-lucid merozoite capsule which has imbibed serum protein in a fashion analogous to the imbibition of serum protein by the pneumococcal capsule.

In the present studies we observed that merozoites (like virulent smooth bacteria) were rarely bound to or ingested by macrophages in the absence of immune serum, whereas trophozoites (like rough avirulent bacteria) were both bound to and ingested by macrophages in the presence of normal serum or even in the absence of serum. Trophozoites do not have a surface coat after exposure to serum and thus probably do not have a capsule. The trophozoite is normally intracellular and thus does not need an antiphagocytic defense, whereas the merozoites must pass from cell to cell and would find an antiphagocytic capsule of considerable utility. This work thus indicates that the function of the merozoite capsule is that of an antiphagocytic defense. If this capsule is impregnated with normal serum, it could provide a further disguise for the parasite. On the other hand, when the capsule is impregnated with specific antibody, its antiphagocytic defense is overcome.

Ladda et al. (14), Miller et al. (18), and Bannister et al. (1) have shown that the surface coat is pushed back and released during penetration of erythrocytes by merozoites. When high parasitemias are present and large numbers of merozoites are released, released surface coat material from penetrating merozoites could react with cytophilic antibody on the macrophage membranes and with opsonic antibody and block macrophage function.

Cochrane et al. (4) found that surface coat material on plasmodial sporozoites migrated to the posterior pole and called this phenomenon capping. Doyle et al. (7) suggested that a similar process occurred with plasmodial merozoites. Our data (Table 3) suggests the possibility that merozoites whose capsules are not stabilized by antibody may disassociate from their surface coats and so escape phagocytosis. Use of the term capping by Doyle et al. (7) and Cochrane et al. (4) to describe the movement of the surface coat material of protozoa is generalizing the term extensively. DePetris and Raff (6) coined the

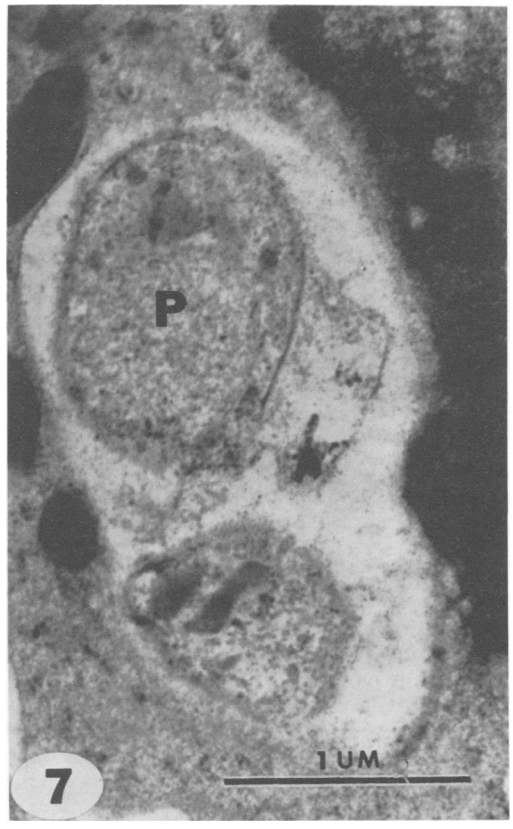

FIG. 7. *Loose phagosome in which a wide space exists between the membrane of the phagosome and perimeter of the ingested organism (P). The space is occupied by fluocculent material, but is mostly electron lucid.*

term "capping" to describe a process of agglutination of elements in a fluid plasmalemma. The process observed with plasmodial merozoites would better be called agglutination of shedding of capsular material.

The infrequency with which macrophages in in vitro preparations ingest erythrocytes from animals with malaria was noted before (3, 12). As erythrocyte ingestion occurs in vivo during acute malaria, some factor other than simple recognition of surface alteration must play a role in this ingestion. A reduction in cell elasticity (24), a necessary requirement for passage of cells through reticuloendothelial organs, may be in part responsible for erythrophagocytosis in vivo.

Our attachment studies were performed in the cold to disassociate attachment from ingestion. We studied ingestion by raising the temperature of the cultures. It was because of this technique that we were able to observe that cytophilic antibody does not facilitate ingestion, only adhesion. A discussion of the characteristics and roles of opsonic and cytophilic antibody in immunity to rodent plasmodia can be found in the paper by Green and Kreier (10). In that paper the immunoglobulin classes of the antibodies involved in stimulation of phagocytosis of plasmodia and the effects of absorption by parasites and macrophages on the phagocytosis-stimulating capacity of the immune serum are described. After attachment, ingestion of antibody-coated parasites occurred very rapidly when the culture temperature was raised. Some of the parasites appeared in tight, and some in loose, phagosomes. Parasites in tight phagosomes appeared relatively unaltered.

Whether loose phagosomes represent deficient phagocytosis is unclear; Dumont and Robert (8) found that when the fungus *Histoplasma* was enclosed in loose phagosomes, it was morphologically unaltered and oftentimes had undergone budding, which would indicate that this type of phagosome rendered the host cell defenseless against the organism. Belcher et al. (2), studying *Candida albicans*, found disrupted organisms within both tight and loose phagosomes. We did not observe any correlation between phagosome type and presence of capsular material or antibody. The roles of the loose and tight phagosomes in phagocytic clearance of plasmodia need more study.

An electron-dense zone was seen to surround some ingested plasmodia. The electron-dense zone surrounding some of the ingested parasites is not peculiar to plasmodia. Belcher et al. (2) suggest that in *Candida* it is of parasite origin. They suggested that it was parasite material which separated from the *Candida* and accumulated on inner surface of the phagosome. Dumont and Robert (8) considered the electron-dense zone to be of macrophage origin and postulated that it was composed of lysosomal contents.

In an earlier study we showed that the washings from free parasites provided an immunizing agent against challenge with blood origin parasites (23). In these studies we have demonstrated an antiphagocytic function for the merozoite capsule, the presence of capsular material on schizonts, and a role for humoral antibody in overcoming the capsular antiphagocytic function. These observations thus explain why capsular material is an antigen for immunization and why merozoites (20) and schizonts (22) are important in vaccines against plasmodia.

ACKNOWLEDGMENTS

This work was supported by grant DADA 17-7202072 from the U.S. Army Medical Research and Development Command to Julius P. Kreier and by the National Fellowship Fund of Atlanta, Ga.

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