Surface Properties of Extracellular Malaria Parasites: Morphological and Cytochemical Study¹

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Morphological and cytochemical surface characteristics of isolated malaria parasites (*Plasmodium berghei*) and host erythrocytes were compared by electron microscopy by using thin section and carbon replica techniques. Erythrocytes were uniform in shape and had fine, granular surfaces. In contrast, free parasites exhibited a variety of sizes, shapes, and surface textures. Fine surface stippling was a common topographical feature of isolated parasites. Small, infective forms often had patterned surfaces resulting from the protuberance of an underlying thick intermediate layer. Results of cytochemical analysis using a sialophilic colloidal iron stain indicated that the malaria parasite's surface lacked exposed sialic acid groups which would normally give rise to a net negative surface charge common to erythrocytes. Biochemical assay demonstrated that malaria parasites contained about one-half the amount of sialic acid per unit weight as did control red cell extracts. Similarly, external acidic mucopolysaccharide coats of free parasites, as revealed by ruthenium red staining, were extremely thin as compared with the thick glycocalyx layer of red cells. Lipid plaques at the surface of parasites and red cells were localized by lipophilic iron colloid staining. Although the gross patchwork distribution of plaques was somewhat similar for the two cell types, the parasites were stained more intensely and had a closer-knit patchwork pattern than those exhibited by the erythrocytes. Such findings indicate that there are slight differences in the arrangement of phospholipids at the surfaces of limiting membranes of host cells and parasites. The significance of the above cytochemical surface properties of the malaria parasite (which are seemingly akin to those of intracellular organelles) is discussed in relation to certain host-parasite interactions, such as parasite adhesion to target cells and enhanced clearance of extracellular parasites.

Obligate intracellular parasitism is a curious biological phenomenon. The dependency of certain groups of microorganisms on an intracellular environment in order to grow and reproduce remains unexplained for the most part. With respect to the malaria parasite, it is thought that this dependency is partially the result of a number of metabolic deficiencies. Such metabolic lesions would include the lack of a *de novo* biosynthetic pathway for purines (27), requirements of the parasite for high internal concentrations of acetyl coenzyme A (28), need for *p*-aminobenzoic acid and, in turn, folic acid for folic acid synthesis (29), etc. Although workers have made a concerted effort to delineate the metabolic requirements responsible for

the intracellular habit, little has been done to evaluate the possible role that membranes play in exerting control on such functions as osmoregulation and cation transport.

A structurally deficient cytoplasmic membrane might impart controls on cellular metabolism. Moulder (18), over a decade ago, suggested that the obligate intracellular microorganisms might be differentiated functionally from extracellular microorganisms by having limiting membranes with "permeability defects." The postulated inability of these organisms to accumulate and to retain essential metabolites extracellularly necessitates an internal growth environment high in metabolite concentration. This "defective membrane" hypothesis remains virtually untested because of the technical difficulties in isolating large quantities of well-preserved plasmodia, free from entrapping host cell membranes, for definitive biochemical or

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physiological studies. As a result, relatively little is known about the structure and function of the malarial parasite's limiting membrane. Ultrastructurally, the thinly sectioned bounding membrane of the parasite closely resembles the host cell plasma membrane. In spite of such morphological similarities, there are definite compositional differences between the limiting cytoplasmic membranes of the parasite and the host cell with respect to lipid composition (22). Other types of chemical differences have not been reported.

By isolating free plasmodia with a newly developed continuous-flow sonic oscillation procedure (20, 21) and by treating these preparations with chemical group-specific electron stains, we were able to compare membrane structure of red cells and parasites by electron microscope examination, thus circumventing many of the intrinsic problems which beset previous workers. It is, therefore, the intent of this article to compare the surface structure of extracellular malaria parasites and host red cells and to examine the ionogenic properties and mucopolysaccharide and phospholipid distribution within the respective plasma membranes.

MATERIALS AND METHODS

General. The NYU-2 strain of *Plasmodium berghei* utilized in this study was maintained in young (4- to 6-week-old), female albino rats by weekly syringe passage of infected blood. When heavily parasitized blood samples were needed for the fractionation procedures outlined below, rats (six to eight) were injected intraperitoneally with *P. berghei*-infected red cells. Rats with 40 to 50% parasitemia were anesthetized and bled by cardiac puncture by using heparinized syringes (50 U/ml blood). Collected blood samples were pooled and centrifuged at $450 \times g$ for 10 min. Plasma supernatants, along with the buffy coats, were aspirated off, and the red cells were resuspended in Alsever solution (pH 7.3). The cells were washed two additional times and resuspended to a final 10% packed cell volume concentration.

Isolation. "Erythrocyte-free" malaria parasites were isolated from parasitized red cells by a continuous-flow sonication method (20, 21). Cell suspensions were sonically treated at maximal intensity (125 W; Artek Sonic Dismembrator; 20,000 c/s) while being pumped through a flow chamber (0.003 by 0.014 cm) at a rate of 14.0 ml/min. Free parasites were collected from the sonically treated suspensions by differential centrifugation. Low-speed centrifugations ($300 \times g$ for 15 min), designed to remove whole red cells, were followed by centrifuging the parasite-containing supernatants at $600 \times g$ for 30 min. Parasite pellets were resuspended in Alsever solution and washed twice by centrifugation ($600 \times g$ for 20 min).

Isolated parasites appeared well preserved when

viewed in an electron microscope. Preliminary infectivity studies suggested that a high proportion of these sonically freed parasites were viable, since groups of rats infected with equal numbers of either infected red cells or free plasmodia exhibited similar prepatent periods. Similar findings have been reported previously (21).

Although free plasmodia preparations which were relatively free of contaminating host cell membranes could be obtained readily, we felt it was advantageous to utilize crude contaminated preparations for cytochemical study since such materials served as internal staining controls. In these preparations, the sonically treated samples were centrifuged initially at a lower g force ($150 \times g$) than the above described $300 \times g$ centrifugation.

Electron microscopy. Erythrocyte-free parasite preparations were fixed routinely with 1.25% glutaraldehyde in a 4% sucrose-0.05 M phosphate buffer (pH 7.3) for several hours. After fixation, the samples were washed in buffer (0.05 M phosphate buffer, pH 7.3) for about 30 min and then postfixed in buffered 1% osmium tetroxide. Samples were dehydrated in ascending concentrations of ethanol and finally in propylene oxide, and embedded in Epon 812 (1). Ultrathin sections were cut and stained with uranyl acetate and lead citrate.

Carbon replicas of cell samples were prepared by standard methods (2). Droplets of glutaraldehyde-fixed cell suspensions were placed on Formvar-coated copper grids and allowed to air dry. Sample grids were shadowed first with platinum and then coated uniformly, by rotation, with evaporated carbon. Formvar coats were removed in a chloroform bath. Replicas were freed from organic elements by incubation in Clorox. After 3 to 4 h in Clorox, the replicas were transferred to distilled water baths. Samples were removed and air-dried.

All prepared specimens were examined with a Siemens Elmiskop 101 electron microscope.

Preparation and application of colloidal iron reagents. Colloidal iron-staining reagents were prepared and utilized in close accord to the methods of Gasic et al. (8). A 67-mg amount of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in 5.0 ml of distilled water was added dropwise to 60 ml of boiling distilled water. The iron solution was cooled rapidly in a cold water bath. A 20-ml amount of this reagent was added to an equal volume of 0.0012 N HCl. Ten milliliters of the above stock hydrosol was mixed with 10 ml of acetic acid and 20 ml of distilled water to produce the acetic acid hydrosol (acetic acid-iron [AI] colloid). A lipophilic, positively charged ferric oxide sol (propionic acid-iron [PI] colloid) was prepared in a similar fashion except that equal volumes (10.0 ml) of propionic acid replaced the acetic acid (G. W. Cooper and L. H. Miller, J. Histochem. Cytochem., in press). The pH of both types of undialyzed colloids was 1.65. Aldehyde-fixed cell samples (0.1 ml) were incubated with 10.0 ml of these reagents for 15 min. After incubation, excess colloid was removed by either a 12% acetic or propionic acid rinse, according to the hydrosol used in staining, followed by a distilled water rinse prior to postfixation in osmium tetroxide.

Although the specificity of the AI colloid (8) and the PI colloid (G. W. Cooper and L. H. Miller, in press) has been determined previously, we felt it necessary to repeat such studies to confirm the reports and to assure ourselves of the specificity of these staining reagents. Therefore, the specificity of the AI and PI colloids was determined by treating 0.1 ml of the packed glutaraldehyde-fixed parasite preparations with the following reagents under the said conditions: (i) chloroform-methanol (2:1), twice extracted; (ii) 0.1 N HCl in methanol at 60 C for 24 h (14); (iii) trypsin (EC 3.4.4.4), 0.25% (wt/vol) in phosphate-buffered saline, pH 7.3 (Difco Laboratories Inc.; pancreas source); (iv) phospholipase A (EC 3.1.1.4), 1.54 U total activity (Sigma Chemical Co.; *Vibrio* sp. source); (v) phospholipase D (EC 3.1.4.4), 3.74 U total activity (Sigma Chemical Co.; cabbage source); (vi) neuraminidase (EC 3.2.1.18), 22.0 U total activity (Sigma Chemical Co.; *Clostridium perfringens* source). All of the chemical and enzymatic treatments listed above, except methylation, were carried out at 37 C for 2 h. Control preparations were incubated and treated similarly except that (i) the organic solvents were replaced with distilled water, or (ii) the equivalent amounts of enzyme protein were replaced by equal weights of bovine serum albumin or by equal amounts of homologous heat-inactivated enzymes.

Mucopolysaccharide localization. The distribution of surface mucosubstance was examined by ruthenium red staining following the procedures outlined by Luft (16). Purified ruthenium red was incorporated into the primary glutaraldehyde fixative and also into the osmium tetroxide postfixing reagent at the concentration of 20 mg/ml. All fixation steps were carried out in the dark at 4 C.

Sialic acid assay. A 0.1-ml volume of packed parasite preparations was incubated with 3.0 ml of 0.1 N H₂SO₄ at 80 C for 1 h. Fractions of these hydrolyzed samples were dried at 70 C in a hot air oven under vacuum for 24 h and weighed on an analytical balance to determine the dry gross weight per milliliter of sample. The remaining hydrolyzed samples were assayed for sialic acid by the thiobarbituric acid method of Warren (31). As suggested by Warren, the determined micromolar concentration of sialic acid per milligram of sample was adjusted for interfering deoxyribose sugars by taking optical density readings

at 532 nm as well as at 549 nm, the absorption maximum for sialic acid. Red cells from rats recovering from malaria, which were mainly reticulocytes (as were the parasitized cell samples), were processed by similar isolation procedures and served as control cell extracts for these chemical determinations.

RESULTS

Ultrastructure. Isolated malaria parasites exhibited a number of different sizes and shapes (Fig. 1) and ranged in size from 1 to 4 μ m in diameter. Surface protuberances and deep invaginated areas were common topographical features (Fig. 1). The variable morphologies of isolated parasites were in contrast to normal erythrocytes which are characterized by their uniform size, shape, and surface topography. Red cell surfaces had a fine granular appearance (Fig. 4), whereas the parasite plasma membranes were usually stippled (Fig. 1). The gross morphology of the microorganisms varied due to different developmental stages within the sampled preparation. Infective forms, the merozoites, were uniformly spherical to oval in shape and were about 1.5 μ m in diameter (Fig. 1). Although not directly evident in Fig. 1, the surface texture of isolated carbon-replicated merozoites often were quite variable—some had surface contours which were relatively smooth, and others had a coarsely patterned lace-like texture. Collapse of the merozoite thin (8 nm), limiting plasma membrane over the thick (13 nm), discontinuous intermediate layer of the parasite pellicle (Fig. 2) resulted in a roughly textured but seemingly structured surface. Growth forms, the trophozoites, were quite pleomorphic, ranging in size from 2 to 4 μ m in diameter. The cytoplasm of the trophozoite was limited by a single membrane (Fig. 3) and lacked the form restraining intermediate (Fig. 2) and microtubular layers common to the merozoite.

Ionogenic surface properties. The ionogenic

Fig. 1. Electron micrograph of carbon-replicated extracellular malaria parasites (*P. berghei*). Large parasites (growth forms) are quite pleomorphic, often exhibiting prominent surface protrusions and deep invaginated areas, whereas small infective forms (merozoites) are uniformly spherical to ovoid in shape. The characteristically stippled plasma membrane of the free parasite is distinct in texture from the fine granular surface of the erythrocyte (see Fig. 4). Abbreviations: TR, trophozoite; M, merozoite; SP, surface protuberance; SV, surface invagination. Line marker represents 1.0 μ m.

Fig. 2. Portion of a thinly sectioned extracellular merozoite. A thin, limiting plasma membrane covers a thick (13 nm) underlying layer of the parasite pellicle. Abbreviations: PM, plasma membrane; IM, intermediate pellicular layers; N, nucleus. Line marker represents 0.2 μ m.

Fig. 3. Thinly sectioned extracellular trophozoite. The cytoplasm of a young trophozoite (growth form) is limited by a single unit membrane. Abbreviations: PM, plasma membrane; SV, surface invaginations. Line marker represents 0.2 μ m.

Fig. 4. Portion of external surface of a carbon-replicated red blood cell (RBC) viewed with an electron microscope. The uniform and fine granular surface texture is in contrast to the stippled plasma membranes of malaria parasites (see Fig. 1). Line marker represents 1.0 μ m.

properties of erythrocytes and parasites, which are attributed to charged glycosidic groups, were evaluated and compared easily by electron microscopy after staining preparations with the positively charged colloidal iron (AI) reagent. Erythrocytes, erythrocyte ghosts, leukocytes, and platelets readily took up the positively charged AI colloid and appeared to be stained heavily in sectioned samples (Fig. 5). The high density of negatively charged surface groups on replicated red cells appeared to be distributed randomly (Fig. 7). This is in contrast to the isolated extracellular malaria parasites which did not stain (Fig. 5 and 6). The lack of staining reactivity was characteristic of merozoites, as well as the isolated intracellular growth forms (Fig. 5).

With respect to staining characteristics, the isolated free parasites were similar to the cell-free organelles derived from ruptured host cells, since mitochondria and nuclei also fail to bind significant amounts of AI colloid (26).

As determined by the inhibition of staining of chemically extracted and enzymatically digested fixed cells, the AI colloid bound to cell surface chemical groups which were sensitive to neuraminidase and acidic methanol treatments. Binding was not affected noticeably by prior proteolytic (trypsin) enzyme digestion. Total colloid uptake was decreased only slightly by enzyme treatments with phospholipase A or D. This demonstrates that a substantial portion of the AI colloid, utilized above, specifically localized charged neuraminic (sialic) acid sites.

Sialic acid content. Total sialic acid content of isolated free parasites, measured biochemically, was markedly less than that of comparable control red cell extracts derived from rats recovering from *P. berghei* infection (Table 1).

Distribution of mucopolysaccharides. Correlated with the lack of AI colloid uptake by parasites and low concentrations of sialic acid as compared with red cells, free *P. berghei* lacked a prominent exterior mucopolysaccharide coat. Only an extremely thin layer, approximately 4 nm thick, could be demonstrated with

an electron microscope by specific ruthenium red staining (Fig. 8). In contrast, contaminating red cells within the standard preparation had a rather thick (25 nm) acidic mucopolysaccharide layer (Fig. 8).

Membrane lipid distribution. When the solvent of the basic colloidal iron reagent was shifted from acetic to propionic acid, the specificity of the stain also shifted. Unlike the standard sialophilic colloidal iron (AI) reagent, the binding of the PI colloid was not affected greatly by prior neuraminidase treatment, whereas phospholipase A and, to a lesser extent, phospholipase D digestion inhibited staining. Prior chloroform-methanol extraction of fixed cells completely inhibited PI colloid uptake. The stain intensity of PI colloid-treated membranes was not altered noticeably by prior proteolytic digestion. These results are similar to those reported previously (G. W. Cooper and L. H. Miller, in press).

As previously noted, free *P. berghei* parasites were not stained by the sialophilic AI colloid. In contrast, parasites treated with the lipophilic PI colloid reagent were stained heavily (Fig. 9). Erythrocyte surfaces, unlike free malaria parasites, bound both types of iron colloid (Fig. 6 and 9).

The patterns of PI colloid staining of parasites and erythrocytes differed. Surfaces of free malarial parasites were stained by large focal aggregates of colloid (Fig. 9). In a tangential section of a PI colloid-stained parasite, the irregular patchwork pattern of colloid-reactive and -nonreactive binding sites was shown (Fig. 10). Again in a surface view of a PI colloid-stained carbon-replicated free parasite, large aggregates of colloid were bound to the cell surface, leaving little membrane unstained (Fig. 12). Host erythrocytes, on the other hand, generally bound the PI colloid to their surfaces in a thin, uniform fashion, with little colloid aggregation (Fig. 9). The erythrocyte-bound PI colloid again formed a definite patchwork pattern at the cell surface. This staining pattern was shown on both tangentially sectioned (Fig.

FIG. 5. Thinly sectioned free malaria parasite preparation stained prior to embedding with the sialophilic colloidal iron reagent (AI colloid). AI colloid stains all host blood cells, i.e., platelets (PT), red cells (RBC), and red cell ghosts (G). Free parasites lack negatively charged sialic acid residues which characterize the surfaces of host blood cells. Abbreviations: M, merozoites; TR, portions of trophozoites. Line marker represents 1.0 μ m.

FIG. 6. Initial contact and orientation of the extracellular merozoite (M) at the surface of the erythrocyte (RBC) prior to its penetration. The preparation was treated with the sialophilic iron colloid prior to embedding to show the presence of negatively charged sialic acid groups on the red cells surface (arrows) and the absence of such groups on the extracellular parasite. The initial contact between the parasite and its host cell might be enhanced nonspecifically by such differences in charged surface components. Line marker represents 0.5 μ m.

FIG. 7. Surface view of a AI colloid-stained, carbon-replicated red cell (RBC). Membrane-bound AI colloid particles localize negatively charged sialic acid groups at the erythrocyte surface. Membrane sites (arrows) are dense and appear to be dispersed randomly. Line marker represents 0.5 μ m.

11) and carbon-replicated red cells (Fig. 13). However, in slight contrast to the PI colloid-stained free parasite, the matrix of bound colloid on the red cell surface was loosely knit and had large areas of unstained membrane (Fig. 11 and 13).

DISCUSSION

The continuous-flow sonic oscillation technique (20), which has been used here to isolate intracellular malaria parasites from infected blood, is a much improved method compared to older isolation methods such as immune, saponin, and French pressure cell lysis, and osmotic shock. Electron microscope examinations of final parasite isolates prepared by these older techniques have shown them to leave either large numbers of parasites entrapped in ghost red cells, badly damaged, or the preparations grossly contaminated with host cell debris (5). The use of the continuous-flow sonic oscillation method, which allows for the isolation of large numbers of well-preserved parasites that are free from entrapping ghost cells, permits us to use non-erythrocyte-penetrating heavy metal stains as effective cytochemical reagents. Secondly, host cell membranes which invariably contaminate isolated parasite preparations serve as internal staining controls rather than an uncontrollable factor with which investigators must contend when using standard biochemical procedures.

In the present report, a comparison of the morphology of the parasite and red cell surfaces has been made. The surface texture of replicated red cells was fairly uniform, with a fine granular appearance. The surface of parasites differed from erythrocyte surfaces in that it lacked such uniformity. The variable surface texture may have been in part the result of the adsorption of cellular debris by free parasites after the continuous sonic oscillation procedure. However, the coarsely patterned surfaces of young infective forms probably were due to the collapse of their thin plasma membranes over the discontinuous intermediate pellicular layers. Many of the isolated parasites were observed to have stippled surfaces. A similar type of stippling has been noted on the bounding plasma membranes of freeze-cleaved intraerythrocytic *P. gallinaceum* parasites (24). It is possible that this stippling is simply the aggregate of enlarged intramembranous particles, analogous to a smaller variety of particles purported to be common to unit membranes from many different sources (3).

The surface structure of the malaria parasite, with respect to its ionogenic properties, appears

to differ from blood cells of the host. The characteristic net negative surface charge associated with a variety of mammalian cell types is well known. Blood cells such as erythrocytes, leukocytes, and platelets have been described as being electrophoretically active (30). The negative charge is associated mainly with sialic acid or, more specifically, with the carboxylic group of *n*-acetyl-neuraminic acid (4, 9, 10, 17, 23). These charged sialic acid groups can be localized with the aid of an electron microscope at the cell surface by the use of AI colloid as an electron-dense staining reagent (8, 17). In preliminary studies, when fractions of free *P. berghei* isolates suspended in physiological salt solutions were examined by light microscopy, a high proportion of the cells were observed to be clumped, indicating that they had agglutinated spontaneously after their isolation from infected cells. Similar observations have been made by others (21). Such autoagglutination might result from a reduced or normally low net replusing surface charge, although other factors, such as the parasite's hydrophobic surface character, which increases adhesiveness, probably play a role. The former suggestion is supported by the results of Gasic's cytochemical procedure, which seem to indicate that the surface of free *P. berghei*, in contrast to host erythrocytes, lack negatively charged sialic acid residues. This does not imply that malaria parasites are devoid of a net negative surface charge, only that they lack charge groups arising from neuraminic acid (sialyl groups). Plasmodia, in all likelihood, probably do have a minimal net surface charge which may be attributed, not to sialic acid groups, but rather to charged phospholipids. This is suggested because it has been noted that free parasites as well as red cells are adsorbed readily to cationically charged resins (J. Kreier and T. Seed, unpublished observations).

Correlated with the results of the AI colloid cytochemistry is the biochemical finding that free-*P. berghei* preparations contain low amounts of sialic acid as compared with control red cell extracts. It is, however, surprising that the free parasites contain as much sialic acid as they do, since no surface sialic acid groups were demonstrated by the AI colloid staining technique. Perhaps sialyl residues are present but buried within the membrane and therefore not available to react with the sialophilic colloid. However, it is most likely that a proportion of the biochemically assayed sialic acid within the parasite preparation is due simply to contamination with host cell membranes.

It is curious that obligate intracellular ma-

laria parasites lack negatively charged surface sialyl groups, as do intracellular organelles of blood cells. One wonders whether other obligate intracellular parasites exhibit similar properties and if there is any general relationship between the lack of these surface components and an organism's intracellular habit. Other intracellular parasites will have to be isolated and studied by utilizing these cytochemical methods before any generalizations can be made. It does seem, however, that the intracellular forms (amastigotes) of *Trypanosoma cruzi* have less surface charge, possibly because of fewer charged glycosidic groups, than do subsequent extracellular developmental forms, i.e., epimastigotes. The latter was demonstrated, not by specific cytochemical staining techniques as we have used here, but by the different elution patterns of the

two developmental forms from ion exchange columns (S. N. Al-Abbassy, J. P. Kreier, and R. M. Pfister, Abstr. Annu. Meet. Amer. Soc. Microbiol., p. 39, 1973). In the same respect, it would be interesting to examine, by cytochemistry and column chromatography, the ionogenic properties of malarial sporozoites which are basically extracellular in nature.

It has been suggested that, in certain aspects, the bounding membranes of malarial parasites resemble, to a greater extent, the membranes of intracellular organelles than plasma membranes of blood cells. This is certainly true when one compares the lipid composition of the respective membranes. Nuclear membranes as well as other bounding membranes of internal organelles have lower concentrations of cholesterol per unit weight, resulting in a higher phospholipid to cholesterol ratio (P/C ratio) than parent cell plasma membranes (32). Isolated plasma membranes from extracellular and/or free-living protozoa such as *Acanthamoeba castellanii*, have high cholesterol content and P/C ratios (12) which somewhat resemble the values determined for mammalian cells (19). In contrast, isolated plasmodial (*P. knowlesi*) plasma membranes have been reported to have significantly more phospholipid and less cholesterol (P/C ratio = 5.2) than host erythrocyte membranes (P/C ratio = 2.15) (22). Although it has been observed earlier that intraerythrocytic growth of the parasite is re-

TABLE 1. Sialic acid content of free malaria parasites and noninfected erythrocytes processed by continuous-flow sonic oscillation

Determination	Sialic acid (μm)/tissue (g)	Preparations (no.)
Noninfected erythrocyte extract	1.52 ± 0.21 ^a	9
Free malaria parasite extract	0.89 ± 0.09	10

^a Average value ± standard deviation.

FIG. 8. Thinly sectioned free parasite preparation stained with ruthenium red prior to embedding. The red cell's thick surface coat (SC) is in contrast to the extremely thin, external layers of mucosubstance on the isolated parasites (TR). Non-erythrocyte-penetrating ruthenium red fails to stain the cytoplasmic membranes of intracellular parasites (IP). Line marker represents 1.0 μm.

lated directly to noted increases in total lipid concentration of infected cells, the relation of specific lipids to total increases was not apparent. Through the work of Rock et al. (22) and later DeZeeuw and colleagues (7), it was shown that phospholipids comprise the major portion of the membrane lipids of the malarial parasite and that the two major classes of phospholipids are phosphatidyl choline and phosphatidyl ethanolamine. Whereas phosphatidyl serine comprises about 10% of the red cell membrane, it is negligible in the parasite plasma membrane. Similarly, sphingomyelin content is considerably lower in the parasite membrane. In spite of these excellent biochemical works, nothing is known about the distribution of lipids within the parasite bounding membrane.

By using a lipophilic colloidal iron reagent, we have been able to localize exposed lipoidal areas of plasma membranes. Demonstration of the lipophilic nature of the propionic acid colloid, by the inhibition of stain uptake by phospholipase-treated membranes, confirms earlier observations made by Copper and Miller (J. Histochem. Cytochem., in press). These workers proposed that lipid sites bound the PI colloid by a direct intercalation of the acyl region of propionic acid, and thus the iron colloid, into the hydrophobic regions of the adjacent phospholipid molecules. With the plasma membranes of the malarial parasite being high in phospholipid content, one would expect the parasite membrane to be stained heavily when incubated with the PI colloid. This we have noted. Free parasites bound colloid in large focal aggregates which appear as a tightly knit patchwork pattern when viewed either in tangential section or by a surface view. This suggests that the membrane is composed of a mosaic of islands of phospholipid which are immersed in a matrix. The erythrocyte membrane similarly possesses a patchwork of stained (phospholipid areas) and unstained areas. The lipoidal plaques, however, appear smaller and

more dispersed. This would be expected, since we already mentioned that the erythrocyte membrane per square unit area has a lower concentration of phospholipid than does the malarial parasite membrane. Although the plasma membranes of the host cell and the parasite seemingly differ with respect to the size and numbers of exposed phospholipid areas, the membranes of the two cell types resemble each other because of their patchwork pattern. This mosaic pattern is consistent with a number of current suggestions concerning membrane structure (11, 25).

There is little doubt that the surface ultrastructure of the malaria parasite differs from that of its host cell and that these differences might be of considerable consequence in the biology of the parasite and, in turn, the disease process. The intracellular parasite seemingly lacks a characteristic glycocalyx layer common to most mammalian cells. There is an absence of specific constituents of coat materials as well as a reduction in the total amount of mucosubstance present. The surface of the parasite is characterized by a high density of phospholipids. Each of these surface properties influences the adhesive character of the parasite. Adhesiveness and, in turn, adhesion are essential factors in successful parasitism (6). The invasiveness of malaria parasites might be enhanced nonspecifically through an increase in adhesiveness of the organisms to their target cells, i.e., erythrocytes. This characteristic would be most important in the parasitic process during the initial contact between parasite and the erythrocyte. This would be prior to the orientation of the merozoite on its host cell and prior to the activation of specific conoid enzymes thought to be functional in the penetration processes (14). Attractive forces and subsequent adhesion of parasite to its host cell would be greater if the parasite surface had a low density of charged glycosidic groups (Fig. 6). Adhesiveness of two cell types is not only the

FIG. 9. Extracellular malaria parasites and erythrocytes stained with lipophilic PI colloid. A high proportion of free parasites (P) bind lipophilic colloid in large focal aggregates (arrows), localizing high affinity phospholipid plaques. Red cell (RBC) surfaces are stained in a more uniform linear fashion. Line marker represents 1.0 μ m.

FIG. 10 and 11. Tangentially sectioned extracellular malaria parasite (Fig. 10) and erythrocyte (Fig. 11) stained with lipophilic iron colloid. A close-knit patchwork pattern of lipid plaques at the surface of the malaria parasite plasma membrane is suggested by the mosaic of bound colloid. This is in contrast to the loose-knit patchwork of lipid plaques at the red cell surface (Fig. 11). Drawn circles are to aid envisioning lipid plaque patterns. Line markers represent 0.2 μ m.

FIG. 12 and 13. Surfaces of lipophilic colloid-stained, carbon-replicated free parasite (Fig. 12) and erythrocyte (Fig. 13). Note in Fig. 12 the intensely stained surface with large and closely bound aggregates of colloid, which is in contrast to the more loosely dispersed islands of bound colloid which are within unstained matrix of the erythrocyte plasma membrane (Fig. 13). Line markers represent 0.2 μ m.

function of the sign of the net surface charge but also the total strength of the charge associated with each cell (6). Non-electrostatic properties may be equally important. The hydrophobic nature of the malarial parasite surface, which results from its high lipid content, might be sufficiently strong to overcome weak static repulsive charges which may be present.

We have speculated that the adhesive surface character of the malaria parasites promotes survival of extracellular forms by aiding them in their attachment to and entrance into susceptible host cells. This trait, however, might equally decrease their chances of survival within the hostile environment of the blood stream by nonspecifically aiding defense mechanisms of the infected host. This surface characteristic might be responsible in part for their rapid removal from the circulation after rupture of infected cells. For a long time, it has been recognized that the number of progeny produced by segmenting parasites is in great excess to that which actually go on to infect new host cells (13). As a consequence of the malarial infection, the reticuloendothelial system of the host becomes activated and the phagocytic rate of foreign particulates nonspecifically increases (W. Cantrell, and E. E. Elko, Fed. Proc. 32:139, 1964). Activated phagocytic cells are more likely to engulf particles, cells, etc., whose surfaces have greater adhesive properties than those with opposite surface characteristics. It has been suggested that these membrane properties are involved in the recognition mechanism of the phagocyte for engulfing certain types of cellular debris such as extruded nuclei from basophilic erythroblasts (26). Therefore, only those parasites which adhere to and quickly penetrate red cells would be expected to survive. The rapidity of parasite attachment and subsequent invasion is apparent as one rarely observes extracellular merozoites in blood fixed quickly after sampling.

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