Inhibitory Effects of Erythrocyte Membrane Proteins on the In Vitro Invasion of the Human Malarial Parasite (*Plasmodium falciparum*) into Its Host Cell

MARGARET PERKINS
Department of Parasitology, The Rockefeller University, New York 10021

ABSTRACT The intracellular development of the erythrocytic stage of the malarial parasite (merozoite) is initiated by the attachment of the parasite to the erythrocyte surface. This paper describes an assay system to investigate *Plasmodium falciparum* merozoite entry into the host cell and reports on three observations regarding this interaction. (*a*) Merozoites do not invade human erythrocytes treated with either trypsin or neuraminidase, and both enzymes partially cleave glycophorin A, the major erythrocyte surface sialoglycoprotein. (*b*) A membrane protein fraction containing glycophorin A will, at low concentrations, inhibit the invasion of isolated merozoites into erythrocytes; no other fractions of membrane proteins have appreciable effects on the reinvasion. (*c*) Merozoites do not reinvade erythrocytes preincubated with F ab' fragments of antibody prepared against glycophorin A. Together, these three observations imply a role for glycophorin A in the attachment of the malarial parasite to the erythrocyte surface.

The development of the human malarial parasite *Plasmodium falciparum* occurs inside the erythrocyte, where it undergoes a process known as schizogony. The mature shizont ruptures, releasing into the serum individual merozoites which are then free to reinvade other erythrocytes. The attachment of the merozoite to the erythrocyte initiates reinvasion and a second cycle of intraerythrocytic growth. Entry has been observed by interference (1) and electron microscopy (2) and is a rapid event, being complete in 1 min. Biochemical characterization of the attachment phase has proved difficult due to the inherent problems in isolating viable merozoites free from host material. Miller and colleagues (3, 4) have studied the invasion of both *P. falciparum* and the simian parasite *Plasmodium knowlesi*. They concluded from a number of investigations that both parasites recognize specific receptors on the surface of the erythrocyte, but that the receptors are different for each parasite; only *P. knowlesi* parasites interact with Duffy blood groups (3), whereas *P. falciparum* appears to interact with a glycoprotein on the erythrocyte surface (4). Miller et al. (4) and Butcher et al. (5) have proposed that the existence of specific receptors on the different erythrocytes and parasites could explain host-parasite restriction of invasion. Biochemical determinants of the specificity of interaction of erythrocytes with different species of parasites has been reviewed recently by Sherman (6). In this work, the attachment of merozoites to the erythrocytes was studied by investigation of the entry of isolated merozoites. In preliminary studies it was found that merozoites will not invade erythrocytes treated with trypsin, chymotrypsin, or neuraminidase, treatments which partially remove glycophorin A, known also as PAS I, the major sialoglycoprotein on the human erythrocyte surface. This prompted experiments to test the effects of different fractions of erythrocyte membrane proteins on merozoite invasion of human erythrocytes.

MATERIALS AND METHODS

Cultivation of P. falciparum

The Gambian strain of *P. falciparum* was cultured in vitro according to the method of Trager and Jensen (7). The medium used was RPMI-1640-HEPES(RPMI) supplemented with 10% human serum (RPS). Synchronous cultures were obtained by first treating an asynchronous culture with sorbitol (8) and then repeated separations of rings and trophozoites by flotation in gelatin (9). Treatment of successive gelatin supernates for four or five cycles resulted in a parasite population synchronous within 4–5 h. The parasitemia was allowed to reach 12–15% before the collection of merozoites.

Collection of Merozoites

When it was apparent from blood smears that the mature shizonts in the synchronous culture were beginning to rupture, the medium was removed and replaced with 4 ml of medium (RPS). The cultures were kept in the candle jar under low oxygen and shaken every 10 min over a 1-h period to minimize reinvasion of released merozoites. After 1 h, the erythrocytes were removed from the culture by centrifugation at 2,000 rpm for 5 min. The pellet was returned to the candle jar and mixed with fresh medium for a second collection. The

supernate, containing merozoites, damaged shizonts, residual bodies, and membrane debris, was mixed with Sepharose-4B (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.) conjugated to antierythrocyte membrane antibody for 3 min at 37°C. The Sepharose-4B was removed by centrifugation at 1,000 rpm for 2 min. The supernate containing the merozoites was then centrifuged at 3,000 rpm for 5 min. The pellet containing merozoites and residual bodies was resuspended in RPMI and added to fresh erythrocytes. There was some variation in the yield of merozoites obtained in the seven experiments described here. Generally, 1–3 × 10^8 merozoites could be collected during a 1-h period from 10 ml of packed erythrocytes with 12–15% parasitemia. Based on the criteria of ability to reinvade, 6–11% of the merozoites were viable after the isolation procedure.

Merozoite Reinvasion Assay

The reinvasion of isolated merozoites was assayed in 10-mm microtiter wells. Washed incubated human erythrocytes were suspended at a concentration of 10^8/ml medium. 1 ml of the suspension was added to each well and incubated at 37°C for 2–3 h, after which time the medium was removed. Merozoites were resuspended in RPMI at a concentration of 5–10 × 10^7/ml; 0.1 ml of this was added to each well. The cells were kept at 37°C for a further 4 h, after which they were removed and washed three times with RPS. Blood smears were made of the cells, and the number of intracellular ring stages of parasites were counted per 10,000 erythrocytes. Typically, of 10,000 erythrocytes counted, 80–100 contained rings, i.e., ~1% were invaded. The percentage of merozoites invading ranged from 6 to 11%. To test the effects of erythrocyte-membrane fractions on reinvasion, the merozoites were mixed with the various fractions and added immediately to the fresh erythrocytes. After 1 h, the medium, containing the protein fractions, was removed and replaced with 1 ml of RPS. The erythrocytes were kept at 37°C for an additional 3 h before the ring stages were counted.

Enzymatic Treatment of Erythrocytes

Conveniently, the major erythrocyte-surface glycoproteins are selectively sensitive to trypsin, chymotrypsin, and neuraminidase (10, 11). The sialoglycoprotein glycophorin A (PAS I) and its monomer (PAS II) are sensitive to trypsin and chymotrypsin, and the sialic acids are removed by neuraminidase. The major protein, referred to as Band 3, is resistant to trypsin and neuraminidase, and glycophorin B (PAS III) is resistant to trypsin only (11). Thus, specific enzyme treatment of the erythrocyte surface can selectively remove the major proteins. The removal of these glycoproteins was confirmed using enzyme concentrations used in subsequent experiments. The degree of proteolysis was assessed by SDS PAGE (12) of ^{125}I-labeled erythrocytes. Enzymaticaly modified erythrocytes for the reinvasion assay were prepared by washing fresh erythrocytes three times in RPMI and once in 0.1 M phosphate-buffered saline (PBS) supplemented with 5 mM $CaCl_2$ and 5 mM $MgCl_2$, then resuspending them in 1-ml samples containing 2 × 10^8 cell/ml. To separate aliquots were added trypsin (Miles Laboratories Inc., Miles Research Products, Elkhart, Ind.; three times crystallized 4611 U/mg) at a concentration of 0.025 mg and 0.1 mg/ml; chymotrypsin (Miles; twice crystallized, 1240 U/mg) at concentrations of 0.05 mg and 0.2 mg/ml; neuraminidase from *Vibrio cholerae* (Grand Island Biological Co., Grand Island, N. Y.; 500 U/ml) at concentrations of 5, 10, 20, and 50 U/ml. The erythrocytes were incubated with the enzymes for 30 min at 37°C. The cells were washed once in RPMI and twice in RPMI supplemented with human serum. A half of each sample of 10^8 cells was placed in each microtiter well for the assay. Each assay was performed in duplicate.

Isolation of Erythrocyte Membrane Proteins

The erythrocyte membrane proteins were fractionated by chloroform:methanol extraction according to the method of Hamaguchi and Cleve (13). By this procedure, glycophorin A partitioned into the aqueous phase after extraction. It was further purified on a Sephadex G-100 column without detergents. It was routinely possible to obtain 10 mg of glycophorin A from 200 ml of packed erythrocytes. There was a loss of protein during purification on the G-100 Sephadex column, and this was attributed to the absence of detergents in this step. In addition, glycophorin B, which accounted for 10% of the protein applied to the Sephadex column, could not be eluted in the absence of detergents. Liposomes containing the glycophorin A were prepared by mixing lecithin (4 mg), phosphatidic acid (2 mg), and cholesterol (0.4 mg) dissolved in chloroform. The lipids were dried in a rotary evaporator and resuspended in 1 ml of the aqueous phase, containing 1 mg of glycophorin A. The remainder of the membrane proteins, spectrin, Band 3, and many minor proteins separating at the interphase were not soluble in either the aqueous or chloroform methanol phase. The only means available to render this material accessible to merozoites was to resuspend it with lipids. This was done by mixing the dried interphase material (2 mg) with lecithin (4 mg), phosphaditidylcholine (2 mg), and cholesterol (0.4 mg) dissolved in chloroform. The mixture was dried and resuspended in 1 ml of PBS. Liposomes containing the chloroform:methanol phase were prepared by dissolving 1 mg in chloroform and mixing with the lipids present in the same ratios as described above. The mixture was dried and resuspended in 1 ml of PBS. The fractionation of the erythrocyte ghosts was repeated using erythrocytes labeled with ^{125}I, and the purity of the separate fractions was assessed by x-ray autoradiography of SDS polyacrylamide gels (12). It also seemed desirable to test effects of glycophorin from an erythrocyte that *P. falciparum* does not normally invade. Rhesus monkey erythrocytes seemed appropriate and glycophorin A was purified from these cells also by the method of Hamaguchi and Cleve (13).

Preparation of F ab' Fragments

Antibodies to glycophorin A were raised in rabbits. The IgG fraction from immune serum agglutinated intact erythrocytes, indicating that the antibodies recognized an antigenic site on glycophorin A exposed on the surface. F ab' fragments were prepared from the IgG (10 mg) fractions by proteolysis with protease (1 mg) according to the method of Brackenbury et al. (14). In the invasion assay the F ab' fragments were incubated with erythrocytes for 1 h before the addition of isolated merozoites. Glycophorin A was immunoprecipitated with antiserum and staphylococcus A conjugated to Sepharose-4B (Bio-rad Laboratories, Richmond, Calif.).

RESULTS

Enzymatic Treatment of Erythrocytes

Table I summarizes the effects of enzymatic treatment of erythrocytes on invasion by isolated merozoites. The results represent the average of three separate experiments. Trypsin treatment at a concentration of 115 U/ml reduced invasion to 7% of control and 460 U/ml completely inhibited reinvasion. The external tryptic fragment of glycophorin was removed by 115 U/ml of trypsin. ^{125}I-labeled Band 3 and glycophorin B were resistant to this trypsin treatment. Chymotrypsin at 62 U/ml reduced reinvasion to 60% of control and 248 U/ml reduced it to 5% of control. The lower concentration of chymotrypsin partially removed glycophorin A, B, and Band 3, while the higher concentrations removed all proteins labeled with ^{125}I. Treatment with 10 U/ml of neuraminidase reduced invasion to 10% of control. This was reduced to 2% when 50 U/ml was used. ^{125}I-labeled glycophorin A, treated with 20 U of neuraminidase, was reduced to a single band migrating with a molecular weight of 50,000 daltons, indicating that the neuraminidase did not have protease activity.

TABLE I

Effects of Enzymatic Treatment of Human Erythrocytes on Invasion by P. falciparum Merozoites

Treatment	Invasion
U/ml	%
Untreated	100
Trypsin	
115	10
460	0
Chymotrypsin	
62	64
248	6
Neuraminidase	
5	89
10	10
20	8
50	2

Values are expressed as a percentage of invasion into the untreated control cells. The results represent the average of three separate experiments. In control experiments, typically 5–10 × 10^8 merozoites were added to 10^8 erythrocytes. Of these, 6–11% were able to reinvade fresh erythrocytes. The enzyme concentrations refer to the amount added to 2 × 10^8 erythrocytes, suspended in 1 ml of PBS supplemented with $CaCl_2$ and $MgCl_2$ (0.5 mM).

Effect of Isolated Erythrocyte Membrane Fractions on Merozoite Reinvasion

Fig. 1 shows the protein fractions isolated from erythrocyte ghosts, labeled with ^{125}I. The identification of the major proteins was based on their known molecular weights and migration pattern in SDS polyacrylamide gels (10, 11, 13, 15). The membrane fraction containing glycophorin A used in the invasion assay is shown in Fig. 1 *a*. Greater than 98% of the radioactivity in this fraction is found in a single band; the band has a molecular weight of ~90,000 daltons and comigrates with the band in Fig. 1 *e* (referred to as protein 3), which, based on sensitivity to trypsin and neuraminidase, was identified as glycophorin A. Very small amounts of glycophorin A monomer (referred to as protein 4), ~60,000 daltons, could be detected. Other ^{125}I-labeled bands were not apparent, nor were additional proteins detected in this fraction when the gel was stained with Coomassie Blue. Table II summarizes the effects of the different membrane protein fractions on reinvasion of merozoites. As little as 10 μg/ml of the fraction containing glycophorin A reduced invasion to 21% of normal. This fraction mixed with liposomes appeared to be almost as effective. Interphase material, which contains Band 3, spectrin, actin, and other membrane proteins, including some glycophorin A, is shown in Fig. 1 *f*. At low concentrations, this did not appear to inhibit reinvasion; at higher concentrations, it reduced invasion to 64% of normal. The chloroform:methanol phase, which contains mainly lipids and glycolipids, did not appear to have any effect. The effect of glycophorin A was abolished when the protein was first incubated with F ab′ fragments of IgG prepared against glycophorin A. Glycophorin A prepared from Rhesus monkey erythrocytes was also effective in blocking invasion, although 20 μg of this glycophorin reduced invasion to 69% of control, whereas the equivalent amount of glycophorin A from human cells reduced invasion to 15%. Antiserum to glycophorin A immunoprecipitated glycophorin A and minor amounts of its monomer (protein 4) and glycophorin B (protein 5) shown in Fig. 1 *b*. Low concentrations of F ab′ fragments blocked reinvasion by ~50%, while it was necessary to add 1 mg/ml to block reinvasion completely.

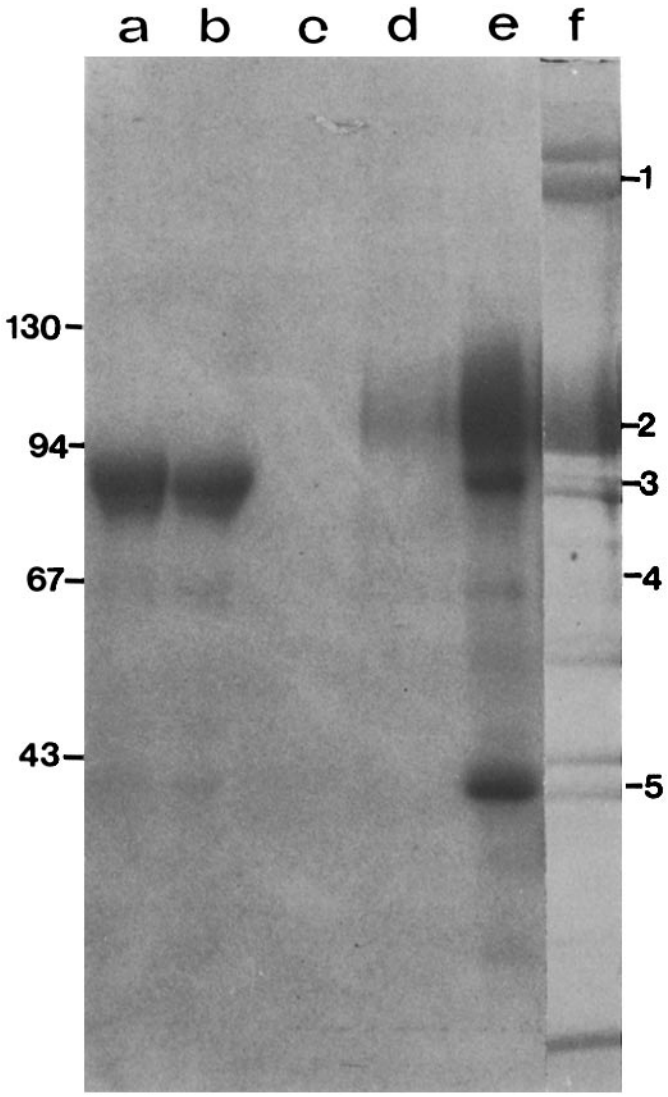

FIGURE 1 Fractionation of membrane proteins of ^{125}I-labeled human erythrocytes by partition in chloroform-methanol (13). Fractions were analyzed on a SDS polyacrylamide gel (5–15% gradient) using the buffers of Laemmli (12). (*a*) ^{125}I-labeled glycophorin A in the aqueous phase after elution from G-100 sephadex column, 100 μg protein; (*b*) immunoprecipitate of glycophorin A from the aqueous phase; (c) chloroform-methanol phase, 100 μg of protein; (*d*) ^{125}I-labeled interphase, 100 μg protein: (*e*) ^{125}I-labeled erythrocyte ghosts, 300 μg of protein: (*f*) Coomassie Blue stain of interphase material, 150 μg of protein. From the molecular weight markers the proteins could be identified as: (*1*) spectrin; (*2*) Band 3; (*3*) glycophorin A dimer (*4*) glycophorin A monomer; (*5*) glycophorin B. The molecular weight markers were β-galactosidase (130,000), phosphorylase b (94,000), serum albumin (67,000), and ovalbumin (43,000).

DISCUSSION

Earlier reports by Miller and co-workers (3, 4) and Butcher et al. (5) have suggested the presence of specific "receptors" on the erythrocyte surface for the malarial parasite. This study was designed to identify the erythrocyte surface components which could act as attachment sites for the human malarial parasite *P. falciparum*. In preliminary experiments it was shown that merozoites did not invade erythrocytes treated with neuraminidase or trypsin; neuraminidase removes sialic acid solely from the sialoglycoproteins (10); trypsin cleaves a fragment from glycophorin A but does not affect glycophorin B (11). On the basis of these experiments, glycophorin A was isolated from human erythrocyte membranes and its effect on reinvasion into fresh erythrocytes was investigated. A fraction of sialoglycoproteins which was >98% pure with respect to glycophorin A dimer was extremely effective in blocking reinvasion; as little as 10 μg/ml of this fraction reduced reinvasion of *P. falciparum* merozoites to 21% of normal, while 50 μg/ml appeared to completely block invasion. The specificity of inhibition is attested by the fact that neither the remainder of the membrane proteins nor the membrane lipids appreciably reduced reinvasion. F ab′ fragments of antibody prepared against glycophorin A were also effective in blocking reinvasion. This antibody, in addition to reacting with glycophorin A (Fig. 1 *b*) and its monomer (protein 4 in Fig. 1 *b*), also cross-reacts weakly with glycophorin B (protein 5 in Fig. 1 *b*). This is to be expected as glycophorin A and B share a common N-terminal of 22 amino acids (11) and common antigenicity in this region (15). These experiments do not eliminate the possible involvement of glycophorin B in merozoite invasion. An antibody specific for the external fragment of glycophorin A, not common to glycophorin B, would be an advantage in defining the contribution of glycophorin A and B to an attachment site. In the purifica-

TABLE II

Inhibition of Merozoite Invasion by Erythrocyte Membrane Fractions

Additions to Merozoites	Invasion
μg	%
Control	100
Liposomes*	
132	100
330	93
660	91
1,320	90
Aqueous phase	
10	21
20	15
50	0
100	0
200	0
Aqueous phase + liposomes‡	
20	46
50	6
100	0
200	0
Interphase + liposomes§	
50	93
100	100
200	81
500	64
Chloroform:methanol phase + liposomes ‖	
20	100
50	100
100	85
200	90
Preimmune serum	
100	100
200	84
500	83
1,000	71
Fab' fragments of anti-glycophorin antibody	
50	40
100	62
200	25
500	15
1,000	0
Glycophorin A from Rhesus erythrocytes	
20	69
50	51
100	19
200	0
Glycophorin A + Fab' fragments	
20	81
50	80
100	100
200	93

The results represent the average of four separate experiments. The membrane fractions were added to the merozoites immediately before their addition to fresh erythrocytes as described in the Methods. The erythrocytes were washed and the intracellular ring stages of the parasite counted to estimate the number of invaded merozoites.

* Liposomes were prepared by adding 4 mg of phosphatidylcholine, 2 mg of phosphatidic acid, and 0.4 mg of cholesterol. The lipids were dried and resuspended in 1 ml of PBS. Of this, 20, 50, 100, and 200 λ were added to the merozoites, amounts equivalent to the lipid in the liposomes containing 20, 50, 100, and 200 μg protein of the membrane fractions.

‡ Liposomes were resuspended in 1 ml of PBS containing 1 mg of the aqueous phase shown in Fig. 1 *a*.

§ Interphase fraction (2 mg) was added to the lipids before drying and then resuspended in 1 ml of PBS.

‖ The chloroform:methanol phase (1 mg) was added to the lipids before drying and resuspended in 1 ml of PBS.

tion method used in this work, it was not possible to obtain pure glycophorin B, free of glycophorin A or its monomer. For this reason, its effect on invasion could not be determined. However, L. H. Miller et al. (personal communication) propose that both glycophorin A and B can act as receptors for *P. falciparum* merozoites. This conclusion is based on experiments using erythrocytes lacking either glycophorin A or glycophorin B and would not be surprising in view of the structural similarities of the two molecules. One result in the present work not consistent with a role for glycophorin B is that isolated merozoites did not invade erythrocytes treated with trypsin in which glycophorin B is intact. The differences in results may be a consequence of the different assay systems used to determine reinvasion; Miller et al. monitor the reinvasion of merozoites from rupturing schizonts into modified erythrocytes in culture rather than isolated merozoites used in the present experiments. It is possible that isolated merozoites, although able to reinvade, are less viable and more sensitive to the considerable modification of the erythrocyte surface. Another consideration is the specific involvement of sialic acids in the binding of merozoites; neuraminidase treatment of erythrocytes reduced invasion to 2% of that found in untreated cells. Sialic acids have been implicated as receptors for a variety of myxo- and paramyxoviruses (16), the best characterized being those for Sendai virus (17). Further experiments will be required to determine whether the merozoites are binding specifically to the sialic acids. It is possible that the removal of sialic acids which constitute 28% (wt/wt) of glycophorin A could alter the tertiary structure of the polypeptide, lowering its affinity for the merozoites.

It is not easy at this point to describe a role for glycophorin in invasion. A simple hypothesis would be that the merozoites recognize and attach to glycophorin and that this, by some yet unknown mechanism, triggers the subsequent events of membrane invagination and endocytosis. However, glycophorin A isolated from Rhesus monkey erythrocytes is also active in blocking *P. falciparum* merozoite reinvasion, albeit, higher concentrations are required, suggesting that it has a lower affinity for the *P. falciparum* merozoites. If glycophorin A is the sole attachment site for merozoites and regulates entry, it would be expected that, based on this result, *P. falciparum* merozoites would invade Rhesus monkey erythrocytes, but with reduced infectivity. But this is not observed. *P. falciparum* parasites do not invade Rhesus cells either in vivo or in vitro (5). Therefore although glycophorin may be the primary attachment site, secondary factors must be involved in regulating entry. Alternatively, the topographical distribution of glycophorin on the surface may be important in its interaction with the merozoite, and this may be different in human and Rhesus erythrocytes.

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