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 glycoporphin 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')2 fragments of antibody prepared against glycoporphin A. Together, these three observations imply a role for glycoporphin 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 schizont 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 glycoporphin 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 schizonts 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
were kept at 37°C for an additional 3 h before the ring stages were counted. 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 with lipids. The lipids were dried in a rotary evaporator and resuspended in 1 ml of the lipid suspension.

**Merozoite Reinvasion Assay**

The reinvasion of isolated merozoites was assayed in 10-μl microtiter wells. Washed incubated human erythrocytes were suspended at a concentration of 10^9/ml medium. 1 ml of the suspension was added to each well and incubated at 37°C for 2-3 h, after which the medium was removed. Merozoites were resuspended in RPMI at a concentration of 5-10 x 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 parasitemia was counted per 1,000 erythrocytes. Typically, 10,000 erythrocytes counted, 80-100 containing merozoites, 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. The dialyzable glycoproteins, 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. Thus, specific enzymic treatment of the erythrocyte surface selectively removes 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 251-labeled erythrocyte ghosts. Enzymatically modified erythrocytes for the reinvasion assay were prepared by washing fresh erythrocytes three times in RPMI and once in 0.1M phosphate-buffered saline (PBS) supplemented with 5 mM CaCl₂ and 5 mM MgCl₂, then resuspending them in 1-ml samples containing 2 x 10^8 cell/ml. To separate aliquots were added trypsin (Miles Laboratories Inc., Miles Research Products, Elkhart, Ind.; three times crystallized 4611 U/ml) at a concentration of 0.025 mg and 0.1 mg/ml; chymotrypsin (Miles; twice crystallized, 12400 U/ml) 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 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 routine possible to obtain 7-10 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. Lipoproteins containing the glycophorin A were prepared by mixing lecithin (4 mg), phosphatidylcholine (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), phosphatidylcholine (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 lipids in chloroform and mixing with the lipids present in the same ratio 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 ^125I, 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 agglutinates 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 proteinase and neuraminidase. The F ab' fragments were incubated with erythrocytes for 1 h before the addition of isolated merozoites. Glycophorin A was immunoprecipitated with antisera 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 trypptic fragment of glycophorin was removed by 115 U/ml of trypsin. ^125I-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 ^125I. Treatment with 10 U/ml of neuraminidase reduced invasion to 10% of control. This was reduced to 2% when 50 U/ml was used. ^125I-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**

| Treatment | Invasion % |
|-----------|------------|
| Untreated | 100        |
| Trypsin   | 100        |
| 115       | 10         |
| 460       | 0          |
| Chymotrypsin | 64    |
| 62        | 6          |
| 248       | 6          |
| Neuraminidase | 89    |
| 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. Control experiments, typically 5 x 10^7 merozoites were added to 10^7 erythrocytes. Of these, 6-11% were able to invade fresh erythrocytes. The enzyme concentrations refer to the amount added to 2 x 10^8 erythrocytes, suspended in 1 ml of PBS supplemented with CaCl₂ and MgCl₂ (0.5 mM).
Effect of Isolated Erythrocyte Membrane Fractions on Merozoite Reinvansion

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. 1a. 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. 1e (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 $\mu$g/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. 1f. 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 $\mu$g 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. 1b. 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 $\mu$g protein; (b) immunoprecipitate of glycophorin A from the aqueous phase; (c) chloroform-methanol phase, 100 $\mu$g of protein; (d) $^{125}$I-labeled interphase, 100 $\mu$g protein; (e) $^{125}$I-labeled erythrocyte ghosts, 300 $\mu$g of protein; (f) Coomassie Blue stain of interphase material, 150 $\mu$g 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 $\beta$-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 $\mu$g/ml of this fraction reduced reinvasion of *P. falciparum* merozoites to 21% of normal, while 50 $\mu$g/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. 1b) and its monomer (protein 4 in Fig. 1b), also cross-reacts weakly with glycophorin B (protein 5 in Fig. 1b). 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.
Interphase fraction (2 mg) was added to the lipids before drying and then resuspended in 1 ml of PBS containing 1 mg of the aqueous phase shown in Fig. 1a. 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 μg 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.

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

| Additions to Merozoites | Invasion |
|------------------------|---------|
| Control                | 100     |
| Liposomes*             | 100     |
| 132                    | 100     |
| 330                    | 93      |
| 660                    | 91      |
| 1,320                  | 90      |
| Aqueous phase          |         |
| 10                     | 21      |
| 20                     | 15      |
| 50                     | 0       |
| 100                    | 0       |
| 200                    | 0       |
| Aqueous phase + liposomes‡ | 46 |
| 20                     | 6       |
| 50                     | 0       |
| 100                    | 0       |
| 200                    | 0       |
| Interphase + liposomes§ | 93 |
| 50                     | 100     |
| 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 μg 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. 1a.

‡ 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.

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 invasion, 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.

I would like to thank Dr. W. Trager for his support during this work and Dr. Louis Miller for his advice and communication of results before publication.

This investigation received financial support from the United Nations Development Programme/World Bank/World Health Organization Special Programme for Training in Tropical Diseases.

Received for publication 6 April 1981, and in revised form 11 May 1981.

REFERENCES

1. Dvorak, J. A., L. H. Miller, W. C. Whitehouse, and T. Shirahashi. 1975. Invasion of erythrocytes by malaria merozoites. Science (Wash. D. C.). 187:748-749.
2. Aikawa, M., L. H. Miller, J. Johnson, and J. Rabbridge. 1978. Erythrocyte entry by malaria parasites. A moving junction between erythrocyte and parasite. J. Cell Biol. 77:73-82.
3. Miller, L. H., S. J. Mason, J. A. Dvorak, M. McGinniss, I. K. Rothman. 1975. Erythrocyte
receptors for *Plasmodium knowlesi* malaria: Duffy blood group determinants. *Science* (Wash. D. C.) 189:561-563.

4. Miller, L. H., J. D. Haynes, F. M. McAuliffe, T. Shiroishi, J. R. Durocher, and M. McGinnis. 1977. Evidence for differences in erythrocyte surface receptors for the malarial parasites, *Plasmodium falciparum* and *Plasmodium knowlesi*. *J. Exp. Med.* 146:277-281.

5. Butcher, G. A., G. H. Mitchell, and S. Cohen. 1973. Mechanism of host specificity in malarial infection. *Nature* (Lond.). 244:40-41.

6. Sherman, I. W. 1979. Biochemistry of Plasmodium (malarial parasites). *Microbiol. Rev.* 43(No. 4):453-495.

7. Trager, W., and J. B. Jensen. 1976. Human malaria parasites in continuous culture. *Science* (Wash. D. C.). 193:674-675.

8. Lambros, C., and J. P. Vanderberg. 1979. Synchronization of *Plasmodium falciparum* erythrocytic stages in culture. *J. Protozool.* 26:418-420.

9. Pavol, G., R. J. M. Wilson, M. E. Smalley, and J. Brown. 1978. Separation of viable schizont-infected red blood cells of *Plasmodium falciparum* from human blood. *Ann. Trop. Med. Parasitol.* 72:87-88.

10. Steck, T., G. Fairbanks, and D. F. H. Wallach. 1971. Disposition of the major proteins in isolated erythrocyte membranes. *Biochemistry*. 10:2617-2627.

11. Furthmayr, H. 1978. Glycophorins A, B and C - a family of sialoglycoproteins. Isolation and preliminary characterization of trypsin-digested peptides. *J. Supramol. Struct.* 9:79-95.

12. Laemmli, U. K. 1970. Cleavage of structural genes during the assembly of the head of bacteriophage T4. *Nature* (Lond.). 227:680-685.

13. Hemaguchi, H., and H. Clev. 1972. Solubilization of human erythrocyte membrane glycoproteins and separation of the MN glycoprotein from a glycoprotein with I, S and A activity. *Biochem. Biophys. Acta* 278:271-280.

14. Brackenbury, R., J.-P. Thiery, U. Rathinshasil, and G. M. Edelman. 1977. Adhesion among neural cells of the chick embryo. *J. Biol. Chem.* 252:6833-6840.

15. Furthmayr, H. 1978. Structural comparison of glycoporphin A and immunological analysis of genetic variants. *Nature* (Lond.). 271:519-524.

16. Bach, T., J. E. Dass, and C. Howe. 1977. Virus-erythrocyte membrane interactions. In *Cell Surface Reviews: Infection and the Cell Surface*. G. Poste, and G. L. Nicholson, editors. North Holland, Amsterdam. 2:83-127.

17. Mackwell, M. A. K., and J. C. Paulson. 1980. Sendai virus utilizes specific sialyloligosaccharides as host cell receptor determinants. *Proc. Natl. Acad. Sci. U. S. A.* 77:5693-5697.