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### Research Article

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## Falciparum Malaria Parasites Invade Erythrocytes That Lack Glycophorin A and B (M<sup>k</sup>M<sup>k</sup>)

### Strain Differences Indicate Receptor Heterogeneity and Two Pathways for Invasion

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#### Abstract

To determine the ligands on erythrocytes for invasion by *Plasmodium falciparum*, we tested invasion into M<sup>k</sup>M<sup>k</sup> erythrocytes that lack glycophorins A and B and enzyme-treated erythrocytes by parasites that differ in their requirement for erythrocyte sialic acid. The 7G8 strain invaded M<sup>k</sup>M<sup>k</sup> erythrocytes and neuraminidase-treated normal erythrocytes with > 50% the efficiency of normal erythrocytes. In contrast, the Camp strain invaded M<sup>k</sup>M<sup>k</sup> erythrocytes at 20% of control and neuraminidase-treated normal erythrocytes at only 1.8% of control. Invasion of M<sup>k</sup>M<sup>k</sup> erythrocytes by 7G8 parasites was unaffected by treatment with neuraminidase but was markedly reduced by treatment with trypsin. In contrast, invasion of M<sup>k</sup>M<sup>k</sup> cells by Camp parasites was markedly reduced by neuraminidase but was unaffected by trypsin. We conclude that the 7G8 and Camp strains differ in ligand requirements for invasion and that 7G8 requires a trypsin sensitive ligand distinct from glycophorins A and B.

#### Introduction

Malaria parasites are obligate intracellular parasites and their ability to invade erythrocytes is crucial to their survival. Several lines of evidence indicate that malaria parasites invade erythrocytes by binding to specific ligands on the erythrocyte surface (1). Different species of *Plasmodium* use different ligands. Erythrocyte glycophorin A has been proposed as a ligand for invasion by *Plasmodium falciparum* (2-7). Evidence for this derives in part from the fact that En(a-) erythrocytes, which lack glycophorin A, are not invaded as well as normal erythrocytes (2, 7). The invasion that does occur with En(a-) erythrocytes may be due to the presence of glycophorin B because glycophorins A and B are homologous for the 28 N-terminal amino acids and both contain many O-linked oligosaccharides (8). Sialic acid on the erythrocyte membrane is required for optimal invasion by all strains reported to date (2,

9, 10). The importance of glycophorins A and B in invasion may be due to the fact that they contain sialic acid residues with which the parasites interact. Recently, Mitchell et al. observed that the degree of dependence on erythrocyte sialic acid for invasion varied between two strains of *P. falciparum* tested (11). One strain, the Thai-Tn, invaded neuraminidase-treated erythrocytes with 50% efficiency compared with untreated erythrocytes. The other strain, the Camp strain, invaded neuraminidase-treated erythrocytes with < 5% efficiency compared with untreated erythrocytes. By testing invasion by a sialic acid dependent parasite and a relatively sialic acid independent parasite into M<sup>k</sup>M<sup>k</sup> erythrocytes that lack glycophorin A and B and into enzyme-treated erythrocytes, we begin to discriminate among the relative importance of sialic acid, peptide backbone of glycophorin and other erythrocyte ligands for invasion by *P. falciparum*. The data suggest that the ligand on glycophorin is primarily the sialic acid and possibly other sugars and may not require the peptide backbone. Furthermore, the receptor heterogeneity includes differing requirements for sialic acid, as previously published (11), and the requirement for a trypsin-sensitive ligand distinct from glycophorins A and B.

#### Methods

**Cultures and parasites.** *P. falciparum* parasites were cultured in vitro as previously described (12, 13). Clones from three strains of *P. falciparum* were studied: the Malayan Camp strain, the 7G8 clone of the Brazilian (IMTM22) strain (14); and the T9/96 clone from Thailand (15). The Camp strain was taken from a patient, adapted to growth in *Aotus trivirgatus* and then adapted to in vitro culture in human erythrocytes. The other two strains were taken directly from patients and adapted to in vitro culture in human erythrocytes.

**Erythrocytes.** M<sup>k</sup>M<sup>k</sup> erythrocytes were obtained from a Japanese patient (K.M.) whose blood was referred to the Osaka Red Cross Blood Center, Osaka, Japan. Blood containing M<sup>k</sup>M<sup>k</sup> erythrocytes and erythrocytes from two controls were drawn at the same time into acid-citrate-dextrose and sent on wet ice to NIH, Bethesda, MD where they arrived still on ice 48 h later. Another donor (R.S.), the sister of K.M. who is also M<sup>k</sup>M<sup>k</sup>, was sent to D. Anstee and tested for invasion by *P. falciparum* clone T9/96 by G.P.

**Surface labeling.** M<sup>k</sup>M<sup>k</sup> erythrocytes and normal erythrocytes were surface-labeled using oxidation with periodate followed by reduction with tritiated borohydride (Amersham Corp., Arlington Heights, IL; 10 Ci/mmol) as described by Gahmberg and Andersson (16), with these modifications:  $1 \times 10^9$  cells in 1 ml of phosphate-buffered saline

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(PBS) containing 2 mM sodium periodate for 8 min at 0°C in the dark with occasional gentle mixing.

**Serologic testing.** Serologic testing was done by standard agglutination methods used by blood banks.

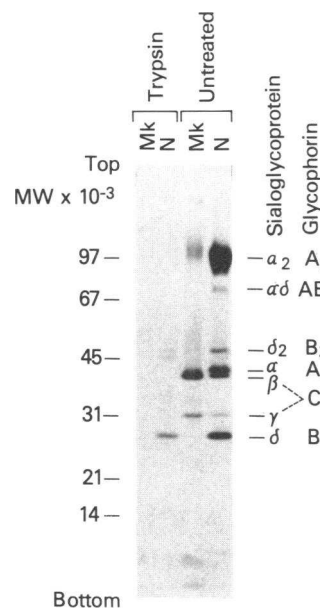
**Treatment of erythrocytes with enzymes.** Treatments of erythrocytes with trypsin (Sigma Chemical Co., St. Louis, MO; 1 mg/ml) and neuraminidase (Gibco Laboratories, Grand Island, NY; 50 U/ml) were described previously (11). Dose-response studies with neuraminidase indicated that the concentration of neuraminidase and treatment-conditions used in these experiments was more than sufficient to obtain the optimum reduction in invasion by the parasites tested (data not shown). The neuraminidase treatment by G.P. was with 0.01 U/ml (Behringwerke AG, Marburg, West Germany) which was of equal activity to the treatment with Gibco enzyme.

**Invasion assay.** Malaria parasites (merozoites) invade erythrocytes and develop sequentially into ring forms, trophozoites and schizonts. Each schizont produces ~ 10 to 20 new merozoites, which are released when infected erythrocytes rupture and invade other erythrocytes. Invasion assays were performed as described previously (11). Schizont-infected erythrocytes ( $1 \times 10^6$ ) were incubated in microtiter wells with  $1 \times 10^7$  uninfected erythrocytes and 200  $\mu$ l of medium. During overnight incubation merozoites were released from infected erythrocytes and invaded uninfected erythrocytes to form characteristic ring forms. At the end of the overnight incubation period (20–24 h), the percentage of erythrocytes infected with ring-forms was determined on Giemsa stained thin blood films. A minimum of 1,000 erythrocytes was counted. At low parasitemia, the number of ring-infected erythrocytes per 10,000 erythrocytes was determined. Rhesus or rabbit erythrocytes that are not invaded by *P. falciparum* were included in each experiment as a control for normal human erythrocytes introduced with the schizont-infected erythrocytes. Invasion of rhesus erythrocytes was always < 3% of control.

## Results and Discussion

The absence of glycoporphins A and B on the  $M^kM^k$  erythrocytes was confirmed by surface labeling using periodate oxidation followed by reduction with tritiated borohydride (Fig. 1) and by serological studies. Human anti-M, lectin anti-N (*Vicia graminea*), anti-S, -s, and -U, anti-Pr1 (Christensen), anti-EnaFS (monoclonal antibodies 453 [17] and 177.1.2), anti-Wra, anti-Wrb (monoclonal antibody 179.9.1 and serum from M. Fra) and a serum from G.W. containing a mixture of anti-Ena and anti-Wrb did not react with these erythrocytes when tested by agglutination. Anti-EnaFS and anti-Wrb (EnaFR) react with amino acids 40–60 and 60–70, respectively, of glycoporphin A (18) and anti-S and -s react with the peptide around amino acid 29 of glycoporphin B (8). Therefore the extracellular peptide backbone of glycoporphin A( $\alpha$ ) and at least a segment of B( $\delta$ ) are missing as previously described (19). Trypsin-treatment of normal erythrocytes cleaved glycopeptides from  $\alpha$ ,  $\beta$ , and  $\gamma$ , but left some of glycoporphin B( $\delta$ ) intact (Fig. 1). Trypsin treatment of  $M^kM^k$  cleaved glycopeptides from glycoporphin C ( $\beta$  and  $\gamma$ ), leaving the cells with no remaining intact glycoporphins.

Invasion studies were performed with cloned parasites from three strains. The Camp and 7G8 strains were tested simultaneously under identical conditions. Differences in invasion efficiencies obtained with each strain therefore relate to differences in their biological behavior rather than differences in erythrocytes or culture conditions. The T9/96 strain was tested separately in a different laboratory (G.P.). The results of these studies indicate that the 7G8 and T9/96 strains differ from the Camp strain in their requirements for erythrocyte



**Figure 1.** Fluorograph after SDS-polyacrylamide gel electrophoresis of  $M^kM^k$  cells ( $M^k$ ) and normal cells (N) that were surface-labeled by treatment with periodate followed by [ $^3$ H]borohydride under conditions that label glycoporphins A ( $\alpha$ ), B ( $\delta$ ), and C ( $\beta$  and  $\gamma$ ), glycoporphin A<sub>2</sub> dimer ( $\alpha_2$ ), glycoporphin B<sub>2</sub> dimer ( $\delta_2$ ), glycoporphin AB heterodimer ( $\alpha\delta$ ). Cells were left untreated or treated with trypsin after [ $^3$ H]borohydride label. Note the absence of  $\alpha_2$ ,  $\alpha$ , and  $\delta$  on untreated  $M^kM^k$  cells. The faint band above  $\alpha_2$  in untreated  $M^kM^k$  cells is superimposed on the Coomassie Blue-stained band 3.

sialic acid. Invasion rates of the Camp strain into neuraminidase-treated erythrocytes were always < 3% of invasion rates obtained with control untreated erythrocytes. This finding was similar to what has generally been reported by others using various strains cultured in vitro (4, 9, 10). In contrast, invasion rates of 7G8 and T9/96 parasites into neuraminidase-treated erythrocytes were at least 50% of invasion rates obtained with control untreated erythrocytes (Table I). Thus, our findings confirm and extend the recent observations of Mitchell et al. (11) that some *P. falciparum* parasites are able to invade by a pathway that is probably independent of sialic acid. The uncloned strain tested by Mitchell et al., the Thai-Tn strain, was continuously cultured in erythrocytes that were deficient in sialic acid (11). It was therefore possible that the unusual culture conditions induced the capability of invading sialic acid-deficient erythrocytes in these parasites. The finding in the present study that cloned parasites (7G8 and T9/96) continuously cultured in normal erythrocytes have the same receptor phenotype as Thai-Tn parasites (that is, the ability to invade neuraminidase-treated erythrocytes with 50% efficiency) indicates that this phenotype exists in unselected parasite populations. It is, therefore, clear from the results reported here and from the results reported previously by Mitchell et al. (11) that different strains of *P. falciparum* differ in terms of their requirements for sialic acid for erythrocyte invasion.

A major question we sought to address in the present study was whether glycoporphin A and/or B are required for invasion. We also attempted to further define the receptor requirements of different strains. As can be seen in Table I, all three strains tested, Camp, 7G8, and T9/96, invade  $M^kM^k$  erythrocytes, albeit at reduced levels. Thus, *P. falciparum* parasites can invade erythrocytes by a pathway that is independent of glycoporphins A and B. The efficiency of invasion into  $M^kM^k$  erythrocytes was higher for the strain (7G8) that was less affected by neuraminidase-treatment of normal erythrocytes than it was for the strain (Camp) that was greatly affected by neuraminidase. This finding suggests that the important ligand on glycoporphins A and B is sialic acid. Glycoporphins A and B contain 70–80% of the erythrocyte membrane sialic acid and thus it is

Table I. Invasion of Untreated and Enzyme-treated M<sup>k</sup>M<sup>k</sup> Erythrocytes by Three Clones of *Plasmodium falciparum* (Camp, 7G8, and T9/96)\*

	Treatment	June 10, 1986		June 18, 1986		June 25, 1986		T9/96 <sup>‡</sup>
		Camp	7G8	Camp	7G8	Camp	7G8	
M <sup>k</sup> M <sup>k</sup>	None	20	86	14	51	18	61	77.6 ± 7.1 n = 5
	NANase	0.8	55	2.1	40	1.8	65	63.5 n = 2
	STI	27	75			16	70	
	Trypsin	27	14			19	9.5	
Control 1	None	93	81	100 (11.7)	100 (35.3)	97	98	100 (3.1 to 7.5)
	NANase	1.5	56	3.0	68	1.5	40	54.5 n = 2
Control 2	STI	100 (6.0)	83			65	89	
	Trypsin	9.3	1.5			9.7	2.2	
	None	93	100 (17.0)			50	100 (5.8)	
	NANase	1.6	70			1.8	40	
	STI	100	99			65	89	
FWK	Trypsin	8.5	0.6			5.9	1.5	
	None	87	80	89	97	74	75	102.2 ± 18.8 n = 5
	NANase	2.2		1.2	56	1.8	86	
	STI	100				100 (3.4)	99	
	Trypsin	6.7				4.7	0.3	

Abbreviations used in this table: NANase, neuraminidase; STI, soybean trypsin inhibitor; FWK, fresh control cells obtained in Bethesda.

\* The data are expressed as the percentage of the maximum invasion for each study. The maximum invasion is in parentheses and is expressed as the percentage of invaded erythrocytes. <sup>‡</sup> The invasion by clone T9/96 was performed by G.P. at Oxford, England, on different Japanese control cells and fresh English control cells. The data are given as mean ± SD.

not surprising that parasites that are heavily dependent on sialic acid for invasion show a more marked reduction of invasion into M<sup>k</sup>M<sup>k</sup> erythrocytes. Whether or not *P. falciparum* parasites recognize a specific linkage of sialic acid remains to be determined. At present there is no convincing evidence that *P. falciparum* interacts with peptide domains of glycoporphins A and B during invasion and the present study clearly indicates that extracellular peptide domains of glycoporphins A and B are not required for invasion.

Ligand requirements for Camp and 7G8 parasites were further characterized by studying invasion of neuraminidase- and trypsin-treated M<sup>k</sup>M<sup>k</sup> erythrocytes. Invasion of M<sup>k</sup>M<sup>k</sup> erythrocytes by Camp parasites was markedly reduced by neuraminidase but unaffected by trypsin. Camp parasites therefore utilize sialic acid for invasion of M<sup>k</sup>M<sup>k</sup> erythrocytes. Sialic acid used by Camp parasites on M<sup>k</sup>M<sup>k</sup> erythrocytes is located on components other than glycoporphin C, since trypsin-treatment of M<sup>k</sup>M<sup>k</sup> erythrocytes (Fig. 1) removed the sialoglycopeptides of glycoporphin C ( $\beta$  and  $\gamma$ ) but had no additional effect on invasion. The finding that Camp parasites can utilize sialic acid on M<sup>k</sup>M<sup>k</sup> erythrocytes is further evidence that the interaction between *P. falciparum* parasites and sialic acid on glycoporphins A and B on normal erythrocytes does not require a specific peptide sequence. The location of the sialic acid used by Camp parasites on M<sup>k</sup>M<sup>k</sup> erythrocytes is not known but, as it is not affected by trypsin, it is linked to a glycolipid or a trypsin-insensitive glycoprotein.

In contrast to its lack of effect on the invasion of Camp parasites into M<sup>k</sup>M<sup>k</sup> erythrocytes, trypsin inhibited invasion of Camp parasites into normal erythrocytes. This effect can be attributed to the loss of sialic acid from these erythrocytes, as trypsin cleaves sialoglycopeptides from all of glycoporphin A and some of glycoporphin B (Fig. 1). Glycoporphin B has generally been considered to be resistant to trypsin when intact erythrocytes are treated but under the conditions used in these experiments [1 mg/ml trypsin-tosylphenylchloromethyl ketone (TPCK) for 1 h at 37°C with an erythrocyte concentration of  $2.5 \times 10^8$  per ml] some cleavage of glycoporphin B was obtained. Similar results have been reported previously (20). The requirement of Camp parasites for erythrocyte sialic acid does not entirely explain the specificity of invasion. For example, *P. falciparum* parasites (including the Camp strain) cannot invade erythrocytes from old world monkeys or from most subprimates despite the presence of sialic acid on their erythrocytes. The specificity may be determined by the type of sialic acid, the linkage of sialic acid to other sugars, the nature of the neighboring sugars, or the requirement for a second as yet uncharacterized ligand.

Another conclusion, based on the biological data reported here, is that 7G8 parasites interact with at least one ligand-site that is different from that used by Camp parasites. In contrast to the findings obtained with Camp parasites, invasion of M<sup>k</sup>M<sup>k</sup> erythrocytes by 7G8 parasites was unaffected by neuraminidase-treatment of M<sup>k</sup>M<sup>k</sup> erythrocytes but was markedly

reduced by trypsin-treatment of M<sup>k</sup>M<sup>k</sup> erythrocytes (Table I). The effect of trypsin was specific, since trypsin treatment of M<sup>k</sup>M<sup>k</sup> erythrocytes had no effect on invasion by Camp parasites. Thus trypsin removes a ligand on M<sup>k</sup>M<sup>k</sup> erythrocytes, and probably on normal erythrocytes, which contains a neuraminidase-insensitive binding site that is utilized by 7G8 parasites but not by Camp parasites. 7G8 parasites, like Thai-Tn parasites previously described, appear to have at least two different receptors for two different ligands on the erythrocyte membrane. One receptor interacts with sialic acid on the erythrocyte membrane and may be the same or similar to the receptor for sialic acid on Camp parasites (21). The other receptor interacts with a neuraminidase-insensitive, trypsin-sensitive ligand that is located on a molecule other than glycoporphins A and B. 7G8 parasites are therefore able to invade erythrocytes that are deficient in sialic acid (e.g., neuraminidase-treated erythrocytes and M<sup>k</sup>M<sup>k</sup> erythrocytes) with roughly 50% efficiency by binding to the neuraminidase-insensitive ligand. This ligand has not yet been identified.

The finding that *P. falciparum* malaria parasites can invade erythrocytes by pathways which are independent of glycoporphins A and B has potential importance for vaccine development. One approach to vaccine development has been to identify parasite receptors and to use them as immunogens to induce antibodies that block invasion. Glycophorin A has been used to affinity-purify parasite molecules that are considered possible receptors (22, 23). One or more of these molecules may be the sialic acid-dependent receptor. The evidence presented here indicates, however, that some *P. falciparum* parasites also have receptors that bind to erythrocyte ligands other than sialic acid and other than glycoporphins A and B. These receptors need to be identified if receptors are to be used effectively as immunogens.

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