CONCISE REPORT

# Invasion of Erythrocytes by *Plasmodium falciparum* Malaria Parasites: Evidence for Receptor Heterogeneity and Two Receptors

By Graham H. Mitchell, Terence J. Hadley, Mary H. McGinniss, Francis W. Klotz, and Louis H. Miller

***Plasmodium falciparum*** **malaria parasites with different capabilities of invading sialic acid-deficient erythrocytes were identified. Thai-2 parasites cultured in Tn erythrocytes invaded neuraminidase-treated and Tn erythrocytes twice as efficiently as Thai-2 parasites cultured in normal erythrocytes and seven to ten times more efficiently than a cloned line of Camp parasites cultured in normal erythrocytes. All three parasite lines required sialic acid for optimal invasion, but Thai-2 parasites cultured in Tn erythrocytes invaded neuraminidase-treated erythrocytes with 45% efficiency whereas Camp parasites invaded neuraminidase-treated erythrocytes with less than 10% efficiency. *P falciparum* malaria parasites probably possess two receptors: one that binds to a sialic acid-dependent ligand and another that binds to a sialic acid-independent ligand. Parasites may differ in the quantity or affinity of their receptors for the sialic acid-independent ligand.**

MALARIA is caused by protozoa (*Plasmodium*) that parasitize erythrocytes. *Plasmodium falciparum* is especially important as a cause of morbidity and mortality in tropical areas of the world. Invasion of erythrocytes by malaria parasites involves specific interactions between parasite receptors and erythrocyte ligands.[1] Sialic acid on the erythrocyte membrane is important for optimal invasion by *P falciparum* parasites. Neuraminidase-treated erythrocytes and Tn erythrocytes, which lack sialic acid and galactose on the O-linked tetrasaccharides of the glycophorins,[2-4] are less susceptible to invasion than normal erythrocytes.[5-9] The present study was done to determine whether the requirement for erythrocyte sialic acid varies among strains of *P falciparum* and whether erythrocyte ligands other than sialic acid are important for invasion.

*P falciparum* parasites from an uncloned isolate were cultured in Tn erythrocytes and examined for their ability to invade neuraminidase-treated erythrocytes, Tn erythrocytes, and trypsin-treated erythrocytes. Comparisons were made with the parent line cultured in normal erythrocytes and with a cloned line from another strain. The observations reported here indicate that *P falciparum* parasites vary in their abilities to invade sialic acid-deficient erythrocytes and that in addition to sialic acid, another erythrocyte ligand is required for invasion.

## MATERIALS AND METHODS

*Cultures and parasites.* Tn erythrocytes from donor C.L. were provided by Mary L. Thomas, United Blood Services, Billings, Mont. *P falciparum* parasites were cultured in vitro as previously described.[10,11] Human serum used in the culture medium was preabsorbed overnight at 4 °C with 4% vol/vol C.L. erythrocytes in order to remove naturally occurring anti-Tn antibodies. An uncloned isolate (Thai-2) of *P falciparum* from Thailand was cultured in C.L. erythrocytes and designated Thai-Tn. Separate cultures of the Thai-2 line were maintained in normal erythrocytes. Cultures of the Malayan-Camp strain were also maintained in normal erythrocytes. This line was derived from a cloned parasite (J.D. Haynes, MD, unpublished data, April, 1982) and did not survive when cultured in Tn erythrocytes.

*Quantitation and purification of Tn erythrocytes.* Erythrocytes from donor C.L. were agglutinated by *Salvia sclarea* lectin, which binds to N-acetylgalactosamine residues exposed on Tn erythrocytes.[12] About 10% of C.L. erythrocytes contained the M blood group antigen as determined by immunofluorescence with an anti-M monoclonal antibody, indicating that approximately 90% of C.L. erythrocytes were of the Tn phenotype.[4] To remove normal erythrocytes, C.L. blood was incubated with the anti-M monoclonal antibody (a gift of J.J. Moulds, Gamma Biologicals, Houston, Tex) and passed over an immunoaffinity column containing goat antimouse immunoglobulin covalently coupled to Sepharose 4B (Cappel, Cochranville, Penn). Fewer than 1% of the effluent cells possessed M antigens as determined by immunofluorescence.

*Treatment of erythrocytes with enzymes.* Erythrocytes ($1 \times 10^8$/mL) were treated with various concentrations of neuraminidase (*Vibrio cholerae*,, GIBCO, Grand Island, NY) in RPMI 1640, 23 mmol/L Hepes, pH 7.2, for one hour at 37 °C. Concentrations from 5 to 100 U/mL were equally effective at reducing invasion of erythrocytes by the malarial parasites tested. Results of the first experiment are expressed as averages of the results obtained at each of the concentrations of neuraminidase (5, 10, 25, 75, and 100 U/mL). In all subsequent experiments, neuraminidase was used at a concentration of 50 U/mL. Erythrocytes ($1 \times 10^8$/mL) were treated with trypsin-TPCK (L-1-tosylamide-2-phenylethyl-chloromethyl ketone; type XIII, Sigma; 1 mg/mL) at 37 °C for one hour; trypsin-treated erythrocytes were washed in RPMI 1640, 23 mmol/L Hepes, and the remaining trypsin was inhibited with soybean trypsin inhibitor (Sigma; 1 mg/mL) prior to final washes.

*Invasion Assay.* Malaria parasites (merozoites) invade erythrocytes and develop sequentially into ring-forms, trophozoites, and schizonts. Each schizont in culture produces approximately ten to 20 new merozoites, which are released when infected erythrocytes rupture and invade other erythrocytes. Schizont-infected erythrocytes were obtained[13] and purified (99% purity) on Percoll-sorbitol

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gradients according to the method of Aley.[14] Invasion assays were done in flat-bottom microtiter wells containing 175 μL culture medium, $1 \times 10^6$ schizont-infected erythrocytes, and $1 \times 10^7$ uninfected erythrocytes. Sera used in the culture media were absorbed with Tn and neuraminidase-treated erythrocytes to remove anti-Tn and anti-T antibodies, respectively. Cultures were harvested after 18 hours for the preparation of blood films, which were fixed with methanol, stained with Giemsa, and examined by light microscopy. For each culture, the percent of erythrocytes infected with ring-forms (invasion rate) was determined by examining 1,000 erythrocytes. Efficiency of invasion was defined as the invasion rate obtained with enzyme-treated or variant erythrocytes expressed as a percentage of the invasion rate obtained with normal erythrocytes using the same line of parasite.

## RESULTS

In preliminary experiments four different lines of *P. falciparum*, including the cloned Camp line, were cultured in erythrocytes from donor C.L. (90% Tn). Only the Thai-2 line survived and multiplied. Thai-2 parasites maintained in culture with Tn erythrocytes (Thai-Tn) were compared with Thai-2 parasites maintained in normal erythrocytes and with Camp parasites maintained in normal erythrocytes. Thai-Tn parasites invaded neuraminidase-treated erythrocytes with an average efficiency of 45%, in contrast to Camp parasites, which invaded neuraminidase-treated erythrocytes with an average efficiency of about 5% (Table 1). Thai-2 parasites invaded neuraminidase-treated erythrocytes with an average efficiency of 28% (Table 1). All three lines tested required sialic acid for optimal invasion. Similar differences were obtained with Tn erythrocytes (Table 2). Thai-Tn parasites invaded Tn erythrocytes seven to eight times more efficiently than did Camp parasites and two to three times more efficiently than did Thai-2 parasites. Furthermore, treatment of Tn erythrocytes with neuraminidase increased their susceptibility to invasion by Thai-Tn parasites by about 40% but had no effect on the susceptibility of these erythrocytes to invasion by Camp parasites. Treatment of normal erythrocytes with trypsin decreased susceptibility to invasion by all three lines tested to about 10% of normal (Table 1).

**Table 1. Invasion of Enzyme-Treated Normal Erythrocytes by Various Lines of *Plasmodium falciparum***

| Treatment | Experiment No. | Invasion Expressed* as % of Control | | | Ratio† of Thai-Tn:Camp |
|---|---|---|---|---|---|
| | | Camp | Thai-2 | Thai-Tn | |
| Neuraminidase | 1 | 1 | | 25 | 25 |
| | 2 | | 13 | 47 | |
| | 3 | 7 | 33 | 54 | 7.7 |
| | 4 | 6 | 28 | 53 | 8.8 |
| Trypsin | 1 | 12 | | 10 | 0.8 |
| | 2 | | 15 | 4 | |

*Invasion rates obtained with test RBCs are expressed as a percentage of invasion rates obtained with normal, untreated RBCs and are calculated as follows:

$$\frac{\text{\% of test RBCs infected}}{\text{\% of normal RBCs infected}} \times 100$$

Invasion rates obtained with normal RBCs were greater than 10% in most experiments. The data are presented for three lines of parasites: Camp, Thai-2, and Thai-Tn (Thai-2 parasites cultured in Tn RBCs).

†The ratios of the invasion rates of Thai-Tn parasites to Camp parasites in the same population of RBCs.

**Table 2. Invasion of Purified Tn Erythrocytes and Purified Tn Erythrocytes Treated with Neuraminidase by Various Lines of *Plasmodium falciparum***

| Treatment | Experiment No. | Invasion Expressed* as % of Control | | | Ratio† of Thai-Tn:Camp |
|---|---|---|---|---|---|
| | | Camp | Thai-2 | Thai-Tn | |
| None | 3 | 2 | 5 | 15 | 7.5 |
| | 4 | 2 | 8 | 19 | 9.5 |
| | 5 | 3 | | 20 | 6.7 |
| Neuraminidase | 4 | 2 | 15 | 31 | 15.5 |
| | 5 | 1 | | 29 | 29 |

*†Same as Table 1.

## DISCUSSION

Thai-Tn parasites invaded neuraminidase-treated erythrocytes with 45% efficiency and Thai-2 parasites invaded neuraminidase-treated erythrocytes with 28% efficiency Although both parasites required sialic acid on the erythrocyte membrane for optimal invasion, their ability to invade neuraminidase-treated erythrocytes suggest that they can also utilize an erythrocyte ligand which is independent of sialic acid. The low efficiency of invasion of neuraminidase-treated erythrocytes obtained with Camp parasites may be due to residual sialic acid on the erythrocyte membrane after neuraminidase treatment, or to a low affinity interaction with another ligand. Treatment of Tn erythrocytes with neuraminidase enhanced their susceptibility to invasion by Thai-Tn parasites but not by Camp parasites, further indicating that Thai-Tn and Camp parasites have different receptors and that Thai-Tn parasites can utilize a sialic acid-independent ligand. The increased invasion of Tn erythrocytes by Thai-Tn parasites following neuraminidase treatment may be due to the decrease in negatively charged residues (sialic acid), making a second erythrocyte ligand more accessible.

The data presented here is the first reported indication that there is receptor heterogeneity among *P falciparum* parasites. Previous reports suggested that two receptors may be involved in invasion.[7,15,16] Friedman et al reported that the effect of neuraminidase on invasion could be partially reversed by incubating neuraminidase-treated erythrocytes with $\alpha_1$ acid glycoprotein.[7] This effect, which required sialic acid on $\alpha_1$ acid glycoprotein, did not occur when $\alpha_1$ acid glycoprotein was incubated with trypsin-treated erythrocytes. Other investigators suggested that, in addition to sialic acid, the hydrophobic $T_6$ peptide of glycophorin A may be a ligand necessary for invasion because the $T_6$ peptide inhibits invasion in vitro.[15,16] However, the specificity of inhibition by the hydrophobic $T_6$ peptide has been questioned by Breuer et al, who found that hydrophobic haptens coupled to bovine serum albumin also inhibit invasion.[17] The $T_6$ peptide of glycophorin A remains associated with the membrane after trypsin treatment of intact erythrocytes and therefore is probably not the trypsin-sensitive ligand utilized by Thai-Tn, Thai 2, and possibly Camp parasites.

The data presented here suggest that *P falciparum* ma-

laria parasites express at least two different receptors for two different ligands on the erythrocyte membrane. One receptor binds to a ligand that is dependent on sialic acid, and a second receptor binds to a ligand that is independent of sialic acid and is sensitive to cleavage by trypsin. Our observations suggest that Thai-Tn parasites have either a greater number of receptors for the sialic acid-independent ligand than Camp parasites or receptors of higher affinity. The location of the sialic acid-independent ligand is unknown. The biologic evidence for two receptors and for receptor heterogeneity may be important to future biochemical characterization of receptors and to vaccine development if receptors are to be used as immunogens.

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