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Binding of *Plasmodium falciparum* 175-kilodalton erythrocyte binding antigen and invasion of murine erythrocytes requires *N*-acetylneuraminic acid but not its *O*-acetylated form

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Sialic acid on human erythrocytes is involved in invasion by the human malaria parasite, *Plasmodium falciparum*. Mouse erythrocytes were used as a reagent to explore the question of whether erythrocyte sialic acid functions as a nonspecific negative charge or whether the sialic acid is a necessary structural part of the receptor for merozoites. Human erythrocytes contain *N*-acetylneuraminic acid (Neu5Ac), whereas mouse erythrocytes, which are also invaded by *P. falciparum* merozoites, contain 9-*O*-acetyl-*N*-acetylneuraminic acid (Neu5,9Ac$_2$) and *N*-glycoloylneuraminic acid (Neu5Gc), in addition to Neu5Ac. We compared the effects of sialidase and influenza C virus esterase treatments of mouse erythrocytes on invasion and the binding of a 175-kDa *P. falciparum* protein (EBA-175), a sialic acid-dependent malaria ligand implicated in the invasion process. Sialidase-treated mouse erythrocytes were refractory to invasion by *P.falciparum* merozoites and failed to bind EBA-175. Influenza C virus esterase, which converts Neu5,9Ac$_2$ to Neu5Ac, increased both invasion efficiency and EBA-175 binding to mouse erythrocytes. Thus, the parasite and EBA-175 discriminate between Neu5Ac and Neu5,9Ac$_2$, that is, the C-9 acetyl group interferes with EBA-175 binding and invasion by *P.falciparum* merozoites. This indicates that sialic acid is part of a receptor for invasion.

Key words: *Plasmodium falciparum*; Red cell; Merozoite; Receptor; Sialic acid

Introduction

Sialic acid on human erythrocytes is involved in the invasion by the human malaria *Plasmodium falciparum* [1–4] based on the following observations: (a) sialidase-treated human erythrocytes are invaded less efficiently than untreated cells, and (b) human

erythrocytes deficient in the major erythrocyte sialic acid-containing glycoproteins, glycophorin A (Ena−), or glycophorins A and B (M^kM^k), are less efficiently invaded than normal erythrocytes. Furthermore, a putative *P. falciparum* sialic acid-binding ligand, a 175-kDa erythrocyte binding antigen (EBA-175), exhibits similar sialic acid-dependent binding properties to erythrocytes [5]; that is, EBA-175 fails to bind sialidase-treated erythrocytes or glycophorin A- or glycophorin A/B-deficient erythrocytes [5].

Friedman et al. [6] found that α_1-acid glycoprotein, a highly negatively charged plasma protein, binds to sialidase-treated

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human erythrocytes and restores their susceptibility to invasion by *P. falciparum* merozoites. They argued from these indirect data that sialic acid on glycoproteins are involved in a relatively nonspecific interaction. In the present study, we use mouse erythrocytes as a reagent to demonstrate that sialic acid is, indeed, part of the structure of a receptor for invasion, and not just a contributor to nonspecific charge interaction. Mouse erythrocytes are susceptible to invasion by *P.falciparum* [7] and contain 3 forms of sialic acid, all with the same negative charge: *N*-acetylneuraminic acid (Neu5Ac), the only form of sialic acid found on human erythrocytes; 9-*O*-acetyl-*N*-acetylneuraminic acid (Neu5,9Ac$_2$) [8]; and *N*-glycoloylneuraminic acid (Neu5Gc). Invasion and EBA-175 binding are enhanced when mouse erythrocytes are pretreated with influenza C virus containing an esterase which specifically removes the C-9 acetyl ester from sialic acid, converting Neu5,9Ac$_2$ to Neu5Ac [9]. From these data, it is concluded that Neu5Ac is part of a receptor for *P. falciparum* merozoites.

Materials and Methods

Parasites and culture.   Clones of the Malayan Camp and the Brazilian 7G8 isolates of *P. falciparum* were used in this study [10, 11]. In vitro culture and purification of schizont-infected erythrocytes were previously described [12].

Erythrocyte invasion assay.   Mature schizont-infected erythrocytes and uninfected erythrocytes were mixed at a ratio of 1:10 schizonts to targets. Erythrocytes (5×10^6) were cultured in 100 μl of media containing heat-inactivated A + human serum preadsorbed against the panel of target erythrocytes.Merozoites released from schizont-infected erythrocytes invaded target cells during a 24 h incubation. The proportion of newly invaded erythrocytes was determined on giemsa-stained thin blood films [7]. If the parasitemia was above 5%, 1000 erythrocytes were counted from each culture. If the parasitemia was less than 5%, 2000 to 10 000 erythrocytes were counted.

Mice.   Erythrocytes were obtained from female C57Bl/6 and DBA/2 mice. Mice were obtained from the Jackson Labs, Bar Harbor, ME.Cells were processed for the erythrocyte invasion as previously described [7].

Erythrocyte binding antigen assay.   The assay has minor modifications from that described previously by Haynes et al. [13].Purified schizont-infected erythrocytes were metabolically labeled with 50 μCi ml^{-1} [^{3}H]isoleucine. Schizont-infected erythrocytes were cultured 12–16 h to permit schizont rupture/merozoite release without target erythrocytes to invade.Supernatants containing radiolabeled EBA-175 were clarified by centrifugation and adsorbed to target erythrocytes (100 μl supernatant per 100 μl packed cells). After centrifuging once through silicone fluid SF50 (General Electric), bound antigens were eluted by high salt (20 μl of 1 M NaCl per 100 μl packed erythrocytes) and fractionated by SDS-PAGE (7.5% acrylamide). Radiolabeled polypeptides were detected by fluorography and quantitated by laser densitometry.

Cell treatments.   Erythrocytes (5% packed cell volume) were treated with *Arthrobacter ureafaciens* sialidase as previously described [14,15] or with purified influenza C/JHG/66 virus [16] (2 μg ml^{-1}) in RPMI-1640, pH 7.2. Erythrocytes were incubated at 37°C for 3 h on a rocking platform. The endpoint for deacetylation was cessation of agglutination [9]. Mock-treated cells were included as controls. 1 μg of influenza C virus corresponds to approximately 2.5×10^6 plaque-forming units (pfu).

Sialic acid analysis.   Erythrocytes from DBA/2 and C57BL/6 mice (after influenza C virus treatment and untreated controls) were washed in PBS and incubated in 1.0 mM diisopropyl fluorophosphate for 10 min at room temperature. The erythrocytes were washed twice in PBS. Packed erythrocytes were lyophilized,

followed by acid hydrolysis in formic acid for 1 h at 70°C, pH 2, dialysis, and a second hydrolysis of the non-dialysable material in HCl for 1 h at 80°C, pH 1, with subsequent dialysis. The diffusates were kept separately and passed over cation- and anion-exchange resin; adsorbed sialic acids were eluted with 1 M formic acid [17]. After lyophilization, the sialic acids were dissolved in a small volume of water and analyzed by a fluorometric HPLC assay [18] using a RP-18 cartridge (25 × 0.4 cm; Merck, Darmstadt, F.R.G.), water/methanol/acetonitrile (84/7/9, by vol), as mobile phase at a flow rate of 1 ml/min and fluorometric detection (Applied Biosystems 980 fluorescence detector; Weiterstadt, F.R.G.) at an excitation wavelength of 343 nm and a cutoff emission filter of 389 nm.

Although this hydrolytic procedure is known to destroy up to 50% of sialic acid O-acetyl groups [8], it is the only method to release all sialic acids from their glycosidic linkages.

Results

P. falciparum malaria merozoites invaded mouse erythrocytes in vitro [7] (Table I). The soluble *P.falciparum* protein 175 kDa (EBA-175) which binds to human erythrocytes [5] also bound to untreated mouse erythrocytes (Fig. 1). To test whether EBA-175 binding and invasion were sialic acid-dependent, C57BL/6 and DBA/2 mouse erythrocytes were treated with *Arthrobacter ureafaciens* sialidase to remove sialic acids from the erythrocyte surface. Human erythrocytes which contain only Neu5Ac were used as a control. After treatment of human erythrocytes, the Camp strain invaded at a low rate, and 7G8 invaded at approximately 50% efficiency. These parasite strain-dependent differences in invasion of human erythrocytes were similar to previous studies with Camp and 7G8 parasites [4]. In contrast, invasion of sialidase-treated mouse erythrocytes was markedly reduced for both Camp and 7G8 parasites (Table I), suggesting that the alternate pathway of invasion that 7G8 can use in human erythrocytes (Hadley et al., 1987) is absent in mouse erythrocytes. Neither mouse nor human erythrocytes treated with sialidase bound EBA-175 (Fig. 1), indicating the requirement of sialic acid for binding of EBA-175.

To determine whether the form of sialic acid

TABLE I

Effects of influenza C virus esterase and *A. ureafaciens* sialidase on erythrocyte invasion by *P. falciparum*

Cell type	Treatment	% Infected RBCs			
		Expt. 1		Expt. 2	
		Camp	7G8	Camp	7G8
Human	Untreated	16.6	11.5	16.8	5.4
	Influenza C	16.9 (+2)[a]	11.3 (−2)	16.6 (−2)	5.6 (+4)
	Sialidase	1.7	7.4	0.3	2.9
DBA/2	Untreated	5.5	4.1	7.9	2.6
	Influenza C	7.8 (+42)	5.6 (+37)	9.8 (+19)	3.9 (+50)
	Sialidase	0.1	0.2	0.2	0.1
C57BL/6	Untreated	4.8	2.7	6.1	1.9
	Influenza C	9.5 (+77)	5.2 (+93)	9.3 (+52)	3.2 (+68)
	Sialidase	0.1	0.1	0.1	0.1

Each target cell type was tested in triplicate and the data presented are the arithmetic means from triplicate samples. Mice were 56 and 63 weeks of age for Expts. 1 and 2, respectively.

[a]The numbers in parentheses are the percentage change in invasion after treatment of erythrocytes with influenza C relative to the untreated cells. The difference between the untreated and influenza C-treated cells was compared by a paired t-test. Influenza C had no effect on invasion of human erythrocytes and significantly increased invasion of DBA/2 ($P < 0.005$) and of C57BL/6 ($P < 0.05$).
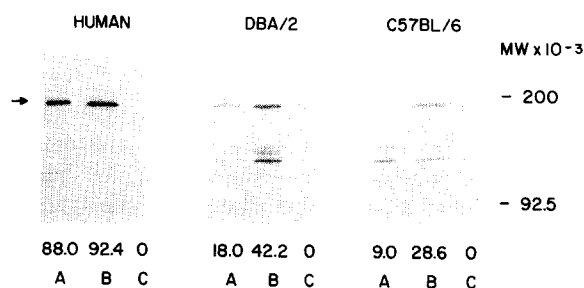
Fig. 1. Binding and elution of *P. falciparum* metabolically-labeled proteins from human erythrocytes and DBA/2 and C57BL/6 mouse erythrocytes. Lane A, untreated cells; lane B, influenza C virus-treated cells; and lane C, *Arthrobacter ureafaciens* sialidase-treated cells. EBA-175 is marked with an arrow.Densitometric measurements for EBA-175 are listed below each lane.Molecular weight markers are indicated on the right.

per se influenced invasion of mouse erythrocytes and binding of EBA-175, we treated the erythrocytes with influenza C virus, which has been shown to have a sialate 9-O-acetyl esterase [9]. Since human erythrocytes do not contain Neu5,9Ac$_2$ residues, treatment of these

TABLE II

The effect of influenza C virus treatment on sialic acid residues of DBA/2 and C57BL/6 mouse erythrocytes

	Neu5Ac	Neu5,9Ac$_2$	Neu5Gc
DBA/2 − virus	86%	7%	7%
DBA/2 + virus	94%	0%	6%
C57BL/6 − virus	73%	14%	13%
C57BL6 + virus	86%	2%	12%

The data are presented for each sialic acid residue as the percentage of total sialic acid in the sample.

TABLE III

Increase in binding of EBA-175 after treatment of mouse erythrocytes with influenza C virus

Expt.	Ratio of + virus / − virus[a]	
	DBA/2	C57BL/6
1	2.3	3.1
2	not done	2.6
3	1.9	3.3
4	1.5	4.6

[a]The densitometric measurements of EBA-175 after treatment of erythrocytes with influenza C (+ virus) were divided by the densitometric measurements of the control, untreated erythrocytes (− virus). Expt. 1 is derived from data in Fig. 1. The erythrocytes in Expt. 4 were used for analysis of sialic acids (Table II).

erythrocytes with the virus had no effect on invasion (Table I) or EBA-175 binding (Fig. 1). Mouse erythrocytes, however, do contain Neu5,9Ac$_2$, as well as Neu5Ac and Neu5Gc [8,19] (Table II).Treatment of mouse erythrocytes with influenza C virus decreased the percentage of Neu5,9Ac$_2$ with a corresponding increase in the percentage of Neu5Ac (Table II). This treatment of mouse erythrocytes also increased invasion (Table I) and binding of EBA-175 (Fig. 1; Table III). Thus, we find that the removal of O-acetyl groups from the C-9 position of Neu5,9Ac$_2$, without changing the charge density, converts a relatively refractory mouse erythrocyte into one that is more efficiently invaded and binds more of the sialic acid-binding ligand, EBA-175. It has to be stated that the original amount of Neu5,9Ac$_2$ on mouse erythrocytes is higher due to the known loss of sialic acid O-acetyl groups by acid hydrolysis [8].

In addition to EBA-175, two, and sometimes 3, other *P.falciparum* proteins are seen at around 120 to 145 kDa (Fig. 1 and unpublished data). The upper band was not seen to bind to human erythrocytes but, in some experiments, the lower 2 bands bound also to human erythrocytes (data not shown). The binding was reduced after sialidase treatment of mouse erythrocytes, but the binding after treatment with influenza C was unchanged in some experiments and increased in others (Fig. 1 and data not shown). These proteins around 135 to 145 kDa may be the same as those described by Sam-Yellowe and Perkins [20]. As the binding of these proteins to human erythrocytes is variable (refs. 5 and 21, and the present study), their role in invasion is unknown.

Discussion

We have shown that mouse erythrocytes treated with influenza C esterase, which removes the 9-O-acetyl group from Neu5,9Ac$_2$ and converts it to Neu5Ac, are invaded more efficiently by *P. falciparum* than untreated erythrocytes.From this, it is evident

that the parasite discriminates between Neu5Ac and Neu5,9Ac$_2$. This indicates that sialic acid is a receptor and not just involved in charge-dependent, nonspecific interactions, as suggested previously [6]. The only other data implicating sialic acid as a receptor came from studies on Cad human erythrocytes, which have an additional neutral sugar (N-acetylgalactosamine) linked to the galactose on the O-linked oligosaccharides that is penultimate to the terminal sialic acid.Studies using these cells demonstrated decreased invasion [22]. The additional sugar does not change the charge of the erythrocyte, but probably affects accessibility of adjacent sialic acids, which is similar to ganglioside GM2 sialic acid that is released by many sialidases only at a very slow rate [23]. Although these studies with Cad erythrocytes were consistent with a possible role of sialic acid as a receptor, other abnormalities of these cells could have affected invasion. In the present study, removal of the C-9 acetate by the influenza C virus esterase increased invasion efficiency of mouse erythrocytes that have the 9-O-acetyl group, and had no effect on human erythrocytes that lack this group.

In light of the present study, the data by Friedman et al. [6] may be reinterpreted as follows. They found that α$_1$-acid glycoprotein can replace the requirement for sialic acid on sialidase-treated erythrocytes. The terminal sugar on α$_1$ acid glycoprotein is sialic acid linked α2,3 or α2,6 to the penultimate galactose [24]. If the structure of the sugar is the critical factor, then the parasite would specifically recognize the structure of the sialic acid on α$_1$-acid glycoprotein.

Camus and Hadley [5] demonstrated that a 175-kDa protein (EBA-175) bound to untreated human erythrocytes, but not to sialidase-treated erythrocytes. Again, the question was unresolved whether the binding was charge dependent or receptor specific. We found that EBA-175 discriminates between 2 forms of sialic acid differing solely in a substitution at the C-9 carbon. Similarly, the majority of influenza viruses whose receptor on the cell is Neu5Ac failed to bind to

Neu5,9Ac$_2$ [25]. Thus, the parasite ligand (EBA-175), like influenza hemagglutinin, is a sialic acid ligand.

Perkins and Rocco [21] have also studied binding of soluble P.falciparum proteins to human and mouse erythrocytes. Their results are difficult to compare with the present work, because they find gp195 (Pf200, MSA1, MSP1) [21] as the binding protein to human erythrocytes, and little or no binding of this protein to mouse erythrocytes [20, 21]. The intact gp195 is not present in culture supernatants from our laboratory; instead, multiple monoclonal antibodies to gp195 only immunoprecipitated proteolytic fragments between 67 and 83 kDa [26,27]. Furthermore, antisera to a peptide derived from the gene encoding EBA-175 [28] immunoprecipitated a protein of identical mobility to the protein that bound to human erythrocytes [27]. Differences in the parasite concentration from which the supernatants were isolated may explain the differences in the erythrocyte binding studies.

Many pathogenic organisms use host cell sialic acids as receptors for attachment. Among these are the influenza viruses [29]. Influenza A and B virus hemagglutinin binds to Neu5Ac. Further specificity is conferred in the glycosidic linkages to galactose: different strains of influenza A bind preferentially to either Neu5Ac-α-2,3-galactose or Neu5Ac-α-2,6-galactose [25].Falciparum EBA-175 also binds preferentially to Neu5Ac-α-2,3-galactose [30]. Influenza C virus hemagglutinin, in contrast, binds to Neu5,9Ac$_2$ and contains a receptor-'destroying' esterase which, we now show, acts as a receptor-'creating' enzyme for P. falciparum merozoites.These results clearly indicate that the sialic acid-dependent binding and invasion by P. falciparum malaria parasites is significantly influenced by the chemical structure of sialic acid present on the erythrocyte surface.

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