Recognition and Invasion of Human Erythrocytes by Malarial Parasites: Contribution of Sialoglycoproteins to Attachment and Host Specificity

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ABSTRACT The receptivity of human erythrocytes to invasion by *Plasmodium falciparum* merozoites can be decreased by neuraminidase or trypsin treatment, an observation that supports a role for the erythrocyte sialoglycoproteins (glycophorins) in invasion. We have found that α_1-acid glycoprotein (AGP), added to in vitro cultures, can restore invasion of enzyme-treated human erythrocytes. AGP is structurally different from the glycophorins although it does carry 12% sialic acid. Its ability to restore receptivity to desialylated cells is dependent on its sialic acid complement, its concentration, and its binding to the erythrocyte surface. We present evidence that AGP forms a bridge between the merozoite and the enzyme-treated erythrocyte that allows the stronger and more complex interactions of invasion to proceed. We suggest that the glycophorins play the same role on the surface of the intact erythrocyte.

The ability of the malaria parasite to recognize and infect erythrocytes is essential to its existence. By living within the erythrocyte, the parasite is protected from interaction with the immune system, and by being in the blood, it can infect its vector, the mosquito, and travel to new hosts. The human erythrocyte is the only cell that is naturally infected by *Plasmodium falciparum* merozoites. Neither the mechanism of recognition nor of invasion has been explained.

Microscopic (1) and ultrastructural (2, 3) observations of invasion of rhesus erythrocytes by *P. knowlesi* suggest that two independent interactions contribute to the endocytic uptake of the malaria parasite by the erythrocyte. These observations provide clues about the molecular nature of invasion. In the first interaction, attachment, the parasite binds passively and without specific orientation to the erythrocyte surface. Attachment is reversible and must be mediated by weak binding interactions. The second interaction, junction formation, involves specific orientation, strong adherence, and a marked rearrangement of the erythrocyte cytoskeleton. The junction is formed at the apical end of the parasite. Freeze fracture studies of the junction zone (4) show that the parasite organizes the intramembranous particles of the erythrocyte membrane first into a cap and then into a ring with a central particle-free domain. The parasite appears to move the junction zone, with its associated constricting ring of erythrocyte membrane components, from its anterior to its posterior end and in doing so enters a newly formed intracellular vacuole. The bonds formed between the parasite and erythrocyte surface must compete with and be stronger than the bonds that maintain normal erythrocyte shape and that prevent both endocytic and exocytic vacuolization of the erythrocyte membrane. In addition, at least one element of the erythrocyte membrane must be responsible for transferring energy expended at the outer surface of the erythrocyte to the underlying cytoskeleton.

Several lines of evidence implicate the erythrocyte sialoglycoproteins (glycophorins) in one or both of the events of invasion by *P. falciparum*. Enzymatic hydrolysis (by trypsin or neuraminidase) (5–7), genetic deficiency (5, 8), or antibody masking (7, 8) of the glycophorins all decrease invasion to some extent. The questions remain, however, of what role glycophorins play in the invasion process and whether their structure determines the host cell specificity demonstrated by these organisms. In this report, we present evidence that glycophorins mediate the first event, attachment, that they are involved in a weak relatively nonspecific interaction,

which allows a second, as yet unidentified, interaction to occur.

MATERIALS AND METHODS

Assay of P. falciparum Invasion and Merozoite Binding: We measured the susceptibility of both human and baboon erythrocytes to invasion by *P. falciparum* merozoites by mixing them with infected human erythrocytes containing late stage parasites (schizonts) (9). During a 16-h incubation, the schizonts complete development, merozoites are released by host cell lysis, and free merozoites invade susceptible cells. The schizonts were prepared from a standard erythrocyte culture of *P. falciparum* in human erythrocytes by gelatin enrichment (10, 11). During this procedure, most uninfected cells and cells with small parasites (rings) aggregate and settle. The still suspended cells containing 30–70% schizonts are washed free of gelatin by low speed centrifugation, counted by Giemsa staining, and used immediately in the invasion assay.

10^8 test cells were mixed with $1–2 \times 10^6$ schizont-infected cells and incubated in 1-cm multiwell plates in a total volume of 0.4 ml RMPI-1640 (Gibco Laboratories Inc., Grand Island, NY), 25 mM HEPES (Sigma Chemical Co., St. Louis, MO) with 10% compatible serum, and 40 μg/ml gentamycin (Schering Corp., Kenilworth, NJ). After 16 h, the percentage of newly invaded cells containing ring forms of the parasite was determined either by microscopic examination or by [^{3}H]hypoxanthine incorporation. In the latter procedure, 1 μCi of [^{3}H]hypoxanthine (New England Nuclear, Boston, MA) was added to each well and the incubation was continued for 24 h. The settled cells were then resuspended, filtered through GF/C filters (Whatman Laboratory Products Inc., Clifton, NJ) and precipitated in 10% trichloroacetic acid. The filters were washed with 5% trichloroacetic acid and methanol, dried, and counted. Control wells contained either normal human erythrocytes or normal cells plus 50 μM dextran sulfate (5,000 mol wt, Sigma Chemical Co.), which completely blocks invasion (9). Test experiments showed that the average count from two duplicate wells correlated with the level of invasion determined by microscopic examination.

Baboon erythrocytes were obtained from normal *Papio papio*. When tested in the invasion assay, stained cells showed very few ring forms, but some cells had recognizable merozoites bound to their surfaces. These bound merozoites were counted only if the merozoite had a distinct nucleus and cytoplasm and was not located near a site of schizont rupture on the slide.

Enzyme Treatment of Erythrocytes: Freshly drawn human erythrocytes (type A+) were washed three times in 0.14 M NaCl, 25 mM HEPES, pH 7.4 (HEPES-buffered saline [HBS]),[1] and suspended in HBS at a concentration of 10^9/ml. *Vibrio cholerae* neuraminidase (1 IU/ml; Calbiochem-Behring Corp., La Jolla, CA) and *N*-tosyl-L-phenylalanine chloromethyl ketone-treated trypsin (227 U/mg; Worthington Biochemical Corp., Freehold, NJ) were added to the indicated concentrations with CaCl$_2$ at 10 and 5 mM, respectively. The cells were incubated for 1 h at 37°C with shaking (or as noted). Neuraminidase-digested cells were washed five times in 100 vol of HBS. Tryspin-digested cells were washed twice in 100 vol of HBS, incubated for 20 min in 1 mM phenylmethylsulfonyl fluoride (Sigma; from a 100 mM stock solution in ethanol) and washed four more times. Controls showed no inhibitory effects from the incubation in HBS with CaCl$_2$ or phenylmethylsulfonyl fluoride.

For partial desialylation of erythrocytes, 1×10^{-3} IU/ml neuraminidase was used. 200-μl aliquots of the incubating cell suspension were removed at 10-min intervals, added to 20 μl 100 mM EDTA, and centrifuged for 5 s in a Beckman microfuge (Beckman Instruments, Inc., Palo Alto, CA). 100 μl of the supernatant was assayed for sialic acid by the thiobarbituric acid method (12), and the cells were washed three times with 1 ml HBS and 5 mM EDTA and once in RPMI-1640.

Fractionation of Serum: Undiluted human serum was fractionated by slowly adding solid ammonium sulfate to 1.56, 2.34, and 3.12 M in sequence. At each concentration, the serum was allowed to equilibrate for 30 min, and the precipitated protein was removed by centrifugation (10,000 rpm × 10 min), dissolved in HBS, and dialyzed for 24 h against distilled H$_2$O. The supernatant containing 3.12 M salt was also dialyzed. All fractions were then lyophilized and redissolved in a volume of HBS equal to the original volume of serum.

Preparation of α$_1$-acid Glycoprotein (AGP): AGP was purified as described earlier (9). The fraction used was AGP X, which was electrophoretically pure and contained 16 sialic acid residues per molecule of AGP.

AGP-depleted serum was prepared by immunoprecipitation. Equal volumes of human serum and affinity-purified goat anti-human AGP antibody (Litton Bionetics, Kensington, MD) were combined and dialyzed against HBS for 16

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[1] *Abbreviations used in this paper:* AGP, α$_1$-acid glycoprotein; HBS, HEPES-buffered saline.

h at 4°C. The immunoprecipitate was removed by centrifugation and the serum was concentrated to its original volume by dialysis against Sephadex G-200 (Pharmacia Inc., Piscataway, NJ). Dialyzed serum was used as a control and had normal activity.

Sialic acid was removed from AGP by neuraminidase. 5 mg AGP in 1 ml 50 mM sodium acetate, 23 mM CaCl$_2$, pH 5.5, was incubated with 0.1 IU of neuraminidase for 5 h at 37°C. The neuraminidase was removed by affinity chromatography at 4°C on a 1-ml column of fetuin-agarose (Sigma) in the same buffer. Eluted fractions containing protein were dialyzed against H$_2$O and concentrated in the dialysis bag against Sephadex G-200.

Cell Electrophoresis: Cellular electrophoretic mobilities were measured with a Zeiss cytopherometer (Carl Zeiss, NY) equipped with a Zn/ZnSO$_4$ electrode assembly (13). The rectangular cytopherometer chamber was thermostated at 25.0 ± 0.1°C during the measurements. Electrophoretic velocities of individual cells were recorded at each of the two stationary layers within the cytopherometer chamber (14), using an applied electric field level of 3.7 or 5.6 V/cm. During electrophoresis the erythrocytes were suspended in a solution consisting of 90 parts of 0.154 M NaCl and 10 parts of Sorensen phosphate buffer (pH 7.4, ionic strength = 0.15). At this ionic strength, the Debye length, which is a measure of the thickness of the ionic double layer associated with fixed charges at the membrane surface (15, 16), is 8 Å. Calculation of the cellular surface charge density, and the changes induced by enzyme treatment or AGP binding, followed procedures described previously (17). In calculating the number of AGP molecules bound to the erythrocyte surface, it was assumed that the outer aspect of each bound molecule contributes 15 negative charges detectable by cell electrophoresis (from a total net charge of 30 negative ionized groups per molecule at the pH and ionic strength used in these experiments, derived from data presented in reference 18). The human erythrocyte surface area was taken to be 134 μm^2 (19) in these calculations.

RESULTS

In our initial experiments, we found that baboon erythrocytes were not efficiently invaded by *P. falciparum* merozoites. With 10% baboon serum in the culture medium, merozoites did not even attach to baboon cells, but they did attach in the presence of human serum (Table I). The attachment activity in human serum was associated with a factor that was highly soluble in ammonium sulfate and nondialyzable (Table II). Thus a macromolecule of human serum appeared capable of either enhancing an endogenous receptor activity of the baboon erythrocytes or, by binding to the surface of the cell, acting as a receptor itself. Even with the attachment factor present, however, baboon cells were not invaded efficiently. In contrast, attachment to and invasion of human erythrocytes proceeded well if not optimally in baboon serum. The normal host cell of *P. falciparum*, then, did not require human serum for invasion.

After treatment with neuraminidase, human erythrocytes lost almost all of their susceptibility to invasion in baboon serum. However, when human serum was included, invasion did occur (Table III), suggesting that the same human serum factor that conferred attachment on baboon cells could restore attachment to neuraminidase-treated human cells. One candidate for this factor was AGP, which is a prominent component of the 3.12 M ammonium sulfate fraction of human

TABLE I

*Effect of Baboon and Human Sera on the Ability of P. falciparum Merozoites to Bind to or Invade Baboon and Human Erythrocytes**

Serum	Merozoites bound to 1,000 baboon cells	Rings in 1,000 baboon cells	Rings in 1,000 human cells
10% baboon	1.0 ± 0.4	0.6 ± 0.5	67
10% human	8.4 ± 1.3	1.8 ± 0.6	92
20% human	23 ± 2.1	7.0 ± 1.2	92

* 5,000 erythrocytes were counted from each culture. Data ± SE ($n = 3$) are shown from a typical experiment.

serum. When added to cultures containing either human or baboon serum, purified AGP increased invasion of neuraminidase-treated human erythrocytes severalfold (Table III). In other experiments, neither human serum lacking AGP (AGP-depleted) nor desialylated AGP in baboon serum could restore invasion (Table IV). The first result confirms the identity of

TABLE II

Effect of Ammonium Sulfate-fractionated Human Serum Components on Merozoite Binding to Baboon Cells

	Merozoites bound to 1,000 baboon cells*	
Serum	Exp. 1	Exp. 2
5% human	2.0	6.0
5% human + 20% human fraction‡		
0-1.56M P	1.2	1.4
1.56-2.34M P	1.6	1.8
2.34-3.12M P	6.8	5.4
3.12M S	7.8	7.2
20% human	9.8	8.8

* 5,000 erythrocytes were counted from each culture. Exps. 1 and 2 are from separate fractionations.
‡ Precipitated (P) and soluble (S) serum protein fractions were prepared as described in Materials and Methods.

TABLE III

Effects of Human and Baboon Sera and AGP on Merozoite Invasion of Neuraminidase-treated Human Erythrocytes

Cells	Serum	AGP	Rings/1,000 erythrocytes
		mg/ml	
Experiment 1—Baboon serum + AGP			
Control	10% baboon	—	43.0
	10% human	—	61.3
Neuraminidase-treated	10% baboon	—	1.0
	10% human	—	6.0
Neuraminidase-treated	10% baboon	0.19	5.2
		0.38	17.8
		0.75	27.3
Experiment 2—Human serum + AGP			
Neuraminidase-treated	10% baboon	—	2.0
	10% human	—	23
	20% human	—	35
Neuraminidase-treated	5% human	—	14
		0.25	14
		0.5	20
		1.0	35

AGP as the attachment factor in serum serum, and the second points to its complement of sialic acid as necessary for attachment activity.

AGP has a strikingly high content of sialic acid-bearing carbohydrate (55%) and is nonspherical (18). The carbohydrate chains contribute to the asymmetry and are thought to be located at one end of an oblong structure. We hypothesized that the peptide portion of the molecule bound to the cell and that the carbohydrate end presented a negatively charged surface to the merozoite, a surface that was sufficient for parasite attachment. We attempted to measure the binding of ^{125}I-labeled AGP to neuraminidase-treated cells, but the small ratio of bound molecules to free molecules and the impossibility of washing out free molecules without losing bound ones contributed to high experimental error. This difficulty was overcome by measuring the electrophoretic mobility of erythrocytes in the absence and presence of AGP (Table V).

After neuraminidase treatment, the negative surface charge density of human erythrocytes was reduced by 88.1% relative to the untreated cells. Upon the addition of 1 mg/ml AGP, the negative surface charge of the neuraminidase-treated cells increased approximately twofold, indicating that the glycoprotein did indeed bind to the cell surface. AGP restored 15% of the surface charge removed by neuraminidase, and, from the increase in negative charge density, it was calculated that 9.0×10^4 electrophoretically detectable AGP molecules were bound at the surface of the desialylated human erythrocyte. The bound AGP was completely removed by one wash in electrophoresis buffer, thus indicating the weak nature of the bond formed between this glycoprotein and the desialylated cell surface. Normal erythrocytes did not exhibit a change in mobility upon addition of AGP to the medium, suggesting that the binding of the glycoprotein to the cell surface is facilitated by the removal of native sialic acid residues. Binding was not saturated at 0.2 mg/ml, but was near or at saturation at 0.5 mg/ml, the same concentration range over which invasion was conferred to desialylated cells.

The amount of erythrocyte sialic acid needed for parasite attachment and invasion in the absence of AGP was measured by treating human erythrocytes with neuraminidase for increasing times, determining the amount of sialic acid removed, and testing the cells for infectivity. Neuraminidase concurrently decreased sialic acid content, surface charge density, and infectability of the erythrocytes, and removal of ~50% of the cell's sialic acid completely destroyed susceptibility to infection (Fig. 1).

TABLE IV

Effects of AGP-depleted Human Serum and Desialylated AGP on Merozoite Invasion of Neuraminidase-treated Human Erythrocytes

Cells	Serum	AGP	Rings/1,000 erythrocytes
Experiment 1—AGP-depleted human serum			
Control	10% human	—	39.2 ± 3.7*
	10% AGP-depleted human	—	40.7 ± 1.1
Neuraminidase-treated	10% human	—	15.4 ± 2.5
	10% AGP-depleted human	—	4.5 ± 0.5
Experiment 2—Human serum + desialylated AGP			
Control	5% human	0.5	50.0 ± 4.5
		0.5 (desialylated)	54.8 ± 5.2
Neuraminidase-treated	5% human	0.5	20.3 ± 1.0
		0.5 (desialylated)	3.7 ± 1.5

* 5,000 erythrocytes were counted in each sample. Mean ± SE (n = 3).

TABLE V

Effects of P. falciparum Infection, Enzyme Treatments, and AGP Binding on Human Erythrocyte Surface Charge Density

Treatment	AGP	Electrophoretic mobility	Surface charge density	Charges per cell	Charges added by AGP
		$(\mu m/s)/(V/cm)$	$(10^{-3}\ C/m^2)$	$\times 10^6$	
None	—	1.09 ± 0.02 (120)*	12.57	10.51	—
	1.0 mg/ml	1.10 ± 0.01 (40)	12.68	10.61	—
Neuraminidase (37°C, 0.05 IU × 30 min)	—	0.13 ± 0.01 (20)	1.50	1.25	—
	1.0 mg/ml	0.27 ± 0.01 (34)§	3.11	2.60	1.35
	wash‡	0.13 ± 0.01 (16)	1.25	1.25	—
Trypsin (37°C, 0.075 mg/ml × 60 min)	—	0.59 ± 0.03 (40)	6.80	5.69	—
	1.0 mg/ml	0.69 ± 0.02 (40)‖	7.92	6.66	0.97
	wash	0.60 ± 0.03 (40)	6.92	5.79	—
Trypsin (37°C, 1 mg/ml × 60 min)	—	0.58 ± 0.03 (40)	6.69	5.69	—
	1.0 mg/ml	0.66 ± 0.02 (40)‖	7.61	6.36	0.67
	wash	0.58 ± 0.03 (40)	6.69	5.69	—
P. falciparum infected	—	1.10 ± 0.02 (40)	12.68	10.61	—

* Mean ± SE (number of measurements).
‡ Washed samples were incubated in 1 mg/ml AGP for 20 min and washed once in electrophoresis buffer.
§ $P < 0.001$ for neuraminidase-treated cells ± AGP.
‖ $0.02 < P < 0.03$ for trypsin-treated cells ± AGP.

Erythrocyte sialic acid is carried primarily on the glycophorins, a family of sialoglycoproteins. There are three well characterized glycophorins containing 68% (A or α), 13% (B or δ), and 6% (C or β) of the surface sialic acid (20, 21). The remainder of the sialoconjugates are poorly characterized. Trypsin cleaves most of the exposed sialoglycopeptide from glycophorins A and C, and removes about half of the total sialic acid. We treated erythrocytes with a concentration of trypsin (0.075 mg/ml) that was sufficient to remove 95% of the trypsin-sensitive sialic acid. These treated cells lost infectability in baboon serum, showing that the cleaved sialoglycopeptides at least contribute to the normal susceptibility of the cell. Again, AGP restored invasion (Fig. 2). Cells treated with 0.075 mg/ml trypsin bound a similar amount of AGP as did neuraminidase-treated cells (Table V), and the bound AGP restored 20% of the surface charge removed by trypsin. Washing the cells free of AGP reversed both the increase in surface charge and the restoration in infection (Table V and Fig. 2). Treatment of cells with 1 mg/ml trypsin reduced the surface charge to the same degree as did the lower concentration of this enzyme, but decreased invasion even more. Unlike cells treated with neuraminidase or 0.075 mg/ml trypsin, invasion was not restored by AGP after treatment with 1 mg/ml trypsin (Fig. 2), even though binding of the glycoprotein to the cell surface could be detected electrophoretically (Table V).

Comparative measurements were also made of baboon erythrocyte electrophoretic mobility and changes resulting from enzyme treatments or AGP binding. Neuraminidase and 1 mg/ml trypsin treatments decreased the mobility of baboon cells by 44.8 and 19.8%, respectively, which was approximately one-half of the reduction of human erythrocytes by these enzymes (Fig. 3). The sialic acid released by neuraminidase and trypsin from the baboon erythrocyte surface was correspondingly low in comparison with human cells (Table VI). Similar to the native human erythrocyte, the
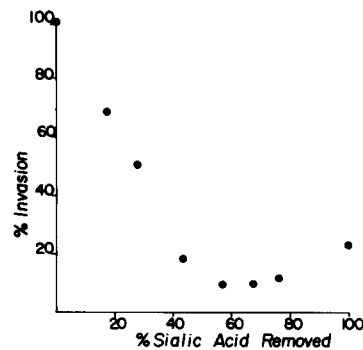

FIGURE 1   Effect of gradual sialic acid removal on invasion of human erythrocytes by P. falciparum. To decrease the infuence of AGP, the medium contained only 5% human serum (~0.035 mg/ml AGP). The slight rise in the curve after complete removal may be caused by AGP binding.
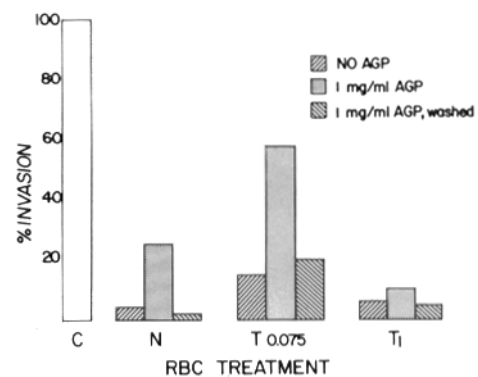

FIGURE 2   Influence of AGP during invasion of human erythrocytes (RBC) treated with neuraminidase (N), 0.075 trypsin ($T_{0.075}$), or 1 mg/ml tryspin (T_1). Human serum was present at 3% in the medium. To test the reversibility of AGP activity, erythrocytes were incubated in 1 mg/ml AGP for 20 min at 20°C, washed once in HBS, and tested for susceptibility to invasion.

baboon erythrocyte surface did not bind electrophoretically detectable amounts of AGP (data not shown).

Electrophoretic measurements were also made on schizont-infected human erythrocytes (isolated by gelatin enrichment)
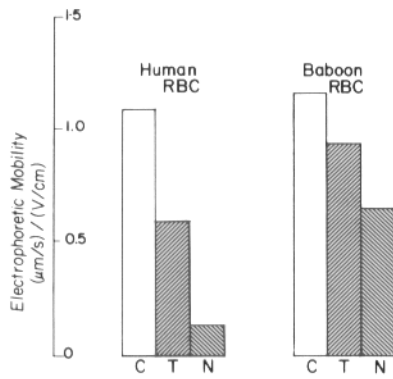
FIGURE 3 Comparison of the effects of neuraminidase and trypsin on the electrophoretic mobilities of human and baboon erythrocytes (RBC). Neuraminidase and trypsin concentrations were 0.01 IU/ml and 1 mg/ml, respectively.

TABLE VI

Sialic Acid Removed by Neuraminidase or Trypsin from Human and Baboon Erythrocytes

Enzyme treatment	Sialic acid	
	Human	Baboon
	$\times 10^{-8}$ µg/cell	
Neuraminidase (0.01 IU/ml)	1.36	0.34
Trypsin (0.075 mg/ml)	0.76	n.d.
Trypsin (1 mg/ml)	0.80	0.26

n.d., not determined.

in order to determine whether parasitization produces an alteration in the electrical charge properties of the host cell membrane. As shown by the data in Table V this does not occur, since the electrophoretic mobilities of infected and control cells are nearly identical.

DISCUSSION

We have found that human serum can mediate the attachment of P. falciparum merozoites to baboon erythrocytes and restore the susceptibility to infection of neuraminidase or 0.075 mg/ml trypsin-treated human erythrocytes. The factor in serum that is responsible for these activities is AGP, and the ability of AGP to restore infection is dependent on its sialic acid content and its concentration. This finding clarifies some of the puzzling results of previous investigations in which the effect of AGP, a normal component of serum, was not understood. Attempts to infect enzyme-treated cells (5–7) or cells deficient in glycophorin A (En(a)-) (5, 8, 22) have given highly variable invasion rates in the presence of human or fetal bovine serum. This variation may be partly due to variable AGP binding. The AGP content of baboon serum has not been determined, but it has little activity in the invasion assay.

We investigated the binding of AGP to enzyme-treated erythrocytes by measuring the electrophoretic mobility of cells in the presence and absence of the glycoprotein. The advantage of this technique is that only bound molecules are detected, so the measurement can be made under ideal equilibrium conditions. We found that AGP bound reversibly to all cells whose sialic acid was depleted by enzyme hydrolysis. By measuring electrophoretic mobility at different AGP concentrations, we were able to show that AGP binding to enzyme-treated cells occurred within the same range of concentrations in which AGP was effective at restoring infection to these cells (Table 3). Thus, AGP appeared to act by serving as a substitute parasite binding site on the surface of the depleted erythrocyte. Support for this hypothesis comes from the dem-

onstration that AGP can also bind to the merozoite. In earlier work, it was shown that AGP inhibits merozoite invasion of normal human erythrocytes by 30% at 1 mg/ml and by 75% at 2.5 mg/ml (9). We know from the present study that AGP does not interact with the normal erythrocyte (Table V), and therefore, AGP must inhibit invasion by binding to the merozoite. By binding to both the enzyme-treated erythrocyte and the merozoite, AGP can form a bridge between these two cells.

We could not detect AGP binding to baboon erythrocytes even though these cells acquired the ability to bind merozoites in the presence of AGP. Since both AGP and the baboon erythrocytes surface are negatively charged, they would be expected to repel each other. The ability of AGP to serve as a binding factor in this instance may depend on AGP first binding to the merozoite. With some of its negative charge neutralized, it may then be able to mediate the binding of the merozoite to the baboon erythrocyte surface.

Both the peptide and carbohydrate structures of AGP (18) differ from those of erythrocyte sialoglycoprotein (23). The ability of AGP to serve as an attachment site for the merozoite suggests that the initial attachment event is relatively nonspecific. Contrary to suggestions that glycophorin A is the erythrocyte receptor for P. falciparum, there is no evidence that any single glycophorin is specifically required for invasion. Soluble glycophorin A inhibits invasion (7, 24, 25), but this is a property common to other polyanions (9). Antibodies to glycophorin A block invasion (7, 8) but are also known to rigidify the erythrocyte membrane (26), and their ability to inhibit invasion may not depend on receptor blockade. In fact, abnormal cells (En(a)-), totally lacking glycophorin A, retain up to 48% infectability (5, 8). (These same cells lack the Wr_b antigen which has also been suggested to be essential for invasion [27].) In addition, other experiments (22, 27) have shown that glycophorins A and B independently contribute to the level of invasion in the other's absence. These studies and our observations indicate that glycophorins in general or AGP can provide a recognizable surface for merozoite binding and therefore that the requirement for attachment must be limited to the property that these proteins share, a high content of sialic acid. The total surface charge per se does not correlate with invasion, but there is a correlation between invasion and superficial sialic acid at the cell surface. Removal by neuraminidase of the most accessible 15% of the sialic acid decreased invasion by 25% (Fig. 1), while the addition of the same amount of sialic acid by AGP to neuraminidase-treated cells (determined by electrophoretic mobility) restored 23% of control invasion (Fig. 2).

AGP on the surface of the enzyme-treated erythrocyte is weakly bound; it is unlikely to serve as a mediator of junction formation and entry since these events appear to require transmembrane communication. Instead, we propose that an AGP bridge formed between merozoite and erythrocyte stabilizes the apposition of the two cells and allows the formation of a stronger and more complex attachment. The life of a merozoite is very short (28), and if a merozoite does not enter a cell within a few minutes, it will die. Therefore, the final level of invasion and parasite multiplication is determined by the kinetics of junction formation and entry; if entry does not occur rapidly, it will not occur at all. The junction is only formed at the anterior end of the merozoite, and the junction bond must be strong and multivalent to exert an influence on the erythrocyte cytoskeleton. The random formation of this

bond would be infrequent between two freely colliding cells. In the absence of AGP, the level of invasion of neuraminidase-treated cells is very low, indicating that the rate of junction formation in the absence of attachment must be low. An attachment event mediated by glycophorins or AGP could increase the probability of junction formation by obviating the dependence on random collisions and by orienting the cell surfaces in a contrafacial position. The kinetic contribution of attachment to invasion efficiency is demonstrated by the addition of AGP to neuraminidase-treated erythrocytes. Infection is increased severalfold.

This first attachment event, although relatively nonspecific, may still contribute to host specificity. Human erythrocytes have been shown to carry a higher percentage of neuraminidase-sensitive sialic acid (Fig. 3; references 16, 29, and 30), a higher density of glycoprotein-bound sialic acid (31), and less ganglioside-bound sialic acid (16) than erythrocytes of all other species studied. The less dense sialylation of nonhuman glycophorins could explain why the baboon cell requires AGP for merozoite binding and why rhesus (7) or sheep (25) glycophorins are less efficient inhibitors of invasion than human glycophorin. The only other *Plasmodium* species whose invasion requirements have been studied is *P. knowlesi*, which can invade both rhesus and human erythrocytes but which does not require erythrocyte sialic acid for invasion (5). *P. falciparum*, which does not invade rhesus erythrocytes, appears to depend on the highly charged sialoglycoproteins of human erythrocytes for host cell recognition and entry. The importance of the type of sialic acid (*N*-acetyl or *N*-glycolyl neuraminic acid) or the linkage of sialic acid to its conjugate have not been evaluated, but the sensitivity of invasion to noncarboxylate polyanions (9) suggests that electrostatic bonds are the primary components of the attachment event.

1 mg/ml trypsin destroyed the susceptibility of human erythrocytes to invasion, but did not destroy AGP binding. Therefore, this high concentration of trypsin may have degraded the site of the second merozoite-erythrocyte interaction, junction formation. If so, we may be able to identify the junction formation site by studying the relative effects of high and low trypsin. This site may prove not to be host specific since the baboon cell could be invaded by *P. falciparum* merozoites (albeit at low levels) when AGP was present.

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