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A lectin-like receptor is involved in invasion of erythrocytes by *Plasmodium falciparum*

(malaria/glycophorin/sialoglycoprotein/merozoites)

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ABSTRACT **Glycophorin both in solution and inserted into liposomes blocks invasion of erythrocytes by the malaria parasite *Plasmodium falciparum*. Furthermore, one sugar, *N*-acetyl-D-glucosamine (GlcNAc), completely blocks invasion of the erythrocyte by this parasite. GlcNAc coupled to bovine serum albumin to prevent the sugar entering infected erythrocytes was at least 100,000 times more effective than GlcNAc alone. Bovine serum albumin coupled to lactose or bovine serum albumin alone had no effect on invasion. These results suggest that the binding of *P. falciparum* to erythrocytes is lectin-like and is determined by carbohydrates on glycophorin.**

It is becoming increasingly clear that carbohydrates expressed on cell surface molecules can act as receptors for many agents. The receptors for many viruses (1), bacteria (2–4), and parasites (5, 6) are carbohydrate determined and are present on membrane-bound glycolipids and glycoproteins. It seems probable that such membrane-anchored carbohydrate-rich molecules play an important role as bridging ligands between cells in cell–cell interactions. The binding of these ligands appears to resemble the binding of plant lectins, which occurs via highly specific interactions between carbohydrates and protein.

There is evidence that a stage of recognition and attachment occurs prior to invasion of erythrocytes by the human pathogenic protozoan *Plasmodium falciparum*. A likely ligand for the merozoites of *P. falciparum* on the erythrocyte is glycophorin, which is known to have lectin-like properties (7). Although these molecules are known to carry the M and N blood group antigens (8) and virus receptor sites (1), their biological function is not known and their importance has been questioned because individuals whose erythrocytes lack glycophorin are hematologically normal. Nevertheless, these deficient cells appear to be relatively resistant to invasion by *P. falciparum* when compared with normal erythrocytes (6, 9). The glycophorin molecule is therefore of special interest in investigation of the recognition of the host erythrocytes by this parasite.

If carbohydrate–protein interactions are important in parasite invasion of erythrocytes, certain monosaccharides should selectively inhibit parasite invasion. This report describes a series of studies which show that certain glycoproteins, both in solution and inserted into liposomes, block invasion of erythrocytes by *P. falciparum*. Furthermore, one sugar, *N*-acetyl-D-glucosamine, completely blocks the invasion of the erythrocyte by the parasite. The blocking of invasion was particularly effective when the sugar was coupled to bovine serum albumin. These results suggest that the binding of *P. falciparum* to its recognition unit on the target erythrocyte is lectin-like and is determined by carbohydrates on surface-bound glycoproteins such as glycophorin.

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MATERIALS AND METHODS

Synchronous continuous cultures of the Uganda–Palo Alto strain of *P. falciparum* were maintained in human erythrocytes as described (10, 11). Stock 300 mM solutions of the following sugars (Sigma) were made in double-distilled water and the pH was adjusted to approximately 7.0 with 0.1 M NaOH: D-mannose, D-fucose, D-galactose, D-glucose, D-glucosamine, D-galactosamine, *N*-acetyl-D-glucosamine (GlcNAc), *N*-acetyl-D-galactosamine (GalNAc), and *N*-acetylneuraminic acid (NeuNAc). GalNAc and GlcNAc were also obtained from Aldrich. Doubling dilutions of these solutions (i.e., 150, 75, 38, and 19 mM) were prepared in complete medium (RPMI 1640 medium supplemented with 0.2% sodium bicarbonate and 10% human type AB serum).

In a second set of experiments bovine serum albumin (hereafter referred to as albumin) was coupled to two sugars, GlcNAc and lactose, after the method of Monsigny and co-workers (12). Briefly, albumin at 1 mg/ml in phosphate-buffered saline was added to the *p*-aminophenyl derivative of GlcNAc or lactose at 1 mg/ml, also in phosphate-buffered saline. Glutaraldehyde (25%, vol/vol), was added to the mixture to give a final concentration of 2%. After incubation for 30 min at room temperature, 1 M glycine was added to give a final concentration of 50 mM. The mixture was incubated for a further 30 min at room temperature. The yellow product was dialyzed with four changes of phosphate-buffered saline (until the yellow-brown color had disappeared). The efficiency of the coupling was determined by using the method of Schacterle and Pollack for measuring the albumin concentration (13) and the resorcinol method for measuring the concentration of sugar (14).

For use in the blocking-of-invasion experiments the sugars coupled to albumin were diluted in complete medium to give final sugar concentrations of 5, 2.5, 1, 10^{-1}, 10^{-2}. . . 10^{-6} mM. The molarity of the sugar was calculated on the basis of a coupling efficiency of 20 molecules of sugar per molecule of albumin (i.e., 5 mM with respect to sugar, equivalent to 0.25 mM albumin). Albumin alone at the same concentrations was also included as a control.

Total erythrocyte sialoglycoprotein was prepared by butanol extraction and subsequent dialysis by the method of Anstee and Tanner (15). Sialoglycoprotein-bearing liposomes were prepared by incubating 500 μl of dimyristoyl phosphatidylcholine at 2 mg/ml in 10 mM Tris·HCl with 1% sodium cholate buffer, pH 7.4, together with 17 μl of glycoproteins at 15.3 mg/ml. This mixture was dialyzed for 36 hr in 10 mM Tris·HCl with 0.9%

Abbreviations: GlcNAc, *N*-acetyl-D-glucosamine; GalNAc, *N*-acetyl-D-galactosamine; NeuNAc, *N*-acetylneuraminic acid.

NaCl. The liposomes were centrifuged for 1 hr at 10,000 × g and stored at 4°C in RPMI 1640 medium (16).

Schizonts of *P. falciparum* were harvested from culture by means of a 3% gelatin solution (Plasmagel, Roger Bellon Laboratories, Neuilly, France) (17) and mixed with uninfected human erythrocytes so as to obtain a 2% cell suspension with 4–5% of the total cells containing schizonts. One hundred and fifty microliters of the mixture was pipetted into the required number of wells of a micro tissue culture plate to which 50 μl of the required sialoglycoprotein or particular sugar-containing solution was added, making a 1:4 dilution of each solution used. The plates were incubated for 18–20 hr at 37°C in 5% O_2/7% CO_2/88% nitrogen and smears were made and stained with Giemsa stain. Cultures with albumin or albumin-sugars were washed three times in RPMI 1640 medium, smeared, and stained. Parasite numbers were assessed by counting the number of ring forms present in at least 1,000 cells.

To detect whether the sialoglycoproteins or sugars had any effect on intracellular maturation of parasites, ring forms obtained from continuous culture were incubated in the presence of the sialoglycoprotein extract or sugar for about 24 hr. At this time smears were made and the numbers of schizonts (mature parasites with two or more nuclei) per 100 singly infected erythrocytes were determined and compared to the controls. In some experiments an extended period of time was allowed for maturation. The protocol for these and appropriate control experiments is summarized in Fig. 1.

RESULTS

Inhibition of Invasion by Glycoprotein. Addition of glycoprotein in medium at high final concentration (2.8 mg/ml) completely blocked the invasion of erythrocytes by *P. falciparum*. Invasion inhibition decreased with increasing dilution of the glycoprotein but was nevertheless persistent: 75% blocking of invasion was observed at 2 mg/ml and 45% at 1 mg/ml, and invasion was still 20% less than in control cultures at 0.25 mg/ml (Fig. 2).

The ability of this protein preparation to inhibit invasion was also tested with the protein inserted into liposomes. The presence and orientation of protein in the liposomal membrane was tested with monoclonal antibody directed to the trypsin-sensitive (outer) portion of glycophorin A (8). In such a binding assay these liposomes were able to reduce binding between the monoclonal antibody and normal cells by 70–80%. Preliminary results showed that when added to the parasite in culture these glycoprotein-containing liposomes competitively inhibited the invasion of uninfected erythrocytes (data not shown). More quantitative studies are required to confirm this finding.

Inhibition of Invasion by Monosaccharides. In a series of six experiments, addition of monosaccharides that are represented on membrane glycoproteins such as glycophorin showed that only a few of these sugars were able to block invasion (Fig. 3). One sugar in particular, GlcNAc, obtained from both sources blocked invasion in a remarkably specific and effective manner. At higher concentrations (38–75 mM), no invasion was observed. At 19 mM the sugar blocked 60% of the invasion and even at 5 mM 35% blocking compared with normal controls was observed (Fig. 3).

With glucosamine the invasion rate was less than 1% of the control at the highest concentration (75 mM) (data not shown). The inhibitory effect decreased so that invasion rates approached normal control levels at 19 mM. GalNAc also showed a slight effect at 75 mM, but this was lost on subsequent dilutions.

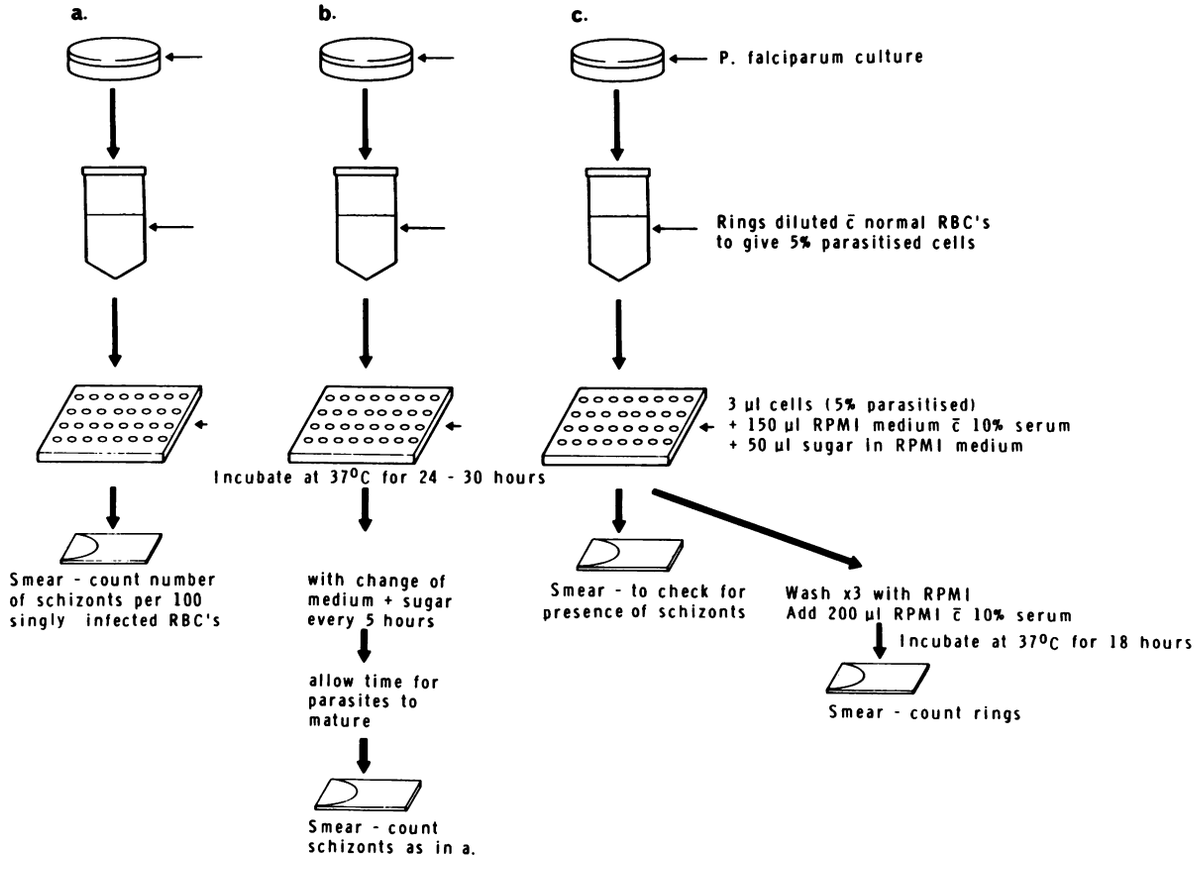

FIG. 1. Outline of procedure used to determine effect of sugars on *P. falciparum* development. RBC, erythrocyte; c̄, with.

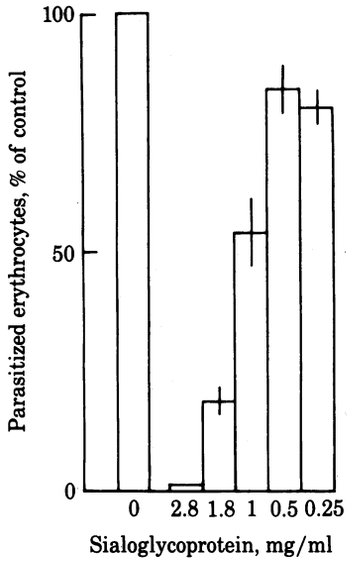

FIG. 2. Inhibition of *P. falciparum* invasion in the presence of sialoglycoprotein in solution. Error bars are SD.

Most of the sugars demonstrated little or no blocking activity. Galactose and fucose showed slight inhibitory activity at the higher concentrations (20% at 75 mM) only in some of the experiments, whereas mannose showed a greater degree of inhibition (up to 45% at 75 mM) in some experiments. No blocking occurred with this sugar at 38 and 19 mM, but it appeared again at 10 mM (data not shown). Two separate experiments using NeuNAc showed some blocking of parasite invasion at the higher concentration.

Inhibition of Invasion by Monosaccharides Coupled to Albumin. The effect of GlcNAc and lactose on merozoite invasion was tested in experiments designed to prevent the sugars from passing through the more permeable membrane of schizont-infected erythrocytes or into the free merozoite by coupling them to bovine serum albumin. The ability of the albumin-coupled GlcNAc to block invasion was greatly increased and was still present at 10^{-6} mM (Fig. 4). The GlcNAc-albumin inhibition was 100,000 times more effective than that with GlcNAc alone (30% of control invasion of erythrocytes at 20 mM with GlcNAc vs. 30% of control invasion at 10^{-4} mM with GlcNAc-albumin). The lactose-albumin showed some inhibition at the higher concentrations (2.5 and 5 mM). However, this effect was soon lost on dilution. Merozoite invasion rate was normal (15 ring forms per 100 erythrocytes) in the albumin control culture compared to the glucose control.

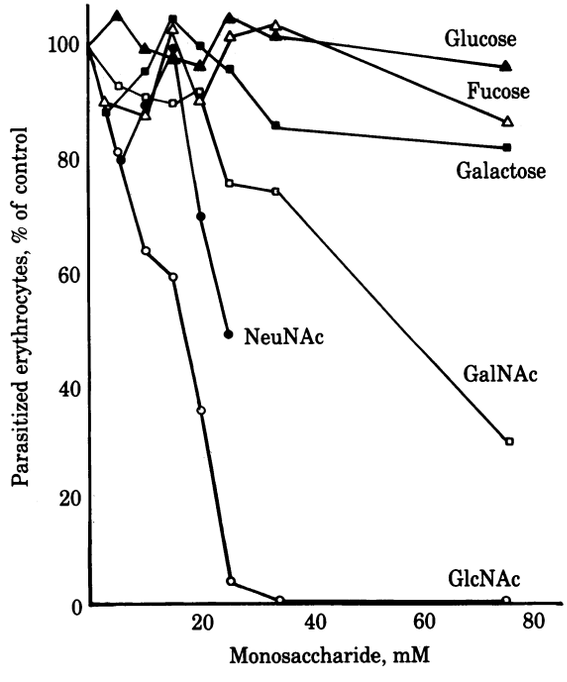

FIG. 3. Inhibition of *P. falciparum* invasion by various monosaccharides in solution at concentrations between 5 and 75 mM.

Development of Parasite in the Presence of Sugars. In the sugar experiments described above it was important to exclude nonspecific toxic effects due to contaminating substances in the sugar preparations or toxic effects of the sugars themselves on the parasites. To eliminate the first possibility the most inhibitory sugar, GlcNAc, was recrystallized. To eliminate the second and more important possibility, studies of parasite development from ring forms to schizonts were carried out in the presence of the sugars.

A delay of development of ring forms to schizonts was observed in the higher concentrations of most of the sugars tested. Table 1 shows the results of experiments in which smears were made on cultures of rings incubated for 24 hr in the presence of the sugars as outlined in Fig. 1*a*. There seemed to be a marked decrease in the number of mature schizonts in certain of the sugar cultures: GlcNAc and fucose cultures had few mature schizonts at the higher concentrations. It was found that in the cultures the pH of the media fell after 18 hr and the erythrocytes became crenated. When the procedure in Fig. 1*b* was followed, we found that allowing a longer incubation time and changing the medium containing the appropriate sugar concentration every 5 hr restored the development of parasites, at sugar concentrations 20 mM and higher (results shown in parentheses in Table 1).

There still remained a great difference in the time required for the maturation of schizonts in certain of the sugar preparations. Therefore experiments were done to determine whether parasites matured in the presence of sugar could produce viable merozoites capable of invading new erythrocytes. The results of these experiments are shown in Table 2. All of the cultures

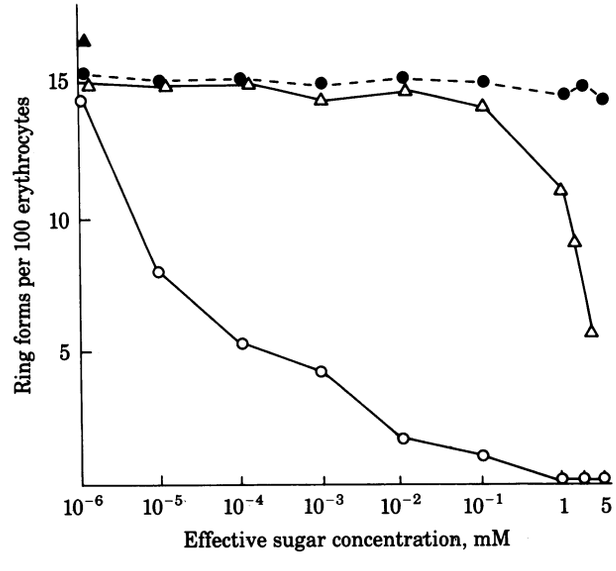

FIG. 4. Inhibition of *P. falciparum* invasion by albumin-coupled sugars. ▲, Glucose; ●, albumin; ○, GlcNAc-albumin; △, lactose-albumin.

Table 1. Development of *P. falciparum* ring forms to schizonts in the presence of sugars

Sugar	Development, % of control						
	75 mM	38 mM	25 mM	20 mM	10 mM	5 mM	2 mM
GlcNAc	0	0	7	25	98	101	99
	(50)	(87)		(91)	(98)		
GalNAc	101	107	93	102	101	104	108
Galactose	93	104	104	106	105	105	108
Glucose	95	97	104	101	107	106	108
Fucose	0	2	109		99	102	102
	(27)	(81)		(100)	(100)		
Mannose	95	106		102	100	111	112
NeuNAc	103	96		100		98	104

In duplicate control experiments there were 88 and 75 schizonts in 100 singly infected erythrocytes. Numbers in parentheses are results from experiments in which cells were incubated for longer period of time than control cells and the medium was changed every 5 hr.

in which the schizonts had been allowed to mature to the two- to four-nuclei state (up to 60 hr in some cases) were washed to remove sugars and incubated in complete medium without sugar for a further 18 hr (Fig. 1*c*). All the cultures showed essentially the same number of rings as the controls. Even the culture with 20 mM GlcNAc showed that a normal number of merozoites was released from schizonts that had matured in the presence of that sugar. Therefore the inhibition of invasion of merozoites in cultures with GlcNAc at concentrations less than 25 mM appears not to be due to a toxic effect on schizonts.

Subtle differences in development were noted depending on the sugar used. There appeared to be more trophozoites than schizonts in glucosamine cultures at higher dilutions of the sugar at 48 hr, while most of the parasites were in the schizont stage in the control and glucose cultures. The fucose cultures showed normal parasite development in the 5 and 10 mM concentrations but development was arrested in the 38 and 75 mM cultures. The development of the parasites in the presence of GlcNAc, which inhibited invasion most, was normal at concentrations less than 25 mM, and development was also normal in the presence of NeuNAc. Albumin-coupled sugars, especially GlcNAc, had no toxic effect on parasites, which developed and invaded new cells normally in both the albumin and the lactose-albumin controls.

DISCUSSION

This study suggests that the interaction between erythrocytes and merozoites of the malaria parasite *P. falciparum* involves highly specific, lectin-like binding of parasites to complementary carbohydrate determinants on target cell surface molecules. It has been suggested that erythrocyte glycophorin is such a complementary molecule (6). There is good evidence that many parasites attach to host cells by sugar-binding proteins complementary to molecules on the target cell's surface.

Table 2. Effect of sugars on multiplication of *P. falciparum* in cultures

Sugar	Rings, % of control			
	20 mM	15 mM	10 mM	5 mM
GlcNAc	100	119	81	103
GalNAc	90	88	104	96
Galactose	103	96	91	98
Fucose	111	104	96	94
Mannose	89	88	83	103
NeuNAc	85	98	94	96

Duplicate control experiments had 12.1 and 13.3 ring forms per 100 erythrocytes.

Recently, Perkins (18) has reported that glycophorin A in solution can block *P. falciparum* invasion of erythrocytes in a concentration-dependent manner. In the experiments described here we have shown that butanol-extracted sialoglycoproteins can similarly completely inhibit *P. falciparum* invasion. No agglutination of merozoites by the glycophorin was observed. The concentration of glycophorin in solution required to produce total blocking was high. The necessity for such a high concentration can be explained in several ways. When glycoproteins are in solution the relevant parasite binding sites may not be as accessible as on the membrane-bound molecule. Alternatively, molecules that have the appropriate receptors may interfere with one another when in solution. Glycophorin inserted into liposomes was also able to block invasion of *P. falciparum* into uninfected erythrocytes, showing that membrane-bound glycophorin was able to competitively inhibit parasite invasion.

Of the six sugars represented on glycophorin, GlcNAc, NeuNAc, and GalNAc at 50 mM showed significant inhibition of invasion, whereas other sugars showed little effect. Weiss *et al.* (19) recently achieved similar results with the same sugars in blocking merozoite invasion. Others have found that these concentrations of the sugars are toxic to parasites in culture because the more mature stages are selectively permeable to many types of small molecules, including sugars such as GlcNAc (20). We have shown that some of the sugars (GlcNAc, fucose) can delay maturation of parasites at high concentrations. However, when culture conditions were altered—i.e., medium changed frequently and parasites allowed an extended time in culture—equivalent numbers of mature schizonts were seen in both "toxic" and "nontoxic" sugar preparations at concentrations less than 20 mM. Finally, when schizonts in these cultures were washed free of the sugar and allowed to incubate a further 18 hr, viable merozoites, in numbers equivalent to normal cultures, invaded uninfected erythrocytes. Ultimately, the only way to unequivocally preclude "toxic" effects on developing parasites in the sugar experiments is to use merozoites. However, viable merozoites in sufficient quantity or purity have as yet not been obtained.

GlcNAc was coupled to albumin to exclude the entry of the sugar into the schizont-infected erythrocytes, which are more permeable. Remarkably, GlcNAc coupled to albumin was at least 100,000-fold more effective than the free sugar in inhibiting invasion of erythrocytes. Several explanations are possible for the difference in blocking seen with the monosaccharide, GlcNAc, compared with the albumin-coupled GlcNAc. The most reasonable one is that the sugar molecules are presented to the parasite in a conformation or at a focal concentration that allows them to compete with membrane-bound molecules such as found on glycophorin. A similar effect is seen in the sugar-binding lectin,

wheat germ agglutinin. Bhavanandan and Katlic (21) suggest that clusters of saccharides (glycoconjugates) are important for stable binding of the wheat germ agglutinin. Kobiler and Mirelman (22) found that the lectin binding of *Entamoeba histolytica*, which is GlcNAc specific, was most effectively blocked by oligosaccharides of GlcNAc, particularly trimers and tetramers. *P. falciparum* may also avidly bind to clusters of specific sugars present on the glycophorin molecule. The dramatic inhibition of invasion by some of the sugars present on erythrocyte glycoproteins that we have shown suggests that *P. falciparum* merozoites recognize and bind by lectin-like structures in the course of entry into erythrocytes.

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