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Role of the Carbohydrate Domains of Glycophorins as Erythrocyte Receptors for Invasion by *Plasmodium falciparum* Merozoites

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Solubilized preparations of purified glycophorins and specific domains of these molecules were assessed for their effects as inhibitors of *Plasmodium falciparum* invasion of human erythrocytes in vitro. The ability of newly invaded merozoites to continue developing and incorporating [^{3}H]hypoxanthine during a 24-h period after their invasion was used as an assay for merozoite invasion. Glycophorins A, B, and C were found to be equally effective as inhibitors. Previous studies had shown *N*-acetylglucosamine, a sugar component of glycophorins A and C but not B, to be an effective inhibitor. Accordingly, molecular domains common to all of the glycophorins were further assessed. Sialic acid was shown to act almost as effectively as *N*-acetylglucosamine, presumably because of the structural similarities between these sugars. The inhibitory ability of sialic acid is considerably enhanced when presented to the parasite in a clustered form, as in an oligosaccharide. The acetyl group of these sugars does not appear to play an essential role in this inhibition. How the *P. falciparum* merozoite recognizes and interacts with the sugar domains of the glycophorin molecule remains to be determined.

Plasmodium species have a high degree of host specificity, and much of this specificity can be accounted for by merozoite recognition of specific membrane characteristics of the erythrocyte (RBC) being invaded (10, 35). A series of recent studies have implicated the glycophorins (Gp's) as the RBC surface components that interact with *Plasmodium falciparum* merozoites. Gp's are sialic acid-rich glycoproteins that traverse the RBC membrane; the major ones have been isolated and characterized as Gps A, B, and C (GpA, GpB, and GpC, respectively by Furthmayr (18).

The types of studies that have been used to suggest a receptor role for Gp's have been on (i) the susceptibility of genetically defined variant RBCs deficient in one or more Gp's (14, 24, 36, 39, 40), (ii) the susceptibility of RBCs treated with enzymes to remove Gp components (6, 7, 14, 24, 36, 39, 40, 42), (iii) the binding affinity between isolated Gp and merozoites or their components (26, 43), (iv) a receptor blockade in which monoclonal antibodies or lectins against specific determinants of the Gp molecule were used (39, 40, 43), (v) a receptor blockade in which exogenous soluble Gp (or its components) was used as a competitive inhibitor of invasion (12, 27, 42, 43, 45, 46, 52; S. K. Gupta, S. Schulman, and J. P. Vanderberg, J. Protozool., in press).

Our own approach to this problem has focused on the last of these, namely competitive inhibition of merozoite invasion by means of substances that might mimic the actual RBC receptor molecule. These include isolated Gp's, components of the Gp molecule, and compounds resembling active domains of the receptor molecule.

MATERIALS AND METHODS

Culture of parasites. Clone A-2 of the FCR-3 strain (51) of *P. falciparum* was cultured in petri dishes (60 by 15 mm; Becton Dickinson Labware) by the procedure of Trager and Jensen (50). The dishes were maintained at 37°C in a gas-controlled Heraeus Incubator at 5% O_2 and 3% CO_2. Infected cells were standardly maintained at a 5% cell suspension in a complete medium made up of RPMI 1640 (GIBCO Laboratories) with TES [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; Sigma Chemical Co.] supplemented with hypoxanthine (50 μg/ml; Sigma), reduced glutathione (600 μg/ml; Sigma), glucose (2 mg/ml; Sigma), gentamycin (40 μg/ml; Schering Corp.), and 10% (vol/vol) human serum (54). Type A^+ RBCs and serum were obtained from the Greater New York Blood Center.

Effects of exogenous substances on schizont rupture and merozoite invasion of new RBCs. Each experiment was initiated with schizont-infected RBCs that had been sharply synchronized by repeated sorbitol treatments (29) and plasmagel separations (41). We determined the inhibitory effects of candidate substances by assessing [^{3}H]hypoxanthine incorporation into the cultures. Accordingly, we omitted unlabeled hypoxanthine from culture media (modified complete medium) used in these assay experiments. RBCs infected with the synchronous schizonts were mixed with freshly washed uninfected RBCs and modified complete medium to yield culture concentrations of 0.5% parasitemia, 2.5% cell suspension, and 20% (vol/vol) serum. The cell suspension was added to flat-bottom 96-well microtiter plates (Becton Dickinson Labware) in portions of 0.1 ml per well. Substances being tested were dissolved in modified medium at appropriate dilutions, and 0.1-ml portions were added to the cell suspensions in the microtiter wells to yield final concentrations of 0.5% parasitemia, 1.25% cell suspension, and 10% serum. In each experiment, triplicate cultures for each substance and concentration were allowed to incubate overnight; during this time, parasites in control cultures pass from the schizont stage to the formation of new rings. Assessment of the degree of merozoite invasion and new ring formation was done by measurement of [^{3}H]hypoxanthine incorporation during the subsequent 24-h incubation period, as previously described (45). Thus, parasite incorporation of [^{3}H]hypoxanthine reflects the number of merozoi-

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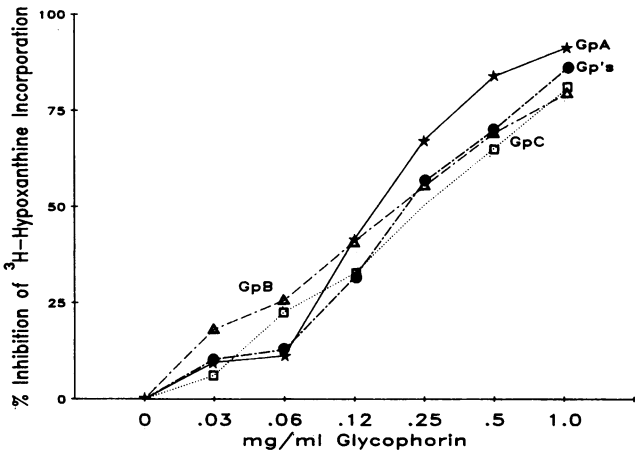

FIG. 1. Inhibitory effects of purified Gp's on *P. falciparum* cultures during schizont to ring stage differentiation. Gp's, mixture of glycophorins.

tes that are released from mature schizonts and that are successful in invading and developing in new RBCs.

The experiments were replicated on at least two subsequent occasions. The mean and standard deviation of the mean inhibition of [^{3}H]hypoxanthine incorporation were calculated for each concentration of each substance and subsequently plotted on a graph. To compare different substances, we determined their 50% inhibition values; this is defined as the amount of material (interpolated from the plot) that will inhibit 50% incorporation of [^{3}H]hypoxanthine into the cultures. The significance of differences between mean 50% inhibition values for different inhibitors was tested by an analysis of variance.

Assessment of merozoite invasion also was made by a morphological determination of parasite differentiation by microscopic examination of Giemsa-stained slides. Slides were examined to determine the effects of inhibitory agents on parasitemia, which is presented as parasites per 100 RBCs. To determine the stage distribution of parasites, at least 100 parasites per slide were examined and scored as rings, trophozoites, or schizonts. The indicator of transition to the trophozoite stage was the presence of a pigment granule that could be readily visualized by polarized light microscopy.

The intracellular toxicity of materials found to be inhibitory was determined as previously described (Gupta et al., in press). In brief, synchronized cultures containing ring stages were incubated with various concentrations of the test materials together with [^{3}H]hypoxanthine for the 24-h period leading to the formation of mature schizonts. The cultures were then harvested, and [^{3}H]hypoxanthine incorporation was determined as described above. Parallel morphological observations were made on the ability of parasites to differentiate into schizonts and to release merozoites in the presence of these agents.

Sugars and glycoproteins. *N*-Acetylglucosamine (GlcNAc), *N*-acetylneuraminic acid (NeuNAc), *N*-glycolylneuraminic acid (NeuNGc), and *N*-acetylneuramin-lactose (NeuNAc-Lac) were from Sigma. Colominic acid was purified from *Escherichia coli* K-235 culture media (4). Fetuin (type IV) and ovomucoid (type III-O) were from Sigma.

Gp's. Gp from human RBCs was prepared from outdated units of O^+ RBCs (kindly supplied by the New York University Hospital Blood Bank) by the procedure of Marchesi and Andrews (32). Gp's were similarly isolated from sheep and horse RBCs obtained from Rockland Inc., Gilbertsville, Pa.

Purified Gp's from human RBCs. GpA, GpB, and GpC were isolated and purified as previously described (18). In brief, a crude glycoprotein fraction was prepared by suspension of RBC membranes in a solution of lithium diiodosalicylate (LIS), followed by partition in a phenol-water mixture. GpA was separated from the other sialoglycoproteins in this fraction by gel filtration in Ammonyx-LO (Onyx Chemical Co.) on a Bio-Rad A 1.5. The first peak (A) was collected; the subsequent peaks (B and C) were further resolved by preparative polyacrylamide gel electrophoresis in sodium dodecyl sulfate. After cutting out the gel strips containing the desired protein bands, the glycoproteins were extracted from the gel, and contaminating materials were removed by filtration, dialysis, and washes in acetone and ethanol.

Desialyzation. The various Gp preparations were desialylated enzymatically rather than by acid hydrolysis to avoid denaturation of these molecules. Ten milligrams of Gp in 5 ml of 0.1 M acetate buffer (pH 5.0) was treated with 5 U of insoluble neuraminidase (from *Clostridium perfringens*, Sigma type X-A, attached to beaded agarose) for 20 h at 37°C. The beads had been washed and equilibrated in the buffer before this use. After enzyme treatment, the Gp was separated from the beads by suction filtration and further washing of the beads with acetate buffer. The Gp-containing washes were pooled and concentrated to a 5-ml volume by ultrafiltration (PM-10 membrane; Amicon Corp.). The concentrate was then loaded and run on a 50-ml Sephadex G-25 (Pharmacia Fine Chemicals) column, which was equilibrated and eluted with water. Fractions containing protein were pooled and lyophilized. Determination of sialic acid levels before and after enzyme treatment of Gp's (25) showed that 75 to 80% of the sialic acid was removed. Desialyzation of fetuin was done in a similar manner.

Preparation of tryptic peptides. Tryptic peptides (AT1, AT2, and CT1) were prepared and isolated as previously described (18, 19). In brief, the sialoglycoproteins (GpA and GpC), prepared as described above, were incubated with trypsin (treated with tosylamido-2-phenyl ethyl chloromethyl ketone) for 20 h at 37°C. After removal of insoluble peptide material, the released soluble peptides were separated by column chromatography on Ultrogel AcA 54 (LKB Instruments, Inc.), and the resulting peaks were further purified on a desalting Biogel P2 column and by ion-exchange chromatography on DEAE-cellulose. Alternatively, tryptic peptides were purified from supernatants after digestion of intact RBCs with trypsin. The major peptides obtained and used were the amino-terminal peptide products from GpA (T1 and T2) and GpC (T1). The purity of these peptides was confirmed by amino acid analysis, amino-terminal analysis, and partial amino acid sequence studies.

RESULTS

We found that a mixture of Gp's prepared from human RBCs by the LIS extraction procedure (32) gave inhibitory results similar to those obtained with the electrophoretically separated and purified glycophorins, GpA, GpB, and GpC, which comprise this mixture (Fig. 1; Table 1). No significant differences were found between the mean 50% inhibition values of any of the purified Gp's. However, the amino-terminal tryptic peptides of these glycophorins (T1 and T2 of GpA and T1 of GpC) had considerably reduced inhibitory ability (Fig. 2; Table 1). The 50% inhibition levels of these

TABLE 1. Inhibitory effects of glycoconjugates on *P. falciparum* cultures during schizont to ring stage differentiation

Glycoconjugates	Concn needed[a] for 50% inhibition (mg/ml)
Human Gp mixture (Gp's)	0.21 ± 0.07 (6)
GpA	0.16 ± 0.09 (4)
GpB	0.20 ± 0.13 (10)
GpC	0.25 ± 0.15 (4)
GpA-T1	>1.0 (3)
GpA-T2	>1.0 (3)
GpC-T1	>1.0 (3)
Sheep Gp mixture	0.27 ± 0.08 (3)
Horse Gp mixture	>1.0 (3)
Desialylated human Gp	0.70 ± 0.21 (3)
Desialylated sheep Gp	0.68 ± 0.12 (3)
Desialylated horse Gp	0.54 ± 0.10 (3)
Fetuin	>1.0 (3)
Desialylated fetuin	>1.0 (3)
Ovomucoid	>1.0 (3)

[a] ± standard deviation of means of individual experiments. The number of experiments is within parentheses.

peptides could not be measured since these levels were not reached by the highest concentrations of peptides tested (1 mg/ml). Human Gp was not significantly more inhibitory than sheep Gp, whereas horse Gp was strikingly less inhibitory than either of these (Fig. 3; Table 1). The comparative 50% inhibitory values for these similarly prepared materials were 0.21 mg/ml for human Gp, 0.27 mg/ml for sheep Gp, and >1 mg/ml for horse Gp. Desialyzation of human and sheep Gp's significantly reduced ($P < 0.001$) but did not entirely eliminate their inhibitory ability (Fig. 3; Table 1). Curiously, desialyzation of horse Gp considerably enhanced its inhibitory ability. The serum sialoglycoprotein, fetuin, as well as desialylated fetuin, showed no significant inhibitory ability. Similar noninhibitory results were obtained with the GlcNAc-rich glycoprotein, ovomucoid (Table 1).

All of the sugars tested (GlcNAc, NeuNAc, NeuNGc, and the trisaccharide, NeuNAc-Lac) showed high inhibition (Fig. 4; Table 2). GlcNAc was significantly more inhibitory than NeuNAc ($P < 0.01$), but no significant differences were found between the two types of sialic acid (NeuNAc versus NeuNGc).

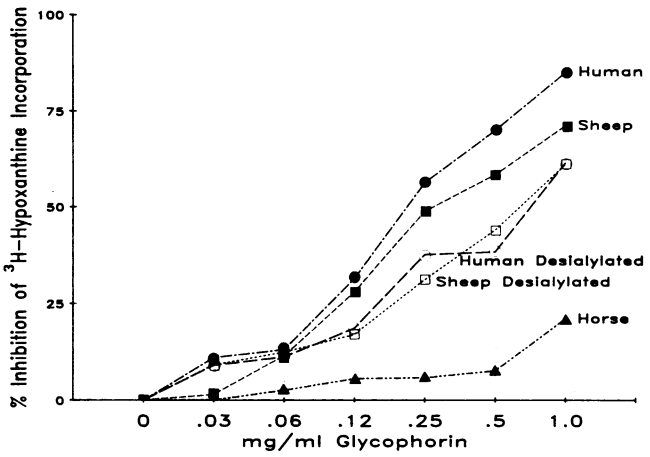

FIG. 3. Inhibitory effects of different species of Gp on *P. falciparum* cultures during schizont to ring stage differentiation. Human, sheep, and horse Gp and desialylated Gp's as indicated.

The NeuNAc polymer, colominic acid, had a 50% inhibitory value of 1.13 mg/ml (Fig. 5; Table 2); this amount of material, if completely reduced to the NeuNAc monomer, would have a concentration of 3.7 mM. Inasmuch as the 50% inhibitory value for the actual free monomer was found to be 23.0 mM, it follows that the inhibitory ability of NeuNAc is considerably enhanced when presented to the parasite as a polymer. When the actual molecular weight of colominic acid (3,000 to 4,000) was taken into consideration (34), the 50% inhibitory value of this polymer was shown to be 280 to 370 μM.

Studies on intracellular toxicity established that NeuNAc caused only a 12% inhibition of [^{3}H]hypoxanthine incorporation at 20 mM. No intracellular toxicity was detected at lower concentrations of the sugar. Morphological observations showed that ring stage parasites cultured in 20 mM NeuNAc for 24 h matured normally into schizonts that released merozoites.

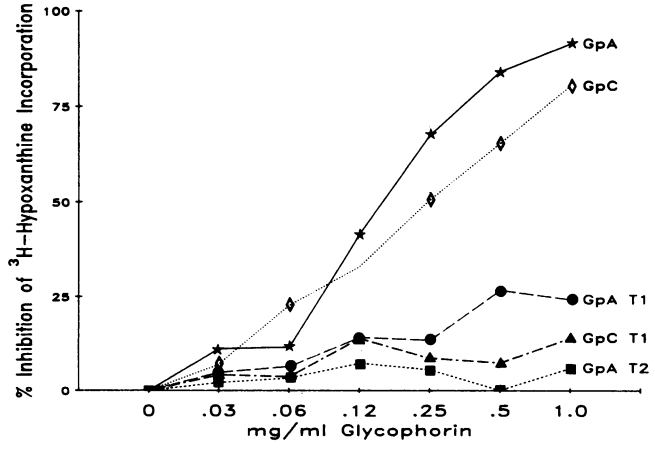

FIG. 2. Inhibitory effects of tryptic glycopeptides of Gp's on *P. falciparum* cultures during schizont to ring stage differentiation.

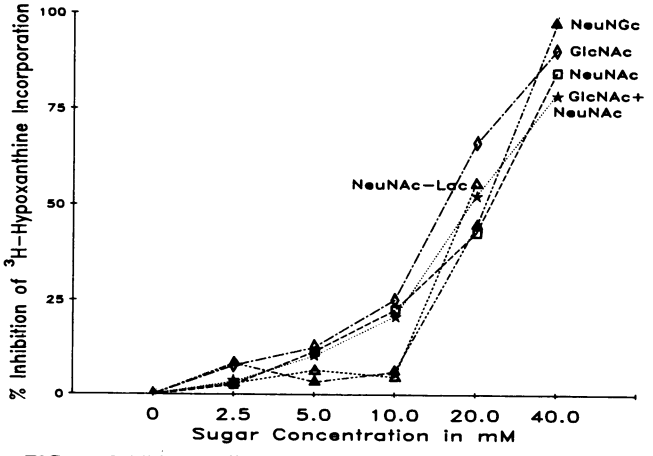

FIG. 4. Inhibitory effects of sugars on *P. falciparum* cultures during schizont to ring stage differentiation. GlcNAc + NeuNAc, Concentration = total molarity of equimolar amounts of two sugars.

TABLE 2. Inhibitory effects of simple and complex saccharides on *P. falciparum* cultures during schizont to ring stage differentiation

Saccharides	Concn needed[a] for 50% inhibition
GlcNAc	15.4 ± 3.9 mM (13)
NeuNAc	23.0 ± 3.2 mM (7)
GlcNAc + NeuNAc	19.2 ± 4.1 mM[b] (3)
NeuNGc	22.0 ± 2.0 mM (3)
NeuNAc-Lac	19.0 ± 6.9 mM (2)
Colominic Acid (actual concn)	1.13 ± 0.4 mg/ml (5)
Colominic Acid (based on mol wt of monomer)	3.7 mM
Colominic Acid (based on mol wt of polymer)	280–370 μM

[a] ± standard deviation of means of individual experiments. The number of experiments is within parentheses.
[b] Total molarity of mixture.

DISCUSSION

Previous studies implicating the Gp's as receptors. Gp's are sialic acid-rich glycoproteins that traverse the RBC membrane. Furthmayr (18) demonstrated that human RBC membranes contain at least three different sialoglycoproteins, which he named GpA, GpB, and GpC.

(i) Differences in susceptibility among different RBC variants. The first indication that sialoglycoproteins might be implicated in the invasion of RBCs by *P. falciparum* was the finding that En(a−) cells lacking the major sialoglycoprotein of the RBC, GpA, had a reduced susceptibility to *P. falciparum* (36). A number of other studies with variant RBCs have been used to further implicate the Gp's. S-s-U- cells (lacking GpB) have been shown to have a reduced susceptibility (14, 39), thus suggesting a role for this sialoglycoprotein in the invasion process. However, results with these variants have not been uniform; some workers (24, 36) found S-s-U- cells to be as susceptible as a normal RBCs in their studies. One also must recognize that these variant RBCs are altered in more ways than merely lacking a given Gp molecule. En(a−) cells, for instance, not only lack GpA but also have a reduced amount of GpB. Furthermore, there are concomitant abnormalities in the glycosylation of the membrane glycoprotein, band 3, as well as abnormalities in the exposed membrane glycolipids of En(a−) cells. Similarly, S-s-U- cells have modifications other than a mere lack of GpB (2, 14). Finally, the relative insusceptibility of variant RBCs may be due to factors unrelated to their surface components, such as reduced deformability, an unfavorable cytoplasmic environment, etc.

An additional variant, Wr^{b-} cells (which lack the Wr^b determinant on the GpA molecule, has been reported to be refractile to *P. falciparum* invasion (39). However, more recent work has failed to confirm this finding (47).

(ii) Removal of receptor components by enzymatic treatment of RBCs. Enzymatic treatment of RBCs also has been used to implicate sialoglycoproteins as receptors for *P. falciparum* merozoites. Both neuraminidase treatment of normal RBCs (which removes sialic acid residues) and trypsinization of normal RBCs (which removes components of GpA and GpC) reduced the susceptibility of human RBCs to invasion by this parasite (36). Further studies on the enzymatic treatment of RBCs confirmed these findings (6, 7, 14, 42). Enzyme digestion studies (14) have been used to suggest a receptor role for GpC in the following way. The partial susceptibility of En(a−) RBCs has been argued as being due to the presence of GpB acting as a receptor in these GpA-lacking cells (40). Nevertheless, trypsinization (which removes GpA and GpC but not GpB) further reduces the susceptibility of En(a−) cells by another 50% (14). Because these cells have no GpA to start with, Facer (14) suggested that components present on GpC also are involved in invasion.

However, these enzyme digestion results can be regarded as circumstantial rather than conclusive evidence of a receptor role for Gp's. Experimental results have varied considerably among different laboratories. For instance, some laboratories have reported that chymotrypsin treatment of RBCs results in a considerable reduction of susceptibility (14, 42); other laboratories have reported no reduction (6, 36). It is too simplistic to suggest that enzyme treatment of RBCs does nothing more than surgically remove specifically defined domains of Gp molecules. Other surface components of the RBC presumably also are affected. In addition, a reduction in susceptibility is as likely due to changes in the tertiary structure and topography of the components remaining after enzyme treatment.

Soluble Gp as a competitive inhibitor. That soluble Gp's from RBC membranes could inhibit *P. falciparum* merozoite penetration of RBCs, presumably by competitive inhibition, was first shown by Weiss et al. (52). These workers suggested, however, that in view of the relatively high levels of Gp required for inhibition, their findings could not be considered to be definitive evidence that Gp was the receptor. Other studies similarly have reported the inhibitory effects of soluble Gp on merozoite invasion (7, 12, 27, 46). In these studies, soluble Gp was allowed to remain in the culture medium for the period from schizont rupture to new ring formation. The degree of merozoite invasion was measured either by a morphological assessment of new rings formed or by the ability of these newly invaded parasites subsequently to incorporate a labeled metabolic precursor. These results are difficult to compare with one another directly, in view of the different procedures used to isolate the Gp's and the different inhibition assay methods used. However, on the order of 0.1 to 1.0 mg/ml was generally required for a ca. 50% inhibition level. Using a different procedure, in which isolated merozoites were incubated with soluble Gp's before adding them to uninfected RBCs, Perkins (42, 43) reported a comparable level of inhibition with as little as 10 μg of Gp

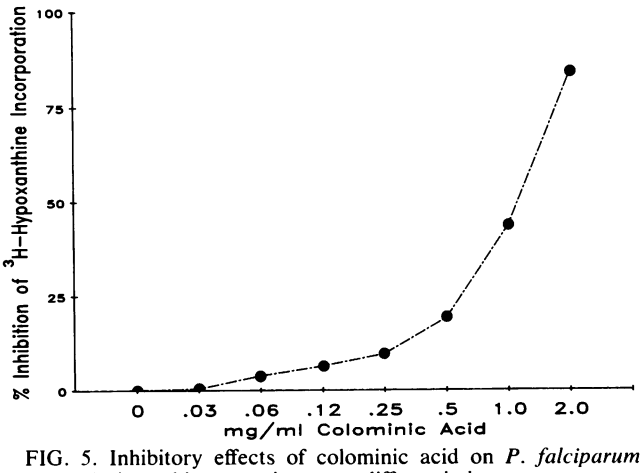

FIG. 5. Inhibitory effects of colominic acid on *P. falciparum* cultures during schizont to ring stage differentiation.

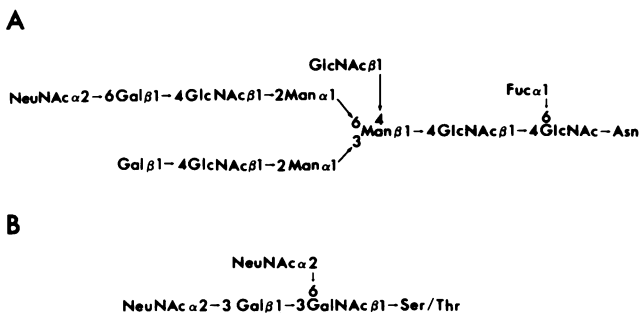

FIG. 6. Structure of chief oligosaccharides associated with GpA. (A) *N*-linked oligosaccharide; (B) major *O*-linked oligosaccharide. Gal, Galactose; Man, mannose; Fuc, fucose; Ser, serine; Thr, threonine; Asn, asparagine.

per ml. However, in view of the procedures used to assay merozoite invasion, the validity of these latter findings are questionable (Gupta et al., in press).

Furthmayr (18) demonstrated that GpA, GpB, and GpC from human RBC membranes could be isolated and characterized by a sequence of procedures, including gel filtration in the presence of a detergent, followed by preparative sodium dodecyl sulfate-gel electrophoresis. When we tested materials isolated in this manner, we found no significant differences between the inhibitory effects of a mixture of Gp's and those of the isolated, purified GpA, GpB, or GpC, thus suggesting that all of these Gp's may have the capacity to serve as receptors for *P. falciparum*; this confirms the previous findings made with variant RBCs and with enzymatically altered RBCs.

Previous studies that compared the inhibitory capacities of what were described as purified Gp's (42, 43) must be interpreted with caution, since the separation procedures used are not known to isolate these distinct Gp types, as defined by Furthmayr (18). Various agents have been used to extract sialoglycoprotein fractions from solubilized RBC membranes. These include chloroform-methanol (23), butanol (3), and LIS extraction (32). When the LIS procedure was first described, the resulting sialoglycoprotein fraction was assumed to be a single molecular species, which was given the name erythrocyte Gp (33). It later became clear that this preparation contained a mixture of distinct sialoglycoproteins. The major one, which could be separated out by gel filtration only in the presence of a detergent, was designated as GpA (23). There is no evidence that the procedure used by Perkins (42), chloroform-methanol extraction, followed by Sephadex G-100 filtration without detergents, can be used to prepare purified GpA. The evidence used to establish the purity of this GpA fraction was an electrophoretogram that showed multiple sialoglycoprotein bands in a whole RBC membrane extract, but only bands characteristic of authentic GpA in the column-purified fraction. However, 300% more whole membrane extract was applied to the gel to demonstrate the non-GpA bands. Thus, the purified GpA fraction was likely contaminated with other Gp's that could not be resolved because insufficient quantities were applied to the gels. Similarly, a later study (43) that purported to test GpB obtained this material by chloroform-methanol extraction of membranes from trypsin-treated RBCs. This material presumably includes the trypsin-insensitive portions of GpA and GpC as well and should more accurately be referred to as a GpB-enriched fraction.

Structure and domains of the Gp's. A brief review of the structures of Gp's is essential before a consideration of how these Gp's may interact with the *P. falciparum* merozoite. The Gp's of the human RBC have been shown to have a number of similarities as well as differences in their molecular structure.

GpA, the major sialoglycoprotein fraction (glycoprotein alpha according to the terminology of Anstee [1]) consists of a single polypeptide chain of 131 amino acids, with a tripartite molecular structure (49). The amino terminus of this protein is exposed to the outside of the RBC membrane; this segment, consisting of ca. 70 amino acids, possesses all of the carbohydrate (33). The protein then traverses the lipid bilayer through a hydrophobic region of ca. 20 amino acids, and finally there is a hydrophilic segment at the carboxy terminus on the cytoplasmic face of the membrane. GpA is made up of ca. 60% carbohydrate, which consists of 15 *O*-glycosidically linked oligosaccharides and a single *N*-linked one. The *O*-linked oligosaccharide (linked to serine or threonine residues in the polypeptide chain) has the structure originally proposed by Thomas and Winzler (48) (Fig. 6B). The *N*-linked oligosaccharide (present at the asparagine-26 position) has been shown (53) to have predominantly the structure illustrated in Fig. 6A. There appears to be some degree of heterogeneity in these structures. For example, some forms of the *N*-linked oligosaccharide have a terminal NeuNAc residue on both of the chains branching from mannose (53).

When RBCs are treated with trypsin, they release two main amino-terminal glycopeptides (T1 and T2) from the GpA molecule (18). These tryptic glycopeptides are identical to corresponding T1 and T2 glycopeptides released after trypsinization of isolated soluble GpA (19). T1 consists of amino acids 1 to 39, and T2 is made up of amino acids 1 to 31 (both with accompanying glycosylation).

GpB (glycoprotein delta according to the terminology of Anstee [1]) has an amino-terminal amino acid sequence identical to that found in the blood group N form of GpA for the first 26 amino acid positions (18, 19). The glycosylation sites of both GpA and GpB also are similar, except for the important difference that GpB lacks the *N*-linked oligosaccharide at asparagine 26. This is consistent with the absence of mannose and GlcNAc from GpB (18). Another significant

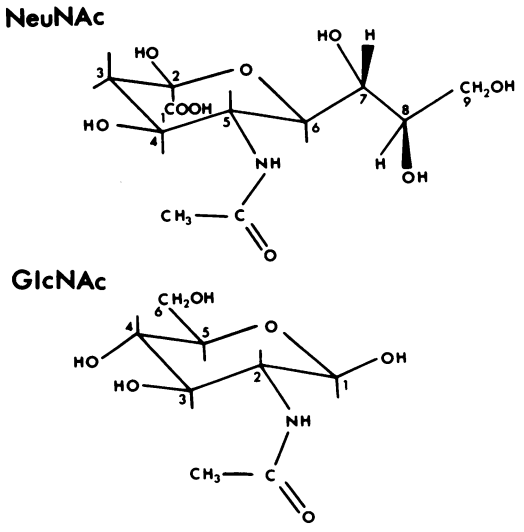

FIG. 7. Comparative structures of GlcNAc and NeuNAc in the pyranose form.

difference is the insensitivity of GpB on intact RBCs to tryptic digestion.

GpC (glycoproteins beta and gamma according to the terminology of Anstee [1]) remains poorly characterized. The amino-terminal sequence is entirely different from that found in GpA and GpB. The molecule, however, is sensitive to trypsin. Trypsinization of intact RBCs releases an amino-terminal glycopeptide identical to the T1 glycopeptide released by trypsinization of isolated soluble GpC (18). GpC contains fucose, mannose, and GlcNAc (18), thus suggesting that presence of a *N*-glycosidically linked oligosaccharide similar to that found at position 26 in GpA. Lectin-binding studies suggest the presence of *O*-linked oligosaccharides similar to those characterized in GpA (1).

Exogenous sugars as competitive inhibitors. The specificity of cell surface interactions involving glycoproteins is often determined by the carbohydrate moieties of the glycoprotein. This specificity can be taken advantage of by using exogeneous carbohydrates to competitively inhibit the binding between cell surface and the macromolecules that interact with them. Accordingly, a study (52) was designed to test the ability of sugars that are components of the Gp molecule to inhibit invasion of RBCs by *P. falciparum* merozoites. The findings showed that GlcNAc had a striking inhibitory effect, which we attributed to competitive inhibition. It was concluded that "*N*-acetylglucosamine may be an important component of the glycoprotein receptor involved in recognition and penetration of human erythrocytes by *Plasmodium falciparum* merozoites." Other workers have confirmed the inhibitory effects of GlcNAc on *P. falciparum* cultures (7, 24, 27).

Interpretation of these results has been rendered somewhat more difficult by the toxic effect shown by high concentrations of GlcNAc on intracellular stages of the parasite (24). However, more recent work has demonstrated that this toxic effect of the sugar on intracellular parasites can be separated from its clear effect on inhibition of merozoite invasion. The evidence for this is as follows.

(i) We have shown that the intracellular toxic effects of GlcNAc is stage related and is seen at high levels of this sugar. At lower levels of the sugar (20 mM), parasites are capable of developing intracellularly and releasing merozoites. The block at these lower concentrations of GlcNAc is clearly at the level of merozoite penetration (Gupta et al., in press). These results are almost identical to the confirmatory results of Jungery et al. (27).

(ii) The intracellular toxicity observed at high levels of the sugar is fully compatible with a more specific effect on inhibition of merozoite penetration at lower levels. There are many biological precedents for a molecular species to act specifically at one concentration, yet nonspecifically at a higher concentration. For instance, cyanide is considered to specifically inhibit cytochrome oxidase at levels of 10^{-3} M. At levels above this, it begins to inhibit multiple metabolic activities and enzyme systems. Thus, the fact that cyanide is a general metabolic poison at high concentrations in no way argues against its specific anti-cytochrome oxidase levels at lower concentrations.

(iii) The first paper on intracellular toxicity of GlcNAc was that of Howard et al. (24), who reported that schizonts do not mature and release merozoites even at low concentrations of GlcNAc. These results, however, are open to question, since the investigators used chimpanzee RBCs that had been infected, frozen, and thawed before exposure to GlcNAc. Such treatment of RBCs results in abnormal fragility of the cells.

(iv) The methyl glycoside of GlcNAc, which presumably does not penetrate the intracellular parasite, has been found to be inhibitory to merozoite invasion but not toxic to the intracellular parasite (P. Hermentin and B. Enders, Behringer Inst. Mitt., in press).

(v) Poly-GlcNAc-neoglycoproteins, which also presumably do not penetrate the parasite, have a high degree of inhibition of merozoite invasion (45). The specificity of the sugar was shown by the fact that poly-GalNAc-neoglycoproteins have no such inhibitory effect.

(vi) Sialic acid, which appears to compete for the same blocking site on the merozoite, is as inhibitory to merozoite invasion as GlcNAc but has virtually no intracellular toxic effect, presumably because of its failure to enter the intracellular parasite (this study).

(vii) The final argument in favor of the intracellular toxicity hypothesis is the report of Perkins (43) that GlcNAc has no inhibitory effects in her invasion system. However, as pointed out previously (Gupta et al., in press), there are serious questions about the validity of the assay system used by Perkins to arrive at her conclusions.

Tests with sugar components of Gp other than GlcNAc have not always yielded clear-cut results among different investigators. For instance, GalNAc has been reported to have significant inhibitory effects by some workers (24, 27) and no significant inhibition by others (7, 52). Of particular interest is NeuNAc, the most prevalent sugar component of Gp and the sugar that gives the RBC its negative surface charge. In studies with *P. falciparum* cultures, NeuNAc has been reported to have no inhibitory effects (12), "only negligible inhibition" (7), and "significant inhibition" (27). Accordingly, we tested this sugar in its monomeric form and in its glycosidic form as a component of oligosaccharides. Our results with NeuNAc (Fig. 4) were remarkably similar to those reported by each of the above groups of researchers, the results being entirely dependent upon the concentration of NeuNAc used. At the extremely low μM levels tested by Deas and Lee (12), we too found no inhibition of *P. falciparum* cultures. At 10 mM, we found a 26% inhibition, compared with the 20% inhibition reported by Breuer et al. (7) at this concentration of NeuNAc. Finally, we found a 50% inhibition level at 24 mM, compared with 25 mM reported by Jungery et al. (27). These findings demonstrate that conclusions based on the testing of a single concentration of an inhibitor may be misleading and that dose-response curves, using a series of dilutions, generally give more useful information. The inhibitory results that we obtained with oligosaccharides containing NeuNAc (including NeuNAc-Lac and colominic acid) confirm the likely importance of NeuNAc in merozoite-RBC interactions.

Native versus desialylated Gp's from other mammalian species. Our finding that sheep Gp's had only slightly less inhibitory ability than human Gp's suggests that both have inhibitory (and presumably receptor) components that are relatively nonspecific in nature. The refractoriness of sheep RBCs to *P. falciparum* merozoites indicates that invasion and subsequent growth of the parasite involve a multiplicity of steps and that mere possession of a receptor-like molecule by a target cell is not sufficient for invasion. Similarly, human K562 erythroleukemic cells, which express GpA or a closely related molecule on their surface, are refractory to invasion (14, 38). It would be interesting to determine whether the initial steps of merozoite recognition and attachment take place with sheep RBCs or K562 cells. Perhaps the block to subsequent development of the parasite takes place at a later step. Breuer et al. (6) also have reported that sheep

Gp's have inhibitory ability in vitro, but these authors did not present any data on the degree of this inhibition. Our results, and those of Breuer et al. (6), are different from those of others (12, 43), who reported that sheep Gp's have no inhibitory ability.

The significantly lower inhibitory effects that we have shown for horse Gp's suggest that horse Gp's are fundamentally different from those of humans and sheep. Accordingly, a comparison of the structure of horse Gp's with those of humans and sheep may be helpful in defining the role of specific domains of Gp's in their recognition of *P. falciparum* merzoites. One of the chief differences is the presence of NeuNGc rather than NeuNAc residues as the major sialic acid of horse Gp's (9). However, when we tested these sialic acids as exogenous monomers, we found no significant differences between NeuNAc and NeuNGc, thus indicating that the reason for the low inhibitory ability of horse Gp's must lie elsewhere. We observed that the inhibitory ability of horse Gp's is enhanced by removal of sialic acid; this is similar to observations made with horse Gp's used to inhibit the agglutination of human group N RBCs induced by the anti-N lectin derived from *Vicia graminea* (30, 31). Desialyzation of horse Gp's greatly increases their ability to inhibit this hemagglutination. Presumably, the removal of sialic acid from horse Gp's uncovers domains that have an enhanced capacity to inhibit *P. falciparum* merozoite invasion as well as *Vicia*-induced hemagglutination. Initial studies characterizing the oligosaccharide units of horse Gp have been reported (17). Further work on elucidation of these structures may give useful clues in identifying those domains of the Gp molecule that are capable of interacting with the *P. falciparum* merozoite ligand.

Role of sialic acid. Our results on desialyzation of isolated Gp's together with our results on sialic acid and sialic acid-containing oligosaccharides point to the important role of sialic acid domains in invasion of RBCs by *P. falciparum* merozoites. The significant reduction, but not total elimination, of the inhibitory ability of human and sheep Gp's by enzymatic removal of sialic acid residues suggests that sialic acid contributes to, but may not be exclusively responsible for, the receptor capability of Gp. These results are compatible with previous results showing that variant RBCs expressing a reduced amount of sialic acid on their surface had a reduced susceptibility to *P. falciparum* (38). Furthermore, there is a general relationship between the amount of sialic acid removed from RBCs by neuraminidase and the reduction in susceptibility of these RBCs (6, 16). Breuer et al. (6) have suggested that this is a threshold phenomenon, with no significant inhibition occurring until more than 50% of the sialic acid has been removed. Friedman et al. (16) have reported that orosomucoid, a sialoglycoprotein found in serum, can restore much of the susceptibility lost by desialylated RBCs; they have proposed that this is attributable to a restoration of the negative charge of the RBCs lost during desialyzation.

Sialic acid has been suggested by most workers to play only a minimal role in interactions between the RBC and the parasite (7, 12, 14). To some degree, these conclusions were arrived at by acceptance of the reports of Deas and Lee (12) that exogenous sialic acid does not inhibit *P. falciparum* cultures. Unfortunately, the micromolar amounts of NeuNAc tested by Deas and Lee (12) were insufficient to support this conclusion. The data we have presented indicate that sialic acid likely plays a significant role as a component of a receptor for *P. falciparum* merozoites. There are various ways in which this negatively charged sugar could act as a component of the Gp molecule. Cabantchik (11) has pointed out the need to distinguish between the overall charge of the cell imparted by the sugar residue (charge density) and a charged group that may be necessary for merozoite-RBC interaction at a specific membrane site. Cabantchik (11) and Pasvol and Jungery (38) have discounted the likelihood that charge density of the RBC plays a major role in invasion by the merozoite. Sialic acid may be playing a more specific role in acting as a component of a RBC receptor. This could be due not only to the charge effect of the sialic acid as a component of a receptor site but also to its specific configuration.

Binding studies with the lectin, wheat germ agglutinin (WGA), may offer some instructive analogies. WGA has long been known to bind to GlcNAc and its oligomers. However, there also are some well-substantiated interactions that can involve NeuNAc in this binding. For example, the binding affinity of Gp's to WGA is reduced by two orders of magnitude after desialyzation of the Gp's (5). Furthermore, the monomer NeuNAc, as well as NeuNAc-containing oligosaccharides such as NeuNAc-Lac (without GlcNAc), are highly effective in inhibiting WGA-induced hemagglutination (5, 37). This activity of NeuNAc may be partially a function of this charge effect (37) but seems most likely due to the structural similarities between GlcNAc and NeuNAc (5, 37).

The structures of GlcNAc and NeuNAc are shown in Fig. 7. It has been postulated that the similar components most likely involved in binding are the ring oxygen, the equatorial acetamido group on C-2 of GlcNAc (corresponding to C-5 of NeuNAc), and the equatorial hydroxyl group on C-3 of GlcNAc (corresponding to C-4 of NeuNAc) (reviews in references 5 and 37). GalNAc, which shares all of these features, possesses a hydroxyl group on C-4 in axial position, which partially impairs its binding to WGA. This sugar has been reported also to have some inhibitory activity in the *P. falciparum* system (24, 27).

The inhibitory effects that we have shown for GlcNAc, NeuNAc, and their oligomers in our *P. falciparum* merozoite system are in many ways similar to the results demonstrated in the WGA binding studies and give further support to the hypothesis that a lectin-like parasite protein is involved in merozoite binding to RBCs (26, 27, 52). Although this putative lectin has some binding characteristics similar to those of WGA, there are also some notable differences.

(i) The dimer and trimer of GlcNAc (chitobiose and chitotriose) bind dramatically better to WGA than does the monomer. In the case of *P. falciparum*, these oligosaccharides are somewhat more inhibitory than GlcNAc itself, but the difference is not so great as is the case with WGA (24; Schulman et al., unpublished data). More recent studies indicate, however, that longer chain polymers of GlcNAc do have a more enhanced inhibitory effect (Schulman et al., unpublished data).

(ii) NeuNGc has no significant binding affinity to WGA (5, 37), whereas it is highly effective in the *P. falciparum* system. This suggests that there is no rigid requirement for an acetyl group in *P. falciparum* binding, as there seems to be for WGA binding. This relatively relaxed specificity of *P. falciparum* for sialic acids is similar to that of viral hemagglutinins, which also are capable of recognizing both acetylated and nonacetylated sialic acids (28).

(iii) The GlcNAc-rich glycoprotein, ovomucoid, has a high binding affinity to WGA but was ineffective in our *P. falciparum* system.

(iv) Colominic acid, a linear polysaccharide of NeuNAc, is highly effective as an inhibitor in our *P. falciparum* system but has no significant binding affinity to WGA (5). Whether this inhibitory effect of colominic acid is specifically due to the configuration of the NeuNAc residues or to the overall charge of this molecule (as postulated for other polyanions by Friedman [15]) remains to be determined.

Studies with glycopeptides of Gp. Our results showing that the tryptic amino-terminal glycopeptides of GpA and GpC had little inhibitory ability are similar to those reported by Breuer et al. (7) and were not entirely unexpected. In a study of the reactivity of these glycopeptides with their antibodies, Furthmayr (19) showed that the glycopeptides had a three to fourfold reduction of MN-blood group serological activity, on a molar basis, as compared with intact GpA and GpB molecules. This blood group activity is associated entirely with the five amino-terminal amino acids found on the peptides and intact Gp. The greater serological activity of the intact Gp is a consequence of interactions between the hydrophobic domains of Gp's, which results in the formation of micellar-like structures in aqueous solutions (22). This permits a clustering of the outward facing amino-terminal portions of these molecules, which enhances their ability to bind anti-M and -N antibodies. The lack of hydrophobic domains in the tryptic peptides prevents these peptides from clustering and exhibiting their full multivalent effect. A similar phenomenon seems to occur with regard to the ability of Gp to bind to influenza and encephalomyocarditis viruses (8). Intact soluble Gp's are able to competitively inhibit the hemagglutinating ability of both viruses. However, after chymotryptic digestion, the only Gp peptide that retained this ability was the one that included a hydrophobic segment; this led the authors to suggest that the aggregating ability of these peptides allowed strong multivalent binding with virus particles. The same phenomenon is likely responsible for the decreased ability of T1 and T2 glycopeptides to bind to *P. falciparum* merozoites.

Conclusions. Our results are compatible with the original suggestion (52) and with the findings of subsequent workers (7, 26, 27, 45; Gupta et al., in press) that lectin-like interactions between the *P. falciparum* merozoite and an RBC surface glycoprotein are involved in recognition and penetration of human RBCs by the parasite. One of the puzzling features of our original suggestion about the participation of GlcNAc in this interaction was that GpB, which does not possess this sugar, also may act as a receptor molecule. It now appears likely that the sugar specificity for this parasite-RBC interaction is a relatively relaxed one and that NeuNAc (as also suggested by Pasvol and Jungery [38]) also may participate in this interaction. Indeed, our findings indicate that NeuNAc is almost as effective an inhibitor as GlcNAc. Because GlcNAc and NeuNAc used together have an additive rather than a synergistic inhibitory effect, these sugars probably compete for the same sites on the *P. falciparum* merozoite. In a clustered configuration (as in colominic acid), NeuNAc is significantly more inhibitory than in its monomeric form. Since there is approximately six times as much NeuNAc as GlcNAc per molecule of GpA, NeuNAc may well play a more important role in merozoite-RBC interactions. The inhibitory activity of NeuNAc is not restricted to its free monomeric form; it also is expressed in the natural glycosidic form of the sugar (as in NeuNAc-Lac and colominic acid).

This relaxed specificity for sugar residues also may help to explain why the inhibiting ability of soluble Gp's and the susceptibility of whole RBCs are reduced but not entirely eliminated by neuraminidase treatment. Even after the removal of significant quantities of NeuNAc, the remaining GlcNAc and NeuNAc residues seem capable of interacting with the parasite. However, the mere presence of these sugars is not sufficient to confer receptor (or inhibitor) status on a molecule. Some components of the RBC membrane are rich in GlcNAc residues (macroglycolipids [13]; band 3 [20]), yet have no significant inhibitory ability (46). Other GlcNAc-rich glycoproteins, such as ovomucoid, show a similar lack of inhibitory ability (7; this paper). Likewise, glycoconjugates rich in NeuNAc, such as fetuin, also may fail to have inhibitory ability (7; this paper). Fetuin has been shown to exhibit an anomolous behavior in its interactions with WGA as well; it interacts quite efficiently with this lectin but is a poor inhibitor of cell to cell interactions, as in WGA-induced hemagglutination (5).

How the specific configuration of GlcNAc and NeuNAc determines their ability to interact with the *P. falciparum* merozoite remains to be resolved. Presumably, the manner in which the sugar residues are clustered within the glycoconjugate influences their interaction with complementary sites on the merozoite. This has been demonstrated in a positive manner by showing the enhanced inhibitory ability of GlcNAc residues clustered on albumin molecules (27, 45), and the enhanced inhibitory ability of NeuNAc residues clustered within the colominic acid molecule (this paper). It has been demonstrated in a negative manner by showing the reduction in inhibitory ability that occurs when the amino-terminal tryptic glycopeptides of Gp molecules are tested.

The way in which the sugar moieties of Gp present themselves on the surface of the RBC is unknown. Although the Gp molecule has been sequenced, little is known about its secondary or tertiary structure, its orientation on the RBC surface, or its interactions with other RBC surface molecules. Furthermore, there may be dynamic changes in its orientation after contact with the invading parasite. Prohaska et al. (44) have proposed a secondary structure for GpA, in which the most important feature is the grouping of the amino-terminal amino acid and one NeuNAc residue much above the rest of the molecule, thereby allowing NeuNAc to be readily accessible to the invading parasite; the parasite must come in contact with many such receptor molecules during this invasion. Assuming that each RBC has ca. 600,000 Gp molecules over a surface area of roughly 100 μm^2, there are ca. 6,000 Gp molecules per μm^2 of surface membrane in normal RBCs. Thus, a merozoite interacting with this surface may potentially come in contact with several thousand Gp molecules. A better understanding of how the parasite recognizes and interacts with the sugar domains of these molecules may lead to a way of isolating the parasite ligand responsible for this affinity, as well as a rational approach to interfering with this interaction by pharmacological means.

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