

# Terminal Sialic Acid Residues on Human Glycophorin A Are Recognized by Porcine Kupffer Cells

Christopher Burlak, Lisa M. Twining, and Michael A. Rees

**Background.** We have previously shown that recognition of human erythrocytes by porcine Kupffer cells is mediated by a carbohydrate-dependent mechanism. The present study explores the possible ligands existing on human glycophorin A and tests their ability to inhibit erythrocyte rosette formation.

**Methods.** Human erythrocytes were tested for ABO and MN specificity and used as targets in a <sup>51</sup>Chromium quantitative erythrocyte rosette assay. Monosaccharides present on human glycophorin A, neuraminyl lactoses, bovine and porcine submaxillary mucins (BSM and PSM), and hyaluronic acid as well as proteoglycan N-linked glycosidase F(PNGaseF)- and sialidase A-treated human erythrocyte glycoproteins (hEGP) and human erythrocytes were all tested for inhibitory potential in the rosetting assay.

**Results.** Porcine Kupffer-cell recognition of human erythrocytes was insensitive to differences in blood groups A, B, O, or MN. At 30 mM, the monosaccharide, N-acetylneuraminic acid, and the trisaccharide mixture, neuraminyl lactoses, disrupted human erythrocyte recognition by 25% and 30%, respectively. A dilution of BSM but not PSM inhibited the rosetting assay by 17% (.2 mg/mL), 33% (1 mg/mL), and 53% (2 mg/mL). The same dilution of hyaluronic acid had no effect on rosetting. Removal of N-linked oligosaccharides from hEGP with PNGaseF did not impair its ability to inhibit the rosetting assay. In contrast, removal of sialic acid completely abrogated its inhibitory ability. Treatment of whole human erythrocytes with sialidase A likewise prevented recognition by porcine Kupffer cells.

**Conclusions.** Terminal sialic acid on human erythrocytes is a target recognized by porcine Kupffer cells, suggesting a role for a sialic-acid receptor in innate cellular recognition of xenogeneic epitopes. Inasmuch as this work reveals a carbohydrate-recognition mechanism for cellular rejection, we shed light on a potential new boundary that will need to be overcome within xenotransplantation.

**Keywords:** Extracorporeal perfusion, Glycophorin A, Xenogeneic, N-acetylneuraminic acid, Sialic acid.

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From the discovery of Concanavalin A to the more recent identification of Toll-like receptors, lectins and their carbohydrate ligands are recognized as playing an increasing role in immunity. The carbohydrate epitope expressed by pigs, galactose alpha-1,3-galactose, has proven to be a formidable barrier to xenotransplantation. Recent studies, however, suggest that transgenic technology may facilitate the transplantation of porcine organs into humans (1, 2). It is likely that there are other epitopes and mechanisms of rejection that will be revealed as the vigorous natural antibody reaction to porcine alpha-gal epitopes is silenced (3). We have previously demonstrated that porcine Kupffer cells bind human erythrocytes and that this binding can be inhibited by glycoproteins, predominantly glycophorin A, purified from the membranes of human erythrocytes (3a). The present work examines which carbohydrate on human erythrocyte glycoproteins (hEGP) is the epitope recognized by porcine Kupffer cells. Human glycophorin A (hGA) is an intrinsic membrane protein with a molecular mass of 37 kD and is the second most prevalent protein expressed on human erythrocytes, with approximately 1 million molecules per cell (4, 5). Approximately 67% of all of the sialic acid on the human erythro-

cyte is borne on hGA. This is not surprising because hGA is roughly 60% carbohydrate and represents 1.6% of the total erythrocyte protein mass (6, 7). hGA is composed of 131 amino acids with 15 O-linked oligosaccharides attached to either serine or threonine within positions 1 to 50 and 1 N-linked oligosaccharide on the asparagine of position 26 (7–10).

O-linked oligosaccharides on hGA occur in many forms and are typically either tri-, tetra-, penta-, or hexasaccharides varying in composition and linkage (11–14). The O-linked oligosaccharides have been found to bear blood group antigens such as the ABO, MN, and Cad types (7, 15, 16). With the exception of the MN blood group that occurs on peptide positions 2 to 4, the ABO and Cad blood groups and other saccharides bound to either serine or threonine appear to vary in position. The N-linked oligosaccharide is a bi-antennary oligosaccharide with a branched mannose (Man) core. The structure is unique because it has only one terminal  $\beta$ -N-acetylglucosamine (GlcNAc) residue at the C-4 position of the  $\alpha$ -mannosyl residue of the core portion and one  $\alpha$ -fucose residue linked at the C-6 position of the proximal GlcNAc residue (17). Interestingly, structural features such as blood-group antigens and substitutions are likely targets in our search for a macrophage lectin-binding epitope.

Different functions have been ascribed to the varied oligosaccharides found on the surface of erythrocytes. Rat Kupffer cells have been shown to bind aging rat erythrocytes through lectin recognition of a terminal galactose (18). This syngeneic binding occurs as sialic acid is cleaved from the surface of aging erythrocytes and galactose molecules become exposed. Rat Kupffer cells recognize the aged rat erythrocytes

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through the galactose particle receptor (19). Using xenogeneic erythrocytes, rat Kupffer cells have been shown to recognize both sheep and human erythrocytes independent of antibody and complement opsonization (20). Human erythrocyte binding to rat Kupffer cells was inhibited by N-acetylgalactosamine (GalNAc) and lactose (Lac), suggesting that the GalNAc/galactose (Gal) particle receptor was responsible. Sheep erythrocytes were best inhibited by a combination of heat aggregated immunoglobulin (Ig)G, gangliosides, and fucose (Fuc). However, neuraminidase-treated sheep erythrocytes could be completely inhibited from binding when treated with saccharides specific for the GalNAc/Gal particle receptor. This evidence set a precedent for the involvement of carbohydrates in xenogeneic recognition.

We have previously shown that human erythrocytes are recognized by porcine Kupffer cells during a xenoperfusion event (21). Porcine Kupffer cells recognized the human erythrocytes independent of antibody and complement (22) and by way of a carbohydrate-dependent mechanism (3a). This study explores the nature of the carbohydrate recognition of human erythrocytes by porcine Kupffer cells. We provide evidence that although the N-linked oligosaccharide, human ABO blood groups, hyaluronic acid, and various monosaccharides are unrelated to binding, a terminal sialic acid of possibly either  $\alpha$ 2-3 or  $\alpha$ 2-6 linkage is responsible for porcine Kupffer cell recognition of human erythrocytes.

## MATERIALS AND METHODS

### Isolation of Porcine Kupffer Cells

Porcine Kupffer cells were isolated as reported by Rees et al. (22).

### Rosetting Assay

Kupffer cells, from primary culture, were washed in CMRL media (without fetal calf serum [FCS]) three times and pelleted at 600g to remove FCS and cell debris. Kupffer cells ( $1 \times 10^5$ ) were allowed to adhere to 13 mm round cover glasses in a humidified, CO<sub>2</sub>, 37°C incubator for 2 hours in CMRL media (without FCS). The media was then removed and replaced with 250  $\mu$ L of fresh CMRL media (without FCS) or a previously prepared sample for 15 minutes. Human or porcine <sup>51</sup>Cr-labeled erythrocytes (500  $\mu$ L of 0.01% in CMRL media) were added to each well (23). Erythrocytes and Kupffer cells were incubated for 2 hours at 37°C to allow rosette formation. Cover glasses were removed and gently washed to remove nonadherent erythrocytes before being detected on a gamma counter (Beckman Gamma 5500b, Beckman Instruments Inc., Fullerton, CA). Percent binding was calculated as the mean gamma counts for triplicate wells of porcine Kupffer cells incubated with potential inhibitory erythrocyte glycoproteins and human erythrocytes divided by the mean gamma counts of triplicate wells for porcine Kupffer cells incubated with human erythrocytes without other treatment (our positive control) multiplied by 100%.

### Isolation of Human Erythrocyte Glycoproteins

Human and porcine erythrocyte membrane fragments were prepared according to Dodge et al. (24). Human and porcine membrane fragments were subjected to lithium diio-

dosalicylate solubilization, as described by Marchesi et al. (25, 26). Isolated hEGP have been described previously (3a).

### ABO Blood-Group Specificity

Human blood was drawn into lithium heparin tubes and tested for blood type by agglutination with monoclonal antibodies for a specific blood type (Gamma Biological, Houston, TX). Blood-typed samples were centrifuged, and the plasma and buffy coat were removed. The erythrocytes of each blood type were labeled separately with <sup>51</sup>Chromium (23). <sup>51</sup>Chromium-labeled human erythrocytes were used in the rosetting assay, as we have previously described (3a). Human blood type AB was excluded because it contains blood type A and blood type B oligosaccharides as well as the H antigen.

### Monosaccharides in the Rosetting Assay

N-acetylneuraminic acid (Neu5Ac), Gal, Fuc, GalNAc, GlcNAc, Man, and arabinose (Ara) were used at the described concentrations. Each monosaccharide was solubilized in CMRL media (CMRL 1066 medium, Invitrogen, Grand Island, NY) with 100 U/mL of Penicillin streptomycin (Invitrogen, Grand Island, NY) and 2 mM L-Glutamine (Invitrogen, Grand Island, NY), and the pH was adjusted to pH 7.0 as necessary. The monosaccharide solutions were incubated with porcine Kupffer cells for 15 minutes before the addition of <sup>51</sup>Chromium-labeled human erythrocytes. The monosaccharides at each concentration were tested in the rosetting assay three times in triplicate.

### Neuraminyl Lactoses in the Rosetting Assay

Neuraminyl lactoses (V-Labs Inc., Covington, LA) were solubilized in CMRL media. Neuraminyl lactoses were tested in the rosetting assay three times in triplicate.

### Enzymatic Deglycosylation by proteoglycan N-linked glycosidase F (PNGaseF) or Sialidase A

Sialidase A and PNGaseF (Prozyme Inc., San Leandro, CA) were used according to the manufacturer's instructions. Either 100 units of PNGaseF or 0.1 units of sialidase A were incubated with 2 mg of hGA for 4 days. A "sham"-treated hEGP sample, without enzyme, was incubated under the same conditions. Enzyme treated, sham, and untreated hGA samples were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to verify a change in migration derived from removal of N-linked oligosaccharides or desialation. Removal of sialic acid from human erythrocytes was confirmed by measuring released sialic acid (data not shown).

### Hyaluronic Acid in the Rosetting Assay

Hyaluronic acid (Sigma Inc., St. Louis, MO) at concentrations of 0.2, 1.0, and 2.0 mg/mL was prepared in CMRL media and incubated with porcine Kupffer cells for 15 minutes before the addition of <sup>51</sup>Chromium-labeled human erythrocytes in the rosetting assay.

### Salivary Mucins in the Rosetting Assay

Porcine submaxillary mucin (PSM) was isolated from porcine submaxillary glands freshly excised and immediately chilled. The fat and connective tissue were removed, and the

gland was cut into small pieces and macerated in a Waring blender with 3 volumes of water. After centrifugation, the supernatant was added to 3 volumes of 95% ethanol with stirring. Flocculation was facilitated by adding a few drops of a saturated solution of potassium acetate until a visible precipitate formed. The precipitate was collected by centrifugation and allowed to dry after washing with ice-cold acetone (27). The sialic acid content was determined for both bovine submaxillary mucin (BSM) and PSM using the orcinol method and detected by absorbance at 572 nm. BSM was obtained from Sigma Inc. (St. Louis, MO). Mucins were incubated at the concentrations of 0.2, 1.0, and 2.0 mg/mL with porcine Kupffer cells for 15 minutes before the addition of <sup>51</sup>Chromium-labeled human erythrocytes.

**PNGaseF-Treated hEGP**

PNGaseF (100 units, Prozyme Inc. San Leandro, CA) was incubated with 2 mg of hEGP for 4 days in accordance with the manufacturer's instructions. A "sham"-treated hEGP sample, without enzyme, was incubated under the same conditions. Enzyme-treated, sham, and untreated hEGP samples were analyzed by SDS-PAGE to verify a change in migration derived from removal of N-linked oligosaccharides.

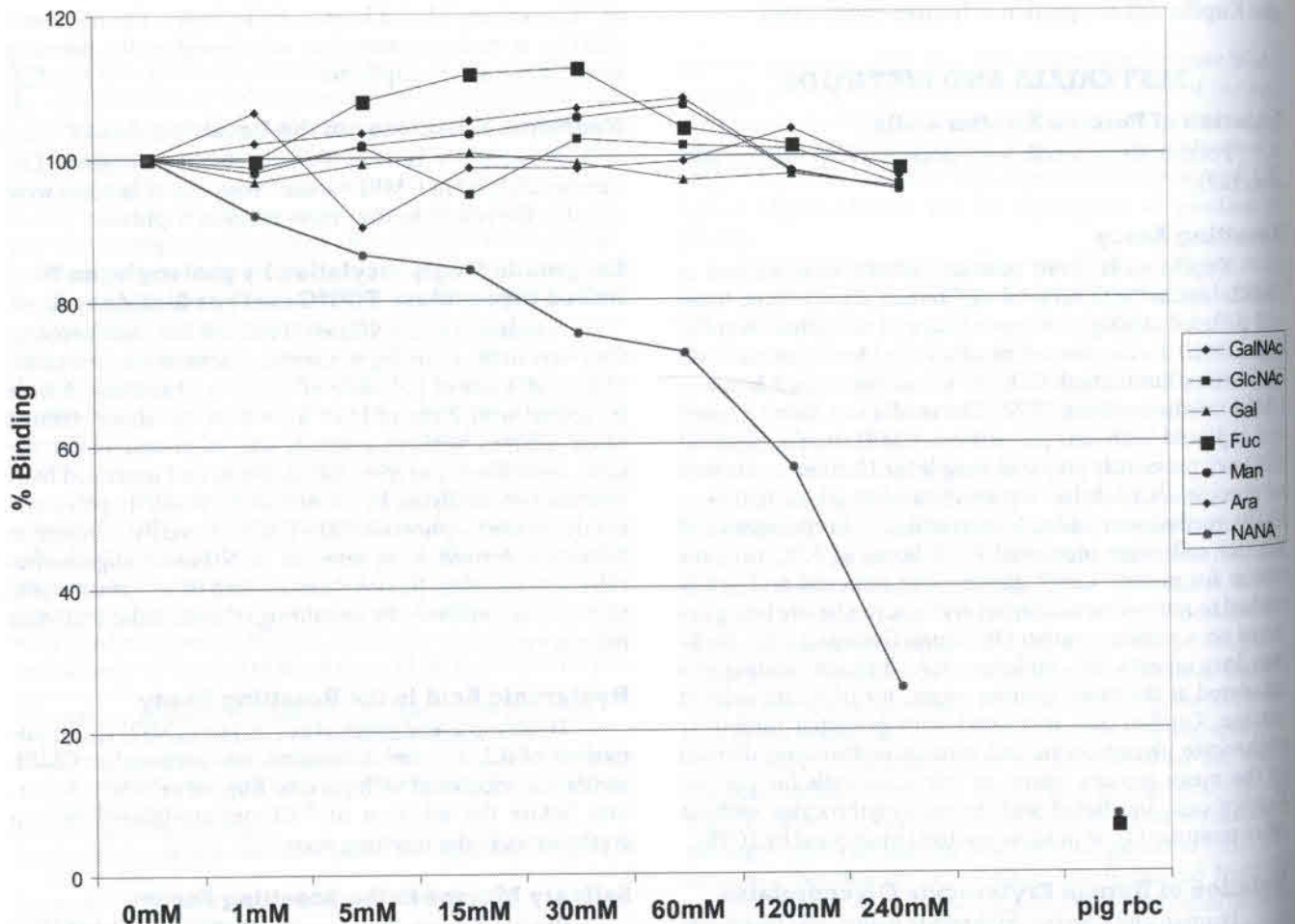
**RESULTS**

**ABO Blood-Group Specificity**

Having previously established that carbohydrate epitopes can be responsible for Kupffer cell binding of human erythrocytes, we wanted to explore the likelihood of blood-group oligosaccharides as potential xenoantigens. <sup>51</sup>Chromium-labeled human erythrocytes of blood types A, B, and O were used in a quantitative rosetting assay that demonstrated no significant difference in the recognition of erythrocytes bearing the blood types A and B, and O.

**Monosaccharides in the Rosetting Assay**

Monosaccharides used in the rosetting assay were chosen on the basis of the known glycosylation pattern of hGA so that carbohydrates both present and absent were evaluated. Neu5Ac, Gal, Fuc, GalNAc, GlcNAc, and Man are monosaccharides known to occur in N- and O-linked oligosaccharides of hGA, whereas Ara has not been found. Gal, Fuc, GalNAc, GlcNAc, Man, and Ara at concentrations up to 240 mM had no effect on rosetting (Fig. 1). Neu5Ac, however, revealed dose-dependent inhibition beginning at 5 mM and reached 75% inhibition at 240 mM (Fig. 1).



**FIGURE 1.** Monosaccharide inhibition of human erythrocyte binding to porcine Kupffer cells. GlcNAc, N-acetylglucosamine; GalNAc, N-acetylgalactosamine; Gal, galactose; Fuc, fucose; Man, mannose; Ara, arabinose.

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### Neuraminyl Lactoses in the Rosetting Assay

Having revealed that Neu5Ac significantly inhibited the rosetting assay, we wished to explore the effects of Neu5Ac linked to a disaccharide substrate as it is found on hGA. Neuraminyl lactoses prepared from bovine milk are approximately 80% Neu5Ac linked  $\alpha$ -2,3 to Lac and 20% Neu5Ac linked  $\alpha$ -2,6 to Lac, with no more than 5% of the total composition being unbound Lac. From 0 mM to 30 mM, neuraminyl lactoses showed dose-dependent inhibition and reaching a maximum of 30% inhibition at 30 mM (Fig. 2).

### Hyaluronic Acid in the Rosetting Assay

It has been suggested that the effect of sialic acid on binding is caused by the large overall negative charge of the Neu5Ac (28, 29). Hyaluronic acid is composed of a peptide backbone that contains numerous substituted uronic acids. Concentrations of 0.2, 1.0, and 2.0 mg/mL hyaluronic acid were unable to inhibit the recognition of human erythrocytes by porcine Kupffer cells (Fig. 3).

### Sialidase A-Treated hEGP and Human Erythrocytes

To address the role of Neu5Ac as a terminating saccharide of hEGP, we used the enzyme sialidase A to cleave sialic acid residues. Sialidase A-treated hGA analyzed by SDS-PAGE showed an increased mobility when compared with sham-treated hGA (data not shown). Sialidase treatment was found to completely remove the ability of hGA to inhibit rosetting when it was compared with the ability of sham-treated hGA to inhibit rosetting (Fig. 4A). Similarly, sialidase A-treated human erythrocytes were not bound by porcine Kupffer cells as compared with untreated human erythrocytes (Fig. 4B). Sialidase A-treated intact human erythrocytes were incubated with the sialic acid  $\alpha$ -2,3-specific lectin *Mackia ameurensis* (1 mg/mL) for 2 hours and evaluated for agglutination. Sialidase A-treated cells did not agglutinate, whereas untreated erythrocytes agglutinated, suggesting that

treatment with sialidase A removed sialic acid from the surface of intact human erythrocytes (data not shown).

### Salivary Mucins in the Rosetting Assay

Within the saliva of mammals are glycoproteins secreted from the submaxillary glands that have been found to bear sialic acids of varying substitutions depending on the species of origin. PSM, which are heavily substituted with Neu5Gc in contrast with BSM, which bear up to 12% Neu5Ac, were tested in the rosetting assay. At concentrations of 0.2, 1.0, and 2.0 mg/mL, PSM had no effect on rosetting, whereas BSM revealed maximal inhibition of 53% at 2.0 mg/mL (Fig. 5).

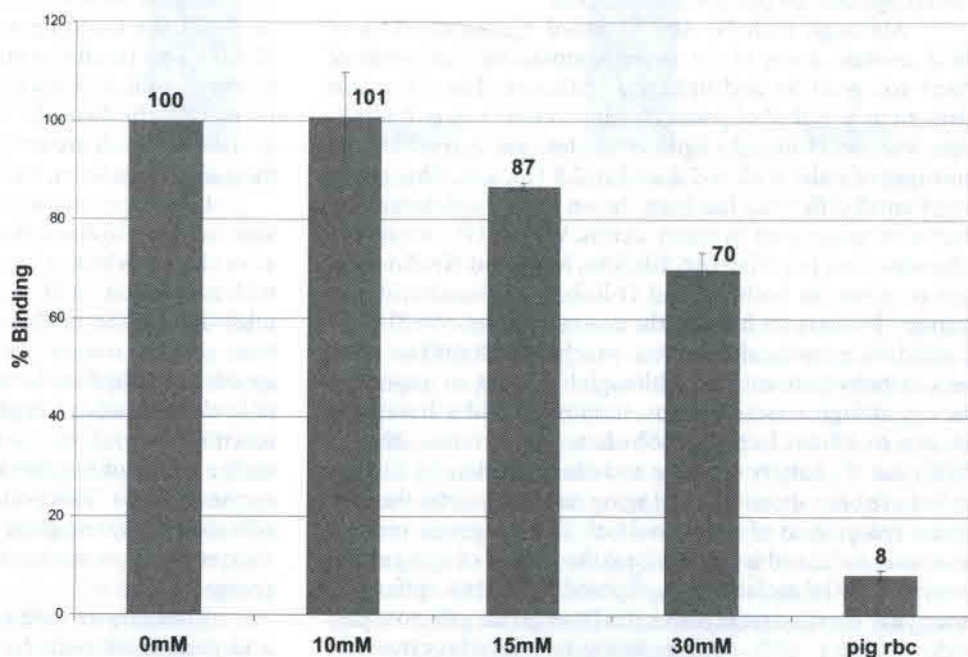
### PNGaseF-Treated hEGP

Removal of the single N-linked oligosaccharide on each hEGP glycoprotein had no effect on the ability of porcine Kupffer cells to bind human erythrocytes. When analyzed by SDS-PAGE, PNGaseF-treated hEGP migrated at approximately 55 kDa, whereas sham-treated hEGP migrated at 62 kDa as expected.

## DISCUSSION

We have previously demonstrated that a carbohydrate moiety is recognized on the surface of human erythrocytes by porcine Kupffer cells during in vitro coculture (3a). Given this finding, we wanted to explore the possible carbohydrate epitopes on hGA and assess their importance in the rosetting assay.

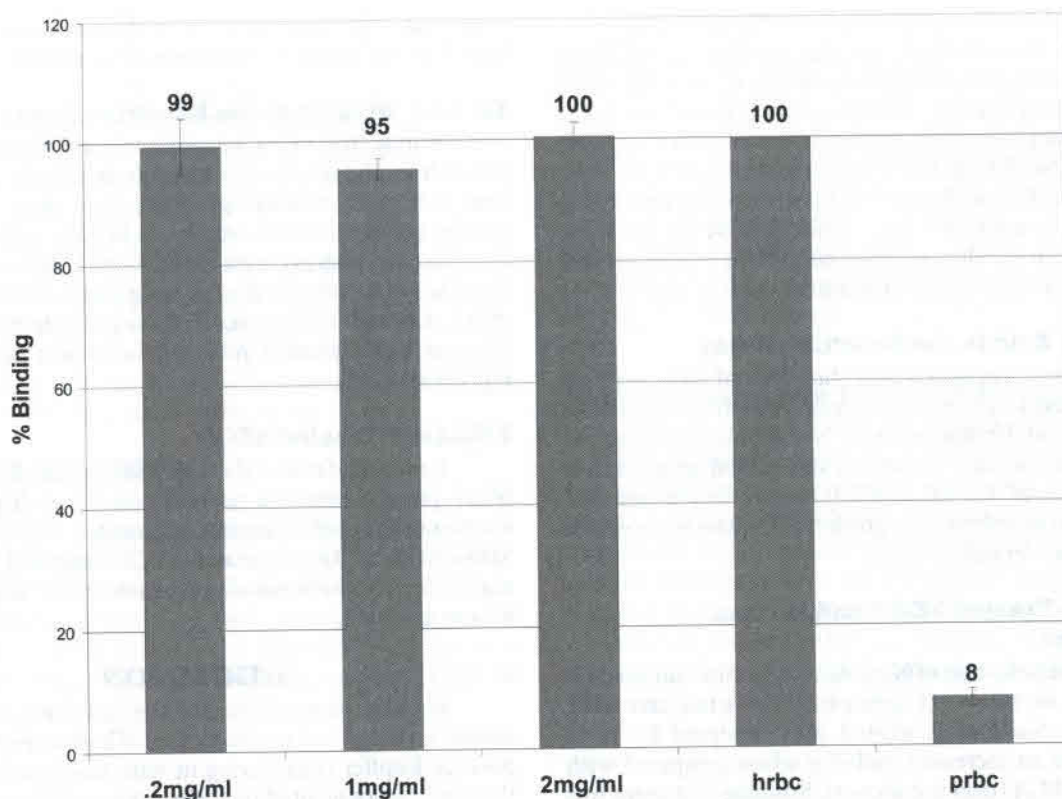
It is well known that ABO blood groups are immunologically recognized between individuals within a species by circulating antibodies. It has been theorized that the reason we have these antibodies directed toward other blood groups is that the offending blood group epitope may also exist on intestinal flora to which we are continuously exposed (30). We tested A, B, and O type human erythrocytes for their affinity to porcine Kupffer cells in the previously described



**FIGURE 2.** Neuraminyl lactose inhibition of human erythrocyte binding to porcine Kupffer cells. RBC, red blood cells.

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 ■ GlcNAc  
 ▲ Gal  
 ■ Fuc  
 ● Man  
 ◆ Ara  
 ● NANA

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**FIGURE 3.** Hyaluronic acid inhibition of human erythrocyte binding to porcine Kupffer cells. HRBC, human red blood cells; PRBC, pig RBC.

rosetting assay. A, B, and O erythrocytes were bound equally by porcine Kupffer cells. This suggests that differences in the GalNAc (type A) or Gal (type B) substitution associated with that blood group is not involved in binding. However, the Fuc  $\alpha$ -1,2 substitution at the blood-group core that comprises the H antigen also is present on blood groups A and B. Thus, blood group O cannot be ruled out as a potential candidate for recognition by porcine Kupffer cells.

Although both N- and O-linked oligosaccharides of hGA contain many of the same monosaccharides, some of their composition and linkage is different. The sialic acid present in N-linked oligosaccharides occurs in an  $\alpha$ -2,6 linkage, whereas O-linked oligosaccharides bear two additional linkages of sialic acid,  $\alpha$ -2,3 and  $\alpha$ -2,8 (13, 17). This seemingly small difference has been shown to be the determining factor in recognition by many lectins. We used the monosaccharides, Gal, Fuc, GalNAc, GlcNAc, Man, and Neu5Ac that are common to both N- and O-linked oligosaccharides in various linkages on hGA in the rosetting assay. We also included the monosaccharide Ara, which is not found on hGA, as a carbohydrate control. Although linkage is an important factor, at high concentrations, monosaccharides have been shown to inhibit lectin-carbohydrate interactions (20, 31). With use of a battery of mono and disaccharides, rat Kupffer cells have been shown to bind aging rat erythrocytes through lectin recognition of a terminal Gal. This syngeneic binding occurs as sialic acid is cleaved from the surface of aging erythrocytes and Gal molecules are exposed (18). Rat Kupffer cells recognize the aged rat erythrocytes through the galactose particle receptor (19). Using xenogeneic erythrocytes, rat

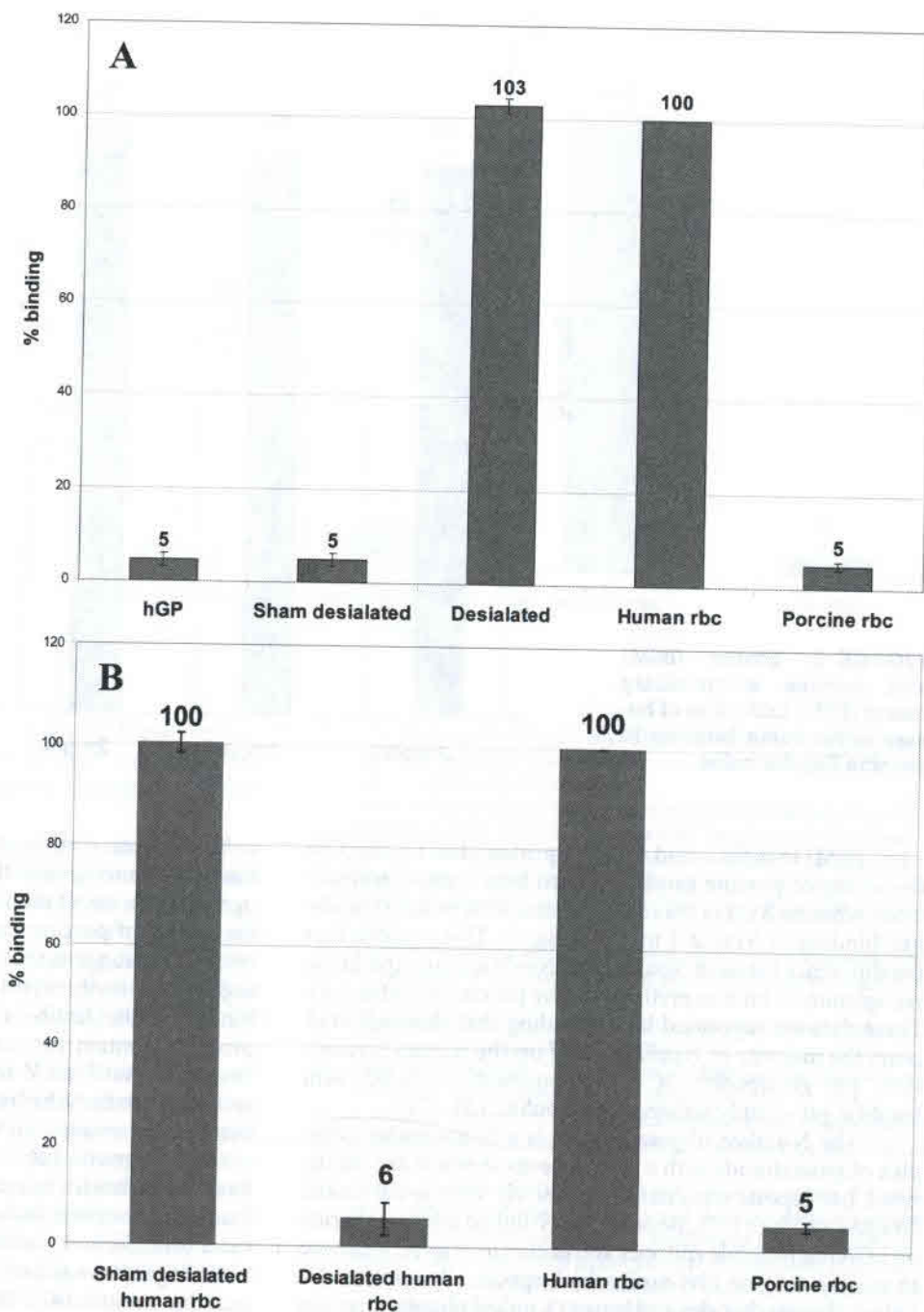
Kupffer cells have been shown to recognize both sheep and human erythrocytes independent of antibody and complement opsonization (20). Human erythrocyte binding to rat Kupffer cells was inhibited by GalNAc and Lac, whereas sheep erythrocyte binding was best inhibited by an assortment of oligo- and monosaccharides (20). In our study, the monosaccharides, Gal, Fuc, Ara, GalNAc, GlcNAc, and Man showed no ability to inhibit the rosetting assay. Neu5Ac, however, inhibited the rosetting assay in a dose-dependent manner (Fig. 1). This result was not surprising considering the prominence of sialic acid as a terminal saccharide on human erythrocytes. Of the 40 acidic 9-carbon alpha-keto sugars, known as sialic acids, all are enzyme-catalyzed modifications of the most abundant form, Neu5Ac (32).

It has been suggested that some or all of the affinity of sialic acid to a ligand is based on the saccharide's overall negative charge, which is increased when in high concentration such as is found on hGA. Therefore, we addressed the possibility that human erythrocytes were being recognized on the basis of their overall negative charge and not by way of a specific carbohydrate lectin interaction. We tested a dilution of hyaluronic acid, a negatively charged glycoprotein, in the rosetting assay. Unlike Neu5Ac, hyaluronic acid had no effect on the ability of porcine Kupffer cells to bind human erythrocytes (Fig. 3). This evidence suggests that porcine Kupffer cells specifically recognize terminal Neu5Ac on human erythrocytes using a mechanism not solely based on negative charge.

Thus far, we have examined the role of terminal sialic acid present on both N- and O-linked oligosaccharides of

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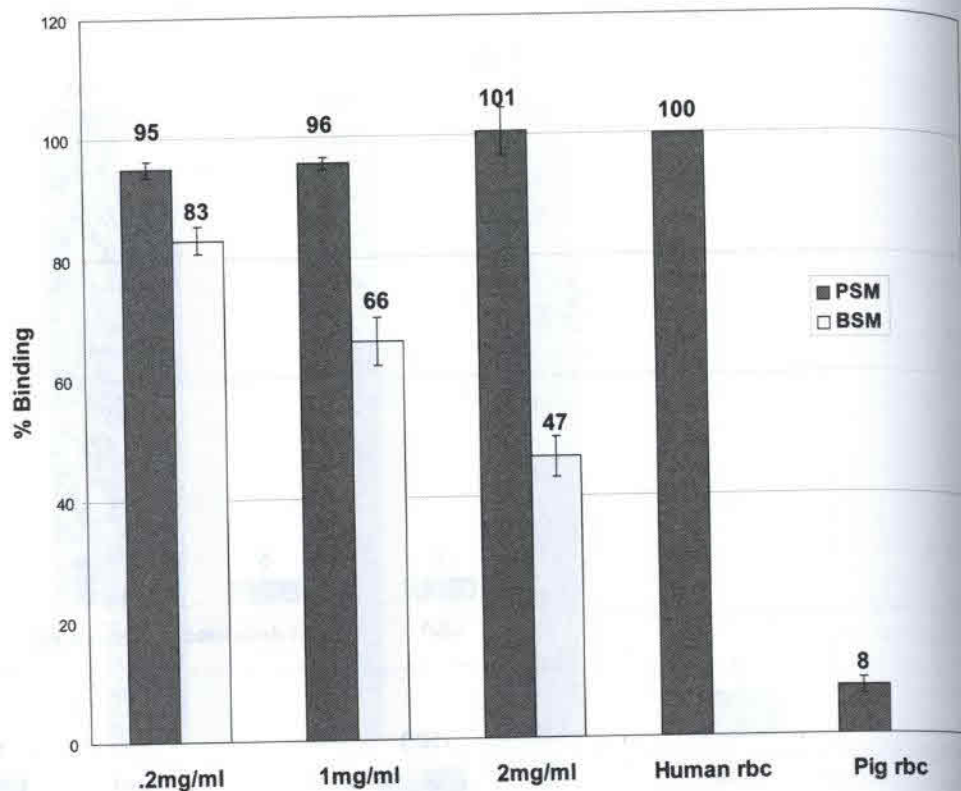
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**FIGURE 4.** Evaluation of desialated human erythrocyte glycoproteins (EGP) to act as inhibitor of human erythrocyte binding to porcine Kupffer cells (A) and desialated human erythrocytes to be bound to porcine Kupffer cells.

hGA and total sialic acid from the surface of intact erythrocytes. Using the enzyme sialidase A, we removed sialic acid from both hEGP and human erythrocytes. The sham-treated hEGP inhibited the ability of porcine Kupffer cells to bind human erythrocytes. However, the sialidase A-treated hEGP was no longer able to inhibit the rosetting assay (Fig. 4). Similarly, sham-treated intact human erythrocytes were recognized by porcine erythrocytes, as we have seen previously. The sialidase A-treated intact human erythrocytes went completely unrecognized by porcine Kupffer cells in the rosetting assay. Taken together, these data further emphasize the importance of sialic acid in porcine Kupffer-cell recognition.

To evaluate the role of sialic acid further, we also tested BSM and PSM. Through a point mutation, humans have lost the ability to produce the sialic acid called N-glycolylneuraminic acid (Neu5Gc) (33). Neu5Gc differs from Neu5Ac in that the acetyl group on the 5<sup>th</sup> carbon of Neu5Ac has been additionally substituted with a hydroxyl group. This small difference has given rise to a significant immunologic barrier between species. Human colon carcinoma cells have recently been shown to be immunologically recognized based on their expression of Neu5Gc (34). Pigs, on the other hand, express both Neu5Ac and Neu5Gc in amounts specific to the tissue analyzed and stage of development (35–37).



**FIGURE 5.** Bovine (BSM) and porcine submaxillary mucin (PSM) inhibition of human erythrocyte binding to porcine Kupffer cells.

PSM (16% sialic acid and 47% protein) had no effect on the ability of porcine Kupffer cells to bind human erythrocytes, whereas BSM (12% sialic acid and 42% protein) inhibited binding by 53% at 2 mg/mL (Fig. 5). This suggests that the difference between Neu5Ac and Neu5Gc may explain the recognition of human erythrocytes by porcine Kupffer cells. These data are supported by the finding that although hGA bears the majority of Neu5Ac found on the human erythrocyte, pig glycoprotein A is predominantly sialated with Neu5Gc yet scantily sialated with Neu5Ac (35–37).

The N-linked oligosaccharide is a bi-antennary complex oligosaccharide with a Man core upon which are substituted Lac repeats terminated exclusively with  $\alpha$ -2,6-linked Neu5Ac residues (17). Because the N-linked oligosaccharide on hGA has multiple epitopes and occurs only once, we chose to use the enzyme PNGaseF, which specifically removes N-linked oligosaccharides and leaves O-linked oligosaccharides unchanged, to treat hEGP. PNGaseF or sham treatment of hEGP had little effect on the ability of hEGP to inhibit the rosetting assay (Fig. 6). Both the PNGaseF- and sham-treated hEGP were able to inhibit binding in the rosetting assay. This suggests that by removing the N-linked oligosaccharide, the epitope recognized by Kupffer cells was either unaffected or not diminished by an effective degree. Although we present evidence that sialic acid is responsible for porcine Kupffer-cell recognition, the Neu5Ac-linked  $\alpha$ -2,6 to Lac on the N-linked oligosaccharide is either at an insignificant concentration to the total Neu5Ac on hGA or is not the correct linkage for recognition. Thus, the data presented here raises the question of linkage as a decisive factor in recognition of Neu5Ac.

We have previously shown that opsonization by antibody or complement is not necessary for porcine Kupffer cells

to bind human erythrocytes during in vitro coculture (38). It has been demonstrated that binding involves the direct recognition of a target on human erythrocytes by a receptor on the surface of porcine Kupffer cells. The latest evidence presented here suggests that the receptor necessary for recognizing human erythrocytes recognizes sialic acid. Sialic-acid-binding Ig-like lectins, siglecs, which are membrane-bound proteins, contain two common structural motifs. The first structural motif is a V-set Ig-like domain that is directly responsible for carbohydrate recognition (39). The second domain has a varying number of C-2 set Ig-like domains located at the N-terminus (40). Porcine sialoadhesin appears to be a likely candidate for human erythrocyte recognition. First, sialoadhesin has been shown to recognize xenogeneic erythrocytes (41). Second, sialoadhesin has been shown to bind but not phagocytose the erythrocytes, which is consistent with our observations (41). Third, sialoadhesin is differentially expressed on only a subpopulation of macrophages, which includes Kupffer cells (42).

Because of the relative prominence of sialic acids as terminal carbohydrates and their large structural variety, it seems obvious that they are involved in mediating and modulating various intercellular interactions. Siglec-3, also known as CD33, has been suggested to function as a negative signaling receptor by its immunoreceptor tyrosine-based inhibitory motif-like structural motif on the cytoplasmic region (39). It has been proposed that when Siglec-3 binds sialic acid, it sends a signal to the cell that it has bound self (43). Together with the data revealing sialic-acid differences between humans and pigs, it is possible that the absence of appropriate sialic-acid-bearing ligands identifies a pathogen, thus distinguishing self from nonself (33, 44). It has been previously

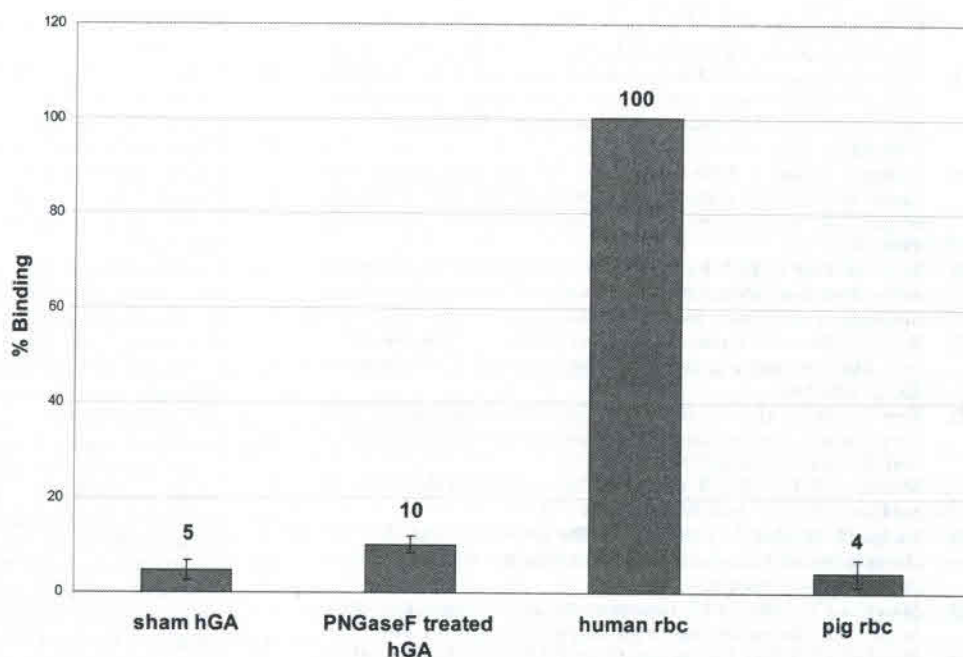
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**FIGURE 6.** Evaluation of proteoglycan N-linked glycosidase F (PNGaseF)-treated human EGP to inhibit human erythrocyte binding to porcine Kupffer cells.

suggested that the lack of appropriate sialic acid epitopes may prevent inhibitory signaling by way of siglec receptors and lead to immune activation followed by xenograft rejection (43, 45–47).

In spite of the evidence presented above that Neu5Ac is an inhibitor of porcine Kupffer-cell recognition of human erythrocytes, a di- or trisaccharide and their linkage may compose the pertinent binding domain. Better understanding of the specific inhibitory oligosaccharide will facilitate the development of inhibitory molecules that could be used to prevent the loss of human erythrocytes during extracorporeal porcine liver perfusion.

We have provided evidence that, of the various monosaccharides tested, sialic acid inhibited porcine Kupffer-cell binding of human erythrocytes. Neuraminyl lactoses, in a mixture of  $\alpha$ -2,3 and  $\alpha$ -2,6 linkages, inhibited rosetting. It appears that the overall negative charge of the sialated hGA is not the characteristic responsible for binding based on the inability of hyaluronic acid to inhibit the rosetting. Finally, when both hEGP and intact human erythrocytes were treated with sialidase A, hEGP lost the ability to inhibit the rosetting assay, and treated human erythrocytes were no longer bound by porcine Kupffer cells. Thus, we have shown that the target epitope on the surface of human erythrocytes recognized by porcine Kupffer cells is a sialic-acid-containing oligosaccharide. The significance of this finding is that although humans have been shown to have natural antibodies against N-glycolylneuraminic acid, this is now the first demonstration that pigs also have an innate immune receptor capable of recognizing the predominant sialic acid of humans, namely Neu5Ac.

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