The Role of Sialic Acid in the Expression of Human MN Blood Group Antigens*

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Human MN blood group activity has been restored to neuraminidase-treated erythrocytes by resialylation with different homogeneous sialyltransferases. The antigenicity of the resialylated cells always corresponded to that of the native cells from which they were prepared. In no case was a cell converted to an MN phenotype different from that of the blood donor. Either one of two sialyltransferases could restore the native MN phenotype to asialoerythrocytes, although some antisera appear to recognize selectively the products of only one of these enzymes. One sialyltransferase forms the structure, NeuAca2 → 3Galβ1 → 3GalNAca1 → O-Thr/Ser, and the other forms the structure, Galβ1 → 3[NeuAca2 → 6]GalNAca1 → O-Thr/Ser. Both linkages are found in the Thr/Ser-linked tetrasaccharides of glycoporphin, confirming the participation of alkali-labile oligosaccharides in MN blood group activity. A third sialyltransferase that forms the structure, NeuAca2 → 6Galβ1 → 4GlcNAc, with asparagine-linked oligosaccharides did not restore MN blood group activity. Together these three sialyltransferases replace ~60% of the sialic acid removed by neuraminidase. Radioactivity profiles obtained following gel electrophoresis of the 14C-labeled glycoproteins of ghosts from resialylated MM or NN cells were virtually identical. In each case, about 85 to 90% of the incorporated sialic acid was found associated with the major erythrocyte glycoprotein, glycoporphin A.

Since the modification of cell surface oligosaccharides by either of two sialyltransferases can restore both M and N activity, specific carbohydrate structures cannot be responsible for the difference between M and N antigens. These results are consistent with the proposal that the MN alleles specify the polypeptide sequence of glycoporphin A. However, the selectivity of some antisera for erythrocytes modified by only one sialyltransferase shows that specific sialic acid structures may still be important for antibody recognition.

The human MN blood group antigens are known to be carried by glycoporphin, the major sialoglycoprotein of the erythrocyte membrane (1), but the structural basis for M and N antigenicity remains incompletely understood. Modification of the oligosaccharide portion of glycoporphin by peridate oxidation (2-5), or alkaline β elimination (6-8) abolishes both M and N reactivity. In addition, the removal of sialic acid from intact erythrocytes (9, 10), as well as from glycoporphin (11) and its proteolytic fragments (12, 13), destroys M and N activity. These observations suggest that O-linked oligosaccharides which contain sialic acid contribute to the antigenicity of M and N active structures. Two types of sialylated oligosaccharides have been described in glycoporphin, the tetrasaccharide, NeuAca2 → 3Galβ1 → 3GalNAcβ1 + 0-Thr/Ser and a small number of related but less complete structures (14), and a more complex oligosaccharide containing several sialylated branches with the terminal oligosaccharide sequence, NeuAca2 → 6[3Galβ1 → 4GlcNAc→R]Aan (II) where R represents the remainder of the oligosaccharide (7, 15, 16). Approximately 15 tetrasaccharides of type I are found on the NH2-terminal half of glycoporphin. A single complex chain of type II is found linked to asparagine at position 26 (17). Erythrocytes of phenotype MM contain a small amount of N antigen (18) and neuraminidase treatment of type MM erythrocytes produces a transient rise in N activity before all M and N antigenicity is destroyed (19). These observations have prompted the proposal that the N antigen is a precursor substance which can be sialylated to form the M antigen (20). In one report (21), M and N antigenicity was apparently conferred upon neuraminidase-treated M and N antigens by reaction with CMP-NeuAc and human serum, which contains low sialyltransferase activities. The antigenic activity produced was reported to always correspond to the M or N phenotype of the serum donor irrespective of the initial phenotype of the erythrocytes from which the asialo-M or N antigen had been prepared. Furthermore, native N antigen was reported to be converted to M antigen by a similar reaction with serum from M individuals, but not with serum from N individuals (22). These observations support the view that specific carbohydrate structures determine M and N

* The abbreviations used are: NeuAc, N-acetylneuraminic acid; CMP-NeuAc, cytidine-5'-monophospho-N-acetylneuraminate; PAS, periodic acid-Schiff reagent; Gal2 → 6 sialyltransferase, CMP-NeuAc:β-galactoside a2 → 6 sialyltransferase; GalNAc2 → 6 sialyltransferase, CMP-NeuAc-N-acetylgalactosaminide a2 → 6 sialyltransferase; Gal2 → 3 sialyltransferase, CMP-NeuAc:β-galactoside a2 → 3 sialyltransferase; TF, Thomsen-Friedenreich antigen; CHO, carbohydrate.
antigenicity, but at present, no consistent differences between the oligosaccharide structures of M and N active compounds have been reported (7, 14).

Covalent modification of the glycoprotein polypeptide has also been shown to alter the M and N antigenicity detected by antisera of both human and rabbit origin as well as by N-specific lectins (2, 23-25). Consequently, it has been proposed that the M and N alleles specify different polypeptides (7, 14, 23-25), and the recent demonstration that the amino acid sequences of M- and N-reactive glycoporin peptides are different is very strong evidence in favor of this viewpoint (26-28). The smallest peptide fragments that have been shown to retain M or N activity contain only eight amino acids, three of which are glycosylated. The structures of these M- and N-active peptides, obtained by cyanogen bromide cleavage of glycoporin (12, 29), differ by two amino acids as follows.

\[
\begin{align*}
\text{CHO} & \quad \text{CHO} & \quad \text{CHO} \\
\text{NH}_2-\text{Ser-Ser} & \quad \text{Thr} & \quad \text{Thr}-\text{Gly}-\text{Val-Ala-Hse-COOH} & \quad \text{M active} \\
\text{CHO} & \quad \text{CHO} & \quad \text{CHO} \\
\text{NH}_2-\text{Leu-Ser} & \quad \text{Thr} & \quad \text{Thr}-\text{Glu-Val-Ala-Hse-COOH} & \quad \text{N-active}
\end{align*}
\]

The carbohydrate moieties (CHO) of both peptides appear to be identical (12, 14, 29) and correspond to Structure I above. An average of one oligosaccharide per peptide may be involved, but at present, no consistent differences between these fragments are in the amino acid sequences, the corresponding MN blood group activities retain sensitivity to both the removal of sialic acids by neuraminidase and to modification of the \( \text{NH}_2 \) terminus by acetylation (12). Thus, it seems likely that the glycoporin polypeptide sequence is determined by the MN alleles, but the contribution of oligosaccharide structures to the antigenicity of both M and N glycophorins remains unexplained.

In this report, three homogeneous sialyltransferases have been used to restore selectively each of the sialic acid linkages in Structures I and II to the erythrocyte membrane. Resialylation of the asparagine-linked oligosaccharides (type II above) does not restore M or N antigenicity, but addition of either sialic acid found in the Thr/Ser-linked structure (type I) can restore MN antigenicity to desialylated red cells. However, striking variability is observed in the reactivity of individual antisera with resialylated erythrocytes, suggesting that the native M and N antigens may contain several determinants.

**EXPERIMENTAL PROCEDURES**

**Materials**—Bovine serum albumin was obtained from Sigma Chemical Co. Neuraminidase (Vibrio cholera) was purchased from Grand Island Biological Co. Bio Beads SM 2 were obtained from Bio Rad Laboratories. CMP-[\(^{14}C\)]NeuAc (\( \sim 1.0 \) mCi/mmol and \( \sim 300 \) mCi/mmols), Protosol, and Aqueous II were purchased from New England Nuclear. Neomycin sulfate and chloramphenicol were manufactured by Upjohn and Parke-Davis, respectively.

**Buffers**—The buffers used for the handling of red cells are identified in the text by these abbreviations: NaCl/P, (phosphate-buffered saline), 10 mM sodium phosphate, pH 7.0 (25°C), 0.15 M NaCl; BBS (barbitial-buffered saline), 10 mM sodium diethylbarbiturate, pH 7.0 (25°C), 0.15 M NaCl; AMAB (modified Alsever’s medium containing 0.15 M NaCl, resuspended in AMAB to a concentration of 50% of the native M and N antigens may contain several determinants.

**Preparation of Blood Cells**—Blood was collected by venipuncture from blood type MM or NN individuals and anticoagulated with \( \sim 30 \) vol of 3.8% (w/v) sodium citrate. The red cells were washed by centrifugation, and the plasma and buffy coat were removed by aspiration. The red cells were washed thoroughly before use with 0.15 M NaCl, resuspended in AMAB to a concentration of 50% (v/v), and stored at 4°C.

**Preparation of Asialoerythrocytes**—Red cells were washed in BBS and 4 mL of a 50% suspension of cells in BBS, containing 4 mM CaCl\(_2\), 25 mg of bovine serum albumin, and 150 units of \( V. \) cholera neuraminidase, were incubated for 1 h at 37°C. One unit of enzyme is defined as the amount that releases 1 ng of sialic acid from human \( \alpha \)-acid glycoprotein in 15 min at 37°C. After reaction, the suspensions were centrifuged and the supernatants were removed for quantitation of the sialic acid released. Cells were then washed three times with 15 mL of BBS and stored at 4°C as a 50% suspension in AMAB. Packed cell volume was determined by a standard hematocrit method in capillary tubes. The sialic acid released was quantitated by the thiobarbituric acid procedure (31) and the results have been expressed as nanomoles of sialic acid/ml of packed cells. In a separate experiment, the conditions of neuraminidase digestion were shown to result in maximal release of sialic acid, as described by others (2, 33).

**Preparation of Antisera**—Human anti-M, No. 96-X, were donated by the DADE Division of American Hospital Supply Corp., Miami, FL. Similarly, Hyland Laboratories, Los Angeles, CA, provided rabbit anti-M, No. 12787, and rabbit anti-N, No. 5157. In addition, rabbit anti-M, No. 162, was a gift from Ortho Diagnostics, Inc., Raritan, NJ.

**Immunoaffinity Chromatography**—To remove anti-TF and other interfering antibodies, all sera were absorbed with equal volumes of packed, neuraminidase-treated MM or NN cells (washed with NaCl/P). Rabbit antisera were incubated for 30 min at 25°C. The human anti-M was incubated for 30 min at 0°C. If necessary, absorption was repeated until no agglutination of either neuraminidase-treated MM or NN cells was observed. Aliquots of the absorbed rabbit anti-M (No. 12787) and anti-N (No. 5157) were in addition absorbed with the native MM and NN cells.

**Preparation of Enzymes**—Three purified sialyltransferases were prepared in the cell incubation buffer (CIB) as specially required for these studies in the following ways.

The Gal\(_{a2-6}\) sialyltransferase was purified to homogeneity from bovine colostrum as previously described (30). A solution of 1.2 units of sialyltransferase (25 units/mg of protein) in 2 mL of 25 mM sodium cacodylate, pH 6.0, 25% (w/v) glycerol was applied at a flow rate of \( \sim 1 \) mL/h to a column (0.3 X 25 cm) filled to a height of 20.5 cm with Sephadex G-50 (fine), overlayered with 4 cm of CDP-Sephadex (30) (0.9 \( \mu \)mol of CDP-hexosyl/m of agarose) and equilibrated with 25 mM sodium cacodylate, pH 6.0. The enzyme was adsorbed quantitatively. To determine affinity, the enzyme was eluted at a flow rate of 0.4 cm of column. After washing with 4 mL of CIB, the sialyltransferase was eluted and desalted in a single step by developing the column with CIB containing 1 M NaCl. Concentrated enzyme emerged ahead of the 1 M NaCl front, and the most active fractions were pooled to obtain 0.4 unit of Gal\(_{a2-6}\) sialyltransferase in 0.8 mL of CIB. One unit will transfer 1 pmol of sialic acid to asialo-\( \alpha \)-acid glycoprotein per min at 37°C with saturating concentrations of both substrates as previously defined (30).

The Gal\(_{NaC2-6}\) sialyltransferase was purified to homogeneity from porcine submaxillary glands \(^2\) was prepared in CIB by chromatography of 0.4-mL aliquots containing 2.7 mg of bovine serum albumin and either 2.0 or 0.7 units of enzyme (41.5 units/mg) on a column (0.9 X 11.6 cm) of Sephadex G-50 (fine) equilibrated with CIB. Fractions (0.2 mL each) were pooled to obtain final concentrations of 1.82 and 0.57 units/mL of Gal\(_{NaC2-6}\) sialyltransferase. One unit transfers 1 pmol of sialic acid to asialo-\( \alpha \)-acid glycoprotein per min at 37°C with saturating concentrations of both substrates as previously defined.

The Gal\(_{a2-3}\) sialyltransferase from porcine submaxillary glands \(^2\) was concentrated on a column similar to that employed above for the Gal\(_{a2-6}\) sialyltransferase except that the concentration of CDP-hexosyl was 14 \( \mu \)mol/mL of agarose, and all buffers contained 1% (w/v) Triton X-100. About 1.5 units of enzyme (11 units/mg) consisting of a mixture of purified enzyme forms A and B in a total volume of 12 mL of 10 mM sodium cacodylate, pH 6.5, 50 mM NaCl, 1% Triton X-100, and 1 mg/mL of bovine serum albumin was applied to the column...
at a flow rate of ~1.5 ml/h. The column was washed with 4 μl of CIB containing 1% Triton X-100 and eluted with CIB containing 1% Triton X-100 and 1 M NaCl. Concentrated enzyme emerged in a peak ahead of the 1 M NaCl buffer. Fractions (1.30 μl each) were pooled to obtain 1.4 units of Gal α2 → 3 sialyltransferase in 0.82 μl of CIB containing 1% Triton X-100. One unit of enzyme transfers 1 μmol of sialic acid to antifreeze glycoprotein per min at 37°C with saturating concentrations of substrates as previously described.1 Detergent was removed immediately before incubation with red cells by gently rocking the enzyme for 3 h at 4°C with ~350 μg of Bio-Beads SM-2 (washed with CIB) according to the method of Holloway (34).

The following solutions in H2O of CMP-[14C]NeuAc were prepared: I, 5.86 mm, 5,250 cpm/nmol; II, 1.29 mm, 13,200 cpm/nmol. The concentrated CIB of CMP NeuAc was determined from absorbance at 273 nm assuming a molar extinction coefficient of 8,600 at pH 12. Aliquots were lyophilized and redissolved directly in CIB containing sialyltransferase for the resialylation of cells as described below.

Sialylation of Red Blood Cells—Asialo-NN and asialo-MM erythrocytes (50% in CIB) were resialylated in reaction mixtures (0.4 ml) containing one of three sialyltransferases, (a) Galα2 → 6, 100 milliunits; (b) GalNAcα2 → 6, 114 milliunits; or (c) Galα2 → 3, 11 milliunits; and either 0.65 mm CMP-[14C]NeuAc, 13,150 cpm/nmol (a), or 2.9 mm CMP-[14C]NeuAc, 5,250 cpm/nmol (b and c). As controls, reaction mixtures containing either native or asialoerythrocytes were prepared without enzymes. The cell suspensions were incubated at 37°C in polystyrene tubes and at appropriate time intervals duplicate 15-μl aliquots were removed and either diluted into 1 ml of 20 mM sodium phosphate, pH 7.0, 2 mM CTP or quantitated of [14C]NeuAc incorporation or into 1 ml of NaCl/Pi, containing 2 mm CTP for determination of hemagglutination titer. After reaction for 4 h at 37°C, 90-μl aliquots of each reaction mixture were diluted with 3 ml of NaCl/Pi and stored at 4°C as a 10% suspension in AMAB. Sialylation of red blood cells was performed as described in the following paper (35) and sodium dodecyl sulfate-gel electrophoresis was performed as described by Laemmli (36).

RESULTS

Specificity of Antiserum—Both human and rabbit antisera contain antibodies reactive with cryptic antigens such as the TF antigen that are exposed on red cells by digestion with neuraminidase (37). Consequently, such antibodies must be removed before anti-M and anti-N sera can be used to specifically discriminate between partially resialylated MM or NN cells. Because both M and N antigens are abolished by the removal of sialic acid from red cells, interfering antibodies can be removed by absorption with neuraminidase-treated cells. As shown in Table I, the absorbed sera retain their original specificity for native M or N cells and in addition will no longer agglutinate neuraminidase-treated cells. The anti-M serum does not agglutinate NN cells; however, a small amount of N antigen is detected on MM cells, as others have reported (18). The antigens that react with anti-N serum on MM cells are similar to those on NN cells, since adsorption of anti-N with either MM or NN cells essentially abolishes hemagglutination with both cell types. As expected, the reactivity of anti-M with MM cells is also abolished by absorption with MM cells.

Preparation of Enzymes—The purified sialyltransferases are usually stored in 50% glycerol, sometimes containing 0.5% Triton X-100. Before they can be incubated safely with cells, this storage buffer must be replaced with a detergent-free buffer that is isosomotic to serum. To accomplish this, each of the three enzymes used in these studies was concentrated on a small affinity column and desalted by gel filtration on a small column of Sephadex G-50 equilibrated with the cell incubation buffer (CIB). To conserve the enzymes, the Gal α2 → 3 and the Gal α2 → 6 sialyltransferases were concentrated and desalted in a single step on a column containing both CDP-agarose and Sephadex G-50. The Gal α2 → 6 and GalNAc α2 → 6 sialyltransferases can be prepared in good yield without the use of detergent-containing buffers. However, the Gal α2 → 3 sialyltransferase requires high concentrations of Triton X-100 for efficient elution from CDP-Sepharose, and detergent must be removed separately with Bio-Beads SM-2 prior to the addition of red cells. In the presence of bovine serum albumin (7 mg/ml), the recovery of Gal α2 → 3 sialyltransferase is virtually quantitative. Because the enzyme looses activity slowly in the absence of detergent, this step was performed immediately before addition to cells.

The incubation buffer (CIB) chosen for resialylation is a compromise between the conflicting needs of the cells for those with the human anti-M serum were performed at 0°C. Titers have been reported as the reciprocal of the lowest dilution of serum giving complete or partial agglutination after 30 min with rabbit sera or 45 min with human sera. The dilution of serum is taken to be that present before the addition of the cell suspension.

Gel Electrophoresis—Solubilized erythrocyte ghosts were prepared as described in the following paper (30) and sodium dodecyl sulfate-gel electrophoresis was performed as described by Laemmli (36).

| Table I |
| --- |
| Characterization of anti-M and anti-N sera |
| Antiserum (rabbit) | Cell type for adsorption | Cell type for hemagglutination |
| | | MM | NN |
| Anti-M1 | None | 256 | ≤2 |
| MM | <2 | ≤2 |
| Anti-N1 | None | 32 | 1024 |
| MM | <2 | 32 |
| NN | <2 | <2 |

4. J. I. Reaick, J. F. Sadler, and R. L. Hill, unpublished observations.
optimum stability and of the enzymes for maximum catalytic activity. At NaCl concentrations lower than 75 mM, extensive lysis of erythrocytes occurs at 37°C, even though the buffer is adjusted to ~300 mosm by the addition of glucose; however, both porcine submaxillary gland sialyltransferases are inhibited by increasing ionic strength. Nevertheless, because erythrocytes are almost completely stable for up to ~6 h at 37°C in cell incubation buffer (CIB), this buffer was selected for the resialylation of red cells despite the significant inhibition of sialyltransferase activities (~50%) by 75 mM NaCl.

**Enzymatic Purity of the Sialyltransferases**—The levels of contaminating enzyme activities in the purified sialyltransferase preparations are shown in Table II. Also shown are the products that can be formed with glycoprotein oligosaccharides based on the known specificity of each enzyme. Each of the sialyltransferases is substantially free of each of the other two enzymes. Contaminating levels were typically not detectable with assays sensitive enough to detect 0.1% of the major activity. The one exception was a 1.5% contaminant of the Galα2 → 3 sialyltransferase with the GalNAcα2 → 6 enzyme. Although the Galα2 → 3 enzyme can be prepared completely free of the GalNAcα2 → 6 enzyme by gel filtration on Sephadex G-200, the small contamination observed does not alter the interpretation of the results described in this report.

**Restoration of M and N Antigenicity to Asialoerythrocytes by Resialylation**—The time course of incorporation of sialic acid by each of the three sialyltransferases into asialo-MM and asialo-NN cells and the resulting changes in antigenicity with a single antiserum are shown in Fig. 1. For each enzyme, no significant differences in the rates of incorporation of sialic acid were found between asialo-MM and asialo-NN cells. However, with each cell type, the three enzymes differed markedly in the extent to which sialic acid was incorporated into the erythrocyte surface. The GalNAcα2 → 6 and Galα2 → 6 enzymes incorporated sialic acid at an initial rate of only 1 to 4% of their maximal velocity, while the rate of incorporation with the Galα2 → 3 sialyltransferase was nearly 80% of maximal velocity. Under the conditions employed, ~9%, 17%, and 27% of the sialic acid present in native cells was restored with the Galα2 → 2, GalNAcα2 → 6, and Galα2 → 3 enzymes, respectively, after 4 h at 37°C. In a separate reaction using 20-fold more of the Galα2 → 3 enzyme, incorporation in 4 h was further increased to ~40% of the sialic acid initially present (Table III). Resialylation by the combined action of all three enzymes restored 53 to 62% of the sialic acid content of native cells (Table III). Reaction of the three enzymes with native MM or NN cells resulted in low levels of incorporation of about 12, 13, and 29 nmol of sialic acid/ml packed cells by the GalNAcα2 → 6, Galα2 → 3, and Galα2 → 6 enzymes, respectively.

The development of hemagglutinating activity with anti-M and anti-N sera as a function of sialic acid incorporation shows a striking dependence upon the sialic acid linkage that is formed as well as upon the amount of sialic acid that is incorporated. There was no change in either M or N antigenicity after transfer of up to 50 nmol of sialic acid/ml of cells by the Galα2 → 6 sialyltransferase (Fig. 1, a and d). This enzyme forms the structure NeuAcα2 → 6Galβ1 → 4GlcNAcα1 → O-Thr/Ser with the corresponding β-galactoside structures of asparagine-linked oligosaccharides (38). In contrast, resialylation by the GalNAcα2 → 6 sialyltransferase restored N antigenicity to asialo-NN cells (Fig. 1e) and reaction with anti-N serum was already evident at levels of sialic acid incorporation below the maximum transferred by the Galα2 → 6 enzyme. However, with the anti-M serum (No. 12787) used here, no increase in M antigenicity was observed at any point in the time course of sialic acid incorporation by the GalNAcα2 → 6 sialyltransferase (Fig. 1b). In contrast, the Galα2 → 3 sialyltransferase restored both M and N antigenicity to asialoerythrocytes (Fig. 1, c and f), although the N titer with antiserum No. 5157 began to change after very little sialic acid had been transferred to asialo-NN cells, while the M titer with antiserum No. 12787 increased only after nearly maximal amounts of sialic acid had been transferred to asialo-MM cells. Thus, a single highly purified enzyme that forms the structure, NeuAcα2 → 3Galβ1 → 3GalNAcα1 → O-Thr/Ser, with glycoprotein substrates can restore either M or N antigenicity to asialoerythrocytes of the same initial phenotype. In addition, an enzyme that forms the structure, NeuAcα2 → 6GalNAcα1 → O-Thr/Ser, can restore N antigenicity to asialo-NN cells.

As shown in Table III, the antigenicity of resialylated cells was further examined with three specific anti-M sera, designated M-1, M-2, and M-3, and two anti-N sera, designated N-1 and N-2. To facilitate comparisons between the different sera, the titers of each with both native and asialoerythrocytes have been included. These titers were unchanged upon incubation of the cells for 4 h at 37°C. With the exception of the aliquot of anti-N-1 serum used here, which retained some ability to agglutinate the asialo-NN cells, none of the antisera reacted with asialo-MM or asialo-NN cells.

A comparison of the titers observed with all five antisera shows that the sialyltransferases used could restore only the original antigenic character of the cells. Specifically, asialo-MM cells regained only N titer, while asialo-MM cells regained only M titer and the very low N titer observed in native MM cells. In no case was the MN phenotype of the resialylated cells different from that of the corresponding native cells.

All of the antisera were specific for the native cell type against which they were raised, but quantitative differences could be demonstrated in their reactivity with different resialylated cells. For example, incubation of asialo-NN cells with

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**Table II**

| Enzymatic purity of three homogeneous sialyltransferases | Sialyltransferase (ST) activities → Galα2 → 6ST | Galα2 → 6ST | GalNAcα2 → 6ST | Galα2 → 3ST |
|--------------------------------------------------------|-----------------------------------------------|-------------|----------------|-------------|
| Galα2 → 6ST: NeuAcα2 → Galβ1 → 4GlcNAcα1 → O-Thr/Ser   | 100 <0.003                                   | <0.003      | 100            | <0.09       |
| GalNAcα2 → 6ST: Galβ1 → 3GalNAcα1 → O-Thr/Ser 6↑       | <0.001                                       | 100         | 1.5            |             |
| NeuAcα2: Galα2 → 3ST: NeuAcα2 → 3Galβ1 → 3GalNAcα1 →  |                             |             |                |             |
| O-Thr/Ser                                                     | <0.003                                   | 100         |                |             |

*Activities are expressed relative to 100 units of the major activity where 1 unit forms 1 pmol of product/min at 37°C.

β-Galactoside α2 → 6 sialyltransferase was assayed as described earlier (30) with α,β-cyclodextrin to quantitate the purified enzyme, or with lactose as substrate followed by separation of the 3' and 6' isomers of sialyllactose to detect contaminating levels in the other enzyme preparations.

α-N-Acetylgalactosaminide α2 → 6 sialyltransferase was assayed as previously described with ovine asialomucin treated with Streptococcus pneumoniae endo-β-N-acetylgalactosaminidase to serve as a specific substrate.

β-Galactoside α2 → 3 sialyltransferase was assayed specifically with either lactose in the presence of GalNAcα2 → 6 enzyme or with antifreeze glycoprotein in the presence of the Galα2 → 6 enzyme.

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*J. I. Rearick, J. F. Sadler, J. C. Paulson, and R. L. Hill (1979) J. Biol. Chem. 254, in press.*
Fig. 1. Resialylation of asialoerythrocytes with restoration of M or N antigenicity. Blood group MM (A, B, C) and NN (D, E, F) asialoerythrocytes were reacted with CMP-[\(^{14}\)C]NeuAc and either the Gal \(\alpha2 \rightarrow 6\) sialyltransferase (A, D), the GalNAc \(\alpha2 \rightarrow 6\) sialyltransferase (B, E), or the Gal \(\alpha2 \rightarrow 3\) sialyltransferase (C, F) as described under "Experimental Procedures." At the indicated times, aliquots from each reaction mixture were removed and quantitated for incorporation of \([^{14}\text{C}]\text{NeuAc}\) into membrane glycoproteins (○) or assayed for the restoration of M or N antigenicity by hemagglutination with antisera M-1 or anti-serum N-1, respectively (●).

### Table III

| Cell type | Sialyltransferase (ST) added | Concentration | Time at 37°C | NeuAc content | Anti-M sera | Anti-N sera |
|-----------|-----------------------------|---------------|-------------|---------------|-------------|-------------|
| Native MM | Native MM                   |               |             |               |             |             |
| Asialo-MM | Gal\(\alpha2 \rightarrow 6\)ST | 250           | 4           | <2            | <2          | <2          |
|           | GalNAc\(\alpha2 \rightarrow 6\)ST | 285           | 4           | <2            | <2          | <2          |
|           | Gal\(\alpha2 \rightarrow 3\)ST | 575           | 0.5         | <2            | <2          | <2          |
|           | Gal\(\alpha2 \rightarrow 6\)ST | 125           | 4           | <2            | <2          | <2          |
|           | GalNAc\(\alpha2 \rightarrow 6\)ST | 425           | 4           | <2            | <2          | <2          |
| Native NN | Gal\(\alpha2 \rightarrow 6\)ST | 250           | 4           | <2            | <2          | <2          |
| Asialo-NN | Gal\(\alpha2 \rightarrow 6\)ST | 205           | 4           | <2            | <2          | <2          |
|           | Gal\(\alpha2 \rightarrow 3\)ST | 575           | 0.5         | <2            | <2          | <2          |
|           | Gal\(\alpha2 \rightarrow 6\)ST | 125           | 4           | <2            | <2          | <2          |
|           | GalNAc\(\alpha2 \rightarrow 6\)ST | 425           | 4           | <2            | <2          | <2          |
|           | Gal\(\alpha2 \rightarrow 3\)ST | 257           | 4           | <2            | <2          | <2          |

* The prefix, asialo-, refers to cells that have been treated with *V. cholerae* neuraminidase.

** The NeuAc content of native cells was determined colorimetrically (31) after release by neuraminidase. The NeuAc content of resialylated cells was determined from the incorporated radioactivity of \([^{14}\text{C}]\text{NeuAc}\).

* Sera used were: M1, Hyland No. 12787; M2, Ortho No. 162; M3, Dade No. RH 9688; N1, Hyland No. 5157; N2, Dade No. 96 X.

* The titers reported here for native and asialo-MM and NN cells are unchanged by incubation of the cells for 4 h at 37°C.
the Galα2 → 3 sialyltransferase alone (Table III) restored the titer with anti-N-2 to only one-fourth of that of native NN cells while the titer of the same cells with anti-N-1 was equal to that of native cells. The much lower anti-N-1 titer of native MM erythrocytes was also restored to asialo-MM cells by the Galα2 → 3 sialyltransferase. The variability among anti-M sera is more striking. Resialylation of asialo-MM cells by 28 milliunits/ml of the Galα2 → 3 sialyltransferase (Table III) restored the titer with anti-M-3 to at least equal the anti-M-3 titer of native MM erythrocytes. In contrast, the titer of the same cells with anti-M-1 was only 2-fold compared to the titer of native MM cells of 512. Resialylation of asialo-MM erythrocytes with the GalNAcα2 → 6 sialyltransferase did not restore any measurable M activity with anti-M-1 or anti-M-3, but the titer with anti-M-2 increased to 2. Incubation with all three sialyltransferases (Table III) restored the titer of asialo-MM cells with both antisera M-1 and M-3 to a higher titer than that of native MM cells, but the titer with anti-M-2 remained only one-fourth that of the native MM cells. Thus, the structures formed by the Galα2 → 3 sialyltransferase alone were sufficient to restore the titer with serum M-3 to equal that of native erythrocytes, while resialylation with all three enzymes restored complete reactivity with anti-M-1.

Sequential incubations with individual sialyltransferases revealed further differences between the three anti-M sera. The GalNAcα2 → 6 sialyltransferase alone did not restore any anti-M-1 titer to asialo-MM cells (Table III) and the Galα2 → 3 sialyltransferase restored only a small fraction of the native cell titer (Table III). To demonstrate that the GalNAcα2 → 6 sialyltransferase could enhance the anti-M-1 titer of cells that had already been sialylated with the Galα2 → 3 sialyltransferase, cells were first reacted with 28 milliunits/ml of Galα2 → 3 sialyltransferase, washed free of the remaining enzyme, and incubated with the GalNAcα2 → 6 sialyltransferase. As shown in Table IV, the incorporation of an additional 60 nmol of sialic acid/ml of cells, all in NeuAcα2 → 6GalNAc linkages, increased the titer with serum M-1 by 256-fold and doubled the titer with serum M-2, but did not change the titer with serum M-3. The anti-M sera can therefore be distinguished by their reactivity with specific resialylated cells. Anti-M-3 requires only the product of the Galα2 → 3 sialyltransferase to restore antigenicity, anti-M-2 will react with cells treated with either the Galα2 → 3 or GalNAcα2 → 6 enzyme, and anti-M-1 reacts most strongly with the cells modified by both sialyltransferases. In this regard, anti-N-1 is similar to anti-M-2, recognizing the product of either the Galα2 → 3 or the GalNAcα2 → 6 sialyltransferase with asialo-NN cells.

**Gel Electrophoresis of [3H]NeuAc-labeled Glycoproteins of MM and NN Erythrocyte Membranes**—Fig. 2 shows that reaction of asialo-MM or asialo-NN erythrocytes with a mixture of the three sialyltransferases produced virtually identical patterns of incorporation of [3H]NeuAc as judged by sodium dodecyl sulfate-gel electrophoresis of the membrane glycoproteins. The electrophoretic patterns obtained by labeling asialo-MM erythrocytes separately with each of the three sialyltransferases differed from one another (35), but there were no differences in the labeling of asialo-MM and asialo-NN erythrocytes by each enzyme (data not shown). The major peaks of radioactivity in Fig. 2 correspond to the positions of the three predominant bands observed upon staining separate gels with the periodic acid-Schiff (PAS) reagent which is specific for glycoproteins. Since PAS Bands 1 and 2 are primarily the dimer and monomer forms, respectively, of glycophorin A (39), and PAS 3 is thought to be monomeric glycophorin B (29, 40, 41), these two related glycoproteins account for more than 90% of the [3H]sialic acid incorporated.

**DISCUSSION**

The studies reported here show that either one of the two sialyltransferases, the β-galactoside α2 → 3 sialyltransferase or the β-N-acetylgalactosaminide α2 → 6 sialyltransferase, can restore M activity only to asialo MM erythrocytes and N activity only to asialo-NN erythrocytes. Glycoporphin A, the major erythrocyte glycoprotein has been shown to carry the M and N antigens (1). Together these two enzymes incorporate sialic acid into the O-linked oligosaccharides of glycoporphin to form the structure NeuAcα2 → 3Galβ1 → 3(NeuAcα2 → 6)GalNAc. Indeed, as shown in Fig. 2, 85 to 90% of the sialic acid incorporated into asialo-MM or NN erythrocytes is found associated with glycoporphin A. The third enzyme, the β-galactoside α2 → 6 sialyltransferase, incorporated a small amount of sialic acid into glycoporphin A (35) presumably into its asparagine-linked oligosaccharide (Structure II), but no antigenicity was restored. These results
confirm the importance of the sialic acid linked to the alkali-labile oligosaccharides of glycophorin in the reaction of anti-MM and anti-NN sera with red cells (6-8).

Since the serotype of the resialylated cells always corresponded to the phenotype of the cell donor, regardless of the sialyltransferase used, the fundamental difference between MM and NN erythrocytes is preserved in neuraminidase-treated erythrocytes. The previous suggestion that N antigen may be converted to M antigen by transfer of an additional sialic acid residue (20, 21), is not consistent with the studies reported here since a single sialyltransferase can restore either M or N antigenicity to asialo-MM or NN erythrocytes, respectively. Instead, the results support the view that sialic acid is required for antibody recognition of M and N antigens and that the M and N alleles code for the polypeptide sequence of type M and type N glycophorin A, which are known to differ by two amino acids (26-28).

It is especially noteworthy that the binding activities of most M and N antisera are strongly dependent on the sialic acid in the antigen. The removal of sialic acid has been proposed to change the conformation of the antigen to make it either inaccessible or unrecognizable to antibody (24). However, the carbohydrate exposed by neuraminidase digestion is sufficiently accessible to be removed by glycosidases (20), recognized by anti-TF antibody (37) or Vicia graminia lectin (42), and to be used as a substrate for purified sialyltransferases as described herein. Alternatively, the change in antigenicity that results upon the removal of sialic acid from MN antigens may simply reflect the close proximity of the sialic acids to the variable amino acid residues that are responsible for M and N specificity. M- and N-active glycopeptides have identical amino acid sequences except at residues 1 and 5, and residues 2 to 4 are glycosylated, containing a total of about 5 residues of sialic acid (29). These structural features may be responsible for the striking differences in the reactivity of the three anti-M sera used in these studies with resialylated erythrocytes. One serum, anti-M-3, reacts strongly with cells that have been resialylated by the Gal a2→3 sialyltransferase but does not recognize the product of the GalNAc a2→6 sialyltransferase, while anti-M-2 reacts with the product of either enzyme alone, and anti-M-1 prefers the product of both enzymes together. A similar phenomenon has been demonstrated with human anti-A sera. A given preparation of anti-A may be very specific for blood type A cells, but the antibodies can be fractionated into subgroups that bind to different features of the A-active structure (43). However, common to all such anti-A sera is the dependence of specificity upon the structural moiety that is indirectly determined by the A-allele; namely, an α-N-acetylgalactosaminide residue. In the present case, different features of the oligosaccharide structures may be recognized by various anti-M and anti-N sera even though the M or N specificity remains a property of the glycophorin A polypeptide.

Given the diversity of the human and rabbit immune responses, it is reasonable to expect that some individuals will produce antibody that is directed primarily against the polypeptide structure of glycophorin. In fact, antibodies to glycophorin which do not require sialic acids for reactivity are occasionally reported. For example, Lisowska and Kordowicz (13) found that asialoglycophorin could induce M- or N-specific antibodies that would react only with the asialo glycophorin of the same serotype. In another report Sturgeon et al. (44) characterized an interesting rabbit anti-N serum (Dde anti-N) that agglutinated asialo-NN and asialo-MN erythrocytes as well as native NN and MN cells, but did not agglutinate either native or asialo-MM cells. Furthermore, Dde anti-N agglutinated the erythrocytes of an individual with geno-

type NN who had the rare phenotype, Tn, characterized by abnormal glycophorin oligosaccharides with the structure, NeuAc2→5GalNAca2→O-Thr/Ser, instead of the usual asialo-glycosyltetrasaccharide (Structure I). Thus, Dde anti-N could apparently recognize native or asialo-type N glycophorin A as well as a variant type N glycophorin which was missing the terminal NeuAc2→3Gal sequence from its O-linked oligosaccharides. Clearly, this excellent anti-N serum would have been destroyed by routine absorption with neuraminidase-treated MN or NN cells. A specific search for similar antisera is likely to reveal many more anti-M and anti-N sera that will react with asialoerythrocytes.

As noted in Tables I and III, MM cells contain a small amount of N-antigen that is destroyed by neuraminidase but can be restored by specific sialyltransferases. Since the discovery of the MN blood group (45, 46), the presence of some N antigen on MM cells has been difficult to incorporate into a simple model for the genetic basis of MN antigenicity. However, it now appears that this N antigen is due to a minor erythrocyte glycoprotein, glycophorin B, which has its first 23 amino acids in a sequence identical with that of glycophorin A of the N phenotype (28). Glycophorin B is inherited separately from glycophorin A and is found both in MM and NN erythrocytes (28, 41, 42). Thus, the ability of MM cells to adsorb anti-N antibodies from anti-N sera (Table I) is probably due to the N antigenicity of glycophorin B. This conclusion is supported by the observation that the restoration of the minor N activity by resialylation of asialo-MM cells is accompanied by the incorporation of sialic acid into PAS Band 3 (Fig. 2) previously shown to be glycophorin B (28, 40).

The studies reported here and in the preceding paper (35) illustrate the use of glycosyltransferases for assessing the structure-function relationships of oligosaccharides on cell surfaces. As additional homogeneous glycosyltransferases become available, they may prove valuable reagents for probing, at the molecular level, the roles of cell surface oligosaccharides on animal cells even more complex than erythrocytes.

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