J. Biochem. 123, 468-478 (1998)

A Streptavidin-Based Neoglycoprotein Carrying More Than 140 GT1b Oligosaccharides: Quantitative Estimation of the Binding Specificity of Murine Sialoadhesin Expressed on CHO Cells[1]

Yasuhiro Hashimoto,*,[2] Minoru Suzuki,* Paul R. Crocker,† and Akemi Suzuki*

Department of Membrane Biochemistry, Tokyo Metropolitan Institute of Medical Science, Honkomagome, Bunkyo-ku, Tokyo 113; and †Imperial Cancer Research Fund Laboratories, Molecular Haemopoiesis Laboratory, Institute of Molecular Medicine, University of Oxford, John Radcliffe Hospital, Headington, Oxford OX3 9DU, UK

Received for publication, October 16, 1997

We prepared a streptavidin-based neoglycoprotein which carries more than 140 GT1b oligosaccharides. GT1b oligosaccharides were covalently coupled to streptavidin by reductive amination, yielding a monomer form of streptavidin carrying 13 oligosaccharides. The monomer form of glycosylated streptavidin was polymerized with biotinylated-bovine serum albumin, which yielded a polymer carrying more than 140 oligosaccharides. Both the monomer and the polymer bound to Chinese hamster ovary cells expressing murine sialoadhesin. The relative binding potencies determined with the polymer, monomer, and free GT1b oligosaccharides were 3,500, 83, and 1, respectively, indicating that an increase in the number of oligosaccharide ligands is critical for high avidity. The high avidity of the polymer enabled us to develop a sensitive and quantitative binding assay, and the assay was applied to characterization of the binding specificity of sialoadhesin. The polymer binding was inhibited by various gangliosides, the order of the inhibitory potencies being GM3 (IC_{50} = 40 μM) > GD1a (100 μM) > sialylparagloboside (120 μM) > GT1b (310 μM) > GM2 (640 μM) > GM4 (2,100 μM) > GD1b > LacCer = GM1 = paragloboside (no inhibition). These results indicate that the binding specificity is comparable to that reported, *i.e.* the determinant structure is NeuAcα2-3Galβ1-linked to either 3GalNAc, 3(4)GlcNAc, or 4Glc, and that the oligosaccharide structure on the polymer is properly presented to sialoadhesin on the cell surface. To determine the precise requirement of the NeuAc structure for binding, NeuAc of GM3 was converted into various derivatives, the inhibitory potencies of which were examined; *i.e.* GM3 containing NeuAc, IC_{50} = 40 μM; C7- or C8-aldehyde, 500 μM; C7- or C8-alcohol, 700 μM; C1-alcohol, 2,000 μM; C1-amide, 2,200 μM; and NeuGc, > 3,000 μM. These results confirmed the requirement of the hydroxyl group at C9 and/or C8, the carboxyl group at C1, and the methyl group of the *N*-acetyl residue of NeuAc in a quantitative manner. Thus, this streptavidin-based neoglycoprotein is a useful multivalent glycoprobe, which exhibits high affinity and specificity to murine sialoadhesin on the cell surface.

Key words: binding specificity, glycoprobe, GT1b ganglioside, neoglycoprotein, sialoadhesin.

Various kinds of neoglycoproteins have been developed to characterize the binding specificities of lectins or sugar binding proteins (*2*). These neoglycoproteins have several advantages over natural glycoconjugates, *e.g.* their carbohydrate structures are homogenous and chemically defined, and their core molecules are easily modified or labeled for use as probes in binding assays. Another important but often unemphasized advantage is the multivalency of carbohydrate ligands, which usually enhances the apparent affinity of neoglycoproteins to lectins (*3*). One such example is a neoganglioprotein, in which GT1b gangliosides are coupled to bovine serum albumin (BSA) in a multivalent manner (*4*). Tiemeyer *et al.* utilized such a neoganglioprotein as a probe to demonstrate the presence of a "ganglioside receptor" in myelin membranes of the central nervous system (*5, 6*), and they hypothesized that the neoganglioprotein presents its oligosaccharide ligands to the receptors

[1] This work was partly supported by Grants-in-Aid for Scientific Research, No. 09680605, and Grants-in-Aid for Scientific Research on Priority Areas, Nos. 04250105 and 04206102, from the Ministry of Education, Science, Sports and Culture of Japan, and by the Yamanouchi Foundation for Research on Metabolic Disorders.

[2] To whom correspondence should be addressed. Phone: +81-3-3823-2101 (Ext. 5483), Fax: +81-3-5685-6607, E-mail: yhashimoto@rinshoken.or.jp

Abbreviations: CHO cells, Chinese hamster ovary cells; GalNAc, *N*-acetylgalactosamine; IC_{50}, concentration for half maximal inhibition; MALDI-TOF/MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; NeuAc, *N*-acetylneuraminic acid; NeuGc, *N*-glycolylneuraminic acid; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Sia, sialic acid; Sn cells, Chinese hamster ovary cells which stably express murine sialoadhesin. The nomenclature for gangliosides is based on the system of Svennerholm (*1*), and the trivial names used for neutral glycolipids and oligosaccharides are listed in Table I.

in a multivalent manner and that this is critical for its high affinity. This may also be the case for other neoglycoproteins when a large number of lectins or receptors are present on the cellular surface. In this *trans*-type of recognition, we examined whether or not the multivalency obtained with BSA-based neoglycoproteins is enough to generate maximal affinity. BSA contains 58 lysine residues in its primary sequence, which are often utilized as target sites for reductive amination, but the actual number of lysine residues coupled with complex oligosaccharides on reductive amination is usually less than 20, probably due to the limited accessibility of the amino groups. In order to obtain much higher valency, we have currently prepared a streptavidin-based neoglycoprotein which carries more than 140 mol of GT1b oligosaccharide per molecule. This paper reports that the neoglycoprotein has been successfully applied to a binding assay, in which sialoadhesin is stably expressed on the cell surface and used as a model receptor molecule.

Sialoadhesin is a macrophage-restricted sialic acid-dependent cell adhesion molecule with 17 immunoglobulin-like domains, and the eponymous member of the sialoadhesin subset of I type lectins (*7-11*). The sialoadhesin family was originally defined on the basis of sequence similarities between sialoadhesin, CD22, CD33, myelin-associated glycoprotein (MAG), and Schwann cell myelin protein (SMP). All family members have been shown to exhibit lectin activity toward sialoglycoconjugates (*12-14*). The oligosaccharide structures recognized by sialoadhesin are NeuAcα2-3Galβ1-linked to either 3GalNAc, 3(4) GlcNAc, or 4Glc (*12-15*), whereas those recognized by CD22 and MAG are Siaα2-6Galβ1-4GlcNAc (*16, 17*) and NeuAcα2-3Galβ1-3GalNAc (*12, 14, 18, 19*), respectively. An absolute requirement of the exocyclic glycerol side chain (C7-C9) of the sialic acid was reported for CD22 binding (*16, 17, 20-22*). Collins *et al.* recently demonstrated that the binding of sialoadhesin, MAG, and SMP also requires the glycerol side chain of the NeuAc in addition to carboxylic acid at the C1 position (*14, 19*). In the present paper, we report the precise binding specificity of sialoadhesin determined with our newly developed method, which gives quantitative results.

MATERIALS AND METHODS

Glycosphingolipids and Oligosaccharides—The structures of the glycosphingolipids and oligosaccharides discussed in this paper are listed in Table I. GM1, GD1a, GD1b, and GT1b were purified from bovine brain by the same methods as described previously (*23*). Sialylparagloboside, paragloboside, GM2, GM3, and GM3(NeuGc) were purified from hog muscle, human erythrocytes, Tay-Sachs brain, bovine adrenal medulla, and horse erythrocytes, respectively. GM2(NeuGc), GM1(NeuGc), and GD1a(NeuGc) were purified from mouse liver (*24*). GM4-(NeuAc) and GM4(NeuGc) purified from frog liver were kindly provided by Dr. Masako Ohashi, Ochanomizu Women's College. Lactosylceramide and asialo GM1(GA1) were purchased from Wako Pure Chemical Industries (Tokyo). LSTa, LSTb, LSTc, DSLNT, α2-3sialyllactose, and α2-6sialyllactose were purchased from Seikagaku Kogyo (Tokyo). GT1b-, GD1a-, and GM1-oligosaccharides were prepared by ozonolysis of gangliosides according to Wiegandt (*25*). Briefly, a ganglioside (*e.g.* GT1b) dissolved in methanol (1 mg/ml) was treated with ozone at 4°C for 3 min. The resulting ozonides were treated with 200 mM sodium carbonate at ambient temperature for 18 h. The oligosaccharides generated were purified by Folch's partitioning followed by ion exchange column chromatography on a HiLoad 26/10 Q Sepharose HP column (Pharmacia Biotech, Uppsala, Sweden).

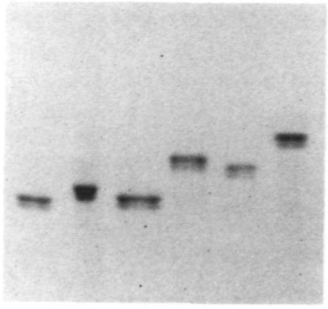
GM3 Derivatives—A thin-layer chromatogram and the structures of GM3 derivatives are shown in Fig. 1. The sialic acid of GM3 containing NeuAc was converted to the corresponding methyl ester by the method of Handa and Nakamura (*26*), and then the resulting methyl ester was converted to either the corresponding alcohol or amide according to Collins *et al.* (*19*). Briefly, 500 μg of GM3-(NeuAc) was incubated in 0.5 ml of dimethylsulfoxide/methyliodine (5:1) at ambient temperature for 15 min. The resulting methyl ester was purified by reversed-phase column chromatography using a SEP-PAK C18 cartridge (Millipore, Milford, MA) as described previously (*27*). One

TABLE I. **Glycosphingolipids and oligosaccharides used in this study.**

Glycosphingolipids[a]	
GM4	Siaα2-3Galβ1-1'Cer
GM3	Siaα2-3Galβ1-4Glcβ1-1'Cer
GM2	GalNAcβ1-4Galβ1-4Glcβ1-1'Cer Siaα2-3
GM1	Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1'Cer Siaα2-3
GD1a	Siaα2-3Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1'Cer Siaα2-3
GD1b	Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1'Cer Siaα2-3 Siaα2-8
GT1b	Siaα2-3Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1'Cer Siaα2-3 Siaα2-8
Sialylparagloboside (SPG)	Siaα2-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1'Cer
Paragloboside (PG)	Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1'Cer
AsialoGM1 (GA1)	Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1'Cer
Lactosylceramide (LacCer)	Galβ1-4Glcβ1-1'Cer
Oligosaccharides	
α2-3Sialyllactose (2-3SL)	Siaα2-3Galβ1-4Glc
α2-6Sialyllactose (2-6SL)	Siaα2-6Galβ1-4Glc
LSTa	Siaα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc
LSTb	Siaα2-6 Galβ1-3GlcNAcβ1-3Galβ1-4Glc
LSTc	Siaα2-6Galβ1-4GlcNAcβ1-3Galβ1-4Glc
DSLNT	Siaα2-6 Siaα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc

[a]The nomenclature of gangliosides is based on that of Svennerholm (*1*) except in the case of GM4 and sialylparagloboside.

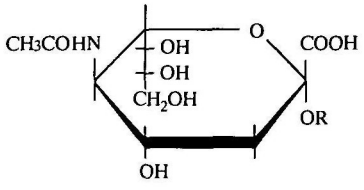
a



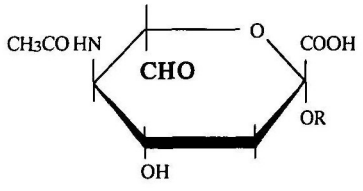
1 2 3 4 5 6

b

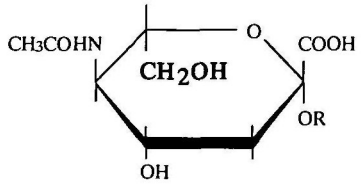
1. GM3(NeuAc)



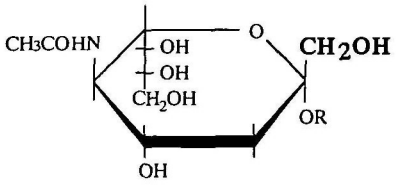
2. GM3(C7-aldehyde)



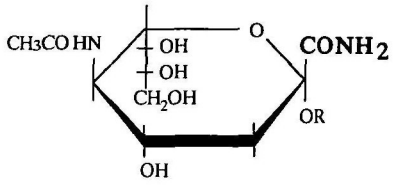
3. GM3(C7-alcohol)



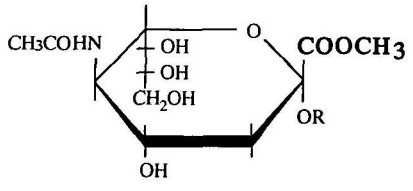
4. GM3(C1-alcohol)



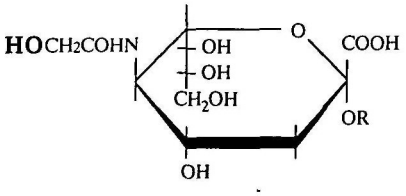
5. GM3(C1-amide)



6. GM3(C1-methyl ester)



7. GM3(NeuGc)



R=3Galβ1-4Glcβ1-1'Cer

portion of the methyl ester was reduced in 1% sodium borohydride in methanol for 2 h. Another portion of the methyl ester was treated with 4 M ammonium hydroxide in methanol/water (5:2) for 12 h at ambient temperature. The resulting GM3(C1-alcohol) and GM3(C1-amide) were purified by reversed-phase column chromatography. The glycerol side chain of the sialic acid of GM3(NeuAc) was oxidized to the C7- or C8-aldehyde, followed by reduction to the corresponding truncated primary alcohols as described (*28*). Briefly, 500 μg of GM3(NeuAc) was treated with 1 ml of ice-cold 150 mM NaCl, 50 mM sodium phosphate, pH 7.4, containing 2.5 mM sodium periodate at 4°C in the dark for 90 min. The resulting GM3(C7- or C8-aldehyde) was purified by reversed-phase column chromatography. A portion of the aldehyde was dissolved in 1 ml of 50 mM sodium bicarbonate containing 10 mM sodium borohydride and then incubated at 37°C for 2 h. The resulting GM3(C7-or C8-alcohol) was purified by reversed-phase column chromatography. The purity of GM3 derivatives synthesized was examined by thin-layer chromatography, using chloroform/methanol/0.2% $CaCl_2$ in water (55:45:10) as the developing solvent. The bands of the derivatives were detected with orcinol reagent. The structures of the GM3 derivatives were confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS), using a Finnigan MAT VISION 200 (Finnigan MAT, Bremen, Germany).

Preparation of GT1b-Oligosaccharyl Streptavidin—GT1b oligosaccharides were coupled to streptavidin (Pierce, Rockford, IL, USA) by reductive amination according to Roy *et al.* (*29*), using pyridine borane (Aldrich Chemical, Milwaukee, WI, USA) as a reducing reagent (*30*). Briefly, GT1b oligosaccharides (1.6 μmol) were mixed with streptavidin (10 nmol) in 43 μl of 200 mM sodium borate, pH 9.0. After the mixture had been incubated at 50°C for 15 min, 7 μl of methanol/pyridine borane (5:2) was added. The reaction was allowed to proceed at 50°C for 2 days. The resulting GT1b-oligosaccharyl streptavidin was purified by

gel filtration column chromatography or ultrafiltration using an ultra free cartridge (Millipore) When the former method was employed, the incubation mixture was applied to a Sephadex G-25 column (2 cm × 5 cm) and 500 μl fractions were collected. The elution profile of streptavidin was monitored as either the absorbance at 282 nm or by the bicinchoninic acid assay (Pierce), whereas that of sialocompounds was monitored by the resorcinol method (*31*). An aliquot of each fraction was spotted onto a PVDF membrane, Immobilon-P^{SQ} (Millipore), using a Bio-Dot apparatus (Bio-Rad Laboratories, Mercules, CA, USA). After the membrane had been blocked with 1% bovine serum albumin (BSA) (Fraction V, Sigma, St. Louis, MO, USA) in phosphate-buffered saline (PBS), it was incubated with neuraminidase from *Clostridium perfringens* (Type X, Sigma) in 100 mM sodium acetate, pH 4.0, at 37°C for 18 h. The membrane was further incubated with horse radish peroxidase-labeled cholera toxin B sub- unit (List Biological Laboratories, Campbell, CA, USA) in PBS containing 1% BSA at ambient temperature for 1 h. Signals were visualized with 4-chloro-1-naphthol reagent.

Sugar Compositional Analysis—Gas-liquid column chromatography (GLC) was carried out by the method of Chaplin (*32*). Briefly, an aliquot of the purified oligosaccharyl streptavidin was lyophilized in a Reacti-Vial (Pierce). The lyophilized sample was treated with 0.5 N methanolic hydrogen chloride in the presence of 20% methyl acetate at 70°C for 16 h. The resulting methylglycosides were treated with methanol/pyridine/acetic anhydride (10:1:1) at ambient temperature for 15 min, and then derivatized to trimethylsilyl methylglycosides with a Sylon HTP kit (Supelco, Bellefonte, PA). The resulting derivatives were analyzed by GLC, using a GC-7A (Shimadzu, Kyoto) equipped with a capillary column of cross-linked methyl silicone (0.31 mm × 25 m). Inositol was used as the internal standard for GLC analysis. For sugar compositional analysis, purified oligosaccharides were used as authentic standards.

Polymerization of GT1b-Oligosaccharyl Streptavidin—Biotinylated-BSA was prepared as a core molecule for the polymerization of GT1b-oligosaccharyl streptavidin. BSA was extensively biotinylated with ImmunoPure NHS-LC-Biotin (Pierce) according to the manufacturer's protocol. The number of biotin molecules coupled to BSA was determined by MALDI-TOF/MS. Biotinylated-BSA was radioiodinated with Na^{125}I (Daiichi Pure Chemicals, Tokyo) by the chloramine T method, yielding an initial specific radioactivity of 200–800 Ci/mmol. [^{125}I]Biotinylated-BSA was mixed with GT1b-oligosaccharyl streptavidin in a molar ratio of 1 to 25–30 in the presence of 50 mM Hepes-NaOH, pH 7.3. The mixture was analyzed by either SDS-PAGE or gel filtration column chromatography as follows.

For SDS-PAGE, an aliquot of the mixture was diluted with a buffer consisting of 62.5 mM Tris-HCl, pH 6.8, 0.1% SDS, 0.001% bromophenol blue at ambient temperature. The resulting mixture was analyzed on a gradient gel of from 3 to 20% (w/v) polyacrylamide (Wakamori, Tokyo) with the buffer system of Laemmli (*33*). The prestained marker proteins used were myosin (205 kDa), β-galactosidase (116.5 kDa), bovine serum albumin (80 kDa), and ovalbumin (49.5 kDa) (Bio-Rad Lab.). After electrophoresis, the protein bands were stained with a Quick CBB kit (Wako Pure Chemical Industries). For autoradiography, the gel was dried with a Model 583 gel dryer (Bio-Rad Lab.) and then exposed to an imaging plate (Fuji Photo Film, Tokyo). Radioactive bands were visualized with a Bio Imaging Analyzer 2000 (Fuji Photo Film).

For gel filtration chromatography, an aliquot of the mixture of [^{125}I]biotinylated-BSA and GT1b-oligosaccharyl streptavidin was diluted with 200 mM ammonium acetate and then applied to a Superose 6 HR 10/30 column (1 cm × 30 cm) (Pharmacia Biotech), which was connected to a FPLC system (Pharmacia Biotech). The effluent was collected in 1 ml fractions and the radioactivity was measured with a gamma counter. Catalase (232 kDa), ferritin (440 kDa), thyroglobulin (669 kDa), and a dimeric form of thyroglobulin (1,338 kDa) were used as molecular mass markers.

Cell Culture and Transfection—CHOK1 cells were maintained in HAM's F10 medium (Life Technologies, Grand Island, NY, USA) supplemented with 5% heat-inactivated fetal calf serum (Andard-Momt, London, UK), 100 units/ml penicillin, 100 μg/ml streptomycin, and 10 mM Hepes-NaOH, pH 7.3.

The cells were co-transfected by electroporation with a plasmid containing full-length sialoadhesin cDNA (7) and pcDNA-3 (British Biotechnology, UK) as a source of the neomycin resistance gene, and resistant colonies were selected in 1.0 mg/ml of G418. After 2 weeks culture, the resistant colonies were pooled, and sialoadhesin-positive cells were purified by magnetic sorting using a VarioMACS apparatus (Miltenyi Biotec) and 3D6 anti-sialoadhesin monoclonal antibody. Positive cells were cloned by limiting dilution and colonies expressing sialoadhesin uniformly were detected by immunocytochemical staining of duplicate colonies plated on 96-well plates. Fluorescence-activated cell sorter (FACS) analysis showed that the clone selected for the present experiments (9B12) was uniformly positive for sialoadhesin and expressed the molecule on the cell surface at a level comparable to that seen on macrophages in tissues such as the spleen (data not shown).

For the standard binding assay, the clonal cells expressing sialoadhesin were dispensed into each well of a microtiter plate (2×10^4 cells/100 μl/well). After cultivation for 2–3 h, 100 μl of 10 mM sodium butyrate in the culture medium was added to each well, and then the cells were cultivated at least for 10 days before being subjected to the binding assay.

Binding Assay—[^{125}I]Biotinylated-BSA was mixed with GT1b-oligosaccharyl streptavidin as described above, and the resulting polymer (see "RESULTS") was separated from free GT1b-oligosaccharyl streptavidin on a small column of Sepharose 6B (1 cm × 10 cm), using 200 mM ammonium bicarbonate, pH 8.0, containing 0.1% BSA as the column buffer. The effluent was collected in 0.5 ml fractions and the radioactivity in each fraction was determined with a gamma counter. The radioiodinated polymer that was eluted around the void fractions was lyophilized, and then dissolved in HAM's F10 medium supplemented with 1% BSA and 10 mM Hepes-NaOH, pH 7.3 (assay medium). The radioiodinated polymer in 15 μl of the medium was overlaid onto CHO cells, which were washed two times with the medium just prior to the assay. After incubation at 20°C for 3 h, the cells were washed 6 times with the medium and then solubilized with 200 μl of medium containing 1% Triton X-100. Each well was washed once again with Triton

X-containing medium and then the medium was combined with the initial washing. Radioactivity in the combined medium was measured with a gamma counter. Nonspecific binding was determined by adding either 2 mM GT1b ganglioside or a mixture of the monoclonal antibodies, SER-4 (*34*) and 3D6 (*15*), at a final concentration of 20 μg/ml each. GT1b-containing liposomes, as an inhibitor, were prepared according to MacDonald *et al.* (*35*), using Liposo-Fast-Basic (AVESTIN, Ottawa, Canada).

When human erythrocytes were used as the ligand, the assay was performed according to Kelm *et al.* (*12*) with slight modifications. Briefly, human erythrocytes suspended in the assay medium (4×10^4 cells/30 μl) were added to each well of a microtiter plate, and then the plate was incubated at ambient temperature for 1 h. After the incubation, 100 μl of PBS was added to each well and the plate was shaken for 10 s on a microtiter plate orbital shaker. One hundred microliters of 0.2% glutaraldehyde in PBS was added to each well and then the plate was shaken for 10 min. The plate was washed two times with PBS, and then 100 μl of ethanol was added to each well to permeabilize erythrocytes. The amount of hemoglobin was determined by the pseudoperoxidase assay (*12*).

RESULTS

Glycosylation of Streptavidin—GT1b oligosaccharides were coupled to streptavidin by reductive amination. After the coupling reaction, the glycosylated streptavidin was separated from free GT1b oligosaccharides by Sephadex G-25 column chromatography. As shown in Fig. 2, glycosylated streptavidin was eluted in the void fractions (fractions 5-8), whereas free oligosaccharides were eluted in fractions 14-24. An aliquot of each fraction was spotted onto a PVDF membrane to immobilize the glycosylated streptavidin, and then the membrane was incubated with neuraminidase to convert GT1b oligosaccharide-streptavidin into GM1 oligosaccharide-streptavidin. The membrane was probed with cholera toxin B subunit, which specifically reacts with GM1 oligosaccharides. Since free oligosaccharides were not immobilized on the membrane, no signal was detected for fractions 14-24. Positive signals were detected for fractions 5-8, corresponding to a protein peak. These signals were not observed without neuraminidase treatment even when 10 times greater aliquots were applied to the PVDF membrane, suggesting that degradation of the oligosaccharide structure from GT1b to GM1 does not occur during the coupling reaction. The structural integrity of GT1b oligosaccharides, except glucose at the reducing end, was further confirmed by sugar compositional analysis involving gas-liquid column chromatography (GLC). Glycosylated streptavidin contained galactose, *N*-acetylgalactosamine, and *N*-acetylneuraminic acid in the molar ratio of 2 to 0.96 to 3.12. No glucose was detected, suggesting that glucose at the reducing terminal of GT1b oligosacchrides was reduced to form a covalent bond with an amino group of streptavidin, and that the linkage was not cleaved under the conditions we employed. Thus, we concluded that GT1b oligosaccharides were successfully coupled to streptavidin without any detectable degradation of the hexasaccharide structure in the non-reducing terminal portion. The quantitative determination of galactose by GLC revealed that the molar ratio of galactose to streptavidin was 1 to 26.2, indicating that the mean number of oligosaccharides coupled to streptavidin was 13.1. The glycosylated streptavidin was desig-

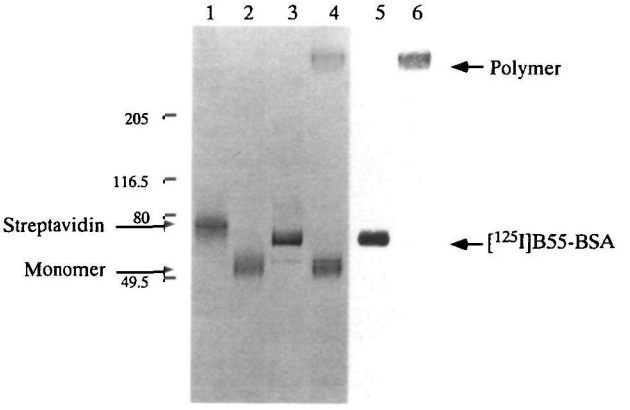


Fig. 3. **SDS-PAGE analysis of the (GT1b oligo)13-streptavidin monomer and polymer.** Lane 1, underivatized streptavidin; lane 2, glycosylated streptavidin carrying 13 mol of GT1b oligosaccharide; lane 3, [^{125}I]biotinylated-BSA; lane 4, a mixture of [^{125}I]biotinylated-BSA and the glycosylated streptavidin in a mixing ratio of 1 to 30. Lanes 5 and 6 are autoradiograms of lanes 3 and 4, respectively. For SDS-PAGE, an aliquot of a sample solution was diluted with a buffer consisting of 62.5 mM Tris-HCl, pH 6.8, 0.1% SDS, 0.001% bromophenol blue at ambient temperature. The resulting mixture was analyzed on a gradient gel of from 3 to 20% (w/v) polyacrylamide. The positions of prestained marker proteins are indicated by bars; myosin (205 kDa), β-galactosidase (116.5 kDa), bovine serum albumin (80 kDa), and ovalbumin (49.5 kDa).

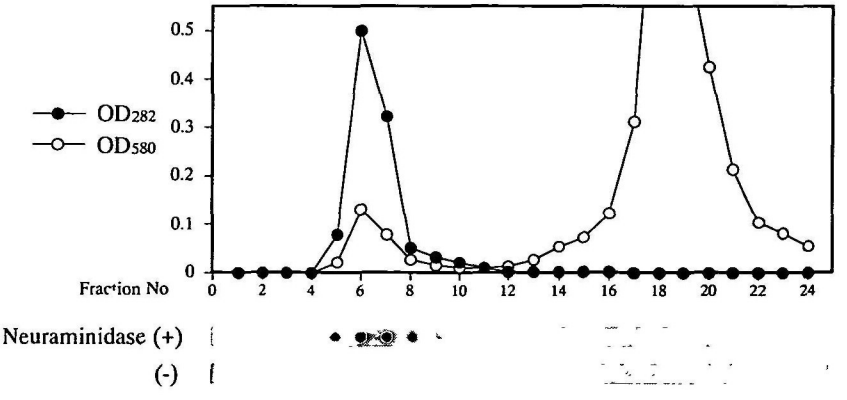


Fig. 2. **Gel filtration of (GT1b oligo)13-streptavidin on a Sephadex G-25 column.** GT1b oligosaccharides were coupled to streptavidin by reductive amination. An incubation mixture was applied to a Sephadex G-25 column. The elution profile of protein was monitored as to the absorbance at 282 nm (closed circles). The elution profile of sialo-compounds was monitored by the resorcinol method (open circles). An aliquot of each fraction was spotted onto a PVDF membrane, with or without following neuraminidase treatment. The membrane was then probed with the colera toxin B subunit.

nated as (GT1b oligo)13-streptavidin.

Polymerization of (GT1b Oligo)13-Streptavidin—(GT1b oligo)13-streptavidin was polymerized with biotinylated-bovine serum albumin, which had been extensively biotinylated and estimated to carry 55 mol of biotin by MALDI-TOF/MS (B55-BSA). The mixing ratio of [^{125}I]-B55-BSA to (GT1b oligo)13-streptavidin was 1 to 25-30. When the mixture was analyzed by SDS-PAGE, a large molecular weight band was observed in addition to a band of the monomer (GT1b oligo)13-streptavidin (Fig. 3). In the mixture, all the radioactivity of [^{125}I]B55-BSA was detected in the large molecular weight band, indicating that all the [^{125}I]B55-BSA was included in complexes with (GT1b oligo)13-streptavidin. Based on the GLC data mentioned above, the molecular mass of (GT1b oligo)13-streptavidin was calculated to be 80.5 kDa. The molecule, however, migrates to a much lower position than expected on SDS-PAGE with ordinary mass marker proteins. This mobility shift was probably due to the negative charge of sialic acids of the oligosaccharide portion. The molecular mass of the polymer was determined by gel filtration chromatography on a Superose 6 column, from which the polymer was eluted at a position around 1,000 kDa (Fig. 4). Since the polymer was expected to be composed of one molecule of biotinylated BSA (84 kDa) and surrounding (GT1b oligo)13-streptavidin molecules (80.5 kDa), the number of the latter molecules was calculated to be 11.4. Thus, more than 140 oligosaccharides were carried by the polymer [designated as (GT1b oligo)13-streptavidin-polymer].

Binding of the (GT1b Oligo)13-Streptavidin-Polymer to CHO Cells Expressing Sialoadhesin—The (GT1b oligo)-13-streptavidin-polymer was expected to interact with sialoadhesin, which recognizes the terminal trisaccharide structure of GT1b or GD1a ganglioside, Siaα2-3Galβ1-4GalNAc (*14*). The polymer bound to CHO cells which stably expressed murine sialoadhesin (Sn cells), but not to wild type cells (Fig. 5). The binding to Sn cells was inhibited by a mixture of monoclonal antibodies, SER-4 (*33*) and 3D6 (*14*), both of which have been shown to block sialoadhesin-dependent binding in several assay systems (*11*). The binding was also inhibited by 2 mM GT1b ganglioside. Thus, the binding of the polymer to Sn cells was shown to depend on the interaction between sialoadhesin and the oligosaccharide portion of the polymer. The addition of sodium azide to the incubation mixture reduced the binding by only 5-7%, suggesting that only a little internalization of the polymer, if any, occurred. The binding was half maximal at 30 min incubation and was near equilibrium at 3 h (data not shown), which was used as the standard incubation time. The specific binding of the polymer was saturable, and the apparent K_d and B_{max} values were 12 nM and 15 fmol/2×10^4 cells, respectively (Fig. 6).

Inhibition of the Polymer Binding by Various GT1b-Related Compounds—Various GT1b-related compounds were examined as to their ability to inhibit the binding. The IC_{50} value of GT1b oligosaccharides was 5.5 mM, indicating poor inhibitory activity (Fig. 7). The micellar and liposomal forms of GT1b gangliosides were more potent inhibitors than the corresponding oligosaccharides, the IC_{50} values being 300 and 60 μM, respectively. This suggests that the multivalency of oligosaccharide determinants on micelles or liposomes is critical for high inhibitory potency. The

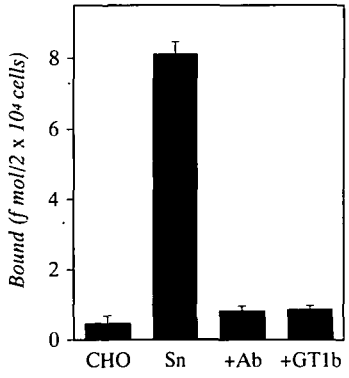


Fig. 5. **Binding of (GT1b oligo)13-streptavidin-polymer to CHO cells expressing murine sialoadhesin.** CHO cells expressing murine sialoadhesin (Sn) and wild type cells (CHO) were dispensed in the wells of a microtiter plate (2×10^4 cells/well). After induction of sialoadhesin with 5 mM sodium butyrate, the cells were subjected to a binding assay, in which they were incubated with [^{125}I](GT1b oligo)13-streptavidin-polymer at 20°C for 3 h. The radioactivity bound to the cells was determined as described under "MATERIALS AND METHODS." Inhibition of the binding was examined with either a mixture of monoclonal antibodies, SER-4 and 3D6 (+Ab), or 2 mM GT1b ganglioside (+GT1b). The values are the means±SD for triplicate determinations.

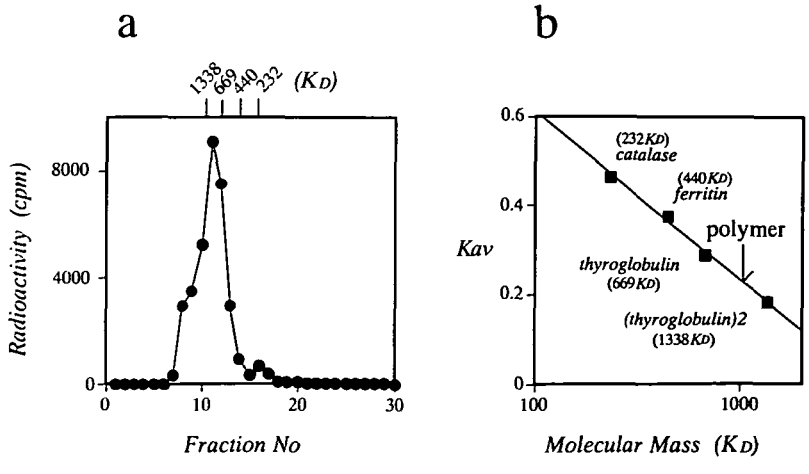


Fig. 4. **Gel filtration of (GT1b oligo)13-streptavidin on a Superose 6 column.** A mixture of [^{125}I]biotinylated-BSA and (GT1b oligo)13-streptavidin was applied to a Superose 6 column. The effluent was collected in 1 ml fractions and the radioactivity in each fraction (closed circles) was monitored with a gamma counter. The positions of marker proteins are indicated by bars at the top of panel a, *i.e.* catalase (232 kDa), ferritin (669 kDa), thyroglobulin (669 kDa), and a dimeric form of thyroglobulin (1,338 kDa). Based on the elution positions of the above marker proteins (closed squares), a calibration line was generated, and the position of the radioactivity peak is indicated by an arrow in panel b.

monomer form of (GT1b oligo)13-streptavidin, carrying 13 oligosaccharides, also exhibited high inhibitory potency, with an IC_{50} value of 10 μM. The most potent inhibitor was the non-radioiodinated polymer, the IC_{50} value being 11 nM, which was 900-fold less than that of the monomer. Thus, the relative inhibitory potencies of the polymer, monomer, and free oligosaccharides were estimated to be 3,500, 83, and 1, respectively, on the basis of their IC_{50} values. These results suggest that the inhibitory potencies of GT1b-related compounds mainly depend upon the multivalency of their oligosaccharides and that the polymer is the most potent.

Inhibition of the Polymer Binding by Various Glycosphingolipids and GM3 Derivatives—Various glycosphingolipids were examined as to their inhibitory activity in the polymer binding assay. When the micellar forms of glycosphingolipids were examined at a concentration of 1 mM, GM3, GD1a, and sialylparagloboside completely inhibited the binding (Fig. 8), suggesting that the terminal disaccharide structure, NeuAcα2-3Galβ1-, shared by these gangliosides and GT1b is a critical determinant. GM4 exhibited weak inhibitory potency, suggesting that only the terminal disaccharide structure is not enough for the maximum binding, and that an additional neutral sugar, or a "spacer" molecule to present the determinant, is required at the internal position. The nature of the internal sugar and its linkage to the disaccharide, however, do not appear to be critical, because GM3, GD1a, and sialylparagloboside, which have 1-4Glc, 1-3GalNAc, and 1-4GlcNAc, respectively, as the internal structure, exhibited fairly strong inhibitory activity, the IC_{50} values being 40, 100, and 120 μM, respectively (Table II). GM2, having an additional GalNAcβ1-residue at the 4-position of Gal(I) of GM3, exhibited some inhibitory potency, but GM1, having Galβ1-3GalNAcβ1-, exhibited no inhibition, suggesting that these substituents hinder the determinant due to their bulky structures. The presence of NeuAc at the non-reducing terminus appears to be essential, since almost no inhibitory activity was observed with lactosylceramide, GM1, GD1b, or paragloboside, which are generated by removing NeuAc from GM3, GD1a, GT1b, and sialylparagloboside, respectively (Fig. 8). In order to determine which portion of the terminal NeuAc is recognized by sialoadhesin, we chemically modified the NeuAc of GM3, the most potent ganglioside, and examined its inhibitory potency. Initially, the carboxylic acid of NeuAc was converted to a methylester by

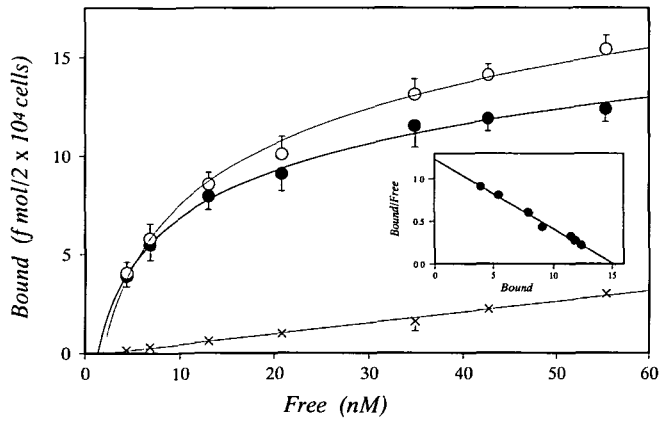


Fig. 6. **Saturation isotherm for (GT1b oligo)13-streptavidin-polymer binding to Sn cells.** Sn cells were incubated with increasing concentrations of [^{125}I](GT1b oligo)13-streptavidin-polymer in the presence or absence of a mixture of monoclonal antibodies, SER-4 and 3D6. Specific binding (closed circles) was defined as total binding (open circles, binding in the absence of the antibodies) minus nonspecific binding (crosses, binding in the presence of the antibodies). The values are the means±SD for triplicate determinations (SD values which fall within the symbols are not shown). Scatchard analysis was performed with the data derived from the saturation isotherm (inset).

TABLE II. **IC_{50} values of various gangliosides and GM3 derivatives.**

Inhibitors[a]	Ligands used for binding assay	
	(GT1b oligo)13-streptavidin-polymer	Human erythrocytes
	(μM)	(μM)
GM3	40	80
GD1a	100	100
SPG	120	—[b]
GT1b	310	200
GM2	640	—
GM4	2,100	—
GM3(C7- or C8-aldehyde)	500	—
GM3(C7- or C8-alcohol)	700	—
GM3(C1-alcohol)	2,000	—
GM3(C1-amide)	2,200	—
GM3(NeuGc)	>3,000	—

[a]The sialic acid species, except NeuAc, are indicated in parentheses. [b]—, not determined.

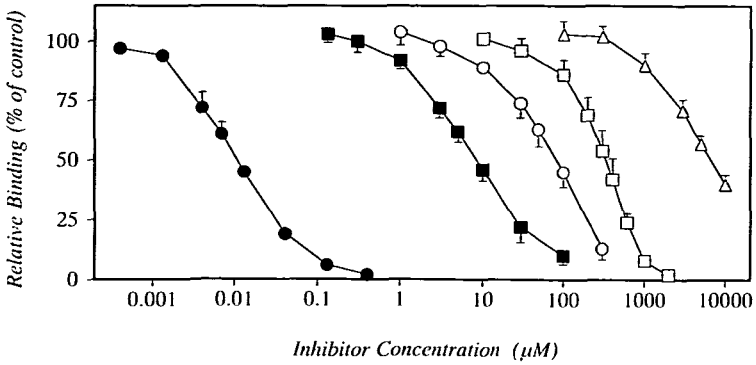


Fig. 7. **Inhibition of (GT1b oligo)13-streptavidin-polymer binding by various GT1b-related compounds.** Sn cells were incubated with increasing concentrations of various GT1b-related compounds as inhibitors, and then [^{125}I](GT1b oligo)13-streptavidin-polymer was added. The binding in the presence of an added inhibitor is expressed as a percentage of the binding in the absence of the added inhibitor. Nonspecific binding was determined in the presence of a mixture of monoclonal antibodies, SER-4 and 3D6, and subtracted from all data points. The values are the means±SD for triplicate determinations (SD values which fall within the symbols are not shown). The inhibitors tested were: (GT1b oligo)13-streptavidin-polymer, closed circles; a monomer form of (GT1b oligo)13-streptavidin, closed squares; the liposome form of GT1b ganglioside, open circles; the micellar form of GT1b ganglioside, open squares; GT1b oligosaccharides, open triangles.

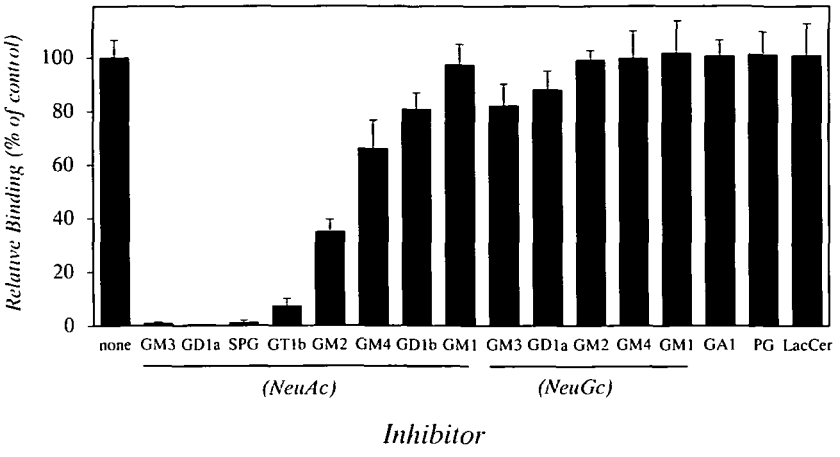


Fig. 8. **Inhibition of (GT1b oligo)13-streptavidin-polymer binding by various glycosphingolipids.** Sn cells were incubated with 1 mM of various glycosphingolipids as inhibitors, and then [^{125}I](GT1b oligo)13-streptavidin-polymer was added. The binding in the presence of an added glycosphingolipid is expressed as a percentage of the binding in the absence of the added glycosphingolipid. Nonspecific binding was determined in the presence of a mixture of monoclonal antibodies, SER-4 and 3D6, and subtracted from all data points. The values are the means±SD for triplicate determinations. The glycosphingolipids tested are indicated under the panel using the abbreviations in Table I. Sialic acid species in the gangliosides are indicated in parentheses.

the method of Handa and Nakamura (*26*), but the methyl ester was partially hydrolyzed during the incubation for the binding assay. The methyl ester, therefore, was converted into either the C1-alcohol by reduction with sodium borohydride or the C1-amide by ammonia treatment. Both the conversions reduced the inhibitory activity to less than 2% of the original level (Table II), suggesting that the carboxylic acid of NeuAc is essential for the binding. The periodate treatment of GM3 reduced the inhibitory activity to 8% and the following reduction with sodium borohydride reduced it to less than 6%, suggesting that the presence of the glycerol side chain of NeuAc is required for binding. The methyl group of the *N*-acetyl residue of NeuAc appears to be critical, since NeuAc-containing GM3 was 75-fold more potent than the NeuGc-containing counterpart. The other gangliosides containing NeuGc were also very poor inhibitors. Taken together, we conclude that the carboxylic acid, the exocyclic glycerol side chain, and the methyl group of the *N*-acetyl residue of NeuAc are strictly required for recognition by murine sialoadhesin.

Binding of Polymers Carrying Various Oligosaccharides—The ligand binding specificity was further characterized by using several kinds of streptavidin-polymers carrying various oligosaccharides. The (GD1a oligo)14-streptavidin-polymer bound well to Sn cells as did the (GT1b oligo)13-streptavidin-polymer, whereas the (GM1 oligo)15-streptavidin-polymer did not (Fig. 9). The (LSTa)13-streptavidin-polymer bound well to the cells, suggesting that the NeuAcα2-3Galβ1- residue attached at the 3-position of the internal GlcNAc is a potent ligand. The (DSLNT)13-streptavidin-polymer exhibited less than half the binding of the (LSTa)13-streptavidin-polymer, indicating that an additional NeuAcα2-6 residue at the internal GlcNAc of LSTa decreases the binding potency. Neither the (LSTb)14- nor the (LSTc)13-streptavidin-polymer bound to the cells, indicating that the substitution of NeuAcα2-6 at either Gal of lactosamine or GlcNAc of the neolactosamine structure does not generate a determinant, and that the NeuAcα2-3 linkage is essential for the binding. The requirement for NeuAcα2-3 but not NeuAcα2-6 was also shown by the binding of the (α2-3sialyllactitol)14-streptavidin-polymer and the lack of binding of the (α2-6sialyllactitol)14-streptavidin-polymer.

Inhibition of Human Erythrocyte Binding by Various Glycosphingolipids and the Polymer—The ligand structure

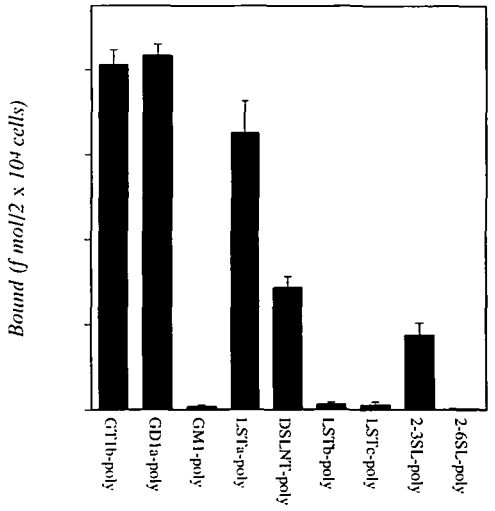


Fig. 9. **Binding of various oligosaccharyl-streptavidin-polymers to Sn cells.** Binding was tested with a concentration of 15 nM. Nonspecific binding was determined in the presence of a mixture of monoclonal antibodies, SER-4 and 3D6, and subtracted from all data points. The values are the means±SD for triplicate determinations. The oligosaccharide structure carried by each polymer is indicated under the panel using the abbreviations in Table I.

for sialoadhesin was initially reported to be NeuAcα2-3βGalβ1-3GalNAc or NeuAcα2-3Galβ1-3(4)GlcNAc. The binding potency of GM3, carrying the NeuAcα2-3Galβ1-4Glc structure, was estimated to be only 64% of that of GT1b (*14*), when these gangliosides were immobilized on a TLC plate and probed with the purified sialoadhesin. With our assay system, on the contrary, GM3 was the most potent inhibitor. In order to determine whether or not this minor but obvious difference is due to the structure of the ligand used in the present study, we set up an assay using an authentic cellular ligand, human erythrocytes. As shown in Table II, GM3 was a potent inhibitor of the binding of human erythrocytes. The IC_{50} values of GM3, GD1a, and GT1b were 80, 100, and 200 μM, respectively, indicating that the inhibitory potencies of these gangliosides as to erythrocyte binding are essentially the same as those as to polymer binding. This suggests that the apparent difference between the present study and a previous one (*14*) is mainly due to the difference in the assay systems, *i.e.* either

the isolated or membrane-bound form of sialoadhesin were used and/or either ganglioside immobilized on a TLC plate or a micellar form were used. The binding of human erythrocytes was inhibited by the (GT1b oligo)13 streptavidin-polymer, with an IC_{50} value of 100 nM.

DISCUSSION

The interaction of carbohydrate chains with lectins on the cell surface comprises an initial step in the process of cellular attachment or recognition. The best characterized families of such lectins are the selectin (E-, P-, and L-selectin) (*36*), and sialoadhesin or I type lectin families (sialoadhesin, CD22, CD33, MAG, and SMP) (*8-11*). Their interactions with sugar chains are believed to occur in a multivalent manner, *i.e.* an array of lectins on the cell surface can bind to multiple oligosaccharide ligands of glycoconjugates on the opposing surface of the recognized cells. Actually, the clustering of sialoadhesin on murine macrophages has been demonstrated at contact sites with myelomonocytic cells on electron microscopy (*37*).

In order to characterize the multivalent interaction of sialoadhesin on the cell surface, CHO cells expressing murine sialoadhesin were used as a model system. As a multivalent ligand, we used a streptavidin-based neoglycoprotein carrying more than 140 GT1b oligosaccharides. Preparation of the neoglycoprotein comprised two steps. The first step was the coupling of GT1b oligosaccharides to streptavidin by reductive amination, which had been established previously for the preparation of neoglycoproteins, especially those based on BSA. By this method we coupled 13 mol of oligosaccharide to streptavidin [(GT1b oligo)13-streptavidin, which maintained its ability to bind biotin molecules]. The next step was the polymerization of (GT1b oligo)13-streptavidin with biotinylated BSA. Although the biotinylated BSA carried 55 mol of biotin (B55-BSA), it bound only 11 mol of (GT1b oligo)13-streptavidin to form a polymer. Even when a 100-fold molar excess of (GT1b oligo)13-streptavidin was mixed with B55-BSA, the number did not increase, suggesting that the rest of the biotin molecules on BSA were not accessible, probably due to steric hindrance due to the 11 molecules of (GT1b oligo)13-streptavidin. The polymer, however, still exhibited a valency of more than 140 oligosaccharides and could be successfully applied to the binding assay.

A glycoprobe carrying more than 140 complex oligosaccharides is not easy to prepare, and only a few successful cases have been reported so far. One is the "glycopolymer" reported by Fan *et al.* (*38*). They coupled high mannose-type oligosaccharides with 3-(*N*-acryloylamino)propyl through a transglycosylation reaction with endo-β-*N*-acetylglucosaminidase, and the resulting compounds were polymerized with acrylamide to form a glycopolymer, generating a valency of 300-400. The glycopolymer exhibited high affinity to the recombinant mannose binding protein due to its multivalency. Their method is quite efficient for the preparation of a multivalent glycoprobe carrying complex oligosaccharides, since the use of the enzyme results in a high yield of the product under mild conditions even for bulky complex oligosaccharides (*39*). The enzyme, however, strictly requires a GlcNAcβ1-4GlcNAc residue of the high mannose-type or hybrid-type *N*-linked sugar chains as an oligosaccharide donor. In contrast, our method can be applied to almost all oligosaccharides as long as they contain a terminal reducing sugar. We believe that the present method is very useful for the preparation of glycoprobes, especially when a high valency of complex oligosaccharides is required.

We were able to develop a sensitive and quantitative assay using a radioiodinated form of the polymer. A quantitative inhibition assay with several compounds carrying different numbers of GT1b oligosaccharide ligands revealed that an increase in the number of the ligands is critical for generating high avidity, and supported the assumption that not a few but a large number of the oligosaccharides on the polymer interact with an array of sialoadhesins on the cell surface. This may also be the case for other sialoadhesin family proteins, when they are expressed on the cell surface. Powell *et al.* reported that when CD22 molecules were stably expressed on CHO cells, they formed a cluster on the cell surface even before the addition of multivalent ligands, and that the cluster could interact with multiple oligosaccharide ligands on a carrier molecule (*17*). In addition, Schnaar and his colleagues reported that CHO or COS cells expressing MAG could adhere to the wells of a microtiter plate when the wells were coated with ganglioside ligands, *e.g.* GT1b, at higher concentrations than a certain threshold level, suggesting that an array of MAG on the cell surface interacted with polyvalent gangliosides on the wells (*18, 19*). This adhesion was inhibited by GT1b micelles, representing a multimeric form of ligands, but not by GT1b oligosaccharides, a monomeric ligand. They hypothesized that a similar polyvalent interaction takes place at the interface between the periaxonal myelin membrane and the axolemma, *i.e.* the former contains MAG and the latter presents sialo-glycoconjugates as ligands. These results as well as ours suggest that the multivalent interaction of lectins with oligosaccharide ligands on opposing cell surface is a common mechanism for generating high avidity or tight attachment, at least for sialoadhesin family proteins.

Collins *et al.* recently utilized the above assay for COS cells expressing sialoadhesin to characterize its binding specificity (*14*). Their results were consistent with ours except that sialoadhesin bound well to GM4 and GD1b in their assay system. In our assay system, these two gangliosides exhibited much weaker inhibitory potency than GM3, GD1a, and GT1b (Fig. 8). There are two possible explanations for this difference. One is the presence of cholesterol and phosphatidylcholine, which they used to coat the microtiter wells with the gangliosides. These lipids might have enhanced the apparent binding activity of gangliosides by generating better accessibility of oligosaccharide ligands to sialoadhesin especially when the sialic acids of gangliosides are located at an internal position (GD1b) or proximal to the lipid layer (GM4). This mechanism is involved in the better accessibility of oligosaccharide ligands when gangliosides are integrated on liposomes. This may partially account for our observation that the liposome form of GT1b was 5-fold more potent than the micellar form of GT1b (Fig. 7). Alternatively, a large amount of sialoadhesin on the cell surface might be required to generate high multivalency of the lectins especially when the ligands exhibit weak binding activity, *i.e.* they used COS cells, which might express higher levels of sialoadhesin than the Sn cells used in the present study.

The present assay was utilized to determine the precise requirement of the NeuAc structure for sialoadhesin binding. GM3 derivatives containing various NeuAc were employed for this experiment. Conversion of the carboxylic acid into the primary alcohol or carboxylamide markedly decreased the inhibitory potency, indicating that the carboxylic acid is required for the binding. Mild periodate treatment of GM3 markedly decreased the inhibitory activity and the subsequent reduction step did not restore it, suggesting that the presence of the glycerol side chain is required for the binding. These results are consistent with recent observations by Collins *et al.*, *i.e.* requirement of the glycerol side chain and the carboxylic acid of NeuAc was demonstrated by using chemically modified GD1a (*14*). The methyl group of the *N*-acetyl residue of NeuAc is also critical for the binding, since NeuAc-containing GM3 was a 75-fold more potent inhibitor than NeuGc-containing GM3, which is consistent with the observation reported by Kelm *et al.* (*40*). Thus, the requirement for the carboxylic acid, the exocyclic glycerol side chain, and the methyl group of the *N*-acetyl residue of NeuAc was confirmed by means of our quantitative assay. Nath *et al.* reported that the sialic acid-binding site of sialoadhesin is located within the NH_2-terminal (membrane-distal) V-set domain (*41*). Site-directed mutagenesis of this domain was recently carried out by Vinson *et al.* (*42*), and their results have been applied to a molecular model of sialoadhesin, in which the amino acids involved in the binding are located at a contiguous site on one of the two β-sheets of the V-set domain. It is anticipated that the direct interaction of some of these amino acids with the above residues of sialic acid will be demonstrated by X-ray crystallography, which is currently being undertaken by May *et al.* (*43*).

The expression of sialoadhesin is restricted to macrophages in tissues such as the spleen, lymph nodes and bone marrow, and the molecule is assumed to mediate the attachment of macrophages to lymphohematopoietic cells by recognizing sialoglycoconjugates on the cell surface. In these processes, modifications of sialic acid may be involved as one of the regulatory mechanisms. Shi *et al.* reported that the *O*-acetylation of sialic acid masks sialoadhesin ligands on murine erythroleukemia cells, and affects their homing or colonization to mouse spleen and liver (*44*). It is also interesting to note that the conversion of NeuAc into NeuGc, the most effective modification for abolishing the sialoadhesin binding, is a physiological event in murine lymphocytes and often associated with cellular activation (*45*). An important question arising is whether or not the conversion is really involved in cellular recognition *in vivo*. A monoxygenase, which catalyzes the conversion of CMP-NeuAc to CMP-NeuGc, is assumed to be the rate-limiting enzyme for the conversion (*46, 47*), and the enzyme has recently been purified from mouse liver (*48*) and its cDNA cloned in our laboratory (*49*). It would be interesting to analyze regulation of the enzyme and changes in sialic acid species of lymphocytes during the process of cellular activation or differentiation, with particular reference to cellular adhesiveness to sialoadhesin. Another important question to be addressed is what kinds of molecule carry high affinity oligosaccharide ligands *in vivo* and how their expression is regulated. The isolation and structural characterization of natural ligands for sialoadhesin, and analysis of mechanisms controlling their expression would be important steps for understanding the biological relevance of sialoadhesin.

We wish to thank Dr. Takaomi Saido, Department of Molecular Biology of our institute, and Dr. Koji Nagata, Department of Molecular Physiology, for the valuable suggestions regarding peptide synthesis, Drs. Masato Umeda and Mizuho Kaneda, Department of Inflammation Research, for their assistance in preparing the liposomes, Dr. Masako Ohashi, Ochanomizu Womens' College, for providing the GM4(NeuAc) and GM4(NeuGc), Drs. Michiko Sekine, Kyoko Nakamura, Hideo Kubo, and Atsushi Irie, of our laboratory, for the helpful discussions and for providing glycolipid standards, Ms. Eiko Kobayashi, Ochanomizu Womens' College, for her technical assistance, and Dr. Tamio Yamakawa, for his encouragement throughout the study. Kazuko Hashimoto is also acknowledged for her excellent secretarial assistance.

REFERENCES

1. Svennerholm, L. (1963) Chromatographic separation of human brain gangliosides. *J. Neurochem.* **10**, 613-623
2. Lee, Y.C. and Lee, R.T. (1994) *Neoglycoconjugates*, Academic Press, San Diego
3. Lee, Y.C. (1992) Biochemistry of carbohydrate-protein interaction. *FASEB J.* **6**, 3193-3200
4. Mahoney, J.A. and Schnaar, R.L. (1994) Ganglioside-based neoglycoproteins. *Methods Enzymol.* **242**, 17-27
5. Tiemeyer, M., Yasuda, Y., and Schnaar, R.L. (1989) Ganglioside-specific binding protein on rat brain membranes. *J. Biol. Chem.* **264**, 1671-1681
6. Tiemeyer, M., Swank-Hill, P., and Schnaar, R.L. (1990) A membrane receptor for gangliosides is associated with central nervous system myelin. *J. Biol. Chem.* **265**, 11990-11999
7. Crocker, P.R., Mucklow, S., Bouckson, V., McWilliam, A., Willis, A.C., Gordon, S., Milon, G., Kelm, S., and Bradfield, P. (1994) Sialoadhesin, a macrophage sialic acid binding receptor for haemopoietic cells with 17 immunoglobulin-like domains. *EMBO J.* **13**, 4490-4503
8. Powell, L.D. and Varki, A. (1995) I-type lectins. *J. Biol. Chem.* **270**, 14243-14246
9. Crocker, P.R., Kelm, S., Hartnell, A., Freeman, S., Nath, D., Vinson, M., and Mucklow, S. (1996) Sialoadhesin and related cellular recognition molecules of the immunoglobulin superfamily. *Biochem. Soc. Trans.* **24**, 150-156
10. Crocker, P.R. and Feizi, T. (1996) Carbohydrate recognition systems: functional triads in cell-cell interactions. *Curr. Opin. Struct. Biol.* **6**, 679-691
11. Kelm, S., Schauer, R., and Crocker, P.R. (1996) The sialoadhesins—a family of sialic acid-dependent cellular recognition molecules within the immunoglobulin superfamily. *Glycoconjugate J.* **13**, 913-926
12. Kelm, S., Pelz, A., Schauer, R., Filbin, M.T., Tang, S., de Bellard, M.E., Schnaar, R.L., Mahoney, J.A., Hartnell, A., and Bradfield, P. (1994) Sialoadhesin, myelin-associated glycoprotein and CD22 define a new family of sialic acid-dependent adhesion molecules of the immunoglobulin superfamily. *Curr. Biol.* **4**, 965-972
13. Freeman, S.D., Kelm, S., Barber, E.K., and Crocker, P.R. (1995) Characterization of CD33 as a new member of the sialoadhesin family of cellular interaction molecules. *Blood* **85**, 2005-2012
14. Collins, B.E., Kiso, M., Hasegawa, A., Tropak, M.B., Roder, J.C., Crocker, P.R., and Schnaar, R.L. (1997) Binding specificities of the sialoadhesin family of I type lectins. *J. Biol. Chem.* **272**, 16889-16895
15. Crocker, P.R., Kelm, S., Dubois, C., Martin, B., McWilliam, A.S., Shotton, D.M., Paulson, J.C., and Gordon, S. (1991) Purification and properties of sialoadhesin, a sialic acid-binding receptor of murine tissue macrophages. *EMBO J.* **10**, 1661-1669
16. Powell, L.D. and Varki, A. (1994) The oligosaccharide binding specificities of CD22 beta, a sialic acid-specific lectin of B cells. *J. Biol. Chem.* **269**, 10628-10636
17. Powell, L.D., Jain, R.K., Matta, K.L., Sabesan, S., and Varki, A.

(1995) Characterization of sialyloligosaccharide binding by recombinant soluble and native cell-associated CD22. Evidence for a minimal structural recognition motif and the potential importance of multisite binding. *J. Biol. Chem.* **270**, 7523-7532
18. Yang, L.J., Zeller, C.B., Shaper, N.L., Kiso, M., Hasegawa, A., Shapiro, R.E., and Schnaar, R.L. (1996) Gangliosides are neuronal ligands for myelin-associated glycoprotein. *Proc. Natl. Acad. Sci. USA* **93**, 814-818
19. Collins, B.E., Yang, L. J., Mukhopadhyay, G., Filbin, M.T., Kiso, M., Hasegawa, A., and Schnaar, R.L. (1997) Sialic acid specificity of myelin-associated glycoprotein binding. *J. Biol. Chem.* **272**, 1248-1255
20. Powell, L.D., Sgroi, D., Sjoberg, E.R., Stamenkovic, I., and Varki, A. (1993) Natural ligands of the B cell adhesion molecule CD22 beta carry N-linked oligosaccharides with α-2,6-linked sialic acids that are required for recognition. *J. Biol. Chem.* **268**, 7019-7027
21. Sgroi, D., Varki, A., Braesch-Andersen, S., and Stamenkovic, I. (1993) CD22, a B cell-specific immunoglobulin superfamily member, is a sialic acid-binding lectin. *J. Biol. Chem.* **268**, 7011-7018
22. Sjoberg, E.R., Powell, L.D., Klein, A., and Varki, A. (1994) Natural ligands of the B cell adhesion molecule CD22 beta can be masked by 9-*O*-acetylation of sialic acids. *J. Cell Biol.* **126**, 549-562
23. Hashimoto, Y., Otsuka, H., and Yamakawa, T. (1982) The occurrence of GM4 and GM2 in erythrocytes from inbred strains of mice. *J. Biochem.* **91**, 1039-1046
24. Suzuki, M., Nakamura, K., Hashimoto, Y., Suzuki, A., and Yamakawa, T. (1986) Mouse liver gangliosides. *Carbohydr. Res.* **15**, 213-223
25. Wiegandt, H. (1968) The structure and the function of gangliosides. *Angew. Chem. Int. Ed. Engl.* **7**, 87-96
26. Handa, S. and Nakamura, K. (1984) Modification of sialic acid carboxyl group of ganglioside. *J. Biochem.* **95**, 1323-1329
27. Hashimoto, Y., Suzuki, A., Yamakawa, T., Miyashita, N., and Moriwaki, K. (1983) Expression of GM1 and GD1a in mouse liver is linked to the H-2 complex on chromosome 17. *J. Biochem.* **94**, 2043-2048
28. Spiegel, S., Ravid, A., and Wilchek, M. (1979) Involvement of gangliosides in lymphocyte stimulation. *Proc. Natl. Acad. Sci. USA* **76**, 5277-5281
29. Roy, R., Katzenellenbogen, E., and Jennings, H.J. (1984) Improved procedures for the conjugation of oligosaccharides to protein by reductive amination. *Can. J. Biochem. Cell Biol.* **62**, 270-275
30. Cabacungan, J.C., Ahmed, A.I., and Feeney, R.E. (1982) Amine boranes as alternative reducing agents for reductive alkylation of proteins. *Anal. Biochem.* **124**, 272-278
31. Svennerholm, L. (1957) Quantitative estimation of sialic acids. II. A colorimetric resorcinol-hydrochloric acid method. *Biochim. Biophys. Acta* **24**, 604-611
32. Chaplin, M.F. (1982) A rapid and sensitive method for the analysis of carbohydrate components in glycoproteins using gas-liquid chromatography. *Anal. Biochem.* **123**, 336-341
33. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685
34. Crocker, P.R. and Gordon, S. (1989) Mouse macrophage hemagglutinin (sheep erythrocyte receptor) with specificity for sialylated glycoconjugates characterized by a monoclonal antibody. *J. Exp. Med.* **169**, 1333-1346
35. MacDonald, R.C., MacDonald, R.I., Menco, B.P., Takeshita, K., Subbarao, N.K., and Hu, L.R. (1991) Small-volume extrusion apparatus for preparation of large, unilamellar vesicles. *Biochim. Biophys. Acta* **1061**, 297-303
36. Lasky, L.A. (1995) Selectin-carbohydrate interactions and the initiation of the inflammatory response. *Annu. Rev. Biochem.* **64**, 113-139
37. Crocker, P.R., Werb, Z., Gordon, S., and Bainton, D.F. (1990) Ultrastructural localization of a macrophage-restricted sialic acid binding hemagglutinin, SER, in macrophage-hematopoietic cell clusters. *Blood* **76**, 1131-1138
38. Fan, J.Q., Quesenberry, M.S., Takegawa, K., Iwahara, S., Kondo, A., Kato, I., and Lee, Y.C. (1995) Synthesis of neoglycoconjugates by transglycosylation with *Arthrobacter protophormiae* endo-β-*N*-acetylglucosaminidase. Demonstration of a macro-cluster effect for mannose-binding proteins. *J. Biol. Chem.* **270**, 17730-17735
39. Fan, J.Q., Takegawa, K., Iwahara, S., Kondo, A., Kato, I., Abeygunawardana, C., and Lee, Y.C. (1995) Enhanced transglycosylation activity of *Arthrobacter protophormiae* endo-β-*N*-acetylglucosaminidase in media containing organic solvents. *J. Biol. Chem.* **270**, 17723-17729
40. Kelm, S., Schauer, R., Manuguerra, J.C., Gross, H.J., and Crocker, P.R. (1994) Modifications of cell surface sialic acids modulate cell adhesion mediated by sialoadhesin and CD22. *Glycoconjugate J.* **11**, 576-585
41. Nath, D., van der Merwe, P.A., Kelm, S., Bradfield, P., and Crocker, P.R. (1995) The amino-terminal immunoglobulin-like domain of sialoadhesin contains the sialic acid binding site. Comparison with CD22. *J. Biol. Chem.* **270**, 26184-26191
42. Vinson, M., van der Merwe, P.A., Kelm, S., May, A., Jones, E.Y., and Crocker, P.R. (1996) Characterization of the sialic acid-binding site in sialoadhesin by site-directed mutagenesis. *J. Biol. Chem.* **271**, 9267-9272
43. May, A.P., Robinson, R.C., Aplin, R.T., Bradfield, P., Crocker, P.R., and Jones, E.Y. (1997) Expression, crystallization, and preliminary X-ray analysis of a sialic acid-binding fragment of sialoadhesin in the presence and absence of ligand. *Protein Sci.* **6**, 717-721
44. Shi, W.X., Chammas, R., Varki, N.M., Powell, L., and Varki, A. (1996) Sialic acid 9-*O*-acetylation on murine erythroleukemia cells affects complement activation, binding to I-type lectins, and tissue homing. *J. Biol. Chem.* **271**, 31526-31532
45. Portner, A., Peter-Katalinic, J., Brade, H., Unland, F., Buntemeyer, H., and Muthing, J. (1993) Structural characterization of gangliosides from resting and endotoxin-stimulated murine B lymphocytes. *Biochemistry* **32**, 12685-12693
46. Shaw, L. and Schauer, R. (1988) The biosynthesis of *N*-glycolylneuraminic acid occurs by hydroxylation of the CMP-glycoside of *N*-acetylneuraminic acid. *Biol. Chem. Hoppe Seyler* **369**, 477-486
47. Kawano, T., Kozutsumi, Y., Takematsu, H., Kawasaki, T., and Suzuki, A. (1993) Regulation of biosynthesis of *N*-glycolylneuraminic acid-containing glycoconjugates: characterization of factors required for NADH-dependent cytidine 5′monophosphate-*N*-acetylneuraminic acid hydroxylation. *Glycoconjugate J.* **10**, 109-115
48. Kawano, T., Kozutsumi, Y., Kawasaki, T., and Suzuki, A. (1994) Biosynthesis of *N*-glycolylneuraminic acid-containing glycoconjugates. Purification and characterization of the key enzyme of the cytidine monophospho-*N*-acetylneuraminic acid hydroxylation system. *J. Biol. Chem.* **269**, 9024-9029
49. Kawano, T., Koyama, S., Takematsu, H., Kozutsumi, Y., Kawasaki, H., Kawashima, S., Kawasaki, T., and Suzuki, A. (1995) Molecular cloning of cytidine monophospho-*N*-acetylneuraminic acid hydroxylase. Regulation of species- and tissue-specific expression of *N*-glycolylneuraminic acid. *J. Biol. Chem.* **270**, 16458-16463