Identification of Oligo-N-glycolylneuraminic Acid Residues in Mammal-derived Glycoproteins by a Newly Developed Immunochemical Reagent and Biochemical Methods*

(Received for publication, September 17, 1997, and in revised form, November 11, 1997)

Chihiro Sato‡§, Ken Kitajima‡, Sadako Inoue‡, and Yasuo Inoue**

From the ‡Department of Biophysics and Biotechnology, Graduate School of Science, University of Tokyo, Hongo-7, Tokyo 113, Japan, and the §Department of Applied Biological Sciences, School of Agricultural Sciences, Nagoya University, Chikusa, Nagoya 464-01, Japan, and the Institute of Biological Chemistry, Academia Sinica, Nankang, Taipei 115, Taiwan

The occurrence of the α2→8-linked oligomeric form of N-glycolylneuraminic acid (oligo-Neu5Gc) residues in mammalian glycoproteins was unequivocally demonstrated using a newly developed anti-oligo/poly-Neu5Gc monoclonal antibody as well as by chemical and biochemical methods. First, the antibody, designated mAb.2→4B, which specifically recognized oligo/poly-Neu5Gc with a degree of polymerization of >2, was developed by establishing a hybridoma cell line from P3U1 myeloma cells fused with splenocytes from an MRL autoimmune mouse immunized with dipalmitoylphosphatidylethanolamine-conjugated oligo/poly-Neu5Gc. Second, oligo-Neu5Gc was shown to occur in glycoproteins derived from pig spleen by Western blot analysis using mAb.2→4B, which was also confirmed by fluorometric high performance liquid chromatographic analysis of the product of periodate oxidation/reduction/acid hydrolysis of the purified glycopeptide fractions and by TLC and 600-MHz 1H NMR spectroscopic analysis of their mild acid hydrolysates. Finally, the ubiquitous occurrence of oligo-Neu5Gc chains as glycoproteinaeous components in Wistar rat tissue was immunochemically indicated. This is the first example demonstrating the diversity in oligo/poly-Sia structure in mammalian glycoproteins, where only poly-N-acetylneuraminic acid is known to occur. Such diversity in oligo/poly-Sia structure also implicates a diverged array of biological functions of this glycan unit in glycoproteins.

Oligo/poly-sialic acid structure represents a group of glycan chains consisting of N-acetyleneuraminic acid (Neu5Ac),1 N-glycolylneuraminic acid (Neu5Gc), and deaminoneuraminic acid (KDN) (1, 2). α2→8-Linked oligo/poly-Neu5Gc structure was first found in polysialoglycoprotein (PSGP) isolated from the unfertilized eggs of rainbow trout (Oncorhynchus mykiss) (3). Following this discovery, α2→8-linked poly-Neu5Ac structure was shown to occur in various animal glycoproteins from insect to human by using immunochemical and enzymatic probes specific to α2→8-linked oligo/poly-Neu5Ac glycoproteins (4–11). Recently, we demonstrated that oligo/poly-Sia chains on PSGP isolated from Salvelinus fish eggs exhibit a remarkable degree of diversity in their building blocks arising from the different substitution at C-5, i.e. Neu5Ac, Neu5Gc, and KDN, and in the presence of either O-acetyl or O-lactyl substitution (2). These structural diversities in oligo/poly-Sia may be potentially relevant to the functional diversity that may be required for multiple cellular recognition processes on the cell surface in biological events, such as fertilization and early embryogenesis (2, 12).

Rather extensive debates have long been going on regarding the biological significance of the structural diversity in Sia residues that are expressed in species-specific, tissue-specific, developmental stage-specific, and tumor-specific manners (1, 13–17). We were motivated to confirm the diversity in oligo/poly-Sia structure not only in teleost fishes, but also in mammals because the components of Sia in most animal tissues consist of not only Neu5Ac and Neu5Gc (18), but also KDN (19). The amount of oligo/poly-Sia expressed on mammalian cells was anticipated to be so tiny that establishment of highly sensitive immunochemical probes and chemical methods was of first priority. As shown by previous studies (20, 21), development of anti-oligo/poly-Sia antibodies appeared to be difficult because of their structural similarity to endogenous glycolipids and glycoproteins present in neural and extraneural tissues (1, 22, 23), and the precise determination of the immunospecificity of the anti-oligo/poly-Sia antibodies was troublesome because immobilization of the oligo/poly-Sia chains on plastic plates, membranes, and TLC plates was difficult (24).

In this study, we developed a new monoclonal antibody, mAb.2→4B, specific to oligo/poly-Neu5Gc by immunization of MRL/MpJUmm-lpr autoimmune mice with dipalmitoylphosphatidylethanolamine (PE)-conjugated oligo/poly-Neu5Gc and determined its immunospecificity using these PE-conjugated oligo/poly-Sia chains for solidification on the plastic surface (24). Subsequently, using this antibody, the presence of oligo/...
poly-Neu5Gc structure on glycoproteins derived from mamma-
lian tissues was suggested. To confirm this, chemical detec-
tion was carried out by the new fluorometric high performance
liquid chromatography (HPLC) method (19, 25, 26) in conjunc-
tion with periodate oxidation (C6/C8 analysis) and the mild acid
hydrolysis/TLC method (2, 27). The Neu5Gc-α refuse to 9 thus obtained were separately desalted by chromatography
excision, and the cells were dissociated. Spleen cells (3 × 106) were
used with 4.1 × 108 P3-X63 Ag8.11 (P3U1) mouse myeloma cells (39) ac-
cording to the procedure of Kohler and Milstein (40). The fused cells
were suspended in Dulbecco’s modified essential medium (Life Tech-
nologies, Inc.) containing 20% fetal bovine serum (lot 8409, Filtron,
Brooklyn, Australia), 500 μg hypoxanthine, 20 μg aminopterin, and
5000 units methylthiouracil. The hybridomas were cultured on 10 cm
Petri dishes (Nunc, Roskilde, Denmark). The hybridoma cells were screened by measuring titers of the supernatant
with oligo/poly-
Neu5Gc-PE and oligo/poly-Neu5Ac-PE. Antibody titers were monitored
by the solid-phase enzyme-linked immunosorbent assay (ELISA) as
described previously (24). The oligo/poly-Neu5Gc-PE-positive and oligo/
poly-Neu5Ac-PE-negative hybridoma cells were cultured twice by
limiting dilution, and a clone named 2–4B was obtained.

**Experimental Procedures**

**Materials—**High molecular mass PSGPs, O. mykiss PSGP containing
only α-2–8-linked oligo/poly-Neu5Gc structure and Sulvelinus namay-
cush PSGP containing exclusively α-2–8-linked oligo/poly-Neu5Ac structure
were isolated from the unfertilized eggs of rainbow trout (O. mykiss) and lake trout (S. namaycush), respectively, as described pre-
viously (2). KDN-rich glycoprotein containing α-2–8-linked KDN chains
was isolated from the ovarian fluid of rainbow trout as described pre-
viously (31). Clostridium perfringens exosialidase and Arthrobacter ure-
ofaciens 3-endo-α-fucosidase were purchased from Boehringer Mann-
heim (Mannheim, Germany) and Nacalai (Kyoto, Japan), respectively. Peps-
tide/N-glycanase F was obtained from Seikagaku Kogyo Co. (Tokyo,
Japan). Colomicin in sodium salt form and PE were purchased from Sigma.
Affinity-purified peroxidase-conjugated goat anti-mouse IgM + IgG antibody was obtained from American Qualex. Alkaline
phosphatase-conjugated goat anti-mouse IgM antibody was purchased from Jackson ImmunoResearch Laboratories, Inc. 5-Bromo-4-chloro-3-
indolyl phosphate p-toluidine salt and nitro blue tetrazolium chloride
were purchased from Life Technologies, Inc. Prestained molecular mass
markers were obtained from Bio-Rad. 1,2-Diamino-4,5-methylenedioxy-
benzene (DMB) was a product of Dojindo Laboratories (Kumamoto,
Japan). Colominic acid in sodium salt form and PE were purchased from Jackson ImmunoResearch Laboratories, Inc. 5-Bromo-4-chloro-3-
indolyl phosphate p-toluidine salt and nitro blue tetrazolium chloride
were purchased from Life Technologies, Inc. Prestained molecular mass
markers were obtained from Bio-Rad. 1,2-Diamino-4,5-methylenedioxy-
benzene (DMB) was a product of Dojindo Laboratories (Kumamoto,
Japan).

**Chemical Analysis—**Neu5Ac and Neu5Gc were quantitated by the
resorcinol method (32) and the thiobarbituric acid method as described
previously (35).

**Preparation of Oligo/poly-Neu5Ac, Oligo/poly-Neu5Gc, Oligo/poly-
KDN, and a Series of Oligo/poly-Neu5Gc Chains with Defined Degrees
of Polymerization (DPs)—**These oligo/poly-Sia chains were prepared as
described previously (24). For preparation of oligo/poly-Neu5Ac, oligo/poly-
oligo/poly-Neu5Gc chains with known DPs, α-2–8-linked Neu5Gc-PE chains
were serially diluted in ethanol (1.6–50 ng of Sia/well) by incubation at 37 °C for 1 h. The wells were then incubated with 1% BSA/PBS at 37 °C for 2 h and then incubated with mAb.2-4B (2.5 μg/well) at 4 °C overnight. Antibody binding was detected using peroxi-
dase-conjugated goat anti-mouse IgG + IgM antibody as described previously (24).

**Antibody Binding Assay—**Antibody binding to various oligo/poly-
Sia-PE chains, a series of oligo-Neu5Gc-PE chains (DP = 1–9), O. mykiss PSGP, and S. namaycush PSGP was determined using the
ELISA method (24). A 96-well Aminoplate (Sumitomo Bakelite, Tokyo,
Japan) was used. For the ELISA test, oligo/poly-Sia-PE and oligo-
Neu5Gc-PE chains were serially diluted in ethanol (1.6–50 ng of Sia/well
and 19–75 pmol of Neu5Gc/well), respectively, on the plate. mAb.2–4B was used at 1–200 μg/ml and was dissolved in PBS contain-
ing 0.05% Tween 20 and then incubated with the secondary antibody, alkaline phosphatase-conjugated anti-mouse IgG (50–100
μg/ml) and nitro blue tetrazolium chloride (see Ref. 41) were placed at 65 °C for 15 min. After
incubation at 37 °C for 2 h. Fifty microliters of mAb.2–4B (2.5 μg/ml) and nitro blue tetrazolium chloride (see Ref. 41) were placed at 65 °C for 15 min. After
incubation at 37 °C for 2 h. Fifty microliters of mAb.2–4B (2.5 μg/ml) were added and incubated at 4 °C overnight. Antibody binding was
carried out as described above.

**SDS-Polyacrylamide Gel Electrophoresis and Immunoblotting—**Pig
spleen and various Wistar rat tissues were homogenized on ice in PBS
containing 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and
1% aprotinin. The homogenates (5 mg of protein/ml) dissolved in Læ-
mml buffer (see Ref. 41) were placed at 65 °C for 15 min. After
–50–100 μg of protein were electrophoresed per lane on 3–15% gradient gels
(10) or 10% polyacrylamide gels, immunoblotting on nitrocellulose or
polyvinylidene fluoride membrane was performed (41) using a semidry
blotting apparatus. Briefly, after transfer, the membrane was incu-
baded in 0.1% NaOH at 37 °C for 30 min, blocked for 1 h with PBS
containing 1% BSA and 0.05% Tween 20, and then incubated with
mAb.2–4B (50–100 μg/ml diluted with the same solution) at 4 °C over-
night. The membrane was serially washed with PBS containing 0.05% Tween 20 and with Tris-buffered saline (0.1 M Tris-HCl (pH 7.5) and
0.15 M NaCl) containing 0.05% Tween 20 and then incubated with the sec-
ondary antibody, alkaline phosphatase-conjugated anti-mouse IgG
antibody (50 μg/ml diluted with Tris-buffered saline containing 1% BSA
and 0.05% Tween 20), at 37 °C for 45 min. The membrane was
serially washed with Tris-buffered saline containing 0.05% Tween 20, Tris-
buffered saline, and 0.1 M Tris-HCl (pH 9.5) containing 50 μM MgCl2
and 150 mM NaCl. The membrane was developed with 5-bromo-4-
chloro-3-indolyl phosphate p-toluidine salt (165 μg/ml) and nitro blue
tetrazolium chloride (330 μg/ml) in 0.1 M Tris-HCl (pH 9.5) containing

---

**Downloaded from http://www.jbc.org/ by guest on July 26, 2018**

**2576 Oligo-Neu5Gc Unit in Mammalian Glycoproteins**
50 mM MgCl₂ and 150 mM NaCl.

Exosialidase and Peptide-N-Glycanase F Treatments of the Blotting Membrane—Tissue homogenates were electrophoresed and transferred to the nitrocellulose or polyvinylidene fluoride membrane as described above. After alkaline treatment of the transblotted membrane, the membrane was treated with C. perfringens exosialidase (0.1 unit/ml) in 50 mM sodium acetate buffer (pH 5.0) containing 1% BSA at 37 °C for 18 h for rat tissue homogenates or with A. ureafaciens exosialidase (2.5 units/ml) in 50 mM sodium acetate buffer (pH 5.5) containing 1% BSA at 37 °C for 24 h or peptide:N-glycosidase F (5 units/ml) in 250 mM phosphate buffer (pH 8.25) at 37 °C for 24 h for pig spleen homogenates.

Purification of Oligo-Neu5Gc-containing Glycopeptide(s) from Pig Spleen—Eight-hundred grams of pig spleen (Shibaura Zouki, Tokyo, Japan) were homogenized in 2.4 liter of cold acetone and filtered. The acetone powder was delipidated with chloroform/methanol extraction (42). After washing with ethanol, the delipidated acetone powder (300 g) was incubated with actinase E (2.0 g; Kokusan Kagaku, Tokyo, Japan) in 2 liter of 0.1 M Tris-HCl (pH 8.0) containing 10 mM CaCl₂ and 0.5% Nonidet P-40 at 37 °C for 3 days (17). The solution was centrifuged at 5,700 × g for 20 min. The supernatant was then mixed with 0.5 volume of 90% phenol and centrifuged at 2100 × g for 15 min. The aqueous phase was removed, and the lower phase was mixed with 0.5 volume of 0.1 M Tris-HCl (pH 8.0) and centrifuged. The aqueous phase thus obtained was dialyzed against water and evaporated to 300 ml (2). The concentrated solution was then mixed with 600 ml of cold ethanol at −80 °C for 1 h and centrifuged at 5700 × g for 15 min. The supernatant was subjected to chromatography on a DEAE-Sephadex A-25 anion-exchange column (2.1 × 42 cm) with a linear gradient of NaCl (0-1.0 M) in 10 mM Tris-HCl (pH 8.0). Fraction A-IV (see Fig. 4) was further purified by chromatography on a Sephacryl S-100 column (1.2 × 103 cm; eluted with 0.1 M NaCl) and desalted by passage through a Sephadex G-25 column (1.2 × 98 cm; eluted with 5% ethanol).

Detection of α2–8-Linked Oligo-Sia by the Periodate Oxidation/Fluorometric HPLC Method (C₄–C₆ Analysis)—Samples (~1 µg of Sia) were dissolved in 25 µl of 40 mM sodium acetate buffer (pH 5.5) and after addition of 2 µl of 0.25 M NaIO₄ left at 0 °C for 3 h in the dark. Then, 5 µl of 3% ethylene glycol and 32 µl of 0.5 M NaBH₄ dissolved in 0.2 mM sodium borate buffer (pH 8.0) were added successively and left at 0 °C overnight. During these procedures, nonreducing terminal Neu5Ac or Neu5Gc residues were oxidized to give rise to the C₄ analog of Neu5Ac (5-acetamido-3,5-dideoxy-L-2-heptulosonic acid; C₄-(Neu5Ac)) or Neu5Gc (5-hydroxylacetamido-3,5-dideoxy-2-2-heptulosonic acid; C₄-(Neu5Gc)), whereas internal residues in α2-8-linked oligo/poly-Sia chains remained intact: Neu5Ac, C₅(Neu5Ac); or Neu5Gc, C₅(Neu5Gc). The hydrolysate was then dissolved in 0.1 M trifluoroacetic acid at 80 °C for 1 h and dried up. A 7 mM solution of DMB was freshly prepared by dissolving DMB dihydrochloride in 50 mM trifluoroacetic acid containing 0.75% 2-mercaptoethanol and 18 mM sodium hydroxulfite. The dried sample was dissolved in 20 µl of 10 mM trifluoroacetic acid and incubated at 50 °C for 2 h after addition of 20 µl of the DMB solution. The reaction mixture (2–20 µl) was analyzed by a Jasco LC-900 HPLC system equipped with a Jasco FP-920 fluorescence detector (wavelengths for excitation set at 373 nm and emission at 448 nm), operating isocratically at 1.0 ml/min at a column temperature of 28 °C. A TSK-gel ODS-120T (250, inner diameter, × 4.6 mm) was used. Methanol/acetonitrile/water (7:9:84, v/v/v) was used as eluent (19, 28). Retention times and response factors on HPLC for the C₄ and C₅ analogs of Neu5Ac and Neu5Gc were determined by the concomitant derivatization of the following compounds: fraction S6 derived from chum salmon egg PSGP (45) for C₄(Neu5Ac)-DMB and C₅(Neu5Ac)-DMB and OP-glycopeptide derived from trout ovarian fluid glycoproteins (44) for C₄(Neu5Ac)-DMB and C₅(Neu5Ac)-DMB.

Identification of α2–8-Linked Neu5Gc Oligomer by the Mild Acid Hydrolysis/TLC Method—Fraction A-IV (see Fig. 6a) was incubated with 50 mM sodium acetate buffer (pH 4.8) at 37 °C for 48 h and subjected to chromatography on a Sephacryl S-100 column (1.2 × 113 cm; eluted with 0.1 M NaCl). The free oligosaccharide acid fraction was collected and desalted by passage through a Sephadex G-25 column (1.2 × 108 cm; eluted with 5% ethanol). One micromolar of sialic acid was detected on a TLC plate (Silica Gel 60, Merek; developed in 1-propanol, 25% NH₄OH, and water (6:1.2.5, v/v/v) for 12 h; and visualized by the resorcinol reagent (2).

600-MHz 1H NMR Spectroscopy—The free sialic acid fraction was subjected to preparative TLC on a Silica Gel 60 plate as previously reported (2). The dimeric sialic acid was further purified by passage through a Sephadex G-25 column and denoted A-IV-MH. A-IV-MH and authentic Neu5Gc2α–8Neu5Gc prepared from O. mykiss PSGP (2) were spotted on a TLC plate (Silica Gel 60, Merek); developed in 1-propanol, 25% NH₄OH, and water (6:1.2.5, v/v/v) for 12 h; and visualized by the resorcinol reagent (2).

RESULTS

Preparation of a Monoclonal Antibody (mAb.2–4B) Specific to α2–8-Linked Oligo-Neu5Gc—Three MRL/MpUmmCrg-1pr autoimmune mice were immunized with oligo/poly-Neu5Gc-PE for 24 days as described under “Experimental Procedures,” and splenocytes prepared from one of the mice were fused with the P3U1 cells.

Hybridoma colonies were screened for antibodies by assaysing the binding affinity toward oligo/poly-Neu5Gc-PE but not toward oligo/poly-Neu5Ac-PE, and finally, one of the clones was established after subcloning by limiting dilution. The antibody released from the clone was designated mAb.2–4B. mAb.2–4B was prepared by precipitation of the serum-free culture supernatant with 50% saturated ammonium sulfate and gel filtration on a Sephacryl S-300 column, yielding 37 mg of immunoglobulin/2 liters of the culture supernatant.

Characterization of mAb.2–4B—The class of mAb.2–4B was determined to be IgM. A number of papers reported that the class of antibodies raised against glycolipids was dominantly IgM, but not IgG (see, for example, Refs. 46 and 47). Fig. 1 shows the results of binding of mAb.2–4B with oligo/poly-Neu5Gc-PE on an ELISA plate. As little as 3 ng (as Sia) of oligo/poly-Neu5Gc-PE was detectable when 20–200 µg/ml mAb.2–4B was used. As shown in Fig. 2a, mAb.2–4B reacted only with oligo/poly-Neu5Gc-PE. No reaction was observed with oligo/poly-Neu5Ac-PE, oligo/poly-KDN-PE, or PE, even at higher concentrations of these neoglycolipids (50 ng of Sia/well). Acid treatment (0.1 M HCl, 37 °C, 20 h) or exosialidase digestion (A. ureafaciens, 2.5 microunits, 37 °C, 20 h) of oligo/poly-Neu5Gc-PE resulted in complete loss of binding (Fig. 2b), indicating the requirement of oligo/poly-Neu5Gc structure for the reactivity of mAb.2–4B.

To determine the DP required for recognition by mAb.2–4B, α2–8-linked oligo/poly-Neu5Gc-PE chains (DP = 1–9) were coated separately on the wells (24) and tested for immunoreactivity (Fig. 2c). Neu5Gc-PE and di-Neu5Gc-PE showed no immunoreactivity, and oligo/poly-Neu5Gc-PE samples with DPs larger than 3 were positive for reactivity. All these reac-
Fig. 2. Characterization of mAb.2–4B. a, shown is the reactivity of mAb.2–4B (2.5 μg/well) with oligo/poly-Neu5Gc-PE (●), oligo/poly-Neu5Ac-PE (▲), oligo/poly-KDN-PE (square), and PE (○) on wells coated at 1.5–50 ng of Sia/well or their equivalent amount of lipid. b, plastic wells were coated with oligo-Neu5Gc-PE (7.5–125 ng of Sia/well) and treated with PBS (■), 0.1 μ HCl (▲), and 2.5 microunits of exosialidase/well (●) at 37 °C for 20 h. Each well was assayed for binding with mAb.2–4B (2.5 μg/well) as described under “Experimental Procedures.” c, shown is the reactivity of mAb.2–4B (2.5 μg/well) with a set of oligo-Neu5Gc-PE chains with DP = 1–9 (75 pmol/well). d, shown is the reaction of mAb.2–4B (2.5 μg/well) with O. mykiss PSGP (PSGP(0m)); ■ containing exclusively α2–8-linked oligo/poly-Neu5Gc and S. namaycush PSGP (PSGP(Sn)); ○ containing exclusively α2–8-linked oligo/poly-Neu5Ac on the well coated with various amounts (31–1000 ng of Sia/well).

Fig. 3. Western blot analysis of pig spleen homogenates using mAb.2–4B. Homogenates were run on 10% polyacrylamide gel and transferred to polyvinylidene fluoride membranes. Lane 1, membrane incubated with mAb.2–4B and visualized as described under “Experimental Procedures”; lane 2, exosialidase-treated membrane; lane 3, peptide:N-glycanase F-treated membrane.
derivatives was higher in fraction A-IV (0.07–0.09) than in fraction A-III (0.01).

Identification of Di-Sia Structure (Neu5Gcα2→3Neu5Gc) in Fraction A-IV by Mild Acid Hydrolysis/TLC—Fraction A-IV (1.5 mg of Sia) was treated with 50 mM sodium acetate buffer (pH 4.8) at 37 °C for 2 days, and the free oligo-Sia fraction was separated from major glycopeptide fractions by Sephadryl S-100 chromatography (Fig. 6b). After desalting, A-IV-MH was analyzed by TLC (Fig. 7). The band in lane 3 marked by the arrowhead was identical in mobility to the authentic sample of the dimer, Neu5Gcα2→3Neu5Gc (Fig. 7), indicating that fraction A-IV has a 2–3-linked oligo-Neu5Gc structure with DP ≥ 2. To confirm this band as Neu5Gcα2→3Neu5Gc, the material eluted from the band was further purified for 3H NMR measurement by preparative TLC.
modified by alkali-resistant substitution, although specificity of mAb.2–4B for such structures is unknown.

DISCUSSION

To investigate the diversity in oligo/poly-Sia structure not only in fish eggs, but also in mammalian tissues, a monoclonal antibody that is highly specific to α2→8-linked oligo/poly-Neu5Gc was developed using lipid-conjugated oligo/poly-Neu5Gc as an immunogen and the MRL autoimmune mouse as a host. Based on the ELISA method using lipidated oligo/poly-Sia, mAb.2–4B was shown to react only with α2→8-linked oligo/poly-Neu5Gc, but not with α2→8-linked oligo/poly-Neu5Ac or α2→8-linked oligo/poly-KDN. The DP of oligo/poly-Neu5Gc required for recognition by the antibody was ≥2.

The use of mAb.2–4B for immunohistochemical detection enabled us to detect the presence of oligo/poly-Neu5Gc chains in pig spleen glycoproteins. Western blot analysis revealed that several glycoprotein components were mAb.2–4B-positive in pig spleen homogenate (50- and 52-kDa glycoproteins). In these glycoproteins, oligo/poly-Neu5Gc was shown to reside on N-linked glycans. Thus, the bands of these glycoproteins became mAb.2–4B-negative on peptide:N-glycanase F digestion. Some components contained mAb.2–4B-positive but sialidase-resistant structures even after alkali treatment of the membrane. These components may contain modified sialic acid residue(s) with alkali-resistant substituent or KDN-capping structure.

The presence of α2→8-linked oligo-Neu5Gc structure in pig spleen glycoproteins was also confirmed by chemical and biochemical methods. Based on the fluorescence-assisted peridinin C−Cα analysis, the sialoglycopeptide fraction A-IV, which was eluted at higher NaCl concentrations on DEAE-Sephadex A-25 chromatography, was found to be rich in Cα derivatives of Neu5Gc and Neu5Ac. The proportion of the internal Sia residues involved in the formation of oligo/poly-Sia structure to the total Sia residues in pig spleen was estimated to be 1.7%. This band was unequivocally identified to be Neu5Gc(2→8)Neu5Gc by 1H NMR measurement. No clear band due to the presence of Neu5Ac dimer or hybrid dimers of Neu5Ac and Neu5Gc was observed by TLC analysis, probably because these dimer structures were present less frequently as compared with di-Neu5Gc structure. Notably, no di-Neu5Ac was observed (Fig. 7, lane 3). Considering the occurrence of the comparable amount of internal Neu5Ac and Neu5Gc residues in fraction A-IV (Table I), this result suggests that most internal Neu5Ac

| Assigned proton | Authentic Neu5Gc dimer (2) | ppm | A-IV-MH’ (2) | ppm |
|----------------|--------------------------|-----|--------------|-----|
| H-3***       | 1.74                     | 1.79| 1.74         | 1.79|
| H-3**        | 2.80                     | 2.26| 2.80         | 2.26|
| H-4          | 3.78                     | 4.11| 3.78         | 4.11|
| H-5          | 3.94                     | 4.00| 3.94         | 4.00|
| H-6          | 3.70                     | 3.73| 3.70         | 3.73|
| H-7          | 3.60                     | 3.92| 3.60         | 3.92|
| H-8          | 3.85                     | 3.44| 3.85         | 3.44|
| H-9          | 3.88                     | 3.99| 3.88         | 3.99|
| H-9’         | 3.96                     | 3.51| 3.96         | 3.51|

Oligo-Neu5Gc Unit in Mammalian Glycoproteins

**Table II**

Proton chemical shifts of authentic Neu5Gc dimer and A-IV-MH’ (Neu5Gc(2→2a→8)Neu5Gc(1))

---

**Fig. 7.** TLC analysis of the free oligosialic acid fraction (A-IV-MH) derived from fraction A-IV by pH 4.8-catalyzed hydrolysis at 37 °C for 2 days. About 1 μg of A-IV-MH was spotted on a silica gel 60 plate and developed in 1-propanol, 25% NH4OH, and water (6:1:2.5, v/v/v) for 12 h. The bands were visualized by heating the plate at 100 °C for 30 min after spraying with the resorcinol reagent. As standards, the partial acid hydrolysates of colominic acid (α2→8-linked oligo/poly-Neu5Ac) and O. mykiss PSGP (α2→8-linked oligo/poly-Neu5Gc), supplemented with Neu5Ac and Neu5Gc, respectively, were run in lanes 1 and 2. Lane 3, A-IV-MH. The numbers in lanes 1 and 2 represent the corresponding DPs of oligo/poly-Sia. O, origin.
residues may be involved in the formation of Neu5Gcα2→8Neu5Ac structure, but not Neu5Acα2→8Neu5Ac structure. The failure to detect oligomers with DP ≥ 3 by TLC strongly suggests that the chain length of the oligosialyl chain in pig spleen glycoproteins is not large, but is most likely solely a dimer.

Furthermore, we also identified several mAb.2–4B-reactive glycoprotein components in various rat tissue homogenates. Fig. 8 shows a common occurrence of oligo/poly-Neu5Gc structure in various tissue glycoproteins. Although a2→8-linked di-Neu5Gc structure is known to occur in various mammalian gangliosides (46, 47), this is the first demonstration of the ubiquitous presence of oligo/poly-Neu5Gc-containing glycoproteins of mammalian origin. The DPs of these oligo/poly-Neu5Gc chains are presently unknown. However, they appear low, as found for pig spleen glycoproteins, because most mAb.2–4B-reactive bands were not so broad (Fig. 8) as usually observed for those of polysialylated neural cell adhesion molecules (4–11). Most intriguing is the elucidation of the biological functions of these oligo-Neu5Gc-containing glycoproteins, and it is therefore important to identify and characterize newly detected oligo-Neu5Gc-containing glycoproteins and to study if the expression of these glycoproteins is developmentally regulated. Some speculation on the biological functions of oligo-Neu5Gc can be allowed if one considers that oligo-Sia is now the common structural unit in both glycoproteins and gangliosides in mammals. Higher gangliosides such as G_{0,0}^{2}, G_{5,5}^{2}, G_{0,4}^{2}, and G_{1,1}^{2} are considered to be involved in cell adhesion (52), differentiation (47, 53, 54), signal transduction (55), ADP-ribosylation (56), and specific oncodevelopmental markers (57, 58), where the sialic acid species of these gangliosides is, however, largely of the Neu5Ac type at present. Interestingly, (Neu5Gc)GD_{1c} has recently been identified as a marker for rat CD4^{+} T lymphocytes that produce interleukin-2 (47), where some di-Neu5Gc-specific functions were suggested, including regulation of differentiation of this type of T cells. As far as the biological importance of Neu5Gc is concerned, much discussion has been made regarding species-specific, tissue-specific, developmental stage-specific, and tumor-specific functions of this sialic acid species (1, 13–17). CD22, a sialic acid-binding lectin that is involved in B cell maturation and activation, is known to have a species-dependent preference in the recognition of sialic acid species. Mouse CD22 preferentially recognizes Neu5Gcα2→6Galβ1→4GlcNAc over the corresponding Neu5Acα2→6Galβ1→4GlcNAc over the corresponding Neu5Acα2→6Galβ1→4GlcNAc (59, 60), whereas human CD22 equally recognizes both forms of sialic acid (61). The differential specificity of these CD22 proteins directly indicates the importance of Neu5Gc residues in this cell adhesion process in mouse. Neu5Gc residues are also known not to occur so frequently in human glycoconjugates (18), and Hanganutziu-Deicher (HD) antigens are well known to be one of the oncofetal antigens in human (62). In this regard, it would be a strong possibility that oligo-Neu5Gc units could be identified as an oncofetal antigen in human using our mAb.2–4B antibody.

In summary, we show here for the first time that there exists a structural diversity in oligo/poly-Sia in mammalian glycoproteins other than fish egg glycoproteins. Recently, mAb.kdn5A (28), which specifically recognizes a2→8-linked oligo/poly-KDN structure (DP ≥ 2) (24), and deaminoneuraminase, which hydrolyzes only KDN ketosidic linkages (50, 51), were developed.
and by combination of these sensitive and specific probes, the presence of oligo-KDN sequence was indicated in mammalian tissues (28, 29) and in some lung carcinoma cells (30). Furthermore, using a sensitive chemical method, KDN residues were confirmed unequivocally in mammalian tissues (19), although the chemical identification of oligo-KDN structure still remains to be elucidated. In Table III, the occurrence of α2→8-linked oligo/poly-Sia in mammalian glycoproteins and the immunospecificity of the presently available anti-α2→8-linked oligo/poly-Sia antibodies are summarized. The significance of the diversity in sialic acid structure is now considered to reside in variants of the ligand determinants that are specifically recognized by cognate sialic acid-binding proteins such as selectin, CD22, sialoadhesin, a complement regulatory protein (H-protein), and influenza virus hemagglutinin (14, 16, 17). Accordingly, the functional importance of the diversity in oligo/poly-Sia structure in mammalian tissue should be exemplified by identification of specific binding proteins that may be species-, tissue-, and developmental stage-specifically expressed on the cell surface.

Acknowledgments—We thank Dr. K. Furukawa (Tokyo Metropolitan Institute of Gerontology, Tokyo) for constant encouragement and helpful discussions throughout this research. Two of us (C. S. and I. Ichiba and Professor M. Isobe (Nagoya University, Nagoya, Japan) for the kind gift of P3U1 cells, and Dr. I. Ichiba and Professor M. Isobe (Nagoya University, Nagoya, Japan) for obtaining the 600-MHz 1H NMR spectra. Finally, two of us (Y. I. and S. I.) express sincere thanks to Professor Rick Troy (University of California, Davis, CA) for constant support and understanding of our work research on oligo- and polysialic acids from a very early stage.

REFERENCES

1. Troy, F. A., II (1992) Glycobiology 2, 5–23.
2. Sato, C., Kitajima, K., Tazawa, I., Inoue, Y., Inoue, S., and Troy, F. A., II (1993) J. Biol. Chem. 268, 23675–23684.
3. Inoue, S., and Iwasaki, M. (1978) Biochem. Biophys. Res. Commun. 83, 1018–1023.
4. Vinm, E. R., McCoy, R. D., Vollger, H. F., Wilkison, N. C., and Troy, F. A. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 1971–1975.
5. Roth, J., Taatjes, D. J., Bitter-Suermann, D., and Finne, J. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 1971–1975.
6. James, W. M., and Agnew, W. S. (1987) J. Biol. Chem. 262, 8443–8448.
7. Livingston, B. D., Jacob, J. L., Glick, M. C., and Troy, F. A. (1988) J. Biol. Chem. 263, 8443–8448.
8. Roth, J., Zuber, C., Wagner, P., Taatjes, D. J., Weisgerber, E. P., Heitz, P. U., Gerdis, C., and Bitter-Suermann, D. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 2999–3003.
9. Angata, T., Kitazume, S., Terada, T., Kitajima, K., Inoue, S., Troy, F. A., II, and Inoue, Y. (1994) Glycoconjugate J. 11, 481–499.
10. Nishino, S., Kuroyanagi, H., Terada, T., Inoue, S., Troy, F. A., II, and Inoue, Y. (1996) J. Biol. Chem. 271, 32667–32677.
11. van Lenten, L., and Ashwell, G. (1971) J. Biol. Chem. 246, 1899–1894.
12. Ushida, Y., Taguchi, S., Sato, C., Kitajima, K., Inoue, S., Morris, H. R., Dell, A., and Inoue, Y. (1996) J. Biol. Chem. 271, 32667–32677.
13. van der Merwe, P. A., Crocker, P. R., Vinson, M., Barclay, A. N., Schauer, R., and Kelm, S. (1996) J. Biol. Chem. 271, 9273–9280.
14. Schauer, R., Manuguerria, J.-C., Gross, H.-J., and Crocker, P. R. (1994) Glycoconjugate J. 11, 576–585.
15. Svennerholm, L. (1957) Nature 179, 505–508.
16. Kudo, M., Kitajima, K., Inoue, Y., Shiokawa, K., Dell, A., and Inoue, Y. (1993) Biochim. Biophys. Acta 1183, 317–322.
17. Ozawa, H., Kowashima, I., and Tai, T. (1992) Arch. Biochem. Biophys. 294, 427–433.
18. Yamaguchi, S., and Kuroyanagi, H. (1996) J. Biol. Chem. 277, 28994–28995.
19. Angata, T., Kitazume, S., Terada, T., Kitajima, K., Inoue, S., Troy, F. A., II, and Inoue, Y. (1994) Glycoconjugate J. 11, 481–499.
20. Hara-Yokoyama, H., Kurihara, H., Yokoyama, S., and Inoue, Y. (1996) J. Biol. Chem. 271, 12951–12955.
21. Tachibana, Y., Suzuki, T., and Ohkura, Y. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 1971–1975.
22. Nakayama, J., Katsuyama, T., Sugiyama, F., and Hirabayashi, Y. (1993) J. Histochem. Cytochem. 41, 2405–2414.
23. Kasahara, K., Watanabe, Y., Yasuyama, T., and Inoue, K. (1997) J. Biol. Chem. 272, 29984–29993.
24. Hara-Yokoyama, H., Kurihara, K., Tomita, K., Hibaraya-ishi, Y., Irie, F., Sugiyama, H., Furuyama, S., and Kataba, T. (1996) J. Biol. Chem. 271, 12951–12955.
25. Hakomori, S. (1985) Cancer Res. 45, 2405–2414.
26. Nakayama, J., Katsuyama, T., Sugiyama, F., and Hirabayashi, Y. (1993) J. Histochem. Cytochem. 41, 1563–1572.
27. van der Merwe, P. A., Crocker, P. R., Vinson, M., Barclay, A. N., Schauer, R., and Kelm, S. (1996) J. Biol. Chem. 271, 9273–9280.
28. Schauer, R., Manuguerria, J.-C., Gross, H.-J., and Crocker, P. R. (1994) Glycoconjugate J. 11, 576–585.
29. Schauer, R., Manuguerria, J.-C., Gross, H.-J., and Crocker, P. R. (1994) Glycoconjugate J. 11, 576–585.
30. Schauer, R., Manuguerria, J.-C., Gross, H.-J., and Crocker, P. R. (1994) Glycoconjugate J. 11, 576–585.
31. Hara-Yokoyama, H., Kurihara, H., Yokoyama, S., and Inoue, Y. (1996) J. Biol. Chem. 271, 28994–28995.
Identification of Oligo-N-glycolylneuraminic Acid Residues in Mammal-derived Glycoproteins by a Newly Developed Immunochemical Reagent and Biochemical Methods

Chihiro Sato, Ken Kitajima, Sadako Inoue and Yasuo Inoue

J. Biol. Chem. 1998, 273:2575-2582.
doi: 10.1074/jbc.273.5.2575

Access the most updated version of this article at http://www.jbc.org/content/273/5/2575

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 59 references, 31 of which can be accessed free at http://www.jbc.org/content/273/5/2575.full.html#ref-list-1