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Synthesis of a Neu2en5Ac analog hapten and isolation of monoclonal antibody to Neu2en5Ac

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Abstract

Neu2en5Ac is a minor component of body fluids and is abundant in sialuria, but no antibody to detect it has been reported. 5-Acetamido-2,6-anhydro-9-glutaramido-3,5,9-trideoxy-D-*glycero*-D-*galacto*-non-2-enonic acid has been synthesized and conjugated with keyhole limpet hemocyanin (KLH) for immunization. A hybridoma named SIC172 was obtained that produces a monoclonal antibody (MAb) to Neu2en5Ac. SIC172 MAb in culture supernatant bound strongly to the hapten conjugated to BSA in ELISA, but slightly to fetuin, a glycoprotein which is rich in Neu5Ac. SIC172 MAb (IgG3(κ)), purified with a protein A/G affinity column, bound strongly to fetuin. Neu2en5Ac competed with the MAb in binding in amounts as low as 3 μM, while the competition of Neu5Ac appeared at amounts of more than 300 μM. SIC172 MAb is a unique MAb specific to Neu2en5Ac and might be useful for detecting Neu2en5Ac, which occurs naturally and in sialuria.

Keywords: Monoclonal antibody; Sialic acid; 5-Acetamido-2,6-anhydro-3,5-dideoxy-D-*glycero*-D-*galacto*-non-2-enonic acid; Neu2en5Ac; *N*-Acetylneuraminic acid; 5-Acetamido-2,6-anhydro-9-glutaramido-3,5,9-trideoxy-D-*glycero*-D-*galacto*-non-2-enonic acid

1. Introduction

Neu2en5Ac (5-acetamido-2,6-anhydro-3,5-dideoxy-D-*glycero*-D-*galacto*-non-2-enonic acid, 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid (**1**) is a transition-state analog of the hydrolysis of sialoglycoconjugates (**2**) that liberates Neu5Ac (*N*-acetylneuraminic acid (**3**) by sialidase; it acts as an inhibitor against sialidase [1–5]. Neu2en5Ac was first detected in the urine, saliva, and blood of a patient with sialuria [6]. After that, it was found in low concentrations in the serum, saliva, and urine of healthy individuals [7] and was further detected as a metabolite in mammalian brain [8]. Current methods of detecting Neu2en5Ac rely on chemical analysis, TLC, GC or MS [9,10]. If a specific antibody to Neu2en5Ac could be produced, it might facilitate the detection and analysis of Neu2en5Ac in various organisms, and it might also be used for screening for sialuria. Here, we describe the synthesis of an effective hapten that induces antibodies to Neu2en5Ac and the finding of a MAb that recognizes Neu2en5Ac in a specific manner.

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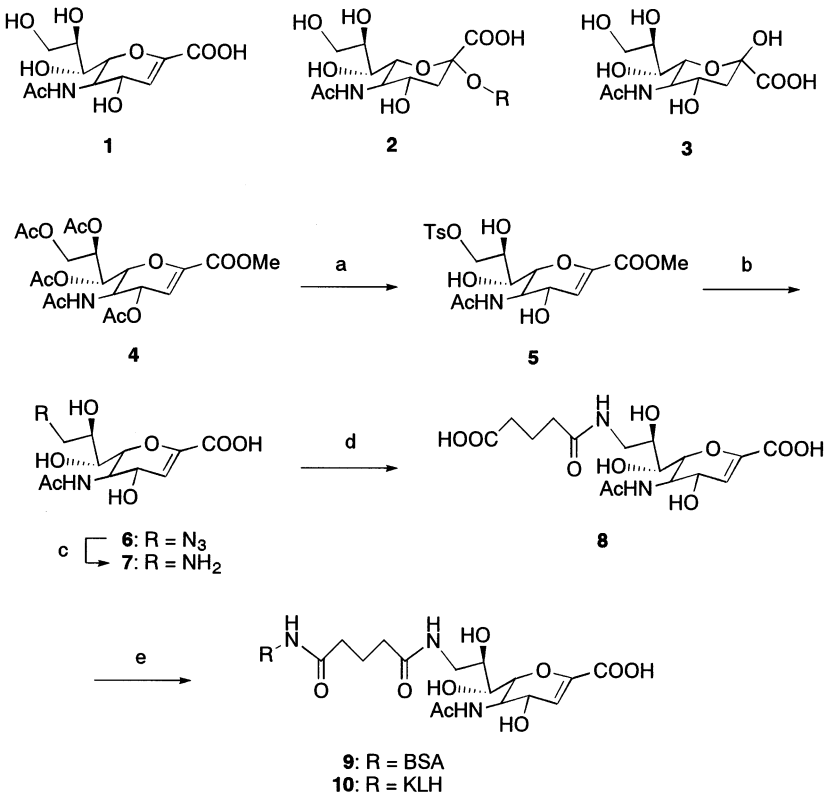
2. Results and discussion

Synthesis of hapten.—The carboxylate group of Neu5Ac has the special characteristic of exhibiting a negative charge. In order to synthesize a potential hapten, a linker was attached at the 9 position, which is remote and may have no relation to the characteristic functional group. Hapten **8** was synthesized from methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-2,6-anhydro-3,5-dideoxy-D-*glycero*-D-*galacto*-non-2-enonate (**4**) [11] (Scheme 1). Compound **4** was treated with NaOMe and then with *p*-toluenesulfonyl chloride to produce tosyl derivative **5** in 42% yield. Compound **5** was converted to azide derivative **6** by treatment with sodium azide, and then saponificated (78% yield). The reduction of azide derivative **6** by triphenylphosphine produced amino derivative **7** in 59% yield. In order to attach a linker at C-9, amino derivative **7** was treated with glutaric anhydride to produce hapten **8** (59%). For the coupling of hapten **8** with bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH), a mixture of hapten **8** and either BSA or KLH was treated with 1-ethyl-3-(3-diaminopropyl)-carbodiimide HCl salt (EDC·HCl). The conjugate was purified by gel permeation chromatography.

Hybridoma.—Spleen cells from mice immunized with conjugate **10** were hybridized with myeloma X63-Ag8.653 cells [12] and plated directly on 0.5% agar plates containing a culture medium with HAT (hypoxanthine, aminopterin, and thymidine) [13]. Colonies grown on the agar plates were isolated and the culture supernatant was assayed by enzyme-linked immunosorbent assay (ELISA) using both conjugate **9** and fetuin as screening antigens. From 394 clones isolated from the agar plates of three hybridization trials, 24 hybridomas were obtained that produced ELISA-positive monoclonal antibodies (MAbs). Most of the positive clones produced antibodies that also bound to fetuin as well. However, one clone named SIC172 produced an antibody that bound strongly to conjugate **9** but weakly



Scheme 1. (a) (1) NaOMe, MeOH, rt; (2) TsCl, Py, rt, 42% (2 steps); (b) (1) NaN_3, DMF, 70 °C; (2) 0.3 M NaOH–MeOH, rt, 78% (2 steps); (c) PPh_3, dioxane–MeOH–H_2O, rt, 80%; (d) glutaric anhydride, MeOH–H_2O–triethylamine, rt, 59%; (e) EDC·HCl, BSA or KLH, H_2O.

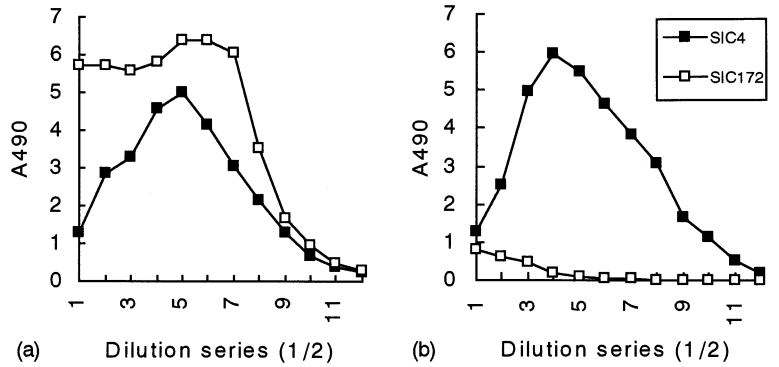


Fig. 1. Binding of SIC172 MAb and SIC4 MAb, both in culture supernatant, to conjugate **9** (a) and fetuin (b). No. 1 of 1:2 dilution series is undiluted culture supernatant of each hybridoma.

to fetuin. Therefore, SIC172 was selected for further study.

The SIC172 MAb was IgG3(κ). The recloning of the SIC172 hybridoma produced subclones essentially similar to the mother clone: 22 subclones out of 23 subclones, isolated as colonies grown on an agar plate, produced MAbs that bound strongly to conjugate **9** but weakly to fetuin.

SIC172 MAb in culture supernatant.—Fig. 1 shows the binding activities of SIC172 MAb at various dilutions to conjugate **9** and fetuin. For comparison, data obtained with SIC4 MAb that bound strongly to conjugate both **9** and fetuin are included. SIC172 MAb shows strong binding to conjugate **9,** and in a 7-level dilution series (i.e., dilutions of 1:1–1:64), it is beyond the upper limit of the assay (A_{490} of about 6). But it binds a little to fetuin, and no increase is found after dilution, which might dilute out some interfering materials, as seen in the case of the binding of SIC4 MAb in both conjugate **9** and fetuin.

The effects of Neu2en5Ac and Neu5Ac on the binding of SIC172 MAb in culture supernatant to conjugate **9** were examined to see if Neu2en5A competes specifically with conjugate **9** (Fig. 2). High doses of Neu2en5Ac of over 10 μg/well (0.34 mM) interfered with the binding, as expected, and at 100 μg/well, it almost completely suppressed the binding. However, low doses of Neu2en5Ac, such as 1 μg/well, increased the binding of SIC172 MAb to conjugate **9**. As for Neu5Ac, it had little effect on binding; and at 100 μg/well (3.2 mM) it slightly increased the binding, just like a low dose of Neu2en5Ac.

The increased binding of SIC172 MAb to conjugate **9** at low doses of Neu2en5Ac may be due to the presence, in culture supernatant, of large amounts of materials that can cross-react with the MAb. Since high doses of Neu5Ac also produce a similar phenomenon, sialic acid-containing materials, which might be abundant in culture supernatant, may be the ones—they would trap some of the SIC172 MAb in culture supernatant. Low

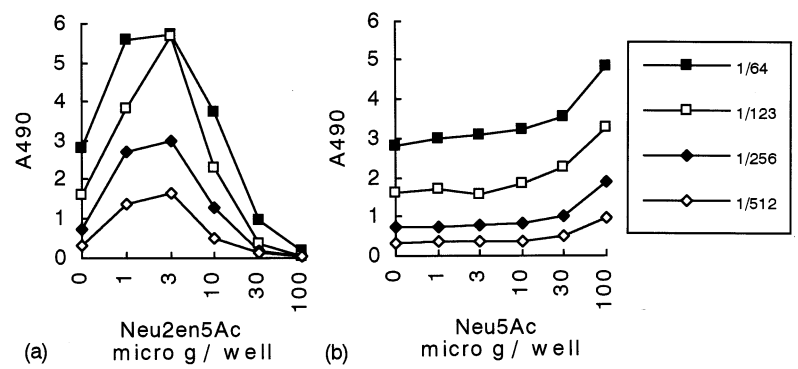


Fig. 2. Effects of Neu2en5Ac (a) and Neu5Ac (b) on the binding of SIC172 MAb in a culture supernatant to conjugate **9**. Numbers in the box indicate dilution factors of the culture supernatant of SIC172 hybridoma.

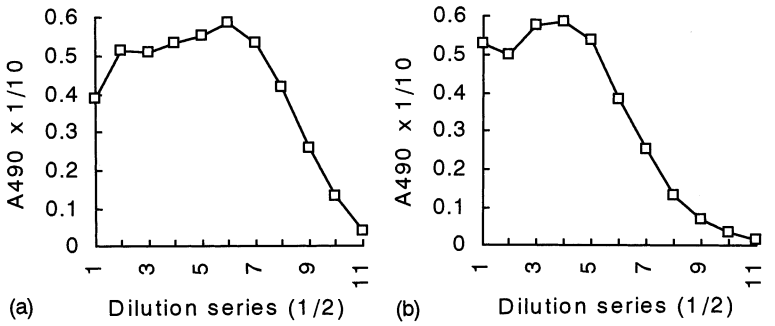


Fig. 3. Binding of SIC172 MAb of purified IgG to conjugate **9** (a) and to fetuin (b). No. 1 of the 1:2 dilution series is 200 nM of IgG.

doses of Neu2en5Ac may release such trapped MAb and increase the opportunity to bind to an ELISA antigen plate. Therefore the purification of MAb should eliminate such contradictory effects of hapten; this was found to be true, as described below.

Purified SIC172 MAb.—To clarify the binding activity of SIC172 MAb, it was purified by using a protein A/G plus-agarose affinity column. The binding activity of the purified SIC172 MAb with regard to conjugate **9** and to fetuin is shown in Fig. 3. Purified MAb bound to conjugate **9** strongly just as MAb in culture supernatant (Fig. 3(a)). Furthermore, as is clearly seen in Fig. 3(b), it also bound well to fetuin. The binding to fetuin is, however, weaker than that to conjugate **9**, as seen at higher dilutions.

The effects of haptens on the binding of purified SIC172 MAb both to conjugate **9** and to fetuin were then examined. In binding to conjugate **9** (Fig. 4(a,b)), at a high dose (1 mM or more) Neu2en5Ac competes strongly with purified SIC172 MAb, but Neu5Ac does not. This clearly shows that the binding is specific to Neu2en5Ac. In binding to fetuin

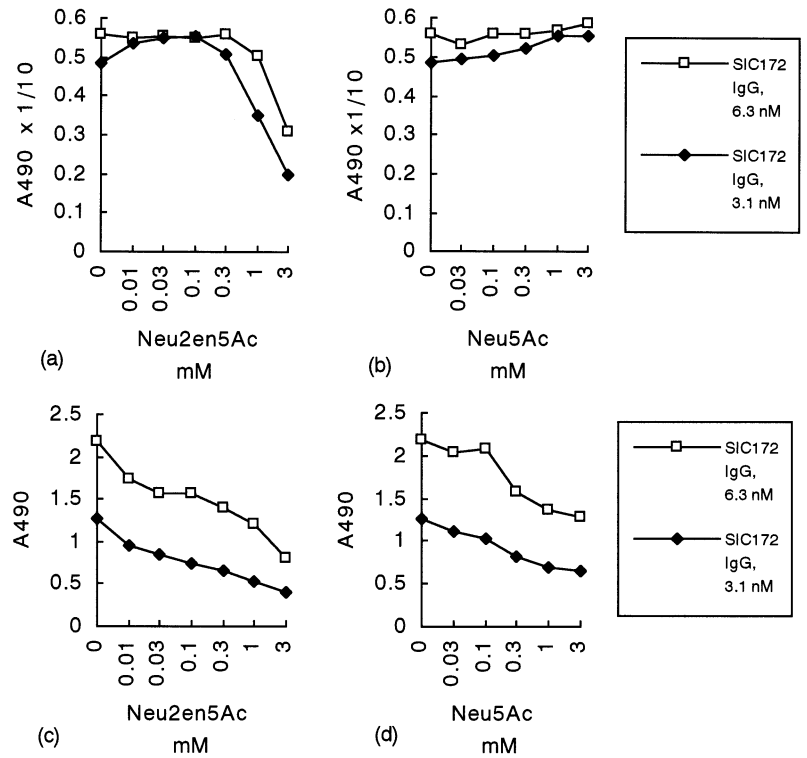


Fig. 4. Effects of Neu2en5Ac (a and c) and Neu5Ac (b and d) on the binding of SIC172 MAb of purified IgG to conjugate **9** (a and b) and to fetuin (c and d). The IgG used is indicated in parentheses.

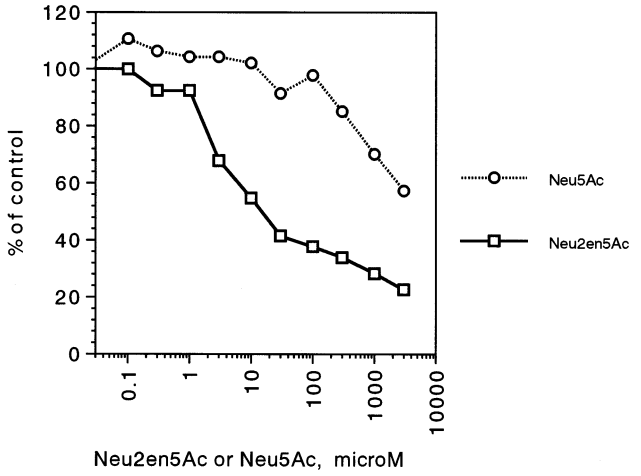


Fig. 5. Comparison of the effects of Neu2en5Ac with those of Neu5Ac on the binding of SIC172 MAb of purified IgG to fetuin. SIC172 IgG, 6 nM, was used. HCl-treated BSA was used to dilute IgG and to block a fetuin-coated plate. A control without hapten shows an A_{490} of 0.75 in this experiment.

(Fig. 4(c,d)), on the other hand, both Neu2en5Ac and Neu5Ac compete with the MAb. But the effect of Neu2en5Ac appears at doses as low as 0.01 mM, while that of Neu5Ac appears at above 0.3 mM. To determine more precisely the effect of low doses of haptens on binding to fetuin, IgG and haptens were preincubated with shaking and applied to an ELISA plate (Fig. 5). The BSA used here for the dilution of IgG and the blocking of a fetuin-coated plate was pretreated with dilute HCl in order to remove sialic acids from any sialo-compounds contaminating the BSA. Neu2en5Ac competes strongly and as little as 3 μM of it had a significant effect, while 300 μM or more of Neu5Ac was required to get a similar result, as before.

The purification of SIC172 MAb thus revealed its ability to bind to fetuin. Since it competes with Neu5Ac, SIC172 MAb recognizes the Neu5Ac residues present in fetuin. Neu2en5Ac competes about 100–300 times more strongly than Neu5Ac, indicating that SIC172 MAb has more affinity to Neu2en5Ac than to Neu5Ac. Since concentrations of Neu2en5Ac as low as 3 μM can be detected by this MAb as a competitor in binding to fetuin, this MAb may be useful for the detection of a small amount of Neu2en5Ac in natural biological samples, though adequate separation of Neu2en5Ac from Neu5Ac, such as by TLC on silica gel [7], is necessary to eliminate possible cross reactions with Neu5Ac, which is usually present at much higher levels than Neu2en5Ac.

It is interesting that a terminal Neu5Ac residue, bound to the next residue at the C-2 position, in a carbohydrate moiety of fetuin can be recognized by SIC172 MAb, which was generated by immunization with Neu2en5Ac that was bound to a linker at the C-9 position. Determination of the amino acid sequence of SIC172 MAb and 3-D modeling of the MAb molecule would offer some clues to this problem.

3. Experimental

General procedure.—^{1}H NMR spectra were recorded with a Jeol EX 270 (Japan) or Bruker DMX 500 (Germany) instrument at 298 K. Chemical shifts are expressed in ppm and referenced to HOD (4.81 ppm) as an internal standard. Optical rotations were measured with a Jasco DIP-4 (Japan) polarimeter. High-resolution mass spectra (HR-MS) were recorded on a Shimadzu/Kratos concept-IIH (Japan/UK) instrument under Fab conditions. All reactions were monitored by TLC (Silica Gel 60-F_{254}, E. Merck, Germany) by charring after spraying with 5% H_2SO_4 in MeOH.

Chemicals.—5-Acetamido-2,6-anhydro-3,5-dideoxy-D-*glycero*-D-*galacto*-non-2-enonic acid, Neu2en5Ac, Neu5Ac, and fetuin were purchased from Sigma (USA).

*Methyl 5-acetamido-2,6-anhydro-3,5-dideoxy-9-*p*-toluenesulfonyl*-D-glycero-D-galacto-*non-2-enonate* (**5**).—To a solution of methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-2,6-anhydro-3,5-dideoxy-D-*glycero*-D-*galacto*-non-2-enonate (**4**) (1.12 g, 2.36 mmol) [11] in dry MeOH (10 mL) was added NaOMe (13 mg, 0.25 mmol), and the mixture was stirred for 1 h at room temperature. The mixture was neutralized by the addition of Dowex 50W × 8 resin. To this mixture was added water (2 mL), and then the mixture was filtered and concentrated. The residue was dried in a desiccator overnight. To a solution of this residue in pyridine (20 mL) was added *p*-toluenesulfonyl chloride (415 mg, 2.17 mmol) at room

Table 1
^{1}H NMR data[a] of synthesized Neu2en5Ac derivatives

	H-3	H-4	H-5	H-6	H-7	H-8	H-9a	H-9b	Others
5	5.87 (2.6)	4.35 (2.6, 9.2)	4.06–4.00	4.24 (1.3. 7.9)	3.44 (1.3, 8.6)	3.89	4.06–4.00	3.86 (8.6, 10.6)	7.74 (7.9, Tosyl) 7.39 (7.9, Tosyl) 3.71 (OMe) 2.41 (Ac)
6	5.75 (2.0)	4.53 (2.0, 8.6)	4.11 (8.6, 10.5)	4.27 (10.5)	3.67 (8.6)	4.19 (2.6, 5.9, 8.6)	3.71 (2.6, 13.2)	3.58 (5.9, 13.2)	2.14 (Ac)
7	5.72 (2.0)	4.49 (8.6)	4.05 (8.6, 10.6)	4.24 (10.6)	3.58 (9.2)	4.13 (3.3, 9.2)	3.47 (13.2)	2.99 (9.9, 13.2)	2.07 (Ac)
8	5.77 (2.0)	4.46 (2.6, 8.9)	4.05 (8.9, 11.6)	4.22 (11.2)	3.50 (9.9)	3.96 (2.6, 7.3, 9.9)	3.59 (2.6, 13.9)	3.32 (7.3, 13.9)	2.41 (7.3, CH_2) 2.32 (7.3, CH_2) 2.05 (Ac) 1.88 (CH_2)

[a] The chemical shift of ^{1}H NMR data is expressed relative to HOD (4.81 ppm, 298 K). Vicinal hydrogen–hydrogen coupling constants (Hz) are shown in parentheses.

temperature, and the mixture was stirred for 4 h. Next MeOH (5 mL) was added to the mixture, which was then concentrated in vacuo. Purification of the residue on a column of silica gel (19:1 EtOAc–MeOH) yielded **5** (460 mg, 42%); $[\alpha]_D^{25}$ −12.0° (*c* 0.5, MeOH); HR-MS *m/z* Anal. Calcd for $C_{19}H_{25}NO_{10}SNa$: 482.1097 $[M+Na]^+$, Found: 482.1109. ^{1}H NMR data of this and some other compounds are shown in Table 1.

5-Acetamido-2,6-anhydro-9-azido-3,5,9-trideoxy-D-glycero-D-galacto-*non-2-enonic acid* (**6**).—To a solution of **5** (450 mg, 0.98 mmol) in DMF (2 mL) was added sodium azide (241 mg, 3.71 mmol) and the mixture was stirred at 70 °C. After 7 h, it was allowed to cool to room temperature. Purification of the mixture on a column of silica gel (49:1 EtOAc–MeOH) yielded azide derivative **6** (280 mg), {^{1}H NMR (D_2O): δ 5.91 (d, 1 H, *J* 2.64 Hz, H-3), 3.76 (s, 3 H, OMe), 3.54 (dd, 1 H, *J* 2.64, 13.2 Hz, H-9a), 3.37 (dd, 1 H, *J* 6.6, 13.2 Hz, H-9b), 2.03 (s, 3 H, Ac)}. The azide derivative was dissolved in 1:1 0.3 M NaOH–MeOH (8 mL) and the mixture was stirred for 2 h at room temperature. The mixture was neutralized by the addition of Dowex 50 W × 8 resin, then filtered. The filtrate was concentrated in vacuo. Purification of the residue on a column of silica gel (1:1 EtOAc–MeOH) yielded **6** (241 mg, 78%); $[\alpha]_D^{25}$ + 33.1° (*c* 0.87, MeOH); HR-MS *m/z* Anal. Calcd for $C_{11}H_{16}N_4O_7Na$ (Na salt): 339.0917 $[M+H]^+$, Found: 339.0944.

5-Acetamido-9-amino-2,6-anhydro-3,5,9-trideoxy-D-glycero-D-galacto-*non-2-enonic acid* (**7**).—To a solution of azide derivative **6** (22 mg, 70 μmol) in 4:3:1 dioxane–MeOH–H_2O (400 μL) was added triphenylphosphine (27 mg, 104 μmol) and the mixture was stirred at room temperature. After 12 h, the mixture was concentrated in vacuo. Purification of this residue on a column of silica gel (3:2:1 EtOAc–MeOH–H_2O) yielded amino derivative **7** (16 mg, 80%); $[\alpha]_D^{25}$ + 30.0° (*c* 0.6, H_2O); HR-MS *m/z* Anal. Calcd for $C_{11}H_{19}N_2O_7$: 291.1192 $[M+H]^+$, Found: 291.1206.

5-Acetamido-2,6-anhydro-9-glutaramido-3,5,9-trideoxy-D-glycero-D-galacto-*non-2-enonic acid* (**8**).—To a solution of amino derivative **7** (5 mg, 17 μmol) in 300:100:3

MeOH–H_2O–Et_3N (403 μL) was added glutaric acid anhydride (3.5 mg, 30 μmol) and the mixture was stirred at room temperature. After 20 min the mixture was concentrated in vacuo. Purification of this residue on a column of silica gel (3:2:1 EtOAc–MeOH–H_2O) yielded amino derivative **8** (4 mg, 59%); $[\alpha]_D^{25} + 25.5°$ (*c* 0.4, MeOH); HR-MS *m/z* Anal. Calcd for $C_{16}H_{24}N_2O_{10}Na$ (Na salt): 427.1329 $[M + H]^+$, Found: 427.1354.

Conjugate **9**.—The reaction was carried out in a centricon tube. Compound **8** (4 mg, 10 μmol) was dissolved in H_2O (250 μL), and the pH of this solution was adjusted to 6.0 by the addition of aq $NaHCO_3$. To this solution was added BSA (15 mg, ca 0.2 μmol) and EDC·HCl (3 mg, 16.5 μmol), and the mixture was incubated at room temperature. After 12 h, the mixture was directly loaded onto a gel permeation column (Sephadex G-50, H_2O). The fraction containing BSA was pooled and lyophilized.

Conjugate **10**.—Prior to the reaction, KLH was dissolved in H_2O (100 mg/0.5 mL), and the precipitate was removed by filtration. The filtrate containing KLH was lyophilized and used for the synthesis of conjugate **10**. The conditions for the reaction were the same as those for conjugate **9**.

Hybridoma.—Conjugate **10** (390 μg) was dissolved in 1 mL of PBS and mixed into one vial of GERBU adjuvant containing 10 μg of GMDP, 40 μg of *N*,*N*′-dimethyl-*N*,*N*-dioctadecylammonium chloride, and 1.68 mg of Zn-proline complex (Gerbu Biotechnik, Germany). Female MRL/MpJ-*lpr*/*lpr* mice [10] (Nihon Slc, Japan), 8-weeks-old, were each immunized subcutaneously with 50 μL of the antigen–adjuvant mixture, which contained 19.5 μg of conjugate **10**. The mice were further immunized with the same dose at 2, 5, and 8 weeks after the first immunization. The titer of antibodies that bound to conjugate **9**, as detected by ELISA, increased gradually after 2 weeks in all the mice immunized. Three or 4 days after immunization at 5 or 8 weeks, spleen cells from two or three mice were obtained and hybridized with myeloma X63-Ag8.653 cells [12] by using polyethylene glycol 6000, and were plated on 0.5% agar plates containing RPMI medium (in a later experiment, DMEM medium with a high glucose level was used instead of RPMI medium), HAT (0.1 mM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine, Sigma, USA) and 20% FBS [13]. From 2 weeks to 1 month later, colonies grown on the agar plates were isolated in liquid culture medium containing HT (0.1 mM hypoxanthine and 16 μM thymidine, Sigma, USA). Culture supernatants were assayed by ELISA for the presence of MAb that can bind to conjugate **9** and/or to fetuin. ELISA-positive clones were selected and transfered to normal medium for further study.

Typing of immunoglobulin subclass.—This was done by using a Mouse MonoAB ID Kit (Zymed, USA).

Affinity purification of IgG.—IgG in the culture supernatant was precipitated with ammonium sulfate at 50%, dissolved and dialysed against the application/binding buffer, and applied to a protein A/G plus-agarose affinity column (Calbiochem., USA). IgG was eluted with acetate buffer (pH 3.0) and glycine–HCl buffer (pH 3.0), and adjusted to a pH of 7.0 with Tris base.

ELISA.—A total of 100 μg of conjugate **9**, or 200 μg (145 μg in a later experiment) of fetuin containing 5.5% Neu5Ac, was dissolved in 1 mL of PBS; 100 μL of the solution was used to coat each well of a 96-well test plate at room temperature for 2 h. Antigen-coated plates were blocked with 1% BSA in PBS, washed with 0.1% Tween 20 in PBS, and used for ELISA. The culture supernatant of hybridoma or purified IgG, undiluted or diluted with 0.1% BSA in PBS, was used as the source of MAb. In some experiments, as shown in Fig. 5, the BSA used to dilute samples and block ELISA plates was pretreated with 0.1 M HCl for 1 h at 80 °C, and neutralized and dialysed against PBS to remove any sialic acids from sialo-compounds contaminating the BSA. To test the effects of Neu2en5Ac and Neu5Ac, they were dissolved in PBS; and the solution was adjusted to a pH of 7.2–7.5 with 0.1 M NaOH by using pHBOY-P2 (Shindengen, Japan), and diluted with PBS. Culture supernatant, purified IgG, or their diluted solutions were preincubated with various

concentrations of either Neu2en5Ac or Neu5Ac at 37 °C for 1 h (or at room temperature with shaking in the experiments for Fig. 5), and then the mixture was applied to an antigen-coated well. POD-labeled sheep anti-mouse-immunoglobulins antibody was used as a secondary antibody. Peroxidase activity was detected with *o*-phenylenediamine and H_2O_2. The absorbance at 490 nm, A_{490}, was read on a microplate reader (Thermo max, Molecular Devices, USA). Assays were carried out in duplicate.

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