enzymatic cleavages of sLacNAc-C12 by neuraminidases were examined. The neuraminidases employed in this study were *Arthrobacter ureafacience* neuraminidase, which hydrolyzes α-(2→3), α-(2→6), and α-(2→8) linkages,[10] and *Macrobdella decora* neuraminidase, which hydrolyzes α-(2→3) linkage.[11] Though *N*-acetylneuraminic acid of A1 was cleaved by both neuraminidases, that of A2 was cleaved by the *A. ureafacience* neuraminidase but not by the *M. decora* neuraminidase. The hydrolyzed products showed the same mobility as synthetic Galβ1-4GlcNAc-C12. Next, ESI (electrospray ionization)-CID (collision-induced dissociation) was employed to distinguish between A1 and A2. The ESI-CID spectra of A1 and A2 showed peaks of *m/z* 887.5 ($[M+2Na-H]^+$) corresponding to NeuNAc-Gal-GlcNAc-C12, and *m/z* 574.3 ($[(M\text{-anNeuNAc})+Na]^+$) corresponding to Y_2 fragment (Gal-GlcNAc-C12). The relative intensity of Y_2 (*m/z* 574.3) to the parent peak (*m/z* 887.5) showed significant differences between A1 and A2, and was 0.68 for A1 and 0.03 for A2. It has been reported that α-(2→3) sialyl linkage was distinguished from α-(2→6) sialyl linkage based on the ESI-CID spectra.[12] In the literature, the fragmentation ions produced by the cleavage of the α-(2→3) sialyl linkage showed much higher intensity than those produced by the cleavage of the α-(2→6) sialyl linkage. Therefore, from the results of enzymatic digestions and ESI-CID spectra, A1 and A2 were determined to be NeuNAcα2-3Galβ1-4GalNAc-C12 and NeuNAcα2-6Galβ1-4GalNAc-C12, respectively.

The MALDI-TOFMS spectra of A3 and A5 (Table 1) revealed peaks of *m/z* 1230.1 ($[M-H]^-$) corresponding to NeuNAc-(Gal-GlcNAc)$_2$-C12 and *m/z* 1571.7 ($[M-H]^-$) corresponding to NeuNAc-(Gal-GlcNAc)$_3$-C12. A3 was considered to be produced by the sialylation of N4. The MALDI-TOF-MS spectra of A4 and A6 revealed peaks of *m/z* 1352.7 ($[M-H]^-$) corresponding to fucosylated A3 and *m/z* 1779.0 ($[M-H]^-$) corresponding to fucosylated A5. The positive-ion mode MALDI-PSD spectrum of A4 (Table 2) revealed peaks at *m/z* 558.9 corresponding to $Y_{1\beta}$ (Fuc-GlcNAc-C12+Na^+) and *m/z* 720.9 corresponding to Y_2 (Fuc+Gal-GlcNAc-C12+Na^+). The positive-ion mode MALDI-PSD spectrum of A6 (Table 2) also revealed a peak at *m/z* 720.2 corresponding to Y_2 (Fuc+Gal-GlcNAc-C12+Na^+), *m/z* 923.9 corresponding to Y_3 (Fuc+GlcNAc-Gal-GlcNAc-C12+Na^+), and *m/z* 1085.7 corresponding to Y_4 (Fuc+Gal-GlcNAc-Gal-GlcNAc-C12+Na^+). These MALDI-PSD spectra suggested that the fucose moieties in A4 and A6 were linked to the innermost GlcNAc residue. It has been reported that HL60 cells express α-(1→3)-fucosyltransferase, and fucosylated monosialyl glycolipids having similar structures to A4 and A6 were detected in HL60 cells.[13] Though the linkages of *N*-acetylneuraminic acid in A3, A4, A5, and A6 could not be determined in the present study, they were inferred to be α-(2→3) from the structural analysis of the sialyl linkage of sialylpolylactosamine expressed in HL60 cells.[14]

2.3. Glycosylation of LacNAc-C12 by HL60 cells

Next, the glycosylation of LacNAc-C12 by HL60 cells was examined. After incubation of HL60 cells with 50 μM LacNAc-C12 for 2 days, glycosylated products and unreacted primer were isolated from the culture medium. The glycosylated products collected using a Sep-Pak C_{18} column were analyzed by HPTLC. One neutral product and six acidic products were detected. The analyses of mobility on HPTLC and the mass spectrum indicated that the products glycosylated from LacNAc-C12 were the same as those from GlcNAc-C12. The neutral product was N2 and the acidic products were A1–A6.

2.4. Comparison of GlcNAc-C12 and LacNAc-C12 as glycosyl acceptors in B16 cells

After incubation of 50 μM GlcNAc-C12 or LacNAc-C12 with B16 cells for 2 days, glycosylated products were isolated from the culture medium. The glycosylated products were analyzed by HPTLC and MALDT-TOF-MS. Using GlcNAc-C12, two glycosylated products were detected. One was Gal-GlcNAc-C12, whose mobility on HPTLC was the same as that of synthetic LacNAc-C12. The other was considered to be NeuNAc-Gal-GlcNAc-C12 (sLacNAc-C12) from the mass spectrum. For LacNAc-C12, the detected product was also suggested to be sLacNAc-C12 from the mobility on HPTLC and from the mass spectrum. To determine the linkage of the sialic acid, the product was treated with neuraminidases from *A. ureafaciens* and *M. decora*. Since the glycosylated product sLacNAc-C12 was hydrolyzed by both sialidases, the linkage of NeuNAc-Gal was determined to be α-(2→3). The amount of sLacNAc-C12 derived from GlcNAc-C12 was two times higher than that from LacNAc-C12, when the dose of saccharide primers was 50 μM (Fig. 3). The glycosylation efficiency of GlcNAc-C12 in cells was higher than that of LacNAc-C12 in cells. When the dose of GlcNAc-C12 was 50 μM (250 nmol), the amount of sLacNAc-C12 was determined to be 7.5 nmol by quantitative analysis using GM1 as standard.

2.5. Cell growth in the presence of saccharide primers

B16 cells were cultured in the absence and the presence of 50 μM GlcNAc-C12 and LacNAc-C12 for 2 days. The cell growth in the presence of the saccharide primers was almost similar to that of control (Fig. 4). Cell growth of HL60 cells was also investigated in the presence of 50 μM GlcNAc-C12 for 2 days (data not

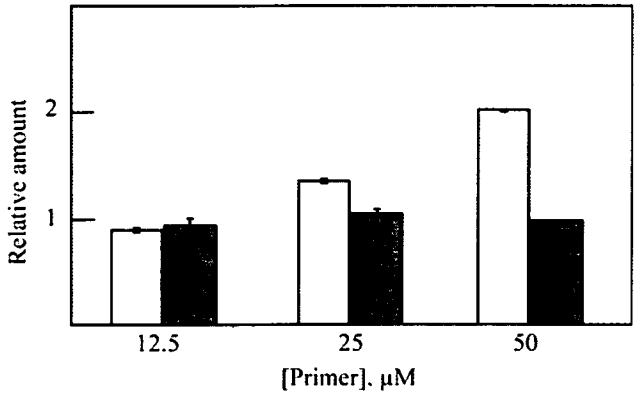

Figure 3. Relative amounts of NeuNAc-Gal-GlcNAc-C12 glycosylated from GlcNAc-C12 (white column) and Gal-GlcNAc-C12 (black column) by B16 melanoma cells (2×10^6 cells). The relative amounts were analyzed by densitometry at 540 nm followed by staining with resorcinol–HCl. The dose of saccharide primers was 50 μM.

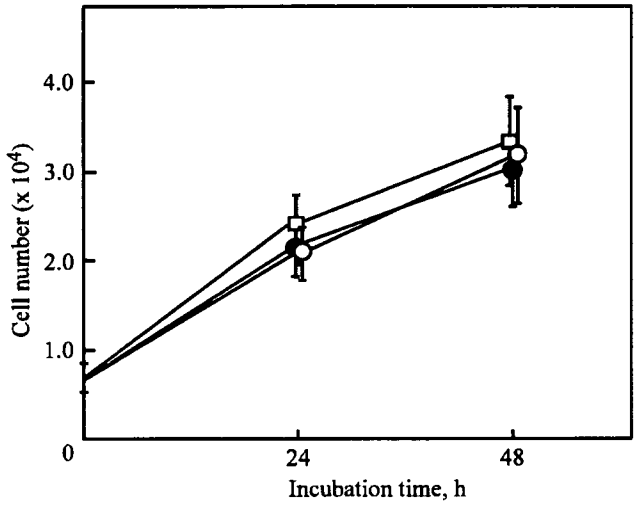

Figure 4. Growth of B16 cells cultured in the absence (closed circle) and the presence of 50 μM GlcNAc-C12 (open circle) and LacNAc-C12 (open square).

shown). The primers showed no cytotoxicity at the present experimental conditions.

3. Discussion

Convenient synthesis of glycan structures present on cells is important for the study to elucidate glycan function. Since saccharide primers can act as substrates for glycosyltransferases present in cells, they are useful for the synthesis of oligosaccharides expressed in cells. Saccharide primers are building blocks for constructing an oligosaccharide library by biocombinatorial synthesis that is combination of different saccharide primers and a variety of cells. It has been reported that Lac-C12, which is a mimicry of lactosylceramide, was useful to synthesize the oligosaccharides of glycosphingolipids (GSL). For example, Lac-C12 gave GM3 oligosaccharide when incubated with B16 melanoma cells. Furthermore, 12-azido dodecyl-β-lactoside (Lac-C12-N3) was synthesized with the aim of preparing glycan arrays or glycopolymers. Lac-C12-N3 was also glycosylated by cells as well as Lac-C12[15] and could be conjugated to solid supports by the modified Staudinger reaction or condensation reaction followed by reduction to the amino group for detecting carbohydrate recognition.[16]

For the construction of oligosaccharide libraries, it is important to synthesize various oligosaccharides. In our ongoing studies, it has been found that Lac-C12 gave rise to various oligosaccharides of ganglio- and globo-series gangliosides. Then, in the present study, we synthesized novel saccharide primers to selectively obtain neolacto-series oligosaccharides. In the biosynthesis of neolacto-series glycans, the lactosamine unit of Galβ1-4GlcNAc is the precursor region for sugar elongation. Thus, saccharide primers containing GlcNAc and LacNAc would be substrates for glycosyltransferases synthesizing neolacto-series oligosaccharides. In the literature, Esko and co-workers have reported that disaccharide primers such as peracetylated Galβ1-4GlcNAc-NM were fucosylated to Galβ1-4(Fucα1-3)GlcNAc-NM, and peracetylated GlcNAcβ1-3Gal-NM was converted to Galβ1-4(Fucα1-3)GlcNAcβ1-3Gal-NM, NeuNAcα2-3Galβ1-4GlcNAcβ1-3Gal-NM, and NeuNAcα2-3Galβ1-4(Fucα1-3)GlcNAcβ1-3Gal-NM by U937 human histiocytic lymphoma cells.[6] Those peracetylated primers were glycosylated after deacetylation in cells. In our study, deacetylated saccharide primer was used for the synthesis of oligosaccharides by cells. GlcNAc-C12 and LacNAc-C12 gave Le^X, sLe^X, polylactosamine, sialylated polylactosamine, and sialylated/fucosylated polylactosamine by incubating with HL60 cells. These oligosaccharides were similar to endogenous glycans observed in HL60.[17] The complex glycosylated products were clearly separated by HPLC, and their chemical structures were determined by enzymatic digestion and mass spectrometry. Separation and structural elucidation of the products were very convenient compared to the endogenous GSLs because the saccharide primers had a uniform aglycon structure.

Since GlcNAc-C12 gave similar glycosylated products to LacNAc-C12, we could conclude that monosaccharide primers as well as disaccharide primers are useful for the synthesis of oligosaccharides. It has been reported that the glycosylation efficiencies of saccharide primers were dependent on their hydrophilic–hydrophobic balance.[2,18] More hydrophilic saccharide primers cannot be internalized into cells, while more hydrophobic ones are strongly adsorbed to the cell membrane. Although the glycosylation efficiency of GlcNAc-C12 was higher than that of LacNAc-C12 in the present study, the structure for giving optimum glycosylation

efficiency would be determined by varying the hydrocarbon chain length.

In conclusion, saccharide primers such as GlcNAc-C12 and LacNAc-C12 were developed to synthesize neolacto-series oligosaccharides using mammalian cells. The glycosylated products were separated by HPLC, and the sequences were determined by enzymatic digestion and mass spectrometry. The saccharide primers employed in this study are expected to be useful for synthesizing oligosaccharides expressed in mammalian cells.

4. Experimental

4.1. Synthesis of dodecyl 2-acetamido-2-deoxy-β-D-glucopyranoside (GlcNAc-C12)

2-Acetamido-1,3,4,6-tetra-*O*-acetyl-2-deoxy-D-glucopyranoside (Ac_4-GlcNAc) was prepared by reacting *N*-acetylglucosamine (2.5 g, 11.3 nmol, GlcNAc, Sigma) with Ac_2O (15 mL, 159 mmol, Wako Pure Chemicals) in 30 mL of pyridine according to the literature.[19] Ac_4-GlcNAc (3 g, 7.71 mmol) was mixed with TMS-OTf (4.0 mL, 21.9 mmol, E. Merck) in CH_2Cl_2 under nitrogen.[20] The solution was refluxed at 50 °C with stirring for 7 h. After evaporation followed by neutralization with Et_3N, the product was chromatographed on silica gel to examine the progress of the reaction. The reaction mixture was mixed with 1-dodecanol (3.6 mL, 15.5 mmol, Wako Pure Chemicals), $BF_3{\cdot}OEt_2$ (21 mL, 7.9 mmol, Wako Pure Chemicals) in the presence of 4 Å molecular sieves (2.5 g), and stirred at room temperature for 22 h.[21] $BF_3{\cdot}OEt$ (20.1 mL, 0.79 mmol) was added at 18 h to complete the reaction. The mixture was neutralized with Et_3N. After evaporation, the product was purified by column chromatography (Silica Gel 60, E. Merck, 7 × 30 cm, 1:1 *n*-hexane–EtOAc). Yield: 63.8% (2.53 g). ^1H NMR($CDCl_3$): δ 5.51 (d, 1H, $J_{2,NH}$ 8.8 Hz, NH), 5.31 (dd, 1H, $J_{2,3}$ 10.1 Hz, $J_{3,4}$ 9.5 Hz, H-3), 5.06 (dd, 1H, $J_{3,4}$ 9.5 Hz, $J_{4,5}$ 9.9 Hz, H-4), 4.65 (d, 1H, $J_{1,2}$ 8.4 Hz, H-1), 4.26 (dd, 1H, $J_{5,6b}$ 4.7 Hz, $J_{6,gem}$ 12.3 Hz, H-6a), 4.12 (dd, 1H, $J_{5,6a}$ 2.4 Hz, $J_{6,gem}$ 12.3 Hz, H-6b), 3.89–3.75 (m, 2H, $J_{2,NH}$ 8.8 Hz, $J_{1,2}$ 8.4 Hz, H-2, $OCH_2CH_2(CH_2)_9CH_3$), 3.69 (ddd, $J_{4,5}$ 9.9 Hz, $J_{5,6a}$ 2,4 Hz, $J_{5,6b}$ 4.7 Hz, H-5), 3.50–3.42 (m, 1H, $OCH_2CH_2(CH_2)_9CH_3$), 1.94, 2.02, 2.02, and 2.08 (s, each 3H, Ac), 1.60–1.50 (m, 2H, OCH_2CH_2-$(CH_2)_9CH_3$), 1.35–1.14 (m, 18H, $OCH_2CH_2(CH_2)_9CH_3$), 0.87 (t, 3H,$OCH_2CH_2(CH_2)_9CH_3$).

Dodecyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranoside (2.5 g, 4.85 mol) in 100 mL of MeOH was deacetylated in the presence of NaOMe (270 mg, 5.0 mmol, Wako Pure Chemicals). Deprotection was carried out with stirring for 40 min. After decolorization on charcoal in EtOH, the product GlcNAc-C12 was obtained by recrystallization in ethanol. Yield: 1.70 g (88.1%). Mp 160–162 °C, lit.[22] mp 161 °C, $[\alpha]_D$ −18.8 (*c* 0.12, CH_3OH). ^1H NMR (CD_3OD): δ 4.38 (d, 1H, $J_{1,2}$ 6.1 Hz, H-1), 3.91–3.83 (m, 2H, H-6a, NH), 3.70–3.58 (m, 2H, H-2, H-5), 3.48–3.40 (m, 2H, H-3, H-6b), 3.34–3.27 (m, 3H, H-4, OCH_2), 1.97 (s, 3H, Ac), 1.53–1.51 (m, 2H, $OCH_2CH_2(CH_2)_9CH_3$), 1.34–1.22 (m, 18H, $OCH_2CH_2(CH_2)_9CH_3$), 0.89 (t, 3H, OCH_2CH_2-$(CH_2)_9CH_3$). MALDI-TOFMS: calcd for $C_{20}H_{39}NO_6$: $(M+Na)^+$, 412.3, Found: $(M+Na)^+$, 412.3. Anal. Calcd for $C_{20}H_{39}NO_6{\cdot}0.3H_2O$ (398.68): C, 60.82; H, 10.11; N, 3.55. Found: C, 60.81; H, 10.04; N, 3.54.

4.2. Synthesis of dodecyl β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranoside (LacNAc-C12)

2,3,4,6-Tetra-*O*-acetyl-β-D-galactopyranosyl-(1→4)-2-acetamido-1,3,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranoside (Ac-LacNAc) was prepared by mixing *N*-acetyllactosamine (982 mg, 2.56 nmol, LacNAc, Yaizu Suisankagaku Industry Co. Ltd, Japan) with Ac_2O (5 mL, 52.9 mmol) in 10 mL of pyridine. Ac-LacNAc (0.799 g, 1.88 mmol) was mixed with TMS-OTf (0.24 mL, 1.33 mmol) in CH_2Cl_2 under nitrogen. The solution was refluxed at 50 °C with stirring for 12 h. After evaporation, followed by neutralization with Et_3N, the product was chromatographed on silica gel to examine the progress of reaction. After evaporation, the product was collected by column chromatography (Silica Gel 60, 2 × 23 cm, 1:2:0.01 toluene–EtOAc–Et_3N). The collected products were mixed with 1-dodecanol (1.3 mL, 5.89 mmol), (*R*,*S*)-camphor sulfonate (27 mg, 0.12 mmol, Wako Pure Chemicals) in the presence of 4 Å molecular sieves (350 mg), and refluxed for 6 h. The mixture was neutralized with Et_3N. After evaporation of the solvent, the product was purified by column chromatography (Silica Gel 60, 2 × 35 cm, 2:3 *n*-hexane–EtOAc). Yield: 50% (478 mg). ^1H NMR ($CDCl_3$): δ: 5.63 (d, 1H, $J_{NH,2}$ 9.3 Hz, NH), 5.35 (d, 1H, $J_{3',4'}$ 2.9 Hz, H-4′), 5.11 (dd, 1H, H-2′), 5.06 (dd, 1H, $J_{3,4}$ 8.1 Hz, H-3), 4.97 (dd, 1H, $J_{2',3'}$ 10.3 Hz, H-3′), 4.51–4.46 (m, 2H, H-1′, H-6a), 4.43 (d, 1H, $J_{1,2}$ 7.3, H-1), 4.15–4.09 (m, 3H, H-6b, H-6b′, H-6a′), 4.03 (dd, 1H, $J_{2,3}$ 9.3 Hz, H-2), 3.87 (ddd, 1H, H-5′), 3.78 (dd, 1H, H-4), 3.62 (ddd, 1H, $J_{4,5}$ 5.6 Hz, H-5), 3.41 (dd, 2H, $OCH_2CH_2(CH_2)_9CH_3$), 2.15–1.96 (m, 21H, Ac), 1.60–1.46 (m, 2H, $OCH_2CH_2(CH_2)_9CH_3$), 1.30–1.18 (m, 18H, $OCH_2CH_2(CH_2)_9CH_3$), 0.87 (t, 3H, $OCH_2(CH_2)_{10}CH_3$).

Dodecyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-(1→4)-2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranoside (478 g, 0.56 mmol) in 25 mL of MeOH was deacetylated by the addition of NaOMe (160 mg, 2.97 mmol) with stirring for 3 h. The reactant was concentrated after treating with Amberlite IR-120B (Organo Co., Japan). LacNAc-C12 was purified by distilling with EtOH, toluene, and $CHCl_3$. Yield: 326 mg

(99%). mp 246 °C, $[\alpha]_D$ −7.6 (*c* 0.2, DMSO). ^1H NMR(DMSO-d_6): δ 7.74 (d, 1H, NH), 4.28 (d, 1H, $J_{1,2}$ 7.8 Hz, H-1′), 4.19 (d, 1H, $J_{1,2}$ 8.1 Hz, H-1), 1.7 (s, 3H, Ac), 1.42–1.41 (m, 2H, $OCH_2CH_2(CH_2)_9CH_3$), 1.17–1.29 (m, 18H, $OCH_2CH_2(CH_2)_9CH_3$), 0.85 (t, 3H, $OCH_2CH_2(CH_2)_9CH_3$). MALDI-TOFMS: calcd for $C_{26}H_{49}NO_{11}$: $(M+Na)^+$, 574.3, found: $(M+Na)^+$, 574.6. Anal. Calcd for $C_{26}H_{49}NO_{11}\cdot 1.5H_2O$ (578.35): C, 53.96; H, 9.06; N, 2.42. Found: C, 54.24; H, 8.77; N, 2.30.

4.3. Cell culture

HL-60 cells (Riken Cell Bank) were grown in RPMI 1640 medium (Nissui Pharm. Co., Ltd) supplemented with 10% heat-inactivated fetal bovine serum (JRH Biosciences Inc.) at 37 °C in humidified 5% CO_2. B16 cells (Riken Cell Bank) were grown in DMEM (Gibco BRL) supplemented with streptomycin 0.1 g/L, penicillin G potassium 50,000 unit/L, and 10% heat-inactivated fetal bovine serum (JRH Biosciences Inc.) at 37 °C in humidified 5% CO_2.

4.4. Glycosylation of saccharide primers in cells

Stock solutions of 20 mM saccharide primers in DMSO were diluted to 50 μM with serum-free and phenol red-free culture medium consisting of RPMI 1640 medium (Gibco BRL) containing 5 mg/L of transferrin, 5 mg/L of insulin, and 30 nM selenium dioxide.

Glycosylation by cells was carried out as follows: HL60 cells (2×10^6) were incubated with RPMI 1640 medium containing 50 μM saccharide primer for 48 h. The glycosylated products secreted in the culture medium were collected with a Sep-Pak C_{18} column (Waters Co.). The water-soluble compounds were removed with water and 3:7 MeOH–H_2O. The glycosylated products were eluted with MeOH. The eluate containing the glycosylated products was evaporated under reduced pressure. The obtained products were dissolved in 100 μL of 2:1 $CHCl_3$–MeOH, and an aliquot was separated on an HPTLC plate (Silica Gel 60, E. Merck) using $CHCl_3$–MeOH–0.2% $CaCl_2$. Acidic and neutral products on the HPTLC plate were stained with resorcinol–HCl reagent and orcinol–H_2SO_4 reagent, respectively. B16 cells (2×10^6) were similarly incubated with saccharide primers in serum-free DMEM/F-12 medium (Gibco BRL) containing 5 mg/L of transferrin, 5 mg/L of insulin, and 30 nM selenium dioxide.

4.5. TLC blotting

TLC blotting was carried out as follows: Glycosylated products separated on an HPTLC plate were sprayed with primuline reagent, and the spots were marked with a red pencil under UV light. Then, the HPTLC plate was dipped in a blotting solvent of 40:7:20 2-PrOH–MeOH–0.2% $CaCl_2$ for 20 s and placed on a glass fiber filter (ATTO Co.). The plate was covered with a PVDF membrane (ATTO Co.), a PTFE membrane (ATTO Co.), and another glass fiber filter. These layers were subjected to pressure at 180 °C for 30 s using a TLC thermal blotter (ATTO Co.). The PVDF membrane was washed with pure water, and glycolipid fractions were extracted with MeOH and 2:1 $CHCl_3$–MeOH.

4.6. High-performance liquid chromatography (HPLC)

Neutral products and acidic products separated using a Sep-Pak C_{18} column were purified by HPLC. The crude products dissolved in 70:28:2 $CHCl_3$–MeOH–H_2O were injected into an HPLC system equipped with an Iatrobead column (6RSP-8005, 4.6 × 250 mm, Iatron Laboratories Inc.) and a light scattering detector (SEDEX75, Sedere). Neutral products were separated with 70:28:2 $CHCl_3$–MeOH–H_2O. Acidic products were separated with 70:28:2 $CHCl_3$–MeOH–H_2O and 60:35:5 $CHCl_3$–MeOH–H_2O. The flow rate was 2 mL/min. The fractions were collected at 30-s intervals for 40 min.

4.7. Mass spectrometry

The structural analyses of glycosylated products were carried out by a MALDI-TOF mass spectrometer (Autoflex, Bruker Daltonics) and an ESI mass spectrometer (Esquire 3000, Bruker Daltonics). 2,5-Dihydroxybenzoic acid (DHB, Aldrich) was employed as a matrix.

4.8. Digestion of glycosylated products by enzymes

Enzymatic digestion of glycosylated products was carried out in 50 mM NaOAc buffer (pH 4.8) containing 50 mU of neuraminidase from *A. ureafaciens* (EC.3.2.1.18, Sigma), or in 50 mM sodium acetate butter (pH 5.5) containing 10 mU of neuraminidase from *M. decora* (EC. 3.2.1.18, Calbiochem). The reactions were carried out in the presence of 0.6 mg/mL sodium taurodeoxycholic acid. The products were collected using a Sep-Pak C_{18} column, separated on an HPTLC plate with 60:35:8 $CHCl_3$–MeOH–0.2% $CaCl_2$, and were stained with orcinol–H_2SO_4.

4.9. MTT assay

Cells (2×10^4) in a 96-well microplate were incubated with 50 μM GlcNAc-C12 or LacNAc-C12 for 48 h. Ten μL of WST-1 dye solution (10 mM WST-1 and 0.2 mM 1-methoxy PMS, Dojindo Laboratories) per well was added to each well. After 2 h, absorbance at 450 nm with a reference wavelength of 690 nm was measured using a microplate reader (Multiskan, Labsystem).

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carres.2008.01.022.

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MINIREVIEW

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Syntheses of Oligosaccharides Using Cell Function

細胞機能を利用したオリゴ糖鎖の合成

Sato, Toshinori[1] ; Hatanaka, Kenichi[2]; Hashimoto, Hironobu[3] ; and Yamagata, Tatsuya[4]

[1]Department of Biosciences and Informatics, Keio University, Hiyoshi, Kouhoku-ku, Yokohama 223-8522, Japan
FAX:81-45-566-1447, E-mail: sato@bio.keio.ac.jp;

[2]Center for Collaborative Research (CCR)& Institute of Industrial Science (IIS), The University of Tokyo, Komaba, Meguro-ku, Tokyo 153-8505, Japan; [3]National Institution for Academic Degrees and University Evaluation, FCCA, Chief Executive Secretary; [4]Laboratory of Tumor Biology and Glycobiology, Shenyang Pharmaceutical University, 103 WenHua Rd, P.O. Box 29, Shenyang 110016,P.R. China

Key Words: *saccharide primer, glycan synthesis, biocombinatorial synthesis, oligosaccharide, glycan array*

Abstract

The authors developed a method of synthesis of oligosaccharides using cell function. By administering the saccharide primer that is amphiphilic alkylglycoside into cell culture medium, glycosylated products were collected from the medium after 1–2 days. The sequences of the products were dependent on the biosynthetic pathway of sugars in the cells. By combining the saccharide primer and cell lines, it was possible to synthesize many kinds of oligosaccharides. The introduction of a functional group in an alkyl chain of saccharide primer made it possible to polymerize and immobilize to solid support. The saccharide primer method is expected to become a new methodology for glycomics.

要　約

筆者らは動物細胞の機能を利用したオリゴ糖鎖の合成法を開発した。両親媒性の擬似糖脂質である糖鎖プライマーを培養液中に加えることで、１－２日後には糖鎖伸長生成物を培養液から回収できた。得られたオリゴ糖鎖の配列は細胞での糖鎖の生合成経路に依存しており、糖鎖プライマーと細胞の種類を変えることで、多種類のオリゴ糖鎖の合成が可能であった。糖鎖プライマーのアグリコン部分に官能基を導入することで、高分子化や基板への固定化を可能にした。糖鎖プライマー法はグライコミクスの新たな手法として期待される。

A. Introduction

The application of glycans to biomaterials and medicines has been anticipated. However, these applications have not been developed to the degree expected. The researches into genes and proteins are supported by the technology of sequencer, automatic synthesizer, amplification with *E. Coli*, and library. Remarkable improvements in research on them were achieved by these instruments and techniques. If there is an idea for research, it is possible to perform experiments by purchasing the required materials. Thus, for the research and development of glycans, it is necessary to develop the technology for improving them drastically. In the development of such technology, the supply of glycans would be required to perform the potential carbohydrate research.

Glycans available as materials are mainly polysaccharide that is abundantly included in natural resources. Polysaccharide such as cellulose, chitin, chitosan, pullulan, and mannan are commercially available and inexpensive. However, for oligosaccharides which are not abundant in nature, the supply and variety is limited because the resources

序　論

糖鎖は生体材料や医薬品への応用が期待されている。しかしながら、糖鎖の応用は期待された程には発展してはいない。遺伝子やタンパク質の研究を支えるのは、シーケンサー、自動合成機、大腸菌での増幅、さらにはライブラリーの技術である。遺伝子やタンパク質の研究の目覚ましい進展は、これらの装置や手法の出現により達成されてきた。研究のアイデアがあれば、材料を購入して実験を行うことができた。そこで、糖鎖の研究や開発においても、それを飛躍的に進展させるための技術の開発が必要である。その様な技術開発の中でも、研究材料としての糖鎖の供給は新たな糖鎖研究を開拓するためにも必要である。

材料として使うことのできる糖は、主に資源として豊富な多糖である。セルロース、キチン、キトサン、プルラン、マンナンのような多糖は市販されており安価である。しかしながら、自然界での存在量の少ないオリゴ糖鎖の供給源は多くの場合動物の臓器であることから、種類や供給量は限られて

Trends in Glycoscience and Glycotechnology
Vol.19 No.105 (January 2007) pp.1–17

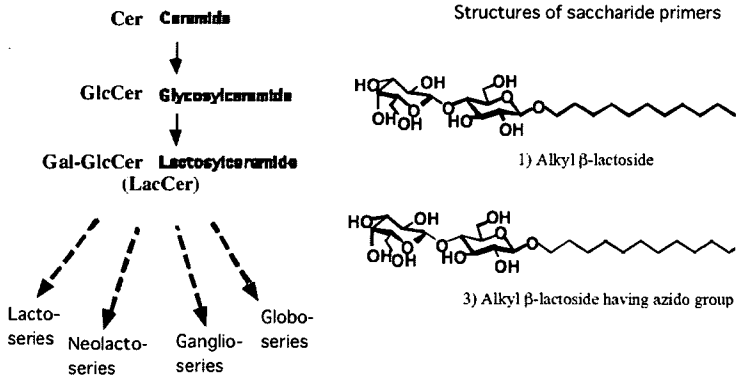

Fig. 1. Biosynthetic pathway of glycolipids and the typical structures of saccharide primers.

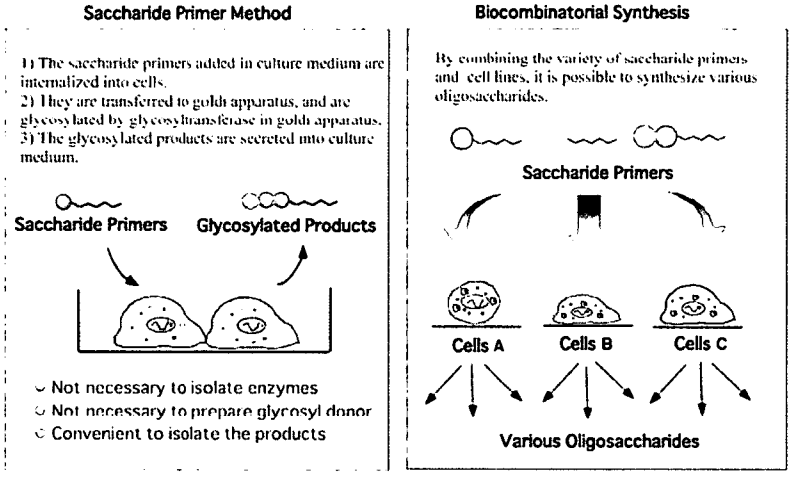

Fig. 2. The principle of saccharide primer method and biocombinatorial synthesis.

are organs of animal in many cases. Then, organic synthesis and enzymatic synthesis have been developing as a method which does not depend on natural resources. The authors have been developing a novel method for production of oligosaccharide as the fourth method. The cell synthesizes glycans in follicle and Golgi. That is to say, the cell is the small factory which synthesizes glycans. Therefore, we aim to establish the synthetic method of the oligosaccharide utilizing such a cell function for sugar biosynthesis

B. The Principle of Saccharide Primer Method

To synthesize oligosaccharides utilizing glycan biosynthesis system in cells, molecules as substrate for glycosyltransferase in cells are required. We named such a molecule "saccharide primer." Glycolipids are synthesized by connecting sugar molecules one by one to ceramide. This lactosylceramide (LacCer) is a common precursor of many series of glycolipids, including the ganglio series (NeuAcα2-3Lac), globo series (Galα1-4Lac), lacto series (GlcNAcβ1-

いる。そこで、天然資源に頼らない有機合成や酵素合成法が発展してきている。これに加えて、筆者らは４つ目の手法として、新たなオリゴ糖鎖の生産方法を開発している。細胞は小胞やゴルジにおいて糖鎖の生合成を行っている。すなわち、細胞は糖鎖を合成する小さな工場である。そこで、我々は、そのような糖鎖生合成における細胞機能を利用したオリゴ糖鎖の合成法の確立を目指している。

糖鎖プライマー法の原理

細胞の糖鎖生合成能力を利用してオリゴ糖鎖を合成するためには、細胞内の糖鎖合成酵素の基質となる分子が必要となる。このような分子を「糖鎖プライマー（　　）」と呼んでいる。糖脂質の糖鎖生合成経路では、糖鎖伸長反応は一定の規則に従って行われている。糖脂質の多くはラクトシルセラミド　　が前駆体となり、ガングリオ系列　α　、グロボ系列　α　、ラクト系列　β　、あるいはネオラクト系列

3Lac), and neo lacto series (GlcNAcβ1-3Lac)(Fig.1). Therefore, it is possible to define the sequence of lactose as a primer for sugar chain elongation reaction.

Dodecyl β-lactoside (Lac-C12) that mimics LacCer is one of the useful saccharide primers (1–3). Lac-C12 is an amphiphilic pseudo-glycolipid, and was a substrate for glycosyltransferase. Because of its amphiphilic property, the saccharide primer supplemented in culture medium could internalize into cells. The products glycosylated in the cells were secreted in the culture medium. Since the glycosylated products existed in the culture medium, the isolation of them was very convenient. This is the most important advantage of the method of synthesizing oligosaccharides using cells. Besides this, the features of the saccharide primer method are as follows: Saccharide primers have monosaccharide or disaccharide structure that can be conveniently synthesized. Cells are treasuries of glycosyltransferases, and also synthesize glycosyl donor. By utilizing the intact cells, it is not necessary to prepare the glycosyltransferase and glycosyl donor (Fig. 2).

The cells were cultured in the serum-free medium supplemeted with the saccharide primer for 1–2 days. The unreacted primer and the glycosylated products in the culture medium were collected with reversed phase column chromatography. The glycosylated products were separated on a HPTLC, and their molecular masses and sequences were analyzed by MALDI-TOF-MS/MS and ESI-MS/MS. The products could be isolated by a HPLC.

The primers as substrates for glycosyltransferase in cells have been already reported. In ongoing research, it has been found that β-D-Xyloside was an initiator (primer) for the biosynthesis of glycosaminoglycan (4,5). After this, Esko *et al.* investigated that GalNAcα-*O*-benzyl 、Xylβ1-6Gal-*O*-2-naphthol、peracetylated Galβ1-4GlcNAcβ-*O*-naphthalenemethanol(6), peracetylated Galβ1-4GlcNAcβ-*O*-naphthalenemethanol (7) were substrates for the glycosyltransferase in cells, and they determined the structures of the glycosylated products. Furthermore, those primers were also found to be inhibitors for endogenous glycan biosynthesis (6–10). The primers having bezyl and naphtyl group as aglycon tend to stay in cells because of their hydrophobicity. Therefore, the activity as inhibitor for biosynthesis would be induced. On the contrary, as the ability of dodecylglycoside to remain in the cells is poor, the glycosylated products are secreted into culture medium. As the glycosylated products could be isolated without the lysis of the cells, the saccharide primers employed in our study have an advantage from the viewpoint of carbohydrate synthesis.

C. Idea of Biocombinatorial Synthesis of Saccharides Using Cells Is Born

In the early 1990 s we were struggling to create new

β などの糖鎖へと伸長している(図)。よって、ラクトシドの配列は糖鎖伸長反応におけるプライマーであると定義することができる。

ラクトシルセラミドを模倣した分子であるドデシルラクトシド( )は糖鎖プライマーの代表的な例である 。

は両親媒性の擬似糖脂質であり、糖転移酵素の基質となる。両親媒性であることから、培養液に溶解しておくと、細胞内に取り込まれ、細胞内で糖鎖伸長を受けた生成物は細胞外に分泌される。生成物が培地中に存在するので単離の操作は簡便である。このことは、細胞を用いて糖鎖を合成することの最も重要な利点である。これ以外にも、糖鎖プライマー法の特徴としては次のような点があげられる。糖鎖プライマーは合成の簡単な単糖あるいは二糖構造を有している。細胞は糖転移酵素の宝庫であり、糖ドナーも合成している。細胞を生きたまま利用することで、糖転移酵素や糖ドナーを準備する必要がない(図)。

糖鎖プライマー法では、糖鎖プライマーを添加した培養液で細胞を1－2日間培養したのち、培養液を逆相カラムに通して未反応の糖鎖プライマーと糖鎖伸長生成物を回収した。回収した生成物を 上で展開し、さらに および により分子量の測定および構造解析を行った。糖鎖伸長生成物は により単離できた。

細胞内での糖鎖生合成酵素の基質となるプライマーはこれまでにも報告されている。最も初期の研究において、β はグリコサミノグリカンの生合成のイニシエーター(プライマー)であることが見出されている 、。その後、らは α 、β 、β β 、β β が細胞内での糖鎖伸長反応の基質になり、伸長した糖鎖構造の決定を行っている。さらに、それらプライマーは内在性の糖鎖の生合成の阻害剤になることも見出されている 。アグリコンに 基や 基を有したプライマーでは疎水性が高いために細胞内にとどまる傾向がある。そのために、生合成阻害剤としての活性が誘導されるのであろう。これに対してドデシルグルコシドでは細胞内にとどまる能力が乏しいので、糖鎖伸長生成物は細胞外に分泌されることになる。そこで、糖鎖伸長生成物は細胞を分解することなく分離することが出来るので、我々が用いている糖鎖プライマーは糖鎖合成という観点において利点がある。

ドデシルラクトシドが糖鎖プライマーであることの発見

私たちは当時糖脂質の機能を知るために様々な方法を考

approaches for elucidation of glycosphingolipid functions. If we synthesize glycosphingolipid mimicking precursor molecules with which cells are to be incubated, there may exist a possibility that cells incorporate the molecules for the synthesis of their own glycosphingolipid. The glycolipid thus synthesized and transported to the outer leaflet of the plasma membrane must be distinct from the natural ones in terms of stability and functions and cell physiology will be affected and thereby functions of glycosphingolipids may be understood. An extensive search for the presence of this idea in the list of publications led us to think that this was a new idea and that artificial glycolipid precursor would become a powerful tool in the study on the elucidation of glycolipid.

A chart of glycosphingolipids with special reference to biosynthesis tells us that lactosylceramide is a key molecule in the glycosphingolipid family; except for galactosylceramide all species of glycosphingolipids in mammals are synthesized through lactosylceramide. Therefore we aimed at the synthesis of lactosylceramide analogues whose structure is simplified for the chemical synthesis and that can be incorporated by cells in culture and used in parallel with, or better than, lactosylceramide for the synthesis of complex glycolipid for the cells. Ceramide consists of two long hydrophobic chains. It would be easy to synthesize a lactoside with a single hydrophobic chain. If the length of the chain were not long enough to give hydrophobicity to the lactoside, it would not be incorporated. If the chain length were too long to be soluble in the medium, the lactoside would fail in utilization. By that time, synthesis of alkyllactoside of different chain length was reported, but we ourselves wanted to synthesize them for our own use. Conjugation of lactose with alkyl chain resulted in alpha and beta isomers which were separated by column chromatography. Synthesis of alkyllactoside according to the published method did not bring us the joy of performing an experiment and thus we synthesized a chromogenic amphiphilic lactoside, 2-(*N*-hexadecanoylamino)-4-nitrophenyl

えていた。糖脂質の構造を真似た化合物を細胞に与えて、細胞の糖脂質生合成経路がその化合物を利用できれば、細胞の正しい糖脂質がおかしな糖脂質に置き換えられてしまうであろう。そうなると細胞の機能や刺激に対する反応が変わるであろう。この方法が糖脂質の機能を調べるという新しいアプローチになると思って文献を調べてみると、このようなアイデアの研究はそれまでに全くないことがわかった。

糖脂質の構造を見ると、ラクトシルセラミドが基本であることが分かる。ガラクトシルセラミド以外の哺乳動物の殆どの糖脂質はラクトシルセラミドを経由して合成される。それで私たちは、二糖であるラクトースを出発物質として、ごく簡単な合成操作で出来る簡単な化合物を合成して、これが細胞に利用されるかどうかを調べ始めた。糖脂質のセラミドは疎水性の長い鎖が二本ある。疎水性のアルキル鎖1本をラクトースに付ける方が簡単であろう。疎水性が低ければ（アルキル鎖が短い）、水には良く溶けても細胞には入らないだろうし、アルキル鎖が長いと水溶性の細胞培養液には溶けないだろうと考えた。実際には、様々な長さのアルキルラクトシドがそれまでに合成されていたが、手に入らないので自分たちで合成を行った。ラクトシドとアルキル鎖の結合ではαとβ結合が出来るのでこの二つはカラムで分離した。すでに知られている構造のアルキルラクトシドを合成しても、合成自身は新しいことではないので論文発表は出来ない。それで単純なアルキル鎖ではなく、

β　　を合成した。これはエンド型のエンドグリコセラミダーゼ　　の活性検出の発色試薬として使

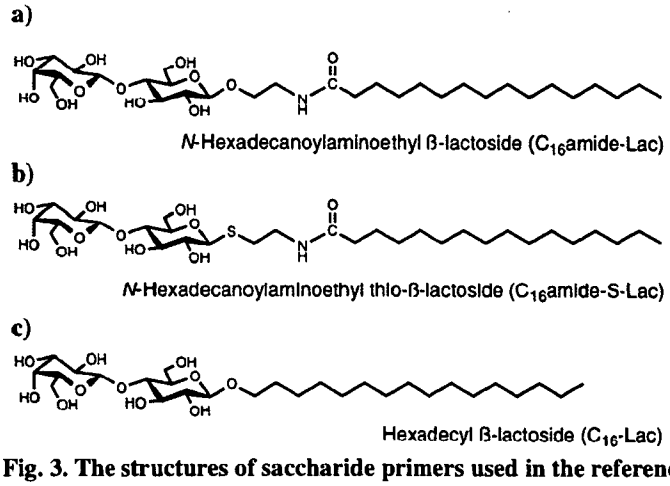

Fig. 3. The structures of saccharide primers used in the reference 2.

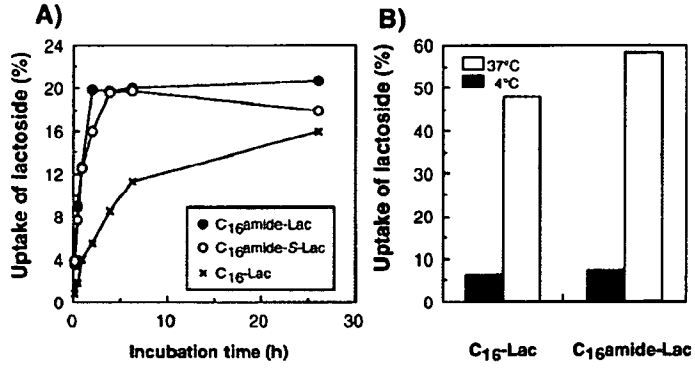

Fig. 4. Saturated temperature-dependent uptake of lactosides by B16 cells(see reference 2).

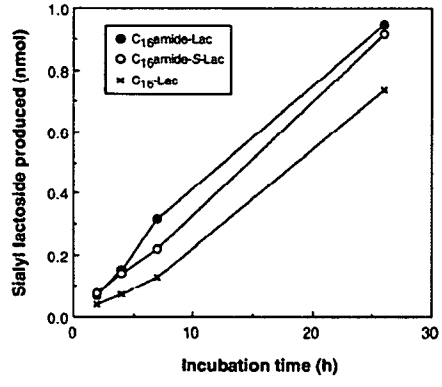

Fig. 5. Priming of putative GM3 analogues by lactosides in B16 cells (see reference 2).

β-lactoside that can be used as a substrate for endo-type glucosylceramidase such as endo-type glycosylceramidases such as EGCase. We further synthesized novel lipophilic alkylamidelactosides whose *N*-acyl structures were introduced via ethanolamine. These lactosides, *N*-acylaminoethyl β-lactosides partially mimicked the ceramide structure and it was expected these lactosides should prove to be more suitable precursor in the glycolipid synthesis following incorporation into cells (2) (Fig. 3).

We wondered if these lactosides were utilized by cultured animal cells. To begin with we used mouse melanoma B16 cells synthesizing the simplest ganglioside GM3, and administered lactosides in the medium at the 25 μM concentration. In order to facilitate the trace of lactosides, these molecules were titrated with galactoside oxidase followed by reduction with ^{3}H-$NaBH_4$ at the C-6 of Gal residue. Following the incubation of B16 cells with the radiolabeled lactoside, cells were washed and incorporated radioactivity was determined. The uptake of C16-series lactosides was dependent on incubation time and proceeded to saturation. When cells were fed with 25 μM lactosides, the C16-alkylamidelactoside amount was much larger than that of C16-alkyllactoside. In 20 h incubation, 20% of

える。次に、エタノールアミンを介して様々な長さの脂肪酸と結合した　アシル基をラクトースに導入した化合物を合成した。セラミドはスフィンゴシンの　アシル化合物で２本足である。私たちの合成した　アシル化合物は１本脚のスフィンゴシンと見なすことが出来るので、より本物に近いものになる　(図)。

このようにして合成したアルキルラクトシドとアルキルアミドラクトシドが細胞に取り込まれるかどうかを調べた。糖脂質合成系の一番簡単なマウスメラノーマ　細胞を培養して培地にこれらの化合物（ μ ）を添加した。取り込みの追跡を容易にするために糖鎖のガラクトースの６位を酸化してから　で還元することで放射標識をした。一定時間のあと細胞に取り込まれた放射能を測ると、アルキルアミドラクトシドの取り込み速度の方が速かったが、どちらも細胞に取り込まれ　時間後には与えた放射能の　%が取り込まれていた　(図)。培養温度を４℃にすると取り込みが見られ

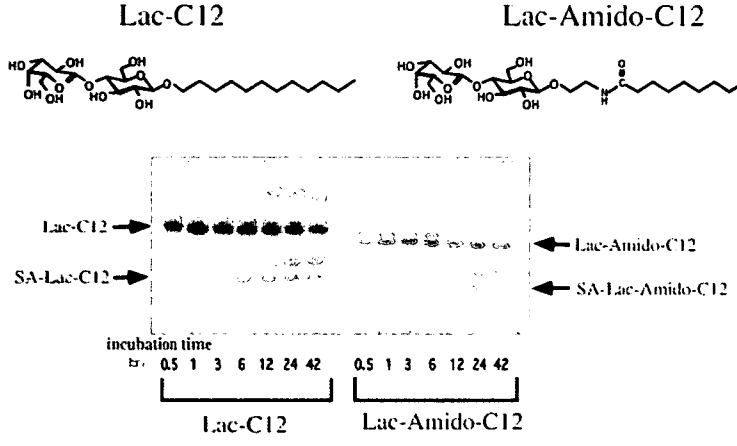

Fig. 6. HPTLC analysis of oligosaccharide released from Lac-C12 and Lac-amido-C12 (see reference 2).

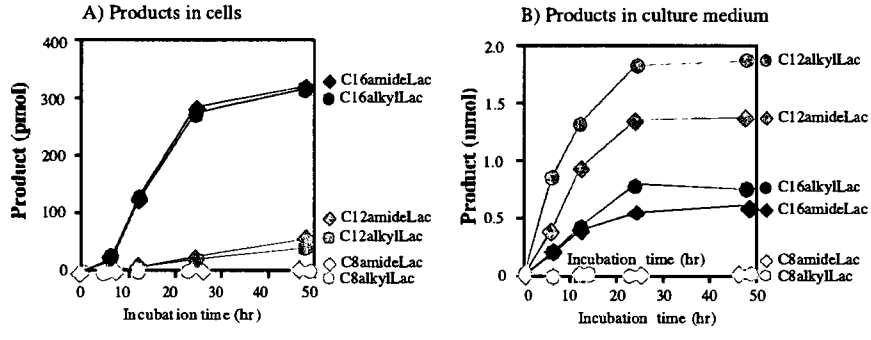

Fig. 7. The amount of the products derived from the several saccharide primers (see reference 3).

radioactivity was incorporated into the cell with incubation with either lactosides (2) (Fig.4). Lactosides subsequent to incubation were far fewer in number at 4 °C compared to 37 °C, suggesting that accumulation of lactosides was due to incorporation but not the diffusion of lactosides. Glycosylated lactosides were found present in the ganglioside fraction of cell extracts as confirmed by HPTLC, and it was shown that the glycosylated lactoside had the same sugar sequence as GM3 by EGCase treatment (2) (Fig. 5).

Saccharide moiety of the glycosylated lactoside was found to be the same as that of GM3, except that they were different from the native GM3 in terms of hydrophobic moiety. 23% of the molecular surface of bovine serum albumin (BSA) is hydrophobic and thus it attracts the hydrophobic moiety to the surface and serves as a lipid carrier in serum. Lipid-removed BSA can in turn be used as an extractor from the membrane of hydrophobic molecules with weak interactions with other molecules in the plasma membrane. The process is called back exchange. B16 cells were incubated with C16-alkyllactoside or C16-alkylamidelactoside for 18 h and

ないので、取り込みは培地からの拡散ではなく、エネルギー依存の積極的取り込みであると思われた。細胞に取り込まれた放射能を頼りに構造を調べると、細胞の中でシアル酸の付加を受けて糖鎖部分は　　と同じ構造になっていた　(図　)。

細胞に添加したアルキルラクトシドとアルキルアミドラクトシドはシアル酸の転移を受けて糖鎖部分は　　と同じになって細胞に発現しているが、２本足であるセラミドの場合に比べて１本足である。　　はタンパク質粒子表面の　%が疎水性でありここに疎水性物質を惹きつけるので脂質キャリアーとして知られている。この　　分子を洗って吸着している疎水性物質を除くと、今度は疎水性物質を細胞表面から引き抜くのに使える。この操作を　　という。細胞をアルキルラクトシドやアルキルアミドラクトシドと　時間保温し、　　で　　をする前後で細胞の　　発現を調べたところ、アルキルラクトシドを取り込んだ細胞で

surface expression of GM3 was examined before and after the back exchange. About 10% and 40% of the cell surface GM3 was decreased from C16-alkyllactoside and C16-alkylamidelactoside treated cells, respectively. The results indicated that 10% and 40% of the cell surface GM3 was replaced with glycosylated lactosides in C16-alkyllactoside and C16-alkylamidelactoside treated cells, respectively. C16-alkylamidelactoside was incorporated into the cells faster than C16-alkyllactoside and it tended to remain within the cells better than C16-alkyllactoside (Fig. 6).

Lactosides having different lipid moiety structures affected the incorporation and glycosylation efficiency, though each of these was not segregated. The amounts of glycosylated lactosides accumulated within the cell and culture medium fractions were determined (3) (Fig. 6 and 7). C8 lactosides (C8amideLac and C8alkylLac) were not glycosylated, indicating that there were not incorporated into cells. C16 series lactosides were glycosylated 5 times as much compared to C12 series lactosides. Alkylamidelactoside showed better glycosylation than alkyllactoside in the cell, because the former could stably be present in the membrane due to possible formation of hydrogen bonding with other molecules. Glycosylation within the cell reached a plateau, while accumulation of significant amounts of glycosylated lactosides in the culture medium was found. C12 series lactosides were found to be more effectively glycosylated than C16 series lactosides. Since C16 lactosides can be packed more tightly than C12 lactosides in the plasma membrane due to their enhanced hydrophobic interactions, they tended to remain in the membrane, while C12 lactosides, following glycosylation in the Golgi apparatus and transportation to the membrane, faced a shorter life span in the membrane due to their weak hydrophobicity. This was also the case within alkylamidelactoside and alkyllactoside; amidelactosides had stronger interactions than alkyllactosides with other molecules and the former tended to remain in the membrane.

The ratio of glycosylated lactosides between the medium and the cell fractions was 1.5-2 and 5-6 in the case of C16 lactosides and C12 lactosides, respectively. Cells used in the experiment were mouse melanoma B16 expressing GM3 as a sole ganglioside. B16 cells were sialylating C12 lactosides as a precursor for the synthesis of GM3, but sialylated C12 lactosides were easily shed from the membrane and thus ganglioside-synthesizing machinery worked perpetually when C12 lactosides were fed and thus glycosylated C12 lactosides were accumulated in the medium far faster than the cells fed with C16 lactosides.

Africa green monkey kidney cell COS7 is known to express ganglio a series ganglioside. By supplementing Lac-C12 in the culture medium, oligosaccharides of ganglio a series are synthesized by the cells. Analysis of the products

は　%が、アルキルアミドラクトシドを取り込んだ細胞では
の発現が　%減った。つまりアルキルラクトシドを取り込んだ細胞では表面　の　%がアルキルラクトシドにシアル酸が付加したものであり、アルキルアミドラクトシドを取り込んだ細胞では表面　の　%がアルキルアミドラクトシドにシアル酸が付加したものであった。細胞への取り込みもアルキルアミドラクトシドの方が速かったし、細胞に留まるという相性もアルキルアミドラクトシドの方が良かった（図　）。

ラクトシドの鎖の長さが細胞への取り込みにどのように影響するかを調べると、　ラクトシドでは細胞には取り込まれず、　ラクトシドでは取り込みが見られるが、　ラクトシドの方が5倍良く取り込まれた　（図　および図　）。アルキルラクトシドとアルキルアミドラクトシドを比べると、後者の方が高かった。これはセラミドと同じようにアミド結合を持っているので細胞膜上で水素結合を形成して安定に存在できるからであろう。細胞への取り込みは時間の経過と共に一定になるが、培地を調べてみると、培地の中に糖鎖の伸長したラクトシドが時間と共に増えていくことが分かった。このときは、　アルキルラクトシド（　）、　アルキルアミドラクトシドが　ラクトシドよりもはるかに効率よく
様化合物を培地に蓄積した。細胞に取り込まれたラクトシドは恐らくゴルジ体に運ばれてシアリル化を受け細胞膜に運ばれるのであろう。細胞膜では　の方が　よりも足の長い分だけ留まりやすいのだろう。　ラクトシドではアルキルアミド鎖の方がアルキル鎖よりも分子間相互作用が強いので膜により多く留まるようである。

培地に溜まる生成物と細胞に留まる生成物の比を見ると、では　～　である一方、　では　～　で程度であった。細胞は　ラクトシドを原料として　を合成しているつもりだが、細胞膜からはどんどん出ていって蓄積しないので、全力を挙げてこれを合成し続けるのであろう。用いている細胞は　を合成する細胞である。ラクトシドを与えられた細胞は、一生懸命に糖鎖構造は　と同じ人工糖脂質を作り、せっせと培地に排泄しているようである。

細胞を変えても同様な挙動がみられた。サルの腎臓細胞　7細胞は、ガングリオシド　系列の糖鎖合成系を有していることが知られている。そこで、　を糖鎖プライマーとして培養液に添加することで、ガングリオ　系列のオリゴ

secreted into the culture medium by a mass spectrometer indicated that the saccharide primer was connected with *N*-acetylneuraminic acid, *N*-acetylhexosamine, hoxose, and second *N*-acetylneuraminic acid in this order. These results suggested that the products were GM3, GM2, GM1, and GD1a of ganglio a series. When we started this research project, the sequence of the glycosylated products has been determined in combination with the substrate specificity by sugar hydrolase and the binding specificity for sugar-binding protein such as cholera toxin. Recently, many oligosaccharide structures can be determined by mass spectrometry using MALDI-TOF-MS/MS and ESI-MS/MS.

D. Biocombinatorial Synthesis

Looking at the above mentioned experimental results, the idea of constructing a saccharide library by biocombinatorial synthesis of saccharides using cells in culture emerged. Artificial lactosides were first developed to modify the glycolipid synthesis of cells and thereby expected to obtain a clue to the glycolipid functions. But with a different point of view, lactosides can be used as a primer to synthesize artificial glycolipids using cells. Purification of glycolipids from tissues and cells is rather easier than that of glycoproteins, but still the purification procedure contains a collection of cells from which glycopids are extracted using lipophilic reagents. Administration of the artificial lactosides results

糖鎖が細胞により合成される。培養基中に分泌された糖鎖伸長生成物の配列を質量分析装置で解析することで、プライマーに　アセチルノイラミン酸、　アセチルヘキソサミン、ヘキソース、および　アセチルノイラミン酸が順番に結合した生成物が得られていることが示された。これによりの生成物はガングリオシド　系列の　、　、　および　であることが推察された。我々が本研究プロジェクトを開始した頃には、糖鎖構造は加水分解酵素による基質特異性、糖認識タンパク質（コレラ毒素）との結合特異性を調べる方法と組み合わせて確認していた。最近では　および　用いた質量分析法により多くの糖鎖構造が決められるようになってきた。

バイオコンビナトリアル合成

これまでの結果を見て「細胞による糖鎖のコンビナトリアル合成」というアイデアが浮かんだ。ラクトシドを細胞に与えて細胞の糖脂質の構造を変えることで糖脂質の機能を調べようというのが研究の初期の目的だったが、視点を変えて人工糖脂質の合成に使えるという考えに至った。糖脂質は糖タンパク質に比べれば細胞からの精製は容易である。それでも、

Table I. Example of glycan structures expressed in the established cell lines.

C□□□□□□□□	G□□□□□□	□□□□□□□□□□
□ □□□□□ □□□□□□□ □B16	G□3, GD3	J. B□□□C□□□. 260, 13328 (1985)
A□□□□□□ G□□□□ □ □□□□□ □□□□□ C□□7	G□3, G□2, G□1, GD1□	J. B□□□C□□□., 261, 15755(1986)
□□□□□□□□□□□□□□□□ □□□□□□ □□C12	G□3, G□4, G□□1-3G□3	B□□□□□□. B□□□□□□ □□□ C□□ □□□, 162, 1093(1989)
C□□□□□ □ □□□□□ E□□□□□□□□□□□□ □DC□	G□3, G□3, G□4, G□□A□□G□4 (F□□□□□□□), G□□C□□ G□□C□□ G□□G□□C□□ □□□C□□ G□□C□□3-□□□□□□□ □□□C□□3-□□□□□□	J. B□□□□□□ (□□□□□), 100, 825 (1986)
H□□□ F□□ □□ B□□□□ □□□ □□□□□□□ □□□□H□60	G□3, GD3, GD1□ G□1□ G□□1-4G□□A□□-3□□□C□□(□□□□□□□□□□□□), □□G□ A □□□□□□ B □□□□□□ □□G□ □□□□□ □□□□□□□□□□□□ □□G□ G□□□A□□1-3□□□C□□	B□□□□ 64, 534(1984)
H□□ □□F□□□□□□□□	G□3, GD3, G□3, G□4, G□□C□□ □□□C□□	J. B□□□C□□□., 247, 5944 (1972)
□ □□□□ □□□ □□□□□□□□□ □ □A□ 117-□ (□□□-□ □□□□□□□), □A□ 117-H10 (□□□-□ □□□□□□□)	G□3, G□4, G□ 1□ GD1□ □□□C□□	C□□□□□□□ 57, 1882-, (1997)
H□□ □□C□□□C□□□□□C□□□201	G□□□-3G□□□A□□-□□□C□□ □A□2-3G□□□-3G□□□A□□-□□□C□□ □□□□ □□□ □ □□□□□□ □□□ G□□□-4G□□□A□□-□□□C□□ □□□□□ □□□□□□□□□□□□ □□□□ □□□	J. B□□□C□□□., 274, 12499(1999)
H□□ □□C□□□□□□C□□□□□H□□	G□, G□3	C□□□. □□□□. B□□□54, 522 (2006). J. C□□□□□117, 1421 (2004).
H□□ □□H□□□□□□□□□□□ □H□□G2	G□3, □□□□□ G□2, G□1, 3-□□□□□□ □□□□□□□□ G□□□□□□□□□□□□□□□□ G□□C□□ □□□C□□	A□□□ B□□□□□□. B□□□□□□□73, 578 (1989)
B□□□□□ □ □□ □□□ E□□□□□□□□ C□□□ B□EC	G□3, G□2, G□1, GD3, GD2, GD1□ G□1□ G□3	□□□□ 13, 59 (1978), J. B□□□C□□□. 261, 5652 (1986)

in glycosylation and the release of glycosylated lactosides in the medium and purification of these products must be simpler than the purification of glycolipids from the cell. The lactosides served as primers for the synthesis of artificial glycolipids. Provided with the suitable primer structures, they can be used for the synthesis of proteoglycan saccharides and glycoprotein saccharides.

The principle of combinatorial chemistry has been utilized for producing chemical libraries such as medicines. Through the improvement of such research techniques, the time to know the correlation between structure and function of the medicine article was rapidly shortened. The technique of combinatorial chemistry has been adopted for the preparation of a glycan library by organic synthesis. We utilize the priciple of combinatorial chemistry for the preparation of an oligosaccharide library using the living animal cells and saccharide primer method (Fig. 2).

Each established cell line has specific glycan biosynthesis pathways (Table 1). By administering Lac-C12 to those cells, it was found that Lac-C12 was elongated with oligosaccharides equal to the endogenous glycolipids. Besides Lac-C12, saccharide primers having monosaccharides and other disaccharides also received the glycosylation in cells. Besides the cells described above, there are enormous kinds of cell lines that can be subcultured. In addition to Lac-C12, several saccharide primers having monosaccharide and disaccharides were synthesized, and were found to be glycosylated in the cells. By combining the culture cells and the saccharide primers, it is possible to produce many kinds of oligosaccharides that are synthesized by the cells. This is the principle of "biocombinatorial chemistry."

E. Various Saccharide Primers

In order to produce many carbohydrate chains by the method proposed by this project, the development of various types of saccharide primers is required. On designing the novel primers, we were interested in the following two, a basic and a challenging subject: what are the suitable structures for the transfer of the primer from the medium to the carbohydrate chain-synthesizing machinery in Golgi apparatus? how do the carbohydrate chain-synthesizing enzymes recognize the primer structure? For the former subject, we planed to examine the effect of the aglycon structure on the behavior of various novel lactosides, because some lactosides have been proved by our college to work as an effective saccharide primer as described below. For the latter and rather challenging subject, if we consider the unique biosynthetic pathway of *N*-glycan, several novel types of primer candidates having disaccharide or two monosaccharide units were synthesized and tested as primer.

The elongation of carbohydrate chain on the lactoside

細胞を集め脂質溶媒で抽出しなくてはならない。ところがこの人工糖脂質は培地に溜まるのである。この方法は人工糖脂質の生産に使える。今のターゲットは細胞の合成する糖脂質だが、プライマーを選べばプロテオグリカン、糖タンパク質の合成も可能かもしれない。

医薬品など多くの有機化合物のライブラリーの作製においてコンビナトリアルケミストリーの原理が利用されている。このような研究手法の進歩により、医薬品の構造一機能相関の研究に要する時間は飛躍的に短縮されるようになった。有機合成による糖鎖ライブラリーの開発でもコンビナトリアルケミストリーの手法はすでに取り入れられている。我々は、組合せの方法によるライブラリーの作製原理を細胞と糖鎖プライマー法を用いた糖鎖ライブラリーの作製において適用している 図 。

樹立されている細胞株は特定の糖鎖生合成経路を発現していることが多くの論文で報告されている 表 。これらの細胞に糖鎖プライマー を投与すると、内在性の糖脂質と同様の糖鎖伸長反応を受けた。また、ラクトース以外でも単糖や二糖を有する糖鎖プライマーは細胞内で糖鎖伸長を受けることを明らかにしている。前述の細胞以外にも継代培養できる細胞の種類は膨大である。ラクトシド以外にも種々の単糖あるいは二糖のプライマーが合成され、細胞での糖鎖伸長されることが見出されている。最近では、 グリカンの糖鎖を合成できる糖鎖プイライマーの開発にも成功している。よって細胞と糖鎖プライマーの組み合わせの方法により種々の糖鎖を得ることができる。細胞と糖鎖プライマーの種類をいろいろと掛け合わせることで、動物細胞が合成できるオリゴ糖鎖を多種類作り出すことができる。これが「バイオコンビナトリアル合成法」の原理を利用した糖鎖の合成法である。

種々の糖鎖プライマー

本プロジェクトが提案する手法により多くの糖鎖を得るには、より多様な糖鎖プライマーを開発することが必要である。新しい糖鎖プライマーのデザインに際して、次の２つの課題、基礎的な課題および挑戦的な課題に興味を抱いた。１つはプライマーが細胞膜を通過して糖鎖合成系が存在する の糖鎖生合成系に到達するには、どのような化学構造が必要とされるか。また、もう１つは糖鎖生合成に関与する酵素が基質として認識する構造は何か。前者については糖脂質糖鎖のプライマーとして機能することが知られており、実験例も多いラクトシドに注目し、アグリコンの異なる多様なラクトシドを合成し、 細胞を用いてその糖鎖プライマーとしての性質を明らかすることを目標とした。一方、後者の観点からは、その特異な生合成経路を考えるとかなり挑戦的ではあるが、これまでに知られていない糖タンパク質の 型糖鎖を伸長させうる、新しい糖鎖プライマーを見いだすべく二糖グリコシドをデザインした。

ラクトシドをプライマーとする糖鎖伸長は、中島ら に

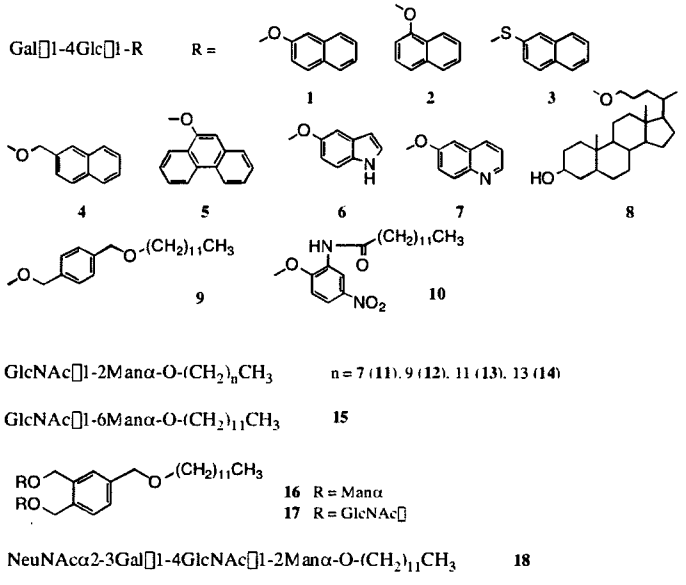

Fig. 8. The structures of saccharide primers used in section E.

primers was examined by Nakajima *et al.* (3) using those with long chain alkyl residue as aglycon from C8 to C16 (Fig. 7). The best result as a primer, was obtained in the case of C12. On the other hand, the chain elongation on *N*-acetylglucosamine-containing disaccharide was reported using its peracetylated α-naphtylmethyl glycoside (6). Therefore, several lactosides having an aromatic ring in their aglycon moiety (Fig. 8, lactoside **1–7**), having also an alkyl chain and so on (Fig. 8, lactoside **8–10**) were synthesized and the chain elongation was examined in B16 cell. The results indicate that the lactoside having the aglycon whose calculated logP (partition coefficient) value by the fragmentation method (ChemDraw, CambridgeSoft Corporation) as the corresponding alcohol is around **5–7** seems to be able to behave as the primer. Although it might be criticized as a hasty decision based on the result of a single cell, it is very interesting that the behavior of a glycoside as a primer could be predicted by the logP value calculated by the empirical data.

It emerged very recently that the less complicated but mysterious mechanism of the *N*-glycan biosynthesis is a part of the quality control of protein. While the carbohydrate chain of glycolipid elongates simply on the proper primers, that of the *N*-linked one does so via a unique two-step construction, the so-called tetradecasaccharide glycosylation followed by processing, to form a variety of branched chain structures. Thus it seems to be challenging to develop a novel primer for the *N*-glycan, and we designed a new type of two monosaccharide units-primer. At first, based on the assumption

より　から　までの鎖長の異なるアルキル鎖をアグリコンとして検討され、　において最大の糖鎖伸長が見られることが報告されている（図　）。一方、　アセチルグルコサミンを含む二糖では α　ナフチルメチルグリコシドにおいて糖鎖伸長が報告されている　。そこで、これまでプライマーとして検討がおこなわれていない、アグリコン部位に芳香環（図 **1–7**）やさらにアルキル鎖を有するラクトシド（図　**8–10**）などを中心に、類縁体を合成しそれらに対する糖鎖伸長を、　細胞を用いて調べた。その結果、部分構造法（　）で算出したアグリコンのアルコールとして　（分配係数）が5から7程度の時に、糖鎖が伸長したと思われる生成物が　で観察された。1種の細胞における結果を持って判断するのは早計であろうが、これらの経験値に基づく　の値から細胞におけるプライマーの挙動が予測できるとすれば大変興味深い。

糖タンパク質の　型糖鎖の複雑なというか、一見不可思議な生合成の機構はタンパク質の品質管理を行うための仕組みの一環であったことが明らかとなったのは、近年のことである。単純に糖残基が伸長する糖脂質とは異なり、多様な枝分かれ構造を創製するプロセシングという糖鎖の伸長過程をもつ　型糖鎖にプライマーという手法が適用できるかについては疑問があるが、興味のある課題であり、　型糖鎖の基幹構造に特徴的な二糖単位に注目してプライマーをデザイン

that glycosyltransferase may recognize the disaccharide unit, the alkyl glycosides of *N*-acetylglucosamine and mannose with β-1,2 (Fig. 8, **11–14**) and β-1,6 linkage (Fig. 8, **15**) were synthesized. While the carbon numbers of the alkyl residue were **8**, **10**, **12** and **16** for the former, for the latter, based on the finding that the glycoside **13** has proved to be a good primer, the carbon number **12** was selected.

On the other hand, considering the glycosyltransferases which may recognize two monosaccharides on the different chains, the glycosides having an alkylated benzene as aglycon as well as two typical monosaccharides in the *N*-linked chain, that is, mannose (Fig. 8, **16**) and *N*-acetylglucosamine (Fig. 8, **17**) were synthesized. Cultivation of the above described glycosides in B16 cell revealed that the additional carbohydrate chains elongated on the disaccharide glycosides **13** and **15**. Further, in the case of **16**, the tetrasaccharide structure **18** with the additional sialic acid and galactose was confirmed by NMR and high mass data and so on. Further, the elongation of the same two monosaccharides on **15** was also confirmed by high mass. The finding would be very exciting, if the result indicates not only the novel primer but also the elongation by a novel, that is, *N*-glycan-synthesizing machinery. To our regret, the observed chain elongation on **13** may be constructed by the biosynthetic machinery of *O*-glycan. No elongation was observed for the parallel two units-type primers **16** and **17** in three kinds of cells (B16, CHO, and HL60). Because the validity of the novel aglycon has not yet been proved, the parallel recognition could not be completely excluded. In order to develop the *N*-type saccharide primer a breakthrough in the approach would be

することとした。まず、糖転移酵素が直接結合した二糖単位を認識することを想定して、　型糖鎖の分枝構造の起点となる、　アセチルグルコサミンとマンノースが、β　（図**11–14**）およびβ　（　**15**）結合した二糖のアルキルグリコシドを合成した。アグリコンの炭素数は、前者では**8**、**10**、**12**、および**16**、後者ではそれらの細胞実験の結果を踏まえて**12**とした。

一方、糖転移酵素が異なった糖鎖の二糖（単糖＋単糖）を認識する場合を想定して、デザインして合成したアルキル鎖を有する芳香核に２つの単糖が結合した２種のグリコシド**16**（マンノース）および**17**（　アセチルグルコサミン）を合成した。細胞実験の結果、二糖グリコシド**13**および**15**は細胞によって糖鎖が伸長することが観測され、また、単離した前者の糖鎖伸長生成物の　およびマススペクトルのデータなどより、シアル酸とガラクトースが伸長した四糖構造を確認した。このように新たにデザインしたプライマーを用いて糖鎖を調製できる可能性は拡大されたことは大いなる成果であるが、この構造は決して　型糖鎖特有ではないので、

型糖鎖の生合成系により糖鎖伸長が起きたと断定するのは、実際には詳細な実験が必要とされた。残念ながらこの糖鎖伸長は　型糖鎖の生合成経路によると考えるのが妥当なようである。並列型の二糖**16**および**17**については、検討した３種の細胞（　、　細胞、　）において糖鎖伸長は観察されなかった。アグリコン部位の構造が全く新しいこともあり、この結果から異なった糖鎖にまたがる認識が否定されるということではないが、　型糖鎖プライマーへのアプローチには

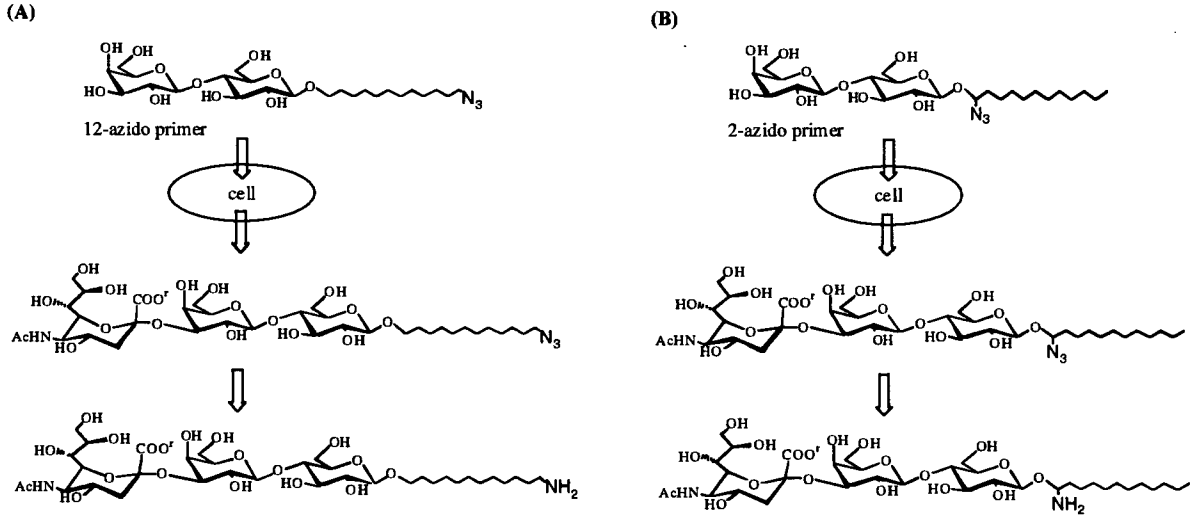

Fig. 9. Saccharide elongation on azido primer and conversion to the functional compounds.

required.

F. Design of Aglycon of Saccharide Primers

It is one of the advantages of the saccharide primer method that the functional group can be introduced to the saccharide primer which is used for the subsequent chemical reaction after the saccharide elongation. Azido (11,12), vinyl (13), and fluoro alkyl (14,15) groups are usable for the saccharide primer method. The azido group is used as a precursor of the amino group. It is well known that the azido group is used instead of the acetamide group in the aminosugar which is often unstable in the cell (or chemically). The position of the azido group in the aglycon of dodecyl lactoside is the farthest (12-azidododecyl) or the nearest (2-azidododecyl) place (Fig. 9). 12-Azidododecyl lactoside (12-azido primer) can be reacted with the other compound after the saccharide elongation, and the dodecyl chain is used as a spacer (16). On the other hand, 2-Azidododecyl lactoside (2-azido primer) is expected to be converted to an amino compound after the saccharide elongation and reacted with fatty acid to give a glycolipid analog.

Both 12-azido and 2-azido primers were investigated to produce similar amounts of oligosaccharides as the non-substituted primer (dodecyl lactoside). It is worth nothing that the azido group remains intact. Both 12-azido and 2-azido primers are taken up to the B16 cells to provide the sialylated product which is the saccharide moiety of GM3, and provide the trisaccharide, tetrasaccharide, and pentasaccharide of ganglioside (saccharide moieties of GM3, GM2, and GM1). The differences among these three primers are the amount of uptake to the cell and the cytotoxicity. 2-Aazido primer is easier to be taken up to the cell than 12-azido and non-substituted primers. Critical micelle concentrations were almost the same among these primers, while the difference of surface activity (2-azido primer is most active) may affect the amount of uptake to the cell (12).

The optimum primer concentration for oligosaccharide production depends on the kind of aglycon, for example, the optimum concentration of 12-azido primer which is added to the culture medium of B16 is 200 μM and the optimum concentration of 2-azido primer is 100 μM. When the optimum concentrations of these two kinds of primers are added to the culture medium, the concentrations in the cell are similar and the amounts of sialyl lactose derivatives produced are almost the same. Therefore, it is indicated that the structure of the aglycon affects the amount of uptake but not the release of the elongated primer. Since the above optimum concentration of the primer shows cytotoxicity and the amount of production of oligosaccharide decreases, it is estimated that the primer concentration in the cell is related to the cytotoxicity (12).

The saccharide-elongated product can be isolated from

更なるブレークスルーが求められていると言える。

糖鎖プライマーのアグリコンの分子設計

糖鎖プライマー法の特長の一つとして、予め分子内に官能基を導入しておき、細胞による糖鎖伸長後にその官能基を化学反応の拠点として使えることが挙げられる。糖鎖プライマー法に適応可能な官能基としてはこれまでにアジド基　、ビニル基　、フッ化炭素　、　が用いられてきた。このうち、アジド基はアミノ基の前駆体として用いられる。アミノ糖に存在するアセトアミド基が細胞内にて（あるいは化学的に）不安定な場合、アジド基がその前駆体として用いられることは良く知られることである。ドデシルラクトシド（ラクトースプライマー）のドデシル基にアジド基を導入する場合、ラクトースから最も遠い位置（　　）とラクトースに最も近い位置（　　）のアジド基がこれまで用いられている　図　。　　（以下、　アジドプライマー）は、糖鎖伸長後にアジド基と別の化合物とを反応させ、長鎖アルキル（ドデシル）部分はスペーサーとして使われる　。一方、　　（以下、アジドプライマー）は、糖鎖伸長後にアジド基をアミノ基に変換して脂肪酸でアシル化することにより糖脂質類似の化合物を合成しようというねらいがある。

アジドプライマーも　アジドプライマーも無置換のプライマー（ドデシルラクトシド）とほぼ同様の糖鎖生産をすることがこれまでの研究により分ってきた。しかも、アジド基は無傷のまま回収されるのである。　細胞に投与すればシアリル化体である　型糖鎖を主に生産し、　細胞に投与すれば、ガングリオ系列の３糖、４糖、５糖（それぞれ　の糖鎖部分）を合成して、細胞外へ（培地中へ）放出する。これらのプライマー間で異なる点は、細胞への取り込み量と細胞に対する毒性である。無置換プライマーやアジドプライマーと比較して　アジドプライマーは細胞内に取り込まれ易い。水中での臨界ミセル濃度はほぼ同じ程度であるが、界面活性能の違い（　アジドプライマーが最も大きい界面活性能を持っている）が細胞内への取り込まれ易さに影響していると思われる　。

糖鎖生産のための最適濃度はアグリコンの種類によっても異なり、　アジドプライマーを　細胞に投与する場合の最適濃度は　μ　、　アジドプライマーでは　μ　である。これらの最適濃度で培地中に投与された２種類のプライマーの細胞内濃度はほぼ等しく、生産されるシアリルラクトース化合物の量もほぼ等しい。即ち、糖鎖プライマーにおけるアグリコンの構造が細胞内へのプライマー取り込み量に影響しているのに対して、細胞外への放出時にはアグリコン構造の違いがそれほど影響していないことが分る。しかも、最適濃度以上の量を投与するといずれも細胞毒性を示して糖鎖生産量が減少することから、細胞内プライマー濃度が細胞毒性に関与していると思われる　。

アジド基の代わりにビニル基（　　）を

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Vol.19 No.105 (January 2007) pp.1-17

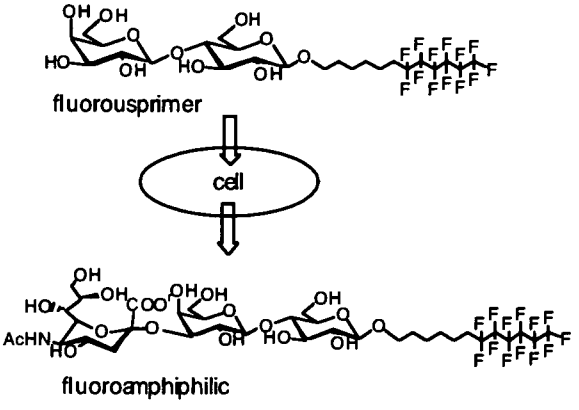

Fig. 10. Saccharide elongation on fluorous primer.

the culture medium by using 10-undecenyl lactoside which has a vinyl group instead of an azido group. The 10-undecenyl lactoside is taken up to B16 cells to afford the same sialylated product as other primers (13).

It is noteworthy that the long alkyl chain which is substituted by several fluorine atoms can be used for the primer to produce the oligosaccharides. Since the fluorous compound which contains many fluorine atoms has an affinity for other fluorous compounds, it is usable for the separation process organic synthesis. Therefore, the isolation of the product becomes easy by using the fluoroalkyl lactoside as a saccharide primer (Fig. 10).

The dodecyl (C12) group is most effective for oligosaccharide production with saccharide primer method. It is estimated that the fluoroalkyl chain whose length is C12 is suitable for carbohydrate production. Partially fluorinated dodecyl lactosides which are $C_6F_{13}C_6H_{12}$-lactoside and $C_{10}F_{21}C_2H_4$-lactoside are synthesized and added to the culture medium, showing the same behavior as non-fluorinated primers (14). However, a high concentration of highly fluorinated lactoside primer ($C_{10}F_{21}C_2H_4$-lactoside) shows the cytotoxicity. On the other hand, $C_{10}F_{21}C_2H_4$-glucoside has low toxicity while dodecyl glucoside shows strong cytotoxicity. Such strong cytotoxicity may depend on the surface activity which is caused by the balance of hydrophilic and hydrophobic parts.

It is expected that the $C_6F_{13}C_6H_{12}$-sialyl lactoside which is produced by incubating B16 with $C_6F_{13}C_6H_{12}$-lactoside is selectively extracted with fluorous solvent. However, $C_6F_{13}C_6H_{12}$-sialyl lactoside is insoluble in fluorous solvents such as perfluorohexane and perfluorotoluene, while it is soluble in the polar solvents such as methanol which does not contain the fluorine atoms. It is caused by the high polarity of hydroxyl and carboxyl groups in the saccharide

用いても、培地中から糖鎖伸長化合物を単離することが可能である。これらのプライマーを　細胞に投与すると、ラクトースプライマーの場合と同様にシアル酸が伸長した化合物を得ることができる　。

置換アグリコンの中で特筆すべきは複数のフッ素原子置換アグリコンである。多数のフッ素原子で置換したアルキル基（フッ化炭素）はフルオラス化合物と呼ばれ、フッ素化合物同士の親和性を示すので、有機合成における化合物の精製に用いられる。そこで、複数のフッ素原子で置換したアグリコンを有するラクトシドを細胞培養系に投与することによって、生成物の単離精製を容易にすることが可能となる 図　。

糖鎖プライマー法による糖鎖生産には炭素数　の長鎖アルキル基が有効であり、水素原子をフッ素原子で置換したアグリコンにおいても　の化合物が適すると思われる。そこで、炭素　個分および　個分をフッ素で置換した　および　をアグリコンとしてラクトシドを合成して細胞に投与すると、フッ素を持たないプライマーと同様の挙動を示した　。但し、フッ素含量の多い　ラクトシドを高濃度で投与すると細胞毒性を示すようになる。その一方で、ドデシルグルコシドや　グルコシドの非常に強い細胞毒性が　をアグリコンとすることで緩和されることは興味深い。このような長鎖アルキルグリコシドの細胞毒性は、親水部分と疎水部分の微妙なバランスによる界面活性能の違いによるものと推測される。

ラクトシドを　細胞に投与した際の生成物である　シアリルラクトシドは、フッ素を含む溶媒（フルオラス溶媒）によって選択的な抽出が可能であると考えられる。しかしながら、パーフルオロヘキサンやパーフルオロトルエンには溶解せず、フッ素を含まないメタノール等の極性溶媒に溶解する。これは、糖鎖部分のヒドロキシル基やカルボキシル基の極性が高いためと考えられる。そこで、ヒドロキシル基を有するフルオラス溶媒であるペンタフルオロ

portion. Fluorous solvents having hydroxyl group such as pentafluoro penthanol and nonafluoro hexanol are used for the selective extraction of $C_6F_{13}C_6H_{12}$-sialyl lactoside. Pentafluoro penthanol and nonafluoro hexanol do not dissolve the dodecyl lactoside and its sialylated compound. Therefore, both hydrophilic interaction and fluorous interaction occur (15), and it can be called "Fluoro-amphiphilic."

As well as the kind of aglycon, the configuration of the glycoside linkage in the saccharide primer is effective on the saccharide elongation by the cell. Both dodecylα- and β-lactosides can be used as primers for oligosaccharide production, while only β-isomers of dodecyl galactoside is usable for saccharide elongation (17).

G. Glycan Array

Though the research on the function of oligosaccharides in cells and tissues has developed, the elucidation of the whole picture of the function of glycan is not yet sufficient. To achieve high-throughput analysis of carbohydrate recognition, it is necessary to construct the screening system using the glycan library. Fundamental research for the development of glycan array has been increasing recently (18–21). For the development of glycan array, the preparation of glycan library and the method of immobilization to solid support and detection are required. In many papers, the adsorption and chemical immobilization of glycans to solid supports have been described. As we established the methodologies to obtain saccharide library using cell function, in the next step we are developing the immobilization method for glycan library produced by cells. For example, we investigated the utilization

ペンチルアルコール、ノナフルオロヘキシルアルコールを用いると、選択的な抽出が可能となる。一方、フッ素を含まない通常のラクトースプライマー（　　ラクトシド）およびその糖鎖伸長化合物　　シアリルラクトシドはペンタフルオロペンチルアルコールとノナフルオロヘキシルアルコールには溶解しない。即ち、親水性の相互作用とフルオラスの相互作用の両方が働いており　、フルオラス両親媒性（　　）と呼んでよさそうである。

アグリコンの種類ではないが、アグリコン（長鎖アルキル基）と糖鎖の間の結合の立体配置も糖鎖伸長に影響する。ラクトースプライマーはα体とβ体の両方に糖鎖伸長が起こるが、ガラクトースプライマーの場合にはβ体のみに糖鎖伸長が観察される　。

糖鎖アレイ

生体中のオリゴ糖鎖の機能に関する研究が進展してきているが、糖鎖の機能の全貌を明らかにしたわけではない。糖鎖認識のハイスループットな解析を達成するには、糖鎖ライブラリーを利用したスクリーニングシステムを構築する必要がある。糖鎖アレイの開発のための基礎研究はこの数年で急速に増えてきている　－　。糖鎖アレイの開発には、糖鎖ライブラリーの作製と基板への固定化と検出手法の開発が要求される。多くの論文では、オリゴ糖鎖を基板に吸着もしくは化学的に固定化する方法の開発について述べている。我々は細胞を用いた糖鎖ライブラリーの構築の方法論を構築したので、次のステップとして細胞に作らせた糖鎖ライブラリーを基板に固定化する手法を開発している。たとえば、糖鎖プラ

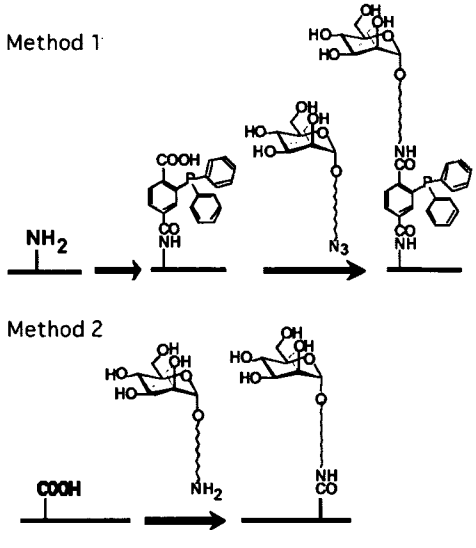

Fig. 11. Immobilization method of azido glycoside and amino glycoside

of saccharide primer having reaction active groups such as the azido group. 12-Azidododecyl glycosides were successfully immobilized onto a sensor chip by either the Staudinger reaction (22, 23) or reduction of the azido group followed by condensation reaction (Fig. 11). Specific bindings of lectin or antibody to the immobilized glycan were achieved by the both methods (16). Since the saccharide primers having a functional group such as the azido group were also glycosylated in cells, it is expected that the saccharide primer method will be useful for the development of glycan array.

A consortium for functional glycomics of United States of America urged the research and development on glycan array (24, 25), and we can freely browse the analysis for carbohydrate recognition of lectins, antibodies, and influenza virus on the website (26). It is very worthwhile to share such research results for the researchers of glycobiology and glycoengineering. Henceforth, the improvement of glycan library will be carried out by the development of new techniques for the immobilization method, detection method and production of glycan library. Here, There is knowledge to be considered in the development of glycan array. The quantitative analysis on the carbohydrate recognition using glycolipid monolayer by Sato *et al.* indicated that the optimum glycolipid density for carbohydrate recognition was dependent on the variety of sugar-binding molecules and lipid matrices (27–30). This fact suggested the problem such as missing the molecules that could recognize the glycan becauset the sugar density on the glycan array was not optimum. Furthermore, in the development of glycan array, improvement of the immobilization method as well as the quality of the glycan library will become important. By utilizing the merit of saccharide primer method that synthesizes the oligosaccharide by cells, we have planed the development of the glycan array that covers the oligosaccharides appearing in specific cells and organs.

H. Conclusion

The several methods to obtain glycans have been established as described in the introduction. However, in practice it is difficult to obtain all glycans by one method. By utilizing the individual features, it is possible to construct a comprehensive glycan library. The cells produce various glycoconjugates using complex biosynthetic pathways. Though the glycans expressed in cells should have important functions individually, our understanding about them is insufficient. Thus, the construction of a library of glycans expressed on cells will certainly be behind the glyco-research.

The libraries of genes, peptides and proteins have already become important tools for many researchers. The research for selection of functional molecules from those libraries has been frequently carried out. Therefore, the

イマーにアジド基の様な官能基を導入した誘導体を利用する手法について検討した。アジド基を有したアルキルグルコシドの基板への固定化は、アジド基をアミノ基に還元して縮合する方法、あるいはトリフェニルホスフィン誘導体をリンカーとして　　反応　　により固定化する方法を行っている（図　）。いずれの方法でも固定化した糖鎖はレクチンや抗体分子と特異的に結合することが示されている　。官能基を有する糖鎖プライマーも細胞内で糖鎖伸長を受けることから、糖鎖プライマー法は糖鎖アレイの開発において有用な手法になると期待される。

米国の機能グライコミクスのコンソーシアムは、糖鎖アレイに関する研究と開発を推進しており　、　、ウエブサイトにおいてレクチン、抗体あるいはインフルエンザウイルスの認識等の解析結果を公開している　。このような研究成果が共有できることは糖鎖生物学や糖鎖工学の研究者にとって大変有意義である。今後、固定化方法や検出方法の改善や糖鎖ライブラリーの増大など、糖鎖アレイの改良が行われて行くであろう。糖鎖アレイにおいて考慮すべき知見がある。佐藤らが行ってきた単分子膜を用いた糖鎖認識の定量的な解析では、糖鎖認識に最適な糖鎖密度は糖鎖を認識する分子の種類やマトリックス脂質に依存していた　－　。これは、作製された糖鎖アレイ上の糖鎖密度の違いにより、糖鎖を認識する分子を見逃してしまう危険性を示唆している。今後の糖鎖アレイの開発においては、糖鎖ライブラリーの質を向上させることのみならず、固定化法の改良が重要になってくるであろう。細胞に糖鎖を作らせるメリットを利用することで、特定の細胞や臓器に発現している糖鎖を網羅した糖鎖アレイを開発することを計画している。

結　論

序論で述べたように糖鎖を得るために種々の方法が確立されてきている。しかしながら全ての糖鎖をひとつの手法で手に入れることは現実的に困難である。それぞれの特徴を利用することで広範囲の糖鎖ライブラリーの構築が可能になるであろう。細胞は複数の生合成経路を使って多種類の複合糖質を作り出している。細胞で発現している糖鎖はそれぞれに重要な機能を有しているはずであるが、それに対する我々の理解は十分ではない。よって、細胞上で発現している複雑なオリゴ糖鎖のライブラリーの構築は、糖鎖の研究を確実に後押しすることになるであろう。

遺伝子、ペプチドあるいはタンパク質のライブラリーは既に多くの研究者の重要な技術の一つになっている。そのようなライブラリーの中から機能性の分子を取り出す研究が活発