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Monoclonal antibody GOM-2 binds to blood group B-Le^y active glycolipid antigens on human gastric cancer cells, KATO-III

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The antigen structure of a mouse monoclonal antibody, GOM-2, established by immunization with KATO-III human gastric cancer cells, was examined. GOM-2 reactive glycolipids were prepared from KATO-III cells and treated with endoglycoceramidase. Structural studies of ten GOM-2 reactive oligosaccharides by a combination of glycosidase digestions, methylation, and affinity chromatography on an *Ulex europeus* agglutinin I (UEA-I) column revealed that nine of them had a Y-related B-active difucosylated determinant (B-Le^y) and one had a B-active determinant. Affinity chromatography of the purified and modified oligosaccharides on an immobilized GOM-2 column demonstrated that GOM-2 has a novel binding specificity: it binds tightly to the biantennary structure carrying the B-Le^y determinant at the termini or the branched structure carrying the B-Le^y structure at two nonreducing termini.

Keywords: B-Le^y active glycolipids, monoclonal antibody.

Abbreviations: UEA-I, *Ulex europeus* agglutinin I; PNA, *Arachis hypogaea* agglutinin; Fuc, L-fucose; Gal, D-galactose; Glcol, glucitol; GlcNAc, *N*-acetyl-D-glucosamine; TBS, 10 mM Tris-HCl, pH 7.8, containing 150 mM NaCl; PBS, 10 mM sodium phosphate buffer, pH 7.5, containing 150 mM NaCl; HPLC, high performance liquid chromatography.

The human gastric cancer cell line KATO-III expresses receptors for peanut (*Arachis hypogaea*) lectin (PNA). PNA receptor glycoproteins have been isolated by affinity chromatography on an immobilized PNA column, and shown to contain clusters of sugar chains [1]. Shimoda *et al.* reported the isolation of two oligosaccharide alditols, Galβ1-3GalNAcol and Galβ1-4GlcNAcβ1-6(Galβ1-3)-GalNAcol, from the PNA receptor glycoproteins by alkaline $NaBH_4$ treatment, and suggested the presence of both N- and O-glycosidically linked sugar chains with high molecular weights [2].

To obtain molecular probes for investigation of the carbohydrate structures of the PNA receptor glycoproteins, we immunized mice with KATO-III cells and established several hybridomas producing monoclonal antibodies that reacted with PNA receptor glycoproteins. One of them, named GOM-2, bound specifically to KATO-III cells but not to more than 40 other cancer cell lines tested, and did not bind to Le^a, Le^b, Le^x, Le^y, or T determinants (unpublished results). In preliminary studies, we found that GOM-2 reacted strongly with neutral glycolipids rather than glycoproteins on KATO-III cells (unpublished results). In this paper, we describe the isolation and structure of GOM-2 reactive oligosaccharides released from the membrane glycolipids of KATO-III cells, and studies on the binding of the oligosaccharides to a GOM-2 affinity column.

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Materials and methods

Materials

Protein A-Sepharose CL-4B and *Ulex europeus* agglutinin I (UEA-I) agarose were purchased from Pharmacia LKB Biotechnology (Uppsala, Sweden) and Hohnen Oil Co. (Tokyo, Japan), respectively. Endoglycoceraminidase (*Rhodococcus* species G-74-2) and endo-β-galactosidase (*Escherichia freundii*) were from Seikagaku Kogyo Co. (Tokyo, Japan). α-L-Fucosidase and β-*N*-acetylglucosaminidase (bovine kidney) and α-galactosidase (coffee bean) were from

Boehringer Mannheim Biochemicals (Indianapolis, IN, USA). β-Galactosidase (jack bean) was from Sigma Chemical Co. (St. Louis, MO, USA). KATO-III cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum. Mouse IgG3 monoclonal antibody GOM-2 was purified from ascitic fluid by chromatography on DEAE-cellulose [3], and immobilized on Protein A-Sepharose CL-4B. For this, 6 mg GOM-2 in TBS (1 ml) was loaded on a column (0.55 cm × 12.5 cm) of Protein A-Sepharose CL-4B at a flow rate of 4 ml h^{-1} at 4 °C, and the column was washed with TBS.

Isolation of GOM-2 reactive glycolipids

Total lipids were extracted from 100 g packed KATO-III cells with 5 volumes (w/v) isopropanol:hexane:water, IHW (55:25:20 by vol). The extract was evaporated to dryness and fractionated by Folch's partitioning [4]. The upper phase containing glycolipids was evaporated, dialyzed against water, lyophilized, and subjected to DEAE-Sephadex A-25 chromatography [5]. The neutral fraction obtained was further fractionated by HPLC on a column (0.8 cm × 60 cm) of Iatrobeads 6RS-8010 (10 μm diameter, Iatron, Tokyo, Japan) using an LKB model 2150 pump. The sample dissolved in 0.1 ml IHW, 55:20:25 by vol, (solution B) was loaded on the column previously equilibrated with IHW, 55:40:5 by vol, (solution A). Elution was performed at a flow rate of 0.5 ml min^{-1} with solution A for 10 min, and then with a linear gradient of increasing concentration of solution B (30% for 10 min, 37% for 60 min, 50% for 20 min, and 100% for 10 min), and fractions of 0.2 ml were collected.

Enzyme-linked immunosorbent assay for antigen activity

An aliquot was taken from each fraction to determine its reactivity with GOM-2. Aliquots were dried in wells of Linbro/Titertek 96-well plates (Flow Laboratories Inc., McLean, VA, USA) and the wells were blocked with 1% bovine serum albumin in PBS for 30 min at 37 °C. The wells were then washed three times with PBS, incubated with 20-fold dilution of hybridoma supernatant (0.1 ml per well) overnight at 4 °C, washed again, and incubated with 0.1 ml horseradish peroxidase conjugated goat anti-mouse IgG (×1000 dilution, Cappel, West Chester, USA) for 4 h at 4 °C. After washing, 0.1 ml *o*-phenylenediamine (10 mg ml^{-1} in 70 mM potassium citrate buffer, pH 5.0) was added for 30 min at 25 °C. The reaction was stopped by the addition of 0.1 ml 2 M sulfuric acid, and the absorbance at 492 nm was measured with a Titertek Multiskan MCC plate reader (Flow Laboratories).

Liberation of oligosaccharides from glycolipids

Each neutral glycolipid fraction that reacted with GOM-2 was treated with endoglycoceraminidase (5 munits per fraction) for 65 h at 37 °C in 0.3 ml 50 mM sodium acetate buffer, pH 5.5, containing 1 mM sodium taurodeoxycholate. The liberated oligosaccharides were recovered by the method of Hansson *et al.* [6]. One-fifth of each fraction was reduced in 0.1 ml of 10 mM NaOH with 2 mCi NaB^3H_4 (600 mCi $mmol^{-1}$, New England Nuclear), and the other four-fifths were reduced with $NaBH_4$ (2 mg, Merck) for 4 h at room temperature. The reaction was stopped by the addition of acetic acid, and the oligosaccharide alditols were separated on a column (1.0 cm × 35 cm) of Sephadex G-25 (Pharmacia-LKB) eluted with water.

Fractionation of oligosaccharide alditols

Both radioactively labeled and unlabeled oligosaccharide alditols were fractionated by HPLC on Bio-Gel P-4 and Asahipak NH_2P-50. Gel permeation chromatography was performed on two columns (0.8 cm × 60 cm) of Bio-Gel P-4 (>400 mesh, Bio-Rad, Richmond, CA, USA) in a high performance liquid chromatograph (Tri-Rotor, Jasco, Tokyo, Japan). Elution was carried out with water at a flow rate of 0.4 ml min^{-1} at 55 °C. HPLC on a column (0.4 cm × 25 cm) of Asahipak NH_2P-50 (Asahi Chemical Industry, Tokyo, Japan) was performed using an LKB model 2249 pump at a flow rate of 1.0 ml min^{-1}. The sample was dissolved in 0.1 ml acetonitrile:water, 50:50 by vol, and injected into the column equilibrated with acetonitrile. The column was developed with a linear gradient of decreasing concentration of acetonitrile to 70% for 10 min and then to 30% for 120 min.

Glycosidase digestion

Oligosaccharide alditols were treated with glycosidases (0.05–0.2 units) in 0.1 ml of an appropriate buffer for 18–48 h at 37 °C. The mixture was then heated at 100 °C for 3 min to terminate the reaction, desalted by passage through a small column of AG50W-X8 and AG1-X8 (Bio-Rad), and analysed by gel permeation chromatography on Bio-Gel P-4. Digestions with α- and β-galactosidases were carried out in 50 mM sodium citrate buffer, pH 5.0, containing 50 mM γ-galactonolactone, and 50 mM sodium citrate buffer, pH 4.0, respectively. For digestions with β-*N*-acetylglucosaminidase and α-L-fucosidase, 50 mM sodium citrate buffer, pH 5.0, was used. endo-β-Galactosidase treatment was carried out in 50 mM sodium acetate buffer, pH 5.6, for 24 h at 37 °C with 50 munits of the enzyme. The preparation was then neutralized with NaOH and labeled with 0.1 mCi NaB^3H_4 as described.

Methylation analysis

Oligosaccharide alditols were methylated by the method of Ciucanu and Kerek [7]. The permethylated products were purified on a small column of silica gel [8], hydrolysed with 3 M HCl for 3 h at 80 °C, and reduced and acetylated as described by Stellner *et al.* [9]. Partially methylated alditol acetates were analysed in a gas chromatograph-mass spectrometer (Shimadzu QP 2000) on a middle bore column of

HiCap-CBP1 (0.2 mm ID × 50 cm, 100–180 °C, 10 °C min^{-1} and 180–260 °C, 3 °C min^{-1}).

Mild acid hydrolysis

Oligosaccharide 1H was subjected to mild acid hydrolysis [10] to obtain the monofucosylated derivative(s). The product was purified by HPLC on Asahipak NH_2P-50 and its structure was determined by glycosidase digestion. The de-α-galactosylated product obtained by α-galactosidase treatment was further digested with α-L-fucosidase with a shift corresponding to 0.8 glucose unit (data not shown). The de-α-galactosylated product was retained on an UEA-I agarose column and eluted with 50 mM L-fucose (see Fig. 5(h)). Therefore, the product obtained by mild acid hydrolysis was proposed to be a B active oligosaccharide and was designated as 1H-B.

Affinity chromatography

Radioactive oligosaccharide alditols (1.5–6.0 × 10^3 disintegrations min^{-1}) in TBS were applied to a GOM-2 column (0.5 cm × 11.5 cm) and eluted with TBS at a flow rate of 3 ml h^{-1} at 4 °C. Fractions of 0.23 ml were collected and the radioactivity of each fraction was measured with a liquid scintillation counter (Aloka LSC-1050). Lectin affinity chromatography on a UEA-I column (0.5 cm × 12.0 cm) was carried out at 4 °C. Radioactive oligosaccharide alditols (0.5–3.0 × 10^3 disintegrations min^{-1}) in PBS were applied to the column and eluted with PBS and then with PBS containing 50 mM L-fucose at a flow rate of 5 ml h^{-1}. Fractions of 0.45 ml were collected.

Results

Isolation of GOM-2 reactive glycolipids

The neutral glycolipids prepared from KATO-III cells were subjected to HPLC on an Iatrobeads column, and five GOM-2 reactive fractions, 1–5, were eluted from the column as shown in Fig. 1. Even after repeated column chromatography on Iatrobeads, each fraction was still structurally heterogeneous as judged on high performance thin layer chromatography by the orcinol-H_2SO_4 reaction and immunostaining [11] with GOM-2 antibody (data not shown). Thus each fraction was treated with endoglycoceraminidase [12] and the structures of the liberated oligosaccharides were investigated.

Fractionation of the released oligosaccharides

The endoglycoceraminidase digests were reduced with NaB^3H_4 or $NaBH_4$ and subjected to gel permeation chromatography on Bio-Gel P-4 to remove the enzyme and other contaminants. Each of the oligosaccharide alditols was recovered and subjected to HPLC on Asahipak NH_2P-50. The mixture of oligosaccharide alditols released from fraction 1 from the Iatrobeads column was divided into

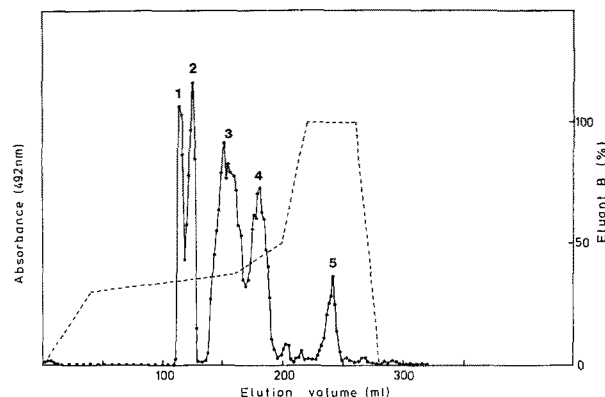


Figure 1. HPLC of the neutral glycolipids on a column (0.8 cm × 60 cm, 10 μm) of Iatrobeads 6RS-8010. Elution was performed at a flow rate of 0.5 ml min^{-1} by the gradient program described in the text. Aliquots (10 μl) of each fraction (0.2 ml) were subjected to enzyme-linked immunosorbent assay.

eight components, 1A–1H, as shown in Fig. 2(a). Similarly, 11 (2A–2K), 8 (3A–3H), 14 (4A–4N), and 11 (5A–5K) components were obtained from fractions 2–5, respectively, from the Iatrobeads column (Fig. 2(b–e)). The oligosaccharide alditols with the same mobilities on both Bio-Gel P-4 and Asahipak NH_2P-50 (for example, 2I and 3F) were combined. Of the 43 oligosaccharide alditols thus obtained, 10 interacted with the GOM-2 affinity column as described later.

Structural analysis

The 10 GOM-2 reactive oligosaccharide alditols appeared to be structurally homogeneous judging from their elution profiles on Bio-Gel P-4, Asahipak NH_2P-50, and GOM-2 Sepharose. Therefore, their structures were investigated by sequential glycosidase digestion, methylation, and lectin affinity chromatography. The product at each step of exoglycosidase treatment of a radioactive oligosaccharide alditol was analysed by gel permeation chromatography on Bio-Gel P-4 (Figs 3 and 4). Four unlabeled oligosaccharide alditols (1H, 2I3F, 2J3G, and 2K3H4I) and their enzymatic degradation products were analysed by methylation, but the other six oligosaccharide alditols were not analysed because their concentrations were too low.

Oligosaccharide alditol 1H

Methylation analysis of the intact oligosaccharide alditol 1H (Table 1) indicated that it is composed of fucose, glucose, galactose, and *N*-acetylglucosamine in a molar ratio of 2:1:3:1, and that it has two terminal fucose residues, a nonreducing terminal and a branching (substituted at C-2 and C-3) galactose residue, a C-3 and C-4 disubstituted *N*-acetylglucosamine residue, and a reducing terminal -3Gal1-4Glcol sequence. The sugar sequence and the anomeric configuration of each monosaccharide were

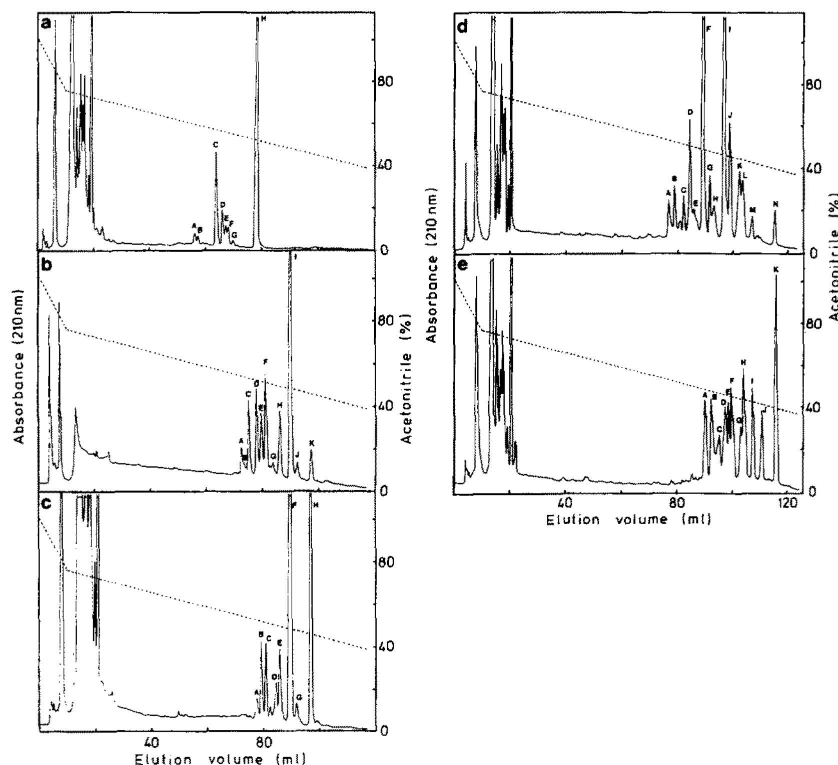


Figure 2. Elution profiles of oligosaccharide alditols on a column (0.4 cm × 25 cm) of Asahipak NH_2P-50. The oligosaccharide alditols released from Iatrobeads fractions 1–5 were recovered from Bio-Gel P-4 columns and applied to an Asahipak NH_2P-50 column. Elution was performed at a flow rate of 1.0 ml min^{-1} and fractions of 0.4 ml were collected. (a) fraction 1 (scale of the ordinate, 0.08); (b) fraction 2 (0.04); (c) fraction 3 (0.04); (d) fraction 4 (0.02); and (e) fraction 5 (0.01).

Table 1. Methylation analysis of intact and modified oligosaccharide alditols.

Methylated sugar	*Molar ratio*[a]						
	1H	*1H* (-α*Gal*)	*1H* (-α*Gal*, -α*Fuc*)	*2I3F*	*2J3G*	*2K3H4I*	*2K3H4I* (-α*Gal*, -α*Fuc*)
1,2,3,5,6-Penta-*O*-methylglucitol	1.0	1.0	1.0	1.0	1.0	1.0	1.0
2,3,4-Tri-*O*-methylfucitol	1.6	1.7	—[b]	1.5	0.8	2.4	—
2,3,4,6-Tetra-*O*-methylgalactitol	1.1	—	1.1	1.0	1.1	1.2	1.1
2,4,6-Tri-*O*-methylgalactitol	0.8	1.1	1.2	1.6	1.7	1.9	1.6
3,4,6-Tri-*O*-methylgalactitol	—	1.0	—	—	—	—	—
4,6-Di-*O*-methylgalactitol	0.8	—	—	0.9	0.8	0.7	—
3,6-Di-*O*-methyl-2-(methylacetamido)-glucitol	—	—	0.8	0.8	1.5	—	1.4
6-*O*-methyl-2-(methylacetamido)-glucitol	0.8	0.9	—	0.7	—	1.6	—

[a] Molar ratios are expressed taking 1,2,3,5,6-Penta-*O*-methylglucitol as 1.0.
[b] Not detected.

estimated by glycosidase digestions. 1H was resistant to β-galactosidase treatment (data not shown), but was digested by α-galactosidase with a decrease in size corresponding to one galactose residue. The product, 1H(-αGal), was not digested with β-galactosidase, but was digested sequentially to the tritiated glucitol with α-L-fucosidase, β-galactosidase, β-*N*-acetylglucosaminidase, and β-galactosidase (Fig. 3(a)). Therefore, the sugar sequence of 1H was concluded to be αGal-(αFuc-)βGal-(αFuc-)βGlcNAc-βGal-Glcol. The decrease in size corresponding to 1.3 glucose units after α-L-fucosidase treatment suggests that one fucose residue is α(1-2)-linked to the penultimate β-galactose residue and the other is α(1-3)- or (1-4)-linked to the β-*N*-acetylglucosamine residue, since the α-fucose

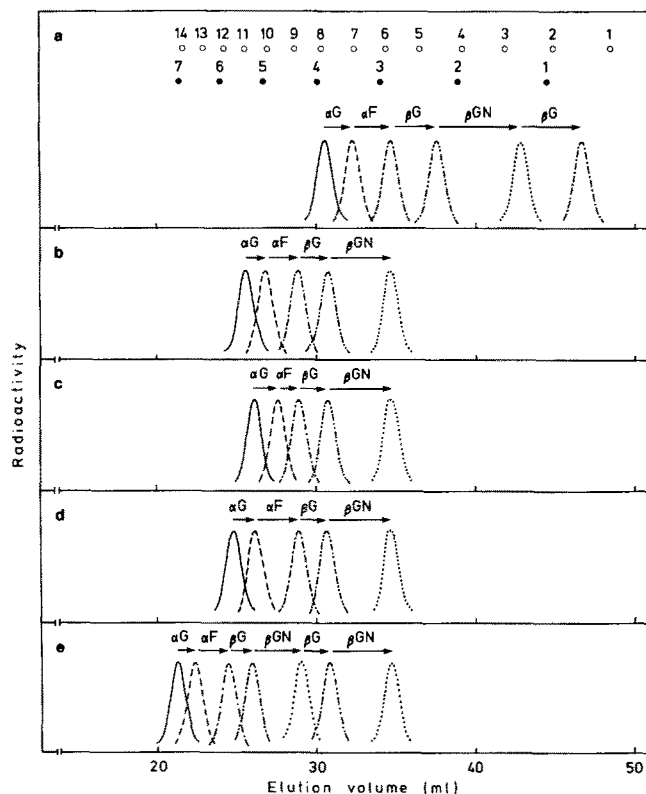


Figure 3. Sequential glycosidase digestions of the oligosaccharide alditols. Labeled oligosaccharide alditols (1–2 × 10^4 disintegrations min^{-1}) were treated with glycosidases as described in the text. The original oligosaccharide alditol (———) was treated with glycosidases and the products with α-galactosidase (αG, – – – – – –), α-L-fucosidase (αF, –·–·–), β-galactosidase (βG, –··–··–··–), and β-*N*-acetylglucosaminidase (βGN, ······) were analysed by gel permeation chromatography on Bio-Gel P-4. The results of successive treatments with β-galactosidase, β-*N*-acetylglucosaminidase, and β-galactosidase of the intermediate products eluted at the same position as 1H(-αGal, -αFuc) are not shown because they were identical to those for 1H(-αGal, -αFuc) (b–e). (a) 1H; (b) 2I3F; (c) 2J3G; (d) 2K3H4I; and (e) 4L5H. ○ (1–14) and ● (1–7) indicate the elution positions of glucose and *N*-acetylglucosamine oligomers, respectively.

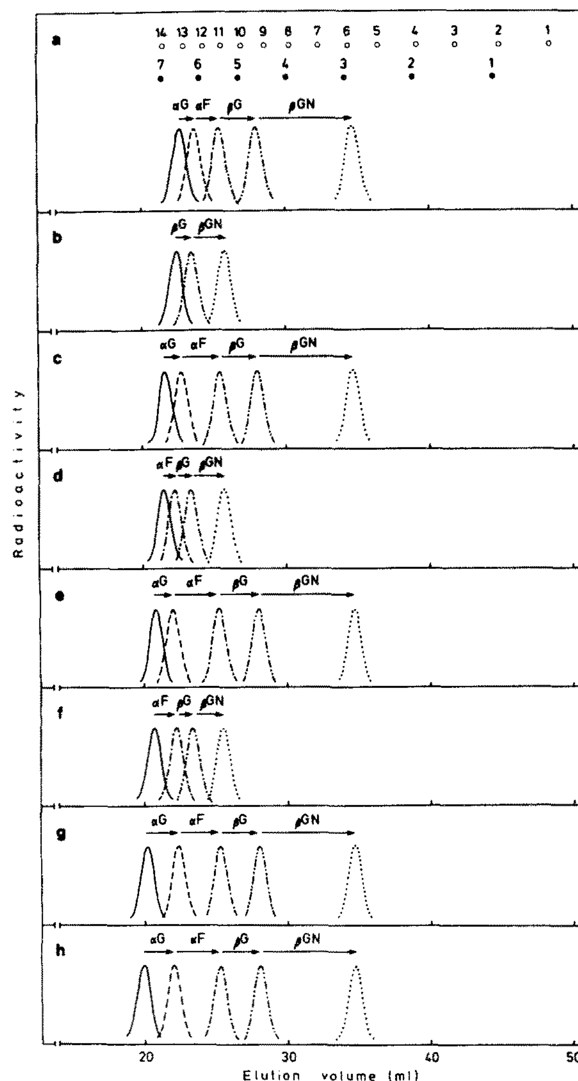


Figure 4. Sequential glycosidase digestions of 4J5F (a, b), 4K5G (c, d), 4M5I (e, f), 5J (g), and 4N5K (h). The explanation is as for Figure 3.

residue in a Fucα1-2Gal group behaves as about 0.9 glucose unit and that in a Galβ1-3 or 4(Fucα1-4 or 3)GlcNAc group behaves as 0.5 glucose unit [13].

To confirm the linkage positions of the two α-fucose residues in 1H, we analysed its enzymatic degradation products, 1H(-αGal) and 1H(-αGal, -αFuc) by methylation (Table 1). The removal of either an α-galactose or two α-fucose residues was confirmed by Bio-Gel P-4 chromatography. The conversion of the galactose derivative originally detected as 4,6-di-*O*-methylgalactitol to 3,4,6-tri-*O*-methylgalactitol identified in 1H(-αGal) indicated that one α-fucose residue is linked at the C-2 position of the β-galactose residue. Detection of 3,6-di-*O*-methyl-2-(methylacetamido)glucitol with concomitant loss of 6-*O*-methyl-2-(methylacetamido)glucitol in 1H(-αGal, -αFuc) indicated that the other is linked at the C-3 position of the *N*-acetylglucosamine residue.

The linkage positions were also estimated by its interaction with an immobilized *Ulex europeus* agglutinin I (UEA-I) column. UEA-I interacts most strongly with an H active type 2 structure, Fucα1-2Galβ1-4GlcNAcβ1-, much less with the Le^y active structure, Fucα1-2Galβ1-4(Fucα1-3)GlcNAcβ1-, and poorly with the H active type 1 structure, Fucα1-2Galβ1-3GlcNAcβ1-, or Le^b active structure, Fucα1-2Galβ1-3(Fucα1-4)GlcNAcβ1- [14, 15]. 1H was eluted from the column without any interaction (Fig. 5(a)), but α-galactosidase treated 1H, 1H(-αGal), was retarded on the column (Fig. 5(b)). After α-galactosidase treatment, the B active oligosaccharide alditol, 1H-B, prepared by mild acid hydrolysis as described in the Materials and methods

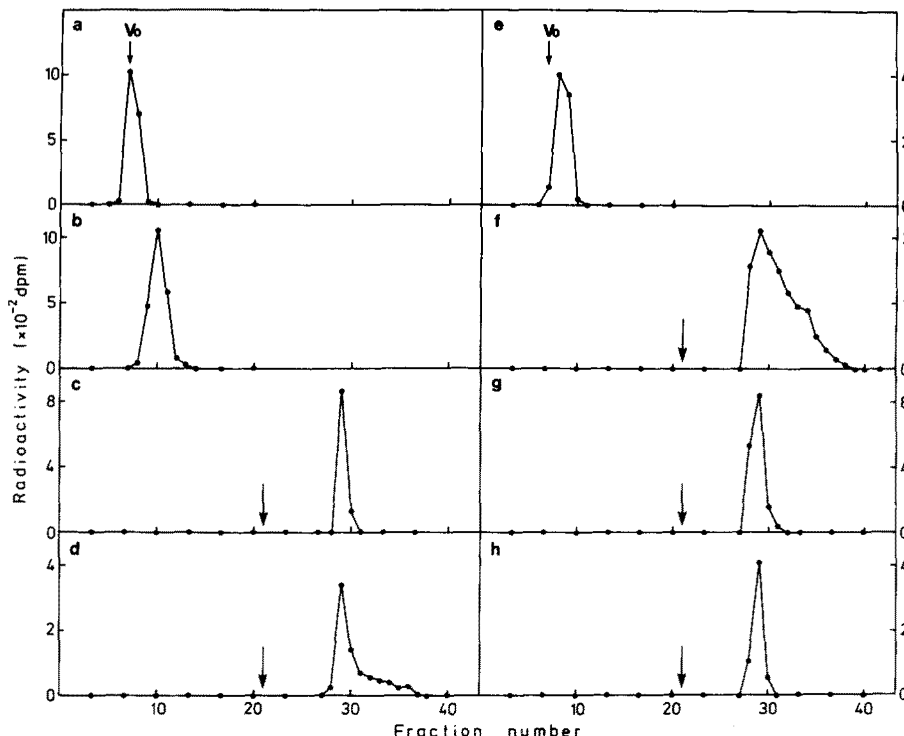


Figure 5. Affinity chromatography of the oligosaccharide alditols on a column (0.5 cm × 12.0 cm) of UEA-I agarose. Elution was performed at a flow rate of 5 ml h^{-1} with PBS, followed by PBS containing 50 mM L-fucose from the position indicated by an arrow. Vo indicates the void volume fraction. (a) 1H; (b) 1H(-αGal); (c) 2J3G(-αGal); (d) 4K5G; (e) 4M5I; (f) 5J(-αGal); (g) 4N5K(-αGal); and (h) 1H-B(-αGal).

section was tightly bound by the column and eluted with 50 mM L-fucose (Fig. 5(h)). From these results we concluded that 1H is the Y-related B-active heptasaccharide alditol shown in Fig. 6.

Oligosaccharide alditols 2I3F, 2J3G, 2K3H4I, and 4L5H

The nonreducing terminal galactose residue of 2I3F was removed by α-galactosidase treatment, and the product was digested with α-L-fucosidase with decrease in size corresponding to 1.3 glucose units. The digest was then treated with β-galactosidase and β-*N*-acetylglucosaminidase, giving a product with the same mobility as 1H(-αGal, -αFuc) (Fig. 3(b)). Further digestion to the tritiated glucitol was achieved by a sequential treatment with β-galactosidase, β-*N*-acetylglucosaminidase, and β-galactosidase (data not shown). The results of glycosidase digestions and the detection of 2,3,4-tri-*O*-methylfucitol (1.5 mol), 4,6-di-*O*-methylgalactitol (0.9 mol), and 6-*O*-methyl-2-(methylacetamido)glucitol (0.7 mol) demonstrated that 2I3F has a linear heptasaccharide structure with two repeated Gal-GlcNAc units, a penultimate β-galactose residue C-2 substituted by an α-fucose residue, and one *N*-acetylglucosamine residue substituted by the other α-fucose residue at the C-3 or C-4 position. The localization and linkage positions of the two α-fucose residues were confirmed by the interaction of 2I3F with a UEA-I column. α-Galactosidase treated 2I3F, 2I3F(-αGal), was somewhat retarded on the column in the same manner as 1H(-αGal), suggesting the exposure of the Le^y determinant after α-galactosidase treatment of 2I3F (data not shown). The overall structure of 2I3F was deduced to be B-Le^y active (Fig. 6).

Gel permeation chromatography showed that the molecular size of 2J3G was 0.5 glucose unit less than that of 2I3F. 2J3G was digested with α-galactosidase and subsequent α-L-fucosidase treatment with decreases in size corresponding to 1 and 0.9 glucose unit, respectively, and the product was further digested with exoglycosidases as in the case of 2I3F(-αGal, -αFuc) (Fig. 3(c)). These results suggest that 2J3G contains the same heptasaccharide sequence as 2I3F, and has only one fucose residue attached to the penultimate β-galactose residue by an α(1-2)-linkage. 4,6-Di-*O*-methylgalactitol (0.8 mol) and 3,6-di-*O*-methyl-2-(methylacetamido)-glucitol (1.5 mol) were detected, but 6-*O*-methyl-2-(methylacetamido)glucitol was not found on methylation analysis. In addition, α-galactosidase treated 2J3G, 2J3G(-αGal), was tightly bound on the UEA-I column and eluted with 50 mM L-fucose (Fig. 5(c)). From these results, 2J3G was deduced to be a B active oligosaccharide alditol (Fig. 6).

2K3H4I was 0.5 glucose unit larger than 2I3F, and de-α-galactosylated 2K3H4I was digested by α-L-fucosidase with decrease in size corresponding to 1.9 glucose units. The digest, 2K3H4I (-αGal, -αFuc), was eluted at the same

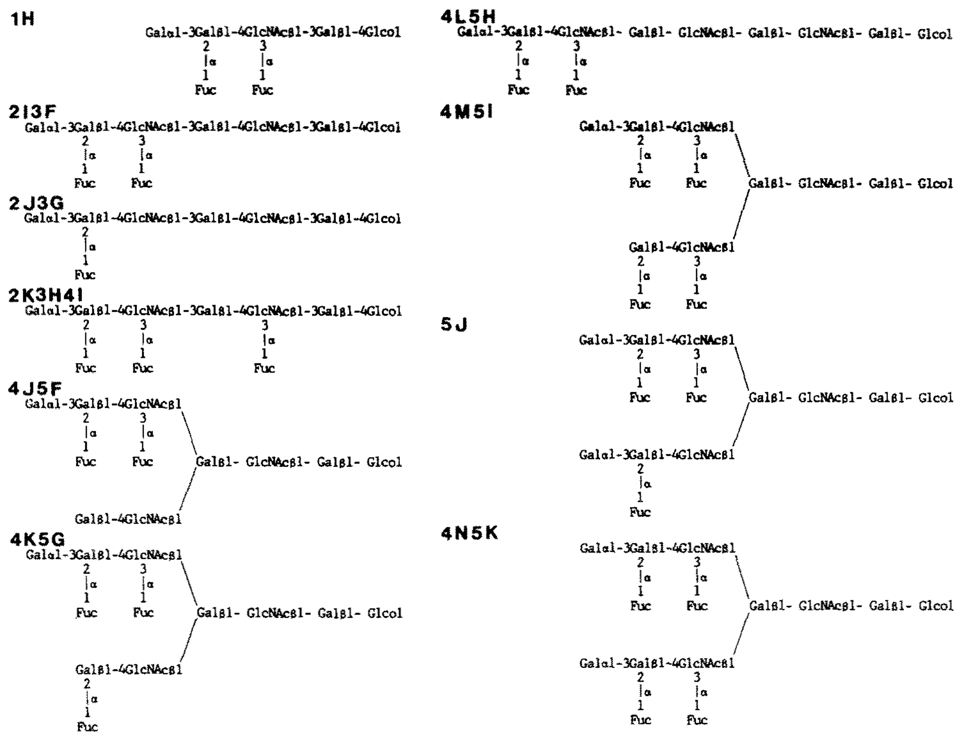


Figure 6. Proposed structures of GOM-2 reactive oligosaccharide alditols.

position as 2J3G(-αGal, -αFuc) and further digested by exoglycosidases as shown in Fig. 3(d). On methylation analysis of 2K3H4I, 2,3,4-tri-*O*-methylfucitol (2.4 mol), 4,6-di-*O*-methylgalactitol (0.7 mol) and 6-*O*-methyl-2-(methylacetamido)glucitol (1.6 mol) were detected, but 3,6-di-*O*-methyl-2-(methylacetamido)glucitol was not. Methylation of the degradation product 2K3H4I(-αGal, -αFuc) revealed that both *N*-acetylglucosamine residues were substituted at the C-3 position by an α-fucose residue, because 6-*O*-methyl-2-(methylacetamido)glucitol disappeared with the concomitant appearance of 3,6-di-*O*-methyl-2-(methylacetamido)glucitol. These results suggest that 2K3H4I contains one fucose residue α(1-2)-linked to the penultimate β-galactose residue and two other fucose residues both α(1-3)-linked to two *N*-acetylglucosamine residues. The elution profile of 2K3H4I(-αGal) was similar to those of 1H(-αGal) and 2I3F(-αGal) on the UEA-I column (not shown).

The oligosaccharide alditol 4L5H was eluted from the UEA-I column in the void volume without any retardation, but was retarded after α-galactosidase treatment (data not shown), suggesting the presence of a B-Le^y active determinant. After treatment with α-galactosidase and α-L-fucosidase, decreases in size corresponding to 1 and 1.4 glucose units, respectively, were observed. The product, 4L5H(-αGal, -αFuc), was sequentially digested by repeated treatments with β-galactosidase and β-*N*-acetylglucosaminidase with decreases in size corresponding to 1 glucose and 1 *N*-acetylglucosamine unit, respectively (Fig. 3(e)). These results demonstrated that 4L5H contains a non-reducing terminal B-Le^y active structure and three repeated βGal-βGlcNAc units. The proposed structure of 4L5H is shown in Fig. 6.

Oligosaccharide alditols 4J5F, 4K5G, 4M5I, 5J, and 4N5K

The structures of these oligosaccharide alditols were studied by a combination of glycosidase digestions and UEA-I affinity chromatography.

Oligosaccharide alditol 4J5F did not react with the UEA-I column but was retarded on the column after α-galactosidase treatment (data not shown). 4J5F was digested with α-galactosidase and subsequent α-L-fucosidase treatment with decreases in size corresponding to 1 and 1.4 glucose units, respectively. When the digest was treated with β-galactosidase, a decrease in size corresponding to two glucose units was detected. Furthermore, this product was digested by β-*N*-acetylglucosaminidase with a decrease in size corresponding to 2 *N*-acetylglucosamine units to give a product with the same mobility as 1H(-αGal, -αFuc), which was sequentially digested by β-galactosidase and β-*N*-acetylglucosaminidase as other oligosaccharide alditols (Fig. 4(a)).

The intact oligosaccharide alditol 4J5F was susceptible to β-galactosidase digestion and one galactose residue was removed without prior α-galactosidase treatment. The product was digested with β-*N*-acetylglucosaminidase to give a product eluted at the same position as 2I3F (Fig. 4(b)). These results suggested the presence of a

βGal-βGlcNAc- branch in addition to a B-Le^y^ active branch.

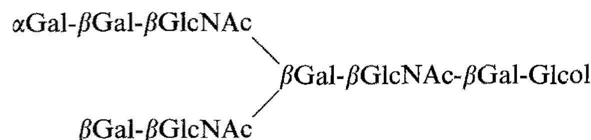
After treatments with α-galactosidase and α-L-fucosidase, four other oligosaccharide alditols, 4K5G, 4M5I, 5J, and 4N5K, were digested by β-galactosidase and β-*N*-acetylglucosaminidase with decreases in size corresponding to two glucose and two *N*-acetylglucosamine units, respectively (Fig. 4(c, e, g, h)), suggesting that they have a branched structure like 4J5F.

Treatment of 4K5G with α-galactosidase and then α-L-fucosidase resulted in decreases in size corresponding to 1 and 2.4 glucose units, respectively (Fig. 4(c)). 4K5G was also digested by sequential treatments with α-L-fucosidase, β-galactosidase, and β-*N*-acetylglucosaminidase with shifts corresponding to 0.9 glucose, 1 glucose, and 1 *N*-acetylglucosamine unit, respectively (Fig. 4(d)). Intact 4K5G was tightly bound on the UEA-I column and eluted with 50 mM L-fucose like 2J3G(-αGal) (Fig. 5(d)), suggesting that it has H active type 2 and B-Le^y^ active branches.

After α-galactosidase treatment, oligosaccharide alditol 4M5I was digested with α-L-fucosidase, β-galactosidase, and β-*N*-acetylglucosaminidase with shifts corresponding to 2.8 glucose, 2 glucose, and 2 *N*-acetylglucosamine units, respectively (Fig. 4(e)). In addition, 4M5I was sequentially digested with α-L-fucosidase, β-galactosidase, and β-*N*-acetylglucosaminidase with decreases in size corresponding to 1.4 glucose, 1 glucose, and 1 *N*-acetylglucosamine units, respectively, without prior α-galactosidase treatment (Fig. 4(f)). The molecular size of 4M5I was about 0.5 glucose unit more than that of 4K5G, and 4M5I was not retained but somewhat retarded on the UEA-I column like 1H(-αGal) (Fig. 5(e)), suggesting that the *N*-acetylglucosamine residue is substituted by an α-fucose residue at the C-3 position and the production of the Le^y^ active branch in 4M5I weakens its interaction with the lectin column.

Both 5J and 4N5K were digested by α-galactosidase with shifts corresponding to two glucose units, indicating that they have two nonreducing terminal α-galactose residues. After α-galactosidase treatment, 5J and 4N5K were digested by α-L-fucosidase with decreases in size corresponding to 2.2 and 2.7 glucose units, respectively. Both 5J(-αGal, -αFuc) and 4N5K (-αGal, -αFuc) were sequentially digested by treatments with β-galactosidase, β-*N*-acetylglucosaminidase, β-galactosidase, β-*N*-acetylglucosaminidase, and β-galactosidase with shifts corresponding to 2 glucose, 2 *N*-acetylglucosamine, 1 glucose, 1 *N*-acetylglucosamine, and 1 glucose units, respectively (Fig. 4(g, h)). These results indicated that both compounds have the same sugar sequence composed of α-galactose (2 mol), β-galactose (4 mol), β-*N*-acetylglucosamine (3 mol), and reducing terminal glucitol, but have different numbers of α-fucose residues. 5J(-αGal) was retained on the UEA-I column and eluted with 50 mM L-fucose, whereas 4N5K(-αGal) was retained but eluted in a sharp peak with 50 mM L-fucose, showing that 5J(-αGal) interacted more strongly than 4N5K(-αGal) with the lectin column (Fig. 5(f, g)). These results demonstrate that 5J(-αGal) has Le^y^ active and H active branches, and 4N5K(-αGal) has two Le^y^ active branches.

To determine the branching position in 4J5F, 4K5G, 4H5I, 5J, and 4N5K, a portion of each oligosaccharide alditol was digested successively with α-galactosidase, α-L-fucosidase, and endo-β-galactosidase. The oligosaccharides released by endo-β-galactosidase treatment were labeled by reduction with NaB^3H_4 and subjected to exoglycosidase digestions (data not shown). In each case, three radioactive peaks were detected at positions corresponding to 7.9, 3.2, and 1.4 glucose units. The first peak was sequentially digested by β-galactosidase and β-*N*-acetylglucosaminidase with the release of two galactose and two *N*-acetylglucosamine residues, respectively, to give a tritiated galactitol. The second peak was digested by β-*N*-acetylglucosaminidase with the release of one *N*-acetylglucosamine residue to produce a tritiated galactitol, and the last peak was judged to be initially labeled glucitol. These results indicate that all five oligosaccharide alditols have the common branching structure:



The structures of the branched oligosaccharide alditols are given in Fig. 6.

Binding specificity of GOM-2

Binding specificity of monoclonal antibody GOM-2 to carbohydrate antigens was studied by immunoaffinity chromatography. Seven oligosaccharide alditols, 1H, 2I3F, 2K3H4I, 4L5H, 4J5F, 4K5G, and 4M5I, carrying one B-Le^y^ determinant, were retarded in the same manner on the GOM-2 affinity column as shown in Fig. 7(a, b, f, g, h) (results for 2K3H4I and 4L5H are not shown). When the terminal α-galactose residue was removed with α-galactosidase treatment, 1H was eluted without any retardation (Fig. 7(d)), suggesting that GOM-2 has no ability to bind to the Le^y^ structure and that the terminal α-galactose residue is essential for the binding. This possibility is supported by the elution profile of the branched oligosaccharide alditol 4M5I. The affinity of GOM-2 to the B-Le^y^ determinant on one branch was not affected by the presence of the Le^y^ determinant on the other (Fig. 7(h)). However, the presence of a B determinant with a B-Le^y^ determinant forming a branched structure slightly increased the affinity (5J, Fig. 7(i)). In addition, 4N5K interacted so strongly with the GOM-2 column that it could not be eluted with 15 column volumes of PBS of 0.1 M methyl-α-galactoside, which was

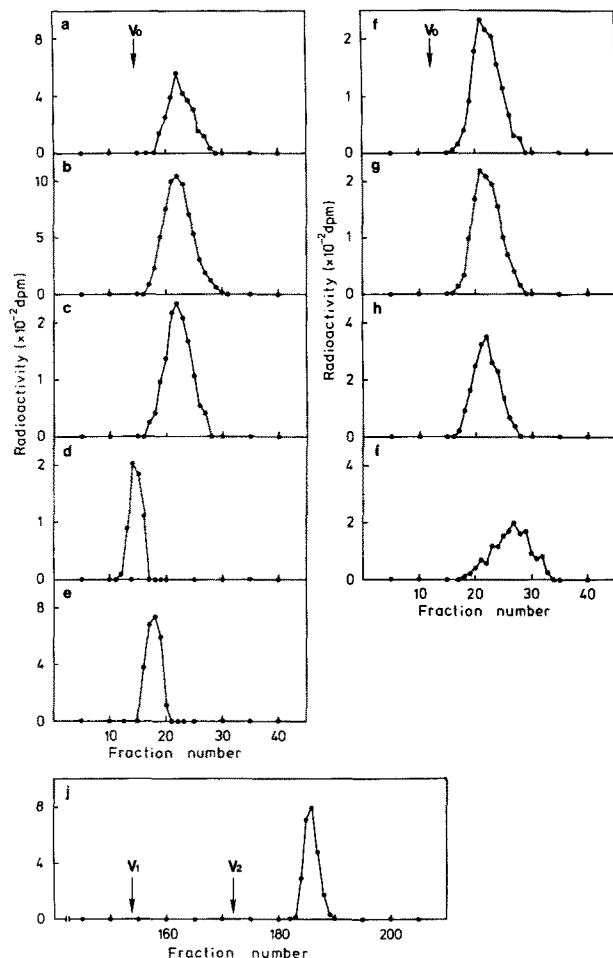


Figure 7. Affinity chromatography of the oligosaccharide alditols on a column (0.55 cm × 11.5 cm) of GOM-2-Protein A-Sepharose. Elution was performed with TBS at a flow rate of 3 ml h^{-1}. Arrows V1 and V2 indicate the positions where the buffer was changed to TBS containing 0.1 M methyl-α-galactoside and 0.2 M sodium acetate buffer, pH 3.5, respectively. (a) 1H; (b) 2I3F; (c) 2J3G; (d) 1H(-αGal); (e) 1H-B; (f) 4J5F; (g) 4K5G; (h) 4M5I; (i) 5J, and (j) 4N5K.

expected to inhibit the binding. This result suggests that methyl-α-galactoside was not inhibitory or that a concentration of 0.1 M was too low to be inhibitory. Bound 4N5K was eluted by changing the elution buffer to 0.2 M sodium acetate, pH 3.5, and its elution was associated with that of GOM-2 antibody noncovalently linked to Protein A-Sepharose (Fig. 7(j)). GOM-2 showed the same affinity for B active octasaccharide alditol 2J3G (Fig. 7(c)) as B-Le^y active oligosaccharide alditols bearing one B-Le^y determinant. However, hexasaccharide alditol 1H-B, prepared by mild acid hydrolysis of 1H, was eluted earlier than 2J3G (Fig. 7(e)), probably due to decreased antigenicity because of the absence of a βGal-βGlcNAc unit. These results suggested that GOM-2 binds strongly to the cluster of B-Le^y structures, and has almost the same binding affinity to a singly B-Le^y and B active determinant on a sugar chain.

Discussion

We have obtained a mouse monoclonal antibody GOM-2 that recognizes the cell surface carbohydrate antigens that are highly expressed on the human gastric cancer cell line KATO-III. A preliminary study suggested that this antibody has novel binding specificity. GOM-2 did not bind to Le^a, Le^b, Le^x, Le^y, or T determinants, and the reactivity of GOM-2 was not correlated with those of the monoclonal antibodies FH-2, FH-6, or AH-6 [16–18]. We purified GOM-2 reactive oligosaccharide alditols from the glycolipids on KATO-III cells, determined their structures, and found that GOM-2 binds strongly to the biantennary structure carrying the B-Le^y determinant at the termini or the branched structure carrying B-Le^y structure at two non-reducing termini.

Blaszczyk-Thurin *et al.* [19] generated a monoclonal antibody, BR55-2, by immunizing mice with HCF-7 human breast carcinoma cells. They isolated a series of glycolipids with Y and blood group B type 2 determinants from KATO-III cells, which react with BR55-2 and an anti-blood group B monoclonal antibody PA83-52 [20]. They tested the reactivity of BR55-2 with various glycolipids of Y-related structures by solid-phase radioimmunoassay and liposome inhibition assay, and found that BR55-2 specifically detected glycolipids with the Le^y determinant and also recognized a difucosylated B type 2 (B-Le^y) determinant to a lesser extent. BR55-2 was not reactive with other glycolipids bearing blood group B type 1, Le^a, Le^b, Le^x, B-Le^b, or A-Le^y determinants. GOM-2 is different from BR55-2 in that it shows no affinity to the Le^y structure and binds strongly to the cluster of B-Le^y determinants. However, there is a possibility that this apparent difference in the specificity is due to the difference in the methods of assay.

Blaszczyk-Thurin *et al.* [19] discussed two possible biosynthetic pathways for production of the B-Le^y determinant in KATO-III cells: tumor-associated expression of α(1-3)-galactosyltransferase specific for the Le^y structure in KATO-III cells and α(1-3)-fucosylation of the B type 2 structure. In the present study, B type 2 oligosaccharide alditol (2J3G), which might be the substrate for α(1-3)-fucosyltransferase, was detected even at low concentration. No galactosyltransferase specific for α(1-3)-galactosylation of the Le^y structure has been reported, and α(1-3)-fucosylation of *N*-acetylglucosamine in the Galβ1-4(Fucα1-3)GlcNAcβ1 structure completely blocked α(1-3)-galactosylation [21]. These facts suggest that fucosylation of B type 2 structure (2J3G) produces B-Le^y structure (2I3F). Moreover, the detection of a series of branched oligosaccharides by GOM-2 suggests a possible biosynthetic pathway of glycolipids carrying these sugar chains. 4K5G

structure might be the product of α(1-2)-fucosylation of 4J5F, so the galactose residue in the Galβ1-GlcNAc side chain of 4J5F is postulated to be (1-4)-linked to the *N*-acetylglucosamine residue, although this was not determined in the present study. Conversely, 4K5G might be the precursor for both α(1-3)-fucosylation and α(1-3)-galactosylation, the products being 4M5I and 5J, respectively, and 4N5K might be produced by α(1-3)-fucosylation of 5J. However, further studies are necessary by using different substrates to determine the acceptor specificity of α(1-3)-fucosyltransferase.

GOM-2 was screened by assay of binding activity with PNA receptor glycoproteins prepared from KATO-III cells. However, the reactivity of GOM-2 with the PNA receptor glycoproteins was much less than that with membrane glycolipids prepared from KATO-III cells (unpublished results). Shimoda *et al.* [2] isolated two *O*-glycosidically linked oligosaccharide alditols from the PNA receptor glycoproteins Galβ1-3GalNAcol and Galβ1-4GlcNAcβ1-6(Galβ1-3)GalNAcol, and suggested the presence of a number of structurally heterogeneous sugar chains both *N*- and *O*-glycosidically linked to the glycoproteins. We subjected the PNA receptor glycoproteins to alkaline-$NaBH_4$ treatment, and fractionated the liberated oligosaccharide alditols on an Asahipak NH_2P-50 column and analysed them by methylation. The data obtained indicated that blood group B determinants were abundantly expressed on the *O*-glycosidically linked sugar chains in accordance with the fact that KATO-III originated from a patient of blood group B. The presence of a small amount of B-Le^y determinant was also suggested by the detection of both 4,6-di-*O*-methylgalactitol and 6-*O*-methyl-2-(methylacetamido)glucitol together in several methylated oligosaccharide alditols (data not shown). These results suggest that B-Le^y determinants can be more abundant in glycolipids than PNA receptor glycoproteins in KATO-III cells.

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