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# Note

## Production of monoclonal antibodies against oligosaccharides coupled to protein *

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Altered glycosylation is widely observed in the glycoproteins produced by tumors. Comparative studies of the sugar chains of glycoprotein produced by malignant cells and their normal counterparts will provide information on their alterations. It has already been shown that such information is useful for the development of the new diagnostic methods of tumors. For the detection of altered sugar chains, two groups of reagents, lectins and monoclonal antibodies, have been widely used[1–4]. Although some immobilized lectin columns have been found to be useful for such purpose, the number of lectins is limited. Because innumerable monoclonal antibodies can be obtained, this reagent can afford more progress for the diagnosis of tumors than lectins. So far, monoclonal antibodies directed to the sugar chains of glycoproteins have been produced by immunizing mice with whole tumor cells. Because various antigenic determinants are expressed on tumor cells, it was necessary to screen tremendous number of antibodies to obtain a useful one.

For the more effective production of monoclonal antibodies directed to the altered sugar chains produced in tumors, we have designed a new strategy. This includes elucidation of the exact structural alterations of the sugar chains of tumor glycoproteins, pick up several of the alterations that are considered to be effective for the diagnosis of tumors, and prepare monoclonal antibodies against them.

Comparative study of the sugar chains of human chorionic gonadotropins (hCGs) isolated from the urine of pregnant women[5] and patients with trophoblastic diseases including hydatidiform mole[6], invasive mole[7], and choriocarcinoma[6,8,9]

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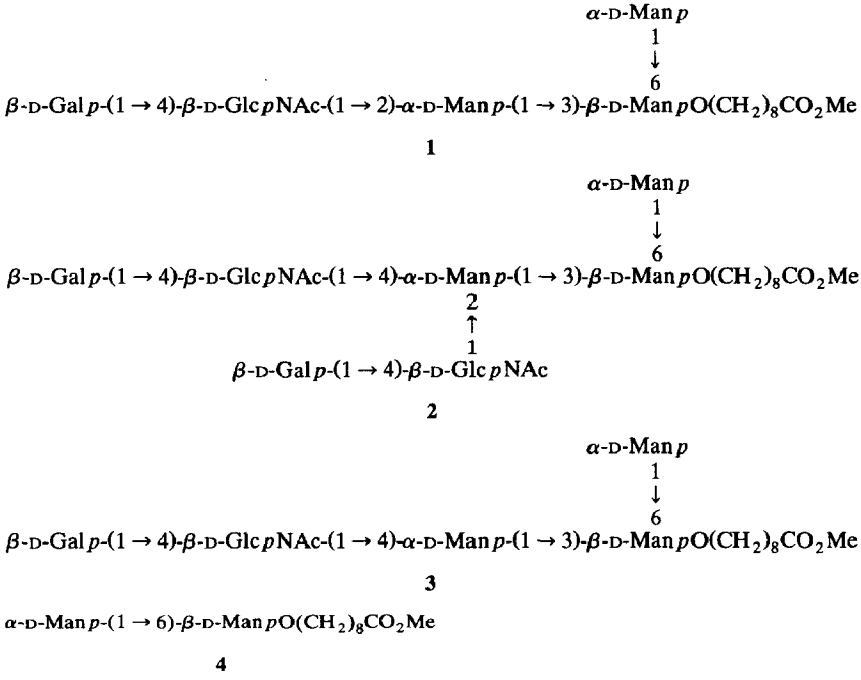
revealed that the structural changes of the sugar chains were induced in the glycohormone produced by malignant tissues. Although the hCGs from hydatidiform mole patients have exactly the same neutral portion of oligosaccharides as normal hCGs, the hCGs from invasive mole patients have 2,4-branched triantennary oligosaccharides, in addition to the sugar chains found in normal hCG. Abnormal biantennary oligosaccharides, as well as 2,4-branched oligosaccharides, newly appear as the sugar chains of hCGs from patients with choriocarcinoma. With use of this altered glycosylation of hCG, we could successfully discriminate invasive mole or choriocarcinoma hCG from normal pregnancy or hydatidiform mole hCG by a *Datura stramonium* agglutinin (DSA)[10]. In order to discriminate choriocarcinoma from invasive mole, development of a monoclonal antibody that recognizes specifically the abnormal biantennary oligosaccharides is required. As the first step of this line of study, the usefulness and the limit of a novel method to produce monoclonal antibodies directed to *N*-linked sugar chains of glycoproteins with use of chemically synthesized oligosaccharides will be reported in this paper.




In general, a single sugar chain is a weak immunogen. Therefore, glycoproteins with high density of sugar chains would be required for the effective production of monoclonal antibodies. In order to obtain the highly glycosylated protein, the coupling-efficiency of various proteins with synthetic oligosaccharide **1** was investigated. Lemieux *et al.*[11,12] reported that synthetic di- and tri-saccharides having the same spacer portion, 8-methoxycarbonyloctyl group, as that of oligosaccharide **1** could be conjugated to bovine serum albumin (BSA), by conversion of the methyl ester group of the synthetic oligosaccharide to an hydrazide and then to an azide

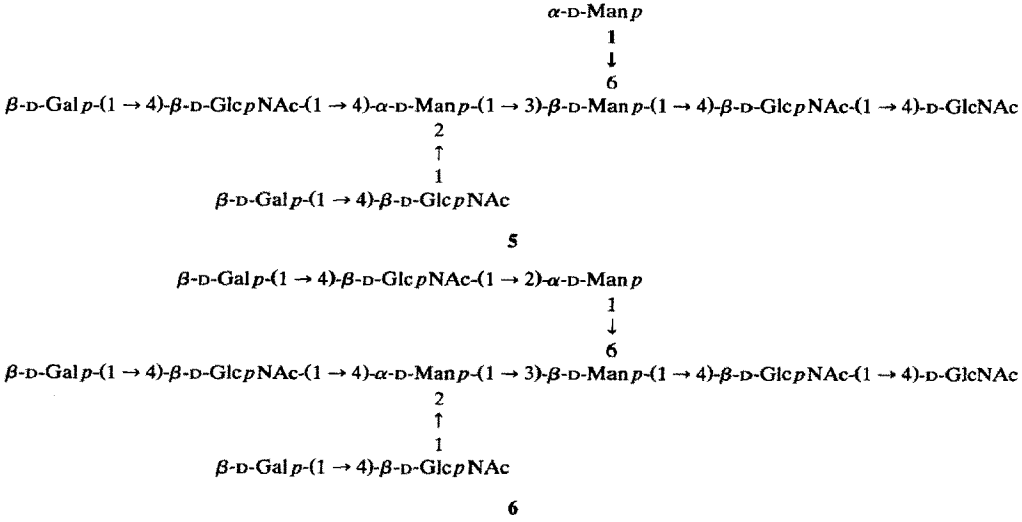
TABLE I

Characteristics of artificial glycoproteins

| Carrier protein [a] | Yield (%) | Molar ratio of hapten to carrier |
|---|---|---|
| BSA | 7.2 | 2.2 |
| KLH [b] | 3.1 | 1.5 |
| Poly-D-lysine | 57.7 | 23.6 |
| mBSA | 55.0 | 16.6 |

[a] 0.2 mg. [b] Keyhole limpet hemocyanin.

group for coupling. Since their method is rather complicated, it could not be applied for the synthesis of 100–200 $\mu$g of synthetic oligosaccharide. Therefore, we used the reaction between the activated carboxyl group of the synthetic oligosaccharide and the $\epsilon$-amino group of a protein to obtain the conjugate efficiently. The results in Table I indicated that the efficiency of coupling depends on the carrier proteins. The great difference found between BSA and methylated BSA (mBSA) suggested that the carboxyl groups in the protein decrease the reaction because mBSA has no carboxyl group. The high efficiency of glycosylation observed in the case of poly-D-lysine also supported this estimation. Oligosaccharides **2**, **3**, and **4** showed an efficiency similar to that of oligosaccharide **1** (data not shown). Accordingly, we decided to use mBSA-conjugates for the immunogen, and poly-D-lysine-conjugates for the screening of hybridomas and for the determination of specificity of monoclonal antibodies. These procedures were proved to be a good system to select hybridomas producing monoclonal antibodies against sugar chains.




Two hybridomas, C2-100 and C2-59, were established, and the antibodies they produced were confirmed to be IgG2a and IgM, respectively. Although both monoclonal antibodies strongly reacted with oligosaccharide **2** conjugated to poly-D-lysine, their reactivities with other oligosaccharides were quite different (Fig. 1).

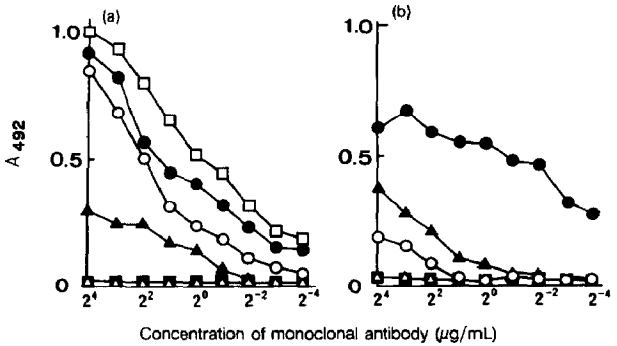



Fig. 1. Reactivities of monoclonal antibodies from C2-59 (a) and C2-100 (b) with various oligosaccharides coupled with poly-D-lysine. Each well contained 500 ng of one of the following samples, and was allowed to react with serially diluted monoclonal antibodies: oligosaccharide **1** (○); oligosaccharide **2** (●); oligosaccharide **3** (▲); oligosaccharide **4** (△); and oligosaccharide **6** (□) coupled with poly-D-lysine; poly-D-lysine (■).

The antibody from C2-59 reacted with oligosaccharide **1** to the same extent as oligosaccharide **2** (Fig. 1a). Weak but significant reactivity was observed with oligosaccharide **3**, but no reactivity with oligosaccharide **4**. The antibody also reacted strongly with oligosaccharide **6**. After treatment with β-D-galactosidase, reactivity of oligosaccharide **2** with the antibody from C2-59 was completely abolished (data not shown). These results indicated that the antibody from C2-59 recognizes the β-D-Gal*p*-(1 → 4)-D-GlcNAc group.

While the reactivity of the antibody from C2-100 with oligosaccharide **3** was about one-third as strong as with oligosaccharide **2**, only weak reactivity was observed with oligosaccharide **1** at concentrations higher than 2 μg/mL (Fig. 1b). The antibody did not react with oligosaccharide **4** and oligosaccharide **6**. These results suggested that the antibody from C2-100 reacted with the 2,4-branched biantennary structure but not with the triantennary and the monoantennary structures.

The results obtained by the affinity chromatography of these synthetic oligosaccharides on a column containing immobilized antibody from C2-100 also supported this conclusion (Fig. 2). While oligosaccharide **1** did not show any interaction, oligosaccharides **3** and **2** showed weak and strong retardation, respectively. However, when radioactively-labeled oligosaccharide **5** was applied to the immunosorbent column, it did not show any interaction with the column (Fig. 2). This result indicated that the spacer of synthetic oligosaccharide was included in the epitope structure. It showed great contrast to the previous study, which found that polyclonal antibodies against sialyloligosaccharides of human milk coupled to protein did not react with the spacer arm at all[13]. This might be due to the different structures of spacer arms because Smith and Ginsburg[13] used the phenethylamine group instead of the 8-methoxycarbonyloctyl group. Presence of the D-galactosyl residues on the *N*-acetyllactosamine unit of the 2,4-branced

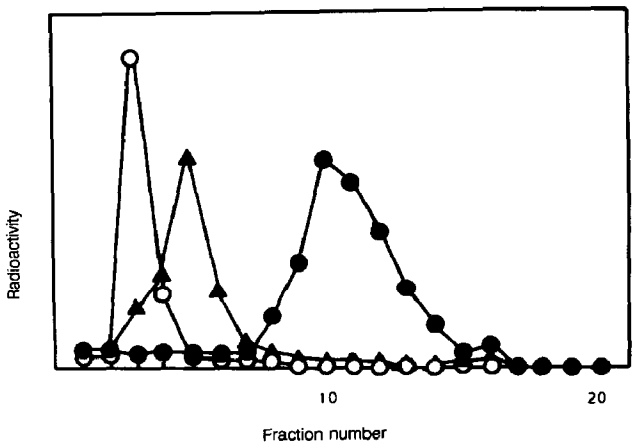



Fig. 2. Affinity chromatography of radioactively-labeled oligosaccharides on a column containing immobilized antibody from C2-100. Chromatography was carried out by elution with PBS at a flow rate of 12 mL/h. [$^3$H]xylitol, which should show no interaction with the column, was eluted in Fraction 3: oligosaccharide **1** and **5** (○); oligosaccharide **2** (●); and oligosaccharide **3** (▲).

biantennary structure was also essential for the binding to the antibody because the reactivity of oligosaccharide **2** was abolished after digestion with β-D-galactosidase (data not shown).

These results indicated that a monoclonal antibody could be prepared by use of a synthetic oligosaccharide analog, but a native oligosaccharide containing *N,N'*-diacetylchitobiose unit should be used to produce monoclonal antibody that reacts effectively with the branched structure of asparagine-linked sugar chains of glycoproteins.

## EXPERIMENTAL

*Materials.*—$NaB^3H_4$ (12.6 mCi/mmol) and UDP-D-[U-$^{14}$C]galactose (12.5 GBq/mmol) were purchased from New England Nuclear, Boston, MA. Poly-D-lysine (average mol wt 100000), methylated bovine serum albumin (mBSA), and bovine milk D-galactosyltransferase were purchased from Sigma Chemicals Co., St. Louis, MO. 3-(3-Dimethylaminopropyl)-1-ethylcarbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide, and $NaBH_3CN$ were purchased from Nacalai Tesque, Inc., Kyoto. Sephadex G-50 and CNBr-activated Sepharose 4B were purchased from Pharmacia LKB Biotechnology, Uppsala, Sweden. Biotinylated sheep anti-mouse Ig(G + M + A) and peroxidase-conjugated streptavidin were purchased from ZYMED Laboratories, Inc., San Francisco, CA. D-Galactose oxidase was purchased from Toyobo Co., Ltd., Osaka. β-D-Galactosidase was purified from jack bean meal by the method of Li and Li[14].

*Oligosaccharides.*—The synthetic oligosaccharides (**1–4**) used in this study were prepared as described previously[15,16]; compound **6** was obtained by sialidase

digestion of the oligosaccharide fraction of bovine fetuin[17], and reduced (with $NaB^3H_4$) **5** from choriocarcinoma hCG as previously described[8].

*Labeling of synthetic and native oligosaccharides.*—Synthetic oligosaccharides (0.1 mg) and D-galactose oxidase (2.45 units) were incubated at 25° for 66 h in 100 μL of buffer containing 0.02 M sodium phosphate, 4.5 mM sodium acetate, 0.15 M NaCl, pH 7.0, and toluene (1 drop). The reaction was stopped by immersing the mixture in boiling water for 3 min. The mixture was passed through a column containing 0.5 mL of AG 50-X12 ($H^+$) and 0.5 mL of AG 3-X4 ($OH^-$) ion-exchange resins. The column was washed with distilled water (4 mL) and the washing was combined with the eluate. After evaporation to dryness, the oligosaccharide fraction was reduced with $NaB^3H_4$ (15 MBq) in 0.05 M NaOH (100 μL) at 30° for 4 h to obtain the $^3H$-labeled synthetic oligosaccharides. They were then purified by paper chromatography in 4:1:1 butanol–EtOH–water.

Oligosaccharide **6** (10 nmol), obtained from bovine fetuin, was digested with jack bean β-D-galactosidase (0.5 unit) in 0.1 M sodium citrate buffer (pH 4.0) at 37° for 18 h. The reaction was stopped by heating the mixture in boiling water for 3 min. After desalting as described above, 0.1 M sodium cacodylate buffer (100 μL), pH 7.3, containing UDP-D-galactose (40 nmol), UDP-D-[U-$^{14}$C]galactose (0.6 nmol), bovine milk galactosyltransferase (2.5 munits), and $MnCl_2$ (1 μmol), was added and the mixture was incubated at 37° for 30 min. It was then passed through a column containing AG 1-X2 ($Cl^-$) (1 mL) ion-exchange resin. The column was washed with distilled water (4 mL) and the washing was combined with the eluate and evaporated to dryness.

*Preparation of oligosaccharide–protein conjugates.*—In order to convert the methyl ester group of synthetic oligosaccharides **1–4** into a carboxyl group, the oligosaccharide (0.2 mg) was incubated in 0.1 M NaOH (100 μL) for 3 h. The mixture was passed through a column containing AG50-X12 ($H^+$) (0.5 mL) cation-exchange resin. The column was washed with distilled water (2 mL), and the washing was combined with the eluate and evaporated. The carboxyl group of the synthetic oligosaccharide and the amino group of the carrier protein were coupled as follows. A solution (100 μL) containing the synthetic oligosaccharide (0.1 mg), the $^3H$-labeled synthetic oligosaccharide ($4 \times 10^4$ cpm), *N*-hydroxysuccinimide (0.15 mg), EDC (2 mg), and a carrier protein (0.1–0.5 mg), was incubated at room temperature for 18 h. In order to separate the synthetic oligosaccharide-conjugated to protein from the remaining uncoupled oligosaccharide, the mixture was applied to a column ($1.6 \times 17$ cm) of Sephadex G-50, equilibrated with phosphate–buffered saline, pH 7.4 (PBS), and eluted with the same buffer; fractions (2 mL) were collected. Absorbance of each fraction at 230 or 280 nm was measured and the radioactivity was determined with an Aloka liquid-scintillation spectrometer (model LSC-1000). Fractions in the void volume, which contained the conjugates, were pooled and concentrated by ultrafiltration (UFP1 LGC BK, Nihon Millipore Kogyo K. K., Yonezawa) and used as immunogen and for the screening of monoclonal antibody. The yields of the oligosaccharides conjugated to the protein were calculated by the radioactivities incorporated in these fractions.

*Preparation of poly-D-lysine-conjugated oligosaccharide.*—Poly-D-lysine (0.5 mg), oligosaccharide **6** (100 nmol), $^{14}$C-labeled oligosaccharide **6** (2 nmol, $4 \times 10^4$ cpm), and $NaBH_3CN$ (2 mg) were dissolved in 50 mM sodium carbonate buffer (100 $\mu$L), pH 9.5. The solution was then incubated at room temperature for 5 days. The poly-D-lysine-conjugated oligosaccharide was purified by Sephadex G-50 column chromatography as described above.

*Preparation of monoclonal antibody.*—BALB/c mice were immunized with mBSA coupled with synthetic oligosaccharide **2**. A solution of the conjugate (100 $\mu$g in 0.1 mL), emulsified with an equal volume of Freund's complete adjuvant, was injected subcutaneously on day 1 and day 14, and a solution of 100 $\mu$g without adjuvant was injected intravenously on day 21. Three days after the last immunization, spleen cells were harvested and fused according to the method of Köhler and Milstein[18,19] with mouse myeloma NS-1. After 12–14 days, culture supernatants from hybridomas were assayed by the solid-phase, enzyme-linked immunosorbent assay (ELISA) as described below. The reactive and stable hybridomas were selected and cloned three times by limiting dilution.

*Enzyme-linked immunosorbent assay (ELISA).*—Flat-bottomed, polystyrene microtiter plates, Nunc-Immuno Plate-1 (Nunc Japan Inter Med, Tokyo), were coated with 50 $\mu$L of the synthetic, oligosaccharide-conjugated poly-D-lysine (10 $\mu$g/mL) in 50 mM $Na_2CO_3$ buffer, pH 9.5, at 4° for 16 h. After washing with the same buffer, 5% acetic anhydride in satd $NaHCO_3$ (100 $\mu$L) was added, to block by *N*-acetylation, the remaining active amino groups of poly-D-lysine. After 5 min, the plate was washed with distilled water. PBS containing 1% BSA was added to each well and the mixture was incubated for 2 h at room temperature. Then it was washed with PBS and then incubated with culture supernatant for 1 h. The plate was washed with PBS, and biotinylated sheep anti-mouse Ig(G + M + A), diluted 1 : 2000 with PBS containing 1% BSA, was added, and the mixture was incubated for another 30 min. The plate was washed with PBS, peroxidase-conjugated streptavidin, diluted 1 : 2000 with PBS, was added, and the mixture was incubated for 15 min. Then it was washed with PBS, and *O*-phenylenediamine (1 mg/mL) in 0.1 M citrate buffer, pH 4.5, containing 0.003% $H_2O_2$ was added to each well. The reaction was stopped after a 30-min incubation at room temperature by the addition of 2 M $H_2SO_4$ (50 $\mu$L). The absorbance at 492 nm was measured with an ELISA reader (Bio-Rad Model 2550 EIA Reader, Richmond, CA).

In order to remove the D-galactosyl groups from the artificial glycoproteins adsorbed to the well, sodium citrate buffer, pH 4.0, containing 0.5 unit of jack bean $\beta$-D-galactosidase (50 $\mu$L) was added to the well after *N*-acetylation and BSA-blocking procedures, and the plate was incubated at 37° for 18 h. It was then washed with PBS and assayed with monoclonal antibodies as described above.

*Affinity chromatography of radioactively-labeled oligosaccharides on an immobilized-antibody column.*—Purified monoclonal antibody (10 mg) was coupled with CNBr-activated Sepharose 4B (1 mL). The amount of monoclonal antibody bound to Sepharose was ~ 9.5 mg/mL of resin. The $^3$H-labeled oligosaccharides ($2 \times 10^3$

cpm), dissolved in PBS (50 μL), were applied to a column containing 1 mL of immobilized antibody, and kept at 4° for 30 min. The column was then washed with the same buffer. Fractions (0.5 ml) were collected and assayed for radioactivity.

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