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Note

Characterization of rabbit antibodies that recognize determinants on naturally occurring glycoproteins

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Glycoproteins constitute a diverse group of natural polymers that occur in cells both in soluble and in membrane-bound form, and that may act as a signal for recognition by other proteins and whole cells[1–3]. Many investigators have reported syntheses of potential antigenic markers in glycoproteins[4–11]. If synthetic antigens can be used to raise antibodies that will recognize the presence of such structures on naturally occurring glycoproteins, knowledge of the fine-specificity of such preparations is important. We had previously reported[8] on the specificity of antibodies raised against the capsular polysaccharide of *C. neoformans* serotype D. We have now prepared and characterized a polyclonal antibody pool raised against the synthetic antigen *p*-diazophenyl *O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→2)-α-D-mannopyranoside linked to the KLH protein (GNM-KLH).

The IgG fraction obtained from the rabbit serum by chromatography on DEAE-Sephadex A-25 was further fractionated by chromatography on immunoadsorbents, as shown in Table I. The various antibody fractions thus obtained were tested for precipitin-band formation against GNM-BSA, and KLH alone. It was found that four fractions (XV-22, XV-37, XV-55, and XV-58) gave bands with GNM-BSA. Three of the *unadsorbed* antibody fractions (XV-22, XV-37, and XV-47) also showed a precipitin band with KLH alone. None of the antibody fractions showed observable ligand-induced fluorescence change. Consequently, binding studies of these fractions was done by using a number of natural and neo-glycoproteins, and employing a radioimmunoassay as shown in Table II. It was found that all the antibody fractions bound to antigens 3, 4, 5, and 6, all of which contain a terminal (non-reducing) β-D-GlcNAc-(1→2)-α-D-Man*p* group, the determinant used as the immunogen. Fractions XV-55 and XV-58 initially showed the presence of some antibody to KLH by solid-phase radioimmunoassay. These were therefore passed through a column of KLH-Sepharose immunoadsorbent. The unadsorbed antibodies (obtained in 90% yield) had no KLH-binding activity, but retained their

TABLE I

AFFINITY CHROMATOGRAPHY OF PURIFIED IgG

Adsorbent	*Total absorbance*	*Eluant*	*Absorbance*	*Recovered fraction*
α-D-Man*p*-BSA-Sepharose 4B	73.5	PBS, pH 7.4	65.1	XV-22
		methyl α-D-Man*p* (0.02M) in PBS	0.5	XV-26
		methyl α-D-Man*p* (0.1M) in PBS	1.7	XV-27
β-D-GlcNAc-BSA-Sepharose 4B	59.2	PBS, pH 7.4	51.8	XV-37
GNM-BSA-Sepharose 4B	51.8	PBS, pH 7.4	26.9	XV-47
		GlcNAc (0.02M) + methyl α-D-Man*p* (0.02M) in PBS, pH 7.4	1.3	XV-65
		GlcNAc (0.1M) + methyl α-D-Man*p* (0.1M) in PBS	1.9	XV-60
		GlcNAc (0.5M) + methyl α-D-Man*p* (0.5M) in PBS	3.0	XV-58
		2% Acetic acid	12.1	XV-55

capability to bind to antigens 3, 4, 5, and 6. It has recently been reported that KLH itself may actually be a glycoprotein[12].

The initial IgG fraction from the polyclonal antibody pool gave a strong precipitin band against both GNM-BSA and GNM-KLH. Anti-KLH antibody in this fraction was expected, as KLH had been used as the carrier protein for immunization. The precipitin band against GNM-BSA indicates the presence of antibody directed against the immunizing disaccharide. The fact that several antibody frac-

TABLE II

ANTIGEN-BINDING SPECIFICITY OF AFFINITY-CHROMATOGRAPHED ANTIBODY FRACTIONS, AS DETERMINED BY SOLID-PHASE RADIOIMMUNOASSAY[a]

Fraction	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*
XV-26	5.2	0.5	28.0	44.0	15.0	25.0	2.0	0.05	0.04
XV-27	11.3	2.5	31.2	44.8	17.0	27.0	2.0	0.08	0.05
XV-47	0.12	0.15	23.3	34.5	23.0	34.0	45.0	0.12	0.09
XV-55	1.9	0.08	35.5	37.5	42.0	33.0	31.0	0.18	0.12
XV-58	0.07	0.21	35.5	31.0	31.0	30.0	23.0	0.15	0.08
XV-60	0.21	0.37	35.0	37.5	19.0	28.0	2.0	0.13	0.12
XV-65	0.25	0.55	35.3	36.0	17.0	27.0	3.0	0.06	0.09

[a]Activity expressed in counts per minute (c.p.m.) × 10^{-3}. Antigens used: 1, α-D-Man*p*-BSA; 2, β-D-GlcNAc-BSA; 3, GNM-BSA; 4, GNM-KLH; 5, bovine IgG; 6, bovine IgG treated with β-D-galactosidase; 7, KLH; 8, human serum transferrin; and 9, fetuin.

tions could be eluted from the same affinity column by using ligand solutions of increasing molarity indicates the presence in the pool of antibodies having various affinities for the same ligand*. It may be seen from the results of the solid-phase radioimmunoassay (see Table II) that the antibody fractions showed binding to both neo- and naturally occurring glycoproteins having the terminal (nonreducing) disaccharide β-D-GlcNAc-(1→2)-D-Man*p* group. It had been reported[13] that a part (**A**) of the glycopeptide (**B**) in bovine IgG is incompletely D-galactosylated (see Fig. 1). Our finding that *both* bovine IgG and β-D-galactosidase-treated bovine IgG bind to our antibody preparations is thus in agreement with this report. Two of the antibody fractions (XV-26 and XV-27) also recognized terminal α-D-Man*p* residues in a neoglycoprotein.

It may be seen from Table I that (after normalization) a total of 43.9 absorbance units of specific antibodies were *adsorbed* by the affinity columns. The total amount of IgG used (73.5 absorbance units) originated from 7.8 mL of rabbit serum. Thus, the serum contains 43.9/7.8, or 5.6 absorbance units/mL of serum. Although this computation is somewhat inaccurate, due to losses caused by handling (which are inevitable), this number is comparable to the quantity produced by monoclonal antibody procedures. Purified antibody fractions bound to the immunizing disaccharide only when this occurred as a terminal unit, and not when the sequence occurred internally, as in fetuin[14] and human serum transferrin. It is particularly noteworthy that two antibody fractions (XV-26 and XV-27), making up ~18% of the total response, both bound to a neoglycoprotein carrying only the nonterminal carbohydrate residue of the immunizing antigen, indicating that a subsite with high binding affinity[15] for the *interior* residue of the immunogen occurs in those immunoglobulins. Thus, certain antibody fractions obtained from the pool

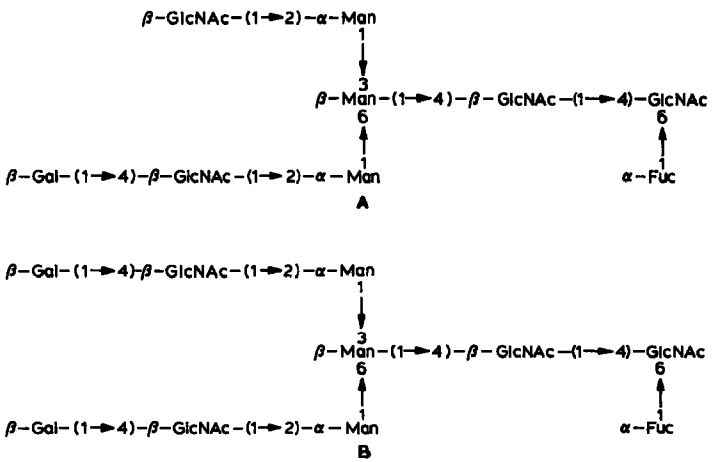

Fig. 1. Structures[13] of asparagine-linked oligosaccharides of bovine IgG.

*Some antibodies were bound to the column with very high affinity, and could not be eluted by saccharide concentrations of 0.1 and 0.5M. In our work on antigalactans, we routinely elute monoclonal antibody of Ka ML/M with 0.2M monosaccharide solution.

can be used as a diagnostic reagent for the presence of *either* residue of the immunizing antigen if it occurs terminally, but all antibody fractions are capable of showing the presence of the disaccharide determinant if it occurs terminally on the glycoprotein tested. One application for this antibody preparation would be as a diagnostic tool in the detection of the oligosaccharide storage-disease known as Sandhoff's disease[16], where the terminal GlcNAc-(1→2)-β-D-Man*p* of the oligosaccharide unit is not broken down, due to lack of *N*-acetyl-β-D-glucosaminidase.

EXPERIMENTAL

General methods. — *p*-Nitrophenyl *O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→2)-α-D-mannopyranoside was a gift from Dr. Goran Ekborg[6]. It was linked to KLH by diazotization[9], and to bovine serum albumin (BSA) by the phenyl isothiocyanate reaction[10]. *p*-Nitrophenyl α-D-mannopyranoside (Sigma Chemical Co., St. Louis, Mo.) was converted into the *p*-aminophenyl derivative by catalytic hydrogenation as described before[6], and then linked to BSA by diazotization. *p*-Aminophenyl-2-acetamido-2-deoxy-β-D-glucopranoside (Sigma) was also linked to BSA by diazotization[9]. Protein A, bovine IgG, fetuin, and human serum transferrin were obtained from Sigma, and KLH from Calbiochem, La Jolla, CA. Methyl α-D-mannopyranoside and 2-acetamido-2-deoxy-D-glucopyranose were obtained from Aldrich Chemical Co., Milwaukee, WI. Sepharose 4B was obtained from Pharmacia Fine Chemicals, Piscataway, NJ. ^{125}I-Labelled protein A was either obtained from New England Nuclear, Boston, MA, or by labelling protein A with Na^{125}I using iodobeads (Pierce Chemical Co., Rockford, IL). The double-diffusion test in agar gel was performed by the method of Ouchterlony[17]. Ion-exchange chromatography was performed on a column (1.6 × 31 cm) of DEAE-Sephadex A-25 (Pharmacia) using 15mM sodium phosphate buffer, pH 7.4, as the eluant. Size-exclusion chromatography was performed on a column (2.5 × 90 cm) of Sephadex G-200 using phosphate-buffered saline (PBS), pH 7.4, as the eluant. Affinity chromatography was performed using three different immunoadsorbents, as shown in Table I.

Preparation of immunoadsorbents. — *p*-Diazophenyl α-D-mannopyranosyl-BSA (α-D-Man*p*-BSA) was linked to cyanogen bromide (CNBr)-activated Sepharose 4B (Pharmacia) as follows. The CNBr-activated gel (3 g) was suspended in 10mM HCl (200 mL), and washed on a sintered-glass funnel with the same solution (400 mL). The α-D-Man*p*-BSA (78 mg) was dissolved in coupling buffer (0.1M sodium hydrogencarbonate, 0.5M sodium chloride, pH 8.3). The washed gel was suspended in this ligand solution (5 mL), and stirred for 2 h at room temperature. Unreacted ligand was washed out with coupling buffer (2 × 40 mL), and excess reactive groups were blocked by reaction with 0.1M Tris·HCl, pH 8.05 (30 mL) for 2 h. The immunoadsorbent was washed with three cycles (3 × 50 mL) of alternating pH 4.0 buffer (0.1M sodium acetate, 0.5M sodium chloride) and coupling buffer. It was finally washed with PBS, and kept at 5° until used. *p*-Diazophenyl 2-acetamido-2-deoxy-β-D-glucopyranosyl-BSA (β-D-GlcNAc-BSA), KLH, and *p*-isothiocyanato-

phenyl *O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→2)-α-D-mannopyranosyl-BSA (GNM-BSA) were linked to CNBr-activated Sepharose 4B by the same procedure, except that, in the last case, excess active groups were blocked by reaction with 0.2M glycine.

Immunization of rabbits. — New Zealand white rabbits (2–2.5 kg) were injected intramuscularly with GNM-KLH (3 mg) in 67% complete Freund's adjuvant (CFA, 1.5 mL), followed by a booster shot of the same immunogen (1.5 mg) in 67% CFA (0.75 mL) two weeks later, and a second booster shot (1.5 mg) in water without adjuvant after three weeks. Sera were collected 10 days after the second booster shot.

Fractionation of antibodies. — Serum (10 mL) was diluted with PBS (10 mL), and cooled to 5°. Saturated ammonium sulfate was added to a final concentration of 40%, and the mixture was gently stirred for 3 h at 5°. The precipitate was collected, dissolved in PBS (6 mL) and the solution dialyzed against 15mM sodium phosphate, pH 7.4, and then chromatographed on a column of DEAE-Sephadex A-25. The material representing the IgG fraction was chromatographed on a column of Sephadex G-200; it gave a single symmetrical peak. This monomeric IgG was next fractionated by affinity chromatography as shown in Table I.

Solid-phase radioimmunoassay. — Assays were performed at room temperature in triplicate, using flexible poly(vinyl chloride) microtiter plates (Dynatech, Alexandria, VA). Glycoprotein solutions (60 μg/mL; 45 μL) were added to each well of the microtiter plate, and kept overnight at room temperature. The wells were emptied, and then filled with Tris-BSA buffer (0.05M tris, 0.15M NaCl, 1.0% BSA, and 0.1% NaN_3; pH 7.8) and kept for 1 h, to prevent nonspecific binding in the next step. The cups were emptied again and antibody solution (45 μL) was added to each well. After 1 h, the wells were emptied, and washed twice with Tris-BSA buffer. ^{125}I-Labeled protein A (50 μL, 50,000 c.p.m.) in the same buffer was then added to each well, and allowed to stand for 1 h. Finally, the wells were washed five times with PBS, cut out, and counted on a Packard model 5260 gamma scintillation counter.

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