Binding Properties of a Mouse Immunoglobulin M Myeloma Protein with Carbohydrate Specificity*

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ABSTRACT: The IgM protein from the murine plasmacytoma MOPC 104E was prepared by a simple immunospecific procedure involving precipitation by dextran, dissociation by hapten, and anion-exchange chromatography. The binding properties of the purified IgM were examined by equilibrium dialysis. The IgM molecule has ten binding sites for the hapten D-glucopyranosyl-(1→3)-α-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl-(1→3)-α-D-glucitol. The sites are homogeneous with an association constant for the hapten of 3.6×10^4 M^{-1} at 4° and 0.8×10^4 M^{-1} at 30°. Quantitative inhibition studies with various nigerosyl derivatives confirm the dominant role of the terminal nigerosyl group in the reactions with MOPC 104E IgM.

The induced mouse plasmacytoma MOPC 104E secretes an IgM protein (McIntyre *et al.*, 1965) which precipitates with dextrans and displays other properties typical of antibodies, *viz.*, complement fixation, hapten specificity, and agglutination of dextran-coated erythrocytes (Leon *et al.*, 1970). The major specificity of the IgM is directed against α(1 → 3)D-glucosyl units (nigerose and its derivatives) but the IgM also shows weak affinity for other α-glucobioses as well as methyl α-D-glucoside.

The MOPC 104E IgM provides, therefore, a model for investigation of an antibody binding site specific for uncharged hydrophilic ligands. As a first step, we have examined by equilibrium dialsysis the association of hapten with MOPC 104E IgM, prepared by a simple immunospecific procedure.

Materials and Methods

Sera were obtained from BALB/c mice bearing the MOPC 104E tumor and were stored at −60°. The tumor line was established from mice kindly supplied by Dr. K. R. McIntyre. Dextrans were obtained through the courtesy of Dr. Allene Jeanes. The linkage composition of the dextrans were B1254L, 69% α 1→6, 31% α 1→4 + α1→2; B1299S, 50% α 1→6, 50% α 1→4 + α 1→2; B1355S1,3, 57% α 1→6, 8% α 1→4 + α 1→2, 35% α 1→3. The tetrasaccharide *O*-α-D-glucopyranosyl-(1→3)-*O*-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→3)-D-glucopyranose (nigerosyl-α(1→4)-nigerose) was a gift from Dr. J. H. Nordin, and the α(1→3)-linked oligosaccharides, nigerotriose and nigerotetraose (Johnston, 1965) were gifts of Dr. I. R. Johnston. Precipitin reactions and hapten inhibition experiments were carried out as previously described (Leon *et al.*, 1970).

Immunospecific Preparation of MOPC 104E IgM. MOPC 104E serum (1 ml) containing 22 mg/ml of IgM was mixed with a solution of dextran B1254L (5 ml of a 50-mg/ml solution in phosphate-buffered EDTA–saline—0.14 M NaCl–0.01 M phosphate–0.01 M EDTA, pH 7.3) or with a solution of dextran B1299S (5 ml of 20 mg/ml in phosphate-buffered EDTA–saline). After standing for 1 hr at room temperature and overnight at 4°, the specific precipitate was centrifuged and washed three times with cold phosphate-buffered EDTA–saline. The specific precipitate was extracted with 5 ml of 1.5 M methyl α-D-glucoside (Corn Products, recrystallized from 85% isopropyl alcohol) in 0.05 M phosphate, pH 8.0. The extract was clarified by centrifugation and chromatographed at room temperature on a DEAE-cellulose column (Whatman DE-52, 2.5 × 35 cm) equilibrated with the above glucoside buffer. The dextran does not bind to the column and was recovered for reuse in the first eluate. The dextran absorbs at 280 nm and its elution is easily monitored. The DEAE-cellulose was then washed with several column volumes of 0.05 M phosphate, pH 7.2, to remove methyl α-D-glucoside and the IgM was eluted with 0.1 M phosphate buffer (pH 6.2), containing 0.9 M NaCl. The product was dialysed to remove the last traces of methyl α-D-glucoside.

Preparation of Alditols. Oligosaccharide, (10 mg), dissolved in 1 ml of water, was treated with 5 mg of sodium borohydride at 0° for 4–5 hr. A second 5-mg portion of sodium borohydride was then added, and the mixture was allowed to stand at 0° overnight. Excess borohydride was decomposed with acetic acid, and the mixture was chromatographed on a Sephadex G-15 column (1.5 × 48 cm, exhaustively washed with water). Fractions containing hexose, detected by an orcinol–H_2SO_4 procedure (Kabat and Mayer, 1961), were pooled. The products contained less than 0.5% of reducing sugars detectable by the Park–Johnson technique (Park and Johnson, 1949). The concentration of glucose in the pooled materials was measured by the orcinol–H_2SO_4 procedure using twice-recrystallized methyl α-D-glucoside as standard, and the molarity of the alditols was calculated. The alditols were obtained in over 90% yields.

Tritium-labeled D-glucopyranosyl-(1→3)-α-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl-(1→3)-α-D-glucitol (nigerosyl-α(1→4)-nigeritol) was prepared from nigerosyl-α(1→4)-nigerose (10 mg in 0.5 ml of water) in the same manner, except that the first portion of sodium borohydride was replaced by

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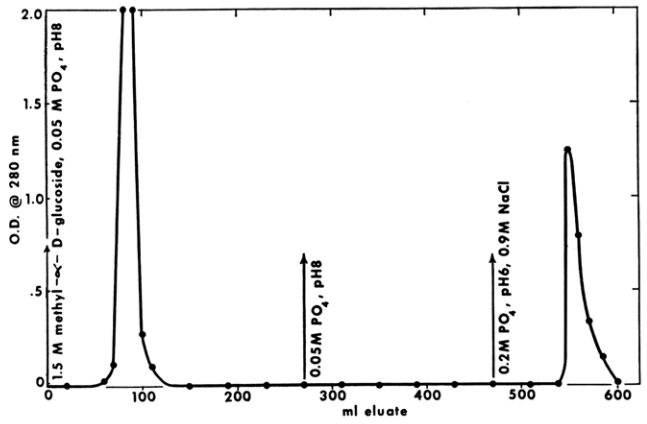

FIGURE 1: Chromatography on DEAE-cellulose of a solution, in 1.5 M methyl α-D-glucoside, of the complex formed between MOPC 104E IgM and dextran B1254L.

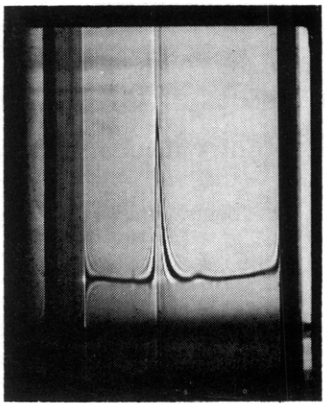

FIGURE 2: Ultracentrifugation of immunospecifically prepared MOPC 104E IgM. Concentration, 4.3 mg/ml; speed, 59,780 rpm, temperature 20°, bar angle 55°, 8 min after reaching speed. Sedimentation to the right.

6 mCi of sodium borotritide (Amersham/Searle 500 mCi/mmole) in 0.3 ml of water.

Equilibrium Dialysis. Purified MOPC 104E IgM was dialyzed against several changes of a Tris–NaCl buffer (pH 7.0, 0.02 M Tris–0.15 M NaCl–0.1% sodium azide) to ensure complete removal of methyl α-D-glucoside. Stock solutions of [^{3}H]nigerosyl-α(1→4)-nigeritol and MOPC 104E IgM were prepared by dilution with the Tris–NaCl buffer to which gelatin (0.05%) had been added.[1] The concentration of IgM used was 2.3 mg/ml, determined spectrophotometrically using $E^{1\%}_{280\,nm}$ 16.0 (Leon *et al.*, 1970). Equilibrium dialyses were carried out in cells of 0.1-ml nominal capacity per compartment (Gateway Immunosera, Cahokia, Ill.). Each compartment received 0.12 ml of protein or hapten solution. The cells were left to reach equilibrium, without agitation, at 4° for 70 hr or at 30° for 45 hr. Samples (100 μl) were withdrawn and counted in a Picker nuclear scintillation counter, using 10 ml of a scintillation mixture consisting of toluene, Bio-Solv BBS-3 (Beckman Instruments), and Liquifluor (New England Nuclear) in the proportions 500:50:23.

Ultracentrifugation. Samples were centrifuged in a Beckman Model E analytical ultracentrifuge at 20° in 0.1 M Tris (pH 8.0). Sedimentation constants were calculated as described by Schachman (Schachman, 1957).

Antisera. Rabbit footpads were injected with 1 ml of a mixture (1:1) of Freund's complete adjuvant (Difco) and pooled normal mouse serum. After 3 weeks, the rabbits were boosted with three weekly intravenous injections of 0.5 ml of mouse serum. Serum was collected 1 week after the last injection.

Results

Immunospecific Purification of MOPC 104E IgM. The complexes, formed between MOPC 104E IgM and dextrans with different linkage compositions, vary greatly in the ease with which they are dissociated by haptens (Leon *et al.*, 1970). The precipitates formed with dextran B1254L or dextran B1299S were readily soluble in 1.5 M solution of the weak hapten, methyl α-D-glucoside, Chromatography of the solution on DEAE-cellulose (Figure 1) permitted separation of the dextran from the IgM. An intermediate wash removed essentially all of the weak hapten, avoiding the contamination of the immunoglobulin product by small amounts of strongly bound hapten (Stevenson *et al.*, 1970) common to other immunospecific preparative methods. Yields by this rapid procedure were usually greater than 90% based on the IgM originally present in the serum, as determined by precipitation with dextran B1355S1,3.

The product was completely precipitated by dextran B1355-S1,3 and at a concentration of 3 mg/ml showed a single line in Ouchterlony and immunoelectrophoresis tests with rabbit antiserum to whole mouse serum. In the ultracentrifuge (Figure 2) at 4.3-mg/ml concentration, the preparation showed a major component with a sedimentation rate $s_{20,w}$ = 17.6 S in agreement with previous results (McIntyre *et al.*, 1965) and a 27S minor component (<5%). Since the preparation was entirely precipitable by dextran, the latter component must also be reactive with dextran B1355S1,3 and may be an IgM dimer, often present in IgM preparations (Suzuki and Deutsch, 1966; Merler *et al.*, 1968).

Equilibrium Dialysis. Binding studies were conducted with tritium-labeled nigerosyl-α(1→4)-nigeritol prepared from nigerosyl-α(1→4)-nigerose by mild reduction with sodium borotritide (Katz and Pappenheimer, 1969). The data are plotted in Figure 3 according to the equation $r/c = nK - rK$, where r is the number of moles of hapten bound per mole of protein, c is the concentration of free hapten, n is the number of combining sites per molecule of protein, and K is the association constant. The molecular weight of the MOPC 104E IgM has not yet been accurately determined and was assumed to be 850,000, a value toward the lower end of the range reported for IgM molecules (Metzger, 1970) which was chosen in view of the $s_{20,w}$ reported above.

At both 4 and 30° the binding data fall on straight lines over the range of saturation studied. The binding sites are therefore homogeneous with respect to nigerosyl-α(1→4)-nigeritol. The association constants are 3.6 × 10^4 M^{-1} at 4° and 0.8 × 10^4 M^{-1} at 30°. From these values, $-\Delta F°$ at 4° is 5.8 kcal/mole, $\Delta H°$ is −9.6 kcal/mole, and $\Delta S°$ is −13.7 eu/mole. The data at both temperatures indicate that there are ten binding sites on each IgM molecule.

Hapten Inhibition. In order to relate the binding data obtained with the [^{3}H]nigerosyl-α(1→4)-nigeritol to the inhibition data previously reported (Leon *et al.*, 1970) quantitative hapten inhibition studies were carried out with unlabeled nigerosyl-α(1→4)-nigeritol and other nigeritols as well as the

[1] We thank Dr. H. N. Eisen for suggesting the use of gelatin to stabilize the myeloma protein during equilibrium dialysis.

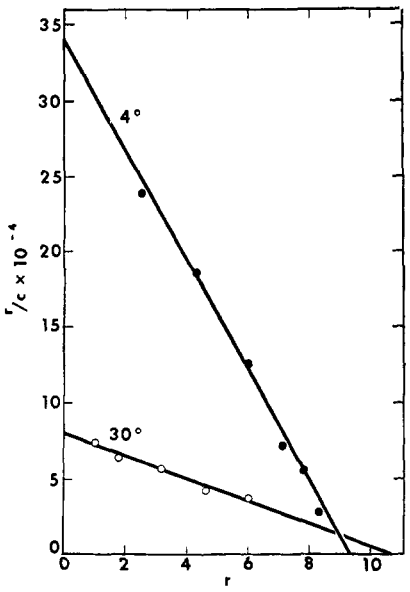

FIGURE 3: Equilibrium dialysis of MOPC 104E IgM at 4° (●) and 30° (○) with [^{3}H]nigerosyl-α(1→3)nigeritol. The points shown at 4° are the means of quadruplicate determinations, and those at 30° are the means of duplicate determinations.

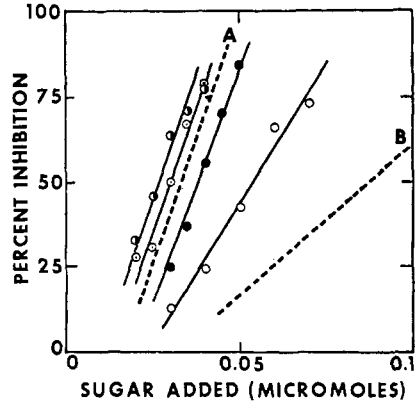

FIGURE 4: Inhibition of precipitation of MOPC 104E serum with dextran B1254L by nigerosyl-α(1→4)-nigeritol (◑), nigerosyl-α-(1→4)-nigerose (⊙), nigerosyl-α(1→3)-nigeritol (●), and nigerotriitol (○). The broken lines represent data obtained previously (Leon *et al.*, 1970) for nigerosyl-α(1→3)-nigerose (A) and nigerose (B).

tetrasaccharide nigerosyl-α(1→4)-nigerose (Figure 4). The quantities of the various haptens required for 50% inhibition of the precipitation of dextran B1254L by MOPC 104E IgM are listed in Table I. The association constants are derived by comparison of the efficiencies of the haptens with that of nigerosyl-α(1→4)-nigeritol, utilizing the association constant obtained with [^{3}H]nigerosyl-α(1→4)-nigeritol.

These data emphasize the dominant role of the α-nigerosyl group in the binding of the haptens to MOPC 104E IgM. The fourth saccharide unit plays little role as the two tetraoses, nigerosyl-α(1→4)-nigerose and nigerosyl-α(1→3)-nigerose, and the tetritols derived from them all have similar association constants to nigerotriose. The third saccharide unit contributes to the binding since nigerotriose and nigerotriitol are superior inhibitors to nigerose. It is also noteworthy that the two tetraoses have similar association constants despite the third saccharide unit being attached quite differently, *viz.*, either α(1→3) or α(1→4).

Discussion

The equilibrium dialysis data reported here demonstrate that the valence of the MOPC 104E IgM is ten and that the binding sites are homogeneous. Presumably there is one binding site on each of the ten Fab portions visible in electron microscopy photographs of the protein (Shelton and McIntyre, 1970; Parkhouse *et al.*, 1970). The decavalence of the IgM is in accord with previous studies of two other IgM myeloma proteins that show antibody activity. The protein γM_{Wag} has ten binding sites for nitrophenyl derivatives (Ashman and Metzger, 1969) and γM_{Lay} has one binding site per Fab′ fragment, for IgG, though the intact molecule is only pentavalent (Stone and Metzger, 1968). All these data are consistent with the IgM model of a circular pentamer of IgG-like subunits proposed by Miller and Metzger (1965). However, this model may require some modification in view of recent data suggesting that J chains are released from IgM by sulfitolysis (Mestecky *et al.*, 1971).

Experiments with the heterogeneous IgM preparations purified from immune sera are technically more difficult and conflicting results have been obtained. Pentavalence (Frank and Humphrey, 1968), decavalence (Merler *et al.*, 1968), and the occurrence of five of each of two kinds of subunits, one having a much lower affinity for hapten than the other (Onoue *et al.*, 1968; Kishimoto and Onoue, 1971) have been found for IgM molecules reactive with different haptens. These and other data have been reviewed by Metzger (1970). It may be significant that the only other carbohydrate binding IgM studied, prepared from rabbit antisera to *Salmonella typhi* 012, also had a valence of ten (Merler *et al.*, 1968).

The association constant for the binding of nigerosyl-α(1→4)-nigerose by MOPC 104E IgM, 3.6×10^4 at 4°, falls in the lower end of the range 10^4 to 10^9 recorded for antibody affinities (Eisen and Pearce, 1962). Carbohydrate antibodies in general have association constants below 5×10^5 (Table II), and in fact the MOPC 104E IgM has a higher association constant than the rabbit IgM antibody to *S. typhi*. Both IgM proteins have lower affinities than the IgG anticarbohydrate antibodies. However, the values reported for anticarbohydrate IgG may represent above average antisera, chosen for experimental convenience.

The thermodynamic parameters for MOPC 104E IgM are

TABLE I: Association of Haptens with MOPC 104E IgM.

Hapten	Molarity for 50% Inhibition[a]	$K \times 10^{-4}$ M^{-1}
Nigerosyl-α(1→3)-nigerose	0.000066[b]	3.1
Nigerosyl-α(1→4)-nigerose	0.000060	3.4
Nigerosyl-α(1→3)-nigeritol	0.000076	2.7
Nigerosyl-α(1→4)-nigeritol	0.000056	3.6
Nigerotriose	0.000070[b]	2.9
Nigerotriitol	0.00011	1.8
Nigerose	0.00017[b]	1.2

[a] Inhibition of the reaction between 1 mg of dextran B1254L and approximately 0.17 mg of MOPC 104E (Leon *et al.*, 1970). [b] Values from Leon *et al.* (1970).

TABLE II: Association Constants of Anticarbohydrate Immunoglobulins.

Antigen	Immunoglobuln	$K \times 10^{-4}$, M^{-1}	Reference
Lactosyl–protein conjugate[a]	Rabbit IgG	29–45	Karush (1957)
Pneumococcal SIII polysaccharide[b]	Rabbit IgG	14–32	Katz and Pappenheimer (1969)
Pneumococcal SVIII polysaccharide[c]	Rabbit IgG (homogeneous)	25	Haber (1970)
S. typhi endotoxin[d]	Human IgM	2.7	Merler *et al.* (1968)
Dextran	Mouse IgM MOPC 104E	3.6	This paper

[a] *p*-(*p*-Dimethylaminobenzeneazo)phenyl β-lactoside used as ligand. [b] $\frac{}{}$ 3)-β-D-GlcA(1→4)-β-D-Glc(1$\frac{}{}$$_n$, $\frac{}{}$ 4)-β-D-Glc(1→3)-β-D-GlcA(1$\frac{}{}$$_3$ used as ligands. [c] Octasaccharide fragment of SVIII used as ligand. [d] Tetrasaccharide fragment of *S. typhi* endotoxin used as ligand.

comparable to those reported for the antilactosyl system (Karush, 1957). For antilactosyl IgG the values were $-\Delta H^\circ = 9.7$ kcal/mole and $\Delta S^\circ = -8.8$ eu/mole while the values for MOPC 104E IgM are 9.6 and −13.3. The enthalpy value suggests that several hydrogen bonds are involved in the binding of hapten by MOPC 104E IgM.

The quantitative inhibition data (Table I) confirm previous observations (Leon *et al.*, 1970) that an increase in ligand size from the disaccharide to trisaccharide significantly improves binding to MOPC 104E IgM, whereas addition of a fourth saccharide unit has no effect. However, these new data indicate that the third saccharide unit can be attached to nigerose *via* either an α(1→3) linkage or an α(1→4) linkage to give this higher affinity. It is possible that the third saccharide unit is not directly bound to the IgM but instead plays a role in fixing the conformation of the nigerosyl group.

The ultimate biological effectiveness of the various classes of antibodies, regardless of specificity, may not be simply related to association constants determined by equilibrium dialysis with small ligands. Secondary factors, such as the presence of multiple binding sites in an IgM antibody, multiple repeating units in a carbohydrate antigen, or the ability to fix complement (Maurer and Talmage, 1953) (Sage *et al.*, 1963) may play significant roles in determining final energy of binding of antigen to antibody *in vivo*.

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