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Mapping of Subsites in the Combining Area of Monoclonal Anti-Galactan Immunoglobulin A J539†

Cornelis P. J. Glaudemans,* Pavol Kováč, and Kjeld Rasmussen

ABSTRACT: Monoclonal immunoglobulin A J539 binds β-(1→6)-D-galactopyranans. Measurement of the affinity of its Fab′ fragment for a series of galacto oligosaccharides—some of which carried deoxyfluoro groups—has made it possible to assign a binding mode of the polysaccharide that has the reducing end oriented from the heavy (H) chain toward the light (L) chain. In addition, the values obtained for the affinity constants of the immunoglobulin with these oligosaccharides, as well as the maximal values obtained for the intrinsic ligand-induced fluorescence, permit a deduction about the relative affinity of the protein's four subsites for each galactose residue of the tetrasaccharide fragment it can bind. If these subsites are labeled C, A, B, and D, going from the H-chain toward the L-chain across the face of the immunoglobulin combining area, then the order of decreasing affinity is A > B > C > D.

Although monoclonal immunoglobulins have been affinity labeled (Givol et al., 1971), no detailed information of the interaction of a homopolysaccharide and its monoclonal immunoglobulin is available to date. Monoclonal immunoglobulin A (IgA) J539 has as its homologous antigen a β-(1→6)-D-galactopyranan, a neutral homopolysaccharide whose interaction with the antibody has been studied [Glaudemans, 1975; Jolley et al., 1974; Manjula & Glaudemans, 1976; Feldmann et al. (1981) and references cited therein; Roy et al., 1981; Ekborg et al., 1983]. IgA J539 Fab′ has been crystallized, but Navia et al. (1979) have observed that the cavity associated with the hypervariable clusters on the frontal surface of J539 is spatially blocked by the constant region of a neighboring, symmetry-related, molecule in the crystal lattice and that, indeed, soaking of these crystals in a solution of 6-*O*-β-D-galactopyranosyl-D-galactose, or of the corresponding tetrasaccharide, did not cause significant changes indicative of binding. Thus, it becomes important to endeavor to map the combining area of this IgA by means other than X-ray diffraction studies.

In order to evaluate the importance of H bonding between ligand and antibody, we have previously synthesized a number of deoxyfluorogalactosides and have already reported on the binding studies of some of these (Ittah & Glaudemans, 1981). In this paper we report the binding affinities of a larger number of such derivatives, which permit a conclusion as to important details of the binding mode of the homologous polysaccharide to IgA J539.

Materials and Methods

Ligands. The preparation of methyl 2-deoxy-2-fluoro-β-D-galactopyranoside (**1**) (Ittah & Glaudemans, 1981), methyl 3-deoxy-3-fluoro-β-D-galactopyranoside (**2**) (Kováč & Glaudemans, 1983a), methyl 4-deoxy-4-fluoro-β-D-galactopyranoside (**3**) (Ittah & Glaudemans, 1981), and methyl 6-deoxy-6-fluoro-β-D-galactopyranoside (**4**) (Kováč & Glaudemans, 1983b) has already been reported. The preparation of methyl 6-*O*-(β-D-galactopyranosyl)-β-D-galactopyranoside (**7**) (Kováč et al., 1984), methyl 6-*O*-(3-deoxy-3-fluoro-β-D-galactopyranosyl)-β-D-galactopyranoside (**8**) (Kováč & Glaudemans, 1983c), methyl 6-*O*-(6-*O*-β-D-galactopyranosyl-β-D-galactopyranosyl)-β-D-galactopyranoside (**9**) (Kováč et al., 1984b), and methyl 6-*O*-[6-*O*-(3-deoxy-3-fluoro-β-D-galactopyranosyl)-β-D-galactopyranosyl]-β-D-galactopyranoside, (**10**) has also been reported (Kováč & Glaudemans, 1983c, 1985), or their reports are in press. The preparation of methyl 6-deoxy-β-D-galactopyranoside (**6**) was as follows: D-Fucose (2 g) was dissolved in pyridine (10 mL) and cooled in ice, and acetic anhydride (10 mL) was added over a period of 1.5 h. The solution was left to warm up to room temperature and left overnight. The reagents were evaporated at 65 °C under vacuum, and traces were removed by flash evaporation with toluene under vacuum (3 times). ^{13}C

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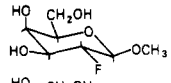
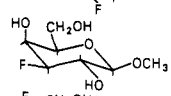
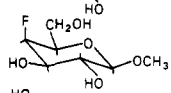
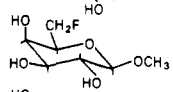
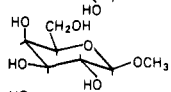
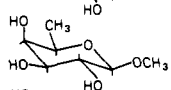
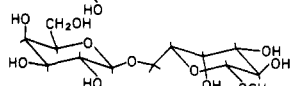
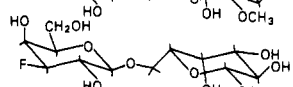
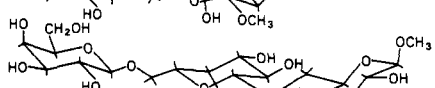
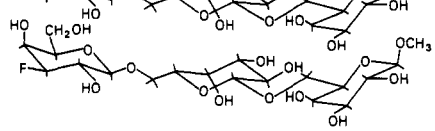
Table I: Binding Constants (K_a) and Maximal Fluorescence Change (ΔF_{max}) for IgA J539 FAB' with Galactose Derivatives

	compound	Ka	ΔFmax
1		0	0
2		0	0
3		0.75×10^3	23%
4		2.5×10^3	23%
5		1.0×10^3	21%
6		2.8×10^3	20%
7		4.3×10^4	36%
8		0.8×10^4	21%
9		3.8×10^5	40%
10		3.5×10^5	40%

NMR spectroscopy of the residual D-fucose tetra-*O*-acetate showed it to be the fucopyranose tetraacetate, the ratio of β to α anomer being 2:1. It was dissolved in dichloromethane (10 mL), and hydrobromic acid (32%) in acetic acid (8 mL) was added. After 1.5 h, thin-layer chromatography (silica gel; toluene–acetone, 9:1) showed conversion to the 2,3,4-tri-*O*-acetyl-D-fucopyranosyl bromide to be complete. Reagents were removed by flash evaporation under vacuum; traces were chased by coevaporation with toluene (3 times). A solution of the bromide in toluene (10 mL) was added to a suspension of barium oxide in methanol (careful, exothermic reaction) and left for 1.5 h. The base was neutralized by the addition of CO_2 (dry ice). The entire mixture was evaporated, dissolved in water, and filtered through Celite (Johns Manville Corp.), and the filtrate was concentrated to a residue that was dried by coevaporation with ethanol under vacuum (3 times). This residue was next extracted with hot ethyl acetate and filtered, and the filtrate was evaporated to yield some 600 mg of crystalline material that was recrystallized from hot ethyl acetate to give approximately 320 mg of pure methyl β-D-fucopyranoside (=methyl 6-deoxy-β-D-galactopyranoside) (**6**): mp 122–124 °C; $[\alpha]^{23}_D$ −12.2° (*c* 2.3, H_2O). The enantiomer has the following: mp 117–119 °C, $[\alpha]_D$ +16° (Minsaas, 1932).

Immunoglobulin. J539 Fab′ was obtained as described before (Rudikoff et al., 1972).

Fluorescence Titration. Association constants (K_a) for the equilibrium Fab′ + ligand ⇄ Fab′···ligand were determined in phosphate-buffered saline (PBS), pH 7.4, with IgA J539 Fab′ at a concentration having OD_{280} = 0.05 from ligand-induced changes in the tryptophanyl fluorescence of the IgA Fab′, as described before (Jolley & Glaudemans, 1973; Glaudemans & Jolley, 1980), with excitation and emission wavelengths of 295 and 340 nm, respectively. 6-*O*-β-D-Galactopyranosyl-D-galactose showed a maximal ligand-induced fluorescence change of 33% with J539 Fab′.

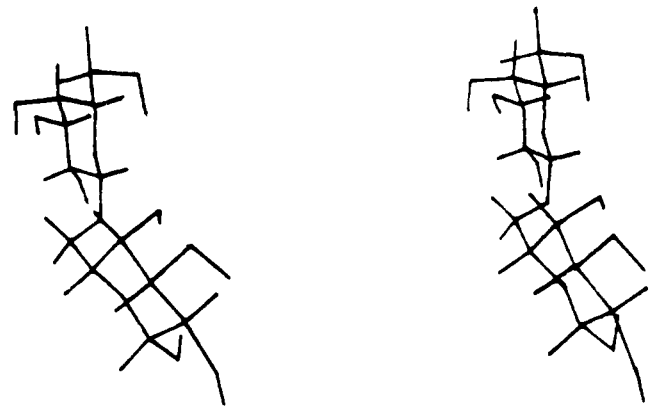


FIGURE 1: Stereoview of conformation of 6-*O*-β-D-galactopyranosyl-β-D-galactopyranose showing ϕ, ψ, and ω intersaccharidic angles of 159, −115, and −173°, respectively. The reducing galactose unit is at the bottom.

Results

Table I lists the association constants and the maximal immunoglobulin fluorescence changes between the ligands tested and IgA J539 Fab′ at 25 °C.

Discussion

IgA J539 binds $\beta(1\rightarrow6)$-D-galactopyranosyl oligo- and polysaccharides (Manjula & Glaudemans, 1976). It has been shown that the antibody combining site can accomodate four sequential galactopyranosyl residues and the antibody appears to bind to interchain $\beta(1\rightarrow6)$-linked tetrasaccharide segments of polysaccharides (Roy et al., 1981; Jolley et al., 1973). It is to be expected that these four antibody subsites each have their own, differing affinity for a galactosyl residue since the protein in general, including the combining area, has an anisotropic nature. Examination of the data in Table I permits an assignment of the relative strength of interaction of each antibody subsite with its galactosyl residue and also permits a conclusion about the direction of the polysaccharide chain as it is bound in the antibody combining area.

A conformation for a $\beta(1\rightarrow6)$-D-galactopyranan having the ring oxygen of each residue in alternate fashion on opposite sides of the polysaccharide chain was proposed (Jolley et al., 1974). We have made energy-minimization calculations for the corresponding disaccharide, 6-*O*-β-D-galactopyranosyl-β-D-galactopyranose (βGal_2), similarly to the calculations used for gentiobiose before (Melberg & Rasmussen, 1980) and have arrived at the three intersaccharidic angles ϕ, ψ, and ω for the $\beta(1\rightarrow6)$-linked galactobiose as having values of 159, −115, and −173°, respectively. This conformation (which will be reported on in more detail in the future) also shows the staggered position of the rings along the chain proposed earlier and is used in this paper to rationalize the binding mode (shown in Figure 1). A tetrasaccharide fragment of a $\beta(1\rightarrow6)$-D-galactopyranan having this conformation has a linear dimension of ca. 17 Å.

The subsite with the highest affinity for a galactose residue is likely to be near a solvent-exposed tryptophanyl residue, since even the simplest ligand—methyl β-D-galactopyranoside (**5**)—shows ligand-induced antibody tryptophanyl fluorescence change (Glaudemans & Jolley, 1980). On the basis of an examination of the coordinates of J539 Fab′ obtained by X-ray diffraction studies at 2.7-Å resolution (S. W. Suh, T. M. Bhat, G. H. Cohen, S. Rudikoff, D. N. Rao, and D. R. Davies,

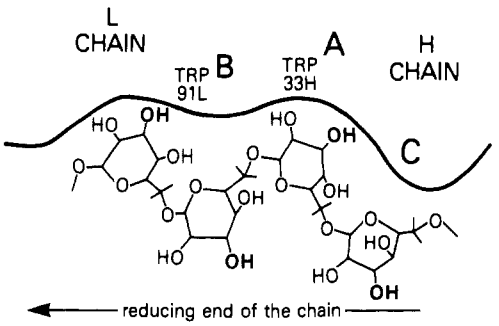


FIGURE 2: Schematic representation of binding mode of a $\beta(1\rightarrow 6)$-D-galactopyranan segment to IgA J539, with the reducing end of the polysaccharide oriented toward the L chain. If the Fc portion of the IgA is called the bottom, the general view is from the top of the protein molecule, looking down along the face of the antibody combining area. The 3-OH groups of each galactosyl residue are boldface. Protein subsites are labeled C, A, and B. The general positions of the two solvent-exposed tryptophanyl residues (91L and 33H) are indicated.

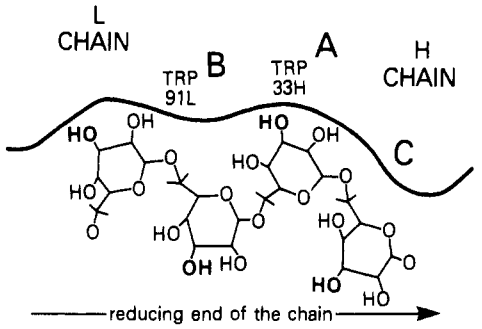


FIGURE 3: Schematic representation of the binding mode of a $\beta(1\rightarrow 6)$-D-galactopyranan segment to IgA J539, with the reducing end of the polysaccharide oriented toward the H chain. For other particulars, see legend to Figure 2.

personal communication), it can be seen that the two tryptophanyl residues in the complementarity determining regions (CDRs) of J539, namely, TRP 33H and TRP 91L (Rao et al., 1979; Rudikoff et al., 1980; Kabat et al., 1983), appear solvent exposed and are at the H/L interface in the center of the immunoglobulin combining area. These two TRP residues are separated by ca. 9–10 Å, and the only other tryptophanyl residues near the general combining area, TRP 36H and TRP 35L, are not in the CDR and are buried internally where they would not be expected to be perturbable by ligands bound on the protein surface. The first antibody subsite to attract a ligand appears to be near tryptophan-33H, as a heterologous H/L-chain recombinant immunogloulin derived from the two monoclonal antigalactan IgAs S-10 and J539 showed fluorescence characteristics upon binding methyl β-D-galactopyranoside similar to those of the H-chain donor protein (Manjula et al., 1979). Both these proteins have the invariant tryptophane-33H residue (Rao et al., 1979; J. Pumphrey, and S. Rudikoff, unpublished results). We label that subsite as A and will show evidence that—when three subsites are labeled B, A, and C as shown in Figures 2–4 across the combining area, with the H-chain on the right—the polysaccharide binds with its reducing end projected toward the L chain and that the order of affinities for the subsites is A > B > C.

Two modes of binding for the polysaccharide chain are a priori possible (as shown in Figures 2 and 3). It has previously been shown that the side of the first galactosyl residue to bind is that face of the pyranosyl ring bearing the 2- and 3-OH groups (Jolley et al., 1974; Ekborg et al., 1983; Glaudemans et al., 1975) while the C-6 (hydroxymethyl group) projects away from the protein toward the solvent (as indicated in

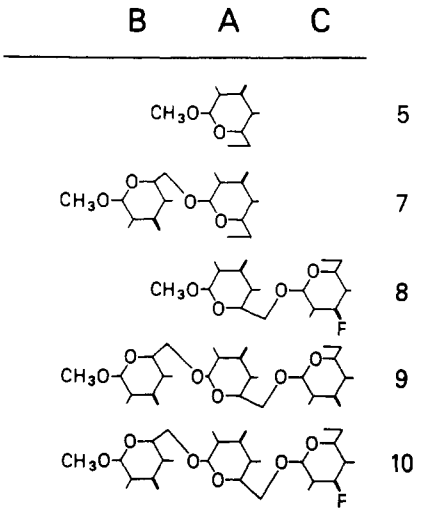


FIGURE 4: Schematic representation of binding of a number of galactosyl derivatives to the subsites B, A, and C of IgA J539. The 3-OH or 3-F is indicated by a boldface line in each galactosyl residue.

Figures 2–4). It has also been reported that replacement of the 2-OH group by fluorine causes nonbinding (Ittah & Glaudemans, 1981), and the present work shows that replacement of the 3-OH group by fluorine also causes cessation of binding (Table I). This agrees with our earlier observation (Das et al., 1979) that, of the three nitrophenyl β-D-galactopyranosides, the *o*-nitrophenyl galactoside binds least strongly to IgA J539. This because the *o*-nitro group is perfectly located to form an intramolecular hydrogen bond with the 2-OH group of the galactosyl residue, thus competing with the antibody site for this hydroxyl group. Presumably, hydrogen bonding between the 2- and 3-OH groups on the one hand and the protein on the other hand is required for this subsite. Thus from these data the polysaccharide can be placed in the combining site as shown in either Figure 2 or Figure 3. Now we will show that the evidence supports the binding mode shown in Figure 2 and not that of Figure 3. Table I lists the maximum changes in the protein tryptophanyl fluorescence inducible by a number of ligands. It can be seen that for the monosaccharides (**3**–**6**) this value is ca 20%. When the ligand increases (approximately doubles) its length to that of 6-*O*-β-D-galactopyranosyl-D-galactose (Gal_2), the value goes up substantially (33%) and even more so for the methyl β-glycoside of this galactobiose **7** (36%) or for methyl galactotrioside **9** (40%). 6-*O*-β-D-Galactopyranosyl-D-galactopyranose (Gal_2) in the conformation having the energy-minimized intersaccharidic ϕ, ψ, and ω angles of 159, −115, and −173° has a length of 9.2 Å. The X-ray diffraction studies of J539 at 2.7 Å (S. W. Suh, T. M. Bhat, G. H. Cohen, S. Rudikoff, D. N. Rao, and D. R. Davies, personal communication, see above) show the solvent-exposed tryptophane-33H and -91L residues to be ca. 9.5 Å apart. Therefore, the above disaccharide Gal_2, with its nonreducing residue in subsite A (and so interfacing the protein with its 2- and 3-OH groups), would reach toward Trp-91L (with the second galactose unit) and perturb that tryptophanyl's fluorescence *only* if in the mode shown in Figure 2, and *not* as in Figure 3, where the second galactose unit would project away from Trp-91L and orient more toward the H-chain side of the protein. Thus, the increased maximal fluorescence shown by Gal_2 is in agreement with the binding mode of Figure 2 only. We therefore conclude that the reducing end of the antigen projects toward the L chain as shown in Figure 2. Hence, we will discuss the binding by using the former mode (and will show additional supporting evidence for this deduction). Turning to Table I, we can interpret the

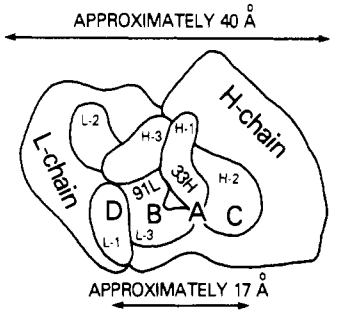


FIGURE 5: Schematic representation of structure of J539 (S. W. Suh, T. M. Bhat, G. H. Cohen, S. Rudikoff, D. N. Rao, and D. R. Davies, personal communication) with the H chain on the right and the L chain on the left, looking into the combining area. The three hypervariable regions for each of the H (H-1, H-2, H-3) and L (L-1, L-2, L-3) chains are shown, and the general location of the solvent-exposed tryptophanyl residues at positions 33H and 91L are indicated. These two residues are ca. 9.5 Å apart. The proposed galactose binding subsites of the antibody are indicated (in decreasing order of affinity) as A, B, C, and D.

binding data as follows: Methyl galactoside **5** binds at subsite A (see Figure 4 for a schematic representation). The methyl galactobioside **7** shows a large increase in affinity and a large increase in the maximally induced protein fluorescence, thus indicating that it is now perturbing both solvent-exposed tryptophanyl residues. We therefore place that bioside, **7**, in subsites A and B (reaching toward the Trp in position 91L). Replacing the 3′-OH in **7** with fluorine, to obtain **8**, brings about a 5–6 times *reduction* in binding affinity (Table I). Since it would be impossible to accommodate the terminal residue bearing the 3′-deoxy-3′-fluoro group of **8** in subsite A (subsite A requires protein contact with the 2- or 3-OH groups), the methyl deoxyfluorobioside **8** shifts to subsites C and A so that the 2- and 3-OH groups (of the methyl galactosyl moiety in **8**) are presented for contact with subsite A, and this allows the 3′-deoxy-3′-fluoro group of **8** to project away from the protein as that galactosyl residue binds in subsite C (Figure 4). If that is so, then that methyl deoxyfluorogalactobioside **8** (*un*like the methyl galactobioside **7**) should cause a maximum fluorescence change in the protein like a *mono*saccharide (it does not come any nearer the tryptophanyl residue at position 91L than does **5** and thus only perturbs the original Trp-33H), namely, ca. 20%. Table I shows that this is so. The evidence above suggests an order of affinity for the subsites as A > B > C. To verify this, we measured the affinity of the methyl galactotrioside **9**. If the above argument is correct, it must follow that this trioside should associate with subsites A, B, and C (with an increased affinity compared to that of **7**) and that the 3″-deoxy-3″-fluoro derivative of **9**, namely, **10**, should bind with the same affinity as **9**, because in the terminal 3″-deoxy-3″-fluorogalactosyl residue associated with subsite C the fluoro group—turned toward the solvent—would not interfere with binding (see Figure 4). Examination of the K_a for **10** (Table I) shows this to be essentially the case. In addition, if the saccharides **9** and **10** bind in the same three subsites A, B, and C, they should show very nearly the same ligand-induced maximal fluorescence change. The values found (Table I) bear this out.

Finally, it must be pointed out that—although the C-6 group of a monogalactoside when binding (to the subsite with highest affinity) appears turned toward the solvent (Ekberg et al., 1983; Glaudemans et al., 1975)—replacement of the 6-OH group with fluorine in methyl galactopyranoside **5** (to give **4**) causes a modest increase in binding strength. This *could* be due to H bonding (Murray-Rust, 1983); however, its magnitude suggests it is not. Also, simply replacing the 6-OH in **5** with hydrogen (to give **6**) causes the same effect (Table I). We have no particular explanation for this observation.

It has been shown that IgA J539 can bind four sequential galactosyl residues of the polysaccharide chain (Jolley et al., 1974). The final protein subsite (D) could be placed on the outside either of C or of B. We prefer the latter, because examination of the structure of J539 (S. W. Suh, T. M. Bhat, G. H. Cohen, S. Rudikoff, D. N. Rao, and D. R. Davies, personal communication) reveals that in the former position a galactosyl residue would be in the solvent. In summary then, the binding of the tetrasaccharide fragment is proposed as shown in Figure 5 with the polysaccharide chain running from the H chain toward the L chain with its reducing end and with the four subsites of the protein each binding to a galactosyl residue, the order of affinity being A > B > C > D. This is the only study of a polysaccharide–monoclonal antibody system using nonbinding moieties on a sequential ligand for the study of their interaction with this degree of detail.

Figures 2 and 5 are fairly schematic, but the structure of J539 Fv (S. W. Suh, T. M. Bhat, G. H. Cohen, S. Rudikoff, D. N. Rao, and D. R. Davies, personal communication) does show a cavity between the H and L domains around the positions 33H and 91L, and that does agree with our earlier observation (Bhattacharjee et al., 1981) that 6-*O*-β-D-galactopyranosyl-D-galactose shows a negative entropy of binding with J539 Fab′, as this can be rationalized by poor contact between the second galactosyl residue and the antibody site, allowing the saccharide to remain largely hydrated.

Acknowledgments

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Registry No. **1**, 79698-13-8; **2**, 31001-23-7; **3**, 51385-54-7; **4**, 35521-88-1; **5**, 1824-94-8; **6**, 1198-82-9; **7**, 77790-33-1; **8**, 93426-89-2; **9**, 77735-11-6; **10**, 89185-98-8; D-fucose, 3615-37-0; D-fucose tetra-*O*-acetate, 93528-14-4; 2,3,4-tri-*O*-acetyl-D-fucopyranosyl bromide, 69483-13-2.

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Interaction of the Fluorescence-Labeled Secretory Component with Human Polymeric Immunoglobulins†

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ABSTRACT: The secretory component (SC) isolated from human milk was labeled with 2 mol of the fluorescent thiol reagent *N*-[7-(dimethylamino)-4-methylcoumarinyl]maleimide (DACM) per mol of SC through the reactive disulfide bond of SC. The binding of the labeled SC to polymeric immunoglobulins was examined by gel filtration by measuring the fluorescence of DACM at 478 nm. The labeled SC was bound to immunoglobulin M (IgM) and its $(Fc)_{5\mu}$ fragment and to dimeric immunoglobulin A (IgA). When the labeled SC was bound to IgM or the $(Fc)_{5\mu}$ fragment, the fluorescence of DACM increased about 30%. By use of this fluorescence change, quantitative studies were made on the equilibrium and kinetics of the reversible interactions of the labeled SC with two IgM proteins and their $(Fc)_{5\mu}$ fragments at pH 7.0 and 25 °C. All the IgM proteins and their $(Fc)_{5\mu}$ fragments had one binding site per mole of polymers. The affinity constant (6×10^8 M^{-1}), the association rate constant (7×10^7 M^{-1} min^{-1}), and the dissociation rate constant (0.1 min^{-1}) of one IgM were different from those of the other IgM (1.7×10^9 M^{-1}, 1.0×10^8 M^{-1} min^{-1}, and 0.06 min^{-1}, respectively). However, the values for the $(Fc)_{5\mu}$ fragments of the two proteins were the same (1.9×10^9 M^{-1}, 1.1×10^8 M^{-1} min^{-1}, and 0.06 min^{-1}, respectively) and were very similar to those of the IgM with the higher affinity constant. These results suggest that, although SC is bound to Fc regions of the IgM molecule, Fab' regions influence the interaction so as to weaken the affinity and the extent of this influence differs in different IgM proteins.

The secretory component (SC)[1] is a glycoprotein that is synthesized by a variety of glandular epithelial cells (Lamm, 1976; Cunningham-Rundles, 1978) and hepatocytes (Zvenbergen et al., 1980). It is found in external secretions associated with polymeric IgA and IgM and also in the free form. Biochemical and cytochemical studies (Cunningham-Rundles, 1978; Brandtzaeg, 1981; Nagura et al., 1979; Takahasi et al., 1982) have shown that SC functions as a receptor in the transcellular transport of polymeric immunoglobulins across epithelial cells and hepatocytes. Subsequently, SC has been found to be synthesized as a larger precursor transmembrane protein which undergoes intracellular processing by cleavage of SC from the precursor during transcellular transport (Mostov et al., 1980; Kühn & Kraehenbuhl, 1981; Mostov & Blobel, 1982). The finding of the precursor of SC has provided a basis for its function as a receptor. More recently, Mostov et al. (1984) have reported the complete primary structure of the precursor of rabbit SC and proposed that SC consists of multiple immunoglobulin-like domains.

The association of polymeric immunoglobulins with the receptor SC molecule is essential for their transport, and there have been many studies of the interaction of SC with polymeric IgA and IgM [reviewed by Brandtzaeg (1981)]. Results have shown that while the interchain disulfide bonds are important for stabilizing the human SC-IgA complex, the specific noncovalent interactions between SC and the Fc regions of J chain containing polymeric IgA and IgM, with an affinity constant of about 10^8-10^9 M^{-1}, reflect the potential receptor

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[1] Abbreviations: SC, secretory component; IgM, immunoglobulin M; IgA, immunoglobulin A; DACM, *N*-[7-(dimethylamino)-4-methylcoumarinyl]maleimide; DACM-SC, SC in which one disulfide bond is reduced and is coupled with 2 mol of DACM; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

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