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Complex Carbohydrate–Lectin Interaction at the Interface: a Model for Cellular Adhesion. II. Reactivity of both the Oligosaccharide Chain and Sugar-binding Domain of a Glycoprotein Lectin

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We describe studies of a new model cell adhesion system involving liposomes bearing lectins and the glycosphingolipid, asialomonosialoganglioside (asialoGM_1). The model provides a simple analysis of experimental data to elucidate the mechanism of heterophilic cell–cell adhesion mediated by multiple protein–carbohydrate interactions. Phospholipid vesicles bearing the fatty acid conjugate of a glycoprotein lectin from *Ricinus communis* (RCAI vesicle) are shown to react with vesicles bearing the fatty acid conjugate of Concanavalin A (Con A) and asialoGM_1 (Con A vesicle). The kinetics of aggregation and monosaccharide-induced disaggregation of the two types of vesicles were followed by monitoring the time-dependent change in turbidity. Depending on the surface density of the asialoGM_1, 40–60% of the resulting precipitin complex was dissociable only in the presence of both RCAI-specific galactose and Con A-specific α-methyl-D-mannoside. Results indicate simultaneous participation of both the saccharide-binding domain and carbohydrage sequence of RCAI, a model cell adhesion molecule, to stabilize the encounter complex by two types of interactions. These findings support the possibility of stable cell–cell adhesion *in vivo* occurring via interactions between cell adhesion molecules on apposing cell-surface membranes.

INTRODUCTION

Cell adhesion molecules (CAM) are cell-surface glycoproteins. Carbohydrate chains modulate adhesion as found in the case of the neural cell adhesion molecule (NCAM) where the extent of sialylation affected the rate of homophilic NCAM–NCAM interaction when isolated at two different developmental stages (Hoffman and Edelman, 1983). The cDNA-derived amino acid sequences show multiple structural domains in CAM (Edelman, 1988; Stoolman, 1989), one of which is lectin-like. Amino acid sequences of adhesion molecules controlling lymphocyte migration e.g., MEL-14 antigen, GMP-140 and ELAM-1 (Bowen *et al.*, 1990; Johnston *et al.*, 1989; Hession *et al.*, 1990) reveal a lectin-like domain. A large body of data implicates a family of receptors, the selectins (lectin-complement binding CAM), in the initial interactions between leukocytes and vascular endothelia (Lawrence and Springer, 1991; Watson *et al.*, 1991). Direct functioning of the lectin-like domain is first evidenced in the lymphocyte homing receptor, where an anionic polysaccharide is shown to play a role in lymphocyte adhesion (Bowen *et al.*, 1990; Coombe and Rider, 1989). Recently, the three members of the selectin receptor family have been shown to recognize a common carbohydrate epitope, the sialylated Lewis^x oligosaccharide (Foxall *et al.*, 1992).

We have designed a liposomal system bearing complementary lectin and carbohydrate molecules to study the molecular mechanism of heterophilic cell–cell adhesion. The kinetics of the aggregation of these two types of vesicles were interpreted in the light of cellular adhesion mediated by a single specific protein–carbohydrate interaction (Chakrabarti and Podder, 1990). The present study shows the reactivity of both the carbohydrate-reactive domain and the oligosaccharide chain of the glycoprotein lectin, a model CAM, involved in multiple interactions in liposomes.

EXPERIMENTAL

Dimyristoylphosphatidylcholine (DMPC), sodium cholate, octyl-β-D-glucopyranoside, galactose (Gal), α-methyl-D-mannopyranoside (Me-Mann), cholesterol and latex particles (305 nm diameter) were obtained from Sigma (St Louis, MO, USA). DEAE-Sephadex, Sephadex G-100, Sepharose 4B and Sephacryl S-1000 were obtained from Pharmacia Co. (Uppsala, Sweden). The other chemicals used were all of analytical grade and locally available.

Asialomonosialoganglioside (asialoGM_1) was prepared by hydrolysing a mixture of di- and trisialogangliosides with 1.0 M formic acid and collected after passing through the same DEAE-Sephadex column with methanol as discussed earlier (Chakrabarti and Podder, 1990).

Ricinus communis agglutinin (RCAI) was isolated from castor seeds using DEAE-Sephadex followed by

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Abbreviations used: CAM, cell adhesion molecules: NCAM, neural cell adhesion molecule; DMPC, dimyristoylphosphatidylcholine; Gal, galactose; Me-Mann, α-methyl-D-mannopyranoside; asialoGM_1, asialomonosialoganglioside; RCAI, *Ricinus communis* agglutinin; Con A, Concanavalin A; PNA, peanut agglutinin.

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Table 1. Molecular weights and diameters of lectin vesicles by gel filtration on Sephacryl S-1000

Vesicles	Diameter (nm)	Molecular weights (Da)	K_D[a]
Latex particle	305	—	0.063
Sonicated vesicles of egg yolk lecithin	25	2.06×10^6	0.704
DMPC with 33% cholesterol prepared by the dialysis of octylglucoside	180	1.20×10^8[c]	0.327
DMPC with fatty acid conjugate of RCAI	300	2.21×10^8	0.068
DMPC with fatty acid conjugate of Con A alone or with asialoGM$_1$	150	9.80×10^7	0.423

[a] K_D is the partition coefficient given by $(V_e - V_0)/(V_t - V_0)$, where V_0 is the void volume (16 mL) and V_t is the total volume (38 mL) of the Sephacryl S-1000 column and V_e is the elution position of the sample.
[b] From Huang (1969)
[c] Determined from the plot of diameter and molecular weight data published by Zumbuehl and Weder (1981)

Sephadex G-100 column chromatography (Lappi *et al.*, 1978). Concanavalin A (Con A) was isolated from Jack bean meal by affinity chromatography (Agrawal and Goldstein, 1967). Peanut agglutinin (PNA) was isolated from locally available seeds (*Arachis hypogaea*) using lactamylsepharose 4B affinity column chromatography (Maiti and Podder, 1989). Protein concentrations were determined spectrophotometrically at pH 7.4 in a buffer containing 10 mM Tris–HCl, 0.1 M NaCl using an absorbance ($A^{1\%}_{280\,nm}$) of 11.8 for RCAI, 11.4 for Con A and 7.7 for PNA.

Preparation of the conjugated lectins and the two types of vesicles have been described previously (Chakrabarti and Podder, 1990). Briefly, the lectins were conjugated with stearoyl chloride in a buffer containing sodium cholate (2%). Phospholipid vesicles were made by the dialysis of cholate from a mixture of the conjugated proteins and lipids. AsialoGM$_1$ was cosolubilized with DMPC in chloroform and dried under a stream of nitrogen.

The rate constants were determined from the slope of the linear semilogarithmic plots of the change in

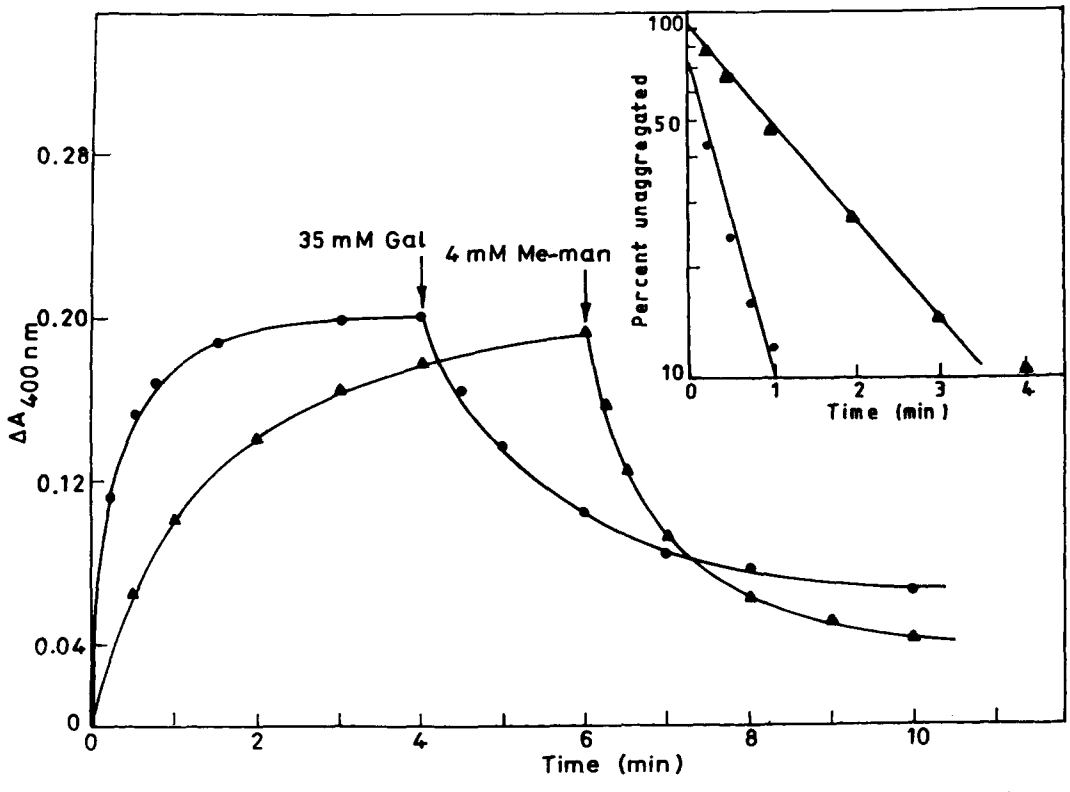

Figure 1. Kinetics of aggregation of two types of vesicles. Con A vesicles bearing 0.1% fatty acid conjugate of Con A and 25% asialoGM$_1$ with the lectin vesicle bearing fatty acid conjugate of PNA (●) and aggregation between vesicle-bound Con A and RCAI (△). Arrow indicates addition of dissociating monosaccharidess. The inset shows the semilogarithmic plot of the aggregation profile. Concentrations of the reacting components are given in Table 2.

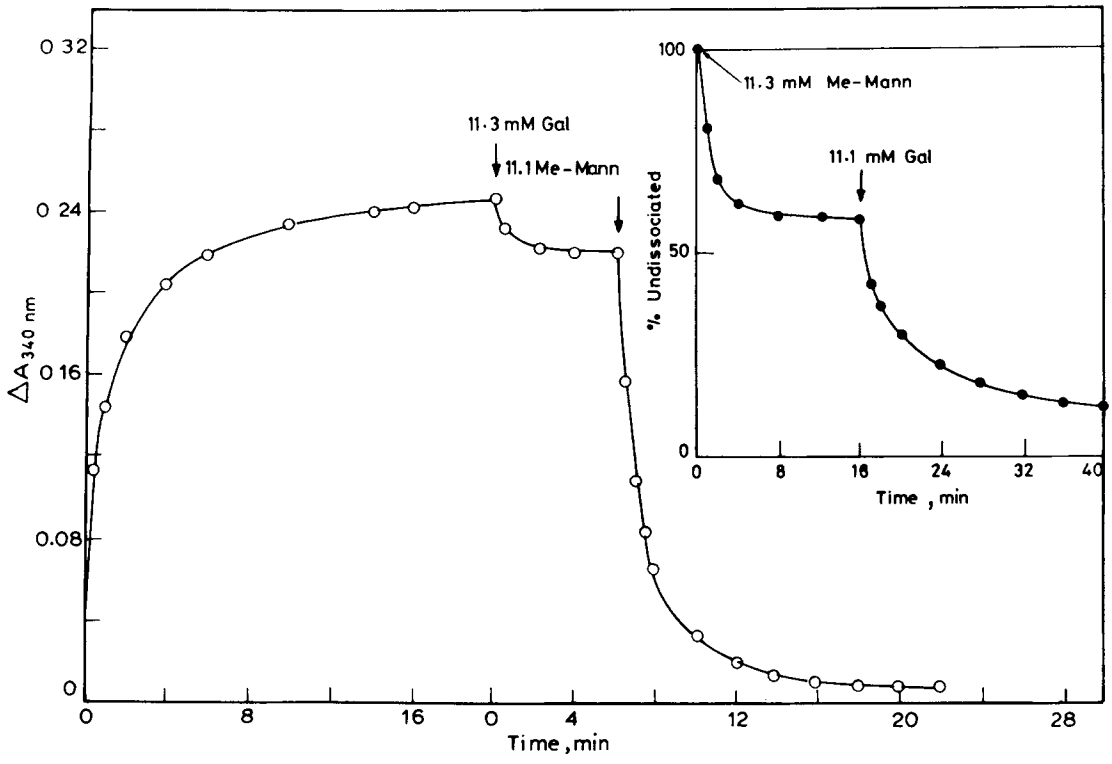

Figure 2. Kinetics of aggregation between RCAI vesicles and Con A vesicles bearing 5% asialoGM$_1$ in addition to 0.1% Con A. Arrow indicates addition of dissociating monosaccharides. Inset shows the dissociation profile when the order of addition of the two monosaccharides was reversed. In both cases the asialoGM$_1$ content of the vesicles is the same.

absorbance with time both for the association and sugar-induced dissociation processes under pseudo-first order reaction conditions (Chakrabarti and Podder, 1990).

The change in turbidity was measured in a Shimadzu UV-190 double beam spectrophotometer equipped with a U-135 recorder at a wavelength of 340 m or 400 nm. The 90° light scattering was measured in a Hitachi-650-60 spectrofluorimeter at 400 nm.

Characterization of vesicles

Average diameters and molecular weights of RCAI and Con A vesicles (with and without asialoGM$_1$) were determined by gel filtration on a Sephacryl S-1000 column (48 × 1 cm) according to the method described by Reynolds *et al.* (1983). The liposome-containing fractions were detected by measuring the 90° light scattering in a spectrofluorimeter. The column was calibrated with (i) latex particles (305 nm diameter) and (ii) sonicated vesicles of egg yolk lecithin (25 nm diameter). The sonicated egg phosphatidylcoline vesciles (2.06×10^6 Da), as described by Huang (1969), and DMPC vesicles containing 33% cholesterol (1.2×10^8 Da) were used as the molecular weight markers.

Table 1 lists the molecular weights and diameters of the different vesicles determined from the elution profile on the Sephacryl S-1000 column. Liposomes with asialoGM$_1$ alone were smaller (30 nm diameter), and were prepared by bath sonication.

For vesicles containnig RCAI and Con A and/or asialoGM$_1$, average diameters were found to be 300 and 150 nm with molecular weights of 2.21×10^8 and 9.8×10^7 Da, respectively.

Table 2. Rate constants of formation (k_f) and dissociation (k_d) of the aggregation reaction between two types of DMPC vesicles containing complementary protein and carbohydrate molecules

Vesicle 1	Vesicle 2	k_f ($\times 10^{-7}$) [/M (ves.) s]	k_d ($\times 10^3$)[a] (/s)	K_{eq} ($\times 10^{-10}$)[b] (/M)
RCAI (2.1 μM)	AsialoGM$_1$ (12.4 μM)	1.71	2.090	0.82
RCAI (0.5 μM)	Con A (1.5 μM)	1.26	0.284	4.44
PNA (0.29 μM)	AsialoGM$_1$ (185 μM) Con A (1.3 μM)	2.62	0.245	10.69

[a] The k_d values were calculated using values for the equilibrium constant of 1.4×10^4/M for the Con A–mannose system (Podder *et al.*, 1974) and 0.94×10^3/M (Neurohr *et al.*, 1981) for the PNA–Gal system. In all cases, the densities of the conjugated proteins were kept constant at 0.1% with respect to DMPC.

[b] $K_{eq} = k_f/k_d$.

Table 3. Extent of sugar-induced dissociation of aggregates formed by the RCAI vesicle and Con A (asialoGM$_1$) vesicle

RCAI vesicle	Con A vesicle	Concentration of sugars Me-Man (mM)	Gal (mM)	Dissociation[a] (%)
RCAI (0.25 μM)	Con A (1.5 μM)	11.3	—	44.0 (34)
	AsialoGM$_1$	11.1	11.1	48.0 (140)
	(105 μM)	—	11.3	10.0 (<10)
		11.1	11.1	88.0 (58)

[a] Values in parentheses are $t_{0.5}$ values

RESULTS

Con A did not hinder accessibility of asialoGM$_1$ in phospholipid vesicles (Con A vesicle). They reacted with vesicles bearing the fatty acid conjugate of PNA, a Gal-binding protein, but not a glycoprotein, instead of RCAI. Figure 1 shows the time-dependent change in absorbance due to the aggregation and monosaccharide-induced disaggregation of (i) PNA vesciles and Con A vesicles bearing asialoGM$_1$ and (ii) RCAI vesicles with the Con A vesicle in the absence of asialoGM$_1$. The presence of 0.1 mol% Con A in the Con A vesicle did not mask asialoGM$_1$s, and PNA vesicles reacted with them giving rise to a precipitin reaction. The addition of Gal decreased absorbance dissociating >80% of the total complex. Similarly vesicle-bound RCAI and Con A also caused precipitation, dissociable by Me-Mann showing the specificity of interaction (Fig. 1). The semilogarithmic plot of these associations was characterized by a single time constant as shown in the inset in Fig. 1.

Figure 2 shows the change in absorbance with time upon aggregation of the RCAI vesicle and the Con A vesicle containing 5 mol% asialoGM$_1$ with respect to DMPC and the monosaccharide-induced disaggregation. The time-dependent disaggregation profiles, induced by the individual monosaccharides, Gal and Me-Mann, show remarkable differences in the kinetic properties. Even though asialoGM$_1$ is present in a 50-fold excess over Con A, only 10–15% of the total complex is dissociable by Gal. The inset shows the differences both in the extent and time constants of the dissociation processes when the order of the addition of the respective monosaccharides was reversed. Moreover, the semilogarithmic plot of the association process is biphasic (data not shown) indicating the formation of two different types of complexes. Table 2 summarizes the rat constants and equilibrium constants of associations and monosaccharide-induced dissociations for the individual RCAI–Con A, PNA–asialoGM$_1$ and RCAI–asialoGM$_1$ systems.

From the differences in the extent of dissociation by Gal and Me-Mann two types of complexes could be detected, as shown in Fig. 3 One type could be dissociated by either of the individual lectin-specific sugars, Me-Mann and Gal (Type I), and the other only in the presence of both (Type II). The exent of sugar-induced dissociation and the time (in seconds) corresponding to 50% total dissociation, $t_{0.5}$, brought about by the individual specific monosaccharide, is also remarkably different (Table 3).

The extent of formation of the Type II complex was determined from the differences of the extent of Gal-induced dissociation in the presence of methyl mannoside from the extent of formation in its absence. The extent of formation could also be determined from the difference of the extent of methyl mannoside-induced dissociations by reversing the order of additions of the respective monosaccharides (Table 3). The formation of Type II complexes, listed in Table 4, is the mean of two such determinations. Table 4 also shows the increase in Type II complex formation with increase in density of asialoGM$_1$ in the Con A vesicle.

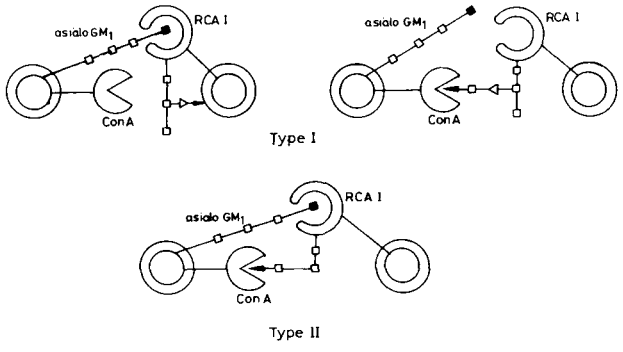

Figure 3. Schematic representation of the two types of complexes as detected by the distinct differences in the kinetics of monosaccharide-induced dissociation.

Table 4. Density-dependent formation of Type II complexes

RCAI vesicle	Con A vesicle	Density of asialoGM$_1$ (%)	Type II complex (%)
RCAI (0.25 μM)	Con A (1.5 μM) AsialoGM$_1$ (105 μM)	5.0	40.0
RCAI (0.26 μM)	Con A (1.45 μm) AsialoGM$_1$ (360 μM)	10.0	45.0
RCAI (0.30 μM)	Con A (1.1 μM) AsialoGM$_1$ (540 μM)	16.0	60.0

DISCUSSION

The main objective in using liposomes carrying surface-bound complementary protein and carbohydrate molecules is to demonstrate the formation of multiple bonds leading to stable adhesion between two such surfaces. The formation of multiple bonds that strengthen adhesion between two apposing surfaces carrying complementary molecules could be achieved in two ways. More than one valency of the multifunctional ligand might be involved in binding as shown in the present study or a number of monovalent protein–carbohydrate bonds could form in order to stabilize the adduct (Chakrabarti and Podder, 1990). The model allows the simple estimation of contributions of indivi-

dual carbohydrate-specific interactions, which is relevant for understanding the molecular mechanism of such processes.

Podder *et al.* (1974) showed that the mannose-rich oligosaccharide chains of RCAI were reactive to Con A giving rise to a precipitin reaction. We have studied the kinetics of the same reaction when RCAI and Con A were bound to DMPS liposomes. We added excess asialoGM$_1$ in Con A vesicles in order for the sugar-binding domain of RCAI to react. In the presence of 5–15 mol% asialoGM$_1$ in Con A vesicles, an aggregate is formed where both the carbohyrate chains and the carbohydrate-reactive domain of the RCAI molecule are involved simultaneously in the formation of bonds. Figure 3 shows the possibility that RCAI can act as a model CAM in a reconstituted system by forming an adduct involving both the functional domains, the oligosaccharide chain and the carbohydrate-binding domain (Type II).

Assuming uniform distribution of Con A and asialoGM$_1$ on the spherical surface of the Con A vesicle bearing asialoGM$_1$, we have calculated the surface density of asialoGM$_1$ to be 10^5 per μm^2 and 2×10^3 per μm^2 for Con A, and the average distance between Con A and asialoGM$_1$ to be 14 nm. This distance is too large for the formation of the Type II complex. This can only be achieved by the lateral diffusion and redistribution of components subsequent to the formation of the encounter complex. The formation of 35–40% Type II complex was favoured by the facile stereochemical orientation of Con A anmd asialoGM$_1$ on the surface of the vesicle.

An estimate of the relative stability of the bonds formed (Chakrabarti and Podder, 1990) is obtained from the ratio of k_f and k_d (see Table 2). The ratio (K_{eq}) is $\sim 10^{10}$–10^{11}/M in the formation of Type I complexes mediated by the individual complex carbohydrate–lectin interactions indicating the formation of multiple bonds.

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