Congregation of Gangliosides at the Junction between Two Model Membranes†

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ABSTRACT: The diversity and distribution of gangliosides in vertebrate tissue suggests an important role in cellular recognition. Two types of experiments are reported to test the hypothesis that gangliosides can congregate to form an adhesive junction between two membranes. First, to monitor ganglioside distribution and mobility in different regions of two large spherical bilayer membranes, fluorescent derivatives of natural gangliosides were synthesized. Second, the cation carrier nonactin was used as a conductance probe to measure the membrane surface potential, which would be altered if there were a redistribution of the charged gangliosides. These studies were conducted in large spherical artificial membranes made from egg phosphatidylcholine or oleoylpalmitoylphosphatidylcholine with 0–12 mol % bovine brain gangliosides dissolved in *n*-decane. The fluorescent gangliosides utilized were lucifer yellow adducts to the sialic acids (LY-gangliosides) or a *cis*-paranaric acid substitution of the *N*-acyl moiety in the ceramide portion of gangliosides GM1 and GD1a (paranaryl-GM1 and paranaryl-GD1a). The polarized fluorescence from the adhesive junction between two membranes containing LY-gangliosides or either paranarylganglioside was compared to that in nonadhesive regions. For LY-gangliosides, total fluorescence in the junction decreased with time, possibly due to electrostatic repulsion of this highly charged derivative. For paranarylgangliosides, fluorescence in the junction increased 7-fold with time, suggesting congregation of this ganglioside. In both cases, a measure of rotational mobility, fluorescence anisotropy, increased dramatically, about 2-fold, as expected for restricted mobility of adhesive compounds. Independent evidence for congregation of charge-bearing gangliosides was found with the conductance probe nonactin. In marked contrast to no change in adhesive membranes without gangliosides, with gangliosides the increase in nonactin conductance across the junction suggested a doubling of the surface potential in this region, consistent with ganglioside congregation. A total of seven independent experiments now suggest a structural rearrangement of gangliosides in an adhesive junction. A molecular model for ganglioside-mediated contact sensation in biological membranes is presented.

The diversity of gangliosides, glycosphingolipids containing sialic acid, their enrichment in the brain, and their surface localization suggest involvement in cellular recognition phenomena (Hakomori, 1981). Intercellular adhesion is a necessary aspect of this unproven proposition. In order to test the direct interaction of carbohydrates in membrane adhesion, we devised a model membrane system composed of large spherical bilayers attached to the tips of two syringes (Brewer & Thomas, 1984, 1986). When the membranes contain a mixture of brain gangliosides at low concentration, initial contact leads spontaneously to an enlarged circular zone of adhesion of the two bilayers. Associated with the adhesion is an increase in electrical conductance between the interiors of the two membrane spheres; the junctional region forms a low-resistance pathway for ionic mobility. While adhesion is still observed without gangliosides, i.e., with phosphatidylcholine alone, the junctional conductance does not change. These ganglioside membranes could provide a simple mechanism of ganglioside-mediated contact sensation.

Two alternative mechanisms could explain this ganglioside-mediated contact sensation. Adhesion of ganglioside-containing membranes could involve direct interactions of gangliosides in the junction or, alternatively, the segregation of gangliosides away from the junctional region (Figure 1). Either mechanism could explain the observed increase in junctional conductance. This is because (A) membranes without gangliosides have higher conductance than those with gangliosides (Brewer & Thomas, 1984) or (B) new ganglioside structures could form in the junction that lower the overall energy barrier for ion flux across two membranes. Recent experiments with the fluorescent probe PRODAN reported a lower polarity in the junctional region only when gangliosides were included, suggesting congregation of gangliosides in the junction (Brewer, 1992). To test this proposal of carbohydrate-mediated adhesion more directly, fluorescent derivatives of gangliosides were utilized in this report to look for an increase in fluorescence in the junctional region and a restriction in mobility that would be expected for compounds in adhesion.

Also reported here are independent measurements of the concentration of the charge-bearing gangliosides in the junctional region. These studies were made possible by incorporating the ion carrier nonactin into our membranes. Since the surface potential, which is directly related to charge density, controls the rate-limiting step for potassium filling the pore in nonactin, conductance across these membranes is proportional to the concentration of gangliosides in the membrane (McDaniel et al., 1984). Therefore, we studied nonactin-mediated conductance across membranes to infer surface concentrations of charged gangliosides.

Experimental Procedures

The apparatus and materials for forming and monitoring large spherical artificial membranes have been described (Brewer & Thomas, 1984; Brewer, 1992). Membranes were formed in buffer containing 100 mM NaCl, 10 mM KCl, 1 mM $CaCl_2$, 1 mM Tris-HCl, pH 7.4. Lucifer yellow gang-

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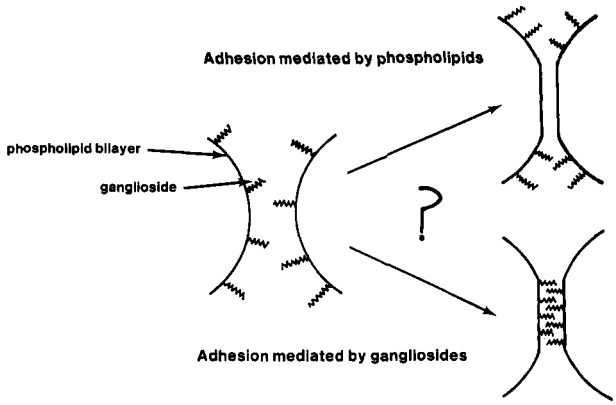

FIGURE 1: Does the adhesion of two bilayers containing gangliosides result from the congregation or dispersion of gangliosides in the junction?

liosides were prepared from lucifer yellow CH (Aldrich, Milwaukee, WI) and mixed bovine brain gangliosides as previously described (Spiegel, 1985). LY-ganglioside fluorescence was excited from the 442-nm line of a He–Cd laser (Liconix, Sunnyvale, CA) and monitored at 530 nm. Nonactin was the generous gift of S. J. Lucania (Squibb 15859, Princeton, NJ). It was diluted 1:1000 into the aqueous phase from a 1 mM ethanolic solution (McDaniel et al., 1984). FCCP [carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazine, Aldrich, Milwaukee, WI] was used at the same concentrations.

For synthesis of paranaryl-GD1a, GD1a was isolated by preparative scale thin-layer chromatography from 90 mg of mixed bovine brain gangliosides by development with $CHCl_3/CH_3OH$/15 mM aqueous $CaCl_2$ (65:35:8). GD1a (20 mg) was deacylated in methanolic 1 M KOH for 20 h at 100 °C (Neuenhofer et al., 1985). The product was N-acetylated with Fmoc-HCl in *n*-hexane and selectively deblocked in liquid ammonia (Neuenhofer et al., 1985). *cis*-Paranaric acid (Molecular Probes, Eugene, OR) was esterified with dicyclohexylcarbodiimide and *N*-hydroxysuccinimide (Schwarzmann et al., 1983). The lyso-GD1a was re-N-acylated with a 5-fold excess of *N*-succinimidylparanaroate in triethylamine, followed by reacetylation of the Fmoc-carbohydrates with acetic anhydride (Neuenhofer et al., 1985). The slower migrating fluorescent band extracted from the thin-layer chromatography plate showed fluorescent emission at 415 nm when excited at 318 nm in an Amino-Bowmen spectrofluorimeter, identical to that of *cis*-paranaric acid. The UV absorption spectrum of paranaryl-GD1a was also identical to that of the fatty acid. The paranaryl-GM1 was synthesized using the method described by Sonnino et al. (1985) and modified by Acquonti et al. (1986). For deacylation and blocking, 120 mg of GM1 (FIDIA) was dissolved in butan-1-ol (11 mg/mL), mixed with tetramethylammonium hydroxide in water, and refluxed at 100 °C for 13 h. After dialysis against distilled water for 2 days at 4 °C, the product was lyophilized and redissolved in methanol. Deacylated ganglioside was separated by preparative HPTLC using the solvent system chloroform/methanol/2.5 M ammonia (60:40:9). *cis*-Paranaric acid (Molecular Probes) was dissolved in tetrahydrofuran and then activated by triethylammonium and ethyl chloroformate and added to deacylated GM1 dissolved in tetrahydrofuran/water (20:1). The products of the reaction were separated by preparative HPTLC in chloroform/methanol/water (60:35:5). Sialic acid residues were re-N-acetylated with dry methanol and acetic anhydride. Products were evaluated by HPTLC using chloroform/methanol/2.5 M ammonia (60:40:9). Lyophilized paranaryl-GM1 was dissolved in chloroform/methanol (2:1) and stored at −20 °C under nitrogen. Fluorescence was excited by the 325-nm line of a He–Cd laser (Liconix) reduced to 2.4 mW to minimize bleaching.

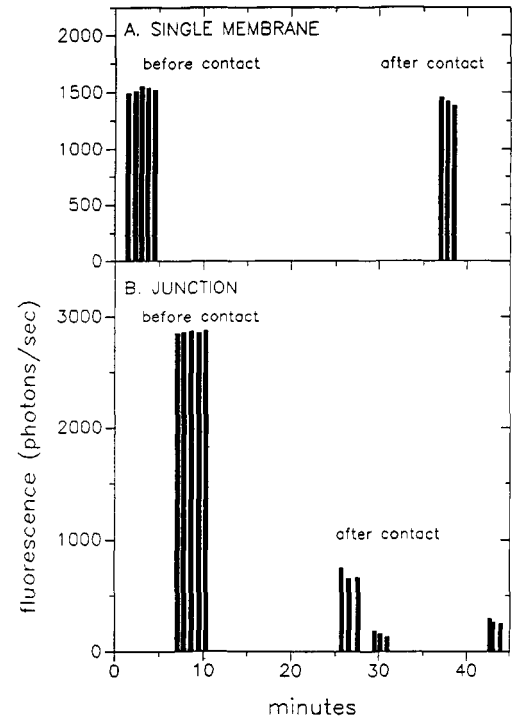

FIGURE 2: Fluorescence of LY-gangliosides monitored in a single membrane (A) and the junctional region before contact (B). At 15 min, the two membranes were moved into contact. Following adhesion, the junction was again monitored for fluorescence (25–31 min), followed by recordings from a nonadherent region of a single bilayer (37–39 min) and a return to the junctional region (42–45 min).

As a measure of rotational mobility, the fluorescence anisotropy was calculated as $(I_{VV} - GI_{VH})/(I_{VV} + 2GI_{VH})$, where I_{VV} and I_{VH} are the observed fluorescence intensities excited by the vertically polarized He–Cd laser and measured through a vertically or horizontally oriented polarizer and G is a correction factor determined beforehand for differential sensitivity of the photomultiplier and optical system to the two polarizations (Lakowicz, 1983). Mean anisotropies were evaluated for statistical significance by Student's two-tailed t-test with rejection of the null hypothesis at $p < 0.05$.

Results

Fluorescnece of Lucifer Yellow GD1a (LY-GD1a). On the basis of the ease of labeling ganglioside GM1 with lucifer yellow and the ability of this fluorescent ganglioside to still act as a receptor for cholera toxin (Spiegel, 1985), we initially investigated lucifer yellow labeled GD1a in our model membranes as a probe of ganglioside mobility and distribution. GD1a causes membranes to adhere more rapidly than GM1 (Brewer & Thomas, 1986). Figure 2 displays the time course from one of several such experiments in which fluorescence was monitored from the junction of two such membranes (Figure 3). The initial reading on the two membranes in close proximity before adhesion (Figure 2B) was roughly double that on a single membrane (Figure 2A). This validates the linearity of the measurement. The membranes were next brought into contact from which an adhesive junction developed, as usual. However, now the fluorescence in the junction was found to decline 90% in 15 min (Figure 2B). A control measurement on a nonadhering portion of a single membrane was the same

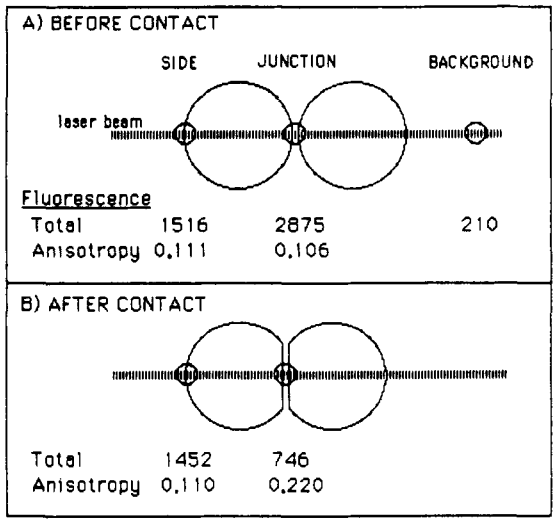

FIGURE 3: Fluorescence of LY-gangliosides measured from different regions of two membranes containing 4 mol % gangliosides and 0.4 mol % LY-gangliosides.

as that before adhesion (Figure 2A). Measurements of fluorescence anisotropy were made at the same time to provide information about the rotational mobility of the LY-ganglioside (Figure 3). After adhesion, the mean anisotropy from the region of the single membrane was 0.111 ± 0.014. In the junction, the mean anisotropy was significantly higher, 0.161 ± 0.021 ($p < 0.02$). This value is probably an understatement of the anisotropy of the junctional lamellae of the two membranes, since the inner lamellae would not be directly involved in adhesion. A simple linear accounting would suggest an anisotropy for the molecules in the junctional lamellae of 0.21, double that in the single membrane and halfway to the theoretical maximum of 0.4.

Fluorescence of Paranarylgangliosides. Because the LY-ganglioside is derivatized in the carbohydrate region, which may interfere with normal interaction of the oligosaccharides, we synthesized a fluorescent ganglioside derivative in which the oligosaccharide is not altered. We exchanged the N-acyl moiety on the sphingosine base with a fluorescent polyene fatty acid, *cis*-paranaric acid (Figure 4). Measurements similar to those for LY-ganglioside were performed with paranaryl-GM1 and paranaryl-GD1a. Again the fluorescence of two membranes in close apposition before adhesion was approximately double that of a single membrane. After adhesion (Figure 5), the total fluorescence in the junction increased exponentially with time with an extrapolated maximum at least 7 times the initial fluorescence. Also, the previously reported increases in capacitance and conductance were observed (data not shown; Brewer & Thomas, 1984). The increase in fluorescence suggests that gangliosides are congregating in the adhesion zone. In further support of aggregation, the paranaryl-GM1 fluorescence anisotropy in the junction rose from 0.14 ± 0.05 (mean ± SD, $n = 12$) to 0.23 ± 0.04 ($n = 15$). In a similar experiment with paranaryl-GD1a, a similar increase was observed (data not shown). This suggests relative immobilization of the fluorescent ganglioside in the adherent lamellae of the junctional bilayers.

Nonactin as a Probe for Surface Potential. If gangliosides congregate in the junction between two adherent membranes, then the increased surface density of the charged sialic acids would cause an increase in the membrane surface potential. This potential can be measured with the ion carrier nonactin, which conducts potassium across the membrane in proportion to surface potential (McDaniel et al., 1984). To calibrate the nonactin conductance, we made single membranes with 0, 4, and 12 mol % gangliosides to impart defined surface potentials. As expected, the conductance increased with increased gangliosides (Table I). Calibration (Table I, legend) produced a change in surface potential of −2 to −4 mV/mol % GD1a over the range of 4–12 mol %. These values compare favorably to the values of −1.3 to −2.4 mV/mol % GM1 reported by McDaniel et al. (1984), especially considering that the charge on GD1a is double that on GM1.

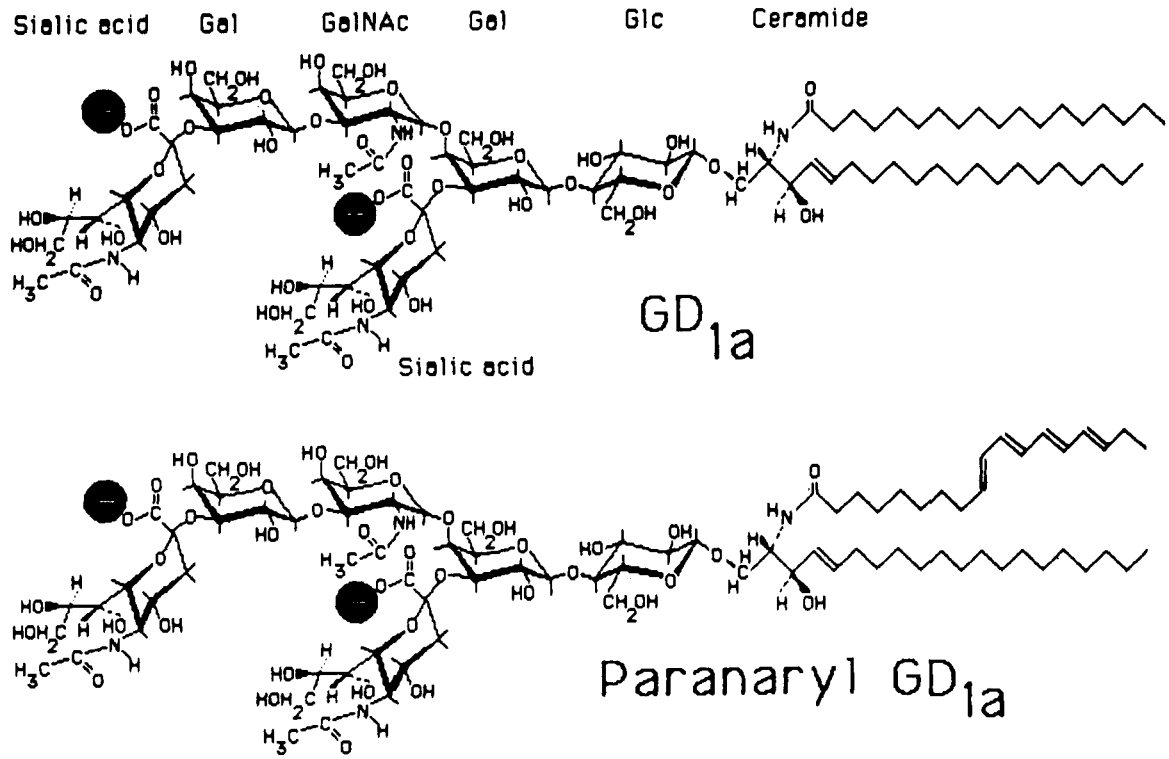

FIGURE 4: Structure of ganglioside GD1a and *cis*-paranaric acid derivative of GD1a. Note the unchanged carbohydrate structure and the minimal perturbation to the ceramide moiety.

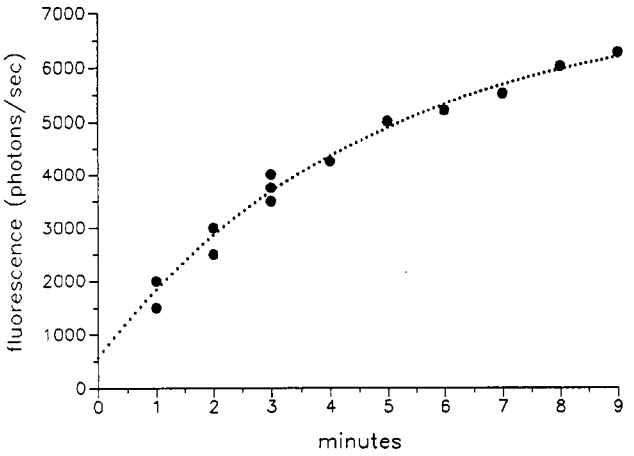

FIGURE 5: Increase in fluorescence of paranaryl-GM1 in the junction between two membranes containing 4 mol % GM1. One of these membranes also contained 0.5 mol % paranaryl-GM1. The dotted curve is a nonlinear regression fit to the equation photons/s = 7110 − 6540 exp(−0.215 min), with a coefficient of determination of 0.983. Multiple points at a given time are from three different experiments.

Table I: Nonactin K^+ Conductance as a Probe for Membrane Surface Potential, $\Delta\psi$[a]

	mol % gangliosides		
	0	4	12
nonjunction (nS/mm^2)	39 ± 6	85 ± 11	111 ± 6
$\Delta\psi$ (mV)		−19	−26
junction (ns/mm^2)	35	153	318
ratio (junction/nonjunction)	0.9	1.8	2.9
increase in $\Delta\psi$ (mV)	+0.3	−15	−26

[a] $\Delta\psi = -(RT/F) \ln (G/G_0) = -25$ mV ln (G/G_0) where G_0 = conductance for reference membrane (e.g., without gangliosides) and G = conductance for membrane containing gangliosides.

With two membranes made with either 0, 4, or 12 mol % gangliosides, the nonactin-mediated junctional conductance was compared to that across single regions of the bilayer (Table I). As expected, the adhesion of two membranes made from phosphatidylcholine without gangliosides produced no significant increase in junctional conductance. In contrast, the junction formed from the adhesion of two membranes containing 4 mol % gangliosides produced nearly a 2-fold increase in conductance. For 12 mol % gangliosides, the junctional increase in conductance was nearly 3-fold. Conversion of conductance values to surface potentials yields a doubling of surface potential in either case. If the adherent lamellae, ψ_J, are considered separately from those on the inside, ψ_0, and the inside surface potential in the junction is the same as that in nonadherent parts of the bilayer, then the total potential averaged over the four lamellae, ψ_T, will be

$$\psi_T = (\psi_j + \psi_0)/2 = 2\psi_0$$

Therefore,

$$\psi_J = 3\psi_0$$

Thus, nonactin conductance suggests that gangliosides congregate in the junctional region of adhesion to an extent 3 times that in the nonjunctional region. This value is surprisingly coincidental with the 3-fold increase in conductance observed across the junction of membranes made with gangliosides without nonactin (Brewer & Thomas, 1984).

As a control for artifacts in the nonactin experiments, we also performed analogous experiments with the anion-conductance probe FCCP (McDaniel et al., 1984). This probe again showed no change in conductance for membranes made without gangliosides. It caused a decrease in conductance across the junction for membranes containing gangliosides as expected for an anion carrier. These results validate the use of these probes to suggest an increase in (negative) surface potential due to congregation of gangliosides.

Discussion

There are now seven independent measurements that suggest a structural change or rearrangement in the adhesive junction between two membranes containing gangliosides: (1) a 3-fold increase in conductance across the junction (Brewer & Thomas, 1984), (2) a 3-fold increase in capacitance across the junction (Brewer & Thomas, 1984), (3) a decrease in polarity or dielectric in the junction (Brewer, 1992) and, from this work, (4) an increase in the anisotropy of LY-ganglioside and (5) paranarylganglioside, (6) an increase in total fluorescence of paranarylganglioside, and (7) a 2- or 3-fold increase in surface potential measured with the conductance probe nonactin.

The dramatic decrease in LY-ganglioside fluorescence in the junction so far below that of a single nonadhesive bilayer merits further consideration. In considering the two mechanisms depicted in Figure 1, a depletion of LY-ganglioside from the junction would explain the data even while underivatized GD1a congregated in the junction. This interpretation is further supported by the increase in anisotropy observed for the diminished amount of probe still in the junction. The depletion could be driven by electrostatic repulsion due to the two extra negative charges on lucifer yellow itself. A second possibility seems less likely. If diffusion of LY-GD1a is restricted, then the decreased fluorescence might be explained by photobleaching of the probe or an increased dynamic quenching (decreased fluorescence lifetime). Photobleaching appears unlikely, since continued exposure did not continue to reduce the fluorescence. Increased dynamic quenching could by itself explain the increased anisotropy without more ordering, but this would be inconsistent with the paranaric acid results where both an increase in anisotropy and intensity were observed. Because the lucifer yellow moiety is so large and alters the charge in the region expected to participate in binding, further experiments with this derivative are not recommended for carbohydrate-mediated interactions.

The weight of these experiments suggest congregation of gangliosides in the junction between two membranes. The finding with nonactin that the surface potential changes by a factor of 3 in the junctional membranes, whether the membranes are made from 4 or 12 mol % gangliosides, may relate to the equilibrium between bound and free gangliosides. The finding of a 2-fold increase in steady-state anisotropy suggests relative rotational immobilization within the excited state lifetime of the paranarylgangliosides. One intriguing possibility is that the acyl chains of the ceramide interdigitate to form a collapsed bilayer (Figure 6). Precedent for such a structure has been reported for sphingomyelin (Levin et al., 1985) and for lysophosphatidylcholine (Hui & Huang, 1986). The distribution of van der Waals interactions between molecules anchored in two lamellae in an interdigitated structure would reinforce structure with greatly reduced rotational mobility.

Given that the gangliosides congregate in the junction, what is the thermodynamic driving force? The oligosaccharides at the membrane surface are heavily hydrated and charged. Perhaps electrostatic repulsion is overcome at the inferface by protonation of the ganglioside carboxyls or complex coordination with calcium ions. Calcium does increase the rate of adhesion of membranes containing gangliosides (Brewer & Thomas, 1984). Probes sensitive to interfacial pH may detect

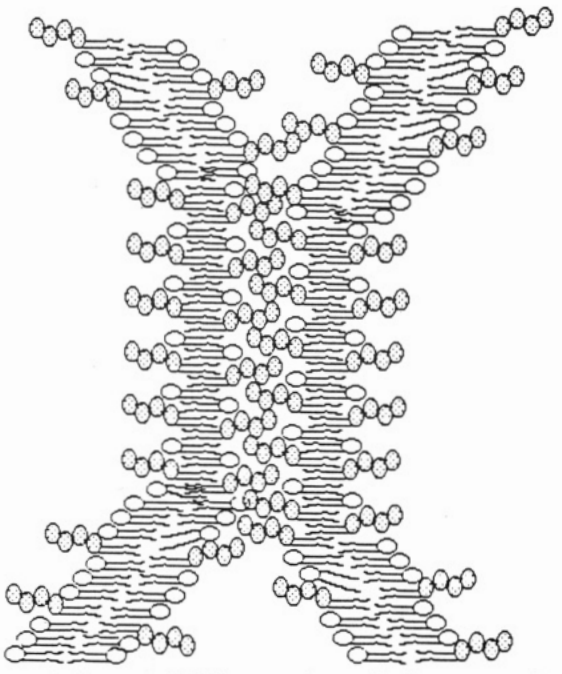

FIGURE 6: Proposed 3-fold increase in ganglioside concentration and interdigitation of ceramide acyl chains in the adhesion zone between two membranes. The polar headgroup of phosphatidylcholine is represented with an open oval, while that of ganglioside is represented as four speckled ovals for the sugars.

a junctional rise in pH. The apposition of homologous oligosaccharides that occurs in our system might result in the displacement of many of the waters of hydration by hydroxyl groups of apposing carbohydrates. The released water would increase the entropy of the system and result in a lower free energy of the adherent membranes based on van der Waals bonding between carbohydrates. In support of this proposition, LeNeveu et al. (1976) measured by X-ray diffraction the spacing between multilayers of EPC in the presence of increasing concentrations of aqueous monosaccharides. At concentrations above 30% by weight, these sugars were found to decrease the spacing between bilayers. This was interpreted as an increase in adhesive force or a decrease in the hydration force that keeps bilayers separated. Calculations demonstrated that only part of the attractive energy could be attributed to van der Waals forces; entropy was not considered but could be the major driving force. A clear example of entropy-driven binding is an antibody to carbohydrate recognition (Cygler et al., 1991). A similar displacement of water seems likely to occur along with the congregation of gangliosides in an adhesive contact. Specific charged carbohydrate–carbohydrate interaction was recently demonstrated for liposomes containing sialosyllactosylceramide (GM3) and gangliotriaosylceramide (Gg3) bound to plastic by Kojima and Hakomori (1989). Furthermore, these same glycolipids appear to mediate adhesion of a melanoma cell line to a lymphoma cell line. In biological cells, such a phenomenon could occur at the tip of a microvillus or in the neuronal growth cone. The structural rearrangement of gangliosides could then trigger changes in the activities of membrane proteins or even transduce a non-protein-mediated signal for contact sensation (Brewer & Thomas, 1984; Thomas & Brewer, 1990).

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