Ultraweak Sugar-Sugar Interactions for Transient Cell Adhesion

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ABSTRACT Carbohydrate-carbohydrate interactions are rarely considered in biologically relevant situations such as cell recognition and adhesion. One Ca^{2+}-mediated homotypic interaction between two Lewis^x determinants (Le^x) has been proposed to drive cell adhesion in murine embryogenesis. Here, we confirm the existence of this specific interaction by reporting the first direct quantitative measurements in an environment akin to that provided by membranes. The adhesion between giant vesicles functionalized with Le^x was obtained by micropipette aspiration and contact angle measurements. This interaction is below the thermal energy, and cell-cell adhesion will require a large number of molecules, as illustrated by the Le^x concentration peak observed at the cell membranes during the *morula* stage of the embryo. This adhesion is ultralow and therefore difficult to measure. Such small interactions explain why the concept of specific interactions between carbohydrates is often neglected.

INTRODUCTION

Molecular features governing the selectivity in cell-cell recognition and adhesion are key elements to understanding morphogenesis and organogenesis. All living organisms are characterized by the presence of glycoproteins and glycosphingolipids on the cytoplasmic membrane. Carbohydrate chains, as exposed structures at the cell surface, should play a key role in early events of cell-cell recognition. Indeed, they have firmly been recognized as interaction sites in cell adhesion processes such as leukocyte recruitment or host-pathogen interaction. Such events are commonly attributed to lectin or lectin-like proteins and the corresponding specific carbohydrate ligands. It has been suggested that specific carbohydrate-carbohydrate interactions may also play this important role (Hakomori, 1991). Several direct measurements of adhesion between surfaces decorated with glycolipids have been reported in the past (Marra, 1985, 1988; Evans, 1987; Rand and Parsegian, 1989; Luckham et al., 1993; Yu et al., 1998; Ricoul et al., 1998). These strong adhesions, obtained through various techniques (surface force apparatus, osmotic stress method, and vesicle micropipette aspiration), were due to van der Waals or other nonspecific forces. However, some carbohydrate-carbohydrate interactions found in biological processes have been proposed to be specific (Hakomori, 1991; Sharon and Lis, 1993; Bovin, 1996). One of them, the Lewis^x determinant (Le^x), has been identified as presumably playing a biological role by means of intermembrane interactions. According to a current hypothesis, it has a calcium-mediated specific interaction with itself.

Le^x has been shown to be involved in murine and human embryogenesis. This carbohydrate is present on the cell membrane in the polar headgroup of glycolipids. It is not expressed at the cell surface until the eight-cell stage, shows maximal expression at the *morula* stage of mouse embryogenesis, and declines after compaction (Solter and Knowles, 1998; Fenderson et al., 1986). This compaction stage could be inhibited either by anti-Le^x antibodies or Le^x itself, or by inactivating the calcium with EDTA. As Le^x is not charged, the role of the calcium is not electrostatic in nature. These results (Eggens et al., 1989; Kojima et al., 1994) led the authors to propose a calcium mediated Le^x-Le^x-specific homotypic interaction as a basis of cell adhesion in preimplantation embryos, and in various tumor cells. Other observations also strongly suggest that Le^x-Le^x interactions exist in the presence of Ca^{2+} (Boubelik et al., 1998; Siuzdak et al., 1993; Henry et al., 1999; Geyer et al., 2000).

Here we report the first direct quantitative measurements of this putative interaction in a physicochemically well defined system and in conditions similar to the natural environment of the glycolipid, i.e., lipid bilayers. This was done through the adhesion between two giant vesicles functionalized with synthetic glycolipids. Sufficient to promote cell-cell adhesion, this ultraweak interaction explains why this concept is often underestimated.

EXPERIMENTAL SECTION

We have measured directly the adhesion between electrically neutral giant vesicles that included synthetic lipids bearing Le^x groups at the vesicle surface. These vesicles were made of stearoyl-oleoylphosphatidylcholine (SOPC; purchased from Avanti Polar Lipids, Alabaster, AL) as the main component.

Received for publication 12 May 2000 and in final form 15 December 2000.

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0006-3495/01/03/1354/05 $2.00

Measurements of vesicle adhesion

To measure their adhesion, two vesicles of cellular dimensions (10–50 μm) were aspirated in micropipettes and micromanipulated into contact. The (negative) pressure, ΔP, in each pipette controlled the (positive) hydrostatic pressure in the vesicle and thus the mechanical tension, τ_m, in its membrane:

$$\tau_m = \frac{\Delta P}{2\left(\frac{1}{r_p} - \frac{1}{r_v}\right)} \quad (1)$$

where r_p and r_v are, respectively, the radius of the micropipette and that of the vesicle. The two osmotically controlled vesicles are observed in interference contrast microscopy. One of them is pressurized into a tight, rigid sphere with large bilayer tension, whereas the adherent vesicle is held with low pressure and remains deformable. The adhesion energy W_{adh} is obtained by determining the contact angle θ_c of the two vesicles (cf. Fig. 1) and the tension τ_m of the flaccid vesicle membrane (Evans, 1990):

$$W_{adh} = \tau_m(1 - \cos\,\theta_c) \quad (2)$$

By combining Eqs. 1 and 2, it is easy to relate ΔP to W_{adh}:

$$\Delta P = C \cdot W_{adh} \quad (3)$$

where C depends only on the geometry of the system:

$$C = \frac{2\left(\frac{1}{r_p} - \frac{1}{r_v}\right)}{1 - \cos\,\theta_c} \quad (4)$$

The measurement of θ_c was deduced numerically from geometrical parameters as indicated in Evans (1990).

Glycolipids

The Le^x determinant is a trisaccharide (Galβ1 $\rightarrow$ 4 [Fucα 1 $\rightarrow$ 3] GlcNAc). It is neutral in physiological conditions. In classical natural sphingolipids, the Le^x trisaccharide is attached to the ceramide through a lactose group. Although they are available in large quantities by chemical synthesis, they have not been used in this study. With their large hydrophilic pentasaccharide headgroups and their relatively small hydrophobic chains, they are rather soluble in water and their distribution in vesicles cannot be controlled. We therefore synthesized a less water-soluble lipid (Esnault et al, 2001) with a Le^x headgroup attached via a so-called spacer group to ensure its mobility (Fig. 2 *a*) that we will refer to as Le^x lipid.

In this synthetic glycolipid, a rather low solubility in water combined with a good bilayer cohesion were obtained by using three hydrophobic chains, instead of two as in ceramide. To ensure good accessibility of the Le^x groups for interaction, they were provided with translational mobility by branching the chains. This hinders the bidimensional crystallization of the chains and keeps the layers in a fluid state. An orientational mobility was provided by means of a flexible spacer between the chains and the Le^x. This allowed the Le^x groups to take the exact orientation and position for which the specific interaction can occur.

For the controls, a glycolipid with the same features but without Le^x was synthesized (Fig. 2 *b*); we will refer to it as Lac lipid.

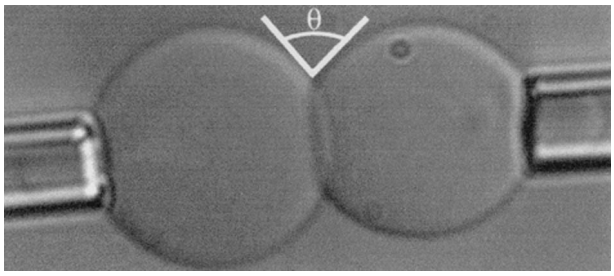

FIGURE 1 The two osmotically controlled vesicles held in micropipettes by aspiration are observed in interference contrast microscopy. The suction pressure applied to the micropipettes allows control of the tension of the vesicle bilayers. One of them (*left*) is pressurized into a tight-rigid sphere with large bilayer tension, whereas the adherent vesicle (*right*) is held with low pressure and remains deformable. The adhesion energy W_{adh} is obtained by determining the contact angle θ_c of the two vesicles and the tension τ_m of their membrane (θ): $W_{adh} = \tau_m(1 - \cos\theta_c)$.

Experimental procedure

Giant vesicles were formed by lipid hydration after evaporation from chloroform solution in 320 mOsm sucrose solution (Needham, 1993; Needham and Evans, 1988). The Le^x-functionalized vesicles, hereinafter referred to as Le^x vesicles, were made from a mixture of SOPC and the synthetic Le^x lipid (90:10 by mol). Two types of control vesicles were prepared: one made of pure SOPC and the other made of SOPC and Lac lipid (90:10 by mol) that we call Lac vesicles.

The vesicle suspension was added to an aqueous glucose solution chamber of a slightly higher osmolarity (360 mOsm) than that of the vesicles, in order to deflate them and make them micromanipulable. Two vesicles were then transferred into another chamber filled with salt solution, either NaCl or $CaCl_2$, at 360mOsm. Both vesicles were micromanipulated into tangential contact. The contact angle was measured for several tension values of the flaccid vesicle membrane by decreasing the aspiration and then increasing it in order to check the reversibility of the adhesion (Evans, 1990).

The experiments consisted of comparing the adhesion of two Le^x vesicles in NaCl and in $CaCl_2$ (Le^x/Le^x experiments). As calcium is known to produce sometimes peculiar effects on bilayer interactions (Marcelja, 1992), many controls were required. First, it was necessary to compare Le^x/Le^x experiments with experiments in which the Le^x groups from one of the vesicles were absent (Le^x/Lac experiments). Second, it was useful to replace the Le^x groups with another sugar in both vesicles (Lac/Lac

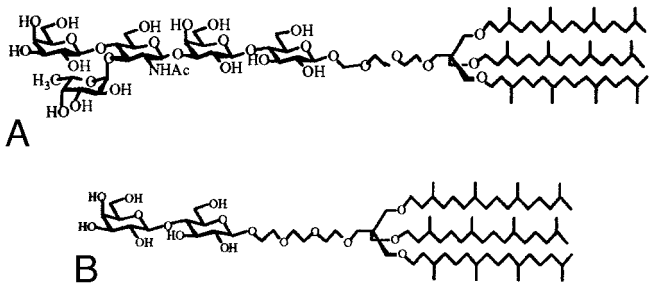

FIGURE 2 (*a*) The Le^x determinant is a trisaccharide (Galβ1 $\rightarrow$ 4 [Fucα 1 $\rightarrow$ 3] GlcNAc). It is neutral in physiological conditions. In a classical natural sphingolipid, the Le^x trisaccharide is attached to the ceramide through a lactose group. In the synthetic glycolipid, a rather low solubility in water together with a good bilayer cohesion were obtained by using three hydrophobic chains instead of two, as in ceramide. To ensure good accessibility of the Le^x groups for interaction, they were provided with translational and orientational mobilities by branching the chains, thus keeping the layers in a fluid state by hindering their crystallization, and by means of a flexible spacer between the chains and the Le^x. (*b*) The same lipid without the Le^x group.

experiments). Third, as SOPC was the main component of our vesicles, it was interesting to compare the adhesions obtained with that of pure SOPC vesicles.

RESULTS AND DISCUSSION

Fig. 3 shows the aspiration pressure as a function of C (cf. Eq. 4) for the controls and Le^x/Le^x experiments. The slopes are equal to the vesicles' adhesion free energies. These slopes are independent of the vesicle size. Adhesion energy values for the controls and for the Le^x/Le^x experiments are given in the table. The effect of adding calcium on the Le^x vesicles is clearly seen in Table 1 and Fig. 3. The adhesion energy in $CaCl_2$ is 2.5 times higher than in NaCl, whereas the Le^x/Lac and Lac/Lac experiments showed a small decrease of the adhesion energy in calcium (3.10^{-6} J/m^{-2}). For comparison, calcium has no influence on the adhesion of pure SOPC vesicles. These results show unambiguously that Le^x groups are necessary on both vesicles for the calcium-induced adhesion enhancement to occur. This is in agreement with the specific interaction scheme advocated by Hakomori (1991).

Because a fraction of the vesicles produced in this way are multilamellar (about 40%; Kwok and Evans, 1981), it is necessary to check that what was seen was really adhesion and not fusion, which would look the same under a microscope. Adhesion experiments were performed in which one of the adhering Le^x vesicles contained fluorescent phospholipids added to its membrane, while the other did not. The vesicles were left in contact 30 min and then observed. It was checked that the fluorescence of the first vesicle did not diffuse into the membrane of the second vesicle.

TABLE 1 Adhesion energy of vesicles in aqueous media

	Adhesion energy (10^{-6} J/m^2)	
Left vesicle/right vesicle	in NaCl 0.2 M	in $CaCl_2$ 0.11 M
Le^x/Le^x	4.5 ± 2	11 ± 2
Le^x/Lac	5.4 ± 1	2.5 ± 2
Lac/Lac	9.5 ± 0.5	6 ± 1
pure SOPC vesicles	14 ± 2	15 ± 4

Adhesion energy of vesicles (10^{-6} J/m^2) in aqueous media between Le^x- or Lac-functionalized vesicles (Le^x means that the vesicle composition is SOPC:Le^x 90:10; same for Lac), and between pure SOPC vesicles, in NaCl and in $CaCl_2$.

The adhesion, in the case of NaCl and for all types of vesicles, is the result of a balance between van der Waals attractions and short-range repulsions that include entropic and structural contributions (Rand and Parsegian, 1989; Israelachvili and Wennerström, 1990). Because 90 percent of the surface of the Le^x and Lac vesicles is made of SOPC, van der Waals forces may be expected to be the same for all

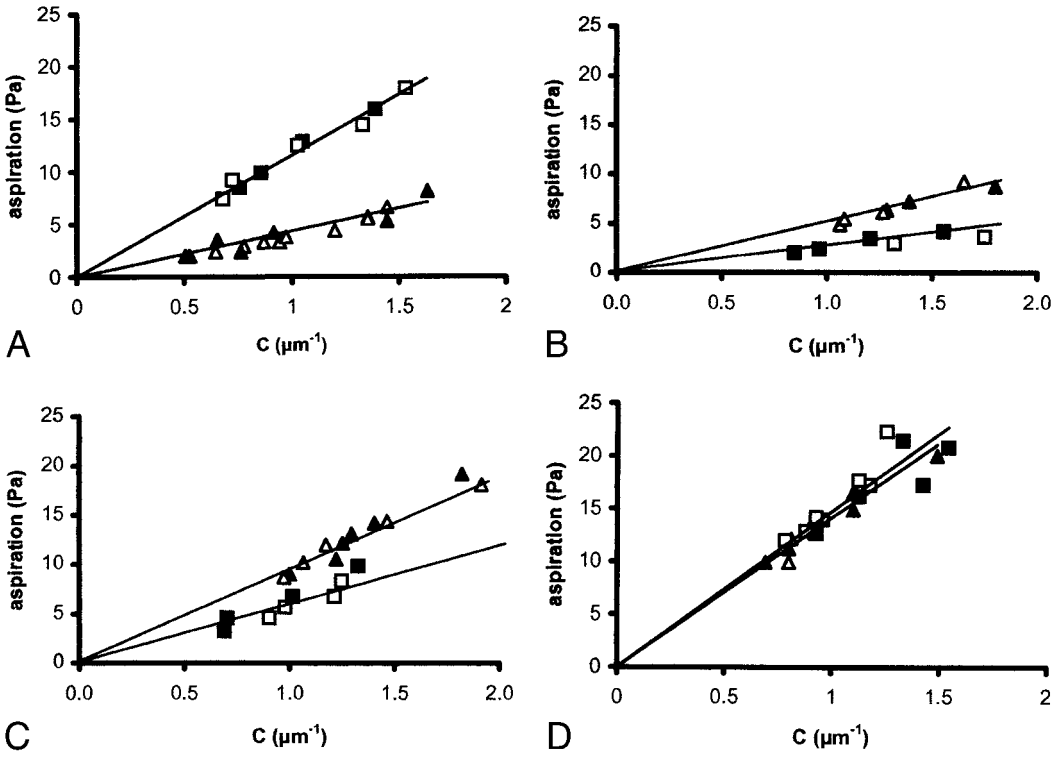

FIGURE 3 Aspiration pressure as a function of parameter C given in Eqs. 3 and 4. (*a*) Le^x/Le^x experiment (two vesicles with SOPC:Le^x of 90:10); (*b*) Lex/Lac experiment (one vesicle is SOPC:Le^x 90:10; the other is SOPC:Lac 90:10); (*c*) Lac/Lac experiment (two vesicles with SOPC:Lac 90:10); (*d*) pure SOPC vesicles. The closed symbols represent decreasing aspiration, and open ones represent increasing aspiration. *Triangles* are for NaCl solution and *squares* for $CaCl_2$. The *straight lines* are least-squares fits.

types of vesicles. Therefore, the higher adhesion in NaCl with pure SOPC, as compared to SOPC/Le^x, may seem surprising. However, one may note that the sugar groups are bound to the lipid chains by a flexible spacer (Fig. 2). The thermal fluctuations of this spacer can give rise to steric repulsions, as polymer brushes do (Taunton et al., 1990). We have performed measurements that show that our Le^x lipid spacers fluctuate like an ideal polymer chain, generating steric repulsions (in preparation). This explains why the adhesion of SOPC vesicles is reduced by the presence of sugars. In agreement with these considerations, Table 1 shows that the adhesion energies vary according to the size of the sugar headgroup present on the vesicle: the larger the headgroup (Le^x), the larger the steric repulsion, and therefore the smaller the adhesion energy ($Le^x/Le^x < Le^x/Lac <$ Lac/Lac $<$ SOPC/SOPC). This difference between Le^x and control vesicles is not relevant for our purpose, since van der Waals forces do not play much role in cell membrane interactions. Usually the adhesions are governed by cell adhesion molecules such as proteins or lipids, given that there are many steric repulsions produced by the glycocalix. For this reason, two biomembranes will not adhere in the absence of specific adhesion sites.

The specific contribution W_{spe} of the Le^x groups to the adhesion energy of our vesicles is equal to the difference between the effects of calcium on adhesion energy in the Le^x/Le^x and in the Le^x/Lac experiments (i.e., the control that is the most similar to the experiment). The table shows that $W_{spe} = 10 \pm 5\ \mu J/m^{-2}$. Even though it is usually delicate to deduce molecular information about a single bond from global adhesion energy measurements (Evans, 1985a,b), the case of Le^x is favorable because of the weakness of the interactions. A single slope for the contact formation and separation of the vesicles features a continuous and reversible adhesion. This implies that the lifetime of the involved bonds is so short that the association/dissociation process can be considered instantaneous compared to the time scale of the distribution of the lipids in the vesicle membrane. The Le^x vesicles thus constitute a highly dynamic system, in contrast to vesicles that bear much stronger binding sites (Noppl-Simson and Needham, 1996). The adhesion molecules are expected to diffuse toward the contact zone, which therefore should contain a higher density of them, leaving the non-contacting part of the vesicle depleted, especially for large contact areas. According to the theoretical approaches proposed in the mid-eighties (Bell et al., 1984; Evans, 1985b), it is possible to deduce directly this enrichment $\Delta\rho$ of the contact zone from W_{spe}:

$$\Delta\rho = W_{spe}/k_BT$$

which gives, in our case, an enrichment $<2\%$. Therefore, the density can be considered constant. This has the consequence that the bonds between the vesicles come from Le^x groups that randomly face each other. The density of such sites is equal to p^2/A, where A is the average area per lipid (here 0.7 nm^2, obtained from Rand and Parsegian (1989) and from monolayer compression isotherms; strictly speaking, A is the area over which the field of attraction of one site is felt by a site on the other vesicle) and p the proportion of Le^x lipids in the vesicle (here 0.1). W_{spe} is equal to the actual number of bonds per unit area in the contact zone times the bond energy e. The weakness of $\Delta\rho$ indicates that e is small. Assuming that it is smaller than k_BT, only half of the facing sites are actually bound. This leads to

$$W_{spe} = (p^2/2A)e \quad (5)$$

This gives an upper estimate of e (because this would underestimate the real number of bonds).

In Eq. 6, assumption is made that the glycolipids are not clusterized in the vesicle membrane. From this equation, e ranges between 0.17 and 0.5 k_BT (i.e., 0.1 and 0.3 kcal/mol). Previous glycolipid studies showed strong van der Waals interactions (Rand and Parsegian, 1989; Yu et al., 1998; Evans, 1987; Marra, 1985, 1988; Ricoul et al., 1998; Luckham et al., 1993). In the present work, the calcium-induced interaction is specific and comes in addition to van der Waals forces. It is worthwhile to note that these molecules are neutral and, therefore, this interaction is not intrinsically electrostatic.

This ultraweak interaction energy validates the assumption that the e is substantially below k_BT. A rough estimate of the bond lifetime can be obtained by comparison with the streptavidin/biotin system (30 k_BT bond energy and lifetime of several days) and assuming an Arrhenius law. It is well below 1 μs. It is most unusual to measure molecular interaction energies well below the thermal energy. This only shows that cell-cell adhesion will require a large number of these molecules, as is, indeed, observed during the *morula* stage of mouse embryogenesis. The molecular mechanism of the Le^x interaction remains mysterious. The mediation by Ca^{2+} could result from the setting up of an appropriate coordination shell around the cation (Bugg, 1973).

These measurements with synthetically tailored Le^x containing neoglycolipids directly confirm the involvement of neutral cell surface oligosaccharides in cell-cell adhesion and illustrate why, when the Le^x sites are blocked, the compaction stage of the embryo does not occur (Eggens et al., 1989). Le^x can, in the presence of Ca^{2+}, substantially enhance the deformation and adhesion of lipid vesicles, i.e., objects with mechanical features akin to those of the cell membranes. The interaction scaled down to one molecule is well below thermal energy. It produces transient and dynamic adhesion that is indeed what cell differentiation requires.

In contrast to these neoglycolipids, the natural glycolipids bearing Le^x are generally glycosphingolipids based on ceramide. This may noticeably influence the adhesion energy, either because of their arrangements in the membrane or

because of their functionalities. For example, in our case, a bidimensional clustering of Le^x lipids in the vesicles' contact region would generate a higher adhesion energy, because a higher number of sites would be involved in the adhesion. It is likely, therefore, that in their biological environment, the Le^x groups would be distributed unevenly in the membranes and generate a higher adhesion than the one measured here on our model vesicles. Here, we have quantified the Le^x-Le^x interaction regardless of the rest of the lipid, which may modulate the resulting adhesion.

This first direct measurement of biologically relevant ultraweak carbohydrate-carbohydrate interaction shows that it is now possible to quantify them even if they are smaller than the thermal energy and opens up a new, promising field of inquiry in biology.

F. P. and E. P. thank E. Evans and J. Wolfe for helpful discussions

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