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A comparison of physical behaviour amongst four glycosphingolipid families

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In the general search for information regarding glycosphingolipid behaviour and arrangement in membranes there has arisen the question of whether the answers may be very different for different glycosphingolipid families. We show here that in fact considerable basic similarity exists at least amongst a certain number of families as sensed by a spin label probe covalently attached to the glycosphingolipid fatty acid. This potential for similarity may be as important to take account of as is the clearly demonstrated potential for differences based on headgroup structure (Maggio, B., Albert, J. and Yu, R.K. (1988) Biochim. Biophys. Acta 945, 145–160). The ganglioside, G_{M1}, was examined, as were the neutral species, galactosyl ceramide, lactosyl ceramide, and globoside, in phospholipid bilayers at 2 mol% and 10 mol%. In each case EPR spectra of a spin probe at carbon 16 of the single glycolipid fatty acid chain were compared. This was done for both 18-carbon and 24-carbon species in order to test the phenomenon over the range of common glycosphingolipid fatty acid chain length. The most telling result was that spectra of these four families were identical within experimental error in both rigid gel phase and semi-fluid non-cooperative bilayer matrices as sensed by a nitroxide spin probe near the centre of the membrane hydrophobic interior. No glycolipid family showed a detectable difference in organization either laterally or vertically within the membranes, nor in membrane dynamics. In addition, the membrane phase transition sensed by each family fell within the same narrow range. Freeze-etch visualization of glycolipid lateral distribution was attempted using a variety of native lectins and antibodies as markers. Success was severely limited by low affinity of marker attachment to neutral species; however, where testable, no differences were observed amongst families.

Introduction

Glycosphingolipids have been a subject of study by groups with diverse interests because of their dual function – as membrane structural elements, and as cell surface recognition sites that may have a role in differentiation, cell cycle, and transformation. Hence the question has arisen in a variety of contexts as to whether different families of glycosphingolipids may behave differently within cell membranes [1–4]. Certainly there is reason to anticipate some differences since headgroup oligosaccharides vary widely in hydrogen-bonding potential, size, charge, and configuration. It has been pointed out that in addressing such questions it will be important to examine bilayer membrane systems which mimic the low overall glycosphingolipid concentrations and non-cooperative, semi-fluid nature of cell plasma membranes [1,2]. We have derived a series of four different glycosphingolipids (GalCer, LacCer, globoside, and G_{M1}) with 18-carbon or 24-carbon spin labelled fatty acids [5–7]. These species are representatives of simple neutral glycosphingolipids (GalCer and LacCer), complex ones (globoside), and gangliosides (G_{M1}). In this article we examine such information to get some idea of the possible extent of behavioural differences amongst these common major families.

The literature offers some divergent opinion in this area, especially with regard to the question of lateral distribution of glycosphingolipids [1–4]. Interpretation is complicated by the fact that different techniques have been used in arriving at published conclusions, and that

Abbreviations: GalCer, Galβ1 → 1Cer; LacCer, Galβ1 → 4Glcβ1 → 1Cer; G_{M1}, Galβ1 → 3GalNAcβ1 → 4Gal(3 → 2αNeuAc)β1 → 4Glcβ1 → 1Cer, globoside, GalNAcβ1 → 3Galα1 → 4Galβ1 → 4Glcβ1 → 1Cer; egg PC, egg phosphatidylcholine; DMPC, L-α-dimyristoylphosphatidylcholine; DPPC, L-α-dipalmitoylphosphatidylcholine; EPR, electron paramagnetic resonance; EM, electron microscopy.

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fatty acid composition is a significant variable. Here we have controlled for fatty acid composition of the glycosphingolipid in substituting known 18- and 24-carbon fatty acids for the natural ones. At the same time we have covalently attached a probe molecule in a known location that samples glycosphingolipid microenvironment even at extremely low concentrations in any membrane. The spin label probe on the fatty acid chain is sensitive to motion, orientation, and the presence of other nearby spin labels. In addition, within the recently determined limitations of the technique, we have used freeze-etch electron microscopy with modest success to test for distribution differences amongst G_{M1}, globoside, and LacCer by marking their locations with lectins or antibodies.

Materials and Methods

L-α-Dipalmitoylphosphatidylcholine (DPPC) was from Avanti Polar Lipids, Birmingham, AL. Galactosylceramide (GalCer) Type II and *N*-lignoceroyldihydrolactocerebroside (LacCer) were from Sigma, St. Louis, MO as were cholera toxin, soybean agglutinin, lima bean lectin, *Helix pomatia* lectin and RCA_{60}. Cholesterol was from Serdary Research, London, Canada. Our methods of isolation and purification of globoside from porcine erythrocytes and of G_{M1} from bovine brain have been recorded previously [6]. Synthesis of spin labelled fatty acids, and their incorporation into the four different (lyso-) glycosphingolipids have also been described elsewhere [5,7]. Glycerophosphocholine with palmitic acid at the 3-position and 16-nitroxystearic acid at the 2-position of the glycerol backbone was synthesized as described by Hubbell and McConnell [13]. Thin-layer chromatography to assess lipid purity and reaction progress was performed on Merck silica gel 60 plates eluted with 65 : 25 : 4 (by vol.) $CHCl_3/CH_3OH/H_2O$, and developed with 1 : 2.75 sulfuric acid/ethanol spray.

Polyclonal antisera to globoside prepared as described previously [7] were purified by two successive ammonium sulfate precipitations and redissolved in isotonic saline at the original serum concentration. Prior to use, aliquots of stock IgG solutions were preadsorbed to remove non-specific liposome binding fractions. Monoclonal IgM anti-LacCer was a generous gift from S. Hakomori. IgM from the mouse ascites fluid was precipitated using 2% boric acid, centrifuged at 14500 × *g* for 10 min, dissolved in borate saline (pH 8.6) and dialysed against borate saline (pH 8.6) at 4°C for 4 days. Small aliquots of the monoclonal antibody were stored at −20°C at a final concentration of 0.9 mg/ml.

Lipid bilayer membranes for these experiments were prepared by dissolving all components at the final desired ratio in 1 : 1 $CHCl_3/CH_3OH$, and removing the solvent under a N_2 atmosphere. Resultant films were further dried by pumping in vacuum (rotary pump) for 2 h at 22°C. Liposomes were prepared by hydration of such films with 10 mM phosphate-buffered normal saline (pH 7.4) containing Ca^{2+} and Mg^{2+}. Free Ca^{2+} and Mg^{2+} concentrations in the samples were 0.96 mM and 0.55 mM, respectively, as determined by chemical analysis. All samples were incubated 10°C above their transition temperatures for 15 min to assure diffusional equilibrium within the bilayer, before being allowed to cool to the temperature of study.

EPR spectra of samples were run on a Varian E12 spectrometer equipped with a TM_{110} cavity and variable temperature accessory (Varian Association, Palo Alto, CA). For this purpose vesicle suspensions were held in 50 μl Dade® disposable glass micropipettes sealed at one end and supported in a plastic sleeve that permitted gas flow.

Liposomes for freeze-etch electron microscopy were harvested by centrifugation. Droplets of liposome pellets were incubated at appropriate temperatures on either (3 mm) gold alloy planchets prior to quenching in a slurry of freon cooled in liquid nitrogen (conventional approach), or on slices of fixed rat lung on the aluminum sample holders supplied with the instrument prior to slam freezing using a Heuser-type Cryopress from Med-Vac Inc., St. Louis, MO (Developed by Dr. J. Heuser and colleagues). In each case freeze-etch replicas were prepared by platinum shadowing in a Balzers BAF 301 apparatus equipped with electron beam guns. Etching was for 2 min at −103°C. Replicas were cleaned in $NaClO_4$, rinsed with distilled water, and exposed to ethanol/acetone (1 : 1, v/v) for 1 hour to remove lipid traces. They were picked up on 400 mesh copper grids and viewed in a Philips EM300.

Results

Probes

Fig. 1 illustrates the structures of the probe-labelled glycolipids used in this work. Two fatty acid chain lengths were selected for study: 18-carbon stearic acid and 24-carbon lignoceric acid, covering the extremes of common fatty acid chain length found in glycosphingolipids. The EPR spectrum of a nitroxide spin label is known to be highly sensitive to environment, motional characteristics, and orientation of structures to which it is attached [8,9]. In each case the probe was rigidly attached to C-16 of the single glycosphingolipid fatty acid chain. Via this choice the potentially perturbing nitroxide probe was localized to a membrane region of relative disorder, and away from the more spatially constrained headgroup region. By the same token of course the probe may not be as sensitive to primary headgroup events as if it were closer to the membrane surface. LacCer has a dihydrosphingosine backbone and thus lacks the double bond shown in Fig. 1.

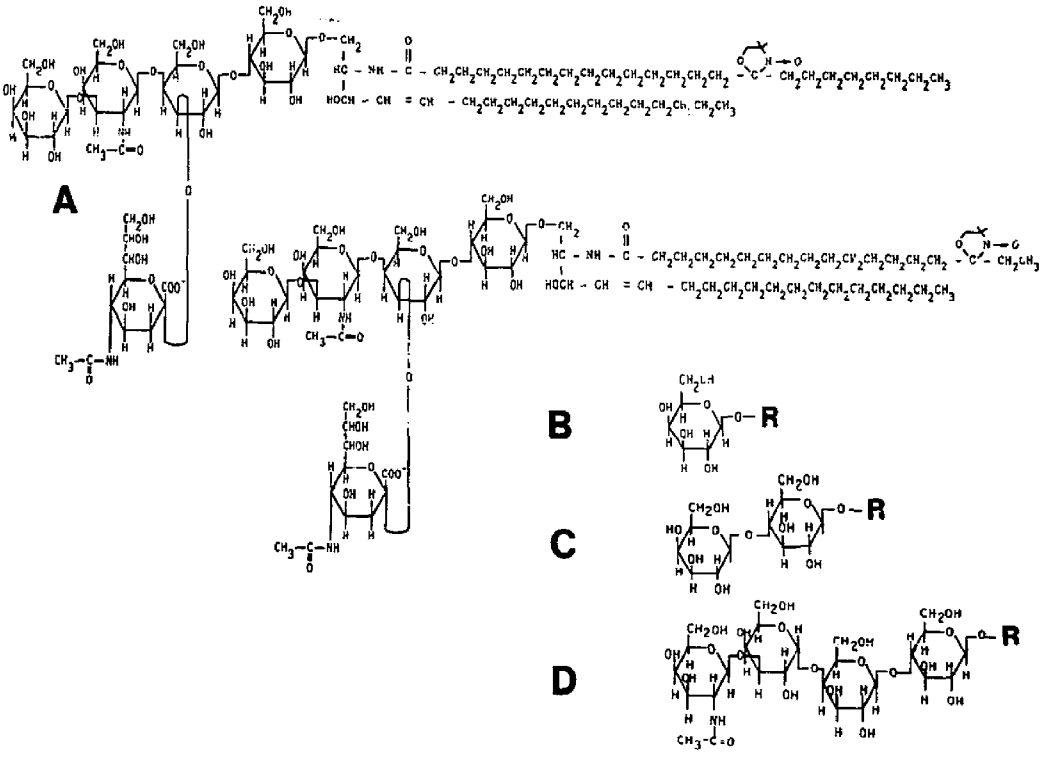

Fig. 1. Structures of the probe molecules studied in this work: (A) G_{M1}, (B) GalCer, (C) LacCer (note this species did not have the double bond shown in the sphingosine portion), (D) globoside.

Spectral analysis

In principal a sensitive method of testing for the existence of physical differences in membrane behaviour amongst the families of glycosphingolipids is to examine their EPR spectra for differences in appearance. For instance motional rate is a major determinant of spectral linewidth and line position [8–13]. Environment polarity and motional anisotropy (directionality) exert control over peak position and shape. In addition collision between spin-label nitroxide radicals produces a characteristic change in which spectral lines broaden and shift inward (so-called 'spin exchange broadening'): as a result, at high spin label concentration EPR spectra collapse to a single line [14] (such a result might be expected in our experiments if there were phase separation of what glycolipid there is within the bilayer into regions of *local* enrichment).

Thus we compared the EPR spectrum of each of the four glycolipid families having 18-carbon fatty acid to that of each other family. We have done the same for the 24-carbon analogues. Samples were studied at 2 mol% and at 10 mol% glycolipid in unsonicated multilamellar phospholipid liposomes. This spans a reasonable range of glycolipid concentration while avoiding concentrations high enough to lead to bilayer breakdown. Both highly ordered gel phase dipalmitoylphosphatidylcholine (DPPC) and 2:1 (mol ratio) DPPC/cholesterol which provides a non-cooperative liquid crystal membrane of intermediate fluidity were examined. Four of the eight resultant sets of spectra are shown as examples in Fig. 2: the 24-carbon fatty acid species at 2 mol% (A,B) and the 18-carbon fatty acid species at 10 mol% (C,D) in DPPC and DPPC/cholesterol, respectively. Incorporation into a matrix of pure DPPC might be expected to emphasize any differences in membrane 'fit' of the four glycolipid families; while the cholesterol-containing system more closely approximates several key features of cell membranes. The 2 mol% glycolipid samples address the low concentration of such species generally present in cell membranes: and the 10 mol% samples represent an attempt to maximize any tendency to glycolipid self-association.

As anticipated, when glycolipid comprised 10 mol% of the total membrane lipid there was greater evidence of spin exchange broadening in the EPR spectra than at 2 mol%, reflecting a statistically greater likelihood of nitroxide-nitroxide collision. It will be seen however that, as we noted previously for globoside [7] at least 80% of the glycolipid was uniformly dispersed in the membrane rather than segregated into regions of rela-

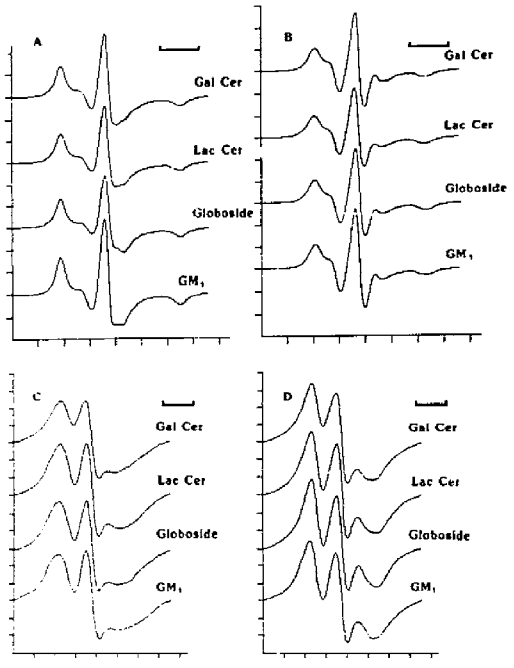

Fig. 2. Examples of spectral comparison amongst different spin-labelled glycosphingolipid (GSL) families in rigid host membranes (dipalmitoyl phosphatidylcholine (DPPC)) and in membranes of intermediate fluidity (2:1 mol ratio DPPC/cholesterol) at 22°C. In each case the spin label was at C-16 of the single 18-carbon or 24-carbon fatty acid attached to the GSL (Fig. 1). Spectra were normalized for direct comparison within each group. (A) 2 mol% GSL with 24-carbon fatty acid in DPPC, (B) 2 mol% GSL with 24-carbon fatty acid in DPPC/cholesterol, (C) 10 mol% GSL with 18-carbon fatty acid in DPPC, (D) 10 mol% GSL with 18-carbon fatty acid in DPPC/cholesterol. All samples in phosphate-buffered saline (pH 7.4) with Ca^{2+} and Mg^{2+}. Bar represents 20 gauss.

tive enrichment (see below and Fig. 3). In our opinion the most important conclusion to be drawn from these data is simply that there was no appreciable difference amongst the series, GalCer, LacCer, globoside, G_{M1} in spite of the known sensitivity of the EPR technique to molecular behaviour and environment. This was true both for the series with 18-carbon fatty acid and that with 24-carbon fatty acid. For any given glycolipid fatty acid chain length and host matrix it could be demonstrated by stepwise spectral subtraction that the spectra were identical within 10–20%. That is, only when 80–90% of the spectrum of any one glycolipid in a given membrane was subtracted from the spectrum of the corresponding member of another family in the same host matrix did the resultant show peak inversion [15]. As described below we consider this to be within the limits of accuracy of our sample composition.

Although in our opinion the most telling observation here was *similarity* amongst spectra of the different families, it is possible to interpret any individual spectrum in terms of glycolipid distribution. Since a variety of phenomena contribute to observed spectral characteristics, we have used comparison with standards in

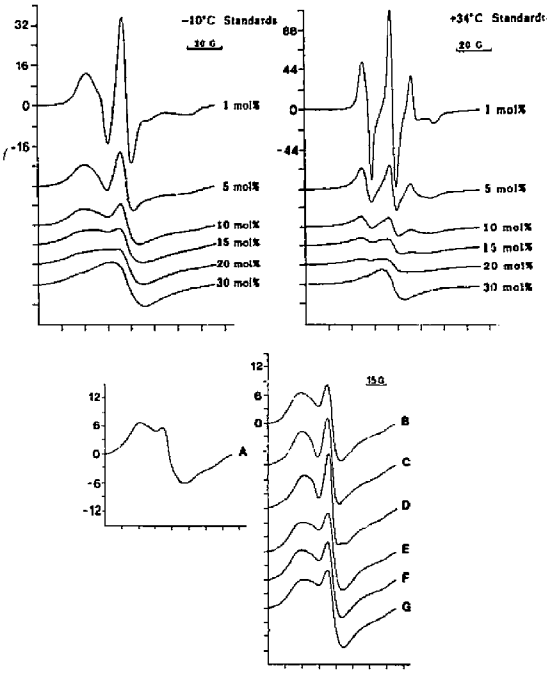

Fig. 3. Demonstration of the sensitive quantitative relationship between EPR spectral features and lipid spin label environment in the system studied here. The upper two composites are 'standard' spectra of *phospholipid* spin labels as a function of concentration in two different fluidity ranges. Fluidity was controlled by temperature effect on the host matrix: −10°C and +34°C being shown here. All spectra were normalized to correspond to the same total number of spin-labelled molecules for direct comparison; but note that the vertical scales are different for each composite in this presentation in order to permit them to be shown in their entirety with adequate peak height. Note the clear effect (within each standard composite) of forcing spin-labelled lipids into closer proximity in both low fluidity (−10°C) and high fluidity (+34°C) membranes. Each standard sample consisted of DPPC bilayers into which 25 mol% cholesterol had been incorporated to remove the phase transition and make fluidity a smoothly controllable function of temperature. The nitroxide spin label in standards was covalently attached to C-16 of a *phosphatidylcholine* fatty acid to minimize likelihood of phase separation. The lower composite (spectra A–G) illustrates application of the approach to lactosylceramide, with its 24-carbon fatty acid spin labelled at C-16, at 10 mol% in a DPPC/cholesterol host matrix at 26°C (spectrum A). Shown for comparison are the following phospholipid spin-label standards and linear combinations of standards plotted at the same vertical scale: (B) 10 mol%, (C) 0.5 of 5 mol% +0.5 of 15 mol%, (D) 0.2 of 1 mol% +0.8 of 20 mol%, (E) 0.1 of 30 mol% +0.9 of 10 mol%, (F) 0.8 of 10 mol% +0.2 of 20 mol%, (G) 0.3 of 30 mol% +0.7 of 10 mol%. All standards run at −9°C. Vertical scale is shown for the uppermost spectrum of each group only.

attempting to interpret and quantitate the spin exchange-associated features seen in Fig. 2 and related experiments. The basic idea is that phase separation of glycolipids into regions of selective enrichment will be quantitatively reflected in line broadening and collapse of the spectrum to a single line [14]. The approach was described in detail earlier for globoside [7] and will only be summarized here. We would simply like to emphasize that exactly the same result was obtained for GalCer, LacCer and G_{M1}. Standards consisted of the 18-carbon fatty acid spin label attached at the β-position of phosphatidylcholine dispersed at known concentrations in bilayers of DPPC/cholesterol. Thus our library of standards comprised 128 spectra recorded over the temperature range, $-30°C$ to $+55°C$, for 1-palmitoyl-2-(16-nitroxy)stearoylphosphatidylcholine at concentrations of 1, 2, 5, 10, 15, 20, 25 and 30 mol% (see Fig. 3). Spin-labelled phospholipids have been studied extensively and shown to exhibit random dispersion in phosphatidylcholine matrices of similar chain length (see, for example, Refs. 14 and 16). The presence of 25 mol% cholesterol is known to completely suppress the cooperative transition [17] so that spectral features were a smooth function of temperature; and made it possible to record standards representing a continuous range of mobilities for matching to glycolipid spectra. Fig. 3 illustrates the strong influence of spin label concentration on EPR spectra in two different fluidity ranges, and the approach taken to interpreting the spectral appearance in terms of glycolipid distribution within the membrane. We demonstrated earlier for globoside [7] that the closest match to a given glycolipid sample was a standard having the same overall concentration of spin labelled phospholipid. In other words, there was no evidence for spin exchange broadening suggestive of glycolipid phase separation. We now report that this was also true for each of the other glycosphingolipid families examined here: GalCer, LacCer and G_{M1}. Moreover, glycolipid samples from different families having the same fatty acid length, concentration, and host matrix were more similar to one another than to any of the phospholipid standard spectra. Blind matching of a given glycolipid sample to phospholipid standards elicited the same standard as the corresponding sample from a different glycolipid family (e.g., 2 mol% LacCer with 24-carbon fatty acid in DPPC elicited the same standard match as 2 mol% globoside with 24-carbon fatty acid in DPPC when done by different investigators). Thus the greatest limitation to quantitation of the degree of similarity amongst physical behaviour of the four glycolipid families studied here was the 10–20% accuracy limit of glycolipid concentration in our model membranes. This was dictated by the difficulty of quantitating milligram total stock amounts of any given spin-labelled derivative.

Evidence of basic similarity in membrane characteristics amongst GalCer, LacCer, globoside and G_{M1} was also seen in their behaviour as a function of temperature within phospholipid bilayers. Once again this was true for glycolipids having 18-carbon fatty acids and for those having 24-carbon fatty acids. The logic upon which we based this experiment was that differences amongst glycolipids might be manifest at some particular fluidity or organization of the surrounding membrane. For this work samples of 2 mol% glycolipid in DPPC were warmed from $25°C$ to $50°C$ at $0.2°C$/min and spectra recorded at 1.5–2°C intervals. The peak height ratios, low-field/middle and high-field/middle, were measured and plotted vs. temperature. A sharp change in these ratios was apparent in each case related to the $41.5°C$ phase transition of pure DPPC from gel (crystal) to fluid (liquid crystal). Typical such curves have been presented previously for globoside [7]. In each glycolipid family the curves were very similar. For GalCer, LacCer, globoside and G_{M1} the phase change in surrounding phospholipids sensed by a spin probe at C-16 of the 18-carbon glycolipid fatty acid fell within the range 39.6–41.2°C. For the corresponding glycolipids having 24-carbon fatty acid it fell within the range 37.6–39.9°C. Accuracy of absolute temperature measurement in our sample was $\pm 0.6°C$.

Position and orientation within membranes

As already indicated, the nitroxide spin-label probe is highly sensitive to orientation and directionality of motion of the molecule to which it is attached. For any given CH_2 group of the fatty acid chain this is in turn sensitive to the depth at which it sits in the membrane and to side-to-side interaction with surrounding lipids. Thus one may address in detail how the fatty acyl chain of any given glycosphingolipid family (and hence the entire molecule) is stationed in the membrane. The ideal approach to this would involve detailed spectral analysis and spectral synthesis taking into account the slow motional and anisotropic properties of molecules in membranes, as described by Freed [12], Seelig [10], McConnell [8] and Griffith and Jost [9]. Using a Freed-type analysis in particular one could derive values for correlation times and distribution of orientations for the glycolipids studied. We have not attempted such a sophisticated analysis, as our interest was in comparing one family to another under identical conditions. The limiting factor was not interpretation of differences in spectral appearance, but rather that within experimental error our spectra were identical amongst the four families studied. We have however quantitated order parameters for different spin-labelled glycosphingolipid families in a variety of membranes to emphasize certain similarities amongst the glycosphingolipids studied here.

The concept of 'order parameter' was introduced to permit quantitation of alkyl chain anisotropic motion in bilayer membranes. The order parameter, S,associated

with a given fatty acid carbon in a membrane is a measure of the degree of motional alignment of that carbon relative to the plane of the membrane. It was measurement of this EPR parameter for spin-labelled phospholipids and fatty acids that originally led to the concept of fluidity gradient in membranes. S varies between 1 (perfect alignment for a given segment of the methylene chain) and 0 (random arrangement). A complete description of order parameter calculations may be found in the works of Hubbell and McConnell [18] and Seelig [16], and in more recent reviews [9,13,19].

Order parameter differences amongst the four glycosphingolipid families might be anticipated following several lines of reasoning. Firstly, the ability of glycosphingolipid sugars to fit amongst membrane phospholipid headgroups [20] would be expected to influence how deeply the acyl portion penetrates the membrane. Esmann et al. recently suggested that depth of fatty acid penetration is different between phospholipids and glycosphingolipids as reflected in the greater order parameter seen by a spin label on the latter [21]. We first recorded this for GalCer in egg PC, and also offered the alternative explanation of increased phospholipid ordering about glycosphingolipids [22] (see Refs. 1, 3, 4 and 23 for the effects of glycolipids on surrounding phospholipids). Carl Alving introduced a similar concept from a different perspective in suggesting that host phospholipid matrix acyl chain length will exercise control over glycosphingolipid headgroup exposure (i.e., how high it sits in the membrane) [24] as one aspect of crypticity. We have examined existing data for GalCer in egg PC and DPPC (Refs. 6, 22, Jakiwczyk and Grant unpublished) to derive an increase or decrease of 0.05 to 0.07 order parameter units per $-CH_2-$ group up or down the fatty acid chain in the region of the membrane occupied by the C-16 spin probe. Secondly the well-known effects of glycosphingolipids upon alignment of surrounding phospholipids would dictate that any change in headgroup sugars or lateral distribution from one family of glycosphingolipids to another might well be expected to alter local phospholipid order as sensed by a spin probe on the glycosphingolipid.

Our compiled results of order parameter measurements on the four glycosphingolipid families in various bilayer host matrices are listed in Table I. The most striking aspect of these data is the similarity amongst S values for the different families. Within experimental error there is no difference. Note that the values compiled represent GalCer (monosaccharide) vs. LacCer (disaccharide) vs. globoside (neutral tetrasaccharide) vs. G_{M1} (acidic pentasaccharide) with both 18-carbon and 24-carbon fatty acid chains. The host matrices range from highly fluid (low order parameter) to intermediate fluidity to quite rigid synthetic cooperative matrices. One might have predicted that if any matrix were to show up a difference in arrangement/order based upon different headgroup fit, it would be in the most conformationally restricted (gel phase) membranes. These have the smallest area per phospholipid and manifest highly ordered packing [25].

TABLE I

Summary of order parameter data for a nitroxide spin label attached at C-16 of the single fatty acid chain of four different glycosphingolipid families at low concentration in lipid bilayer model membranes

The spin-label code, [1,14], signifies 18-carbon stearic acid while [7,14] signifies that the nitroxide was at C-16 of 24 carbon lignoceric acid (see Fig. 1). Glycosphingolipid concentration was 2 mol% in bilayers of egg phosphatidylcholine (egg PC), dimyristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine (DPPC), or 2:1 mol ratio DPPC/cholesterol. Order parameters were calculated using Method II of Ref. 14 (see also Refs. 12, 13 and 15). Values shown are averages of 2–4 measurements with a precision of ± 0.02–0.04. All samples in phosphate-buffered saline with a supernatant Ca^{2+}, Mg^{2+} concentration of 0.96 and 0.55 mM, respectively. n.d., not determined.

GSL Fatty Acid	Host Bilayer Matrix	T (°C)	Order parameter, S			
			GalCer	LacCer	Globoside	G_{M1}
[1,14]	egg PC	10	0.12	0.13	0.13	0.13
[7,14]	egg PC	10	0.39	0.40	0.42	0.41
[1,14]	egg PC	22	0.08	0.08	0.09	0.08
[7,14]	egg PC	22	0.22	0.24	0.24	0.25
[1,14]	DMPC	3	0.60	0.62	n.d.	0.56
[7,14]	DMPC	3	0.93	0.94	n.d.	0.94
[1,14]	DMPC	22	0.33	0.33	0.31	0.34
[7,14]	DMPC	22	0.69	0.68	0.64	0.70
[1,14]	DPPC	22	0.43	0.42	0.42	0.43
[7,14]	DPPC	22	0.76	0.84	0.86	0.85
[1,14]	DPPC/chol	22	0.35	0.34	0.33	0.33
[7,14]	DPPC/chol	22	0.64	0.65	0.65	0.66

Direct visualization

In principle it would be informative to have direct visualization data for correlation with studies by other techniques in dealing with the question of differences in membrane behaviour amongst glycolipids. This has proven to be a challenging problem at the level of resolution of the electron microscope. Two groups have brought the technique of freeze-etching to bear upon it. The glycolipid molecule itself is not massive enough to cast a platinum shadow that would make its location apparent in a membrane, so various higher molecular weight markers have been tested. The earliest approaches were our use of influenza virus, and later sonicated vesicles with receptor and bound lectin, as markers of the surface presence of gangliosides on large liposomes [22,26]. Via these approaches gangliosides were found in both rigid and fluid regions of coexisting phospholipid domains, unlike the glycoprotein, glycophorin, in the same system. It was demonstrated by Tillack and Thompson that lectins and antibodies with covalently attached ferritin permitted more detailed lo-

calization of glycosphingolipids via freeze-etch EM [27,28]. In these initial ground-breaking experiments Tillack and Thompson recorded a strikingly aggregated distribution of bound marker for the neutral glycolipids, globoside and Forssman antigen, in trypsinized erythrocytes [27], and for asialo-G_{M1} in liposomes [28]. We subsequently demonstrated that a variety of native lectins could be used satisfactorily, and employed them to mark ganglioside location in liposomes more accurately than had been possible with viruses or sonicated vesicles [29–31]. Native lectins bound to gangliosides in liposomes were diffusely distributed, but with a tendency to exist in small groups. We and Tillack and Thompson have attempted to gauge the importance of the following factors that may limit the interpretation of such experiments. Firstly glycolipids have not been chemically 'fixed' in membranes the way that proteins can be prior to labelling. Thus it can be difficult to rule out possible redistribution during sample preparation (in the quenching step). Secondly the affinity of specific polypeptide ligands for most glycolipids is modest, so that excessively high concentrations may have to be used to achieve good surface coverage with marker. Thirdly each glycolipid molecule only occupies a membrane surface area of some 0.5 nm^2 while a platinum shadowed marker protein may cover roughly 80 nm^2. The last two points dictate that in most cases one will be labelling a subpopulation of glycolipids. With regard to the first point above, Tillack and Thompson have found that the lateral distribution of G_{M1} as marked by cholera toxin was essentially random when liposomes were frozen by a very rapid quenching process ('slam frozen'), while it was significantly clustered as prepared by the conventional approach [32].

There remains in the literature the suggestion that neutral glycolipids show a much greater tendency to phase separate into domains of local enrichment than do (charged) gangliosides [3]. Our results detailed in the previous sections do not support this concept, although there is evidence that G_{M1} may behave in some ways like neutral glycolipids [33–35]. Keeping in mind the above limitations of the freeze-etch technique, we summarize here our attempts to directly visualize some of the key systems studied by EPR spectroscopy. Using slam freezing to minimize glycolipid redistribution during quenching, we experimented with a range of unmodified lectins and antibodies as marker molecules for glycolipid location. Certainly lectins such as WGA and RCA_{60} work well for gangliosides, and their pattern of labelling at least via conventional quenching techniques is known [29–32]. The major concern was to examine neutral glycolipids for comparison. The globoside headgroup is similar to that of G_{M1}, but for the NANA group of the latter. Thus we tested RCA_{60} (at 0.5 and

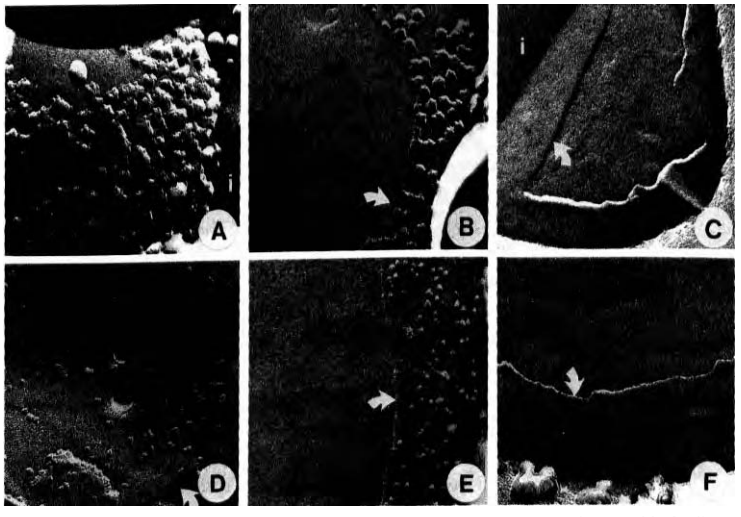

Fig. 4. Examples of the most successful markers for freeze-etch localization of neutral glycosphingolipids in liposomal membranes. (A–C) Monoclonal IgM used to mark the location of spin-labelled lactosylceramide at 10 mol% in 2:1 mol ratio DPPC/cholesterol: (A) lactosylceramide had 18-carbon fatty acid, (B) lactosylceramide had 24-carbon fatty acid, (C) liposomes contained no lactosylceramide. (D–F) *Ricinus communis* agglutinin (RCA_{60}) used to mark the location of spin-labelled lactosyl ceramide (D) or G_{M1} (E) at 10 mol% in 2:1 mol ratio DPPC/cholesterol: (D) lactosylceramide had 18-carbon fatty acid, (E) G_{M1} had 18-carbon fatty acid, (F) liposomes contained no glycosphingolipid. IgM was 0.9 mg/ml final concentration. RCA_{60} was 0.3 mg/ml final concentration in D, F and 0.15 mg/ml in E. IgM was preadsorbed with liposomes without lactosylceramide to remove non-specific binding proteins. All samples in 30 mM saline containing Ca^{2+} and Mg^{2+} buffered at pH 7.4 with 10 mM phosphate. Samples warmed to 50°C for 15 min, then cooled slowly prior to adding marker and incubating at 22°C. Arrows point from fracture face (membrane hydrophobic interior) to junction with etch face (liposome outer surface). i = ice. Shadow direction from bottom to top of micrograph. Magnification ×100000, bar represents 100 nm.

1.27 mg/ml), soybean agglutinin (at 0.5 mg/ml), *Helix pomatia* lectin (at 0.25 and 1.0 mg/ml), lima bean lectin (at 0.25, 1.0 and 2.0 mg/ml), cholera toxin (claimed to bind to globoside at high concentration [36], 0.03 mg/ml), and a polyclonal IgG antiserum to mark the location of globoside at 10 mol% in liposomes of DPPC/cholesterol at 22°C. Blanks consisted of the same liposome and lectin or antibody, without receptor. The high end of marker concentration was the point beyond which significant binding to the blank was seen. None of the markers tried for globoside proved to be satisfactory since the affinities of binding were too low to produce extensive surface coverage with bound marker. However, the markers which did bind did so in a dispersed fashion; and there was no difference noted between the 18-carbon and 24-carbon fatty acid globoside derivatives. We also used a monoclonal IgM and RCA_{60} to label LacCer. These both worked acceptably, although the antibody appeared as large particles of uniform size. The result of a comparison between long and short fatty acid LacCer is shown. There was no significant difference, and we have not seen a fatty acid-related difference in our other samples. Clearly there was no tendency to large regions of clustered receptor. Fig. 4 shows a comparison of G_{M1} and LacCer both labelled with the same marker (RCA_{60}) and appearing identical in distribution. Thus what information we have been able to obtain from direct visualization shows no evidence of a different distribution between charged vs. neutral glycosphingolipids in a phosphatidylcholine model membrane. The EM results are consistent with the EPR experiments of the previous section.

Discussion

The question of carbohydrate headgroup effects on glycosphingolipid structural and functional roles in membranes is an extraordinarily complex one which has been addressed in review articles and numerous specific experiments (references in Refs. 1–4 and 35). We have described here an attempt at directly measuring the physical effect of headgroup differences via an EPR spin probe deep within the hydrophobic interior. The observation that most warrants discussion is the similarity sensed by the nitroxide probe at C-16 of the single glycosphingolipid fatty acid chain amongst GalCer, LacCer, globoside and G_{M1}. Similarity was seen in spectral fingerprint as well as in order parameter, thermal effects and evidence of lateral distribution. One possibility that should not be ignored is that probe techniques can be misleading: conceptually at least the nitroxide spin probe might perturb the glycosphingolipid to such an extent as to be the major determinant of its arrangement relative to surrounding phospholipids. In such a case the observation that spectra of different (spin-labelled) glycosphingolipids were similar would not be surprising. This seems unlikely to us based upon past experience with spin labelled phospholipids in membrane systems [8,9,13], however, ideally one could repeat similar experiments with essentially non-perturbing probes such as deuterium (NMR). In this regard a comparison of GalCer and GlcCer by ^{2}H-NMR is in agreement with our spin-label findings (Grant and Jarrell, unpublished). If one is to hypothesize that galactose vs. lactose vs. globoside vs. G_{M1} headgroups have large differential effects, sufficient to alter fatty acid depth of penetration by as much as 1.5 Å, then this should show up in EPR order parameters in spite of spin label perturbation. Concerns over spin-label effects may be greatest with regard to measuring tendency to phase separate, since if the spin label prevents glycosphingolipid phase separation there would be no spin exchange difference amongst the glycosphingolipid families as seen here. Once again there is no historical evidence of such interference with spin-labelled lipids in membranes. Unfortunately, although sensitive to fatty acid order and motional characteristics, NMR spectra are not sensitive to close approach of labelled molecules. Fluorescent probes are sensitive to close approach (quenching effects) but suffer from the same concern of possible disruptive influence by the probe itself.

It is not appropriate to present here a systematic review of the literature bearing on headgroup effects on glycolipid structure/function relationships in membranes (but see Refs. 1–4 and 34). However, it seems abundantly clear from studies of species suspended as micelles, lamellar phases or monolayers in buffer, that their different headgroup properties are reflected in different arrangements in the pure state. The question is whether differences seen for the pure systems are significantly less manifest when 'diluted' into a membrane bilayer in which sugar headgroups are not forced into close proximity with one another. Presumably at very least there would be local differences due to steric factors, H-bonding, and ionic or Van der Waals effects with phospholipid headgroups. For instance conformational effects have been demonstrated by ^{2}H-NMR for glucose vs. mannose in pure glycosyldiacylglycerols [20]. Extensive DSC and monolayer work has laid a clear basis for headgroup effects amongst pure glycosphingolipids [33,37]. Furthermore, these workers have systematically examined the DSC heat uptake profiles of a range of glycosphingolipids in DPPC showing that supramolecular differences result from headgroup alterations [34]. Interestingly they found that neutral glycolipids and G_{M1} behaved similarly in significant respects as in our work; although their data was consistent with phase separation of these glycolipids while ours is not. Greater differences in DSC behaviour were observed from other glycolipids including G_{D1a}. A report from our laboratory of differences in WGA labelling pattern

between G_{M1} and G_{D1a} in liposomes examined by freeze-etch EM presumably also reflects local surface effects [30]. Given the considerable background of accumulated information on glycosphingolipids, it has become important to design experiments to test specific details of interpretation uncategorically in forming the existing data into a clear overall picture.

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