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Globoside with spin-labelled fatty acid: bilayer lateral distribution and immune recognition

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We have critically addressed the question of lateral distribution of glycolipids in bilayer membranes, and the effect of glycolipid fatty acid chain length upon such distribution. For this purpose we synthesised the complex neutral glycosphingolipid, globoside, with spin-labelled fatty acid. Base hydrolysis to remove the natural fatty acid was found to deacetylate the GalNAc residue concomitantly, necessitating application of the synthetic route described for gangliosides by Neuenhofer et al. (Biochemistry 24, 525–532 (1985)). Globosides were produced with 18-carbon and 24-carbon fatty acids bearing a spin label at the C-16 position. Spin-labelled globosides were incorporated at 2 and 10 mol% into rigid, highly cooperative bilayer matrices of 1,2-dipalmitoylglycerophosphocholine (DPPC) and also into semi-fluid, non-cooperative membranes of DPPC/cholesterol. Recorded electron paramagnetic resonance (EPR) spectra were analysed by comparison with a library of standards representing samples of known composition. Spectra were manipulated using a computer program which permitted linear combination of standards to simulate coexistence of laterally separated domains of different composition. The most important conclusions were as follows: (1) at least 80% of the globoside was definitely not confined to domains highly enriched in glycolipid, although there was evidence of binary-phase separation in the rigid DPPC/globoside matrix; (2) the presence of 33 mol% cholesterol reduced the evidence of globoside phase separation; (3) there was remarkably little difference in results whether the globoside fatty acid chain length was similar to that of the phospholipid host matrix or eight carbons longer. Temperature profiles derived over the phase-transition region of DPPC using spin-labelled globoside or an unattached amphiphilic spin label were consistent with these findings. The same systems lent themselves to consideration of the role of glycolipid fatty acid chain length and cholesterol in determining glycolipid crypticity in membranes: (1) polyclonal anti-globoside IgG bound to globoside in DPPC liposomes without inducing agglutination. (2) The same antibodies did agglutinate DPPC/cholesterol liposomes bearing globoside. (3) The effect of cholesterol probably was upon glycolipid dynamics or attitude in the membrane, rather than upon distribution. (4) These observations were basically unaffected by the choice of 18-carbon vs. 24-carbon glycolipid fatty acid. (5) It appears that the (tetrasaccharide) globoside sugar chain is sufficiently long that the importance of fatty acid chain length in determining headgroup 'accessibility' to antibodies is greatly reduced relative to the situation of monosaccharide species such as galactosylceramide.

Abbreviations: globoside, GalNAcβ1→3Galα1→4Galβ1→4Glc→Cer; G_{M1}, Galβ1→3GalNAcβ1→4(NeuAcα2→3)Galβ1→4Glc→Cer; G_{M2}, GalNAcβ1→4(NeuAcα2→3)Galβ1→4Glc→Cer; G_{D1a}, NeuAcα2→3Galβ1→3GalNAcβ1→4(NeuAcα2→3)Galβ1→4Glc→Cer; DPPC, 1,2-dipalmitoylglycerophosphocholine; TEMPO, 2,2,6,6-tetramethylpiperidine-*N*-oxyl; EPR, electron paramagnetic resonance.

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Introduction

In addition to serving as recognition sites that mediate interaction of cell with surroundings, glycosphingolipids in the cell membrane are thought to play a significant structural role. It is an observed fact that glycolipid receptor function is critically modulated by 'arrangement' in the membrane (reviewed in Refs. 1–4); and one would presume that any structural involvement will also depend strongly upon this factor. Hence, there has been widespread interest in glycolipid physical behaviour. Studies of pure glycolipids hydrated to form micelles or lamellar structures comprise an important part of research in this area. However, while experiments with pure glycolipids have provided valuable insight into the forces controlling their behaviour, this approach does not permit direct extrapolation to membranes. As a result, techniques have been devised to assemble glycolipids into phospholipid bilayers that mimic selected aspects of cell membranes [5]. Bilayers comprised totally of synthetic phospholipids with highly homogeneous headgroup and fatty acid composition offer the advantage that effects related to the host matrix phase transition can be studied to provide information regarding glycolipid arrangement and receptor function. Indeed, there has been some considerable success in analysing such systems (reviewed in Ref. 1). However, cell membranes and their closest model membrane analogues do not show sharp phase transitions. Lipid heterogeneity, and the presence of proteins or cholesterol, suppress the cooperativity of temperature-induced phospholipid bilayer fluidisation. It has been pointed out that the results from highly cooperative synthetic systems cannot be extrapolated to the non-cooperative, intermediate fluidity situation of cell membranes [1]. In consequence, a number of attempts have been made at determining the physical arrangement of glycolipids in systems more closely approximating the features of cell membranes, and in cell membranes themselves (reviewed in Refs. 3 and 4). Perhaps foremost amongst the questions left incompletely answered in such work has been that of glycolipid lateral distribution. There has been great disagreement regarding the possibility that glycolipids may 'cluster' in membranes, i.e., form domains of high local concentration. Evidence exists from experiments with both cells and model membranes that local glycolipid enrichment may occur. However, in most cases contradictory suggestions from other experiments exist. Complicating the picture is the view that glycolipid behaviour may be a sensitive function of headgroup sugars and ceramide fatty acid. In this article, we focus on development of a specific family of glycosphingolipids with attached spectroscopic probes in the hope of overcoming some of the difficulties that have occurred. The probe chosen was the nitroxide spin label at carbon 16 of 18-carbon and 24-carbon fatty acids. These fatty acids were used to replace the natural fatty acid of globoside. Covalent attachment of the probe assures maintenance of spectral sensitivity even at the low glycolipid concentrations typical of cell membranes.

The complex neutral glycolipid, globoside, (GalNAcβ1 → 3Galα1 → 4Galβ1 → 4Glc → Cer) provides an important example for questions currently being asked about glycosphingolipids. It was demonstrated very early on that antibodies to globoside bind much more extensively to fetal erythrocytes than to those of later developmental stages, although antigen quantities are similar in the membranes [6]. The term 'crypticity', has come to be applied to such phenomena – in which glycolipids show an apparently decreased recognition function toward exogenous macrostructures – and has been noted for a number of glycolipids in both cell and model membranes [4,7]. Globoside was also identified early on as having the capacity to alter the physical properties of a host phospholipid matrix [8]. It seems a general property of glycosphingolipids that they can decrease the fluidity of membranes, and no doubt influence macroscopic properties including bilayer curvature [4,9]. We show here that globoside with probe-labelled fatty acids may be used to address these phenomena in molecular terms.

Experimental procedures

Materials

1,2-Dipalmitoylglycerophosphocholine and galactosylceramide (type II) were from Sigma, St. Louis, MO, U.S.A. Cholesterol was from Serdary

Research, London, Canada. Stearic acid was from P-L Biochemicals, Milwaukee WI, U.S.A. *N*-Tetracosanoic acid (lignoceric acid) was from Supelco, Bellefonte, PA, U.S.A. The above lipids ran as single spots on thin-layer chromatography (TLC) plates (Merck silica gel 60) eluted with 65:25:4 (v/v) $CHCl_3/CH_3OH/H_2O$, and developed with 1:2.75 sulfuric acid/ethanol spray. The unattached spin label, TEMPO, was from Aldrich. Spin-labelled fatty acids employed in this work were prepared following the general method of Hubbell and McConnell [10]. We have described synthesis of the long chain fatty acid spin label previously [11].

Globoside was isolated from porcine red blood cells according to the method of Hakomori and Siddiqui [12], except that the crude sphingolipid extract was run on a silicic acid column (BioSil A, 3 × 70 cm eluted with 1 l of 1:9 (v/v) $CH_3OH/CHCl_3$ and 1.5 l of 1:4 $CH_3OH/CHCl_3$, followed by a gradient of 1:4–2:3). The globoside fraction was further purified on a small silicic acid column eluting with 60:40:4 (v/v) $CHCl_3/CH_3OH/NH_4OH$. It ran as a single spot on TLC plates eluted with 55:25:4 $CHCl_3/CH_3OH/H_2O$.

Glycolipid and phospholipid synthesis

In synthesizing globosides with selected fatty acid composition we adapted the procedure developed by Neuenhofer et al. for producing lysoG_{M1} [13]. The various stages of the synthesis were monitored by ^{1}H-NMR. Typically, we started with 50–100 mg quantities of native globoside. Hydrolysis in methanolic KOH at 100 °C in tightly sealed Kimax (Kimble) culture tubes produced only one (slower running-R_F value, 0.08) spot, that was ninhydrin-positive on TLC plates eluted with 60:40:9 $CHCl_3/CH_3OH/2M$ NH_4OH. After neutralization of excess KOH, this material could be isolated by column chromatography on silicic acid (Bio-Sil A 200–400 mesh) eluting successively with 30, 40, 50 and 60% $CH_3OH/CHCl_3$. ^{1}H-NMR spectroscopy showed that the *N*-acetyl function of the sugar headgroup had been lost along with the fatty acid. It is hydrolytic loss of sugar *N*-acetyl groups that necessitates the sequence of blocking, reacetylation and deblocking developed by Neuenhofer et al. for G_{M1}. The same sequence of reactions led to successful replacement of the galactosamine *N*-acetyl group in lysogloboside as demonstrated by ^{1}H-NMR. This material was purified by column chromatography as described above. Appropriate fatty acids were attached to lysogloboside by the procedure we originally described for lysogalactosylceramide [14]. The final fatty acid-replaced globosides were purified on the same type of column, coming off in 40–50% $CH_3OH/CHCl_3$. Spin-labelled globosides ran with identical R_F values to native globoside on the TLC system described above. Yield was 10% based on native globoside starting material. Fatty acid-substituted galactosylceramide was synthesized as previously described [14]. Glycerophosphocholine with palmitic acid at the 1-position and 16-nitroxy stearic acid at the 2-position was synthesized by the method of Hubbell and McConnell [10]. The synthesis of globoside with a spin label on the terminal GalNAc residue has been reported by us earlier [15].

EPR spectroscopy

Spectra were run on a Varian E12 EPR spectrometer equipped with TM_{110} microwave cavity and Varian variable temperature accessory. Samples were sealed in disposable 50 μl glass capillary micropipettes and held in the Dewar insert using the device originally described by Gaffney and McNamee [16]. Sample temperature was monitored with a copper/constantan thermocouple whose tip rested just above the cavity sweep coils. For TEMPO studies, liposome suspensions were mixed with a small aliquot of the spin label to give a lipid-to-spin label molar ratio of 125:1 (spin label added from a $5 \cdot 10^{-3}$ M aqueous stock solution). Data treatment was as recommended by Shimshick and McConnell [17,18]. EPR spectra of phospholipid and glycolipid spin labels were digitised on a Tektronix 4954 Graphics Tablet interfaced with a Cyber 835 mainframe computer and stored on disk. Digitised spectra were then manipulated according to programs written to run on the above computer system. Manipulation included double integration with a wedge baseline correction, and subsequent normalisation of all spectra to a fixed, arbitrary sample quantity. The programs written for this work permitted addition

of pairs of spectra in selected ratios to mimic coexistence of domains with different properties.

Sample preparation

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 stream of nitrogen gas. 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 appropriate buffer. All samples were incubated 10°C above their transition temperatures for 15 min to assure bilayer diffusional equilibrium, before being allowed to cool to the temperature of study.

Immunological studies

Antisera to globoside and galactosylceramide were raised in New Zealand White rabbits by subcutaneous and intradermal injection according to the procedure of Alving et al. [19]. The immunoglobulin fractions were purified by two successive ammonium sulfate precipitations and then dissolved in isotonic saline at the original serum concentration. Prior to use, aliquots of stock IgG solutions were preadsorbed to remove nonspecific liposome-binding fractions. This involved incubating 1 ml of stock IgG solution with liposomes (10–20 mg of DPPC/cholesterol 2:1 molar ratio) for a period of 2 h at 22°C with gentle agitation on a rocker table: liposomes with bound nonspecific antibodies were subsequently removed by centrifugation for 15 min at 13000 × *g*. Antibody-induced agglutination of liposomes bearing glycolipid hapten was assayed by two different methods. The first involved use of microtiter plates (Dynatech Labs, Alexandria, VA, U.S.A., polystyrene – U wells) and the classic hemagglutination approach, but with liposomes replacing erythrocytes (see legend to Table I for details). Alternatively, the method of Utsumi et al. was used, which involves determination of the agglutination endpoint by monitoring the effect of 2-fold serum dilutions on droplets of liposome suspensions under the light microscope [20].

In order to measure antibody binding activity for a given type of liposome, immune sera were first preadsorbed for nonspecific binding as described above. Subsequently, 50-μl aliquots of sera were adsorbed with measured quantities of the (glycolipid-bearing) liposome suspensions being studied. This was done by incubating serum plus liposomes for 24 h at 22°C on a rocker table, followed by centrifugation for 5 min at 13000 × *g*. The amount of anti-globoside or anti-galactosylceramide antibody remaining in the clear supernatant was then quantitated in a microtitre assay using standard liposomes known to agglutinate readily in the presence of specific antibody.

Results and Discussion

Spin-labelled globoside

The simple neutral glycolipids, galactosylceramide and lactosylceramide, with spin-labelled fatty acids have been reported previously [11,20,21]. However, in both cases, the native fatty acid could be replaced via a two-step process. A different approach may be generally required for more complex species with amino sugars, since sugar *N*-acetyl groups can be sensitive to the base hydrolysis step used to remove the natural fatty acid. Neuenhofer et al. reported virtually complete removal of acetyl groups from the sialic acid residues of gangliosides [13]. Interestingly though, they saw minimal hydrolysis of acetate from *N*-acetylgalactosamine residues of G_{M1}, G_{M2} and G_{D1a}. Globoside, of course, is without sialic acid. However in globoside we observed that the *N*-acetylgalactosamine residue was completely deacetylated by the initial hydrolysis step, presumably due to differences in accessibility. Hence, in generating lysoglobоside we followed the protocol that Neuenhofer et al. developed to produce lysoG$_{M1}$ [13]. This protocol involves base hydrolysis of amide linkages, specific blocking of the sphingosine -NH_2 group, reacetylation of sugar -NH_2 groups and subsequent deblocking. The product was then reacylated with selected fatty acids. Typical overall yield based on starting native glycolipid was 10%. Fig. 1 shows the spin-labelled globosides used in this work. Note that in each case the nitroxide ring is located at the same position in the fatty acid, i.e., on carbon-16, and, thus, should probe the same region of the bilayer membrane: that of the methyl termini of the phospholipids comprising the bilayer.

Implications of spectral analysis to glycolipid distribution

The spectral features of spin labels attached to molecules in membranes reflect polarity of environment, motional rate, motional anisotropy (i.e., preferential motional directionality) and frequency of collision between labelled molecules. Slow motion broadens all spectral lines due to relaxation effects. Environment polarity and motional anisotropy alter EPR spectral line position (the latter has formed the basis of order parameter calculations in membranes [10,22]). Collision between spin label nitroxide radicals produces a characteristic change in which spectral lines broaden and shift inwards (so-called 'spin exchange broadening'): as a result, at high spin label concentration EPR spectra collapse to a single line [23]. High spin label concentration in the experiments described here might result from the initial addition of large amounts of glycolipid to phospholipid membranes, or from phase separation of what little glycolipid there is within the bilayer into regions of locally high concentration.

Since the question of glycolipid distribution in membranes has proven elusive, we address it here based upon observations with this new family of spectroscopic probes. Although a variety of forms of data manipulation are possible, the most concrete method of spectral interpretation in this situation is comparison with standards. Thus, in addressing the question of how spin-labelled glycolipids behave in bilayers composed of phosphatidylcholine, we chose a set of standards consisting of spin-labelled phospholipids in bilayers. We used the same 18-carbon fatty acid spin label shown attached to globoside in Fig. 1, but attached to phosphatidylcholine in a matrix of DPPC. Spin-labelled phospholipids have been tested by other workers and shown to exhibit essentially random distribution in phosphatidylcholine bilayers (e.g., Ref. 22). 25 mol% cholesterol was added to the standards to suppress the cooperative phase transition. Inclusion of cholesterol also made it possible to alter spin label mobility in the standards smoothly by varying temperature, as necessary, to match optimally this variable in glycolipid spectra. The library of standards consisted of 128 spectra recorded over a temperature range from -30 to $+55\,^{\circ}C$ for 1-palmitoyl-2-(16-nitroxy)-stearoylphosphatidylcholine at concentrations of 1, 2, 5, 10, 15, 20, 25, 30 mol% (examples in Fig. 2). The process of analysing spectra of spin-labelled glycolipids involved comparison with standards of different overall (phospholipid) spin label concentration. It was made more reliable by using a computer program to normalise all spectra via double integration. The program was written to permit correction for sloping base lines, and linear combination of pairs of standards to simulate coexistence of domains with different composition (see Materials and Methods). Thus, using known standards, one could test theories of glycolipid arrangement. The technique is illustrated in Figs. 3 and 4.

We were primarily interested in examining globoside spectra from samples of relatively low glycolipid content, since it is in such samples that the spin label technique has most to offer relative to other approaches, and since this is the concentration region of greatest physiological relevance. Some idea of the questions which should be asked is obtained from the following examples of recent literature observations. There is evidence from freeze-etch electron microscopy of Forssman antigen and globoside in erythrocytes that these species are sharply phase separated into domains

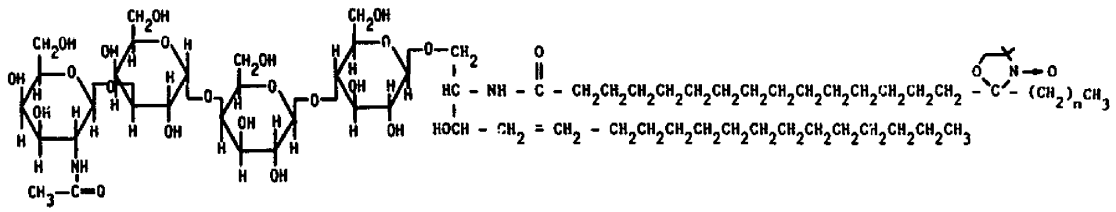

Fig. 1. Structures of the 18-carbon and 24-carbon fatty acid spin labelled globosides: stearoyl-16-nitroxyglobоside ($n = 1$) and lignoceroyl-16-nitroxyglobоside ($n = 7$).

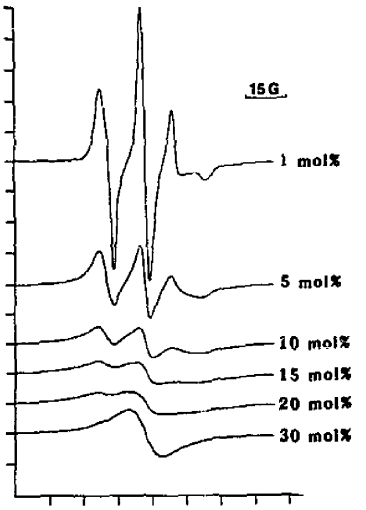

Fig. 2. Typical spectra from the library of standards used to estimate glycolipid local concentrations within membranes. Each standard sample consisted of DPPC/cholesterol (3:1 molar ratio), in which a portion of the phosphatidylcholine was spin-labelled (1-palmitoyl-2-(16-nitroxy)stearoylphosphatidylcholine). All spectra have been normalised to a constant total amount of spin label, but reflect different concentrations within the membrane: the spin label concentration is recorded to the right of each spectrum. Spectra shown were run at 34.4° C. Liposomes were hydrated in phosphate-buffered saline (pH 7.4), incubated at 50° C for 15 min and cooled slowly to 22° C prior to placement in the spectrometer.

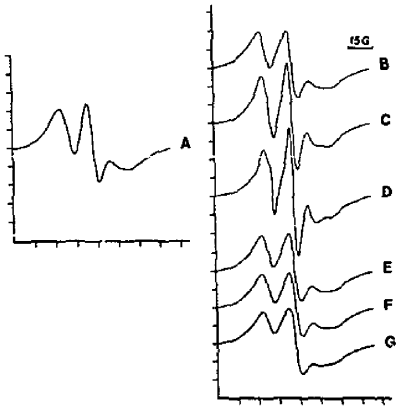

Fig. 3. Normalised EPR spectrum of globoside (with 18-carbon spin-labelled fatty acid) at 10 mol% in a DPPC host matrix (A). Shown for comparison are the following phospholipid spin label standards: (B) 10 mol%; (C) 0.5 of 5 mol%+0.5 of 15 mol%; (D) 0.2 of 1 mol%+0.8 of 20 mol%; (E) 0.1 of 30 mol%+0.9 of 10 mol%; (F) 0.2 of 20 mol%+0.8 of 10 mol%; (G) 0.3 of 30 mol%+0.7 of 10 mol%. Sample preparation as in Fig. 2. Globoside spectrum run at 26° C.

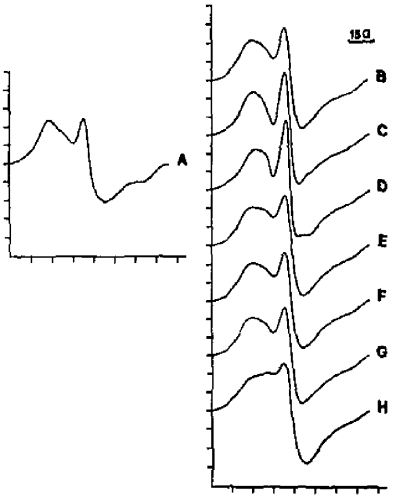

Fig. 4. Normalised EPR spectrum of globoside (with 24-carbon spin-labelled fatty acid) at 10 mol% in a DPPC host matrix (A). Shown for comparison are the following phospholipid spin label standards: (B) 10 mol%; (C) 0.5 of 5 mol%+0.5 of 15 mol%; (D) 0.2 of 1 mol%+0.8 of 20 mol%; (E) 0.1 of 30 mol%+0.9 of 10 mol%; (F) 0.2 of 20 mol%+0.8 of 10 mol%; (G) 0.2 of 15 mol%+0.8 of 10 mol%; (H) 0.5 of 30 mol%+0.5 of 10 mol%. Sample preparation as in Fig. 2. Globoside spectrum run at 26° C.

of up to 1-μm diameter [24]. Electron microscopy and glycolipid transfer rates measured in liposomes suggest that asialo-G_{M1} (whose structure, Galβ1 → 3GalNAcβ1 → 4Galβ1 → 4Glc → Cer, is similar to globoside) phase separates into distinct microdomains up to 0.1 μm in diameter [25]. There have been indications that neutral glycolipids in general phase separate in phospholipid bilayer membranes (reviewed in Refs. 3 and 4). Existing calorimetry measurements of galactosylceramide in dipalmitoylphosphatidylcholine showed evidence of relative dispersion when it bore a 16-carbon fatty acid, and dramatic exclusion from the bilayer when the fatty acid was a natural brain mixture (reviewed in Ref. 1). Thus, one should be looking both for evidence of distinct phase separation of globoside, and for evidence that the length of the fatty acid may be a key determinant of such separation. Based on the striking sensitivity to spin label concentration indicated in the standard spectra of Fig. 2, it should be possible to distinguish amongst at least various possible major scenarios for globoside arrangement. For instance the existence of significant

domains with very low mol% glycolipid will give rise to (three) intense, narrow lines. On the other hand domains with 20 mol% or more glycolipid should produce a spectral feature approaching one broad transition. Between 5 and 20 mol% lines broaden and shift inward with increasing spin label concentration. Note from Fig. 2 that broad spectral peaks corresponding to concentrated spin label are of low intensity and difficult to appreciate; while the sharp lines of dilute label are visually emphasised. Since spin label motional rate and order affect spectral appearance, it was important to choose standard spectra of equivalent 'fluidity'. This was done by first selecting the single standard spectrum of the same overall spin label concentration that most closely resembled the 'unknown'. The temperature corresponding to this standard was then used in subsequent selection of pairs of standards for matching. This approach seems justified because in general a single standard (of the same overall spin label concentration) exists that makes a good first approximation to the unknown.

We have focused our study on glycolipids with overall membrane concentrations of 2 mol% and 10 mol%. The appearance of 10 mol% globoside with 18-carbon spin-labelled fatty acid is shown in Fig. 3, A, along with selected standards for comparison. Certainly with the stearic acid spin label, the spectrum of 10 mol% globoside is quite similar to that of the 10 mol% spin-labelled phospholipid standard (Fig. 3, B). Adding any significant fraction of more dilute standard made the fit noticeably worse. The 1 : 1 combination of 5 mol% with 15 mol% standards (Fig. 3, C) and the 1 : 4 combination of 1 mol% with 20 mol% (Fig. 3, D) are illustrated. Another important possibility to consider is that globoside may be totally excluded from some regions of DPPC bilayer, to produce coexisting domains of considerable enrichment and total absence of globoside. Clearly, as already demonstrated by the basic similarity of 10 mol% globoside and 10 mol% standard, there cannot be a large fraction of DPPC without glycolipid. In order to estimate the upper limit of domains concentrated in globoside we have tried manipulations such as computer addition of a 1 : 4 or 1 : 9 ratio of the broader standard spectra, 20 mol% and 30 mol% spin label, to the 10 mol% standard (e.g., Fig. 3, E and F). This did not improve the fit to the 10 mol% globoside spectrum, and probably worsened it. Certainly, adding more than this fraction of broad component did reduce the fit (e.g., Fig. 3, G), thus strongly arguing against the great majority of 18-carbon fatty acid globoside being in phase-separated domains containing greater than 15 mol% glycolipid. Our data do not, however, exclude the possible presence of up to 1/10 or even 1/5 of the glycolipid in domains of concentration considerably higher than the overall concentration. This possibility – that as much as 1/5 of the globoside could be in concentrated domains or pure clusters without our being able to positively identify the resultant spectral features – represents a practical estimate of our experimental uncertainty. It is attributable largely to the fact already mentioned that spectral features are controlled not only by spin exchange, but also by motional rate and anisotropy which are expected [21] to differ independently between (phospholipid) standards and (glycolipid) unknowns even at identical concentrations and distributions. Thus, peak positions, widths and relative intensities cannot be perfectly matched. Attempts to test for the presence of underlying broad spectral components by serial subtraction of sharp standard spectra [26] from glycolipid spectra were not advantageous for the same reason.

For the globoside with 24-carbon fatty acid incorporated at 10 mol% into DPPC, a somewhat better fit to linewidth was achieved by adding in a minor component of a more concentrated standard such as 15 or 30 mol% to the 10% mol standard (e.g., in a 1 : 9 or 1 : 4 ratio (Fig. 4, E, F and G)) to simulate coexistence of domains with different composition. Once again though the 10 mol% phospholipid standard on its own (Fig. 4, B) was a reasonable first approximation to the 10 mol% globoside (with 24-carbon fatty acid) (Fig. 4, A). A poorer match was obtained in attempting to simulate phase separation with combinations of more dilute standards plus more concentrated standards (Fig. 4, C and D). As in the case of the 18-carbon derivative, we are unable to exclude the possibility that up to 1/5 of the globoside may be in highly concentrated domains in the overall 10 mol% sample, although larger quantities cannot be involved.

10 mol% globoside would be a high glycolipid concentration in a cell membrane. Figs. 5 and 6 summarise the results obtained with 2 mol% globoside spin label in DPPC bilayers. As was observed with the 10 mol% samples, reasonable approximations to the spectra of 2 mol% globoside in DPPC were obtained from single phospholipid standards of the same concentration (Fig. 5, A and Fig. 6, A). However, a somewhat better fit was obtained in each case by using a combination of standards with higher (5 mol%) and lower (1 mol%) spin-labelled phospholipid concentrations – as would be expected from a simple phase-separation of globoside into domains of higher and lower concentrations (Fig. 5, B and C and Fig. 6, B).

With both 10 mol% and 2 mol% globoside in DPPC, the two most important observations were that (1) the results are completely inconsistent with dramatic exclusion of glycolipid from the bilayer host matrix into domains enriched in glycolipid and (2) the 18-carbon and 24-carbon fatty acid species show basically similar behaviour.

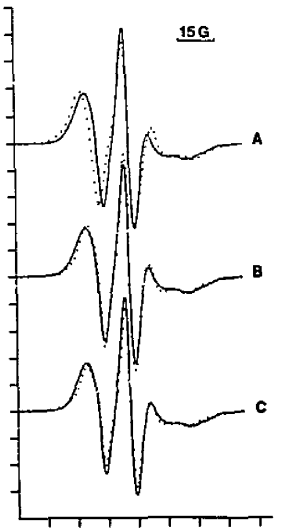

Fig. 5. Normalised EPR spectrum of globoside (with 18-carbon spin-labelled fatty acid) at 2 mol% in a DPPC host matrix. The spectrum of globoside (solid line) is shown superimposed upon the following three normalised standards or composites of standards (dotted lines) for comparison: (A) 2 mol%; (B) 0.7 of 1 mol% + 0.3 of 5 mol%; (C) 0.5 of 1 mol% + 0.5 of 5 mol%. Sample preparation as in Fig. 2. Globoside spectra run at 22° C.

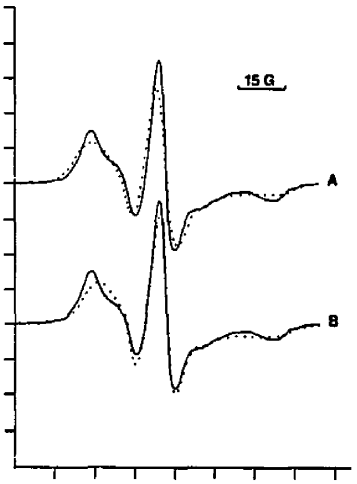

Fig. 6. Normalised EPR spectrum of globoside (with 24-carbon spin-labelled fatty acid) at 2 mol% in a DPPC host matrix. The spectrum of globoside (solid line) is shown superimposed upon the following two normalised standards or composites of standards (dotted lines) for comparison: (A) 2 mol%; (B) 0.7 of 1 mol% + 0.3 of 5 mol%. Sample preparation as in Fig. 2. Globoside spectra run at 22° C.

Effect of temperature variation

The response of any crystal to temperature variation reflects its molecular arrangement. This is particularly true when the temperature region scanned encompasses a phase transition/phase separation. Bilayer membranes have crystal properties, and may undergo phase transitions if they possess a high proportion of a single phospholipid type. The presence of an 'impurity' (globoside in this case) alters melting behaviour in ways that depend upon the 'impurity' arrangement in the bilayer membrane. This logic has formed the basis for some excellent studies via differential scanning calorimetry of pure glycolipids and their mixtures with dipalmitoylphosphatidylcholine (reviewed in Ref. 1). Sample response to temperature variation may be followed in the EPR spectrometer in our case, since spectral features of membrane-associated spin labels are sensitive to the melting process. An important aspect of the work described here is that, since the nitroxide probe is covalently attached to the glycolipid, any spectral changes

reflect conditions at the location of the glycolipid, even at the low concentrations typical of cell membranes. Alternatively, of course, one may use the spin label TEMPO, that partitions generally throughout lipid membranes [17,18], to achieve a result more directly comparable to the calorimetry approach. Thus, by monitoring a given spectral feature such as peak height ratio as a function of temperature, it is possible to record sample melting behaviour. We were particularly interested to test whether the melting process experienced by small amounts of glycolipid in DPPC bore a close relationship to that of the bulk host matrix, and whether there was obvious sensitivity to fatty acid chain length. Bilayers of pure DPPC, when warmed from the gel state, manifest a premelting phenomenon at about 35°C related to phospholipid reorientation, followed by a sharp phase change to the fluid ('liquid crystal') state at 41.5°C. This behaviour is well known from calorimetric, spin label [18], NMR and fluorescence studies (reviewed in Ref. 26). Any spin label dispersed in such a membrane should register dynamic changes associated with melting of the surrounding phospholipid matrix. Thus, if a DPPC membrane containing a very small amount of globoside spin label homogeneously distributed throughout were warmed through the phase transition region, one would expect to see the EPR spectrum change in a fashion closely reflecting the behaviour of DPPC itself. This is the basis of using trace quantities of the spin label, TEMPO, to monitor membrane phase behaviour. Typical results of experiments with the small, unattached, TEMPO spin label are illustrated in Fig. 7A and B where it was used to monitor the melting of DPPC host matrices bearing non-spin-labelled globosides with 24-carbon, and 18-carbon fatty acids, respectively. The curves are quite similar to one another and to the curve for pure DPPC (Fig. 7, C): they show small inflections corresponding to the host matrix pre-transition between 32 and 35°C, with main transition at 41°C. In contrast to the situation with TEMPO, when the spin label is covalently attached to globoside, any dramatic glycolipid phase separation would lead to the spin label preferentially sampling domains with relatively high glycolipid concentration. Domains of high glycolipid concentration should show broadened

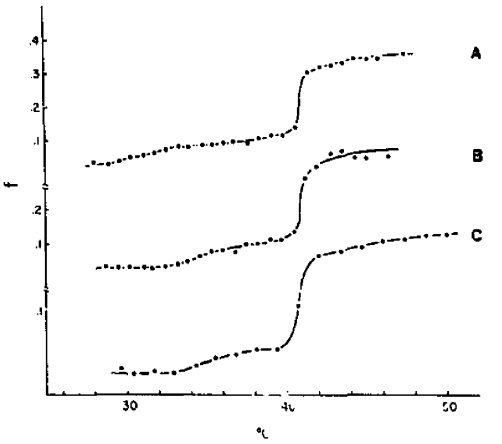

Fig. 7. TEMPO-derived melting curves for dipalmitoylphosphatidylcholine liposomes bearing unlabelled globoside with either 24-carbon or 18-carbon fatty acid. The TEMPO spin label spectral parameter, f, of Shimshick and McConnell [18] has been plotted vs. temperature (°C). Data are shown for (A) 5 mol% globoside with 24-carbon fatty acid in DPPC; (B) 5 mol% globoside with 18-carbon fatty acid in DPPC; and (C) pure DPPC. Sample preparation as in Fig. 2. Samples were run from low to high temperatures.

and altered melting ranges relative to the DPPC host matrix. From the results in the previous section, one would probably anticipate that DPPC melting monitored via small amounts of spin-labelled globoside should to a first approximation produce a picture similar to that seen with TEMPO. Indeed, this is the result obtained (Fig. 8). The major curve feature in each case is associated with the DPPC main transition. Significant curve inflections in the range of 30–35°C are absent, in keeping with suggestions by some workers that it is the headgroup region of the membrane that is most involved in the pre-transition phenomenon [27]. In other words, the primary observation is that over the range 1–15 mol%, the melting behaviour recorded by a spectral probe covalently attached to globoside is similar to that of a probe dispersed randomly throughout the membrane; rather than showing distinctly different behaviour such as might be expected if the glycolipid were sharply phase separated. Upon closer examination there is, however, a difference in phase behaviour between the 24-carbon and 18-carbon globoside: the effect of glycolipid con-

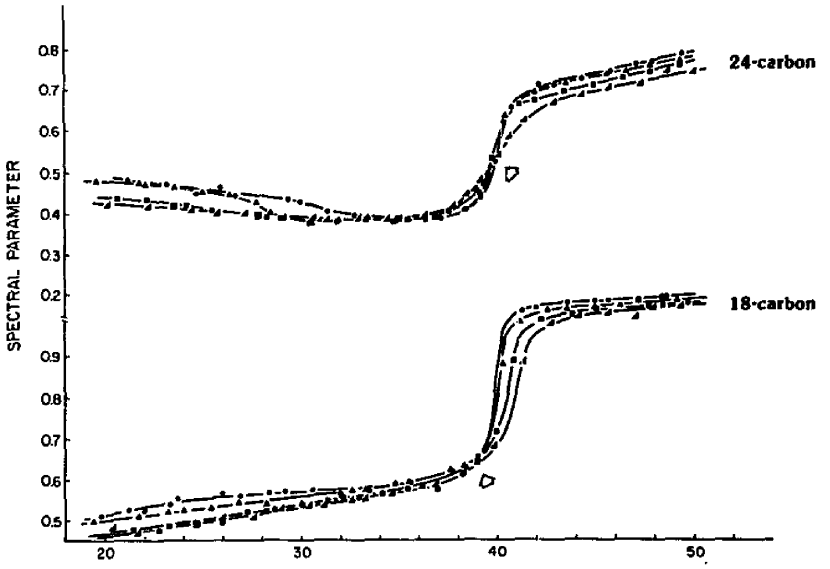

Fig. 8. Spin-labelled-globoside-derived melting curves for dipalmitoylphosphatidylcholine liposomes bearing controlled quantities of globoside. The height ratio of low field-to-mid field spectral peaks was plotted as a function of temperature (° C). Data are shown for 1 mol% globoside in DPPC (●), 5 mol% globoside in DPPC (▲), 10 mol% globoside in DPPC (■) and 15 mol% globoside in DPPC (▲). In each case, 1 mol% globoside was spin labelled while the remainder of the globoside was unlabelled. Upper curves are for globoside with 24-carbon fatty acid; lower curves are for the 18-carbon analogue. The arrow associated with the upper curves indicates the center of the melting region ('transition temperature'); the arrow associated with the lower curves indicates the relatively constant point of melting onset. Sample preparation as in Fig. 2. Samples were run from low to high temperature.

centration on the temperature region of melting is different. The point of melting onset (39° C) remains quite constant between 0 and 15 mol% globoside for the 18-carbon chain species, while the completion of melting shifts some 1.5 C° to higher temperature. For the 24-carbon analogue increasing the glycolipid concentration simply broadens the melting region uniformly, while leaving the transition temperature unchanged.

Prior to the development of synthetic approaches that permit introduction of a spin label into the fatty acid of globoside, we had attached spin labels to the headgroup sugars [15]. These labelled species were incorporated into various phospholipid bilayer systems in order to study headgroup sugar behaviour. One observation we made was that when bilayers of DPPC containing a low percentage of headgroup-labelled globoside were warmed from 0° C to 50° C, the major spectral change was in the range of 30–35° C for DPPC, with a lesser inflection at the 41.5° C main transition. The result of a similar experiment with 10 mol% headgroup spin-labelled globoside in DPPC is illustrated in Fig. 9A. We were unable to distinguish between two major explanations: that the bulk of the globoside was in concentrated domains that melted at a temperature some 8 C° lower than the host matrix, or that the lower curve inflection was somehow associated with a pretransition phenomenon. From our more recent experiments with fatty acid-substituted globosides, we now can assert that the latter is the definitive explanation. The marked effect of the pretransition phenomenon on sugar headgroup vs. fatty acid spin label is interesting (compare, for example, the melting curve for 10 mol% headgroup-labelled globoside in Fig. 9, A to the curves for fatty acid-labelled globoside in Fig. 8). It has been claimed that pretransition phenomena involve the bilayer surface (reviewed in Ref. 27). Our observation of strong pretransition effects of the DPPC host matrix upon glycolipid headgroup sugars is reminiscent of the observation of Myers et al., that ganglioside headgroup 'accessibility' to neur-

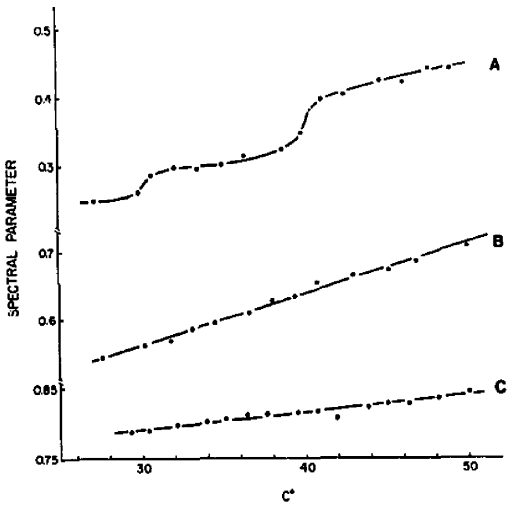

Fig. 9. Spin-labelled-globoside-derived melting curves for DPPC liposomes and DPPC/cholesterol liposomes bearing globoside. The height ratio of low or high field-to-mid field spectral peaks was plotted as a function of temperature (°C). The curve, A, was derived for a sample of 10 mol% globoside with native fatty acid (i.e., fatty acids ranging in length from 18 to 24 carbons on the ceramide backbone) in a DPPC host matrix. Each native globoside molecule was labelled with one spin label covalently attached to the GalNAc residue [15]. In this case, the ratio of high field-to-mid field peaks was plotted vs. temperature (°C). The curves, B and C, were derived for host matrices of 2:1 (molar ratio) DPPC/cholesterol bearing 5 mol% of spin-labelled globoside with 24-carbon fatty acid chain (B) or 18-carbon chain (C). For B or C the globoside fatty acid chain bore a spin label covalently attached at C-16 and the ratio of low field-to-mid field peak heights was plotted. Sample preparation as in Fig. 2. Samples were run from low to high temperature.

aminidase attack rises markedly in the pretransition temperature region, while showing less sensitivity to the host matrix 41°C main transition [28].

Effect of cholesterol

Although studies of phenomena related to the phase transition in model membranes have proven very informative, systems that most closely mimic cell membranes do not show distinct melting behaviour. The membranes of cells are generally (semi) fluid, contain more than 20 mol% cholesterol and manifest considerable lipid heterogeneity – all of which prevent the application of physical techniques that rely upon the measurement of cooperative melting [27,29]. Cell membranes usually display only a steady increase in fluidity with increasing temperature. Several typical temperature profiles for cholesterol-bearing bilayers containing spin-labelled globoside appear in Fig. 9. Note the smooth and featureless change in spectral parameter as a function of temperature. Such profiles provide little insight into membrane molecular arrangement. However, the spectral analysis approach described earlier, in which a set of standards with a known spin label distribution has been normalised for comparison with 'unknown' glycolipid samples, is equally applicable to any membrane. In order to permit comparison with our work using DPPC bilayers, we have examined samples containing the same 10 mol% and 2 mol% globoside spin label. The host matrix in this case was 2:1 (molar ratio) DPPC/cholesterol. The same library of standard spectra was employed. 'Unknowns' were first compared to standards of the same overall spin label concentration, and the single closest fit was chosen. The temperature corresponding to this standard was used in further selection of standards for spectral synthesis. Once again, this approach should be valid, since to a first approximation the unknowns could be adequately matched without difficulty from the standard library of the same overall spin label concentration. Higher temperature standards were required to match the 2 mol% and 10 mol% glycolipid samples in the DPPC/cholesterol host matrix – as expected from the fact that cholesterol fluidises a DPPC matrix below the phase transition temperature [27]. Results are illustrated in Figs. 10 and 11. Once again, the most straightforward approach to analysis of the data is comparison with standards. It is also informative, in this case, to compare the spectra in Figs. 10 and 11 with those in Figs. 5 and 6, respectively. The most striking observation is the similarity between the spin-labelled globoside spectra in a host matrix with or without cholesterol (i.e., compare Fig. 10, A with Fig. 5, A and compare Fig. 11, A with Fig. 6, A). Thus, the primary conclusion would have to be that there is remarkably little difference in lateral distribution of globoside in the cholesterol-containing matrix as sensed by the spin label at C-16. A secondary observation, however, is that whereas adding fractions of different standards usually improved the

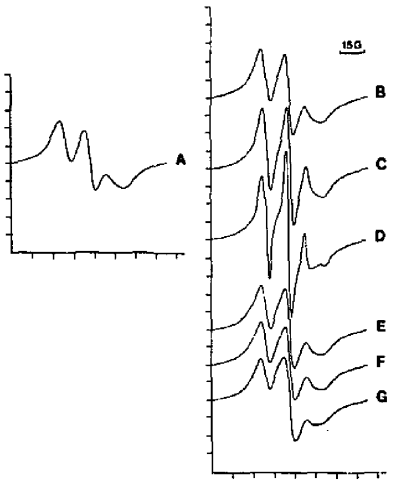

Fig. 10. Normalised EPR spectrum of globoside (with 18-carbon spin-labelled fatty acid) at 10 mol% in a DPPC/cholesterol (2:1 molar ratio) host matrix (A). Shown for comparison are the following phospholipid spin label standards: (B) 10 mol%; (C) 0.5 of 5 mol% + 0.5 of 15 mol%; (D) 0.2 of 1 mol% + 0.8 of 20 mol%; (E) 0.1 of 30 mol% + 0.9 of 10 mol%; (F) 0.2 of 20 mol% + 0.8 of 10 mol%; (G) 0.3 of 30 mol% + 0.7 of 10 mol%. Sample preparation as in Fig. 2. Globoside spectrum run at 26 °C.

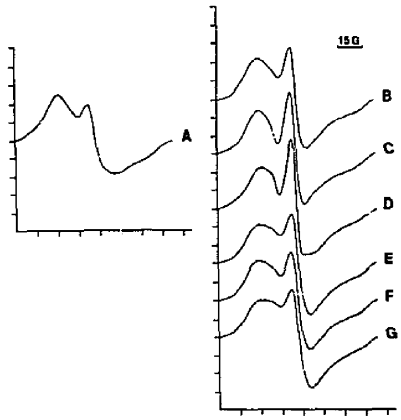

Fig. 11. Normalised EPR spectrum of globoside (with 24-carbon spin-labelled fatty acid) at 10 mol% in a DPPC/cholesterol (2:1 molar ratio) host matrix (A). Shown for comparison are the following phospholipid spin label standards: (B) 10 mol%; (C) 0.5 of 5 mol% + 0.5 of 15 mol%; (D) 0.2 of 1 mol% standard + 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%. Sample preparation as in Fig. 2. Globoside spectrum run at 26 °C.

spectral fit to the samples without cholesterol, this was not the case in the samples with cholesterol. Such an observation is consistent with the concept that some degree of phase separation existed in the DPPC host matrix, which was decreased in the cholesterol-containing matrix. Qualitatively, this is the same result obtained by Utsumi et al. for galactosylceramide in DPPC vs. DPPC/cholesterol [20], although the degree of phase separation in our case is less striking.

Receptor function of globoside spin labels

Naturally occurring globoside is one of the earliest recorded examples of glycolipid crypticity in membranes [6]. The lipid bilayer systems studied here involve phenomena thought to be capable of inducing crypticity; hence, we have considered this aspect using the labelled globosides. A classic crypticity-related observation has been that lipid haptens in a host matrix of rigid DPPC demonstrate relatively poor 'interaction' with specific macromolecules directed against them, while the addition of cholesterol improves such interaction. With regard to glycolipids, this phenomenon has been studied by Inoue and Nojima [20,30–32] who have taken the view that cholesterol may cause glycolipid haptens to disperse laterally in the DPPC membrane, thus increasing their spatial accessibility to macromolecules. In particular they have studied the simple glycolipid, galactosylceramide with spin label in the fatty acid chain [20,31,32]. They observed that, in the absence of cholesterol, DPPC liposomes bearing galactosylceramide failed to be agglutinated by antibodies directed against the glycolipid. EPR spectra of the spin-labelled glycolipid were interpreted in terms of the latter being in close proximity to one another (clustered) at 10 mol% in DPPC. Addition of cholesterol was seen to sharpen the spectral peaks of the spin label (consistent with reduced glycolipid-glycolipid proximity, reduced dipolar interaction and/or increased mobility). It also permitted marked specific antibody-induced liposome agglutination. The authors favoured the interpretation that a lateral phase separation of galactosylceramide was occurring, sufficient to induce clustering and headgroup sugar crowding in (rigid) DPPC bilayers at 15–20 °C, but that there was relative dispersion of the glycolipid in the pres-

ence of cholesterol [20,31,32]. Interestingly, under similar circumstances, anti-Forssman antibody bound equally well to its antigen (a pentasaccharide-bearing complex glycolipid) in a DPPC host matrix with or without cholesterol [30].

We have measured the immune receptor function of our new family of spin-labelled complex glycolipids. Anti-globoside serum was produced in rabbits, and the globulin fraction was isolated (see Materials and Methods). Liposomes bearing globoside were tested directly for agglutinability by anti-globoside IgG in a microtitre plate assay, and in an assay similar to that of Inoue and Nojima that involved light microscope visualisation of agglutination endpoints on glass slides [20]. Antibody binding was assayed by preadsorbing solutions of known antibody concentration with aliquots of a given liposome preparation: after centrifugation to remove liposomes (with any bound antibody) the supernatants could be tested for residual antibody concentration by assaying their agglutinating potency against a standard liposome preparation that has been shown to agglutinate strongly in the presence of specific antibody. Typical results are summarised in Tables I and II. Liposomes formed of DPPC/globoside (without cholesterol) were not agglutinated by antisera in our assays. This was true for native globoside, and also for globoside spin-labelled with the 18-carbon or 24-carbon fatty acid. When the liposomes contained 33 mol% cholesterol, they were subject to striking agglutination by specific immune sera. Once again, however, there was no obvious difference between the result obtained with 18-carbon vs. 24-carbon fatty acid.

Interestingly, although agglutination of liposomes bearing globoside in a host matrix of DPPC alone did not occur, antibody binding did. This was readily demonstrated, for instance, in the preadsorption assay (Table II). One should note that this is different from the result of Inoue's group mentioned above for the monosaccharide species, galactosylceramide, in an identical assay [20,31,32]. It is, however, in agreement with their observation that the pentasaccharide glycolipid, Forssman antigen, in rigid DPPC bilayers binds antibody specifically [30]. We therefore tested galactosylceramide in our microtitre assay system and Inoue's system and found the same result as that which they reported (Tables I and II). It

TABLE I

EFFECTS OF GLYCOLIPID FATTY ACID CHAIN LENGTH AND PRESENCE OF CHOLESTEROL UPON ANTIBODY-MEDIATED AGGLUTINATION OF LIPOSOMES BEARING GLOBOSIDE OR GALACTOSYLCERAMIDE

The occurrence of agglutination is indicated by +, and the endpoint is given. DPPC/cholesterol data shown are for a 2:1 molar ratio; however, we also employed a 1:1 molar ratio with similar results. Galactosylceramide data shown correspond to liposomes with dicetylphosphate (DCP) (DPPC/cholesterol/DCP in a 2:1:0.22 molar ratio) as employed by previous workers. Inclusion of dicetylphosphate in our liposomes bearing globoside did not alter the endpoints. The 18-carbon and 24-carbon fatty acids attached to glycolipids were spin labelled at C-16. Galactosylceramide natural fatty acids are primarily 24-carbon. γ-Globulin fractions of polyclonal antisera were used after preadsorption with DPPC/cholesterol liposomes (1 ml of antibody solution incubated with 10 mg of lipid overnight on a rocker at 22°C) to reduce nonspecific binding.

Sample	Agglutination	Endpoint (serum dilution)
DPPC	–	–
DPPC/5 mol% native globoside	–	–
DPPC/5 mol% 18-carbon globoside	–	–
DPPC/5 mol% 24-carbon globoside	–	–
DPPC/chol (2:1 molar ratio)	–	–
DPPC/chol/5 mol% native globoside	+	1:64–1:128
DPPC/chol/5 mol% 18-carbon globoside	+	1:64–1:128
DPPC/chol/5 mol% 24-carbon globoside	+	1:64–1:128
DPPC/chol/DCP/5 mol% native galactosyl ceramide	+	1:512–1:1024
DPPC/chol/DCP/5 mol% 18-carbon galactosylceramide	+	1:64–1:128
DPPC/chol/DCP/5 mol% 24-carbon galactosylceramide	+	1:256–1:512

TABLE II

EFFECTS OF GLYCOLIPID FATTY ACID CHAIN LENGTH AND PRESENCE OF CHOLESTEROL UPON SPECIFIC ANTIBODY BINDING TO LIPOSOMES BEARING GLOBOSIDE OR GALACTOSYLCERAMIDE

Binding of antibody by a given liposome/glycolipid combination (indicated by +) was demonstrated by titering antibody solutions before and after incubation with the liposomes in question. γ-Globulin fractions were first preadsorbed as already described to remove nonspecific lipid-binding portions. They were subsequently titered by noting the agglutination endpoint using liposomes of DPPC/cholesterol bearing 5 mol% of either galactosylceramide or globoside as appropriate. 50 μl of this antibody solution was then incubated at 22°C for 24 h with an aliquot of the liposomes whose binding function was to be measured (a total of 1 μmol of phospholipid for samples containing globoside or 5 μmol for samples containing galactosylceramide). The liposome suspension was centrifuged at 13000×*g* for 5 min, and supernatant was carefully removed for serial dilution to obtain the new titer (shown in the table as Residual agglutinating potential (endpoint dilution)). Note that an endpoint of 1:2 indicates the first well of the microtiter plate.

Serum incubated with:	Residual agglutinating potential (endpoint dilution)	Binding
DPPC	1:16	–
DPPC/5 mol% native globoside	1:2	+
DPPC/5 mol% 18-carbon globoside	1:2	+
DPPC/5 mol% 24-carbon globoside	–	+
DPPC/chol (2:1 molar ratio)	1:16	–
DPPC/chol/5 mol% native globoside	–	+
DPPC/chol/5 mol% 18-carbon globoside	–	+
DPPC/chol/5 mol% 24-carbon globoside	–	+
DPPC/5 mol% native galactosylceramide	1:128–1:256	–
DPPC/chol/5 mol% native galactosylceramide	–	+

would appear that binding of antibody, and crosslinking of liposomes by bound antibody, can be isolated as distinct phenomena in these systems. Apparently, if bidentate IgG binding occurs in the DPPC/globoside liposomes, it is mainly intraliposomal rather than interliposomal.

The fact that the tetrasaccharide species, globoside, binds antibodies when the monosaccharide species, galactosylceramide, does not may well be a reflection of reduced spatial constraint at the bilayer surface for the former glycolipid class. The concept of spatial constraint controlling glycolipid headgroup 'accessibility' to macromolecules has been clearly laid down by Carl Alving [33–35]. He has suggested the need for a lipid hapten to protrude adequately above surrounding membrane components in order to be bound. Harden McConnell and his colleagues emphasized that a number of factors may be involved, and are difficult to separate from one another [36–38]. Thus, McConnell has remarked that cholesterol might affect hapten properties such as surface area, lateral mobility and exposure [37]. In one example, binding of an antibody to a lipid hapten in DPPC was clearly enhanced by the presence of cholesterol, without any change in hapten distribution (which remained apparently random) [37]. They point out that such effects should be most crucial when hapten headgroup extension above the bilayer plane is limited. Owicki and co-workers used antibody binding and fluorescence spectroscopy to monitor phospholipid hapten crypticity in bilayers. They suggest that cholesterol strongly influences headgroup conformation, thereby determining accessibility to IgG [39,40].

Another important observation related to glycolipid crypticity is the effect of acyl chain length. Alving and co-workers noted that incorporation of galactosylceramide into bilayer membranes comprised of short chain phospholipids resulted in greater antibody binding than in longer chain host matrices (e.g., 14-carbon vs. 16-carbon and 18-carbon [34]). This effect was only seen at low temperatures such as 20–25°C. Alving's suggestion is that a given glycolipid headgroup may project farther from the surface in a shorter chain host matrix and thus be more 'accessible' to macromolecules. In an apparently analogous fashion, it was shown by Alving and Richards that galactocerebroside with short chain fatty acid provides

a less effective receptor for antibodies than does galactocerebroside with a long chain fatty acid in a given host matrix [33]. A similar effect was recently reported for sulfate-substituted galactocerebroside (sulfatide) by Crook et al. [41]. The phenomenon has been considered with regard to intact cells by Gahmberg and by Hakomori (Ref. 42 and Refs. therein). Indeed in our hands galactosylceramide with 24-carbon spin label conferred greater antibody-mediated agglutinability upon liposomes bearing it than did the 18-carbon spin label (by three serum dilutions, Table I). However, there was no detectable difference in agglutinability conferred by globoside with 24- vs. 18-carbon fatty acid (although Table II shows marginally more antibody binding by the long chain globoside). Once again, this observation seems adequately explained by the fact that globoside's longer oligosaccharide headgroup is more spatially accessible than that of galactosylceramide so that fatty acid chain length of glycolipid or host matrix is less critical to receptor function. Uemura et al. have recorded a difference in immune globulin binding by Forssman antigen (a pentasaccharide species), depending upon the hydrophobic portion of the glycolipid [43]. However, the effect was modest in liposomes with phospholipid and cholesterol, and required total replacement of the sphingosine acyl chain with an -OH group.

Acknowledgements

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