Organization of Glycosphingolipids in Phosphatidylcholine Bilayers: Use of Antibody Molecules and Fab Fragments as Morphologic Markers†

Philip Rock, Margaretta Allietta, William W. Young, Jr., Thomas E. Thompson, and Thomas W. Tillack*
Departments of Pathology and Biochemistry and the Cancer Center, University of Virginia Health Sciences Center, Charlottesville, Virginia 22908

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ABSTRACT: The techniques of ultrafast freezing and freeze–etch electron microscopy have been successfully employed to visualize IgG molecules and Fab fragments specifically bound to the neutral glycosphingolipids Forssman and asialo-G_{M1} incorporated into phosphatidylcholine liposomes. Monovalent Fab is the superior marker because of its small size and because it does not cause liposomal aggregation with concomitant glycolipid reorganization. Analysis of Fab labeling of liposomes containing these neutral glycosphingolipids leads to the conclusion that the Forssman glycosphingolipid is dispersed in clusters of not more than several molecules when present at low mole fraction in fluid-phase 1-palmitoyl-2-oleoylphosphatidylcholine liposomes. In contrast to this, asialo-G_{M1} under the same conditions is present in clusters of about 15 molecules in this phospholipid matrix.

Glycosphingolipids are ubiquitous eukaryotic cell surface components. Their location in the external leaflet of the membrane lipid bilayer and their heterogeneous carbohydrate and fatty acid composition suggest important biological roles for these molecules in cell membranes. Although there is much information available on the chemistry of glycosphingolipids, relatively little is known about their biological functions; there is evidence that certain gangliosides can serve as receptors for viruses, bacteria, or toxins [for reviews, see Hakomori (1981), Curatolo (1987), and Thompson and Tillack (1985)].

Very little is known about whether specific glycosphingolipids on the surfaces of cells are individually dispersed in the lipid matrix or are organized into domains that might enhance their effectiveness as receptors. Biological membranes usually contain many different types of glycosphingolipids, and these molecules often bear carbohydrate groups that are similar to or identical with those on membrane glycoproteins (Tonegawa & Hakomori, 1977). Also, membrane glycoproteins may mask underlying glycosphingolipids (Hakomori, 1981). The organization of glycosphingolipids and glycoproteins on the surfaces of cells has been inferred from the patterns of macromolecular markers used to label these cell membrane components; however, the utility of these markers may be compromised by their size, tendency to aggregate because of multivalency, variation in affinity, and specificity. An assessment of the magnitude of these problems encountered in studies on biological membranes can be made by examining the organization of glycosphingolipids in the phospholipid bilayers of liposomal preparations using the same morphological techniques. Liposomes of defined chemical composition containing glycosphingolipids can be easily prepared, and the development of ultrafast freezing techniques permits their examination by freeze–etching over a rather wide temperature range. The prequench temperature can be precisely controlled by using a humidified environmental chamber for the labeling and freezing steps, thus allowing examination of the effects of the phase state of the phospholipid matrix on glycosphingolipid organization. Freeze–etching permits the examination of surfaces of liposomes at very high resolution, and individual molecules or molecular fragments such as IgG, Fab, and cholera toxin can be used as direct labels for glycosphingolipids without the need for secondary markers such as colloidal gold or ferritin.

In this study of glycosphingolipid organization in phosphatidylcholine bilayers, we have used the neutral glycosphingolipids Forssman antigen and asialo-G_{M1}, with known fatty acid and sphingoid base composition, incorporated into phosphatidylcholine multilamellar liposomes to study glycosphingolipid organization in different phase states of the matrix phospholipid. Fast freezing techniques and freeze–etching were employed to examine the surface distribution of these glycosphingolipids as determined by visualization and quantitation of individual IgG molecules with binding specificity for Forssman glycolipid or asialo-G_{M1} and by binding of Fab fragments prepared from these IgG molecules. A comparison of the efficacy of both labels as molecular markers for glycosphingolipids leads to the conclusion that the Fab is a more reliable indicator of the organizational pattern of glycosphingolipids, because of its monovalency, small size, and high specificity. It is concluded that the neutral Forssman glycosphingolipid is dispersed in clusters of no more than a few molecules in the phosphatidylcholine bilayer, both in the liquid-crystalline and in the gel states. In contrast, asialo-G_{M1} is organized into clusters of about 15 molecules under similar conditions.

Materials and Methods

Dipalmitoylphosphatidylcholine (DPPC),[1] 1-palmitoyl-2-oleoylphosphatidylcholine (POPC), and dimyristoylphosphatidylcholine (DMPC) were obtained from Avanti Polar Lipids, Inc., Pelham, AL, and used without further purification. Forssman glycolipid was isolated from canine small intestine by the method of Sung et al. (1973). Glycosphingolipid fatty acid and sphingoid base composition were determined by the combined gas chromatography/mass spectroscopy method of Aaronson and Martin (1983). The Forssman preparation was found to contain greater than 95%

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*To whom correspondence should be addressed at the Department of Pathology.

[1] Abbreviations: DPPC, dipalmitoylphosphatidylcholine; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine.

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C-18 sphingosine long-chain base and to have a mixture of amide-linked fatty acids as follows: C-16:0, 11%; C-18:0, 14%; C-20:0, 12%; C-21:0, 3%; C-22:0, 17%; C-23:0, 12%; C-24:0, 21%; and C-24:1, 9%. Asialo-G_{M1} was prepared from G_{M1}, obtained from Sigma Chemical Co., St. Louis, MO, by formic acid desialation as described earlier (Tillack et al., 1982). The fatty acid composition was 81% C-18:0 and the long-chain base composition was approximately 70% C-18 sphingosine:30% C-20 sphingosine.

Multilamellar liposomes were prepared by spraying a chloroform solution of the mixed lipids onto a glass plate as described earlier (Thompson et al., 1985). Liposomes were hydrated in a pH 7.4 buffer containing 10 mM sodium phosphate and 10 mM NaCl at least 10 °C above the transition temperature of the matrix lipid, usually to a concentration of 5 mM total lipid.

Rabbit polyclonal anti-Forssman and anti-asialo-G_{M1} sera were prepared by the method of Laine et al. (1974). Fab fragments of IgG fractions were prepared by using established procedures (Coulter & Harris, 1983).

For labeling experiments, 40 μL of liposomes (200 nmol of lipid phosphorus) was mixed with 150 μL of a solution of IgG (1.8 mg/mL) or Fab (0.8 mg/mL) and incubated at the appropriate temperature for 1–2 h. Labeled liposomes were pelleted briefly (1 min at 3000*g*) in a microfuge. In order to determine that there was excess IgG or Fab available for labeling the liposomes, the supernatant was then used to label an additional aliquot of liposomes. Such secondary labeling experiments confirmed that enough IgG and Fab remained to completely label additional Forssman-containing liposomes even after labeling liposomes containing 7 mol % Forssman, the highest glycolipid concentration used. Similar results were obtained with asialo-G_{M1}-containing liposomes.

The relative affinity of the IgG and Fab preparations was assessed by repeated washing of the labeled liposomes. No reduction in the amount of IgG bound per unit area of external liposomal surface was detectable after three washes. However, about 30% of the bound Fab was removed after a single wash. The reduced affinity of the Fab fragment relative to the intact IgG molecule is a consequence of its monovalency (Petrossian & Owicki, 1984). We concluded that the amount of Fab visualized on the surfaces of unwashed liposomes was a result of specific binding, since control experiments using anti-Forssman Fab to label asialo-G_{M1}-containing liposomes or anti-asialo-G_{M1} Fab to label Forssman-containing liposomes revealed no binding (see Figure 2).

The pellet of labeled liposomes was resuspended in 20 μL of buffer. A total of 10 μL of this suspension was loaded onto a 3 mm^2, 0.8 mm thick piece of fixed, washed rabbit lung and placed on the sample head of a rapid freezing apparatus (Heuser, 1983). The cryopress was located in a temperature- and humidity-controlled environmental room to allow cryofixation above or below the T_m of the matrix phospholipid. Humidification of the room was necessary to prevent lowering of the temperature of the specimen prior to freezing due to evaporational cooling. The sample was frozen by slamming it onto a liquid helium cooled copper or silver block and then immediately stored under liquid nitrogen. Freeze–etching and platinum/carbon rotary shadowing were performed as described previously, using a Balzers BAF-300 freeze–etching apparatus (Thompson et al., 1985). Replicas were examined with a Zeiss 902 electron microscope.

Results

Neutral Glycosphingolipids in Fluid-Phase POPC Multilamellar Liposomes. Figure 1 shows a POPC liposome in the fluid phase containing 1.4 mol % Forssman glycolipid labeled with anti-Forssman IgG. The external surface of the liposome, exposed by deep-etching, is labeled with randomly distributed individual IgG molecules with an approximate diameter of 150 Å. The variable appearance is due to different orientations of the IgG molecules binding to the underlying glycolipid. The random distribution of the IgG molecules indicates that the Forssman glycolipid is not organized into domains larger than the diameter of the IgG.

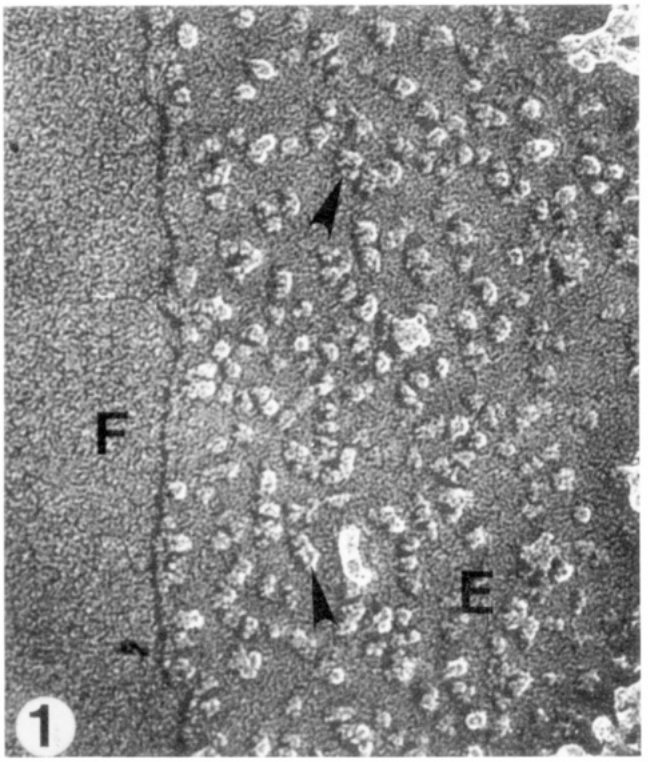

FIGURE 1: Freeze–etch micrograph of fluid-phase POPC liposome containing 1.4 mol % Forssman glycolipid labeled with anti-Forssman IgG and quenched from 18 °C. The external surface of the liposome (E) contains IgG molecules (arrows) while the bilayer fracture face (F) is smooth. Magnification is 200000×.

Figure 2 shows a replica of a POPC liposome containing 2.7 mol % Forssman glycolipid labeled with IgG directed against asialo-G_{M1}, an antigenically unrelated glycosphingolipid. The external surface of the liposome is smooth, indicating that there is no nonspecific binding of IgG to POPC liposomes.

A replica of a fluid-phase POPC liposome containing 4.5 mol % Forssman glycolipid, labeled with Fab prepared from anti-Forssman IgG, is shown in Figure 3. The Fab fragments appear on the liposomal surface as spheroidal particles with an average diameter of 50 Å. The Fab labeling pattern is similar to the IgG distribution in that there is no evidence for domain formation greater than the size of the 50-Å Fab fragment. A replica of a POPC liposome containing 3.7 mol % asialo-G_{M1}, labeled with Fab prepared from anti-asialo-G_{M1} IgG, is shown in Figure 4. There is less than half the density of Fab label on the surface of the asialo-G_{M1}-containing liposomes than on the Forssman-containing liposomes, even though the concentration of glycosphingolipid in the two preparations is about the same (see Table I).

Aggregation of POPC Liposomes by IgG. Forssman-containing liposomes incubated with anti-Forssman IgG rapidly form large aggregates that cannot be separated by mechanical means. Liposomes containing asialo-G_{M1} treated with anti-asialo-G_{M1} IgG form loose aggregates that are more easily dispersed by shaking. Liposomes treated with monovalent Fab

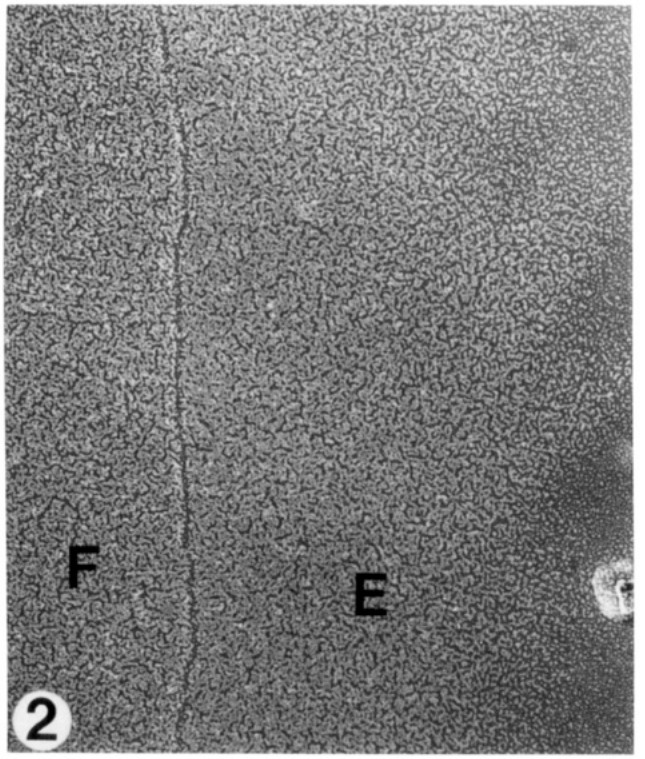

FIGURE 2: Freeze–etch electron micrograph of fluid-phase POPC liposome containing 2.7 mol % Forssman glycolipid labeled with anti-asialo-G_{M1} IgG. The external surface (E) of the liposome contains no IgG molecules. F = bilayer fracture face. Magnification is 200000×.

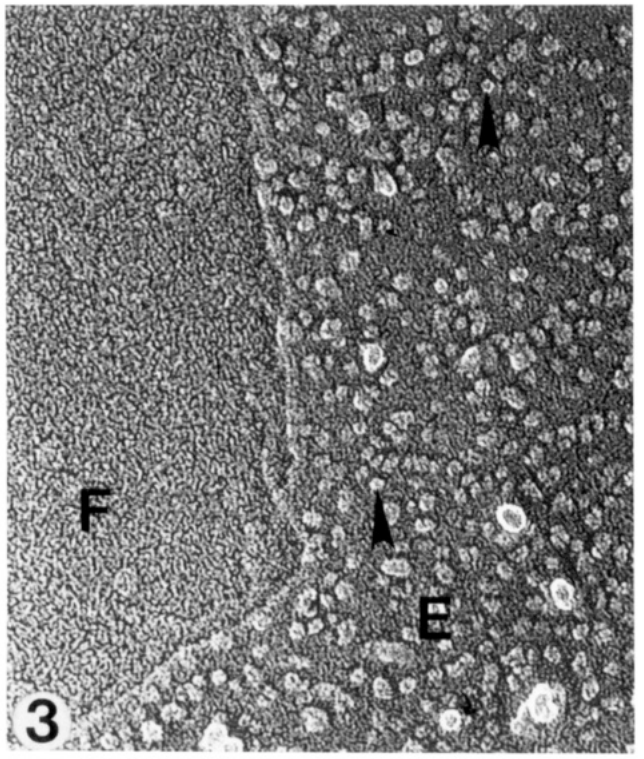

FIGURE 3: Freeze–etch micrograph of fluid-phase POPC liposome containing 4.5 mol % Forssman glycolipid labeled with anti-Forssman Fab fragments and quenched from 18 °C. The external surface of the liposome (E) contains Fab fragments (arrows). F = bilayer fracture face. Magnification is 200000×.

do not aggregate. The numbers of IgG molecules or Fab fragments bound to the liposomal surfaces were obtained by counting surface areas exposed by deep-etching. In the case of Forssman-containing liposomes treated with IgG, the marked agglutination of the liposomes limited counting to the outside surfaces at the periphery of the aggregates.

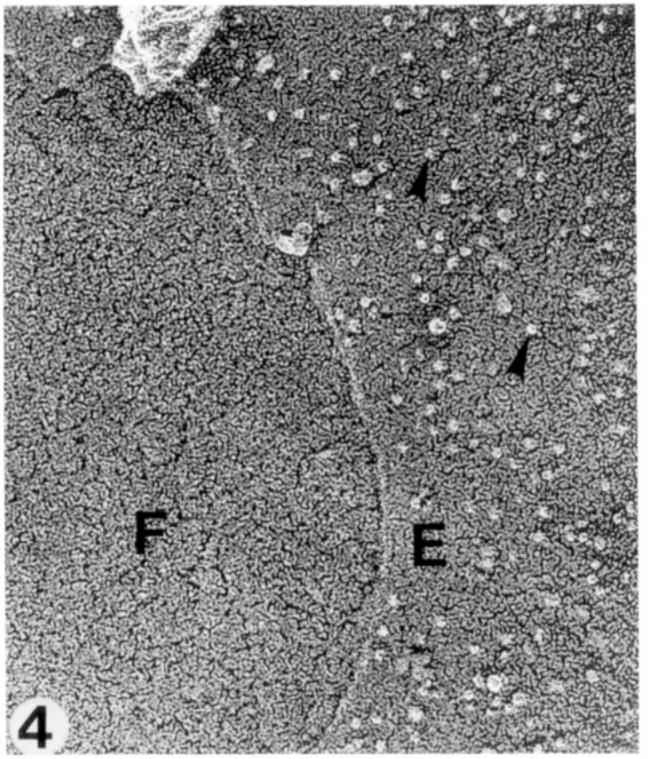

FIGURE 4: Freeze–etch micrograph of fluid-phase POPC liposome containing 3.7 mol % asialo-G_{M1} labeled with anti-asialo-G_{M1} Fab fragments and quenched from 18 °C. The external surface of the liposome (E) contains Fab fragments (arrows). F = bilayer fracture face. Magnification is 200000×.

Table I: Density of Forssman and Asialo-G_{M1} in POPC Liposomes at 20 °C Labeled with IgG and Fab[a]

mol % Forssman	anti-Forssman IgG/μm^2	anti-Forssman Fab/μm^2
0.5	964 ± 61	2164 ± 169
1.0	1240 ± 112	2640 ± 165
2.5	2110 ± 125	4289 ± 180
4.5	3591 ± 197	5005 ± 144

mol % asialo-G_{M1}	anti-asialo-G_{M1} IgG/μm^2	mol % asialo-G_{M1}	anti-asialo-G_{M1} Fab/μm^2
0.2	580 ± 32	0.3	880 ± 25
0.4	1350 ± 58	0.7	1125 ± 235
0.9	1530 ± 105	1.1	1729 ± 126
3.1	2106 ± 36	3.7	2133 ± 37
6.4	3400 ± 330	8.2	2719 ± 218
12.8	3400 ± 148	16.0	3641 ± 274

[a] Values are the averages ± one standard error of the mean. The number of different liposomal surfaces examined ranged from 5 to 11.

POPC liposomes aggregated by Forssman IgG have consistently less IgG per unit area of free surface as compared with liposomes treated with Fab, as shown in Table I. The data in Table I show, however, that with asialo-G_{M1}-containing liposomes the amount of IgG and Fab per unit area is about the same.

In addition to a lower density of marker, liposomes aggregated by anti-Forssman IgG, in general, have an altered morphology. Flattened liposomes are commonly seen pressed against each other in large arrays. Nonbilayer structures are also observed, and there sometimes appears to be IgG on the "surfaces" of interior lamellae that have been exposed by the freeze–fracture, deep-etching procedure, but presumably would not be accessible to externally added IgG (data not shown).

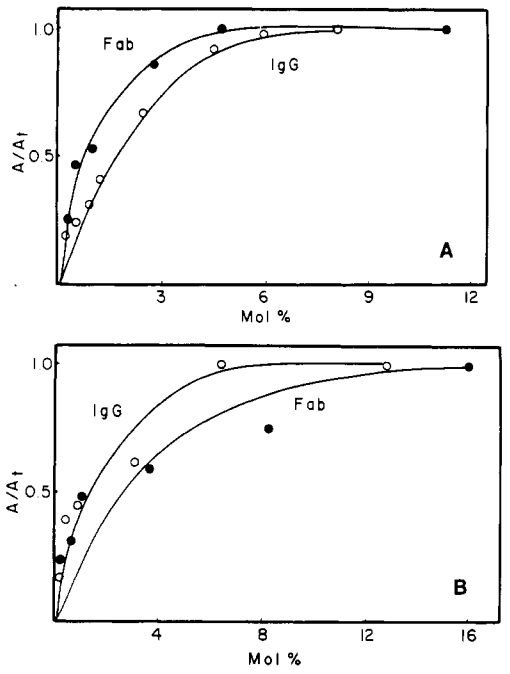

FIGURE 5: Fractional POPC liposomal coverage by Fab (●) and IgG (○) as a function of Forssman (A) and asialo-G_{M1} (B) mole percent at 20 °C. The average number of glycolipid molecules under each macromolecular label was determined by the method of Thompson et al. (1985) using the equation $A/A_t = 1 - (1 - c)^{n/m}$, where A/A_t = fractional surface coverage, c = mole fraction of glycolipid, n = cross-sectional area (in Å^2) of the marker molecule and is the inverse of A_t, and m = cross-sectional area (in Å^2) of the glycolipid molecule using 92 Å^2 for Forssman (based on the value reported for globoside) and 55 Å^2 for asialo-G_{M1} at 25 mN/m, determined from monolayer studies by Maggio et al. (1985).

	Forssman		asialo-G_{M1}	
	IgG	Fab	IgG	Fab
n (Å^2)	24.5×10^3	20.4×10^3	29.4×10^3	27.5×10^3
n/m_{th}	266	222	535	500
n/m_{exp}	45	92	60	32
$(n/m_{th})/(n/m_{exp})$	5.9	2.4	8.9	15.6

The addition of anti-Forssman or anti-asialo-G_{M1} Fab to glycosphingolipid-containing liposomes does not lead to any structural alterations of the liposomes or to the labeling of interior surfaces.

Estimation of the Size of Glycosphingolipid Domains. Since the cross-sectional areas of both Fab fragments and IgG molecules are much larger than that of a glycosphingolipid molecule, either label could cover more than 100 glycosphingolipid molecules. To determine the average number of glycosphingolipids under a marker, the approach of Thompson et al. (1985) was employed. Binding curves were generated, plotting the fraction of liposomal surface covered by IgG or Fab as a function of the mole fraction of glycosphingolipid in the liposomes (Figure 5). The area occupied by each marker was determined from the inverse of marker density at maximal surface coverage as described in the legend to Figure 5.

At maximal surface density, the IgG molecules pack closely and the cross-sectional area estimated for an individual IgG molecule is consistent with its known molecular dimensions. The Fab molecules do not closely pack. The maximum Fab density on liposomal surfaces cannot be increased by increasing the mole fraction of glycolipid or by using more Fab in the labeling procedure. Hence, the area commanded by an Fab molecule is greater than would be predicted on the basis of its molecular dimensions. This may be due to charge interactions between Fab fragments, which would be exaggerated in the low salt buffer used. A similar discrepancy was noted between the predicted and experimentally determined area of a ferritin–cholera toxin conjugate used to label the ganglioside G_{M1} in a similar analysis (Thompson et al., 1985).

Table II: Fab Labeling of Forssman Glycolipid in Gel- and Fluid-Phase DMPC Liposomes[a]

mol % Forssman	T (°C) (phase)	Fab/μm^2
0.34	18 (gel)	1240 ± 131
0.34	32 (fluid)	530 ± 38
1.6	18 (gel)	2471 ± 146
1.6	32 (fluid)	1151 ± 137

[a] Values are the averages ± one standard error of the mean from ten different surfaces for each preparation.

Dividing the cross-sectional area determined for IgG and Fab by the area of a glycosphingolipid molecule gives an estimate of the number of glycolipids that could be covered by either marker (n/m theoretical). This value can also be determined from the equation $A/A_t = 1 - (1 - c)^{n/m}$ [see Thompson et al. (1985) and legend to Figure 5]. A/A_t and c are experimentally determined, and the value of n/m (experimental) comes from a best fit of the data to the equation. Figure 5A compares the computer-generated best fit for Forssman IgG and Fab. The ratio of n/m derived by these two methods for IgG is 5.9; that for Fab is 2.4. This indicates that, on the average, about 6 Forssman molecules reside under an IgG molecule and 2.5 under a Fab fragment.

In contrast to this result, the data shown on Figure 5B for liposomes containing asialo-G_{M1} yield cluster sizes of 8.9 and 15.6 for anti-asialo-G_{M1} IgG and Fab, respectively. This result is in agreement with our earlier qualitative observations on this system (Tillack et al., 1982). For reasons discussed below, we believe the correct domain size is given by the calculation based on Fab density.

Glycolipid Distribution in DMPC Liposomes. Labeling of DMPC liposomes containing Forssman or asialo-G_{M1} glycosphingolipid allows a comparison of glycosphingolipid distribution in two different phases of the same phospholipid matrix by varying the temperature. Table II compares the Fab surface density in gel-phase (18 °C) and liquid-crystalline-phase (32 °C) DMPC liposomes containing two different concentrations of Forssman glycosphingolipid. In liposomes containing the same amount of glycosphingolipid there is approximately twice as much Fab on gel-phase liposomal surfaces as on liquid-crystalline surfaces.

The use of anti-Forssman IgG to label Forssman glycolipid in liquid-crystalline DMPC liposomes results in marked aggregation of the liposomes, as seen with POPC liposomes containing Forssman glycolipid. In contrast, labeling of gel-phase Forssman-containing DMPC liposomes with IgG does not cause extensive aggregation. The loose aggregates that do form are readily broken apart by shaking the tube containing them.

A gel-phase DMPC liposome containing 1.6 mol % Forssman glycolipid that has been labeled with anti-Forssman Fab is shown in Figure 6. The characteristic ridged pattern of gel-phase DMPC is present on both the external surface of the liposome as well as on the freeze–cleaved bilayer face. The labeling pattern of the anti-Forssman Fab is randomly distributed and shows no correlation with the underlying ridged structure. A similar distribution is seen in gel-phase DMPC liposomes containing 12.0 mol % asialo-G_{M1} labeled with Fab directed against this glycosphingolipid, as shown in Figure 7.

It should be noted that the electron micrographs shown in Figures 1–4, 6, and 7 are representative of each of the lipo-

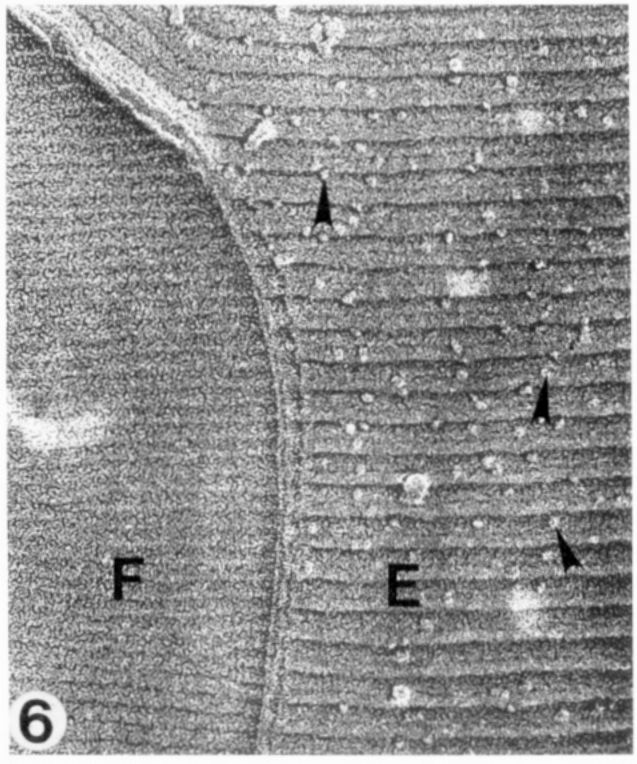

FIGURE 6: Freeze–etch electron micrograph of $P_{\beta'}$-phase DMPC liposome containing 1.6 mol % Forssman glycolipid labeled with anti-Forssman Fab and quenched from 18 °C. The external surface (E) and bilayer fracture face (F) both contain ridges. Fab fragments (arrows) are present on the external surface. Magnification is 200000×.

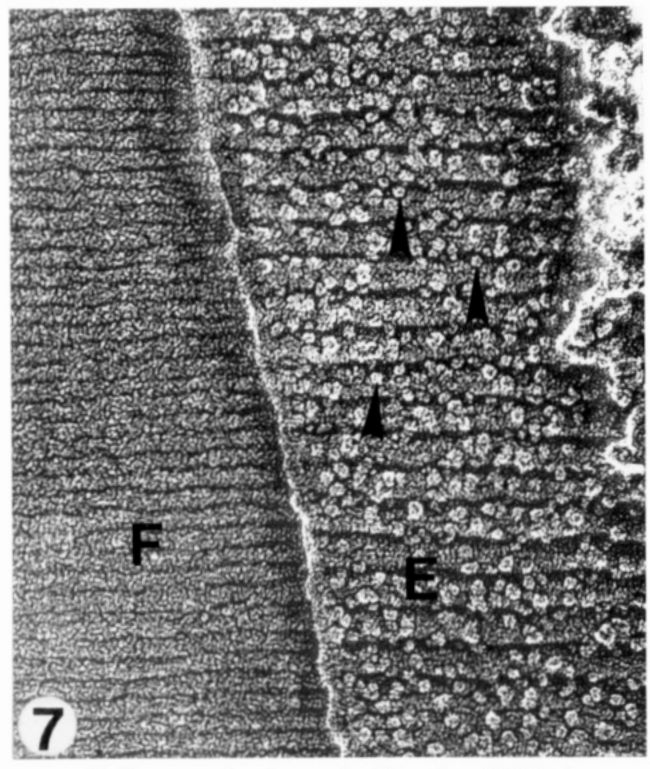

FIGURE 7: Freeze–etch micrograph of $P_{\beta'}$-phase DMPC liposome containing 12.0 mol % asialo-G_{M1} labeled with anti-asialo-G_{M1} Fab and quenched from 18 °C. The ridged external surface (E) contains Fab fragments (arrows); the fracture face (F) also contains ridges. Magnification is 200000×.

somal preparations examined by freeze–etch electron microscopy with very little variation in labeling density from one liposome to another. There is also reflected in the statistical evaluation of the data shown in Tables I and II.

Discussion

The results presented here demonstrate that specific anti-glycosphingolipid antibodies and Fab fragments of antibodies can be used as labels for glycosphingolipids in liposomal systems using ultrarapid freezing combined with freeze–fracture and deep-etching techniques. However, because it is monovalent for Forssman glycolipid, anti-Forssman Fab proved to be the clearly superior marker in that it did not cause aggregation with concomitant reorganization of the liposomes. Asialo-G_{M1}-containing liposomes did not aggregate to the marked extent that Forssman-containing liposomes aggregated; therefore, both IgG or Fab proved to be satisfactory labels in this system.

The Fab labeling data suggest that, at low mole percent Forssman, glycosphingolipid is dispersed in fluid-phase POPC liposomes at room temperature and is not organized in a macrodomain pattern. This is in agreement with the findings of Mehlhorn et al. (1988), who found that the closely related glycosphingolipid globoside (with a spin-labeled fatty acid) was dispersed in gel- and fluid-phase DPPC liposomes.

For quantitative purposes, monovalent or functionally monovalent ligands are the preferred labels for freely diffusible bilayer components, in order to better reflect the organization of the surface at the instant of addition of the label. An earlier study (Thompson et al., 1985) examining the distribution of the ganglioside G_{M1} using cholera toxin, a pentavalent ligand, led to the conclusion that G_{M1} was molecularly dispersed in the plane of the bilayer, a finding consistent with the results of independent physical studies. The multivalent cholera toxin did not cause liposomal aggregation. This is presumably due to the asymmetric nature of the binding subunit (Dwyer & Bloomfield, 1982), which is thought to have all five binding domains on one face of the planar molecule. That feature of the marker, coupled with its presence in great excess relative to G_{M1}, made it functionally monovalent.

There are at least two possible explanations for the aggregation of Forssman-containing liposomes in the liquid-crystalline state caused by anti-Forssman IgG. One is the formation of aggregates induced by interliposomal binding of bivalent IgG; another is aggregation through the interaction of the Fc regions of IgG molecules bound to different liposomes. Our evidence strongly supports the concept of interliposomal binding of IgG. This may result in an enrichment of glycolipid at points of interliposomal contact with a concomitant relative depletion of glycolipid from the free liposomal surfaces. The recruitment of glycosphingolipid at points of interliposomal contact may proceed by the initial binding of one arm of the IgG molecule to the surface of a liposome, followed by the approach of a second liposome that would effectively double the number of glycosphingolipids accessible to the second arm. The apposition of the liposomes would then further enhance interliposomal cross-linking by additional IgG molecules. In a fluid liquid-crystalline state, the liposomal surfaces in close contact with each other would now constitute a relatively immobile fraction of the total lipid. This mechanism would not be expected to operate in gel-phase bilayers.

Other investigators have studied liposomal aggregation by immunoglobulin molecules with the general conclusion that aggregation depends on the fluidity of the bilayer and on the concentration of antigen. Luedtke and Karush (1982), using a fluorescent ligand linked to dipalmitoylphosphatidylethanolamine, found that aggregation of DMPC vesicles by IgG was enhanced by the presence of cholesterol, increased

ligand concentration, and increased temperature. Mehlhorn et al. (1988) reported that anti-globoside IgG did not agglutinate gel-phase DPPC liposomes containing globoside unless cholesterol was also present to "fluidize" the gel-phase DPPC bilayer. This is in keeping with our result noted above that the aggregation of fluid-phase DMPC liposomes containing Forssman by anti-Forssman IgG is much more pronounced than that of gel-phase DMPC liposomes.

Aggregation by interliposomal binding of IgG results in an enrichment of Forssman glycolipid at points of interliposomal contact with a concomitant relative depletion of glycolipid in the free liposomal surfaces. Since it is on the free liposomal surface after freeze–etch that the surface density of IgG is determined, the net result is an overestimation of Forssman glycolipid domain size. The use of monovalent Fab eliminates aggregation and therefore gives the correct domain size. In fact, the domain size calculated using Forssman IgG is more than twice as large as that calculated for the Fab fragment used as a glycolipid marker. In contrast to this situation, for asialo-G_{M1}, the domain size determined using IgG is actually somewhat smaller than that obtained with the Fab fragments. The domain sizes calculated from Fab data for both Forssman and asialo-G_{M1} are clearly the better estimates of the correct sizes.

The alternative explanation for liposomal aggregation by IgG directed against a glycosphingolipid component of the bilayer is through interactions of the Fc regions of the IgG molecules. Direct interaction of the Fc region with the bilayer does not occur since there is no nonspecific binding of immunoglobulin molecules to the liposomes. Fc–Fc interactions, however, remain a possible mechanism leading to aggregation of liposomes. Intraliposomal binding of IgG to multivalent domains of glycosphingolipid could lead to the formation of patches of IgG on liposomal surfaces. These patches of IgG could then interact through Fc–Fc associations to cause aggregation of the liposomes. Fc–Fc interactions have previously been shown to enhance immune precipitation (Moller & Christiansen, 1983) and cooperative binding of antibodies to antigens (Greenspan et al., 1989). We believe that Fc–Fc interactions are unlikely to play a major role in promoting agglutination of Forssman-containing liposomes. The data on anti-Forssman Fab binding to POPC liposomes containing Forssman glycolipid indicate that the glycosphingolipid is essentially molecularly dispersed in the plane of the membrane. It is unlikely that a bivalent IgG molecule could induce the formation of patches of a dispersed monovalent glycosphingolipid in a fluid phospholipid matrix. Also, patches of IgG are not observed in the freeze–etch replicas of liposomal surfaces at a distance from the regions of apposition; only individual IgG molecules are observed on these surfaces.

The data obtained with asialo-G_{M1}-containing liposomes show that either IgG or the Fab fragment directed against this glycosphingolipid can be employed as a label. This result is in direct contrast to that obtained with Forssman glycolipid, which indicates that only the Fab fragment is a reliable label.

A plausible explanation for the differences in the behavior of these two neutral glycosphingolipids may rest in their organization in the matrix phospholipid bilayer. The data in Figure 5 show that whereas the Forssman antigen is essentially molecularly dispersed in the phosphatidylcholine bilayer, asialo-G_{M1} is clustered in small domains. A bivalent IgG encountering a domain of asialo-G_{M1} has a high probability of liganding both binding sites in a single domain. A Fab fragment will also bind to a single domain. Thus, with IgG or Fab, the surface density of either label will be the same at a given concentration of asialo-G_{M1} in the surface. The data in Table I show that this is indeed the case. Furthermore, aggregation of liposomes by the binding of an IgG to two liposomes will be less frequent.

The situation with the molecularly dispersed Forssman glycolipid is, however, different. A bivalent IgG that encounters a single Forssman glycolipid will still have one free binding site. The free site may recruit a second glycolipid in the same bilayer or in the bilayer of an adjacent liposome. If a glycolipid is recruited in the same bilayer, two Forssman molecules will reside under the IgG molecule. However, since a Fab fragment has only a single binding site, at a given concentration of Forssman glycolipid the surface density of the IgG label should be half the surface density of the Fab label. The data in Table I support this explanation. Aggregation by anti-Forssman IgG cross-linking of liposomes would be expected to occur to a greater extent in this system than in the asialo-G_{M1} system.

The differences in behavior between the Forssman glycosphingolipid and asialo-G_{M1} in the same matrix phosphatidylcholine must be due to differences in the molecular structure of these two sphingolipids. The oligosaccharide structure of Forssman is GalNAcα1→3GalNAcβ1→3Galα1→4Galβ1→4Glcβ1→1Cer and that for asialo-G_{M1} is Galβ1→3GalNAcβ1→4Galβ1→4Glcβ1→1Cer. The ceramide portion of our Forssman preparation contains primarily C-18 sphingosine but has a very heterogeneous fatty acyl composition with 62% of the acyl chains longer than C-20. This is in contrast to asialo-G_{M1} which has a 70:30 ratio of C-18:C-20 sphingoid bases but contains about 80% C-18 acyl chains. Thus both polar and nonpolar moieties of these two glycosphingolipids differ markedly. Studies of the spontaneous interbilayer transfer of glycosphingolipid show that desorption from a phosphatidylcholine bilayer is strongly dependent on the nonpolar moiety and much less dependent on the polar portion of these molecules (Jones & Thompson, 1990). A similar conclusion can be drawn from studies of glycosphingolipid partitioning between gel and liquid-crystalline phosphatidylcholine phases (Rock et al., unpublished observation). These studies suggest that glycosphingolipid aggregation in phosphatidylcholine bilayers should also depend primarily on interactions between the nonpolar moiety of the glycosphingolipid and the acyl chains of the matrix phosphatidylcholine. Clearly, understanding of the detailed molecular interactions must await studies utilizing pure molecular species of glycosphingolipids.

The data in Table II show that the density of Fab label is substantially larger in gel-phase (18 °C) than in liquid-crystalline-phase (32 °C) DMPC liposomes containing low concentrations of Forssman antigen. One factor operating to cause this difference is the decrease in cross-sectional area of phosphatidylcholines in the gel phase relative to the liquid-crystalline state. The area per molecule of DMPC decreases from 0.68 nm^2 at 32 °C to about 0.60 nm^2 at 18 °C (Cevc & Marsh, 1987). This decrease in area per molecule accounts, however, for only about 24% of the increase in the surface density of Fab at 18 °C seen in Table II. The increase may also reflect a change in the aggregation state of the Forssman antigen in the DMPC bilayers at the two temperatures. If this is the case, the cluster size must increase at the higher temperature. Since conditions were chosen to saturate the glycolipid with Fab at both temperatures (see Materials and Methods), it seems unlikely that the increase in Fab label at low temperature reflects the increase in the binding constant with decreasing temperature resulting from the negative en-

thalpy associated with antigen–antibody binding (Biltonen & Langerman, 1979).

Earlier work from this laboratory (Tillack et al., 1982) had suggested a linear localization of asialo-G_{M1} in the troughs between the $P_{\beta'}$ ridges of gel-phase DMPC liposomes. Using a much more rapid freezing method (the liquid helium cryopress) and a smaller label with higher affinity (Fab anti-Forssman or anti-asialo-G_{M1}), we now find that the distribution of Forssman and asialo-G_{M1} bears no discernible spatial relationship to the ridged gel-phase structure (Figures 6 and 7). It is possible that the linear localization of ferritin conjugates of ricin lectin seen in the earlier study was a result of redistribution of the asialo-G_{M1} during the slower freezing method used at that time. Alternatively, there may have been preferential loss of the low-affinity ferritin–lectin label from the peaks of the ridges with preservation of binding in the troughs of the $P_{\beta'}$ ripples due to interaction of the label with glycolipids extending from both sides of the trough. Also, as pointed out in the previous study (Tillack et al., 1982), ferritin–ricin conjugates showed relatively poor affinity for DMPC or POPC liposomes containing asialo-G_{M1} in the fluid phase, resulting in a very low density of label on the surface of fluid-phase liposomes. In contrast, the IgG and Fab labels used in this study showed good affinity and accurate quantitation of the label density on liposomal surfaces was achieved.

Registry No. POPC, 26853-31-6; DMPC, 18194-24-6; Forssman glycolipid, 60267-39-2; asialo-G_{M1}, 71012-19-6.

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