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EVIDENCE OF A DISTRIBUTION DIFFERENCE BETWEEN TWO GANGLIOSIDES IN BILAYER MEMBRANES

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Freeze-etch electron microscopy, a platinum shadowing technique, has been used to compare the lateral distribution of several gangliosides in bilayer model membranes by directly visualizing bound lectin molecules. In particular, GM_1 and GD_{1a}, major components of brain ganglioside, were studied in phase-separated mixtures of dipalmitoyl- and dielaidoylphosphatidylcholines exposed to *Ricinus communis* agglutinin and wheat germ agglutinin. The distribution of glycolipid showed evidence of microheterogeneity in that bound lectin tended to occur in clusters of several or more molecules. With GD_{1a} as receptor such clusters were small and very uniformly distributed over the membrane surface. Somewhat larger, irregularly spaced clusters of up to a dozen lectin particles were more typical of membranes bearing GM_1 and, in addition, there were occasional extensive patches of bound lectin coexisting with areas apparently devoid of glycolipid receptor in phase-separated mixtures of dipalmitoyl- and dielaidoylphosphatidylcholine. Gangliosides in the latter mixtures were not obviously influenced in their lateral distribution by the presence of coexisting fluid and rigid domains. These basic observations seem to extend to bilayer membranes containing mixtures of two gangliosides. The patterns of lectin binding were not grossly affected by incubation time or history of warming and cooling. This study was extended to bilayers of pure dipalmitoylphosphatidylcholine in expectation that the distinctive features characteristic of the $P_{\beta'}$ phase of this lipid might accentuate any behavioural differences between GM_1 and GD_{1a}. GM_1 was found to exist preferentially in the 'trough' regions between $P_{\beta'}$ ripples, while GD_{1a} showed no apparent preferential arrangement. Given that bound lectins adequately reflect glycolipid distribution in membranes, it would appear that structurally different glycolipids from the same host membrane can assume different distributions on the basis of interactions with defined lipid host matrices.

Introduction

Because the lipid bilayer has been demonstrated to be an integral feature of cell membranes, the use of bilayer models has become a popular method of probing questions relating to membrane structure and function. In this article we address the subject of glycolipid lateral distribution in phosphatidylcholine model membranes. Glycosphingolipids possess a special significance as membrane components. Not only are they candidates for various structural roles and a role in synaptic transmission [1–5] but they are also strongly implicated as specific receptors for exogenous contacts [2,3,6] and are known to be influenced by

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Abbreviations: RCA 60, *Ricinus communis* agglutinin; GM_1, Galβ1 → 3GalNAcβ1 → 4(NeuAcα2 → 3)Galβ1 → 4Glc → Cer; GD_{1a}, NeuAcα2 → 3Galβ1 → 3GalNAcβ1 → 4(NeuAcα2 → 3)Galβ1 → 4Glc → Cer; GT_{1b}, NeuAcα2 → 3Gal1β1 → 3GalNAcβ1 → 4(NeuAcα2 → 8NeuAcα2 → 3)-Galβ1 → 4Glc → Cer.

differentiation and oncogenic transformation [2,3,6–8]. Such properties would be expected to be importantly influenced by lateral distribution of the involved glycolipids. It would be interesting therefore if glycolipid distribution were a function of their structure and family. In this regard we have focussed on several different species which together comprise a significant fraction of bovine brain (grey matter) ganglioside [9].

In spite of an extensive knowledge of lipid dynamics, surprisingly little is known about the details of their lateral distribution in cell membranes. It is widely presumed, however, that lipid distribution is somewhat inhomogeneous: the existence of microdomains being maintained by lipid-lipid and lipid-protein interactions [10]. Generally, glycosphingolipids are restricted to the surfaces of plasma membranes. There have been considerations as to whether they localize predominantly at synapses of neuronal tissue cell bodies and axons [11,12]. Huang [13] observed that, when fluorescently labelled cerebroside was incorporated by exogenous addition into the membranes of cultured cells, the result was a formation of patchy fluorescence with time. On the basis of spin label experiments we originally suggested that a tendency existed for ganglioside [14–16] and globoside [17] to cluster in phospholipid bilayer membranes. This is by no means a subject of universal agreement though. For instance Delmelle et al. [18] have causally linked glycolipid clustering in phosphatidylcholine bilayers to rigidification of the host matrix, Freire et al. [19] have considered headgroup H-bonding as a mechanism of clustering, and Bunow and Bunow [20] have interpreted their calorimetric experiments on the ganglioside, GM_1, as not favouring clustering. Some differences of opinion on this subject may reflect a question of semantics regarding the size of clusters and the coexistence of a non-clustered fraction. Even pure clusters of a given lipid exhibit reduced cooperativity in their melting behaviour when they comprise fewer than some 60 molecules [21,22]. Thus, the calorimetric behaviour of small clusters may have features resembling that of mixtures. Moreover, it is reasonable to anticipate selective enrichment, rather than exclusive clusters of pure material. Recently, freeze-etch electron microscopy has been brought to bear on this problem, and it is this approach that will be considered here.

Freeze-etching is a shadowing technique which offers extensive face views of membrane architecture, resolving details down to 3–4 nm. It also has the unique ability to demonstrate features in the hydrophobic interior. The membrane specimen is rapidly frozen ('quenched') from some temperature of interest, fractured in vacuum to expose membrane hydrophobic regions, etched in vacuum to expose membrane surfaces by sublimation of surrounding ice and then coated with an atomic beam of platinum. When prepared in this way, cell membrane fracture faces appear as smooth surfaces interrupted by well-defined intramembranous particles [23], representing points of membrane penetration by protein. The etch faces of cell plasma membranes are typically smooth and featureless [24] but upon close examination may show an indistinct granularity associated with their very extensive, amorphous layer of surface material. Simple lipid bilayers studied by freeze-etching are characterized by smooth fracture and etch faces, reflecting the lack of protruding structure in these regions [25–27]. Glycolipids incorporated into lipid bilayer membranes at low mol ratios give rise to no identifiable features — presumably because their headgroups are too diminutive to cast a platinum shadow [27]. This has led to several attempts at visualizing their location indirectly by the presence of bound structures. Thus, we demonstrated, using influenza virus as markers for beef brain ganglioside mixtures, that the latter occurred in fluid and rigid regions of model membranes [27] in contrast to glycoprotein receptors for the same virus [26]. Tillack et al. [28] used ferritin-linked RCA 60 to mark asialo-GM_1 in bilayers of pure phospholipids. The same group have extended this important contribution by visualizing ferritin or colloidal gold-linked antibodies bound to globoside and Forssman antigen in erythrocyte ghosts [29]. They demonstrated in each case a clustered distribution of glycolipids. We have experimented with the use of native lectins, and with lipid vesicles bearing glycolipid receptors plus bound lectins, as markers for the distribution of unfractionated beef brain ganglioside in binary phospholipid mixtures [16]. The lack of extensive surface material in such systems makes it possible to resolve unmodified lectin molecules

attached to the otherwise smooth and featureless membrane exterior [16]. In this article we describe the use of native lectins to outline the distribution of several pure glycolipids in model membranes.

Materials and Methods

Dipalmitoylphosphatidylcholine (DPPC), RCA 60 and wheat germ agglutinin were obtained from Sigma. Dielaidoylphosphatidylcholine (DEPC) was from PL Biochemicals. GD_{1a}, GM_1 and GT_{1b} were from Supelco. GT_{1b} contained 10–30% GD_{1b}; all other lipids were pure as judged by thin-layer chromatography on plates coated with silica gel GF_{254} (Stahl). Succinyl wheat germ agglutinin-Ferritin was from E.Y. Laboratories, and was centrifuged for 30 min at $10000 \times g$ to remove aggregates immediately before use.

Liposome preparation. Gangliosides were incorporated at 7 mol% into liposomes of 1 : 1 (mol ratio) DEPC/DPPC by mixing all lipids in chloroform/methanol and drying as a thin film under nitrogen. Films were further dried in vacuum and then hydrated with 10 mM phosphate buffer (pH 7.4) at 41°C followed by vortexing gently with glass beads. All liposomes were warmed to 45°C and allowed to cool slowly to 20°C prior to preparation for freeze-etching. Samples for freeze-etching were suspended at a concentration of 0.33 mg per ml total lipid and incubated with various concentrations of lectin for 15 min at 20°C. Liposomes were then quickly harvested by centrifugation at $120 \times g$ and frozen in freon cooled in liquid nitrogen.

Freeze-etch replicas were prepared by platinum shadowing in a Balzers apparatus. Samples to be directly compared were often handled simultaneously to avoid any differences due to shadow variability. Etching was for 2 min at −103°C. Replicas were cleaned in $NaClO_4$, rinsed with distilled water and then exposed to acetone to remove traces of lipid. Replicas were examined using a Philips EM300.

Results and Discussion

Fig. 1. illustrates the appearance of phase-separated model membranes with ganglioside as receptors after exposure to the monovalent 'lectin', RCA 60. The host phospholipid matrix in all cases was a 1 : 1 (mol ratio) mixture of the individual pure lipids, dielaidoyl- and dipalmitoylphosphatidylcholine. At 20°C such liposomes possess identifiable coexisting fluid and rigid domains. The behaviour and local composition of the host matrix may be understood by having recourse to the phase separation analyses originally described in membranes by Phillips et al. [30] and Shimshick and McConnell [31] (see also Refs. 32, 33). The situation is analogous to the mixing of two pure solids which individually would melt at distinct temperatures. When pure dielaidoylphosphatidylcholine, which has a phase transition temperature of 11°C [34], is mixed with pure dipalmitoylphosphatidylcholine, whose phase transition temperature is 41.5°C [31,35], the resultant hydrated bilayers form laterally separated domains whose properties reflect their selective enrichment in one or the other lipid. At 20°C a 50/50 mixture of these two lipids contains roughly equal amounts of rigid bilayer with a composition of some 1 : 3 DEPC/DPPC and fluid bilayer with the reverse composition [34]. Although the overall significance of this particular phenomenon in cell membranes is unclear, the existence in membranes of microdomains with different composition is generally conceded [10]. Rigid domains in this lipid mixture are identifiable [33,34,36] by virtue of their characteristic ripple pattern associated with the $P_{\beta'}$ phase of Tardieu et al. [37]. These features are preserved by the extremely rapid freezing process which is part of the freeze-etch technique.

In Figs. 1A–C, bound RCA 60 is seen marking the etch face location of the monosialo ganglioside, GM_1 — the only glycolipid present. The fracture faces (membrane hydrophobic interior) show no features attributable to either glycolipid or bound lectin. RCA 60 has a molecular weight of 60000 [38] and, as such, should have a diameter on the order of 10 nm when shadowed with platinum (calculated diameter assuming spherical shape, 5.2 nm). The individual etch face particles resolved are in this size range. RCA 60 has been proposed to be a monovalent species (thus not a true lectin). Nevertheless, it is known to be a weak agglutinin [39] (perhaps due to contamination by multidentate species such as RCA 120), and in this regard Fig. 1B shows some small vesicles bearing

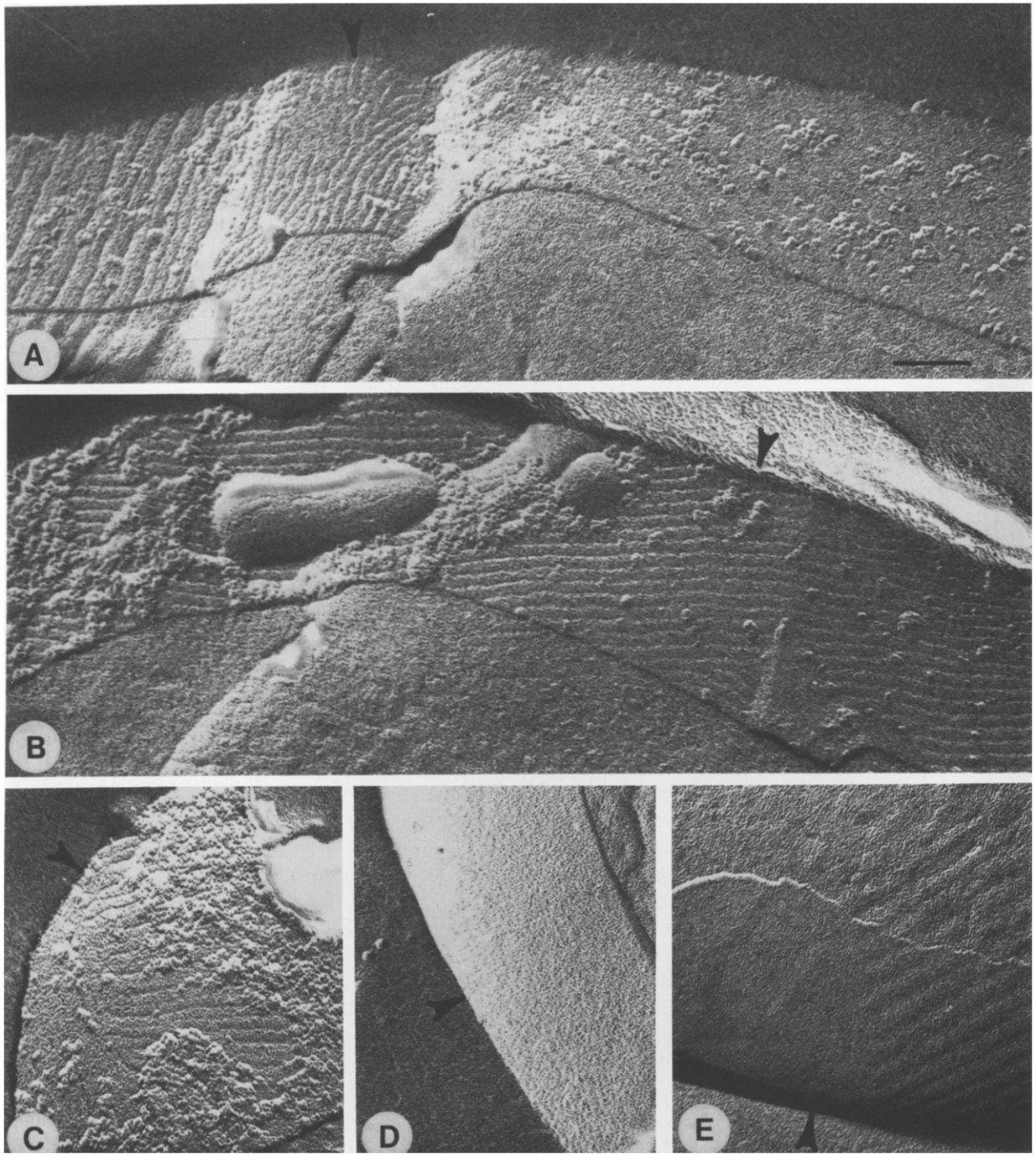

Fig. 1. Freeze-etch electron micrographs of liposomes bearing purified gangliosides, after a 15 min exposure at 20°C to native RCA 60. In each case the lipid composition was 7 mol% ganglioside in a host phospholipid matrix of 1 : 1 (mol ratio) DEPC/DPPC. Under the sample conditions described, the membranes may be seen to possess coexisting fluid and rigid domains (see text for details). 'Ripple' patterns are evident which mark regions of rigid $P_{\beta'}$ phase selectively enriched in the higher melting phospholipid. The concentrations of RCA 60 employed were between 0.067 and 0.130 mg/ml without obvious differences. There are no features permitting direct localization of glycolipid in the membranes, but bound RCA 60 is easily resolved on the etch face as 10-nm particles (note the absence of such particles on the membranes bearing GD_{1a} which is not a receptor for RCA 60 (E)). A–C illustrate the features typical of GM_1 distribution: small clusters and some larger patches. A–C, liposomes bearing GM_1, a known receptor for RCA 60; D, same as above but in the presence of 0.2 M inhibitory sugar (D-galactose); E, liposomes bearing GD_{1a}. Shadow direction is from bottom to top of page. Arrowheads point from the ice toward the membrane outer surface and abut on the outermost edge of the latter. Bar denotes 100 nm.

receptor, adhering to a patch of GM_1 (see also Fig. 3). Bound RCA 60 can be seen in Fig. 1A to exist in small dispersed clusters of anywhere from several to a dozen particles. This pattern is very similar to the asialo-GM_1 distribution reported by Tillack et al. [28] in model membranes labelled with Ferritin-conjugated RCA 60. Fig. 1B and C illustrate a second feature common to these phase-separated membranes bearing GM_1: extensive patches of glycolipid, separated by regions apparently devoid of receptor coexisting with the smaller clusters referred to above. Fig. 1D shows the same liposomes after exposure to RCA 60 in the presence of inhibitory sugar (D-galactose). Fig. 1E is a micrograph of a similar liposome preparation, but with GM_1 replaced by the disialo ganglioside, GD_{1a}, which is not a receptor for *Ricinus communis* agglutinin (RCA 60 is specific for D-galactose and D-GalNAc [38]). Clearly there is no significant non-specific binding. Note (Fig. 1A) that there is no obvious effect of membrane fluidity on the pattern of glycolipid distribution: fluid and rigid membrane domains show much the same etch face appearance.

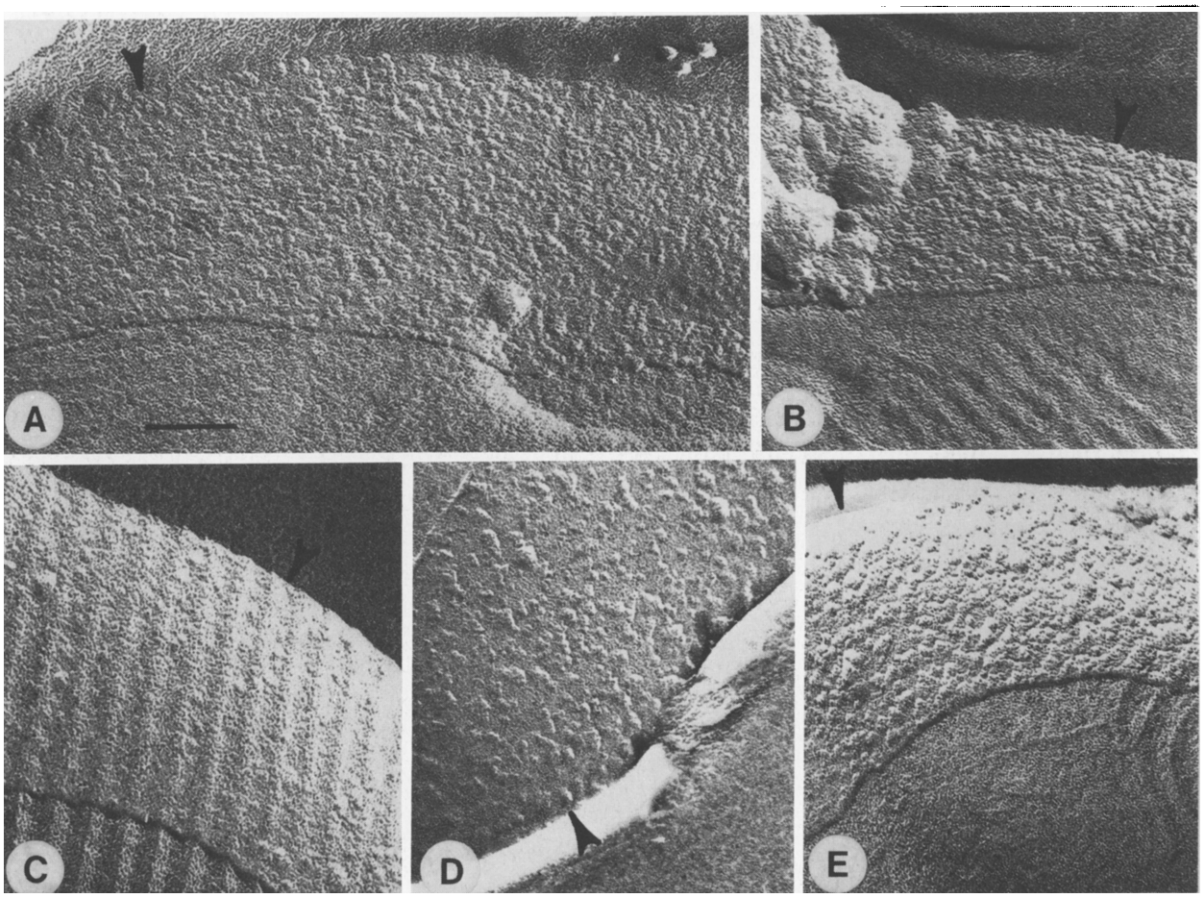

Fig. 2. Freeze-etch electron micrographs of liposomes such as those described in Fig. 1 but after a 15 min exposure at 20°C to native wheat germ agglutinin instead of RCA 60. Lectin concentration, 1.0 mg/ml. Once again, there are no features permitting direct localization of ganglioside in the membranes, but bound wheat germ agglutinin gives rise to 6-nm particles on the etch face (membrane outer surface). Such particles are relatively sparse on the membranes bearing GM_1 as receptor (Fig. 1C). As marked by bound wheat germ agglutinin, GD_{1a} and GT_{1b} exist in small clusters uniformly distributed over the membrane surface. A, B, D, lipsomes bearing GD_{1a} at 7 mol%; C, same as above but with GD_{1a} replaced by 7 mol% GM_1; E, Same as A, but with GD_{1a} replaced by 7 mol% GT_{1b}. Shadow direction is from bottom to top of page. Arrowheads point from the ice toward the membrane outer surface and abut on the outermost edge of the latter. Bar denotes 100 nm.

Fig. 2 illustrates the lateral distribution of glycolipid receptors for the lectin, wheat germ agglutinin, in liposomes such as those described above. With a molecular weight of only 34000 (calculated diameter assuming spherical shape, 4.4 nm), this lectin is evident as poorly resolved particles on membrane etch faces coated with a thin film of platinum. Wheat germ agglutinin has two binding sites for terminal NANA residues [38], and hence should be capable of binding specifically to all gangliosides. In Fig. 2A, B, D the liposomes bore the disialo ganglioside, GD_{1a}. Upon close examination bound wheat germ agglutinin appears to exist in small clusters, however, the gross distribution is uniform and does not show the larger patchy arrays common to GM_1 as in Fig. 1. Evidence of liposome agglutination was common in samples treated with wheat germ agglutinin, which is polyvalent: in Fig. 2B a clump of smaller vesicles may be seen attached by lectin to the large liposome under study. Fig. 2C shows that very little binding under the same conditions of sample exposure occurs to liposomes bearing only the monosialo, GM_1, while extensive binding occurs to the trisialo ganglioside GT_{1b} (Fig. 2E) in a distribution similar to that of GD_{1a}. Once again though, there is no difference in pattern of labelling for rigid vs. fluid lipid domains. Basically similar results were seen when we used succinylated ferritin-conjugated wheat germ agglutinin as opposed to the unmodified native lectin, although, at the lower overall lectin concentrations necessary with the ferritin conjugated material the extent of membrane labelling was less informative (unpublished observation).

In Fig. 3 we have addressed the question of whether the basic patterns of receptor distribution noted above using RCA 60 to mark the location of GM_1 and wheat germ agglutinin to mark the location of GD_{1a} are also found for bilayer membranes containing mixtures of the two receptors. Fig. 3A and B show the RCA 60 binding features typical of liposomes bearing only GM_1, yet the liposomes involved contained both GM_1 and GD_{1a}. Similarly, the wheat germ agglutinin binding pattern for the same mixture (Fig. 3C) is not obviously different from that of GD_{1a} alone. Unfortunately, such experiments approach the limit of interpretability of our present analytical approach. We are currently attempting to develop a detailed quantitative approach to similar lectin binding experiments in the hope of making the technique capable of detecting more subtle differences in receptor distribution.

The results of these experiments are certainly consistent with previous freeze-etch studies of gly-

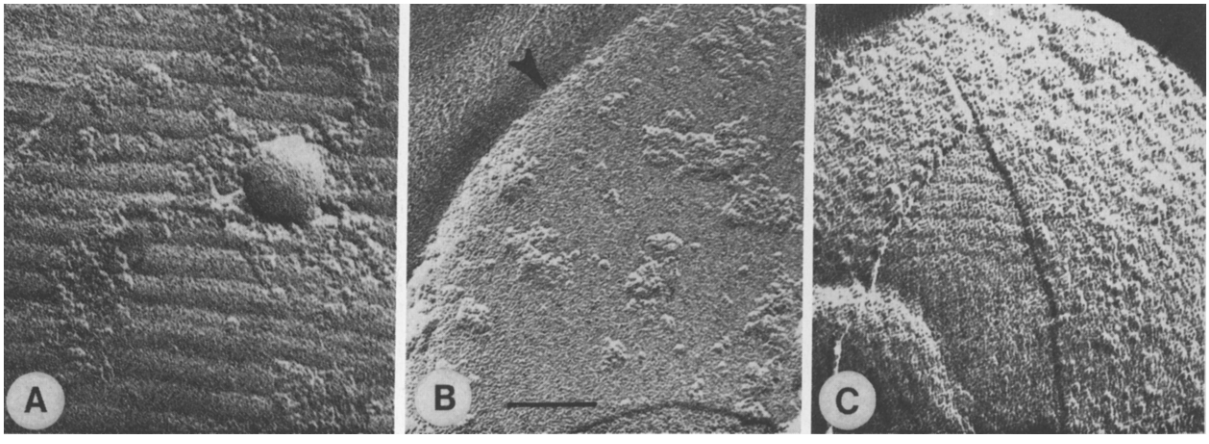

Fig. 3. Freeze-etch electron micrographs of liposomes such as those described in Fig. 1, but bearing a 50:50 mixture of GM_1 and GD_{1a}. Each sample was exposed to lectin (RCA 60 or wheat germ agglutinin) for 15 min at 20°C before quenching. A, B, 7 mol% GM_1 and 7 mol% GD_{1a} exposed to 0.067 mg/ml RCA 60; C, 5 mol% GM_1 and 5 mol% GD_{1a} exposed to 1 mg/ml wheat germ agglutinin. Samples containing 10 mol% or 14 mol% total ganglioside appeared identical. Shadow direction is from bottom to top of page. Arrowheads point from the ice toward the membrane outer surface and abut on the outermost edge of the latter. Bar denotes 100 nm.

cosphingolipids in membranes and support the concept of the existence of forces tending to drive them to cluster. However, it is not the intention of this communication to emphasize these aspects, which will require much more study before a full understanding exists—particularly in host matrices more representative of natural membranes. Rather, the aim is to consider whether the lectin-marked distribution is the same or different for GM_1 and GD_{1a} from the same source: i.e., whether structurally different gangliosides may be made to distribute differently on the basis of host matrix interactions. Toward this end an attractive approach is to choose a system with distinctive glycolipid features. Such a system was reported by Tillack et al. [28]: dramatic linear arrays of ferritin-conjugated lectin bound to asialo GM_1 in single pure phospholipids in the $P_{\beta'}$ phase. These authors suggested that such a pattern could be accounted for by assuming that asialo GM_1, like cholesterol [40], might preferentially inhabit the region between $P_{\beta'}$ ripples. This would be a form of phase separation. In Fig. 4 we have illustrated the appearance of dipalmitoylphosphatidylcholine bilayers bearing 7 mol% GM_1 or GD_{1a} at 20°C. It will be seen from Figs. 4A–4C that there is an overall preponder-

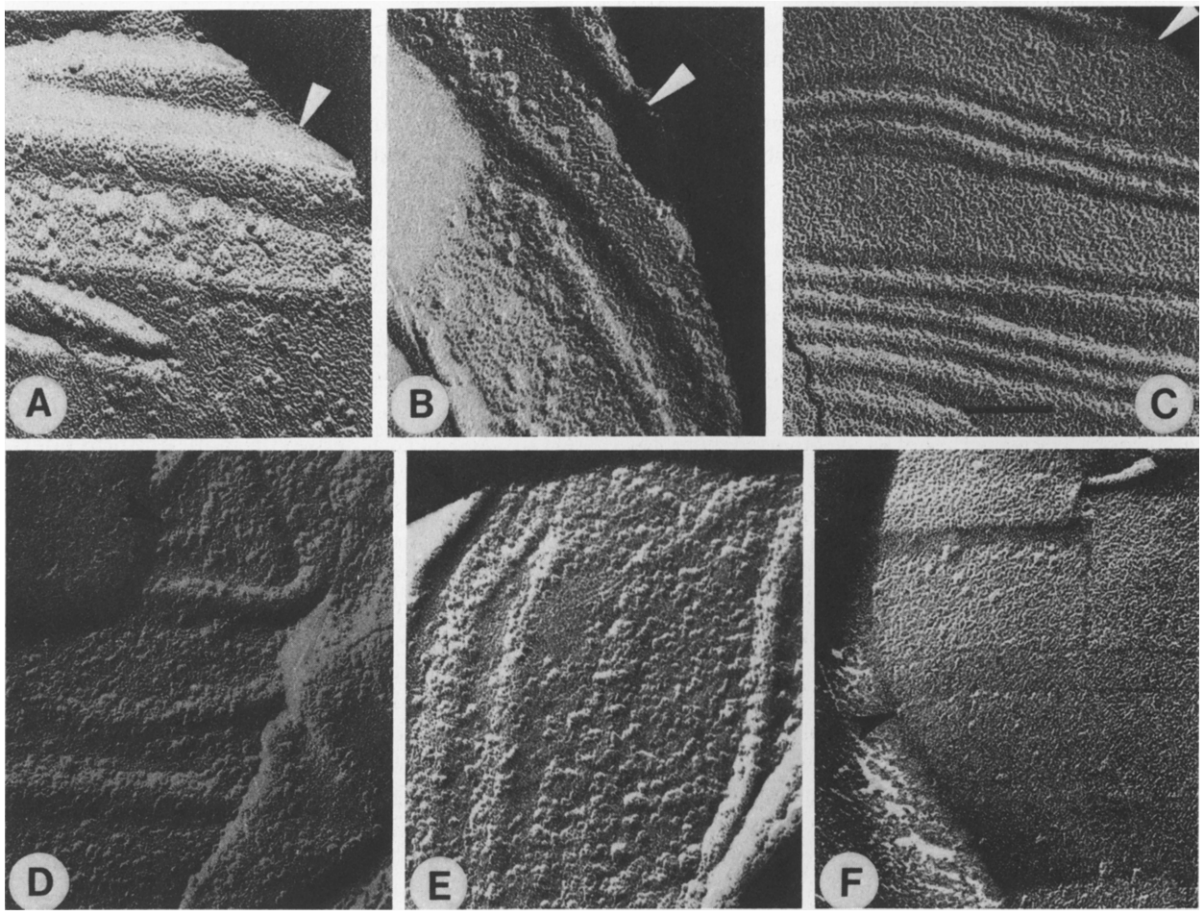

Fig. 4. Freeze-etch electron micrographs of liposomes of pure dipalmitoylphosphatidylcholine bearing 7 mol% GM_1 or GD_{1a} and exposed for 15 min to RCA 60 (0.15 mg/ml) or wheat germ agglutinin (0.5 mg/ml) at 20°C. Striking (elevated) ripples are evident separated by wider smooth areas. With GM_1 as receptor, bound RCA 60 is predominantly localized to the regions between ripples (A, B) — this is also true for wheat germ agglutinin binding to GM_1, although this lectin binds less extensively (F). GD_{1a} does not bind RCA 60 (C) and, as marked by bound wheat germ agglutinin, occurs with equal frequency on or between $P_{\beta'}$ ripples (D, E). Shadow direction is from bottom to top. Bar denotes 100 nm.

ance of RCA 60 bound to GM_1 in the 'troughs' or smooth areas between (raised) ripples typical of the $P_{\beta'}$ phase. This seems particularly so for larger clusters (unpublished observation). Note that there is virtually no non-specific binding to membranes bearing the wrong receptor (Fig. 4C). In contrast, wheat germ agglutinin bound to GD_{1a} shows no evidence of preferentially labelling regions between ripples (Fig. 4D, E). As noted earlier, wheat germ agglutinin binds less extensively to GM_1 (Fig. 4F) — although what binding there is is consistent with the GM_1 distribution marked by RCA 60.

Hence, as marked by the presence of bound RCA 60 and wheat germ agglutinin, respectively, GM_1 and GD_{1a} from bovine brain grey matter can have visibly different distributions in phospholipid bilayer membranes. Neither showed an obvious preference for fluid vs. rigid lipid domains. It is difficult to totally exclude the possibility that the distribution difference seen may be secondary to a redistribution following lectin binding. Unfortunately, prefixation of lipids in model membranes for subsequent lectin binding studies is not as straightforward as in the case of receptor glycoproteins: glutaraldehyde cannot be depended upon to adequately fix even amino lipids, and OsO_4 has poorly understood interactions with sugar headgroups. However, the RCA 60 used here to mark the (patchy) distribution of GM_1 has only one sugar binding site [38]. Furthermore, it is sterically unlikely that several macromolecules can bind simultaneously to the same glycolipid oligosaccharide headgroup and thereby directly induce patch formation (see also Ref. 28). Certainly, from comparison of the patterns of lectin binding to receptors in rigid vs. fluid lipid (where lateral diffusion rates are different by a factor of 10^3 [41]), there is no evidence of lectin-induced glycolipid redistribution. In order to specifically consider the possibility that lateral rearrangement subsequent to lectin binding may contribute to the distribution of RCA 60 bound to GM_1, we have examined the effect of sample history upon freeze-etch appearance. For instance, a preparation of liposomes of dipalmitoylphosphatidylcholine (phase transition temperature 41.5°C) bearing 7 mol% GM_1 was split in half: one half was incubated briefly at 20°C with RCA 60 and quenched immediately, while the other half was warmed with RCA 60 to above 42°C for 15 min prior to cooling and quenching from 20°C. The appearance of these samples was indistinguishable in spite of the fact that one sample had been allowed an opportunity to rearrange, while in the other sample receptor rearrangement should have been minimal. Furthermore, the features seen were identical in all respects to those recorded by Tillack et al. [28] for ferritin-conjugated RCA 60 binding to the neutral glycolipid, asialo GM_1, in dipalmitoylphosphatidylcholine.

As mentioned already, the possibility of microheterogeneity in the membrane distribution of glycosphingolipids has been considered by various workers and recent studies by electron microscopy have supported such a concept. Thus, Tillack et al. [28] have described clustered arrays of asialo GM_1 in phospholipid model membranes exposed to ferritin-conjugated RCA 60. These workers have also described large clusters and extensive patches of Forssman antigen and globoside as marked by bound ferritin-conjugated and colloidal gold-conjugated antibodies, respectively, in erythrocyte ghost membranes [29]. The results reported for the above three neutral glycosphingolipids are similar to those found here for the monosialo ganglioside, GM_1 whereas, in our hands, the polysialo species, GD_{1a} (and GT_{1b}), showed a more uniform lateral distribution as marked by the presence of bound lectin. The molecular interactions governing lateral distribution and phase behaviour of lipids are complex. In the case of glycosphingolipids one might look to the sugar headgroup, the alkyl chains, or the ceramide 'shoulder' region as possible sources of interactions predisposing toward their distribution within a phosphatidylcholine host matrix. The existence of the amide linkage in the 'shoulder' region of sphingolipids has attracted attention because of its potential for hydrogen bonding (reviewed in Ref. 42). Curatolo et al. [43] demonstrated that the amide group can account for the metastable behaviour of glucosyl ceramide in phosphatidylcholine bilayers, suggesting that hydrogen bonding via this group may be a significant source of glycolipid-glycolipid attractive interaction. The possible role of glycolipid oligosaccharide headgroups in mediating their interactions has also been considered (e.g., hydrogen bonding,

charge, dipolar interactions and steric effects) [4,5,19,44–47]. In addition, alkyl chain differences are known to influence miscibility of lipids in bilayers [30–33]; and Yohe et al. [48] have specifically pointed out the possibility that acyl chain length may contribute to the lateral distribution of glycosphingolipids in membranes. With regard to the difference noted in this work between the lateral distribution of GM_1 and GD_{1a} (and GT_{1b}), there exist differences in headgroup and acyl chain length [9]. Maggio et al. [5] have indicated, on the basis of monolayer studies and surface potential calculations, that, as seen here, monosialo gangliosides (i.e., GM_1) might be expected to behave like neutral glycolipids and differently from polysialo gangliosides. These workers found that the excess free energy of mixing with a phosphatidylcholine host matrix was positive (predisposing toward demixing or patching) for neutral glycolipids and GM_1, but negative for polysialogangliosides. It now seems possible to begin to test some of these complex considerations by actual visualization in various membrane systems under controlled conditions.

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