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Influence of phospholipid chain length on verotoxin/globotriaosyl ceramide binding in model membranes: comparison of a supported bilayer film and liposomes

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The importance of the surrounding lipid environment on the availability of glycolipid carbohydrate for ligand binding was demonstrated by studying the influence of phosphatidylcholine fatty acid chain length on binding of verotoxins (VT1 and VT2c) to their specific cell surface receptor, globotriaosylceramide (Gb_3) in the presence of auxiliary lipids both in a microtitre plate surface bilayer film and in a liposome membrane model system. In the microtitre assay, both VT1 and VT2c binding to Gb_3 was increased as a function of decreasing PC acyl chain length likely resulting in increased Gb_3 exposure. In the liposome assay VT1 binding was similarly modulated, however the effect on VT2c binding was more complex and did not follow a simple function of increased carbohydrate exposure. Earlier work established that C22:1 and C18:1Gb_3 fatty acid homologues were the preferred Gb_3 receptor isoforms in the microtitre assay for VT1 and VT2c respectively. This selectivity was maintained in C16PC containing liposomes, but in C14PC liposomes, binding to C22:1Gb_3 (but not C18:1Gb_3) was elevated such that this Gb_3 species now became the preferred receptor for both toxins. This change in verotoxin/Gb_3 homologue binding selectivity in the presence of C14PC did not occur in the microtitre bilayer format. These results are consistent with our proposal that these toxins recognize different epitopes on the Gb_3 oligosaccharide. We infer that relative availability of these epitopes for toxin binding in an artificial bilayer is influenced not only by the exposure due to the discrepancy between the fatty acyl chain lengths of Gb_3 and PC, but by the physical mode of presentation of the bilayer structure. Such acyl chain length differences have a more marked effect in a supported bilayer film whereas only the largest discrepancies affect Gb_3 receptor function in liposomes. The basis of phospholipid modulation of glycolipid carbohydrate accessibility for receptor function is likely complex and will involve phase separation, gel/liquid crystalline transition, packing and lateral mobility within the bilayer, suggesting that such parameters should be considered in the assessment of glycolipid receptor function in cells.

Keywords: glycolipid recognition, carbohydrate exposure, conformation, solid phase binding

Introduction

Verotoxins (VT), also known as Shiga-like toxins [1], comprise a family of toxins that are produced by certain strains of enterohaemorrhagic *E. coli* [2, 3]. Verotoxins are composed of an active (A) subunit and five binding (B) subunits [4, 5]. The family comprises four members, VT1 (identical to Shiga toxin), VT2, VT2c found in human *E. coli* isolates, and the toxin responsible for pig oedema disease, VT2e. The human toxins are the cause of two significant diseases, haemorrhagic colitis [6, 7] and haemolytic uraemic syndrome [8] which is the major cause of acute pediatric renal failure. Both these diseases are characterized by microvascular endothelial cell damage and verotoxins are cytotoxic for certain endothelial cells *in vitro* [9, 10].

Globotriaosyl ceramide (galactoseα1-4galactoseβ1-4glucosyl ceramide: Gb_3) is the functional receptor for verotoxins (11–13). The glycolipid binding specificity is a primary determinant of the sites of pathology in animal studies [14, 15]. VT binding is dependent on the terminal galactoseα1-4galactose disaccharide [11, 12] but is also strongly influenced by the nature of the lipid moiety [16–18].

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Internalization of cell-bound toxin occurs by receptor-mediated endocytosis [19], and is targeted intracellularly to the rough endoplasmic reticulum by a process termed retrogade transport [20–22]. The A subunit of VT1 is proteolytically nicked and reduced to the A1 fragment, the RNA glycanase activity of which suppresses protein synthesis through the inhibition of elongation factor 1 dependent aminoacyl-tRNA binding to the 60S ribosome [23]. However, cytotoxic potential of the holotoxin is also defined by B subunit Gb_3 binding [5].

While the presence of Gb_3 is necessary to confer susceptibility to VT cytotoxicity, the cellular concentration of Gb_3 is not necessarily proportional to that sensitivity [24, 25]. Thus factors in addition to receptor concentration determine sensitivity to VT.

It has been demonstrated that 'exposure' of glycosphingolipid carbohydrate is influenced by their lipid composition and membrane environment [26–31]. Such effects have been ascribed to the ability of the lipid matrix to modulate glycolipid head group exposure according to the relative phospholipid hydrocarbon chain length. The ability of glycolipids to phase separate into domains with a phospholipid bilayer can also affect carbohydrate availability to soluble ligands [32].

Previous studies have established that fatty acid heterogeneity [16], chain length and unsaturation [17] markedly affect verotoxin binding to Gb_3 in a model phospholipid bilayer surface. Two members of the verotoxin family, VT1 and VT2c which show a significant difference in specific cytotoxicity [5], were found to bind preferentially to different Gb_3 fatty acid isoforms under these conditions [17].

Conformational modelling studies of globoseries glycosphingolipids [33, 34] suggest that the orientation and steric presentation of the saccharide chain at the membrane surface for recognition by antibodies or bacterial adhesins is determined, in part, by the combined influence of the linkage between the saccharide head group and the ceramide moiety and the relative plane of the membrane/solvent interface. In a study of synthetic glycolipids, we found that species which were not recognized by VT in tlc overlay could serve as effective receptors when presented in a lipid matrix [18]. Thus the lipid moiety of a glycolipid and the membrane environment can have a major effect on glycolipid carbohydrate receptor function.

The interaction of the *E. coli* elaborated verotoxin with Gb_3 provides a highly manipulable model to study the molecular basis of specific GSLs recognition. In order to approach the question whether our present solid phase assays accurately reflect the regulation of glycolipid receptor function in cells, we have compared verotoxin/Gb_3 binding in phospholipid/cholesterol bilayers in liposomes and as a surface film in microtitre wells. The influence of phospholipid hydrocarbon chain length on the presentation of Gb_3 in these bilayer formats for VT1 and VT2c binding was compared.

Materials and methods

Lipids

Egg phosphatidylcholine (egg PC), dimyristoylphosphatidylcholine (diC14 PC), dipalmitoylphosphatidylcholine (diC16 PC), distearoylphosphatidylcholine (diC18 PC), dibehecicphosphatidylcholine (diC22 PC), fatty acids and cholesterol were purchased from Sigma.

Extraction of human renal Gb_3

Gb_3 was purified as previously using human kidney obtained at autopsy [16].

Semisynthetic Gb_3 homologues

Semisynthetic Gb_3 homologues containing C22:1 or C18:1 fatty acids were synthesized from lyso-Gb_3 [35] as previously described [17].

Toxin preparation

VT1 was purified from the over-producing recombinant *E. coli* strain pJLB28 [36] by the method involving polymxin B extraction, ultrafiltration, hydroxyapatite chromatography, chromatofocusing, and Cibacron Blue chromatography [5].

VT2c was purified by virtually the same method from an *E. coli* clinical isolate, strain E 32511, which was determined to produce VT2c only [37].

VT1 and VT2c iodination

Purified toxins (50–100 μg) were iodinated using Iodobeads (Pierce). Two iodobeads were incubated with 0.5 mCi Na ^{125}I (Amersham) and 100 μl of 0.1 M sodium phosphate, pH 7.2, for 10 min.

Labelled VTs were separated from unreacted iodine using a G-25 column equilibrated with PBS.

VT glycolipid TLC overlay

VT binding was performed as described by Yiu and Lingwood [38].

Microtitre verotoxin binding assay

Based on the method used previously [16, 17] 100 μl of Gb_3 (0.1 μg) in a methanolic solution containing egg PC (or C14 PC, C16 PC, C18 PC, or C22 PC) and cholesterol (1:5:2.5 by weight) was added in triplicate to 96 well flexible microtitre plates and the solution was allowed to evaporate overnight. The plates were blocked with 2% BSA in 50 mM TBS (pH 7.4) for 1 h at room temperature. The wells were washed once with 0.1% BSA in TBS.

Dilutions containing 50–300 ng of ^{125}I-VT1 or ^{125}I-VT2c diluted in 0.1% BSA in TBS were added to the Gb_3-coated wells and incubated for 2 h at room temperature. After five washes with 0.1% BSA in TBS, the wells were cut out and counted in a Beckman 5500 gamma-counter. Control wells containing no Gb_3 were prepared and counted as above. Binding for controls was < 5% of total binding in the presence of Gb_3 and was subtracted. Assays were performed in triplicate.

Preparation of Gb_3 containing liposomes

Multilamellar vesicles containing PC/Chol/GSLs (egg PC, C14 PC, C16 PC, C18 PC, or C22 PC) were used in order to avoid the high surface curvature of small unilamellar liposomes and the possible exclusion of the Gb_3 during preparation of large unilamellar liposomes by extrusion or solvent injection procedures [39]. A chloroform:methanol (2:1 v/v) solution of the lipid mixtures in weight ratio of 1:5:2.5 of Gb_3:PC:Chol (as used in the microtitre plate binding assay) were added to a screwcapped glass tube, and after evaporation of the solvent under stream of nitrogen, was further dried under vacuum for 1 h. Then the lipids were dispersed in 50 mM Tris buffered saline (TBS) pH 7.4 at a concentration of 10 ng μl^{-1} Gb_3 by vortexing. The suspension was dispersed by alternate vigourous vortexing and heating for 30 s at a temperature of 90–95 °C (5–10 °C above the lipid transition temperature) for a period of 10 min. Background binding to control liposomes lacking Gb_3, routinely > 6%, was subtracted. Measurements were made in triplicate.

Electron microscopy

Gb_3 containing phospholipid liposomes were prepared as above, fixed with glutaraldehyde and osmium tetroxide, dehydrated, embedded and ultrathin sections visualized by negative staining with uranyl acetate [40].

VT-binding assay-Gb_3 liposomes

One hundred µl of freshly prepared liposomes containing 1 µg Gb_3 were added in triplicate to 2 ml centrifuge tubes. The liposomes were blocked by incubation with 2% BSA in 50 mM TBS for 1 h at room temperature and washed once with TBS containing 0.2% BSA by centrifugation at 16 000 × **g** for 30 mins. Different concentrations of ^{125}I-VT1 or VT2c were added to each series in order to reach saturation and were incubated for 1 h at room temperature. The vesicles were washed four times with 0.2% BSA in TBS. Bound VT in the liposome pellet was counted using a gamma counter.

All toxin binding experiments were repeated three times. One representative experiment with the means of triplicate determinations is shown in each case.

Results

BINDING OF HUMAN RENAL Gb_3 BY VT1 AND VT2c IN DIFFERENT PC CONTAINING LIPID BILAYERS

Two binding assay systems were used to determine the effect of different fatty acid chain lengths of PC (C14, 16, 18, 22, or egg PC) on VT1 and VT2c recognition of Gb_3 in a phospholipid bilayer.

a) Microtitre binding assay

The binding of both VT1 and VT2c to a fixed concentration of Gb_3 immobilized in a PC bilayer film on the microtitre plate surface was reduced as a function of increasing PC chain length (the binding order was C14 > C16 > C18 = C22). Binding was increased for C14 PC 3 fold as compared with C22/C18 PC (Fig. 1A, B).

b) Liposome binding assay

A representative example of the multilamellar liposomes as determined by electron microscopy is shown in Fig. 2. The surface area of the liposomes did not vary significantly between the different preparations.

For each toxin, overall binding was increased two-to-three-fold as compared to the microtitre assay. Increasing PC chain length had a different effect on the binding of either toxin to liposomes containing Gb_3 (Fig. 1C, D) compared with the microtitre plate format. For VT1 (and VT2 not shown) binding was increased for C14 PC containing liposomes, but increasing the PC chain length beyond C16 had no additional effect (Fig. 1C).

The binding of renal Gb_3 by VT2c in this model membrane system showed a distinct modulatory effect as compared to VT1 (and VT2, not shown) and did not follow a predictable trend. C18 PC containing vesicles showed the maximum binding to VT2c (Fig. 1D), slightly higher than C14 PC liposomes. Liposomes containing C22, C16 or egg PC showed lower binding (C18 PC > C14 >> C22 > C16 = egg PC) and were saturated at a lower VT2c concentration as compared to C18 and C14 vesicles.

BINDING OF SEMISYNTHETIC Gb_3 BY VT1 AND VT2c IN DIFFERENT PC CONTAINING LIPID BILAYERS

a) Microtitre binding assay

Semisynthetic Gb_3 homologues containing C22:1 or C18:1 fatty acid (which are the preferred receptors in the bilayer film microtitre assay for VT1 and VT2c respectively [17]) were prepared from lyso-Gb_3 as described in the Methods. The preferential binding of VT1 and VT2c was confirmed in the microtitre format. Binding of both toxins to the Gb_3 isoforms was increased in the presence of C14 PC relative

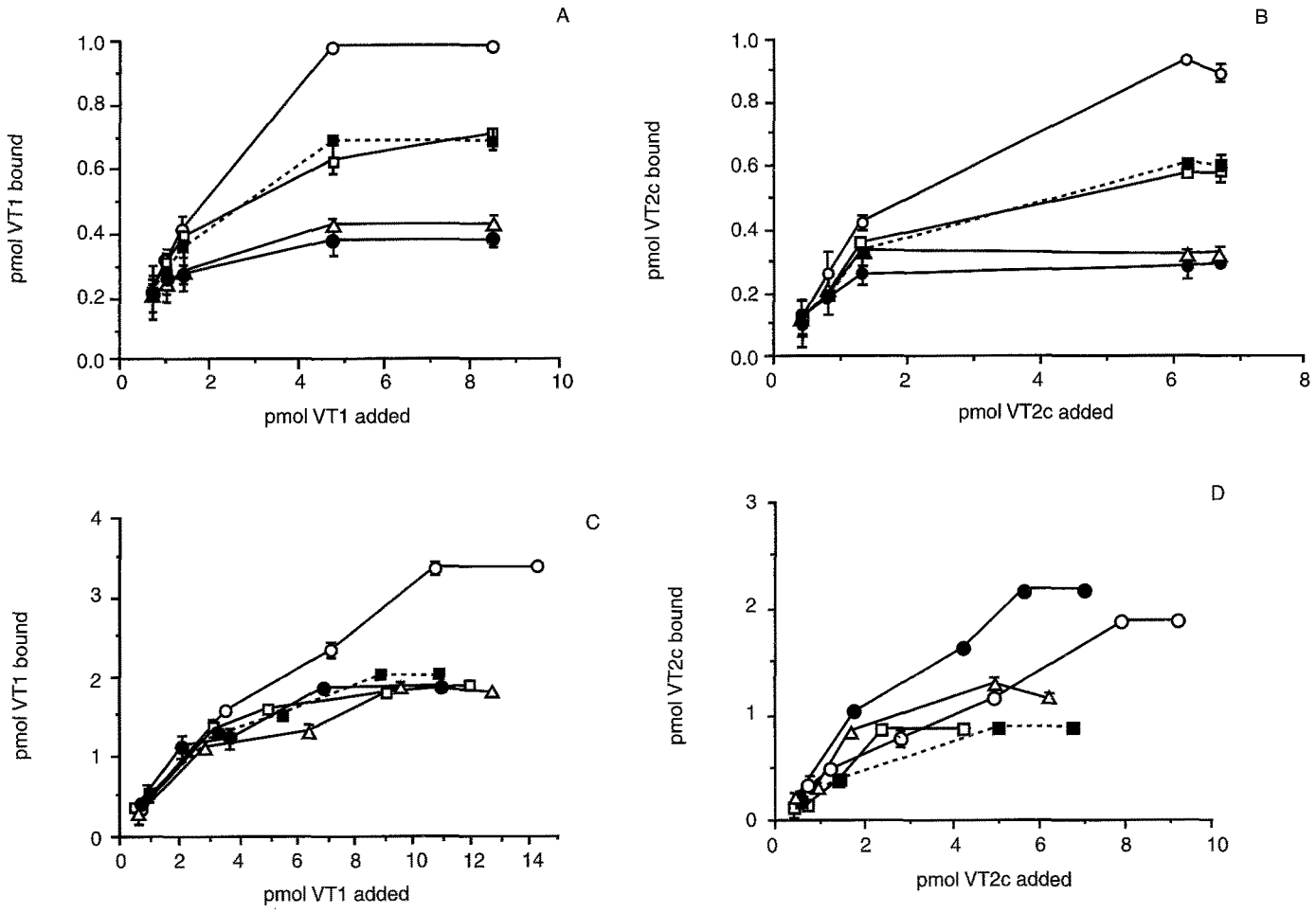

Figure 1. Comparison of the effect of PC chain length on verotoxin binding to Gb_3 in the microtitre bilayer film (A, B) and liposomes (C, D). PC species: (—○—): C14, (—□—): 16, (—●—): 18, (—△—): 22, (···■···): egg PC, A, C: VT1, B, D: VT2c. Standard errors of triplicate determinations are shown.

to C16 PC but in each case maximum binding of VT1 and VT2c was to Gb_3 containing C22:1 and C18:1 fatty acids respectively (Fig. 3A, B).

b) Liposomes

The binding selectivity of VT1 and VT2c for the Gb_3 C22:1 and C18:1 fatty acid homologues was then compared in the multilamellar liposomes. The highest VT1 binding was observed for the C22:1 containing Gb_3 in both a C14 PC environment and in C16 PC liposomes, as observed in the microtitre format above. Liposomal binding for these homologues showed a different effect for VT2c. For VT2c, the C18:1 Gb_3 homologue was the best receptor as compared to C22:1 Gb_3 when in C16 PC-containing liposomes but the C22:1 homologue was the preferred VT2c receptor in C14 PC-containing liposomes. In this case the lipid environment not only influenced the binding of VT2c to Gb_3, but the fatty acid homologue specificity was also altered. Essentially for both VT1 and VT2c, only binding to the C22:1Gb_3 homologue is sensitive to changes in PC chain length in liposomes (Fig. 3C, D).

VT BINDING STOICHIOMETRY TO NATURAL Gb_3 IN LIPOSOMES

The maximum binding was observed by VT1 (Fig. 1C) with natural Gb_3 in vesicles containing C14 PC. The capacity of liposomes containing 1 nmol of Gb_3 to be bound by VT is approximately 4 pmol (or 1000:4, Gb_3:VT mol/mol). VT1 is pentavalent and if all the valencies are bound, this result would suggest that 1:50 Gb_3 are bound by the toxin. In addition, if the maximum exposure of Gb_3 in the outer monolayer of the multilamellar vesicles is about 10% [30], this means 1:5 Gb_3 are bound by the toxin in liposomes containing C14 PC.

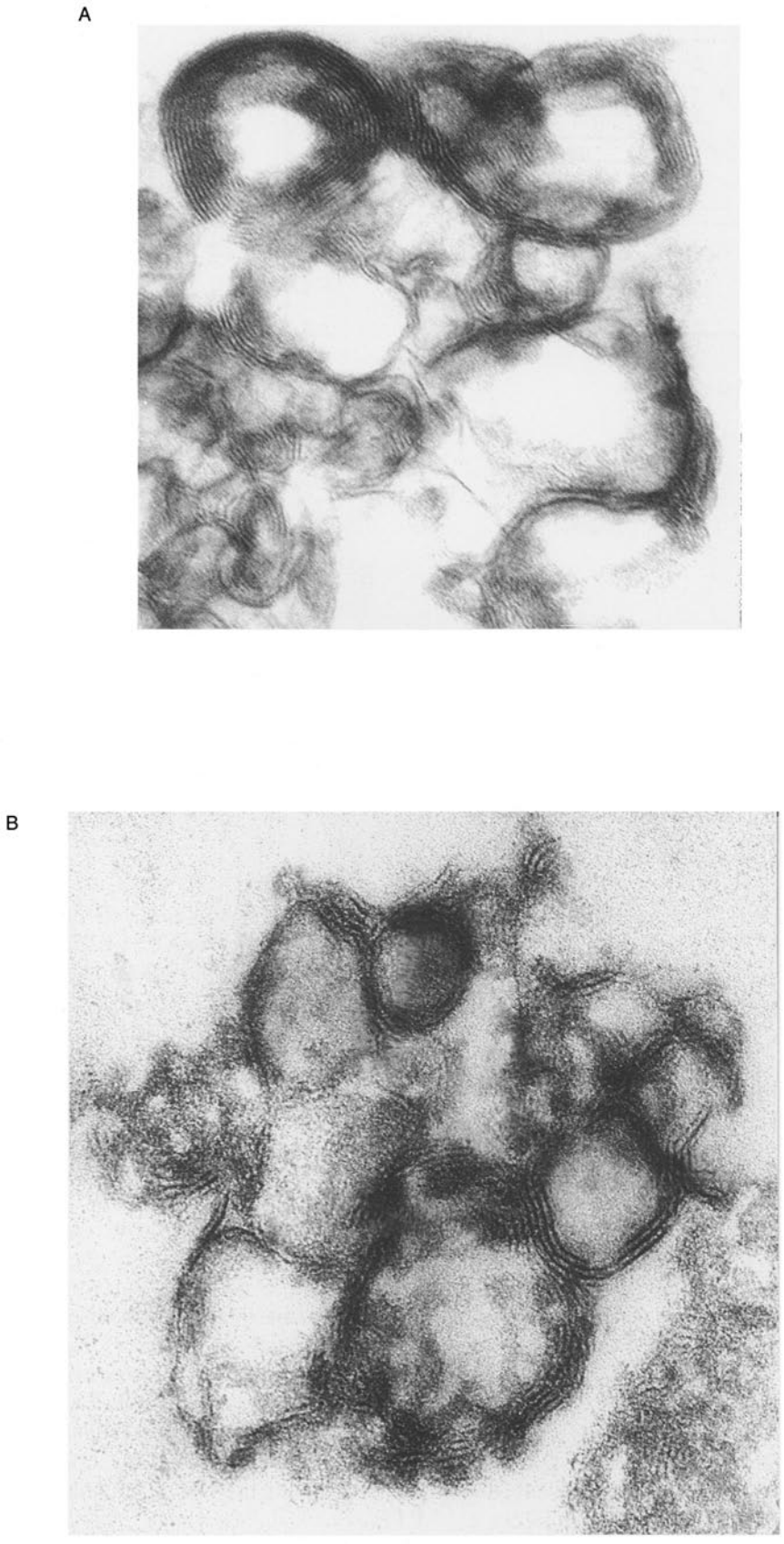

Figure 2. Electron microscopy of multilamellar liposomes. (A) renal Gb_3 in C18 PC, (228 000×). (B) renal Gb_3 in C14 PC (236 000×).

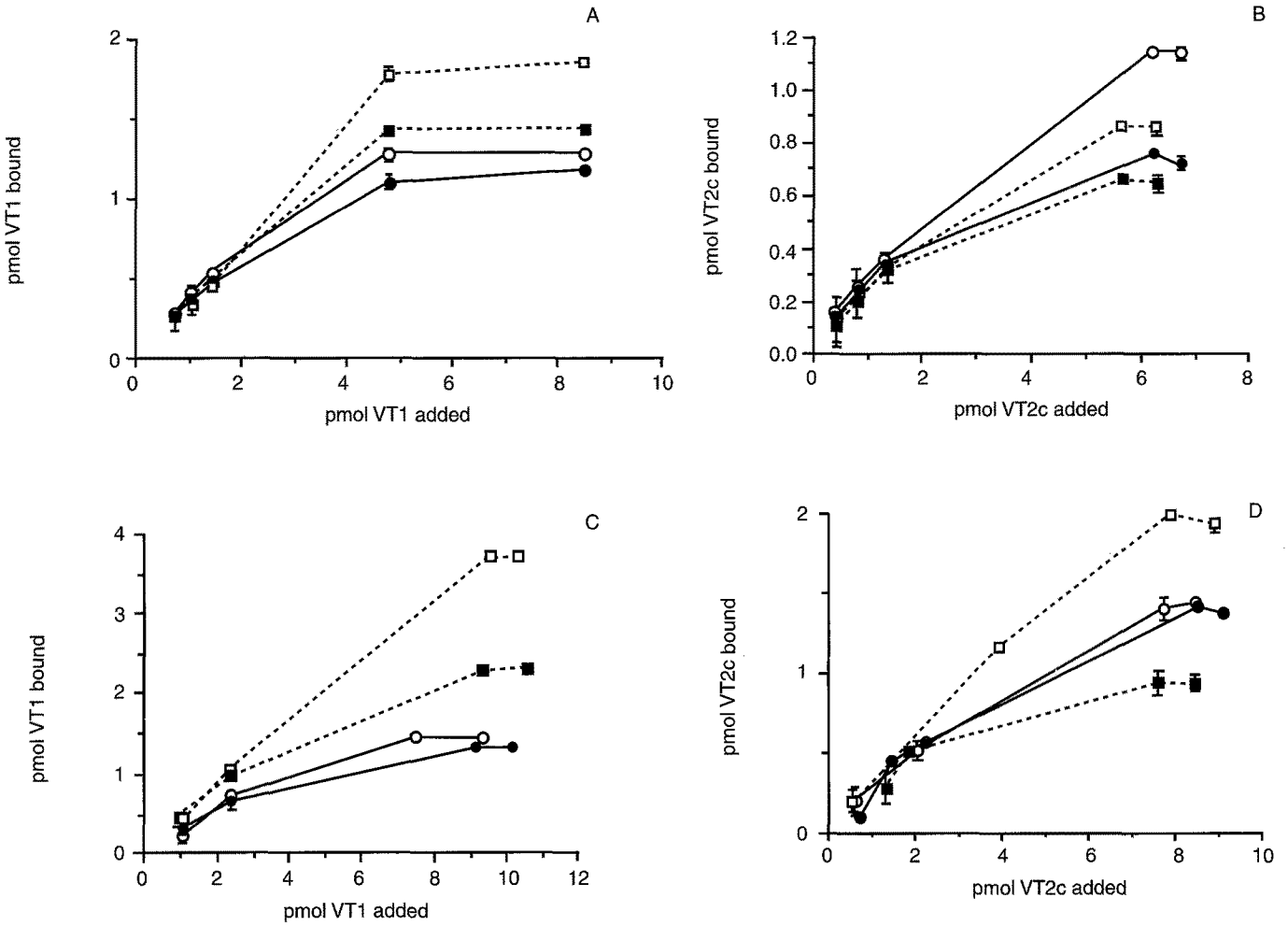

Figure 3. Comparison of the effect of PC chain length on VT/Gb_3 fatty acid homologue binding preference in the microtitre bilayer film (A, B) or liposomes (C, D). C18:1 Gb_3 and C14 PC (—○—), C18:1 Gb_3 and C16 PC (—●—); C22:1 Gb_3 and C14 PC (····□····), C22:1 Gb_3 and C16 PC (····■····). A, C:VT1, B, D: VT2c. Standard errors of triplicate determinations are shown.

Discussion

The use of phospholipid/cholesterol mixtures as a manipulable model of plasma membrane function is common. While the composition of the Gb_3/phospholipid matrices used does not reflect that of biological membranes, the present studies follow on from our demonstration of the importance of the lipid environment on toxin binding [18] and represent a first step to extrapolate *in vitro* solid phase binding to the regulation of glycolipid function in the plasma membrane of susceptible cells. Verotoxin glycolipid binding specificity was established by tlc overlay [11, 41, 42], but although such results in general correlate with cell cytotoxicity there are several exceptions [25, 38, 43] and toxin binding in the solid phase has been shown to vary widely according to the manner of glycolipid immobilization [17, 18]. Thus the interaction of verotoxin with cell surface Gb_3 is likely a complex procedure belied by its ease of assay *in vitro*.

These studies show that for VT1, binding to Gb_3 in the presence of phosphatidyl choline and cholesterol is modulated by the PC hydrocarbon chain length. This result is in agreement with several previous studies [29, 30] which demonstrate that glycolipid exposure for ligand binding in such a lipid environment is promoted by reduction of the PC chain length. The differential effect of PC chain length on the binding of VT2c (as opposed to VT1) to Gb_3 containing liposomes supports our previous hypothesis that these two toxins bind to differing carbohydrate epitopes on the Gb_3 molecule [17, 44]. Binding of VT2c is not solely a function of carbohydrate exposure, increased as a reciprocal of PC chain length. These results are also consistent with our recent thermodynamic calculation of two potential independent Gb_3 binding sites per B subunit monomer [45]. Such sites may be differentially utilized by VT1 and VT2c.

The finding that VT2c binding to Gb_3 is modulated by PC chain length in a manner identical to VT1 in the

microtitre format, provides evidence that the presentation of glycolipid carbohydrate in these two bilayer formats are not equivalent.

The fact that for both VT1 and VT2c, only binding to C22:1, as opposed to C18:1 Gb_3, was increased by reduction in PC fatty acyl chain length (Fig. 3C, D) in liposomes, resulted in a change in Gb_3 homologue binding selectivity for VT2c in C14 PC liposomes. This indicates that the membrane environment can exert a differential effect on glycolipid carbohydrate 'exposure' according to the discrepancy in phospholipid/glycolipid hydrocarbon chain length. Moreover the finding that this differential effect was not observed in the microtitre assay may indicate that the bilayer width may have some 'pliancy' in liposomes, denied in a surface bilayer film. Thus in liposomes, only the largest chain length discrepancy (e.g. C22:1Gb_3 with C14 PC) resulted in increased binding. The smaller discrepancies may be accommodated in liposomes by distortion of the bilayer hydrocarbon chain interface. This might also explain the effect of PC chain length on VT binding to renal Gb_3. For VT1, only the shortest PC had an enhancing effect on binding in liposomes whereas there was a linear inhibitory effect of increasing chain length on VT binding renal Gb_3 in the microtitre assay (Fig. 1A, 1B). Alternatively interdigitation may provide the mechanism of accommodation [46–49].

We have suggested that the differential binding of VT1 and VT2c to different Gb_3 fatty acid homologues is due to a different galabiose conformational requirement for the binding of these two toxins [17]. Using thermodynamic calculations, we identified two possible Gb_3 binding sites per VT B monomer, which accommodated different Gb_3 conformers [45]. Many studies have established a contribution of the conformation of carbohydrate hydroxyls in determining the intermolecular organization of bilayer glycosphingolipids [50], and it is therefore not unlikely that the reverse might also be true.

Although the bilayer structure of the liposomes can be clearly established by standard electron microscopy (Fig. 2), technical limitations prevent similar analysis of the microtitre supported phospholipid film at present. Phospholipid films however, when hydrated may adopt a bilayer format [46, 51] and atomic force electron microscopy [52] would seem ideally suited to confirm this in future studies. The phospholipid film format has often been adopted to quantitate glycolipid receptor binding specificity [53–58]. We have shown that glycolipid receptor function can vary markedly according to the manner of immobilization, particularly when comparing tlc overlay binding with that in a microtitre format with accessory lipids [17, 18].

The biophysical basis for the difference in receptor activity of Gb_3 in phospholipid matrices of PCs of different chain length under the conditions we have described is likely to be complex, requiring consideration of differences in phase separation and possible formation of lipid microdomains, gel/liquid crystalline transition temperatures and molecular area in addition to that of the relative glycolipid/phospholipid acyl chain length. Although such studies are beyond the scope of this report, our present results serve as an initial alert to the potential effect of the solid phase to modify glycolipid receptor function in a phospholipid bilayer. This work also further demonstrates a difference in VT1 and VT2c binding to Gb_3.

Studies with artificial membranes often assume equivalence of phospholipid bilayers immobilized on a surface and in vesicles [59, 60] and little data counter to this assumption is available. However in a few reports, supported bilayer films have been shown to represent a closer functional *in vitro* equivalent of natural plasma membranes than liposomal bilayers [61–63].

Our studies provide further evidence that a liposomal bilayer does not necessarily mimic a bilayer on a surface. Our results imply that the lipid composition and local membrane environment in combination, may have a stereoselective effect on glycolipid carbohydrate receptor function. Presentation of glycolipid carbohydrate in a surface bilayer film is not necessarily equivalent to that in a liposome. These studies serve to further illustrate the limitations of a simple mechanism of glycolipid receptor function in a biological membrane [50].

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