Glycoconjugate Journal 20, 33–38, 2004
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Generation of receptor-active, globotriaosyl ceramide/cholesterol lipid 'rafts' *in vitro*: A new assay to define factors affecting glycosphingolipid receptor activity

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Purified renal globotriaosyl ceramide (Gb_3)/cholesterol mixtures, sonicated and heated in a Triton-containing buffer and placed below a discontinuous sucrose gradient, form glycosphingolipid (GSL)-containing dense lipid structures at the 30/5% sucrose interface after centrifugation. Inclusion of fluorescein-labeled verotoxin 1 B subunit (FITC-VT1 B) within the most dense sucrose layer results in the fluorescent labeling of this Gb_3-containing raft structure. Alternatively, inclusion of ^{125}I-labeled VT1 and fractionation allows quantitation of binding. FITC-VT1 B effectively competes for ^{125}I-VT1/Gb_3 raft binding. This assay will allow the definition of the optimal raft composition for VT1 (or any other ligand) binding. The effect of several potential cellular raft components are reported. Increased cholesterol content increased VT1 binding. Addition of phosphatidylethanolamine had minimal effect while phosphatidylserine was inhibitory. Although inclusion of sphingomyelin increased the Gb_3 content of the 'raft', reduced VT1 binding was seen. Inclusion of other glycolipids can also be inhibitory. The addition of globotetraosyl ceramide had no effect; however, addition of sulfogalactosyl ceramide, but not sulfogalactoglycerolipid, inhibited VT1/Gb_3 raft binding. These results suggest that certain GSLs can disfavour the formation of the appropriate 'raft' structure for ligand binding and that this is dependent on both their carbohydrate and lipid structure. Such "deceptor" GSLs may provide an as yet, unappreciated mechanism for the regulation of cellular GSL receptor activity. This model is an effective tool to approach the dynamics and ligand-binding specificity of GSL/cholesterol-containing lipid microdomains.
Published in 2004.

Keywords: **verotoxin, detergent-resistant lipid microdomains, sulfatide**

Introduction

Plasma membrane glycolipids are central components of lipid microdomains [1,2]. The non-uniform distribution of certain glycolipids at the cell surface [3] is largely consistent with this concept. The use of detergent insolubility to isolate these membrane sub-fractions, variously termed glycolipid-enriched membranes (GEM), detergent-resistant domains (DRM), detergent-insoluble membranes (DIM) or lipid rafts, has been widely used as a prelude to their separation by centrifugation through a discontinuous sucrose gradient [4]. These cholesterol-rich domains float to a characteristic density by ultracentrifugation, routinely monitored via the ganglioside GM1 content, considered a raft marker, assessed by cholera toxin binding [5–7]. Inclusion of proteins within this fraction is used as the criterion for raft association [6]. The basis of the molecular assembly occurring in such detergent resistant rafts and the degree to which this reflects the natural organization of such species within the plasma membrane, remains a matter of debate. Isolated lipid species naturally phase separate [8], and it is therefore likely that a significant proportion of these interactions are determined by the parameters of interlipid assembly [9]. These lipid microdomains provide foci for signal transduction processes [10–12] and a variety of cell surface receptor species, particularly those membrane-bound via a glycophosphoinositide anchor, partition in and out of, or cluster within such domains on ligand binding, to effect transmembrane signaling [13]. The mechanism by which such cell

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surface ligand interactions effect a transmembrane signal to cytosolic surface-associated signal transduction cascade effectors is also unknown.

It is becoming clear that the lipid microdomains, as defined above, are heterogeneous [14–16] and although non-detergent methods have been devised for isolation of some subsets of raft structures [17], it is clear that the isolation of the majority of these domains in the absence of detergent, necessary to access their biological function, is some way off.

The *E. coli*-elaborated verotoxin is an AB_5 subunit toxin, associated with the etiology of the hemolytic uremic syndrome [18], which specifically binds to the neutral GSL, globotriaosylceramide (Gb_3) [19]. This binding is central to its mechanism of cytotoxicity/pathology, and organization of Gb_3 within detergent-resistant membrane domains has been shown to correlate with differential intracellular targeting [5] and verotoxin sensitivity [20].

We have begun to use the interaction of verotoxin1 with Gb_3 to develop a new *in vitro* system, essentially to build raft-like condensed lipid structures—cholesterol/GSL assemblies. This offers the potential to determine the optimum lipid composition of a VT1-binding Gb_3-containing raft and potentially, to identify novel cellular components which interact with them.

Materials and methods

Recombinant VT1 was purified from pJB28 and radio-iodinated as previously described [21]. The VT1B subunit was purified from pJB120 [22] and labeled with fluorescein as described [23]. Gb_3 and Gb_4 were purified from human kidney [24] and SGG from bovine testes [25]. SGC was purchased from Sigma.

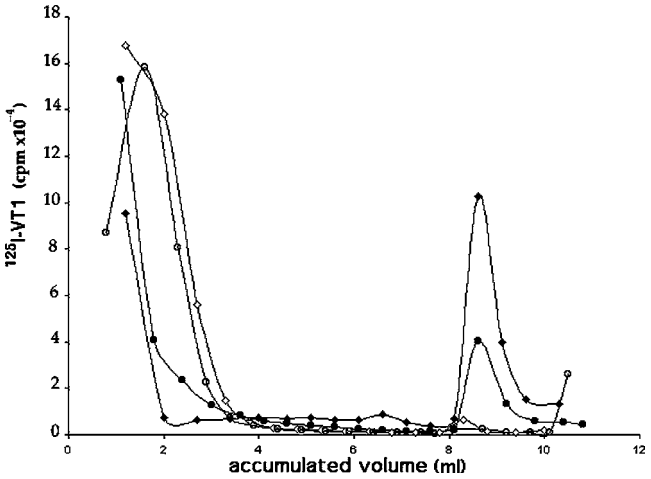
Sucrose-density gradient ultracentrifugation

A 2:1 ratio [8] of Gb_3 (50 μg) and cholesterol (25 μg) in ethanol were dried together and dissolved in 1.5 ml of MES-Triton buffer (25 mM MES, 150 mM NaCl pH 7.2, 1% Triton X-100). The solution was vortexed (1 min), sonicated (1 min), heated at 55°C (5 min) and vortexed (1 min). Then 1.5 ml 73% sucrose solution in MES (pH 7.2) was added, gently mixed and allowed to stand at room temperature for 1 h. The mixture was then overlayed with 2 ml of 30% sucrose containing 10 μg/ml FITC-labeled VT1B or 1 μg/ml ^{125}I-VT1. This was then overlayed successively with 2 mL of 30% sucrose and 3 ml of 5% sucrose and condensed lipid species separated by floatation ultracentrifugation at 34,000 rpm for 66 h at 4°C. FITC-VT1 B-containing tubes were photographed under UV and visible illumination, while a needle was used to puncture the bottom of the ^{125}I-VT1 containing tubes and fractions were collected and counted in a γ-counter.

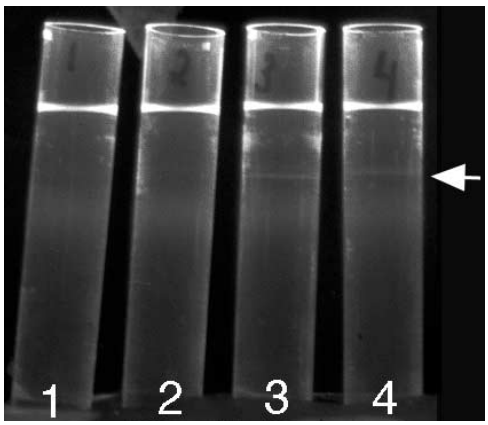
Results

The ^{125}I-VT1 sucrose-gradient separation profile ± FITC-VT1 B is shown in Figure 1(a). Two different Gb_3/cholesterol ratios were used, 2:1 and 2:0.1 (w:w). It can been seen that more ^{125}I-VT1 is associated with the Gb_3 'raft' band at the higher cholesterol concentration. Inclusion of a 60 fold molar excess FITC-VT1B completely prevents this association at both cholesterol concentrations and displaces the ^{125}I-VT1 to the bottom of the gradient. UV illumination of the gradients prior to fractionation (Figure 1(b)) shows the presence of a single fluorescent band in the FITC-VT1B-containing tubes, approximately at the 30/5% sucrose interface. The band was slightly less intense in the 2:0.1 Gb_3/cholesterol containing gradient. A band at this position could be seen in all Gb_3 containing gradients under white light illumination (not shown).

The effect of including PE or PS, potential components of the cytosolic raft surface, in the initial Gb_3/cholesterol



(a)



(b)

Figure 1. Comparison of ^{125}I-VT1 and FITC-VT1 B labeling of *in vitro* generated Gb_3/chlesterol rafts separated by sucrose gradient ultracentrifugation. (a) ^{125}I-VT1 gradient profiles, Gb_3:cholesterol 2:1□◆, 2:0.1 ●■; ^{125}I-VT1 alone □●; ^{125}I-VT1+ FITC-VT1 1B ◆■. (b) UV illumination, ^{125}I-VT1 alone tubes 1,2; ^{125}I-VT1+ FITC-VT1 1B tubes 3,4.; Gb_3:cholesterol 2:1, tubes 1,3, 2:0.1 tubes 2,4. The fluorescent 'raft' band is arrowed.

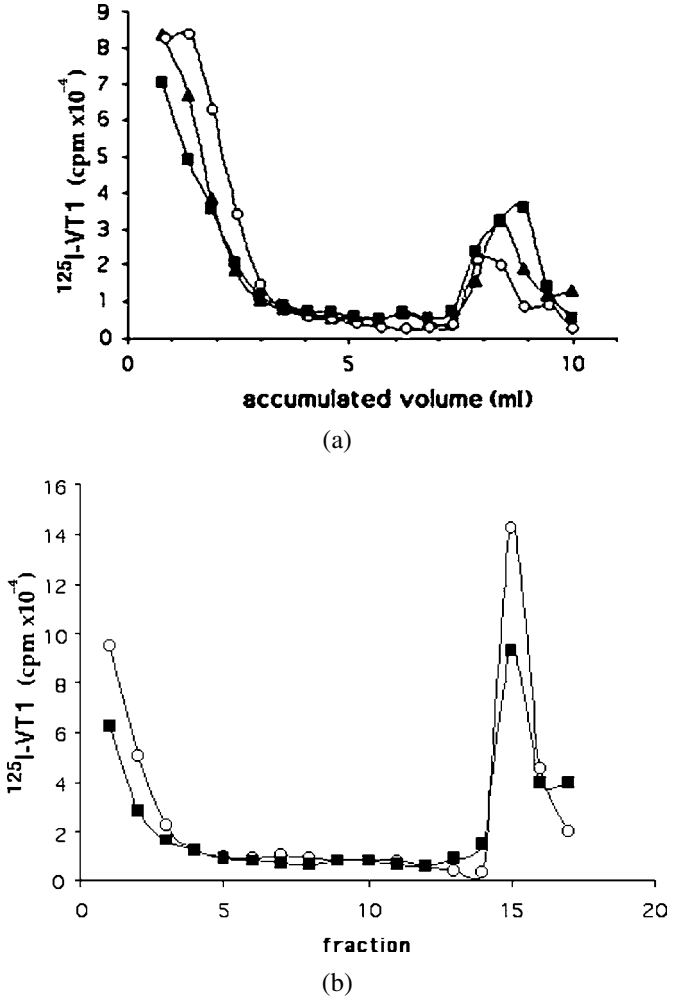


Figure 2. Effect of aminophospholipids on VT1/Gb_3 raft binding. Panel (a) Gb_3/cholesterol 2:1 (▲) was mixed with phosphatidylethanolamine (2:1:1) (■) or phosphatidylserine (2:1:1) (○) and rafts generated tested for ^{125}I-VT1 binding. Panel (b) Gb_3/cholesterol 2:1 (○) was mixed with sphingomyelin (2:1:1) (■) and rafts generated floated through ^{125}I-VT1 by sucrose gradient ultracentrifugation.

mixture was investigated using the ^{125}I-VT1 system (Figure 2(a)). At the ratios studied, neither of these phospholipids promoted ^{125}I-VT1/Gb_3 'raft' binding. Indeed, the inclusion of PS was inhibitory. Similarly, inclusion of sphingomyelin, considered a central sphingolipid component of cellular 'rafts' [26,27], decreased, rather than increased ^{125}I-VT1 raft binding (Figure 2(b)).

Since cellular lipid microdomains contain a variety of glycolipids, the ability of VT1 to bind to these Gb_3 dense lipid structures containing additional glycolipids was initially addressed by mixing Gb_3 with an equal amount of either Gb_4, SGG or SGC prior to cholesterol addition. As visualised by FITC-VT1B binding within the gradient (Figure 3), the addition of either Gb_4 or SGG did not affect VT1B/Gb_3 'raft' binding.

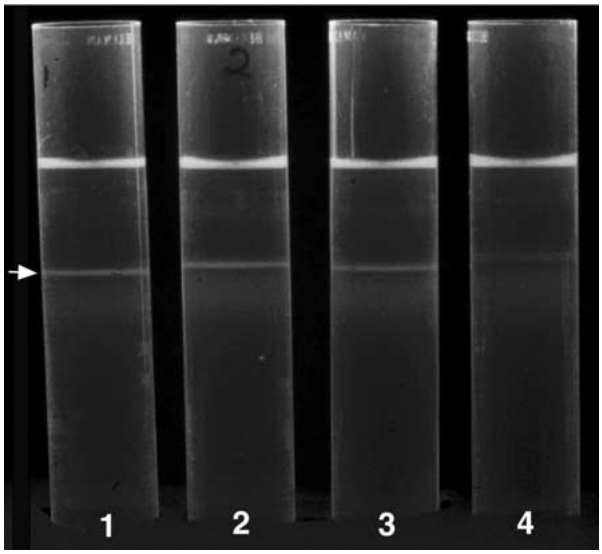


Figure 3. Effect of other glycolipids on VT1/Gb_3 raft binding. 50 μg Gb_3 was mixed with 25 μg cholesterol alone (1) +50 μg Gb_4 (2), +50 μg SGG (3) or +50 μg SGC (4) and rafts generated tested for FITC-VT1 1B binding. The fluorescent 'raft' band is arrowed. The weak band in 4 is not fluorescent.

However inclusion of SGC significantly reduced FITC-VT1B binding.

Discussion

Our results demonstrate the utility of the *in vitro* 'raft' generation and ligand binding system we are developing, to examine the optimal compositional requirements for VT1-binding, Gb_3-containing lipid rafts. The lipid concentrations we have used in this initial study are arbitrary and do not reflect cellular conditions. Nevertheless some new insights into glycolipid receptor function within condensed lipid domains has been obtained. We show VT1 binding is a function of the Gb_3 raft cholesterol content and the inclusion of aminophospholipids is unnecessary, and can be inhibitory. We had previously shown that, while inclusion of sphingomyelin increased the Gb_3 content of the artificial rafts; this did not promote FITC-VT1 B binding [28] and have confirmed this more quantitatively in the current study using ^{125}I-VT1. This suggests that, by some mechanism, sphingomyelin can mask the receptor function of Gb_3. In our earlier work, we showed that sphingomyelin could not substitute for cholesterol in the generation of these artificial rafts. Indeed, in the interaction with HIV gp120, sphingomyelin prevented the cholesterol-mediated enhanced interaction with Gb_3 monolayers [28]. Thus, for both VT1 and gp120, sphingomyelin content in rafts is detrimental to Gb_3 binding.

We have made the novel observation that inclusion of SGC together with Gb_3 inhibits VT1/Gb_3 raft binding. Many cells, particularly those of renal origin, express both SGC and Gb_3. We suggest the term "deceptor" to describe the effect of glycosphingolipid to diminish Gb_3 receptor function. While a similar

effect in cellular rafts has yet to be shown, this could represent an unappreciated mechanism for the modulation of VT1 sensitivity or other glycolipid receptor functions. In this regard, it is of interest to note that while Gb_3-containing rafts are important for B cell receptor signaling [10], incorporation of SGC inhibits B cell Ig production and proliferation [29]. The fact that inclusion of the glycerol-based SGG did not affect VT1/Gb_3 raft binding highlights the importance of the ceramide base in the'deceptor' disturbance of Gb_3 presentation for VT1 binding. The concept that certain glycolipid receptor molecules can inhibit the receptor function of others could have wide implication. SGC can function as a cell surface receptor in its own right [19] and has been reported in lipid rafts [30]. Like Gb_3 [31], the receptor function of SGC varies according to the lipid moiety [32,33], and the lipid-free sugar has little receptor function [34,35]. However, SGC and Gb_3 have distinctive properties (in addition to their charge difference). Though both GSLs are bound by gp120, Gb_3 promotes membrane fusion [36] while SGC prevents it [37].

We have made soluble adamantyl derivatives of both Gb_3 [38,39] and SGC [32,40]. While adamantylGb_3 has a larger molecular area and forms a more rigid monolayer at a water/air interface than Gb_3, adamantylSGC has a significantly smaller area and is less rigid than SGC (Fantini and Lingwood unpublished) and adamantylGb_3 [28]. AdamantylGb_3 is an excellent ligand for gp120 while adamantylSGC is not [28].

We should point out that different glycolipids within rafts do not necessarily interfere with each other. Gb_4 and SGG had no effect on VT1 B binding in the present study and in cells, the binding and internalisation of VT1 and cholera toxin on the same cells are entirely independent [41] indicating the independence of GM1 and Gb_3.

GLS-containing lipid microdomains play a central role in many trans-plasma membrane signaling cascades [13,42,43] and are also essential for the microbial infection of many eukaryotic cells [44–49]. For the most part, such signaling cascades involve cell surface receptors which partition in/out of, or cluster within such rafts on ligand binding [4]. However GSL binding *per se* within such domains can also effect transmembrane signal transduction [11,20,50]. These microdomains are cholesterol-rich assemblies in a more rigidly ordered phase than the bulk bilayer. The regulation of GSL receptor function by the lipid moiety [51] and the phospholipid environment [52] strongly suggest that GSL receptor function is markedly affected within lipid rafts. It is becoming clear that such domains are heterogeneous [14,53] but the nature/composition of these domains is difficult to determine due to the lack of adequate isolation methodology [54]. Their standard isolation by Triton extraction is unlikely to retain the original cellular organization or composition. Indeed, in our experiments the density of the VT1-binding Gb_3 condensed lipid structure varies according to the Triton concentration and type of detergent used (unpublished), indicating that the detergent is a component of the raft thus formed, likely substituting for the membrane lipids missing after extraction.

Gb_3 in the cell membrane can be found within rafts [10,20,55] and this organizational difference is important in determining cell susceptibility to cytotoxicity [5] via differences in intracellular trafficking of the VT1/Gb_3 complex. VT1 binding to cell surface Gb_3 within rafts was internalized and transported retrogradely to the ER. VT1 binding to cells in which Gb_3 was not organized in rafts, resulted in the internalization and trafficking of the complex to endosomes and lysosomes for degradation, suggesting the regulation of intracellular vesicular trafficking can discriminate raft structures.

Gb_3 is also an important component in HIV/host cell fusion [36] which also requires lipid rafts [44,45,47]. The V3 loop of the HIV glycoprotein adhesin, gp120 binds to several glycolipids *in vitro* [56,57]. These include galactosyl ceramide, SGC and GM3 [56,58]. Gb_3 is a relatively poor gp120 receptor. In our studies of insertion into Gb_3 monolayers at an air/water interface, a significant lag phase was seen before gp120 insertion, which then followed sigmoidal kinetics. This lag could be eliminated and binding/insertion became exponential by the inclusion of cholesterol [28]. This is consistent with a requirement for Gb_3 rafts. A V3 loop-derived peptide encompassing the GSL binding domain was found to bind the same Gb_3/cholesterol dense lipid structures as studied in the present report. This peptide competed with VT1 for Gb_3-raft binding [28]. This indicates VT1 and gp120 preferentially bind the same Gb_3 rafts. Analysis of the optimal composition of such rafts is essential to understanding and intervening in these infection processes.

While the current studies fall far short of this goal, this simple assay we have developed has already presented a few unexpected results in the inhibitory effects of sphingomyelin and SGC. The assay should be easily adaptable to other GSL-raft binding ligands, *e.g.* cholera toxin [58], and indeed can be adapted to screen for such species.

Acknowledgments

This work was supported by CIHR grant #MT13073. The authors thank Dr M. Mylvaganam for valuable discussion.

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