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# Spontaneous curvature of ganglioside GM1 – Effect of cross-linking

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ABSTRACT

The membrane-curvature dependent lateral distribution of outer leaflet ganglioside GM1 (GM1) and the influence of GM1 cross-linking induced by fluorophore-tagged cholera toxin subunit B (CTB) plus anti-CTB was analysed in cell membranes by fluorescence microscopy. Data are presented indicating that cross-linked GM1–ligand patches accumulated at the tips of human erythrocyte echinocytic spiculae induced by $Ca^{2+}$/ionophore A23187. However, when lipid fixative osmium tetroxide was added prior to the ligand no accumulation in spiculae occurred. GM1-staining remained here distributed over the spheroid cell body and in spiculae. Similarly, osmium tetroxide completely prohibited CTB plus anti-CTB-induced GM1 patching in representatives for flat membrane, i.e. discoid erythrocytes and K562 cells. Our results demonstrate that GM1 *per se* shows low membrane curvature dependent distribution and therefore holds flexible spontaneous curvature. In contrast, the cross-linked GM1–ligand complex has a strong preference for highly outward curved membrane and possesses overall positive spontaneous curvature. Osmium tetroxide efficiently immobilises GM1.



## 1. Introduction

GM1 is a glycosphingolipid present in the outer leaflet of animal cell plasma membranes and constitutes a major component of cholesterol- and sphingolipid-enriched membrane microdomains (rafts) [1,2]. GM1 is the natural receptor of cholera toxin [3]. Fluorophore-conjugated cholera toxin subunit B (CTB) is commonly used to stain GM1. CTB is a pentamer and may bind five GM1. In human erythrocytes, which lack cytoskeleton and ability to endocytosis, dispersed clusters form on the membrane upon CTB binding to GM1, and these can be further aggregated by anti-CTB to homogenously distributed patches [4]. In cells possessing a cytoskeleton, as K562 cells used in the present study, CTB binding to GM1 results in cross-linking of CTB–GM1 to clusters on the cell surface [5]. Simultaneously, endocytosis of CTB–GM1 complexes occurs [6]. Patching of GM1 also occurs in K562 cells upon CTB plus anti-CTB treatment [5,7].

The present study aimed to clarify the spontaneous curvature of GM1 in biological membranes and how it is affected by cross-linking with CTB plus anti-CTB. Therefore, the lateral mobility and distribution of ligand-bound cross-linked GM1 was monitored by fluorescence microscopy (FLM) in curved membrane as accumulation of GM1-patches in human erythrocyte echinocytic spiculae, and in flat membrane, represented by discoid erythrocyte and K562 cell surface, as patching of GM1. $Ca^{2+}$/ionophore A23187 was used to attain echinocytic erythrocytes. Cross-linking of GM1 was induced by fluorophore-conjugated CTB plus anti-CTB treatment. Fixation with osmium tetroxide laterally immobilised GM1 and revealed the lateral distribution and spontaneous curvature of GM1 prior to the cross-linking.

## 2. Materials and methods

### 2.1. Chemicals

Cholera Toxin subunit B (CTB)-Alexa Fluor$^R$ 594 (C34777) and anti-cholera toxin (antiCTB, V34404) were from Invitrogen (Carlsbad, CA, USA). Ionophore A23187 (C-7522) was obtained from Sigma. Osmium tetroxide (19130) was from Electron Microscopy Sciences.

### 2.2. Isolation of erythrocytes

Blood was drawn from the authors by venipuncture into heparinized tubes. The blood was washed three times with buffer (145 mM NaCl, 5 mM KCl, 4 mM $Na_2HPO_4$, 1 mM $NaH_2PO_4$, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 10 mM glucose, pH 7.4). Erythrocytes were suspended in the buffer at $1.65 \times 10^9$ cells/ml, stored at +4 °C and used within 5 h.

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### 2.3. K562 cells

Erythroleukemia K562 cells were cultured as previously described [8].

### 2.4. Staining

Erythrocytes ($1.65 \times 10^8$ cells/ml, ~1.5% haematocrit) and K562 cells ($2 \times 10^6$ cells/ml in buffer) were incubated with Alexa 594 conjugated CTB (1/250, 60 min, RT). Following three washes, the cell suspension was incubated with anti-CTB (1/50, 60 min, RT) and washed three times. To obtain echinocytic cells the erythrocytes were treated with ionophore A23187 (2 μM, 20 min, 37 °C) plus calcium (final concentration 3 mM). The morphology of treated erythrocytes was followed by transmission light microscopy. The cells were either pre- or postfixed with 5% paraformaldehyde plus 0.01% glutaraldehyde (PFA + GA) (60 min, RT) in buffer, followed where indicated by fixation in 1% osmium tetroxide (10 min, RT) in buffer. Following washing, the cells were settled on polylysine-treated (0.1 mg/ml, 10 min) cover glasses and washed. The cells were mounted on 80% glycerol. The cover slips were sealed with nail polish. Notably, GA efficiently preserves cell morphology and particularly membrane spiculae [4]. However, GA is problematic to use in protocols involving FLM, among other things due to its strong auto fluorescence at high concentrations [4]. In our study 0.01% GA was shown to aid in fixation of spiculae without bringing with too much autofluorescence. Prefixation with 0.01% GA did not affect GM1 staining with CTB.

### 2.5. Microscopy

A large number of cells in several separate experimental samples were studied for GM1 staining using a Leica DM RXA fluorescence microscope (100×/1.4 aperture immersion oil objective, 10× ocular). Images (single-section) were acquired with a Leica DC300F CCD-camera. Images present "flat" surfaces of discoid erythrocytes and mid-plane sections of spheroid K562 cells and echinocytic erythrocytes. Erythrocytes were processed for scanning electron microscopy (SEM, Leo 1530 Gemini) as previously described [9].

## 3. Results

### 3.1. Discoid erythrocytes and K562 cells as representatives for flat membrane

CTB plus anti-CTB treatment cross-linked GM1 to distinct dispersed patches on the cell membrane of discoid human erythrocytes (Fig. 1A) and K562 cells (Fig. 1B). As seen in Fig. 1A the patches formed on the "flat" surfaces of discoid erythrocytes are uniformly distributed over the cell surface. In spheroid K562 cells the patches are seen at the cell rim in mid-plane sections and are indicated with arrows (Fig. 1B). The areas with strong fluorescence in K562 cells, marked with asterisks, are endovesiculated patches (data not shown), and are not further discussed in this study. Cells in the above experiments were post-fixed with PFA + GA.

In cells prefixed with PFA + GA plus osmium tetroxide prior to CTB–anti-CTB treatment, no accumulation of staining, i.e. GM1–CTB–anti-CTB patches, occurred, but the staining remained uniformly distributed over the discoid erythrocyte membrane (Fig. 1C) and in K562 cells (Fig. 1D). Occasionally, minor endovesiculation occurred in K562 cells (Fig. 1D).

Osmium tetroxide did not affect the distribution, size or fluorescence strength of pre-formed GM1–CTB–anti-CTB patches in erythrocytes or K562 cells (insets in Fig. 1A and B, respectively).

### 3.2. Echinocytic erythrocytes as representative for curved membrane

To indicate in detail the morphology of echinocytic spiculae induced in erythrocytes by $Ca^{2+}$/ionophore A23187, scanning electron microscope micrographs are presented in Fig. 3B. Here, spiculae with pro-vesicles at their tips, and spiculae from where vesicles have apparently been already shed, are shown. Morphologically similar echinocytic erythrocytes and spiculae to those presented in Fig. 3B occur in the FLM images in Fig. 2A and B, where the thin spiculae are barely visible in the mid-plane section images. FLM showed that both when GM1–CTB–anti-CTB patches were formed in discoid cells prior to echinocytosis (Fig. 2A), and after echinocytosis (inset in Fig. 2A), they predominantly accumulated at the tips of echinocytic spiculae. However, following prefixation with PFA + GA plus osmium tetroxide, GM1–CTB–anti-CTB complex did not accumulate at the top of echinocytic spiculae, but the staining remained distributed over the cell surface including spiculae (Fig. 2B). Notably, GM1–CTB–anti-CTB complexes did not accumulate in stomatocytic membrane invaginations induced by chlorpromazine (data not shown).

## 4. Discussion

In order to microscopically study the lateral distribution and mobility of a specific membrane component, staining with a marker-conjugated molecule (e.g. ligand/antibody) is an option. However, this binding may either directly or by inducing oligomerisation (clustering) alter the (average) intrinsic molecular shape and thereby the mobility and curvature-dependent lateral distribution (spontaneous curvature) of the membrane component. In this study prefixation with PFA + GA plus osmium tetroxide (1%), was used as a tool to immobilise GM1 prior to staining and cross-linking with fluorophore-conjugated CTB plus anti-CTB.

### 4.1. GM1

Our study shows that GM1, when immobilised by osmium tetroxide prior to staining with CTB plus anti-CTB, mainly resides in the "flat" membrane of the spheroid cell body of echinocytic erythrocytes, but is also found in the saddle-like base and the moderately outward curved membrane of membrane spiculae (Fig. 2B and 3). No accumulation of GM1 was seen at the tips of spiculae. Thus, our microscopic observations could locate GM1 to flat and slightly positive membrane curvature. However, biochemical analysis indirectly show that GM1 also occurs at the tips of echinocytic spiculae induced by $Ca^{2+}$/ionophore A23187, because GM1 was found in shed vesicles [10]. Here, GM1 occurred together with cholesterol in the detergent-resistant membrane fraction (rafts) of shed vesicles. These results pinpoint a crucial property of GM1; the capacity of GM1 to form microdomains with cholesterol. Thus, GM1 can hardly be seen as a separate molecule floating alone in the biological membrane, but rather as a part of a cholesterol-containing microdomain (raft). It can therefore be proposed that GM1 due to its ability to reside in and float with cholesterol-containing microdomains, possesses (indirect) highly flexible spontaneous curvature. In line with our results, studies with model membranes have emphasised the ability of gangliosides for metamorphism, co-operativity and demixing, and suggest different non-negative values for GM1 spontaneous curvature [11]. Ganglioside abundancy and ionic properties of the solution were shown to affect these GM1 properties.

### 4.2. Cross-linked GMI

When GM1 was not immobilised by osmium tetroxide, cross-linking of GM1 with CTB plus anti-CTB resulted in the strong

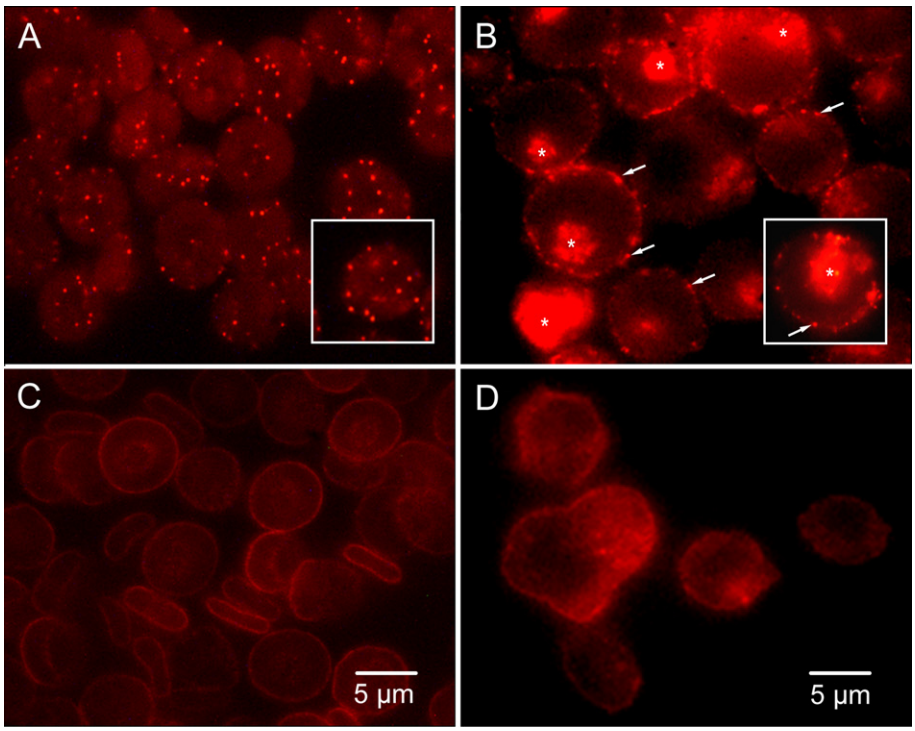


**Fig. 1.** Fluorescence microscopy images showing the lateral membrane distribution of CTB plus anti-CTB stained ganglioside GM1 in flat membrane represented by discoid erythrocytes (A and C) and K562 cells (B and D). (A and B); Cells were stained with CTB plus anti-CTB and fixed with PFA 5% + GA 0.01%. (C and D); Cells were fixed with PFA 5% + GA 0.01% and 1% osmium tetroxide prior to staining with CTB plus anti-CTB. Insets in A and B; cells were stained with CTB plus anti-CTB and fixed with PFA 5% + GA 0.01% and 1% osmium tetroxide. Images in Fig. 1A and inset show GM1-CTB-antiCTB patches distributed over the "flat" surfaces of discoid erythrocytes. Images in Fig. 1B and inset show mid-plane sections of spheroid K562 cells. GM1–CTB–antiCTB patches are seen at the cell rim and are marked with arrows. In K562 cells the areas with strong fluorescence, indicated with asterisks, are patches accumulated following endovesiculation. Scale bars represent 5 μm.

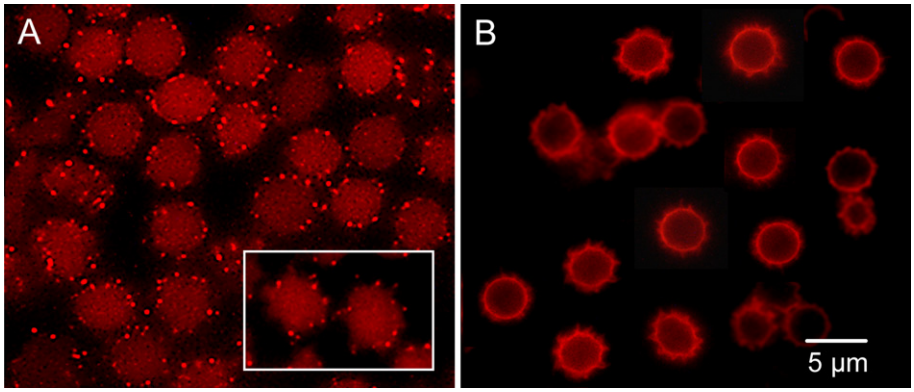


**Fig. 2.** Fluorescence microscopy images showing the lateral membrane distribution of CTB plus anti-CTB stained ganglioside GM1 in curved membrane represented by echinocytic erythrocytes. Echinocytosis was induced by ionophore A23187 (2 μM, 20 min, 37 °C) plus calcium (3 mM). Erythrocytes were stained with CTB plus anti-CTB before (A) and after (A inset) induction of echinocytosis, and fixed with PFA 5% + GA 0.01%. (B); Echinocytic erythrocytes were fixed with PFA 5% + GA 0.01% and 1% osmium tetroxide prior to staining with CTB plus anti-CTB. Images are mid-plane sections of spheroid echinocytic erythrocytes. Scale bar represents 5 μm.

accumulation of the GM1-complexes at the tips of membrane spiculae (Fig. 2A and A inset). This finding is in accordance with previous studies indicating that GM1–CTB–anti-CTB distribute in erythrocyte spiculae [4,12] and in there from released exovesicles (unpublished results). The accumulation of GM1–CTB–anti-CTB complexes at the tips of echinocytic spiculae indicates that the complexes possess high overall positive spontaneous curvature (+/+, Fig. 3) in the human erythrocyte membrane.

It has been reported that cross-linking of GM1 with CTB alone also results in the accumulation of the formed complexes in the tips of spiculae, but this accumulation was less pronounced [13]. This partial accumulation in spiculae of GM1–CTB complexes suggests a more flexible positive spontaneous curvature for these complexes. Other studies have accordingly indicated an altered mobility and membrane curvature dependence of GM1 upon CTB binding. CTB binding was shown to increase the partition of GM1 into the detergent-resistant membrane fraction (rafts) of HL60 cells [14], suggesting that CTB alters the molecular surrounding and local membrane distribution of GM1. Furthermore, the membrane curvature-dependent distribution of GM1 upon cross-linking by CTB in urothelial cells has been related to an attained positive spontaneous curvature [15]. Interestingly, experiments with cho-

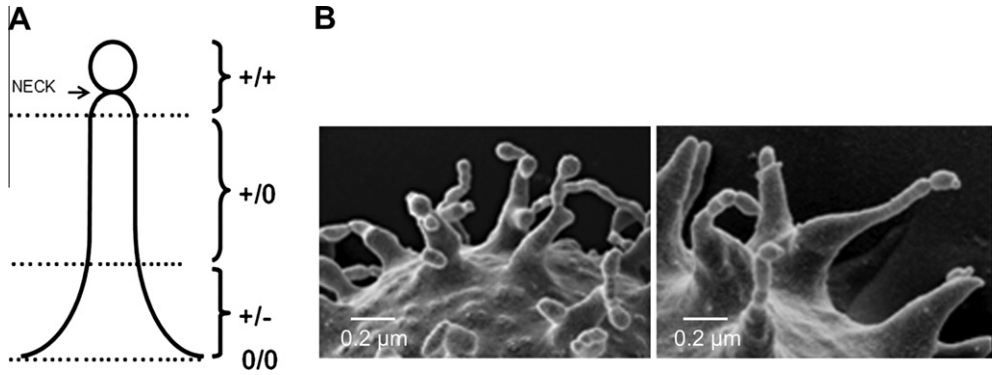


**Fig. 3.** Schematic figure indicating the principal membrane curvatures in two directions (90° to each other) in different parts of a membrane spiculae (protrusion) with a protruding vesicle; top and vesicle +/+, tube +/0, saddle-like base +/–, base 0/0, where + denotes outward (positive) curvature, 0 neutral/zero curvature and – inward (negative) curvature. Furthermore, a tubular membrane neck (+/−) may (transiently) connect the vesicle with the spiculae (A). The scanning electron microscope micrographs indicate the morphology of echinocytic spiculae induced in human erythrocytes by ionophore A23187 (2 μM, 20 min, 37 °C) plus calcium (3 mM) (B). Scale bars represent 0.2 μm.

lesterol-containing giant unilamellar model membrane vesicles of varying phospholipid composition showed that the GM1–CTB complex is sorted away from the positive curvature of pulled tethers into the low curvature of the mother vesicle [16]. Here, increased tether curvature was shown to increase the depletion of GM1–CTB. To summarise, the more pronounced overall positive spontaneous curvature of GM1–CTB–anti-CTB complexes compared to GM1–CTB complexes may be related to the larger size of the former complex. Complex size may in a direct way correlate with an increased effective conical shape [17] what propagates the positive spontaneous curvature of the complex. Noticeably, GM1 complexes appear not to accommodate well in tubular membrane, i.e. structures with positive curvature in one direction only (0/+, Fig. 3).

When accumulating in highly outward curved membrane the GM1–ligand complexes may contribute to the bilayer bending in accordance with the bilayer couple mechanism [18,19], and thereby propagate membrane bending and vesicle shedding. Notably, the composition and intrinsic properties of biological and model membranes may affect GM1–ligand spontaneous curvature, as may also the experimental conditions such as the ionic properties of the experimental solutions.

In conclusion, our study indicates that GM1 as part of cholesterol-containing microdomains in biological membranes possesses flexible spontaneous curvature, while ligand-binding of GM1 with CTB plus anti-CTB results in formation of GM1–ligand complexes with a high overall positive spontaneous curvature. Prefixation with osmium tetroxide efficiently immobilises GM1 prior to CTB-staining.

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