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# Differential endocytic trafficking of neuropathy-associated antibodies to GM1 ganglioside and cholera toxin in epithelial and neural cells

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ABSTRACT

Gangliosides are glycolipids mainly present at the plasma membrane (PM). Antibodies to gangliosides have been associated with a wide range of neuropathy syndromes. Particularly, antibodies to GM1 ganglioside are present in patients with Guillain–Barré syndrome (GBS). We investigated the binding and intracellular fate of antibody to GM1 obtained from rabbits with experimental GBS in comparison with the transport of cholera toxin (CTx), which binds with high affinity to GM1. We demonstrated that antibody to GM1 is rapidly and specifically endocytosed in CHO-K1 cells. After internalization, the antibody transited sorting endosomes to accumulate at the recycling endosome. Endocytosed antibody to GM1 is recycled back to the PM and released into the culture medium. In CHO-K1 cells, antibody to GM1 colocalized with co-endocytosed CTx at early and recycling endosomes, but not in Golgi complex and endoplasmic reticulum, where CTx was also located. Antibody to GM1, in contraposition to CTx, showed a reduced internalization to recycling endosomes in COS-7 cells and neural cell lines SH-SY5Y and Neuro2A. Results from photobleaching studies revealed differences in the lateral mobility of antibody to GM1 in the PM of analyzed cell lines, suggesting a relationship between the efficiency of endocytosis and lateral mobility of GM1 at the PM. Taken together, results indicate that two different ligands of GM1 ganglioside (antibody and CTx) are differentially endocytosed and trafficked, providing the basis to gain further insight into the mechanisms that operate in the intracellular trafficking of glycosphingolipid-binding toxins and pathological effects of neuropathy-associated antibodies.



## 1. Introduction

Gangliosides are acidic complex glycosphingolipids synthesized in the lumen of the Golgi complex. After their synthesis, gangliosides leave the Golgi complex via the lumenal surface of exocytic transport vesicles that move to and fuse with the plasma membrane (PM) [1–4]. At the PM, gangliosides participate in cell surface events such as modulation of growth factor receptors and cell-to-cell and cell-to-matrix interactions [5–14]. It has been also demonstrated that sphingolipids in general and glycosphingolipids in particular are differentially required for clathrin-independent endocytosis [10,15,16]. In addition, the correct balance of glycosphingolipids in endomembranes is essential for the proper function of exocytic and endocytic trafficking [17–20].

Information on intracellular trafficking of endogenously synthesized glycolipids has been lacking owing to the methodological difficulties of studying their transport. Consequently, lipid sorting has been analyzed using fluorescent-labeled lipid analogues, labeled toxins or radioactive-labeled lipids [21]. In this sense, ganglioside GM1 endocytic transport has been studied principally by the use of cholera toxin (CTx) [6,22]. CTx belongs to the family of $AB_5$ toxins and binds up to five molecules of GM1 at a time via its B subunits (CTxB). Ganglioside GM1 has been suggested to be the vehicle that directs the toxin to its final intracellular destination [6,23]. CTx is internalized to endosomes and then sorted to the trans-Golgi network (TGN) and endoplasmic reticulum (ER). If the endocytic pathways of CTx and exogenous added GM1 (fluorescent- or radioactive-labeled GM1) are pathways followed by endogenous GM1 remains to be established.

We recently used an antibody binding technique to study the intracellular fate of internalized ganglioside GD3 [24]. We demonstrated that a mouse monoclonal antibody to GD3 was specifically endocytosed bound to the ganglioside, sorted to early and recycling endosomes and transported back to the PM by clathrin-coated

*Abbreviations:* CHO, Chinese hamster ovary; CTx, cholera toxin; CTxB, cholera toxin B subunit; ER, endoplasmic reticulum; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; FRAP, fluorescence recovery after photobleaching; GalNAc-T, UDP-GalNAc:LacCer/GM3/GD3 N-acetylgalactosaminyltransferase; GBS, Guillain–Barré syndrome; GFP, green fluorescent protein; PBS, phosphate-buffered saline; PM, plasma membrane; RT, room temperature; TGN, trans-Golgi network; Tf, transferrin; YFP, yellow fluorescent protein

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vesicles. Antibodies to gangliosides, in particular to GM1, have been associated with neuropathologic syndromes, like Guillain–Barré syndrome (GBS) [5,25–28]. Neither internalization nor intracellular destination of neuropathy-associated antibodies to gangliosides has so far been extensively explored.

The aim of this study was to investigate cellular binding and endocytic destination of neuropathy-associated antibody to GM1 ganglioside in comparison with CTx. Antibody to GM1 (IgG and IgM classes) was obtained from rabbits with experimental GBS induced by sensitization with GM1 ganglioside [25,26]. Results obtained by biochemical and fluorescent confocal techniques clearly suggest a differential endocytic trafficking of antibody to GM1 ganglioside and CTx in different cell types.

## 2. Materials and methods

### 2.1. Cell culture, plasmids and DNA transfection

The following cells were used: wild-type Chinese hamster ovary (CHO)-K1 cells (ATCC, Manassas, VA); CHO-K1$^{GM1+}$, a double-stable transfectant expressing UDP-GalNAc:LacCer/GM3/GD3 N-acetylgalactosaminyltransferase (GalNAc-T) and UDP-Gal:GA2/GM2/GD2 galactosyltransferase (Gal-T2), which synthesize GM1 and GD1a (clone 2, [29]); neuroblastoma cell lines Neuro2A and SH-SY5Y (ATCC); and COS-7 cells. CHO-K1, COS-7 and Neuro2A cells were grown and maintained at 37 °C in 5% $CO_2$ in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics. SH-SY5Y cells were grown and maintained at 37 °C in 5% $CO_2$ in Dulbecco's modified Eagle's medium-F12 (DMEM/F-12) supplemented with 10% FBS and antibiotics. Expression plasmids for Rab11a-green fluorescent protein (GFP), GFP-Rab5 and GalNAc-T fused to yellow fluorescent protein (YFP) were already described [24]. The major histocompatibility complex class II invariant chain isoform (Iip-33) fused to GFP was gratefully received from H.J.F. Maccioni (CIQUIBIC, National University of Córdoba, Argentina). Expression plasmid for caveolin-1–GFP was generously received from M. Pagac (Institute of Biochemistry, ETH Zurich, Zurich, Switzerland). Transient transfections were carried out with 1 μg of DNA per 35-mm dish using cationic liposomes (Lipofectamine; Invitrogen, Carlbad, CA) essentially according to the manufacturer's instructions and incubated for 24 or 72 h (for caveolin-1–GFP expression) at 37 °C with the transfection reagent and DNA mixture.

### 2.2. Preparation of GM1 affinity column and antibody purification and labeling

For preparation of GM1 affinity column, 1.5 ml methanol-aqueous 0.2 M KCl (1:1) containing 250 nmol GM1 was added to 1 ml octyl-Sepharose CL-4B (Sigma-Aldrich, St. Louis, MO). The mixture was rotated end-over-end at room temperature for 12 h, the solution was removed, and the gel was washed three times with 5 volumes of phosphate-buffered saline (PBS). The remaining steps were performed at 4 °C. Small portions of gel were loaded in calibrated columns and washed with PBS [30]. Antibody to GM1 was obtained from GBS rabbits [25] and purified by affinity chromatography. Briefly, sera were diluted 1:1 with PBS and applied to the GM1-octyl-Sepharose CL-4B column. After washing with PBS, antibody to GM1 (IgG and IgM isotype) was eluted with KSCN 3M, and, immediately, applied to a Sephadex-G25 (Sigma-Aldrich) column for buffer exchange with PBS. Antibody was then concentrated with Centricon-10 (Amicon, MA). For IgG and IgM purification, antibody eluted from Sephadex-G25 column was applied to a protein G column (HITRAP protein G HP, GE Healthcare Bio-Sciences, Piscataway, NJ) essentially according to the manufacturer's instructions. IgM antibody does not bind to protein G, so it passes through the column. IgG antibody binds to the protein G column and is recovered in the elution buffer. After purification, IgG and IgM antibodies were concentrated by Centricon-10. Antibody purity and molecular mass was controlled by SDS-PAGE electrophoresis under reducing and non-reducing conditions and Coomassie-Blue staining and Western blotting. For fluorescent labeling, antibody to GM1 was labeled with Alexa$^{488}$ using the Alexa Fluor® 488 Monoclonal Antibody Labeling Kit (Molecular Probes, Eugene, OR) according to the manufacturer's instructions.

### 2.3. Cell labeling and internalization assays

Cells transiently transfected or not with plasmids indicated above were incubated at 4 °C (on ice) for 10 min to inhibit intracellular transport. Then, cells were incubated for another 45 min at 4 °C with antibody to GM1 in conjunction or not with Alexa$^{555}$–CTxB (Molecular Probes, 0.2 μg/ml), or with CTx (Sigma-Aldrich, 2 μg/ml) in order to label GM1 ganglioside present at the cell surface. Afterwards, cells were washed three times with cold PBS and harvested or fixed or transferred to 37 °C with fresh prewarmed complete DMEM or DMEM/F-12 to allow endocytic transport for different times, and finally harvested by scraping or fixed in 4% paraformaldehyde in PBS for 30 min at room temperature (RT). For transferrin (Tf) internalization, cells were first incubated for 60 min in culture medium without FBS, then incubated at 4 °C in cold culture medium containing 10 μg/ml Alexa$^{647}$–Tf (Molecular Probes) and antibody to GM1 or CTx for 45 min, and then transferred to 37 °C with prewarmed culture medium without FBS, supplemented with 10 μg/ml Alexa$^{647}$–Tf and processed at different times. For lysosome staining, cells were incubated in DMEM without FBS supplemented with 0.2 μM acidotropic probe LysoTracker Red DND-99 (Molecular Probes) for 15 min at 37 °C before fixation. When indicated, antibody to GM1 or CTx remaining at the cell surface was removed by acid stripping with acetate buffer pH 3.6 containing 0.5 M NaCl, at 4 °C for 3 min.

### 2.4. Recycling assay of antibody to GM1

CHO-K1$^{GM1+}$ cells were incubated at 16 °C for 10 min and then with antibody to GM1 (IgG or IgM) for another 60 min at 16 °C to allow endocytosis to early endosomes. Cell surface-bound antibody was then removed by acid stripping at 4 °C, and cells were extensively washed with cold PBS. Then, they were incubated at 37 °C with prewarmed fresh culture medium without FBS in order to restore transport of internalized antibody. Cells and culture medium were recovered at different times. Proteins from the culture medium were precipitated with chloroform/methanol (1:4, v/v) and resuspended in PBS. The presence of antibody (IgG or IgM classes) in cells and culture medium was assessed by Western blotting.

### 2.5. Assay of CTx induced morphological change in CHO-K1$^{GM1+}$ cells

The assay was carried out as previously described [31] with minor changes. Briefly, CHO-K1$^{GM1+}$ cells were harvested and transferred to 35-mm dishes with coverslips. After 3 h, DMEM with 10% FBS was added supplemented or not with CTx (2 μg/ml). Coverslips were fixed at different times with 4% paraformaldehyde at RT during 15 min and examined microscopically to determine the percentage of cells with morphological changes. Untreated or non-responding cells are round while those cells treated with CTx show polymorphic shape changes, as bipolarity and branching [31]. More than 500 cells were counted per each time.

### 2.6. Electrophoresis and Western blotting

Proteins were resolved by electrophoresis in 10% polyacrylamide gels under reducing or non-reducing conditions and then transferred