Histochemical detection of GM1 ganglioside using cholera toxin-B subunit. Evaluation of critical factors optimal for *in situ* detection with special emphasis to acetone pre-extraction

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Abstract

A comparison of histochemical detection of GM1 ganglioside in cryostat sections using cholera toxin B-subunit after fixation with 4% formaldehyde and dry acetone gave tissue-dependent results. In the liver no pre-treatment showed detectable differences related to GM1 reaction products, while studies in the brain showed the superiority of acetone pre-extraction (followed by formaldehyde), which yielded sharper images compared with the diffuse, blurred staining pattern associated with formaldehyde. Therefore, the aim of our study was to define the optimal conditions for the GM1 detection using cholera toxin B-subunit.

Ganglioside extractability with acetone, the ever neglected topic, was tested comparing anhydrous acetone with acetone containing admixture of water. TLC analysis of acetone extractable GM1 ganglioside from liver sections did not exceed 2% of the total GM1 ganglioside content using anhydrous acetone at -20°C, and 4% at room temperature. The loss increased to 30.5% using 9:1 acetone/water. Similarly, photometric analysis of lipid sialic acid, extracted from dried liver homogenates with anhydrous acetone, showed the loss of gangliosides into acetone 3.0±0.3% only. The loss from dried brain homogenate was 9.5±1.1%.

Thus, anhydrous conditions (dry tissue samples and anhydrous acetone) are crucial factors for optimal *in situ* ganglioside detection using acetone pre-treatment. This ensures effective physical fixation, especially in tissues rich in polar lipids (precipitation, prevention of *in situ* diffusion), and removal of cholesterol, which can act as a hydrophobic blocking barrier.

Introduction

Gangliosides are charged glycosphingolipids (GSLs) containing one or more sialic acid residues in their carbohydrate chains. They are highly enriched in the outer leaflet of the plasma membrane and most of their functions are associated with this location. In contrast to glycerolipids, GSLs are more rigid molecules, and therefore protect cells against harmful environmental factors by keeping the outer leaflet of the plasma membrane mechanically stable and chemically resistant.[1-3] Carbohydrate moieties of gangliosides function as surface antigens, receptors for bacteria, bacterial toxins, viruses, and other bioactive molecules. Quantities and types change during development, differentiation, tumorigenesis, cell adhesion, and signal transduction.[4] *In situ* detection of GSLs in tissue sections is important for reliable information on their subcellular distribution. GM1 ganglioside is used as the representative member of the GSL family. The detection of GM1 is based on its strong binding affinity with cholera toxin B-subunit, which is highly sensitive for GM1, thus detecting this predominantly among all other gangliosides.[5-7] It can occur an additional staining due to binding of the other gangliosides, mainly GD1b, but its resolution from GM1 in histochemical detection is not possible. TLC analysis of gangliosides with resorcinol and cholera toxin staining should bring attention to unusual samples with high ratio of other gangliosides to GM1. Comparison of GM1 detection with cholera toxin and with anti-GM1 monoclonal antibody was referred by Kotani *et al.*[8]

GM1 detection is also widely used for detection of glycolipid-enriched membrane micro domains, called rafts. The fluctuations in cell surface glycolipid signaling molecules, at ultramicroscopic level, has been demonstrated in connection with fluorescence-topographic imaging at nano scale optical microscopy.[9]

The use of proper fixation technique is generally considered crucial for immunohistological detection of GSLs.[10] Acetone fixation of cryostat sections is recommended and preferred to formaldehyde, as it provides better accessibility of antibodies or bacterial ligands to GSLs.[10,11] For this reason, acetone fixation has been used in several studies.[8,12,13]

The first report on the effect of water in acetone on extraction of polar lipids was published by an author of our group.[14] In this report, it was demonstrated that the water content of acetone used during the extraction procedure, and even the water content of the treated samples, affects the degree of polar lipid extraction.

Since then, acetone fixation has been used in several studies.[8,12,13] However, these studies did not mention whether water was excluded from the acetone step, as even whether the water content of the studied sample might contribute to significant extraction of highly polar lipids.[15] The aim of the present study was to define the optimal conditions for the detection of GM1 using cholera toxin B-subunit by comparing formaldehyde fixation, acetone pre-extraction or a combination of both (acetone + formaldehyde sequence) in tissues with different polar lipid content; in this study liver and brain were used as representative tissues with significantly different polar lipid content.

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Materials and Methods

Chemicals

Paraformaldehyde, cholera toxin B-subunit biotin-labeled (ChT-B-biotin), streptavidin-peroxidase-polymer, albumin, biotin, and diaminobenzidine (DAB)-tetrahydrochloride tablets were supplied by Sigma (St Louis, MO, USA); avidin was obtained from Fluka (Buchs, Switzerland); cholera toxin B-subunit conjugated with peroxidase (ChT-B-Px) came from List laboratories (USA) and DEAE Sephadex was supplied by GE (Healthcare, UK). All other chemicals were purchased locally from Penta (Czech Republic).

Experimental animals

Female Wistar rats (Anlab, Prague, Czech

Republic) were housed under controlled temperature and a natural light-dark cycle. Liver and brain samples were taken from the Wistar rats and snap frozen.

All aspects of the study met the accepted criteria for the experimental use of laboratory animals and all protocols were approved by the Animal Research Committee of the 1st Faculty of Medicine, Charles University, Prague, Czech Republic.

Preparation of fixatives

Formaldehyde: 4% formaldehyde was freshly prepared by depolymerization of paraformaldehyde.

Dry acetone: 1000 mL of acetone was dried over 100-150 g of anhydrous calcium chloride in a closed glass bottle (with occasional mixing) for several days, then decanted and distilled under elimination of air moisture. The obtained dry acetone was stored in tightly closed bottles.

Quantification of GM1 ganglioside extraction with acetone from liver sections

Twelve 6 μm cryostat sections from normal rat liver were cut and placed on the same microscopic slide. One slide, containing 12 sections, represented one sample for TLC densitometry. The samples (slides) used in each extraction were in triplicates.

The loss of GM1 ganglioside during acetone fixation was studied by comparison of the following extractions:

1) The samples (slides) were extracted directly with chloroform-methanol-water (C-M-W) 10:10:1 v/v/v with shaking on an orbital shaker at room temperature (25°C) for 10 min. These samples were used to measure the total quantity of GM1 in the tissue sections.

2) Other samples were extracted with i) dry acetone at room temperature for 15 min, ii) dry acetone at -20°C for 15 min, and iii) a cold acetone-water 9:1 v/v mixture, at -20°C for 15 min.

The samples from the acetone extraction were re-extracted with C-M-W 10:10:1 v/v/v by shaking at room temperature for 10 min.

All extracts were evaporated under a stream of nitrogen, dissolved in chloroform-methanol 1:1 v/v, and then applied onto HPTLC aluminium sheets of silica gel (Merck; Darmstadt, Germany). Chromatograms from the experiments were developed in a solvent mixture C:M:0.2% aqueous $CaCl_2$ (50:45:11). After drying, the silica gel layer was impregnated with 0.1% polyisobutylmethacrylate in cyclohexane. Non-specific binding was blocked using 1% BSA in PBS. Thereafter, the biotin-labeled cholera toxin B-subunit (diluted 1:300) was bound to the GM1 ganglioside at room temperature for 30 min. After extensive washing, streptavidin-peroxidase-polymer (diluted 1:400) was used for ultra-sensitive binding to biotin and amplification of the peroxidase enzyme signal. After washing, a blue reaction product was formed by reaction of the peroxidase with a solution of 1-chloronaphtol and H_2O_2 in a citrate phosphate buffer (pH=7.2).

Densitometry of the chromatogram in reflectance mode at 580 nm (CAMAG TLC Scanner II, Switzerland) was used for evaluation of the percentage distribution of GM1, in both the acetone and C-M-W extracts.

Quantification of acetone extraction of gangliosides from dried liver and brain homogenates

A very fine-grained homogenates were prepared from 1 g of liver and 0.5 g of brain, dried using a two-day lyophilization and then rapidly transferred into desiccator (to eliminate condensation of air moisture on the cold samples). One group of samples was extracted with C-M-W (4:8:3) repeated extractions (3x) with shaking (extracts A, n=6). The collected extracts represented the total gangliosides in the sample. The second group was extracted with dry acetone at room temperature for 15 min with shaking (extracts B, n=6) and afterwards re-extracted (3x) with C-M-W (4:8:3) using the same extraction technique described for extract A (extracts C, n=6).

Gangliosides from the extracts were isolated with ion exchange chromatography (DEAE Sephadex), base treatment, dialysis and silica gel column chromatography according to the procedure described by Ledeen *et al.*[16] Total gangliosides (total lipid sialic acid) in the extracts were determined using the resorcinol-HCl method.[17]

Histochemical detection of GM1 ganglioside in rat liver sections

Cryostat liver sections 6 μm thick were dried overnight at room temperature, and fixed with freshly prepared 4% formaldehyde at room temperature for 5 min. The parallel sections were fixed in dry acetone at -20°C for 2 or 15 min, then briefly transferred into a desiccator in order to eliminate air moisture condensation on the cold microscopic slides and thereby dilution of the acetone with condensed water. The influence of temperature on GM1 extraction from liver sections was studied by comparing fixation with dry acetone at -20°C for 2 or 15 min and fixation at room temperature for 2 or 15 min. The effect of the water content in the acetone fixative was studied in a further experiment comparing fixation using dry acetone with an acetone-water mixture (9:1 v/v), both at -20°C for 15 min. After acetone fixation, liver sections were dried in desiccator at room temperature and then put into PBS.

Histochemical detection of GM1 ganglioside was performed with ChT-B-biotin, according to the procedure described by Jirkovská *et al.*[18] In brief, the endogenous peroxidase activity was blocked by incubation in PBS, supplemented by 1% H_2O_2 and 0.1% sodium azide. Endogenous biotin was blocked using a blocking kit (DakoCytomation, Denmark). In order to block non-specific binding, sections were treated with 3% BSA in PBS for 15 min. Sections were then incubated with ChT-B-biotin, diluted 1:300 with PBS plus 3% BSA, for 16.5 h at 8°C. After washing with PBS, the sections were incubated with streptavidin-peroxidase-polymer diluted 1:400 with PBS at room temperature for 60 min. Peroxidase activity was visualized using DAB tetrahydrochloride in darkness for 20 min.

In each series two control tests were included. First, ChT-B-biotin was omitted in a negative test; second, fixed sections were extracted with a C:M (1:1 v/v) and C-M-W 1:1:0.3 v/v/v mixtures at room temperature, both for 15 min with shaking before detection of GM1. In order to obtain comparable results, corresponding areas were selected on parallel liver sections and photographed under constant exposure conditions.

Densitometric analysis of GM1 ganglioside in tissue sections

Liver sections obtained from 23 Wistar rats were analyzed. In each animal, the optical density of GM1 staining in formaldehyde-fixed samples represented 100% and the density of GM1 staining after acetone fixation was expressed as a proportion.

The mean optical density of the GM1 ganglioside reaction product in liver parenchyma was measured using an Olympus Cue 2 densitometric program at 70 areas in each section, at a magnification of 500x. Areas of sections containing either large vessels or technical artifacts (disruptions, etc.) were excluded from measurements.

Comparison of histochemical detection of GM1 ganglioside in rat liver and brain sections

Parallel cryostat sections from liver tissues were treated with the following fixatives: 1) 4% formaldehyde; 2) anhydrous acetone. Sections from brain tissue were fixed with: 1) formaldehyde; 2) Baker's solution (formaldehyde containing calcium chloride); 3) acetone followed by formaldehyde (A+F); and 4) acetone followed by Baker's solution (A+B). Acetone followed by formaldehyde gave better-preserved morphology than acetone alone. After acetone fixation, liver and brain sections were dried in a desiccator, put into formaldehyde or Baker's