Trisialosyllactosylceramide (GT3) is a ganglioside of human lung

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Received 1 December 1985

A trisialoganglioside not found before in human tissue was isolated from human adult lung. Structural characterization showed this to be $II^3(NeuAc)_3$-LacCer or GT3. The ganglioside constituted 40% of the trisialogangliosides of human lung or 0.2 nmol ganglioside per g tissue.

Mass spectrometry *(Human lung)* *Ganglioside GT3*

1. INTRODUCTION

In 1979 Avrova et al. [1] demonstrated the presence of a trisialosyl lactosylceramide (GT3) in the central nervous sytem of elasmobranches. The same ganglioside was also isolated from the brain of a teleost (cod) and carefully characterized by Yu and Ando [2]. They suggested that this ganglioside represented the parent compound for a phylogenetically older route of ganglioside biosynthesis.

Yet, ganglioside GT3 was also found to occur in a mammalian tissue, namely hog kidney cortex [3]. The yield was high, 6.8 nmol/g tissue or 13 mol% of total ganglioside sialic acid. Here, we describe the isolation of GT3 from human tissue and confirm its structure by mass spectrometry.

2. EXPERIMENTAL

2.1. *Materials*

Lung tissue was obtained from several subjects aged 40–70 years who had died in accidents. There were no macroscopical signs of lung disease.

Silica gel 60, 70–230 mesh, glass-backed TLC and HPLC plates of silica gel G were obtained from Merck, Darmstadt. Iatrobeads RS 8060, 60 μm, were from Iatron Chemical Co., Tokyo. The anion-exchange resin Spherosil-DEAE-dextran was a gift from Institut Merieux, Lyon, France [4]. All organic solvents and other chemicals were of analytical quality and used without further purification. *Vibrio cholerae* sialidas (EC 3.2.1.18) was from Behringwerke, Marburg-Lahn, FRG. Gangliosides used as standards and references were all isolated in our laboratory.

Abbreviations: gangliosides have been designated according to [16]

2.2. *Isolation of gangliosides*

The lung tissue ($2000 \times g$) was homogenized in 2 vols water in a scissor homogenizer. Then 8 vols methanol followed by 4 vols chloroform were added under continuous stirring [5]. The supernatant was freed from the extracted lung tissue by centrifugation at $2000 \times g$ for 30 min. The lung tissue was re-extracted with 1 vol. water, 2.5 vols methanol and 1.25 vols chloroform to give a final chloroform/methanol/water ratio of 4:8:3 (v/v) [5]. After the tissue residue was removed by centrifugation as before, the combined supernatants were evaporated to dryness and dissolved in 2 l chloroform/methanol (1:2, v/v). Upon standing overnight, a large precipitate appeared, which was removed by centrifugation. The extract was then evaporated and dissolved again in the same solvent

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00145793/86/$3.50

- this procedure was repeated until only negligible precipitate appeared.

The lipid extract was then divided into two equal portions and chromatographed on 2 columns of 200 g silica gel 60, 70–230 mesh. The columns were eluted with 10 vols chloroform, 8 vols chloroform/methanol/water (65:25:4, v/v), 5 vols chloroform/methanol/water (60:35:8, v/v) and 15 vols chloroform/methanol/water (50:40:10, v/v).

The last fraction contained the complex gangliosides [6] which were separated into mono-, di- and trisialogangliosides by anion-exchange chromatography on Spherosil-DEAE-dextran [4].

Individual gangliosides of the trisialoganglioside fraction were isolated by preparative thin-layer chromatography in chloroform/methanol/0.25% aq. KCl (50:40:10, v/v). Two major ganglioside bands of almost equal size were isolated (fig.1). The slow-migrating ganglioside had the same R_F value and composition as authentic GT1b while the fast-migrating ganglioside had an R_F value which did not coincide with any known ganglioside of human origin.

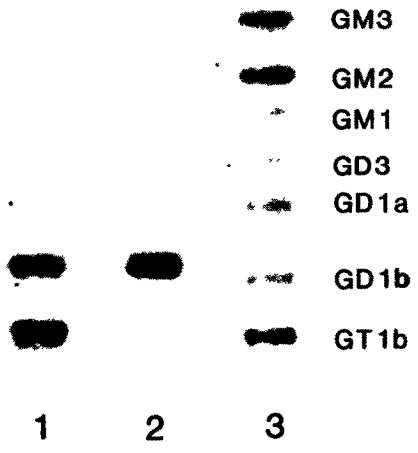

Fig.1. HPLC of trisialogangliosides isolated from human adult lung. Developing solvent: chloroform/methanol/0.25% aq. KCl (50:40:10, v/v). The gangliosides were visualized with the resorcinol reagent. Lanes: 1, total trisialogangliosides; 2, purified ganglioside GT3; 3, reference gangliosides.

2.3. *Structural analyses of the novel trisialoganglioside*

Sialic acid was determined using the resorcinol assay [7] and sphingosine bases with a modified methyl orange method [8]. The carbohydrate composition was quantitatively determined by GLC of the corresponding alditol acetates [9]. Sialidase and acidic hydrolyses were performed as described [10]. The ganglioside was permethylated by a modified procedure of Ciucanu and Kerek [11]. The ganglioside (50 nmol) was dissolved in 100 μl dry dimethyl sulfoxide in a small glass tube with a teflon-lined screw cap, and 4 mg powdered sodium hydroxide and 20 μl methyl iodide was added under nitrogen atmosphere. The capped tube was then sonicated at 25°C for 30 min. The permethylated ganglioside was extracted from the reaction mixture with chloroform after the addition of water, and further purified by repeated partitioning against chloroform. After evaporation, the permethylated ganglioside was dissolved in 100 μl chloroform and applied to 100 mg Iatrobeads packed in a pasteur pipette with a glass-wool plug. The column was eluted with 1.0 ml chloroform and 2.50 ml chloroform/methanol (95:5, v/v). The second fraction contained the permethylated ganglioside and the purity was checked by HPLC in chloroform/methanol (9:1, v/v) as solvent. For checking of the procedure, samples containing 5–100 nmol of ganglioside GM1 radioactively labelled in the ceramide portion were permethyl-

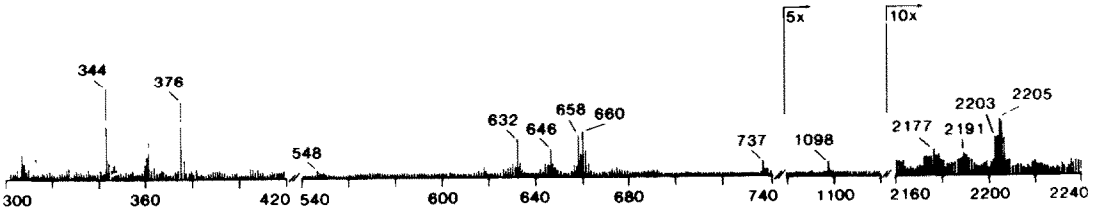

Fig.2. Positive ion FAB-MS of permethylated ganglioside GT3. Significant background peaks from the thioglycerol matrix were subtracted.

ated. The recovery of permethylated ganglioside was approx. 80% when the sample range was between 5 and 15 nmol and 90% between 15 and 100 nmol. Partially methylated alditol acetates from 10 nmol ganglioside were obtained as described [12]. They were analyzed by GLC on a fused silica BP1 capillary column (SGE, Australia) and by GC-MS on a VG 7070 mass spectrometer equipped with a VG 11/250 data system (VG Analytical, Manchester, England). Analyses of partially permethylated sialic acids were performed as described by Rauvala and Kärkkäinen [13].

FAB-MS was performed on a VG ZAB HF (VG Analytical) equipped with a fast atom gun. The permethylated ganglioside was dissolved in chloroform/methanol (1:5, v/v) to give a concentration of 1 nmol/μl. The stainless-steel target was coated with 2 μl of 0.1% sodium acetate in methanol and dried. Afterwards 3 μl thioglycerol and 1 μl of the permethylated ganglioside solution were added to the target [14]. The target was bombarded with xenon atoms having kinetic energies equivalent to ~8 keV. Spectra were recorded in the positive ion mode by downfield mass controlled linear scans of 100–300 s duration. The resolution of the instrument was set to 300 ppm. The spectra were evaluated by manual counting of the spectral lines.

3. RESULTS AND DISCUSSION

The concentration of the unknown trisialoganglioside in the adult human lung was 0.2 nmol ganglioside/g tissue, which is 0.6% of total ganglioside sialic acid or 40% of the trisialogangliosides. The isolated ganglioside contained sphingosine, *N*-acetylneuraminic acid, glucose and galactose in the molar ratio 0.9:2.6:1.0:1.0. Hydrolysis with sialidase or weak acid yielded lactosylceramide. Prolonged acid hydrolysis also produced glucosylceramide. Methylation analysis of the intact ganglioside gave 2,4,6-Me_3-Gal and 2,3,6- Me_3-Glc in approximately equimolar amounts. Analyses of the desialylated substance gave 2,3,4,6-Me_4-Gal instead of 2,3,4-Me_3-Gal. Of the partially methylated sialic acids 4,7,9-Me_3-NeuAcMe_3 and 4,7,8,9-Me_4-NeuAcMe_3 were found in a ratio of 1.0:1.0. Analysis of reference gangliosides GD3 and GD1b at the same time showed a ratio of 0.6:1.0 between the two sialic acids. The low yield of 4,7,9,-Me_3-NeuAcMe_3 might be explained by the destruction of sialic acid during methanolysis [15].

The FAB mass spectra of the permethylated ganglioside showed ions at *m/z* 376, 344 (376 – 32), 737 and 1098, which indicates the presence of 3 sialic acids linked together (fig.2). The ceramide residue was represented by ions at *m/z* 660, 658, 646 and 632. Thus the ceramide consisted mainly of C22–C24 fatty acids in combination with 4-sphingenine. A weak pseudomolecular ion at *m/z* 2205 (M + Na) represented NeuAc$_3$Hex$_2$ Cer$_{24:0}$ + Na, but ions at *m/z* 2203, 2191 and 2177 were also found, corresponding to ceramide portions containing C24:1, C23:0 and C22:0 fatty acids.

The structural analyses of the isolated trisialoganglioside of human lung showed it to be II^{3}NeuAc$_3$LacCer or GT3:

NeuAcα2–8NeuAcα2–8NeuAcα2–3Galβ1–4Glc - β1–1 ceramide.

ACKNOWLEDGEMENTS

This study was supported by a grant from the Swedish Medical Research Council (project no. 03X-627). We thank Dr Jasna Peter-Katalinic for assistance with the mass spectrometric determinations.

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