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Structure of the main ganglioside from the brain of *Xenopus laevis*

Angela Maria Rizzo[1], Bruno Berra[1], Federica Rossi[1], Anna Guerra[1], Rosalba Gornati[2], Giovanni Bernardini[2], Takao Taki[3], Takeshi Kasama[4], Laura Mauri[5] and Sandro Sonnino[5]*

[1] *Institute of General Physiology and Biochemistry, 20134 Milan, Italy,* [2] *Department of Structural and Functional Biology, 21100 Varese, University of Insubria, Italy,* [3] *Molecular Medical Science Institute, Otsuka Pharmaceutical Co. Ltd., Tokushima, Japan,* [4] *Instrumental Analysis Research Center for Life Science, Tokyo Medical and Dental University Tokyo, Japan,* [5] *Center of Excellence on Neurogenerative Diseases, Study Center for the Biochemistry and Biotechnology of Glycolipids, Department of Medical Chemistry, Biochemistry and Biotechnology, 20090 Segrate, University of Milan, Italy*

The main component of the ganglioside[1] mixture from the brain of the adult amphibian *Xenopus laevis* accounts for 35% of the total, as lipid bound sialic acid. This ganglioside has been purified and characterized by thin layer chromatography, partial and exhaustive enzymatic hydrolysis with sialidase, TLC-overlay procedures with anti-Gg_4Cer and anti-Neu5Acα6GalNAcβ specific monoclonal antibodies and mass spectrometry. All together the results suggest the following structure:

Neu5Acα8Neu5Acα3Galβ3(Neu5Acα8Neu5Acα6)GalNAcβ4Galβ4Glcβ1Cer,
or, IV^3-α-Neu5Ac$_2$,III^6-α-Neu5Ac$_2$-Gg_4Cer.

Keywords: **gangliosides, structure, *Xenopus laevis*, amphibia**

Introduction

Xenopus laevis is an animal model representing a powerful and flexible tool in developmental biology researches. *Xenopus* embryos are easily produced in large number by *in vitro* fertilisation, can be kept in plastic Petri dishes, develop at room temperature in fresh water and do not need to be fed during the first days of development. On the other side, a few day old *Xenopus* embryo already has well differentiated and functional organs.

Scant data are available on the lipid biochemistry of the amphibian *Xenopus laevis* even though it is one of the vertebrate species most widely used for research in the fields of molecular, cellular and developmental biology. In previous papers the pattern of neutral glycolipids and gangliosides were studied both in embryonal (during the first six days of development) and in adult tissues of *Xenopus laevis*. The changes of glycolipid expression that rapidly occur during *Xenopus laevis* early development are thought to regulate developmental processes as morphogenetic cell interactions [1]. During this phase a marked increase in ganglioside content was observed, even though the ganglioside pattern remained unchanged [2]. Of the two main gangliosides of *Xenopus laevis* embryos one showed the same chromatographic behaviour of GD3, while the other was highly hydrophilic and with a very low TLC retention time (lower than GQ1b) [3]. A ganglioside with a behavior similar to this latter compound was also one of the main components of the total ganglioside mixture from adult frog brain [3].

Gangliosides are characterized by a very large number of structures differing in both the oligosaccharide and ceramide moieties [4]. Nevertheless, gangliosides of the nervous system mainly belong to the gangliotetrahexosyl series Galβ3-GalNAcβ4Galβ4Glcβ1Cer, differing each other for the number of the sialic acid units and for the chetosidic linkage structures. Very often the sialic acid residues or the sialyl chains are linked to the position 3 of galactose units, but some structures have a sialic acid or a disialyl chain linked also to position 6 of *N*-acetylgalactosamine [4–7], such as the cholinergic neuron gangliosides IV^3-α-Neu5Ac,III^3-α-Neu5Ac,II^2-α-Neu5Ac-Gg_4Cer and IV^3-α-Neu5Ac,III^3-α-Neu5Ac,II^2-α-Neu5Ac$_2$-Gg_4Cer. Gangliosides with sialic acid

*To whom correspondence should be addressed: Sandro Sonnino, Dipartimento di Chimica, Biochimica e Biotecnologie per la Medicina—LITA-Segrate, Via Fratelli Cervi 93, 20090 Segrate (Milano), Italy. Fax: +390250330360; E-mail: Sandro.Sonnino@unimi.it

linked to *N*-acetylgalactosamine and to the external galactose have been suggested to be components of the brain of *Rana catesbeiana* [8,9], an amphibian with characteristics similar to *Xenopus laevis*.

In this study, we show that the main ganglioside from *Xenopus laevis* brain is a tetrasialoganglioside of the ganglio-tetrahexosyl series having two disialosyl chains linked to the external galactose and to *N*-acetylgalactosamine.

Materials and methods

All reagents were of analytical grade, and solvents were redistilled before use. *Vibrio cholerae* sialidase, *N*-acetylneuraminic and *N*-glycolylneuraminic acid were from Sigma; anti-gangliotetrahexosylceramide (Gg_4Cer) monoclonal antibody AG-1, from Seikagaku. KA17 monoclonal antibody recognizing the Neu5Acα6GalNAcβ-disaccharide was from Dr. Yoshio Hirabayashi, RIKEN (Japan) [10]. Standard gangliosides and glycolipids are prepared and are available in the Authors' laboratories. Silica gel plates (Kieselgel 60, HPTLC 10 × 10 cm), LiChroprep RP-18 (40–63 μm) and silica gel 100 (0.063–0.2 mm, 70–230 mesh, ASTM), were purchased from Merck. ECL was from Pierce. *Xenopus laevis* were purchased from a local dealer (Rettili, Varese, Italy); before utilization the animals were housed in aquaria and maintained in controlled condition feeding them once a week with *Xenopus* adult food purchased from Carolina Biological Supply Company [11].

Adult *Xenopus* were anaesthetised by hypothermia and killed. Brain were dissected out and frozen until analysed.

Lipid extraction

Lipids were extracted from *Xenopus laevis* brains by the tetrahydrofuran-phosphate buffer procedure [12]. After partitioning with diethylether and water, the total ganglioside mixture was fractionated by preparative RP18 column chromatography (25 × 2.5 cm) equilibrated and eluted with CH_3CN/5 mM, pH 7, phosphate buffer, 45:55 by vol, at a flow rate of 10 ml/min. Fractions containing the unknown ganglioside were collected and subjected to silica gel 100 column chromatography using the solvent system chloroform-methanol-water, 60:35:8 by vol. After drying, the homogeneous ganglioside was solubilised in a small volume of water, dialyzed and precipitated with acetone.

Analytical procedures

The purified ganglioside solubilised in water, 10 μg/mL, was treated with *Vibrio cholerae* sialidase, 10^{-4} U/μg ganglioside. The reaction was maintained under stirring for up 12 h at 37°C. Aliquots of the the reaction mixture withdrawed after 1, 2 and 3 hours, and, the reaction mixture at the end of incubation were dialyzed against 2 ml of water, lyophilised and the residues, after solubilisation in chloroform/methanol 2:1 by vol, were analysed for lipid composition. The dialysis water was lyophilised and the residue solubilised in a few μL of propan-1-ol/water, 7:3 by vol, was analysed for sialic acid content. Gangliosides and the reaction mixtures obtained by *Vibrio colerae* sialidase treatments were separated by HPTLC using the solvent system chloroform/methanol/0.2 M $CaCl_2$, 50:42:11 by vol and chloroform/methanol/water, 110:40:6 by vol. Gangliosides and sialic acid were visualised with Ehrlich's reagent [13] (the plate is gently sprayed with the reagent, 20 ml of 37% HCl added to 0.6 g of *p*-dimethylaminobenzaldehyde solubilised in 80 ml ethanol, then covered with a pre-wormed glass and heated at 120°C for 10 min); neutral glycosphingolipids were visualised with the anisaldehyde reagent [14] (the plate is sprayed up to be deeply wet with the reagent, 1 mL of 98% H_2SO_4 added to 0.5 ml of anisaldehyde solubilised in 50 mL of glacial acetic acid, then heated at 130°C for 15 min). Immunodetection of the enzyme products was carried out with the anti-tetrahexosylceramide AG-1 monoclonal antibody and with the anti-GD1α KA17 monoclonal antibody, as previously described [15].

For MS analyses a few μg of the ganglioside were purified by TLC, using the solvent system chloroform/methanol/0.2 M $CaCl_2$, 55:45:9 by vol, followed by far-eastern blot [16,17]. After blotting to PVDF, the membrane was subjected to SIMS. The marked ganglioside area was excised and trimmed to form a circle 2 mm in diameter that fit the probe tip of the MS equipment. One microliter of triethanolamine was placed on the PVDF membrane. Negative SIMS spectrum was obtained with a Finnigan TSQ70 mass spectrometer. The ganglioside on the membrane was bombarded with a Cs^+ beam at 20 kV. The ion multiplier was 1.5 kV and the conversion diode was 20 kV.

Gangliosides were quantified by the resorcinol-HCl method [18] using sialic acid (Neu5Ac) as the reference standard.

Results and discussion

Figure 1 shows the HPTLC separation of the total ganglioside mixture extracted and purified from adult *Xenopus laevis* brains. A main ganglioside with high hydrophilic feature showed a chromatographic behaviour characteristic of a polysialoganglioside. Its TLC_{Rf} in the solvent system chloroform/methanol/0.2 M $CaCl_2$, 50:42:11 by vol, was slightly lower than that of standard GQ1b. The total ganglioside content in the brain of adult *Xenopus laevis* was 410 μg Neu5Ac/g fresh tissue. On the basis of this value, after TLC separation and densitometric quantification, the amount of the unknown ganglioside was about 130 μg Neu5Ac/g fresh tissue. Nevertheless, using 5.2 g of fresh brains, only 100 μg of ganglioside, as Neu5Ac, could be obtained in pure form after several chromatographic steps, the largest part of it being absorbed, and lost, on the gel column chromatography. Moreover, the ganglioside was quite unstable and very rapidly formed a series of less polar compounds. Thus, a repurification was necessary within time.

Exhaustive treatment of the unknown ganglioside with *Vibrio cholerae* sialidase yielded a compound weakly stained with the Ehrlich's reagent, but strongly reactive to the anisaldehyde reagent after TLC separation. This compound showed the same TLC_{Rf} of Gg_4Cer in two chromatographic solvent

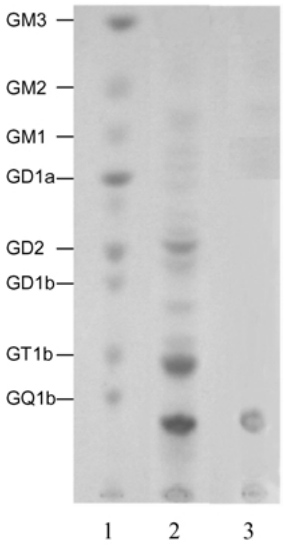

Figure 1. HPTLC separation of gangliosides extracted form *Xenopus laevis* adult brain. Lane 1, standard gangliosides (from top to bottom, are GM3, GM2, GM1, GD1a, GD2, GD1b, GT1b, GQ1b); lane 2, total gangliosides; lane 3, purified ganglioside. Colorimetric detection with Ehrlich's reagent.

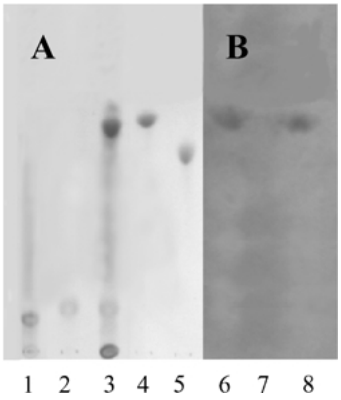

Figure 2. HPTLC analysis of purified ganglioside after 12 hour treatment with *Vibrio cholerae* sialidase. Lane 1, purified ganglioside; lane 2, standard *N*-acetylneuraminic acid; lanes 3 and 6, lipid mixture after sialidase treatment of purified ganglioside; lanes 4 and 8, standard $GgOse_4Cer$; lanes 5 and 7, standard GM1. Panel A, colorimetric detection with anisaldehyde reagent; panel B, immunodetection with anti-$GgOse_4Cer$ monoclonal antibody and visualization with ECL. Solvent system chloroform-methanol-water, 110:40:6 by vol.

systems and was recognized by the monoclonal antibody AG-1, specific for the Gg_4Cer structure. Figure 2 shows the HPTLC separation in the solvent system chloroform/methanol/water, 110:40:6 by vol, of the reaction mixture obtained by exhaustive treatment with sialidase. The colorimetric and immunological stainings are shown in panels A and B of Figure 2, respectively. These results suggest that the neutral oligosaccharide core of this ganglioside is Galβ3GalNAcβ4Galβ4Glcβ-, and that no sialic acid residue is linked to the inner Gal. In fact it is well

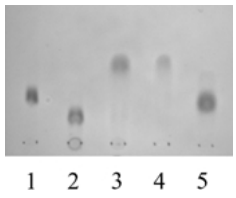

Figure 3. HPTLC analysis of the sialic acid released from the purified ganglioside by sialidase treatment. Lane 1, standard GQ1b, lane 2 purified ganglioside, lane 3 sialic acid composition of the dialysis water obtained from the total sialidase reaction mixture; lane 4, standard *N*-acetylneuraminic acid; lane 5 standard *N*-glycolylneuraminic Solvent system chloroform-methanol-0.2 M $CaCl_2$ 50:42:11 by vol; visualized with Ehrlich's reagent.

known that *Vibrio cholerae* sialidase is not able to hydrolyse the Neu5Acα3Galβ linkage when a GalNAc unit is linked to position 4 of the Gal unit [19].

After sialidase treatment the reaction mixture was dialysed against a small volume of water and the dialysis water analysed for the sialic acid content. Figure 3 shows that the sialic acid released from the purified ganglioside by sialidase had the same TLC_{Rf} of *N*-acetylneuraminic acid. No *N*-glycolylneuraminic acid could be detected. This suggests that all the sialic acid residues have the *N*-acetylneuraminic acid structure.

Figure 4 shows the TLC separation of the reaction mixture obtained by partial hydrolysis with *Vibrio cholerae* sialidase. Within the enzyme mixture a formed compound, coded as A in Figure 4, had the TLC behaviour of standard GD1α, IV^3-α-Neu5Ac,III^3-α-Neu5Ac-Gg_4Cer, and was stained by the monoclonal antibody KA17 that has been described to be very specific for the Neu5Acα6GalNAcβ- epitope [10]. Another compound that we did not characterized, coded as B in Figure 4, with chromatographic behavior intermediate between GD1α and the

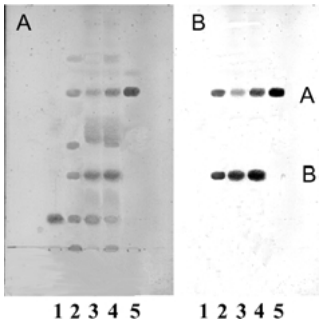

Figure 4. HPTLC analysis of purified ganglioside after partial hydrolysis with *Vibrio cholerae* sialidase. Lane 1, purified ganglioside; lanes 2, 3 and 4, purified ganglioside after partial hydrolysis, 1, 2 and 3 hour treatment, with *Vibrio cholerae* sialidase; lane 3, ganglioside mixture extracted from *Xenopus laevis* brains; lane 4, ganglioside mixture extracted from *Xenopus laevis* brains after partial hydrolysis with *Vibrio cholerae* sialidase; lane 5, standard GD1α. Panel A, colorimetric detection with anisaldehyde reagent; panel B, immunodetection with anti-GD1α monoclonal antibody and visualization with ECL.

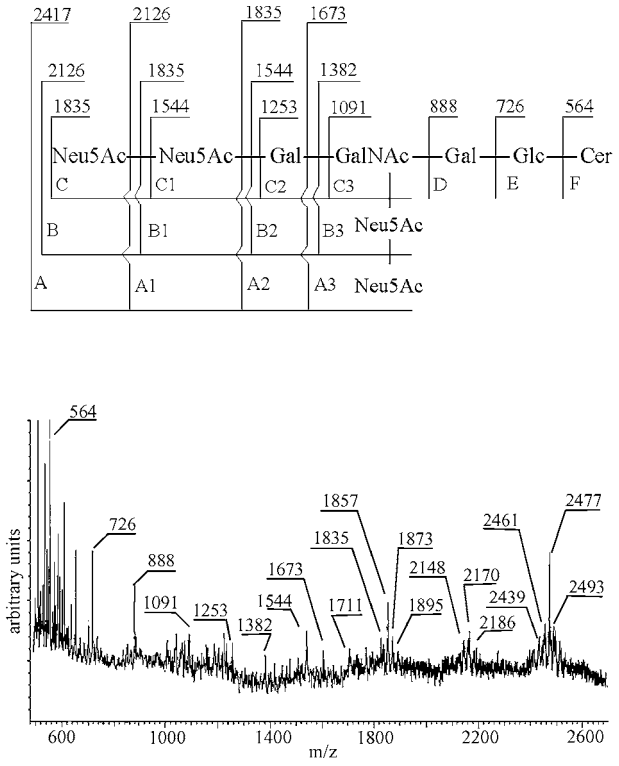

Figure 5. MS spectrum of purified ganglioside together with the proposed fragmentation pattern. The ganglioside purified from the total ganglioside mixture was separated from degradation products, formed during storage, by TLC followlwed by blotting to PVDF, before MS analysis. One microliter of triethanolamine as SIMS matrix was placed on the PVDF membrane. The negative spectrum was obtained by bombarding the ganglioside on the membrane with a Cs^+ beam at 20 kV. The ion multiplier was 1.5 kV and the conversion diode was 20 kV. In the spectrum, the main ions are associated to their *m*/*z* value. According to the proposed scheme, in the spectrum we recognised the following *m*/*z* ions: 564, [F]; 726, [E]; 888, [D]; 1091, [C3]; 1253, [C2]; 1382, [B3]; 1544, [C1 or/and B2]; 1673, [A3]; 1711, [A3 + K]; 1835, [A2 or/and B1]; 1857, [A2 + Na, B1 + Na or/and C + Na]; 1873, [A2 + K, B1 + K or/and C + K]; 1895, [A2 + Na + K, B1 + Na + K or/and C + Na + K]; 2148, [A1 + Na or/and B + Na]; 2170, [A1 + 2Na or/and B + 2Na]; 2186, [A1 + Na + K or/and B + Na + K]; 2439, [A + Na]; 2461, [A + 2Na]; 2477, [A + Na + K]; 2493, [A + 2K].

purified ganglioside was also stained by the monoclonal antibody KA17. Thus, the purified ganglioside should be a polysialoganglioside of the gangliotetrahexosyl series, that contains the structure of the disialoganglioside GD1α. The final structure was definitively established by MS analysis.

Figure 5 shows the MS ganglioside spectrum and the scheme with the proposed fragmentation. A series of ions between *m*/*z* 2439 and 2493, suggests a gangliotetrahexosylceramide containing four sialic acid units. The fragments ions at *m*/*z* 564, 726, 888, 1091 and 1253 corresponding to Cer, Glc-Cer, Gal-Glc-Cer, GalNAc-Gal-Glc-Cer, and Gal-GalNAc-Gal-Glc-Cer sequences, respectively. The ions at *m*/*z* 1382 and 1673, together with those corresponding to their salts, are representative of Neu5Ac- and Neu5Ac-Neu5Ac- containing GalNAc-Gal-Glc-Cer structures. Nevertheless, a disialy chain can be solely linked to GalNAc. In fact, in addition to the fact that by exhaustive sialidase we showed that no sialic acids are linked to the inner Gal, no *m*/*z* ions corresponding to sialyl- and disialyllacosylceramide structures were found in the spectrum. Thus, the mass spectrum suggests that a disialosyl-chain is linked to the GalNAc unit and the other is linked to the external Gal.

All together our data are suggesting that the main ganglioside of the total lipid mixture extracted from the brains of amphibian *Xenopus laevis* is the tetrasialoganglioside Neu5Acα8Neu5Acα3Galβ3(Neu5Acα8Neu5Acα6)GalNAc-β4Galβ4Glcβ1Cer.

Conclusions

Ganglioseries gangliosides that contain an Neu5Acα6GalNAcβ linkage have been described to be component of plasma membrane of both neural and non-neural tissues [5–7]. They are usually minor components of the total ganglioside mixture from nervous system. Two of them are specifically associated to cholinergic neurons and have one residue of sialic acid linked to the external Gal, one to the GalNAc and one, or a disialyl chain, linked to inner Gal [5,6]. Gangliosides with residues of sialic acid linked to the external Gal and to GalNAc have been suggested to be components of the ganglioside mixture from the amphibian *Rana catesbeiana* [8,9]. In this paper we show that the main ganglioside of *Xenopus laevis* brain is a tetrasialoganglioside belonging to the gangliotetrahexosyl series, having a disialosyl chain linked to the external Gal and the other one linked to the GalNAc unit. In particular, the structure of the ganglioside neutral oligosaccharide chain was established by HPTLC immuno blotting with a specific antibody after removal of the sialic acid residues by *Vibrio cholerae* sialidase treatment, while the position of the sialic acids was determined by MS analysis.

The main characteristic of this ganglioside was to be completely desialylated by exhaustive sialidase treatment with *Vibrio cholerae* sialidase. This suggests that the conformation of the Neu5Acα6GalNAc linkage should be very dynamic and not allowing any interaction with the neighbouring saccharides. In fact, it is known that when the sialic acid is linked to Gal, the trisaccharide GalNAc-(Neu5Ac-)Gal forms a very rigid unit that does not allow to sialidase to interact with and to hydrolyse the ketosidic Neu5Acα3Galβ linkage [20,21]. Unlikely, this -Neu5Acα6GalNAcβ- containing tetrasialoganglioside was unstable. The yield of purification was very low, and in solution, in short time, it gave hydrolytic by-products. This did not allow any experiment of high resolution proton NMR spectroscopy [22] necessary to have information on this structural property.

Acknowledgments

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Note

1. Ganglioside nomenclature is according to the IUPAC-IUB [23].

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