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GANGLIOSIDES AND SULPHATIDE IN HUMAN CEREBROSPINAL FLUID· QUANTITATION WITH IMMUNOAFFINITY TECHNIQUES

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SUMMARY

A sensitive micromethod involving extraction, purification and thin-layer chromatography (TLC)–enzyme immunostaining was developed for the quantation of gangliosides and sulphatide, as markers for neuronal disorders and myelin disturbances, respectively, in individual samples of less than 5 ml of cerebrospinal fluid The gangliosides of the gangliotetraose series were individually determined with cholera toxin subunit B by TLC–enzyme-linked immunosorbent assay (ELISA) after chromatography and subsequent sialidase hydrolysis to II^{3}NeuAc-GgOse$_4$Cer (GM1) Other gangliosides and sulphatide were determined with specific monoclonal antibodies by TLC–ELISA The total ganglioside content varied between 100 and 230 nmol/l in ten normal cerebrospinal fluid samples from adults The major gangliosides were of the gangliotetraose series, represented by GM1, IV^{3}NeuAc,II^{3}NeuAc-GgOse$_4$Cer, (GD1a), II^3(NeuAc)$_2$-GgOse$_4$Cer (GD1b) and IV^{3}NeuAc,II^3(NeuAc)$_2$-GgOse$_4$Cer (GT1b), of which the b-series gangliosides dominated, i e , GD1b and GT1b

INTRODUCTION[a]

Glycosphingolipids are important constituents of all mammalian cell membranes, but are particularly abundant in brain tissue The composition of the glycosphingolipids is cell type-specific and undergoes marked changes during development and transformation [1].

[a]Ganglioside abbreviations follow the nomenclature system of L Svennerholm, Eur J Biochem , 79 (1977) 11–21 GA1, GgOse$_4$Cer, GA2, GgOse$_3$Cer, GM3, II^{3}NeuAc-LacCer, GD2, II^3(NeuAc)$_2$GgOse$_3$Cer, GM1, II^{3}NeuAc-GgOse$_4$Cer, GD1a, IV^{3}NeuAc,II^{3}NeuAc-GgOse$_4$Cer, GD1b, II^3(NeuAc)$_2$-GgOse$_4$Cer, GT1b, IV^{3}NeuAc,II^3(NeuAc)$_2$-GgOse$_4$Cer, 3′-LM1, IV^{3}NeuAc-nLcOse$_4$Cer, 3′-isoLM1, IV^3-LcOse$_4$Cer

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The glycosphingolipids are concentrated in the outer plasma membrane and they are released to a certain extent to the intercellular space in the course of normal surface turnover. This process has been termed "surface shedding" [2] and proceeds continuously whether cells are resting or activated, normal or neoplastic. Increased release of plasma membrane glycosphingolipids to the intercellular space might occur during degenerative processes and cell death. The intercellular space of the central nervous system (CNS) is in direct contact with the cerebrospinal fluid (CSF), which will then reflect the shedding of glycosphingolipids from the cells under normal and pathological conditions Determinations of glycosphingolipids in CSF might be a sensitive clinical method for the diagnosis of various degenerative disorders and tumours, particularly the extent and the activity of an ongoing process. We chose to determine gangliosides as a markers for neuronal disorders and CNS tumours and sulphatide as a marker for demyelination.

The ganglioside composition of normal human CSF has been determined by thin-layer chromatography (TLC) [3,4] and the gas chromatographic (GC) determination of sialic acid liberated after acidic hydrolysis [5]. These analyses require large volumes of CSF (25–100 ml), and the methodologies used do not allow the determination of gangliosides in individual CSF samples. Owing to the low content of gangliosides in normal human CSF, the sensitivity of immunological assays, which have been applied in some recent studies, is required In one, a radioimmunoassay was used to measure GM1 ganglioside with cholera toxin subunit B (CT-B) [6]. In another set of studies, gangliosides of the ganglioseries were separated by TLC and hydrolysed on the plates with *Arthrobacter sialidase* to GA1 and GA2, which were determined with specific antibodies [7,8].

We have developed a complete procedure, including extraction, purification and TLC–enzyme immunostaining, for the determination of major gangliosides in individual samples of less than 5 ml of CSF. The gangliosides of the gangliotetraose series, GM1, GD1a, GD1b and GT1b, were individually assayed by TLC–enzyme-linked immunosorbent assay (ELISA) after chromatography and sialidase hydrolysis to GM1. CT-B and a specific monoclonal anti-CT-B antibody were used to determine the GM1 formed. The glycosphingolipids, GD2, 3′-isoLM1, 3′-LM1 and sulphatide, were determined with specific monoclonal antibodies by TLC–ELISA. This paper describes the method and presents the results obtained for the concentrations of individual gangliosides and sulphatide in normal CSF from ten individuals.

EXPERIMENTAL

Chemicals

Silica gel (230–400 mesh) was obtained from Merck (Darmstadt, F.R.G.). Polygrams Sil G precoated plastic sheets (10×20 cm) were supplied by Mach-

erey & Nagel (Duren, F.R.G.). 5-Bromo-4-chloro-3-indolyl phosphate (BCIP) was obtained from Sigma (St Louis, MO, U.S.A.). *Vibrio cholera* sialidase (EC 3.2.18) was purchased from Behringwerke (Marburg-Lahn, F R.G.). CT-B was a gift from the Institute Merieux (Lyon, France). The gangliosides GM1 and GT1b were labelled in the sphingosine base using the sodium borohydride procedure [9] Gangliosides and sulphatide used as references and standards were isolated and characterized in our department.

Antibodies

The monoclonal antibodies (Mab) used against CT-B (CTOB+5), GD2 (SGD2), 3′-isoLM1 (SL50) and 3′-LM1 (TR3) and sulphatide (Sulph-1) were produced in our department (Table I). Affinity-purified alkaline phosphatase-conjugated goat anti-mouse IgG and IgM (H+L) were obtained from Jackson Immunoresearch Labs. (West Grave, PA, U S.A.).

CSF samples

The investigation was performed on CSF samples from ten patients referred to our laboratory for protein determinations for the exclusion of an infectious disorder The inclusion criteria were <300 mg/l albumin and absence of immunoglobulin elevation and M components The mean age of the patients was $50\ 4 \pm 13.7$ years.

Extraction of gangliosides

CSF (5 ml) was centrifuged, lyophilized and extracted twice with 5 ml of chloroform–methanol–water (60 30 4.5, v/v) The supernatants were pooled and applied to a column of 1 g of silica gel packed in chloroform–methanol–water (65 25 4, v/v). Sulphatide was eluted with eight bed volumes of chloroform–methanol–water (65 25 4, v/v), then the gangliosides were eluted with ten bed volumes of chloroform–methanol–water (30 60 20, v/v). The ganglioside fraction was evaporated, dissolved in water and dialysed against 20 μM potassium carbonate buffer (pH 8.5). After dialysis the ganglioside fraction was evaporated and dissolved in 5.0 ml of chloroform–methanol–water (60 30 4 5, v/v) The recovery of gangliosides in the extraction and purification procedure was assessed by liquid scintillation counting of internal standards of [^{3}H]GM1 and [^{3}H]GT1b.

Determination of gangliosides of the gangliotetraose series by sialidase treatment on the TLC plate combined with TLC–ELISA

The gangliosides of the gangliotetraose series, GM1, GD1a, GD1b and GT1b, were determined by a two-step procedure. Standards of GM1 (0 1–0 8 pmol), a reference of brain gangliosides of known composition and 5–20 μl of the CSF extract were applied on 5-mm lanes on a plastic sheet After chromatographic separation in chloroform–methanol–0 25% potassium chloride solution

TABLE I

BINDING SPECIFICITY OF THE MONOCLONAL ANTIBODIES

Antigen		Monoclonal antibodies[b]				
Name	Epitope structure[a]	CTOB+5	SGD2	TR3	SL50	Sulph-1
GM1	Galβ1-3GalNAcβ1-4Galβ1-R α2|3 NeuAc	0 1	–	–	–	–
GT1a	Galβ1-3GalNAcβ1-4Galβ1-R α2|3 α2|3 NeuAc NeuAc α2|8 NeuAc	–	30	–	–	–
GQ1b	Galβ1–3GalNAcβ1-4Galβ1-R α2|3 α2|3 NeuAc NeuAc α2|8 α2|8 NeuAc NeuAc	–	30	–	–	–
GD2	GalNAcβ1-4Galβ1-R α2|3 NeuAc α2|8 NeuAc	–	3	–	–	–
GD3	Galβ1-R α2|3 NeuAc α2|8 NeuAc	–	100	–	–	–
3′-LM1	Galβ1-3GlcNAcβ1-3Galβ1-R α2|3 NeuAc	–	–	5	–	–
3′-isoLM1	Galβ1-3GlcNAcβ1-3Galβ1-R α2|3 NeuAc	–	–	5	5	–
Sulphatide	Galβ1-R_1 3 | SO_3	–	–	–	–	30
Lactosylceramide 3-sulphate	Galβ1-R 3 | SO_3	–	–	–	–	30

[a] R_1 = Cer, R = 4Glcβ1-Cer

[b] Detection limits (pmol) for the binding between the monoclonal antibodies and their antigen The binding was assayed by TLC-ELISA on thin-layer plates as described under Experimental

TABLE II

APPROPRIATE VOLUMES OF CSF FOR DETERMINATION OF GANGLIOSIDES AND SULPHATIDE BY TLC-ELISA

Glycosphingolipid	Detection limit (pmol)	Volume of CSF for TLC-ELISA analyses (μl)
GM1	0.1	20–30
GD1a, GD1b, GT1b	0.1	5–10
GD2	3	400
3′-LM1	2	1000
3′-isoLM1	2	1000
Sulphatide	30	500

(50 40 10, v/v) a 21°C until the solvent was 5 mm from the front, the plate was dipped twice in 0.1% poly(isobutyl methacrylate) in hexane for 1 min, dried and preincubated with Tris–bovine serum albumin (BSA) [0.15 *M* Tris–HCl (pH 8) containing 0.14 *M* sodium chloride and 1% BSA] for 30 min at room temperature.

The plate was overlaid with *Vibrio cholerae* sialidase (0.02 U/ml) diluted in 0.02 *M* Tris–maleate buffer (pH 6.5) containing 4 m*M* calcium chloride placed in a humid chamber at 37°C for a minimum of 16 h. The reaction was terminated by washing the plate in phosphate-buffered saline (PBS) (0.15 *M* sodium chloride, 0.01 *M* sodium phosphate, pH 7.4). All gangliosides of the gangliotetraose series were hydrolysed to GM1, which was detected by incubation with CT-B (1 μg/ml) and subsequently with anti-CT-B antibody by TLC–ELISA [10] Some batches of the plastic-backed TLC plates did not allow the separation of GD1a and GD1b in the neutral solvent described above. On these occasions, a second plate was chromatographed in an alkaline solvent, chloroform–methanol–2.5 *M* ammonia (50 40 10, v/v), which separated GD1a from GD1b and the procedure for determination was repeated.

Determination of gangliosides and sulphatide with specific monoclonal antibodies by TLC-ELISA

The method described by Magnani et al. [11] was applied with some modifications as previously described [10]. The gangliosides and sulphatide were determined with specific monoclonal antibodies. Plastic treatment and preincubation with Tris–BSA were performed as described above. Alkaline phosphatase-linked second antibodies were used as detecting antibodies and BCIP as phosphatase substrate. The volumes of CSF required for the determination of each individual ganglioside are given in Table II. The glycolipids were quantified by densitometric scanning of the TLC plate on a Camag TLC Scanner II at 620 nm. The detection limits for the immunoreaction between sulphatide, GD2, 3′-LM1 and 3′-isoLM1 and the corresponding antibody and for the assay of the gangliotetraose series are summarized in Table II.

RESULTS

Optimization of the extraction and separation procedure

Chloroform–methanol–water (60 30 4 5, v/v) was the solvent system with lowest polarity that extracted all the gangliosides quantitatively. The recovery of GM1 and GT1b was 95–102% based on ten samples Extraction of CSF gangliosides with chloroform–methanol (2 1, v/v) resulted in recoveries of GM1 and GT1b of 70 and 43%, respectively The presence of water in the extraction solvent was therefore essential

Quantitative elution of gangliosides from the silica gel column was obtained using ten bed volumes of chloroform–methanol–water (30 60 20, v/v), and the recovery of GM1 and GT1b was 95–100% ($n=10$). Other mixtures containing chloroform–methanol–water in different proportions were tested for the elution of the silica gel column The recoveries of GM1 and GT1b with ten bed volumes of chloroform–methanol–water (60 35 8, v/v) were 90 and 65% and with 50 40 10, v/v eluent 95 and 60%, respectively

Direct sialidase treatment of gangliosides on TLC plates

A ganglioside reference containing the gangliosides GM1, GD1a, GD1b and GT1b in the proportions 46 20 21 13 was applied to TLC plates in order to determine the optimum conditions for the hydrolysis of gangliosides GD1a, GD1b and GT1b by the enzyme sialidase. The production of GM1 from GD1a and GT1b increased with increasing incubation time and enzyme concentration. Complete hydrolysis of GT1b was obtained first after 16 h using 0.02 U sialidase per ml buffer, whereas 8 h were sufficient for complete hydrolysis of GD1a and GD1b (Fig. 1). As mentioned under Experimental, some batches of plates did not allow the separation of GD1a and GD1b in the neutral solvent system chloroform–methanol–0 25% potassium chloride solution (50 40–10, v/v) and a second plate was run in chloroform–methanol–2.5 *M* ammonia (50 40 10, v/v) to separate GD1a and GD1b (Fig. 2) The values of GM1,

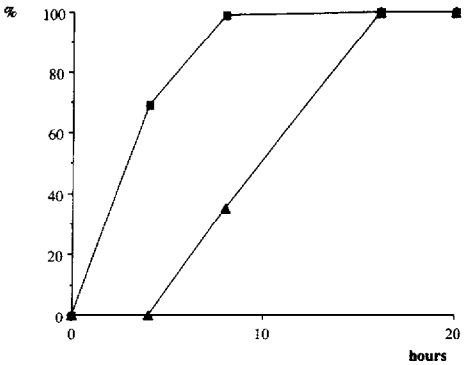

Fig 1 Effect on incubation time of sialidase on the hydrolysis of GD1a, GD1b and GT1b ■, % GD1a+GD1b, ▲, % GT1b

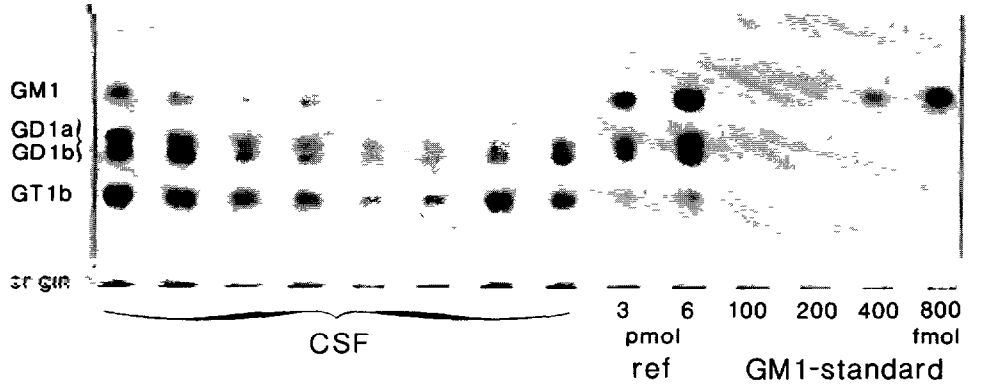

Fig 2 Determination of ganglιotetraose series ganglιosides The ganglιosides were chromatographed in the solvent system chloroform-methanol-0 25% potassium chloride solution The plate was treated with sialidase (0 02 U/ml) and the resulting ganglιoside GM1 was determined with CT-B and an antibody against CT-B in TLC-ELISA The ganglιoside reference (3 and 6 pmol total sialic acid) contained GM1, GD1a, GD1b and GT1b

TABLE III

COEFFICIENTS OF VARIATION FOR DIRECT SIALIDASE TREATMENT ON TLC PLATES COMBINED WITH TLC-ELISA

Ganglιoside	Coefficient of variation ($n=10$) (%)		Ganglιoside standard (fmol)	
	Within-run	Between-run	TLC-ELISA	Resorcinol staining
GM1	10	10	360	366
GD1a	15	20	164	160
GD1b	15	20	164	168
GT1b	12	14	103	108

GD1a+GD1b and GT1b were obtained from plates from the former run and only the value of GD1a was determined from the plates from the latter, because the spot representing GD1b and GT1b was not quantitative under the present conditions.

The imprecisions of the method were 10–20%, calculated on ten samples of the standard ganglιoside mixture for the within- and between-run determinations. The within- and between-run coefficients of variation are given in Table III.

CSF concentrations of ganglιosides and sulphatide in adults

The total ganglioside content in CSF, excluding GM3, from ten individuals varied between 100 and 230 nmol/l (total sialic acid 183–540 nmol/l). The dominating gangliosides were of the ganglιotetraose series, represented by GM1, GD1a, GD1b and GT1b (Table IV). Only 100 μl of CSF were required for the

TABLE IV

GANGLIOSIDES AND SULPHATIDE IN NORMAL CSF

Mean values, standard deviations and ranges for ten CSF samples from normal adults

Glycosphingolipid	Glycosphingolipid content (nmol/l)			Total sialic acid (nmol/l)		
	Mean	S D	Range	Mean	S D	Range
GM1	14	4 3	9-20	14	4 3	9-20
GD1a	26	13	11-50	52	26	22-100
GD1b	39	9 0	22-50	78	18	44-100
GT1b	48	22	27-93	144	44	54-186
GD2	4 2	2 0	2 8-4 6	8 3	3 9	5 5-9 2
Sulphatide	60	26	20-102	-	-	-
Total gangliotetraose series	120	45	100-221	278	98	179-491

determination of these gangliosides, whereas quantitation of, for example, GD2 required a much larger volume of 400 μl. The major part of the oligoganglioside fraction was represented by GD1a, GD1b and GT1b, of which the b-series gangliosides, i e., GD1b and GT1b, dominated

The antibodies TR3 and SL50, which bind to 3′-LM1 and 3′-isoLM1, respectively, did not give any positive reaction in TLC–ELISA for any sample, which means that these antigens constituted < 1 nmol/l.

The mean value for sulphatide concentration in CSF was 60 nmol/l.

DISCUSSION

This method allowed the determination of gangliosides and sulphatide in CSF from individual samples. Determination of the gangliotetraose series gangliosides by direct sialidase treatment on TLC plates, combined with CT-B on TLC–ELISA, required only 100 μl of CSF, as 100 fmol of the respective ganglioside, i.e., GM1, GD1a, GD1b and GT1b, could be assayed. The method is also suitable for determination of the gangliotetraose series gangliosides in other tissues, cell lines and biological fluids, which can be obtained only in small amounts Ginns and French [6] previously determined GM1 ganglioside in CSF with CT-B using a radioimmunoassay, but other gangliosides were not assayed in their study. Another micromethod was recently reported for the assay of the gangliotetraose series gangliosides using CT-B [12]. In that method, the assay was performed in microtitre wells and allowed the analysis only of GM1 and the total amount of gangliosides of the gangliotetraose series. The mean value of the ganglioside content in this study was 143 nmol/l (324 nmol/l sialic acid), but it should be noted that GM3 was not included as we are lacking a monoclonal antibody against this ganglioside

It has been suggested that CSF gangliosides reflect the composition of brain gangliosides [3,5]. The large content of the four major brain gangliosides in our study is further evidence that most of the CSF gangliosides are derived from the CNS by shedding. It could also be assumed that some of the gangliosides would be transported from plasma, as the pattern of the major CSF lipids is the same as that in serum [13]. A possible way to evaluate the influence of plasma gangliosides would be to determine GM3, the dominating ganglioside in serum [14]. This ganglioside could, however, not be determined in CSF from individual samples. We have calculated the GM3 level to be 8 nmol/l, ca. 2 5% of the total sialic acid (unpublished data) in pooled normal CSF based on TLC with resorcinol staining. The ratio between GM3 and GM1 is much higher in serum (11.2) than in CSF (0.6). This finding suggests that only a small proportion of ganglioside is transported from serum to CSF together with other serum lipids. No migration between serum and CSF was found with an intact blood–brain barrier in the study of Svennerholm et al. [15], while a reduced blood–brain barrier function could lead to a leakage of plasma gangliosides into CSF according to a previous study in our laboratory [16]. For this reason, only CSF from patients with an intact blood–spinal fluid barrier function was selected, i.e., having an albumin content <300 mg/l.

The published values for the total ganglioside content in CSF differ markedly. Bernheimer [3], Ledeen and Yu [5] and Nagai et al [4] have reported values for the ganglioside content of CSF to be 3200, 456–1100 and 426 nmol sialic acid, respectively As ganglioside sialic acid comprises only a few percent of total sialic acid in CSF [5], even low contamination with protein-bound sialic acid might give elevated values for total ganglioside sialic acid. In the radioimmunoassay by Ginns and French [6], where the antigens were not identified, it is also possible that the presence of glycoproteins and/or other gangliosides might have given falsely high values for GM1 (161 nmol/l compared with our mean value of 14 nmol/l). Our method eliminates this effect as the ganglioside extract is first chromatographed and each individual ganglioside is identified from its migration rate. Another explanation for the discrepancy in the reported values for the ganglioside content in pooled CSF might be that the CSF was collected from patients with a reduced blood–CSF barrier function. Under such circumstances, one would find an increase in GM3 in particular. The increased values of GM3 and GM1 in pooled CSF from multiple sclerosis patients reported by Nagai et al [4] could therefore be explained by a reduced blood–CSF barrier and an increased transport of serum gangliosides to CSF.

There have been only a few studies concerning CSF gangliosides determined from individual samples [6–8]. In the study by Ginns and French [6], only GM1 was determined. Two papers [7,8] have described a similar type of method to that proposed here. The gangliosides separated by TLC were hydrolysed with *Arthrobacter urefaciens* sialidase to GA1 and GA2, which were then determined with specific antibodies. In those two studies, the normal total gan-

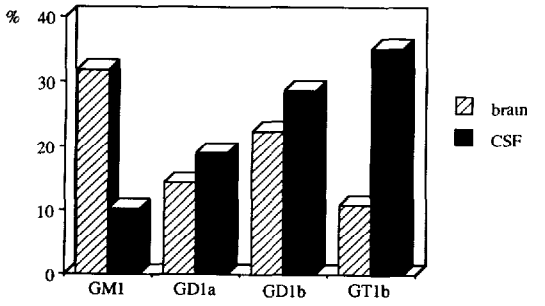

Fig 3 Comparison of the proportions of the ganglιotetraose series ganglιosides ιn normal human CSF and braιn ($n=10$, mean age 50 4 years) and the whole braιn ($n=7$, mean age 51 4 years)

glιoside content was found to be 50–150 nmol/l and GM1 and GD1a to be the major ganglιosides In our study, the dominatιng ganglιosides ınstead were the b-serιes ganglιosides, GD1b and GT1b. Their low recovery of ganglιosides and the dιfferent patterns mιght be explaιned by ıncomplete extractıon of the ganglιosides. They used chloroform–methanol (2 1, v/v), whιch has been shown by us to gıve an incomplete extraction of gangliosides, partıcularly GT1b

In our study all four major braın ganglιosides, GM1, GD1a, GD1b and GT1b, were represented ın CSF, but ın dıfferent proportıons to brain tıssue (Fig. 3). The ratıo between GM1 and the oligoganglιosides ın normal CSF was hıgher than ın normal braın tissue, whereas the ratıo between GD1a and GD1b ın CSF is sımılar to that ın the whole brain. The concentratıon of GT1b was consıderably higher ın CSF than ın normal brain tissue (Fig. 3).

The present method was developed for the study of the CSF ganglιoside pattern ın lysosomal storage dısorders, degenerative diseases such as Alzheımer and Parkınson disease and braın tumours Sıgnıficant changes ın the ganglιoside pattern have been found ın menıngıomas [10]. We have assumed that the assay of sulphatide may give ımportant ınformation about the extent and progress of a demyelinating process such as ın cerebrovascular and senile dementıa [15] and multiple sclerosis. We are at present using the assay to evaluate the effect of bone marrow transplantation on metachromatic leucodystrophy.

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