Cultured cerebellar granule cells, but not astrocytes, produce an ester of ganglioside G_{D1b}, presumably G_{D1b} monolactone, from exogenous G_{D1b}

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Granule cells and astrocytes from rat cerebellum were fed in culture with 2 μM ganglioside [*Gal*-^{3}H]G_{D1b} and then analysed for the presence of carboxyl esters of that ganglioside. Before extraction and purification of gangliosides, cells were treated with $NaBH_4$ under conditions that would allow complete reductive cleavage of carboxyl ester linkages, [*Gal*-^{3}H]G_{D1b} monolactone and dilactone being used as reference esters of G_{D1b}. These conditions, established by adding harvested cells (250 μg of protein) with 0.01–2 nmol of standard [*Gal*-^{3}H]G_{D1b} monolactone or dilactone and [*Gal*-^{3}H]G_{D1b}-1ol or -2ol formed respectively, consisted of an $NaBH_4$/cell protein ratio of 2:1 (w/w). Cerebellar granule cells, but not astrocytes, were able to produce a radioactive compound which was identified as G_{D1b}-1ol. The formation of this compound increased with pulse (up to 4 h) and chase (up to 3 h) time after a 2 h pulse and also occurred when ganglioside endocytosis was blocked. It can be concluded that cerebellar granule cells are able to convert ganglioside G_{D1b} into a carboxyl ester form, presumably G_{D1b} monolactone. The natural occurrence of the same G_{D1b} carboxyl ester in cerebellar granule cells was also demonstrated.

INTRODUCTION

Gangliosides, which are glycosphingolipids particularly abundant in the nervous system, contain one or more sialic acid residues that dissociate at physiological pH, producing a negative charge [2]. This largely contributes to the unusual physicochemical properties [3] and possibly the functional implications of gangliosides [4]. It is assumed that processes capable of modifying the negative charge of gangliosides will be physiologically relevant. For this reason the possibility of gangliosides being involved in the formation and breakdown of ester linkages of the sialic acid carboxy group, carboxyl esters (inner esters; methyl or ethyl esters; intermolecular esters), was investigated. So far, experimental evidence has only been provided for the natural occurrence of inner esters (lactones) of gangliosides ([5,6] and reviewed in [7]). In particular, G_{M3} lactone has been detected in B16 melanoma cells [8], G_{D1b} monolactone (with the ester linkage between the carboxy group of the external sialic acid and the hydroxy group at C-9 of the internal sialic acids) has been isolated from human brain [9], and two lactone forms of G_{M4} have been obtained from the brain of Bryde's whale [10]. Recently, a monoclonal antibody, BBH5, capable of binding the inner ester linkage between two contiguous residues of sialic acid was produced [11], providing a specific and sensitive tool with which to recognize some ganglioside lactones separated by t.l.c.

Detection and quantification of naturally occurring ganglioside lactones is not easy. The inner ester linkages of ganglioside lactones are labile under mild alkaline conditions [12] and are spontaneously produced under mild acidic conditions [13] and possibly under drastic dehydrating conditions. Therefore the procedures used to extract and prepare gangliosides from cells and tissues may either artificially produce lactones or destroy lactones that occur naturally. At present the only method capable of avoiding artifacts in the detection of ganglioside lactones is one developed by Gross et al. [6]. This is based, as the first step of sample treatment, on $NaBH_4$ reductive cleavage of the inner ester linkage with transformation of the sialic acid carboxy group to an alcoholic group. It should be noted that formation of the 'ol' derivative indicates the presence of a carboxyl ester and does not exclude, in principle, the possible occurrence of external ester linkages, although these have never been shown to occur in biological systems.

No information is available on the mechanism of ganglioside lactonization under physiological conditions. A previous paper [14] showed that intracisternal administration of G_{D1b} to rat brain is followed by formation of G_{D1b} monolactone. In the present study we investigated the metabolic formation of G_{D1b} carboxyl esters, particularly G_{D1b} lactones, using primary cultures of cerebellar granule cells and astrocytes fed with [*Gal*-^{3}H]G_{D1b}. Detection and quantification of the ester form of G_{D1b} was accomplished by a perfection of the method of Gross et al. [6]. The results obtained demonstrate that cerebellar granule cells, but not astrocytes, are capable of transforming G_{D1b} to a G_{D1b} carboxyl ester, presumably G_{D1b} monolactone. This compound was also shown to occur naturally in the same cells.

EXPERIMENTAL

Chemicals

Commercial chemicals were of the highest grade available. Basal modified Eagle's medium (BME) and fetal calf serum (FCS) (heat-inactivated before use) were from Biochrom (Berlin, Germany); 1-α-D-arabinofuranosylcytosine, NeuAc and crystalline BSA were from Sigma Chemical Co. (St. Louis, MO, U.S.A.); *Vibrio cholerae* sialidase (EC 3.2.1.18) was from Boehringer (Mannheim, Germany); *Rhodococcus* endoglycoceramidase was from Genzyme (Boston, MA, U.S.A.); NaB^3H_4 (13.7 Ci/mmol) was from Amersham International (Amersham,

Abbreviations used: The ganglioside nomenclature of Svennerholm [1] is used; G_{D1b} carboxyl ester, derivative of G_{D1b} with the carboxy group of sialic acid involved in an ester linkage; G_{D1b}-1L, G_{D1b} monolactone {β-Gal-(1→3)-β-GalNAc(1→4)-[α-NeuAc-(2→8; 1→9)-α-NeuAc-(2→3)]-β-Gal-(1→4)-β-Glc-(1→1)-Cer}; G_{D1b}-2L, G_{D1b} dilactone {β-Gal-(1→3)-β-GalNAc(1→4)-[α-NeuAc-(2→8; 1→9)-α-NeuAc-(2→3; 1→2)]-β-Gal-(1→4)-β-Glc-(1→1)-Cer}; G_{D1b}-1ol, α-nonulosamine-(2→8)-α-NeuAc-(2→3)-containing G_{D1b}; G_{D1b}-2ol, α-nonulosamine-(2→8)-α-nonulosamine-(2→3)-containing G_{D1b}; Sph, sphingosine; h.p.t.l.c., high-performance thin-layer chromatography; BME, basal modified Eagle's medium; FCS, fetal calf serum.

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Bucks., U.K.). Gangliosides G_{M3}, G_{M1}, G_{D1a} and G_{D1b} were prepared and characterized as previously described [15]. The lactone forms of G_{D1b} (G_{D1b}-1L and G_{D1b}-2L) and the reduced forms of G_{D1b} lactones (G_{D1b}-1ol and G_{D1b}-2ol) were prepared from G_{D1b} and characterized as described previously [16,17].

Preparation of radiolabelled gangliosides and ganglioside derivatives

Ganglioside G_{D1b} was isotopically ^3H radiolabelled at C-6 of the terminal galactose ([*Gal*-^{3}H]G_{D1b}) [18], the specific radioactivity being 1.0 Ci/mmol. [*Gal*-^{3}H]G_{D1b}-1L and -2L were prepared from [*Gal*-^{3}H]G_{D1b} [16,17], and [*Gal*-^{3}H]G_{D1b}-1ol and [*Gal*-^{3}H]G_{D1b}-2ol from [*Gal*-^{3}H]G_{D1b}-1L and -2L respectively [17]. G_{M1} was labelled with ^3H at C-3 of the sphingosine moiety ([*Sph*-^{3}H]G_{M1}) [19], and [^{14}C]G_{M3} was radiolabelled at the *N*-acetyl group [20]. The radiochemical purity of all radiolabelled compounds was greater than 99%.

Cell cultures

Cultured granule cells and astrocytes were obtained from the cerebellum of 8-day-old Sprague–Dawley rats. Granule cells were prepared and cultured as previously described [21]. Inhibition of glial cell growth was achieved by addition of 1-α-D-arabinofuranosylcytosine 16–20 h after seeding [22]. Granule cells were used on the 8th day in culture, when they were fully differentiated. Astrocytes were prepared and cultured as described previously [23], and used on the 10th–12th day in culture. Both granule cells and astrocytes constituted about 95% of the cell population in the cultures and were incubated at 37 °C in an atmosphere of CO_2/air (1:19).

Detection of naturally occurring ganglioside carboxyl esters in cultured cells: treatment with NaB^3H_4

Cultured cells were treated with NaB^3H_4 by the procedure of Gross et al. [6]. Ganglioside carboxyl esters, particularly lactones, were thereby transformed to the corresponding '[^{3}H]ol' derivatives. Briefly, harvested cells were suspended in PBS (0.5 mg of cell protein/ml). To 900 μl of this suspension was added 100 μl (2.5 mCi) of NaB^3H_4 dissolved in 0.01 M NaOH; the mixture was incubated at room temperature for 1 h, with mild shaking. The mixture was then pelleted by centrifugation (2000 *g*, 10 min), the pellet washed twice in cold PBS, and then submitted to lipid extraction and fractionation.

Quantitative reduction of G_{D1b} lactones by $NaBH_4$

Using the original method of Gross et al. [6], the optimal conditions were established for the quantitative reductive cleavage of the inner ester linkages of G_{D1b}-1L and G_{D1b}-2L in the presence of cultured cells. To this purpose, control cells were scraped from the plates and suspended in PBS (10 mg of cell protein/ml). To 25 μl of this suspension was added increasing amounts of G_{D1b}-1L or G_{D1b}-2L (0.05–2 nmol, measured as the lactone linkage) containing 10 nCi of [*Gal*-^{3}H]G_{D1b}-1L or [*Gal*-^{3}H]G_{D1b}-2L. The mixtures were immediately treated with 25 μl of a freshly prepared $NaBH_4$ solution (1–20 mg/ml) and incubated at room temperature for 30 min with shaking. The mixtures were then submitted to lipid extraction, and G_{D1b}-1ol and G_{D1b}-2ol formed were separated by high-performance thin-layer chromatography (h.p.t.l.c.) [17] and determined as bound radioactivity.

Metabolic studies after feeding cells with [*Gal*-^{3}H]G_{D1b}

Cells cultured in 60 mm-diameter dishes were washed twice with BME without FCS and incubated for a given period of time (pulse experiments) in the same medium (2 ml/dish) containing 2 μM [*Gal*-^{3}H]G_{D1b} with 2 μCi/ml radioactivity. At the end of the incubation, the medium was removed and the cells were washed (30 min) with BME containing 10% FCS. In the chase experiments, after washing, cells were further incubated with 5 ml of 10% FCS-containing medium for different periods of time. After pulse or pulse–chase, cells were rinsed twice with cold PBS, scraped from the plates and centrifuged (2000 *g*, 10 min). The pellets were resuspended in PBS and immediately submitted to $NaBH_4$ treatment using optimal conditions for quantitative reduction (2 mg of $NaBH_4$/mg of cell protein).

Extraction and quantification of gangliosides and ganglioside derivatives

Total lipids were extracted and partitioned as previously described [24]. The aqueous phase, after lyophilization, was resuspended in 100 μl of chloroform/methanol (1:1, v/v), stirred and centrifuged at 5000 *g* for 5 min. The pellet was washed twice as above and the supernatants pooled. The individual [^{3}H]gangliosides, [^{3}H]ganglioside lactones or [^{3}H]ganglioside-ols present in the pooled supernatants were separated by one- or two-dimensional h.p.t.l.c. [17] and quantified as bound radioactivity.

Isolation and characterization of [^{3}H]G_{D1b}-1ol from cultured granule cells

A radioactive molecule, co-migrating with authentic [*Gal*-^{3}H]G_{D1b}-1ol on h.p.t.l.c., was present in cultured granule cells (a) after treatment with NaB^3H_4 before lipid extraction and (b) after feeding with [*Gal*-^{3}H]G_{D1b} followed by $NaBH_4$ treatment. In both cases the band that behaved chromatographically like standard G_{D1b}-1ol was scraped from the plate and eluted twice from the gel with 1 ml of chloroform/methanol (2:1, v/v) and twice with 1 ml of chloroform/methanol (1:1, v/v). The compound obtained was characterized by *Vibrio cholerae* sialidase treatment [9], endoglycoceramidase treatment [25] and mild acid methanolysis (0.05 M HCl, 80 °C, 20 min) [17], followed by exhaustive dialysis against distilled water.

T.l.c.

One-dimensional h.p.t.l.c. separation of gangliosides and ganglioside derivatives was performed using the following solvent systems: chloroform/methanol/0.2% $CaCl_2$ (45:55:10, by vol.) (solvent A); chloroform/methanol/0.2% $CaCl_2$/32% NH_4OH (60:50:9:1, by vol.) (solvent B). In two-dimensional h.p.t.l.c., solvents A and B were used for the first and second run respectively. The oligosaccharides released after endoglycoceramidase treatment of gangliosides or ganglioside derivatives were separated by one-dimensional h.p.t.l.c. using ethanol/1 M ammonium acetate (7:3, v/v) (solvent C).

Determination of radioactivity

Radioactivity was determined by liquid-scintillation counting or fluorography as previously described [26]. Radiodensitometric

quantification of gangliosides and ganglioside derivatives separated by h.p.t.l.c. was performed using a Digital Autoradiograph (Berthold, Germany) [26].

Colorimetric methods

Ganglioside content, measured as lipid-bound NeuAc, was determined by the resorcinol method [27], using NeuAc as standard. Total proteins were assayed [28] using BSA as standard.

RESULTS

Detection of ganglioside carboxyl esters in cultured granule cells and astrocytes

After treatment of cerebellar granule cells and astrocytes with NaB^3H_4, followed by ganglioside extraction and h.p.t.l.c. separation, a number of radioactive spots were detected (Figure 1). One of these, the most abundant radioactive compound in granule cells, behaved chromatographically like standard $[^3H]G_{D1b}$-1ol (Figure 1a), but differently from $[^3H]G_{D1b}$-2ol (not shown). This substance was isolated and proved to be resistant to *Vibrio cholerae* sialidase, and, on endoglycoceramidase treatment, produced an oligosaccharide that migrated on h.p.t.l.c. in the same position as that obtained from standard G_{D1b}-1ol submitted to the same treatment (Figures 2a and 2b). Moreover, when this substance was subjected to mild acid methanolysis and exhaustive dialysis, the radioactivity became completely dialysable. It is noteworthy that, in cultured astrocytes (Figure 1b), no G_{D1b}-1ol could be detected, excluding the occurrence of G_{D1b} carboxyl esters, whereas a major radioactive band was present in the region possibly corresponding to less polar forms of G_{M3} or G_{D3}. This band remains to be characterized.

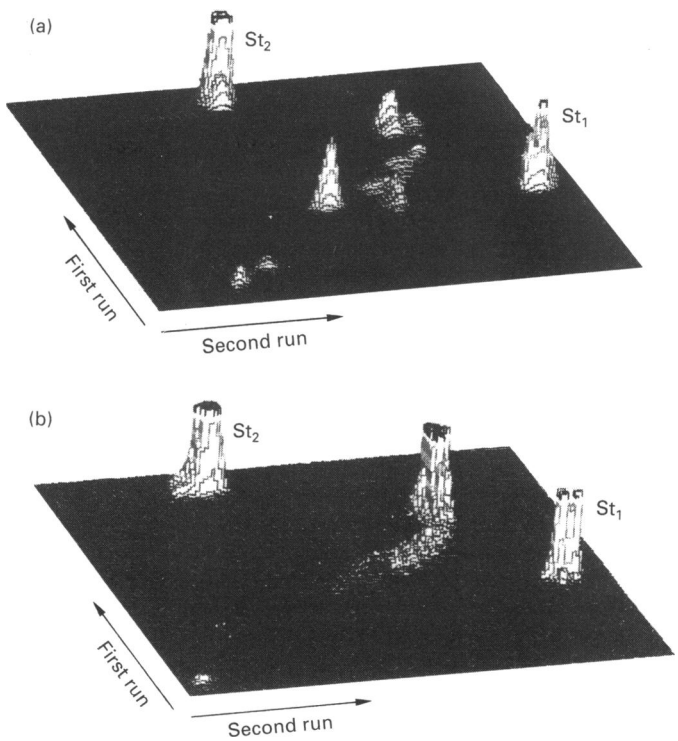

Figure 1 Two-dimensional h.p.t.l.c. of ganglioside extracted from cerebellar granule cells (a) and astrocytes (b) after NaB^3H_4 treatment

First run, solvent A; second run, solvent B. St_1 and St_2, standard $[^3H]G_{D1b}$-1ol subjected only to the first and second run respectively. For details see the Experimental section.

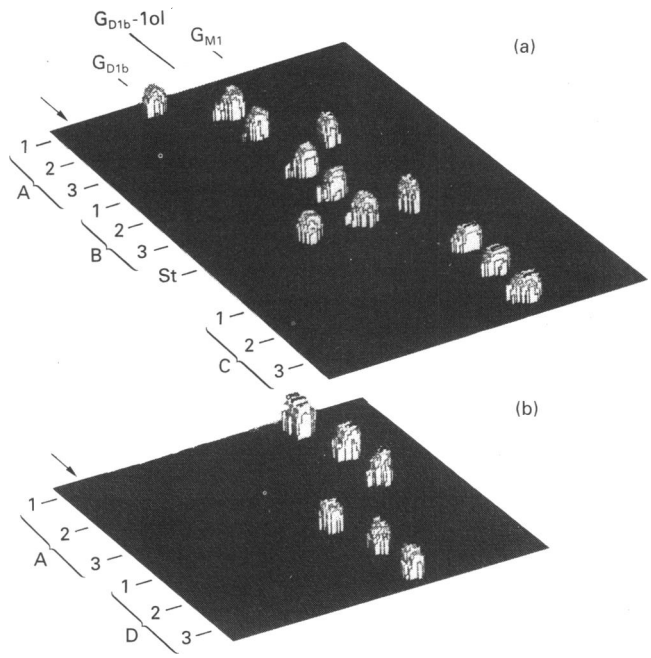

Figure 2 Characterization of G_{D1b}-1ol isolated from cerebellar granule cells after (i) reductive cleavage of carboxyl-ester linkages with NaB^3H_4 (G_{D1b}-1ol from naturally occurring G_{D1b} carboxy ester) or (ii) 4 h pulse with 2 μM [*Gal*-^{3}H]G_{D1b} followed by $NaBH_4$ reduction (G_{D1b}-1ol from G_{D1b} carboxyl ester metabolically formed from G_{D1b})

Digital autoradiographic h.p.t.l.c. patterns are shown of the ^3H-labelled compounds formed after the following treatments: A, no treatment; B, *Vibrio cholerae* sialidase hydrolysis; C, mild acid hydrolysis; D, endoglycoceramidase hydrolysis. 1, standard $[^3H]G_{D1b}$; 2, standard $[^3H]G_{D1b}$-1ol; 3, $[^3H]G_{D1b}$-1ol isolated from cerebellar granule cells [either (i) or (ii) except for C, only (ii)]. The arrow indicates the origin. (**a**) Solvent system A; (**b**) solvent system B. For further details see the Experimental section.

Quantitative reduction of G_{D1b} lactones by $NaBH_4$ in the presence of cultured cells: optimal conditions for G_{D1b} lactone quantification

Efforts were made to optimize the conditions for complete reductive cleavage of the lactone linkage in the presence of cultured cells. It was observed that the $NaBH_4$/cell protein ratio is much more important than the $NaBH_4/G_{D1b}$ lactone ratio. As shown in Figure 3, complete reduction of $[^3H]G_{D1b}$-1L (up to 2 nmol added) to $[^3H]G_{D1b}$-1ol was obtained with an $NaBH_4$/cell protein ratio of at least 1:1 and surely 2:1 (w/w). Identical conditions were required to the quantitative reduction of $[^3H]G_{D1b}$-2L (up to 1 nmol added) to $[^3H]G_{D1b}$-2ol. The same results were obtained for both granule cells and astrocytes. It is noteworthy that, in the absence of $NaBH_4$, a portion of G_{D1b}-1L was hydrolysed to G_{D1b}, indicating that, under the conditions used for lipid extraction and ganglioside purification, G_{D1b}-1L and presumably other ganglioside lactones are unstable, as expected [12]. Using an $NaBH_4$/cell protein ratio of 2:1 and 0.25 mg of cell protein, a linear relationship between $[^3H]G_{D1b}$-1L added and $[^3H]G_{D1b}$-1ol formed was observed from 0.05 to at least 2 nmol of $[^3H]G_{D1b}$-1L (from 0.025 to 1 nmol for $[^3H]G_{D1b}$-

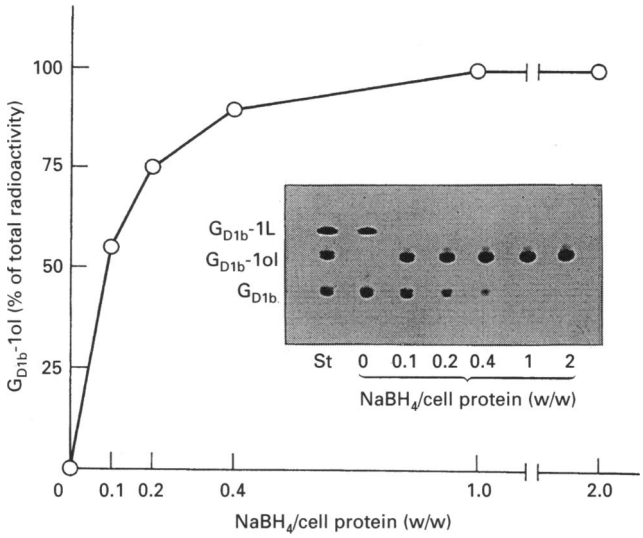

Figure 3 Formation of [*Gal*-^{3}H]G_{D1b}-1ol from [*Gal*-^{3}H]G_{D1b}-1L, added (2 nmol) to cerebellar granule cells (0.25 mg of protein), after reduction with increasing amounts of $NaBH_4$

The inset is the h.p.t.l.c. autoradiographic pattern of compounds formed at different $NaBH_4$/cell protein ratios. St, standard [^{3}H]gangliosides G_{D1b}, G_{D1b}-1ol and G_{D1b}-1L. Solvent system A was used. Analytical details are reported in the Experimental section. Exactly the same results were obtained with equimolar amounts (measured as the lactone linkage) of [*Gal*-^{3}H]G_{D1b}-2L.

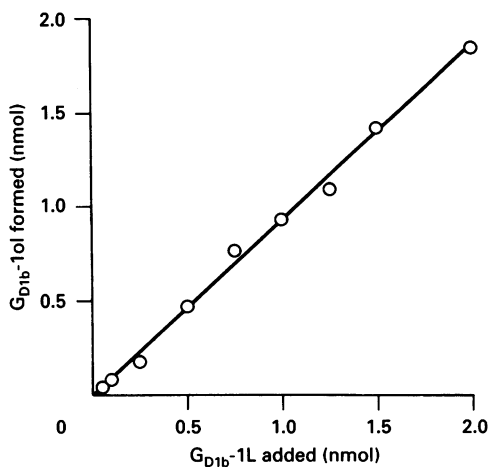

Figure 4 Recovery of G_{D1b}-1L (added to cerebellar granule cells in culture) as G_{D1b}-1ol after $NaBH_4$ treatment

Different amounts (0.05–2 nmol) of G_{D1b}-1L containing 20 nCi of [*Gal*-^{3}H]G_{D1b}-1L were added to 0.25 mg (as protein) of cerebellar granule cells and then treated with 0.5 mg of $NaBH_4$. [^{3}H]G_{D1b}-1ol was extracted and quantified as described in the Experimental section. Each value represents the mean of five determinations; S.D. values never exceeded 5% of the mean. The regression equation was $y = -0.01 + 0.94x$. Exactly the same results were obtained using equimolar amounts (measured as the lactone linkage) of [*Gal*-^{3}H]G_{D1b}-2L.

2L), with a correlation coefficient of 0.98 and 0.96 for cerebellar granule cells and astrocytes respectively (Figure 4). The coefficient of variation of 15 repetitive determinations with 0.05, 0.2 and 2 nmol of added [^{3}H]G_{D1b}-1L was 4.5, 3.2 and 2.3% for cerebellar granule cells and 5.7, 4.4 and 4.0% for cerebellar astrocytes respectively. These values (which were virtually identical for

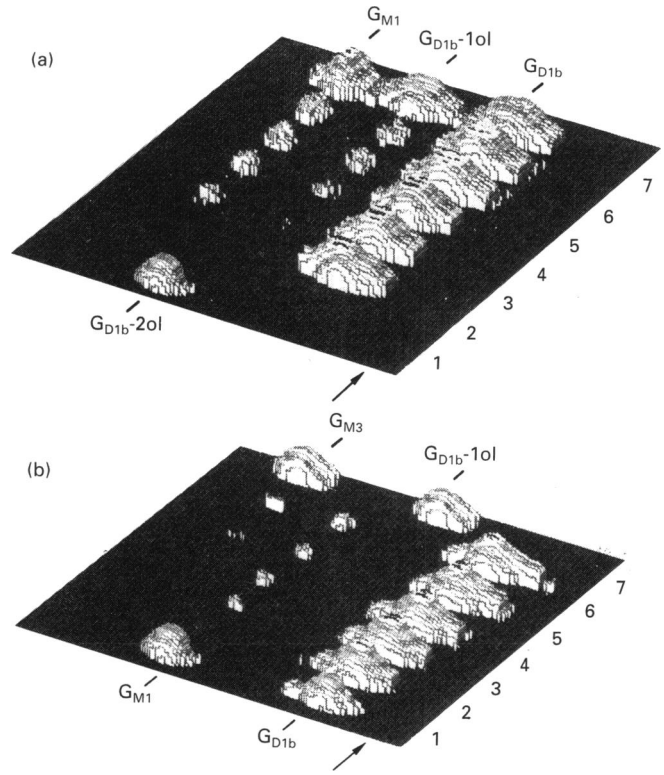

Figure 5 One-dimensional h.p.t.l.c. pattern of radiolabelled gangliosides obtained from cultured cerebellar granule cells and astrocytes after different pulse times in the presence of 2 μM [*Gal*-^{3}H]G_{D1b}, followed by reduction with $NaBH_4$ (2 mg/mg of cell protein)

(**a**) Cerebellar granule cells: lane 1, standard [^{3}H]G_{D1b}-2ol; lane 2, [^{3}H]gangliosides from the incubation medium at 4 h pulse; lanes 3–6, [^{3}H]gangliosides from cells after 0.5, 1, 2 and 4 h pulse respectively; lane 7, standard [^{3}H]gangliosides G_{D1b}, G_{D1b}-1ol and G_{M1}. (**b**) Cerebellar astrocytes: lane 1, standard [^{3}H]G_{D1b} and G_{M1}; lane 2, [^{3}H]gangliosides from the incubation medium at 4 h pulse; lanes 3–6, [^{3}H]gangliosides from cells after 0.5, 1, 2, 4 h pulse respectively; lane 7, standard [^{3}H]GM_3 and G_{D1b}-1ol. The arrow indicates the origin. Solvent system A was used. Analytical details are reported in the Experimental section.

equimolar, measured as the lactone linkage, concentrations of [^{3}H]G_{D1b}-2L) did not significantly change from one cell preparation to another. Control experiments showed that the recovery of [^{14}C]G_{M3}, [^{3}H]G_{M1} and [^{3}H]G_{D1b}, added to the cell preparations and treated under optimal conditions for reduction of [^{3}H]G_{D1b}-L, was over 95% in all cases. This indicates that reductive cleavage does not affect the process of ganglioside extraction and purification.

Metabolic formation of [^{3}H]G_{D1b} carboxyl ester from exogenous [*Gal*-^{3}H]G_{D1b} in cerebellar granule cells in culture

After a 0.5–4 h pulse with [^{3}H]G_{D1b}, cultured granule cells and astrocytes were shown to take up similar amounts of radioactivity in a time-dependent fashion: at 0.5 and 4 h the radioactivity taken up was 160 and 430 nCi/mg of protein in granule cells and 176 and 471 nCi/mg of protein in astrocytes. One-dimensional h.p.t.l.c. autoradiographic patterns of [^{3}H]gangliosides obtained after feeding cerebellar granule cells and astrocytes with 2 μM [*Gal*-^{3}H]G_{D1b}, followed by $NaBH_4$ treatment (2 mg/mg of cell protein), are shown in Figure 5. In granule cells (Figure 5a) two main radioactive spots were present, besides the administered

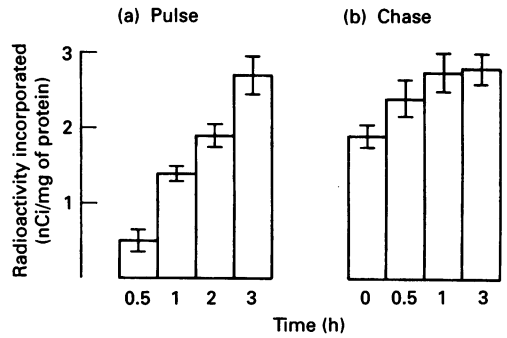

Figure 6 Time course of incorporation of radioactivity into [^{3}H]G_{D1b}-1ol extracted from cerebellar granule cells fed with 2 μM [*Gal*-^{3}H]G_{D1b} for different pulse times (a) and chase times after 2 h pulse (b) and then treated with $NaBH_4$ (2 mg/mg of cell protein)

Data are means ± S.D. of five experiments performed in duplicate. For details see the Experimental section.

[^{3}H]G_{D1b}. The major one was identified as [^{3}H]G_{M1}, derived from metabolic desialosylation of [^{3}H]G_{D1b} [24]. The second one, migrating between G_{M1} and G_{D1b}, co-migrated with standard [^{3}H]G_{D1b}-1ol, but not G_{D1b}-2ol, in different solvent systems, and was absent when $NaBH_4$ treatment was omitted. In astrocytes (Figure 5b), the administration of [^{3}H]G_{D1b} resulted in the formation of [^{3}H]G_{M1} and, at the longest pulse times, of [^{3}H]G_{M3} (possibly derived from biosynthetic recycling of [^{3}H]Gal, liberated during [*Gal*-^{3}H]G_{D1b} degradation). It is noteworthy that no radioactive spots migrating as standard G_{D1b}-ols were detectable in the astrocyte extract. The only radioactive ganglioside also present in the incubation medium after the longest pulse time used (4 h) was G_{D1b} in both granule cells and astrocytes. This indicates that the radioactive gangliosides and ganglioside derivatives (besides G_{D1b}) detected in the cells are authentic cellular metabolites.

The radiolabelled compound co-migrating with standard [^{3}H]G_{D1b}-1ol on h.p.t.l.c. was isolated from cerebellar granule cells pulsed for 4 h and submitted to chemical identification methods. Its radiochemical purity, assessed by h.p.t.l.c., was greater than 98%. It co-chromatographed with standard [^{3}H]G_{D1b}-1ol (Figures 2a and 2b) (but not with standard [^{3}H]G_{D1b}-2ol; not shown) in solvent systems A and C, and, when exposed to *Vibrio cholerae* sialidase under conditions that allowed complete hydrolysis of standard [^{3}H]G_{D1b} to [^{3}H]G_{M1}, was resistant to the enzyme (Figure 2a), equally as well as standard [^{3}H]G_{D1b}-1ol. Mild acid methanolysis resulted in a derivative co-migrating exactly with standard [^{3}H]G_{M1} (Figure 2a). Moreover, it was susceptible to endoglycoceramidase hydrolysis and the oligosaccharide released exhibited chromatographic behaviour different from that of G_{D1b} under the same conditions but identical with that of standard [^{3}H]G_{D1b}-1ol (Figure 2b).

The time course of incorporation of radioactivity into [^{3}H]G_{D1b}-1ol in granule cells fed with 2 μM [Gal-^{3}H]G_{D1b} is shown in Figure 6. [^{3}H]G_{D1b}-1ol was already detectable at 30 min and markedly increased if the pulse time was increased. The amounts of [^{3}H]G_{D1b}-1ol formed at 30 min and 4 h were 0.52 and 2.84 nCi/mg of protein respectively corresponding to 0.33 and 0.66% of total radioactivity taken up. Under the same conditions, the amount of [^{3}H]G_{M1} formed was 1.18 and 7.54 nCi/mg of protein at 30 min and 4 h, corresponding to 0.74 and 1.75% respectively of total radioactivity incorporated. During the chase, after 2 h pulse, the radioactivity incorporated into [^{3}H]G_{D1b}-1ol showed a moderate increase up to 1 h, maintaining a constant level thereafter. Pulse experiments with 2 μM [*Gal*-^{3}H]G_{D1b} were also carried out on cultured granule cells at 4 °C, a condition known to block endocytosis. The amount of firmly bound radioactivity taken up at 4 h was 316 nCi/mg of protein and both [^{3}H]G_{D1b}-1ol and [^{3}H]G_{M1} appeared to be produced: 1.37 and 1.86 nCi/mg of protein corresponding to 0.43 and 0.59% respectively of total radioactivity incorporated.

DISCUSSION

The formation of carboxyl esters may provide a powerful and physiologically relevant way of modifying the chemical properties of gangliosides. So far, only inner esters (i.e. lactones) of gangliosides have been reported to exist in nature [5–11], with the use of two analytical approaches: (a) reductive cleavage of the inner esters with $NaBH_4$, resulting in formation of stable ganglioside-ols (^{3}H-labelled if NaB^3H_4 is used) [6]; and (b) reaction with the monoclonal antibody BBH5 [11], which recognizes the lactone ring between the two sialic acid residues of the sequence NeuAc2→8NeuAcα. The first approach, which recognizes the different inner esters present in ganglioside lactones (NeuAc→Gal; NeuAc→NeuAc), prevents any artificial formation or breakdown of these esters, as $NaBH_4$ reduction is performed before extraction and purification of gangliosides. The second approach [11] recognizes only one lactone linkage, and does not distinguish between natural and artificially produced ganglioside lactones, as it is applied after the steps of ganglioside extraction and purification. The first approach was adopted in our study and optimized to make it suitable not only to verify the presence, but to quantify the metabolic formation, of G_{D1b} carboxyl esters in cultured cerebellar granule cells and astrocytes.

Optimization of the Gross et al. method [6] for quantitative purposes was accomplished by using standard [*Gal*-^{3}H]G_{D1b}-1L and [*Gal*-^{3}H]G_{D1b}-2L added to cultured granule cells and astrocytes. Using an $NaBH_4$/cell protein ratio of 2:1 (w/w), i.e. an $NaBH_4$ concentration 50-fold higher than that reported in the original method, both [^{3}H]G_{D1b}-1L and [^{3}H]G_{D1b}-2L were completely recovered, in a sufficiently wide range of concentrations (from 0.05 to at least 2 nmol, as lactone linkage), as the 1ol and 2ol derivatives, with correlation coefficients very close to 1.00 in both cell types. Therefore $NaBH_4$ reductive cleavage, carried out as described here, can be used for accurate quantification of G_{D1b} lactones as the corresponding G_{D1b}-ols. As the lactone linkages so far detected in gangliosides are present in the reference standards G_{D1b}-1L and G_{D1b}-2L, it seems reasonable to infer that the perfected method is suitable, in general, for quantification of ganglioside lactones as the corresponding ganglioside-ols.

A useful contribution of this work is the evidence that administration of [*Gal*-^{3}H]G_{D1b} to cultured granule cells causes formation of a compound that, on optimized $NaBH_4$ treatment, generates G_{D1b}-1ol. Identification of the G_{D1b}-1ol formed is based on the fact that this substance: (a) co-migrates with authentic G_{D1b}-1ol, but not G_{D1b}-2ol, on h.p.t.l.c.; (b) is resistant to *Vibrio cholerae* sialidase action, as well as standard G_{D1b}-1ol, which is probably due to the absence of a free carboxy group, known to be a necessary requirement for sialidase activity [29]; (c) produces G_{M1} after mild acid methanolysis, exactly like standard G_{D1b}-ol subjected to the same treatment; (d) on endoglycoceramidase hydrolysis releases an oligosaccharide that behaves chromatographically like that released by standard G_{D1b}-1ol but differently from that originated by G_{D1b}. The time course

of formation of G_{D1b}-1ol in the pulse and pulse–chase experiments follows a trend that appears to be typical of a metabolic process. Moreover, cultured granule cells appear to be able to produce [^{3}H]G_{D1b}-1ol from exogenous [^{3}H]G_{D1b} at 4 °C, conditions under which endocytosis as well as the following lysosomal catabolism of exogenous G_{D1b} are blocked [30].

It is noteworthy that the percentage of [^{3}H]G_{D1b}-1ol formed over total [^{3}H]G_{D1b} taken up after a 4 h pulse is similar at 4 °C and 37 °C (0.43 and 0.66 %), suggesting that the metabolic origin of the precursor of G_{D1b}-1ol is presumably the same in both cases. Interestingly, the amount of [^{3}H]G_{M1} formed from exogenous [^{3}H]G_{D1b} is significantly different at the two incubation temperatures – 0.59 and 1.75 % of the total radioactivity incorporated after 4 h at 4 °C and 37 °C respectively – probably indicating that in this case G_{M1} is produced, as already demonstrated [30], at the plasma-membrane level at 4 °C and at the plasma-membrane level and in lysosomes at 37 °C.

All this is consistent with the hypothesis that cerebellar granule cells in culture are able to transform G_{D1b} into a G_{D1b} carboxyl ester of the external sialic acid residue at a subcellular level that is independent of the endocytic flux and lysosomes, presumably the plasma membrane. This observation is particularly important as endocytosis of G_{D1b} taken up and subsequent delivery to the lysosomes implies exposure of G_{D1b} to an acidic milieu with possible formation of G_{D1b} lactone(s). Exclusion of endocytosis and lysosomal processing suggests the realistic hypothesis that an enzyme, linked to the plasma membrane, is responsible for the formation of the G_{D1b} carboxyl ester. It is notable that cultured astrocytes under identical conditions do not produce any G_{D1b}-1ol. It should be remembered that cultured astrocytes do not contain G_{D1b}, whereas granule cells do [24]. Moreover, as shown in the present work, cerebellar granule cells in culture contain a ganglioside derivative that, on NaB^3H_4 treatment, produces [^{3}H]G_{D1b}-1ol. This derivative appears to be identical with that formed after cell feeding with [*Gal*-^{3}H]G_{D1b}. Thus it seems reasonable to conclude that cerebellar granule cells differentiated in culture express both G_{D1b} and a G_{D1b} carboxyl ester, and are metabolically equipped to produce this carboxyl ester from G_{D1b}. Primary cultures of cerebellar astrocytes, which do not contain either G_{D1b} or the G_{D1b} carboxyl ester, do not operate this metabolic route. It will be interesting to know whether the capacity to form G_{D1b} carboxyl ester is linked to the differentiation process of cerebellar granule cells in culture.

At present no direct chemical proof can be provided about the type of ester linkage present in the G_{D1b} carboxyl ester described. However, the facts that (a) the formation of G_{D1b}-1ol from cerebellar granule cells parallels that from standard G_{D1b}-1L, (b) G_{D1b}-1L has previously been reported to occur in brain [9], (c) intracisternal administration of G_{D1b} to rat brain results in formation of G_{D1b}-1L, (d) only inner carboxyl esters of gangliosides have been reported so far support the hypothesis that the G_{D1b} carboxyl ester formed is G_{D1b}-1L.

In conclusion, this work provides the first evidence that a carboxyl ester of G_{D1b}, presumably G_{D1b}-1L, is metabolically formed from G_{D1b} by cerebellar granule cells in culture, probably at the level of the plasma membrane. The fact that cerebellar granule cells contain G_{D1b} lactone and are metabolically equipped to form it from G_{D1b} whereas astrocytes do not indicate that ganglioside lactonization might be a specific process. This evidence is expected to prompt studies on the formation mechanism, the possible dependence on neuron differentiation and the biological significance of the process of ganglioside lactonization.

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