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Synthesis of 2-deoxy-D-galactose containing gangliosides in vivo

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Incorporation of 2-deoxy-D-galactose into the oligosaccharide moieties of different gangliosides of rat liver was examined. After intraperitoneal administration of 2-deoxy-D-galactose it was shown by GLC/MS analysis that this hexose analogue is metabolized and incorporated into all the gangliosides investigated, and predominantly into G_{M3} and G_{D3}. In both of these gangliosides, 25–55% of the galactose residues were substituted by 2-deoxy-D-galactose. The epimer, 2-deoxy-D-glucose, was not detectable.

Ganglioside; 2-Deoxy-D-galactose; Hexose analogue

1. INTRODUCTION

In an earlier study, it was demonstrated that the D-galactose analogue, 2-deoxy-D-galactose (2dGal), is metabolized by the same pathway as the naturally occurring hexose D-galactose in rat liver [1]. The main metabolites were 2-deoxy-D-galactose-1-phosphate (99.3%), UDP-2-deoxy-D-galactose (0.3%) and, after epimerization, UDP-2-deoxy-D-glucose (0.1%). These results suggested that the UDP-hexode analogues may act as substrates for the respective glycosyltransferases involved in the biosynthesis of glycoconjugates. In an earlier study, we presented the first evidence that this assumption is true for glycoproteins [2]. Recently, using GLC/MS analysis, we demonstrated that 2dGal is incorporated into the oligosaccharide moiety of membrane glycoproteins of HepG2 cells. The incorporation of 2dGal was paralleled by a reduced α1-2 fucosylation [3].

In the present study, we investigated the incorporation of 2dGal into the oligosaccharide moiety of glycosphingolipids (GSLs) of rat liver. GSL biosynthesis in rat liver is well documented [4–9]. Recently, it was suggested that the first steps of GSL biosynthesis occur on the cytoplasmic leaflet of the Golgi apparatus. Evidence was provided that UDP-Glc:Cer β1–1 glucosyltransferase and UDP-Gal:Glc-Cer β1–4 galactosyltransferase are located on the cytoplasmic side of the Golgi apparatus, whereas enzymes catalysing subsequent steps occur at the luminal side [10]. These results have been confirmed by Pagano and co-workers [11], but there are also studies which located the biosynthesis of lactosylceramide in the Golgi lumen [12]. If the first steps of GSL biosynthesis have a cytoplasmic location, UDP-2dGal does not need to be specially transported into the Golgi apparatus in order to serve as a substrate. The present study describes the isolation and structural characterization of modified gangliosides occurring in rat liver after treatment of rats with 2dGal. Our data show for the first time that 2dGal is incorporated into G_{M3}, G_{D3}, G_{D1b} and G_{T1b}. The incorporation of 2dGal into gangliosides was higher as compared to the incorporation of 2dGal into glycoproteins [3].

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Abbreviations: Gangliosides are coded according to the nomenclature of Svennerholm [13] and the IUPAC-IUB recommendations [14]; GSL, glycosphingolipid; 2dGal, 2-deoxy-D-galactose; GLC/MS, gas-liquid chromatography/mass spectrometry; FAB/MS, fast atom bombardment/mass spectrometry; EI, electric ionization.

2. MATERIALS AND METHODS

2.1. *Chemicals*

All solvents for GLC were purchased from Promochem (Wesel, Germany) in GLC quality. 2-Deoxy-D-galactose and D-galactose were purchased from Sigma (München). Acetonitrile HPLC Grade E was purchased from Rathburn chemicals Ltd. (Scotland, UK), other solvents from Roth (Karlsruhe, Germany) in p.a. grade. Sephadex G 25 was from Pharmacia (Sweden), sodium borohydride from Serva (Heidelberg, Germany). Standard bovine brain gangliosides were from BioCarb (Sweden). Other chemicals were from Merck (Darmstadt, Germany) and of p.a. grade.

2.2. *Treatment of animals*

Male Buffalo rats with an average body weight between 350 and 400 g were intraperitoneally injected twice a day with 2dGal at a concentration of 200 mg per kg body weight for 8 days. Livers were perfused during narcosis, removed and stored at −20°C.

2.3. *Extraction and purification of glycosphingolipids (GSLs)*

All steps were carried out at 4°C. Samples of rat liver were homog-

enized in distilled water, centrifuged at 70,000 × g and the pellets washed twice by suspension in distilled water and centrifugation. The pellets were then lyophilized to dryness. Total lipids were extracted according to the method Riboni et al. [15]. Neutral GSL and phospholipids were removed by partition in diisopropylether/*n*-butanol/50 mM aqueous NaCl (6:4:5, by vol.) according to the method of Ladisch et al. [16]. The aqueous phase was evaporated to dryness To remove remaining glycerophospholipids the lipid extract was incubated with 0.2 M methanolic NaOH at 37°C for 1 h. After neutralization with 0.2 M acetic acid in methanol, the mixtures were extracted with 10 ml *n*-hexane per g liver wet weight. The samples were desalted by Sephadex G-25 column chromatography Briefly, samples were dissolved in chloroform/methanol/water (60·30.4.5; by vol.) and applied to a Sephadex G-25 column prewashed with the same eluent. GSLs were eluted with chloroform/methanol (2:1, v/v) and chloroform/methanol/water (60:30:4.5; by vol.) [17]. Eluates were pooled and evaporated to dryness. Remaining neutral GSLs and sphingomyelin were removed by silica gel chromatography. The samples were adsorbed on a silica gel column and washed thoroughly with ether. Gangliosides were then eluted with chloroform/methanol (2:1 and 1·4; v/v). Eluates were pooled and dried under vacuo

2.4. *HPLC and HPTLC procedures for analysis and detection of GSLs*

The HPLC system consisted of two pumps (Bischoff Analysentechnik, Leonberg, Germany), a programmer (Kontron Analytik, München), an UV-detector (SPD-6AV, Shimadzu, Japan), an injection valve (Model 7125, Rheodyne Incorp. USA) and a fraction collector (2211 Superrac, LKB, Sweden). Chromatography was performed according to Gazzotti et al. [18] with slight modifications: two series-connected Spherisorb-NH_2-columns (2 × 250 × 4 6 mm, particle size 5 μm; Knauer Säulen-technik, Germany) were used and the time of the gradient was extended to 150 min.

HPTLC was performed on pre-coated high-performance thin-layer plates (Silica Gel 60 for nano-DC; Merck, Germany) Gangliosides were developed in chloroform/methanol/0.2% aqueous $CaCl_2$ (50:45:10; by vol.), and visualized by staining with copper sulfate/phosphoric acid reagent (10% $CuSO_4$ in 8.5% phosphoric acid) according to Touchstone et al. [19].

2.5. *Preparation of samples for GLC*

To ascertain the rate of exchanged galactose residues, the same amounts of isolated gangliosides were hydrolysed under two different conditions: for complete release of galactose of the gangliosides, samples were hydrolysed by heating in 2 M HCl at 100°C for 3 h. Since 2dGal is degraded under these conditions, in a second procedure hydrolysation was performed with 62 5 mmol/l H_2SO_4 as described by Schmidt et al. [20]. Under this condition 2dGal is conserved and could be obtained nearly completely, but only about 10% of the other monosaccharides could be released. From this determination the content of 2dGal could be calculated, whereas the content of D-galactose is calculated under the conditions described first.

Samples were placed on a Dowex AG 3 × 4 column (acetic form, Bio-Rad, München, Germany). After elution with double-distilled water, the sample was evaporated to dryness, and monosaccharides were reduced with 2% sodium borohydride (Serva, Germany) in borate buffer, pH 9.1. Acetylation was performed as described by Geyer et al. [21] with slight modifications. Standard monosaccharides were reduced and acetylated as described above.

2.6 *Capillary gas liquid chromatography mass spectrometry (GLC/MS)*

For quantitative analysis a Carlo Erba HRCG 5300 gas chromatograph, equipped with flame ionisation detection (FID), was used as described earlier [3]. Samples were analysed on a DB210-30N capillary column (J&W Scientific, Rancho Cordova, USA) by on-column injection and on a DB1-60N capillary column (J&W Scientific, Rancho Cordova, USA) using a moving needle injection system (Chrompack, Müllheim, Germany). Hydrogen served as the carrier gas. The temperature program ws from 50°C to 100°C at 40°C/min, then raised to 240°C at 3°C/min.

A Perkin Elmer 8420 gas chromatograph coupled with a Perkin Elmer Ion Trap Detector (ITD) was used for GLC/MS analysis Samples were analysed on a DB1-60N capillary column (J&W Scientific, Rancho Cordova, USA) by on-column injection. Helium served as the carrier gas. The temperature program was from 50°C to 150°C at 30°C/min, then to 250°C at 2 8°C/min.

Peaks were identified by their retention times relative to that of inositol as internal standard, and by their corresponding mass spectra [22,23].

2.7. *Fast atom bombardment mass spectrometry (FAB/MS)*

FAB/MS was performed on a VG ZAB HF mass spectrometer using negative ion desorption [24,25]. The samples were dissolved in acetic acid/methanol 1:1 (v/v) to a concentration of about 5 μg/ml and applied to the target coated with triethanolamine as matrix. The resolution of the instrument was set to 500 ppm and the sample was bombarded with xenon atoms with a kinetic energy equivalent to 9 kV. Spectra were recorded with an AMD-Intectra data system.

3. RESULTS

The gangliosides of 2dGal-treated rat livers were purified and isolated by alkaline methanolysis and different chromatographic procedures as described in section 2. After final separation by HPLC, the purity of the different isolated gangliosides was examined by HPTLC, as shown in Fig. 1. The amounts of isolated gangliosides are summarized in Table I. All isolated gangliosides contained negligible quantities of impurities.

In order to analyse the composition of the oligosaccharide moieties of the isolated gangliosides, samples were submitted to hydrolysis as described above. Data

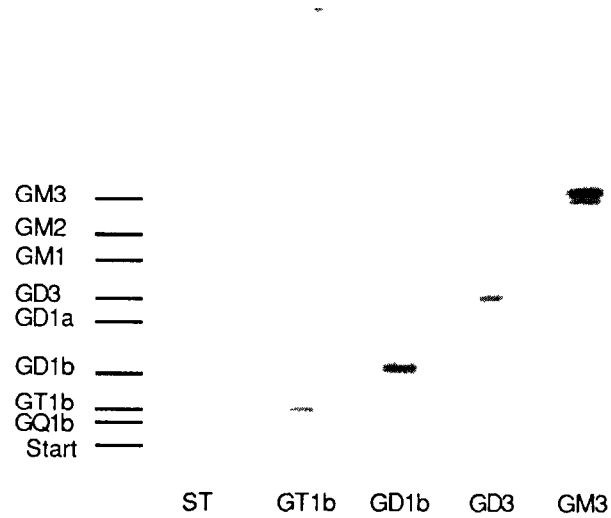

Fig. 1. Purity of the isolated gangliosides G_{M3}, G_{D3}, G_{D1b} and G_{T1b} after separation by HPLC. Gangliosides were purified by HPLC as described. After purification the samples were dissolved in chloroform/methanol (1:1; v/v). 5% of each ganglioside was applied to the HPTLC plate and separated in chloroform/methanol/0.2% aqueous $CaCl_2$ (50:45.10; by vol.) Finally, the chromatogram was stained with copper sulfate reagent. 10 μg of bovine brain gangliosides serve as a standard (ST).

Table I

Amounts of isolated gangliosides after separation by HPLC and the rate of exchange of D-galactose against 2-deoxy-D-galactose

Purified ganglioside	Amount of isolated ganglioside (nmol/g liver wet weight)	Exchange rate (D-galactose/ 2-deoxy-D-galactose)
G_{M3}	6.3	28% (40%)[a]
G_{D3}	0.6	56%
G_{D1b}	2.0	traces only
G_{T1b}	0.2	> 10%

[a] Value obtained from the sum of relative intensities of molecular ions produced by the 2-deoxy-D-galactose-containing G_{M3} isospecies by FAB/MS analysis.

from GLC/MS analysis show clearly that 2dGal is incorporated into every ganglioside investigated, for example into G_{M3} as shown in Fig. 2, but in different amounts, as shown in Table I. The gas chromatogram of the hydrolysed and peracetylated sample, Fig. 2B, shows a peak (2) which co-chromatographs with the peracetylated 2dGal. Under electric ionization conditions the fragmentation pattern of this peak resembles the peracetylated standard. Clearly detectable are the primary fragment ions at m/z 145, 159 and 231. As a secondary fragment the [M+1−60] molecular ion, after elimination of acetic acid, is detectable at m/z 317. The epimer, 2-deoxy-D-glucose, was not detected in any ganglioside.

The negative ion mass spectrum of the G_{M3} fraction derived from 2dGal-modified rat liver gangliosides is shown in Fig. 3 [24]. Major fragment ions are observed for the ceramide moiety at m/z 536 (palmitoylsphingosine) and at m/z 646 and 648 (nor marked) with $C_{24:1}$ and $C_{24:0}$ fatty acids. The corresponding ions for the monohexosylceramides are observed at m/z 698, 808, 810 (not marked) and at m/z 860, 970, 972 (not marked) for the dihexosylceramide ions, respectively. All these ions represent G_{M3} fragments that do not contain 2dGal. Among the more intense molecular ions $[M-1]^-$ that are shown in the insert, two series of ions can be clearly recognized. They may be attributed to G_{M3} isospecies (Table II) carrying C_{16}–C_{24} fatty acids and containing either two hexoses or one hexose and one deoxyhexose. The ions derived from deoxy galactose-containing species appear at 16 a.m.u. lower mass values. Isospecies with $C_{20:0}$, $C_{22:0}$ and $C_{24:0}$ fatty acids in the ceramide portion consistently showed higher relative intensities for the deoxy compound.

The FAB/MS analysis of the G_{D1b} fraction was performed on a very small amount (1 μg). Therefore, only molecular ions were observed, the major ones at m/z 1,907 $[M+2Na^+-3]^-$ and 1,885 $[M+Na^+-2]^-$ pointing to the presence of a C_{38} ceramide with either C_{20} fatty acid and sphingosine or C_{18} fatty acid and eicosasphingosine. The presence of ions of lower intensity at m/z 1,891 and 1,869 point to a partial incorporation of at least one deoxy-galactose into the G_{D1b} fraction.

4. DISCUSSION

This is the first report of the incorporation of the hexose analogue, 2-deoxy-D-galactose (2dGal), into the oligosaccharide moieties of different gangliosides. In

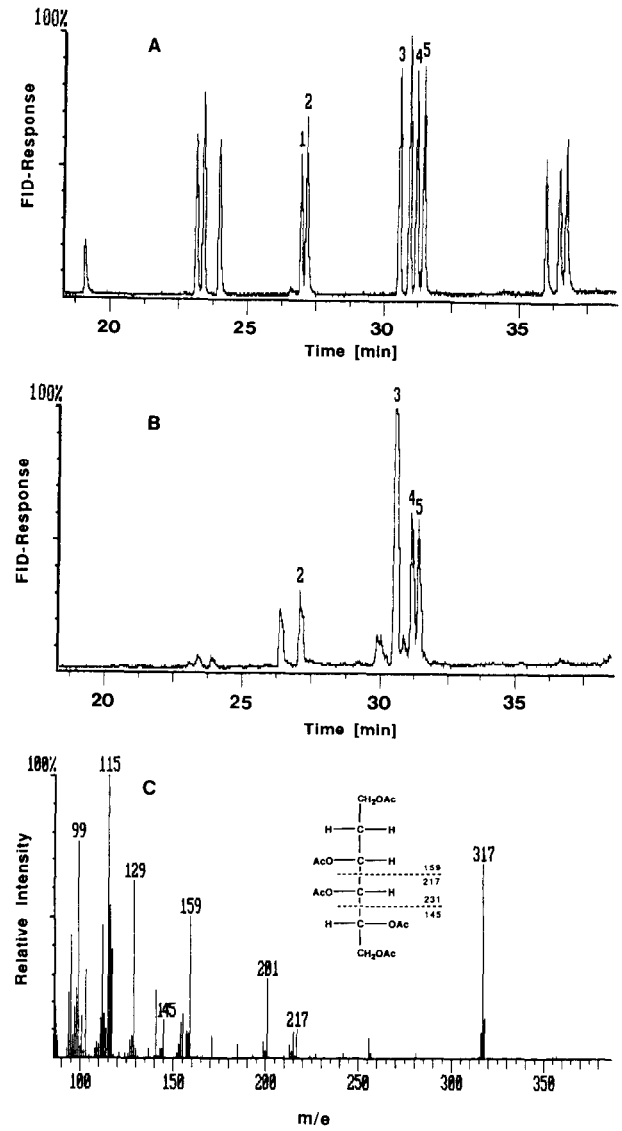

Fig. 2. Gas chromatography/mass spectrometry analysis of liver ganglioside G_{M3} from rats treated with 2dGal. The highly purified ganglioside was subjected to a mild acidic hydrolysis and the released monosaccharides were subsequently reduced and peracetylated as described. Peracetylated derivatives of alditols were analysed by GC. (A) Standrd mixture of peracetylated derivatives from left to right: 2-deoxy-D-ribitol, D-ribitol, L-fucitol, D-xylitol, 2-deoxy-D-glucitol (1), 2-deoxy-D-galactitol (2), inositol (internal standard, 3), D-mannitol, D-glucitol (4), D-galactitol (5), 2-acetamido-2-deoxy-D-glucitol, 2-acetamido-2-deoxy-D-mannitol and 2-acetamido-2-deoxy-D-galactitol. For G_{M3} expected peracetylated alditols are numbered. (B) GC of sample G_{M3}. (C) GC/mass spectrum [M+1−60] from (2) under EI conditions.

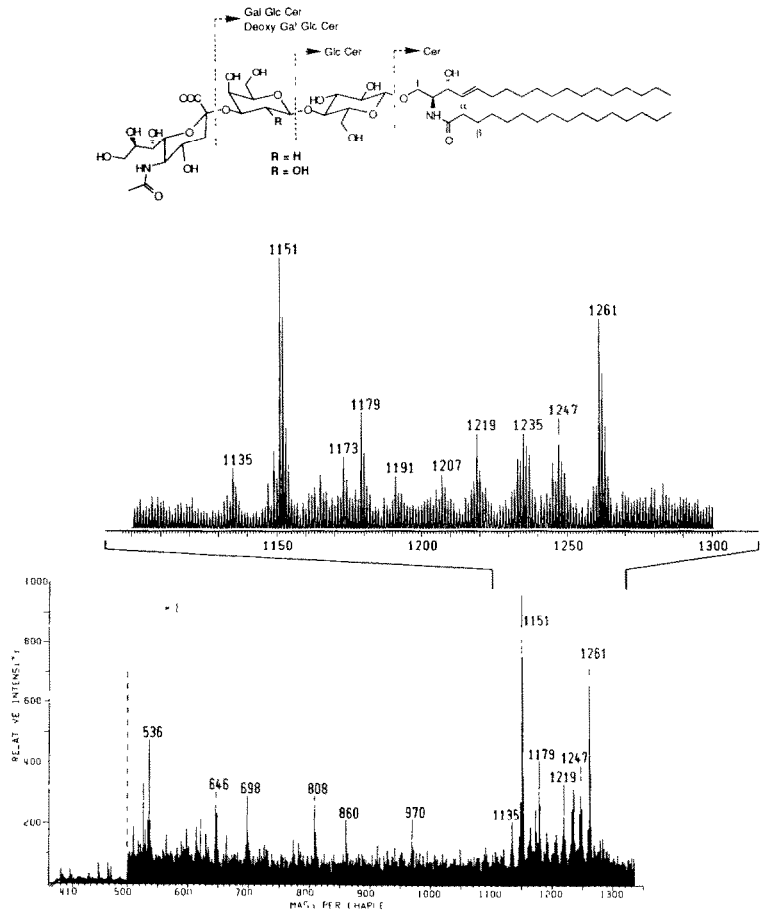

Fig. 3. Negative ion FAB mass spectrum of the G_{M3} fraction containing isospecies with 2dGal. The attribution of the molecular ions $[M-1]^-$ shown in the insert is presented in Table II. The oligosaccharide moiety of the new ganglioside, 2dGal-modified G_{M3}, is composed of D-glucose, 2dGal and *N*-acetyl-neuraminic acid. We suggest the possible substitution of the galactose residue by a 2dGal residue as shown.

earlier studies we used different approaches to demonstrate that 2dGal is incorporated into the oligosaccharide moiety of membrane glycoproteins [2,3]. These findings are in agreement with data on the metabolism of 2dGal, which indicated the formation of UDP-2-deoxy-D-galactose. Since UDP-D-galactose:glucosylceramide galactosyltransferase, which transfers activated D-galactose, is not absolutely specific for its substrate [26], it is reasonable to suppose that it can also transfer UDP-2dGal to gangliosides.

After the administration of 2dGal to rats, this hexose analogue was detected in all of the investigated gangli-

Table II

Molecular ions $[M-1]^-$ and fragment ions observed in the negative ion FAB mass spectrum of the G_{M3} fraction (Fig. 3) containing C_{16}–C_{24} fatty acids and either galactose or 2-deoxy galactose (deoxy-G_{M3})

Fatty acid of ceramide	Fragment ions			Molecular ions $[M-1]^-$	
	Cer	Glc-Cer	Gal-Glc-Cer[a]	G_{M3}	Deoxy-G_{M3}
C_{16}	536	698	860	1,151	1,135
C_{18}				1,179	1,163[b]
C_{20}				1,207	1,191
C_{22}				1,235	1,219
$C_{24:1}$	646	808	970	1,261	1,245[b]
$C_{24:0}$	648[b]	810[b]	972[b]	1,263[b]	1,247

[a] Minor fragment ions not surpassing the noise level 2-fold were not marked.
[b] Not marked in Fig. 3.

osides of rat liver. It should be noted that the difference of incorporated 2dGal into G_{M3} between GLC/MS and FAB/MS data may be due to the hydrolysation conditions prior to GLC/MS analysis. As a proportion of the replaceable galactose residues, the replacement was especially pronounced in G_{M3} and G_{D3}.

At least three reasons may account for these findings. First, the turnover of the G_{M3} and G_{D3} pool is higher than that of the other gangliosides. Second, lactosylceramide is formed on the cytoplasmic side of the *cis*-Golgi leaflet [10–12]; for all the other steps of glycosylation, UDP-D-galactose or UDP-2dGal must be transported into the Golgi apparatus by specific transporters [26]. This is in accordance with recent findings on 2dGal incorporation into membrane glycoproteins of our group [3]. In the study, we have shown that only 10% of the galactose residues of glycoproteins were replaced by 2dGal. No data are available on the selectivity of this transporter for 2dGal. Third, the mild hydrolytic conditions cleave predominantly terminal or subterminal 2dGal residues; 2dGal residues incorporated into higher gangliosides may not be accessible to this treatment.

Finally, further studies are needed to confirm the proposed structures of the 2dGal-modified gangliosides, for example nuclear magnetic resonance (NMR) analysis. Such studies would require the isolation of larger amounts of modified gangliosides. In addition it would be interesting to examine the effects of 2dGal-modified gangliosides on ganglioside metabolism in general.

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