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Acylgalactosylceramides in Developing Dysmyelinating Mutant Mice

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Abstract: Acylgalactosylceramides (AGC) from forebrains of normal and dysmyelinating (quaking and shiverer) mice were purified by Florisil column chromatography and preparative TLC. These procedures resolved the AGC on the basis of their R_f values into two main fractions which comigrate with their homologs from rat forebrains. In control animals, AGC were detectable in mouse forebrains from the eighth postnatal day and reached maximal values within 20 days. The same developmental pattern was obtained in dysmyelinating shiverer mice but the AGC content was reduced to approximately 30% of control values. In quaking mutants, the AGC were hardly detected. They were also present in sciatic nerve of normal mice and to a lesser extent in trembler mice. Gas chromatography-mass spectrometry analysis of both ester- and amide-linked fatty acids isolated from AGC of normal and shiverer mice shows that the shiverer mutant AGC display a chemical structure similar to that of normal AGC. AGC constituents of control myelin are reduced by approximately 70% in shiverer myelin, indicating that these molecules can be considered as early markers of oligodendrocyte differentiation. The early arrest of myelinogenesis in the quaking animals and the near absence of AGC are in good agreement with this proposal. Moreover, the reduced amount of AGC in the trembler PNS indicates that AGC could also be early markers for differentiation of the Schwann cell. **Key Words:** Acylgalactosylceramides—Cerebrosides—Dysmyelinating diseases—Myelinogenesis—Mutant mice. **Theret N. et al.** Acylgalactosylceramides in developing dysmyelinating mutant mice. *J. Neurochem.* **50,** 883–888 (1988).

In order to define the pathogenetic processes leading to defects in myelination, it is essential to have a clear appreciation of the dynamic relationships between different myelin sheath components. Mutant mice are particularly useful in contributing to our understanding of the role of individual myelin components in myelin development and maintenance. Both quaking and shiverer mice carry an autosomal recessive mutation which results in severe impairment in myelin formation. The shiverer mice show a defect in the CNS myelin major dense line (Privat et al., 1979). In the quaking mice, the rare myelin sheaths appear also poorly organized with frequent lack of membrane compaction (Berger, 1971; Wisniewski and Morell, 1971). Trembler mutants present onion bulb formation and alterations of PNS myelin (for review, see Hogan and Greenfield, 1984).

A glycolipid fraction of lower polarity than cerebrosides has been isolated from mammalian brains (Klenk and Lohr, 1967; Kishimoto et al., 1968; Tamai, 1968; Yasugi et al., 1982, 1983; Theret et al., 1987). It comprises three or four galactosylceramides combined with an additional fatty acid through an ester linkage. It has recently been proposed, with regard to skin, that acylgalactosylceramides (AGC) may serve as transmembranal elements involved in linking together two membranes (Wertz and Downing, 1982, 1983; Landman et al., 1984); and it is possible that these molecules might play a similar role in myelin. AGC with an 18 carbon sphingosine chain and a long fatty acyl chain (or an acylated fatty acyl chain), is a highly asymmetric molecule and displays a preferential structural organization within membranes. Asymmetric lipids extend from one mono-

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Abbreviations used: AGC, acylgalactosylceramides; GC-MS, gas chromatography-mass spectrometry; 2.18OH18:0, dihydroxystearic acid; 18OH18:0, hydroxystearic acid.

layer of the membrane bilayer to the other and interdigitate (Hui et al., 1984). So it is worth considering that AGC might play a crucial role in the formation and maintenance of the myelin sheaths.

The present study was undertaken to examine the occurrence of AGC in the myelin sheaths of normal and mutant mice forebrain or sciatic nerve.

EXPERIMENTAL PROCEDURES

Materials

Trembler mutant (B6CBA strain) originated from the Scottish mutation (from the laboratories of Dr. Guenet, Pasteur Institute); shiverer mice (C3H-SWV) were obtained from Washington University (Dr. Bird); and quaking mice (C57-B6 black) originated from Jackson Laboratories. These mutants were kindly provided by Dr. Baumann (INSERM U-134) and bred in our laboratory. Animals were compared with their normal littermates, except for shiverers which were compared with a control strain (heterozygotes are affected).

Myelin preparation

Myelin preparation was performed according to the method of Norton and Poduslo (1973). Its purity was determined by electron microscopy, by assay of marker enzymes, and by protein analysis as previously published (Bourre et al., 1984). Shiverer myelin was prepared using 0.9 *M* sucrose, a procedure which has been shown to be the most appropriate for such animals (Bourre et al., 1980). In the case of controls, myelin was also eventually prepared on 0.9 *M* sucrose in order to obviate the possibility that the altered AGC content in shiverer myelin was due to contamination by a 0.85–0.9 *M* fraction that might have been rich in these molecules.

Isolation and purification of AGC

Isolation and purification of AGC were performed as previously described (Theret et al., 1987). Pooled forebrains (20–30) from mutant and control mice were lyophilized and stored at −20°C. Tissues were extracted with $CHCl_3$/CH_3OH (2:1, vol/vol) according to the procedure of Folch et al. (1957). The pooled Folch's lower phases were evaporated to dryness, dissolved in $CHCl_3$, and applied to a 1 × 10 cm Florisil column. Cholesterol was eluted with $CHCl_3$ (50 ml), whereas more polar lipids appeared following successive washes with (a) 50 ml of $CHCl_3$/CH_3OH (97:3, vol/vol), (b) 50 ml of $CHCl_3$/CH_3OH (96:4, vol/vol), (c) 50 ml of $CHCl_3$/CH_3OH (94:6, vol/vol), and finally (d) 50 ml of pure $CHCl_3$. Purification of AGC was monitored by HPTLC. Fractions b and c were purified further by preparative TLC to isolate glycolipid ester fractions designated as AGC I, II, and III in order of decreasing R_f values. AGC I were not studied and therefore not purified further.

TLC

One dimensional analytical and preparative TLC of AGC was performed on silica gel 60 HPTLC plates using $CHCl_3$/CH_3OH/H_2O (40:10:1, by vol) as solvent (Wertz et al., 1983). The preparative TLC of nonhydroxy and hydroxy fatty acids was accomplished on silica gel 60 HPTLC plates using hexane/ethyl ether/acetic acid (70:30:1, by vol) as solvent system (Wertz and Downing, 1982). Visualization of each component was achieved by spraying the plates with Rhodamine 6G for all lipids and orcinol reagent for hexose-containing lipids.

Densitometric scans

Densitometric scans of cerebrosides and cerebroside esters were obtained after TLC separation of total lipid extracts on plastic TLC sheets developed in $CHCl_3$/CH_3OH/H_2O (40:10:1, by vol) with a Celloprofil apparatus (Sebia) after H_2SO_4 charring. If HPTLC allowed efficient AGC separation into three subfractions, a TLC run gave a single spot of cerebroside ester. Because cerebrosides and AGC gave the same standard curves, cerebrosides (0.5-1 μg) were commonly used as a convenient internal standard.

Mild alkali treatment

Saponification of AGC was performed as described by Wertz et al. (1983) with $CHCl_3$/CH_3OH/10 *M* aqueous NaOH (2:7:1, by vol). After acidification with HCl, the free fatty acids released from the AGC were extracted with hexane and subjected to GLC analysis following methylation. The deacylated AGC were purified further by preparative HPTLC using $CHCl_3$/CH_3OH/H_2O (40:10:1, by vol) as solvent system.

GLC analysis of monosaccharides

Deacylated AGC were methanolyzed in 1 *M* methanolic HCl (Gaver and Sweeley, 1965). After hexane extraction of amide-linked fatty acids, the methanolic phase containing the methylglycosides was neutralized and trimethylsilylated before GLC analysis (Kamerling et al., 1975). GLC analysis was performed with a Girdel 3000 instrument equipped with a flame ionization detector.

The trimethylsilyl derivatives of methylglycosides were analyzed with CpSil 5CB column (Chrompack) using a program which increased the column temperature from 120°C to 240°C at a rate of 2°C/min.

Gas chromatography-mass spectrometry analysis of hydroxy and nonhydroxy fatty acids

Both amide- and ester-linked fatty acids of AGC were analyzed by gas chromatography-mass spectrometry (GC-MS). The ester-linked fatty acid composition was obtained after saponification of AGC. Thereafter, amide-linked fatty acids were analyzed following methanolysis of the deacylated AGC. The trimethylsilyl and methyl derivatives of fatty acid methyl esters were analyzed on a CpSil 5CB column (25 m × 0.25 mm) using a program which increased the column temperature from 100°C to 240°C at the rate of 2°C/min. Identification of all fatty acid derivatives was confirmed by GC-MS on a Ribermag 10-10 apparatus coupled to a Sidar 21 data processor with an electron impact energy of 70 eV and an ionizing current of 0.2 mA.

RESULTS

Mouse brain AGC

The HPTLC pattern (Fig. 1) reveals the heterogeneity of mouse forebrain AGC which comigrated primarily with the two major AGC isolated from rat brain (data not shown) and identified in our previous work as AGC II and III on the basis of their decreasing R_f values (Theret et al., 1987). As was the case for the rat brain AGC, mouse brain AGC were stained with orcinol reagent indicating the presence of hexose. After saponification with alkaline reagent, they

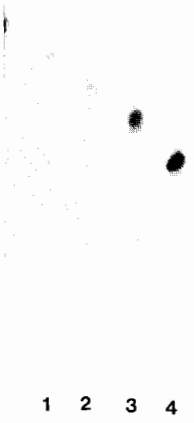

FIG. 1. HPTLC separation of purified AGC II and III in $CHCl_3/CH_3OH/H_2O$ (40:10:1, by vol). Lane 1: ceramides. Lane 2: AGC II. Lane 3: AGC III. Lane 4: cerebrosides.

were converted mainly into cerebrosides and free fatty acids, suggesting the presence of ester-linked fatty acids in their structure.

GLC of trimethylsilylated methylglycosides formed from the carbohydrate portion of AGC demonstrated the presence of galactose as the unique sugar component for mouse AGC.

Table 1 gives the fatty acid distribution in mouse AGC II and AGC III. The ester-linked fatty acid composition was performed after saponification; thereafter, the amide-linked fatty acid composition was achieved after methanolysis of deacylated AGC. Since it is known that alkanes as long as squalene are found in mammalian brains (Nicholas and Bombaugh, 1965; Bourre et al., 1977) causing difficulty in characterizing some fatty acids, the GLC identification of fatty acids was coupled with mass spectrometric analysis. Only unambiguously identified fatty acids are listed in Table 1. Some minor differences were registered between mouse and rat AGC fatty acids (Theret et al., 1987). For both animals, ester-linked fatty acids were composed mainly of palmitic, stearic, and oleic acid, but palmitic acid represented the major fatty acid in mouse AGC. Moreover, mouse AGC contained more myristic and palmitic acid and less stearic acid than rat AGC. In contrast, monounsaturated fatty acid content was similar in both animals. Amide-linked fatty acids were rather longer and more unsaturated than their ester-linked counterparts. However, the high ratio 18:0 + 18:1/16:0 + 16:1 may represent a special feature of mouse amide-linked fatty acids in comparison with rat. The other particularity of amide-linked fatty acids is the presence of hydroxy fatty acids (none of these hydroxy fatty acids was encountered in the ester-linked population). The hydroxy fatty acids were identified by GC-MS as previously described (Theret et al., 1987). The presence of ω-hydroxy stearic acid (18OH18:0) was restricted to AGC II, whereas α,ω-dihydroxystearic acid (2.18OH18:0) was only found in AGC III.

AGC during myelinogenesis

A marked change of AGC levels was observed during the course of myelinogenesis (Fig. 2). This change is clearly demonstrated in the densitometric scan of glycolipids on TLC. Only minute amounts of AGC could be detected in 10-day-old brains; thereafter, the

TABLE 1. *Fatty acid composition of AGC in normal and shiverer mice forebrains*

	Galactocerebrosides Normal mice		AGC							
			Normal mice				Shiverer mice			
			Ester		Amide		Ester		Amide	
	Cer	OH-Cer	II	III	II	III	II	III	II	III
C14:0	3.6	5.3	7.3	5.5	3.5	7.3	2.4	10.0	10.8	5.4
C16:0	3.6	6.0	40.3	40.8	25.0	20.8	44.0	38.7	18.0	22.0
C16:1	1.4	0.8	4.0	4.0	2.8	1.6	5.4	6.6	2.8	2.5
C18:0	7.0	10.5	21.3	13.9	19.0	17.8	20.0	14.1	14.0	18.4
C18:1	6.4	12.0	20.6	23.5	34.5	36.4	17.5	17.5	34.9	35.3
C18:2 n-6	3.0	1.0								
C20:0	1.0	1.8	0.9	4.4	3.0	4.0	1.5	5.8	1.6	3.5
C20:1	2.5	4.2	1.7	3.0	1.5	1.2	1.8	1.6	3.8	1.9
C20:4 n-6	1.8									
C22:0	8.7	6.9	0.5	1.2	4.0	4.1	0.6	1.7	4.8	3.8
C22:1	0.8		0.7	1.0	2.5	2.0	0.4	0.7	2.4	2.5
C24:0	28.7	3.8	1.3	1.2	3.1	4.0	4.3	2.0	4.6	3.5
C24:1	31.5	4.0	1.4	1.5	1.1	0.8	2.1	1.3	2.3	1.2
OHC18		1.4								
OHC20		1.6								
OHC22		20.2								
OHC24		20.5								

Values are expressed as percent distribution. Cer, nonhydroxylated cerebrosides; OH-Cer, hydroxylated cerebrosides.

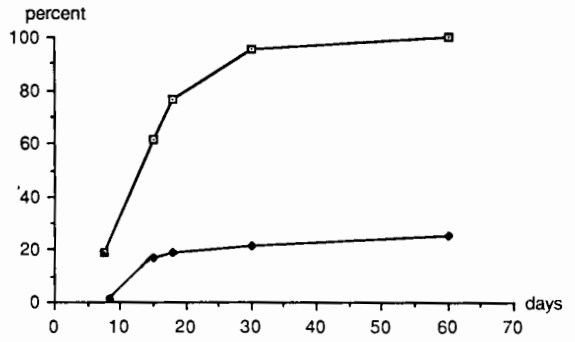

FIG. 2. Evolution of the level of AGC (♦) and nonhydroxylated cerebrosides (□) in normal mouse brain during myelination. The results are expressed as a function of the amount of nonhydroxylated cerebroside in normal mice at 60 days, which was arbitrarily given the value of 100%.

AGC content increased sharply during myelination in the mouse cerebral hemispheres (the AGC content increased 11-fold between days 10 and 30). As myelination was accompanied by proportionate increases of hydroxy and nonhydroxy cerebrosides (Bauman et al., 1973), the ratios of AGC to nonhydroxylated cerebrosides and of AGC to hydroxylated cerebrosides were shown to be constant throughout brain development, with mean values of 0.16 and 0.08, respectively (Table 2).

AGC in dysmyelinating mutants

Quantitative abnormalities were detected in the mutant lipids. The fast-moving spot corresponding to nonhydroxy cerebrosides completely disappeared in quaking mouse brain extracts, and AGC were reduced to undetectable levels (Fig. 3). For shiverer mutants, the level of brain AGC rose with myelinogenesis and began to increase soon after the 10th day (Table 2); however, it was always lower than the level of AGC in normal mice. Globally, the shiverer AGC were reduced to nearly 30% of the control value, as were the other galactolipids.

High amounts of AGC were found in normal sciatic nerve in contrast with the dysmyelinating trembler mutant (Fig. 3).

Compositional analysis of AGC from shiverer and normal mice was performed on the AGC II and III fractions. For both control and shiverer AGC, GLC of trimethylsilylated methylglycosides formed from the carbohydrate portion of AGC indicates the presence of galactose as a unique sugar residue.

Table 1 gives the fatty acid distribution of AGC from shiverer mice as results of GC-MS analysis. It is worth noting that there were only minute amounts of long chain fatty acids in the AGC of both normal and dysmyelinating mice when compared with hydroxylated or nonhydroxylated cerebrosides. Otherwise, the fatty acid compositions of normal and mutant AGC were similar. Ester-linked fatty acids of AGC from shiverer were similar to those from control brains, and were composed mainly of palmitic, stearic, and oleic acids. The same homology was also found with the amide-linked fatty acids, which again contained 18OH18:0 in AGC II, with a ratio 18OH18:0/18:0 of 0.25, and 2.18OH18:0 in AGC III, with a ratio 2.18OH18:0/18:0 of 0.65. No significant differences between control and mutant mice were detected in these lipids.

AGC and myelin

AGC were present in myelin (Fig. 3). The yield of AGC in the 0.9 *M* sucrose myelin fraction was less than from the 0.85 *M* sucrose fraction, confirming that AGC are natural constituents of CNS myelin; their content was increased 1.5-fold between 0.9 *M* and 0.85 *M* sucrose fractions (the 0.85 *M* sucrose fraction corresponding to the more purified myelin). In shiverer myelin, AGC were also present but to a lesser extent, insufficient to be quantified with confidence.

DISCUSSION

Our working hypothesis was that AGC may be involved in maintaining the integrity of myelin mem-

TABLE 2. *Hydroxylated and nonhydroxylated cerebrosides and AGC during myelination in the normal and shiverer mouse*

	OH-Cer	Cer	AGC/OH-Cer × 100	AGC/Cer × 100	AGC shi/AGC control × 100
Control					
10 days	0.2	0.1	8.6	16.5	—
20 days	0.2	0.5	7.3	19.3	—
30 days	2.4	1.0	6.8	16.1	—
Shiverer					
10 days	—	traces	—	7.8	26.7
20 days	—	0.2	—	16.0	40.4
30 days	0.8	0.3	4.9	14.3	24.2

The results are expressed as a function of the amount of cerebroside in normal mice at 30 days, which was arbitrarily given the value of 1. Cer, nonhydroxylated cerebrosides; OH-Cer, hydroxylated cerebrosides; shi, shiverer.

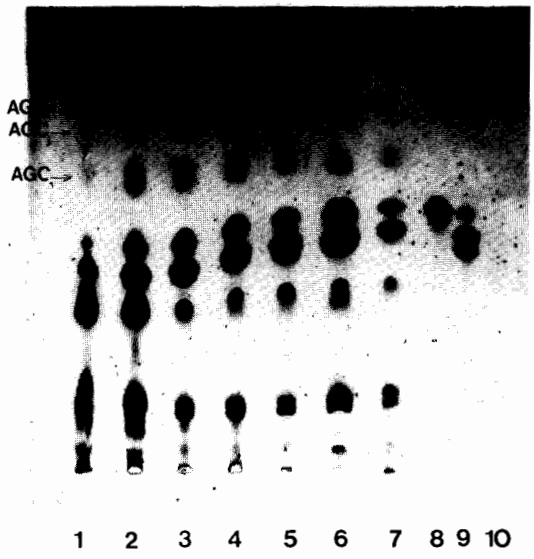

FIG. 3. HPTLC analysis of CNS and PNS crude lipids extracted from the following: Lane 1: quaking forebrain [dry weight (DW): 6 mg]; lane 2: control quaking forebrain (DW: 6 mg); lane 3: myelin:0.9 *M* sucrose control shiverer (DW: 1 mg); lane 4: myelin:0.85 *M* sucrose control shiverer (DW: 1 mg); lane 5: myelin:0.9 *M* sucrose shiverer (DW: 1 mg); lane 6: control trembler sciatic nerves (DW: 3 mg); lane 7: trembler sciatic nerves (DW: 3 mg); lane 8: cerebrosides; lane 9: hydroxylated cerebrosides; and lane 10: ceramides. HPTLC was developed in $CHCl_3/CH_3OH/H_2O$ (40:10:1, by vol).

branes. So it was deemed worthwhile to investigate the presence of AGC in CNS and PNS myelin in order to uncover different features between control normal mice and the dysmyelinating mutants, and to correlate these changes with the eventual role of AGC in myelinogenesis.

AGC in control mice

Mice AGC were purified according to the procedure employed for rat AGC (Theret et al., 1987). It was possible to demonstrate the presence of two major pools of brain AGC comigrating with the AGC II and AGC III of rat brains. GLC compositional analysis did not reveal any major difference between rat and mice AGC. Both contained galactose as unique sugar, and the composition of fatty acids either linked by ester or amide bonds presented no consistent difference. As for rat AGC, the amide-linked fatty acids were not as long as those encountered in epidermal AGC (Wertz and Downing, 1982). GC-MS analysis confirmed, once again, the absence of very long fatty acids in brain AGC.

AGC in myelinogenesis

The developmental increase in the level of AGC is perfectly correlated with the accumulation of myelin in the brain. AGC were detectable first after 10 days of age, and never earlier. Moreover, their variation during development is in step with the accumulation of cerebrosides (Fig. 2). It is, in fact, very interesting to compare the accumulation of AGC and the accumulation of cerebrosides, because their syntheses are closely linked. Cerebrosides are present even in 8-day-old brains (this is not the case for AGC), but they remain in the cytosol until myelination begins. At that point, it appears that there is a transfer of newly synthesized cerebrosides from the endoplasmic reticulum or Golgi apparatus to the myelin sheaths, mediated by cytosolic components.

AGC deposition is closely linked to myelinogenesis in normal mice and starts soon after the eighth postnatal day. From then until mature levels are reached (at 30 days), AGC consistently account for approximatively 15% of the nonhydroxylated cerebrosides. This phenomenon suggests that AGC are derived from the acylation of a precursor ceramide or galactolipid in the myelin sheath.

AGC in dysmyelinating mutants

It is clearly evident that AGC are severely reduced in both shiverer and quaking brains. This sharp drop in AGC content fits well with the proposed involvement of AGC in myelin assembly, which is defective in both mutants.

In quaking mutants, the AGC content is undetectable (Fig. 3). The failure in maturation of myelin, also termed as an arrest of myelinogenesis (Hogan and Greenfield, 1984), is correlated biochemically with a defect in galactosyltransferase activity and with the presence of immature fatty acids in myelin-associated lipids (Baumann et al., 1973). The level of hydroxy fatty acids in quaking mice represents only 30% of the control value. That means that in this mutant the two potential sites of esterification (galactose and ω-hydroxy fatty acids) are reduced or absent. It is tempting to attribute the defect of AGC in quaking mice to a defect in the acylation step, in addition to an impaired synthesis of galactolipids. Possibly the enzymes and/or substrates for the synthesis of AGC may be defective or compartmentalized.

In shiverer mutants, a sharp decrease in AGC and cerebrosides is also evident (Table 2). This is also in good agreement with a decrease in the cerebroside level. We report here that AGC are also reduced to 30% of the AGC content of the control. For these mutants, two mechanisms might be impaired during AGC metabolism. These may be a defect either in the synthesis of AGC precursors or in the esterification step of the precursors which supposes special species of galactolipids (with less 24:0 and 24:1 in the molecule), their segregation to the right place, and the presence of acyltransferase. As the ratio of AGC to nonhydroxylated cerebrosides in the mutants is not significantly impaired and the fatty acid composition not altered, it seems that the reduced levels of AGC in shiverer brain may be due to an initial defect in galactolipid production. The acylation step seems to be constant, but depends on limited availability of AGC precursors.

In order to provide an independent source of evidence, we investigated the structure of sciatic nerves. Once again, AGC were present in controls and reduced sharply in trembler mice which are characterized by a severe hypomyelination of the peripheral nerves (Ayers and Anderson, 1973; Low, 1976*a,b;* Aguayo and Bray, 1982).

AGC—natural constituent of myelin

This study provided evidence that AGC are components of myelin sheaths and are probably involved in the maintenance of myelin membrane stability. Moreover, AGC are probably early markers of oligodendrocyte and Schwann cell differentiation. They are nearly totally absent in quaking mice (in which myelinogenesis is arrested due to an unknown alteration in oligodendrocyte differentiation), dramatically reduced in trembler mice (in which there is an indeterminable alteration in Schwann cell differentiation), and slightly reduced in shiverer mutants (in which myelin is nearly absent, but the oligodendrocyte is normally differentiated). In this mutant, the level of AGC is much higher than expected when taking into account the near absence of myelin and might indicate a probable accumulation in oligodendrocytes.

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