Accumulation of cholesteryl esters associated with cerebellar hypoplasia in jaundiced Gunn rats[1]

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Summary. A marked accumulation of cholesteryl esters in the cerebellum of 20-day-old jaundiced homozygous Gunn rats was shown by quantitative TLC analyses and it was larger than that found in heterozygotes.

Hyperbilirubinemic homozygous (jj) Gunn rats show a marked cerebellar hypoplasia[2-4], and serve as a unique model for the study of pathogenic mechanisms of bilirubin-induced encephalopathy. Morphological observations have shown that Purkinje cells in the hypoplastic cerebellum contain bizarre membranous bodies in their cytoplasm which are suggested to be formed in consequence of an excessive accumulation of phospho- or glyco-lipids[2]. A recent report from our laboratory revealed that the concentration of cerebellar lipids in jj rats was increased compared with that in j+ rats (heterozygotes) and was inversely correlated with the cerebellar wet weight[5]. Thus, further studies were performed to clarify the composition of lipids accumulated in the hypoplastic jj rat cerebellum severely affected by bilirubin.

Materials and methods. 20-day-old jj and j+ (control) Gunn rats of Wistar and Sprague-Dawley[6] strains obtained from the closed colonies of our department were used. Preparation of cerebellar samples and extraction of lipids were done as previously described[5], except that during the extraction procedure the upper (methanol-water) and lower (chloroform) layers were washed twice with equal volumes of the theoretical lower and upper layers, respectively. The lower layer finally obtained was evaporated to dryness with a rotatory evaporator and dissolved in a known amount of chloroform-methanol (2:1, v/v) mixture. The solvent systems used for TLC on pre-coated HPTLC plates (Merck) were as follows: n-hexane/ethyl ether/glacial acetic acid (80:30:1, v/v) for cholesterol (CH), cholesteryl ester (CHE), triacylglycerol (TAG) and free fatty acid (FFA); chloroform/methanol/water (65:25:4, v/v) for cerebroside (CE); and chloroform/methanol/glacial acetic acid/10% aqueous $NaHSO_3$ (50:30:6:1.5, v/v)[7] for phospholipid (PL). All plates were prewashed with the respective solvent mixtures before the separation procedure. After development the plates were dried at 130 °C for 5–10 min before being sprayed with reagent. The spray regents used were the Dittmer reagent[8] for PL and the copper acetate reagent[9,10] for other lipids. In the latter case the plates were heated at 130 °C for 30 min to assure complete color development. Quantitative densitometric determinations were carried out at 720 and 600 nm for PL and other lipids, respectively, with a Shimadzu CS-910 Dual-Wavelength TLC Scanner after the plates were kept at room temperature for 60 min. CH and its palmitate, oleic acid, and natural CE and PL (Serdary Res. Labs, London, Canada) were co-chromatographed on the same plates and served as standards. All measurements were done within the range of the linear relationship between absorbance and the amount of each lipid. Phosphorus contents in the extracted lipids and the standard natural PL preparations were assayed by the method of Bartlett[11]. Total PL in the extracted lipids was calculated according to Dickerson[12]. Statistical significance was evaluated by a t-test or a non-parametric method.

Results. As shown in table 1, the concentrations of lipids in cerebella of 20-day-old jj rats of Wistar and Sprague-Dawley strains were significantly increased by as much as about 40 and 70% compared with those of the respective j+ rats. No differences in cerebellar dry weight on a wet

Table 1. Cerebellar wet weight, dry weight and lipid concentration in 20-day-old homozygous (jj) and heterozygous (j+) Gunn rats of Wistar and Sprague-Dawley strains

Strain	Genotype	No.*	Wet weight (mg)	Dry weight (mg/g of wet weight)	Lipid concentration (mg/g of wet weight)
Wistar	j+	9	160.2 ± 13.3 (137.3–180.1)	193 ± 4 (189–199)	50.5 ± 7.5 (37.8–58.7)
	jj	5	41.3 ± 6.3** (31.9–47.0)	194 ± 14 (179–213)	69.4 ± 12.5*** (55.5–89.1)
Sprague-Dawley	j+	5	158.5 ± 10.2 (144.0–170.4)	194 ± 3 (191–198)	46.5 ± 8.0 (39.3–58.5)
	jj	4	36.5 ± 10.3** (25.6–49.0)	187 ± 6 (180–195)	81.0 ± 7.1*** (72.5–89.8)

Each value is an average ± SD with the range in parenthesis. *Number of animals used. **$p < 0.001$; ***$p < 0.01$ in comparison with the respective j+ rats.

Table 2. Composition of lipids in cerebella of 20-day-old homozygous (jj) and heterozygous (j+) Gunn rats of Wistar strain

Lipid	Genotype	No.*	Composition (% of dry weight) Average ± SD	Range
Cholesterol	j+	9	4.33 ± 0.89	3.29–6.39
	jj	5	5.79 ± 0.60***	4.99–6.52
Cholesteryl	j+	9	0.16 ± 0.07	0.06–0.27
ester	jj	5	2.78 ± 2.31**	1.14–6.23
Free fatty	j+	9	0.59 ± 0.21	0.23–0.90
acid	jj	5	1.45 ± 0.73**	0.93–2.72
Phospholipid	j+	9	17.4 ± 3.4	13.7–24.5
	jj	5	17.5 ± 1.6	15.4–19.2
Cerebroside	j+	6	1.23 ± 0.42	0.73–1.83
	jj	5	1.73 ± 0.52	0.92–2.18

*Number of animals used. **$p < 0.01$; ***$p < 0.02$ in comparison with the respective j+ rats.

Table 3. Composition of phospholipids in cerebella of 20-day-old homozygous (jj) and heterozygous (j+) Gunn rats of Wistar strain

Phospholipid	Genotype	Composition (nmoles phosphorus/mg of dry weight) Average ± SD*	Range
Lysophosphatidyl choline	j+	1.0 ± 0.3	0.7– 1.3
	jj	5.0 ± 3.0**	2.1– 9.1
Sphingomyelin	j+	11.8 ± 2.7	9.1– 15.6
	jj	12.4 ± 3.3	9.1– 16.8
Diphosphatidyl glycerol	j+	4.4 ± 1.3	2.7– 5.9
	jj	3.8 ± 0.9	2.6– 4.0
Phosphatidyl serine +	j+	30.5 ± 4.4	24.6– 34.7
inositol	jj	25.9 ± 0.8	25.2– 28.7
Phosphatidyl choline	j+	92.7 ± 19.2	65.9–109.2
	jj	92.3 ± 11.3	80.7–107.3
Phosphatidyl ethanolamine	j+	98.4 ± 26.8	82.6–129.4
	jj	88.9 ± 6.4	83.3– 95.9

Total phosphorus contents (average ± SD) in the extracted lipids were 258.7 ± 39.5 (n = 4) and 220.0 ± 18.2 (n = 4) nmol/mg of dry weight in j+ and jj rats, respectively, with no statistically significant difference between them. Recoveries of phosphorus contents were 92.3% in j+ rats and 103.8% in jj rats. *Four rats. **$p < 0.05$ in comparison with j+ rat.

weight basis were observed between jj and j+ rats of both strains, implying that water contents in jj and j+ rat cerebella at the postnatal day of 20 were almost at the same level of approximately 80%.

To clarify the composition of lipids accumulated in the hypoplastic cerebellum of jj rats, cerebellar lipids from Wistar strain rats were analyzed by TLC and densitometrically quantitated. As seen in table 2, CE and total PL showed no differences between the 2 groups of rats, but CH and FFA were about 1.3 and 2.5 times higher in jj rats than those in j+ rats, respectively. The CHE content in individual cerebella of jj rats was highly variable but distinctly separated from that of j+ rats. The average value in the former rats was 17 times as high as that in the latter. A similar increase of CHE was also observed in jj rats of the Sprague-Dawley strain (in percent of cerebellar dry weight, 0.67 ± 0.37 (range, 0.11-0.88; $n=4$) in j+ rats and 7.86 ± 4.36 (range, 4.00-12.6; $n=3$) in jj rats). It was also found that in jj rats the content of CHE was roughly proportional to the degree of cerebellar hypoplasia. Under the experimental conditions of the assay, TAG was below the limits of detection in both jj and j+ rat cerebella of Wistar and Sprague-Dawley strains.

Although the content of PL was not different between jj and j+ rats, it was expected from the enhanced level of FFA in jj rats that minor components such as lyso-type phosphoglycerolipids might be increased. Thus, the composition of cerebellar PL from Wistar strain rats was next investigated. As shown in table 3, the composition of PL except lysophosphatidyl choline (LPC) did not differ significantly between the 2 rats. Like the case of CHE, the level of LPC in individual jj rats was markedly scattered, but no values obtained from jj rats overlapped with those from j+ rats. A 5-fold increase of LPC, on average, was noted in jj rats. Lysophosphatidyl ethanolamine and phosphatidic acid were below the limits of detection in cerebella of both jj and j+ rats.

Discussion. The present report clearly showed that there was a marked increase of CHE in the bilirubin-induced hypoplastic cerebellum of jaundiced jj rats. This is consistent with our recent histochemical observations[13]. It is well known that CHE exists only in small amounts in normal adult mammalian brains but is high at the time just prior to the commencement of active myelination[14]. It has also been demonstrated that CHE appears in substantial quantities under various pathological demyelinating conditions[15,16]. The mechanisms by which CH is esterified to such a striking degree in the jj rat cerebellum remain obscure at present. However, it is unlikely that the process of myelin breakdown is involved in the CHE accumulation. This is supported by the observations that there are little or no histological changes in the cerebellar medullae of jj rats[2], and also that the activity of 2′,3′-cyclic nucleotide 3′-phosphohydrolase[17] and the content of CE (table 2) do not differ significantly between cerebella of jj and j+ rats.

Biochemical[17] and histochemical[18] studies in our laboratory revealed that the specific activity of cerebellar arylsulfatase, a marker enzyme for lysosomes, was significantly elevated in jj rats, with a peak around 20 days of life which was about 3 times as high as that in j+ rats, and that the appearance of some cells with the stronger lysosomal acid phosphatase activity was accompanied with the higher degree of cerebellar hypoplasia. These observations would appear to suggest that the accumulation of CHE was closely related to the bilirubin-induced enhancement of lysosomal activities. The source of fatty acids for an increased formation of CHE in some brain diseases has been suggested to be the β-linked fatty acids of phosphatidyl choline[15]. On the basis of the significant increase of LPC (table 3), therefore, the possibility would exist that in the jj rat cerebellum a similar mechanism of CH esterification was, though not entirely accountable in a stoichiometric sense, operating irrespective of myelin breakdown.

Although there is limited information as to the cerebellar lipid composition in weanling rats, and almost none for Gunn rats, the cerebellar level of CHE in j+ (control) rats seems to be considerably higher than that reported for the whole rat brain (see Eto and Suzuki[19]). Since TAG was hardly detectable with the method used here, the contributions of blood lipids to the present data would be, if any, negligible. Furthermore, preliminary qualitative examinations by 2-dimensional TLC with use of a silver nitrate-treated plate for the 2nd dimension showed that fatty acid moieties of CHE from the Gunn rat cerebellum consisted of mainly saturated, monoenoic and polyenoic (arachidonic or longer chain) fatty acids. This pattern is distinct from that of serum[19,20]. On the other hand, it has been reported that FFA is dramatically increased during post-decapitation ischemia[21-23] with a suggestion of phospholipase A activation[21]. A high cerebellar level of FFA in 20-day-old j+ rats compared with that in adult rats[22] may be largely due to such an ischemic process, although the cerebella of animals employed were rapidly removed, weighed and frozen at −80 °C, followed by lyophilization. The increase of FFA and LPC in the jj rat cerebellum appears to suggest that the activation of phospholipases A_1 and A_2[21] in situ as well as in the post-decapitation period occurs more strongly in jj rats than in j+ rats.

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