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Developmental Control of the Expression of Two Ten-sugar Branched Chain Fucolipids in Rat Small Intestine

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Two branched decaglycosylceramides, apparently identical to those identified in the small intestine of adult rats [Breimer ME, Falk K-E, Hansson GC, Karlsson K-A (1982) J Biol Chem 257:50-59], were absent during the three weeks following birth. They appeared abruptly at around 21 days. After their appearance, their tissue concentration and their base composition did not change during development. Their fatty acids were non-hydroxylated and the percentage of C_{22}-C_{24} fatty acids, which was low at 24 days, increased and reached 48.6% by 27 days.

Glycosphingolipids are known to be markers of cellular differentiation [1]. Glycolipids with short sugar chains, namely glucosyl-, lactosyl- and globotriaosyl-ceramide, and hematoside (G_{M3}-ganglioside) undergo qualitative and quantitative modifications during the postnatal development of rat small intestine [2, 3]. These modifications are specific for the ontogenic differentiation and differ from what is observed in the crypt-villus differentiation of the adult intestine [4, 5].

Beside glycolipids with a short glycosidic chain, the intestine of the adult rat contains several glycolipids with a long sugar chain. Breimer *et al.* [6, 7] have investigated two strains of rats and shown that one strain contains blood group A- and H-active glycolipids, whereas the other lacks the A-active glycolipids. In this latter strain, two branched blood group H-active decaglycosylceramides (H-10 glycolipids) have been found in the epithelial cells of small intestine [8]. One of these fucolipids contains exclusively the type 1 (Galβ1-3GlcNAc) carbohydrate chain, while the other contains type 2 (Galβ1-4GlcNAc) and type 1 carbohydrate chains (Fig. 1A and B respectively). They both differ

Nomenclature: Globotriaosylceramide ($GbOse_3Cer$), Galα1-4Galβ1-4GlcCer; G_{M3}-ganglioside, II^{3}NeuAc-LacCer; globoside (globotetraosylceramide, $GbOse_4Cer$), GalNAcβ1-3Galα1-4Galβ1-4GlcCer.

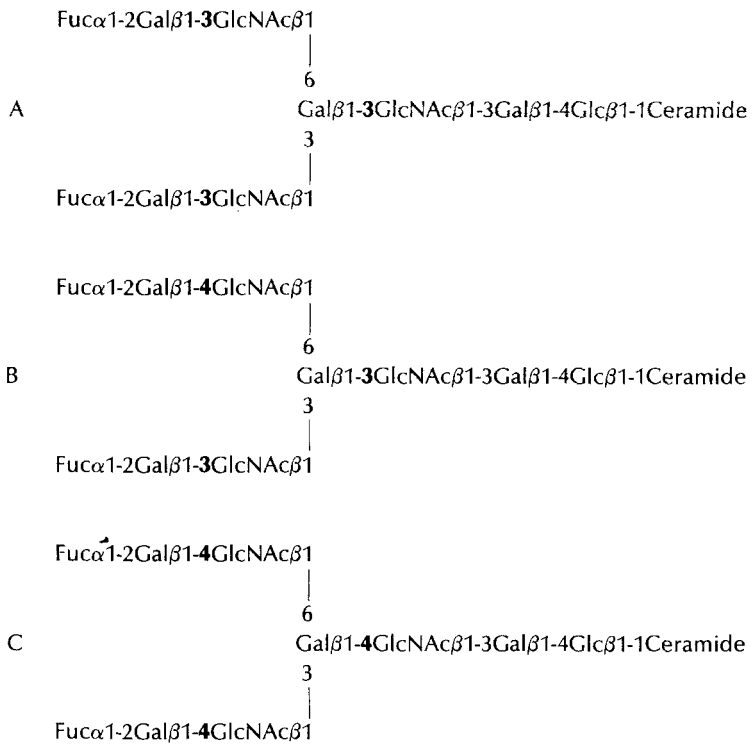

Figure 1. Structure of blood group H-active decaglycosylceramides. A and B: Structures of the decaglycosylceramides of rat small intestine identified by Breimer *et al.* [8] and designated as H-10 glycolipids by these authors. C: Structure of the decaglycosylceramide of human blood group O erythrocytes identified by Watanabe *et al.* [9] and designated as H_3-glycolipid by these authors.

from the blood group H-active decaglycosylceramide (H_3-glycolipid) of human erythrocytes which has the same sugars in sequence but exclusively the type 2 saccharide chain (Fig. 1C) [9].

In human erythrocytes, the concentration of H_3-glycolipid is low before birth and increases during the first year [10, 11]. It was of interest to know whether the branched decaglycosylceramides of rat small intestine were subject to developmental changes. Here, we present evidence that they appeared abruptly on the 21st day of life, a critical time in the maturation of the intestinal epithelium.

Experimental Procedures

Sprague-Dawley rats were obtained from the local animal farm. Day 1 of age was the day after birth. The whole intestine was excised and flushed thoroughly with saline. Standard glycolipids were extracted from the red blood cells of outdated group O blood. Lipids were extracted successively in chloroform/methanol (1/1 by vol) and twice in chloroform/methanol/water (30/60/8 by vol). The dry residue remaining after extraction of the lipids was dissolved in 2 N sodium hydroxide and the protein content was assayed by the method of Lowry *et al.* [12].

H-10-like Glycolipid Purification

A lipid extract enriched in polar glycolipids was obtained by partition of the total lipid extract according to the method of Folch *et al.* [13], and six washings of the lower phase with the Folch theoretical upper phase. The combined upper phases were desalted through a Sep-Pak C18 cartridge (Water Associates, Milford, MA, USA), after saponification of the alkali-labile phospholipids. In some instances, glycolipids were purified by acetylation of the total lipid extract and chromatography on a Florisil column according to the method of Saito and Hakomori [14]. In both cases, the neutral glycolipids were separated from the acidic lipids by chromatography on DEAE-Sephadex (A-25, acetate form, 0.5 g) [15].

Glycolipids were analyzed on Silica Gel 60 high performance TLC plates (Merck, Darmstadt, West Germany) in the following solvents: (A) chloroform/methanol/water (60/35/8 by vol), and (B) chloroform/methanol/water (55/45/10 by vol). They were visualized with the 1-naphthol/sulfuric acid reagent. For preparative purposes, the glycolipid mixture enriched in H-10-like glycolipid was chromatographed in solvent B and the glycolipid position was localised in u.v. light after spraying with a solution of Primuline (ICN Pharmaceuticals, Plainview, NY, USA) [16].

Structural Analysis

The ceramide portion was analyzed after glycolipid hydrolysis in methanol/conc. HCl/water (83/8.6/9.4 by vol). Sphingoid bases were analyzed as aldehydes according to the method of Sweeley and Moscatelli [17]. The methylation of fatty acids was completed in 0.8 N anhydrous methanolic HCl at 80°C for 2 h and the fatty acid methyl esters were analyzed as already described [18].

For carbohydrate analysis, the purified glycolipid was permethylated by the method of Hakomori [19] modified by Finne *et al.* [20]. The permethylated glycolipid was submitted to acetolysis/hydrolysis, reduction and acetylation as described by Stellner *et al.* [21]. The partially methylated alditol acetates were analyzed by GLC on a fused silica capillary column (0.2 mm × 25 m) wall-coated with SP 2100.
The injector temperature was 200°C. The oven temperature was programmed from 150°C to 220°C at 1.5°C/min. The detector temperature was set at 300°C. All GLC analyses were performed on a Hewlett-Packard 5710A chromatograph equipped with a flame ionization detector. The detector signal was recorded on a Hewlett-Packard 3390A integrator.

Results

In preliminary experiments, we checked the presence or absence of H-10-like glycolipids by TLC of the neutral glycolipids of the Folch upper phase obtained by partition of the total lipid extracts from the intestine of 10 individual rats of the same litter at the same age. The H-10-like glycolipid was absent at birth, at 6, 13 and 21 days, and present at 28 days. At 28 days, H-10-like glycolipid had the same position on the thin layer chro-

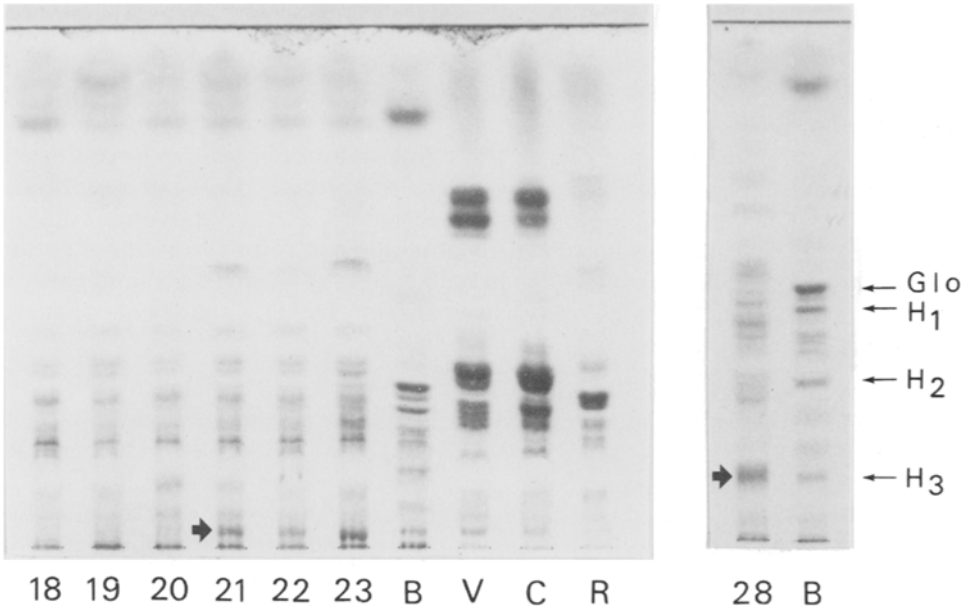

Figure 2. Thin layer chromatogram of the Folch upper phase neutral glycolipids of rat small intestine during development. Numbers refer to the age of rats in days. Glycolipids corresponded to 9.4 mg protein at 18 days, 12.7 mg at 19 days, 10.5 mg at 20 days, 10.3 mg at 21 days, 11.0 mg at 22 days, and 13.3 mg at 23 days. (B), Folch upper phase neutral glycolipids of blood group O human red blood cells; (V), (C) and (R), total neutral glycolipids of villus and crypt cells, and the non-epithelial residue, respectively, of an adult rat intestine. The arrow indicates the position of H-10-like glycolipids. Chromatogram on the left, solvent A; chromatogram on the right, solvent B.
Abbreviations: Glo, globoside; H_1, H_2, H_3, blood group H-active glycolipids according to the nomenclature of Watanabe *et al.* [9].

matogram as the H_3-decaglycosylceramide of human erythrocytes (Fig. 1 and 2), although the spot was more diffuse. Thus, we observed the appearance of H-10-like glycolipid between the end of the third and the fourth week.

We had to take into account the possible tissue specificity of individuals of a mixed population in order to trace the developmental appearance of a glycolipid with a blood group determinant. We observed that more than 90% of the Sprague-Dawley rats we used had an intestinal epithelium with a neutral glycolipid profile characteristic of the strain lacking the blood group A-active glycolipids (white rats) described by Breimer *et al.* [6, 7]. The remainder presented a profile similar to that of the strain with blood group A- and H-active glycolipids (black and white rats) described by the same authors. Therefore, all experiments were conducted on individual intestines. After checking for their presence or absence by TLC, H-10-like glycolipids of rats of the same age were isolated and pooled for structural analyses.

H-10-like glycolipids shown in Fig. 2 were probably identical to H-10 glycolipids described by Breimer *et al.* [8]. However, we used a different rat strain and definitive structural identification was not established in the present study. Thus, the subsequent designation of these glycolipids as H-10, for a matter of simplification, means H-10-like glycolipids.

Table 1. Presence of H-10-like glycolipid in small intestine according to rat age. Analyses were made by HPTLC of the glycolipids in the small intestine of individual rats from a litter taken at one day intervals.

	Days of age										
Litter No	18	19	20	21	22	23	24	25	26	27	28
1					+	+	+	+	+	+	+
2				—	+	+					
3			—	+	+	+					
4	—	—	—	+	+	+					

The appearance of H-10 was found to occur around 21 days (Table 1, Fig. 2). It was always absent at 20 days and always present at 22 days. At 21 days, some rats had, others had not H-10, depending on the litter, but rats of the same litter were either all negative or all positive. These results might be explained by the time interval between birth of distinct litters of the same age. Rats of the same litter were born within 2 h, whereas rats of different litters might be born 8 to 10 hours apart. In any case, our results indicated that H-10 appearance was rapid, in a matter of hours, in 21-day-old rats.

Shorter sugar chain fucolipids occur in small amounts in the intestine of adult rats with the A-negative glycolipid profile [7]. It was not possible to trace them, or possible precursors of H-10, during the postnatal development of rat intestine, and thus to draw any conclusion on a developmental regulation.

After its appearance in the tissue, the concentration of H-10 seemed stable (Fig. 2). We found 6.6 nmol of H-10 per intestine at 23 days and 15 nmol at 28 days, which paralleled the doubling of the intestinal wet weight during this period.

Structure of H-10-like Glycolipid Sugar Chain

GLC of the partially methylated alditol acetates was undertaken in order to determine the composition and linkages of the carbohydrate chains (Fig. 3). Fucose was the only terminal residue observed, there was a disubstituted galactose, and *N*-acetylglucosamine substituted at carbon 3 was more abundant than *N*-acetylglucosamine substituted at carbon 4. These results were compatible with the structures described by Breimer *et al.* [8] (Fig. 1), although the responses of all sugars compared to glucose were lower than theoretical values (Table 2). Low recovery of other sugars than glucose might be due to the small amounts of products submitted to analysis. The most important and erratic losses affected -2Gal1- and -3GlcNAc1-. By comparison with these sugars, the response of Fuc1-, -3Gal1-, -3,6Gal1- and -4GlcNAc1- remained more consistent. Assuming that the two structures A and B (Fig. 1) were present and that the recovery of -4GlcNAc1- was the same as -4Glc1-, one might conclude that H-10 form B (containing the type 2 sequence on the 6-substituted branch) accounted for 43-50% of the total H-10-like glycolipids.

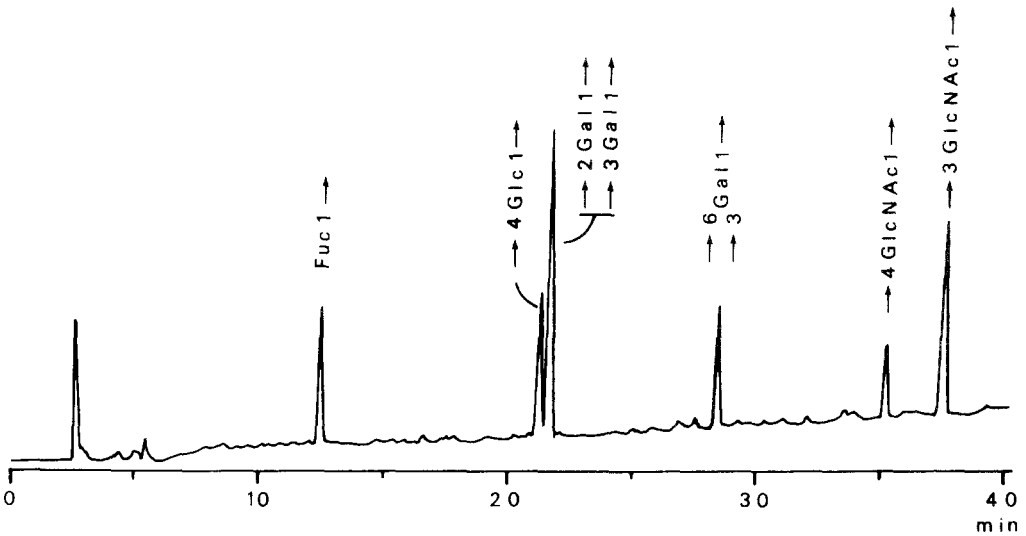

Figure 3. Gas-liquid chromatogram of the partially methylated alditol acetates of H-10-like glycolipids of a 28 days-old rat. Acetates of 2,3,4-trimethyl-fucitol (Fuc1-), 2,3,6-trimethyl-glucitol (-4Glc1-), 2,4,6-trimethyl-galactitol (-3Gal1-), 3,4,6-trimethyl-galactitol (-2Gal1-), 2,4-dimethyl-galactitol (>3,6Gal1-), 3,6-dimethyl-*N*-methyl-2-acetamido-2-deoxyglucitol (-4GlcNAc1-), and 4,6-dimethyl-*N*-methyl-2-acetamido-2-deoxyglucitol (-3GlcNAc1-).

Table 2. Carbohydrate composition of H-10-like glycolipids. H-10-like glycolipids were permethylated, submitted to acetolysis, hydrolysis, reduction and acetylation, then analyzed as described in Fig. 3. 50μg of glycolipid were treated for each age. Sugar amounts are expressed as the ratio of peak areas to glucose peak area. -2Gal1- and -3Gal1- were separated by GLC on a conventional OV-225 column (not shown).

	Days of age					
	23[a]	23[a]	23[a]	28	Adult	Theoretical[b] value
Fuc1-	0.63	0.72	0.80	0.90	0.74	1.73
-4Glc1-	1.00	1.00	1.00	1.00	1.00	1.00
-2Gal1-	0.95	0.89	1.28	1.39	1.27	2.00
-3Gal1-	0.85	0.81	0.91	0.95	0.80	1.00
$\begin{matrix}-6\\-3\end{matrix}$Gal1-	0.74	0.81	0.77	0.82	0.77	1.07
-4GlcNAc1-	0.50	0.57	0.57	0.55	0.49	0.45
-3GlcNAc1-	1.08	1.31	0.85	1.65	1.60	2.95

[a] Analyses were performed on 3 samples extracted from different 23-day-old rats.
[b] Theoretical values were calculated from the carbon content of each acetate. Theoretical occurrence of -4GlcNAc1- and -3GlcNAc1- were calculated on the basis of 60% of form A and 40% of form B according to Breimer *et al.* [8].

Table 3. Fatty acid composition of decahexosylceramide of rat small intestine during development. Results are expressed as percentage of the peak areas after GLC of fatty acid methyl esters.

	% total fatty acids Age (days).	
Fatty acids	24	27
16:0	43.6	29.1
16:1	8.7	2.7
18:0	13.1	9.1
18:1	9.1	6.6
20:0	6.3	2.9
20:1	1.2	0.6
22:0	8.7	12.3
23:0	2.8	2.7
23:1	0	1.3
24:0	4.0	30:6
24:1	2.4	1.7

Ceramide Composition of H-10-like Glycolipids

Sphingoid bases and fatty acids were obtained after hydrolysis of the native H-10-like glycolipids. Phytosphingosine and sphingosine accounted for 80% and 20%, respectively, of the bases at 25 days. These results were fully comparable with data obtained on permethylated H-10 glycolipids of adult rat by mass spectrometry [8]. Therefore, the base composition of H-10 did not change with development. The fatty acid composition was not as consistent as the base composition. At 24 days, there was a majority of C_{16}-C_{18} fatty acids whereas at 27 days, C_{22}-C_{24} fatty acids became major components (Table 3). During this period, 2-hydroxy fatty acids were not detected.

Discussion

The branched chain decaglycosylceramides appear in the rat small intestine around the twenty-first day of life. The timing of H-10 expression was synchronous with the rediffe-rentiation that affects this organ at the beginning of the weaning period [22, 23]. Shorter chain glycolipids also undergo critical changes at 21 days, with fatty acids beginning to be highly 2-hydroxylated in glucosylceramide [2] and hematoside (G_{M3}-ganglioside) [3]. At this time the sialic acid of G_{M3}-ganglioside shifts from *N*-acetylneuraminic to *N*-glyco-lylneuraminic acid [3]. Such changes in the degree of hydroxylation are initiated at around 21 days but are amplified in the following days and weeks. Here, we found that H-10-like glycolipids appeared at 21 days and maintained the same concentration during the following days. During the first week of appearance, H-10 had an increasing percen-tage of long chain fatty acids but 2-hydroxy fatty acids were not detected. As we have found previously that the percentage of 2-hydroxy fatty acids increases in glucosylcera-mide and hematoside even after 28 days, it is possible that the small amounts of C24:0 hydroxy fatty acids detected in H-10 glycolipids of adult rat by Breimer *et al.* [8] appear after the first month of life.

The precisely timed expression of H-10 contrasted with the slow rise in the level of H_3-glycolipid which is observed in human red blood cells during the first year of life [10, 11]. It is likely that such differences result from the different half-lives of the cells, which is 2 months for adult human erythrocytes and less than 2 days for adult rat enterocytes. It is also known that during the first 21 days the intestinal epithelium grows with little renewal and that the high cellular turn-over, which is characteristic of the adult epithelium, is triggered around 21 days [24]. As H-10-like glycolipids were found in both crypt and villus cells of the adult rat small intestine (Fig. 2), in agreement with the findings of Breimer *et al.* [5], they were likely to appear in intestine with a new generation of cells arising from the proliferative cells located in the crypts.

Acknowledgements

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