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Glycosphingolipids in Feces of Germ-Free Rats as a Source for Studies of Developmental Changes of Intestinal Epithelial Cell Surface Carbohydrates

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Non-acid and acid glycosphingolipids were isolated from feces of one litter of germ-free rats from day 17 to day 51. Quantitative and qualitative changes described for small intestine of conventional rats [Bouhours D, Bouhours J-F (1981) Biochem Biophys Res Commun 99:1384-89] were also found in the feces of these germ-free rats. A decrease in lactosylceramide and sialyllactosylceramide excretion and a change from *N*-acetylneuraminic acid to *N*-glycoloylneuraminic acid, as well as an appearance of type 1 chain blood group H-active penta- and decaglycosylceramides were observed during the weaning period. Thus the dramatic changes seen in rat intestinal glycosphingolipids postnatally seem to be primarily regulated by non-microbial factors.

The rat small intestine has been used for studies on glycosphingolipids associated with differentiation of enterocytes both along the crypt-villus axis in adult animals [1] and along the suckling and weaning periods in young animals. In 1981 Bouhours *et al.* reported for the first time on developmental changes of rat intestinal glycolipids occurring during the first month after birth [2]. Later they specifically described the loss of ganglioside, preferentially G_{M3}, during the second and third weeks of life and from day 21 they noted structural modifications of G_{M3} such as a successive replacement of *N*-acetylneuraminic acid by *N*-glycoloylneuraminic acid and non-hydroxylated fatty acids by α-hydroxylated fatty acids [3]. The decrease of ganglioside content of the small intestine was later found to be accompanied by a more general loss of sialic acid and a concomitant increase of fucose [4], as well as a decrease of sialyl- and an increase of fucosyltransferase activities in this tissue during this period of development [5]. The increase of fucosyltransferase activity has also been shown to parallel an increase in blood group H- and A-active glycolipids in the small intestine during the third week of life [6, 7].

Abbreviations: G_{M3}, G_{M3}-ganglioside, II^{3}NeuAc-LacCer or II^{3}NeuGc-LacCer; SPG, IV^{3}NeuAc-nLcOse$_4$Cer; G_{M1}, G_{M1}-ganglioside, II^{3}NeuAc-GgOse$_4$Cer

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We have earlier shown that feces of germ-free rats is rich in non-degraded glycosphingolipids derived mainly from the small , but also from the large, intestine and also from the stomach of these rats [8]. To clarify to what extent the developmentally-associated changes of cell surface carbohydrates described above also took place in germ-free rats we have now analysed the glycosphingolipids of feces of germ-free rats during the first two months of life.

Materials and Methods

One litter, including five males and four females, of the AGUS rat strain [9] kept under germ-free conditions for several generations were housed under sterile conditions initially together with the mother in a cage with grid-like bottom for 16 days and then transferred to a cage with wire-bottom and stainless steel collection-tray for the days 17-40.The rest of the experimental period the rats were kept in ordinary sterile cages. Water and R3 diet was allowed *ad libitum*. Fecal samples were systematically collected from day 17 as stimulated feces or picked from the tray or cage and immediately frozen.

The fecal samples were lyophilized, weighed and extracted in small Soxhlet apparatuses with chloroform/methanol, 2/1 and 1/9 by vol. Non-acid and acid glycosphingolipids were prepared essentially as recently described [10] with the exception that the acetylated non-acid glycolipids were partitioned in chloroform/methanol/water, 8/4/3 by vol, with one extra washing with practical Folch lower-phase, and then only the lower phases were deacetylated using 0.5-1 ml of deacetylating reagent (methanol/toluene/0.2 M KOH in methanol, 3/1/1 by vol), neutralized with acetic acid and analysed without further purification.

Qualitative analyses of glycosphingolipids were done with thin layer chromatography using HPTLC plates (Si 60, Merck, Darmstadt, FRG) with glass support for detection with anisaldehyde [11] and resorcinol [12] and with aluminium support for immunostaining with monoclonal antibodies using a modified procedure [13, 14] of the technique originally described by Magnani *et al.* [15]. Monoclonal anti-blood group A (A581), B (A582) , H (A583) and rabbit anti-mouse antibodies were purchased from Dakopatts A/S (Glostrup, Denmark). Monoclonal anti-blood group B (E83-52) and Le^b and H type 1 chain (NS10) antibodies were kindly provided by Drs. J. Thurin and H. Koprowski, the Wistar Institute, PA, USA [13]. The rabbit anti-mouse antibody was ^{125}I-labelled with Iodo-Gene reagent (Pierce Chemical Co., Rockford, IL, USA), purified on a PD-10 column (Pharmacia, Uppsala, Sweden) and diluted with 2% bovine serum albumin in phosphate buffered saline, pH 7.4, containing 0.1% sodium azide to a final activity of about 1 x 10^6 cpm/ml. TLC solvents for non-acid glycolipids were chloroform/methanol/water, 65/25/4 and 60/35/8 by vol, and for gangliosides chloroform/methanol/acetic acid/water, 60/35/8/10 by vol, chloroform/methanol/2.5 M ammonia or chloroform/methanol/0.2% $CaCl_2$, both 60/40/9 by vol, and methyl acetate/2-propanol/0. 2% $CaCl_2$/2.5 M ammonia, 45/35/15/10 by vol.

Chemical characterization of sialic acids of the dominant gangliosides of the acid glycolipid fractions was performed after treatment with neuraminidase (*Cl. perfringens* neuraminidase, Boehringer Mannheim GmbH, FRG) under conditions [16] known to hydrolyse the G_{M3} but not the G_{M1} ganglioside. The released sialic acids were purified by reversed phase column chromatography (C_{18} Bond Elut prepacked columns, Analytichem International Inc., Harbor City, CA, USA) and finally analysed by TLC [17].

Table 1. Weights of fecal specimens and their glycosphingolipid content obtained from one litter of germ-free rats on days 17 to 51.

	Fecal collection (days)							
	17-18	19	20	21-23	25-27	30-32	44	51
Fecal dry weight (g)	0.30	0.48	1.19	1.52	7.67	7.34	0.26	0.44
Non-acid glycosphingolipids								
mg	6.8	5.4	6.5	7.0	35.0	39.7	1.4	3.2
mg/g dry weight	22.7	11.3	5.5	4.6	4.6	5.4	5.4	7.3
Acid glycosphingolipids								
mg	7.7	6.8	11.4	10.2	26.8	26.6	4.9	5.7
mg/g dry weight	25.7	14.2	9.6	6.7	3.5	3.6	18.9	12.9

Results

Fecal samples were not possible to obtain before day 17, neither as stimulated feces nor as picked specimens from the tray. On days 17-18 the fecal material was greenish and soft, day 19 it had an intermediate character but from day 20 and onwards it looked like ordinary germ-free adult stools. Table 1 gives a summary of the weights of the fecal specimens collected at different ages as well as their contents of sphingolipids. Obviously, the highest content of non-acid glycosphingolipids (22.7 mg/g dry weight) was found in the first fecal portions collected, i.e. from days 17-18, but from day 20 it was stabilized at a level of about 5-6 mg/g dry weight. The acid glycolipid fraction showed similar high concentration in the 17-18 day fecal sample, then fell to a lower level but again rose to higher levels in the two last samples.

The thin layer chromatographic appearance of the non-acid glycosphingolipid fractions are shown in Fig.1 together with a reference of fecal glycolipids of adult (410 days old) AGUS rats [8]. The most obvious changes seen were a shift of cerebrosides into only a few molecular species, a dramatic decrease of the content of diglycosylceramide, a change in the relative amounts of glycolipids in the complex tetra- to hepta-glycosylceramide region and a successive increase of glycolipids in the deca-glycosylceramide region.

Immunostaining with monoclonal antibodies was used to monitor specific changes in blood group active glycolipids found in feces during this period of development (Fig. 2). The blood group A active glycolipids were quantitatively diminished, while the blood group B active glycolipids (assayed with two different monoclonal antibodies) were excreted at a rather constant concentration during this period. The blood group H active glycolipids, were assayed with two antibodies with different molecular specificities (A583 preferentially

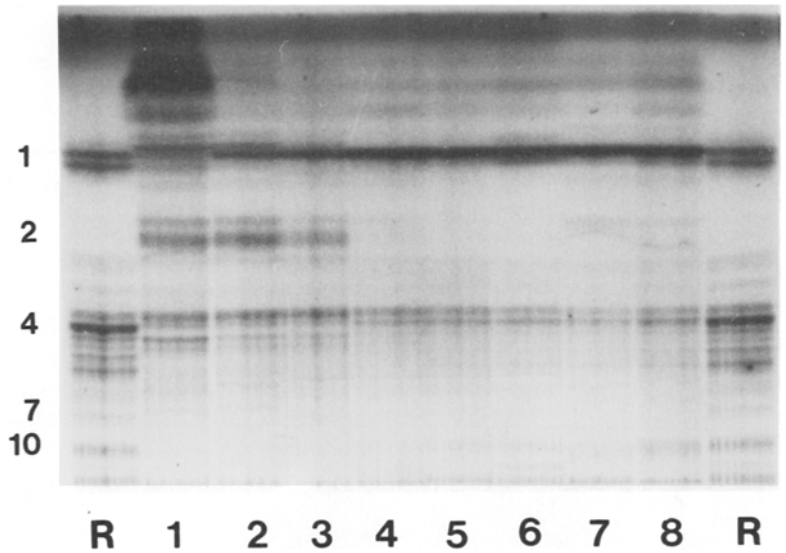

Figure 1. Thin layer chromatogram of non-acid glycosphingolipid fractions of feces of germ-free rats collected at days 17-18 (1), 19 (2), 20 (3), 21-23 (4), 25-27 (5), 30-32 (6), 44 (7), 51 (8) and of adult rats (R). Figures to the left indicate the number of monosaccharides in the carbohydrate chains of the respective glycolipids. The amount spotted on each lane corresponds to 6 mg of dry feces. The solvent used was chloroform/methanol/water, 60/35/8 by vol, and the anisaldehyde reagent [11] was used for detection.

reactive with type 2 chain H and NS10 reactive with type 1 chain H determinants). The blood group H active triglycosylceramide was excreted at a relatively constant level throughout this period but both the penta- and decaglycosylceramides, especially those with type 1 chain H determinants, were increased after weaning, i.e. after 21 days.

The ganglioside contents of the fecal samples are illustrated in Fig. 3 together with standard references. The dominant single ganglioside was $G_{M3,}$ which dramatically decreased in concentration in time and also changed from two fast moving species to three more slowly moving species. The sialic acids released after neuraminidase treatment are shown in Fig. 4 and were identified as being mainly *N*-acetylneuraminic acid during the suckling period i.e. during the first days of collection, with a successive change to a dominance of *N*-glycoloylneuraminic acid during the following two weeks .

Discussion

The qualitative and quantitative changes of intestinal glycosphingolipids observed in these germ-free rats may be correlated with what has been described for conventional animals. Thus, the dramatic decrease of the G_{M3} concentration and the change from *N*-acetylneuraminic acid to *N*-glycoloylneuraminic acid reported for whole small intestines and for small intestinal epithelial cells between days 1 and 60 for conventional rats [3] were here

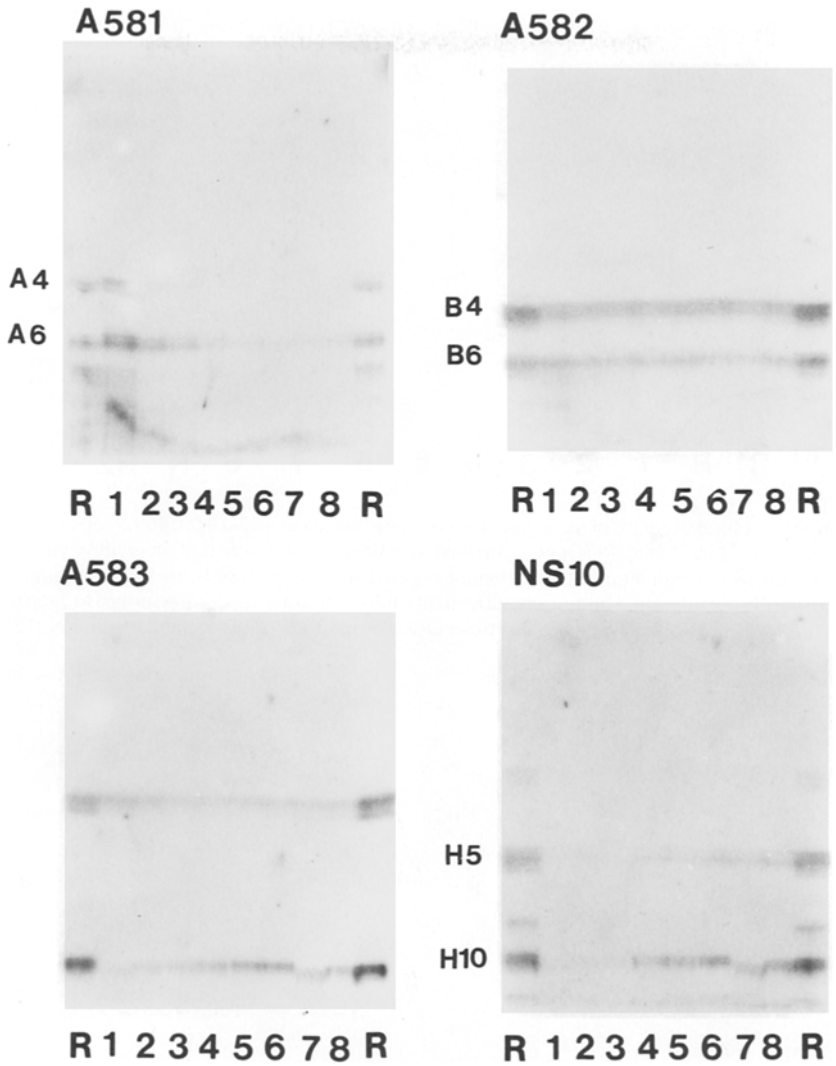

Figure 2. Immunostaining of the non-acid glycolipids of Fig 1 using monoclonal anti blood group A (A581), anti blood group B (A582), and anti blood group H (A583 and NS10) antibodies. Fractions are indicated as in Fig 1, and the amount spotted on each lane corresponds to 2 mg of dry feces. Symbols to the left of each autoradiogram denote the blood group reactivity and the number of monosaccharides in the carbohydrate chains of the respective glycolipids.

confirmed in fecal samples of germ-free rats. The chemically detectable appearance of a blood group H active branched chain decaglycosylceramide around day 21 in small intestine of conventional rats [6] was also confirmed for the germ-free animals. We found immunologically detectable levels of this structure already at 17-18 days but the amounts increased significantly at 21-23 days of age. At this time there also appeared blood group

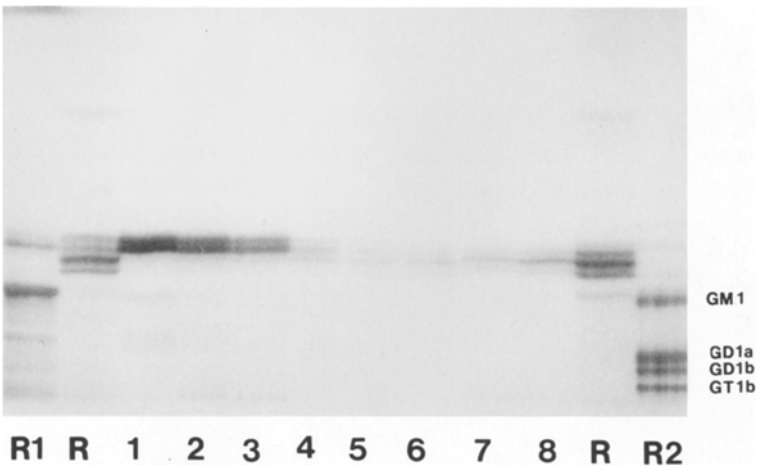

Figure 3. Thin layer chromatogram of fecal gangliosides collected at days 17-18 (1), 19 (2), 20 (3), 21-23 (4), 25-27 (5), 30-32 (6), 44 (7) and 51 (8). Reference gangliosides were prepared from human erythrocytes (R1), human brain (R2) and from feces of adult rats (R). The identities of the various gangliosides are indicated as abbreviations on the sides of the chromatogram. The amount spotted on each of the lanes 1 to 8 corresponds to 2 mg of dry feces. The solvent used was chloroform/methanol/acetic acid/water, 60/35/8/10 by vol, and resorcinol [12] was used for detection.

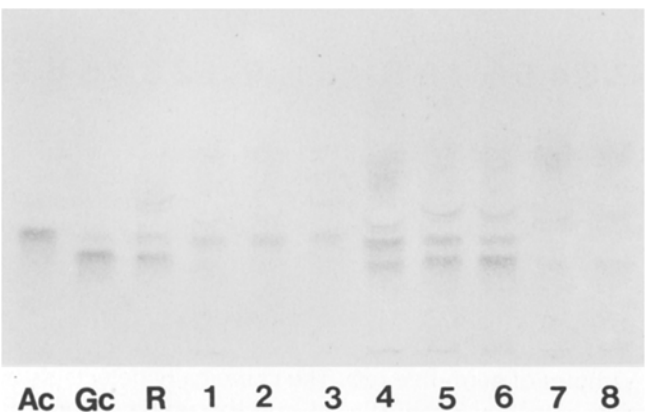

Figure 4. Thin layer chromatogram of sialic acids released by neuraminidase treatment of acid glycolipid fractions of Fig 3, lanes 1-8 and R, and of *N*-acetylneuraminic acid (Ac) and *N*-glycoloylneuraminic acid (Gc). The solvent used was 1-propanol/water/25% ammonia, 70/28/1.5 by vol and resorcinol was used for detection [3].

H determinants on type 1 chain [Galβ1-3GlcNAc) glycolipids both as a pentaglycosylceramide and as a decaglycosylceramide. These findings correlate very nicely with the appearance of type 1 chain blood group A active glycolipids in rats of another strain expressing blood group A but not H active glycolipids in the small intestine [7]. Also the decrease of lactosylceramide content in feces has been described before for whole small intestine of conventional animals [2]. These comparative data indicate that the changes seen in rat intestinal glycosphingolipids postnatally are not primarily dependent on an intestinal microflora. This is in agreement with the developmental changes of the intestinal glycosphingolipids observed for both conventional and germ-free mice [18].

In 1981 Umesaki *et al.* reported that conventionalization of germ-free mice induced the appearance of fucosyl asialo G_{M1} in the intestinal mucosa [19, 20]. The appearance of this glycolipid was transient, reflecting a transient increase in the activity of a specific fucosyltransferase [21] The origin and type(s) of physiological mediator(s) inducing such changes of the glycolipid composition of the intestinal mucosa are not known, although recently several factors were described which could affect the expression of these structures in the mouse small intestine [22]. Whatever the mechanisms might be, however, structural changes of intestinal glycoconjugates may in various ways influence the composition of the microflora in conventional animals. That glycosphingolipids may function as receptors for bacterial adhesion is now well accepted [23] and recently the importance of host-derived nutrients as major ecological determinants for the growth and composition of a complex oral microflora was underlined [24]. Similar factors may thus influence the establishment of a normal intestinal microflora in young mammals.

The present paper illustrates the possibility of using feces of germ-free rats to follow dynamic events of the expression of cell surface carbohydrates. Although not only the small intestinal epithelium but also the epithelia of the stomach and large intestines contribute to the complexity of extruded glycosphingolipids there is no need to kill the animals nor to physically separate epithelial cells from stromal cells to obtain the epithelial material wanted for such studies. However, there seems to be a practical limit as to how young animals can be used for these experiments since it was not possible to obtain stimulated feces from the rats until day 17. The reason for this is probably the nursing and coprophagia of the mother.

Acknowledgements

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