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Screening for the presence of polyglycosylceramides in various tissues: partial characterization of blood group-active complex glycosphingolipids of rabbit and dog small intestines

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Twenty different human and animal tissues were investigated for the presence of polyglycosylceramides. The glycolipids were isolated by peracetylation of dry tissue residues left after conventional lipid extraction, followed by extraction with chloroform and subsequent Sephadex LH-20, Sephadex LH-60 and silica gel chromatography. In most of the cases only trace amounts of complex glycolipids were found. Distinct bands of glycosphingolipids migrating on TLC plates in a region of brain gangliosides and below were observed in bovine erythrocytes, human leukocytes and human colon mucosa. Definite fractions of polyglycosylceramides were isolated from rabbit small intestine, dog small intestine, human placenta and human leukocytes. The polyglycosylceramides of dog and rabbit intestine were characterized by colorimetric analysis, methylation analysis, mass spectrometry and immunological assays. The dog material contained branched carbohydrate chains with repeated fucosylated N-acetyllactosamine units. Rabbit intestine polyglycosylceramides resembled rabbit erythrocyte polyglycosylceramides with Hex-Hex- terminal determinants but were more complex in respect of sugar composition and structure. The material isolated from dog intestine showed A, H, Le^x and Le^y blood group activities. Polyglycosylceramides of human erythrocytes, placenta and leukocytes showed strong binding affinity for *Helicobacter pylori,* while polyglycosylceramide fractions from rabbit and dog intestine were receptor-inactive for this bacterium or displayed only weak and poorly reproducible binding.

***Keywords*: Glycosphingolipids, polyglycosylceramides, rabbit, dog, small intestine, blood groups, helicobacter pylori**

***Abbreviations*: C, chloroform; M, methanol; Hex, hexose; HexNAc, *N*-acetylhexosamine; Fuc, fucose; NeuAc, *N*-acetylneuraminic acid; NeuGc, *N*-glycolylneuraminic acid; TLC-thin layer chromatography; FAB/MS, fast atom bombardment mass spectrometry; GC/MS, gas chromatography-mass spectrometry; PGCs, polyglycosylceramides; EI/MS, electron impact ionization mass spectrometry; PBS, phosphate-buffered saline; BSA, bovine serum albumin. The carbohydrate and glycosphingolipid nomenclatures are according to recommendations of IUPAC-IUB Commission on Biochemical Nomenclature (*Lipids* (1977) 12 : 455–68; *J Biol Chem* (1982) 257 : 3347–51; and *J Biol Chem* (1987) 262 : 13–18)**

Introduction

Glycosphingolipids are present in the outer leaflet of the lipid bilayer of plasma membranes and are of relevance for cell surface recognition phenomena and biotransduction of membrane mediated information [1–4]. A special group of glycosphingolipids constitute polyglycosylceramides (PGCs) which are characterized by the presence of complex carbohydrate chains with many glycosyl units and repeated antigenic structures [5–15]. Polyglycosylceramides have been described as blood group-active substances with ABH [5, 7, 8], B-like [9, 10] and Ii specificities [9, 10, 14, 15]. They seem to be likely candidates as receptor-active membrane components for different types of proteins including bacterial adhesins. Evidence that protein-bound poly-*N*-acetyllactosaminyl chains are functionally related to binding of microbes has been provided [16–19]. Recently we described a high-affinity, sialic acid-dependent binding by *Helicobacter pylori* to PGCs of human erythrocytes [20]. In the present paper we report on the occurrence of PGCs in rabbit and dog small intestines and present general characteristics of the isolated fractions. We also discuss the results of our screening experiments performed on a larger number of human and animal tissues.

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Materials and methods

Lyophilized tissues were extracted with chloroform-methanol [21] prior to preparation of polyglycosylceramides. Red cell membranes were prepared from fresh erythrocytes by toluene flotation [7] and extracted as reported [21]. High performance thin layer plates precoated with silica gel 60, and silica gel 60, 230–400 mesh were purchased from Merck AG (Darmstadt, Germany). Sephadex G-15, LH 20 and LH 60 were from Pharmacia (Uppsala, Sweden), polyisobutylmethacrylate (Plexigum P28) was from Röhm GmbH (Darmstadt, Germany) and XAR-5 films were from Eastman Kodak (Rochester, NY). Anti-A, -B and -H monoclonal mouse antibodies and rabbit anti-mouse antibodies were purchased from Dakopatts a/s (Glostrup, Denmark), and anti-Lewis antibodies from Signet (Histolab Products AB, Frölunda, Sweden). Endoglycoceramidase from *Rhodococcus* was supplied by Genzyme Corporation (Cambridge, USA) and taurodeoxycholic acid (sodium salt) by Sigma (St. Louis, USA). Polyglycosylceramides from human erythrocytes (blood group O) and reference blood group-active glycosphingolipids were obtained in our laboratory. Bovine brain gangliosides (a mixture of GM1, GD1a, GD1b and GT1) were purchased from Calbiochem (USA).

Isolation of complex glycosphingolipids from tissues

Complex glycosphingolipids were isolated from pre-extracted tissues according to the acetylation procedure as described elsewhere [6]. The dry material was kept several hours in formamide (5 ml per 1 g dry tissue) prior to acetylation. The peracetylation was performed overnight at room temperature in formamide/pyridine/acetic anhydride (10 : 5 : 4, by vol.) after which chloroform was added (40 ml per 1 g dry tissue) to the reaction mixture and the suspension was filtered through filter paper. The insoluble material was reextracted (20 ml per 1 g dry tissue) with chloroform. The combined extracts were washed 3 times with water (each time 1/3 volume of water as compared with chloroform volume was used) and evaporated in a rotary evaporator. The acetylated products were purified and separated by Sephadex LH-20 and Sephadex LH-60 chromatography as described previously [6]. Polyglycosylceramide fractions recovered after Sephadex LH-60 were deacetylated overnight at room temperature in 0.1 M NaOH in water, neutralized with acetic acid and dialysed against distilled water for 2 days. The deacetylated PGCs were partitioned in C/M/water (8 : 4 : 3, by vol.), and the material contained in the upper phase was used for analytical tests.

Polyglycosylceramides from rabbit intestine were additionally purified as acetylated derivatives using preparative silica gel chromatography. The acetylated PGCs were dissolved in a small volume of C/M (2 : 1, by vol.) and applied to a silica gel plate after which the plate was developed in C/M/water (20 : 6 : 0.4, by vol.) to 1/3 of the plate height. The plate was divided into several parallel zones which were scraped off and extracted with C/M (1 : 1, by vol.), and C/M/water (65 : 25 : 4, by vol.). Sugar-positive fraction migrating in the region of reference PGCs was deacetylated and used for further analyses.

Polyglycosylceramide fractions from dog intestine were additionally purified as deacetylated glycolipids by extraction with 2-propanol/hexane/water, (50 : 55 : 19, by vol. [11]). The dry material was suspended in the above solvent (2 mg ml^{-1}) and centrifuged. The glycolipids contained in supernatants were used for further analyses.

Endoglycoceramidase digestion of glycosphingolipids [22]

The reaction mixture contained 40 nmol of glycosphingolipid, 200 μg of taurodeoxycholate and 0.5 mU of the enzyme in 80 μl of 50 mM acetate buffer (pH 6.0). The sample was incubated overnight at 37 °C. Next 220 μl of water and 1500 μl of C/M (2 : 1, by vol.) were added, and the sample was shaken and centrifuged. The upper and lower phases were collected separately and analysed for the presence of sugars and ceramides, respectively.

Analytical methods

Hexose was determined according to Dubois *et al.* [23], sphingosine according to Lauter and Trams [24], protein according to the method of Smith [25] and sialic acid according to Svennerholm [26]. FAB-MS analyses were performed on a ZAB 2F/HF mass spectrometer (VG Analytical, Manchester, UK). Negative FAB spectra of ceramides were produced by Xe atoms (8 kV), using triethanolamine as matrix. The ceramides were purified before analyses [27] on silica gel TLC plates which were developed in C/M (9 : 1, by vol.). The zones corresponding to ceramides were scraped off and extracted with C/M/water (40 : 40 : 12, by vol.). Alditol acetates of sugars were prepared as described before [28], permethylation of glycolipids was according to Larson *et al.* [29], partially methylated alditol acetates were obtained as described previously [30, 31]. The GC/MS analysis was performed on a Hewlett Packard 5972 mass spectrometer using a fused silica column (30 m × 0.25 mm) coated with cross linked SE-54, ($d_f = 0.25$ μm), and fused silica column (20 m × 0.22 mm) with poly(arylene-methylphenyl)siloxane as a stationary phase (df = 0.2 μm). Electron-ionization mass spectrometry of permethylated glycolipids was performed as described before [32] using JEOL SX-102 mass spectrometer. Samples were evaporated in the ion source between 150 °C and 410 °C and the spectra were recorded at different points of broad peaks in the end of the run. The electron energy was 70 eV and ion current 300 μA.

Binding of antibodies to glycolipids on thin-layer chromatograms

The details of this procedure are given elsewhere [33, 34]. Briefly: the developed TLC plates were treated with 0.5%

polyisobutylmethacrylate in diethyl ether/hexane for 1 min, dried and incubated in 2% BSA in PBS for 2 h. The plates were then overlaid with an adequate mouse anti-blood group antibody and incubated at room temperature for 2 h. After washing with PBS, the plates were overlaid with a second antibody (^{125}I-iodinated [35] rabbit anti-mouse antibody) and incubated as above for additional 2 h. Finally, the plates were washed with PBS, dried and exposed to Kodak X-ray films for 1–4 days.

Binding of PGCs by Helicobacter pylori

Overlay of PGCs on TLC plates with ^{35}S-methionine-labelled bacteria was done exactly as described [20].

Results

Screening experiments

The following human and animal cells and tissues were investigated for the presence of polyglycosylceramides: human erythrocytes, horse erythrocytes, cat erythrocytes, dog erythrocytes, wild boar erythrocytes, goat erythrocytes, guinea pig erythrocytes, rat erythrocytes, chicken erythrocytes, bovine erythrocytes, human leukocytes, cow stomach, rat small intestine, rabbit small intestine, dog small intestine mucosa, human kidney, rabbit thymus, wild boar small intestine, monkey small intestine, human placenta and human colon mucosa.

Among red cells only human erythrocytes contained larger amounts of polyglycosylceramides [6, 20]. The material isolated from this source was used as reference PGCs in further studies. Bovine erythrocytes contained small amounts of complex glycosphingolipids, which migrated on TLC in the range of brain gangliosides and below, but a definite PGC fraction was not found in this material. These results are in agreement with previous studies performed on these cells [36, 37]. Other animal erythrocytes contained only trace amounts of sugar-positive fractions migrating on TLC plates below brain gangliosides.

The preparations isolated from sources other than erythrocytes contained as a rule more sugar-positive material but in most of the cases complex glycosphingolipids constituted only a trace amounts of the total material. Somewhat higher quantities of polar glycosphingolipids were observed in human colon mucosa (not shown). Well defined fractions of polyglycosylceramides were found in human placenta (which is in agreement with [11]), rabbit small intestine (Figure 1) and dog small intestine mucosa (Figure 2). The PGCs isolated from rabbit and dog small intestines were

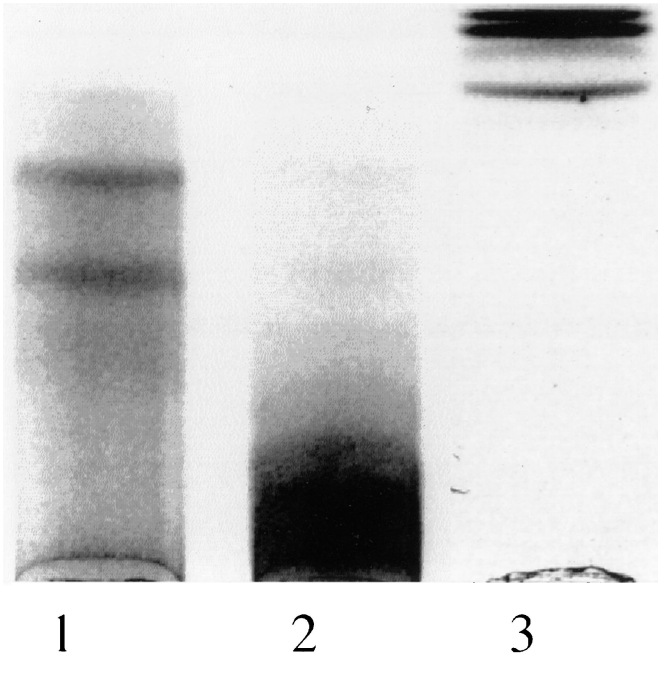

Figure 1. Effect of endoglycoceramidase on PGCs isolated from rabbit small intestine. Lane 1, undigested material; Lane 2, the same material treated with endoglycoceramidase; Lane 3, bovine brain gangliosides. The plate was developed in propanol/0.25%KCl in water/M/C (7 : 5 : 1 : 0.5, by vol.) and stained with 4-methoxybenzaldehyde.

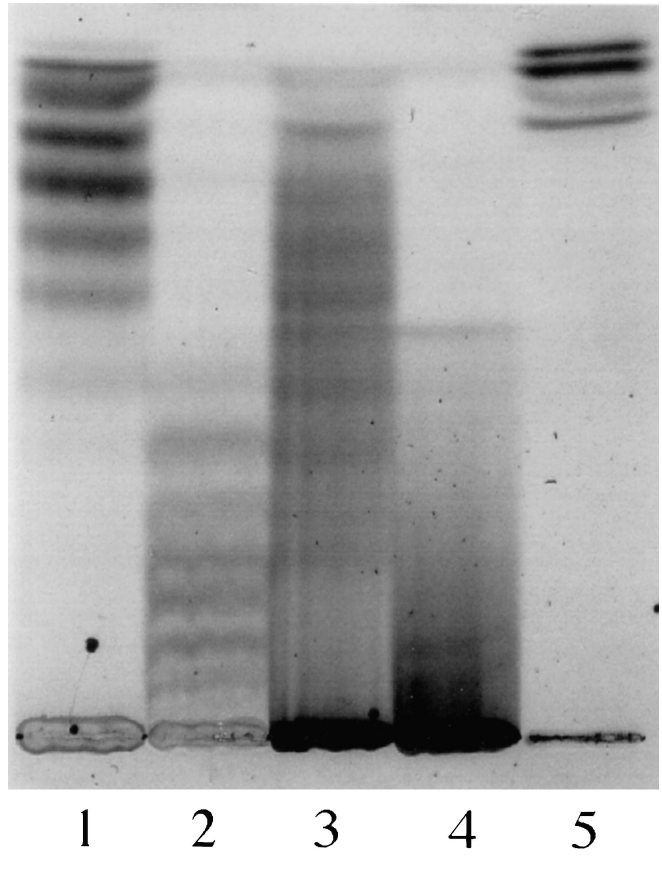

Figure 2. Effect of endoglycoceramidase on PGCs isolated from small intestine of an individual dog. Lane 1, fraction 1 (less complex), untreated; Lane 2, fraction 1 after digestion; Lane 3, fraction 2 (more complex), untreated; Lane 4, fraction 2 after digestion; Lane 5, bovine brain gangliosides. The plate was developed and stained as in Figure 1.

Table 1. Partially methylated alditol and hexosaminitol acetates obtained from hydrolysates of permethylated PGCs.

Methylated derivative	*Human erythrocytes group O*	*Rabbit intestine*	*Dog intestine (pooled material)*	*Dog intestine (individual dog. fr. 1)*	*Dog intestine (individual dog. fr. 2)*
2,3,4-tri-*O*-methyl-1,5-di-*O*-acetylfucitol	+	+	++	+++	+++
2,3,4,6-tetra-*O*-methyl-1,5-di-*O*-acetylgalactitol	+	++	+	+	+
2,3,6-tri-*O*-methyl-1,4,5-tri-*O*-acetylgalactitol	–	(+)	(+)	–	(+)
2,3,6-tri-*O*-methyl-1,4,5-tri-*O*-acetylglucitol	+	+	+	+	+
3,4,6-tri-*O*-methyl-1,2,5,-tri-*O*-acetylgalactitol	+	+	+	+	+
2,4,6-tri-*O*-methyl-1,3,5-tri-*O*-acetylgalactitol	++++	++++	+++	+++	+++
4,6-di-*O*-methyl-1,2,3,5-tetraacetylgalactitol	–	+	+	+	+
2,4-di-*O*-methyl-1,3,5,6-tetra-*O*-acetylgalactitol	+++	+++	++	++	+++
3,4,6-tri-*O*-methyl-1,5-di-*O*-acetyl-2-deoxy-2-*N*-methylacetamidoglucitol	–	(+)	–	–	(+)
3,4,6-tri-*O*-methyl-1,5-di-*O*-acetyl-2-deoxy-2-*N*-methylacetamidogalactitol	–	(+)	(+)	+	+
4,6-di-*O*-methyl-1,3,5-tri-*O*-acetyl-2-deoxy-2-*N*-methylacetamiglucitol	–	(+)	(+)	(+)	(+)
3,6-di-*O*-methyl-1,4,5-tri-*O*-acetyl-2-deoxy-2-*N*-methylacetamidoglucitol	++++	+++	+++	++	+++
6-*O*-methyl-1,3,4,5-tetra-*O*-acetyl-2-deoxy-2-*N*-methylacetamidoglucitol	(+)	(+)	++	+++	++
Unidentified	(+)	(+)	(+)	(+)	(+)

Very small or trace amounts are indicated by (+).

characterized in these studies in greater detail using various analytical procedures. A series of complex glycosphingolipids was obtained also from human leukocytes, which will be discussed in a separate paper.

Complex glycosphingolipids isolated from rabbit small intestine

All analytical tests were performed on a pooled material obtained from several rabbits. The crude fraction after Sephadex LH-20 isolated from rabbit small intestine contained glycosphingolipids of different complexity migrating on TLC plates in the region of reference brain gangliosides and below. The glycolipids were separated as acetylated derivatives by Sephadex LH-60 chromatography into a 'less complex' fraction (glycolipids migrating in a TLC region corresponding to brain gangliosides) and a PGC fraction, which was further purified by silica gel chromatography.

The final PGC material contained 300 nmols of hexose, 15 nmols of sialic acid and 12 nmols of sphingosine per 1 g of the dry tissue residue. When tested by TLC under routine conditions (developing solvent: $C/M/H_2O$, 60:35:8, by vol.) the PGC fraction stayed at the application line. In highly polar solvent system adapted to separate polyglycosylceramides [6], the bulk of the material migrated on the plate underneath reference gangliosides (Figure 1, lanes 1 and 3). The migration rate was changed drastically after treatment of the material with endoglycoceramidase (Figure 1, lane 2).

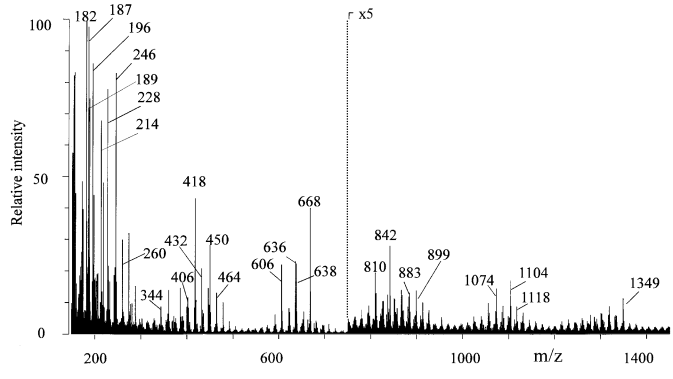

Figure 3. EI/MS of permethylated PGCs prepared from rabbit small intestine (pooled material obtained from several different rabbits).

The analysis of sugars as alditol acetates revealed the presence of galactose, *N*-acetylglucosamine, fucose, glucose and a trace of *N*-acetylgalactosamine and mannose. The pattern of partially methylated alditol acetates obtained from rabbit intestine material was similar to that obtained from human erythrocyte polyglycosylceramides (Table 1).

The electron-ionization (EI) mass spectrometry of the permethylated rabbit intestine PGCs (Figure 3) showed the presence of terminal Hex- (*m/z* 219 and 187), Fuc- (*m/z* 189 and 157), HexNAc- (*m/z* 260), NeuAc- (*m/z* 376 and 344)

and NeuGc- (*m/z* 406 and 374). Distinct fragment ions corresponded to $Hex_2HexNAc_1$- (*m/z* 668 and 636), $Fuc_1Hex_1HexNAc_1$- (*m/z* 638 and 606) and Hex_1-$HexNAc_1$- (*m/z* 464 and 432). Minor fragment ions corresponding to $Fuc_1Hex_2HexNAc_1$- (*m/z* 842 and 810), $NeuAc_1$-Hex_1-$HexNAc_1$- (*m/z* 825 and 793, not shown in the figure), $Fuc_1Hex_1HexNAc_2$- (*m/z* 883 and 851) and $Hex_3HexNAc_2$- (1118) were also found. An intense fragment ion at *m/z* 182 confirmed the presence of neolacto (type 2) carbohydrate chains [38]. Fragment ions corresponding to internal HexNAc, $Hex_1HexNAc_1$ and $Hex_2HexNAc_2$ were found at *m/z* 246 (214) and 450 (418) and 899 (867). These ions appeared in all EI spectra of PGCs and were derived most probably by elimination of sugars from larger ions and attachment of protons (e.g. 638.34 ($Fuc_1Hex_1HexNAc_1$) − 189.11 (Fuc) + 1.01 (H) = 450.24). The fragment ion at *m/z* 899 could be derived from linear structures (e.g. 1087.57 ($Fuc_1Hex_2HexNAc_2$) − 189.11 (Fuc) + 1.01 (H) = 899.46), and branched structures (*eg* 1362.70 ($Hex_3HexNAc_3$) − 464.25 ($Hex_1HexNAc_1$) + 1.01 (H) = 899.46). Fragment ions at *m/z* 1074, 1104 and 1349 could also originate from the branched carbohydrates (Fig. 7). The presence of these fragment ions and the lack of abundant fragment ions corresponding to linear structures with more than 1 lactosamine unit confirm that rabbit intestine PGCs are highly branched.

Ceramides released from PGCs were analysed by TLC and FAB mass spectrometry. The results showed the presence of a series of molecular species composed of fatty acids and sphingosines with different number of carbon atoms and different degree of unsaturation and hydroxylation. The most pronounced TLC band contained ceramides with sphingosine (d18 : 1) and C18-26 fatty acids (*m/z* of ceramides: 563, 593, 621, 635, 645, 647, 649, 661 and 675). The ceramide ion at *m/z* 645 suggested the presence of d18 : 2 sphingosine and C24 : 1 fatty acid.

Polyglycosylceramides isolated from rabbit intestine displayed A, B, H and Le^y blood group activities, as shown by overlay of TLC plates with labelled antibodies.

Complex glycosphingolipids isolated from dog small intestine mucosa

The analyses were performed on PGCs obtained from several different dogs (pooled material) and on PGCs from one individual dog. The crude PGCs (obtained from 1 g of the dry tissue) contained on an average 60 nmols of sphingosine. TLC analysis indicated high heterogeneity of the material, as shown in Figure 2. The two fractions obtained after Sephadex LH-60 chromatography migrated on thin layer plates as multiple, sugar-positive bands (lanes 1 and 3), and changed chromatographic mobility after treatment with endoglycoceramidase (lanes 2 and 4).

These fractions contained sphingosine, hexose and sialic acid in molar proportions 1 : 12 : 0.4 and 1 : 31 : 0.9, respectively. Analysis of sugars as alditol acetates revealed the presence in all dog preparations of galactose, fucose, *N*-acetylglucosamine and minor amounts of *N*-acetylgalactosamine, glucose and traces of mannose. The composition of partially methylated alditol acetates is shown in Table 1. The dog intestine mucose PGCs differed from other analysed PGCs in that they contained higher amounts of terminal fucose (2,3,4-tri-*O*-methyl-1,5-di-*O*-acetylfucitol) and substantial amounts of 1,3,4-linked *N*-acetylhexosamine (6-*O*-methyl-1,3,4,5-tetra-*O*-acetyl-2-deoxy-2-*N*-methylacetamidoglucitol).

Electron ionization mass spectrometry of the permethylated PGCs from pooled dog material and of two fractions from an individual dog (Figures 4–6) showed the presence of terminal fucose (*m/z* 189), hexose (*m/z* 219 and 187), *N*-acetylhexosamine (*m/z* 260) and *N*-acetylneuraminic acid (*m/z* 376 and 344). Trace of *N*-glycolylneuraminic acid was also present (*m/z* 406 and 374). Fragment ions at *m/z* 464 and 432 indicated the presence of terminal disaccharides composed of Hex and HexNAc, and fragment ions at *m/z* 638 (606) and 1088 (1056) showed mono-fucosylated carbohydrate sequences $Fuc_1Hex_1HexNAc_1$ and $Fuc_1Hex_2HexNAc_2$, respectively.

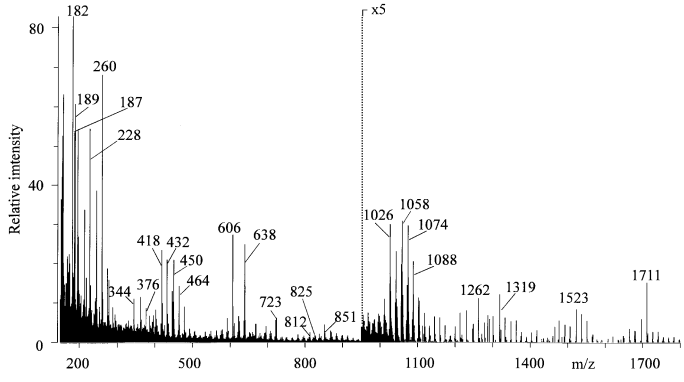

Figure 4. EI/MS of permethylated PGCs prepared from dog small intestine (pooled material obtained from several different dogs).

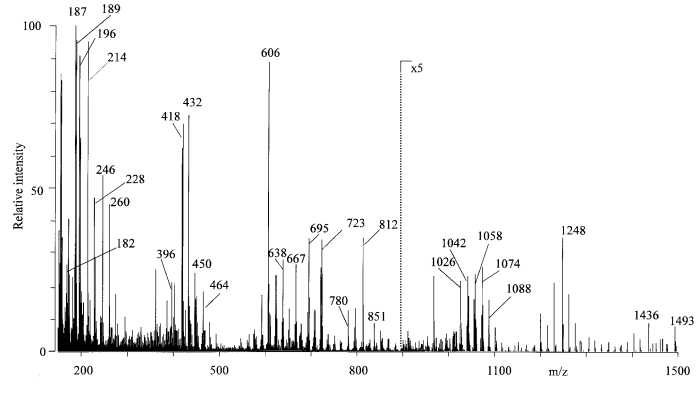

Figure 5. EI/MS of permethylated PGCs prepared from dog small intestine (fraction 1 from an individual dog, see also Table 1 and Figure 2).

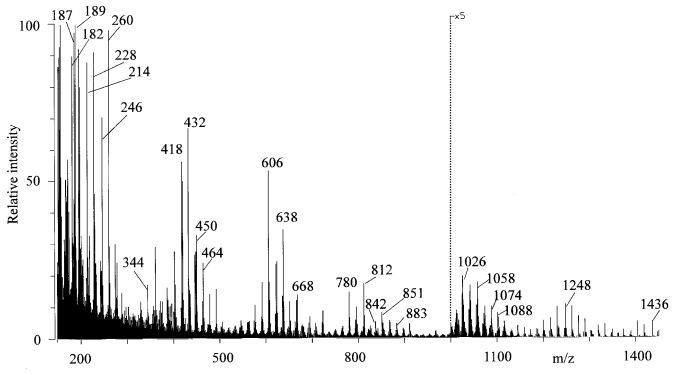

Figure 6. EI/MS of permethylated PGCs prepared from dog small intestine (fraction 2 from an individual dog, see also Table 1 and Figure 2).

Less intense fragment ions at *m/z* 842 and 883 (851) corresponded to $Fuc_1Hex_2HexNAc_1$ and $Fuc_1Hex_1HexNAc_2$. The main difucosylated structures were found at *m/z* 812 (780), $Fuc_2Hex_1HexNAc_1$, 1058 (1026), $Fuc_2Hex_1HexNAc_2$ and 1711, $Fuc_2Hex_3HexNAc_3$. Minor quantities of fragment ions at *m/z* 1436 and 2059 (not shown in the figure) indicated the presence of tri- and tetrafucosylated carbohydrate chains ($Fuc_3Hex_2HexNAc_2$ and $Fuc_4Hex_3HexNAc_3$). Small amounts of a fragment ion at *m/z* 825 ($NeuAc_1Hex_1HexNAc_1$) were also detected in all dog intestine fractions. Fragment ions at *m/z* 1074, 1248, 1493 and 1523 could be obtained by elimination of fucose from larger ions and attachment of protons (e.g. 1710.88 ($Fuc_2Hex_3HexNAc_3$) − 189.11 (Fuc) + 1.01 (H) = 1522.78, or 1435.77 ($Fuc_3Hex_2HexNAc_2$) − 189.11 (Fuc) + 1.01 (H) = 1247.67) or by elimination of lactosamine-based side branches from the branched ions (Figure 7). Generally, the fragmentation pattern indicates the presence of both linear and branched terminal structures in dog PGCs. For example a fragment ion at 1262 ($Fuc_2Hex_2HexNAc_2$) seen in Figure 4 indicates a core chain of Hex-HexNAc-Hex-HexNAc, but a more abundant fragment ion at 1711 ($Fuc_2Hex_3HexNAc_3$) agrees well with the branched structure (Figure 7). The fractions obtained from the individual dog (Figures 5 and 6) were more highly fucosylated than the pooled material (Figure 4).

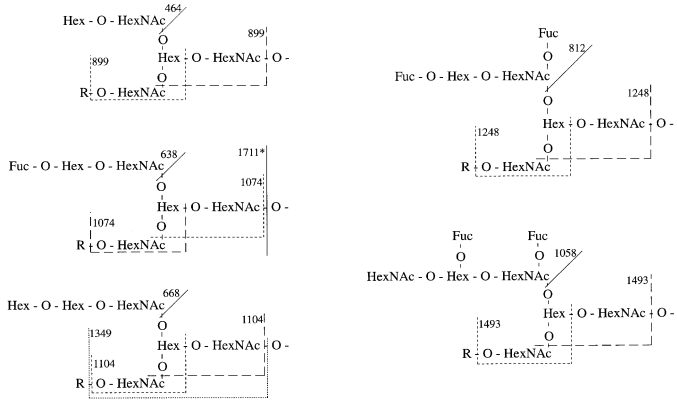

Figure 7. Proposed fragmentation patterns of branched carbohydrate chains (supplement to Figures 3–6). R: terminal carbohydrate sequence of the second branch; *: mass in case of R = Fuc-O-Hex.

Ceramides released from the dog material by endoglycoceramidase were identified by negative FAB/MS. The most intense molecular ions corresponded to ceramides with mainly phytosphingosine (t18:0) and hydroxy fatty acids (the most pronounced ions were at *m/z* 553, 571, 599, 627, 655 and 683, corresponding to t18:0 and 16:1 (and/or d18:1 and h16:0), t18:0 and h16:0, t18:0 and h18:0, t18:0 and h20:0, t18:0 and h22:0, and t18:0 and h24:0, respectively). Fragment ions corresponding to ceramides can be distinctly seen in EI mass spectrum of the less complex PGC fraction from an individual dog (Figure 5), (ions at *m/z* 667, 695 and 723 correspond to ceramides and the ion at 396 corresponds to phytosphingosine).

All PGC fractions isolated from dog small intestine mucosa were A, H, Le^x and Le^y blood group active. The Lewis activities of PGCs from the individual dog are shown in Figure 8.

Receptor activity of *H. pylori*

In a separate paper (20) a high-affinity binding by *H. pylori* of PGCs of human erythrocytes was documented. This binding was absolutely dependent on a sialic acid, since treatment of PGCs with neuraminidase, mild acid or mild periodate eliminated binding, and the detection level of erythrocyte PGCs on TLC plates was 10 pmols based on sialic acid content. Using the same assay conditions, a similar apparently high-affinity and sialic acid-dependent binding was found for PGCs of human leukocytes and human placenta (to be reported separately). However, there was no binding of *H. pylori* to rabbit PGCs, and only weak and poorly reproducible binding to dog PGCs, although all tested fractions contained sialic acid in comparable amounts.

Discussion

The results presented in these studies indicate that rabbit and dog small intestines contain series of complex glycosphingolipids with branched polylactosamine chains terminated with different antigenic structures. The presence of 1,4-linked GlcNAc in the carbohydrate core chains (as indicated by the presence of 3,6-di-*O*-methylated glucosaminitol derivative, see Table 1) is in agreement with an intense fragment ion at *m/z* 182 in EI spectra (Figs 3, 4 and 6) diagnostic for type 2 carbohydrate structures (38). The weak peak at *m/z* 182 in the EI spectrum of the less complex dog intestine fraction (Fig. 5) was probably due to the substitution of a substantial amount of *N*-acetylglucosamine with fucose. The presence of 1,2- and 1,2,3-substituted Gal (represented by 3,4,6- and 4,6-*O*-methylated galactitol derivatives, see Table 1) is in agreement with ABH

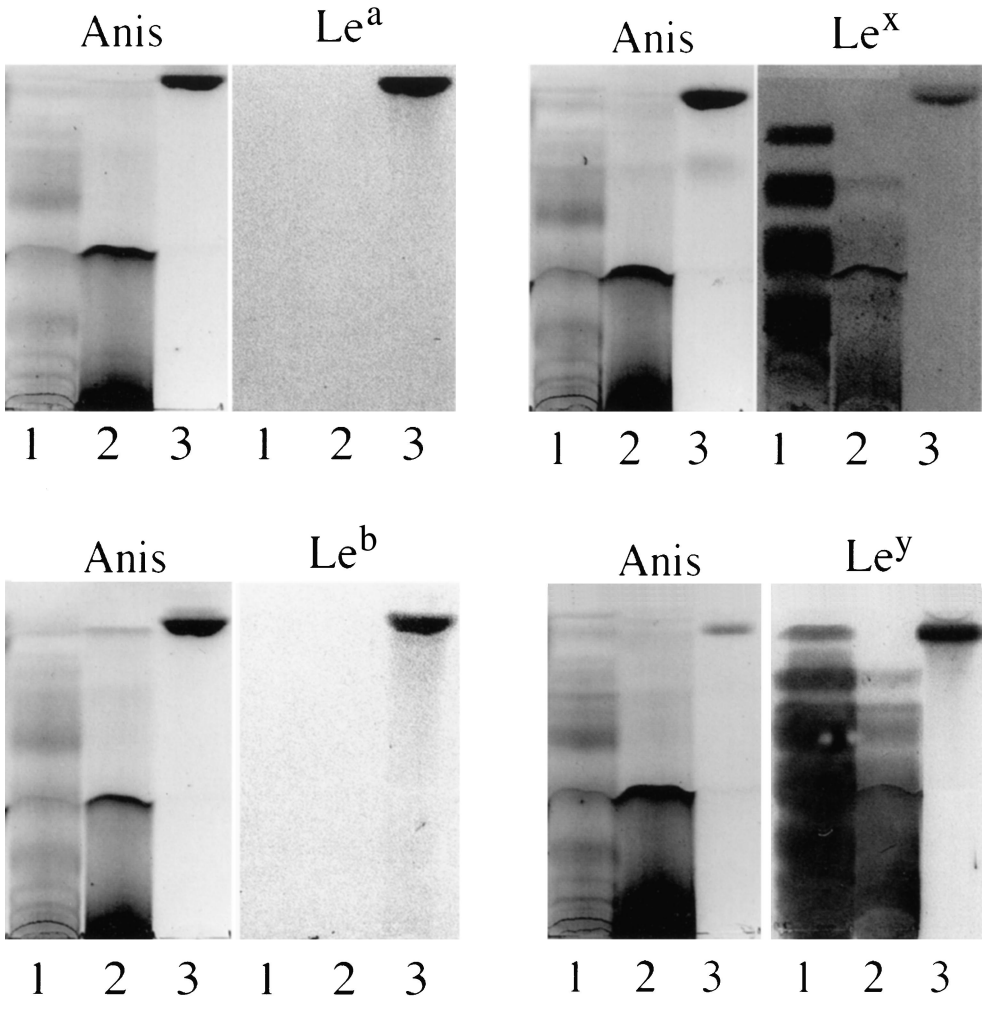

Figure 8. Lewis blood group activities of PGC fractions prepared from an individual dog. TLC plates were developed in C/M/H_2O, (50 : 55 : 19) and visualized by spraying with 4-methoxybenzaldehyde (anisaldehyde) or by overlay with anti-Le^a, anti-Le^b, anti-Le^x or anti-Le^y antibodies. Lane 1, less complex fraction; Lane 2, more complex fraction; Lane 3, reference glycosphingolipid with an appropriate Lewis activity.

and Lewis blood group activities. Small amounts of 1,3-substituted GlcNAc (4,6-di-*O*-methyl-1,3,5-tri-*O*-acetyl-2-deoxy-2-*N*-methylacetamidoglucitol, see Table 1) may indicate the presence of some lacto (type 1) structures in dog and rabbit PGCs. Polyglycosylceramides from rabbit intestine contained terminal carbohydrates similar to those found in PGCs from rabbit erythrocytes ($Hex_2HexNAc$), [9], but the material was more complex and contained also unsubstituted HexHexNAc terminals as well as fucosylated, sialylated and HexNAc-terminated carbohydrate sequences. PGCs from dog intestine contained highly fucosylated sugar chains with fucose attached to both terminal and internal saccharide units. Sialic acid was found in all investigated PGC fractions, as confirmed by fragment ions at *m/z* 376 and 344 (obtained from *N*-acetylneuraminic acid) and at *m/z* 406 and 374 (obtained from *N*-glycolylneuraminic acid), although in small amounts. Larger sialylated fragments were of very low abundance in EI spectra, which hinders interpretation of the structures. It is highly possible that PGCs carry different sialylated carbohydrate sequences. We have found, that sialic acid-dependent binding of *H. pylori* to PGCs differs depending on PGC origin. In contrast with PGCs from human erythrocytes, leukocytes and placenta, which had a strong, sialic acid-dependent affinity for *H. pylori* [20] the PGCs from rabbit and dog intestine were inactive or poorly active for the bacterium, although all of the preparations contained branched polylactosamine chains and sialic acid in comparable amounts. Another possible explanation for this difference in binding in steric interference with access to the binding epitope in non-reactive PGCs.

The PGCs isolated from both rabbit and dog intestine showed ABH and Lewis blood group activities and they might also be carriers of other biological specificities. The PGCs reacted with anti-Le^y and/or anti-Le^x (but not with anti-Le^a and anti-Le^b) antibodies, which is in agreement

with the presence of type 2 (neolacto) carbohydrate chains [39]. Less complex glycosphingolipids with ABH and Lewis activities were isolated previously from dog [40–43] and rabbit [44] small intestines.

All polyglycosylceramides investigated in these studies formed heterogeneous mixtures of components with apparently closely related structures and progressively increasing complexity, as indicated by chemical studies and overlay of TLC plates. The distribution of sialic acid (not shown here, see [20]) and fucose (Table 1 and Figures 5 and 6, see also distribution of Lewis antigens in the dog material, Figure 8) was similar in PGCs of different complexity indicating that this heterogeneity was not an effect of degradation but biosynthesis. All PGC preparations obtained after Sephadex LH-60 contained small amounts of protein as shown by colorimetric tests and confirmed by the presence of trace amounts of mannose. However, the majority of the sugar-positive material was susceptible to ceramide glycanase indicating glycosphingolipid nature of the preparations (Figures 1 and 2).

Screening studies performed on a large number of different tissues indicate that polyglycosylceramides do not generally occur in biological material in high amounts. In most of the cases only traces of sphingosine were detected among highly polar fractions, which made further chemical analyses impossible. The amounts of PGCs in rabbit and dog intestines are also relatively small (the yields of the preparations were at microgram levels as related to 1 g of dry tissue), suggesting a specific biological function rather than a structural function. However the real contents of PGCs in tissues are difficult to evaluate because of the possible incompleteness of the extraction procedure and due to the loss of PGCs at different steps of preparations. The published information about PGC contents in tissues is very limited. In human erythrocytes PGCs constitute only a small part of all glycolipids. They were isolated from these cells with a yield of 2–5 mg per 1 blood unit [6, 8], while globoside (the main neutral glycosphingolipid present in human erythrocytes) was isolated with a yield of about 40 mg per 1 blood unit [45]. On the other hand in human placenta PGCs seem to occur in higher amounts relative to shorter ganglioside species, as judged by TLC plates [11]. These different patterns may reflect different functions; however, nothing is known about the biological role of these ceramide-anchored glycoconjugates and their contribution to cell surface properties.

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