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Intestinal Sphingolipid Excretion Associated with Feeding of Phytohemagglutinin Lectin (*Phaseolus vulgaris*) to Germ-free and Conventional Rats

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Intestinal sphingolipids of feces of germ-free and conventional rats were analyzed during the pair feeding of a complete defined diet containing phytohemagglutinin lectin (PHA) from red kidney beans (*Phaseolus vulgaris*) as 1% dietary protein in comparison to casein fed controls. Phytohemagglutinin in the diet increased the total fecal excretion of sphingomyelins (18-fold for germ-free and 20-fold for conventional rats), of non-acid glycosphingolipids (3.5-fold for germ-free and 9-fold for conventional rats) and also of the gangliosides (2.5-fold) for the germ-free rats compared to controls. For germ-free rats the increase of non-acid glycolipids was ascribed to an effect of the lectin strictly on the small intestinal mucosa, while for conventional rats an effect was seen also on the large intestinal mucosa. Increase of fecal gangliosides of germ-free rats was due mainly to an increased excretion of *N*-acetylneuraminosyl-lactosylceramide, a ganglioside species restricted to epithelial cells of duodenum, of upper jejunum and of large intestines. The effects on glycolipid excretion observed in germ-free rats and the rather similar effects seen in conventional animals suggested that the influence of dietary PHA was due directly to effects elicited by PHA binding to the enterocyte brush border membrane and not to secondary effects induced by increase in the luminal microflora.

Dietary studies of phytohemagglutinin (PHA), a plant lectin derived from red kidney beans, (*Phaseolus vulgaris*), have shown that this lectin binds to the brush border membranes of both conventional and germ-free rat small intestine [1]. Feeding of two plant lectins, PHA and Concanavalin A, have been associated with a proliferation of the small intestinal microbiota [2], variable morphological damage to intestinal villi and loss of microvillus membrane enzyme activity [3]. The effects of both these lectins on brush border membrane structure and function may have been indirect, by inducing a bacterial overgrowth syndrome, similar to that observed in the blind loop animal model [4]. Alternatively, these changes might have been a consequence of direct interaction of lectin with the brush border

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membrane. Recent studies using *in situ* intestinal luminal exposure of loops to two membrane binding lectins, wheat germ agglutinin and Concanavalin A, documented that morphological and functional damage occurred in a dose response fashion to the microvillus membrane in short-term experiments [5]. To study how the small intestine responds to chronic dietary administration of a membrane binding lectin would be of additional interest [6].

Our objectives in the present experiments were to measure the effects of purified PHA ingestion on sphingolipids of the intestinal mucosa of germ-free and conventional rats. We have shown earlier, for germ-free rats, that sphingolipids shed from the intestinal epithelium and excreted in the feces do not show any signs of degradation [7] and that fecal glycolipids of such rats may be used for studying developmental changes of glycosphingolipids expressed in the intestinal epithelium [8]. In fecal samples of conventional rats, gangliosides but not blood group A-, B-, and H-active glycolipids were found to be degraded by bacterial glycosidases [7]. Although previous observations have made it clear that growth impairment is less severe in germ-free animals on lectin containing diets [9], the effect of PHA lectin on small intestinal glycolipids has not been addressed in either germ-free or conventional animals fed diets comparable in nutrient intake to pair-fed controls.

The present studies will define specific biochemical alterations associated with intestinal binding of the PHA lectin. They also provide insight into how dietary lectins alter the fecal sphingolipid excretion and how changes in glycolipid excretion are dependent on interactions with the intestinal microbiota.

Experimental Procedure

Preparation of Phytohemagglutinin

Phytohemagglutinin was prepared from red kidney beans by methods described previously [10]. The PHA yields from several column preparations were combined, assayed for hemagglutination activity and protein content. Hemagglutination titer of dietary purified PHA and PHA extracted from fecal extracts employed a 4% suspension of washed typed O erythrocytes. No separation of PHA isomers was done.

Animals and Diets

Twelve adult germ-free (GF) outbred Sprague Dawley male rats were introduced into sterile flexible plastic isolators and placed in individual wire mesh bottom metabolic cages. Purified PHA was incorporated as 1% dietary protein and mixed with a sterilized basal diet (#2485 Teklad, Madison, WI, USA) which provided all essential nutrients. Additional dietary protein (19%) was supplied as casein for test animals (T) while control animals (C) received a 20% casein diet. Both T and C diets were isonitrogenous and essentially isocaloric. All components of the diets were sterilized by autoclaving, except for the PHA which was irradiated at 3 mRads and then mixed into the sterilized diet. Loss of PHA hemagglutination activity was 5% after irradiation. Groups of six germ-free rats were placed into their respective sterile isolators, and matched for equivalent weights (mean ±SEM; T: 185±11 and

C: 179±9 g). All rats were observed for an initial 24 h period on the C diet. Subsequently, T rats received a daily *ad libitum* diet and C rats were pair fed that amount consumed in a previous 24 h period by the T animals. Bacterial contamination was assessed by bacterial culture of fresh stool. No evidence of bacterial contamination was observed in either T or C animals during the course of the study.

Twelve adult male conventional (CV) rats were housed individually in mesh bottom metabolic cages to minimize coprophagy. All animals were maintained on control diets three days prior to the study with PHA. Diets for T and C groups, respectively, were as described above. During the seven day study period all the rats were weighed daily and fecal and urine output was individually collected. All GF and CV animals were maintained at a constant temperature with a 12 h light-dark cycle and water was allowed *ad libitum*.

Total Fat and Nitrogen Assays

Total fecal fatty acids was measured from pooled fecal collections. A representative aliquot of lyophilized fecal material was measured for total nitrogen using a Coleman Nitrogen Analyzer (Coleman Instruments, Perkin-Elmer, Chicago, IL, USA).

Sphingolipid Analyses

Samples of 0.9 g to 2.1 g of lyophilized pooled fecal material were taken from the four groups of rats (CV-C, CV-T, GF-C, GF-T) to analyze their sphingolipid contents. The procedure for preparation of total alkali-stable gangliosides and sulfolipids, sphingomyelins, and total non-acid glycosphingolipids essentially followed that described for the preparation of sphingolipids of human plasma and intestine [11]. Minor modifications were the change of silicic acid to Kieselgel S (32-63 µm, Riedel-de Haën, Seelze, FRG) reactivated at 120°C for 24 h after six repetitive washings in methanol; and the change of ion-exchange chromatography material to DEAE-Sephadex A-25 (Pharmacia, Uppsala, Sweden) used in the acetate-form [12].

The quantitative determinations of sphingosine contents of non-acid glycosphingolipid and sphingomyelin fractions were performed in triplicate by the method of Naoi *et al.* [13]. Pure sphingosine hydrochloride was a kind gift from Dr. I. Pascher, Department of Medical Biochemistry, Gothenburg University. The relative amounts of gangliosides (sialosyl-lactosylceramide) in feces of germ-free test and control rats were assayed densitometrically on a TLC plate after staining with resorcinol using a dual wavelength scanning mode on a Shimadzu CS 9000 densitometer (Shimadzu Corp., Kyoto, Japan).

Thin-layer chromatographic analyses of glycosphingolipids were done on HPTLC plates (Si 60, Merck, Darmstadt, FRG) with glass support for detection with anisaldehyde [14] and resorcinol [15] and with alumina support for detection with monoclonal antibodies or ^{35}S-labelled bacteria using modified procedures [16-18] of the TLC overlay technique originally described by Magnani *et al.* [19]. Monoclonal anti-blood group A (A581), B (A582), H (A583) and rabbit anti-mouse immunoglobulins were purchased from Dakopatts A/S (Glostrup, Denmark). The 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,

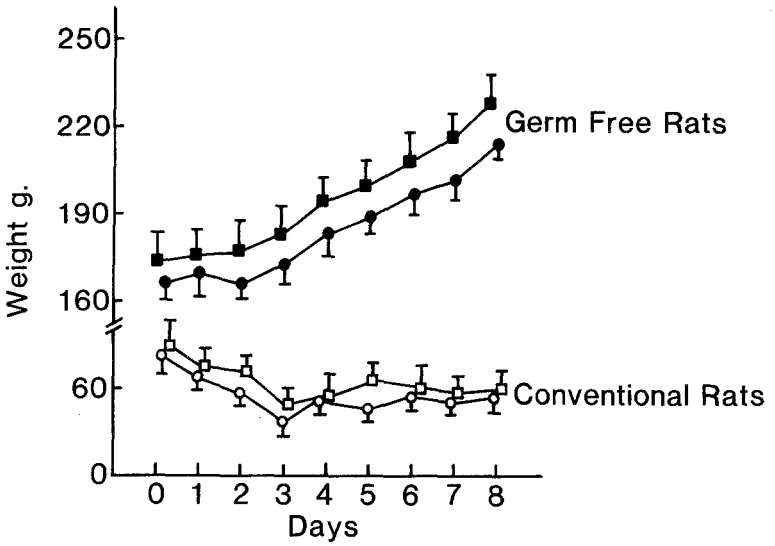

Figure 1. Body weight of pair-fed germ free (test ■; control ●) and conventional (test □; control ○) rats during the seven-day study period. Test animals received 1% of total dietary protein as PHA; control animals, an equivalent quantity of casein protein.

Sweden) and diluted with 2% bovine serum albumin in phosphate buffered saline, pH 7.4, containing 0.1% sodium azide [16] to a final activity of about 1×10^6 cpm/ml. *Escherichia coli* strains 3669 and MR506, which both specifically bind Galα1-4Gal-containing glycolipids [18, 20], were labelled with [^{35}S]methionine to a specific activity of 5×10^5 cpm/ml with 10^8 bacteria/ml and was a kind gift from Dr. C. Svanborg-Edén, Department of Clinical Immunology, Gothenburg University. The TLC solvent used for gangliosides was chloroform/methanol/acetic acid/water, 60/35/8/10 by vol,and for non-acid sphingolipids chloroform/methanol/water, 60/35/8 and 65/25/4 by vol.

For the structural characterization of gangliosides of GF feces an amount corresponding to 20 mg dry feces (about 50 µg) of T and C ganglioside fractions were hydrolysed with 50 µl (50 mU) of *Clostridium perfringens* neuraminidase, EC 3.2.1.18 (Boehringer Mannheim GmbH, FRG) dissolved in pure water and incubated at room temperature overnight [8]. After addition of 10 µl chloroform, 100 µl methanol and 40 µl water, the hydrolysate was applied twice on a 0.1 g pre-packed C18 Bond Elut column (Analytichem International Inc., Harbor City, CA, USA) which had been pre-treated with 5 ml of chloroform/methanol/water, 1/10/9 by vol. Sialic acids were eluted with another 0.75 ml of this solvent and retained lipids were eluted with 2.5 ml of chloroform/methanol, 2/1 by vol. TLC analyses of sialic acids were done on HPTLC plates (Merck) using 1-propanol/water/25% ammonia, 70/28/1.5 by vol, [21] and of retained glycolipids as described above.

Table 1. Analyses of cumulative fecal output (mean ±SEM) for the seven day study period.

Rats	Total fecal output	Test (n = 12)	Control (n = 12)
Germ-free (n = 12)	Wet weight (g)	11.1±1.6[a]	6.5±0.8
	Dry weight (g)	7.0±0.8[a]	4.8±0.5
	Fatty acids (mg)	171.0±26.8[b]	43.0±5.3
	Nitrogen (mg)	316.7±4.3[a]	160.5±15.7
Conventional (n = 12)	Wet weight (g)	7.5±0.8[b]	2.5±0.3
	Dry weight (g)	5.5±0.7[b]	2.2±0.3
	Fatty acids (mg)	n.d.[c]	n.d.
	Nitrogen (mg)	317.0±48[b]	47.8±7.7

[a] $p<0.05$.
[b] $p<0.001$.
[c] n.d. = not determined.

Immunohistochemistry

Histological tissue from jejunal and ileal sites were fixed and 3 μm sections stained with hematoxylin and eosin for light microscopy. Indirect immunoperoxidase staining of PHA adherent to the epithelial surface utilized polyvalent antisera to the PHA-E isolectin [22].

Data for T and C groups are presented as mean ±SEM with 95% interval of confidence. Statistical analyses were performed by Student t test utilizing unpaired analysis.

Results

All GF rats gained weight progressively and pair-fed T and C groups demonstrated no differences in mean weight gain over the study period [T: +5.47±0.57; C: +4.75±0.64 g/rat/day (Fig. 1)]. Food consumption during the study period increased daily, ranging from 6.2 to 14.5 g, with a mean intake of 11.1±1.3 g/rat/day with no statistical difference between T and C rats.

CV rats whose growth characteristics are also depicted in Fig. 1, did not gain weight during the study period (T: -0.8±0.4 g/rat/day, C: -0.5±0.5 g/rat/day). Characteristic changes which occur in the small bowel microflora of these CV animals have already been published [23].

Analyses of the cumulative fecal output are shown in Table 1. For GF rats the cumulative fecal wet weight and dry weight of T exceeded C for the seven day study ($p<0.05$). Average daily output was 1.6±0.2 g for T and 1.0±0.1 g for C. Feces of GF animals are characteristically loose and poorly formed, but those of T animals were observed to be more watery than those of C. The magnitude of the fecal water content was inaccurately reflected by these results due to the air drying of feces during the 24 h collection period in the metabolic cages while exposed in the air inflated isolators. An increase in fecal fatty acid and nitrogen content

Table 2. Sphingolipid contents in pooled fecal samples of conventional (CV) and germ-free (GF) rats being either test-fed with PHA lectin (T) or fed with control diet (C).

Sphingolipid fraction	Sphingosine content (μmol/g dry feces)			
	CV-T	CV-C	GF-T	GF-C
Non-acid glycosphingolipids	12.5±1.3[a]	3.4±0.1	7.6±1.7[a]	3.2±0.3
Sphingomyelin	0.66±0.07[a]	0.08±0.04	1.1±0.2[a]	0.09±0.03

[a] $p<0.001$.

contributed to increased fecal weight in T ($p<0.001$ and $p<0.05$, respectively). Cumulative fecal output was greater in T than C for CV animals and nitrogen excretion was increased similar to that observed in GF-T animals.

The fecal sphingolipid excretion measured as μmol sphingosine per g dry weight, was increased in test animals compared to controls in both groups of rats (Table 2). The non-acid glycolipids were increased almost four-fold in CV-T animals and more than two-fold in GF-T rats. Taking into account the increase of fecal output the total increase during the seven day period would be nine-fold for CV-T rats and 3.5-fold for GF-T rats compared to controls. During the same period the excretion of sphingomyelins (although quantitatively smaller) was increased 20-fold and 18-fold, respectively, for the two groups of rats. Fecal gangliosides could not be quantified with the technique of Naoi *et al.* [13] due to interfering impurities. However, using densitometry it was clear that there was a 2.5-fold higher excretion of gangliosides for GF-T rats compared to controls.

The thin-layer chromatographic appearance of non-acid glycosphingolipids in one dimension (Fig. 2) and in two dimensions (not shown) did not reveal any major qualitative changes of glycosphingolipid structures within each group of CV and GF rats. The quantitative differences were however obvious and in agreement with those of Table 2. With immunostaining of the non-acid glycolipid fractions with monoclonal anti blood group A, B, and H antibodies (Fig. 3) the same quantitative differences were again observed for the blood group H-active glycolipids with three and 10 monosaccharides in the carbohydrate chains, i.e. for H-3 and H-10 produced predominantly in the small intestine of the rat [24]. For the blood group A-active (A-6) and B-active (B-4, B-6) compounds, produced in the rat large intestine [25], an increase in fecal excretion was observed only for CV and not for GF animals. Using chromatographic overlay with [^{35}S]methionine-labelled bacteria, binding specifically to Galα1-4Gal-containing glycolipids, again quantitative but not qualitative differences were observed between the two groups of rats (Fig. 4).

In the acid fractions, the single major ganglioside was G_{M3}, sialosyl-lactosylceramide (Fig. 5). The amount of G_{M3} in feces of CV animals was low as compared to that of GF animals. This decrease of G_{M3} content paralleled an increase in the amount of the more slow moving species of lactosylceramide (see Fig. 2) and was probably an effect of microbial degradation as has already been reported [7]. The acid fractions of feces of GF rats showed chromatographically several species of G_{M3} of which mainly the more fast moving species were

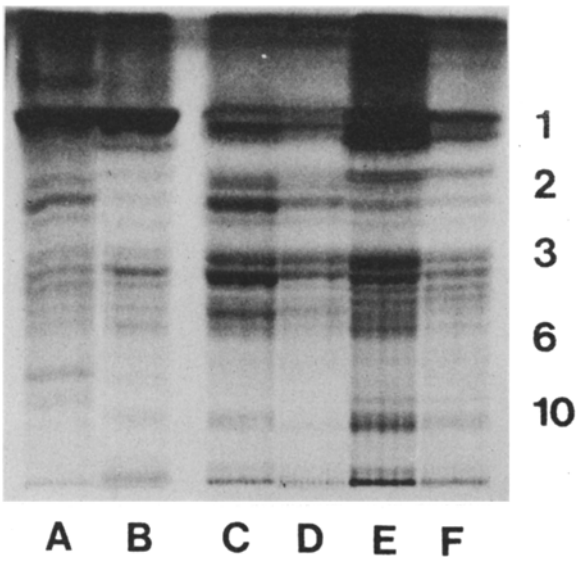

Figure 2. Thin-layer chromatogram of non-acid glycolipid fractions of feces of conventional PHA-fed (lane C) and control (lane D) rats and germ-free PHA-fed (lane E) and control (lane F) rats. An amount corresponding to 12 mg of dry weight of feces was spotted on each lane. Reference lanes A and B correspond to non-acid glycolipids of 40 mg dry feces of, respectively, conventional and germ-free rats of another strain [7]. Anisaldehyde was used for detection. Figures to the right indicate number of monosaccharide units in the respective glycolipids.

quantitatively increased in T rats. The solvent employed separates G_{M3} species both according to ceramide differences and differences in the sialic acid structure i.e. *N*-acetyl- or *N*-glycoloylneuraminic acid [7, 26]. By treating the acid fractions with neuraminidase and analysing the released sialic acids (Fig. 6) it was possible to show that the G_{M3} of GF-T rats had a significant increase only in containing more *N*-acetylneuraminic acid as compared to GF-C rats. Lactosylceramide species obtained after neuraminidase treatment were chromatographically very similar for the GF-T and GF-C rats but again quantitative differences of T over C were observed (Fig. 7).

On histological examination by light microscopy, the epithelia of jejunum and ileum were essentially normal except for some reduction in villus, height, and increase in crypt depth. No significant differences were detected in PHA-exposed tissue when compared to controls. Immunohistochemical activity after PHA-staining was restricted to the brush border of the villus. In general, staining was more intense in the proximal intestine. These findings were similar to previous observations [22] and were therefore not presented again.

Discussion

In the present study we have analyzed sphingolipids of lyophilized feces of both CV and GF rats either PHA-fed or fed on a control diet. A major aim was to reveal whether there might

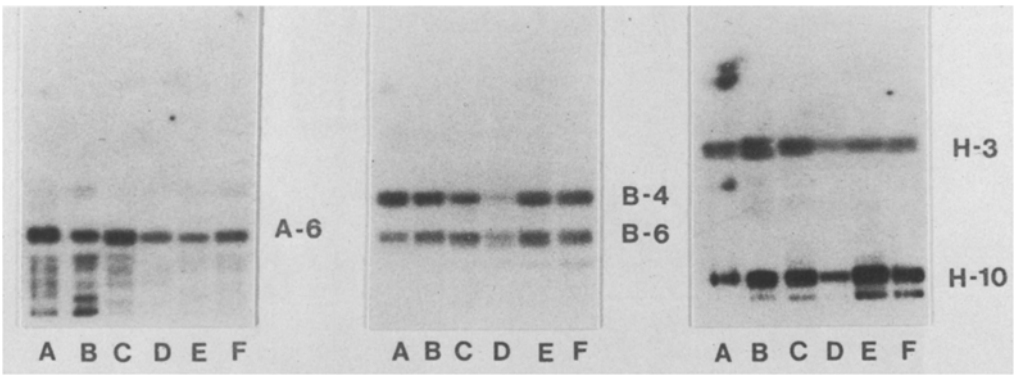

Figure 3. Autoradiograms of fecal non-acid glycosphingolipid fractions identical to those of Fig. 2 after immunostaining with anti blood group A (left), B (middle) and H (right) antibodies. The amount spotted on each lane correspond to 4 mg (lanes C-F) and 13 mg (lanes A, B) dry feces. The abbreviations to the right of the chromatograms give the identities by blood group activities and monosaccharide units of the respective glycolipids.

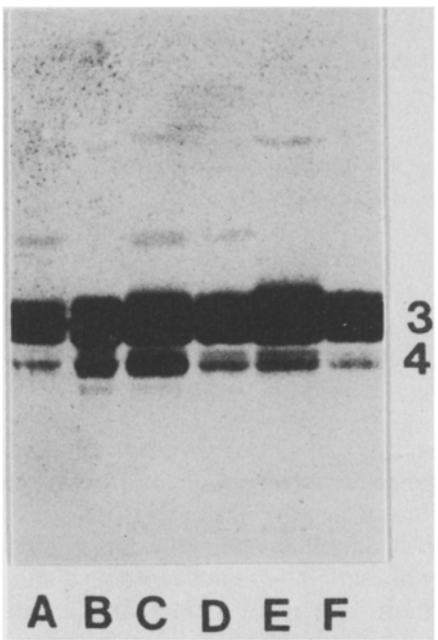

Figure 4. Autoradiogram of fecal non-acid glycosphingolipids identical to those of Figs. 2 and 3 after overlay with ^{35}S-labelled *E. coli* (strain MR506) which specifically binds to Galα1-4Gal-containing glycolipids [20]. Figures to the right indicate the number of monosaccharide units in the respective glycolipids.

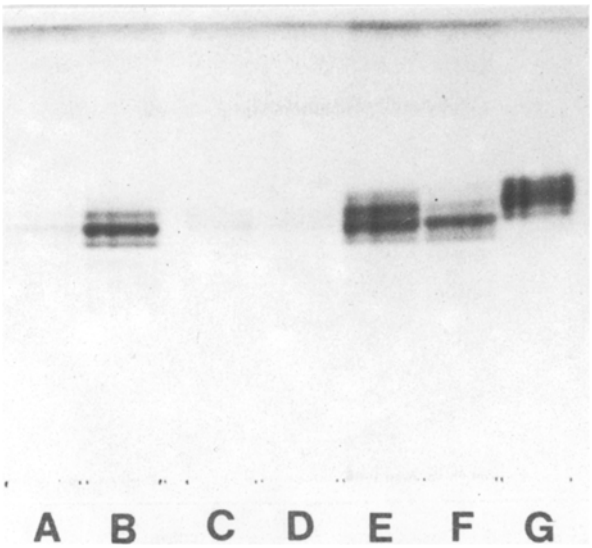

Figure 5. Thin layer chromatogram of acid glycolipid fractions of feces of conventional PHA-fed (lane C) and control (lane D) rats and germ-free PHA-fed (lane E) and control (lane F) rats corresponding to an amount of 2 mg dry feces. Reference lanes A and B correspond to acid glycolipids of 10 mg dry feces of another rat strain [7] and lane G correspond to 3 μmol of *N*-acetylneuraminosyl-lactosylceramide of human meconium [31]. Resorcinol was used for detection of gangliosides.

be changes induced in sphingolipid excretion as a result of the direct effects of PHA on the enterocyte brush border membrane. This would be expected not only for GF animals, where there is no bacterial degradation of glycolipids shed into the gut lumen, but also for CV animals where the luminal degradation is dependent on the specific activities of glycosidases produced by the intestinal microflora [7].

For germ-free animals PHA in the diet induced an increased fecal excretion of all sphingolipid fractions, i.e. sphingomyelins (18-fold during the seven day period), non-acid glycolipids (3.5-fold) and gangliosides (2.5-fold). In an attempt to analyse the site for this action of PHA along the gastrointestinal tract, specific glycosphingolipids were selected for study. With monoclonal anti-blood group A, B, and H antibodies it was shown that only the blood group H active glycolipids, produced mainly in the small intestinal epithelium [24], and not the blood group A and B active glycolipids, produced in the large intestinal epithelium [25] of these rats, were excreted in increased amounts during PHA-feeding. These results favour the major effect of PHA on glycolipid excretion being directed towards increasing the turnover of the small intestinal epithelium to which it is bound.

The increase of *N*-acetylneuraminosyl-lactosylceramide was noticeable since this species of G_{M3} has been found restricted to epithelial cells of rat large intestines [25], and to rat duodenum and proximal jejunum [26]. King and coworkers [3] observed that the most severe histological injury in response to feeding a toxic red kidney bean extract was present in the epithelium of duodenum and upper small intestine. Such a localization would thus agree very well with our chemical findings of an increased excretion of *N*-acetylneuraminosyl-lactosylceramide in feces and point to a direct effect of PHA on these cells.

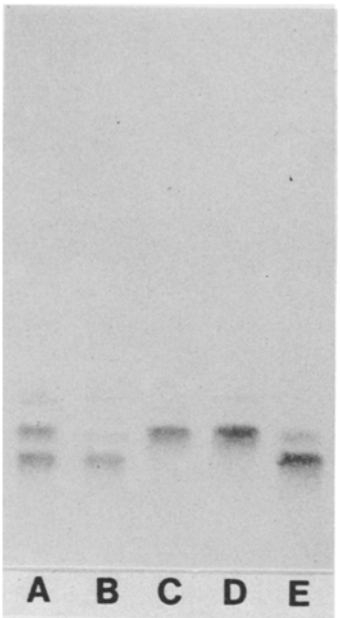

Figure 6. Thin layer chromatogram of sialic acids after neuraminidase treatment of acid glycolipids from feces of germ-free rats fed on PHA-diet (lane A) or on a control diet (lane B). Reference lanes are neuraminidase treated *N*-acetylneuraminosyl-lactosylceramide of human meconium (lane C), 0.5 µg *N*-acetylneuraminic acid (lane D) and 0.5 µg *N*-glycoloylneuraminic acid (lane E). Resorcinol was used for the detection.

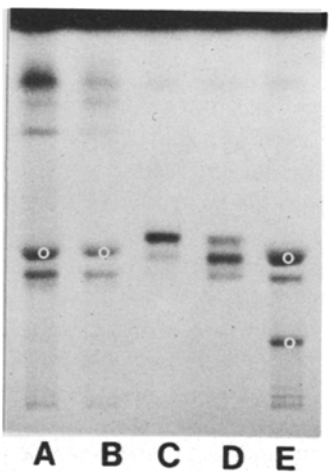

Figure 7. Thin layer chromatogram of lactosylceramide fractions obtained after neuraminidase treatment of acid glycolipids from feces of GF rats fed on PHA-diet (lane A), or on a control diet (lane B), and from two *N*-acetylneuraminosyl-lactosylceramide fractions of human meconium (lanes C and D), and the acid fraction from feces of another GF-rat strain (lane E) [7]. Anisaldehyde was used for detection and the bands marked with an O were not glycolipids.

Recently, a detailed description of oligosaccharide structures interacting with the PHA leukoagglutinating (L-PHA) and erythroagglutinating (E-PHA) isomers was published [27]. Both lectins bound oligosaccharides with terminal sialic acid linked α(2-3)- but not α(2-6)- to galactose. The desialylated *N*-acetyllactosamine-terminated oligosaccharides containing a bisecting *N*-acetylglucosamine residue bound β(1-4)- to the mannose core showed the strongest interaction with both isolectins. In an attempt to elucidate whether there might be any direct interaction of PHA with glycolipids in the plasma membrane we have performed binding studies with ^{125}I-labelled PHA or native PHA and radiolabelled antibodies against PHA but found no specific binding to glycolipids using the thin-layer chromatogram overlay technique [16, 17]. Glycolipids tested were both non-acid and acid fractions from the present study, total gangliosides of bovine brain and purified gangliosides G_{M3}, G_{M1} and NeuAcα(2-3)-neolactotetraosylceramide. Thus, we argue that the changes we have seen in the fecal excretion of glycolipids are probably secondary to the binding of PHA to membrane glycoproteins of intestinal epithelial cells and may be exerted either by the mitogenic or trophic effects of PHA and/or possibly by a direct loss of brush border membranes of these cells.

The differences in the increase between excretion of sphingomyelin and non-acid glycosphingolipids upon PHA feeding is interesting. It was recently shown for rats that nine days of feeding PHA in low doses (0.4% or less of total diet protein) essentially only increased micro-vesiculation or blebbing of microvilli with the enterocytes still morphologically healthy, while at higher doses (2.5% of total diet protein) cell death became the most important effect [28]. Microvesicles exfoliated from cultured cells have earlier been shown to contain significantly increased amounts of sphingomyelin compared to purified plasma membranes of the same cells [29]. Thus, although sphingomyelin is only a minor sphingolipid of rat intestinal microvillus membranes [30] and a small part of the total fecal excretion of sphingolipids, the highly increased rate of excretion of sphingomyelin when 1% PHA was included in the diet support the idea that PHA interacts both with specific parts of the microvillus membranes as well as causes an increased exfoliation of mucosal cells.

The effects of PHA on CV rats were similar to those observed for GF rats. For CV rats however, the increase of fecally excreted non-acid glycolipids was even more pronounced (nine-fold) and in contrast to GF rats not only blood group H, but also blood group A and B active glycolipids were quantitatively increased in the stools of PHA exposed animals. How these changes were generated are unclear, but might indicate an indirect effect through the effects of the overgrowth of specific bacteria of the small and large intestinal microflora.

Some of these opportunistic bacteria have been isolated [23] and their interactions with intestinal glycolipids will be a source for further study since increased luminal shedding of sphingolipids might play a role as nutrient sources and/or adherence sites for luminal commensal bacteria to proliferate in response to exposure of the mucosa to PHA.

Acknowledgements

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