Microbial Pathogenesis 76 (2014) 51–60

Contents lists available at ScienceDirect

Microbial Pathogenesis

journal homepage: www.elsevier.com/locate/micpath

Porcine intestinal glycosphingolipids recognized by F6-fimbriated enterotoxigenic *Escherichia coli*

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ARTICLE INFO

Article history:
Received 5 March 2014
Received in revised form
16 September 2014
Accepted 17 September 2014
Available online 18 September 2014

Keywords:
F6-fimbriated *Escherichia coli*
Porcine intestinal glycosphingolipids
Microbial adhesion
Glycosphingolipid characterization
Mass spectrometry

ABSTRACT

One important virulence factor of enterotoxigenic *Escherichia coli* is their ability to adhere via fimbrial adhesins to specific receptors located on the intestinal mucosa. Here, the potential glycosphingolipid receptors of enterotoxigenic F6-fimbriated *E. coli* were examined by binding of purified F6 fimbriae, and F6-expressing bacteria, to glycosphingolipids on thin-layer chromatograms. When intestinal mucosal non-acid glycosphingolipids from single pigs were assayed for F6 binding capacity, a selective interaction with two glycosphingolipids was observed. The binding-active glycosphingolipids were isolated and characterized as lactotriaosylceramide (GlcNAcβ3Galβ4Glcβ1Cer) and lactotetraosylceramide (Galβ3GlcNAcβ3Galβ4Glcβ1Cer). Further binding assays using a panel of reference glycosphingolipids showed a specific interaction between the F6 fimbriae and a number of neolacto core chain (Galβ4GlcNAc) glycosphingolipids. In addition, an occasional binding of the F6 fimbriae to sulfatide, galactosylceramide, lactosylceramide with phytosphingosine and/or hydroxy fatty acids, isoglobotriaosylceramide, gangliotriaosylceramide, and gangliotetraosylceramide was obtained. From the results we conclude that lactotriaosylceramide and lactotetraosylceramide are major porcine intestinal receptors for F6-fimbriated *E. coli*.

1. Introduction

Infection with F4 (K88)-, F5 (K99)-, F6 (987P)- and/or F18-fimbriated *Escherichia coli* is a common cause of diarrhea or edema disease in young pigs, thereby leading to significant economic losses. The carbohydrate receptors involved in adherence of most of these *E. coli* strains to the porcine intestinal epithelium have been subjected to thorough investigations, leading to identification of carbohydrates involved in $F4^+$, $F5^+$ and $F18^+$ *E. coli* adherence. Thus, $F5^+$ *E. coli* was shown to adhere to NeuGc-GM3 [1,2], whereas $F18^+$ *E. coli* have a specific binding to blood group ABH determinants on type 1 core chains [3], and $F4^+$ *E. coli* recognizes a more diverse set of carbohydrates on sphingolipid or protein backbones [4–6].

The F6 fimbriae are polymeric structures consisting of hundreds of copies of FasA (major subunit), supplemented with the minor subunits FasF and FasG. FasF is the linker between FasA and FasG, and the adhesive subunit is FasG [7–10]. Different chaperone proteins are used for various F6 subunits, with FasB being the periplasmic chaperone for FasA, and FasC being the chaperone for the adhesin FasG [11]. FasD was characterized as the usher involved in transport of the fimbrial subunits across the outer membrane and FasE is a third chaperone-like protein [12], and FasH activates transcription of the structural subunit FasA [13].

Earlier studies have shown that the FasG adhesin recognizes a specific set of protein receptors with sizes of 33–39 kDa, 17 kDa and 14 kDa [9]. The 33–39 kDa proteins were identified as histone H1 proteins, which are recognized by F6 fimbriae in a carbohydrate-independent manner [14]. By immunohistochemistry and electron microscopy these histone H1 proteins were visualized on the microvilli of neonatal piglet intestinal epithelium, and it was suggested that the histone H1 proteins might stabilize the interaction between F6 fimbriae and cell membrane compounds, by binding simultaneously to the membrane and the F6 fimbriae.

Binding of F6 fimbriae to the two major glycosphingolipids of porcine intestinal epithelium, sulfatide (SO_3-3Galβ1Cer) and

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http://dx.doi.org/10.1016/j.micpath.2014.09.009

galactosylceramide (Galβ1Cer), has also been reported [15], and it has been demonstrated that lysine-117 of the FasG subunit is involved in the interaction with sulfatide [16]. Binding of F6 fimbriated *E. coli* to gangliotetraosylceramide (Galβ3GalNAcβ4-Galβ4Glcβ1Cer), lactosylceramide (Galβ4Glcβ1Cer), sulfatide, gangliotriaosylceramide (GalNAcβ4Galβ4Glcβ1Cer), and galactosylceramide has also been reported [17,18].

During our studies of carbohydrate recognition by fimbriae of porcine ETEC we also examined the potential role of carbohydrates as adhesion receptors for F6 fimbriated *E. coli*, by binding of F6 fimbriae to an extensive panel of glycosphingolipids. Thereby a specific interaction between F6-expressing bacteria, and purified F6 fimbriae, and a number of neolacto core chain (Galβ4GlcNAc) glycosphingolipids was detected, in addition to the previously described binding to sulfatide and galactosylceramide. Two minor glycosphingolipids recognized by F6 fimbriae were isolated from piglet intestinal mucosa, and characterized by mass spectrometry and proton NMR as lactotriaosylceramide (GlcNAcβ3-Galβ4Glcβ1Cer) and lactotetraosylceramide (Galβ3GlcNAcβ3Galβ4Glcβ1Cer).

2. Materials and methods

2.1. Bacterial strains, culture conditions and labeling

The prototype F6-positive *E. coli* strain 987 (serotype O9:K103:NM, $F6^+$, STa^+, STb^+) was grown in Mueller Hinton broth (Oxoid, Basingstoke, Hampshire, England) [19].

For metabolic labeling, the culture medium was supplemented with 10 μl ^{35}S-methionine (400 μCi; Amersham Pharmacia Biotech) per 10 ml. Bacteria were harvested by centrifugation, washed three times in phosphate-buffered saline, pH 7.4 (PBS), and resuspended in PBS containing 2% (w/v) bovine serum albumin, 0.1% (w/v) NaN_3 and 0.1% (w/v) Tween 20 (BSA/PBS/TWEEN) to a bacterial density of 1×10^8 colony forming units/ml. The specific activity of bacterial suspensions was approximately 1 cpm per 100 bacteria.

2.2. Purification of F6 fimbriae

E. coli strain 987 was cultured statically in Mueller Hinton broth at 37 °C during 6 days. After growing these bacteria, slide agglutination with an F6-specific monoclonal antibody (CVI Wageningen, Lelystad, The Netherlands) was performed to confirm the presence of F6 fimbriae. Next, the bacteria were harvested by centrifugation at 2750 g for 35 min, and suspended in phosphate-buffered saline (PBS, pH 7.4). Subsequently, the bacteria were washed with ice cold PBS and the bacterial pellet was mixed using an Ultra Turrax for 20 min. The mixed bacterial suspension was then centrifuged twice at 12,500 g to separate the bacterial cell debris from the fimbrial solution. Thereafter, the bacterial pellet was discarded and the fimbriae were precipitated with 60% ammonium sulfate at 4 °C overnight. After centrifugation at 1450 g for 30 min, the precipitated fimbriae are dissolved in PBS and dialyzed against PBS overnight at 4 °C.

2.3. ^{125}I-labeling

Aliquots of 100 μg of fimbrial protein were labeled with ^{125}I, using $Na^{125}I$ (100 μCi/ml; Amersham Pharmacia Biotech, Little Chalfont, U.K.), according to the IODO-GEN protocol of the manufacturer (Pierce, Rockford, IL), giving approximately 2×10^3 cpm/μg protein.

2.4. Reference glycosphingolipids

Total acid and non-acid glycosphingolipid fractions were isolated as described [20]. Individual glycosphingolipids were isolated by repeated chromatography on silicic acid columns and by HPLC, and identified by mass spectrometry [21,22], and ^{1}H NMR spectroscopy [23].

2.5. Thin-layer chromatography

Thin-layer chromatography was done on aluminum- or glass-backed silica gel 60 high performance thin-layer chromatography plates (Merck, Darmstadt, Germany). Glycosphingolipid mixtures (40 μg) or pure glycosphingolipids (4 μg) were applied to the plates, and eluted with chloroform/methanol/water (60:35:8, by volume) as solvent system. Chemical detection was done with anisaldehyde [24].

2.6. Chromatogram binding assay

Binding of radiolabeled F6 fimbriae and F6-fimbriated bacteria to glycosphingolipids on thin-layer chromatograms was done as described previously [3,6]. Dried chromatograms were dipped in diethylether/*n*-hexane (1:5 v/v) containing 0.5% (w/v) polyisobutylmethacrylate for 1 min. To diminish background binding the chromatograms were blocked with 2% (w/v) bovine serum albumin, 0.1% (w/v) NaN_3 and 0.1% (w/v) Tween 20 (BSA/PBS/TWEEN) for 2 h at room temperature. Then the plates were incubated with ^{125}I-labeled fimbriae ($1–5 \times 10^6$ cpm/ml) or ^{35}S-labeled bacteria ($1–5 \times 10^6$ cpm/ml) diluted in BSA/PBS/TWEEN for another 2 h at room temperature. After washing six times with PBS, and drying, the thin-layer plates were autoradiographed for 12 h using XAR-5 X-ray films (Eastman Kodak, Rochester, NY).

Chromatogram binding assays with monoclonal antibodies directed against the blood group A determinant (Dakopatts a/s, Glostrup, Denmark) were done as described [25], using ^{125}I-labeled anti-mouse antibodies for detection.

2.7. Isolation of F6 fimbriae binding non-acid tri- and tetraglycosylceramides from piglet small intestinal mucosa

Non-acid glycosphingolipids were isolated from mucosal scrapings from one blood group O and one blood group A six weeks old piglet small intestines as described [20]. Briefly, the mucosal scrapings were lyophilized, and then extracted in two steps in a Soxhlet apparatus with chloroform and methanol (2:1 and 1:9, by volume, respectively). The material obtained was subjected to mild alkaline hydrolysis and dialysis, followed by separation on a silicic acid column. Acid and non-acid glycosphingolipid fractions were obtained by chromatography on a DEAE-cellulose column. In order to separate the non-acid glycolipids from alkali-stable phospholipids, this fraction was acetylated and separated on a second silicic acid column, followed by deacetylation and dialysis. Final purifications were done by chromatographies on DEAE-cellulose and silicic acid columns. Thereby, 180 mg total non-acid glycosphingolipids were obtained from 24 g dry weight blood group O piglet small intestinal mucosa, while 60 mg was obtained in the case of the blood group A piglet small intestinal mucosa (dry weight 21 g).

The F6 binding tetraglycosylceramide was isolated from the non-acid fraction of the blood group A piglet small intestinal mucosa. Here, 40 mg of the total non-acid glycosphingolipid fraction was first separated on a silicic acid column eluted with increasing volumes of methanol in chloroform. Thereby one fraction containing monoglycosylceramides and one fraction containing

diglycosylceramides and more slow-migrating glycosphingolipids was obtained. The latter fraction (17.5 mg) was separated on an Iatrobeads (Iatrobeads 6RS-8060; Iatron Laboratories, Tokyo) column (2 g) eluted with 20 × 1 ml chloroform/methanol/water 60:35:8 (by volume). Throughout the separation procedures aliquots of the fractions obtained were analyzed by thin-layer chromatography, and fractions that were colored green by anisaldehyde were tested for binding of F6 fimbriae using the chromatogram binding assay.

The F6 binding fractions were pooled, and further separations were achieved by repeating the Iatrobeads column chromatography two times. The first column (2 g) was eluted with 20 × 0.5 ml chloroform/methanol/water 60:35:8 (by volume), while the second column (2 g) was eluted with 30 × 0.5 ml chloroform/methanol/water 65:25:4 (by volume). The F6 binding compound migrating in the tetraglycosylceramide region eluted in fraction 28, and this fraction was designated fraction A-tetra (0.5 mg), and used for structural characterization.

The same strategy was used for isolating the F6 binding triglycosylceramide from the non-acid fraction of the blood group O piglet small intestinal mucosa. Thus, 160 mg of the total non-acid glycosphingolipid fraction was first separated on a silicic acid column eluted with increasing volumes of methanol in chloroform. Thereafter, the triglycosylceramide recognized by the F6 fimbriae was isolated by repeated chromatographies on Iatrobeads columns, and finally, a fraction containing the F6 fimbriae binding compound was obtained. This fraction, designated fraction O-tri (0.7 mg), was used for structural characterization.

2.8. ESI/MS and ESI/MS/MS of native glycosphingolipids

The glycosphingolipids (dissolved in methanol/acetonitrile 75:25, by volume) were separated on a 200 × 0.150 mm column, packed in-house with 5 μm polyamine II particles (YMC Europe GMBH, Dinslaken, Germany), and eluted with a water gradient (A: 100% acetonitrile; B: 10 mM ammonium bicarbonate). Samples were analyzed on an LTQ linear quadrupole ion trap mass spectrometer (Thermo Electron) by LC-ESI/MS at −3.5 kV. Full-scan (*m/z* 500–1800, 2 microscans, maximum 100 ms, target value of 30,000) was performed, followed by data dependent MS^2 scans (2 microscans, maximum 100 ms, target value of 10,000) with normalized collision energy of 35%, an isolation window of 2.5 units, an activation $q = 0.25$, and an activation time of 30 ms.

2.9. Endoglycoceramidase digestion and LC/MS

Endoglycoceramidase II from *Rhodococcus* spp. (Takara Bio Europe S.A., Gennevilliers, France) was used for hydrolysis of glycosphingolipids. Briefly, 50 μg of the F6 binding non-acid glycosphingolipid fractions from piglet small intestine (fractions O-tri and A-tetra) were resuspended in 100 μl 0.05 M sodium acetate buffer, pH 5.0, containing 120 μg sodium cholate, and sonicated briefly. Thereafter, 1 mU of endoglycoceramidase II was added and the mixture was incubated at 37 °C for 48 h. The reaction was stopped by addition of chloroform/methanol/water to the final proportions 8:4:3 (by volume). The oligosaccharide-containing upper phase thus obtained was separated from detergent on a Sep-Pak QMA cartridge (Waters, Milford, MA). The eluants containing the oligosaccharides were dried under nitrogen and under vacuum.

The glycosphingolipid-derived oligosaccharides were analyzed by capillary-LC/MS and MS/MS as described [22]. In brief, the oligosaccharides were separated on a column (200 × 0.180 mm) packed in-house with 5 μm porous graphite particles (Hypercarb, Thermo Scientific), and eluted with an acetonitrile gradient (A: 8 mM ammonium bicarbonate; B: 100% acetonitrile). The saccharides were analyzed in the negative ion mode on an LTQ linear quadrupole ion trap mass spectrometer (Thermo Electron, San José, CA).

3. Results

3.1. Binding of F6 fimbriae and F6 fimbriated E. coli to glycosphingolipid mixtures

In order to expose the F6 fimbriae to a large number of variant carbohydrate structures, mixtures of glycosphingolipids separated on thin-layer plates were used in the initial screening for carbohydrate recognition by the F6 fimbriae and F6 fimbriated bacteria. Among the total acid and non-acid fractions coming from different sources (more than 30 fractions tested), the F6 fimbriae and the F6 fimbriated bacteria bound only to few glycosphingolipids (Fig. 1).

Thus, there was a distinct binding of both the fimbriae and the bacterial cells to a number of compounds in the non-acid fractions of horse erythrocytes (Fig. 1(B), lane 1), bovine erythrocytes (Fig. 1(B), lane 4), rabbit erythrocytes (Fig. 1(B), lane 5; Fig. 1(D) and (E), lane 2) and rabbit thymus (Fig. 1(B), lane 6; Fig. 1(D) and (E), lane 1). This finding suggested a recognition of neolacto-containing glycosphingolipids, since the major glycosphingolipids of several of these fractions are based on neolacto core chains [26–30].

3.2. Binding of F6 fimbriae non-acid glycosphingolipid mixtures of piglet small intestinal mucosa

Two different binding patterns were observed upon binding of F6 fimbriae to non-acid glycosphingolipids of piglet intestinal mucosa. The first pattern was obtained with the glycosphingolipid mixture from a blood group O pig where the F6 fimbriae bound to a compound migrating in the triglycosylceramide region (Fig. 2(B), lane 2). The second pattern, obtained with the glycosphingolipid mixture from a blood group A pig, was binding of the fimbriae to two compounds migrating as tri- and tetra-glycosylceramides, respectively (Fig. 2(B), lane 3).

3.3. Isolation and characterization of the F6 binding glycosphingolipids from piglet small intestinal mucosa

3.3.1. F6 binding triglycosylceramide

The triglycosylceramide recognized by the F6 fimbriae was isolated from the non-acid glycosphingolipid fraction of the blood group O piglet intestinal mucosa by chromatographies on silicic acid and Iatrobeads columns, and the fractions obtained were tested for F6 binding activity using ^{125}I-labeled F6 fimbriae. After several chromatographic steps 0.7 mg of a triglycosylceramide fraction containing the F6-binding glycosphingolipid was obtained (designated fraction O-tri (Fig. 3(A) and (B), lane 3)).

Structural characterization of fraction O-tri demonstrated that it contained two glycosphingolipids, globotriaosylceramide (Galα4Galβ4Glcβ1Cer) and lactotriaosylceramide (GlcNAcβ3-Galβ4Glcβ1Cer). This conclusion is based on the following observations:

I) The binding-active compound migrated in the triglycosylceramide region on thin-layer chromatograms (Fig. 2, lane 2 and 3; Fig. 3(A) and (B), lane 3).
II) By LC/MS analysis of the saccharides obtained by hydrolysis of fraction O-tri with *Rhodococcus* endoglycoceramidase, two $[M-H]^-$ ions were observed at *m/z* 503 (retention time 17.3–18.1 min) and at *m/z* 544 (retention time 20.1–20.7 min), respectively (Fig. 3(C)).

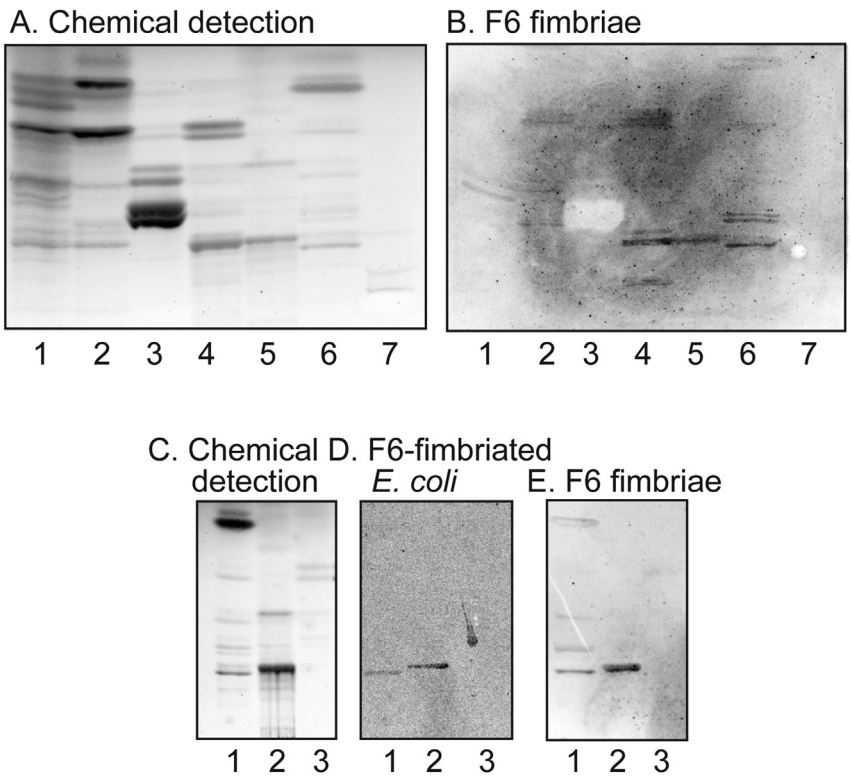

Fig. 1. Binding of F6 fimbriae and F6-fimbriated *Escherichia coli* to glycosphingolipid mixtures. Chemical detection by anisaldehyde (A and C), and autoradiograms obtained by binding of ^{125}I-labeled F6 fimbriae (B and E), and ^{35}S-labeled F6-expressing *E. coli* (D). The glycosphingolipids were separated on aluminum-backed silica gel plates, using chloroform/methanol/water 60:35:8 (by volume) as solvent system, and the binding assays were performed as described under "Materials and Methods". Autoradiography was for 12 h. The lanes on chart A and B were: Lane 1, non-acid glycosphingolipids of horse erythrocytes, 40 μg; Lane 2, non-acid glycosphingolipids of cat erythrocytes, 40 μg; Lane 3, non-acid glycosphingolipids of porcine erythrocytes, 40 μg; Lane 4, non-acid glycosphingolipids of bovine erythrocytes, 40 μg; Lane 5, non-acid glycosphingolipids of rabbit erythrocytes, 40 μg; Lane 6, non-acid glycosphingolipids of rabbit thymus, 40 μg; Lane 7, acid glycosphingolipids of rabbit thymus, 40 μg. The lanes on chart C–E were: Lane 1, non-acid glycosphingolipids of rabbit thymus, 40 μg; Lane 2, non-acid glycosphingolipids of rabbit erythrocytes, 40 μg; Lane 3, non-acid glycosphingolipids of human gastric cancer, 20 μg.

The MS^2 spectrum of the $[M-H]^-$ ion at *m/z* 503 had a series of prominent C-type fragment ions (C_1 at *m/z* 179 and C_2 at *m/z* 341) identifying a triglycosylceramide with Hex–Hex–Hex sequence (Fig. 3(D)). There was also a $^{0,2}A_2$ fragment ion at *m/z* 281 demonstrating an internal hexose substituted on C-4 as in Galα4Galβ4Glc (globotriaose) [22].

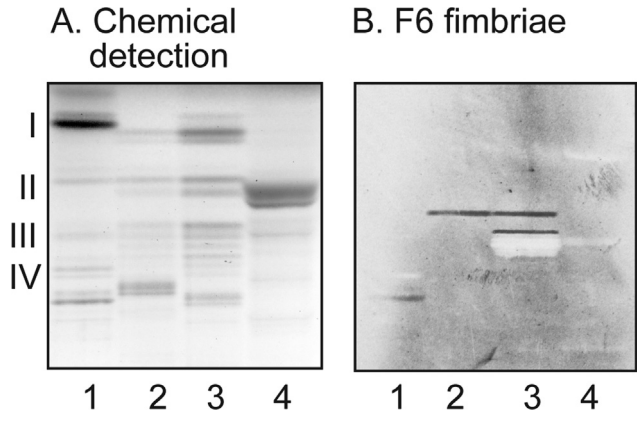

Fig. 2. Binding of F6 fimbriae to non-acid glycosphingolipids of piglet intestinal mucosa. Chemical detection by anisaldehyde (A), and autoradiogram obtained by binding of ^{125}I-labeled F6 fimbriae (B). The glycosphingolipids were separated on aluminum-backed silica gel plates, using chloroform/methanol/water 60:35:8 (by volume) as solvent system, and the binding assays were performed as described under "Materials and Methods". Autoradiography was for 12 h. The lanes were: Lane 1, non-acid glycosphingolipids of rabbit thymus, 40 μg; Lane 2, non-acid glycosphingolipids of blood group O piglet intestinal mucosa, 40 μg; Lane 3, non-acid glycosphingolipids of blood group A piglet intestinal mucosa, 40 μg; Lane 4, acid glycosphingolipids of blood group A piglet intestinal mucosa, 40 μg. The Roman numbers to the left of panel A denotes the approximate numbers of carbohydrates in the non-acid glycosphingolipid fractions in lanes 1–3.

MS^2 of the ion at *m/z* 544 gave C-type fragment ions at *m/z* 220 (C_1) and at *m/z* 382 (C_2), identifying a HexNAc–Hex–Hex sequence (Fig. 3(E)). No $^{0,2}A_2$ fragment ion at *m/z* 322 was found, indicating that the penultimate Hex was 3-substituted. Taken together these spectral features tentatively identified a lacto trisaccharide (GlcNAcβ3Galβ4Glc).

3.3.2. F6 binding tetraglycosylceramide

The binding-active tetraglycosylceramide was isolated from the non-acid glycosphingolipid fraction of the blood group A piglet intestinal mucosa by chromatography on silicic acid and Iatrobeads columns, and the preparative procedure was monitored by binding of radiolabeled F6 fimbriae on thin-layer chromatograms. Finally, 0.5 mg of an F6 binding tetraglycosylceramide fraction was obtained (denoted fraction A-tetra).

Characterization of fraction A-tetra identified lactotetraosylceramide (Galβ3GlcNAcβ3Galβ4Glcβ1Cer) and the A type 1 hexaosylceramide (GalNAcα3(Fucα2)Galβ3GlcNAcβ3-Galβ4Glcβ1Cer). This conclusion was based on the following properties:

I) On thin-layer chromatograms the binding-active glycosphingolipid migrated as a distinct band in tetraglycosylceramide region (Fig. 2(B), lane 3).
II) The base peak chromatogram from ESI/MS of the native fraction A-tetra had one $[M-H]^-$ ion at *m/z* 1241 and one $[M-H]^-$ ion at *m/z* 1574 (data not shown). These ions indicated one glycosphingolipid with one HexNAc and three Hex

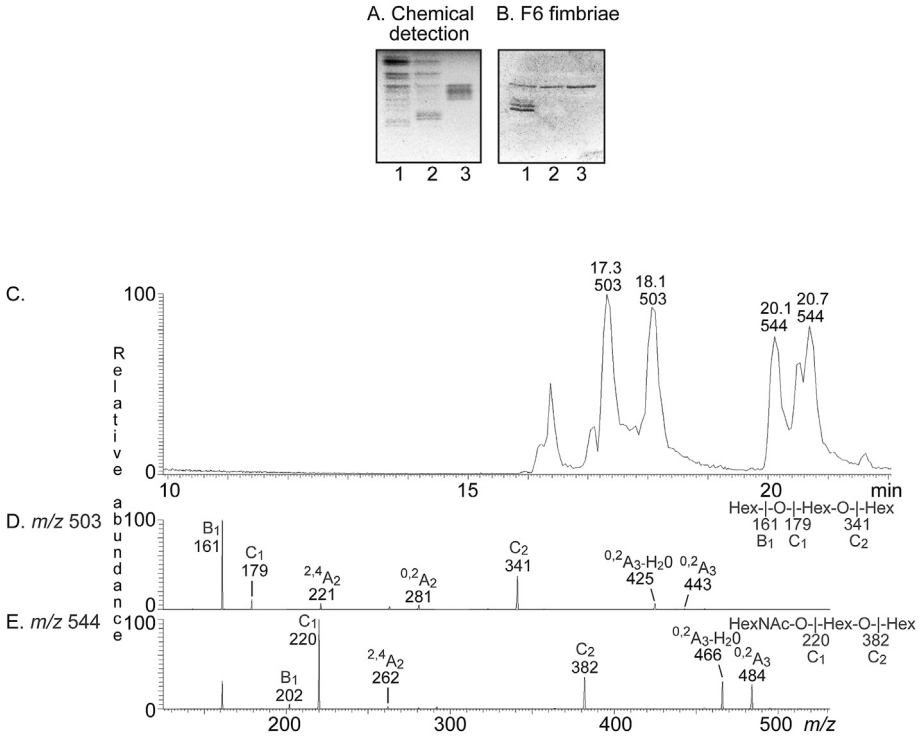

Fig. 3. Characterization of the F6 fimbriae binding triglycosylceramide of piglet intestinal mucosa. Chemical detection by anisaldehyde (A), and autoradiogram obtained by binding of ^{125}I-labeled F6 fimbriae (B). The glycosphingolipids were separated on aluminum-backed silica gel plates, using chloroform/methanol/water 60:35:8 (by volume) as solvent system, and the binding assays were performed as described under "Materials and Methods". Autoradiography was for 12 h. The lanes were: Lane 1, non-acid glycosphingolipids of blood group A piglet intestinal mucosa, 40 μg; Lane 2, non-acid glycosphingolipids of blood group O piglet intestinal mucosa, 40 μg; Lane 3, Fraction O-tri isolated from blood group O piglet intestinal mucosa, 4 μg. (C) Base peak chromatogram from LC-ESI/MS of the oligosaccharides obtained by digestion with *Rhodococcus* endoglycoceramidase II of the F6-binding glycosphingolipid fraction O-tri from blood group O piglet intestinal mucosa. (D) MS^2 spectrum of the ion at *m*/*z* 503 (retention time 17.3 min). (E) MS^2 spectrum of the ion at *m*/*z* 544 (retention time 20.7 min). The interpretation formulas show the deduced carbohydrate sequences.

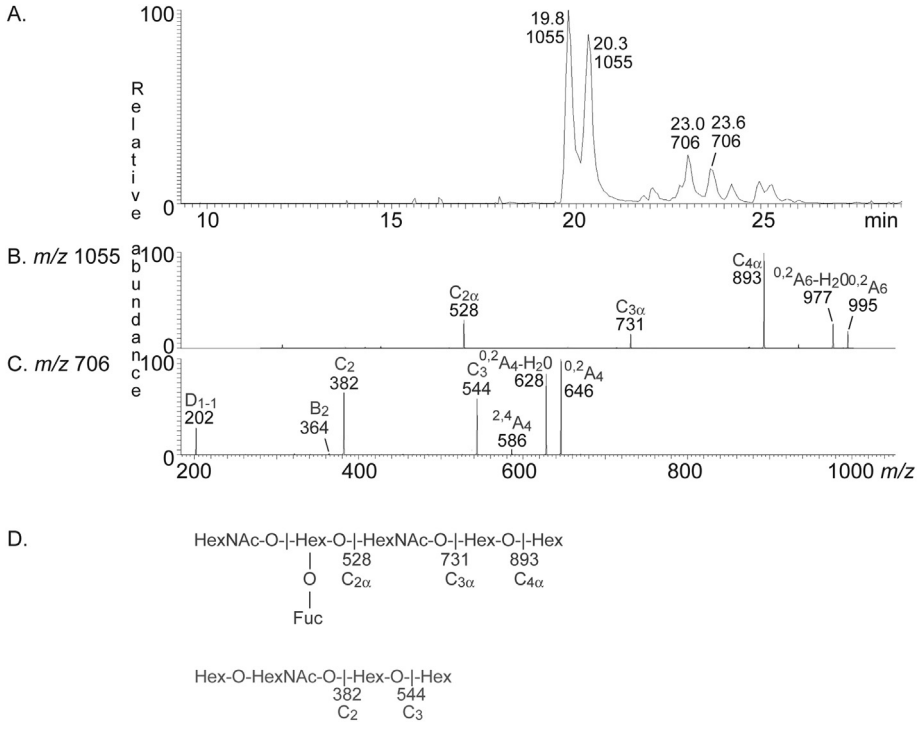

Fig. 4. Characterization of the F6 fimbriae binding tetraglycosylceramide of porcine intestinal mucosa. (A) Base peak chromatogram from LC-ESI/MS of the oligosaccharides obtained by digestion with *Rhodococcus* endoglycoceramidase II of the F6-binding glycosphingolipid fraction A-tetra. (B) MS^2 spectrum of the ion at *m*/*z* 1055 (retention time 19.8–20.3 min). (C) MS^2 spectrum of the ion at *m*/*z* 706 (retention time 23.0–23.6 min). (D) Interpretation formulas showing the deduced carbohydrate sequences.

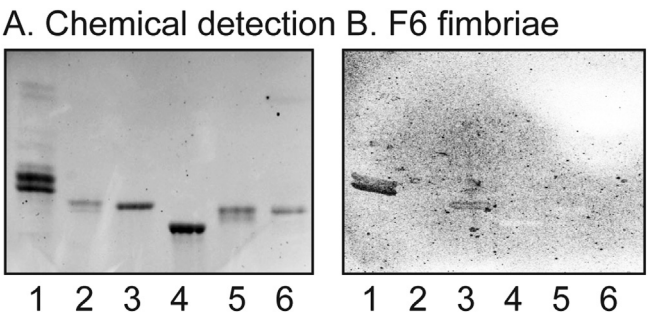

Fig. 5. Binding of F6 fimbriae to reference glycosphingolipids. Chemical detection by anisaldehyde (A), and autoradiogram obtained by binding of ^{125}I-labeled F6 fimbriae (B). The glycosphingolipids were separated on aluminum-backed silica gel plates, using chloroform/methanol/water 60:35:8 (by volume) as solvent system, and the binding assay was performed as described under "Materials and Methods". Autoradiography was for 12 h. The lanes were: Lane 1, neolactotetraosylceramide (Galβ4GlcNAcβ3-Galβ4Glcβ1Cer) of human neutrophils, 4 μg; Lane 2, GM3 ganglioside (Neu-Acα3Galβ4Glcβ1Cer) of human brain, 4 μg; Lane 3, blood group H type 2 pentaglycosylceramide (Fucα2Galβ4GlcNAcβ3Galβ4Glcβ1Cer) of human erythrocytes, 4 μg; Lane 4, blood group B type 2 hexaglycosylceramide (Galα3(Fucα2)Galβ4Glc-NAcβ3Galβ4Glcβ1Cer) of human erythrocytes, 4 μg; Lane 5, blood group A type 1 hexaglycosylceramide (GalNAcα3(Fucα2)Galβ3GlcNAcβ3Galβ4Glcβ1Cer) of human meconium, 4 μg; Lane 6, B5 pentaglycosylceramide (Gal-α3Galβ4GlcNAcβ3Galβ4Glcβ1Cer) of rabbit erythrocytes, 4 μg.

and d18:1-h16:0 ceramide, and one glycosphingolipid with one Fuc, three HexNAc and three Hex and d18:1-16:0 ceramide, respectively.

III) The base peak chromatogram from LC/MS of the saccharides obtained by hydrolysis of fraction A-tetra with *Rhodococcus* endoglycoceramidase had two $[M-H]^-$ ions at *m/z* 1055 (retention time 19.8–20.3 min) and at *m/z* 706 (retention time 23.0–23.6 min) (Fig. 4(A)).

IV) The MS^2 spectrum of the $[M-H]^-$ ion at *m/z* 1055 was weak (Fig. 4(B)). However, the series of C ions ($C_{2\alpha}$ at *m/z* 528, $C_{3\alpha}$ at *m/z* 731, $C_{4\alpha}$ at *m/z* 893) indicated an oligosaccharide with HexNAc(−Fuc−)Hex−HexNAc−Hex−Hex sequence. The absence of a $^{0,2}A_3$ ion at *m/z* 630 suggested that the internal HexNAc was substituted at C3, *i.e.* a type 1 core chain [22]. Thus, a hexasaccharide with HexNAc−(Fuc) Hex−HexNAc−Hex−Hex sequence and an internal HexNAc substituted at C-3 was indicated, most likely a blood group A type 1 hexasaccharide (GalNAcα3(Fucα2) Galβ3GlcNAcβ3Galβ4Glc).

A fragment ion at *m/z* 202 was present in the MS^2 spectrum of the ion at *m/z* 706 (Fig. 4(C)). This fragment ion is obtained by C_2−Z_2 double cleavage (D_{1-2}), and is characteristic for an internal 3-linked GlcNAc [31]. The prominent $^{0,2}A_4$ ion at *m/z* 646, and the $^{0,2}A_4$-H_2O ion at *m/z* 628, were derived from the lactose unit at the reducing end. Taken together with the C_2 ion at *m/z* 382, and the C_3 ion at *m/z* 544, and the absence of a $^{0,2}A_2$ ion at *m/z* 281 and $^{0,2}A_3$ ion at *m/z* 484, this tentatively identified a Hex−3HexNAc−3Hex−4Hex saccharide, most likely a lacto tetrasaccharide (Galβ3GlcNAcβ3Galβ4Glc).

The two F6 binding fractions from piglet intestine both contained two compounds, globo- and lacto-triaosylceramide in the case of fraction O-tri, while fraction A-tetra had lactotetraosylceramide and blood group A type 1 hexaglycosylceramide. However, globotriaosylceramide and the A type 1 hexaosylceramide were also found in the preceding and later-eluting fractions from the final Iatrobeads columns that were devoid of F6 binding activity and, no binding of the F6 fimbriae to reference globotriaosylceramide from human erythrocytes or reference the A type 1 hexaosylceramide from human small intestine was obtained (see below). Thus, lactotriaosylceramide and lactotetraosylceramide were the F6 fimbriae binding glycosphingolipids from piglet small intestinal mucosa.

3.4. Binding of F6 fimbriae to reference glycosphingolipids

Next the binding of the F6 fimbriae to a number of pure glycosphingolipids at defined concentrations was tested in the chromatogram binding assay. The results are exemplified in Figs. 5–7, and summarized in Table 1. Thus, in addition to lactotriaosylceramide and lactotetraosylceramide of porcine small intestinal epithelium, the F6 fimbriae bound to galactosylceramide, sulfatide, lactosylceramide with phytosphingosine and/or hydroxy fatty acids (Galβ4Glcβ1Cer; Fig. 6, lane 4, Fig. 7, lane 3), isoglobotriaosylceramide (Galα3Galβ4Glcβ1Cer; Fig. 7, lane 5), gangliotriaosylceramide (GalNAcβ4Galβ4Glcβ1Cer Fig. 7, lane 8), gangliotetraosylceramide (Galβ3GalNAcβ4Galβ4Glcβ1Cer), neolactotetraosylceramide (Galβ4GlcNAcβ3Galβ4Glcβ1Cer; Fig. 5, lane 1), neolactohexaosylceramide (Galβ4GlcNAcβ3Galβ4GlcNAcβ3-Galβ4Glcβ1Cer), and neolactooctaosylceramide (Galβ4GlcNAcβ3-Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer). Apart from the lactosylceramide binding, which required phytosphingosine and hydroxy fatty acids, there was no apparent dependence on a certain ceramide composition for the binding to occur.

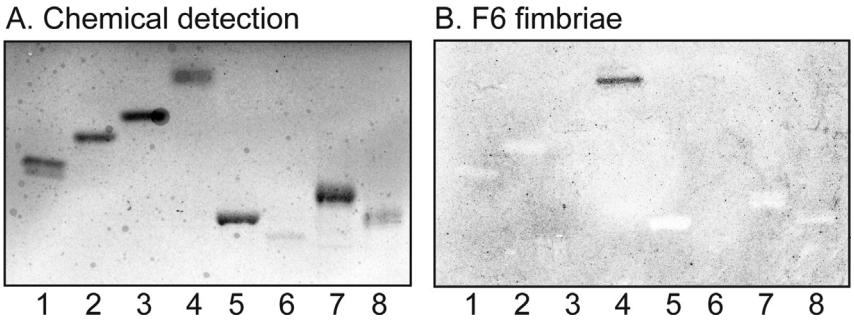

Fig. 6. Binding of F6 fimbriae to reference glycosphingolipids. Chemical detection by anisaldehyde (A), and autoradiogram obtained by binding of ^{125}I-labeled F6 fimbriae (B). The glycosphingolipids were separated on aluminum-backed silica gel plates, using chloroform/methanol/water 60:35:8 (by volume) as solvent system, and the binding assay was performed as described under "Materials and Methods". Autoradiography was for 12 h. The lanes were: Lane 1, Forssman pentaglycosylceramide (GalNAcα3GalNAcβ3Galα4-Galβ4Glcβ1Cer) of dog intestine, 4 μg; Lane 2, globotetraosylceramide of (GalNAcβ3Galα4Galβ4Glcβ1Cer) human erythrocytes, 4 μg; Lane 3, gangliotriaosylceramide (GalNAcβ4-Galβ4Glcβ1Cer) of guinea pig erythrocytes, 4 μg; Lane 4, lactosylceramide (Galβ4Glcβ1Cer) with t18:0-h16:0-h24:0 ceramide of dog intestine, 4 μg; Lane 5, blood group B type 2 hexaglycosylceramide (Galα3(Fucα2)Galβ4GlcNAcβ3Galβ4Glcβ1Cer) of human erythrocytes, 4 μg; Lane 6, blood group B type 1 heptaglycosylceramide (Galα3(Fucα2)Galβ3(Fucα4) GlcNAcβ3Galβ4Glcβ1Cer) of monkey intestine, 1 μg; Lane 7, Le^a pentaglycosylceramide (Galβ3(Fucα4)GlcNAcβ3Galβ4Glcβ1Cer) of human intestine, 4 μg; Lane 8, Le^b hexaglycosylceramide (Fucα2Galβ3(Fucα4)GlcNAcβ3Galβ4Glcβ1Cer) of human meconium, 4 μg.

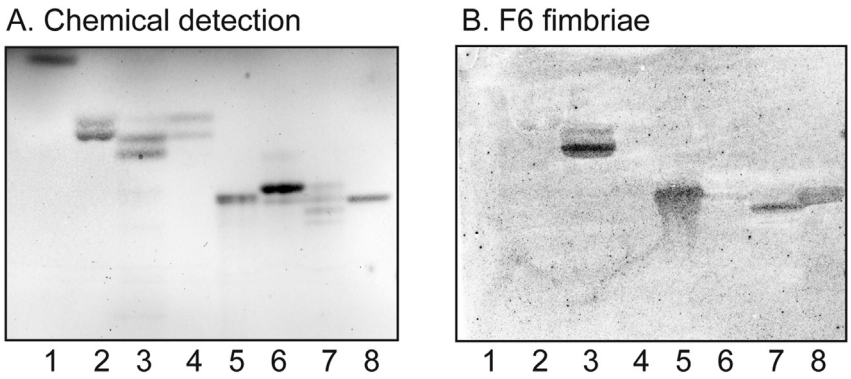

Fig. 7. Binding of F6 fimbriae to reference glycosphingolipids. Chemical detection by anisaldehyde (A), and autoradiogram obtained by binding of ^{125}I-labeled F6 fimbriae (B). The glycosphingolipids were separated on aluminum-backed silica gel plates, using chloroform/methanol/water 60:35:8 (by volume) as solvent system, and the binding assay was performed as described under "Materials and Methods". Autoradiography was for 12 h. The lanes were: Lane 1, galactosylceramide (Galβ1Cer) from Sigma–Aldrich with d18:1-h18:0-h24:0 ceramide, 4 μg; Lane 2, sulfatide (SO_3-3Galβ1Cer) of human intestine with t18:0-h24:0 ceramide, 4 μg; Lane 3, lactosylceramide (Galβ4Glcβ1Cer) of dog intestine with t18:0-h16:0-h24:0 ceramide, 4 μg; Lane 4, lactosylceramide (Galβ4Glcβ1Cer) of human neutrophils with d18:1-16:0-24:1 ceramide, 4 μg; Lane 5, isoglobotriaosylceramide (Galα3Galβ4Glcβ1Cer) of cat intestine with t18:0-h22:0 and h24:0 ceramide, 4 μg; Lane 6, globotriaosylceramide (Galα4Galβ4Glcβ1Cer) of human erythrocytes with d18:1-16:0-24:1 ceramide, 4 μg; Lane 7, lactotriaosylceramide (GlcNAcβ3Galβ4Glcβ1Cer) of pig intestine with d18:1-h16:0 ceramide, 4 μg; Lane 8, gangliotriaosylceramide (GalNAcβ4-Galβ4Glcβ1Cer) of guinea pig erythrocytes with d18:1-16:0 and 24:0 ceramide, 4 μg.

It should be noted that while the binding of the F6 fimbriae to lactotriaosylceramide, lactotetraosylceramide, and the neolacto glycosphingolipids was highly reproducible, binding to galactosylceramide, sulfatide, lactosylceramide, isoglobotriaosylceramide, gangliotriaosylceramide and gangliotetraosylceramide was only occasionally obtained. This is exemplified by gangliotriaosylceramide, which is non-binding in Fig. 6 (lane 3), but recognized by the F6 fimbriae in Fig. 7 (lane 8).

Table 1
Binding of ^{125}I-labeled F6 fimbriae to glycosphingolipids on thin-layer chromatograms.

No. Trivial name	Structure	Binding[a]	Source
Simple compounds			
1. Galactosylceramide	Galβ1Cer	+	Sigma–Aldrich
2. Glucosylceramide	Glcβ1Cer	–	Porcine kidney
3. Sulfatide	SO3-3Galβ1Cer	+	Pig intestine
4. LacCer (d18:1-16:0-24:0)[b]	Galβ4Glcβ1Cer	–	Human granulocytes
5. LacCer (t18:0-h16:0-h24:0)	Galβ4Glcβ1Cer	+	Dog intestine
6. Isoglobotri	Galα3Galβ4Glcβ1Cer	+	Dog intestine
7. Globotri	Galα4Galβ4Glcβ1Cer	–	Human erythrocytes
8. Lactotri	GlcNAcβ3Galβ4Glcβ1Cer	+++	Piglet intestine
Gangliosseries			
9. Gangliotri	GalNAcβ4Galβ4Glcβ1Cer	+	Guinea pig intestine
10. Gangliotetra	Galβ3GalNAcβ4Galβ4Glcβ1Cer	+	Mouse intestine
Neolactoseries			
11. Neolactotetra	Galβ4GlcNAcβ3Galβ4Glcβ1Cer	+++	Human granulocytes
12. H5 type 2	Fucα2Galβ4GlcNAcβ3Galβ4Glcβ1Cer	–	Human erythrocytes
13. Le^x-5	Galβ4(Fucα3)GlcNAcβ3GalI4Glcβ1Cer	–	Dog intestine
14. B5	Galα3Galβ4GlcNAcβ3Galβ4Glcβ1Cer	–	Rabbit erythrocytes
15. B6 type 2	Galα3(Fucα2)Galβ4GlcNAcβ3Galβ4Glcβ1Cer	–	Human erythrocytes
16. A7 type 2	GalNAcα3(Fucα2)Galβ4(Fucα3)GlcNAcβ3Galβ4Glcβ1Cer	–	Human erythrocytes
17. B7 type 2	Galα3(Fucα2)Galβ4(Fucα3)GlcNAcβ3Galβ4Glcβ1Cer	–	Human erythrocytes
18. Neolactohexa (linear)	Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer	+++	Rabbit thymus
19. Neolactohexa (branched)	Galβ4GlcNAcβ6(Galβ4GlcNAcβ3)Galβ4Glcβ1Cer	–	Bovine buttermilk
20. Neolactoocta	Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer	+++	Rabbit thymus
Lactoseries			
21. Lactotetra	Galβ3GlcNAcβ3Galβ4Glcβ1Cer	+++	Piglet intestine
22. Le^b-6	Fucα2Galβ3(Fucα4)GlcNAcβ3Galβ4Glcβ1Cer	–	Human meconium
23. A6 type 1	GalNAcα3(Fucα2)Galβ3GlcNAcβ3Galβ4Glcβ1Cer	–	Human intestine
24. A7 type 1	GalNAcα3(Fucα2)Galβ3(Fucα4)GlcNAcβ3Galβ4Glcβ1Cer	–	Human meconium
Globoseries			
25. Globotetra	GalNAcβ3Galα4Galβ4Glcβ1Cer	–	Human erythrocytes
26. Forssman	GalNAcα3GalNAcβ3Galα4Galβ4Glcβ1Cer	–	Dog intestine
Gangliosides			
27. GM3	NeuAcα3Galβ4Glcβ1Cer	–	Human brain
28. NeuAcα3neolactotetra	NeuAcα3Galβ4GlcNAcβ3Galβ4Glcβ1Cer	–	Human erythrocytes
29. NeuGcα3neolactohexa	NeuGcα3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer	–	Rabbit thymus

[a] Binding is defined as follows: +++ denotes a binding when 1 μg of the glycosphingolipid was applied on the thin-layer chromatogram, + denotes an occasional binding at 4 μg, while – denotes no binding even at 4 μg.

[b] In the shorthand nomenclature for fatty acids and bases, the number before the colon refers to the carbon chain length and the number after the colon gives the total number of double bonds in the molecule. Fatty acids with a 2-hydroxy group are denoted by the prefix h before the abbreviation *e.g.* h16:0. For long chain bases, d denotes dihydroxy and t trihydroxy. Thus d18:1 designates sphingosine (1,3-dihydroxy-2-aminooctadecene) and t18:0 phytosphingosine (1,3,4-trihydroxy-2-aminooctadecene).

All other glycosphingolipids tested were non-binding in the chromatogram binding assay (Table 1). Furthermore, all binding assays in this study were done with both ^{125}I-labeled F6-fimbriae and with ^{35}S-labeled F6-fimbriated *E. coli*. The F6 fimbriae and the F6-fimbriated bacteria gave identical binding patterns, although a high background binding often was obtained when using bacterial cells.

3.5. Comparison of non-acid glycosphingolipids of newborn and adult pig small intestinal mucosa

The amount of the ganglioside NeuGc-GM3, recognized by F5-(K99-) fimbriated *E. coli*, in the porcine small intestine decreases as the age of the pig increases [2]. Here the non-acid glycosphingolipids from the small intestinal mucosa of a three day old piglet and an adult pig were compared. Already the chemical detection indicated a developmental change, since the non-acid fraction of the newborn pig (Fig. 8(A), lane 1) had a major compound migrating in the monoglycosylceramide region, and some glycosphingolipids migrating as di- to tetra-glycosylceramides, while the non-acid fraction of the adult pig intestine (Fig. 8(A), lane 2) was more complex, with a major compound in the triglycosylceramide region, and also glycosphingolipids migrating as penta- and hepta-glycosylceramides. The major triglycosylceramide of porcine intestine is globotriaosylceramide [6], which is not recognized by the F6 fimbriae, and the complex glycosphingolipids have terminal blood group H or A determinants [3]. Here several compounds recognized by monoclonal antibodies directed against the blood group A determinant were present in the non-acid fraction from adult pig intestine (Fig. 8(B), lane 2), while the binding of the anti-A monoclonal antibodies to the non-acid glycosphingolipids from the newborn pig intestine (Fig. 8(B), lane 1) was relatively weak. No binding of monoclonal antibodies directed against the blood group H determinant to either fraction was obtained (data not shown). The F6 fimbriae again bound to a compound migrating in the tetraglycosylceramide region in the non-acid glycosphingolipids from the newborn pig intestine (Fig. 8(C), lane 1).

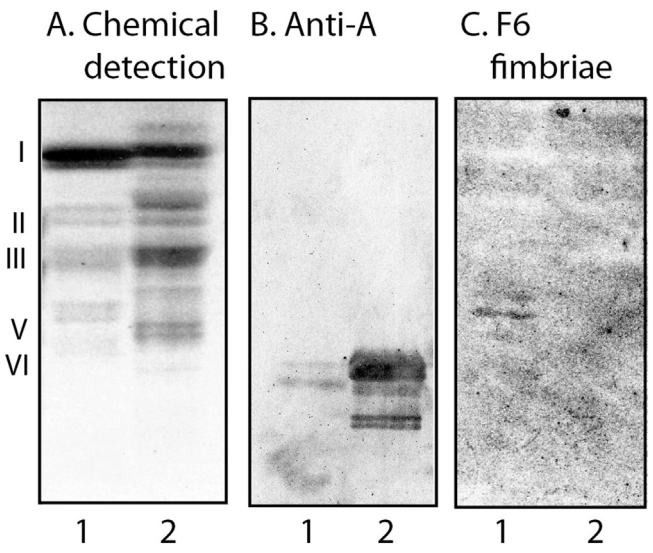

Fig. 8. Comparison of non-acid glycosphingolipids of newborn and adult pig small intestinal mucosa. Chemical detection by anisaldehyde (A), and autoradiograms obtained by binding of monoclonal antibodies directed against the blood group A determinant (B), and ^{125}I-labeled F6 fimbriae (C). The glycosphingolipids were separated on aluminum-backed silica gel plates, using chloroform/methanol/water 60:35:8 (by volume) as solvent system, and the binding assays were performed as described under "Materials and Methods". Autoradiography was for 12 h. Lane 1, non-acid glycosphingolipids of the small intestinal mucosa of a three day old pig, 40 μg; Lane 2, non-acid glycosphingolipids of the small intestinal mucosa of an adult pig, 40 μg. The Roman numbers to the left of the chromatogram in (A) indicate the approximate number of carbohydrate units in the bands.

4. Discussion

In this study we identify two F6 fimbriae binding glycosphingolipids from piglet small intestinal mucosa. These F6-binding compounds were characterized as lactotriaosylceramide and lactotetraosylceramide. When screening a library of glycosphingolipids, we found that the F6 fimbriae also binds to the related glycosphingolipids neolactotetraosylceramide, neolactohexaosylceramide, and neolactooctaosylceramide. However, the presence of neolacto glycoconjugates in the target tissue of F6-fimbriated *E. coli* has not been shown. In our series of studies of the non-acid glycosphingolipids of mucosal scrapings of pig small intestine we have not found any glycosphingolipids with unsubstituted neolacto sequences [3,32,33]. Very little information about the glycosylation of porcine intestinal glycoproteins is available. However, the Galβ4GlcNAc binding lectin from *Erythrina cristagalli* did not bind to brush border membrane proteins from the small intestine of newborn pigs [34], indicating an absence of unsubstituted neolacto sequences.

We have recently characterized the glycosphingolipid binding specificities of F4ab, F4ac and F4ad fimbriae of porcine enterotoxigenic *E. coli* [6]. As shown in Table 2, the carbohydrate recognition profile of the F6 fimbriae has features resembling all three variants of F4 fimbriae. The F4ad fimbriae bound to neolactotetraosylceramide, but this binding was occasional, in contrast to the high affinity binding to lacto/neolacto sequences obtained with the F6 fimbriae.

Since the F6 fimbriae bind to lactotriaosylceramide a terminal GlcNAc is the minimal structural element required for binding to occur. Terminal GlcNAc is also recognized by G fimbria of human uropathogenic *E. coli*, and by fimbriae belonging to the F17 family produced by bovine enterotoxigenic and invasive *E. coli* strains. Crystal structures of the lectins domains of the G and F17 fimbriae in complex with *N*-acetylglucosamine have been reported [35,36]. However, the target cell receptors for these fimbriae have not yet been identified.

The F6 fimbriae also binds to sulfatide, and Lysine-117 of the FasG subunit has been identified as an important factor for the interaction with sulfatide [17]. Interestingly, it was recently reported that in the crystal complexes of the FimH, FedF and F17G fimbrial adhesins with their respective carbohydrate ligands, there is in all three cases also highly charged regions in complex with sulfate present in the vicinity of the reducing end sugars of the ligands in their carbohydrate binding sites [37]. High mutation rates involving arginines and lysines was found in the two ETEC adhesins (10 in 17 of the F17G, and 6 in 8 of the FedF lectin domains, respectively), and it was speculated that this may be a functional adaptation among ETEC strains allowing the bacteria to bind to carbohydrate receptors that are increasingly modified with negative charges downstream the intestinal tract.

Dean et al. have reported that F6 fimbriated *E. coli* colonize the small intestine and cause diarrhea only in neonatal (<6-day-old) piglets [38]. Still the F6 fimbriated bacteria adhered *in vitro* to intestinal epithelial cells from both neonatal and weaned piglets, and the same amounts of F6 binding galactosylceramide and sulfatide was present in the glycosphingolipid preparations from the intestinal epithelium of pigs of both ages. In addition they found glycosphingolipids migrating as sulfatide and lactosylceramide in

Table 2
Comparison of glycosphingolipid binding of F4 fimbriae and F6 fimbriae.

No. Trivial name	Structure	F4ab[a]	F4ac	F4ad	F6	Source
Simple compounds						
1. Galactosylceramide	Galβ1Cer	+++[b]	+++	−	+	Porcine intestine
2. Sulfatide	SO_3-3Galβ1Cer	+++	+	−	+	Human intestine
3. LacCer	Galβ4Glcβ1Cer	+++	+++	+++	+	Dog intestine
4. Lactotri	GlcNAcβ3Galβ4Glcβ1Cer	−	−	−	+++	Human neutrophils
Ganglioseries						
5. Gangliotri	GalNAcβ4Galβ4Glcβ1Cer	−	−	+++	+	Guinea pig erythrocytes
6. Gangliotetra	Galβ3GalNAcβ4Galβ4Glcβ1Cer	−	−	+++	+	Mouse intestine
Neolactoseries						
7. Neolactotetra	Galβ4GlcNAcβ3Galβ4Glcβ1Cer	−	−	+	+++	Human neutrophils
8. Neolactohexa	Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer	ND[c]	ND	ND	+++	Rabbit thymus
9. Neolactoocta	Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer	ND	ND	ND	+++	Rabbit thymus
Lactoseries						
10. Lactotetra	Galβ3GlcNAcβ3Galβ4Glcβ1Cer	−	−	−	+++	Human meconium
Globoseries						
11. Isoglobotri	Galα3Galβ4Glcβ1Cer	−	−	+	+	Cat intestine
12. Globotri	Galα4Galβ4Glcβ1Cer	+++	−	−	−	Rat intestine

[a] Glycosphingolipid binding data for F4ab, F4ac, and F4ad fimbriae are from ref [6].
[b] Binding is defined as follows: +++ denotes an intense and highly reproducible staining when 4 μg of the glycosphingolipid was applied on the thin-layer chromatogram, + denotes an occasional staining, while − denotes no binding even at 4 μg.
[c] ND, not determined.

their porcine intestinal mucus preparations, isolated by separation of intestinal buffer washings on Sepharose CL-4B columns. Therefore, it was proposed that receptors for F6 fimbriated *E. coli* are released into the intestinal mucus of older pigs, and there act as decoys and prevents attachment to the intestinal epithelium [18].

However, the intestinal mucus gel contains mainly the very large oligomeric glycoproteins known as mucins, and a number of associated proteins [39,40]. The amphipathic glycosphingolipids, on the other hand, are found in the cell membrane. Thus, the glycosphingolipids found in the intestinal washings were break down products either from extruded epithelial cells, or epithelial cells extracted during the washing procedure.

The identification of lactotriaosylceramide and lactotetraosylceramide as F6 binding glycosphingolipids of piglet intestine suggests an alternative explanation for the age-dependent diarrhea-inducing effect of F6-fimbriated *E. coli* colonization of piglet intestine. A developmental change of the acid glycosphingolipids of pig intestine has previously been demonstrated [2,41], with very simple composition in the intestine of the newborn pig, and more complex gangliosides found in the adult pig intestine. The comparison of non-acid glycosphingolipids of newborn and adult pig intestine shows that also the non-acid glycosphingolipids are more complex in the adult pig. The majority of the complex non-acid glycosphingolipids of adult pig intestine have blood group H and A determinants on type 1 core chains, *i.e.* are built on lactotetraosylceramide [3,32,33]. However, while lactotetraosylceramide is readily recognized by the F6 fimbriae, the αFuc in 2-position of the terminal Gal of the blood group H type 1 determinant (Fucα2Gal) blocks the F6 binding. Thus, the intestinal amounts of F6 binding lactotetraosylceramide will be reduced by the developmental appearance of glycosphingolipids with blood group determinants.

In summary, these studies implicate lactotriaosylceramide and lactotetraosylceramide as candidate receptors for mediating attachment of F6-fimbriated enterotoxigenic *E. coli* to porcine small intestinal cells. Our findings may be a basis for the rational design of receptor saccharide analogs for inhibition of the intestinal adhesion of F6-expressing *E. coli*.

Funding

This study was supported by the Swedish Research Council/Medicine (S.T., Grant no. 12628), the Swedish Cancer Foundation (S.T.), governmental grants to the Sahlgrenska University Hospital (S.T.), and the Fonds voor Wetenschappelijk Onderzoek Vlaanderen (E.C.; FWO-Vlaanderen (Grant no. G.0389.07, Grant no. G.0263.07, and Grant no. 3G0A4312)).

Acknowledgments

The use of the LTQ linear quadrupole ion trap mass spectrometer (obtained by a grant from the Swedish Research Council (No. 342-2004-4434) to Gunnar Hansson) is gratefully acknowledged.

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