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

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**A B S T R A C T**

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 (Galβ4GlcNAcβ) 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*.

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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 plasmic 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 (SO3-3Galβ1Cer) and...
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 (Galβ3GalNAcβ4Galβ4Glcβ1Cer), lactosylceramide (Galβ4Glcβ1Cer), sulfatide, gangliotriaosylceramide (GalβNAcβ4Galβ4Glcβ1Cer), and galactosylceramide has also been reported [17,18]. During our studies of carbohydrate recognition by fimbiae of porcine ETEC we also examined the potential role of carbohydrates galactosylceramide has also been reported [17,18]. Binding of F6 to gangliotetraosylceramide (Galβ3GalNAcβ4Galβ4Glcβ1Cer), lactosylceramide (Galβ4Glcβ1Cer), sulfatide, gangliotriaosylceramide (GalβNAcβ4Galβ4Glcβ1Cer), and galactosylceramide has also been reported [17,18].

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 35S-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) NaNO3 and 0.1% (w/v) Tween 20 (BSA/PBS/TWEEN) to a bacterial density of 1 × 108 colony forming units/ml. The specific activity of bacterial suspensions was approximately 1 cpm per 100 bacteria.

2.2. Purification of F6 fimbiae

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 fimbiae. 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 fimbral solution. Thereafter, the bacterial pellet was discarded and the fimbiae were precipitated with 60% ammonium sulfate at 4 °C overnight. After centrifugation at 1450 g for 30 min, the precipitated fimbiae are dissolved in PBS and dialyzed against PBS overnight at 4 °C.

2.3. 125I-labeling

Aliquots of 100 µg of fimbrial protein were labeled with 125I using Na125I (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 × 103 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 1H 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 fimbiae and F6-fimbriated bacteria to glycosphingolipids on thin-layer chromatograms was done as described previously [3,6]. Dried chromatograms were dipped in diethyl ether/hexane (1:1 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) NaNO3 and 0.1% (w/v) Tween 20 (BSA/PBS/TWEEN) for 2 h at room temperature. Then the plates were incubated with 125I-labeled fimbiae (1–5 × 104 cpm/ml) or 35S-labeled bacteria (1–5 × 106 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 125I-labeled anti-mouse antibodies for detection.

2.7. Isolation of F6 fimbiae 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 tetrargycosylceramide 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 × 0.5 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 tetracosylceramide 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 polystyrene 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 MS2 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 ml 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 eluates 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 mbriae 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-glycosylnesteramides, 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 125I-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 (Galβ1-4Galβ1-4Glcβ1Cer) 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)).
The MS2 spectrum of the [M−H]− ion at m/z 503 had a series of prominent C-type fragment ions (C1 at m/z 179 and C2 at m/z 341) identifying a triglycosylceramide with HexeHexeHex sequence (Fig. 3(D)). There was also a 0.2A2 fragment ion at m/z 281 demonstrating an internal hexose substituted on C-4 as in Galα4Galβ4Glc (globotriaose)[22].

The MS2 of the ion at m/z 544 gave C-type fragment ions at m/z 220 (C1) and at m/z 382 (C2), identifying a HexNAc−Hex−Hex sequence (Fig. 3(E)). No 0.2A2 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 (GlcNAcb3Galb4Glc).

3.3.2. F6 binding tetracygosylceramide

The binding-active tetracygosylceramide 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 tetracygosylceramide fraction was obtained (denoted fraction A-tetra).

Characterization of fraction A-tetra identified lactotetraosylceramide (Galβ3GalNAcb3Galβ4Glcβ1Cer) and the A type 1 hexosylceramide (GalNAcb3[Fucα2]Galβ3GalNAcb3Galβ4Glcβ1Cer). This conclusion was based on the following properties:

1) On thin-layer chromatograms the binding-active glycosphingolipid migrated as a distinct band in tetracygosylceramide 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 125I-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) MS² spectrum of the ion at m/z 503 (retention time 17.3 min). (E) MS² 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 tetracygosylceramide 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) MS² spectrum of the ion at m/z 1055 (retention time 19.8–20.3 min). (C) MS² spectrum of the ion at m/z 706 (retention time 23.0–23.6 min). (D) Interpretation formulas showing the deduced carbohydrate sequences.
A. Chemical detection

B. F6 fimbriae

Fig. 5. Binding of F6 fimbriae to reference glycosphingolipids. Chemical detection by anisaldehyde (A), and autoradiogram obtained by binding of 125I-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.

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 MS2 spectrum of the [M+H]- ion at m/z 1055 was weak (Fig. 4(B)). However, the series of C ions (C2g at m/z 528, C3g at m/z 731, C4g at m/z 893) indicated an oligosaccharide with an internal HexNAc (Fig. 4(C)). This fragment ion is obtained by Z2 double cleavage (D1g/2g). The two F6 binding fractions from piglet intestine both contain two compounds, globos- 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 hexosylceramide were also found in the preceding and later-eluting fractions from the final latepeaks were not identified. The lanes were: Lane 1, Forssman pentaglycosylceramide (Galb3GalNAcβ3Galβ4Glc[1Cer]) of human meconium, 4 μg; Lane 2, blood group H type 2 pentaglycosylceramide (Fuc2Galβ3GalNAcβ3Galβ4Glc[1Cer]) of human erythrocytes, 4 μg; Lane 5, blood group A type 1 hexaglycosylceramide (GalNAcβ3Galβ2Galβ3Galβ3Galβ4Glc[1Cer]) of human erythrocytes, 4 μg; Lane 6, B5 pentaglycosylceramide (Galα3Galβ4Glc[1Cer]) of rabbit erythrocytes, 4 μg.

A fragment at m/z 202 was present in the MS2 spectrum of the ion at m/z 706 (Fig. 4(C)). This fragment ion is obtained by C2g–2z double cleavage (D1g–2g), and is characteristic for an internal 3-linked GlcNAc [31]. The prominent 0,2A4 ion at m/z 646, and the 0,2A4–H2O ion at m/z 628, were derived from the lactose unit at the reducing end. Taken together with the C2 ion at m/z 382, and the C1 ion at m/z 544, and the absence of a 0,2A2 ion at m/z 281 and 0,2A3 ion at m/z 484, this tentatively identified a Hex–3HexNAc–3Hex–4Hex saccharide, most likely a lacto tetra-saccharide (Galβ3GalNAcβ3Galβ4Glc[1Cer]).

The two F6 binding fractions from piglet intestine both contained two compounds, globos- 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 hexosylceramide were also found in the preceding and later-eluting fractions from the final latepeaks were not identified. The lanes were: Lane 1, Forssman pentaglycosylceramide (Galb3GalNAcβ3Galβ4Glc[1Cer]) of human meconium, 4 μg; Lane 2, blood group H type 2 pentaglycosylceramide (Fuc2Galβ3GalNAcβ3Galβ4Glc[1Cer]) of human erythrocytes, 4 μg; Lane 5, blood group A type 1 hexaglycosylceramide (GalNAcβ3Galβ2Galβ3Galβ3Galβ4Glc[1Cer]) of human erythrocytes, 4 μg; Lane 6, B5 pentaglycosylceramide (Galα3Galβ4Glc[1Cer]) of rabbit erythrocytes, 4 μg.

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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 sphingosine and/or hydroxy fatty acids (Galβ4Glc[1Cer]; Fig. 6, lane 4, Fig. 7, lane 3), isoglobotriaosylceramide (Galβ3Galβ4Glc[1Cer]; Fig. 6, lane 4, Fig. 7, lane 3), galactotriaosylceramide (Galβ3Galβ4Glc[1Cer]; Fig. 6, lane 4, Fig. 7, lane 3), lactotetraosylceramide (Galβ4Glc[1Cer]; Fig. 6, lane 4, Fig. 7, lane 3), lactosylceramide with sphingosine and/or hydroxy fatty acids, there was no apparent dependence on a certain ceramide composition for the binding to occur.
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

<table>
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<th>Structure</th>
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<td>Porcine kidney</td>
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<tr>
<td>3</td>
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<tr>
<td>4</td>
<td>LacCer (d18:1-16:0-24:0)</td>
<td>Galβ4Glcβ1Cer</td>
<td>−</td>
<td>Human granulocytes</td>
</tr>
<tr>
<td>5</td>
<td>LacCer (t18:0-16:0-24:0)</td>
<td>Galβ4Glcβ1Cer</td>
<td>+</td>
<td>Dog intestine</td>
</tr>
<tr>
<td>6</td>
<td>Isoglobotriose</td>
<td>Galβ3Galβ4Glcβ1Cer</td>
<td>−</td>
<td>Dog intestine</td>
</tr>
<tr>
<td>7</td>
<td>Globotriose</td>
<td>Galβ3Galβ4Glcβ1Cer</td>
<td>+</td>
<td>Human erythrocytes</td>
</tr>
<tr>
<td>8</td>
<td>Lactotriose</td>
<td>Glcβ1(Nαc3βGlcβ1Cer</td>
<td>+++</td>
<td>Piglet intestine</td>
</tr>
<tr>
<td>9</td>
<td>Gangliotriose</td>
<td>Glcβ1(Nαc3βGlcβ1Cer</td>
<td>+</td>
<td>Guinea pig intestine</td>
</tr>
<tr>
<td>10</td>
<td>Gangliotetraose</td>
<td>Galβ3Glcβ1(Nαc3βGlcβ1Cer</td>
<td>+</td>
<td>Mouse intestine</td>
</tr>
<tr>
<td>11</td>
<td>Neolactotetraose</td>
<td>Galβ4Glcβ1(Nαc3βGlcβ1Cer</td>
<td>+++</td>
<td>Human granulocytes</td>
</tr>
<tr>
<td>12</td>
<td>αS type 2</td>
<td>Fucα2Galβ3Galβ4Glcβ1Cer</td>
<td>−</td>
<td>Human erythrocytes</td>
</tr>
<tr>
<td>13</td>
<td>Leα5-5</td>
<td>Galβ4Fucβ3Glcβ1(Nαc3βGlcβ1Cer</td>
<td>−</td>
<td>Dog intestine</td>
</tr>
<tr>
<td>14</td>
<td>B5</td>
<td>Galβ3Fucβ2Glcβ1(Nαc3βGlcβ1Cer</td>
<td>−</td>
<td>Rabbit erythrocytes</td>
</tr>
<tr>
<td>15</td>
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<td>Galβ3Fucβ2Glcβ1(Nαc3βGlcβ1Cer</td>
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<td>Human erythrocytes</td>
</tr>
<tr>
<td>16</td>
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<td>Galβ3Fucβ2Glcβ1(Nαc3βGlcβ1Cer</td>
<td>−</td>
<td>Human erythrocytes</td>
</tr>
<tr>
<td>17</td>
<td>B7 type 2</td>
<td>Galβ3Fucβ2Glcβ1(Nαc3βGlcβ1Cer</td>
<td>−</td>
<td>Human erythrocytes</td>
</tr>
<tr>
<td>18</td>
<td>Neolactobetaose (linear)</td>
<td>Galβ4Fucβ3Glcβ1(Nαc3βGlcβ1Cer</td>
<td>+++</td>
<td>Rabbit thymus</td>
</tr>
<tr>
<td>19</td>
<td>Neolactobetaose (branched)</td>
<td>Galβ4Fucβ3Glcβ1(Nαc3βGlcβ1Cer</td>
<td>−</td>
<td>Bovine buttermilk</td>
</tr>
<tr>
<td>20</td>
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<td>Galβ4Glcβ3Glcβ1(Nαc3βGlcβ1Cer</td>
<td>+++</td>
<td>Rabbit thymus</td>
</tr>
<tr>
<td>21</td>
<td>Lactoβtetraose</td>
<td>Galβ3Glcβ1(Nαc3βGlcβ1Cer</td>
<td>+++</td>
<td>Piglet intestine</td>
</tr>
<tr>
<td>22</td>
<td>Leα6-6</td>
<td>Fucα2Galβ3Galβ4Glcβ1Cer</td>
<td>−</td>
<td>Human meconium</td>
</tr>
<tr>
<td>23</td>
<td>A6 type 1</td>
<td>Galβ3Fucβ2Galβ3Galβ4Glcβ1Cer</td>
<td>−</td>
<td>Human meconium</td>
</tr>
<tr>
<td>24</td>
<td>A7 type 1</td>
<td>Galβ3Fucβ2Galβ3Galβ4Glcβ1Cer</td>
<td>−</td>
<td>Human meconium</td>
</tr>
<tr>
<td>25</td>
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<td>Galβ3Galβ3Galβ4Glcβ1Cer</td>
<td>−</td>
<td>Human erythrocytes</td>
</tr>
<tr>
<td>26</td>
<td>Forssmann</td>
<td>Galβ3Glcβ1(Nαc3βGlcβ1Cer</td>
<td>−</td>
<td>Dog intestine</td>
</tr>
<tr>
<td>27</td>
<td>GM3</td>
<td>NeuAcβ3Galβ4Glcβ1Cer</td>
<td>−</td>
<td>Human brain</td>
</tr>
<tr>
<td>28</td>
<td>NeuAc3Neolactotetraose</td>
<td>NeuAcβ3Galβ4Glcβ1(Nαc3βGlcβ1Cer</td>
<td>−</td>
<td>Human erythrocytes</td>
</tr>
<tr>
<td>29</td>
<td>NeuAc2Neolactohexa</td>
<td>NeuAcβ3Galβ4Glcβ1(Nαc3βGlcβ1Cer</td>
<td>−</td>
<td>Rabbit thymus</td>
</tr>
</tbody>
</table>

* 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.

* 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-aminoocatadecene) and t18:0 phytosphingosine (1,3,4-trihydroxy-2-aminoocatadecene).
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-fimbriata and with $^{35}$S-labeled F6-fimbriated E. coli. The F6 fimbriata 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 NeuGe-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 fimbriata, 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 fimbriata 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).

4. Discussion

In this study we identify two F6 fimbriata 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 fimbriata 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 Galβ1-4GlcNAc 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 fimbriata of porcine enterotoxigenic E. coli [6]. As shown in Table 2, the carbohydrate recognition profile of the F6 fimbriata has features resembling all three variants of F4 fimbriata. The F4ad fimbriata bound to neolactotetraosylceramide, but this binding was occasional, in contrast to the high affinity binding to lacto/neolacto sequences obtained with the F6 fimbriata.

Since the F6 fimbriata 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-acetylgalactosamine have been reported [35,36]. However, the target cell receptors for these fimbriata have not yet been identified.

The F6 fimbriata 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

**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 fimbriata (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.
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- mbriated enterotoxigenic 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 amphiphatic glycosphingolipids, on the other hand, are found in the cell membrane. Thus, the glycosphingolipids found in the intestinal washings were break down on the other hand, are found in the cell membrane. Thus, the glycosphingolipids of piglet intestine have blood group H and complex in the adult pig. The majority of the complex non-acid complex gangliosides found in the adult pig intestine. The complex non-acid complex gangliosides found in the adult pig intestine. The complex non-acid complex gangliosides found in the adult pig intestine.

The identification of lactotriaosylceramide and lactotetraosylceramide as F6 binding glycosphingolipids of piglet intestine suggests an alternative explanation for the age-dependent diarrhoea-inducing effect of F6-mbriated 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 (Fuc2Gal) 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-mbriated enterotoxigenic E. coli to porcine small intestinal cells. Our findings may be a basis for the rational design of receptor saccharides analogs for inhibition of the intestinal adhesion of F6-expressing E. coli.

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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.

References


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

<table>
<thead>
<tr>
<th>No.</th>
<th>Trivial name</th>
<th>Structure</th>
<th>F4ab</th>
<th>F4ac</th>
<th>F4ad</th>
<th>F6</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Galactosylceramide</td>
<td>Gal1Cer</td>
<td>+++ b</td>
<td>+++ b</td>
<td>+++ b</td>
<td>+</td>
<td>Porcine intestine</td>
</tr>
<tr>
<td>2</td>
<td>Sulfatide</td>
<td>SO3-3Galβ1Cer</td>
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<td>+++ b</td>
<td>+++ b</td>
<td>+</td>
<td>Human intestine</td>
</tr>
<tr>
<td>3</td>
<td>LacCer</td>
<td>Galβ4Glcβ1Cer</td>
<td>+++ b</td>
<td>+++ b</td>
<td>+++ b</td>
<td>+</td>
<td>Dog intestine</td>
</tr>
<tr>
<td>4</td>
<td>Lactotriota</td>
<td>GlcNAcβ3Galβ4Glcβ1Cer</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>Human neutrophils</td>
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<tr>
<td>5</td>
<td>Gangliotieta</td>
<td>GalNAcβ4Galβ4Glcβ1Cer</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>Guinea pig erythrocytes</td>
</tr>
<tr>
<td>6</td>
<td>Gangliotieta</td>
<td>Galβ3GalNAcβ4Galβ4Glcβ1Cer</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>Mouse intestine</td>
</tr>
<tr>
<td>7</td>
<td>Neolactotriota</td>
<td>Galβ4GlcNAcβ3Galβ4Glcβ1Cer</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>Guinea pig erythrocytes</td>
</tr>
<tr>
<td>8</td>
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<td>-</td>
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<td>Isoglobotriota</td>
<td>Galβ3Galβ4Glcβ1Cer</td>
<td>-</td>
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<td>-</td>
<td>+</td>
<td>Guinea pig erythrocytes</td>
</tr>
<tr>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>Guinea pig erythrocytes</td>
</tr>
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</table>

a Glycosphingolipid binding data for F4ab, F4ac, and F4ad mbriae 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.


