Adhesion of K99 fimbriated *Escherichia coli* to pig intestinal epithelium: correlation of adhesive and non-adhesive phenotypes with the sialoglycolipid content

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Evidence for the existence of two phenotypes of piglets born to experimental herds was obtained based on the susceptibility of intestinal brush borders to adhesion of K99-positive *Escherichia coli*. The enterocytes of the K99-receptive piglets displayed a characteristic sialoglycolipid pattern, with a higher content of the monosialoglycolipids II³NeuGc-LacCer (GM3Gc), IV³NeuGc-nLcOse₄Cer (SPGGc) and IV³NeuAc-nLcOse₄Cer (SPG) and the oligosialogangliosides IV³NeuAc,II³NeuAc-GgOse₄Cer (GD1a), II³(NeuAc)₂-GgOse₃Cer (GD2), II³(NeuAc)₂-GgOse₄Cer (GD1b) and IV³NeuAc,II³(NeuAc)₂-GgOse₄Cer (GT1b) when compared to the gangliosides of non-receptive piglets. The gangliosides from enterocytes of the non-receptive piglets were mainly the monosialogangliosides II³NeuAc-GgOse₃Cer (GM2) and II³NeuAc-LacCer (GM3), only traces of the other sialoglycolipids being detected. Adhesion of ¹⁴C-labelled K99-positive *E. coli* cells to the piglet small intestinal sialoglycolipids, as tested by the thin-layer chromatogram overlay assay, revealed that the receptive enterocyte membrane was richer in glycolipids containing K99 receptor structures than the non-receptive enterocyte. Adhesion of K99-positive *E. coli* correlated with the degree of sialylation of the brush border glycolipids.

Introduction

K99 fimbriae are often found on enterotoxigenic *Escherichia coli* (ETEC) strains isolated from diarrhoeic calves, piglets and lambs (Gaastra & de Graaf, 1982). The K99 fimbrial adhesin is known to be a sialoglyco-conjugate-binding lectin; in the small intestine, it recognizes both mucins (Mouricout & Julien, 1987a; Lindahl & Carlstedt, 1990) and glycolipids (Kyogashima *et al.*, 1989; Teneberg *et al.*, 1990). A preferential affinity of K99 fimbriae for neuraminic acid derivatives has been established, *N*-glycolylneuraminic acid (NeuGc) being twofold more potent than *N*-acetylneuraminic acid (NeuAc); also, the degree of *O*-acetylation of neuraminic acid may influence colonization of the bovine intestine (Lindahl *et al.*, 1987). Recent advances have indicated that the piglet intestine contains a receptor-active glycolipid, the monosialoganglioside II³NeuGc-LacCer (GM3Gc) (Kyogashima *et al.*, 1989; Teneberg *et al.*,

1990), which was earlier identified as a receptor in horse erythrocytes (Smit *et al.*, 1984; Ono *et al.*, 1989). [Glycolipid nomenclature is according to IUPAC-IUB (1986) recommendations; see also Table 1.] Investigations with glycolipids isolated from porcine small intestine suggested that K99 fimbriae preferentially recognized IV³NeuGc-nLcOse₄Cer (SPGGc), which possessed 10 times more affinity than II³NeuGc-LacCer or IV³NeuAc-nLcOse₄Cer (SPG) (Kyogashima *et al.*, 1989). In a previous report, we observed that various receptors were recognized by K99 fimbriae on an epithelial cell line and that the adhesion mechanism was governed by the density of gangliosides (Mouricout & Julien, 1987b).

In the present studies, we noted that K99-positive *E. coli* did not adhere to the intestinal brush borders from all piglets. In order to determine whether the adhesion was linked to sialoglycolipids, we studied the sialoglycolipid pattern of jejunal epithelium isolated from conventional, monoassociated (with *E. coli*) and axenic piglets and examined the binding of radiolabelled K99-positive *E. coli* to separated sialoglycolipids.

Abbreviations: ETEC, enterotoxigenic *Escherichia coli*; ST, heat-stable enterotoxin, infant-mouse-model active; LT, heat-labile enterotoxin.

Table 1. *Abbreviated representation of sialoglycolipids*

Glc, Glucose; Gal, galactose; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine. The sialic acid species of sialoglycolipids are indicated in parentheses; NeuAc, *N*-acetylneuraminic acid; NeuGc, *N*-glycolylneuraminic acid. All monosaccharides have a D-configuration except fucose, which has an L-configuration. Lac, lactosyl; nLcOse₄, neolactosetetraosyl; GgOse₃, gangliotriosyl; GgOse₄, gangliotetraosyl; Cer, ceramide.

Svennerholm system*	IUB nomenclature†	Structure‡
GM3	II ³ NeuAc-LacCer	NeuAc(α2-3)Gal(β1-4)Glc(β1-1)Cer
GM3Gc	II ³ NeuGc-LacCer	NeuGc(α2-3)Gal(β1-4)Glc(β1-1)Cer
GM2	II ³ NeuAc-GgOse ₃ Cer	GalNAc(β1-3)[NeuAc(α2-3)]Gal(β1-4)Glc(β1-1)Cer
GM1a	II ³ NeuAc-GgOse ₄ Cer	Gal(β1-3)GalNAc(β1-4)[NeuAc(α2-3)]Gal(β1-4)Glc(β1-1)Cer
GM1b	IV ³ NeuAc-GgOse ₄ Cer	NeuAc(α2-3)Gal(β1-3)GalNAc(β1-4)Gal(β1-4)Glc(β1-1)Cer
GM1Fuc	IV ³ Fuc, II ³ NeuAc-GgOse ₄ Cer	Fuc(α1-2)Gal(β1-3)GalNAc(β1-4)[NeuAc(α2-3)]Gal(β1-4)Glc(β1-1)Cer
GD3	II ³ (NeuAc) ₂ -LacCer	NeuAc(α2-8)[NeuAc(α2-3)]Gal(β1-4)Glc(β1-1)Cer
GD2	II ³ (NeuAc) ₂ -GgOse ₃ Cer	GalNAc(β1-3)[NeuAc(α2-8)NeuAc(α2-3)]Gal(β1-4)Glc(β1-1)Cer
GD1a	IV ³ NeuAc, II ³ NeuAc-GgOse ₄ Cer	NeuAc(α2-3)Gal(β1-3)GalNAc(β1-3)[NeuAc(α2-8)NeuAc(α2-3)]Gal(β1-4)Glc(β1-1)Cer
GD1b	II ³ (NeuAc) ₂ -GgOse ₄ Cer	Gal(β1-3)GalNAc(β1-4)[NeuAc(α2-8)NeuAc(α2-3)]Gal(β1-4)Glc(β1-1)Cer
GT1b	IV ³ NeuAc, II ³ (NeuAc) ₂ -GgOse ₄ Cer	NeuAc(α2-3)Gal(β1-3)GalNAc(β1-4)[NeuAc(α2-8)NeuAc(α2-3)]Gal(β1-4)Glc(β1-1)Cer
GQ1b	IV ³ (NeuAc) ₂ , II ³ (NeuAc) ₂ -GgOse ₄ Cer	[NeuAc(α2-8)NeuAc(α2-3)]Gal(β1-3)GalNAc(β1-4)[NeuAc(α2-8)NeuAc(α2-3)]Gal(β1-4)Glc(β1-1)Cer
SPG	IV ³ NeuAc-nLcOse ₄ Cer	NeuAc(α2-3)Gal(β1-4)GlcNAc(β1-3)Glc(β1-1)Cer
SPGGc	IV ³ NeuGc-nLcOse ₄ Cer	NeuGc(α2-3)Gal(β1-4)GlcNAc(β1-3)Glc(β1-1)Cer

* Abbreviated representations based on the Svennerholm (1963) system.

† Names and structures of glycolipids represented according to IUPAC-IUB (1986) recommendations.

Methods

Animals. Nineteen Large White piglets were born to three different litters (piglets nos 1–8, litter 1; nos 9–13, litter 2; nos 14–19, litter 3) and came from the Institut National de la Recherche Agronomique, experimental stock farm at La Minière, France (Table 2). Two piglets (nos 5 and 6) were conventional (holoxenic) and 17 were colostrum-deprived piglets (Duval-Iflah & Chappuis, 1984; Chappuis *et al.*, 1985). The colostrum-deprived piglets were either germ-free (axenic; nos 14–19) or monoxenic, i.e. monoassociated with the following *E. coli* strains previously described (Duval-Iflah *et al.*, 1983; Chappuis *et al.*, 1985): strain C5148.1 (O117: K88ab⁻ LT⁻ ST⁻), piglets nos 1, 4, 7 and 9; strain C5148.2 (O117: K88ab⁺ LT⁻ ST⁻), piglets nos 2, 3 and 8; and strain P 2200 (O149: K91 K88ac⁺ ST⁺ LT⁺), piglets nos 10–13. Piglets nos 10–13 were resistant to *in vivo* attachment of K88-positive *E. coli*. They developed a slight and transitory diarrhoea; the other animals received non-enterotoxigenic strains and never developed disease. The inoculated strains were recovered at high levels [$\geq 10^8$ (g wet wt contents)⁻¹] in the lumen of distal segments of the small intestine and at low levels [$\leq 10^6$ (g wet wt contents)⁻¹] in the proximal segments. All 19 piglets, which were healthy, were slaughtered within 12–21 days after birth.

Immediately after death, the small intestine was removed, placed in ice-cold saline, and rinsed with 0.9% (w/v) NaCl to remove the contents; the duodenum was removed and the small intestine was divided into seven equal segments (the sections were identified as 1 to 7, in a stomach to colon sequence). The segments were longitudinally opened along the mesenteric border and then processed. After gentle scraping, preparations contained mostly columnar-shaped enterocytes with identifiable brush borders when observed with a phase-contrast microscope.

Brush border preparation. Brush borders were prepared according to Snodgrass *et al.* (1981). Sections of proximal jejunum (2 cm) were excised and immersed in 10 ml Evans' buffer (8 mM-potassium dihydrogen phosphate, 5.6 mM-disodium hydrogen phosphate, 96 mM-NaCl, 1.5 mM-KCl, pH 6.8) containing 10 mM-EDTA, for 15 min at 20 °C. The mucosa was then scraped off, suspended in 5 ml Evans' buffer supplemented with 0.3 M-sucrose and homogenized with a Teflon-tipped tissue grinder. After centrifugation (300 g for 5 min) the pellet was suspended in 0.5 M-sodium hydrogen carbonate containing 5 mM-EDTA, pH 7.4 (5 ml) and homogenization repeated. Brush borders were washed twice in Krebs' buffer (1 mM-potassium dihydrogen phosphate, 25 mM-sodium hydrogen carbonate, 120 mM-NaCl, 14 mM-KCl, pH 7.4). They were finally suspended in Krebs' buffer at a concentration close to 10^7 brush borders ml⁻¹ (i.e. 1–2 brush borders per microscope field).

ETEC adhesion to brush borders. *E. coli* strain B80 (O20: K17, K99; STa⁺), originally from the Central Veterinary Laboratory, Weybridge, UK and previously used by us (Mouricout & Julien, 1987a), was chosen as it lacks F41 fimbriae. Bacteria were grown for 18 h on Minca agar (Guinée *et al.*, 1977) at 37 °C, then harvested, washed and suspended in Krebs' buffer, pH 7.2. The presence of K99 fimbriae was tested by an agglutination test using specific antiserum (Ifa-Mérieux) (Mouricout & Julien, 1987a) and a haemagglutination test with horse erythrocytes (Tixier & Gouet, 1975). *E. coli* strain B80 grown for 48 h at 18 °C, which did not express K99 fimbriae (Roosendaal *et al.*, 1986), was used for control tests.

ETEC adhesion to brush borders was determined according to Sellwood *et al.* (1975). Brush border suspension (100 µl) was incubated with an equal volume of strain B80 K99-positive (10^9 c.f.u. ml⁻¹) for 30 min at 20 °C with shaking (100 r.p.m.). Adherence was examined by microscopy ($\times 1000$, Leitz microscope with phase-contrast condenser and objective). The assay consisted of recording the proportion of K99-receptive brush borders and the numbers of bound bacteria. Results

were scored as follows: (++++), all the brush borders were receptive, each with 11–20 K99-positive *E. coli* cells attached; (+++), (++) or (+), 75%, 50% or 25% of brush borders with 11–20 bacteria attached, the remainder having 1–5 bacteria. If there were only one or two bacteria attached to a few brush borders, the test was recorded as (±). Results were recorded as (–) when all the brush borders were free of bacteria. Control tests were done with K99-negative bacterial cells (10^9 c.f.u. ml⁻¹) under the same conditions.

Assessment of K99 fimbriae production by bacterial suspension. The fimbrial proteins were isolated from cultures of the K99-positive strain B80 by incubating at 60 °C for 20 min in 0.05 M-sodium phosphate, pH 7.2 and then shearing twice, 1 min each time, in a Waring blender (Poly Labo P. Block). Cells were removed by centrifugation at 27000 g for 15 min, and the supernatant fraction was filtered through a 0.45 µm pore-size filter (Millipore). Protein was determined by the Lowry method. The total protein extract was analysed by SDS-PAGE; gels were stained with Coomassie brilliant blue and scanned at 560 nm.

Glycolipids. Lyophilized scrapings from each segment were processed separately. Scrapings (200–500 mg dry material) were briefly sonicated in 10 vols methanol to destroy esterase activity (Herrler *et al.*, 1987). Lipids were extracted with 50 vols methanol and methanol/chloroform (2:1, then 1:2, v/v) to ensure complete extraction of gangliosides. Insoluble material was removed by filtration. Filtrates were combined, dried under a stream of nitrogen, weighed and redissolved in chloroform/methanol/water (30:60:8, by vol.). Portions of total lipid extract were taken for determination of sialic acid and lipids. The non-lipid residues remaining after extraction of the lipids were assayed in duplicate aliquots for protein and residual lipid.

The neutral and acidic glycolipids were separated by anion-exchange chromatography on Pharmacia DEAE-Sephadex A25, acetate form (Ledeon & Yu, 1982). The hydrophobic peptides present in chloroform/methanol extracts (Benajiba *et al.*, 1982) and neutral glycolipids were eluted with chloroform/methanol/water (30:60:8, by vol.). Total acidic glycolipids were eluted with 0.5 M-sodium acetate in methanol, desalted by reverse phase matrix chromatography (Sep-Pak C18 cartridges, Waters) and dried under nitrogen before analysis. Using these methods, it has been shown by other investigators that recovery of individual glycolipids ranges from 75–90% (Ledeon & Yu, 1982).

Lipids were assayed with phosphoric acid/vanillin reagent (Merck). The amount of gangliosides was determined as lipid-bound sialic acid using resorcinol/HCl reagent (Svennerholm, 1957) as modified by Miettinen & Takki-Lukkainen (1959).

High-performance thin-layer chromatography. HPTLC was done on Silica Gel 60 (Merck). Gangliosides were separated using chloroform/methanol/water (60:35:8, by vol.) containing 0.015 g CaCl₂ (solvent A). Two-dimensional HPTLC was developed with solvent A in the first dimension and solvent B (propan-1-ol/28% w/v ammonia/water; 75:5:25, by vol.) (Tanno *et al.*, 1988) in the second dimension. The sialic-acid-containing spots were visualized with resorcinol/HCl spray reagent (Svennerholm, 1957). Individual gangliosides were quantitated by integration after densitometric scanning (CD60 Desaga) of the ganglioside spots on thin-layer plates at 580 nm and expressed as percentages of total gangliosides. Densitometer detector responses per mol of sialic acid were quantified (Mullin *et al.*, 1983) with standard gangliosides GM3, GM2, GM1, GD1a, GD3 and GD1b in the range 0.05–10 nmols sialic acid for each ganglioside species.

The following glycosphingolipids were isolated in our laboratory: *N*-glycoloyl-GM3 (GM3Gc) from horse erythrocytes (Smit *et al.*, 1984), GD3 from yolk egg; *N*-acetylsialoparagloboside (SPG) from human erythrocytes; and *N*-glycoloylsialoparagloboside (SPGGc) from bovine and porcine erythrocytes (Suzuki *et al.*, 1985). Standard *N*-acetylated forms of bovine gangliosides GM1, GM2, GD3, GD1a and GD1b were purchased from Dr Pallman, Biotechnik, München, Germany.

Structures, sialoglycolipid nomenclature and abbreviations are listed in Table 1.

Analytical procedures. Partial characterization of mono-, di- and polysialogangliosides (GM3, GM3Gc, GM1, GM2, GD3, GD1a, GT1b) was done by sialidase treatment. For preparative purpose, gangliosides were isolated by HPTLC, localized on the plate, scraped and eluted from silica gel with chloroform/methanol/water (30:60:8, by vol.). The isolated gangliosides (45 nmol sialic acid) were dried under nitrogen at the bottom of a test-tube. They were dissolved in 200 μ l 0.1 M-sodium acetate buffer, pH 5.5, and incubated with 0.1 unit of sialidase from *Clostridium perfringens* (Boehringer) at 37 °C overnight. The reaction was stopped by addition of 1 ml chloroform/methanol (2:1, v/v). After evaporation of the solvent, the sample was desalted on a Sep-Pak C18 cartridge. Sialic acids linked to internal galactosyl residues and resistant to the action of sialidase were hydrolysed in 0.5 M-formic acid for 1 h at 100 °C (Saito *et al.*, 1985). *N*-Glycoloyl- and *N*-acetylneuraminic acids were separated by HPTLC on cellulose using butan-1-ol/propan-1-ol/HCl (1:2:1, by vol.) (Schauer, 1978). NeuAc and NeuGc (Sigma) served as reference compounds.

Alkali-labile gangliosides were treated by dissolving material in propan-1-ol/propan-2-ol/conc. ammonia for 1 h at 37 °C (Ghidoni *et al.*, 1980). After evaporation of solvents, gangliosides were dissolved in chloroform/methanol/water (60:35:8, by vol.).

ETEC binding to piglet intestinal glycolipids. Strain B80 was cultured for 18 h at 37 °C or 48 h at 18 °C on Minca agar containing [14 C]sodium acetate (3.4 GBq mol $^{-1}$; CEA, France) (Mouricout & Julien, 1987a). Gangliosides (from 1.5–6 nmol sialic acid) were chromatographed on thin-layer plates in solvent A. Plates were treated with 0.5% (w/v) polyisobutylmethacrylate (Plexigum P28, Röhm) in diethyl ether followed by blocking of non-specific sites with 2% (w/v) BSA in phosphate-buffered saline (0.14 M-NaCl, 0.01 M-sodium phosphate, pH 7.2), containing 0.05% (w/v) NaN $_3$. Then the plates were overlaid with 14 C-labelled bacteria (1.5–2.0 kBq ml $^{-1}$; 10 7 and 10 8 c.f.u. ml $^{-1}$) for 2 h at 20 °C (Hansson *et al.*, 1985). Autoradiography was done using Hyperfilm-MP film (Amersham). Stained controls were made with orcinol/HCl reagent on plates, after removing the plastic film by 15–20 short sequential washings in diethyl ether (Hansson *et al.*, 1985). Autoradiograms were subjected to quantitative densitometry by using an optical scanner.

Results

Brush border adhesion of K99-positive *E. coli*

The abilities of brush borders obtained from the proximal jejunum of 19 piglets to bind to K99-positive *E. coli* were tested by using an *in vitro* adhesion assay. Bacterial adhesion was observed to all, or none, of the brush borders and the piglets fell into two groups, i.e. K99-receptive (nos 1–6) and non-receptive (nos 7–19), independent of sex, age and intestinal flora (Table 2). Variations occurred in the numbers of bound K99-positive *E. coli*. Most of the receptive piglets exhibited both high (10 or more) or low numbers (4–5) of bacteria attached to brush borders (phenotype ++). A highly receptive phenotype with 11–20 bacteria bound to all the brush borders (+++++) was found in piglet no. 6. In contrast, a low number of K99-positive *E. coli* was

Fig. 1. Sialoglycolipid concentrations along the small intestines of K99-receptive and K99-non-receptive Large White piglets. The concentration of sialoglycolipids was measured in the intestines of individual piglets as follows: K99-receptive piglets nos 1 (■) and 4 (●); K99-non-receptive piglets nos 8 (○) and 9 (□).

observed bound mainly to the surface of brush borders (+) from piglet no. 3. In non-K99-receptive piglets (nos 7–19), all the brush borders were free of bacteria.

Further, the adhesion of K99-positive bacteria to the non-receptive piglet phenotypes was similar to that shown by K99-negative bacteria to both piglet phenotypes, indicating that adhesion *in vitro* was mediated by K99 fimbriae.

Relationship between the binding of K99-fimbriated bacteria to enterocytes and glycolipid content of jejunal brush borders

Extraction efficiency was given by sialic acid and lipid assays as described in Methods. No glycolipid was detected in tissue residues. The yield of total lipid extracted was from 92–98%. Bacterial attachment to proximal jejunal brush borders seemed to correlate with the level of sialoglycolipids in the brush borders (Table 2).

The jejunal epithelia receptive to adhesion of K99-positive bacteria contained 344 ± 111 nmol sialic acid (g dry wt brush border) $^{-1}$, i.e. 43 ± 14 nmol sialic acid (g wet wt brush border) $^{-1}$. Total sialoglycolipids ranged from 20–29% of the total lipid extract [mean $\pm$ SD $26.6 \pm 1.9\%$ w/w Table 2]. The level of gangliosides from K99-non-receptive jejunal brush borders was low [209 ± 100 nmol sialic acid (g dry wt brush border) $^{-1}$, i.e. 26 ± 13 nmol (g wet wt brush border) $^{-1}$]. This represented 5–16% of the total lipids [mean $\pm$ SD $10.5 \pm 2.5\%$ w/w].

Table 2. Effect of jejunal membrane sialoglycolipid concentration on adhesion of K99 fimbriated bacteria

Healthy piglets (M, male; F, female) were slaughtered 12–21 d after birth. Animal nos 1–8, 9–13 and 14–19 were offspring produced by three different mating pairs of Large White pigs.

Piglet characteristics				Sialoglycolipids	
No. (sex)	Age (d)	Bacterial status*	K99 phenotype†	Concn‡ [nmol sialic acid (g dry wt brush border) ⁻¹]	% (w/w)§
1 (F)	18	Monoxenic	++	293	28.3
2 (M)	20	Monoxenic	++	259	27.0
3 (F)	19	Monoxenic	+	255	24.2
4 (M)	18	Monoxenic	++	552	29.1
5 (M)	21	Holoxenic	++	375	24.9
6 (M)	21	Holoxenic	+++	334	26.2
Mean . . .				344 ± 111	26.6 ± 1.9
7 (M)	19	Monoxenic	–	259	12.6
8 (F)	20	Monoxenic	–	167	14.9
9 (M)	14	Monoxenic	–	141	12.1
10 (F)	12	Monoxenic	–	167	12.3
11 (F)	14	Monoxenic	–	160	11.3
12 (M)	13	Monoxenic	–	157	10.0
13 (F)	15	Monoxenic	–	139	9.1
14 (M)	14	Axenic	–	192	12.3
15 (F)	14	Axenic	–	135	9.2
16 (M)	14	Axenic	–	249	7.8
17 (M)	14	Axenic	–	318	10.5
18 (M)	14	Axenic	–	151	5.1
19 (F)	14	Axenic	–	485¶	8.7
Mean . . .				209 ± 100	10.5 ± 2.5

* Colostrum-deprived piglets were germ-free (axenic) or monoassociated with one *E. coli* strain (monoxenic). Two piglets were conventional (holoxenic).

† Adhesion of K99 fimbriated *E. coli* strain B80 was assayed on brush borders isolated from the proximal jejunum of piglets by an adhesion test. The system of grading was: (+++), all brush borders receptive, each with 11–20 K99-positive *E. coli* bacterial cells attached, (++) , (+), 50% or 25% of brush borders with 11–20 attached bacteria, the remainder having 1–5 associated bacteria. Results were recorded as negative (–) when all the brush borders were free of bacteria. Control tests were done with K99-negative cells (10^9 c.f.u. ml⁻¹) under the same conditions.

‡ Sialoglycolipid concentrations [expressed as nmol sialic acid (g dry wt brush border)⁻¹] were those of proximal jejunal segments used for the adhesion test.

§ The proportion (% w/w) of sialoglycolipid in the lipid extract.

|| Mean value ± SD; results were analysed by using Statview software (Brain Power, Calabasas, USA). The different values of *P* for the K99-receptive group compared with the non-receptive group were: concentration of sialoglycolipid $0.005 < P < 0.01$; proportion of sialoglycolipid, $P \leq 0.0005$.

¶ High concentrations of sialoglycolipids were observed in the absence of bacterial adhesion.

Analysis of the sialoglycolipids extracted from the intestinal brush borders of colostrum-deprived piglets (Fig. 1) indicated that an acidic glycolipid gradient might exist along the different intestinal segments of the individual piglets of the K99-receptor-positive phenotype. The largest amounts of sialic-acid-containing glycolipids were found in the epithelium of both proximal jejunal and ileal segments (Fig. 1 and Table 2; piglet no. 1) or in the proximal jejunum only (piglet no. 4). However, this finding was not observed in non-receptive piglets. In contrast, the level of sialic acid-containing glycolipids was low and variations in concen-

tration along the intestine were not significant (piglets nos 8 and 9).

Relative distribution of gangliosides in jejunal brush border epithelia

In order to characterize further the apparent relationship between sialoglycolipid content and the susceptibility of each piglet to K99-positive-*E. coli* adhesion, the distribution of the molecular species of acidic glycolipids in all piglets listed in Table 2 was examined by HPTLC. Two-dimensional thin-layer chromatography of sialoglycolipids demonstrated that the ganglioside patterns of K99-

Fig. 2. Two-dimensional HPTLC analysis of total sialoglycolipid extracts of the proximal jejunum of K99-receptive (*a*, piglet no. 5) and K99-non-receptive (*b*, *c* piglets nos 9 and 15) Large White piglets. Sialoglycolipids (3 nmol sialic acid) were spotted at the origin of the plates and separated in chloroform/methanol/water (60:35:8, by vol.) containing 0.015 g CaCl₂ (1st dimension) and then in propan-1-ol/28% (w/v) ammonia/water (75:5:25, by vol.) (2nd dimension). Spots were identified by comparing their migration to that of authentic reference markers.

receptive piglets (Fig. 2*a*) were more complicated than those of K99-non-receptive piglets (Fig. 2*b*, *c*). Receptive intestinal cells contained the gangliosides GQ, GT1, GD1b, GD1a, GD2, GD3, GM1, GM2, GM3Gc and the sialoparaglobosides SPG and SPGGc, identified by their chromatographic mobilities (Fig. 2). Two gangliosides present in very low amounts were identified as GM1Gc and GD3Gc. The conventional piglet no 5 of the receptive group (++) showed the same distribution as the monoxenic receptive piglet of the same phenotype (data not shown).

The gangliosides of three representative individuals from each piglet group, viz. K99-receptive piglets (nos 2, 3 and 6) and non-receptive piglets (nos 7, 8 and 16) were analysed quantitatively (Table 3). Whereas the monosialylated gangliosides varied from 78–165 nmol sialic acid

(g dry wt brush border)⁻¹ and represented 30–49% of the total sialoglycolipid in the K99-receptive jejuna (values derived from piglet nos 1–6 inclusive), they accounted for 62–97% of the total sialoglycolipid in non-K99-receptive jejuna [from 130–241 nmol (g dry wt brush border)⁻¹] (values derived from piglet nos 7–19 inclusive). GM2 was the most abundant ganglioside (Fig. 2*b*; piglet no. 9), comprising $59 \pm 12\%$ (mean $\pm$ SD) of total acidic glycolipids in jejunum from the seven monoxenic piglets to which K99-positive *E. coli* did not adhere. It comprised $80 \pm 9\%$ (mean $\pm$ SD) of the total sialoglycolipid extract from the six non-receptive, axenic individuals (Fig. 2*c*, piglet no. 15; Table 3, piglet no. 16). Even though some endothelial cells contaminated the enterocyte preparations, analysis of the distribution of monosialylated glycolipids from purified non-receptive brush

Table 3. Sialoglycolipid pattern of K99-receptive and non-receptive jejunum

Concentration values in nmol sialic acid (g dry wt brush border)⁻¹ were determined from the amounts of sialic acids (cited in Table 2) and the percentages of sialic acids obtained after calibration of densitometer responses as described in Methods. The values presented are the average of duplicate scans of one of more analyses. Duplicate assays were typically within 5% of the mean. tr., Trace.

Sialoglycolipid	Sialoglycolipid concentration [nmol sialic acid (g dry wt brush border) ⁻¹]					
	K99-receptive piglets			Non-K99-receptive piglets		
	No. 2	No. 3	No. 6	No. 7	No. 8	No. 16
GM3	12.5	13	tr.	36.6	28.9	50.1
GM3Gc	40	26	63	5.1	7.8	0
GM2	0	11	29	123.5	94.6	179.1
SPG	2.6	6.2	tr.	0.9	0	0
SPGGc	9	10	9	0.6	0	0
GM1	10	11.8	22	12.7	14	12.1
GM1Fuc	tr.	18	tr.	0	4	0
GM1Gc	4	1.2	tr.	tr.	0	0
GD3	33.3	30.7	46.5	2.4	0.9	2.7
GD3Gc	19.8	3.1	5	0	0	0
GD3-O-Ac	1.5	tr.	tr.	2.2	tr.	0
GD1a	13	17.6	25.9	2.1	2.8	2.5
GD2	19.5	17	15.7	2.2	1.5	0
GD1b	29.6	17	15.3	9.5	4	4.7
GT1b	31	14.2	16.6	1.6	8.9	0
GQ1b	36	1.7	14.5	9.2	3.3	0

border preparations indicated no differences with that observed in epithelial cells (data not shown).

Furthermore, while the concentration of the ganglioside GM1 was roughly in the same range in both phenotypes [10–22 nmol (g dry wt brush border)⁻¹ [i.e. 1.5–2.7 nmol (g wet wt brush border)⁻¹], the concentrations of the other sialoglycolipids differed considerably (Table 3). Considering the concentration ratios of the various sialoglycolipids isolated from K99-receptive and non-K99-receptive brush borders, it appeared that GD3, SPGGc, GT1b, GD1a, GM3Gc, GD2 and GD1b were, respectively, about 25, 16, 12, 9, 7, 5 and 2 times more abundant in the K99-receptive piglets than in the non-K99 receptive ones.

The amounts of the ganglioside GM3Gc varied from 20–70 nmol (g dry wt brush border)⁻¹ [i.e. 2.5–8.7 nmol (g wet wt brush border)⁻¹] and SPGGc averaged 10 nmol (g dry wt brush border)⁻¹ [i.e. 1.2 nmol (g wet wt brush border)⁻¹] in K99-receptive piglets. GM3Gc levels were lower than 8 nmol (g dry wt brush border)⁻¹ [i.e. 1 nmol (g wet wt brush border)⁻¹] and SPGGc was not detected in non-K99-receptive jejunum.

Binding of the K99-positive *E. coli* strain B80 to individual gangliosides

When total sialoglycolipids were separated and reacted in various concentrations with suspensions of ¹⁴C-

Fig. 3. Autoradiogram after binding of ¹⁴C-labelled K99-positive *E. coli* cells to piglet proximal jejunal sialoglycolipids. Total glycolipids extracted from (a) K99-receptive piglet no. 3 and (b) non-receptive piglet no. 7 (Table 3) were separated in chloroform/methanol/water (30:60:8, by vol.) containing 0.015 g CaCl₂. Two samples of sialoglycolipids, equivalent to 1.5 nmol (lanes 2 and 4) and 3 nmol (lanes 3 and 5) sialic acid, were incubated with K99-fimbriated bacteria (strain B80, 10⁷ c.f.u. ml⁻¹) for 2 h. In a control experiment, ¹⁴C-labelled K99-negative *E. coli* cells (strain B80 grown at 18 °C for 48 h; 10⁷ c.f.u. ml⁻¹) and sialoglycolipids equivalent to 3 nmol sialic acid were incubated under the same conditions (lanes 1 and 6).

labelled K99-positive *E. coli* cells, there were quantitative differences in the binding of K99-positive bacteria to sialoglycolipids extracted from either K99-receptive

Fig. 4. Binding of ^{14}C -labelled, K99-positive or K99-negative *E. coli* cells to piglet proximal jejunal sialoglycolipids. Glycolipids in increasing amounts, 1.5 nmol (lanes 2 and 5), 3 nmol (lanes 3 and 6), 6 nmol (lanes 1, 4 and 7), were extracted from (a) the receptive piglet no. 6 and (b) non-receptive piglet no. 8 and separated as described in Fig. 3. Incubation time was 2 h with K99-positive strain B80 (10^8 c.f.u. ml^{-1}). A control experiment was done by incubating ^{14}C -labelled K99-negative *E. coli* cells (10^8 c.f.u. ml^{-1}) and sialoglycolipid equivalent to 6 nmol sialic acid (lane 1).

or non-receptive piglets (Figs 3 and 4). A suspension of K99-positive *E. coli* containing 10^7 bacteria ml^{-1} (i.e. 0.1–0.15 ng pure pili ml^{-1}), when incubated with 1.5 or 3 nmol glycolipid sialic acid (Fig. 3a, lanes 2 and 3), preferentially bound to GM3Gc among the glycolipids isolated from a K99-receptive piglet (animal no. 3). Furthermore, a bacterial cell suspension containing 10^8 cells ml^{-1} , reacting with 1.5 nmol glycolipid sialic acid (Fig. 4a, lane 2) bound to both GM3Gc and SPGGc among the glycolipids isolated from K99-receptive piglet no. 6. At both densities of bacterial suspensions, K99-positive bacteria, reacting with 1.5 nmol glycolipid sialic acid, weakly bound to the glycolipids isolated from non-K99 receptive piglets nos 7 and 8 (Fig. 3b, lanes 4 and 5; Fig. 4b, lane 5).

The effect of ganglioside concentration on binding was analysed with a K99-positive *E. coli* suspension containing 10^8 bacteria ml^{-1} . The binding patterns were distinct when the bacteria were reacted with 3 nmol glycolipid sialic acids (Fig. 4a, b, lanes 3 and 6). The glycolipids recognized by the bacterial cells were abundant in the K99-receptive piglet, for which a high level of bacterial adhesion to all of the brush borders was found (Fig. 4a, lane 3). Quantitative autoradiography showed that the gangliosides to which ^{14}C -labelled K99-positive *E. coli* bound were 2.5 times more numerous in the mucosa of the K99-receptive piglet than in the mucosa of the non-receptive piglet (Fig. 4b, lane 6). An increase in the amount of ganglioside (6 nmol sialic acid) weakly modified the binding (Fig. 4a, lane 4). An additional phenomenon, especially marked in extracts from non-K99-receptive piglets, was observed. Radioac-

tivity was detected in the di- and polysialogangliosides regions (Fig. 4, lanes 3, 4, 6 and 7), indicating in particular that both GQ and GT1b act as receptors.

No binding occurred with non-fimbriate bacterial cells (grown at 18°C) (Fig. 3, lanes 1 and 6; Fig. 4, lane 1).

Discussion

With the aim of studying the expression of epithelial glycosphingolipids and their role as receptors for K99-fimbriate ETEC, the intestinal glycosphingolipids of young piglets (12–21-d-old) were analysed. It is recognized that piglets are susceptible to infection by K99-positive ETEC before weaning (usually at 3-weeks-old) but that they then develop resistance to these strains (Runnels *et al.*, 1980).

The sialoglycolipid content was close to values previously reported in pig intestinal epithelium, namely 11–14 nmol ganglioside sialic acid (g wet wt mucosa) $^{-1}$ (Holmgren *et al.*, 1975; Kyogashima *et al.*, 1989), although it varied with individuals from 16–57 nmol (g wet wt mucosa) $^{-1}$. On the basis of sialoglycolipid concentration, it appeared that piglets could be classified into two groups (Table 2). Piglets susceptible to K99 adhesion had a higher level of sialylated glycolipids than those resistant to K99 attachment. Therefore, the proneness to K99 adhesion to brush borders from piglet intestinal cells correlated with the degree of sialylation of the intestinal epithelium.

The difference in K99-positive bacterial adhesion may

be explained by some physicochemical characteristics of K99 fimbriae. The K99 adhesin, with a pI of 10, is positively charged in the gut and thus behaves quite differently from most fimbrial adhesins which are negatively charged structures with pI values in the range 3.5–5.6 (Gaastra & de Graaf, 1982). The clusters of negative charges on the sialic acid moieties of sialoglycolipids could bind K99 fimbriae by interacting with the positively charged residues present in the subunits. Specific binding of K99 fimbriae was probably favoured by a high density of negative charges at the enterocyte surface. This hypothesis could be supported by the fact that K99 fimbriae possess the ability to recognize the three or four sialic-acid-containing gangliosides IV³NeuAc,II³(NeuAc)₂-GgOse₄Cer (GT1b) and IV³(NeuAc)₂,II³(NeuAc)₂-GgOse₄Cer (GQ).

The sialoglycolipids seemed to be relatively unaffected by the presence of intestinal flora, since they were chemically very similar, on the one hand, in holoxenic (piglet nos 5 and 6) and monoxenic (nos 1–4) receptive piglets, and on the other hand, in monoxenic (nos 7–13) and axenic (nos 14–19) non-receptive animals. Association with non-enterotoxigenic K88-negative *E. coli* (piglet nos 1, 4, 7 and 9), non-enterotoxigenic K88-positive *E. coli* (nos 2, 3 and 8) or enterotoxigenic K88-positive *E. coli* (piglet nos 10–13) did not apparently affect the epithelial sialoglycolipids. As found in murine intestine, porcine epithelial cells may be protected *in situ* by mucus, the lack of hydrolase activities of bacteria colonizing the surfaces and/or the presence of specific surface-associated enzyme inhibitors (Gustafsson *et al.*, 1986).

Our results have demonstrated the existence of two receptor phenotypes in piglets on the basis of the brush border adhesion test and the distribution of sialoglycolipids (Fig. 2). This observation was supported by the binding of K99-positive ETEC to sialoglycolipids. Two receptor-active glycolipids are known to contribute to bacterial binding, II³NeuGc-LacCer (Teneberg *et al.*, 1990; Smit *et al.*, 1984; Ono *et al.*, 1989) and IV³NeuGc-nLcOse₄Cer (Kyogashima *et al.*, 1989), which both contain the same minimal sequence NeuGcα2-3Galβ1-4Glc (or GlcNAc). At low concentrations of sialoglycolipids, the K99 lectin expressed by *E. coli* preferentially interacted with II³NeuGc-LacCer. However, the interaction between this ganglioside and K99 lectin was not the only contribution to the observed binding to brush borders (Fig. 4), since the K99 fimbriae probably recognized sialoglycolipids with a broad affinity range. Furthermore, cooperative interactions have been observed previously in bacterial adhesion to sialoglycoconjugates (Mouricout & Julien, 1987*a, b*). The non-K99-receptive phenotype could result from the presence, as

receptors, of sialoglycolipids in very low amounts, or even their absence from brush borders.

Even though linkage between bacterial adhesion and infection has often been observed (Cheng & Costerton, 1986; Runnels *et al.*, 1980), it remains to be established whether or not the K99-receptive piglet phenotype predisposes individuals to infection and whether or not the non-receptive piglet phenotype is resistant to infection. Data obtained from infant mice, a model for K99-positive ETEC infection, indicate that differences in susceptibility depend on the parental murine strain, and suggest that resistance is inherited as a dominant trait (Duchet-Suchaux *et al.*, 1990). In piglets, it has not been possible to get an insight into the transmission of susceptibility/resistance. Also it remains to be established that the K99-receptor piglet phenotypes detected by the brush border adhesion test has a genetic basis as reported for the K88-receptor phenotypes (Gibbons *et al.*, 1977; Rapacz & Hasler-Rapacz, 1986). Nevertheless, we suggest that the molecular basis of the two phenotypes, observed in piglets of comparable age, is related to the expression of sialoglycolipids and to the *N*-substitution of neuraminic acid.

During the first 3 weeks of postnatal life before weaning, adult-type enterocytes begin to displace foetal-type cells (Smith & Peacock, 1980). Recently, it was shown that piglet mucosa contains a substantially higher content of acidic glycolipids (about 10 times on a protein basis) than that of adult pigs (Teneberg *et al.*, 1990). It is thus possible that the variation in K99-positive bacterial adhesion could result from age-related differences. However, this seems unlikely in our case, since K99-receptive piglets were 19 ± 1-d-old and non-receptive piglets were 14 ± 2-d-old (mean ± SD; Table 2). Our results could indicate that piglets have different receptive phenotypes due to the individual time-courses of maturation, which are probably dependent on the regulation of expression of the receptors. The classification of piglets into two groups did not take the age-dependent expression of gangliosides into account. Nakamura *et al.* (1988) analysed the gangliosides II³(NeuAc)₂-GgOse₄Cer and IV³NeuAc,II³(NeuAc)₂-GgOse₄Cer, in the livers of several inbred strains of mice aged from 2–20 weeks, and defined phenotypes with respect to genetic polymorphism in ganglioside expression. They demonstrated that age-dependent changes in ganglioside expression do occur, and that strains show different phenotypes with respect to this age-dependent expression. It appears to be necessary to study adhesion to, and to analyse the amounts and distribution of different types of sialoglycolipids of, brush borders of piglets from birth to 3-weeks-old in order, to determine whether ganglioside expression is constant or not.

The highest concentration of the 'a' series ganglioside II³NeuAc-GgOse₃Cer was found in non-receptive piglets, while most gangliosides isolated from K99-receptive animals are of the 'b' series, viz. II³(NeuAc)₂-LacCer, II³(NeuAc)₂-GgOse₃Cer, II³(NeuAc)₂-GgOse₄Cer and IV³NeuAc,II³(NeuAc)₂-GgOse₄Cer. The biosynthesis of gangliosides occurs in the sequence LacCer → II³NeuAc-LacCer → II³(NeuAc)₂-LacCer → II³(NeuAc)₃-LacCer, the sialyltransferases I (EC 2.4.99.-) and II (EC 2.4.99.8) being the starting enzymes of the 'a' and 'b' series, respectively (Pohlentz *et al.*, 1988; Trinchera *et al.*, 1990). Since the two pathways are regulated by the differential expression of these enzymes (Pohlentz *et al.*, 1988), it might be suggested that the control of expression or activity of both enzymes could be a possible genetic basis for the K99-receptive phenotype of piglets.

Moreover, we observed that the presence of NeuGc was one of the principal features which determine K99-positive bacterial adhesion. The hydroxylation of NeuAc was more frequent in the K99-receptive phenotype. Also, the amount of NeuGc in the sialoglycolipid fraction might reflect the situation at the sialoglycoprotein level. This hydroxylation reaction is catalysed by a monooxygenase acting on free NeuAc to yield NeuGc (Shaw & Schauer, 1988). Evidence from studies on two mammals (canine and murine species) has indicated that the polymorphic variation of the sialic acid species of GM3 (NeuAc or NeuGc derivatives) within individuals is also under genetic control (Yamakawa *et al.*, 1986).

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