Receptor-active glycolipids of epithelial cells of the small intestine of young and adult pigs in relation to susceptibility to infection with *Escherichia coli* K99

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Glycolipids from mucosa scrapings of small intestine of neonatal and adult pigs were tested by the thin-layer chromatogram overlay assay for the binding of *Escherichia coli* K99. There was practically no binding to acid or non-acid glycolipids of adult pig, known to be resistant to infection with this bacterium. However, piglets, which are susceptible to infection, showed a clear binding to a doublet band in the acid glycolipid fraction. The receptor-active glycolipid was isolated and shown by mass spectrometry, NMR spectroscopy and degradation methods to be NeuGcα-3Galβ4GlcβCer (NeuGc-GM3), the two bands being due to heterogeneity of the ceramide. When tested against various reference glycolipids, NeuAc-GM3 was shown to be inactive. This ganglioside was dominating in adult pig. The apparent developmental disappearance of *N*-glycolyl groups in glycolipids of intestinal mucosa may have a correspondence in protein-linked sequences as well and thus explain the resistance of adult pigs to infection with *E. coli* K99.

Bacterial adhesion: *E. coli* K99; Pig intestine; Receptor; Glycolipid; Sialyllactosylceramide; *N*-Glycolylneuraminic acid

1. INTRODUCTION

Escherichia coli strains possessing the K99 antigen cause diarrhea in neonatal calves, lambs and piglets [1,2]. Two virulence factors have been identified, the ability to colonize the small intestine, and the production of enterotoxins that cause the diarrhea. The K99 antigen, which is involved in the adherence of the bacteria to the brush border of epithelial cells, has been characterized as a fimbrial structure with a single repeating protein subunit. The purified antigen causes a mannose-insensitive agglutination of horse erythrocytes, but no agglutination of guinea-pig erythrocytes. The receptor on horse erythrocytes was identified as NeuGcα3Galβ4GlcβCer, based on mass spectrometry and NMR spectroscopy [3] and this was later confirmed [4]. This information was recently supplemented by inhibition of agglutination of human erythrocytes using intact bacteria and a series of synthetic sialic acid derivatives [5].

We were interested in knowing the nature of receptor substances in the target cells, the epithelial cells of the small intestine. Furthermore, the known resistance to infection among adult animals [1] was of relevance to test for receptor characteristics. Glycolipids were therefore isolated from mucosa scrapings of piglets and adult pigs and these were tested for binding of *E. coli* K99 using the thin-layer chromatogram overlay assay [6]. Binding of bacteria to glycolipids coated in microtiter wells was also examined [7]. Only piglet mucosa contained a receptor-active glycolipid, which was isolated and shown to be identical with the earlier identified receptor of horse erythrocytes, namely NeuGcα3Galβ4GlcβCer.

The condensed representation of sugar chains follows the IUB-IUPAC JCBN recommendations [(1985) J. Biol. Chem. 262, 13–18].

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2. MATERIALS AND METHODS

2.1. *Growth of bacteria and labeling*

E. coli strain K-12 C600λ−,*ton*A21,*thr*,*leu*-6,*thi*-1,*sup*E44, *lac*γ−1-^{stable} was used as the host for plasmid pFK99, which contains the genes required for the biosynthesis of K99 fimbriae [8]. Cells were cultured overnight at 37°C in liquid Minca medium containing 50 μCi ^{35}S-methionine (Amersham, UK) per 10 ml of medium. At this temperature the cells express K99 fimbriae [9]. They were also grown at 20°C with no expression of fimbriae [2,9]. Labeled cells were collected by centrifugation at 6×10^3 rpm for 10 min and washed twice with phosphate-buffered saline, pH 7.4 (PBS). For overlay the cells were suspended in PBS with 1% mannose giving 1×10^9 cells/ml and approximately 1×10^6 cpm/ml. Cells were also grown without labeled methionine for detection with antibody, see below and Fig. 1.

2.2. *Glycolipid preparations*

Pathogen-free pigs were from the Central Veterinary Institute, Lelystad, the Netherlands. Neonatal pigs were about 3 days old and adult pigs were more than 6 months of age. Mucosa was obtained by

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gentle scraping along the small intestine. Lyophilized cells were extracted with chloroform/methanol and worked up for total acid and total non-acid glycolipids mainly as described [10]. The acid fraction from piglet was fractionated on Iatrobeads (Iatron Co., Tokyo) using a linear gradient of chloroform/methanol/ water, from 50:40:1 to 10:90:2, by vol. Eluted fractions were monitored for receptor-active ganglioside using thin-layer chromatography and overlay with labeled bacteria [6,7]. Purity was checked by thin-layer chromatography. Structural characterization of isolated glycolipids was done with mass spectrometry, NMR spectroscopy and other methods, in principle as described elsewhere [3,11–14]. Reference glycolipids (Table II) were characterized with a similar approach.

2.3. *Thin-layer chromatography*

Thin-layer chromatography was done on aluminum sheets coated with silica gel 60 (HPTLC nano plates, Merck, FRG) using chloroform/methanol/water 60:35:8, by vol. (non-acid fractions) and chloroform/methanol/0.2% $CaCl_2$ 60:40:9, by vol. (acid fractions).

2.4. *Binding assays*

Overlay of ^{35}S-labeled bacteria on thin-layer chromatograms with separated glycolipids and autoradiography, or binding to glycolipids coated in microtiter wells, have been described in detail elsewhere [6,7]. For detection of bound bacteria with antibody [7] a mouse monoclonal antibody (CVI K99-16, kindly provided by F.G. van Zijderveld, CDI, Lelystad, the Netherlands) with specificity for the K99 fimbriae was diluted 1:100 in PBS with 2% bovine serum albumin and 0.1% NaN_3 (solution A). This was overlayered on the plate for 2 h followed by careful washing with PBS and final overlay for 2 h with ^{125}I-labeled rabbit antimouse antibody (Dakopatts A/S, Denmark), diluted 1:100 in solution A to about 2×10^3 cpm/μl, and careful washing.

2.5. *Molecular modeling*

Molecular modeling was based on the HSEA [15] and GESA [16] methods as outlined elsewhere [17]. The ceramide conformation of the monohexosylceramide part was adapted from crystal data [18].

3. RESULTS AND DISCUSSION

Fig. 1 shows a thin-layer chromatogram with acid and non-acid total glycolipid fractions from piglet and adult pig small intestinal mucosa, documenting a clear developmental change in patterns from young to adult stage (left). The autoradiogram (right) after overlayering with bacteria, detection with antibody and careful washing, looks very simple: only binding to a doublet band in the acid fraction of piglet and no binding to adult pig or to non-acid fractions. Identical results were obtained with metabolically labeled bacteria. After growing the bacteria at 20°C, when no K99 fimbriae are expressed, no binding occurred.

The positive doublet band was isolated and subjected to mass spectrometry (Fig. 2) and NMR spectroscopy (Fig. 3), and also degradation studies with sialidase and with acid. The results supported the sequence as NeuGcα3Galβ4GlcβCer. In the mass spectra (permethylated-reduced spectrum not shown) there were diagnostic peaks for NeuGc (in Fig. 2 at m/z 406 and 374, which is 406 minus methanol), and one may interpret the two bands (see Fig. 1) as based on mainly longer-chain hydroxy fatty acid (h22–h24) in the upper band, and mainly shorter-chain hydroxy fatty acid (h16) in the lower band, both combined with sphingosine (18:1).

Table I documents substantially more acid glycolipids (about 10 times) in the piglet mucosa compared to adult pig on a protein basis. The major acid glycolipid was sulphatide (3-*O*-sulphated GalβCer), see Fig. 1. In the adult pig there was in addition an unknown major band which was absent in the piglet and with a mobility in between sulphatide and GM3. The GM3 of adult pig was mainly NeuAc-GM3 as shown by mass spectrometry and NMR spectroscopy (not reproduced). The unusually high levels of acid glycolipids in the piglet means a probably high density of receptors for *E. coli* K99 in the intestinal epithelium.

In Table II binding preferences for a limited series of reference gangliosides are summarized. Of interest is a very similar activity for NeuGc-GM3 (no. 3) and NeuGc-GM2 (no. 4), while NeuGc-GM1 (no. 5) is inactive. GalNAc substituted on the core saccharide does

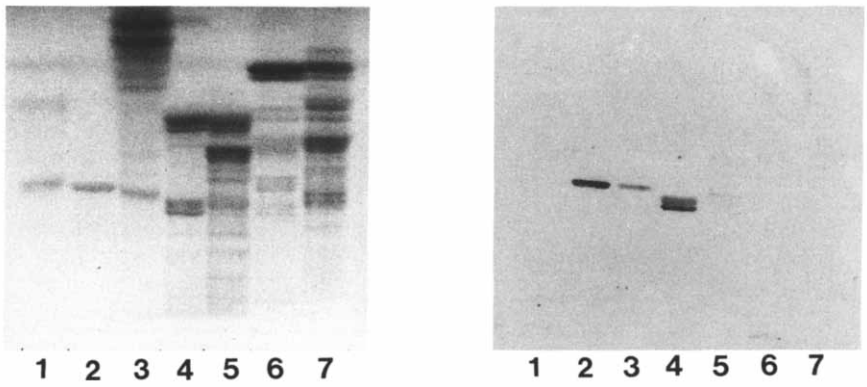

Fig. 1. Thin-layer chromatogram after detection with anisaldehyde (left) and autoradiogram after binding of *E. coli* expressing K99 fimbriae (right). Bound bacteria were detected with anti-K99 antibody and ^{125}I-labeled anti-antibody. The lanes were: 1, 0.7 nmol of NeuAc-GM3 of human brain (no. 2 of Table II); 2, 0.7 nmol of NeuGc-GM3 of horse erythrocytes (no. 3 of Table II); 3, 0.7 nmol of total acid glycolipids of horse erythrocytes; 4, 7 nmol of total acid glycolipids of mucosa of small intestine of 3 weeks old piglets; 5, 7 nmol of total acid glycolipids of mucosa of small intestine of adult pig; 6, 7 nmol of non-acid glycolipids of mucosa of small intestine of piglet; and 7, 7 nmol of non-acid glycolipids of mucosa of small intestine of adult pig. The solvent was chloroform/methanol/water, 60:35:8, by vol.

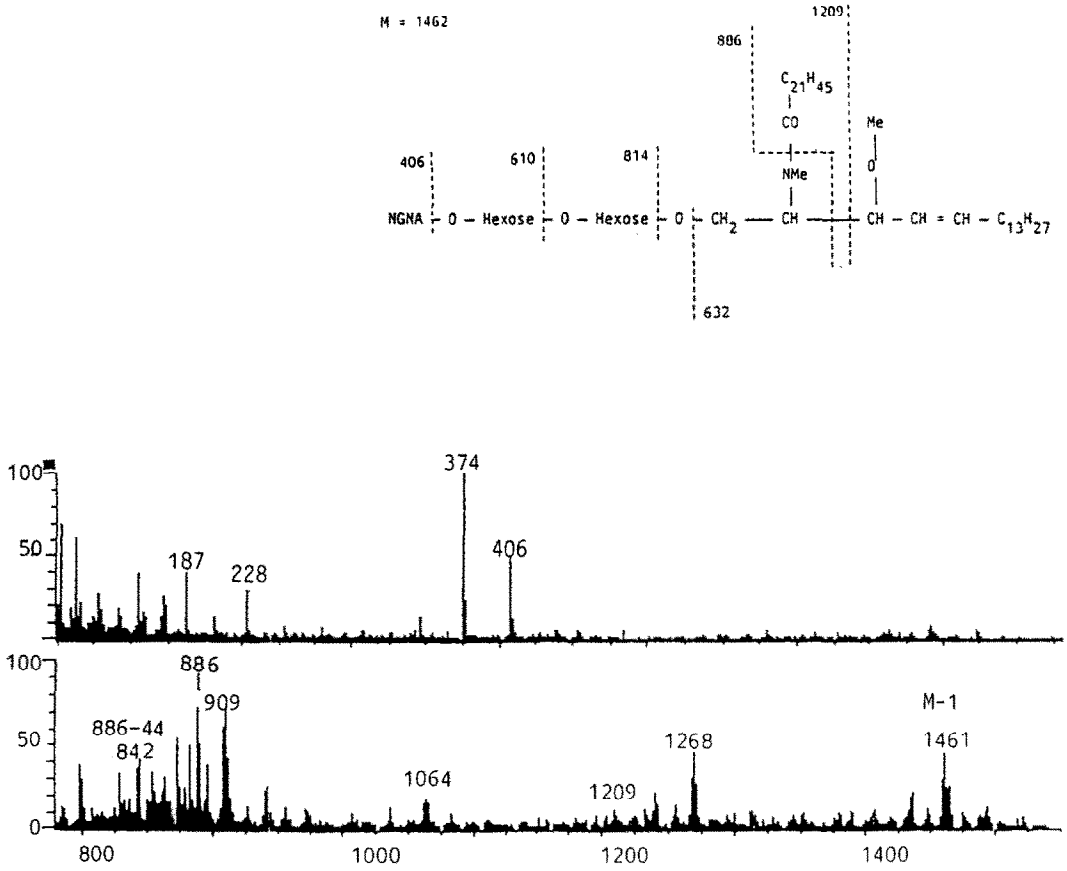

Fig. 2. Mass spectrum and simplified formula of the permethylated, receptor-active ganglioside isolated from piglet small intestine. The instrument used was a ZAB-2F apparatus (VG Analytical, UK), and the electron energy was 46 eV, trap current 500 μA, acceleration voltage 8 kV and probe temperature 200°C.

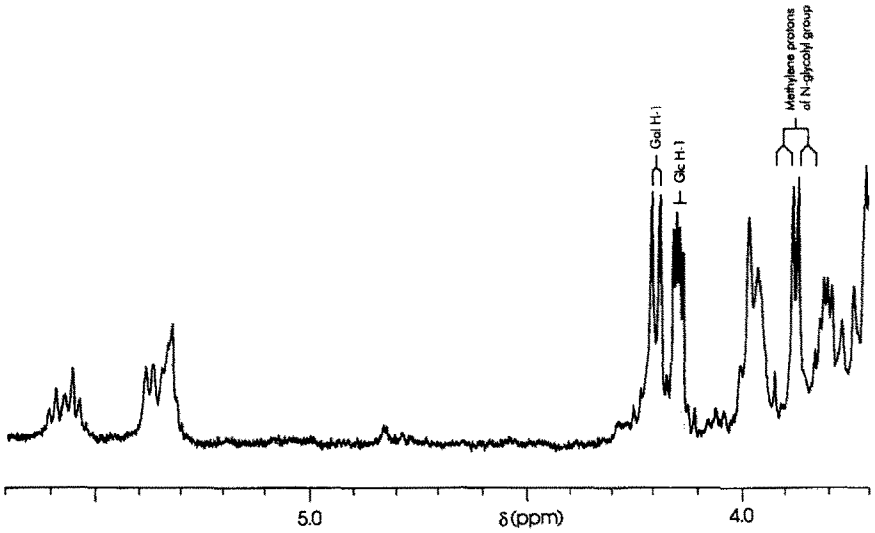

Fig. 3. Proton NMR spectrum of the receptor-active ganglioside isolated from piglet small intestine. The glycolipid was dissolved in dimethylsulphoxide-d6:D_2O 98:2, by vol., and the spectrum was recorded at 30°C on a 400 MHz Varian XL-400 spectrometer. The spectrum is in good agreement with that for the receptor-active glycolipid isolated from horse erythrocytes [3]. Resonances are marked which are indicative of the lactose unit, at 4.14 ppm (J = 5.6 Hz) and 4.19 ppm (J = 5.6 Hz), and of the N-glycolyl group at 3.85 ppm (J = 16 Hz) and 3.90 ppm (J = 16 Hz).

Table I

Quantitative data on intestinal mucosa and glycolipids obtained from piglet and adult pig

	Piglet	Adult pig
Mucosa dry weight (mg)	9.1×10^3	40.3×10^3
Total lipid extract (mg)	3.4×10^3	10.3×10^3
Total acid glycosphingolipids (μmol)[a]	188	98
Total non-acid glycosphingolipids (μol)[a]	25.9	57.2
Acid glycosphingolipids/g protein[b] (μmol)[a]	35.5	3.8
Non-acid glycosphingolipids/g protein[b] (μmol)[a]	4.9	2.2

[a] Assayed as sphingosine [20] in a slight modification [21]
[b] Assayed according to [22]

therefore not affect the binding, while an additional Gal blocks the binding, probably for sterical reasons. This suggests a probable binding site of NeuGc, based on molecular modeling (Fig. 4), which result is in agreement with data from inhibition of hemagglutination with glycolipids [4] and synthetic modified sialic acids [5].

These results based on analysis of the target mucosa cells of piglet small intestine support earlier proposals of NeuGc as the receptor substance for *E. coli* K99, based on hemagglutination studies [3–5]. The absence of receptor-active glycolipids of adult pig, as shown by solid-phase overlay analysis with living bacteria (Fig. 1), may explain the resistance to infection appearing with development and is further evidence for the relevance of NeuGc as the receptor in vivo. Whether glycoproteins of these cells show a similar change in NeuGc with development is not known. After the present work was finished a paper appeared on piglet intestine with a similar approach as used here, in general supporting our findings [19]. However, adult pig was not studied.

We have also investigated intestinal mucosa of calves, which are also prone to infection with *E. coli* K99 [1]. In this case the receptor situation is more complex than in the pig. Data on this as well as the detailed

Table II

Results from binding of *E. coli* K99 to glycosphingolipids

No.		Binding	Tissue source
1	GalβCer	–	dog small intestine
2	NeuAcα3Galβ4GlcβCer	–	human brain
3	NeuGcα3Galβ4GlcβCer	+	horse erythrocytes
4	GalNAcβ4(NeuGcα3)Galβ4-GlcβCer	+	calf small intestine
5	Galβ3GalNAcβ4(NeuGcα3)-Galβ4GlcβCer	–	calf small intestine

Binding was tested on thin-layer plates with 1 μg of glycolipid. A minus sign means no darkening on the autoradiogram. A plus sign means a strong darkening with a detection level at 50–100 ng of glycolipid

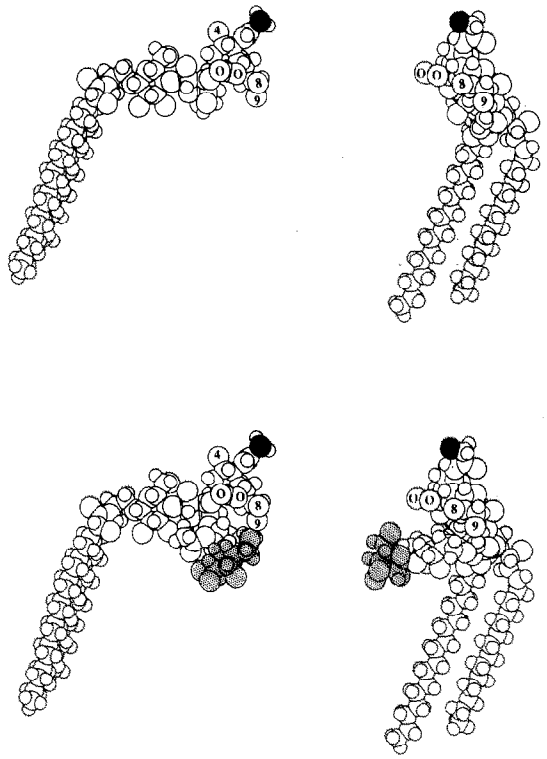

Fig. 4. Calculated preferred conformations of the receptor-active glycolipid isolated from piglet, NeuGc-GM3 (top, sequence 3 of Table II) and the receptor-negative NeuGc-GM1 (bottom, sequence 5 of Table II), to visualize the probable site of NeuGc being involved in the binding of *E. coli* K99. The two left (top and bottom) projections have C2, C3 and C4 of sialic acid in the plane of the paper and with C2 and C4 through the *y*-axis. The two right projections were obtained by a 90° rotation around this *y*-axis through C2 and C4 making the *N*-glycolyl group (oxygen in black) point towards the viewer. This makes the proposed binding site of NeuGc exposed to the viewer in the left projections, while the binding is from the side and left in the two right projections. NeuGc is labeled with O on the carboxyl oxygens and with numbers on O4, O8 and O9, and the essential glycolyl oxygen is in black. That NeuGc-GM1 (lower models) is a non-binder of *E. coli* may be explained by the sterical hindrance for access to NeuGc by the terminal Galβ3 (dotted), which projects from the proposed binding site. Although the hexosylceramide partial sequence was based on its crystal conformation [18], the direction of the paraffin chains may be different than shown due to a possible rotation at the glycosidic bond to sphingosine.

structures of the various pig glycolipids, including the definition of the binding epitope on the receptor, will be published separately.

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