Binding of K99 fimbriae of enterotoxigenic *Escherichia coli* to pig small intestinal mucin glycopeptides

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Binding of purified K99 fimbriae to cryostat sections of pig small intestine was detected. Binding sites were located in the mucus layer, but not in the submucosal connective tissue. High-*M_r* mucin glycopeptides from pig small intestine were found to bind to K99-fimbriated enterotoxigenic *Escherichia coli*, in contrast to non-fimbriated cells. Sialic acid specificity of K99 fimbriae was demonstrated by the significant reduction in binding upon desialylation of mucin glycopeptides. The binding was saturable and the dissociation constant was estimated to be 6×10^{-7} M. Fimbriated bacteria were calculated to possess 2.3×10^3 binding sites per cell.

Introduction

Bacterial adhesion to eukaryotic cells has been studied for decades in order to evaluate the contribution of this property to pathogenicity. A number of factors mediating adhesion, and thus colonization of the host, have been identified and their specificities for various molecules present on eukaryotic cell surfaces have been established (Evans *et al.*, 1979; Jones *et al.*, 1976; Morris *et al.*, 1980; Parkkinen *et al.*, 1983; Smith & Linggood, 1971). Carbohydrate structures acting as receptors for bacterial adhesins have been found on glycolipids as well as on glycoproteins, and efforts have been made to establish the carbohydrate specificity of such adhesins (Bock *et al.*, 1985; Hansson *et al.*, 1985; Lindahl & Wadström, 1986; Murray *et al.*, 1982; Ramphal & Pyle, 1983).

Enterotoxigenic *Escherichia coli* (ETEC) have been isolated from both man and animals (Satterwhite *et al.*, 1978; Smith & Linggood, 1971). Neonates, as well as adults, may suffer from ETEC diarrhoea, although this condition is more severe and may be fatal to the young (Dorner *et al.*, 1980; Sherman *et al.*, 1983). K99 is one of the fimbrial adhesion factors that enables the bacterium to colonize the small intestine of neonatal pigs, calves and lambs (Morris *et al.*, 1980). Adhesion of K99-fimbriated ETEC to enterocytes is dependent on the age

of the host (Runnels *et al.*, 1980), possibly explaining the age-dependent susceptibility to infection (Smith & Halls, 1967).

It has been established that K99 fimbriae specifically bind to sialic acid residues on the surface of erythrocytes (Lindahl *et al.*, 1987). The identification of receptor molecules has so far been restricted to glycolipids since they are easy to purify and analyse (Kyogashima *et al.*, 1989; Smit *et al.*, 1984).

The intestinal surface is covered by a mucus layer which protects the epithelium (Neutra & Forstner, 1987). Mucus glycoproteins (mucins), the macromolecules forming the gel, are very rich in carbohydrate which occurs as oligosaccharides, some of which have blood group antigens (Carlstedt *et al.*, 1985). The mucus layer is thick compared to the size of a bacterium, and the oligosaccharides provide potential receptors for bacterial adhesins (McQueen *et al.*, 1983; Mouricout & Julien, 1987; Murray *et al.*, 1982; Ramphal & Pyle, 1983). The aim of this study was to investigate the interaction between K99-fimbriated ETEC and mucin glycopeptides from pig small intestine in order to elucidate some of the factors involved in the adherence of this pathogen to the intestinal mucosa of neonatal piglets.

Methods

Bacterial cells. *E. coli* strain B117 (O8:K85ab, K99:H⁻), kindly supplied by Dr J. A. Morris, Central Veterinary Laboratory, Weybridge, UK, was cultivated on Minca-agar at 37 °C (Guinée *et al.*,

Abbreviations: ETEC, enterotoxigenic *Escherichia coli*; FITC, fluorescein isothiocyanate; PAS, periodic acid-Schiff.

1977). To assess fimbrial production bacterial suspensions ($1-5 \times 10^9$ c.f.u. ml^{-1}) in phosphate-buffered saline (PBS; 0.14 M-sodium chloride, 0.01 M-sodium phosphate buffer, pH 7.4) were mixed with equal volumes (20 μl) of anti-K99 monoclonal antibodies (kindly supplied by Dr P. Sadowski, Mol. Genetics Inc., USA) on a glass slide. K99-positive cultures, completely aggregated as judged by the naked eye, were subjected to hydrophobic interaction chromatography on Octyl-Sepharose (Pharmacia) to measure the fraction of fimbriated cells. The gel (1 ml) was packed in Pasteur pipettes, equilibrated with 2 ml 1 M-ammonium sulphate. Bacterial suspensions ($100 \mu\text{l}$; 5×10^{10} cells ml^{-1} in PBS) were added and each column was eluted with 5 ml 1 M-ammonium sulphate. Fractions (1 ml) were collected and the OD_{550} measured. A standard curve obtained by using bacterial suspensions with known c.f.u. ml^{-1} values was used to determine the amount of bacteria in the collected fractions. The amount of fimbriated (adsorbed) bacteria in the sample was calculated by subtracting the determined number of unadsorbed (non-fimbriated) bacteria (Wadström *et al.*, 1978).

Purification of fimbriae. A K99-positive culture of strain B117, grown on Minca-agar, was harvested in PBS. The suspension was diluted to about 10^{11} cells ml^{-1} in PBS and sonicated on ice for 90 s (6×15 s) in an MSE sonicator (20 μm amplitude) with the probe end at the surface. The suspension was centrifuged twice at 12000 g for 15 min. and the supernatant collected. Proteins were precipitated at 4°C after the addition of solid ammonium sulphate to a final concentration of 4 M. The precipitate was recovered after centrifugation at 27000 g for 30 min, suspended in PBS and dialysed against PBS at 4°C . Nucleic acids were digested with deoxyribonuclease I and ribonuclease A, (overnight at 20°C), in the presence of 10 mM-magnesium sulphate. Fimbriae were purified on a Sephacryl S-1000 column (Pharmacia) eluted with PBS containing 2 M-urea (de Graaf *et al.*, 1980). Material eluting in the void volume was dialysed against PBS and tested for the presence of K99 by agglutination with anti-K99 monoclonal antibodies as described above. Purity of fimbriae was assessed by SDS-PAGE (Blobel & Dobberstein, 1975). A sample containing 15 μg protein yielded a single band (19 kDa) which was visualized by Coomassie brilliant blue staining.

Preparation of fluorescein isothiocyanate (FITC)-labelled K99 fimbriae. Purified K99 fimbriae (0.5 mg ml^{-1}) were incubated for 1 h in 0.05 M-sodium carbonate buffer, pH 9.0, containing FITC (1 mg ml^{-1}). Excess reagent was separated from conjugated fimbriae by using gel filtration on a PD-10 column (Pharmacia).

Cryostat sections of porcine small intestine. The small intestine of a newly slaughtered, 1-d-old piglet was cut into 0.5 cm segments, mounted on chucks and instantly frozen in cold isopentane. Cryostat sections (10 μm) were mounted on glass slides and dried. No fixative was used. Sections were stained with periodic acid-Schiff (PAS), counterstained with Mayer's haematoxylin and dehydrated with ethanol. Non-stained sections were incubated for 15 min with fluorescein-conjugated K99 fimbriae (50 μg per section) in PBS. After washing (three times in PBS), the sections were examined in the fluorescence microscope.

Isolation and purification of mucin glycopeptides. Mucosal scrapings from pig small intestine were obtained from the local abattoir. Material collected from about the midpoint to approximately 20 cm from the caecum was immediately treated with acetone [10 ml acetone (g mucosa) $^{-1}$] to remove water and lipids as well as to inhibit degradative enzymes. After stirring overnight, the particulate material was recovered by filtration, suspended in acetone [1.5 ml (g mucosa) $^{-1}$], recovered again and treated with chloroform/methanol (2:1, v/v; 0.65 ml per g wet mucosa). After a final filtration step the organic solvents were removed under a stream of nitrogen.

Dried and delipidated-free material was digested with papain in 0.05 M-sodium phosphate buffer, pH 7.0, containing 5 mM-cysteine-HCl and 5 mM- Na_2EDTA at 65°C for 24 h while shaking. After centrifugation, polyanionic material was precipitated from the supernatant by the addition of cetylpyridinium chloride to a final concentration of about 1 g per 100 ml. The ionic strength of the solution was then decreased by diluting with water until no further precipitation occurred. The precipitate was recovered by centrifugation, dissolved in 2 M-NaCl, precipitated with ethanol (final concentration 80%, v/v), dissolved in water and again precipitated with ethanol. The final material was treated with absolute ethanol followed by diethyl ether, and dried under a stream of nitrogen. The crude preparation of mucin glycopeptides (1 g) was digested with DNAase (2 mg) and RNAase (10 mg) in 100 ml 0.05 M-sodium phosphate buffer, pH 6.5, supplemented with 5 mM- MgCl_2 at 37°C overnight. A few drops of toluene were added to inhibit bacterial growth. After digestion the macromolecules were recovered by dialysis against water followed by lyophilization. In order to remove all oligonucleotides the preparation was chromatographed on Sephadex G75 (Pharmacia) eluted with 0.5 M-sodium acetate adjusted to pH 7 with acetic acid. Material eluting with the void volume of the column was pooled, dialysed against water and freeze-dried.

In order to separate the high- M_r mucin glycopeptides from glycosaminoglycans the material was subjected to isopycnic density-gradient centrifugation in CsCl. A sample (105 mg) was dissolved in water, and CsCl and water were added to a starting density of 1.64 g ml^{-1} in a total volume of 40 ml. Centrifugation was done in an MSE 8×25 angle rotor for 70 h at 36000 r.p.m. and 15°C . Fractions were collected from the bottom of the gradient and analysed for uronic acid (Brown, 1946) and for sialic acid (Jourdain *et al.*, 1971). The mucin glycopeptides were recovered from the top of the gradient (density lower than 1.62 g ml^{-1}), dialysed against water and freeze-dried. The material is referred to below as the total population of high- M_r mucin glycopeptides from this tissue.

The glycopeptides chromatographed as a unimodal although polydisperse peak when subjected to ion-exchange HPLC on a Pharmacia Mono-Q column. The amount of sialic acid was determined as 11.6% (w/w) by the method of Jourdain (1971). The weight-average M_r of the mucin glycopeptides was determined with total intensity light-scattering using a Malvern system 4600 equipped with a 15 mW He/Ne laser essentially as described by Sheehan & Carlstedt (1984).

Desialylation of mucin glycopeptides. Freeze-dried mucin glycopeptides (5 mg) were dissolved in water (5 ml), HCl was added to a final concentration of 0.1 M and the solution was incubated for 1 h at 80°C (Wu & Pigman, 1977). Sodium carbonate solution (1.0 M) was then added dropwise until a pH of about 7 was reached. The hydrolysed mucin glycopeptides were dialysed extensively against water and freeze-dried. The amount of residual sialic acid was determined to be less than 10% of the original amount.

Iodination of mucin glycopeptides. Glycopeptides (50 μl ; 1 mg ml^{-1} in 0.1 M-sodium phosphate buffer, pH 7.4) were mixed with 1 mCi (37 MBq) Na^{125}I (Amersham) and 10 μl chloramine T (2 mg ml^{-1} in 0.1 M-sodium phosphate buffer, pH 7.4) and left for 1 min. Sodium bisulphite ($25 \mu\text{l}$; 2 mg ml^{-1}) and potassium iodide (100 μl ; 2 mg ml^{-1}), both in 0.1 M-sodium phosphate buffer, pH 7.4, were then added. The sample was applied to a Pharmacia PD-10 column equilibrated with PBS and the iodinated glycopeptides, eluting in the void volume, were collected (Hunter, 1978). The specific radioactivity was 1.5×10^6 c.p.m. μg^{-1} . The radiolabelled material co-chromatographed with the unlabelled mucin glycopeptides when subjected to ion-exchange HPLC on a Pharmacia Mono-Q column.

Binding assay. Polystyrene and polypropylene tubes were coated with 5% (w/v) BSA in PBS for 1 h at 20°C , washed with water and dried. Bacterial cells (5×10^9) were mixed with 60 ng iodinated mucin

Fig. 1. (a) Binding of FITC-labelled K99 fimbriae to a cryostat section of pig small intestine. FITC-labelled K99 fimbriae binding to mucin give the space between villi a bright appearance in fluorescence microscopy. Black areas represent villi. Bar, 100 μ m. (b) Cryostat section of pig small intestine. A 10 μ m thin section was PAS-stained and counter-stained with Mayer's haematoxylin. Epithelial cells (E), especially goblet cells (G), are intensely stained by PAS, and condensed mucin (M) appears as dark lines between villi (V). Bar, 100 μ m.

glycopeptides in a total volume of 300 μ l PBS, containing 0.02% sodium azide (4.5 ml polystyrene tubes) at 20 $^{\circ}$ C for the indicated periods of time. After incubation, aliquots (200 μ l) were added to 200 μ l of PBS, already layered, on top of 1.0 ml 10% (w/v) Percoll in PBS (1.5 ml polypropylene tubes). The tubes were centrifuged for 30 min at 2300 g (4 $^{\circ}$ C) and the supernatants discarded. The radioactivity of the resulting pellets (containing the bacteria), were measured in a γ -counter to determine the amount of labelled mucin glycopeptides bound to bacteria. Samples without bacteria, but otherwise subjected to the same procedure, showed that non-specific binding of mucin glycopeptides to the tube wall did not occur. To estimate the dissociation constant and the number of binding sites, bacteria were incubated as above but for 1 h and with increasing amounts (1.0–150 μ g) of unlabelled mucin glycopeptide. All determinations were made in duplicate.

Chemicals. Deoxyribonuclease I (type IV), ribonuclease A (type I-A) *N*-acetylneuraminic acid and FITC were purchased from Sigma. Percoll is a product of Pharmacia. Orcinol, resorcinol and chloramine-T were obtained from Merck. All other chemicals were of analytical grade.

Results and Discussion

When cryostat sections of pig small intestinal mucosa were treated with FITC-labelled K99 fimbriae, a bright fluorescence was found in the spaces between the villi (Fig. 1a). At the same location, mucus was visualized as a thin condensed PAS-stained strand (Fig. 1b).

The oligosaccharide clusters in pig small intestinal mucus glycoproteins were isolated as high- M_r mucin

glycopeptides after papain digestion. Removal of contaminating nucleic acids was achieved with nuclease digestion followed by gel chromatography on Sephadex G75 (results not shown). The mucin glycopeptides were finally separated from glycosaminoglycans by using isopycnic density-gradient centrifugation in CsCl (Fig. 2). The peak in the middle of the gradient appeared at the density expected for hyaluronic acid, whereas material eluting in early fractions represents sulphated glycosaminoglycans. The yield of purified mucin glycopeptides was estimated to be 3.5 mg per g mucosal scraping (wet weight) and the weight-average M_r was determined to be 4.4×10^5 .

Iodinated mucin glycopeptides were found to bind K99 fimbriated *E. coli*. In contrast, when the same bacterial strain was grown at 20 $^{\circ}$ C to suppress fimbrial expression, no radioactivity could be detected in the bacterial pellet. The binding was time-dependent and reached a maximum after about 60 min (Fig. 3). Assuming that equilibrium was reached at this time, bacteria were incubated for 1 h with a constant amount of 125 I-labelled glycopeptide and an increasing amount of unlabelled glycopeptide (Fig. 4). The hyperbolic curve (Fig. 4a) indicated the presence of receptors which were saturable with mucin glycopeptides. The data provided a linear Scatchard plot (Fig. 4b), indicating the presence of only one class of binding site (Scatchard, 1949). This

Fig. 2. Isopycnic density-gradient centrifugation of high- M_r mucin glycopeptides and glycosaminoglycans from pig small intestinal mucosa. For full experimental detail, see the text. After centrifugation, fractions were collected from the bottom of the tubes and analysed for uronic acid (●), sialic acid (○) and density (□). Fractions containing high- M_r mucin glycopeptides were pooled as indicated by the horizontal bar.

Fig. 3. Kinetics of binding of ^{125}I -labelled mucin glycopeptides to K99-fimbriated *E. coli*. Bacteria were incubated with 60 ng labelled glycopeptides (94×10^3 c.p.m.) for the indicated periods of time. The data (means of three experiments) refer to 300 μl samples containing 5×10^9 bacteria. Radioactivity representing bound glycopeptides was measured after centrifugation.

implies that K99 is the only adhesin present which specifically binds mucin glycopeptides. The number of binding sites, 2.3×10^3 per fimbriated cell (range 1.7 – 3.0×10^3 ; four experiments) was calculated by using the intercept on the abscissa as the maximum amount of

Fig. 4. (a) Saturability of binding of ^{125}I -labelled mucin glycopeptides to K99-fimbriated *E. coli*. Bacteria (5×10^9) were incubated with 60 ng labelled glycopeptide (94×10^3 c.p.m.) and with unlabelled glycopeptides as indicated. The amount of glycopeptide bound was calculated from the radioactivity associated with bacteria by using the specific radioactivity of mucin glycopeptides in each sample. The data displayed originate from one experiment which is representative of four determinations. (b) Scatchard plot analysis of the data in (a). Binding data were fitted by linear regression using least-squares analysis. The number of binding sites was calculated by using the intercept of the abscissa as the amount of glycopeptide bound at saturation.

glycopeptide bound and the number of fimbriated bacteria (i.e. adsorbed to Octyl-Sepharose), which was found to be 85% of 5×10^9 . From the slope (Fig. 4b), the dissociation constant was calculated to be 6×10^{-7} M (range 2 – 10×10^{-7} M; four experiments). However, only 11% of the glycopeptides bound to the bacteria at the highest bacteria/mucin ratio that could be studied (Fig. 3), possibly reflecting the lack of receptors on the remaining glycopeptides. Alternatively, multivalent binding between the mucin glycopeptides and the fimbriated bacteria could occur at low concentration of glycopeptides, which would impair binding of glycopeptides owing to steric hindrance. The dissociation constant for the bacteria-mucus interaction, *in vivo*, may be lower than our estimate, since the concentration of glycopeptides usually exceeds the concentration of binding sites (8×10^{-8} M) when unlabelled glycopeptides

are added. Multivalent binding, which is likely to occur *in vivo*, will decrease the dissociation constant and enable the bacteria to adhere firmly to mucus. Desialylation of mucin glycopeptides reduced their capacity to bind to bacteria. When bacteria were incubated with 60 ng of desialylated glycopeptides, only 0.3 ng was found to bind. This is a 95% reduction of binding as compared to the untreated glycopeptides (6.6 ng bound). Previous investigations have shown that K99 fimbriae mediate haemagglutination by specifically interacting with sialic acid (Lindahl *et al.*, 1987). Our results confirm the role of this sugar as the K99-receptor in the mucosa of the pig small intestine.

It was recently shown that sialylated glycolipids exist in the small intestine mucosa of pigs (Kyogashima *et al.*, 1989). However, the total amount of sialic acid residues of sialoglycolipids was about 11 nmol (g wet weight mucosa)⁻¹. In contrast, our data show that mucin-bound sialic acid [11.6 g (100 g mucin glycopeptide)⁻¹] would correspond to 1300 nmol (g wet weight mucosa)⁻¹ (see above). Consequently, mucin contains 100 times more binding sites for K99 fimbriae than the membrane-bound sialoglycolipids of pig small intestine.

Studies to reveal receptors for pathogenic *E. coli*, usually by using haemagglutination, have contributed to the knowledge of specific adhesion of these bacteria (Evans *et al.*, 1979; Lindahl *et al.*, 1987; Parkkinen *et al.*, 1983). The use of receptors or receptor analogues as prophylaxis or treatment of bacterial infections has, however, not yet been successful (Aronson *et al.*, 1979; Svanborg-Edén *et al.*, 1982). This may be due to lack of detailed information on the interaction between the bacteria and the epithelium, for example the strength of the bacterial affinity for the epithelium. The mucus blanket has, at least in cholera infection, been regarded as a protective coat which must be penetrated or circumvented by the bacteria (Jones *et al.*, 1976). However, findings suggesting adhesion of other bacteria to mucins from, for example, saliva and tracheal mucus (Murray *et al.*, 1982; Ramphal & Pyle, 1983), have been reported. The binding of fimbriated ETEC to crude mucus preparations from both mice and calves has been studied (Laux *et al.*, 1984; Mouricout & Julien, 1987) and K99-fimbriated ETEC has been found to bind specifically to calf small intestinal mucus (Mouricout & Julien, 1987). Such preparations may, however, contain a variety of components, such as glycolipids, DNA, hyaluronic acid and membrane glycoproteins, which could interact with the bacteria both specifically and unspecifically.

Mucin oligosaccharides are *O*-glycosidically linked to the protein core of the macromolecule, whereas both *O*-glycosidically and *N*-glycosidically linked glycans may be found on cell surface glycoproteins (Wagh & Bahl,

1981). Furthermore, the carbohydrate content of mucins (80%, w/w) is much higher than in most other glycoproteins (Mantle & Allen, 1981), which implies that the receptor density on cell membranes could differ substantially from that of mucus. The receptor density is an important factor for the adhesion of both eukaryotic and prokaryotic cells (Hansson *et al.*, 1985; Weigel *et al.*, 1979).

Histochemical studies have revealed the presence of *O*-acetylated sialic acids in intestinal mucus and it has been proposed that adult man has a higher degree of *O*-acetylation than infants (Culling *et al.*, 1977). The degree of sialic acid *O*-acetylation has been shown to influence the interaction between K99 fimbriae and this sugar, which might explain why only the very youngest animals are sensitive to infection of K99-fimbriated ETEC. Furthermore, K99-fimbriated cells have been shown to adhere preferentially to enterocytes from neonatal animals (Runnels *et al.*, 1980), possibly reflecting an age-dependent decrease in receptor density of porcine enterocytes. A decreased receptor density has been shown to have a dramatic effect on adhesion of cells to a receptor-coated surface (Weigel *et al.*, 1979). However, the results obtained in our assay are not expected to be affected by receptor density, as mentioned above, since the ligands (i.e. mucin glycopeptides) are soluble. The question whether bacterial adhesion to epithelial cells does occur and to what extent it is relevant to the disease cannot be answered here. However, the specific interaction between K99-fimbriated enterotoxigenic *E. coli* and sialic acid residues in the mucus layer has been demonstrated. The location of mucus, a carbohydrate rich gel covering the epithelial cells, may initially prevent bacteria from coming in close contact with the epithelial cell membrane. However, the presence of receptor structures for bacterial adhesins, in the mucus layer, provide excellent conditions for initiation of colonization and the development of ETEC neonatal diarrhoea.

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