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-M₉ 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 $6 \times 10^{-7}$ M. Fimbriated bacteria were calculated to possess $2.3 \times 10^3$ 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.,...
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In order to separate the high-M, mucin glycopeptides from glycans 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 1.46 g ml⁻¹ in a total volume of 40 ml. Centrifugation was done in an MSE 8 × 25 cm 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 ionic 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, mucin glycopeptides from this tissue.

The glycopeptides were dialysed against 5% (w/v) BSA in PBS for 1 h at 20°C, washed with water and 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 µCi (37 MBq) Na¹²⁵I (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 µl; 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 × 10⁶ 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 × 10⁹) were mixed with 60 ng iodinated mucin glycopeptides. 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-Na₂EDTA at 65°C for 24 h while shaking. After centrifugation, polyanionic material was precipitated from the supernatant by the addition of cetlypyridinium chloride to a final concentration of about 1 g per 100 ml. The ion 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-MgCl₂ 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.

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Binding of K99 to pig intestinal mucosa

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 μm. (b) Cryostat section of pig small intestine. A 10 μm 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 μm.

glycopeptides in a total volume of 300 μl PBS, containing 0.02% sodium azide (4-5 ml polystyrene tubes) at 20 °C for the indicated periods of time. After incubation, aliquots (200 μl) were added to 200 μl 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 °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 pg) 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. 1 a). At the same location, mucus was visualized as a thin condensed PAS-stained strand (Fig. 1 b).

The oligosaccharide clusters in pig small intestinal mucus glycoproteins were isolated as high-M, 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, was determined to be 4.4 × 10⁵.

Iodinated mucin glycopeptides were found to bind K99 fimbriated E. coli. In contrast, when the same bacterial strain was grown at 20 °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 ¹²⁵I-labelled glycopeptide and an increasing amount of unlabelled glycopeptide (Fig. 4). The hyperbolic curve (Fig. 4 a) indicated the presence of receptors which were saturable with mucin glycopeptides. The data provided a linear Scatchard plot (Fig. 4 b), indicating the presence of only one class of binding site (Scatchard, 1949). This
Fig. 2. Isopycnic density-gradient centrifugation of high-Μ, 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-Μ, mucin glycopeptides were pooled as indicated by the horizontal bar.

Fig. 3. Kinetics of binding of 125I-labelled mucin glycopeptides to K99-fimbriated E. coli. Bacteria were incubated with 60 ng labelled glycoproteins (94 x 10^3 c.p.m.) for the indicated periods of time. The data (means of three experiments) refer to 300 µl samples containing 5 x 10^8 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 x 10^3 per fimbriated cell (range 1.7–3.0 x 10^3; four experiments) was calculated by using the intercept on the abscissa as the maximum amount of glycopeptide bound and the number of fimbriated bacteria (i.e. adsorbed to Octyl-Sepharose), which was found to be 85% of 5 x 10^9. From the slope (Fig. 4b), the dissociation constant was calculated to be 6 x 10^-7 M (range 2–10 x 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 x 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 mucus 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 [1.6 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., 1983; 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 enteroctyes. 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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References


