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Enterotoxigenic *Escherichia coli* (K88) induce proinflammatory responses in porcine intestinal epithelial cells

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ABSTRACT

Infections with F4$^+$ enterotoxigenic *Escherichia coli* (ETEC) causes severe diarrhoea in piglets, resulting in morbidity and mortality. F4 fimbriae are the key virulence factors mediating the attachment of F4$^+$ ETEC to the intestinal epithelium. Intestinal epithelial cells (IEC) are recently being recognized as important regulators of the intestinal immune system through the secretion of cytokines, however, data on how F4$^+$ ETEC affect this cytokine secretion are scarce. By using ETEC strains expressing either polymeric, monomeric or F4 fimbriae with a reduced polymeric stability, we demonstrated that polymeric fimbriae are essential for adhesion to porcine IEC and the secretion of IL-6 and IL-8 by IEC. Remarkably, this cytokine secretion was not abrogated following stimulation with an F4-negative strain. Since this strain expresses flagellin, TLR5 mediated signalling could be involved. Indeed, porcine IEC express TLR5 and purified flagellin induced IL-6 and IL-8 secretion, indicating that, as for other pathogens, flagellin is the dominant virulence factor involved in the induction of proinflammatory responses in IEC. These results indicate a potential mucosal adjuvant capacity of ETEC-derived flagellin and may improve rational vaccine design against F4$^+$ ETEC infections.

1. Introduction

Enterotoxigenic *Escherichia coli* (ETEC) are an important cause of diarrhoea in man and animals. Mainly children in and travellers to developing countries are affected by ETEC-induced diarrhoea (Clarke, 2001; Ratchtrachenchai et al., 2004; Qadri et al., 2005). In neonatal and recently weaned piglets, ETEC-associated diarrhoea results in morbidity and mortality (Gyles, 1994) and is considered as one of the economically most important diseases in swine husbandry (Chen et al., 2004; Frydendahl, 2002; Van den Broeck et al., 1999c). ETEC express long, proteinaceous appendages or fimbriae on their surface, which mediate adhesion to the gut epithelium. Porcine ETEC strains isolated from diarrheic pigs express 5 different fimbriae of which F4 and F18 fimbriae are the most prevalent (Fairbrother et al., 2005). F4 fimbriae are composed of a major structural subunit, FaeG, and some additional minor subunits. In contrast to most fimbriae, where the adhesin is located at the tip of the fimbriae, the major subunit FaeG also functions as the adhesin (Bakker et al., 1992). Attachment of F4$^+$ ETEC to the host epithelial cells is mediated by an interaction of F4 fimbriae with F4-specific receptors (F4R) present on the brush borders of the small intestinal enterocytes, enabling colonization of the small intestine (Van den Broeck et al., 1999b). Subsequently, heat-labile (LT) and heat-stable (STa/b) enterotoxins are secreted, which induce severe diarrhoea. Since F4 fimbriae are a key virulence factor involved in mediating attachment, they are an important target in vaccination studies against F4$^+$ ETEC (Cox et al., 2002). Indeed, oral immunization of F4R$^+$ piglets with purified F4 fimbriae induces an F4-specific intestinal immune response, which protects them against a subsequent ETEC challenge (Van den Broeck et al., 1999a; Verdonck et al., 2004a). Furthermore, the presence of the F4R is a prerequisite for the successful immunization of piglets, indicating that receptor-mediated binding is important for the induction of a protective intestinal immunity (Van den Broeck et al., 1999a). The strong immunogenicity of F4 fimbriae can be explained by their resistance to digestive enzymes, their pH stability and their polymeric nature (Snoeck et al., 2004; Verdonck et al., 2008). Indeed, oral immunization with F4 fimbriae purified from F4$^+$ ETEC mutants, in which the polymeric stability of the fimbriae is disrupted, resulted in reduced mucosal immune responses (Joensuu et al., 2006; Verdonck et al., 2008).

Intestinal epithelial cells (IEC) are pivotal for the activation of innate immunity and subsequently for the induction of adaptive immune responses (Sansonetti, 2004). IEC function as sensors detecting pathogen-associated molecular patterns (PAMPs) through pathogen-recognition receptors (PRRs), such as Toll-like receptors (TLRs). Upon recognition of these PAMPs, IEC secrete

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Table 1
Overview of the different bacterial strains.

Bacterial strains	Genotype of phenotype	Reference
GIS26	Wild-type F4$^+$ ETEC reference strain (O149:F4ac$^+$LT$^+$STa$^+$STb$^+$)	Verdonck et al. (2004b)
IMM01	Wild-type F4$^+$ ETEC strain which lacks flagellin expression (O147:F4ac$^+$LT$^+$STb$^+$)	Verdonck et al. (2004b)
5/95[a]	Wild-type F4$^+$ ETEC strain which also lacks flagellin expression (O149:F4ac$^+$LT$^+$STb$^+$)	Verdonck et al. (2004b)
GIS26FaeG	F4 deficient mutant GIS26 strain (FaeG::Cm)	Verdonck et al. (2008)
pHMM02[b]	GIS26ΔFaeG transformed with pHMM02 to complement F4 fimbrial synthesis	Verdonck et al. (2008)
HB101[c]	*E. coli* K12 laboratory strain	

[a] Strain 5/95 is a Finnish field isolate and was kindly provided by Dr. J. Joensuu.
[b] Two mutations were inserted into the faeG gene, resulting in a reduced stability of the F4 fimbrial structure.
[c] Genotype: *supE*44, Δ(*mrcC-mrr*), *recA*13, *ara*-14, *proA*2, *lacY*1, *galK*2, *rpsL*20, *xyl*-5, *mtl*-1, *leuB*6, *thi*-1.

several cytokines and chemokines, thereby alerting the underlying mucosal immune cells, such as dendritic cells, to trigger innate immune defences and promote adaptive immune responses (Kagnoff and Eckmann, 1997; Neutra and Kozlowski, 2006). However, studies on the influence of F4$^+$ ETEC on the innate immune functions of IEC are limited. This incited us to elucidate how the polymeric nature of F4 fimbriae influences bacterial adhesion to porcine IEC and subsequently, the cytokine secretion profile of IEC in an *in vitro* IPEC-J2 culture system. IPEC-J2 cells provide a relevant model for intestinal epithelial cells since they form apical microvilli, express tight junction proteins, produce glycocalyx bound mucins and glycoproteins for bacterial adhesins, and are known to express cytokines and chemokines after bacterial stimulation (Burkey et al., 2006; Schierack et al., 2006; Skjolaas et al., 2007). In addition, F4$^+$ ETEC can bind to IPEC-J2 (Koh et al., 2008; Johnson et al., 2009). Moreover, the IPEC-J2 cell line was derived from the porcine jejunum and the jejunal Peyer's patches are the major inductive site for F4$^+$ ETEC specific immune responses (Snoeck et al., 2006).

2. Materials and methods

2.1. Bacterial strains

The bacterial strains used in this study are listed in Table 1. GIS26, IMM01 and 5/95 are all wild type F4$^+$ ETEC strains. The amino acid sequences of the FaeG subunit of GIS26 and IMM01 are 100%, while the FaeG amino acid (AA) sequence differs at 7 positions between the GIS26 and the 5/95 strain. Two of these AA are specific for the 5/95 strain, while the other different AA are also found in other ETEC isolates (Verdonck et al., 2004b). The GIS26ΔfaeG strain was generated by inserting a chloramphenicol (Cm) resistance gene into the faeG gene (faeG::Cm), resulting in a defective F4 fimbrial biosynthesis. In the pHMM02 strain, F4 fimbrial biosynthesis was restored through the introduction of an expression vector containing the faeG gene. However, two mutations were inserted in this sequence, substituting the wild type AA by the 5/95 strain-specific AA (Verdonck et al., 2008).

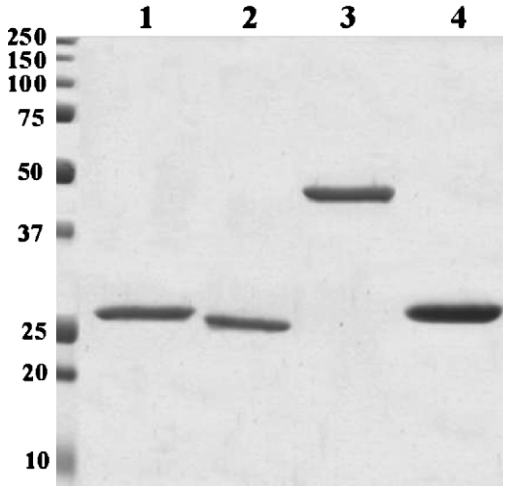

Fig. 1. Analysis of the purity of F4 fimbriae and flagellin preparations. Heat-denatured samples (3 μg total protein) were loaded on a 12% SDS-PAGE in sample buffer. The migrating bands were visualized with Coomassie staining. The molecular weight marker (kDa) is shown on the left. Lanes: (1) $F4_{IMM01}$, (2) $F4_{5/95}$, (3) flagellin, and (4) $F4_{pHMM02}$.

2.2. Agglutination test

The agglutination assay was carried out as previously described (Hu et al., 2009). Briefly, the bacterial strains were grown in Brain Heart Infusion (BHI; Oxoid, Hampshire, UK) overnight at 37 °C, 200 rpm and diluted 1/2 in PBS. Next, 10 μl of the bacterial suspension was applied on a glass side, after which 10 μl of the FaeG-specific mAbs IMM01 and IMM09, generated at our laboratory, were added and mixed. IMM01 detects both FaeG monomers and polymers, whereas IMM09 only detects FaeG polymers. Visible agglutination of the bacteria after 5 min incubation was considered as positive.

2.3. Purification of ETEC virulence factors

F4 fimbriae were purified from the *E. coli* strain IMM01 ($F4_{IMM01}$), 5/95 ($F4_{5/95}$) and pHMM02 ($F4_{pHMM02}$) as previously described (Van den Broeck et al., 1999a; Verdonck et al., 2008). Briefly, bacteria were grown in Tryptone Soya Broth (TSB; Oxoid) for 18 h at 37 °C and 85 rpm. Subsequently, the F4 fimbriae were isolated from the bacteria by mechanical shearing. After precipitation through the addition of ammonium sulphate (40%, w/v), the fimbrial proteins were dialyzed, filtrated and stored at −20 °C. Flagellin was isolated from strain GIS26ΔfaeG using the same protocol as to purify F4 fimbriae (Verdonck et al., 2008). The purity of the isolated fimbriae and flagellin was assessed by SDS-PAGE and Coomassie staining (Fig. 1).

To confirm binding of the purified $F4_{IMM01}$ fimbriae to IPEC-J2 cells, these fimbriae were conjugated with 5(6)-carboxyfluorescein-*N*-hydroxysuccinimide ester (FluoS, 480 Da) using the fluorescein labelling kit (Roche Diagnostics, Basel, Switzerland). Confluent IPEC-J2 cells were dislodged with sterile PBS + 1 mM EDTA (PBS/EDTA) and 5.0×10^5 cells were incubated at 4 °C for 30 min with 0, 5 and 25 μg $F4_{IMM01}$-FluoS. After removal of unbound $F4_{IMM01}$-FluoS by washing, data were acquired on a FACSCanto flow cytometer with a minimum event count of 30,000 and analysed with FACSDiva® software (Becton Dickinson, Erembodegem, Belgium).

2.4. Cell lines and culture conditions

The IPEC-J2 cell line is a non-transformed intestinal epithelial cell line derived from the jejunal epithelium of a neonatal, unsuckled piglet. The cell line is maintained as a continuous culture