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Cholera toxin improves the F4(K88)-specific immune response following oral immunization of pigs with recombinant FaeG

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Abstract

Oral immunization of both humans and animals with non-replicating soluble antigens often results in the induction of oral tolerance. However, receptor-dependent uptake of orally administered soluble antigens can lead to the induction of an antigen-specific immune response. Indeed, oral immunization of pigs with recombinant FaeG (rFaeG), the adhesin of the F4(K88) fimbriae of enterotoxigenic *Escherichia coli* (ETEC), induces an F4-specific humoral and cellular immune response. This response is accompanied with a reduction in the excretion of F4+ *E. coli* following challenge. To improve the immune response against F4, rFaeG was orally co-administered with the mucosal adjuvant cholera toxin (CT). Oral immunization of pigs with rFaeG and CT significantly improved the induction of an F4-specific humoral and cellular immune response and also significantly reduced the faecal F4+ *E. coli* excretion following F4+ ETEC challenge as compared to rFaeG-immunized pigs. Therefore, the present study demonstrates that CT can act in pigs as a mucosal adjuvant for antigens that bind to the intestinal epithelium by a CT-receptor-independent mechanism.
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Keywords: F4(K88) fimbriae; FaeG adhesin; Cholera toxin; Pig; Enterotoxigenic *Escherichia coli*

1. Introduction

F4+ enterotoxigenic *Escherichia coli* (ETEC) induced post-weaning diarrhoea in pigs is a cause of important economical losses. F4 fimbriae consist of several minor subunits and a repeating major subunit FaeG that also constitutes the adhesin (Verdonck et al., 2004b), allowing the bacteria to adhere to F4-receptors (F4R) on the brush border of small intestinal enterocytes and subsequently colonize the intestine (Gyles, 1994). In addition, these strains secrete heat-labile (LT), heat-stable a (STa) and/or heat-labile b

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(STb) enterotoxins that induce the loss of electrolytes and water, resulting in a secretory diarrhoea (Gyles, 1994).

The induction of an antigen-specific mucosal antibody response is needed to protect both human and animals against an intestinal infection (Porter et al., 1974; Bloom and Boedeker, 1996). However, oral administration of most non-replicating antigens (e.g. food antigens) results in oral tolerance (Strobel and Mowat, 1998). One of the exceptions is the oral immunization of F4-receptor positive ($F4R^+$) pigs with F4 fimbriae, which induces a protective F4-specific intestinal antibody response (Van den Broeck et al., 1999a). In $F4R^-$ pigs, F4 fimbriae act as a normal food antigen (Van den Broeck et al., 2002). We recently demonstrated that oral immunization of pigs with recombinantly produced F4 fimbrial adhesin FaeG (rFaeG) induced a systemic and mucosal FaeG-specific immune response (Verdonck et al., 2004a). However, the FaeG-specific response was weaker following oral immunization with rFaeG than with purified F4, resulting in a decreased protection against an $F4^+$ ETEC challenge.

The interest of the present study was to improve the FaeG-specific immune response following co-administration of rFaeG with the mucosal adjuvant cholera toxin (CT) (Ogra et al., 2001). In mice, CT enhances co-stimulation (Cong et al., 1997) and promotes Th2 cytokine responses with induction of antigen-specific serum IgG and mucosal IgA (Marinaro et al., 1995). In pigs, the oral administration of CT is non-toxic at an oral dose of 100 μg (Foss and Murtaugh, 1999). Moreover, Foss and Murtaugh (1999) reported that CT could be used as a mucosal adjuvant in pigs since CT enhanced the induction of an antigen-specific immune response to co-administered CT-B or an antigen that was targeted to the gut-associated lymphoid tissue by coupling to CT-B. However, there is no information whether oral co-administration of an antigen and CT, without conjugation of the antigen to CT or its B-subunit, improves the antigen-specific immune response in pigs.

In the present study, it was determined if the use of CT could improve the induction of an immune response against FaeG following oral co-administration of newly weaned pigs with rFaeG.

2. Materials and methods

2.1. Bacterial inoculum

The ETEC strain GIS 26 (serotype O149:K91, $F4ac^+$, $LT^+STa^+STb^+$) was cultured during 18 h in Tryptone Soya Broth (Oxoid, Basingstoke, Hampshire, England) at 37 °C and 85 rpm. The bacteria were collected by centrifugation and washed with phosphate-buffered saline (PBS) (150 mM, pH 7.4). The concentration of the bacteria was determined by measuring the optical density of 10-fold dilutions of the bacterial suspension at 660 nm (OD_{660}). An OD_{660} of 1 equals 10^9 viable bacteria/ml, as determined by counting colony-forming units.

2.2. Purification of F4 fimbriae

F4 fimbriae were purified as described by Van den Broeck et al. (1999b). These fimbriae were used in the F4-specific antibody ELISA. Isolated F4 fimbriae were also further purified by anion exchange chromatography (AEC) using a Bio-Scale Q5 column (BIORAD, Eke, Belgium) (indicated as AEC-purified F4), subsequently sterilised by filtration through a 0.2 μm filter and used to induce F4-specific proliferation. The F4 fimbrial protein concentration was calculated from the total protein concentration as determined using the bicinchoninic acid (BCA) reaction with bovine serum albumin (BSA) as a standard (ICN Biomedicals, Belgium) and taking into account the percentage of F4 fimbriae on the total protein concentration. This percent purity of the isolated F4 and the AEC-purified F4 fimbriae was assessed using a Coomassie stained 15% SDS-PAGE and the ImageMaster 1D prime software (Amersham Pharmacia Biotech, Belgium).

2.3. rFaeG expression and refolding

Recombinant FaeG (rFaeG) containing a N-terminal fused His- and S-tag was expressed and refolded as described by Verdonck et al. (2004a). Briefly, BL21(DE3)[pETFaeG7] *E. coli* were grown overnight in Luria broth (LB; Life Technologies, Paisley, Scotland) with 30 μg/ml kanamycin at 28 °C. Afterwards, the bacteria were diluted 100 times in fresh LB supplemented with kanamycin and incubated

at 28 °C, 200 rpm to an OD_{660} of 0.2–0.3. rFaeG expression was subsequently induced by adding 1 mM isopropyl-β-D-thiogalactoside (IPTG, Sigma) to the cultures, which were further incubated during 4 h at 37 °C and 200 rpm. Subsequently, the insoluble cytoplasmatic fraction was isolated and resuspended in PBS + 0.5% SDS. Following overnight dialysis against PBS at 4 °C, the concentration of rFaeG was determined using the BCA-reaction and used to immunize animals.

2.4. Experimental procedure

Twenty-one, $F4R^+$ and F4-seronegative, conventionally bred pigs (Belgian Landrace × Piétrain) were weaned at the age of 4 weeks, transported to the faculty and subsequently housed in isolation units were they obtained water and food ad libitum. These pigs were treated orally with colistine (Promycine pulvis, VMD, Berendonk, Belgium, 150,000 U/kg of body weight/day) from 2 days before till 3 days after weaning to prevent *E. coli* infections due to transport and handling.

One week post-weaning, all pigs were orally given 20 mg rabeprazolum (Pariet, Janssen-Cilag, Berchem, Belgium) on three subsequent days to block the gastric HCl production. They also received rabeprazolum 15 days post-primary immunization (dppi). Twenty-four hours following each rabeprazolum ingestion (0, 1, 2 and 16 dppi), the pigs were orally immunized with 25 μg CT (CT group, $n = 7$), 4 mg rFaeG (rFaeG group, $n = 7$) or 4 mg rFaeG + 25 μg CT (rFaeG + CT group, $n = 7$) in 10 ml PBS. Each animal was deprived of food and water from three hours before till 2 h after gastric pH neutralization or immunization. One week following the booster immunization (23 dppi), the F4-specific proliferation of peripheral blood monomorphonuclear cells (PBMC) was determined.

At 24 dppi, the animals were orally challenged with the virulent $F4^+$ ETEC strain GIS26 as previously described (Cox et al., 1991) with minor modifications. Briefly, pigs were orally pre-treated at 21 and 22 dppi with 300 mg florfenicol (Nuflor, Schering-Plough, Brussels, Belgium) to decrease colonization resistance. Pigs were sedated with Stressnil (40 mg/ml; Janssen-Cilag, Berchem, Belgium), after which the gastric pH was neutralized by intragastrical administration of 62 ml $NaHCO_3$ (1.4% (w/v) in distilled water). Fifteen to 30 min later, 10^{10} $F4^+$ ETEC (GIS26) in 10 ml PBS was given intragastrically. Faecal samples were taken daily to determine the excretion of $F4^+$ *E. coli* from challenge till 8 days post-challenge (dpc, 31 dppi). Furthermore, F4-specific IgA, IgG and IgM and CT-specific (total) antibodies were determined in serum at 0, 7, 16, 24, 28, 31, 38 and 49 dppi, whereas F4-specific IgA was analysed in saliva at 0, 16 and 24 dppi. Three weeks following challenge (49 dppi), the remaining pigs were euthanatised and jejunal villi were isolated to confirm the presence of the F4R.

All pigs were weighed at 0, 3, 7, 15, 24, 31 and 49 dppi. The daily weight gain of each pig was calculated at 3, 7, 15, 24, 31 and 49 dppi by subtracting the weight of two subsequent measurements, and dividing the difference by the number of days between both measurements. Subsequently, the average daily weight gain (ADWG) per group was calculated ± standard error of the mean (S.E.M.).

2.5. Samples

To determine antigen-specific serum and mucosal antibodies, serum and saliva were sampled as described by Van den Broeck et al. (1999c) and Van der Stede et al. (2002), respectively.

$F4^+$ *E. coli* were enumerated in faecal samples by dot blotting using the F4-specific MAb IMM01 as previously described (Van den Broeck et al., 1999b). The resulting brown-red dots were counted and the average within each group was calculated. Results are presented as the mean number ± S.E.M. of excreted $F4^+$ *E. coli* per gram faeces.

At the end of the experiment, jejunal villi were isolated from all euthanatised pigs to confirm the presence of the F4R as described by Van den Broeck et al. (1999b). Adhesion of more than five $F4^+$ *E. coli* per 250 μm villous length was noted as positive (Cox and Houvenaghel, 1993).

2.6. ELISA for F4- and CT-specific antibodies

For detection of F4-specific antibodies, the indirect ELISA described by Van den Broeck et al. (1999a) was used. An identical ELISA which only differed in the coating step and conjugate was used to determine CT-specific antibodies: the wells of

microtiter plates (NUNC, Polysorp Immuno Plates, Life Technologies, Roskilde, Denmark) were coated with 5 μg/ml CT (Sigma) in PBS and an optimal dilution of horseradish peroxidase conjugated rabbit polyclonal antibodies (Dako, Denmark) was used as conjugate. The F4- and CT-specific cut-off values were calculated as the mean OD_{405}-value of all sera (dilution 1/10) or saliva (dilution 1/2) samples at day 0, increased with three times the standard deviation (cut-off values of the F4-specific serum IgM, IgA, IgG and mucosal IgA and CT-specific ELISA were 0.32, 0.17, 0.31, 0.28 and 0.21, respectively). The antibody titer was the inverse of the highest dilution that still had an OD_{405} higher than the calculated cut-off value.

2.7. F4-specific proliferation

Blood was collected from the jugular vein and PBMC were isolated as described by Van den Broeck et al. (1999c). The PBMC were diluted to a concentration of 5×10^6 cells/ml in leukocyte medium (RPMI-1640 supplemented with penicillin (100 IU/ml), streptomycin (100 μg/ml), kanamycin (100 μg/ml), L-glutamin (200 mM), sodiumpyruvate (100 mM), non-essential amino acids (100 mM), β-mercaptoethanol (5×10^{-5} M) and 5% (v/v) F4-seronegative serum). Subsequently, the isolated PBMC were incubated in medium (negative control) or medium containing purified F4 (final concentration of 10 μg/ml), or concanavalin A (final concentration of 10 μg/ml, positive control) to determine their F4-specific proliferation as described by Van der Stede et al. (2003). The results are presented using the F4-specific stimulation index (SI) (±standard deviation), obtained by dividing the mean counts per minute (cpm) of the F4-stimulated cultures by the mean cpm of the non-stimulated cultures. Mean background levels of about 700 cpm were obtained in medium samples, whereas positive controls of concanavalin A reached 170,000 cpm.

2.8. Statistical analysis

Statistical analysis (SPSS 10.0 for Windows) of antibody titers and $F4^+$ *E. coli* excretion was done using General Linear Model (Repeated Measures Analysis of Variance). Differences between groups in F4-specific cell proliferation and ADWG were analysed for statistical significance using the one-way ANOVA. $P < 0.05$ was considered as statistically significant.

3. Results

3.1. F4-specific serum antibody response following oral immunization

Oral immunizations of newly weaned pigs with rFaeG (rFaeG group) induced F4-specific serum IgM antibodies (mean log2 titer 3.88) that were significantly higher 7 dppi as compared to the background value in animals immunized with CT (CT group; mean log2 titer 3.40; $P = 0.002$) (Fig. 1). However, oral immunization of pigs with rFaeG in the presence of CT (rFaeG + CT group; mean log2 titer 4.69) resulted in significantly higher F4-specific IgM serum titers than in the rFaeG and CT (16 dppi; $P = 0.004$) groups. The oral booster immunization 16 dppi induced low F4-specific serum IgA and IgG titers in the rFaeG group, whereas the F4-specific IgM titer was significantly higher (mean log2 titer 4.20; $P = 0.011$) 24 dppi as compared to the background value in the animals immunized with CT alone (mean log2 titer 3.5). The highest F4-specific serum antibody titers following booster immunization were obtained in the rFaeG + CT group, with significantly higher titers than in the rFaeG group (IgG; $P = 0.038$) and the CT group (IgM, IgA, IgG; $P \leq 0.024$) 24 dppi.

3.2. CT-specific serum antibody response following oral immunization

The use of CT in the oral immunizations induced a total CT-specific systemic immune response, whereas no CT-specific serum antibodies were detected in the non-CT immunized rFaeG group (Fig. 2). One week following booster immunization (24 dppi), the CT-specific antibody titer of both CT immunized groups (mean log2 titers of 4.98 and 5.32) was significantly higher as compared to the rFaeG group (mean log2 titer 3.32; $P \leq 0.016$). On the other hand, the dose of CT used, did not result in diarrhoea (data not shown) or growth retardation (Fig. 3) of the pigs.

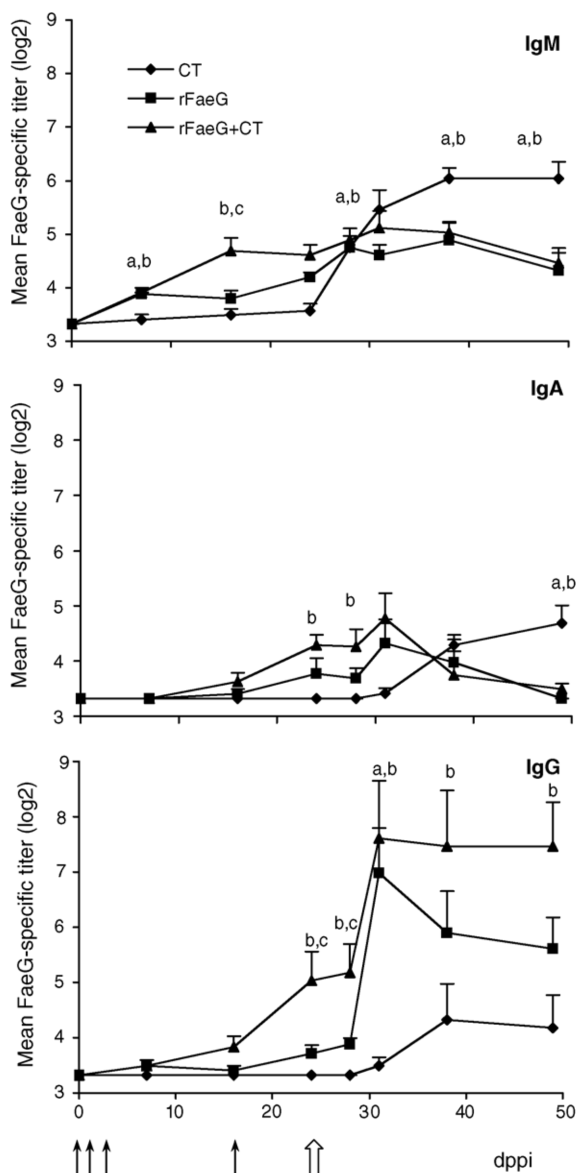

Fig. 1. Mean F4-specific IgM, IgA and IgG serum antibody titers (±S.E.M.) at 0, 7, 16, 24, 28, 31, 38 and 49 days post-primary immunization (dppi) of pigs orally immunized with cholera toxin (CT, $n = 7$), rFaeG ($n = 7$) or rFaeG and cholera toxin (rFaeG + CT, $n = 7$). Significant difference ($P < 0.05$) between CT and rFaeG (a), between CT and rFaeG + CT (b), between rFaeG and rFaeG + CT (c). Black arrow: immunization; white arrow: challenge.

3.3. F4-specific cell proliferation following oral immunization

Oral immunization of pigs with plain rFaeG induced an F4-specific cell proliferation (mean SI 3 ± 1.8) that was significantly higher as compared to the CT immunized pigs (mean SI 1.2 ± 1.8; $P = 0.021$). The observed adjuvanticity of CT on the F4-specific immune response to co-administered rFaeG was not restricted to the antibody response. A significantly higher F4-specific cell proliferation was observed in the rFaeG + CT group (mean SI 4.6 ± 1.18) as compared to both other groups 24 dppi ($P \leq 0.036$).

3.4. F4-specific mucosal antibody response following oral immunization

Oral rFaeG immunization of newly weaned pigs resulted in the secretion of very low amounts of F4-specific antibodies in saliva. In the rFaeG group, an insignificant increase in F4-specific IgA was seen from 16 to 24 dppi (log2 titer 1.08 and 1.25, respectively). In the rFaeG + CT group, significantly higher F4-specific IgA responses were found as compared to both other groups both 16 and 24 dppi (log2 titer 1.33 and 1.74, respectively, $P \leq 0.047$).

3.5. $F4^+$ E. coli excretion following challenge

To determine if the induced F4-specific immune response was able to protect against an $F4^+$ ETEC infection, the pigs were challenged 24 dppi. Daily enumeration of the faecal $F4^+$ *E. coli* excretion (Fig. 4) revealed $>10^7$ bacteria per gram faeces in the CT group till 4 days post-challenge (dpc), whereafter the excretion gradually decreased till 8 dpc. The excretion of the rFaeG-immunized animals was similar to that of the CT group until 2 dpc (more than 10^7 $F4^+$ *E. coli* per g faeces), after which it decreased faster to become significantly lower from 4 till 7 dpc ($P \leq 0.016$). However, animals immunized with rFaeG in the presence of CT already showed a significantly lower $F4^+$ *E. coli* excretion than the CT group as well as the rFaeG group from day 1 post-challenge onwards until 7 and 6 dpc, respectively ($P \leq 0.004$). Despite these differences in $F4^+$ *E. coli* excretion, no significant differences were observed in faecal consistency (data not shown) and ADWG (Fig. 3) between the groups.

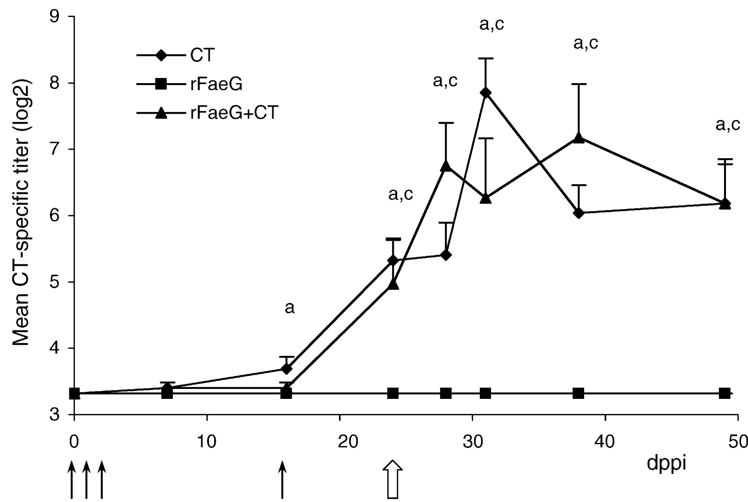

Fig. 2. Mean total CT-specific serum antibody titers (±S.E.M.) at 0, 7, 16, 24, 28, 31, 38 and 49 days post-primary immunization (dppi) of pigs orally immunized with cholera toxin (CT, $n = 7$), rFaeG ($n = 7$) or rFaeG and cholera toxin (rFaeG + CT, $n = 7$). Significant difference ($P < 0.05$) between CT and rFaeG (a), between CT and rFaeG + CT (b), between rFaeG and rFaeG + CT (c). Black arrow: immunization; white arrow: challenge.

There were no pigs with diarrhoea following challenge infection.

3.6. F4-specific serum antibody response following challenge

In agreement with the high F4$^+$ *E. coli* excretion in the CT group, the challenge infection induced a primary F4-specific immune response in the CT immunized group (Fig. 1). Indeed, an F4-specific IgM serum antibody response appeared 28 dppi (4 dpc; mean log2 titer 4.75) and was significantly higher than for both rFaeG-immunized groups on 38 and 49 dppi ($P \leq 0.019$), whereas the F4-specific IgA and IgG serum antibodies (mean log2 titer 4.29 and 4.32, respectively) were only detected from 38 dppi onwards. On the other hand, the F4-specific IgG antibodies of both rFaeG-immunized groups showed a secondary response upon challenge with titers (mean log2 titers 6.98 and 7.61) significantly higher as compared to the CT group 31 dppi (mean log2 titer 3.49; $P \leq 0.043$). Thereafter, IgG titers decreased again in the rFaeG group, but not in the rFaeG + CT group where they remained significantly higher than in the CT group (38 and 49 dppi; $P \leq 0.037$).

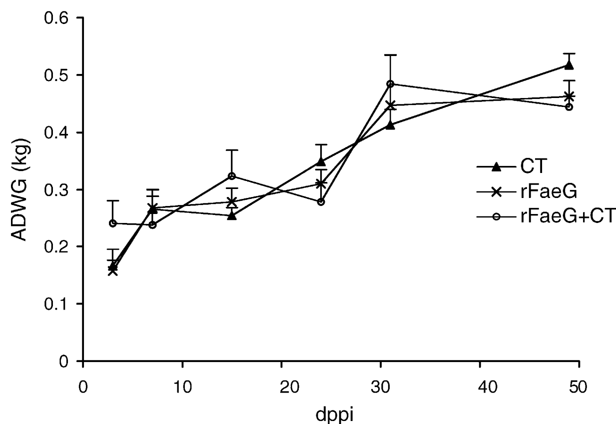

Fig. 3. Average daily weight gain (ADWG) at 3, 7, 15, 24, 31 and 49 dppi of pigs orally immunized with cholera toxin (CT, $n = 7$), rFaeG ($n = 7$) or rFaeG and cholera toxin (rFaeG + CT, $n = 7$).

3.7. CT-specific serum antibody response following challenge

Following challenge infection of CT-immunized animals with the LT-producing F4$^+$ ETEC strain GIS26, CT-specific antibodies remained increasing until 4 or 7 dpc (rFaeG + CT and CT groups, respectively; Fig. 2) and then reached a plateau. The increase of the CT-specific antibody titer following ETEC infection could suggest a booster of the CT-response. On the other hand, challenge infection could

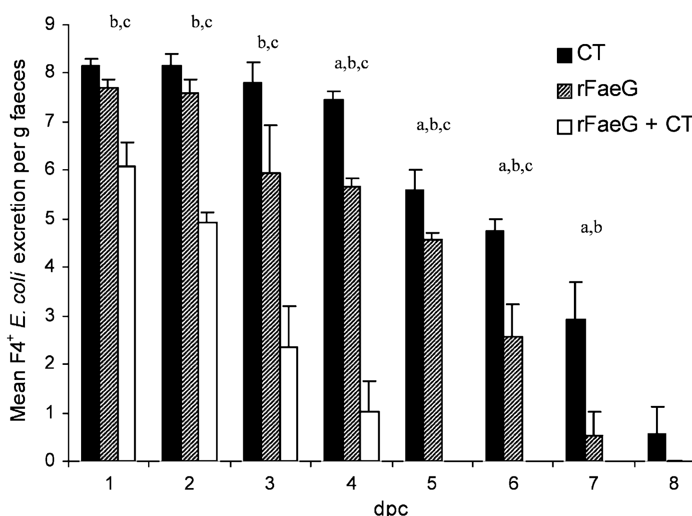

Fig. 4. Mean faecal F4$^+$ *E. coli* excretion per gram faeces (±S.E.M.) of pigs orally immunized with cholera toxin (CT, n = 7), rFaeG (n = 7) or rFaeG and cholera toxin (rFaeG + CT, n = 7). Significant difference (P < 0.05) between CT and rFaeG (a), between CT and rFaeG + CT (b), between rFaeG and rFaeG + CT (c).

not induce CT-specific serum antibodies in animals immunized with rFaeG alone, so that the observed increase in both other groups could still have been due to the CT immunization 16 dppi.

4. Discussion

The results of the present study show that oral immunization of newly weaned pigs with rFaeG results in an F4-specific mucosal and systemic immune response, in agreement with previous experiments (Verdonck et al., 2004a). However, the results of the present study indicate that oral co-administration of rFaeG with CT significantly improves the induction of an F4-specific immune response in pigs. Indeed, the addition of the mucosal adjuvant CT induces faster and significantly higher F4-specific antibody titers in serum as well as F4-specific cell proliferation in the rFaeG + CT group, as compared to the animals immunized with rFaeG alone. The mechanisms underlying the observed mucosal adjuvanticity of CT are not clear, but there is growing evidence that the establishment of an environment leading to an enhanced antigen presentation is important (Porgador et al., 1998; Gagliardi and De Magistris, 2003). In pigs, CT increases CD80–CD86 expression and induces IL-1 expression by macrophages (Foss et al., 1999). In addition, several studies in mice also report a CT-mediated optimised antigen presentation, influencing the induction or regulation of an antigen-specific immune response (Cong et al., 1997; Yamamoto et al., 1999; Jang et al., 2003).

The high F4-specific serum IgG titers and the detection of F4-specific IgA antibodies in saliva samples in the rFaeG + CT group, are in agreement with the induction of antigen-specific IgG (mainly IgG1) and IgA antibodies in the serum and mucosal secretions, respectively, following oral co-administration of different heterologous antigens with CT in mice (Xu-Amano et al., 1994; Marinaro et al., 1995; Cong et al., 1997; Kim et al., 1998). The low levels of F4-specific IgA antibodies in saliva samples suggest diffusion of F4-specific IgA from serum to saliva, instead of a local production of F4-specific IgA (Vaerman et al., 1997). On the hand, oral immunization of pigs with rFaeG has been shown to induce F4-specific antibody secreting cells in the gut-associated lymphoid tissue (Verdonck et al., 2004a). Secretion of more F4-specific IgA antibodies in the small intestinal lumen following rFaeG + CT immunization could occur since CT induces a predominant Th2-response (Xu-Amano et al., 1994; Marinaro et al., 1995) and stimulates IgA isotype switching (Kim et al., 1998) in mice. This could explain the better inhibition of F4$^+$ ETEC colonization following challenge of the rFaeG + CT immunized animals as compared to rFaeG-

immunized animals. Indeed, a correlation between the presence of F4-specific IgA antibodies in the small intestinal lumen and a reduction of the F4$^+$ ETEC colonization has been reported (Porter et al., 1974).

Despite the improved F4-specific immune response, pigs orally immunized with rFaeG and CT are not fully protected against a subsequent F4$^+$ ETEC challenge. Therefore, further improvement of the rFaeG immunization protocol is necessary. Perhaps, a higher rFaeG dose is necessary and/or the refolding of rFaeG must be further optimised. Refolded rFaeG is reported to bind the F4R and to have the conformational epitopes a6, a7 and c in common with purified F4 fimbriae (Verdonck et al., 2004a). However, if the rFaeG folding is not totally identical to that of purified F4, rFaeG could induce less neutralizing antibodies or antibodies with a lower affinity to the native structure. Indeed, conformational changes in antigens are reported to influence its immunogenicity in terms of both affinity and titer (Subramanian et al., 2001; Titball and Williamson, 2001; Joyce et al., 2002). On the other hand, the effect of the challenge infection in the present study was more severe as compared to a previous experiment (Verdonck et al., 2004a) and therefore needs a higher F4-specific immune response to inhibit the F4$^+$ ETEC colonization.

Results of the present study show that oral administration of CT to pigs results in the significant induction of CT-specific serum antibodies. In addition, Foss and Murtaugh (1999) observe CT-B-specific IgA and IgG antibodies in jejunal mucus and saliva of pigs that are orally immunized with CT. As could be expected, the CT-specific immune response did not reduce F4$^+$ ETEC colonization since F4$^+$ *E. coli* excretion resembled that of severely infected non-immunized pigs (Verdonck et al., 2002). In humans, the presence of high anti-LT antibody titers is also shown not to be protective against ETEC infections (Cravioto et al., 1990; Levine et al., 1979). However, since cross-reactivity is reported between antibodies against the related enterotoxins CT and LT (Svennerholm et al., 1983; Clemens et al., 1988), the induced CT-specific antibodies are likely to reduce or even inhibit the toxic effect of F4$^+$ ETEC-produced LT during challenge infection. Indeed, oral vaccines successful in protecting humans against ETEC-induced diarrhoea contain the cholera toxin B-subunit to induce anti-toxin antibodies (Peltola et al., 1991; Savarino et al., 1999; Hall et al., 2001).

Foss and Murtaugh (1999) suggest that the mucosal adjuvanticity of CT in pigs needs mucosal targeting of the added heterologous antigen, as an antigen-specific immune response is only observed when the co-administered heterologue antigen is coupled to CT-B. However, the results of the present study show that CT can act in pigs as a mucosal adjuvant for a heterologous antigen that is targeted to the mucosae by other systems (for instance the F4R) than binding to the CT-receptor GM1.

In conclusion, oral co-administration of rFaeG and CT to pigs induces an improved FaeG-specific immune response that significantly reduces F4$^+$ *E. coli* excretion following F4$^+$ ETEC challenge, as compared to rFaeG-immunized pigs.

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