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Effect of levamisole on the number of intestinal goblet cells in weaned pigs experimentally vaccinated against colibacillosis

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ABSTRACT

Postweaning colibacillosis (PWC) is an etiologically complex disease commonly induced by porcine F4ac$^+$ enterotoxigenic *Escherichia coli* (ETEC) for which no effective vaccine is available. The objective of this study was to determine the nonspecific immunomodulatory effect of levamisole in combination with specific intragastric immunization of weaned pigs with a candidate F4ac$^+$ non-ETEC oral vaccine on the population of intestinal goblet cells (GC). The pigs were immunized with F4ac$^+$ non-ETEC strain, in combination with or without levamisole. Seven days after immunization the pigs were challenged with F4ac$^+$ ETEC strain and 14 days following immunization they were euthanatized for sampling of specimens of the small intestine for immunohistochemistry and morphometric analyses. Samples of the ileum were tested for the presence of acidic and neutral carbohydrates, components of mucus produced and secreted by the intestinal goblet cells (GC). The volume density (V_V) of the PAS$^+$ and AB$^+$/PAS$^+$ GC was determined using the stereological point-counting method. The Vv of the ileal PAS$^+$ GC was lowest (0.130 ± 0.075 mm^3) in the pigs that were immunized with the vaccine candidate F4ac$^+$ non-ETEC strain. Interestingly, AB$^+$/PAS$^+$ GC Vv were found to be highest (0.262 ± 0.091 mm^3) in this group of pigs. The Vv of PAS$^+$ GC was the largest (0.201 ± 0.064 mm^3) in the negative control group of pigs. Pigs from the levamisole pretreated group were shown to have the lowest Vv (0.166 ± 0.051 mm^3) of AB$^+$/PAS$^+$ GC. According to the data presented, there was no significant influence of non-specific/specific immunization on the nonimmune defence mechanism of the intestinal mucosa, as measured by GC Vv in weaned pigs. Hence, it is likely that the production of mucus was not affected and that nonspecific

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protection of porcine intestines provided by mucus layer was not compromised following peroral immunization against PWC. Also, presumably the anti-inflammatory effect of levamisole was observed as the population of ileal mast cells was not considerably affected by non-specific/specific immunization and challenge infection.

Key words: goblet cells, intestine, specific/non-specific immunization, colibacillosis, pigs

Introduction

Gastrointestinal mucosa provides a barrier against potentially dangerous agents such as bacteria and their toxins. Viscoelastic mucus gel layer that acts as a protective barrier against the harsh luminal environment covers the luminal surface of the gastrointestinal tract. It helps lubrication of the epithelial surface for the passage of luminal contents, removal of parasites, and maintenance of constant pH (DUNSFORD, 1991; VIJTIUK et al., 2002). The mucus layer has been confirmed as important non-immune protective factor (CORFIELD et al., 2000). Its importance lies in the prevention of bacterial adherence to the enterocytes and in helping defecation. The intestinal goblet cells (GC) are interspersed among the absorptive cells of the mucosal epithelium. Their main function is synthesis of the water-soluble glycoproteinaceous mucins. Mucins released from GC protect the underlying mucosa from various insults and dehydration. The glycoproteins and proteoglycans, once packaged for export, may not always be transported to the exterior of the cell immediately. It seems that also in the mast cells delayed secretion of mucosubstances can occur, until an appropriate stimulus is received (BARRETT, 1971). Generally, the number of GC and the amount of mucus that they secrete increases under the influence of bacterial presence in the gut (SMIRNOVA et al., 2003). The opposite happens in the gut of recently weaned pigs, implying the role of stress caused by weaning. Other factors may be involved in the change of GC populations at weaning, such as new diet, normal differentiation and maturation. The chemical composition of mucin within the GC also undergoes change during their migration in the gut (DUNSFORD, 1991).

Most intestinal diseases are a result of the malfunction of the intestinal mucosa and are associated with diarrhoea (BARKER and VAN DREUMEL, 1985). Porcine post-weaning colibacillosis (PWC) is characterized by diarrhoea, body mass loss and eventually death. It is an etiologically complex disease commonly induced by $F4^+$ or $F18^+$ enterotoxigenic *Escherichia coli* (ETEC) strains and is among the most common diseases in pigs (FAIRBROTHER et al., 2005). An effective vaccine is still not available, so porcine PWC remains an important cause of morbidity in pigs around the world (VIJTIUK et al., 2005). The loss from colibacillosis provoked by post-weaning stress is high in piglets due to loss of lactogene protection and suppression of the immune system (VALPOTIĆ et al., 1992). Oral immunizations with non-replicating antigens (HUSBAND, 1993) have generally been ineffective in protecting against mucosal pathogens. Live oral vaccines were supposed to offer the best protection against porcine ETEC (ATTRIDGE et al., 1988), while live attenuated *E. coli* oral vaccine expressing the F4ac antigen was found to offer partial

protection against the challenge-induced clinical disease with virulent F4ac$^+$ ETEC in weaned piglets (BOŽIĆ et al., 2002a). In recent years, a number of advances have been made in the fields of gastrointestinal physiology, microbiology and mucosal immunology in relation to weaning and nutrition in young pigs. These include new insights into pre- and probiotic action on intestinal physiology, microbiota and the mucosal immune system (LALLES et al., 2007). Long-term methodologies to control enteric infections, such as colidiarrhea and colienterotoxemia comprise a non-antibiotic approach by manipulating composition of the weaning diet and by stimulating local (intestinal) defence mechanisms with live oral vaccines and non-specific immunomodulators (mucosal adjuvants) applied before weaning (NAGY and FEKETE, 2005).

Levamisole has been shown to express the immunomodulatory effect besides its antihelminthic activity (BOŽIĆ, 2000). The drug seems to act on the cellular immune response, enhancing immune activity specifically in immunocytes whose function is impaired (BRUNNER and MUSCOPLAT, 1980; MULCAHY and QUINN, 1986). Levamisole priming of weaned pigs experimentally vaccinated against colibacillosis stimulated their gut immune system upon virulent challenge and significantly contributed to the effectiveness of a live attenuated oral vaccine against porcine PWC (BOŽIĆ et al., 2002b; 2003a; 2003b; 2006). These studies indicate that the drug may contribute to immune protection from challenge-induced porcine PWC by stimulating recruitment and activation of intestinal T cells and macrophages. However, it is not entirely clear whether levamisole may modulate natural protection mechanisms in the gut of pigs, experimentally vaccinated against PWC. Thus, the objective of this study was to determine the effect of levamisole on the intestinal GC population of early-weaned pigs vaccinated against colibacillosis.

Materials and methods

Pigs. Twenty crossbred Swedish Landrace and Yorkshire pigs were randomly divided into four groups comprising 5 pigs each. Pigs received a commercial weaner diet and had unlimited access to water (SARMIENTO et al., 1988). At the age of four weeks the pigs were immunized with F4ac$^+$ non-ETEC strains in combination with or without levamisole.

Immunization and challenge infection. Levamisole was used as a nonspecific immunostimulator in a concentration of 2.5 mg/kg (BRUNNER and MUSCOPLAT, 1980) and intramuscularly (i/m) given daily over three consecutive days, *i.e.* at day 0, 1 and 2 of the experiment. After levamisole priming, the pigs from the principal group were intragastrically (i/g) vaccinated with 1×10^{10}CFU/mL of F4ac$^+$ non-ETEC vaccinal strain 2407 (serovar O9:K36:H19:F4ac:LT$^-$ STb$^-$) in 60 mL of Trypticase soy broth (TSB). At day 0 the pigs from the negative control group were i/m treated with phosphate buffered saline (PBS) and i/g with 60 mL of TSB as a placebo. The pigs from the positive control groups were given either levamisole and TSB (positive control 1) or PBS as a placebo

and F4ac$^+$ non-ETEC strain 2407 (positive control 2) as aforementioned. Seven days later all the pigs were challenged with F4ac$^+$ ETEC strain 11-800/1/94 (serovar O149:K91: F4ac:F6:Hly$^+$LT$^+$STb$^+$) and euthanatized on post-challenge day 7 (or at day 14 of the experiment) as described earlier (BOŽIĆ et al., 2002a).

Sampling and histochemical procedures. Tissue samples of ileum were fixed in methacarn, dehydrated, embedded in paraplast, and cut into sections 7 μm thick. Detection and characterization of neutral and acidic carbohydrate components of GC were performed by the technique established for carbohydrate histochemistry which apply the combination of alcian blue (AB) staining and periodic acif-Schiff (PAS) reaction (JOHANNES and KLESSEN, 1984). The PAS reaction specifically stains the complex of neutral carbohydrate components of GC with an intensive rose colour (PAS$^+$ GC). The combination of AB and PAS staining techniques stains mixed acidic and neutral carbohydrate components of GC with a violet colour (AB$^+$/PAS$^+$ GC) (FISCHER et al., 1999). Quantitative analysis of the GC by the stereological method based on the point-counting principle was performed on tissue samples of ileum stained with a combination of AB and PAS reactions.

Histomorphometric observations. Using a «Leitz» light microscope with a lens magnification of 40x, the volume density of PAS$^+$ (Vv_G) and AB$^+$/PAS$^+$ (Vv_p) GC in the ileum were calculated, demonstrating the percentage of the goblet cells in the ileal epithelia and lamina propria. An eyepiece (x10) with Weibel's multipurpose test system (M42) served for point counting (WEIBEL, 1979).

Statistics. For statistical analysis, the Mann Whitney U test was used.

Results

The ileal GC were demonstrated with a combination of AB and PAS staining (Fig. 1). It is visible that the PAS reaction specifically stains the complex of neutral carbohydrate components of GC with an intensive rose colour, thus identifying PAS$^+$ GC. The combination of AB and PAS staining demonstrates the mixed acidic and neutral carbohydrate components of GC with a violet colour, and thus identifies AB$^+$/PAS$^+$ GC.

Stereological analysis of PAS$^+$ and AB$^+$/PAS$^+$ ileal GC showed the latter to be most numerous in pigs immunized with F4ac$^+$ non-ETEC strain (0.262 ± 0.091 mm^3) (Table 1).

The Vv of AB$^+$/PAS$^+$ GC in levamisole pretreated pigs was 0.159 ± 0.023 mm^3. The difference between AB$^+$/PAS$^+$ GC populations in the villi and in the intestinal crypts of the levamisole pretreated and non-treated (control) pigs was not significant. The Vv of the PAS$^+$ GC was the lowest (0.130 ± 0.075 mm^3) in the pigs immunized with the F4ac$^+$ non-ETEC strain. The pigs serving as the negative control group had the largest Vv of PAS$^+$ GC (0.201 ± 0.064 mm^3). The Vv of PAS$^+$ and AB$^+$/PAS$^+$ GC, representing the percentage of the entire population of ileal GC, was not significantly different between the groups of

pigs tested (Table 1). The PAS^+ GC were more numerous in the crypts and AB^+/PAS^+ GC were more abundant in the villous epithelium of pigs pretreated with PBS and challenged with $F4ac^+$ ETEC strain seven days following the placebo treatment (Fig. 1).

Table 1. The volume density (V_V) of the ileal total PAS^+ and AB^+/PAS^+, $PAS^+$and AB^+PAS^+ GC in 6 week-old weaned pigs immunized with levamisole (at day 0, 1 and 2)/with or without $F4ac^+$ non-ETEC vaccine candidate strain (at day 0) and challenged with $F4ac^+$ ETEC pathogenic strain at day 7; the pigs were euthanatized at day 14 of the experiment

Group of pigs* (treatment/challenge)	N° of fields «n»	Volume density of ileal GC stained with: ($V_V \pm SE/mm^3$)		
		$PAS^+$and AB^+PAS^+	PAS^+	AB^+PAS^+
Negative control (PBS + TSB/ETEC)	200	0.390 ± 0.019	0.201 ± 0.064	0.189 ± 0.045
Positive control 1 (Levamisole + TSB/ETEC)	200	0.346 ± 0.079	0.181 ± 0.029	0.166 ± 0.051
Positive control 2 (PBS + non-ETEC/ETEC)	155	0.392 ± 0.016	0.130 ± 0.075	0.262 ± 0.091
Principal (Levamisole + non-ETEC/ETEC)	447	0.329 ± 0.014	0.170 ± 0.034	0.159 ± 0.023

*Groups comprised 5 pigs each.

The GC of both staining types were slightly more abundant in the ileal villi of pigs pretreated with levamisole and challenged seven days thereafter with $F4ac^+$ ETEC strain than in the pigs pretreated with PBS (Fig. 2).

Alcian blue also stained glycosaminoglycans in the mast cells (MC) in the ileal Lieberkűhn crypts of pigs perorally immunized with $F4ac^+$ non-ETEC strain and challenged with $F4ac^+$ ETEC strain seven days following the vaccination (Fig. 3). Numbers of GC were similar to those observed in the pigs from the negative control group.

In the pigs non-specifically/specifically immunized and challenged, the number of MC was slightly higher, whereas the presence of GC seems to be less evident as compared to the finding in the negative control pigs (Fig. 4).

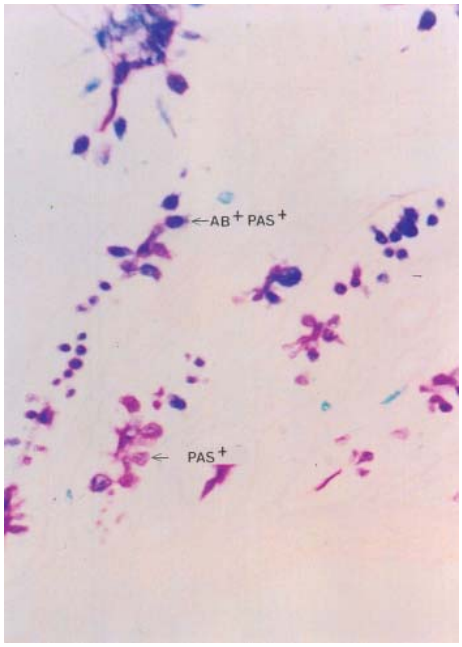

Fig. 1. Differentiated staining of the PAS^+ and AB^+/PAS^+ GC in the ileal Lieberkűhn crypts of a 6-week old pig from the negative control group pretreated with PBS/TSB (at day 0) and challenged with $F4ac^+$ ETEC strain at day 7 of the experiment. ×150.

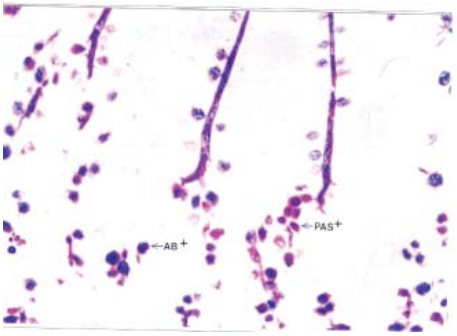

Fig. 2. Differentiated staining of the PAS^+ and AB^+/PAS^+ GC at the basis of the ileal villi of a 6-week old pig from the positive control group 1 pretreated with levamisole (at days 0, 1 and 2)/TSB (at day 0) and challenged with $F4ac^+$ ETEC strain at day 7 of the experiment. ×150.

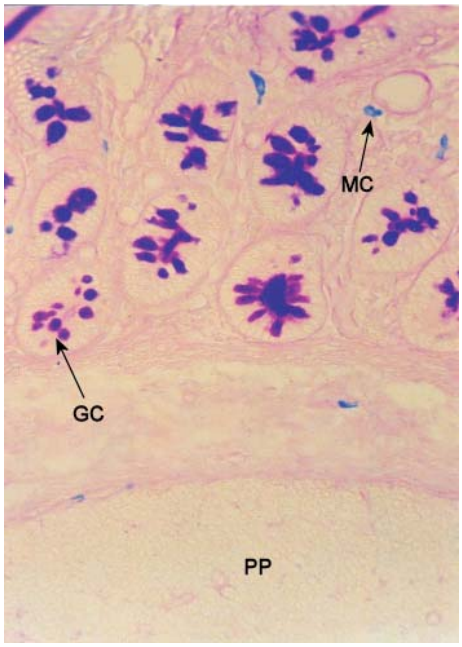

Fig. 3. Visible goblet (GC) and mast cells (MC) in the ileal Lieberkűhn crypts of a 6-week old pig from the positive control group 2 pretreated with PBS/immunized with F4ac$^+$ non-ETEC strain (at day 0) and challenged with F4ac$^+$ ETEC strain at day 7 of the experiment; Peyer's patch (PP) is visible in the submucosis. ×240.

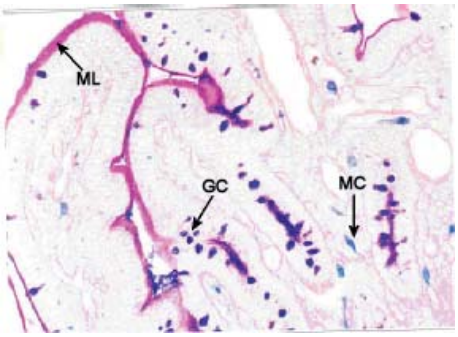

Fig. 4. Visible Goblet (GC) and mast cells (MC) in the ileal villi covered by mucus layer (ML) of a 6-week old pig from the principal group pretreated with levamisole (at days 0, 1 and 2)/ immunized with F4ac$^+$ non-ETEC strain (at day 0) and challenged with F4ac$^+$ ETEC strain at day 7 of the experiment. ×150.

Discussion

Since levamisole is known as an immunomodulator, and may act as a mucosal adjuvant enhancing mucosal cell-mediated immunity in weaned pigs vaccinated against colibacillosis (BOŽIĆ et al., 2003a; 2003b; 2006), this study was performed in order to reveal the possible influence of the drug on numbers of porcine intestinal GC, which are known to play an important role in the nonimmunological defence of the gut mucosa against intraluminal microbiota (ITOH et al., 1999). In the present study, the results obtained by the stereological method for quantification of the GC showed that neither levamisole alone nor its combination with the vaccine candidate non-ETEC strain had a significant influence on the GC population. Slightly smaller numbers of GC found in non-specifically/specifically immunized and challenged pigs confirmed the previous observation that any treatment of weaned pigs may influence the rate of GC proliferation and consequently their role in the defence of the mucosal surfaces of weaned pigs (PERDUE, 1996). Moreover, it would be expected that bacterial infections strongly stimulate activation of GC and induce their hyperplasia (SMIRNOVA et al., 2003). However, our findings implicate that synergistic reactions to stress accompanying weaning and to additional stress caused by the treatment with levamisole and by peroral immunization/challenge procedures, may inconsiderably influence the number of intestinal GC, and thus, any significant changes in their nonspecific defence mediated by the secretion of mucus are not likely to be expected.

In the present study, we observed only a slight decrease of AB^{+}/PAS ileal GC in weaned pigs primed with levamisole. This was observed in both groups of pigs where levamisole was applied, and could be deliberately interpreted in the light of the anti-inflammatory effect of levamisole. Namely, the priming of weaned pigs with levamisole may attenuate mucosal inflammation accompanied by the GC hyperplasia, as overproduction of mucus and mucins is normally associated with inflammation. Since hyperplasia of the intestinal mucus-secreting GC is an important part of the nonspecific defence mechanisms, one can speculate that a decrease in their number may impair their function. However, the priming of vaccinated weaned pigs with levamisole was recently found to stimulate the specific mucosal cell-mediated immune response upon challenge infection, contributing to the effectiveness of a live attenuated oral vaccine against porcine PWC (BOŽIĆ et al., 2003a; 2003b; 2006). Thus, these latter results, together with the results generated from the present study imply that intestinal inflammation is not an essential requirement for the expression of protective immune responses to vaccination against porcine PWC, and that both of these phenomena are influenced by levamisole. Our finding of rare mast cells in the ileum of non-specifically/specifically immunized and challenged pigs was in accordance with the former affirmation, as their roles in mucosal inflammation, host defences, and tissue repair are well known. These cells could be activated following stimulation with intraluminal antigens (such as non-ETEC /ETEC strains applied in the current study)

to release large quantities of histamine, leukotrienes, and a series of cytokines. Thus, it would be relevant to analyze their numbers/distribution and the molecular profiles of their secretions, particularly cytokines, in our further research using this model system.

In conclusion, although the relationship between intestinal dysfunction and immune/nonimmune protection against gastrointestinal pathogens remains controversial, here we present evidence that one of the nonimmunological mechanisms of defence of mucosal surfaces (operating through GC function) is not altered by the application of non-specific (levamisole) and/or specific (non-ETEC/ETEC strains) immunization procedures against porcine PWC. It is well established that levamisole may act as a potent mucosal adjuvant for vaccine candidate non-ETEC (BOŽIĆ et al., 2003a; 2003b; 2006), but its anti-inflammatory properties should not be ignored. It is obvious that the drug needs to be further examined for both its immunostimulatory potentials and eventual deleterious effects in this and other enteric disease models.

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SAŽETAK

Kolibaciloza odbijene prasadi (KOP) etiološki je složena bolest, najčešće izazvana svinjskim F4ac$^+$ enterotoksigenim sojevima bakterije *Escherichia coli* (ETEC), za koju nema djelotvorne vakcine. Namjera ovoga rada bila je da se utvrdi imunomodulacijski učinak levamisola, u kombinaciji sa specifičnom intragastričnom imunizacijom odbijene prasadi pokusnim vakcinalnim F4ac$^+$ ne-ETEC sojem, na populaciju crijevnih vrčastih stanica (VS). Prasad je bila imunizirana F4ac$^+$ ne-ETEC sojem u kombinaciji s levamisolom ili bez njega, sedam dana nakon toga bila je izazivački inficirana F4ac$^+$ ETEC sojem, a 14 dana nakon imunizacije bila je usmrćena radi uzimanja uzoraka crijeva za imunohistokemijske i morfometrijske analize. Uzorci ileuma testirani su na prisutnost kiselih i neutralnih ugljikohidrata, sastavnica sluzi koju proizvode i izlučuju VS. Volumna gustoća (Vg) VS koje se boje kombinacijom alcijanskoga modrila (AM) i periodičnoga kiseloga Schiffovoga (PKS) reagensa određivana je pomoću stereologijske metode brojanja točaka. Vg ilealnih PKS$^+$ VS bila je najniža (0,130 ± 0,075 mm^3) u prasadi imunizirane pokusnim vakcinalnim F4ac$^+$ ne-ETEC sojem. Međutim, zanimljivo je da je utvrđena najveća Vg AM$^+$/PKS$^+$ VS (0,262 ± 0,091 mm^3) u prasadi iz te skupine. U prasadi iz negativne kontrolne skupine utvrđena je najveća Vg PKS$^+$ VS (0,201 ± 0,064 mm^3), dok je prasad iz skupine obrađene levamisolom i vakcinalnim ne-ETEC sojem imala najnižu Vv ukupnih (AM$^+$/PKS$^+$) VS (0,159 ± 0,023 mm^3). Primjereno dobivenim rezultatima, nije utvrđen značajan utjecaj nespecifične/specifične imunizacije na neimunosne obrambene mehanizme crijevne sluznice vrednovane temeljem Vg i distribucije VS u odbijene prasadi. Stoga je vrlo vjerojatno da proizvodnja sluzi nije umanjena i da nespecifična zaštita crijeva prasadi slojem sluzi ne bi bila slabija nakon peroralne imunizacije protiv KOP. Također je zapažen potencijalni protuupalni učinak levamisola s obzirom na činjenicu da populacija ilealnih mastocita nije bila znatnije stimulirana nespecifičnom/specifičnom imunizacijom i izazivačkom infekcijom.

Ključne riječi: vrčaste stanice, crijevo, specifična/nespecifična imunizacija, kolibaciloza, prasad