VETERINARSKI ARHIV 81 (1), 77-90, 2011

De novo differentiation of intestinal villous M cells in weaned pigs immunized with levamisole-adjuvanted vaccine candidate F4ac+ or F18ac+ non-enterotoxigenic *Escherichia coli* strains

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ABSTRACT

Active immunization against porcine postweaning colidiarrhea (CD) and/or colienterotoxemia (CE) caused by F4+ and/or F18+ enterotoxigenic *Escherichia coli* (ETEC) is still an unsolved problem. The intestinal microfold (M) cells play a role in the entry/invasion of intraluminal pathogens (such as ETEC strains), in antigen sampling, and in facilitating the induction of immunity to gut infections. Just as ETEC strains can exploit M cells as the portal of entry for infections, such as CD and/or CE, their high transcytotic ability makes them an attractive target for mucosally delivered vaccines, adjuvants and therapeutics. The objective of our study was to evaluate the effects of levamisole-adjuvanted vaccine candidate F4ac+ and F18ac+non-ETEC strains on incidence/frequency of ileal M cells and up-regulation of antigen delivery by *de novo* formation of these cells in weaned pigs. Conventionally reared 4-week-old pigs were divided into three groups, of which two were parenterally and orally immunized with levamisole (at days -2, -1 and 0) in combination with either vaccine candidate non-ETEC strain (at day 0), respectively. The third group of pigs received saline as a placebo.

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ISSN 0372-5480
Printed in Croatia

Challenge was performed (at day 7) with the F4ac+ ETEC strain, and the pigs were euthanatized (at day 13) and sampled for immunohistology. Distribution patterns of cytokeratin 18 positive M cells revealed that they are interspersed between enterocytes than as small clusters, and most of them were found to be located at the apex of the villi in the ileum of 6-weeks-old-pigs. Morphometric quantification of M cells in the ileal mucosa showed that levamisole-pretreated F18ac+non-ETEC-immunized and challenged pigs had significantly increased numbers (P<0.01) of ileal M cells as compared to the values obtained in the control non-primed and challenged pigs. The proportion of these cells in this group of pigs was increased by 145%. In the levamisole-pretreated F4ac+ non-ETEC-immunized and challenged pigs only a slightly increased (for 7%) proportion of M cells was recorded. However, this increase was not significantly different from the numerical values obtained for control pigs. Our finding that levamisole-adjuvanted F18ac+non-ETEC vaccine may affect *de novo* differentiation of antigen-sampling M cells by increasing their number in the ileum, indicated that the vaccine probably utilizes these cells as a target for entry/delivery to the nearby lymphocytes and induces protective immunity against CE. On the other hand, the failure of levamisole-adjuvanted F4ac+non-ETEC vaccine to produce a similar effect on M cells remains to be elucidated.

Key words: M cells, levamisole adjuvanticity, *E. coli* vaccines, weaned pigs

Introduction

The abnormal colonization pattern of the small intestine by F4ac+ and/or F18ac+ enterotoxigenic *Escherichia coli* (ETEC) strains was recognized as a characteristic of the commonly occurring postweaning colidiarrhea (CD) and/or colienterotoxemia (CE) in newly weaned pigs, aging between 4 and 12 weeks (NAGY and FEKETE, 1999). These infections are the most important cause of economic losses in the swine industry due to morbidity, decreased growth rate, cost of medication, and mortality. To date, none of the preventive strategies applied (FAIRBROTHER et al., 2005) has been able to efficiently protect pigs from CD and/or CE. One of the most promising immunoprophylactic approaches to prevention and control of these infections includes oral immunization of pigs before weaning with live avirulent non-ETEC strains carrying fimbrial adhesins, homologous to those of ETEC strains present in the given swine population (NAGY and FEKETE, 2005).

However, the successful mucosal vaccines against CD and/or CE must circumvent the same barriers that enteric pathogens have, *i.e.* mucus, proteases, nucleases, secretory IgA antibodies, and the epithelial glycocalyx (MILLER et al., 2007). Enteric pathogens themselves, such as ETEC strains, have so far been the most effective immunogens exploited for mucosal vaccination. (COX et al., 2002; VERDONCK et al., 2002). Just as an ETEC can exploit intestinal microfolds (M) as the portal of entry for infection, researchers have investigated the potential of using M cell-specific mechanisms for drug or vaccine delivery to the mucosal immune system (BRAYDEN and BAIRD, 2004; KUOLEE and CHEN, 2008). Upon M cells-mediated antigen sampling at the interface of epithelial surfaces and the intraluminal environment, the intestinal mucosal immune system can actively discriminate between harmful pathogenic bacteria and their products and harmless dietary antigens, resulting in either protective immune responses or oral tolerance, respectively

(STOKES and BAILEY, 1994; STOKES and BAILEY, 1996). Although the intestinal mucosal immune system has the ability to recognize these different groups of antigens and has evolved a battery of immune cells/molecules, which may orchestrate an appropriate response, it is paradoxical that the question as to how this system selects a particular response still remains largely unanswered (STOKES et al., 2004; BAILEY et al., 2005). This is the main obstacle for the development of safe and effective vaccines against porcine post-weaning CD and/or CE. The potential of immunomodulators/adjuvants being used therapeutically, not only to reconstitute defective responses, such as in immunologically compromised or immature newly weaned pigs, but also to enhance normal responses to target antigens at intestinal mucosal sites, is now an attractive approach for mucosal vaccine design (HUSBAND et al., 1999). Also, such an approach includes the use of adjuvants (HOLMGREN et al., 2003), particularly levamisole (CHEN et al., 2008) which has exhibited potentials as an immunomodulator and mucosal adjuvant when applied alone or in combination with live oral vaccines against CD and/or CE (BOŽIĆ et al., 2002; BOŽIĆ et al., 2003a, b; BOŽIĆ et al., 2006; JANJATOVIĆ et al., 2008; KOVŠCA JANJATOVIĆ et al., 2009). More recent findings that levamisole may affect *de novo* differentiation of antigen-sampling M cells by increasing their numbers in the ileum of weaned pigs, make it an interesting adjuvant to study the development of an effective M cell-targeted vaccine against porcine post-weaning CD and/or CE (VALPOTIĆ et al., 2010). The latter studies have implied that levamisole may also affect differentiation and proliferation of porcine M cells by increasing their numbers in the intestinal villous epithelium. Thus, it would be tempting to suggest that our observation of levamisole-stimulated proliferation of the ileal villous M cells may have some relevance to the process of delivery of new mucosal vaccines against porcine CD and/or CE to the immune cells populating the intestinal lamina propria.

Indeed, it has been demonstrated that M cells can transport a diverse array of intraluminal microorganisms across the intestinal epithelial barrier, including *E. coli* (GEBERT et al., 1996), but also a variety of synthetic microparticles/nanoparticles as vehicles for mucosal vaccine delivery (TORCHÉ et al., 2000), beta 1 integrins, pathogen recognition receptors, and specific carbohydrate residues (KUOLEE and CHEN, 2008). Porcine intestinal M cells may be distinguished from the other cells residing in follicle-associated epithelium (FAE) by the expression of cytokeratin peptide 18 (GEBERT et al., 1994). As in rodents and humans (Jang et al., 2004), we have demonstrated (VALPOTIĆ et al., 2010) that cytokeratin 18 peptide enabled identification, localization and quantification of intestinal villous M cells within the epithelial layer in the ileal mucosa of weaned pigs.

Recently we have demonstrated by histomorphometric analyses that levamisole-adjuvanted F18ac+ and F4ac+non-ETEC vaccine candidate strains stimulated lymphoid and myeloid cells residing in the jejunal and ileal mnusosa of weaned pigs (KOVŠCA

JANJATOVIĆ et al., 2009; KOVŠCA JANJATOVIĆ et al., 2010). The current study evaluated the effects of levamisole-adjuvanted vaccine candidate F4ac$^+$ and F18ac$^+$ non-ETEC strains on the incidence/frequency of ileal M cells and up-regulation of antigen delivery by *de novo* formation of these cells in weaned pigs. The main objective was to establish the potential of levamisole-adjuvanted vaccine candidate strains in promoting differentiation of porcine ileal M cell phenotypes as an attractive target for mucosally delivered vaccines against CD and/or CE.

Materials and methods

Pigs. Fifteen crossbred pigs (Swedish Landrace × Yorkshire) weighing 6.5 ± 1 kg, the progeny of three litters, were purchased from a swine farm near Zagreb, Croatia. The pigs were weaned at 4 weeks of age, housed in the animal facility at the Faculty of Veterinary Medicine, University of Zagreb and fed with a standard weaner diet.

*Bacterial strains***.** The recombinant avirulent vaccine candidate F4ac$^+$ non-ETEC strain 2407 (serotype O9: K36: H19: F4ac: LT$^-$ STb$^-$) kindly donated by dr. sc. Thomas A. Casey from NADC, Ames, IA, USA (CASEY and MOON, 1990) and attenuated vaccine candidate F18ac$^+$ non-ETEC strain 2143 (serotype O157:K119:F18ac) kindly donated by dr. sc. Bela Nagy from the Veterinary Medical Institute of Hungarian Academy of Sciences, Budapest, Hungary (KOVŠCA-JANJATOVIĆ et al., 2009), were used for the immunization. The authentic F4ac$^+$ ETEC strain 11-800/1/94 (serotype O19: K91: F4ac: 987P: Hly$^+$ LT$^+$ STb$^+$), isolated from diarrheic pigs aged between 3 and 4 weeks reared on swine farms in Croatia, was used for the challenge infection. Both strains were kept in glycerin broth at -80 ºC until used.

Antibodies. Mouse IgG1 (clone C-04) monoclonal antibody (mAb), reactive with porcine cytokeratin peptide 18 (GEBERT et al., 1994) was used as the primary antibody (Biovendor Laboratory Medicine, Heidelberg, Germany). Polyclonal F(ab')$_2$ rabbit anti mouse IgG:HRP antibody (AbD Serotec, Oxford, UK) was used as the secondary antibody. To uncover the tissue antigens fixed with formalin, stabilized proteolytic enzyme mixture in an antigen retrieval kit (Abcam, Cambridge, UK) were used prior to the application of the primary antibody.

Study design. At the age of 4 weeks the pigs were randomly assigned into three groups comprising 5 animals each. After 2 days of accommodation, the pigs were treated as follows: (1) control pigs received 5 mL of saline intramuscularly (i.m.) at day 0, (2) the pigs from the first group of principals were i.m. primed with levamisole (Nilverm®, Pliva, Zagreb, Croatia) at the immunostimulatory dose of 2.5 mg/kg over three consecutive days (-2, -1, 0) and intragastrically (i.g.) immunized with 10^{10} CFU/mL of vaccine candidate F4ac$^+$ non-ETEC strain 2407 in 60 mL of Trypticase soya bujon (TSB) at day 0, and (3) the second group of principals received levamisole as aforementioned and was i.g.

vaccinated with 10^{10} CFU/mL of vaccine candidate F18ac$^+$ non-ETEC strain 2143 in 60 mL of TSB at day 0. Seven days later all pigs were *i.g.* challenged with 10^{10} CFU/mL of F4ac$^+$ ETEC strain 11-800/1/94, and 2 from each group were euthanatized at day 13 and sampled for immunohistology.

Clinical observations. Clinical observations for signs of colibacillosis, such as diarrhea, dehydration, weight loss, weakness and anorexia were recorded three times daily by a person blinded to given treatments. Pigs were weighed at the beginning of the trial (day -2), and 7 and 10 days after immunization (at day 0) or 4 days after the challenge infection. The diarrhea developed by pigs was graded on a scale of intensity where scores (per pig per day of the experiment) were given based on stool consistency: +, soft feces = mild diarrhea; ++, fluid feces = moderate diarrhea; +++, watery feces = severe diarrhea. Pigs with normal firm feces were scored as diarrhea negative (-).

*Sampling and tissue processing***.** Immediately following euthanasia the samples of ileum were fixed in 10% neutral-buffered formalin (pH 7.0-7.6) containing 4% formaldehyde for 24 hours. After fixation the tissue specimens were dehydrated by graded alcohol solutions (in 75%, 80%, 95%, 100% ethanol), washed with xylene as a clearing agent, and incubated in the paraplast embedding medium (Sigma, Deisenhofen, Germany). Following incubation, paraplast-embedded specimens were cut into 5-6 μm thick serial sections and floated on a water bath containing distilled water, heated to approximately 42 °C. The selected sections were picked up with the precoated slides and dried horizontally on a warming tray overnight at 37 °C.

Immunohistological staining. Paraplast-embedded ileal specimens were dewaxed in xylene, hydrated in graded alcohol solutions (in 100%, 100%, 95%, 80%, 75% ethanol) and immersed in distilled water. Endogenous peroxidase was blocked with 3% aqueous hydrogen peroxide solution for 30 minuntes at room temperature, and nonspecific binding was blocked by 5% rabbit serum and 5% pig serum diluted in phosphate-buffered saline (PBS), for 30 minutes prior to staining. Then the sections were incubated overnight at 4 °C with mouse mAb against cytokeratin peptide 18 (1:50 dilution). The secondary antibody F(ab')$_2$ rabbit anti-mouse IgG HRP conjugate diluted 1:500 in PBS was incubated at room temperature for 1 hour. The reaction was visualized using a 0.05% solution of 3.3-diaminobenzidine tetrachloride (DAB) in 0.05 M Tris-HCl (pH 7.6) containing 0.01% H_2O_2. The slides were dehydrated by graded alcohol solutions and mounted in canada balsam.

Histomorphometric analysis. The number of M cells in immunohistologically stained tissue sections of ileum were determined by computer-assisted quantitative analysis. A light microscope equipped with a video camera was interfaced with a desktop computer containing a live video/computer graphics adapter and commercial imaging Lucia G software.

Statistics. Statistical validation was performed on 12 randomly selected digital image fields of an average area of 700480 μm^2 per 5 tissue sections of 2 pigs from three experimental groups. Antecedent testing showed that when more than 10 fields in each of the 5 tissue samples from one pig per group were analyzed there were no significant deviations in the cell counts of individual pig. Levels of significance of differences between principal and control pigs were determined by the two-tailed Student's *t*-test. The results deviating from the null hypothesis at a level of $P<0.01$ were considered as significant.

Results

Clinical findings. None of the pigs developed signs of colidiarrhea, and all were clinically normal at the time of treatment. The mean weight (kg ± SD) of pigs per group at day -2 were: control = 7.40 ± 0.6; levamisole-pretreated F4ac$^+$ non-ETEC-immunized/challenged = 6.68 ± 0.8; and levamisole-pretreated F18ac$^+$ non-ETEC-immunized/challenged = 6.50 ± 0.8. All these body weights were statistically similar. Seven days following the treatment, levamisole-pretreated F4ac$^+$ non-ETEC-immunized/challenged pigs had lower ($P<0.05$) body weight (5.93 ± 0.5) as compared to that in control pigs (7.75 ± 1.0). Weight gain in levamisole-pretreated F18ac$^+$ non-ETEC-immunized/challenged pigs was slightly (7.32 ± 0.9) but not significantly lower. Ten days after the treatment, average group weights were similar and none of them differ from the corresponding weight on day -2. In the control and levamisole-pretreated F18ac$^+$ non-ETEC-immunized/challenged pigs body weights were slightly, but not significantly increased (8.68 ± 1.0 and 7.40 ± 0.5, respectively). Levamisole-pretreated F4ac$^+$ non-ETEC-immunized/challenged pigs gained weight much faster as compared to day 7 (7.75 ± 0,5) but this increase was not significantly higher than their weight at day -2. Two of the five control pigs became diarrheic three days after the treatment (at day 3) and the third pig developed moderate diarrhea at day 4 (Table 1).

The diarrhea, ranging from mild to severe continued for three to five days. Transient diarrhea was apparent in two levamisole-pretreated F4ac$^+$ non-ETEC-immunized/challenged pig at days 3 and 7 after the treatment, whereas the other three pigs were in poor health and feeding condition during the entire study period. Two of them developed moderate to severe diarrhea at day 1 and one died at day 3. The third pig developed moderate diarrhea at day 2, which continued in a milder form until day 7. Two of three diarrheic pigs in the levamisole-pretreated F18ac$^+$ non-ETEC-immunized/challenged group developed mild diarrhea at day 3, but recovered shortly. The third pig developed moderate diarrhea at day 1 and remained diarrheic until day 8 of the experiment. As in both vaccinated groups 4 of 8 diarrheic pigs developed diarrhea at day 1 or day 2, we assume that it was a consequence of natural infection rather than the vaccination.

Table 1. Extent of diarrhea intensity expressed as scores based on stools consistency: -, firm feces = no diarrhea; +, soft feces = mild diarrhea; ++, fluid feces = moderate diarrhea; +++, watery feces = severe diarrhea.

Treatment of pigs[a]	Pig No.	Day of experiment 1	2	3	4	5	6	7	8	9-13
Saline[b]/ F4ac$^+$ ETEC	1									
	2			++	++	+++	++		++	
	3				++	+	+			
	4			++	+	+				
	5									
Levamisole + F4ac$^+$ non-ETEC/ F4ac$^+$ ETEC	1		++	+	+	+		+		
	2			+				+		
	3	++	++	+++	++	++		++		
	4	+++	+++	c						
	5			+				+		
Levamisole + F18ac$^+$non-ETEC/ F4ac$^+$ ETEC	1			+						
	2	++	++	++	+	+			++	
	3									
	4									
	5			+	+					

[a] Groups comprised five 4-weeks-old pigs each. [b]Control pigs received saline as a placebo. [c]Died at day 3 due to diarrheal disease.

Immunohistological findings. Immunohistological identification of cytokeratin peptide 18$^+$ M cells within the villous epithelium of the ileum of the weaned pigs is shown in Figs. 1, 2 and 3. It is visible that M cells were quite scarce in the villous epithelium of the ileum of the control nonpretreated and challenged pig (Fig. 1). Similarly as in the control nonpretreated and challenged pigs, these cells were rarely scattered in the villous epithelium of ileum of levamisole-primed F4ac$^+$ non-ETEC-vaccinated and challenged pigs (Fig. 2). On the other hand, numerous M cells were found within the villous epithelium of ileum of levamisole-pretreated F4ac$^+$ non-ETEC-immunized and challenged pigs (Fig. 3). Generally, the M cells are rather solitarily interspersed between enterocytes rather than as small clusters (Figs. 1, 2 and 3), and most of them were found to be located at the apex of the villi in 6-week-old pigs.

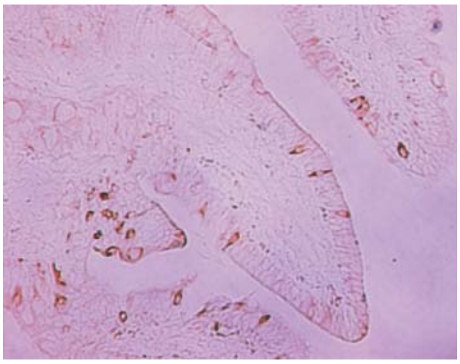

Fig. 1. Cytokeratin peptide 18+ M cells in ileal villous epithelium of control (at day 0 *i.m*. received saline as a placebo) and challenged (at day 7 perorally received pathogenic F4ac+ ETEC strain) pig aging 6 weeks; original magnification ×400.

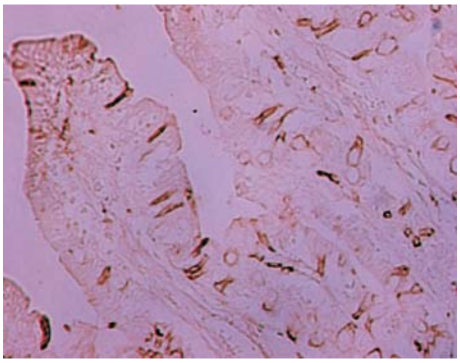

Fig. 2. Cytokeratin peptide 18+ M cells in ileal villous epithelium of levamisole-primed (at days -2,-1 and 0 *i.m*. received the drug) F4ac+ non-ETEC-immunized (*i.g.* at day 0) and challenged (at day 7 *i.g.* received pathogenic F4ac+ ETEC strain) pig aging 6 weeks; original magnification ×400.

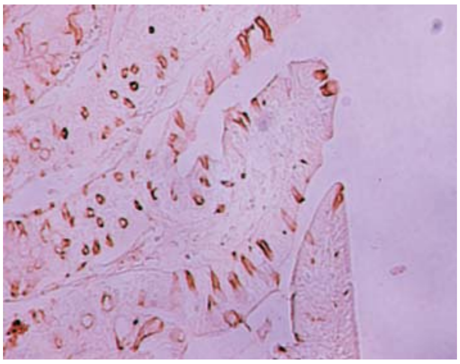

Fig. 3. Cytokeratin peptide 18+ M cells in ileal villous epithelium of levamisole-primed (at days -2,-1 and 0 *i.m*. received the drug) F18ac+ non-ETEC-immunized (*i.g.* at day 0) and challenged (at day 7 *i.g.* received pathogenic F4ac+ ETEC strain) pig aging 6 weeks; original magnification ×400.

Histomorphometric findings. Numerical data on the ileal M cells of the pigs from three experimental groups, as determined by computer-assisted morphometric analyses are shown in Table 2. Also, the proportions of M cells are expressed as a ratio between number of M cells in the principal groups (no. increased or decreased) and that in the control group (where the number of cells = 100% or 1.00). Quantitative phenotypic analyses showed that levamisole-pretreated F18ac$^+$non-ETEC-immunized and challenged pigs had significantly increased number (P<0.01) of ileal M cells as compared to the values obtained from the control nonprimed and challenged pigs. The proportion of these cells in this group of pigs was increased by 145%. In the levamisole-pretreated F4ac$^+$ non-ETEC-immunized and challenged pigs only a slightly increased (for 7%) proportion of M cells was recorded. However, this increase was not significantly different from the numerical values obtained for the control pigs.

Table 2. Numerical values of cytokeratin peptide 18$^+$ M cells in villous epithelium of the ileum from pigs primed with levamisole (days -2, -1 and 0) and immunized with either F4ac$^+$ or F18ac$^+$ non-ETEC vaccine candidate strains (day 0) and challenged with F4ac$^+$ ETEC strain (day 7); the results are expressed as mean values and standard deviations (M ± SD) of number of the cells per μm^2 of tissue section field.

Treatment[a]	M ± SD number of ileal M cells in 6-weeks-old pigs[b]	Index [c]	Increase/ decrease
None[d] + F4ac$^+$ ETEC	$6.12 \times 10^{-5} \pm 9.44 \times 10^{-6}$	1.00	/
Levamisole + F4ac$^+$ non-ETEC + F4ac$^+$ ETEC	$6.53 \times 10^{-5} \pm 9.90 \times 10^{-6}$	1.07	+ 0.07
Levamisole + F18ac$^+$ non-ETEC + F4ac$^+$ ETEC	$1.50 \times 10^{-4} \pm 5.74 \times 10^{-5}$ *	2.45	+ 1.45

[a]Groups comprised five 4-week-old pigs each. [b]As counted in 12 randomly selected fields of the average area of 700480 μm^2 per sample from 2 pigs (euthanatized at day 13) per group.[c]Ratio between no. of M cells in the principal groups and that (no. of the cells = 100% or 1.00) in the control group. [d]Control pigs received saline at day 0 as a placebo.*Significantly higher (P<0.01) than in the control pigs. vv

Discussion

This study demonstrates that (1): cytokeratin 18 peptide, as a specific marker for porcine M cells residing the FAE region (GEBERT et al., 1994) also enabled us to identify these cells within the villous epithelium of the ileum of 6-week-old pigs, regardless of the treatments applied; (2) porcine intestinal villous M cells probably originated from immature enterocytes upon exposure to foreign antigens or pathogens (such as fimbrial and/or toxin antigens of either non-ETEC or ETEC strains applied, respectively), as postulated for rodents and humans (JANG et al., 2004), (3) the M cells are solitarily interspersed between ileal enterocytes rather than as small clusters, and most of them were found to be located at the apex of the villi, (4) levamisole-adjuvanted F18ac$^+$non-ETEC vaccine affected *de novo* differentiation of M cells by increasing their number in the villous epithelium of the ileum, whereas levamisole-adjuvanted F4ac$^+$non-ETEC vaccine failed to produce the same effect, (5) vaccine candidate F18ac$^+$ non-ETEC strain probably utilizes M cells as a target for entry/delivery to the nearby lymphocytes, which is a prerequisite for induction of the intestinal immune responses, and (6) F4ac+-ETEC challenge strain acted synergistically rather than competitively with non-homologous vaccinal strain on the increased rate of *de novo* formation of M cells in weaned pigs. It is well known that non-adherent antigens are frequently poorly internalized by M cells and hence generally induce weak immune responses or even tolerance (BELYAKOV et al., 2004). The failure of levamisole-adjuvanted F4ac+non-ETEC vaccine to produce a similar effect on M cells as levamisole-adjuvanted F18ac+non-ETEC vaccine did, could be explained by its competition for the same enterocyte receptors with F4ac+-ETEC challenge strain, which resulted in flushing of nonadherent bacteria by peristaltic. Such adhesion is a prerequisite for stimulation of the intestinal mucosal immunity preceded by development of M cells from enterocytes upon exposure to enteric pathogens (JANG et al., 2004), such as the F4ac+ ETEC strain. Indeed, it was recently shown that it is possible to differentiate the intestinal immune responses of pigs immunized with levamisole-adjuvanted experimental mucosal vaccines against postweaning CE and CD from those of non-immunized pigs (KOVŠCA JANJATOVIĆ et al., 2009; KOVŠCA JANJATOVIĆ et al., 2010). Also, we have more recently demonstrated that levamisole, already recognized as a potent immunomodulator/adjuvant (BOŽIĆ et. al., 2006; JANJATOVIĆ et al., 2008) may influence the *de novo* differentiation of M cells, by increasing their number in the ileum of weaned pigs (VALPOTIĆ et al., 2010). This finding makes levamisole an interesting adjuvant to study development of the effective M cell-targeted vaccines against porcine post-weaning CD and/or CE in order to circumvent weak or tolerogenic responses to orally applied *E. coli* vaccines as described earlier (FRANCIS and WILLGOHS, 1991). It is now recognized that levamisole-adjuvanted live oral F4ac+ non-ETEC vaccine induced both intestinal secretory (JANJATOVIĆ et al., 2008) and cellular immunity (BOŽIĆ et al., 2006), offering solid protection to pigs vaccinated against CD.

Since the M cells play a central role in initiation of the intestinal immune responses, it has been postulated that cross talk between these cells, and underlying dendritic cells and lymphocytes is crucial for determining the outcome of protective immune responses *versus* tolerogenic responses (EL BAHI et al., 2002; MILLER et al., 2007). MIYAZAWA et al. (2006) suggested that committed M cells differentiate to mature M cells by contact with lymphocytes at the FAE periphery. As it has been recognized that lymphoepithelial interactions and soluble factors provide important signals for differentiation of M cells (NEUTRA et al., 2001; EL BACHI et al., 2002), it would be reasonable to assume that levamisole-adjuvented F18ac+non-ETEC vaccine candidate strain might influence *de novo* formation of porcine intestinal villous M cell phenotype by stimulating the activation of lymphocyte subsets in the lamina propria and Peyer's patches of weaned pigs as we have reported earlier (BOŽIĆ et al., 2003a, b; BOŽIĆ et al., 2006; KOVŠCA JANJATOVIĆ et al., 2009). It is very likely that levamisole is a crucial agent for such up-regulation of M cells proliferation since the drug when given alone produced similar effect in this model system

(VALPOTIĆ et al., 2010). However, the interaction between levamisole-adjuvanted F4ac+ non-ETEC vaccine and porcine intestinal villous M cells did not result in up-regulated proliferation of these cells. The main reason for this differences seen among two non-ETEC strains might be the presence or absence of M cell-targeting gene products, *i.e.* the long polar fimbria which play important role in the selective adherence of *E. coli* to M cells. For example, the rabbit diarrheagenic *E. coli*-1 strain is selective for adherence to M cells (GEBERT et al., 1996), whereas enteropathogenic *E. coli* is not transcytosed by the M cells and remains in the gut lumen (SIEBERS and FINLAY, 1996). Accordingly, it seems likely that F4ac+ non-ETEC strain did not readily attach to the M cells, and thus, was not able to stimulate their proliferation as F18ac+non-ETEC strain did. Indeed, the former *E. coli* strain failed to stimulate increased proliferation of M cells when given alone (VALPOTIĆ et al., 2010) or in the combination with levamisole as an adjuvant.

It is concluded that levamisole-adjuvanted F18ac+non-ETEC vaccine may affect *de novo* differentiation of porcine M cells by increasing their number in the ileal villous epithelium, indicated that the vaccine probably utilizes these cells as a target for entry/delivery to underlying lymphocytes and induces protective mucosal immunity against CE. Since the intestinal villous M cells are functionally analogous to the FAE-residing M cells (JANG et al., 2004) and may compensate for their functions independently of Peyer's patches, we assume that our finding of up-regulated proliferation of these cells following the inoculation of weaned pigs with adjuvanted-vaccine against porcine postweaning CE might be of relevance to the process of delivery to the immune cells populating intestinal lamina propria as well as to its immunogenicity/protective ability.

Acknowledgements

The current study was supported by the grants nos. 053-0532265-2255, 053-0532265-2248 and 053-0481153-1129 from the Ministry of Science, Education and Sport of Croatia. The excellent technical assistance of Mrs. Zrinka Benčina is gratefully acknowledged.

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Received: 22 February 2010
Accepted: 29 October 2010

KOVŠCA JANJATOVIĆ, A., G. LACKOVIĆ, F. BOŽIĆ, H. VALPOTIĆ, M. TOMINAC-TRCIN, S. SLADOLJEV, B. ŠEOL, I. VALPOTIĆ, M. POPOVIĆ: *De novo* diferencijacija M stanica crijevnih resica u odbijene prasadi imunizirane pokusnim cjepnim F4ac+ ili F18ac+ neenterotoksigenim sojevima bakterije *Escherichia coli* s levamisolom kao adjuvansom. Vet. arhiv 81, 77-90, 2011.

SAŽETAK

Aktivna imunizacija odbijene prasadi protiv kolidijareje (KD) i/ili kolienterotoksemije (KE) uzrokovane F4ac+ i/ili F18ac+ enterotoksigenim sojevima bakterije *Escherichia coli* (ETEC) još je uvijek neriješen problem. Crijevne mikronaborane (M) stanice imaju funkciju pri ulasku/invaziji intraluminalnih patogena (kao što su ETEC sojevi), unosu uzoraka antigena i pogodovanju tvorbi imunosti na probavne infekcije. Činjenica da ETEC sojevi rabe M stanice kao ulazna vrata za uzročnike infekcija, kao što su KD i/ili KE, a njihova velika sposobnost transcitoze čini ih ujedno pogodnim stanicama za unos mukoznih cjepiva, adjuvanata i lijekova. Cilj ovoga rada bilo je vrednovanje učinaka pokusnih cjepnih sojeva F4ac+ i F18ac+ ne-ETEC, s levamisolom kao adjuvansom, na pojavnost i brojnost M stanica u ileumu, te na poticanje unosa antigena nakon *de novo* tvorbe tih stanica u odbijene prasadi. Prasad iz uobičajenoga uzgoja, u dobi od 4 tjedna, bila je razvrstana u tri skupine od kojih su dvije parenteralno/oralno imunizirane levamisolom (-2., -1. i 0. dana pokusa) u kombinaciji s jednim od dva pokusna vakcinalna ne-ETEC soja (0. dana pokusa). Treća je skupina primila fiziološku otopinu kao placebo. Izazivačka je infekcija provedena s F4ac+ ETEC sojem (7. dana pokusa), a prasad je usmrćena (13. dana pokusa) radi uzimanja uzoraka za imunohistologiju. Distribucijski obrasci M stanica, pozitivnih na biljeg za citokeratin 18, pokazuju da su pretežito raspršene između enterocita, a manje ih je u malim nakupinama, te da se većina tih stanica nalazi pri vrhu resica ileuma prasadi u dobi od 6 tjedana. Morfometrijska kvantifikacija M stanica u sluznici ileuma pokazuje da prasad koja je prethodno dobivala levamisol i imunizirana F18ac+ ne-ETEC sojem ima značajno veći broj ($P<0,01$) M stanica u usporedbi s vrijednostima dobivenima u kontrolne neimunizirane prasadi. Udjel M stanica u te prasadi bio je povećan za 145%. U skupini prasadi prethodno obrađene levamisolom i imuniziranih F4ac+ ne-ETEC sojem zabilježen je samo blagi porast (za 7%) udjela M stanica. Međutim, taj porast nije bio statistički značajno različit od vrijednosti dobivenih u kontrolne prasadi. Naš nalaz da F18ac+ ne-ETEC vakcina s levamisolom kao adjuvansom može pospješiti *de novo* diferencijaciju M stanica time što povećava njihovu brojnost u ileumu, upućuje na povećanu sposobnost tih stanica da unose pokusni vakcinalni soj i tako dostavljaju imunogen do obližnjih limfocita što potiče zaštitnu imunost protiv KE. Međutim, trebalo bi objasniti izostanak sličnog učinka na M stanice u prasadi koja je primila F4ac+ ne-ETEC vakcinu s levamisolom kao adjuvansom.

Ključne riječi: M stanice, adjuvantnost levamisola, *E. coli*, vakcine, odbijena prasad