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Adjuvant activity of levamisole for experimental F18ac$^+$ *Escherichia coli* oral vaccine against porcine post-weaning colibacillosis

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ABSTRACT

It has been suggested that the *Escherichia coli* strain expressing F18 fimbriae possesses lower capability of stimulation of immune responses in weaned pigs than the F4$^+$ *E. coli* strain. In order to overcome this problem, we used levamisole as a candidate mucosal adjuvant for an experimental F18ac$^+$ *E. coli* oral vaccine. We hypothesized that levamisole may exert its adjuvant activity in weaned pigs vaccinated with experimental F18$^+$ *E. coli* oral vaccine by triggering the immune effector sites of the gut-associated lymphoid tissue (GALT), such as the mesenteric lymph node (MLN). Therefore, flow cytometry was used to analyze CD25, SWC7, and SWC9 activation antigens expression on the surfaces of MLN and spleen T cells, B cells and macrophages, respectively, isolated from levamisole-primed F18ac$^+$ non-enterotoxigenic *E. coli* (ETEC)-vaccinated (N = 5) or sham-vaccinated (N = 5) challenge-inoculated weaned pigs. Our results have shown that levamisole synergizes experimental F18ac$^+$ *E. coli* oral vaccine in stimulating activation of CD25$^+$ T cells, SWC7$^+$ B cells, and SWC9$^+$ macrophages preferentially in the MLN of challenged weaned pigs.

Key words: levamisole**,** cell-mediated immune response, weaned pigs, vaccination, colibacillosis

Introduction

Porcine post-weaning diarrhoea (PWD) and oedema disease (OD) induced by F4$^+$ or F18$^+$ enterotoxigenic *Escherichia coli* (ETEC) strains are economically the most significant diseases of swine in the World (FAIRBROTHER et al., 2005). The effective prevention of these infections is still an unsolved problem. It is desirable to produce a mucosal vaccine that would protect against *E. coli* infections through the stimulation of

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the mucosal immune system (WANNEMUEHLER and GALVIN, 1994; VAN DEN BROECK et al., 1999). However, vaccination can fail not only because of the immaturity of the immune system in the pigs and the added immunodepression induced by stress at the time of weaning (ROTH, 1999), but also because of inappropriate porcine intestinal immune cells activation upon specific oral immunization with the vaccinal F4$^+$ *E. coli* strain (BOŽIĆ et al., 2000 and 2002a). Equally, although fimbriae F18 have been proposed as potential candidates for vaccine antigens against OD (SARRAZIN and BERTSCHINGER, 1997; BERTSCHINGER et al., 2000), oral immunization of weaned pigs with F18 fimbriae did not induce a protective immune response against the challenge infection (VERDONCK et al., 2007).

Several strategies have been devised to overcome these limitations, such as the use of an adjuvant and delivery systems. In this context, almost four decades ago, RENOUX and RENOUX (1971) observed an increased response to *Brucella* vaccination in mice treated with levamisole [2, 3, 5, 6-tetrahydro-6-phenylimidazole (2,1-b) thiazole], suggesting that this anthelmintic drug may also act as an adjuvant for preventive vaccines (VAN WAUWE and JANSSEN, 1991). Indeed, levamisole administered in combination with DNA viral vaccines against foot and mouth disease, porcine respiratory reproductive syndrome or severe acute respiratory syndrome, induced a strong cell-mediated and antibody response (JIN et al., 2004 and 2005; KANG et al., 2005). Levamisole has also been shown to exhibit potent adjuvant activity for DNA vaccine against *Schistosoma japonicum* infection, enhancing the protective immunity induced by the vaccine and reducing liver pathology (WANG et al., 2008). Most importantly, priming by levamisole of weaned pigs vaccinated with experimental F4$^+$ *E. coli* oral vaccine stimulates their mucosal immune system and abrogates the inefficacy of vaccination against colibacillosis induced by the vaccine alone (BOŽIĆ et al., 2003 and 2006; KOVŠCA JANJATOVIĆ et al., 2008). On the other hand, an attempt was made to overcome the inefficacy of experimental F18$^+$ *E. coli* oral vaccine by microencapsulating F18 fimbriae into poly (lactide-co-glycolide) microspheres (FELDER et al., 2001). However, oral immunization of weaned pigs with microspheres containing F18 fimbriae did not induce a significant immune response, nor reduced F18$^+$ *E. coli* colonization following a challenge infection.

A very recent report based on immunohistochemical and histomorphometric data suggests that non-ETEC strain expressing F18 fimbriae have the capability of stimulating cell-mediated immune responses in the gut of weaned pigs primed with levamisole (KOVŠCA JANJATOVIĆ et al., 2009). We hypothesized that levamisole may exert its adjuvant activity in these pigs by triggering immune effector sites of the gut-associated lymphoid tissue (GALT). Therefore, flow cytometry was used to investigate whether levamisole synergizes experimental F18ac$^+$ *E. coli* oral vaccine in stimulating T cell, B

cell and macrophage activation antigens expression in the mesenteric lymph node (MLN) and spleen of weaned pigs.

Materials and methods

*Experimental animals.*Ten commercial crossbred pigs were purchased from a swine farm near Zagreb, Croatia, weighed and randomly assigned to 2 groups of 5 pigs each, immediately after weaning at 4 weeks of age. The piglets were housed in the animal facility at the Veterinary Faculty University of Zagreb and fed with a standard weaner diet.

*Bacterial strains.*The attenuated F18ac$^+$ non-ETEC vaccinal strain 2143 (serotype O157:K119:F18ac) and the challenge F4ac$^+$ ETEC strain 11-800/94 with authentic F4ac plasmid (serotype O149:K91:F4ac:987P: Hly$^+$ LT$^+$ STb$^+$) were used for immunization and challenge infection, respectively (KOVŠCA JANJATOVIĆ et al., 2009). The vaccinal *E. coli* strain was obtained from Dr B. Nagy, Veterinary Medical Institute of Hungarian Academy of Sciences, Budapest, Hungary and the challenge F4ac$^+$ ETEC strain was isolated from diarrheic pigs reared on swine farms in Croatia. Both strains were kept in a glycerine broth at -80 °C until used. The vaccine candidate strain was attenuated by a special culturing procedure as described elsewhere (GORDON et al., 1992; KOVŠCA JANJATOVIĆ et al., 2009).

Experimental design. All treatments of pigs were conducted in accordance with the "Directive for the Protection of Vertebrate Animals used for Experimental and other Purposes" (86/609/EEC). On the second day post-weaning the pigs were intramuscularly primed with levamisole (Nilverm®; Pliva, Zagreb, Croatia) in an immunostimulatory dose of 2.5 mg/kg given daily, over 3 consecutive days (BRUNNER and MUSCOPLAT, 1980). Immediately after the last levamisole dose was given, one group of pigs (experimental group) was intragastrically vaccinated with 10^{10} colony forming units per mL (CFU/ml) of F18ac$^+$ non-ETEC vaccinal strain 2143 in 60 mL of Trypticase soy broth (TSB) and the other one (control group), housed separately, was administered TSB only, according to the same schedule. Seven days later, all pigs were challenge-inoculated with 10^{10} CFU/mL of F4ac$^+$ ETEC isolate 11-800/94 (O149:K91:F4ac:987P, Hly^{+++}, LT$^+$, STb$^+$) isolated from diarrheic pigs reared on swine farms in Croatia. The pigs were euthanatized with T-61® (Hoechst, München, Germany) on post-challenge (p.c.) day 6, and MLN and spleens collected for lymphoid cells isolation.

Isolation of mononuclear cells. Spleens and MLN were aseptically removed from each animal immediately after slaughter for isolation of mononuclear cells, as previously described (BOŽIĆ et al., 2002c). Briefly, immediately after excision, the collected spleens and MLN were placed into modified Eagle's medium with Hanks (MEM-H; Institute of Immunology, Zagreb, Croatia). Mononuclear cells were isolated by perfusing and teasing

tissue in MEM-H medium, followed by density gradient separation over Lymphoprep (Nycomed, Oslo, Norwey) and centrifugation at 750× *g* for 25 min. The cells were than collected and washed once with MEM-H medium. The cells were more than 95% viable as determined by propidium iodide staining.

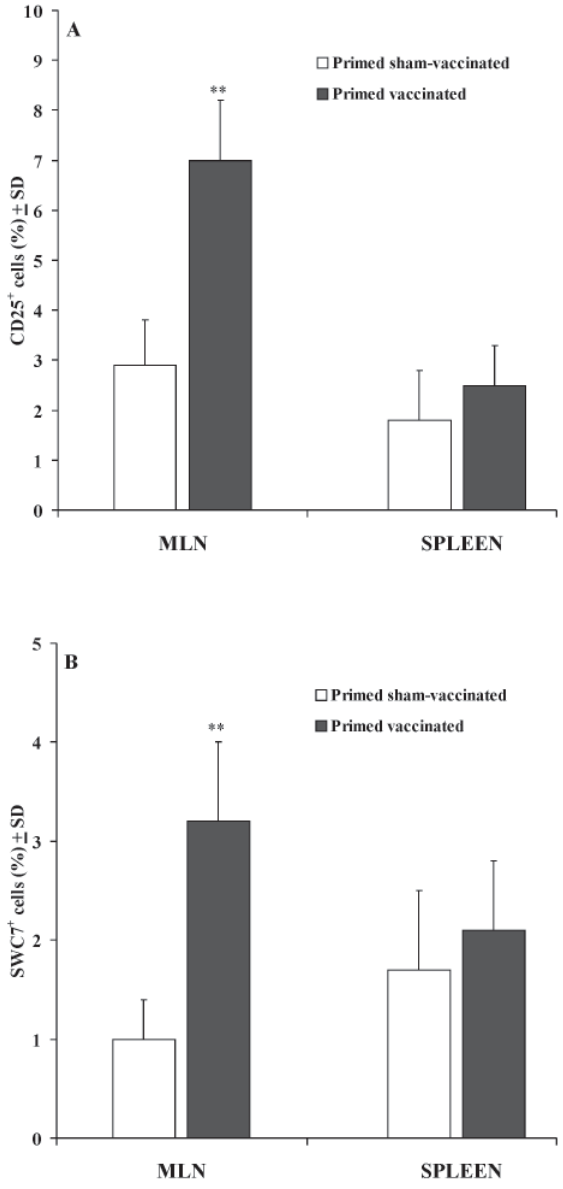
Monoclonal antibodies. Monoclonal antibody K231.3B2 recognising α chain of the porcine interleukin-2 receptor (IL-2R; CD25) (BAILEY et al., 1992) was donated by Dr C. R. Stokes (University of Bristol, Bristol, UK). Anti-swine mAbs to swine workshop cluster (SWC)7 (clone IAHCC55) and SWC9 (clone C4), tested at the 2nd Swine Workshop, Davis, CA, USA, 1995 (DENHAM et al., 1998; DOMÍNGUEZ et al., 1998), were donated by Drs C. Howard (BBSRC, IAH, Compton, Newbury, Berkshire, UK), and J. Dominguez (CISA-INIA, Madrid, Spain), respectively.

Analysis of cells by flow cytometry. Single cell suspensions were prepared and incubated with mAbs (50 μL/10^6 cells) used in single colour flow cytometry to determine the percentage of positively staining cells. The fluorescence of the mAbs labelled cells was analyzed by using an EPICS C flow cytometer (Coulter Electronics, Hialeah, FL, USA), as described earlier (BOŽIĆ et al., 2002c). Flow cytometric analysis of the positively stained cells expressing CD25, SWC7 or SWC9 antigens was performed for each animal and the data presented as arithmetic mean ± standard deviation (mean ± SD).

Statistical analysis. Levels of significance between the levamisole-primed vaccinated and primed sham-vaccinated challenge-infected groups of pigs were determined by the two-tailed Student's *t*-test and a value of $P \leq 0.05$ was considered significant.

Results

Dichotomy in leukocyte activation antigens expression in the MLN and spleen. To gain insight into the activation state of T and B cells as well as macrophages in the MLN and spleen of the two groups, the surface expression of the leukocyte activation markers CD25, SWC7 and SWC9, respectively, was analyzed. The data of the quantitative phenotypic analysis of the isolated cells show that both groups contained a comparable low percentage (< 5%) of $CD25^+$ (Fig. 1a), $SWC7^+$ (Fig. 1b) and $SWC9^+$ spleen cells (Fig. 1c), there being no difference in $CD25^+$ and $SWC7^+$ cells from primed vaccinated, or primed sham-vaccinated, challenge-infected weaned pigs. However, the number of spleen cells expressing the SWC9 activation antigen increased significantly ($P \leq 0.01$) in primed-vaccinated as compared with primed sham-vaccinated pigs (Fig. 1c). In the control group of weaned pigs, the proportions of the MLN $CD25^+$, $SWC7^+$ and $SWC9^+$ activated T and B cells, and macrophages, respectively, were generally as low as those detected in the spleen. However, in primed vaccinated *vs.* primed sham-vaccinated challenge-infected pigs, the number of MLN cells expressing the CD25 (Fig. 1a), SWC7 (Fig. 1b) and SWC9 (Fig. 1c) activation antigens increased significantly ($P \leq 0.001$).


A
Primed sham-vaccinated
Primed vaccinated
**
CD25+ cells (%) ± SD
10
9
8
7
6
5
4
3
2
1
0
MLN
SPLEEN
B
Primed sham-vaccinated
Primed vaccinated
**
SWC7+ cells (%) ± SD
5
4
3
2
1
0
MLN
SPLEEN

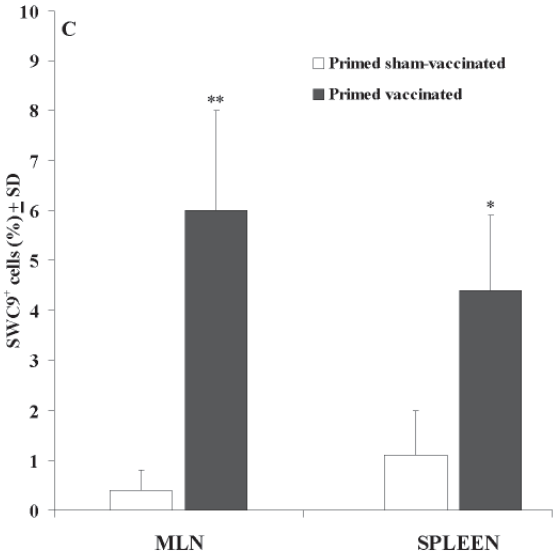


Fig. 1. The proportion of $CD25^+$ (A), $SWC7^+$ (B) or $SWC9^+$ (C) MLN and spleen cells of the levamisole-primed $F18ac^+$ non-ETEC-vaccinated or sham-vaccinated challenge-inoculated weaned pigs. Pigs were treated i.m. with levamisole in a immunostimulatory dose of 2.5 mg/kg given daily, for 3 consecutive days, prior to the vaccination or sham-vaccination (day 0). Seven days later, all pigs were challenged with the $F4ac^+$ ETEC strain and euthanatized on post-challenge day 6. Significant difference between the two groups was at $^{**}P \leq 0.001$ and $^{*}P \leq 0.01$.

Discussion

Whilst ETEC infections of suckling pigs can be best prevented by maternal immunisation and by early supply of immune colostrum, active immunisation against PWD of pigs induced by ETEC strain expressing F4 or F18 fimbriae, both of which are regarded as important in PWD, is much more difficult (MOON and BUNN, 1993; NAGY and FEKETE, 2005). The main reasons for these difficulties are numerous, so that several immunisation strategies exist to circumvent these problems. About decade ago we started to test the immunomodulatory activity of levamisole and what we found was that the drug may act as a potent mucosal adjuvant for the vaccine candidate $F4ac^+$ non-ETEC strain (BOŽIĆ et al., 2003). Interestingly, levamisole only exerts its immunopotentiating activity in vaccinated but not non-vaccinated animals, including weaned pigs (SCHIJNS, 2001; SAJID et al., 2006; BOŽIĆ et al., 2002b and 2006).

Although fimbriae F18 have been proposed as potential candidates for vaccine antigens against PWD and OD, oral immunization of weaned pigs with F18 fimbriae or with microspheres containing them did not induce a protective immune response against challenge infection (FELDER et al., 2001; VERDONCK et al., 2007). These findings, together with work demonstrating the slower and lower induction of a fimbriae-specific immune response after an $F18^+$ verotoxigenic *E. coli* infection, as compared with an $F4^+$ ETEC infection (VERDONCK et al., 2002), indicate that F18 is less immunogenic than F4. It has been proposed recently that since PWD can be caused by $F4^+$ as well as by $F18^+$ *E. coli*, a combined vaccine against both infections would be appropriate (TIELS et al., 2008). The results of the latter study show that conjugating F18 fimbrial antigen to F4 fimbriae may improve the antigen delivery to the intestinal mucosa, subsequently leading to stimulation of a specific, but partly protective immune response against PWD in the pigs. In our model using levamisole as a candidate mucosal adjuvant for experimental $F18ac^+$ *E. coli* oral vaccine in weaned pigs, priming of the vaccinated pigs by the drug has a tendency to trigger the mucosal rather than systemic immune system and abrogates the inefficacy of vaccination against porcine PWD induced by the vaccine alone (KOVŠCA JANJATOVIĆ et al., 2009; this report).

The possible mechanism by which levamisole controls the selective induction of protective mucosal immune responses to a virulent ETEC strain may be the enhanced translocation of F18ac antigen to the major mucosal effector sites, e.g. MLN. In this context, it is well known that an antigen that does not reach the draining lymph node is not responded to and that immune responsiveness in the draining lymph node may be increased by an adjuvant (SCHIJNS, 2001). In the current study, increased CD25 and SWC7 expression observed on cells in the MLN of the primed vaccinated, *vs.* sham-vaccinated, challenge-infected pigs, suggests enhanced T- and B-cell-mediated immunity (BAILEY et al., 1992; DENHAM et al., 1998) induced by the potential synergistic action of the drug and vaccine. It is of note that initiation of immune responses in the porcine gut mucosa occurs in the organized structures of GALT, ultimately leading to T and B cell activation and proliferation within the MLN. The present study also shows that levamisole appeared to prime the macrophages in the draining MLN and spleen for interaction with non-ETEC-derived F18ac antigen. It is well known that macrophages play an important role in the host defence against bacteria immigrating into the MLN (GAUTREAUX et al., 1994). As the SWC9 molecule can be considered as an activation antigen exclusively expressed on porcine mature macrophages (DOMÍNGUEZ et al., 1998), it can be concluded that priming by levamisole eliciting recruitment of $SWC9^+$ macrophages in the MLN and spleens of experimental pigs improves their functions through enhanced cell maturation (AMERY, 1978). Taken together, it is not surprising that levamisole may contribute to immune protection from challenge-induced porcine PWD by stimulating activation of T cells, B cells and macrophages in the MLN of the primed vaccinated weaned pigs.

In conclusion, it has been demonstrated that levamisole co-administered with a viral, bacterial or parasitic vaccines stimulates T cell activation and increases the production of the antibody (JIN et al., 2004 and 2005; KANG et al., 2005; BOŽIĆ et al., 2003 and 2006; WANG et al., 2008; KOVŠCA JANJATOVIĆ et al., 2008). Besides, levamisole as an adjuvant can activate innate immunity via toll-like receptors (TLR)7/8 subsequently leading to enhancement of the adaptive immune responses (ZHANG et al., 2009), and can attenuate inflammatory response in the gut of weaned pigs experimentally vaccinated with a candidate F4ac$^+$ oral vaccine against colibacillosis (VALPOTIĆ et al., 2009). In addition, in spite of the belief that F18$^+$ *E. coli* is less immunogenic than F4$^+$, we have shown that this problem can be overcome by the use of levamisole (KOVŠCA JANJATOVIĆ et al., 2009). Specifically, what we found in the present study was that priming by levamisole of weaned pigs vaccinated with experimental F18$^+$ *E. coli* oral vaccine may improve the antigen delivery to the GALT (e.g. MLN), subsequently leading to stimulation of a specific protective immune response against challenge infection.

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SAŽETAK

Predmnijeva se da soj bakterije *Escherichia coli* koji nosi F18 fimbrije slabije potiče imunosnu reakciju u odbite prasadi nego soj F4$^+$. Kako bismo nadišli potonji problem, uporabili smo levamisol kao potencijalni mukozni adjuvans za pokusno peroralno cjepivo koje sadržava bakteriju *E. coli* koja nosi F18 fimbrije. Pretpostavili smo da će levamisol djelovati adjuvantno u odbite prasadi cijepljene pokusnim peroralnim cjepivom koje sadržava bakteriju *E. coli* koja nosi F18 fimbrije tako što će potaknuti imunosnu reakciju u izvršnim odjeljcima GALT-a (engl. gut-associated lymphoid tissue), primjerice u mezenterijskim limfnim čvorovima (MLČ). Slijedom toga, uporabili smo protočnu citometriju kako bismo analizirali ekspresiju aktivacijskih biljega na površini T limfocita (CD25), B limfocita (SWC7) i makrofaga (SWC9) izdvojenih iz MLČ i slezene odbite prasadi tretirane levamisolom cijepljene neenterotoksigenim sojem bakterije *E. coli* (N = 5) i lažno cijepljene odbite prasadi (N = 5) inficirane izazivačkim sojem. Rezultati su pokazali da levamisol djeluje sinergijski s pokusnim peroralnim cjepivom koje sadržava soj F18ac$^+$ bakterije *E. coli* u poticanju aktivacije CD25$^+$ T stanica, SWC7$^+$ B stanica SWC9$^+$ makrofaga ponajprije u MLČ odbite prasadi nakon izazivačke infekcije.

Ključne riječi: levamisol, stanična imunosna reakcija, odbita prasad, cijepljenje, kolibaciloza