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Modulation by levamisole of CD45RA and CD45RC isoforms expression in the gut of weaned pigs vaccinated against colibacillosis

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Apart from its anthelmintic activity in domestic food animals, levamisole is recognized and employed for its immunomodulatory activity. The drug seems to act primarily on cellular immune responses, enhancing immune activity specifically in immunocytes whose function is impaired (Brunner & Muscoplat, 1980; Mulcahy & Quinn, 1986). When used in a modified lymphocyte stimulation test, levamisole significantly potentiated the *Brucella abortus*-induced lymphocyte blastogenesis from unresponsive cattle (Kaneene *et al.*, 1981). Because in this latter study the potentiation was observed only when the drug was added to lymphocyte cultures prior to the addition of the *Brucella* antigen, the authors concluded that levamisole appeared to prime the lymphocytes and macrophages for interaction with antigen. Levamisole was also found to ameliorate the suppression of lymphocyte function seen in artificially reared pigs enhancing their responses to values comparable with those of sow reared controls (Hennessy *et al.*, 1987). Importantly, when administered before or together with antigen to piglets and young pigs, levamisole enhanced the secondary rather than primary immune responses (Reyero *et al.*, 1979). The authors suggested that levamisole stimulates the development of memory T or B cells in swine; however, this has never been proven directly. With the use of suitable monoclonal antibodies (mAbs) to distinguish naive and memory T cells in swine, this would be clarified. Antiswine mAbs specific for a truly memory phenotype, CD45RO, are not yet available.

Nevertheless, on porcine leukocytes at least four CD45 isoforms (CD45RAC, CD45RA, CD45RC and CD45RO with molecular weights of 240, 226, 210 and 190 kDa, respectively), produced by the alternative splicing of exons A, B and C within a single gene, can be expressed (Schnitzlein & Zuckermann, 1998; Zuckermann *et al.*, 1998). It has been shown that T cells from the normal porcine gut lamina propria (LP) are almost exclusively $CD45RA^-$ and $CD45RC^-$ and, by inference, $CD45RO^+$ (Bailey *et al.*, 1998; Haverson *et al.*, 1999). In the intestinal mucosa of weaned pigs vaccinated with a live attenuated oral vaccine against colibacillosis, cells expressing predominantly the CD45RA isoform are uncommon, and this was correlated with low protection of the pigs from clinical disease. Thus, vaccination probably failed not only because of the immaturity of the immune system in the pigs and immunodepression induced by stress at the time of weaning (Roth, 1999), but also because of weak immunogenicity of the vaccine eliciting an inadequate immune response in the intestinal mucosa dominated by naive $CD45RA^+$ cells. Moreover, in recent years there have been significant increases in the occurrence of antimicrobial resistance in a variety of pathogenic bacteria from pigs, including *Escherichia coli* O149 (Aarestrup *et al.*, 2000). Hence, porcine postweaning colibacillosis (PWC) induced by enterotoxigenic *E. coli* (ETEC) strains remains an important cause of morbidity (Nagy & Fekete, 1999), and another approach in order to protect weaned pigs against *E. coli* infections is needed.

We proposed that this problem could be overcome by the use of immunomodulatory agents, such as levamisole, but little is known about its effect in eliciting cell-mediated immune responses of pigs to vaccination against gastrointestinal pathogens. The aim of the present study, therefore, was to evaluate the priming effect of levamisole on CD45RA and CD45RC isoforms expression in the gut of weaned pigs vaccinated with $F4ac^+$ non-ETEC strain against ETEC-induced PWC. The study was approved by the Croatian Animal Research Authority.

Ten commercial crossbred piglets weighing 6.6 ± 1 kg were purchased from a swine farm near by Zagreb, Croatia. Piglets were randomly assigned to two groups of five pigs each, immediately after weaning at 4 weeks of age, and housed in the animal facility at the Veterinary Faculty University of Zagreb and fed with a commercial weaner diet. On the second day, postweaning pigs were intramuscularly primed with levamisole (Nilverm®; Pliva, Zagreb, Croatia) in immunostimulatory dose of 2.5 mg/kg given daily, in three consecutive days (Brunner & Muscoplat, 1980). Immediately after the last levamisole dose was given, one group was intragastrically vaccinated with 10^{10} CFU/mL of $F4ac^+$ non-ETEC vaccinal strain 2407 (Casey & Moon, 1990) in 60 mL of Trypticase soy broth (TSB) and the other one, 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 postchallenge day 6, and jejunal LP (JLP) and ileal Peyer's patch (IPP) collected for lymphoid cells isolation. The JLP and IPP leukocytes were isolated from pigs gut by Percoll (Sigma, St Louis, MO, USA) density gradient centrifugation as described by Bailey *et al.* (1998) and by Andersen *et al.* (1999a), respectively.

Monoclonal antibody 231.3B2 recognizing 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). Antiswine mAbs to CD45RA (clone STH267) and CD45RC (clone 3a56) isoforms (Zuckermann *et al.*, 1998) were donated by Dr M. Shimizu (Ibaraki, NIAH, Japan) and by Dr A. Saalmüller (Tübingen, FRCVDA, Germany), respectively. 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 positive staining cells. Analysis was performed as detailed by Haverson *et al.* (1999).

Flow cytometric analysis of the positively stained cells expressing CD25, CD45RA or CD45RC molecules was performed for each animal and the data presented as arithmetic mean ± standard deviation (mean ± SD). Levels of significance between 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.

In the gut of weaned pigs primed with levamisole, vaccination with F4ac$^+$ non-ETEC strain against ETEC-induced PWC, vs. sham-vaccination, was followed by dramatic changes in CD45RA and CD45RC isoform expression on the cell surfaces in the present study. Both groups of pigs contained a comparable low percentage (< 10%) of CD45RA$^+$ (Fig. 1a) and CD45RC$^+$ JLP cells (Fig. 1b), there being no difference in cells from primed vaccinated, or sham-vaccinated, challenge-infected pigs. By contrast, the IPP of the levamisole-primed sham-vaccinated ETEC-infected weaned pigs was dominated by naive cells, with approximately 70% CD45RA$^+$ cells (Fig. 1a). When compared with this latter group, the primed vaccinated challenge-infected group of the pigs exhibited a significant decrease in the expression of CD45RA isoform on IPP cells ($P \leq 0.005$). Conversely, CD45RC expression was increased significantly ($P \leq 0.05$) on the surfaces of IPP cells in the primed vaccinated, vs. sham-vaccinated, challenge-infected weaned pigs (Fig. 1b).

In order to assess the activation state of leukocytes in the intestinal mucosa of the two groups of pigs, surface expression of the lymphocyte activation marker CD25 (Bailey *et al.*, 1992) was analysed. As it can be seen from the data depicted in Fig. 2, the density of expression of CD25 on JLP and IPP cells isolated from the gut of levamisole-primed sham-vaccinated ETEC-infected group of the pigs was extremely similar and low. In the gut of primed vaccinated, vs. sham-vaccinated, challenge-infected weaned pigs the number of both JLP and IPP cells expressing the CD25 surface molecule increased significantly ($P = 0.002$ and $P = 0.004$, respectively).

The present study shows that pretreatment of both vaccinated and sham-vaccinated challenge-infected weaned pigs with levamisole induces no significant differences in the proportion of CD45RA$^+$ or CD45RC$^+$ JLP cells. Nevertheless, a relatively small number of these cells (less than 10%) observed among the two groups of pigs suggest that pretreatment of the pigs with levamisole may induce naive JLP cell priming and probably stimulates memory cell generation irrespective of the specific vaccination. A limited expression of CD45RA and CD45RC isoforms by LP lymphocytes in older normal swine suggests that these cells may be antigen experienced (Haverson *et al.*, 1999), and in a memory state, e.g. CD45RO$^+$ (Bailey *et al.*, 1998). Although there is no direct evidence for this speculation (because of lack of mAbs), it can be conceivable, because in mammalian T cells the alternative splicing of CD45 is regulated so that naive T cells predominantly express CD45RA isoform and switch to expression of CD45RO upon activation (Meeusen, 1998). In addition, most human LP T lymphocytes express the memory marker CD45RO (Brandtzaeg *et al.*, 1998). However, practically no JLP T cells express CD45RA, whereas approximately 5% of CD4$^+$ and up to 30% of CD8$^+$ cells express CD45RC in young pigs (Dr K. Haverson, personal communication) which may be in transition from naive to memory phenotype. Some degree of activation of JLP cells in levamisole-primed non-ETEC-vaccinated, vs. both sham-vaccinated (this report) and unprimed vaccinated (Božić *et al.*, 2001), challenge-infected weaned pigs was also supported by detection of low but definite levels of CD25, the lymphocyte activation marker (Bailey *et al.*, 1992, 1998; Haverson *et al.*, 1999).

In contrast to the finding in JLP, in IPP of vaccinated, vs. sham-vaccinated, challenge-infected weaned pigs priming with levamisole was found to influence significantly CD45RA and CD45RC isoforms expression in that more CD45RC$^+$ and fewer CD45RA$^+$ IPP cells can mean that CD45RA$^+$ cells developed CD45RC phenotype. Thus, one might expect levamisole to be able to activate porcine IPP cells when administered prior to specific vaccine. It is also possible that CD45RA$^+$ cells are emigrating or that CD45RC$^+$ cells are immigrating, as CD45RA$^+$ cells have not been proven directly to generate CD45RC phenotype as a consequence of activation in swine. However, it has been discussed that memory T cells can express CD45RC (Haverson *et al.*, 1999). Because CD45 isoforms are expressed on multiple cell types (Zuckermann *et al.*, 1998; Alexander, 2000; Haverson *et al.*, 2000), it is not possible to identify the IPP cell type expressing these isoforms with the use of single labelling. In this context it is important to note that although the pig IPP is a primary lymphoid organ generating B lymphocytes, this site is populated with a relatively high proportion (up to 5%) of CD3$^+$ T cells (Andersen *et al.*, 1999a). Interestingly, CD3 expression was increased on IPP T cells from approximately 5% in both levamisole-primed sham-vaccinated and unprimed F4ac$^+$ non-ETEC-vaccinated to approximately 10% in primed vaccinated weaned pigs (not shown). Also, increased CD25 expression observed on cells in the IPP of the primed vaccinated, vs. sham-vaccinated, challenge-infected pigs suggests enhanced T cell-mediated immunity

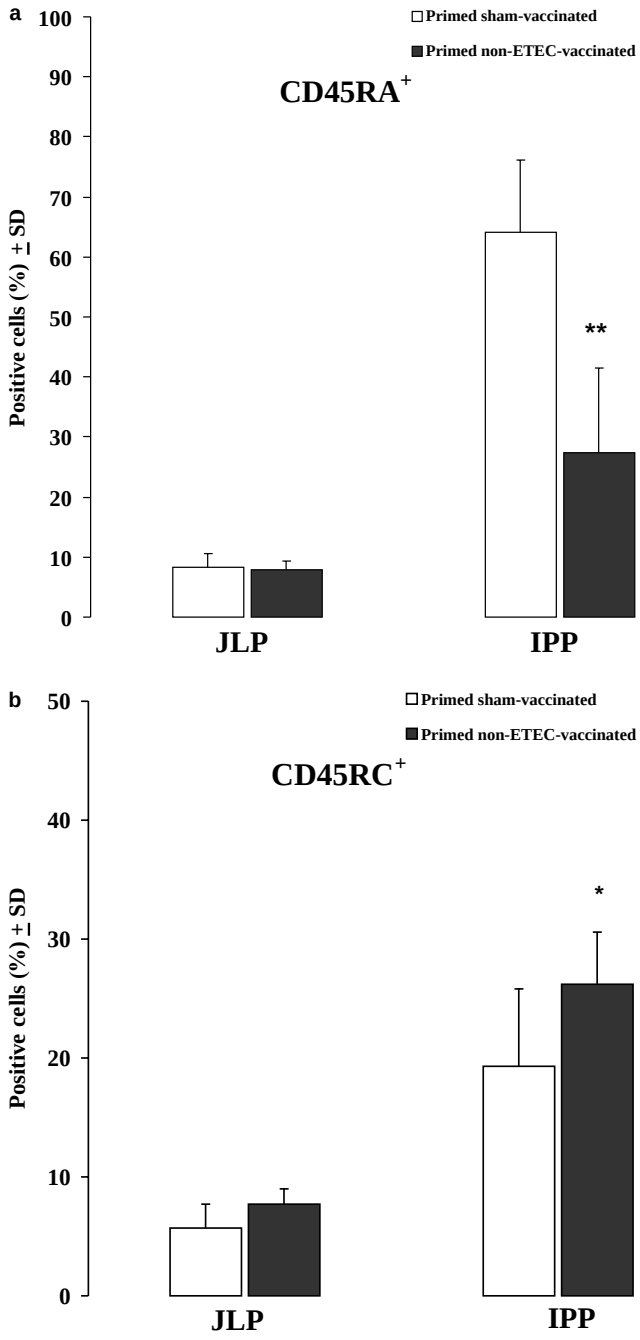

Fig. 1. CD45RA$^+$ (a) and CD45RC$^+$ (b) cells in the jejunal lamina propria (JLP) and ileal peyer's patch (IPP) of the levamisole-primed F4ac$^+$ non-ETEC-vaccinated or sham-vaccinated challenge-infected weaned pigs. Pigs were i.m. treated with levamisole in immunostimulatory dose of 2.5 mg/kg given daily, in three consecutive days, prior to the vaccination or sham-vaccination (day 0). Seven days later, all pigs were challenge-inoculated with F4ac$^+$ ETEC strain and euthanatized on postchallenge day 6. Significant difference between the two groups at $^{**}P \leq 0.005$ and $^{*}P \leq 0.05$.

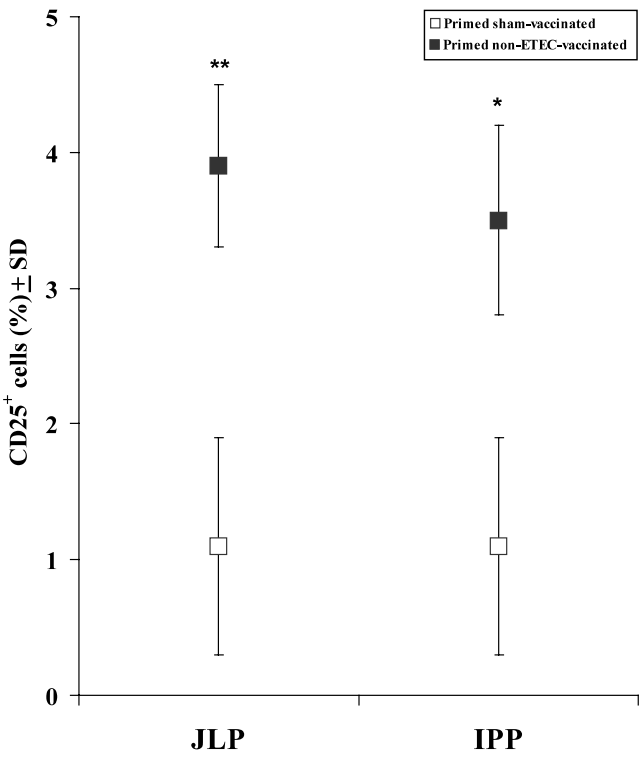

Fig. 2. The proportion of CD25$^+$ JLP and IPPcells of the levamisole-primed F4ac$^+$ non-ETEC-vaccinated or sham-vaccinated challenge-infected weaned pigs. Pigs were i.m. treated with levamisole in immunostimulatory dose of 2.5 mg/kg given daily, in three consecutive days, prior to the vaccination or sham-vaccination (day 0). Seven days later, all pigs were challenge- inoculated with F4ac$^+$ ETEC strain and euthanatized on postchallenge day 6. Significant difference between the two groups at $^{**}P \leq 0.002$ and $^{*}P \leq 0.004$.

in this B cell compartment induced by the potential synergistic action of the drug and vaccine. Apart from its low intensity of expression, it is well known that activated porcine intestinal T cells (Bailey *et al.*, 1998; Haverson *et al.*, 1999) and memory T cells in human intestinal mucosa (Brandtzaeg *et al.*, 1998) express CD25. However, as recently demonstrated by Andersen *et al.* (1999b), porcine IPP follicular B cells rescued from apoptosis also express CD25. Consistent with these results, there is possibility of activating both T and B cells in IPP of the vaccinated pigs by levamisole, and of promoting their expansion ultimately leading to the generation of T and/or B cell memory. Although further studies will clarify lymphoid cell types and their functional significance in eliciting the desired adaptive immune response, our present overall evidence suggest that CD45RA$^+$ IPP cells could develop CD45RC phenotype as a consequence of activation in swine.

In summary, apart from the fact that CD45RA and CD45RC expression is so complex that definitive conclusion regarding the changes observed is difficult, without dissecting the cell populations into T and B cells, the present study indicates that levamisole can reach mucosal immune sites very rapidly after intramuscular application and may act as a potent mucosal adjuvant during a specific vaccination of weaned pigs against PWC. Our results further implicate this potential action of the drug owing to its influence on the CD45 protein expression on the intestinal mucosa cell surfaces because CD45 is a tyrosine-specific phosphatase intimately involved in signal transduction through the T cell antigen receptor and through membrane

immunoglobulin on B cells (Alexander, 2000). Its adjuvanticity linked to the ability to stimulate memory/recently activated cells generation, where secondary contact with a specific antigen leads to a more intense response than the primary response, could be of practical importance in the establishment of protective immunity against ETEC and other gastrointestinal infections.

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