# Intestinal Immune Response of Weaned Pigs Experimentally Vaccinated with F4ac$^+$ Non-enterotoxigenic Strains of *Escherichia coli*

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**Abstract**

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The potential effects of two adjuvants - the copolymer of polyoxyethylene-polyoxypropylene (POE-POP) and N-acetylglucosaminyl-(β,1-4)-N-acetyl-muraminyl-L-alanyl-D-isoglutamine (GMDP) on mucosal humoral and cell-mediated immune responses were tested in weaned pigs immunized with the F4ac$^+$ non-enterotoxigenic *Escherichia coli* (non-ETEC) strain 2407 and challenged with the F4$^+$ ETEC strain 11-800/1/94. The presence of an anti-F4ac secretory IgA (sIgA) antibody in small intestinal washes was detected by the use of an indirect ELISA test, and the tissue-specific distribution patterns of CD3a$^+$ and CD21$^+$ T and B cells, respectively, within the gut mucosa were evaluated by an immunohistochemical *in situ* staining. The results showed that approximately 75% of 2407+POE-POP-treated pigs, and 50% to 60% of pigs that were immunized with either the 2407+GMDP or the 2407 strain alone respectively, were positive for anti-F4ac sIgA antibodies. The T cell antigen CD3a was expressed predominantly in lymphocytes in the ileal epithelium, the dome region of ileal Peyer's patches (IPP) and the extrafollicular areas of the mesenteric lymph node (MLN) in 2407$^+$GMDP or 2407-treated pigs. The B cells expressing a CD21 surface molecule were present in the interfollicular areas of MLN and the germinal centers of IPP in 2407-treated pigs. A strong reaction to sIgA was determined in plasma cells in the lumen of the crypts and in the cells residing the lamina propria (LP) of the jejunum and ileum of pigs immunized with the 2407 strain. The obtained results imply that oral immunization of weaned pigs with the F4ac$^+$ non-ETEC strain may induce the priming of intestinal immune cells, which can be additionally enhanced by the tested adjuvants.

*Weaned pigs, colibacillosis, non-ETEC vaccine, adjuvants*

Protection from porcine enteric colibacillosis is related to either the passive transfer of lactogenic immunity, or the induction of mucosal (intestinal) immunity, to prevent the development of neonatal or post-weaning diarrhea (Moon and Bunn 1993). Protection against intestinal microbial antigens is achieved by a combination of specific and non-specific defense mechanisms mediated by immune cells from the gut-associated lymphoid tissues (GALT) (Bailey et al. 1996; Husband et al. 1999). The normal route by which bacterial antigens such as enterotoxigenic *Escherichia coli* (ETEC) are taken up by the GALT is via the epithelial surface of the small intestine. However, an antigen presented in this way generates predominantly a suppressor response (Husband 1993). High doses of luminal antigens result in a clonal deletion or clonal apoptosis, while lower doses appear to generate active suppression mediated by CD8$^+$ or CD4$^+$ T cells in pigs (Bailey et al. 1997). The initial trigger to generate tolerance rather than an active response is still unclear. Recent studies have indicated that immunological components of porcine small intestinal lamina propria (LP) may also play a role in the maintenance of tolerance. The distribution of T cell subsets is highly compartmentalized, i.e. CD8$^+$ cells are located in the epithelium and CD4$^+$

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cells in the LP (Vega-Lopez et al. 1993; Bailey et al. 1996). Such organization appears to be centered around the capilliary plexus underlying the epithelial basement membrane, which in the pig expresses high levels of major histocompatibility complex (MHC) class II antigens (Wilson et al. 1996). These MHC class II bearing cell populations occur in a close apposition with highly differentiated homogeneous T cells of memory phenotype in the intestinal LP (Stokes et al. 1996). They may present antigenic peptides to $CD4^+$ and $CD8^+$ T cells, and this interaction may result in anergy or apoptosis of a highly commited differentiated $CD45RA^+$ and $CD45RC^+$ isoforms of CD45 (Božić et al. 2002).

Strategies designated to overcome this effect include the use of adjuvants, immune response modifiers, or delivery microsystems such as liposomes and microcapsules (Walker 1994).

Some of the most effective adjuvants used with vaccines have been derived from bacterial components. The glycopeptide N-acetylglucosaminyl-N-acetilmuramyl-L-alanyl-D-isoglutamine (GMDP) is a natural compound isolated from the cell walls of the well-known nutrient microorganism *Lactobacillus bulgaricus*. It is an aqueous phase adjuvant that does not have a depot effect, but also produces a minimal inflammatory response (Britsina et al. 1992).

The copolymers used as adjuvants in parenteral immunization have been shown to act by adherence to lipids, thereby promoting the retention of the protein antigen in local tissue and facilitating the uptake of the antigen by antigen presenting cells (Hunter et al. 1994). POE-POP is nonionic block copolymer composed of hydrophilic polyoxyethylene (POE) and hydrophobic polyoxypropylene (POP) (Zigterman et al. 1987).

In this study we determined the cellular and humoral intestinal immunity in weaned pigs after the inoculation with a recombinant non-ETEC strain as a source of the replicating antigen supplemented with two IRMs (GMDP or POE-POP) given as adjuvants. While microbial derivatives stimulate the Toll-like receptors and activate nonspecific immunity (Janeway and Medzhitov 2002), GMDP was applied before the treatment and POE-POP shortly after the priming in order to enhance humoral immunity.

## Materials and Methods

### Pigs

Thirty-two 4-week-old crossbred pigs (Swedish Landrace × Yorkshire) of both sexes, weighing 4.0 kg on average, were purchased from a commercial swine farm in Croatia. According to the previously determined adhesive phenotype, the pigs were classified in the group with a high occurrence of F4 receptors on the small intestinal enterocytes, and thus were considered susceptible to the infection with $F4^+$ *E.coli* strains (Valpotić et al. 1992). They were selected at weaning from the litters of either second or third parity sows. The pigs were randomly assigned to four experimental groups comprising eight animals each. They were managed, housed and fed as described previously (Sarmiento et al. 1988).

### Vaccination and pathogenic strains of *E. coli*

The non-ETEC fimbriated ($F4ac^+$) strain 2407 (O9:K36:H19:K88ac, LT-, STb-) was used as the vaccine candidate strain. A 60 ml of freshly grown culture medium (Trypticase soy broth (TSB) supplemented with 1.2% sodium bicarbonate) containing $1 \times 10^{10}$ viable *E. coli* per ml was given via orogastric tube to twenty-four 4-week-old pigs. Eight control animals received 60 ml of TSB only. On the post-vaccination Day 4, principal and control pigs were orally challenged with the $F4ac^+$ ETEC strain (O149:K91:K88ac:987P $Hly^+$ $LT^+$ $STb^+$).

### Adjuvant priming

Two vaccinated groups of pigs were primed with either N-acetylglucosaminyl-(β,1-4)-N-acetyl-muraminyl-L-alanyl-D-isoglutamine administered intramuscularly at the dose of 50 μg/2 ml (GMDP, Biotech, USA) on Day 0, or with 10 ml orally given copolymer of polyoxyethylene and polyoxypropylene (POE-POP, USA patent No. 5,234,683/1993) on Day 2 prior to the vaccination.

### Sampling

Small intestinal washes were obtained immediately after euthanasia, 8 days after the specific immunization, by taking 1 m of a jejunal segment. The segment was tightened at one end, filled with 100 ml of phosphate buffered saline (PBS) supplemented with 10 mM of EDTA and 1% of bovine serum albumin (BSA; Sigma, St. Louis, USA), and tightened at the other end. After incubation and shaking for 30 min, the intestinal wash was collected. After

removing larger components by centrifugation, the obtained supernatant was further centrifuged at 7000 *g* for 30 min in a refrigerated centrifuge at 4 °C (WKF, Brandau/Odw, Germany). The supernatant was supplemented with 0.01% sodium azide and frozen at -80 °C until used in the ELISA test.

Simultaneously, specimens of the jejunum, ileum (1 cm), and MLN (1 $cm^3$) were taken and fixed in methanol-chloroform-acetic acid (methacarn; 6:3:1, Kemika, Croatia) for 12 hours at room temperature, embedded in paraplast, cut into 6 ± 1 µm thick serial sections, and stored until processed for immunohistochemical staining of immune cells using monoclonal antibodies (mAbs) reactive with swine leukocyte CD antigens (Lacković et al. 1997; 1999).

ELISA

The presence of anti-F4ac secretory IgA (sIgA) antibody was detected by the use of a modification of the ELISA test described previously (Chandler et al. 1986; Sarmiento et al. 1988; Valpotić et al. 1989). Plates were prepared according to the method described previously (Place and Schroeder 1982). 200 µl of *E. coli* cells (2407 and 11-800/1/94) suspended in carbonate-bicarbonate buffer (15mM $Na_2CO_3$, 3.49 mM $NaHCO_3$; pH 9.6) at the concentration of $2 \times 10^9$ cells/ml were added in each well. Thereafter the plates were incubated for 2 hours at 37 °C, followed by incubation of 18 h at 4 °C. The plates were washed three times with PBS supplemented with 0.05% Tween-20 (PBS-T; Sigma, St. Louis, USA) and incubated with 100 µl of jejunal wash samples at 37 °C for 1 h. Secretory anti-F4ac IgA (sIgA) was detected by incubating the plates with 100 µl of goat anti-swine IgA-specific antibody labeled with peroxidase (Sigma, St. Louis, USA) diluted at 1:100 with PBS-T supplemented with 0.5% bovine serum albumine (BSA; Sigma, St. Louis, USA) at 37 °C for 30 min. The plates were washed once with PBS-T and three times with distilled water. Finally, the substrate 2-2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS; National Veterinary Service Laboratories, Ames, USA) was added and the 405nm absorption was spectrophotometrically measured after 1 hour incubation.

Immunohistochemistry

The monoclonal antibodies reactive with cell surface molecules in porcine lymphocytes, i.e. $CD3a^+$ T cells (FY2A11) and $CD21^+$ B cells (IAH CC51), kindly provided by Dr. Yang (IAH Pirbright Laboratory, Ash Road, Pirbright, Woking, GU24 ONF, UK) and Dr. Howard (BBSRC, IAH, Compton, Newbury, Berkshire, RG20 7NN, UK), were used for the immunohistochemical detection of $CD^+$ T and B cells by the avidin-biotin complex (ABC) method (Larrson 1989; Lacković et al. 1999). Rabbit anti-swine IgA antibody (DAKO, Copenhagen, Denmark) was used in an indirect peroxidase-antiperoxidase (PAP) method.

## Results

The indirect ELISA test revealed the presence of specific sIgA in intestinal washes of both principal and control pigs (Table 1). After testing with $F4ac^+$ ETEC, 75% of intestinal washes were positive in both the controls and the pigs immunized with the non-ETEC 2407 strain and primed with POE-POP. In pigs immunized non-specifically with GMDP and specifically with the vaccinal strain 2407, 50% of intestinal washes were found positive.

Table 1. The presence of anti-$F4ac^+$ sIgA antibody as detected by ELISA in the intestinal washes of weaned pigs non-specifically immunized with POE-POP or GMDP (Day -2 and Day 0, respectively) and specifically immunized (Day 0) with the $F4ac^+$ non-ETEC strain 2407. The control group received TSB only. Pigs were orally challenged (Day 4) with $F4ac^+$ ETEC strain and sacrificed following the immunization. Groups comprised 8 pigs each.

| | Specific sIgA (%)* | | | | | |
|---|---|---|---|---|---|---|
| Groups/Treatment | $F4ac^+$ ETEC** | | | $F4ac^+$ non-ETEC strain 2407 | | |
| | Positive | Suspect | Negative | Positive | Suspect | Negative |
| TSB+ETEC | 75 | 25 | 0 | 75 | 25 | 0 |
| 2407+ETEC | 40 | 40 | 20 | 60 | 0 | 40 |
| 2407+POE-POP+ETEC | 75 | 0 | 25 | 75 | 0 | 25 |
| 2407+GMDP+ETEC | 50 | 25 | 25 | 50 | 25 | 25 |

*Of totally screened samples obtained from intestinal washes.

**$2 \times 10^9$ of *E. coli* cells /ml were used as the antigen.

Similar data were obtained when the $F4ac^+$ non-ETEC 2407 strain was used as an antigen. Differences between the bindings to tested antigens were found in pigs vaccinated with the 2407 strain only. Namely, the specific sIgA was determined in 40% and 60% of samples when the ETEC or non-ETEC strains, respectively, were used as coating antigens.

Numerous $CD3a^+$ T-lymphocytes were visible mostly on the luminal surface

(intraepithelial lymphocytes) and in clusters between the Peyer's patch follicles of the ileum (Plate IX, Fig. 1). This T-lymphocyte subpopulation was more numerous in pigs that were specifically immunized with the 2407 vaccinal strain and primed with GMDP.

Equally distributed $CD3a^+$ T-lymphocytes, i.e. more numerous in paracortical and scarce in follicular regions of MLN, were found in both principal and control pigs (Plate X, Fig. 2).

On the contrary, $CD21^+$ B-lymphocytes were more numerous in MLN of pigs vaccinated with the 2407 strain as compared to the control pigs (Plate XI, Fig. 3).

A strong reaction to sIgA was exhibited by plasma cells in the lumen of the crypts and by these cells residing the lamina propria of the jejunum/ileum of the pigs immunized with the 2407 strain (Plate XII, Fig. 4).

## Discussion

In this study, the immunogenicity and protective properties of the $F4ac^+$ non-ETEC strain 2407 enhanced with the adjuvants GMDP and POE-POP in a specific intra-gastric immunization of weaned pigs followed by a challenge infection with the $F4ac^+$ ETEC strain were investigated.

The main hypothesis of this investigation is in concordance with the main strategies for the development of bacterial vaccines (Husband et al. 1999), and controlling of mucosal infections induced with ETEC strains (Husband 1993). Namely, we assumed that the F4 fimbrial antigen would elicit a protective intestinal immune response mediated by cooperative T and B cell subpopulations in IPP and MLN of pigs primed with selected adjuvants and immunized with the 2407 strain.

IgA antibodies provide an important first line of defense against mucosal pathogens by preventing their adherence to the epithelium. Since the rate of IgA secretion is directly proportional to the number of IgA plasma cells located in the submucosa (Husband et al. 1996), it is of an outmost importance to understand the factors which are responsible for the induction of IgA precursor cell recruitment. Majority of IgA plasma cell precursors arise in Peyer's patches, enter to circulation and then migrate to a variety of mucosal subepithelial sites. The activated (antibody secreting) lymphocytes in the intestinal mucosa show the $\alpha 4\beta 7$ integrin expression which redirects these cells to the mucosa (Kantele et al. 1996). It was found that both gut derived T and $Ig^+$ lymphocytes migrated to the lamina propria of the pigs, but only $IgA^+$ cells reached the intestinal mucosa (Rothkötter et al. 1999). There are well documented evidences that the IgA expression in the mucosa depends on cytokine induction in different stages: the isotype switching, selective localization, and proliferation among precursor cells in the induction sites (Husband et al. 1999). Therefore, the upregulation in the production of selected cytokines may be an important method to enhance the mucosal response. It was determined that peptidoglycan induces an interleukin-12 (IL-12) mRNA production and IL-12 secretion by mouse macrophages (Lawrence and Nauciel 1998). The smallest isolated structure of a bacterial cell wall with immunoadjuvant properties – MDP, when administered orally to mice, enhanced the production of inflammatory cytokines in response to various bacterial components (Takada et al. 2002). The proposed mechanism for the adjuvant activity of microbial derivatives is the stimulation of Toll-like receptors and the subsequent activation of signalling pathways that result in the induction of antimicrobial genes and inflammatory cytokines (Janeway and Medzhitov 2002). It is therefore reasonable to assume that the GMDP used in our study (which is chemically a derivative of MDP) acts through a similar pathway. Copolymers such as POE-POP evaluated in our study, have been used extensively as adjuvants for parenteral immunization (Brey 1995) and can elicit both cellular and humoral immunity as well as modulate the produced antibody isotype (Hunter et al. 1994). Our results showed the presence of anti-F4ac sIgA antibodies in 75% of 2407+POE-POP-treated

pigs, and 50% to 60% of pigs that were immunized with either the 2407+GMDP or the 2407 strain alone. A specific sIgA antibody was also found in 75% of the non-immunized, but challenged pigs. This implies a previous colonization of the piglets. Although the animals were supposed to belong to a swine population highly susceptible to the infection with $F4^+$ *E. coli* strains (Valpotić et al. 1992), and it is possible that they were already infected, the intention of this approach was to use farm animals, and not germ-free or specific pathogen-free ones, in order to project the future field efficacy of immunization. On the other hand, clinical data which are not shown here depict the beneficial role of immunization with the non-enterotoxigenic strain 2407 in preventing diarrhea. Namely, while the $F4ac^+$ ETEC strain induced diarrhea in 4/8 inoculated pigs, the 6/8, 7/8 and 8/8 pigs immunized with 2407 alone, 2407+ POE-POP and 2407+GMDP, respectively, remained healthy.

The phenotypic/functional expression of immune cell subsets and their distribution in porcine GALT following the specific immunization with the $F4ac^+$ non-ETEC strain 2407 against post-weaning colibacillosis and the challenge infection with the $F4ac^+$ ETEC strain 11-800/1/94 seems to be an antigen(s) driven process comprising both cellular and humoral protective immune responses at the site of the enteric infection, i.e. the jejunal/ileal mucosal surfaces or in the ileal MLN. The activation of T cells results in the release of a number of distinct cytokines or chemokines and the recognition of antigenic epitopes involving MHC class 1 or 2 molecules. Both the T-cell activation and the release of specific cytokines are involved in the process of the B-cell activation. Our results showed that the distribution of $CD21^+$ B cells in MLN correlated well with the presence of numerous plasma cells with a strong reaction to sIgA in the lumen of the crypts and the lamina propria of the jejunum/ileum of pigs immunized with the 2407 strain.

In 2407+GMDP treated pigs we observed more $CD3a^+$ T cells in the ileal epithelium and in the dome region of ileal Peyer's patches (IPP) than in the respective tissues of pigs of other groups. The CD3 molecule is a complex of four polypeptide chains γ, δ, ε and ζ, which provide the coupling between the antigen specific signal delivered by the T cell receptor and the intracellular activation pathways. According to the results of the Second International Swine CD Workshop (Pescovitz et al. 1998), anti-CD3a moAb used in our study reacts with CD3ε chain which is identified as an important cell surface protein involved in the signal transduction of activation signals in porcine T cells. The *in situ* increase of $CD2a^+$, $CD8a^+$ and $SWC1^+$ populations of jejunal and MLN T cells and the *in vitro* activation of lymphocytes from IPP and MLN of 2407 vaccinated pigs (Vijtiuk et al. 2002) additionally confirm the important role of T cells in regulating the mucosal response to the bacterial replicating antigen. We propose that the observed elevated presentation of CD3 molecule plays a role in the surveillance of the *in situ* suppressor response.

In the present study, the vaccinal candidate strain of *E. coli* might cause the priming of the gut immune cells, particularly when the adjuvants POE-POP or GMDP were given through the adherence of the fimbrial antigen. It can be recommended as a potential source of the replicating antigen(s) for the development of a live oral vaccine against swine coli-diarrhoea.

## Imunitní odpověď buněk střeva odstavených selat experimentálně vakcinovaných $F4^{ac+}$ enteronetoxigenním (non-ETEC) kmenem *Escherichia coli*

Byl testován možný vliv dvou adjuvans, kopolymeru polyoxyethylenu-polyoxypropylenu (POE-POP) a N-acetylglukosaminyl-(β,1-4)-N-acetyl-muraminyl-L-alanyl-D-izoglutaminu (GMDP), na humorální a buněčnou slizniční imunitní odpověď u selat imunizovaných enteronetoxigenním $F4^{ac+}$ kmenem *Escherichia coli* (non-ETEC) 2407 a čelenžovaných kmenem ETEC $F4^+$ 11-800/1/94. Přítomnost sekrečních anti- F4ac protilátek IgA (sIgA) ve výplašcích z tenkého střeva byla zjišťována pomocí nepřímého ELISA testu. Tkáňově-specifický způsob distribuce $CD3^{a+}$ a $CD21^+$ T a B buněk ve střevní sliznici byl zjišťován pomo-

cí imunohistochemického barvení in situ. Výsledky ukázaly, že pozitivních na přítomnost anti-F4$^{ac}$ sIgA protilátek bylo přibližně 75 % selat ošetřených 2407 + POE-POP a 50-60 % selat imunizovaných buď 2407 + GMDP nebo samotným kmenem 2407. U selat ošetřených 2407 + GMDP nebo 2407, byl v lymfocytech sliznice, místu Peyerových plaků (IPP) a v extrafolikulárních částech mezenteriálních lymfonodů (MLN), exprimován hlavně CD3$^{a}$ antigen T buněk. Mezi folikuly MLN a v germinativních centrech IPP u selat ošetřených 2407 byly přítomny B buňky exprimující povrchovou molekulu CD21. Silná reakce na sIgA byla zjištěna u plazmatických buněk ve střevních kryptách a v buňkách lamina propria (LP) jejuna a ilea u selat imunizovaných kmenem 2407. Získané výsledky naznačují, že perorální imunizace ostavených selat F4ac+ non-ETEC kmenem může aktivovat imunitní buňky střeva, a testované adjuvans může jejich počet ještě více zvýšit.

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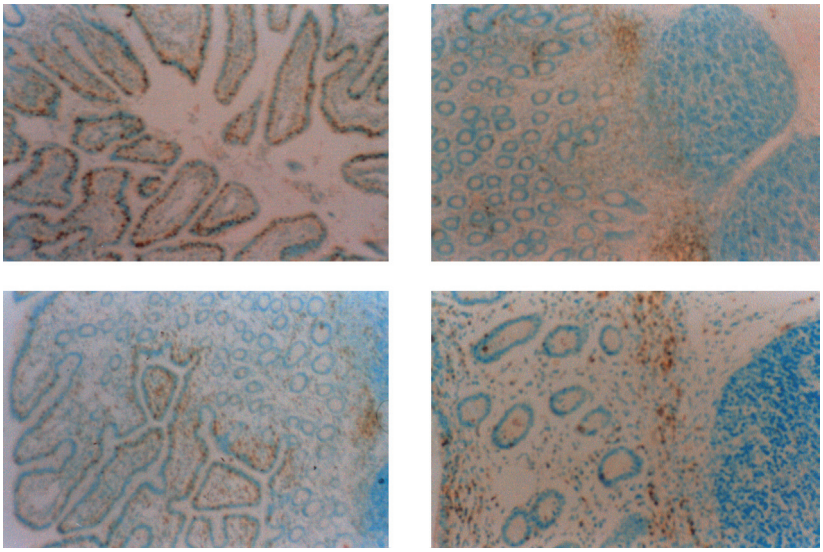

Fig. 1. The intraepithelial localization of $CD3a^+$ T cells in the ileum (IEL) of the pig eight days after the immunization. The aggregates of these cells are also present between the Peyer's patches (PPL). The ileal samples were stained with a CD3a antibody (mAb FY2A11) using the ABC method with 3.3-diaminobenzidine tetrachloride (DAB) as a chromogen; × 60.
a) Pigs non-specifically immunized with GMDP, specifically immunized with the $F4ac^+$ non-ETEC strain 2407 (Day 0), and orally challenged (Day 4) with the $F4ac^+$ ETEC strain 11-800/1/94.
b) TSB-treated pigs on Day 0 and orally challenged with the $F4ac^+$ ETEC strain 11-800/1/94 on Day 4 (control group).

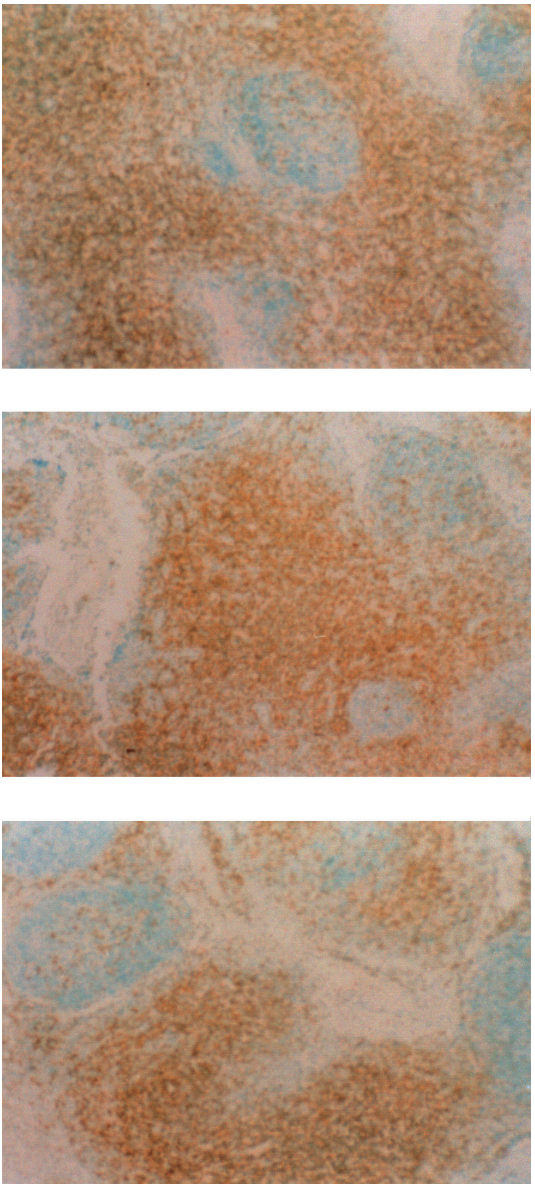

Fig. 2. CD3a$^+$ T cells detected by mAb FY2A11 in the MLN of pig eight days after the immunization. Numerous CD3a$^+$ T cells are visible in the extrafollicular areas of MLN, several T lymphocytes are visible in the lymphatic follicles; × 60. Staining was performed with the ABC method using DAB as a chromogen.
a) Pigs non-specifically immunized with GMDP, specifically immunized with the F4ac$^+$ non-ETEC strain 2407 (Day 0), and orally challenged (Day 4) with the F4ac$^+$ ETEC strain 11-800/1/94.
b) Pigs specifically immunized with the F4ac$^+$ non-ETEC strain 2407 (Day 0), and orally challenged (Day 4) with the F4ac$^+$ ETEC strain 11-800/1/94.
c) TSB-treated pigs at Day 0 and orally challenged with the F4ac$^+$ ETEC strain 11-800/1/94 on Day 4 (control group).

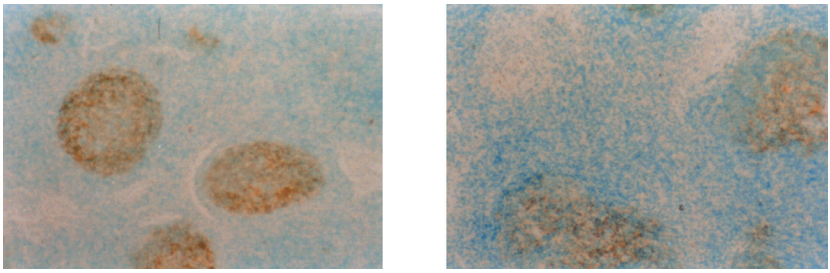

Fig. 3. The intrafollicular localization of $CD21^+$ B cells in the MLN of the pig eight days after the immunization, detected by mAb IAHCC51 by the ABC method using DAB as a chromogen; $\times 60$.
a) Pigs specifically immunized with the $F4ac^+$ non-ETEC strain 2407 (Day 0), and orally challenged (Day 4) with the $F4ac^+$ ETEC strain 11-800/1/94.
b) TSB-treated pigs on Day 0 and orally challenged with the $F4ac^+$ ETEC strain 11-800/1/94 at Day 4 (control group).

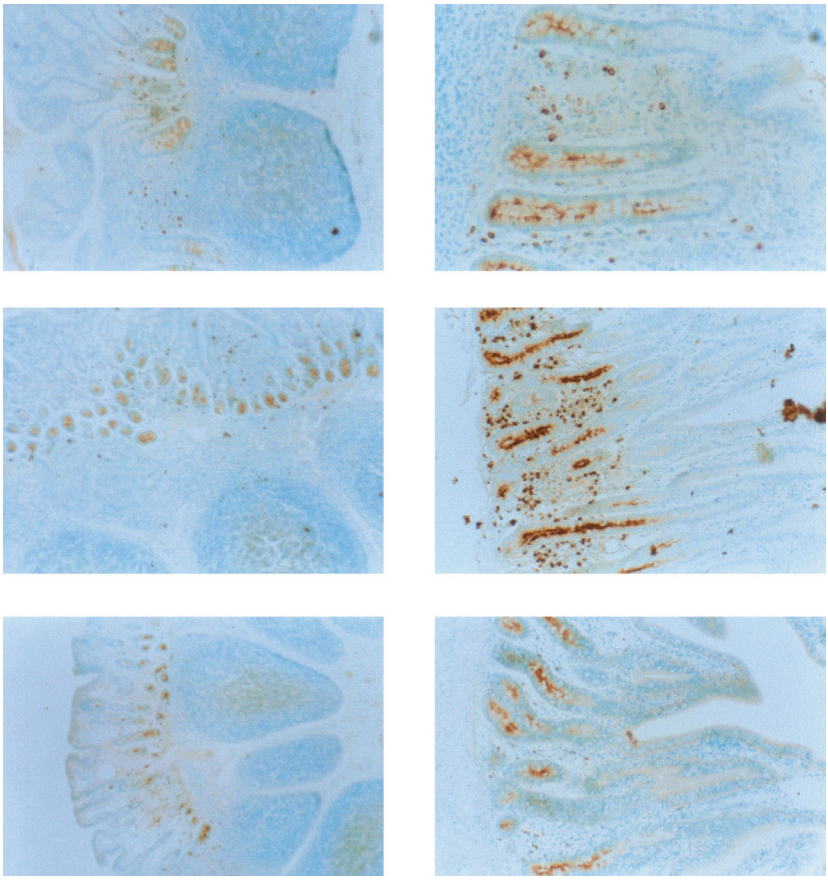

Fig. 4. sIgA and IgA$^+$ plasma cells demonstrated by rabbit-anti swine IgA polyclonal antibody in the ileum (I) and jejunum (J) of the pig eight days after the immunization, by the PAP method using DAB as a chromogen. Positive sIgA reaction is visible in the lumen of crypts; numerous IgA+ plasma cells are present in the lamina propria around the crypts; × 150.
a) Pigs non-specifically immunized with GMDP, specifically immunized with the F4ac$^+$ non-ETEC strain 2407 (Day 0), and orally challenged (Day 4) with the F4ac$^+$ ETEC strain 11-800/1/94.
b) Pigs specifically immunized with the F4ac$^+$ non-ETEC strain 2407 (Day 0), and orally challenged (Day 4) with the F4ac$^+$ ETEC strain 11-800/1/94.
c) TSB-treated pigs at Day 0 and orally challenged with the F4ac$^+$ ETEC strain 11-800/1/94 on Day 4 (control group).