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Immunostimulatory effects of anionic alkali mineral complex solution Barodon in porcine lymphocytes

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The anionic alkali mineral complex solution, Barodon (Barodon-S.F. Corp., Korea), was evaluated for its effectiveness as a nonspecific immunostimulator in pigs. The effects of Barodon were determined by analysis of feed efficiency, growth rate, and phenotype of leukocyte subpopulations using monoclonal antibodies specific to porcine leukocyte differentiation antigens and flow cytometry (FC). The study was focused to investigate the change in proportion of the $CD4^+CD8^+$ double positive T lymphocyte subpopulation (dpp) which exists uniquely in pigs. In addition, the mitogen-stimulated lymphoproliferative response, tissue distribution in lymphoid organs and the adjuvant effect of Barodon on hog cholera vaccine efficiency were determined. The study has revealed the average daily gain rates and feed conversion rates were significantly ($p<0.05$) improved in either group of pigs fed with 0.05% Barodon-spray feed (Tx-1) or pigs fed with 3% Barodon-fermented feed (Tx-2) in comparison with group of pigs fed with feed containing no Barodon (control). The proportion of cells expressing $CD4^+$ antigen in Barodon-treated group increased from 3 weeks posttreatment and was significantly higher ($p<0.05$) than that of control at 8 weeks posttreatment. Particularly, the significantly higher proportion was maintained from 8 weeks through 13 weeks posttreatment in Tx-1 group ($p<0.05$). The proportion of cells expressing $CD8^+$ antigen was significantly higher at 3 weeks posttreatment in Tx-2 ($p<0.01$). Proportion of MHC class II-expressing cells was significantly higher in Tx-1 and Tx-2 group at 11 weeks and 8 weeks posttreatment ($p<0.05$), respectively. In addition, the proportion of Non T/Non B (N) cells was also significantly higher in Tx-2 at 3 weeks posttreatment ($p<0.01$) and maintained to 13 weeks posttreatment ($p<0.1$). Between Barodon-treated groups, the proportion of MHC class II-expressing cells was observed to be larger in Tx-2 than Tx-1 from 3 weeks to 8 weeks posttreatment ($p<0.05$). However, there were no significant difference in the proportions of $CD2^+$ cells, B cells, monocytes and granulocytes between Barodon-treated and control group during the experiment. Dual-color FC analysis, study has revealed an increased proportion of dpp present in lymphocytes obtained from peripheral blood (PB) and mesenteric lymph node (MLN) of Barodon-treated group at 8 and 11 weeks posttreatment. The proportion of dpp in PB was 27.5% and 32.1% in Tx-1 and Tx-2, respectively, but only 2.2% in control group at 8 weeks posttreatment. In MLN, the proportion was 45.1% and 52.1% in Tx-1 and Tx-2, respectively, otherwise 16.5% in control group at 8 weeks posttreatment. The mitogen-stimulated activity was significantly higher in Tx-1 than in the control group at 11 weeks posttreatment when cells were stimulated with Con A and PHA, respectively ($p<0.01$). Also, Con A-, PHA- and PWM-stimulated activity was significantly higher in Tx-2 than in the control group at the same time ($p<0.05$). The tissue distribution of $CD4^+$, $CD8^+$ and $CD4^+CD8^+$ dpp in MLN and spleen was significantly larger in Tx-1 and Tx-2 than in the control group ($p<0.01$). Also, a larger proportion of dpp was observed in Tx-2 than Tx-1 in spleen between Barodon-treated groups ($p<0.01$). In conclusion, the study has demonstrated that Barodon had an immunostimulatory effect on pigs through proliferation and activation of porcine immune cells, specially $CD4^+CD8^+$ dpp lymphocytes.

Key words: Barodon, Immunostimulator, porcine immune cells

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Introduction

There has been an increasing demand in the food animal industry for drugs which leave no residue in meat because of concern about antibiotic-resistance problems in humans [1, 17]. Alternative methods such as nonspecific immunostimulators (NIS), synthetic peptides, natural herbs and fermentative microorganism are being evaluated with new interest [4, 8, 10, 13, 21]. Recently, anionic alkali mineral complex solution, Barodon, was introduced to animal farms to improve the productivity. The composition and characteristics of Barodon are based on minerals including Si, Ag and Na, K ions as an alkali (pH 13.5) solution. Although Barodon was patented in US as an anionic solution and also registered in Korea, the exact mechanism of Barodon and its effect to host animal is unknown. This study was designed to evaluate Barodon as a nonspecific immunostimulating agent in pigs. A set of monoclonal antibodies specifically reactive with porcine leukocyte differentiation antigens and flow cytometry were used to determine the proportion of leukocyte subpopulations. Lymphoproliferative responses of immune cells from peripheral blood, mesenteric lymph node and spleen were examined in pigs treated with Barodon. To investigate the specific cell types which may respond to Barodon, two-color fluorescence flow cytometry and immunohistochemical analysis using monoclonal antibodies of different isotypes were used to react with lymphocytes from peripheral blood and lymphoid tissues.

Our studies show that Barodon has an immunostimulatory effect on porcine immune cells and in particular, porcine $CD4^+CD8^+$ double positive T lymphocytes, the population which is important in activation in the porcine defensive system.

Materials and Methods

Experimental animals and experimental design

A total of fifty healthy feeder pigs at 15 weeks age were used for the study. The pigs were three breed-mixed (Yorkshire × Landrace × Duroc), and were divided into three groups. Ten heads were control group fed with feed without Barodon (Agribrands Purina Korea Inc., Korea). Twenty heads were fed with 0.05% Barodon-spray in the same animal feed as the control (Tx-1). Another 20 heads were fed with 3% Barodon-fermented animal feed (Tx-2). Each group of animals was fed for 9 weeks for the study. Daily weight gain, feed conversion rate and feed consumption were measured for 6 weeks in each group.

Collection of peripheral blood and lymphoid tissues

About 20 ml of blood were collected from each animal using vacuum tube (Becton Dickenson Vacutainer System, Rutherford, NJ) to measure leukocyte subpopulations and mitogenesis assay. A total of 9 pigs were sacrificed for the collection of mesenteric lymph nodes and spleen for mitogenic assay and immunohistochemistry (Fig. 1).

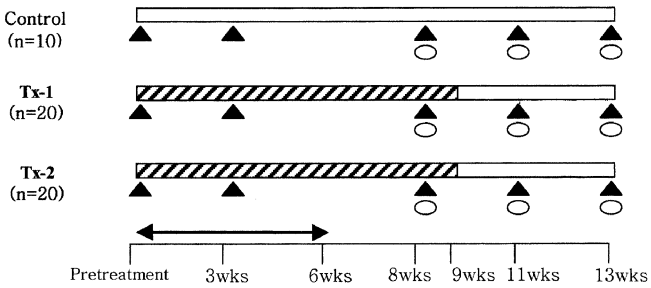

Fig. 1. Experimental design.
Control : Barodon-Nonfed
Tx-1 : Barodon 0.05% spray feed
Tx-2 : Barodon-additive 3% added feed
▨ : Barodon added feed supplementation
▲ : Blood collection for analysis of leukocyte subpopulation
○ : Mesenteric lymph nodes & spleen collection for mitogenesis and immunohistochemistry
↔ : Average daily gain & Feed efficiency check

Nonspecific immunostimulator Barodon

Composition of anionic mineral complex solution, Barodon, is shown in Table 1. The product was patented in US (patent No. 005571460-) and in Korea (patent No. 128110). The specific gravity of the product was 1.43 and pH was 13.5.

Proportion of porcine leukocyte subpopulations

A set of monoclonal antibodies specifically reactive with porcine leukocyte differentiation antigens and flow cytometry was used to examine the proportion of leukocyte subpopulations in peripheral blood from each group.

Preparation of peripheral blood leukocytes : Separation of peripheral blood leukocytes was done by the method of Davis *et al*. [7]. Briefly, collected blood was mixed with

Table 1. Composition of major ingredients for Barodon

Ingredient	Amount
Na_2SiO_3	600 g
K_2CO_3	300 g
Na_2CO_3	9 g
$Na_2B_4O_7$	9 g
$C_{12}H_{22}O_{11}$	q. s.*
$AgNO_3$	q. s.
NaCl	q. s.
$Na_2S_2O_3$	0.12 g
H_2O	1000 ml

*q.s.: quantum satis

Table 2. A panel of monoclonal antibodies specifically reactive with swine leukocyte differentiation antigens

mAb[a]	Isotype of mAb	Molecules[b]	Cell type[c]	Reference
PT85A	IgG_{2a}	MHC class I	All nucleated cells	[7]
H42A	IgG_{2a}	MHC class II	Antigen presenting cells	〃
TH81A5	IgG_{2a}	MHC class III	Antigen presenting cells	〃
MSA4	IgG_{2a}	Po CD2	T cells	〃
PT90A	IgG_{2a}	Po CD4	Th/i cells	〃
PT81B	IgG_{2b}	Po CD8	Tc/s cells	〃
PIg45A	IgG_{2b}	sIgM	B cells	〃
PT79A	IgG_{2a}	γδTCR	N cells	〃
DH59B	IgG_1	Granulocyte+Monocyte	Granulocyte+Monocyte	〃

[a]mAb: Monoclonal antibodies specifically reactive with leukocyte differentiation antigen
[b]Molecules: Porcine leukocyte differentiation molecules
[c]Cell type: Cells expressing molecules

equal volume of acid-citrate dextrose (ACD)-ethylenediamine tetraacetic acid (EDTA) and leukocytes were separated using Hypaque Ficoll (d:1.086, Sigma, St. Louis, MO, USA) density gradient centrifugation at 1,500 rpm for 30 min and cells were counted by the trypan blue exclusion technique and final concentration was adjusted to 1×10^7 cells/ml.

Monoclonal antibodies (mAbs) specific to porcine leukocyte differentiation antigens : A panel of mAbs specifically reactive with porcine leukocyte differentiation antigens is shown in Table 2. The mAbs specific to major histocompatibility complex (MHC)- class I, class II, porcine (Po)-CD2, PoCD4, PoCD8, surface (s)IgM, NonT/NonB (γδTCR), granulocyte and monocyte were used to examine the proportion of leukocyte subpopulations. Mouse anti-pig CD4-FITC conjugate (isotype; IgG_2b, Southern Biotechnology Associates Inc. 4515-02) was used in dual color analysis.

Flow cytometry (FC) analysis : The proportion of leukocyte subpopulations was determined by FC (FACSCalibur, Becton Dickinson, USA) using CellQuest program. About 50 μl (15 μg/ml) of mAbs was reacted with 100 μl of cells at 1×10^7 cells/ml concentration in a V-bottomed 96 well microplate. After the first incubation on the ice for 30 min, plates were washed three times with first washing buffer [PBS 450 ml, ACD 50 ml, 20% NaN_3 5 ml, gamma globulin free horse serum (Sigma) 10 ml, 250 mM EDTA 20 ml, and 0.5% phenol red 1 ml] with centrifugation at 1,700 rpm for 5 min. The pellet was disrupted by vortexing and mixed with 100 μl of ×200 dilution of FITC-conjugated goat anti-mouse IgG + IgM antibody (Caltag Lab, Burlingame, CA, USA) and incubated on the ice for 30 min in the dark. The pellets were then washed 3 times with second washing buffer (same as the first washing buffer excluding horse serum) by centrifugation at 1,700 rpm for 5 min. After final washing, the pellets were mixed with 200 μl of 2% PBS-formaldehyde (38% formalin 20 ml, PBS 980 ml) and kept at refrigerator for FC analysis.

For the dual color analysis, a pair of FITC or pycoerythrin (PE) conjugated-PoCD4 or -PoCD8 mAbs (Southern Bio-

Table 3. Growth performance of pigs fed with experimental diets

Growth Performance	Groups		
	Control(10[a])	Tx-1(10)	Tx-2(10)
Initial wt.(kg)	70.80±6.63[b]	71.35±4.87	68.20±7.14
Final wt.(kg)	106.20±8.38	108.75±5.13	105.20±6.11
Wt. Gain(kg)	35.40±2.58	37.40±1.76	37.00±2.28
ADG(g)	842.86±61.42	890.48±41.92	880.95±54.29
Feed intake(kg)	113.95	116.30	111.17
ADFI(g)	2713.10	2769.05	2647.62
Feed/gain	3.22	3.11	3.01

Control: Leantec grower feed (Product manufactured by Agribrands Purina Korea, Inc.).
Tx-1: Barodon 0.05% spray feed
Tx-2: 3% Barodon-fermented feed
[a]No. of pigs
[b]Mean±SD

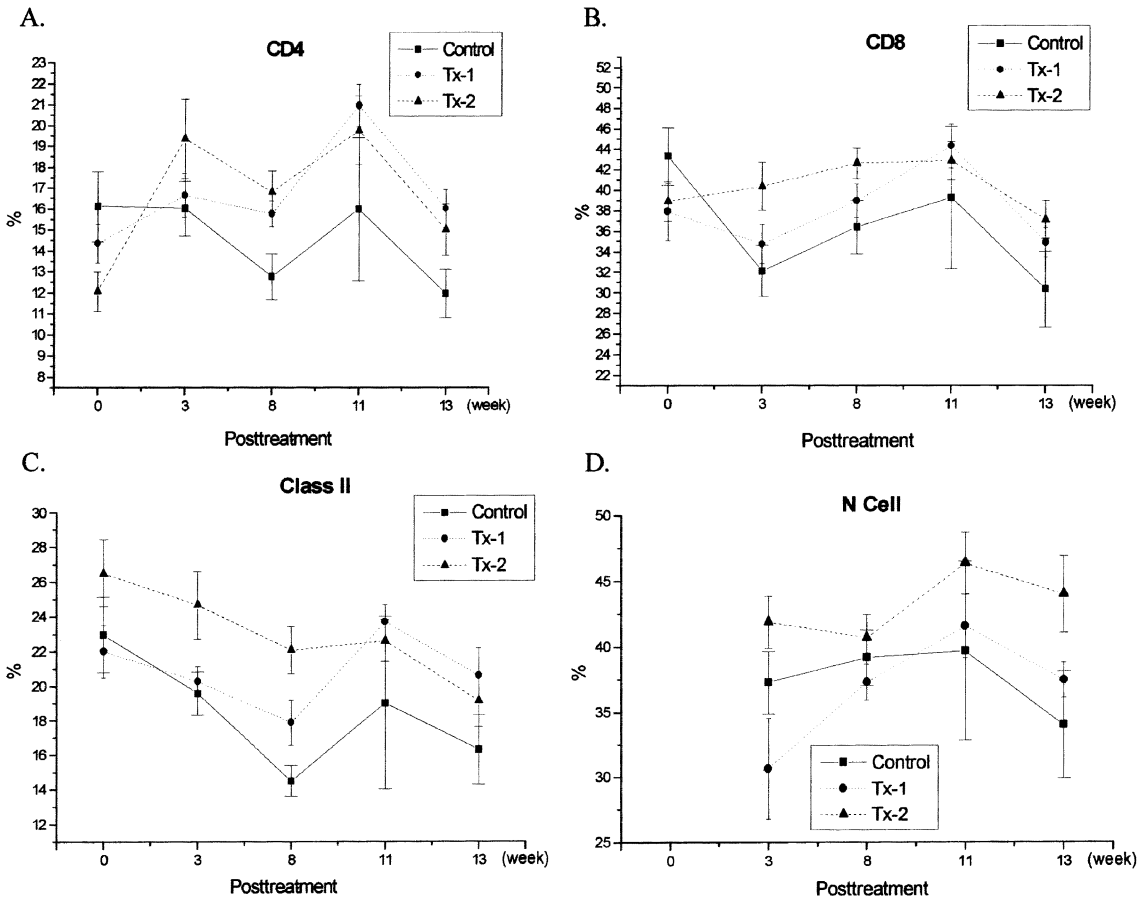

Fig. 2. Changes of proportion of porcine $CD4^+$(A), $CD8^+$(B), MHC-Class II(C), and N Cell(D) lymphocyte subpopulation at posttreatment with Barodon-spray feed (Tx-1), Barodon-fermented feed (Tx-2) and Barodon-nonfed group.

technology Associates Inc., Birmingham, AL, USA) with different isotypes were used as second step reagents.

Mitogen-stimulated lymphoproliferative responses

Peripheral blood leukocytes : Porcine peripheral blood leukocytes were prepared by Davis *et al.* and Salack-Johnson *et al.* methods [7, 20]. The final concentration of cells was adjusted to 1×10^7cells/ml.

Mesenteric lymph node leukocytes : Mesenteric lymph node was separated and fat was removed before mincing and passing through a 40 mesh sterile screen. The cells were washed 2-3 times with PBS and final concentration of cells was adjusted to 1×10^7cells/ml.

Lymphoproliferative response assay : Concanavalin A (Con A, Sigma), phytohemagglutinin (PHA, Sigma), pokeweed mitogen (PWM, Sigma) and *Salmonella typhimurium* lipopolysaccharide (LPS, Sigma) were diluted to optimal concentration. One hundred μl of 1×10^7cells/ml of each cells was reacted with the same volume of Con A (5 μl/ml), PHA (50 μl/ml), PWM (2.5 μl/ml) or LPS (10 μl/ml) in 96-well flat-bottomed microplates. After incubation at 37°C, 5% CO_2 for 72 hrs, 1 μCi [^{3}H]-thymidine (6.7 Ci/mmol, New England Nuclear Co., Boston, MA, USA) was added and the plates were incubated another 18 hrs. Cells were harvested at glass fiber filter strips (BRANDEL Inc., Gaithergurg, MD, USA) using a cell harvester (Cambridge Techonology, Inc., Watertown, MA, USA) and transferred to the scintillation counter (Wallac Oy, Turku, Finland) after being mixed with 3 ml of scintillation cocktail. Lymphoproliferative responses were measured by stimulation index (SI) described by the previous reports [5, 12, 14, 19].

Immunohistochemistry

Mesenteric lymph nodes and spleen were removed from pigs in each group and fixed for about 12 hrs at 10% buffered formaldehyde solution followed by another 12 hrs fixation at fresh 10% buffered formaldehyde solution. Fixed tissues were dissected and embedded in paraffin by auto-processor. The tissues were mounted at 'probe-on plus' slides (Fisherbiotech, Pittsburgh, PA, USA), deparaffinized using xylene (Sigma) and then subsequently dehydrated with 100%, 95%, 80%, 70%, and 60% ethyl alcohol solution. Fresh 0.3% Hydrogen peroxide was added to inhibit

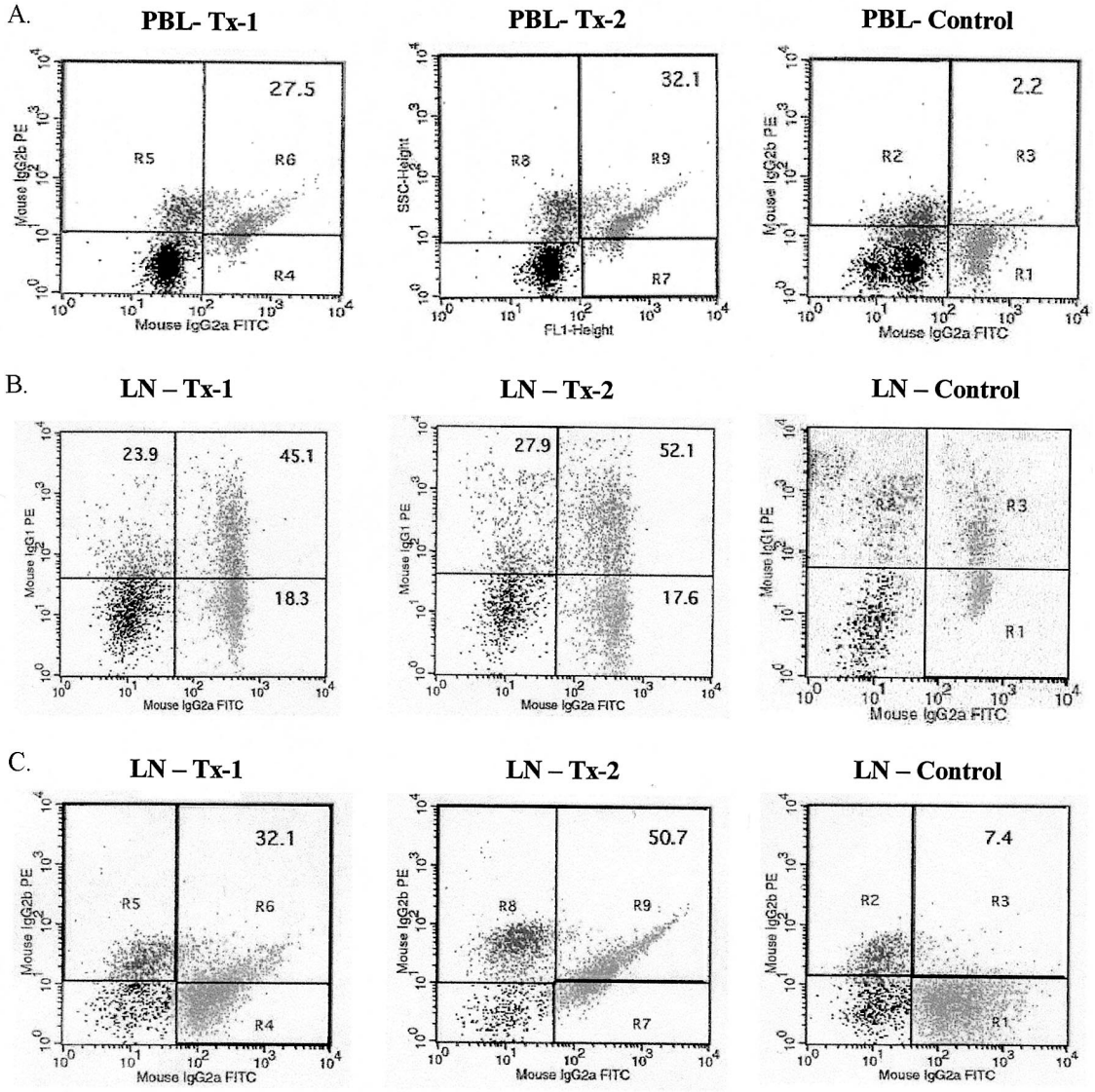

Fig. 3. Proportion of $CD4^+CD8^+$ double positive populations (dpp) in peripheral blood at 8 weeks posttreatment (A), mesenteric lymph nodes at 8 weeks (B), and mesenteric lymph nodes at 11 weeks (C) with Barodon-spray, Barodon-fermented feed or in Barodon nonfed group. PBL-Tx-1 (LNTx-1); lymphocyte subpopulations in peripheral blood (mesenteric lymph nodes) in group fed with Barodon-spray feed, PBL-Tx-2 (LNTx-2); lymphocyte subpopulations in peripheral blood(mesenteric lymph nodes) in group fed with Barodon-fermented feed, PBL-Control (LNControl); lymphocyte subpopulations in peripheral blood(mesenteric lymph nodes) of Barodon nonfed group

*Each number in quadrants indicates the proportion of $CD4^+CD8^+$ (upper right), $CD4^+$ (upper left), $CD8^+$ (lower right) and $CD4^-CD8^-$ (lower left) subpopulations

endogenous peroxidase activity in the tissues. The slides were washed using TTB (Tris buffer with 0.5% Triton-X 100) solution followed by blocking with TTB with 3% normal horse serum (NHS/TTB) and Avidin-Biotin solution (Vector Labs, Burlingame, CA, USA). The mAbs PT81B and PT90A (5 μg/ml) were added to the slides and incubated at room temperature for about 1 hr before being washing and blotted with TTB. Secondary antibody (biotinylated horse anti-mouse IgG, Vector Elite Mouse ABC kit) diluted in TTB was added and incubated at room temperature for 40 min. ABC reagent (avidin DH & biotinylated horseradish peroxidase, reagent A & B) was diluted to 1 : 250 in TTB 30 min prior to washing and blotting. Tissue slides were reacted with the diluted ABC reagent for 40 min. The slides were washed with TTB and then reacted either with AEC substrate (Vector Labs) for CD4 or Nikel-added DAB substrate (Vector Labs) for CD8 for 10-15 min and washed with distilled water (D.W). Slides were counterstained using hematoxylin for 2 min and washed with D.W. Aqueous (Biomeda Corp. Foster, CA,

USA) and non-aqueous mounting medium (Vector Labs) for AEC and DAB substrates were added to slides, respectively.

In simultaneous staining of one tissue for CD4 and CD8 antigen, reaction was made for CD8 first and CD4 second, and slides were mounted with aqueous mounting medium (Biomedia Corp). Thirty different portions of mesenteric lymph node and spleen were randomly selected for measuring the proportions of $CD4^+$ or $CD8^+$ or $CD4^+CD8^+$ dpp T lymphocyte populations using Optima 6.5 Program with Image analyzer (Olympus, USA) [18].

Statistical analysis

ANOVA and Students *t*-test were used to compare the difference among Tx-1, Tx-2, and control group during the entire period of experiment using Microcal™ Origin™ 5.0 (Microcal Software Inc., Northampton, MA, USA).

Results

Feed efficiency, weight gain and productivity

In comparison with control, daily weight gain was improved to 5.65% in Tx-1 and 4.52% in Tx-2 group, respectively. Feed efficiency rate was also improved and the rate was 3.22, 3.11 and 3.01 in control, Tx-1 and Tx-2 group, respectively (Table 3).

Flow cytometry analysis

The proportion of porcine leukocyte subpopulations : The change of proportion of porcine leukocyte subpopulations was investigated using mAbs and FC. The proportion of cells expressing $CD4^+$ antigen in Barodon-treated group increased from 3 weeks posttreatment and was significantly higher ($p<0.05$) than that of the control group at 8 weeks posttreatment. Particularly, the significantly higher proportion was maintained from 8 weeks through 13 weeks posttreatment in the Tx-1 group ($p<0.05$) (Fig. 2A). The proportion of cells expressing $CD8^+$ antigen was significantly higher at 3 weeks posttreatment in Tx-2 ($p<0.01$), however, no significant difference was observed from 8 weeks posttreatment (Fig. 2B).

The proportion of MHC class II-expressing cells was significantly higher in the Tx-1 and Tx-2 groups at 11 weeks and 8 weeks posttreatment ($p<0.05$), respectively (Fig. 6).

In addition, the proportion of Non T/non B (N) cells was also significantly higher in Tx-2 at 3 weeks posttreatment ($p<0.01$) and remained high at 13 weeks posttreatment

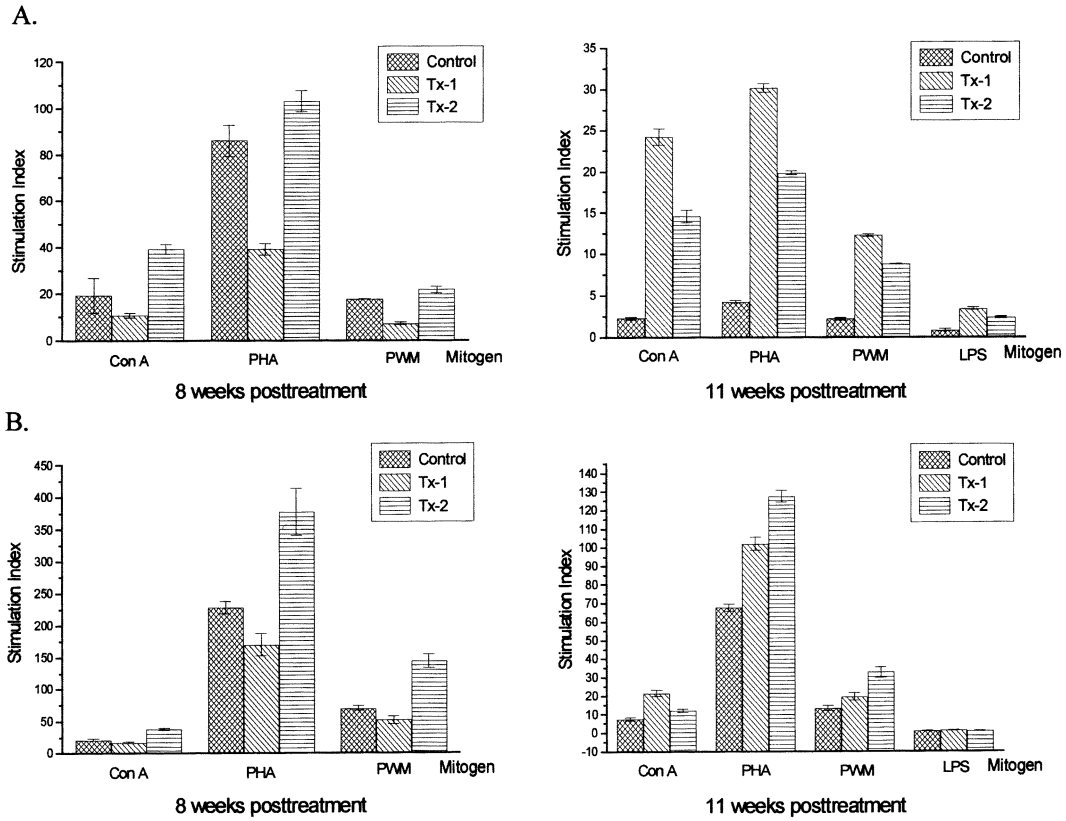

Fig. 4. Comparison of mitogen (Con A, PHA, PWM, and LPS)-stimulated lymphocyte activation determined by mitogenesis stimulation index (SI) in peripheral blood lymphocytes (A) and mesenteric lymph node lymphocytes (B) at 8 and 11 weeks posttreatment of Barodon-spray (Tx-1), Barodon-fermented (Tx-2) or in Barodon-nonfed pig group

Table 4. T-cell subsets per field (0.06 mm^2) in mesenteric lymph nodes from the pigs.

Group	$CD4^+$	$CD8^+$	$CD4^+CD8^+$ dpp
Control	32 ± 5	29 ± 2	10 ± 1
Tx-1	35 ± 4	39 ± 5	32 ± 3
Tx-2	40 ± 4	47 ± 5	35 ± 4

Table 5. T-cell subsets per field (0.06 mm^2) in spleen from the pigs.

Group	$CD4^+$	$CD8^+$	$CD4^+CD8^+$ dpp
Control	11 ± 1	8 ± 1	3 ± 1
Tx-1	11 ± 1	11 ± 1	6 ± 1
Tx-2	14 ± 1	17 ± 1	11 ± 1

(p<0.1) (Fig. 2D). Between Barodon-treated groups, the proportion of MHC class II-expressing cells was higher in Tx-2 than Tx-1 from 3 weeks to 8 weeks posttreatment (p<0.05) (Fig. 2C). The proportion of cells expressing $CD4^+$ or $CD8^+$ antigen was also higher in Tx-2 at 3 weeks posttreatment (p<0.1) and the proportion of Non T/Non B (N) cells was higher in Tx-2 at 13 weeks posttreatment (p<0.1).

There were no significant differences in proportion of $CD2^+$ cells, B cells, monocytes and granulocytes between Barodon-treated and control group during the experiment (Data not shown).

Proportion of $CD4^+CD8^+$ double positive T lymphocytes (dpp) in peripheral blood and mesenteric lymph nodes : The proportion of dpp in peripheral blood (PB) and mesenteric lymph nodes (MLN) was examined using two mAbs with different isotypes in dual color FC analysis. An increased dpp was observed in lymphocytes obtained from PB and MLN of Barodon-treated group at 8 and 11 weeks posttreatment. The proportion of dpp in PB was 27.5% in Tx-1 and 32.1% in Tx-2, respectively, and only 2.2% in control group at 8 weeks posttreatment (Fig. 3A). In MLN, the proportion was 45.1% and 52.1% in Tx-1 and Tx-2, respectively, and 16.5% in control group at 8 weeks posttreatment (Fig. 3B). Although a slightly reduced proportion was observed at 11 weeks posttreatment in Tx-1 and Tx-2, with 32.1% and 50.7%, these proportions were still higher than those of control group, 7.4% (Fig. 3C).

Mitogen-stimulated lymphoproliferative activity of cells from PB and MLN

To examine the mitogen-stimulated lymphoproliferative responses, lymphocytes of PB and MLN obtained from Barodon-treated and nontreated groups and the nontreated control group at 8 and 11 weeks posttreatment were stimulated using Con A, PHA, PWM and LPS. The SI of PB lymphocytes stimulated with all four mitogens was significantly higher in Tx-1 and Tx-2 than that of control at 11 weeks posttreatment (p<0.01). At 8 weeks posttreatment significantly higher SI was only observed with PB of Tx-2 stimulated with PWM as compared to controls at 8 weeks posttreatment (p<0.05). In MLN, the activity was significantly higher in Tx-2 when lymphocytes were stimulated with PHA (p<0.05) and PWM (p<0.01), respectively. The activity was significantly higher in Tx-1 than that of control at 11 weeks posttreatment when cells were stimulated with Con A and PHA, respectively (p<0.01), otherwise $Con\ A^-$, PHA^- and PWM-stimulated activity was significantly higher in Tx-2 than in the control at the same time point (p<0.05) (Fig. 4A and 4B).

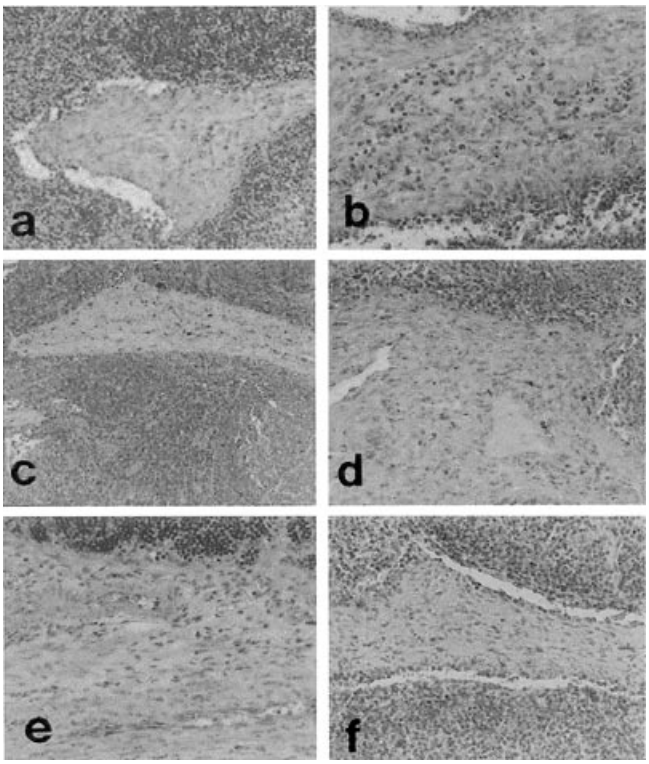

Fig. 5. Immunohistochemical analysis of mesenteric lymph nodes from Barodon-nonfed pigs in lymphatic vessels of mesenteric lymph nodes. $CD4^+$, $CD8^+$, $CD4^+CD8^+$ dpp were stained as red, grayish black and grayish brown color, respectively.
a, b: CD4 single staining in which PT90A (mAb) and AEC substrate were used a; ×100, b; ×200. c, d: CD8 single staining in which PT81B (mAb) and DAB+Ni substrate were used. c; ×100, d; ×200. e: N cell single staining as control in which PT79A (mAb) and AEC substrate were used. ×200. f: Dual staining in which CD8 staining was followed by CD4 staining. ×200.

Distribution of $CD4^+CD8^+$ double positive T lymphocytes (dpp) in MLN and spleen in immunohistochemistry

The distribution of dpp in MLN and spleen was investigated by ABC method and image analysis to compare the difference between Barodon-treated and nontreated control

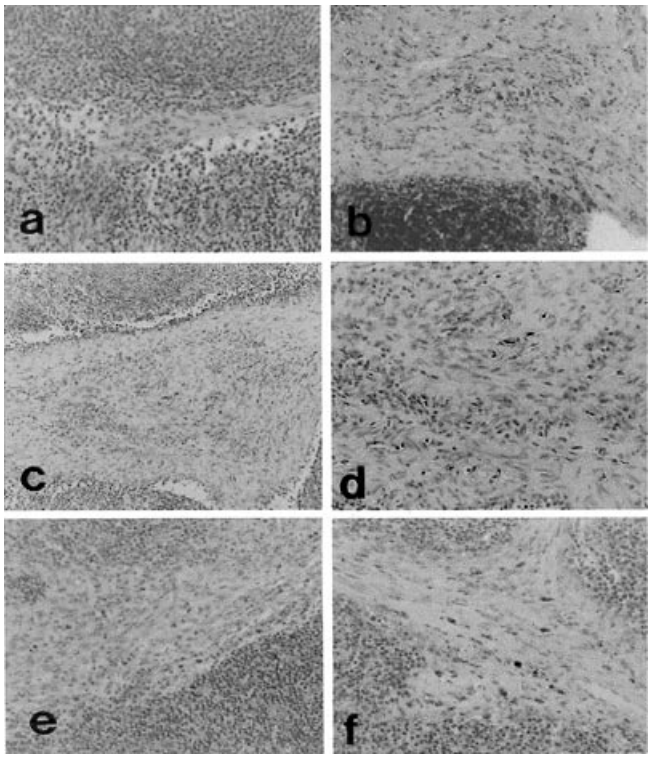

Fig. 6. Immunohistochemical analysis of mesenteric lymph nodes from Barodon-fed pigs in lymphatic vessels of mesenteric lymph nodes. $CD4^+$, $CD8^+$, $CD4^+CD8^+$ dpp were stained as red, grayish black and grayish brown color, respectively. a, b: CD4 single staining in which PT90A (mAb) and AEC substrate were used. a; ×100, b; ×200. c, d: CD8 single staining in which PT81B (mAb) and DAB+Ni substrate were used. c; ×100, d; ×200. e: N cell single staining as control in which PT79A (mAb) and AEC substrate were used. ×100. f: Dual staining in which CD8 staining was followed by CD4 staining. ×200.

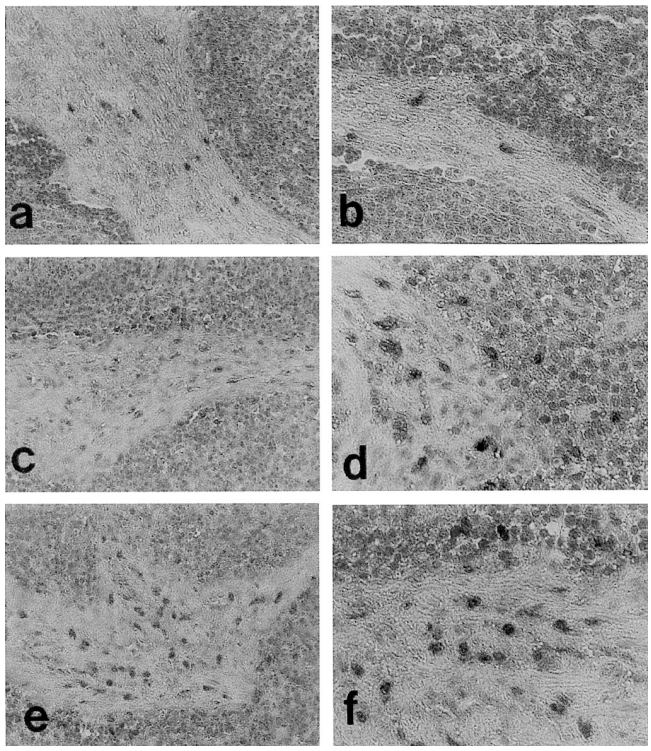

Fig. 7. Immunohistochemical analysis of $CD4^+CD8^+$ dpp of mesenteric lymph nodes from Barodon-nonfed (a; ×200, b; ×400), Barodon-spray (Tx-1, c; ×200, d; ×400) and Barodon-fermented (Tx-2, e; ×200, f; ×400) pigs. The same methods of analysis were used as in dual staining of Fig. 16 and 17. Barodon-fed (Tx-1 and Tx-2) pigs had more $CD4^+CD8^+$ dpp in mesenteric lymph nodes than Barodon-nonfed pigs. Compared to Tx-1 and Tx-2 exhibited higher expression of $CD4^+CD8^+$ dpp, resulting in grayish brown color development.

groups. The proportion of $CD4^+$, $CD8^+$ and $CD4^+CD8^+$ dpp of MLN and spleen were significantly higher in Tx-1 and Tx-2 than in the control group ($p<0.01$) (Tables 4 and 5, Figs. 5-7). Also, a higher proportion of dpp observed in Tx-2 than in Tx-1 in spleen between Barodon-treated group ($p<0.01$) (Table 4).

Discussion

The recent development of mAbs specific to leukocyte differentiation antigens of various animals make it possible to define the host immune system more completely [2, 3, 16, 22, 23]. By monitoring the animal immune system, the efficacy of vaccines and new drugs can be evaluated *in vivo* by comparing the host response before and after application of reagents [6, 9, 11]. Likewise, the porcine immune system was defined using various mAbs specific to porcine leukocyte differentiation antigens. A unique cell population, $CD4^+CD8^+$ dpp, is present in pigs and has an important role in host defense. The dpp population was increased in peripheral blood by antigen stimulation, and a larger increase was observed in lymphoid organs [24]. The increased dpp in peripheral blood, mesenteric lymph nodes and spleen in Barodon-treated pigs indicates Barodon has effects on porcine immune system. Although total $CD2^+$ T lymphocyte population was not increased after Barodon application, $CD4^+$ or $CD8^+$ T lymphocyte populations were significantly increased in the blood. Zuckermann and Husmann have indicated $CD4^+CD8^+$ dpp T lymphocyte have a specific memory cell marker CD29 at the same time, so the dpp may play a role in inducing secondary immune responses in the host [24]. Further *In vitro* studies using restimulation of dpp with the same antigen used *in vivo* are necessary to clarify the role. Also, comparison of the dpp population in animals with infectious and in healthy controls would be instructive, since the lymphoid organ can be the first target activated by antigen or reagents. The dpp increase was more evident in lymphoid organs from Barodon-treated groups. This result has indicates Barodon may induce antigenic stimulation in the immune tissues. The proportion of dpp was increased and might influence the

activated mitogen-stimulated lymphoproliferative responses in the tissues. Increased lymphoproliferative responses stimulated by PHA or PWM in early stages and Con A-stimulated responses at later stage might be attributable to initial specific stimulation of immature $CD1^+$ T or B cells and later stimulation of mature T cells, maybe $CD4^+CD8^+$ dpp. Further studies using purified dpp cell populations will elucidate the activity of Barodon more specifically.

Barodon's effect in animal herds has been characterized by improvement of immune responses of pigs to *Actinobacillus pleuropneumoniae* vaccine (unpublished data, 1999). The immunoenhancing effect of Barodon as an adjuvant has also been proved in hog cholera vaccinated pigs by an increase of antibody titers and immune cell proportions after treatment [15]. The major ingredient of Barodon is minerals, which may affect vital biological processes including immune responses. Barodon's effect on enhancement of productivity and activation of immune responses in pigs may be attributable to the anionic aqueous solution of Barodon, which can penetrate into the body fluid easier than other similar products with powder formula.

Although more studies are needed to elucidate the exact mechanism of Barodon and its synergic effect with vaccines or antibiotics in the porcine immune system, this study has shown that Barodon can be a candidate immunostimulator to improve productivity and host immune responses as an alternative method to antimicrobial feed additives.

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