ペプチドグリカン投与による離乳期子豚小腸粘膜固有層の免疫増強

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Immunopotentiation of the Mucosa of the Small Intestine of Weaning Piglets by Peptidoglycan

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ABSTRACT. Peptidoglycan (PG) derived from *Bifidobacterium thermophilum*, an intestinal flora of swine, was given orally to suckling piglets, and a comparison was made of the immunoresponsiveness of the lamina propria in the small intestinal mucosa and the numbers of *E. coli* in various parts of the intestines of treated and non-treated piglets 5 and 6 weeks old. After PG treatment, the numbers of IgA-bearing cells of the lamina propria in the middle of the jejunum and in the ileum were significantly higher than those of the non-treated group at 5 and 6 weeks of age ($p<0.01$), respectively. The number of IgA-bearing cells in the lamina propria was much higher than that of IgM-bearing cells in the treated piglets, whereas the number of IgM-bearing cells in the lamina propria was much larger than that of IgA-bearing cells in the control. As a result of these findings, it was concluded that local immunoresponsiveness developed with the oral administration of PG. The lower numbers of *E. coli* found in various portions of the small intestines of the treated animals as compared with the control group suggested that the count of *E. coli* was inversely proportional to the IgA-bearing cells in both the PG administration group and the non-treated group.—KEY WORDS: immunopotentiation, peptidoglycan, piglet, post weaning diarrhea.

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Recently, several reports [6, 15, 17, 18, 20, 36] have emphasized that the incidence of post weaning diarrhea syndrome (PWDS) in piglets of 4-6 weeks is gradualy increasing in commercial pig farms. Studies on PWDS have revealed that enteropathogenic and enterotoxigenic *E. coli* with pili might be a major causative agent of this diarrhea [22, 26, 31, 33, 34].

It is well known that peptidoglycan (muramyl dipeptide), derived from the cell walls of Gram positive organisms, is an immunopotentiator, and this substance is applied to various kind of immunodeficiencies, especially in man [7, 19, 29, 30, 38].

The purpose of the present study was to observe the immunological consequences of the administration of peptidoglycan derived from *Bifidobacterium thermophilum* to suckling piglets and to clarify whether or not an augmentation of local immunoresponsiveness is manifested in weaning piglets aged 5–6 weeks, as well as to investigate changes in the numbers of *E. coli* in various parts of the small intestine.

MATERIALS AND METHODS

Animals: Sixteen suckling piglets derived from 4 sows (Duroc × Landrace) were employed as shown in Table 1. They were all sired by the same boar and the sows themselves were closely related. Of these, 8 piglets from 2 sows were treated with peptidoglycan, and the remaining 8 piglets were used as non-treated controls. The herd was managed conventionally throughout the experimental period. At 4 weeks (28 days) of age, all piglets employed were separated from the sows and remaining littermates and they fed with artificial milk throughout the experiment.

Table 1. Experimental animals

Sow No.	Piglet No.					
	Litter size	PG-treated	Control	Sacrificed	Age when sacrificed	
					5 weeks	6 weeks
1	11	4	0	4	2	2
2	9	4	0	4	2	2
3	13	0	4	4	2	2
4	12	0	4	4	2	2

Peptidoglycan (PG): PG, a substance containing muramyl dipeptide [27], was prepared using *Bifidobacteriuim thermophilum* which exists predominantly in the large intestines of adult swine.

Briefly, *Bifidobacterium thermophilum*, strain P2–91, was cultivated in Brigges liver broth under anaerobic conditions for 16 h at 37°C, harvested by centrifugation and washed with saline [25]. The bacterial cell walls were disrupted in a French press. They were centrifuged and washed twice with saline and suspended in the phosphate buffer of Sörensen (pH 6.2) in an equal volume of native cells (10^{12} cells/ml). Enzymatic digestion of cell walls was performed by adding 0.01% egg white lysozymes and 0.05% pronase, and then incubating them at 37°C for 48 hr. The optical density of the digested solution, 640 nm, was decreased to 5% of the initial value. The supernatant of the fluid was considered to consist of digested cell walls. One ml of the substance was derived from 10^{12} native cells. For more details about the method, see Namba *et al* [27].

Collection of Samples: Eight piglets were given 5 doses of 0.5 m*l* PG orally from the day of birth to 5 days of age, and were also given 3 doses of 0.1 m*l* PG orally from 3 weeks of age. Each administration was done under blind conditions, while the control group was given a water suspension of lactose as a placebo.

At 5 or 6 weeks of age, the animals were sacrificed by bleeding, the entire intestinal tract was carefully removed and packed gently in crushed ice. Attention was given to preventing the movement of the intestinal fluid from one area of the intestinal tract to another. Then 10 cm long sections of the duodenum, the middle portion of the jejunum, and the ileum were ligated and isolated as soon as possible. The isolated small intestines were each placed in liquid nitrogen immediately for freezing and kept at −80°C until use.

Counting Ig-bearing cells in the lamina propria of the small intestine: The immunohistochemical technique employed in these experiments was the enzyme-linked antibody method.

Frozen tissues (the duodenum, the middle part of the jejunum and the ileum of piglets aged 5 and 6 weeks) were cut into 6μm sections and placed on glass slides. Then the sections were dried immediately and fixed in periodate-lysine-paraform aldehyde (PLP) at 4°C for 8 hr.

After washing 3 times in 0.01 M cold phosphate saline (PBS, pH 7.2), the sections were incubated in 98% methanol and 0.3% H_2O_2 for 30 min at room temperature to block endogenous peroxidase activity. They were then washed in 0.01 M cold PBS for 30 min at room temperature and incubated in normal serum diluted with PBS for 1 hr at room temperature. Normal rabbit

serum was employed to count IgA-bearing cells and normal goat serum for counting IgM- and IgG-bearing cells. After the sections were washed in 0.01 M cold PBS, primary antibody was applied and the sections were incubated at 4°C overnight. Anti-swine IgA goat serum (Miles Lab. Inc., U.S.A.) was employed to determine the number of IgA-bearing cells, anti-swine IgM rabbit serum (Nordic Immuno. Lab. Inc., Netherlands) to count IgM-bearing cells and anti-swine IgG serum (Cappel Lab. Inc., U.S.A.) for counting IgG-bearing cells. Next, the rabbit sections were then washed in 0.01 M cold PBS for 30 min and incubated for 2 hr in secondary antibody serum at room temperature. Anti-goat IgG rabbit serum was used to count IgA-bearing cells and anti-rabbit IgG goat serum (Cappel Lab. Inc. U.S.A.) to measure the number of IgM- and IgG-bearing cells. Sections were then washed in 0.01 M cold PBS for 30 min at room temperature and incubated for 2 hr with peroxidase-antiperoxidase (PAP) complex (Miles Lab. Inc., U.S.A.). After being washed twice in 0.01 M cold PBS, the sections were stained in 0.05 M Tris buffer (pH 7.6) containing 0.02% diaminobenzidino-tetrahydrochloride (DAB) (Sigma, St. Louis, MO.) and 0.005% H_2O_2 for 5–10 min. Sections were then washed several times in distilled water and stained with Mayer's hematoxylin. Finally, the sections were washed and dehydrated in alcohol/xylene and mounted in Permount (Fisher).

Counting plasma cells in the lamina propria of the small intestine: Samples were stained with methylgreen pyronin stain, which enabled plasma cells to be clearly distinguished from other pyroniophilic cells. All the samples were examined with an Olympus microscope, type BH-2. Cell counting was done using the method of Allen Porter [4]; the count was made by estimating the number of stained cells present in 20 fields selected at random using a ×40 objective with a ×10 eyepiece.

Isolation and count of viable E. coli: Immediately after euthanasia, 5-cm long sections of the duodenum, the middle portion of the jejunum and the ileum of 5-and 6-week-old piglets were isolated. These tissues were opened aseptically and their contents collected by scraping the mucosa lightly with a surgical knife. The contents were diluted with a 10-fold dilution in phosphate buffer (pH 7.4), and each dilution was streaked on McConkey agar (Difco) and then incubated overnight at 37°C. The count per 1 g was expressed logarithmically.

Clinical findings: Sixteen piglets derived from 4 sows were observed for clinical findings, body weight and the components of the colon.

Statistical analysis: Data derived from the counting of Ig-bearing cells and plasma cells were analyzed by Student's *t*-test.

RESULTS

Plasma cells: The number of plasma cells in the lamina propria of the duodenum, middle of the jejunum and the ileum of 5- and 6-week-old piglets, with and without treatment by PG, are shown in Fig. 1.

This figure shows a significant difference ($P<0.05$–$P<0.01$) between the PG-treated and the control group in the number of plasma cells in various parts of the intestinal mucosa.

Proportion of Ig-bearing cells: In piglets aged 5 and 6 weeks which were treated with PG, the proportion of IgA-bearing cells of the lamina propria in the duodenum, middle of the jejunum, and ileum was higher than that of IgM-bearing cells in the corresponding parts and weeks. As shown in Fig. 2, these proportional trends were significant in comparisons between the treated and the untreated piglets in the lamina propria of

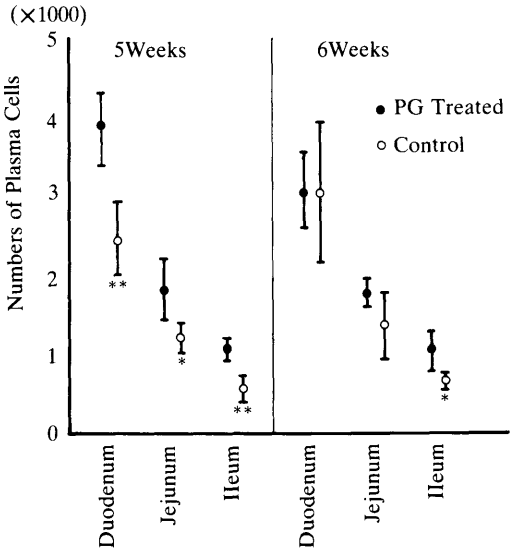

Fig. 1. Number of plasma cells in the lamina propria of the small intestine of piglets treated with PG and in controls. The number of plasma cells is expressed as the mean (±S.D.) of total counts of cells per 20 fields (×400) *=P<0.05 of probability with Student's *t*-test. **=P<0.01.

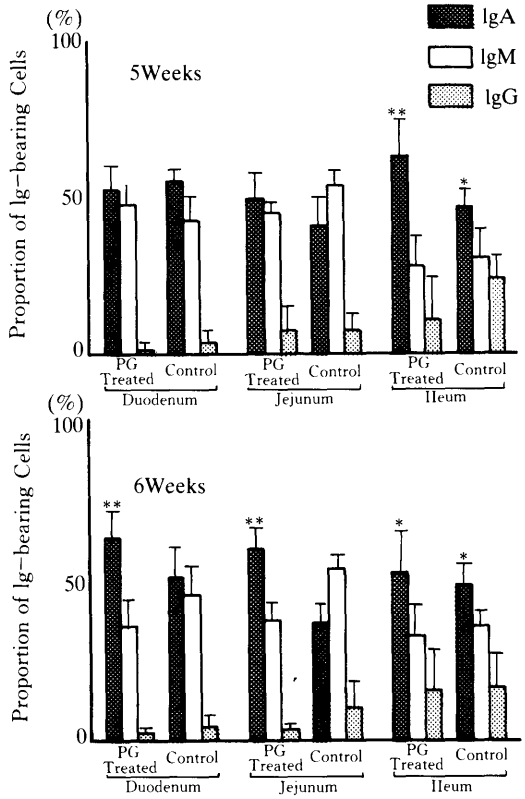

Fig. 2. Proportion of Ig-bearing cells of the lamina propria in the small intestine of piglets treated with PG and controls. Ig-bearing cells expressed as the mean (±S.D.) of the numbers of Ig-A, Ig-M and Ig-G bearing cells per 20 fields (×400) *=P<0.05 of probability with Student's *t*-test. **=P<0.01.

the ileum (P<0.01) in piglets aged 5 weeks and that of the duodenum (P<0.01), the middle of the jejunum (P<0.01), and the ileum (P<0.05) of the animals aged 6 weeks.

In the control group, the proportion of IgM-bearing cells was higher than that of IgA-bearing cells in the 5- and 6-week-old piglets. In other words, the proportion of IgA- and IgM-bearing cells in the intestinal mucosa was reversed in the PG-treated piglets and the non-treated ones.

Number of Ig-bearing cells: The counts of IgA-bearing cells of the lamina propria of the small intestines of the piglets aged 5 and 6 weeks, PG-treated and non-treated, are shown in Fig. 3. A significantly larger number (P<0.01) of IgA-bearing cells was clearly demonstrated in the mucosa of the middle of the jejunum and the ileum of the piglets treated with PG as compared to the animals without treatment at 5 and 6 weeks of age. In both the treated and the control groups, the count of IgA-bearing cells was higher for the duodenum than for either the jejunum or the ileum.

Viable E. coli: In Table 2, the number of viable *E. coli* in various portions of the small intestines of the animals treated or untreated with PG are shown. The number of organisms in each portion of the small intestine was higher for the untreated groups than for the PG-treated groups. The results show clearly that the number of *E. coli* was inversely proportional to the IgA-bearing cells in both the treated and the control groups.

Clinical findings: The piglets treated with PG were apparently healthy throughout the

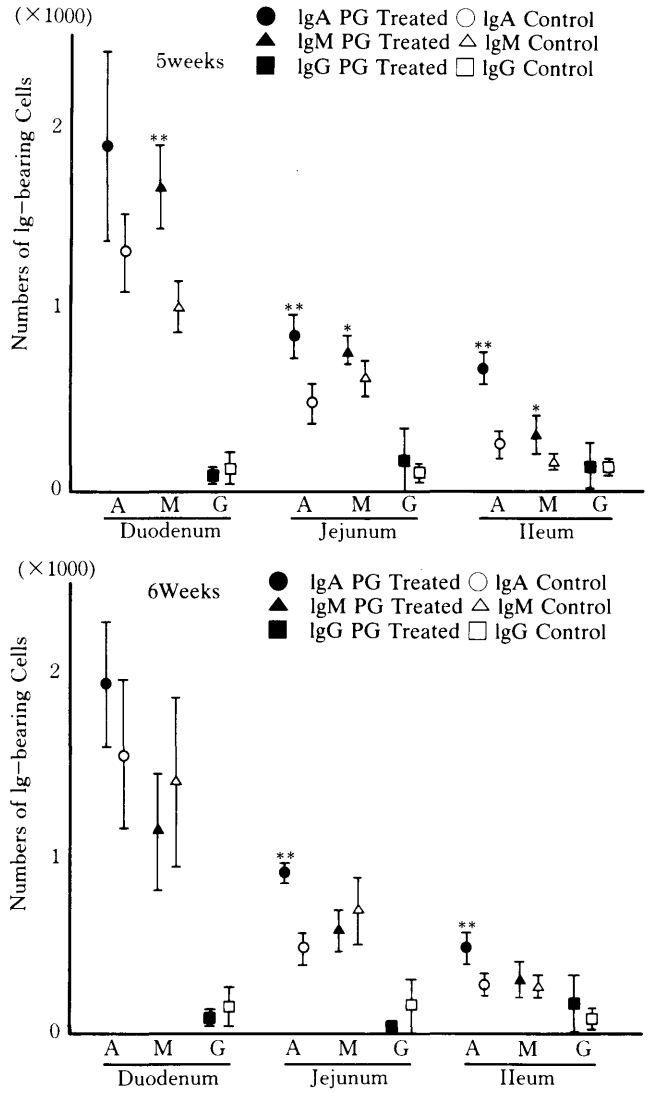

Fig. 3. Number of Ig-bearing cells of the lamina propria in the small intestine of 5-and 6-week-old piglets treated with PG and in controls. Number of Ig-bearing cells expressed as the mean (±S.D.) of total counts of cell per 20 fields (×400) *=P<0.05 of probability with Student's *t*-test. **=P<0.01.

experimental period, while the untreated piglets had diarrhea occasionally. In both groups these conditions were revealed by changes of body weight (P<0.01) at the age of sacrifice. As shown in Table 3, the contents of the colon were different in both groups: toothpaste-like (normal) contents were seen in the group treated with PG, while a watery stool was observed in the control group.

DISCUSSION

A high incidence of PWDS frequently occurs in piglets age 5 to 6 weeks on commercial farms when sow milk is com-

Table 2. Comparison of *E. coli* counts in the small intestines of piglets treated with PG and controls

Portion	*E. coli* count			
	5 weeks		6 weeks	
	PG-treated	Control	PG-treated	Control
Duodenum	0.0[a)]	1.2 (0.0– 4.6)	0.0	1.1 (0.0–4.3)
Jejunum	0.0	2.0 (0.0– 4.0)	0.0	1.4 (0.0–5.6)
Ileum	4.6 (3.3–6.3)	7.0 (3.6–10.0)	4.5 (0.0–8.0)	6.9 (5.3–9.3)

a) Logarithmic count per g of contents. 0 indicates$<10^{3.3}$ organisms.

Table 3. Condition of the experimental animals

Group	Age in weeks	Sex	Body Weight (kg)		Content of the colon	Clinical findings
			Newborn	Age at sacrifice		
PG-treated	5	Male	1.5	8.0	Toothpaste-like	Nomal
	5	Male	1.3	8.5	Toothpaste-like	Nomal
	5	Male	1.2	9.0	Toothpaste-like	Nomal
	5	Female	1.5	10.0	Toothpaste-like	Nomal
	6	Male	1.6	11.0	Toothpaste-like	Nomal
	6	Female	1.4	10.0	Toothpaste-like	Nomal
	6	Female	1.4	11.0	Toothpaste-like	Nomal
	6	Female	1.3	10.0	Toothpaste-like	Nomal
Control	5	Female	1.4	4.0	Watery	Diarrhea
	5	Male	1.2	5.0	Watery	Mild diarrhea
	5	Male	1.5	6.0	Rabbit-feces-like	Constipation
	5	Female	1.5	6.5	Watery	Diarrhea
	6	Female	1.3	7.0	Watery	Diarrhea
	6	Male	1.4	8.0	Watery	Diarrhea
	6	Female	1.3	5.0	Watery	Diarrhea
	6	Male	1.5	7.5	Watery	Diarrhea

pletely changed to artificial milk, *i.e.*, at the period of complete weaning. This results in a number of diseased piglets that often become stunted. Though the causal agent of PWDS remains unclear, in a majority of cases the disease is considered to be caused by some kind of enteropathogenic *E. coli* [8] and/or the association of rotavirus with *E. coli* [9. 37. 39].

In this study, it was clear that the number of IgA-bearing cells in the lamina propria of the jejunum and the ileum was significantly higher in weaning piglets, aged 5 and 6 weeks, which were administered PG than in non-treated piglets. These results suggest that enhancement of local immunoresponsiveness occurs due to the administration of PG in piglets in the weaning period. Moreover, the development of local immunity with the administration of PG was also indicated; the number of IgM-bearing cells which appeared in immunologically immature piglets decreased in the lamina propria, while the number of IgA-bearing cells increased.

On the other hand, the number of *E. coli*

organisms in a given part of the intestine in a given age group was significantly smaller for the PG-treated groups than for the controls; the number of *E. coli* was inversely proportional to the number of IgA-bearing cells in both the treated and the control groups. Moreover, significant differences were seen between the PG-treated and the control groups in the area of clinical findings; no clinical signs were recognized in the PG-treated groups, while diarrhea and decreasing weight gain were seen in the control groups.

Since the study on the cell walls of *Mycobacteriuim tuberculosis* by Freund [14], many studies on adjuvant activity or immunopotentiation with cell walls derived from Gram positive bacteria have been carried out.

Ellouz et al. [13] demonstrated that the N-acetylmuramyl-depeptide (MDP) in the cell wall was the minimum structure possessing adjuvant activity, and Kotani *et al.* [16] also analysed and proved the efficacy of the substance. Concerning the immunopotentiation of PG, Namba et al. [27] reported that intestinal becteria might be solubilized by oral administration of bacteriolytic enzymes and that the absorbable fragment of PG released, as well as the bacterial cell walls, might be responsible for the enhancement of host immune responses.

Michalek *et al.* [23] reported that with the oral administration of PG derived from *Streptococcus mutants,* protection against dental caries was seen, as well as a significant increase of IgA-bearing cells in the spleen and of the IgA in the serum and the saliva. Other studies [21, 24, 32] have also revealed the enhancement of oral immune response with PG administration. According to Namioka *et al.* [28], a larger but not significant number of IgA-bearing cells was observed in the lamina propria of the mucosa of the small intestines in PG-treated groups, aged 3 and 4 weeks, than in the control groups. Suganuma *et al.* [35] reported that, in piglets of less than 4 weeks of age, maturation of the differentiation ability of B lymphocytes was suppressed by T lymphocytes.

Judging from these facts, in piglets less than 4 weeks of age, an increase of IgA-bearing cells in the intestinal mucosa may not occur readily in spite of PG administration. However, as was shown in this study, at the weaning stage, *i. e.*, after 5 weeks of age, the administration of PG seems to be very effective for the enhancement of local immune response. Suganuma *et al.* [35] also reported that the suppressor activity of T lymphocytes gradually decreased with aging, and at 5 weeks of age or later, the suppressor activity of T lymphocytes was not consistently demonstrated. Therefore, the administration of PG might to some degree, be efficacious in the prevention of PWDS.

Allen and Porter [1-4], Atking et al. [5], Brown and Bourne [10, 11] and Butler *et al.* [12] reportred that the proportion of IgM- and IgA-bearing cells in the lamina propria of the intestinal mucosa reversed with aging, and that, in the lamina propria of the duodenum, the number of IgA-bearing cells became dominant in 30-day-old piglets.

In our experiment, the number of IgA-bearing cells in the lamina propria of the duodenum, the jejunum and the ileum was dominant in the PG-treated groups, while in the non-treated control groups, a much higher number of IgM-bearing cells was seen in the middle part of the jejunum compared to that of IgA-bearing cells at the age of either 5 or 6 weeks. This fact also suggests that enhancement of immune response occured with PG treatment of weaning piglets.

The results mentioned above confirm that PG may play a role in enhancing non-specific immunopotentiating activities at the weaning stage and may consequently lead to

a decrease in the incidence of PWDS. This fact suggests the potential use of PG as a treatment for PWDS caused by *E. coli* and other agents like rotavirus.

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ペプチドグリカン投与による離乳期子豚小腸粘膜固有層の免疫増強：佐々木隆志・前出吉光・波岡茂郎（北海道大学獣医学部家畜内科学教室）——豚腸内菌叢のひとつ *Bifidobacterium thermophilum* 由来ペプチドグリカン（PG）を子豚の生下時から5日齢まで，および3週齢に1日1回3日間経口投与し離乳後の5および6週齢の小腸各部粘膜固有層における免疫グロブリン（Ig）保有細胞数および小腸内大腸菌数をしらべ，PG非投与豚におけるそれらの成績と比較した。PG投与後の5および6週齢における小腸中部および回腸粘膜固有層 Ig 保有細胞数は非投与群のそれに比べて有意（$P < 0.01$）に多かった。また，PG投与および非投与群の該部における IgA および IgM 保有細胞数の比率をみると，前者では IgA 保有細胞数が IgM のそれを上回った反面，後者ではその比率が逆転していた。さらに，小腸各部位における大腸菌数では，PG投与群のそれらは非投与群に比べて少なかった。以上の成績から，哺乳期子豚における PG 投与後子豚の消化管局所の免疫応答応が増強されることが示唆された。