Enhancement of Cytotoxic Activity of Lymphocytes in Mice by Oral Administration of Peptidoglycan (PG) Derived from *Bifidobacterium thermophilum*

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ABSTRACT. A preparation of peptidoglycan (PG) of *Bifidobacterium thermophilum* (*B. thermophilum*) of swine was orally administered to SPF-C57BL/6CrSlc mice in order to confirm the enhancement of the cytotoxic activity of natural killer cells (NK), intraperitoneal cytotoxic T lymphocytes (CTL) and lymphocytes stimulated by concanavalin A (Con A-stimulated lymphocytes). The NK cells from the spleen and the mesenteric lymph node (MLN) of mice that were continuously fed with PG-mixed feed for three weeks showed a significantly higher rate of cytolysis than those from the control group. However, a single oral administration of PG had no significant effect on NK activity. The activity of peritoneally sensitized CTL of the mice that were continuously fed with PG-mixed feed was assayed. The PG-mixed feed administered group showed a higher CTL activity than that of the control group. The cytotoxic activity of Con A-stimulated lymphocytes in the PG-mixed feed administered group was higher than that of the control group. These results indicate that the cytotoxic activity of mice was enhanced by orally administered PG.—**KEY WORDS:** *Bifidobacterium*, ConA, CTL, lymphocyte, NK, peptidoglycan.

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It is well known that peptidoglycan (PG), derived from the cell walls of gram positive bacteria, is an immunopotentiator, and this substance has been applied as immunomodulator in various forms, especially in human.

Previously we orally administered PG of *Bifidobacterium thermophilum* (*B. thermophilum*) to specific pathogen free (SPF)-ICR mice in order to confirm the enhancement of the defense activity of the mice against *Escherichia coli* infection. It was observed that the survival rates of the PG-administered group were significantly higher than those of the non-treated control group after the single oral administration of PG mice [14].

The purpose of this study was to determine whether the cytotoxic activity in mice was enhanced by the oral administration of a PG preparation derived from *B. thermophilum*. We observed augmentation of NK cells and CTL activities of the mice by the oral administration of PG. In addition, the cytotoxic activity of lymphocytes stimulated by concanavalin A (Con A-stimulated lymphocytes) was also seen to be enhanced.

MATERIALS AND METHODS

Animals: C57BL/6CrSlc male specific pathogen free (SPF) mice 5 weeks old were purchased from Japan SLC (Shizuoka Prefecture, Japan). The mice were kept in plastic cages in barrier system rooms and sterile mats were used for breeding. Mice were fed with commercial solid feed (Oriental Yeast, Japan) by feeder bottle and sterile water ad libitum. Mice aged 6–9 weeks were used for the study.

Preparation of PG: The preparation method for PG was exactly the same as that used in previous studies [8]. Briefly, the P2–91 strain of *B. thermophilum* was cultured for 16 hr at 37°C in Briggs liver broth under anaerobic

conditions. The bacteria were then disrupted by a French press and washed twice with Sorensen's phosphate buffer (pH 6.2) by centrifugation at 5,000 rpm for 30 min and precipitate was suspended in the same buffer. Then this suspension was treated with 0.01% lysozyme and 0.05% pronase for 48 hr at 37°C. One ml of PG-preparation contained the cell wall digest from 10¹² native *B. thermophilum* cells. The PG solution was freeze-dried to make PG powder.

Administration: The PG preparation was stirred for 30 min with a stirrer, and sonicated for 15 min. Then the solution was adjusted to 500 µg (content of hexosamine: 2.9%) / 0.5 ml with PBS and was administered directly to the stomach with a stainless steel canula. Sterile PBS was orally administered to the control group in the same manner. After 24 hr, 1 week, 2 week and 3 week of administration, the NK activity was determined. Fifty µg of the freeze dried PG preparation was mixed with 5 g of commercial powder feed (Oriental Yeast, Japan) and fed to mice daily for three weeks. The control group was fed only with commercial feed in the same manner. NK activity was determined on the last day of feeding.

In vivo generation of cytotoxic lymphocytes: On the second week of feeding, mice were inoculated in the peritoneal cavities with 5 × 10⁶ mouse mastocytoma P815 cells (P815 cells) for purpose of stimulating the peritoneal cytotoxic T lymphocyte (CTL) [13]. P815 cells were treated with mitomycin C in order to interfere with DNA synthesis before the inoculation. The control group was treated in the same way.

Preparation of lymphocytes: (1) Preparation of lymphocytes derived from the spleen and mesenteric lymph node (MLN); The spleens and MLNs were mashed on a #200 mesh, and cells were suspended in RPMI 1640 medium. Cells were carefully layered onto the HISTOPAQUE-

1077 (Sigma, Ltd., U.S.A.) and centrifuged at $400 \times g$ for 30 min at room temperature. After centrifugation, the layer of lymphocytes was transferred into clean tube. Lymphocytes were washed twice with the RPMI 1640 culture medium supplemented with 10% fetal calf serum (FCS), suspended in the same medium at a concentration of 2×10^6 cells/ml, and then used for the effector cells in the cytotoxicity test.

(2) Preparation of the peritoneal cavity CTL; The lymphocytes sensitized with P815 cells *in vivo* were harvested from the peritoneal cavities in 10 ml of RPMI 1640 culture medium containing 1 unit/ml sodium heparin with an injection pump. The peritoneal cells were washed with the RPMI 1640 culture medium, transferred to a plastic petri dish, and then incubated at 37°C for 2 hr under 5% CO₂ in air. Only the plastic adherent cells were used for the effectors of the CTL activity test.

(3) Stimulation of lymphocytes with mitogens; Lymphocytes derived from the spleen or MLN were suspended at 2×10^6 cells/ml and mixed with an equal volume of 1 µg/ml ConA solution. The mixtures were cultured for 72 hr at 37°C under 5% CO₂, washed three times with RPMI 1640 culture medium and then used for the effector cells in the cytotoxicity test.

Cytotoxicity assay: (1) Cytotoxicity assay of NK cells; K562 cell line derived from chronic leukemia in human and Mitsukaïdo cells of B lymphoid oncocyte of pigs [11] were used as target cells. The ratio of effector cells to target cells (E:T) was used at 40:1. The target cells were washed once with RPMI 1640 culture medium and suspended in 1 ml of the same medium. Ten µl of 2,7-ABIS (2-CARBOXY-ETHYL)-5(AND 6)-CARBOXYFLUORESCIN, ACETA-XYMETHYLESTER (BCECF-AM) [5, 21] was added to the cell suspension and the cells were incubated at 37°C for 1 hr under 5% CO₂. After that the cells were washed 3 times with RPMI 1640, adjusted to 1×10^5 cells per ml and suspended in the same medium supplemented with 10% FCS. The target cells were cocultured with the prepared effector cells at the E:T = 40:1 ratio in a 96-well plate, and incubated at 37°C in 5% CO₂ in air. After 4 hr, latex beads up to 2% of

the medium were added, the medium was stirred lightly, transferred to an Epicon plate (Idexx. Ltd., Portland, Maine, U.S.A.) and fluorescence was determined by fluorescence concentration analyzer (FCA) (Idexx). Triton X 100 was added to the target cells at 0.5% concentration, and they were used as positive control. The target cells in the RPMI 1640 culture solution supplement with 10% FCS were used as a negative control. The cytotoxicity tests of the lymphocytes sensitized with target cells were conducted in the same manner.

(2) Cytotoxicity assay of CTL activity; The CTL activity was determined by the same procedure as for NK activity, except that P815 cells were used as the target cells, and the mixture ratios (E:T) of effector cells (E) to target cells (T) were set at 6:1, 12:1, 25:1 and 50:1.

(3) Cytotoxicity assay of ConA-stimulated lymphocytes activity; The cytotoxicity test was conducted with ConA-induced lymphocytes in the same way as the determination of NK activity.

Calculation of cytotoxic activities: The results of FCA were applied to the following formula and the rates of activities of NK, CTL and ConA-stimulated lymphocytes were determined:

$$\text{Percent lysis} = \frac{\text{Negative control value} - \text{Tested value}}{\text{Negative control value} - \text{Positive control value}} \times 100$$

Statistics: The results were presented as the mean and standard deviations. The differences from the values of the control group were analyzed by Student's *t*-test.

RESULTS

Effect of a single oral administration of PG on NK activity in mice: The augmentation of the NK activity of splenogenic lymphocytes was not observed 1 week after the single administration of PG, and then the cytotoxic activity of NK cells gradually declined (Table 1). Increase

Table 1. Effect of a single oral administration of PG on NK activities of lymphocytes derived from spleen and mesenteric lymph node (MLN)

Target cells	Effector cells	Time after single administration of PG				
		24 hr	1 week	2 weeks	3 weeks	control
K562	Spleen	16.5±20.4	22.5±11.7	13.4±8.7	7.1±8.1	10.0±11.4
	MLN	13.3±18.2	16.4±10.9	10.9±4.6	13.8±12.0	12.0±11.4
Mitsukaïdo	Spleen	11.2±6.9	16.5±9.7	7.5±5.9	7.6±5.4	11.1±11.0
	MLN	9.9±6.8	10.2±10.0	6.0±4.8	14.4±16.0	7.3±5.3

Groups of 10 mice were treated with a single dose of PG (500 µg) or PBS p.o. 24 hr, 1 week, 2 weeks or 3 weeks before assay of NK activities of lymphocytes derived from spleen and mesenteric lymph node (MLN). The ratio of target cells to effector cells was 40:1. Data are presented as mean±S.D. of the percentage of target cell lysis.

in activity was not statistically significant after this single-dose schedule and there was also no significant differences in comparison to control.

Effect of 3-week-feeding of PG-mixed feed on NK activity in mice: The NK activities of lymphocytes derived from the spleen of the mice which had been fed with PG-mixed feed were significantly higher against Mitsukaido cells ($P<0.05$) than those of the control group. However, there was no significant difference in NK activity against K562 cells in the two groups (Fig. 1).

The NK activities of lymphocytes from the MLN of the mice fed with PG-mixed feed were also significantly higher against K562 cells ($P<0.05$) compared with the rate of the

Fig. 1. Effect of dietary PG treatment on NK activity by lymphocytes derived from the spleen. Data are presented as the mean $\pm$ standard deviation (S.D.) of percent lysis of K562 cells or Mitsukaido cells at a 40:1 effector to target cell ratio. ■: PG treated group, □: Non-treated group, *: significantly different ($P<0.05$) from the control (without PG-treatment) according to the Student's *t*-test.

Fig. 2. Effect of PG mixed feed treatment on NK activity by lymphocytes derived from the mesenteric lymph node. Data are presented as the mean $\pm$ standard deviation (S.D.) of percent lysis of K562 cells or Mitsukaido cells at a 40:1 effector to target cell ratio. ■: PG-treated group, □: Non-treated group, *, **: significantly different (*: $P<0.05$, **: $P<0.01$) from the control (without PG-treatment) according to the Student's *t*-test.

Fig. 3. Effect of dietary PG treatment on cytotoxic T lymphocyte activity by peritoneal cytotoxic lymphocytes generated by intraperitoneal immunization with P815 cells 7 days before the assay. Data are presented as the mean $\pm$ standard deviation of percent lysis of P815 cells at 50:1, 25:1, 12:1 and 6:1 effector to target cell ratios. ●: PG-treated group, □: Non-treated group, **: significantly different ($P<0.01$) from the control (without PG-treatment) according to the Student's *t*-test.

control group and were similar to the result against Mitsukaido cells ($P<0.01$) (Fig. 2).

Effect of 3-week-feeding of PG-mixed feed on sensitized CTL activity in the peritoneal cavity: The activity of peritoneally sensitized CTL of the mice that were fed PG-mixed feed showed significantly higher cytolysis rates than those of the control group at various E/T ratios ($P<0.01$). The activities of peritoneal CTL increased with the elevation of the E/T ratio in both PG-fed and control groups (Fig. 3).

Cytotoxic activity of ConA-stimulated lymphocytes of mice fed PG-mixed feed: Cytotoxic activities of ConA-stimulated lymphocytes from spleen of the PG-fed group were higher than those of the control group against Mitsukaido cells ($P<0.05$) but against K562 cells there was no difference between the two groups. ConA-stimulated lymphocytes activity of MLN was not significantly different against K562 cells and Mitsukaido cells. The cytolysis rate of the ConA-stimulated lymphocytes derived from the MLN was higher than that from the spleen (Table 2).

DISCUSSION

The adjuvant effect of bacteria and components of the cell wall has been reported in various studies since Freund's study on killed *Mycobacterium tuberculosis* [3]. It has been shown that the cell wall of *Listeria* [18] and *Norcadia* [1], and PG in *Bifidobacterium* [16, 17] have a carcinostatic effect, and that muramyl dipeptide (MDP) purified from PG is the essential component for activating immunoresponse [2, 4, 8].

Those applications of effective immunopotentiators in the management of domestic animals should be very

Table 2. Comparison of cytotoxic activity (proportion of cell lysis) of ConA-stimulated lymphocytes of mice fed with PG-mixed feed

Target cells	Groups	Spleen	MLN
K562	Pg-treated	28.0±11.2	39.6±13.2
	Control	20.1±12.8	32.4±11.4
Mitsukaido	PG-treated	23.4±16.1 *	62.8±1.8
	Control	5.7±13.3	62.3±5.0

Lymphocytes derived from the spleen and mesenteric lymph node (MLN) of mice fed daily with PG-mixed feed for 3 weeks were stimulated with concanavalin A (Con A). The ratio of target cells to effector cells was 40:1. Data are presented as mean±S.D. *: P<0.05 as determined by t-test.

important, and the use of such agents via oral administration is rather attractive for livestock industries. Previously we have shown that oral administration of PG derived from *B. thermophilum* activates local immunological response in the small intestines of piglets [9, 15]. PG of *B. thermophilum* enhanced immunoresponce not only to swine [9, 15] but also to chickens [7], and mice and guinea pig [8]. From these aspects, it might be considered that PG of *B. thermophilum* possesses an ability of immunopotentialization to hosts of wide variety.

In this study, we conducted a cytotoxicity test with NK cells, peritoneal CTL and ConA-stimulated lymphocytes of mice and demonstrated the enhancement of cell-mediated-immunity by oral administration of PG of *B. thermophilum* derived from swine. The NK cells derived from the spleen and MLN of the mice after three weeks of daily feeding of PG showed significantly higher cytotoxic rates compared with that of the non-treated control group. In addition, the peritoneal CTL activity was also found to be higher in the PG-fed group than in the control group. However, a single-dose administration of PG showed no significant difference between treated and non-treated groups in this experimental condition. These findings indicate that repeated sensitization with PG is necessary for the enhancement of cytotoxic activity. These results are similar to those from the oral administration of OK432, a streptococcal preparation, which augmented NK activity in mice [10].

The cytotoxic activity of lymphocytes stimulated by ConA was elevated with the PG-mixed feed administration compared with control. This seems to indicate that PG increases the cytotoxic activity of lymphokine activated killer (LAK)-like cells as well as the NK and CTL activities. We concluded that immunocompetent cells reacted strongly to stimulation by PG when compared with the cells in the control group.

The cytotoxic activity of lymph derived from the MLN was higher than that of the cells from the spleen, indicating that orally administered PG-mixed feed had a strong effect on the MLN.

Microfold cells (M cells) [12, 20] in the enteric canal are considered as the most hopeful candidates involved in the first process of activation by the oral intake of PG. A likely scenario is that the orally administered PG was phagocytized by M cells and a portion of PG was communicated to antigen-presenting cells such as macrophages in M cells. Activated antigen-presenting cells may secrete cytokines. Then these cytokines would activate the lymphocytes. We consider that the IL-2 is the most important cytokine for cytotoxic lymphocytes. In this regard, it has also been reported that cytotoxic cells, part of CD8⁺ T cells and NK cells possess the IL-2 receptor (IL-2 R β), which is one of the IL-2 binding proteins [19, 22]. Therefore, it can be assumed that NK, CTL and LAK-like cells may be enhanced by IL-2 which the PG activated lymphocytes secreted.

From the results of this study, it is clear that the oral consecutive administration of PG may be a very effective procedure for the enhancement of cellular immunity, and may have a wide and beneficial range of applications in the livestock industry.

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