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Immunostimulation Effects of Cell Wall Components Isolated from *Lactobacillus plantarum*

KANG, KWAN YUEB, SUNG HO PARK AND TAE BOO CHOE*

Department of Microbial Engineering, Kon-Kuk university, Seoul 133-701, Korea

Immunostimulation effects of the cell wall components isolated from *Lactobacillus plantarum* were investigated by studying the macrophage's tumorcidal activity, splenocyte proliferation, anticomplementary activity and the inhibition of peritoneal tumor cell growth measured with ICR mice inoculated with sarcoma 180. The immunopotentiating cell wall components were a complex of peptidoglycan and exopolysaccharides. The tumorcidal activity of macrophage against Yac1 and B16 tumor cells was enhanced when the cell wall components were added into the macrophage's culture medium. They also stimulated splenocytes to proliferate up to the same level as when the concanavalin A was added into the splenocyte's culture medium. The complementary activity was inhibited by 50% when the cell wall components were incubated with the sheep red blood cells treated with hemolysin and guinea pig complement. This result confirmed that the cell wall components had an antitumor effect, because the anticomplementary activity is usually accompanied by an antitumor activity at the same time. This fact was confirmed again by the inhibition of the growth of sarcoma 180 when the cell wall components were injected intraperitoneally into ICR mice inoculated with sarcoma 180. As a result, it is concluded that the cell wall components isolated from *Lactobacillus plantarum* had multifunctional immunostimulation effects *in vitro and in vivo*.

Lactobacillus has been reported to have preventive effects against microbial infection and cancer by stimulating the host's immune system (1, 2). Immunostimulating substances produced from microorganisms previously reported are Bacillus of Calmette Gueria (BCG), corynebacterium of Freund complete adjuvant, lipopolysaccharide in Gram (−) bacteria, glucans of yeast and lentinan of fungi. These substances are called Biological Response Modifier (BRM) because they don't act directly on the infectants or cancer cells like antibiotics or anticancer agent, but function by stimulating the host's immune system (3). In consequence, these substances usually have a merit of low toxicity.

Until now, many workers reported the immunopotentiating activity of the food microorganisms by showing the antitumor activities (4-6), but few works were done on the immunostimulating substances isolated from microorganisms, except the fungal and microbial exopolysaccharides. In this study, cell wall components (CWC) were isolated from *Lactobacillus plantarum* and their immunostimulating activities were investigated by studying the effects of macrophage (Mϕ) stimulation, splenocyte proliferation, anticomplementary activity and inhibition of peritoneal tumor cell growth.

*Corresponding author
Key words: immunostimulation effect, *Lactobacillus plantarum*, macrophage stimulation, antitumor activity

MATERIALS AND METHODS

Microorganism and Culture Media

Lactobacillus used in the study was chosen, for its high immunostimulating capacity, from various microbial strains isolated from *Kimchi* and identified as a *Lactobacillus plantarum* in reference to Bergey's manual and cultured in MRS medium at 30°C for 3 days (7).

Experimental Animal and Tumor Cells

Peritoneal macrophages and splenocytes were separated from ICR mouse. Tumor cells were B16 (melanoma from BALB/C), Yacl (T cell lymphoma from BALB/C) and S180 (sarcoma from ICR) and cultured in Dulbecco's modified essential medium (DMEM) with 10% fetal bovine serum (FBS).

Sarcoma 180 tumor cells were cultured intraperitoneally using ICR mouse for 15 days and washed with 0.75% saline prior to use.

Separation and Purification of Cell Wall Components

Sonication of cell mass—Culture medium of *Lactobacillus plantarum* was centrifuged at 3,000 rpm for 15 min. Cell pellet was washed with phosphate buffer saline (PBS) three times and resuspended in 50 ml PBS.

The cell suspension was sonicated three times for five minutes and centrifuged at 5,000 rpm for 15 min and the supernatant was retreived (8).

Treatment with perchloric acid—Perchloric acid was added to the supernatant up to 5% (v/v). It was then centrifuged at 12,000 rpm for 30 min to sediment coagulated proteins and dialyzed against PBS.

Treatment with periodate—The neutralized supernatant was treated with periodate 1% (w/v) overnight and dialyzed against PBS for 24 hr and concentrated in 10 fold.

Assay of Muramic acid—The amount of CWC was assayed on the base of muramic acid, an essential component of cell wall peptidoglycan of *Lactobacillus plantarum* (8). Separated CWC 0.5 m*l* (PBS) and 2.5 m*l* H_2SO_4 was contained in a sealed cap tube and boiled at 100°C for 30 min and cooled. $CuSO_4 \cdot 5H_2O$ 25 μ*l* (4% in distilled water) and hydroxydiphenyl 50 μ*l* (1.5% in ethanol) was added to each tube and was placed in water bath at 30°C for 30 min. The amount of CWC was determined by its optical denstiy at 560 nm. The amount of protein and saccharide was assayed by the Bradford's method (9) and phenol sulfate method (10), respectively.

Stimulation of Macrophage and Measurement of Tumoricidal Activity

Peritoneal exudate cell (PEC) was separated from peritoneal cavity of ICR mouse and washed with Hank's balanced salt solution (HBSS). $1.0 \sim 2.0 \times 10^5$ cells was loaded to each well of the 96 well plates and incubated in DMEM (10% FBS) for 90 min. using a 5% CO_2 incubator. Unadsorbed cells were discarded by decanting the supernatant after gentle shaking and fresh medium was added. The CWC, muramyl dipeptides (MDP, a commercial immunostimulator) and dried cell mass of *Lactobacillus plantarum* were suspended respectively in PBS at various concentrations and loaded into the macrophage's culture well by 10μ*l*. After 4 hr-incubation, 50μ*l* of tumor cell culture medium was loaded to each well (1.0×10^4 cells/well).

After 36 hrs of incubation, macrophage's tumorcidal activity was measured by MTT (3-(4,5-Dimethylthiazol-2ly)-2,5-diphenyl tetrazolium bromide) assay method. 16 μ*l* of MTT solution (5 mg/m*l*) was added to each well. After 4 hr-reaction, the supernatant was discarded and acidic isopropanol (0.04 N HCl) was added to each well to determine the formazan crystal formation by living cells (11).

Tumoricidal activity of the macrophage was expressed by % cytotoxicity calculated from the optical density measured at 540 nm. All the experiments were triplicated.

% Cytotoxicity

$$= 100 - \frac{[M\Phi + \text{Tumor}] - [M\Phi]}{[\text{Tumor}]} \times 100$$

[MΦ+Tumor]: the mean value of MTT assay measured when the MΦ and tumor cells were cocultured

[MΦ]: the mean value of MTT assay measured when the MΦ's were cultured

[Tumor]: the mean value of MTT assay measured when the tumor cells were cultured

Splenocyte Proliferation

Splenocytes were separated from ICR mouse and washed twice with HBSS and resuspended in DMEM (10% FBS) at the cell density of 4.8×10^7 cells/m*l*. 150 μ*l* of splenocyte suspension was loaded to each well. The CWC, concanavalin A and dried cell mass were suspended respectively in PBS at various concentrations and 10 μ*l* of each was added to well. After 48 hours of incubation, the extent of splenocyte proliferation was measured by MTT assay method (12).

Determination of Anticomplementary Activity

One m*l* of CWC in PBS and 1 m*l* of guinea pig complement diluted by 1 : 140 were incubated at 37°C for 30 min. Sheep Red Blood Cell (SRBC) was suspended in 1 m*l* of PBS at the cell density of 3.0×10^8 cells/m*l* and mixed with 1 m*l* of hemolysin (anti-SRBC antibody) diluted by 1 : 100. The opsonized SRBC was treated with a complement solution and after 12 hr, the extent of hemolysis was measured by determining the released hemoglobin from SRBC at 541 nm (14).

Inhibition of Tumor Cell Growth *in vivo*

ICR mice (5~6 weeks) were divided into two groups, a control and a CWC treatment group, 5 mice for each. 100 μ*l* of 0.75% saline or 3.3 μg (muramic acid base) of CWC was administrated intraperitoneally to the mice.

After 24 hr, sarcoma 180 that was adjusted to the cell density of 1.0×10^7 cells/m*l* in 0.75% saline, was intraperitoneally injected by 100 μ*l* into each mouse. Inhibition effect of the tumor cell growth by CWC was determined by measuring the mean body weight and life span of the experimental mice (12).

RESULTS AND DISCUSSION

Separation and Purification of Cell Wall Components from *Lactobacillus*

As the effective substance for the immunostimulation was found in the supernatant after the sonication of cell suspension, we started to purify the CWC from the supernatant.

Fig. 1 represents the result of Sephadex G-100 gel chromatography of CWC after the treatment with per-

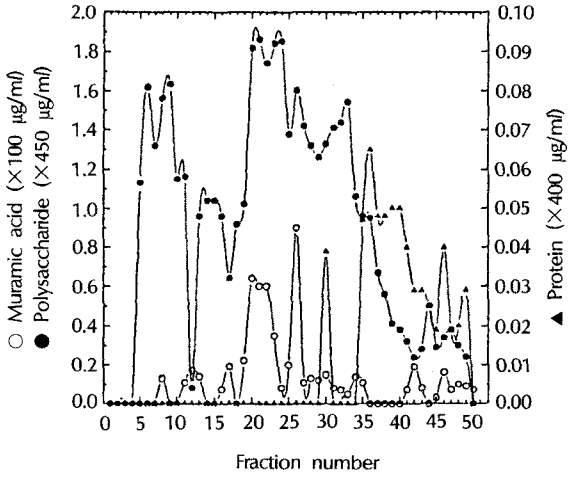

Fig. 1. Sephadex G-100 gel chromatography of the cell wall components after the treatment with perchloric acid.
Flow rate: 4 ml/hr.

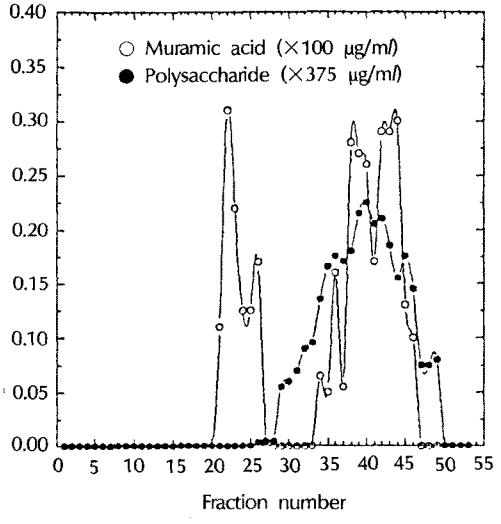

Fig. 2. Sephadex G-100 gel chromatography of the cell wall component after the treatment with periodate.
Flow rate: 4 ml/hr.

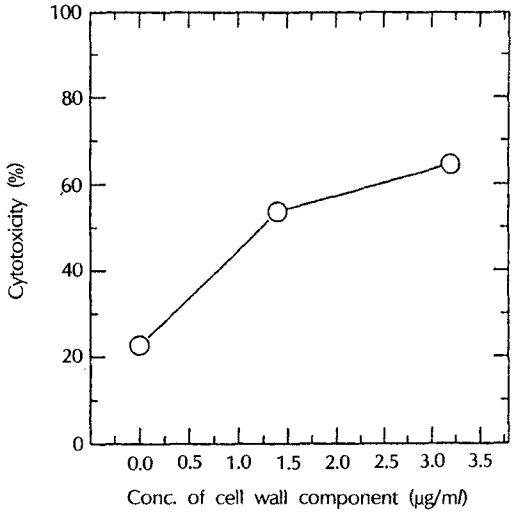

Fig. 3. Effect of the cell wall component (CW) on the tumoricidal activity of macrophage against Yac1 tumor cell (MΦ : Yac1 = 10 : 1).

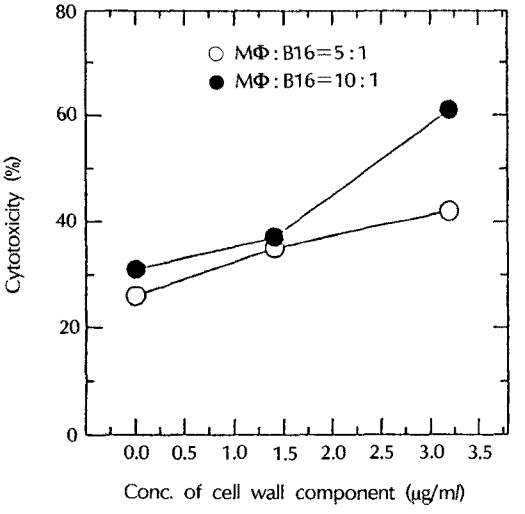

Fig. 4. Effect of cell wall component (CW) on the tumoricidal activity of macrophage against B16.

chloric acid showing that the proteins not removed by perchloric acid can be separated from the peptidoglycan containing polysaccahride. The fractions 19~23, 25, 26 and 30 of Fig. 1 were retrieved and concentrated as the sample of cell wall components containing polysaccharide (CWS).

Fig. 2 is the result of gel chromatography after the periodate treatment showing that the total sugar was reduced by 80%, but the total amount of muramic acid was almost unchanged. By this method, peptidoglycan of the cell wall components could be partially purified. The fractions 21~26, 34 and 35 of Fig. 2 were retrieved and concentrated as the sample of cell wall components without polysaccharide (CW).

Tumoricidal Activity of Macrophage Stimulated by Cell Wall Components

Fig. 3 and 4 show that the addition of CW in the culture medium of macrophage can stimulate macrophage's tumorcidal activity against the tumor cells, Yac1 and B16. The effective cytotoxicity of macrophage stimulated by CW was almost tripled. The effect of CWS on the immunostimulation of macrophage was not so different from that of CW (data not shown). Fig. 5 shows the macrophage's tumorcidal activities against S-180, which could be acquired when CW, MDP or dried cell mass were added to the culture medium. CW showed

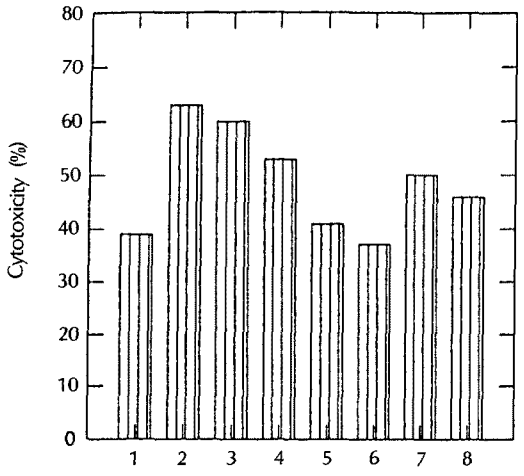

Fig. 5. Tumorcidal activity against S-180 of macrophages treated with cell wall component (CW), MDP and dried cell mass (MΦ : S180=10 : 1).
1: control, 2: CW 20 μg/ml, 3: CW 10 μg/ml, 4: CW 5 μg/ml, 5: dried cell mass 65 μg/ml, 6: dried cell mass 33 μg/ml, 7: MDP 40 μg/ml, 8: MDP 80 μg/ml.

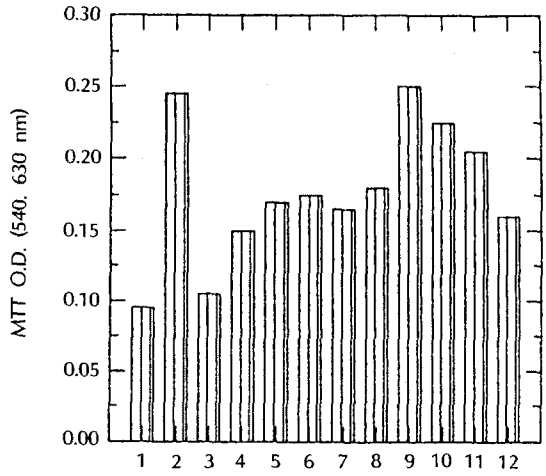

Fig. 6. Splenocyte proliferation by the cell wall component (3.5×10^6 cells/well).
1: control, 2: Con. A 0.5 μg/ml, 3: dried cell mass 12.5 μg/ml, 4: dried cell mass 20 μg/ml, 5: CW 20 μg/ml, 6: CW 10 μg/ml, 7: CW 5 μg/ml, 8: CW 2.5 μg/ml, 9: CWS 20 μg/ml, 10: CWS 10 μg/ml, 11: CWS 5 μg/ml, 12: CWS 2.5 μg/ml.

the greatest stimulation effect than any other components. As all the target tumors, Yac-1 (H-2^a type), B16 (H-2^d type) and S180 (complex type) were sensitive to the macrophages isolated from ICR mice (complex type), the cytotoxicity of macrophages seems to be independent on the MHC (major histocompetability complex) type.

Splenocyte Proliferation by Cell Wall Components

Fig. 6 shows the splenocyte proliferation caused by the CWC as well as concanavalin A and dried cell mass. The CWC, when added to the final concentration of 20 μg/ml, stimulated the splenocyte to proliferate at the same level as the concanavalin A. From this result, it may be concluded that CWC with exopolysaccharide, CWS, has an excellent splenocyte proliferation activity. According to Lee (15), it was the B lymphocytes, not the T lymphocytes, that began to proliferate when the mouse splenocytes were treated with food microoganisms.

Anticomplementary Activity of Cell Wall Component

Complementary factors can remove invading microorganisms by lysis of the invaders combining with host antibodies. Furthermore, some of the complements are converted into immunostimulation factors such as C3, C5, C3b during the activation process. An activation of complement is induced not only by antigen-antibody reaction but also by the nonspecific immunostimulating materials. If the complement factors binds to these nonspecific immunostimulating materials, they lose the activity of lysing the antigen, such as SRBC.

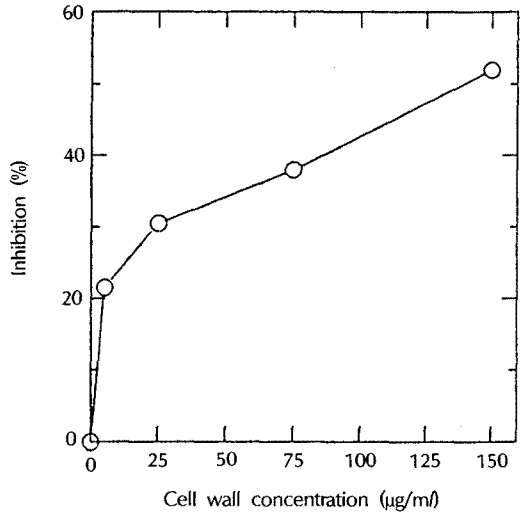

Fig. 7. The inhibition of complement activity by cell wall component (CW).

In this experiment, the serum complement of guinea pig was added to the CW and the mixture was added to the SRBC treated with hemolysin (anti-SRBC antibody) to determine the amount of complement activated nonspecifically by CW. Fig. 7 shows that when the 150 μg/ml of CW was added to the complement solution, the activity of SRBC lysis was reduced by 50%. The reduced complement's activity to lysis SRBC is caused by the convertion of the complement into the nonspecific immunostimulation factors by binding to the CW. The result implies that the cell wall components

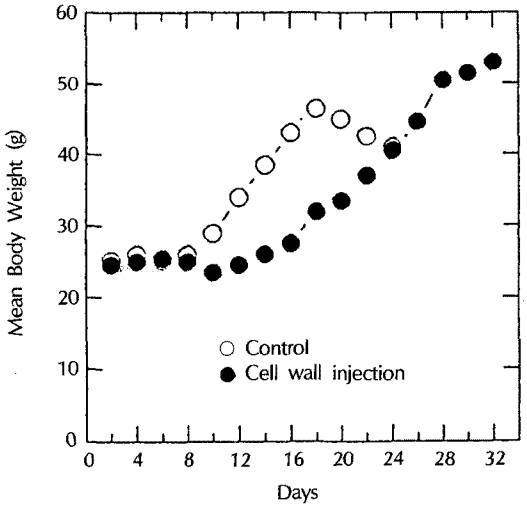

Fig. 8. Effect of intraperitoneal injection of cell wall components (CWS) on the increase of body weight of the ICR mice inoculated with S180 (CWS injection; day 1, 3.3 μg/mouse).

Table 1. The inhibition effect of *L. plantarum* cell wall components on the growth of ascitic tumor S-180 (MA: muramic acid)

Sample	Mean life span± SD	Survival rate (%)
Control	20.0± 1.8	100%
CWS	31.6± 2.0	158%
CW	25.2± 0.8	126%
MA	26.4± 0.9	132%
Crude cell extract	22.2± 1.1	111%

had an antitumor effect as the anticomplementary activity is usually accompanied by antitumor activity at the same time (16).

Inhibition of Peritoneal Tumor Growth by Cell Wall Components

Fig. 8 shows that the growth of sarcoma 180 could be retarded by 7~8 days by intraperitoneal injection of 3.3 μg of CWC (on the base of muramic acid) into mouse and talble 1 shows the effect of CWC on the prolongation of the expected life span of mice inoculated with S180. In this result, it was found that the immunological defence ability of mice could be enhanced with very little amount of CWC (about 3~4 μg), while the dried microorganism has been reported to be required more than 100 μg of cell mass for the same level of effect (4-6).

The immunopotentiating effect of CWS was superior to CW, which means that the polysaccharide group is a key factor for the efficient stimulation of murine immune system. According to Fig. 6, the polysaccharide of CW also enhances the proliferation of splenocytes *in vitro*. However, there was no great difference between the tumoricidal activities of macrophages treated with CW and with CWS.

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