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RESEARCH NOTE

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Effects of Intraperitoneal Administration of *Lactococcus lactis* ssp. *lactis* Cellular Fraction on Immune Response

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Abstract Cellular components of *Lactococcus lactis* ssp. *lactis* (heat-killed whole cells, cytoplasm, and cell walls) were tested for their *in vivo* immunopotentiating activities. Peritoneal macrophages from mice injected intraperitoneally with cell-wall fractions exhibited significantly greater phagocytic activity than groups injected with whole cells or cytoplasm fraction. Cytotoxicity of natural-killer cells was highest in cytoplasm fractions. Production of cytokines (IFN-γ, IL-2, IL-6, and IL-12) in spleen cells was significantly higher when cellular components were injected intraperitoneally, and tended to be higher in whole-cell and cytoplasm groups than in cell-wall group. These results demonstrate lactic acid bacteria whole cells and their cytoplasm and cell-wall fractions have immunopotentiating activities.

Key words: Cytokine, *Lactococcus lactis* ssp. *lactis*, NK cell activity, Phagocytic activity, Immunopotentiating activity

Introduction

An immunopotentiator is a substance that directly or indirectly enhances a particular immunological function by modifying one or more components of the immuno-regulatory networks. Immunopotentiators of bacterial origin have been used in vaccination and immunotherapy (1-2). Many lactic acid bacteria (LAB), which occur widely throughout nature, are beneficial for preventing or treating intestinal disorders and for enhancing host immune responses (3-6). Considerable attention has also been focused on the cancer-preventing activity of LAB, which inhibits tumor formation in the gastrointestinal tract of humans and animals. Because this activity is thought to be mediated by the activation of the host's immune system, many studies have investigated the effects of LAB and fermented milk on the immune system (7). The oral administration of LAB and fermented milk increases mitogenic responses (8), peritoneal macrophage activity (9), antibody responses to sheep red blood cells (10), and protection against intestinal infection (4). Animal studies have confirmed that yogurt and fermented milk containing probiotic bacteria inhibit tumor formation and proliferation (11-12). However, most reports on the antitumor and immunopotentiating activities of LAB have focused on LAB whole cells and their peptidoglycans, with little attention being paid to the soluble fraction of *Bifidobacterium* species (2, 13), despite the potential in food applications differing between soluble and insoluble materials.

Results of a previously described study on the *in vitro* immunopotentiating activity of the cellular component of *Lactococcus lactis*, reported for the first time by our group, suggest that *L. lactis* cytoplasm and cell-wall fractions as well as whole cells are capable of stimulating lymphocytes and macrophages to produce several cytokines (14). Based on these observations, in the present study, we have demonstrated systemic and mucosal immune responses of mice injected intraperitoneally with whole cells, cell-wall, and cytoplasm fractions of *L. lactis* ssp. *lactis*. These data could elucidate the interaction of the cellular components of LAB with the immune system.

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Materials and Methods

Culture of *L. lactis* ssp. *lactis* *L. lactis* was cultured in M17 media (Difco, Detroit, MI, USA) for 18 hr at 30°C. After cultivation, the cells were harvested in a refrigerated centrifuge (Vision, Seoul, Korea), washed three times with distilled water, and lyophilized for storage. The lyophilized cells were resuspended in distilled water at 10 mg/ml and sonicated with a cell disruptor (Sonics and Materials, Danbury, CT, USA) for 30 min on ice. After the suspension was centrifuged at 800 × g for 30 min at 5°C, the pellet was removed. A cell-wall fraction of the pellet and a cytoplasm fraction of the supernatant were obtained from the supernatant using an ultracentrifuge (Hitachi, Tokyo, Japan) at 70,000 × g for 30 min.

Experimental animals Six-week-old male BALB/c mice (Clea Japan, Tokyo, Japan), were housed in plastic cages in an air-conditioned room and given food and water *ad libitum*.

Administration of *L. lactis* cellular components The mice were given the desired dose of cellular components dissolved in PBS. *L. lactis* cellular components were delivered by intraperitoneal injection on days 0, 2, and 4 at 500 μg/mouse/day, and PBS was used for controls. Each mouse was killed 1 day after completing the administra-

tion, after which peritoneal exudate cells (PEC) and spleen cells were isolated.

Evaluation of the phagocytic activity of PEC The phagocytic activity of PEC was evaluated in an *in vitro* culture with fluorescent microparticles (15). PEC was isolated from the peritoneal cavity of the mice by lavage with 5 ml HBSS after the intraperitoneal injection of the cellular component. After centrifugation, the cell pellet was washed twice with HBSS and resuspended in 1 ml HBSS-HEPES. Twenty microliters of Fluoresbrite carboxylate microspheres (2.0 μm; Polyscience, Warrington, PA, USA) diluted 100-fold with HBSS-HEPES was added to the PEC suspension, which was then incubated for 1 hr at 37 °C. After stopping the reaction by adding 2 ml cold EDTA-PBS and collecting a cell pellet by centrifugation, the pellet was resuspended in 300 μl EDTA-PBS, and the phagocytic activity was measured using flow cytometry.

Analysis of natural-killer-cell activity The CytoTox 96 nonradioactive cytotoxicity assay kit (Promega, Madison, WI, USA) was used to assess the natural-killer (NK) activity of spleen cells. The CytoTox 96 assay quantitatively measures lactate dehydrogenase (LDH), a stable cytosolic enzyme that is released upon cell lysis. Concentration of released LDH in culture supernatants was measured with an enzymatic assay. Briefly, the isolated spleen cells were used as effector cells, and cells from the mouse Moloney leukemia cell line, Yac-1, were used as the target cells. Yac-1 cells (2×10^4 cells/ml) in RPMI-1640 without phenol red were seeded in round-bottomed tissue culture plates. Subsequently, 0.1 ml spleen cells treated with cellular components was added at appropriate concentrations. The assay plates were incubated for 4 hr in a humidified chamber at 37°C and 5% CO_2, and, after 4 hr, centrifuged at $250 \times g$ for 4 min. The supernatants were transferred, and the substrate mix was added to each well. After incubation for 30 min, the absorbance was measured at 490 nm. The NK-cell activity was calculated as a percentage of effector-cell-specific lysis. The percentage of specific LDH release was calculated according to the following formula:

Specific lysis (%) = (experimental-effector spontaneous - target spontaneous)/(target maximum - target spontaneous) × 100

Quantification of cytokine production Spleen, Peyer's patch, and mesenteric lymph-node cells were added to each well of a 24-well plate and cultured in the presence of Con A (5 μg/ml) or LPS (20 ng/ml) for 48 hr at 37°C: Con A was used for measuring IFN-γ, IL-2, and IL-4 productions; and LPS was used for IL-6, IL-12, and TNF-α. Cell-free supernatant fractions were harvested and stored at -20°C until assayed.

Concentration of cytokines in culture supernatant was determined using a sandwich ELISA. Briefly, microtiter plates were coated overnight at 4°C with purified rat anti-mouse cytokine-capture antibody at 50 μl/well (Pharmingen, San Diego, CA, USA) in 0.1 M sodium bicarbonate buffer (pH 8.2). The plates were then washed three times with PBS containing 0.2% Tween-20 (PBS-T), blocked with 100 μl of 1% (w/v) bovine serum albumin (BSA) in PBS for 30 min at 37°C, and washed three times with PBS-T. Standard murine cytokines or samples were diluted in PBS-T solution containing 1% BSA, and 50-μl aliquots of the mixture were added to the appropriate wells. The plates were incubated overnight at 4°C and washed four times with PBS-T. Subsequently, 50 μl biotinylated rat antimouse cytokine-capture monoclonal antibody diluted in BSA-PBS was added to each well. The plates were incubated at room temperature for 60 min and washed six times with PBS-T. Fifty microliters of streptavidin–alkaline-phosphatase conjugate diluted in BSA-PBS was added to each well. The plates were incubated for 30 min at room temperature, washed with PBS-T, and, to each well, added with 50 μl substrate (*p*-nitrophenylphosphate). The absorbance was read at 405 nm on a microplate reader (Bio-Rad, Hercules, CA, USA), and cytokine concentrations were quantified using a standard curve.

Statistics Significant differences between the experimental and control groups were determined using Fisher's protected least-significant difference (PLSD) test. All results are presented as means ± SD values. Tests were considered significant at $P<0.05$.

Results and Discussion

Phagocytic and NK cells are the major effectors of natural immunity, and numerous reports have been published on the relation between LAB and natural immunity (16). The phagocytic activity of peritoneal macrophages is shown in Fig. 1 as the phagocytic uptake of fluorescent microparticles by PEC. The microparticles incorporated into cells were counted with a flow cytometer (Fig. 1A). The results are expressed as the mean percentages of cells in which one, two or more than two particles were incorporated. Peritoneal macrophages from mice injected with cell-wall fractions exhibited significantly greater phagocytic activity than the other groups. Although the production of TNF-α and IL-6 in peritoneal macrophages was not affected by LAB cellular components (Fig. 1C), the percentage of cells incorporating three or more particles in the group injected with cell walls was three times higher than that of PEC from the control mice (PBS group). NK activity was assessed using spleen cells; the cytotoxicity of NK cells was about two times higher in cytoplasm groups than in the other groups (Fig. 2). Enhanced NK cytotoxicity has been reported to prevent cancer, and these cells may play an important role in the regulation of tumor development and metastasis (17). The primary target of LAB for their immunostimulatory effect was shown to be NK cells (18), and NK-cell activation by *Lactobacillus casei* has also been reported (19-20).

The production of cytokines by splenocytes in response to Con A or LPS was enhanced by intraperitoneal injection of cellular fractions of *L. lactis*. The production of IFN-γ, IL-2, IL-6, and IL-12 was significantly higher in spleen cells from the cytoplasm-fraction-injected group than in PBS-injected controls (Fig. 3). The injection of the whole-cell preparation enhanced the secretion of IL-2, IL-6, and IL-12, while the cell-wall fraction only enhanced the secretion of IL-6 and IL-12. The production of IL-4 and TNF-α did not differ significantly with the type of

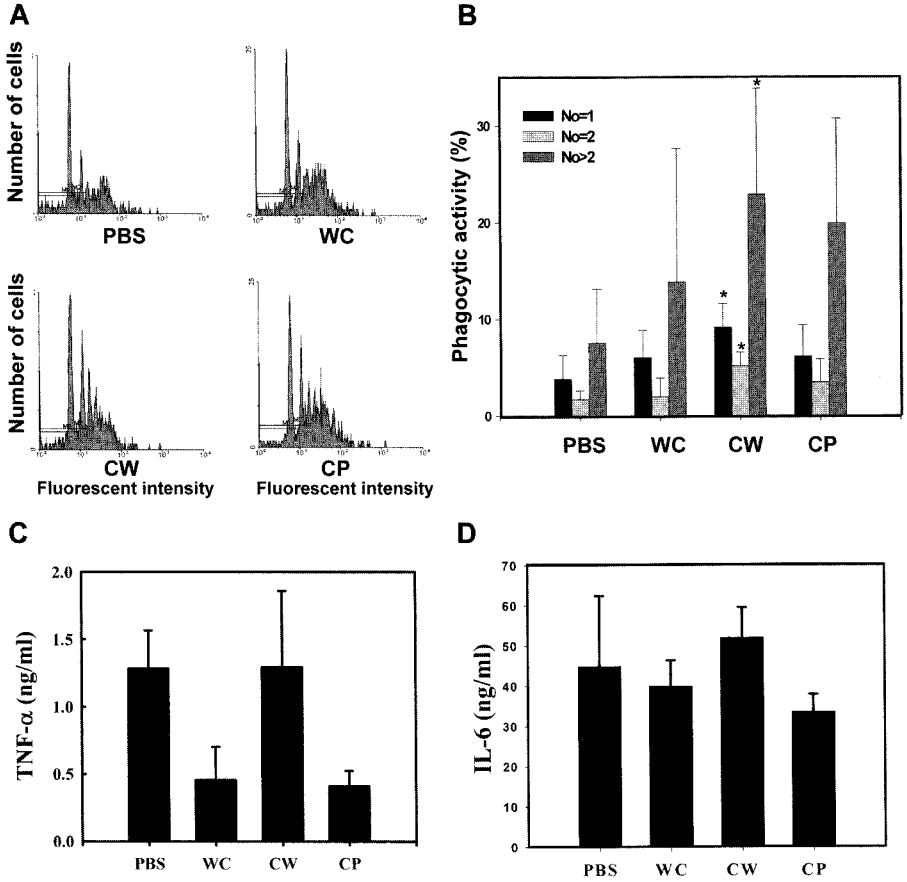

Fig. 1. Phagocytic activity of PEC obtained from mice injected intraperitoneally with heat-killed whole cells (WC), cell walls (CW), and cytoplasm (CP) of *L. lactis* at 500 μg/mouse on days 0, 2, and 4. On day 5, the phagocytic activity was measured (A, B) and cytokine production in PEC was analyzed (C). (A) Typical result of flow cytometry. (B) Course analysis of PEC phagocytic activity ("No." is the number of particles per cell). (C) TNF-α production in PEC. (D) IL-6 production in PEC. Data are shown as mean ± SD values (n=6, $^*P < 0.05$).

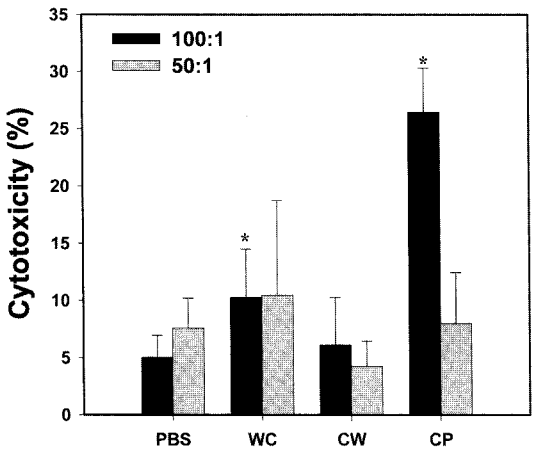

Fig. 2. Percentage cytotoxicity of the natural-killer activity of spleen cells obtained from mice injected intraperitoneally with WC, CW, and CP of *L. lactis* against Yac-1 cells. Effector: target cell ratios were 100:1 and 50:1. The cytotoxicity was measured using LDH assays. Data are shown as mean ± SD values (n=6, $^*P < 0.05$)

fraction injected (Fig. 3). Macrophages are the main IL-12-, IL-6-, and TNF-α-producing cells, and these are important target cells for the antitumor or immunomodulating effects of some microorganisms. In particular, IL-12 potently stimulates cytotoxic T cells and NK cells, and enhances the production of several cytokines, including IFN-γ, IL-2, and TNF-α. In the present study, the injection of cellular components of *L. lactis* mainly augmented the production of IFN-γ, IL-2, IL-6, and IL-12, which is consistent with the results of previous studies (21-25). Most studies to date on the immunopotentiating activity of LAB have focused on whole LAB cells and their peptidoglycans (26), with little attention being paid to the soluble fraction, despite the potential in food applications differing between soluble and insoluble materials. Several recent studies have examined the effects of LAB cytoplasm on immunopotentiating activity (14). For example, the polysaccharide fraction of *B. adolescentis* cytoplasm stimulates Peyer's patches and lymph-nodes lymphocytes *in vitro* (2, 27). Another species of *Bifidobacterium*, *B. breve* (either whole cells or a cell-wall preparation) accelerates the *in vitro* proliferation of Peyer's-patch cells, particularly B cells (13). Takahashi *et al.* (28) investigated the interaction of cell fractions of LAB and the immune system using *B. longum* and *Lb. acidophilus*. Tejada-Simon and Pestka (29) reported that LAB and their cell-free extracts exhibit mitogenic and polyclonal-activating properties when cultured with cells of the immune system. Lee *et al.* (30) also reported that *Bifidobacterium* whole cells and cell-free extracts differentially induce cytokine

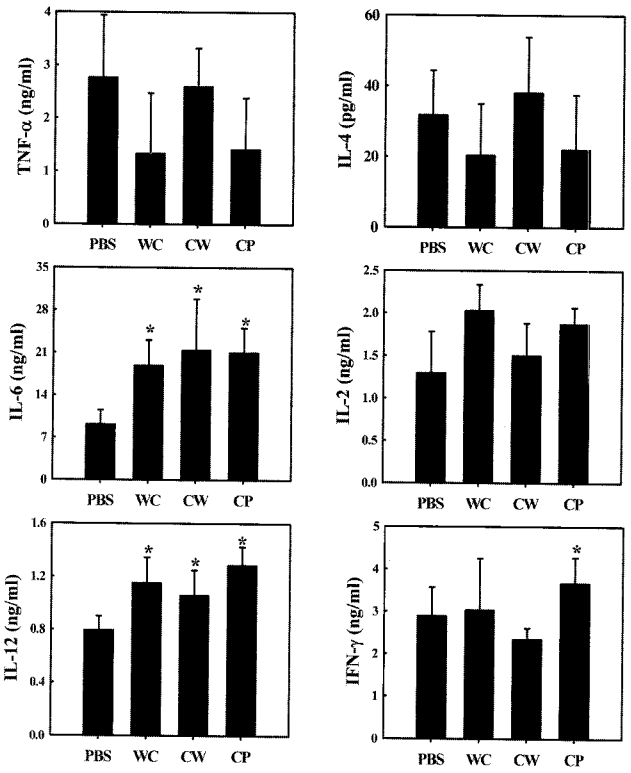

Fig. 3. Production of cytokines by spleen cells from mice injected intraperitoneally with WC, CW, and CP of *L. lactis*. Spleen cells were cultured with mitogen (Con A or LPS) for 48 h. The concentrations of cytokines (IL-2, IL-4, and IFN-γ for Con-A-stimulated cultures, and IL-6, IL-12, and TNF-α for LPS-stimulated cultures) in culture supernatant were measured by ELISA, and the data are shown as mean ± SD values (n=6, $*P < 0.05$)

production in murine macrophages. The present study has demonstrated that LAB whole cells as well as their cytoplasm and cell-wall fractions have immunostimulating activities, a finding which is important for understanding the mechanisms underlying the immunoregulatory function of LAB and their potential applications.

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