Inc, Irvine, CA), 100U/ml penicillin (BANYU Pharmaceutical CO., LTD., Tokyo, Japan), 100μg/ml streptomycin (MEIJI SEIKA KAISHA, LTD., Tokyo, Japan), 3mM L-glutamine, and 50μM 2-ME for the indicated times at 37°C in a humidified atmosphere containing 5% CO_2.

2.4 Measurement of cytokine and IgA production *in vitro*

PP cells and splenocytes were prepared from 6-8 weeks old BALB/c mice. Mouse immune tissue cells (2.5×10^6cells/well in a total volume of 1ml) were cultured with 50-200μg/ml DS-βG, 20μg/ml Lipopolysaccharide (LPS; from Esherichia coli O55:B5) or 100μg/ml zymosan (Sigma, St. Louis, MO) in 48-well flat-bottomed plates. Control cultures were incubated in base medium alone. Culture supernatants were collected after 72 h and assayed for IL-5, IL-6, IL-12(p40/p70) and IFN-γ levels. At the same time, mouse immune tissue cells (2.0×10^5cells/well in a total volume of 200μl) were cultured with mitogens in 96-well flat-bottomed plates and supernatants collected after 1 week and assayed for IgA production. Cytokine and IgA levels were measured by sandwich ELISA.

2.5 Measurement of cell proliferation *in vitro*

PP cells and splenocytes (2.0×10^5cells/well in a total volume of 200μl) were cultured with 50-200μg/ml DS-βG, 20μg/ml LPS or 100μg/ml zymosan in 96-well flat-bottomed plates. Control cultures were incubated in base medium alone. After 40 h incubation, 20μl of CellTiter 96® AQueous One Solution Reagent (Promega Corporation, Madison, WI) was added to each well and the plate incubated for 3 h. Total cell count was measured by the absorbance of light at 490 nm via a microplate reader. Relative proliferation activity was quantified by comparison with control cultures.

2.6 Determination of cytokine and IgA levels by ELISA

Ninety-six-well plate (Immuno Plate; Nunc, Inter-Med, Denmark) were coated with relevant purified anti-mouse cytokine anti-bodies (BD Bioscienses Pharmingen, San Jose, CA) or goat anti-mouse IgA (MP Biomedicals Inc., Irvine, CA) in 0.1M $NaHCO_3$ (pH 8.4) or 0.1M Na_2HPO_4 (pH 9.0) and incubated overnight at 4°C. Wells were washed with phosphate buffered saline (PBS) buffer containing 0.05% Tween-20 (PBS-T). Uncoated binding sites in the wells were blocked with PBS containing 1% bovine serum albumin (Sigma) at room temperature for 2 h.Wells were washed with PBS-T and then either standard recombinant cytokines or IgA dilutions or culture supernatant samples in PBS-T were added to the wells and incubated overnight at 4°C. Wells were then washed and relevant biotinylated anti-mouse cytokine antibodies (Pharmingen) or IgA antibody (Sigma) in PBS-T added and incubated at

room temperature for 2 h. Wells were washed and streptavidin-alkaline phosphatase (Invitrogen Corp., Carlsbad, CA) in PBS-T added and incubated at room temperature for 1 h. Plates were washed and the substrate solutions added (i.e. *p*-nitrophenyl phosphate) and incubated at 37°C for about 30 minutes. The absorbance of each well at 405 nm was measured using an automated spectrophotometer (microplate reader, BIO-RAD, Hercules, CA). Cytokines and IgA concentrations in culture supernatants were calculated from the standard curves produced by dilutions of the recombinant standard.

2.7 Statistical analyses

Results are expressed as means + S.E.M. and were compared using the Tukey test. Differences between control and stimulation groups were considered statistically significant at $p < 0.05$*, $p < 0.01$** or $p < 0.001$***.

3. RESULTS AND DISCUSSION

Beta-(1,3-1,6)-linked glucan (DS-βG) was prepared via specific treatments to reduce its viscosity, improve filtration and recovery. The molecular mass of DS-βG was determined to be between 5.0×10^4 and 3.0×10^5 by gel filtration chromatography, and the average mass approximately 1.0×10^5. The structure consisted of a main chain of β-(1,3)-linked β-D-glucose with β-(1,6)-linked side-chains. The integral ratio of branches of β-1,6 for β-1,3 was estimated to be 50-80% from NMR and emzymatic analyses. Finally, DS-βG was prepared to 85 to 95% purity (Suzuki, T. 2005, Iizuka, M. 2002).

To investigate the effects of DS-βG on mouse immune tissue, cell proliferation, cytokine and IgA production were assessed *in vitro* following stimulation with 0-200μg/ml of DS-βG. As a positive control, LPS or zymosan were simultaneously added to the cultured immune cells. DS-βG stimulated cell proliferation of both PP cells and splenocytes in a dose-dependent manner (Table 1). DS-βG also stimulated IL-5 or IL-6 production by cultured PP cells in a dose-dependent manner (Table 2). A similar result was obtained for IgA production (Table 2). The culture supernatant from DS-βG-treated PP cells showed a significantly higher amount of IL-5, IL-6 and IgA comparable to those from zymosan-treated cultures at the same concentration (100μg/ml) (Table 2). As such, it appears that highly purified DS-βG causes a more effective immune response than zymosan. IL-12(p40/p70) and IFN-γ production were also stimulated by DS-βG (data not shown). These results clearly show that DS-βG stimulates PP

cells to produce cytokines or IgA *in vitro*. In addition, DS-βG stimulated IL-6 production by cultured splenocytes in a dose-dependent manner (data not shown).

We then postulated whether oral administration of DS-βG could stimulate IgA production in PP cells and splenocytes. IgA production was increased in PP cells from mice given DS-βG in comparison to the control group with results for both groups being almost the same (data not shown). IgA production by splenocytes from both groups was not significantly different (data not shown).

Table 1. Effects of DS-βG on cell proliferation by Peyer's patches cells and splenocytes of BALB/cA mice *in vitro*.

		Relative activities	
	(μg/ml)	PP	SPL
Control		1.000	1.000
DS-βG	50	1.523 ± 0.035***	1.243 ± 0.033**
DS-βG	100	1.636 ± 0.086***	1.373 ± 0.026***
DS-βG	200	1.784± 0.018***	1.652 ± 0.048***
LPS	20	2.396 ± 0.072***	1.795 ± 0.023***
Zymosan	100	0.889 ± 0.004	0.811 ± 0.027

PP cells and splenocytes were stimulated with DS-βG (50-200μg/ml), LPS (20μg/ml) or zymosan (100μg/ml). Control cultures were incubated in culture medium with sterile saline/diluent added. Cell proliferation levels show relative activity compared with control cultures as baseline. The results represent the mean ± S.E.M. of triplicate independent assays. Statistically significant differences are shown as $p < 0.01$**, $p < 0.001$***.

Table 2. Effects of DS-βG on IL-5, IL-6 and IgA productions by Peyer's patches cells from BALB/cA mice *in vitro*.

		Cytokine and IgA production (ng/ml)		
	(μg/ml)	IL-5	IL-6	IgA
Control		n.d. (+ / −)	+ / −	+ / −
DS-βG	50	n.d.	+	+
DS-βG	100	+	+	++
DS-βG	200	+	++	+++
LPS	20	n.d.	++	+
Zymosan	100	n.d.	−	−

PP cells were stimulated with DS-βG (50-200μg/ml), LPS (20μg/ml) or zymosan (100μg/ml). Control cultures were incubated in culture medium with sterile saline/diluent added. Supernatant cytokine and IgA levels were measured by ELISA. The results represent + / − (control), − (decreased), + (increased), ++ (increased, $p < 0.05$) and +++ (increased, $p < 0.01$). Statistically significant differences are shown as $p < 0.05$*, $p < 0.01$**.

In this study, we propose that DS-βG stimulates mucosal immune tissues, such as PP. IL-5 and IL-6 production was enhanced in PP cells

stimulated by DS-βG *in vitro*. IL-5 and IL-6 are known to further enhance IgA production. IgA production was enhanced in PP cells by DS-βG *in vitro*. Therefore, we suggest that DS-βG may prevent infection by pathogens through interactions with the intestinal immune system. In addition, IFN-γ production was enhanced in cultured PP cells by DS-βG. This result suggests that elimination of intracellular pathogens may also be effectively induced. We here suggest that DS-βG up-regulates intestinal immune responses and is available as a health food material.

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In Vivo Immunopotentiating Effects of Cellular Components from *Lactococcus lactis* ssp. *lactis*

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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 activity. Peritoneal macrophages from mice orally administered with heat-killed whole cells exhibited significantly greater phagocytic activity than the groups administered with cell-wall fraction or cytoplasm fraction. The cytotoxicity of natural-killer cells was the highest in the group administered with whole cells, and the production of cytokines (IFN-γ, IL-2, and IL-12) in spleen cells was significantly higher, when cellular components were injected, and it tended to be higher in the cell-wall and cytoplasm groups than in the whole-cell group. Interestingly, the cytokine production of Peyer's patch cells was high, when cytoplasm fractions were administered. These results demonstrate that whole cells and cytoplasm and cell-wall fractions of *L. lactis* ssp. *lactis* have immunopotentiating activities, which are related to the stimulation of Peyer's patches.

Key words: Cytokine, immunopotentiating activity, *Lactococcus lactis* ssp. *lactis*, NK cell activity, phagocytic activity

The capacity of certain lactic acid bacteria (LAB) to function as probiotics, when ingested as a part of fermented dairy products or dietary adjuncts, is receiving increasing attention, and an extensive volume of literature on the possible health benefits associated with the consumption of LAB is now available [1, 5, 11, 13, 15]. The mechanisms underlying these favorable effects include changes in viable populations of microorganisms in the intestinal flora, competition for adhesion sites and nutrients between ingested bacteria and potential pathogens, production of antibacterial substances, and stimulation of the immune system. With regards immunity, Perdigon *et al.* [17] observed enhanced macrophage and lymphocyte activities in mice after administration of a mixed culture of *Lactobacillus acidophilus* and *Lb. casei*, and also reported that peritoneal macrophages in mice were activated by the oral administration of *Lb. casei* and *Lb. bulgaricus* [16, 18]. Similar results have been found for *Streptococcus thermophilus* and *Lb. acidophilus* orally administered [19], and heat-killed *Lb. casei* injected into mice [20]. The oral administration of LAB and fermented milk has been demonstrated to increase mitogenic responses [3]. These reports indicate that orally administered LAB and fermented milk stimulate the host's immune system. However, most reports on the immunopotentiating activity of LAB have focused on whole LAB cells and their peptidoglycans, with little attention being paid to the soluble fraction, although the potential in food applications is different between soluble and insoluble materials. We have recently described the *in vitro* immunopotentiating activity of the cellular component, containing the soluble fraction of *Lactococcus lactis* [12]. Those results indicated that *L. lactis* cytoplasm and cell-wall fractions as well as whole cells are capable of stimulating lymphocytes and macrophages to produce several cytokines. Based on these observations, we demonstrated the systemic and mucosal immune responses of mice administered orally with whole cells and cell-wall and cytoplasm fractions of *L. lactis* ssp. *lactis*.

L. lactis was cultured in M17 media (Difco, Detroit, MI, U.S.A.) for 18 h at 30°C. After cultivation, the cells were harvested in a refrigerated centrifuge (Vision, Seoul, South Korea), washed three times with distilled water, and lyophilized for storage. The lyophilized cells were resuspended

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at 10 mg/ml in distilled water and sonicated with a cell disruptor (Sonics and Materials, Danbury, CT, U.S.A.) 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.

To elucidate the immunopotentiating activity of the cellular components of *L. lactis*, whole cells, cell walls, or cytoplasm of *L. lactis* were administered to mice, and the phagocytic function, natural killer (NK) cell activity, and cytokine production were assayed. Oral administration was performed as follows. Six-week-old male BALB/c mice (Clea Japan, Tokyo, Japan) were orally given the desired dose of cellular components dissolved in phosphate-buffered saline (PBS). *L. lactis* cellular components were administered orally for 7 consecutive days at 4 mg/mouse/day, with the solution delivered to the stomach via a stainless steel needle. PBS was used for controls. Each mouse was killed the day after completing administration, and then peritoneal exudate cells (PEC), spleen, and Peyer's patch cells were isolated.

Phagocytic cells are the major effectors of natural immunity, and there are numerous reports on the relationship between LAB and natural immunity [4]. The phagocytic activity of PEC was evaluated in a culture with fluorescent microparticles [23]. Therefore, PEC was isolated from the peritoneal cavity of the mice by lavage with 5 ml of HBSS after the oral injection of the cellular component. After centrifugation, the cell pellet was washed twice with HBSS and resuspended in 1 ml of HBSS-HEPES. Twenty μl of Fluoresbrite carboxylate microspheres (2.0 μm; Polyscience, Warrington, PA, U.S.A.) diluted 100-fold with HBSS-HEPES was added to the PEC suspension, which was then incubated for 1 h at 37°C. After stopping the reaction by adding 2 ml of cold EDTA-PBS and collecting a cell pellet by centrifugation, the pellet was resuspended in 300 μl of EDTA-PBS, and the phagocytic activity was measured using flow cytometry. Figure 1 shows the phagocytic activity of peritoneal macrophages as the phagocytic uptake of fluorescent microparticles by PEC, when orally administered. The microparticles incorporated into cells were counted with a flow cytometer (Fig. 1A), and the results are expressed as the percentages of cells in which one, two, or more than two particles were incorporated (Fig. 1B). The phagocytic activity of peritoneal macrophages was significantly greater in mice administered with heat-killed whole cells than in the PBS, cell-wall, and cytoplasm groups (Fig. 1): For example, the activity was about 1.5 times higher than in the PBS group.

NK activity, another effector of natural immunity, of spleen cells was determined using the CytoTox 96 nonradioactive cytotoxicity assay kit (Promega, Madison, WI, U.S.A.). The CytoTox 96 assay quantitatively measures lactate dehydrogenase (LDH), a stable cytosolic enzyme that is released upon cell lysis. Released LDH in culture supernatants was then 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

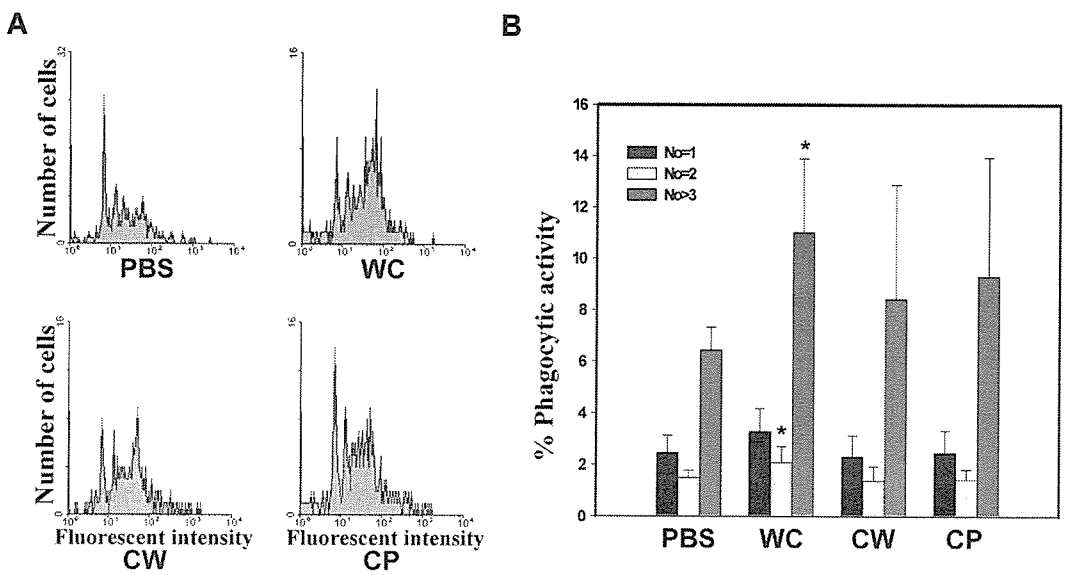

Fig. 1. Phagocytic activity of PEC obtained from mice orally injected with whole cell (WC), cell-wall (CW), and cytoplasm (CP) fractions of *L. lactis* at 4 mg/mouse for 7 consecutive days.

On day 7, the phagocytic activity was measured using flow cytometry. **A**. Typical result of flow cytometry. **B**. Analysis of PEC phagocytic activity based on flow cytometry ("No" is the number of microparticles taken up by PEC). Data are shown as mean±SD values (n=6, *P<0.05).

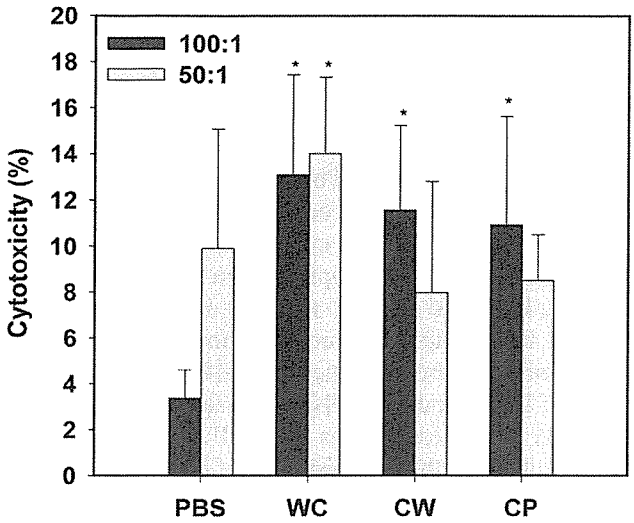

Fig. 2. Percentage cytotoxicity of NK cells in spleen cells obtained from mice orally administered with whole cell (WC), cell-wall (CW), and cytoplasm (CP) of *L. lactis* against Yac-1 cells; effector:target cell ratios were 100:1 and 50:1.
The cytotoxicity was measured using LDH assays. Data are shown as mean±SD values (n=6, *P<0.05).

of the spleen cells suspension treated with cellular components was added at appropriate concentrations. The assay plates were incubated for 4 h in a humidified chamber at 37°C and 5% CO_2 and then centrifuged at 250 ×g for 4 min. The supernatants were transferred, and the substrate mix was added to each well. After incubation for 30 min, absorbance at 490 nm was measured. 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. Enhanced NK cytotoxicity has been reported to be effective in cancer prevention. These cells may play an important role in the regulation of tumor development and metastasis [10]. The primary target of LAB for their immunostimulatory effect was shown to be NK cells [6], and NK cell activation by *Lb. casei* has also been reported [9, 21]. However, in the present study, all mice receiving *L. lactis* cellular fractions showed NK activities similar to those in control mice (Fig. 2), and the group treated with heat-killed whole cells tended to have a slightly higher cytotoxic activity than the controls.

The production of cytokines by spleen and Peyer's patch cells in response to Con A (5 μg/ml) or LPS (20 ng/ml) for 48 h at 37°C was assessed using a sandwich ELISA: Con A was used for measuring IFN-γ, IL-2, and IL-4, and LPS was used for IL-12. Thus, cell-free supernatants

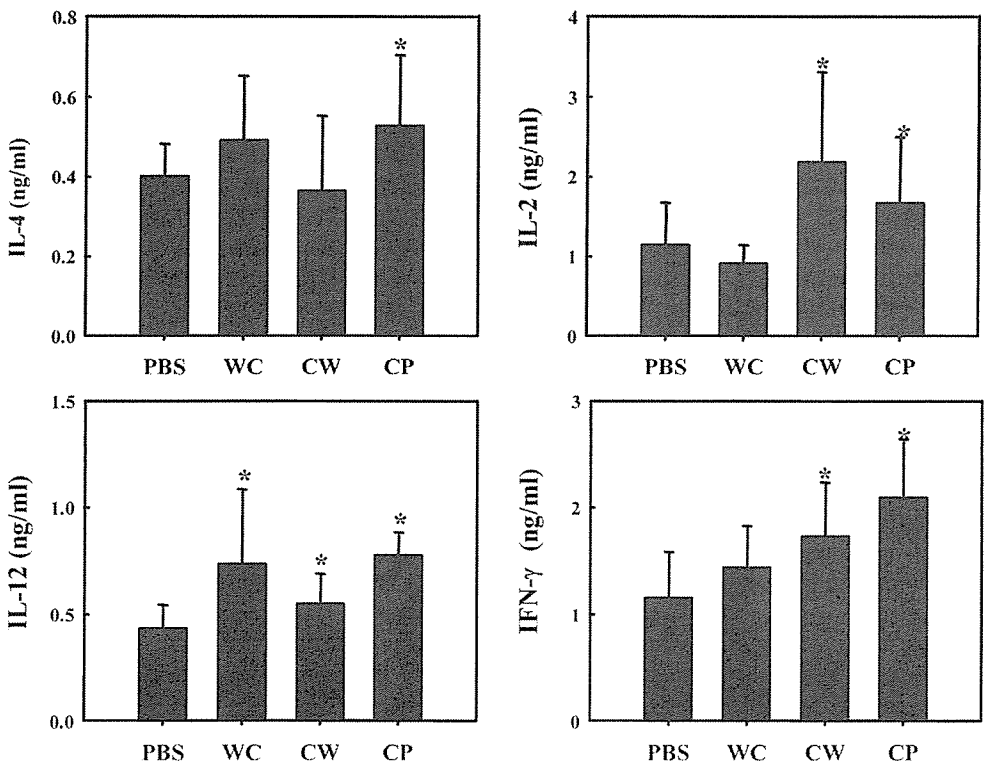

Fig. 3. Production of cytokines by spleen cells from mice orally administered with whole cell (WC), cell-wall (CW) and cytoplasm (CP) fractions of *L. lactis*.
Spleen cells were cultured with mitogen (Con A for IFN-γ, IL-2, and IL-4; or LPS for IL-12) for 48 h. The concentrations of cytokines in culture supernatant were measured using ELISA, and the data are shown as mean±SD values (n=6, *P<0.05).

were harvested and stored at −20°C until assayed. 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, U.S.A.) in 0.1 M sodium bicarbonate buffer (pH 8.2). The plates were washed three times with PBS containing 0.2% Tween-20 (PBS-T). Plates were 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 were added to the appropriate wells. The plates were incubated overnight at 4°C, washed four times with PBS-T, and then 50 μl of biotinylated rat anti-mouse cytokine-capture monoclonal antibody diluted in BSA-PBS was added to each well. Plates were incubated at room temperature for 60 min and washed six times with PBS-T. Fifty μl of streptavidin-alkaline-phosphatase conjugate diluted in BSA-PBS was added to each well, and plates were incubated for 30 min at room temperature. The plates were then washed with PBS-T, and 50 μl of substrate (*p*-nitrophenylphosphate) was added to each well. The absorbance was read at 405 nm on a microplate reader (Bio-Rad, Hercules, CA, U.S.A.), and cytokine concentrations were quantified using a standard curve. As seen in Fig. 3, the production of IL-2, IL-12, and IFN-γ was higher in spleen cells when orally administered with either cytoplasm or cell-wall fractions than with whole cells. The production of cytokines in Peyer's patches was also studied. Interestingly, in Peyer's patch cells, the cytoplasm fraction was more effective than whole cells and the cell-wall fraction: The production of IL-2, IL-12, and IFN-γ by Peyer's patch cells was significantly higher in mice orally administered with cytoplasm fractions than in the other groups (Fig. 4). Macrophages are the main producers of IL-12, IL-6, and TNF-α, and they 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, such as IFN-γ, IL-2, and TNF-α. In the present study, the administration of cellular components of *L. lactis* augmented mainly the production of IFN-γ, IL-2, and IL-12, which is consistent with earlier studies [2, 7, 8, 14, 22]. The results demonstrate that *L. lactis* whole cells as well as their cytoplasm and cell-wall fractions have immunostimulating activities, and that this mechanism is related to the stimulation of Peyer's patches. The reason why the cytoplasm fraction was so effective in stimulating Peyer's patch cells is not clear, but it might have been due to the fact that this fraction is effectively incorporated into the Peyer's patch follicles.

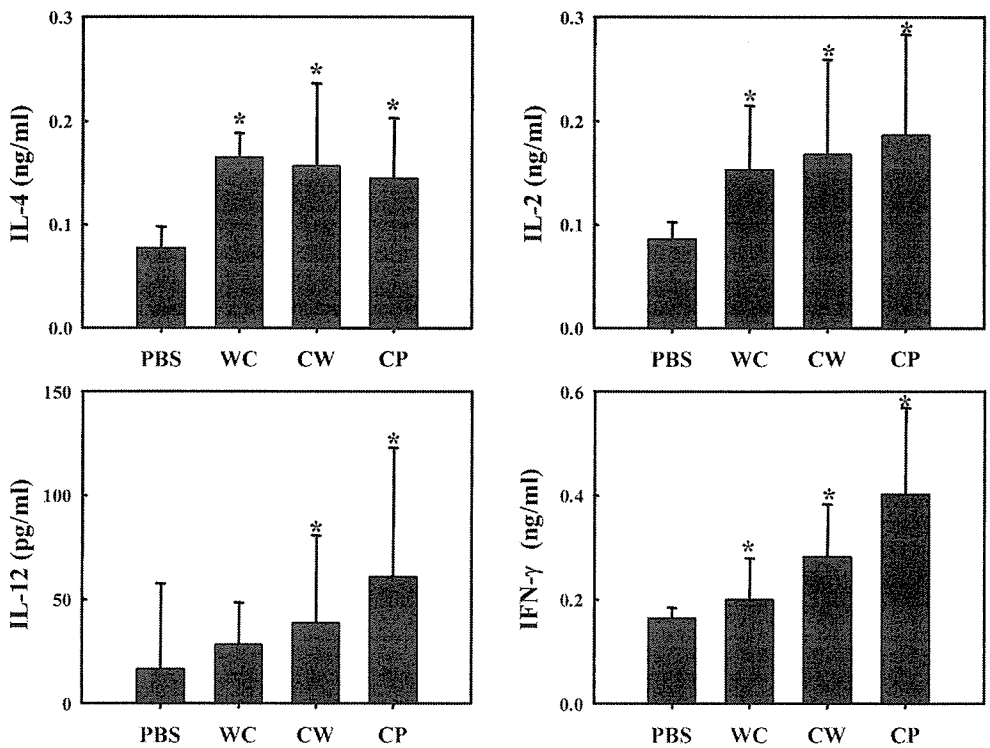

Fig. 4. Production of cytokines by Peyer's patch cells from mice orally administered with whole cell (WC), cell-wall (CW) and cytoplasm (CP) fractions of *L. lactis*.

Peyer's patch cells were cultured with mitogen (Con A for IFN-γ, IL-2, and IL-4; or LPS for IL-12) for 48 h. The concentrations of cytokines in the culture supernatant were measured using ELISA, and the data are shown as mean±SD values (n=6, *P<0.05).

In conclusion, the present results suggest that *L. lactis* whole cells as well as their cytoplasm and cell-wall fractions are capable of stimulating splenocytes, Peyer's patches, and macrophages to produce several cytokines. Elevated cytokine production (particularly of IFN-γ and IL-12) was correlated with NK cell activity, which is important in the understanding of the mechanisms underlying the immunoregulatory function of *L. lactis* and its potential applications.

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Evaluation of Blood Pressure Measured by Tail-Cuff Methods (without Heating) in Spontaneously Hypertensive Rats

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Conventional noninvasive blood pressure in conscious rats or mice are typically measured using the tail-cuff method after heating the animal. The goal of this study was to assess the validity of a novel tail-cuff method without animal heating when compared with the conventional heating tail-cuff method (unanesthetized rats with heating), telemetry method (unanesthetized restrained rats without heating), or carotid arterial catheter method (anesthetized rats, carotid arterial cannulation). The blood pressure and heart rate of spontaneously hypertensive rats were measured at 13:00—17:00 h for all experiments. Experiments demonstrated similar systolic blood pressure measurements when comparing the unheated-animal tail-cuff method and the telemetry method. Further, values obtained by both methods were lower than those obtained by the heated-animal tail-cuff method. Systolic blood pressure measurements obtained through carotid arterial cannulation were lower than those obtained by any other method. The heart rate was highest using the unheated-animal tail-cuff method when compared with the other methods. These data suggest that the novel unheated-animal tail-cuff method is a sensitive and accurate approach for the noninvasive measurement of blood pressure in conscious rats.

Key words blood pressure; spontaneously hypertensive rat; tail-cuff method; telemetry method

Generally, blood pressure (BP) in rats and mice is measured using the tail-cuff method after heating the animal. However, this method yields an indirect measurement of BP, and the requirement for animal heating and restraint may cause stress-induced changes in BP.[1—4] To overcome these limitations, a radiotelemetric monitoring system was developed to continuously measure cardiovascular parameters in freely moving rats.[5] Bazil *et al.*[6] compared the cardiovascular parameters of spontaneously hypertensive rats (SHR) recorded by different methods (*e.g.*, radiotelemetry device, cannulated arterial catheters, and indirect tail-cuff) and suggested that telemetric monitoring was a useful method of cardiovascular study that did not cause stress-induced changes in blood pressure. However, telemetric monitoring requires the implantation of a radiotransmitter in the abdomen, and the apparatus is expensive.

Recently, a novel device was developed for the tail-cuff method that does not require animal heating if the ambient temperature is greater than 23 °C. The goal of this study was to assess the validity of this novel tail-cuff method without animal heating (method A) when compared with the conventional heating tail-cuff method (method B; unanesthetized rats with heating), telemetry method (method C; unanesthetized restrained rats without heating), or carotid arterial catheter method (method D; anesthetized rats, carotid arterial cannulation).

MATERIALS AND METHODS

Animals and Operative Procedures Experiments were performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society and Mukogawa Women's University. Male rats at 11—12 weeks of age were used, and BP and heart rate (HR) were always measured between 13:00—17:00 h.

Tail-Cuff Method without Heating (Method A) Rats (SHR/Izm, Japan SLC, Inc., Shizuoka, Japan) were placed in plastic restrainers. A cuff with a pneumatic pulse sensor was attached to the tail. Rats were allowed to habituate to this procedure for 7 d before experiments were performed. BP and HR values were recorded on a Model MK-2000 (Muromachi Kikai Co., Ltd., Tokyo, Japan) without heating and were averaged from at least three consecutive readings obtained from each rat.

Tail-Cuff Method with Heating (Method B) In this experiment, we measured the blood pressure and heart rate of rats used in the experiment of method A. Rats were preheated in a chamber at 35 °C for 10 min, then placed in plastic restrainers. A cuff with a pneumatic pulse sensor was attached to the tail. Rats were allowed to habituate to this procedure for 7 d before experiments were performed. BP and HR values were recorded on a Model MK-2000 with heating and were averaged from at least three consecutive readings obtained from each rat.

Telemetry Method (Method C) Rats (SHR/NCrj, Charles River Japan, Yokohama, Japan) were implanted with a radio transmitter (TA11PA-C40, Data Sciences, St. Paul, MN, U.S.A.). Individual rats were placed in a plastic cage on top of a receiver (RLA1020, Data Science) for measurement of BP or HR. Data were continuously collected and analyzed by computer software (Data Quest Lab Pro, Data Science). Values measured at 13:30 were shown as results.

Carotid Catheter Method (Method D) Rats (SHR/Izm, Japan SLC, Inc., Shizuoka, Japan) were anesthetized with urethane (1 g/kg, i.p.), and underwent cannulation of the right carotid artery. Catheters were connected to pressure transducers (SBP-105, NEC San-ei Instruments, Ltd., Tokyo,

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Japan), and BP was monitored on a PowerLab/800 (ADInstruments Pty Ltd., NSW, Australia).

Effects of Urethane on Cardiovascular Parameters Rats (Wistar, Japan SLC, Inc., Shizuoka, Japan) were placed in plastic restrainers, and baseline BP and HR values were determined by method A. Rats were then removed from the restrainers and given urethane (1 g/kg) or vehicle (saline) by intraperitoneal injection, and BP and HR were recorded at 30 min after the injection.

Effects of Nicardipine on Cardiovascular Parameters Rats (SHR/Izm, Japan SLC, Inc., Shizuoka, Japan) were placed in plastic restrainers, and baseline BP and HR values (0 h at am 10:00) were determined by method A or B. Rats were then removed from the restrainers and given nicardipine (30 mg/kg) by oral administration using a stomach sonde, and BP and HR were recorded at 1, 2, 4, 6, 8, 23 h after administration.

Drugs Urethane and nicardipine were obtained from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan) and from Yamanouchi Pharmaceutical Co., Ltd. (Tokyo, Japan), respectively. Other reagents were purchased from Wako Pure Chemical Ltd. (Osaka, Japan).

Statistics All values are reported as the mean±S.E.M. For multiple comparisons, statistical analysis was performed by one-way analysis of variance (ANOVA) followed by the Bonferroni/Dunn test. Other data were evaluated for significance by Student's *t*-test. When the variances of two groups were different, the Welch test was used. Probability of less than 0.05 was considered significant. Statistical analyses were performed with a computer software package (StatView 4.5, Abacus Concepts, Cupertino, CA, U.S.A.).

RESULTS

Comparison of the Four Methods Systolic BP (SBP) and HR were measured in SHR using four different methods (Fig. 1). SBP values by method A were similar to those by method C. SBP values by method B were higher than those by the other methods. HR values by method A were higher than those by the other methods. HR values by method C were similar to those by method D.

Effects of Urethane and Nicardipine on Cardiovascular Parameters The influence of anesthesia on SBP and HR of SHR was examined using urethane (1 g/kg, i.p.). Urethane induced a marked decrease in SBP and HR as recorded by method A (Fig. 2). The values of SBP and HR treatment with urethane were similar to those by method D.

BP and HR were also recorded in SHR after the oral administration of nicardipine (30 mg/kg) (Fig. 3). SBP and diastolic BP (DBP) values at 0 and 23 h were higher when using method B than when using method A. In contrast, SBP measured at 1 h (maximal hypotensive time) was significantly lower when measured by method B compared with method A. HR values at 0, 8 and 23 h were higher when using method A than when using method B.

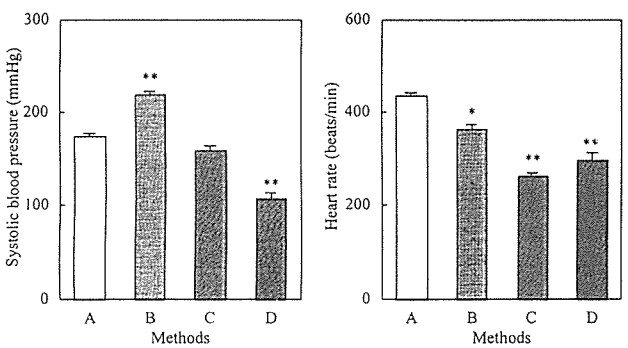

Fig. 1. Baseline Systolic Blood Pressure and Heart Rate in Spontaneously Hypertensive Rats as Recorded by Four Different Methods

(A) Tail-cuff; unheated-animal tail-cuff method (n=6); (B) tail-cuff; heated-animal tail-cuff method (n=6); (C) telemetry; telemetry method (n=15), (D) catheter; carotid catheter method (n=6). Each point represents the mean±S.E.M. *,** p<0.05, 0.01 *vs.* method B.

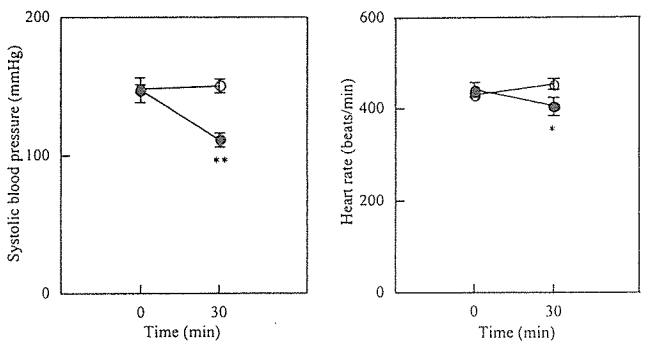

Fig. 2. Effect of Urethane on Systolic Blood Pressure and Heart Rate in Wistar Rats as Measured by the Unheated-Animal Tail-Cuff Method (Method A)

Changes in systolic blood pressure and heart rate were recorded after intraperitoneal injection of urethane (●; 1 g/kg) or saline (○). Each point represents the mean±S.E.M. (n=6). *,** p<0.05, 0.01 *vs.* 0 min.

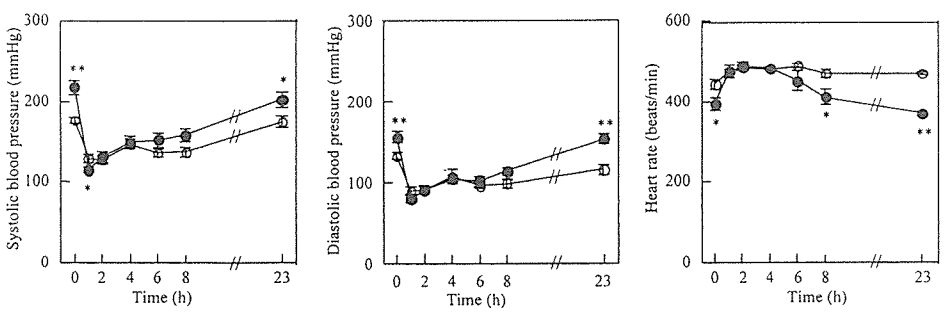

Fig. 3. Effect of the Oral Administration of Nicardipine on Systolic Blood Pressure (Left Panel), Diastolic Blood Pressure (Middle Panel) and Heart Rate (Right Panel) in Spontaneously Hypertensive Rats

Changes in blood pressure and heart rate were recorded by the unheated-animal tail-cuff method (method A; ○, n=6) or by the heated-animal tail-cuff method (method B; ●, n=5). Each point represents the mean±S.E.M. *,** p<0.05, 0.01 *vs.* unheated tail-cuff method.

DISCUSSION

Telemetry is a useful method of performing cardiovascular studies in conscious experimental animals.[5,6] However, this method is limited by the need for radio transmitter implantation and by the cost of the telemetry apparatus. By contrast, the tail-cuff method is simple and inexpensive but carries the potential for stress-induced changes in blood pressure due to animal restraint and heating.[1—4] Several groups have attempted to develop improved tail-cuff methods, such as integrating the use of a lamp lens to localize the heating stimulus to the tail rather than to the whole animal,[7] or by employing electrical impedance to avoid animal heating altogether.[8] However, these methods still require specialized apparatus, which limits their widespread use. The goal of this study was to assess the validity of a novel tail-cuff method that does not require animal heating (method A).

This study demonstrated that SBP values were similar when comparing methods A and C, and the SBP values from these two methods were higher than those obtained with method D and lower than those obtained by method B. By contrast, HR values were higher when measured with method A than when measured with methods B, C or D. The HR value measured by method D was low and almost equal to that measured by method C. The reason why HR values measured by methods C and D were lower may be the influence of sleep and anesthesia. Indeed, urethane decreased blood pressure and heart rate measured by method A. The reason why the HR value measured by method A was higher than that by method B is not clear. Yen *et al.*[1] reported that heating stimulus resulted in increased SBP and decreased HR in SHR, likely because of a primary heat-induced elevation in SBP and a secondary baroreflex-mediated decrease in HR. Baroreflex or vasodilation by heating may be involved. The hypotensive action of nicardipine measured by method B was stronger than that measured by method A. This difference seems due to a difference in the BP level before nicardipine administration. Generally, it is accepted that the efficacy of vasodilator agents depends on the tension of vascular smooth muscle.

In conclusion, these data suggest that the novel unheated-animal tail-cuff method is a sensitive and accurate approach for the noninvasive measurement of blood pressure in conscious rats. Further research would be of benefit.

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Effects of *Ginkgo Biloba* Extract Feeding on Salt-Induced Hypertensive Dahl Rats

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We previously demonstrated that *Ginkgo biloba* extract (GBE) produced vasodilation *via* the nitric oxide synthesis and release by increasing the intracellular calcium level in vascular endothelial cells of rats. The present study aimed to clarify the effects of dietary administration of GBE on the blood pressure and vascular tone of hypertensive Dahl salt-sensitive (Dahl) rats in order to evaluate its therapeutic actions and availability. Dahl rats were fed an 8.0% NaCl diet or an 8.0% NaCl plus 0.5% GBE diet for 24 d. The feeding of GBE did not change the heart rate, but significantly decreased systolic blood pressure. After 24 days' administration, the effects of GBE on the atria and aorta isolated from Dahl rats were examined. The GBE-containing diet did not affect the negative and positive actions of isolated atria that were produced by acetylcholine and isoproterenol, respectively. In the aortic preparations, the relaxation in response to acetylcholine was significantly potentiated by a GBE-containing diet. Sodium nitroprusside-induced relaxation was unchanged by GBE-containing diet. These results demonstrated that GBE reduced salt-related elevation of blood pressure and restored the impaired acetylcholine-induced vasodilation in aortic segments.

Key words *Ginkgo biloba* extract (GBE); Dahl salt-sensitive rat; blood pressure; aorta; relaxation

Ginkgo biloba extract (GBE), which is the leaf extract of *Ginkgo biloba*, has many pharmacological effects. For example, preventing ischemia-induced oxidation,[1—3] improving cerebral blood flow[4] and antagonizing the action of platelet-activating factor[5] have been reported. GBE and its constituents, especially terpenoids and flavonoids, are also reported to possess vasorelaxant properties.[6,7] These findings have led us to consider the possibility that GBE might have protective effects in cardiovascular disease. However, few reports have clarified the effect of GBE on blood pressure, using an animal model of hypertension. Dahl salt-sensitive (Dahl) rats develop high blood pressure when fed salt, and are therefore similar to a subgroup of humans with hypertension.[8,9] In this study, we analyzed the effects of daily-term oral GBE treatment on blood pressure, heart and vascular function in Dahl rats.

MATERIALS AND METHODS

Animals and Materials Experiments were performed in accordance with Guiding Principles for the Care & Use of Laboratory Animals approved by The Japanese Pharmacological Society and Mukogawa Women's University. The GBE powder was supplied by Tama Biochemical Co., Ltd. (Tokyo, Japan) and contained 24.2% flavonoids and 9.4% terpenes; similar to that of EGb 761®[10] used in European countries. Male 6-week-old Dahl salt-sensitive rats ($n=12$) were obtained from Japan SLC, Inc. (Shizuoka, Japan). Rats were initially fed a control diet (8% NaCl addition MR stock (Japan SLC, Inc.) diet without GBE) for 7 d. After that, the animals received 8% NaCl addition MR stock diet (control group, $n=6$) or 8% NaCl plus 0.5% GBE addition MR stock diet (GBE group, $n=6$) for 24 d. The animals had free access to drinking water in the experiment. Rats were then anaesthetized with pentobarbital sodium (60 mg/kg, i.p.), blood was taken from the abdominal aorta, and the heart and thoracic aorta were rapidly removed. Heart rate and blood pressure were measured by the tail-cuff method (Model MK-2000, Muromachi Kikai Co., Ltd., Tokyo, Japan) in unanaesthetized rats between 13.00 and 17.00 h, at 23—25 °C.

Materials Acetylcholine chloride, *R*(−)-isoproterenol (+)-bitartrate salt and noradrenalin were obtained from Daiichi Pharmaceutical Co. (Tokyo, Japan), RBI (Natick, MA, U.S.A.) and Sankyo Co. (Tokyo, Japan). Other reagents used were purchased from Nacalai Tesque, Inc. (Kyoto, Japan).

Chronotropic and Inotropic Effects on Isolated Rat Atria The isolated heart was immediately placed in a Krebs–Henseleit solution of the following composition (mM): NaCl 118.4, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25.0, KH_2PO_4 1.2 and glucose 10.0. After excess blood vessels and the ventricle were removed from the heart, the atria preparation which consists of auricular nodes and left-right atrium were mounted in a 5 ml organ bath filled with Krebs–Henseleit solution. The chronotropic and inotropic changes were measured with a force-displacement transducer (Model T-7, NEC San-ei Instruments, Ltd., Tokyo, Japan) coupled to a PowerLab/800 (ADInstruments Pty Ltd., NSW, Australia) under a resting tension of 1.0 g. The bath solution was maintained at 32 °C to avoid the exhaustion condition of the atria and bubbled with a 95% O_2–5% CO_2 gas mixture. Each preparation was allowed to equilibrate for at least 60 min prior to initiation of experimental procedures, and during this period the incubation medium was changed every 20 min. After the equilibration period, acetylcholine or isoproterenol were cumulatively added to the bath solution.

Relaxation Studies on Isolated Rat Aorta The thoracic aorta was immediately placed in a Krebs–Henseleit solution of the following composition (mM): NaCl 118.4, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25.0, KH_2PO_4 1.2 and glucose 10.0. After removing periaortic fat and connective tissue, the aorta was cut into ring segments of approximately 3 mm length. Each ring preparation was mounted vertically

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under a resting tension of 1 g in a 5 ml water jacketed organ bath filled with Krebs–Henseleit solution and attached to a force-displacement transducer (Model T-7, NEC San-ei Instruments, Ltd., Tokyo, Japan). The bath solution was maintained at 37 °C and bubbled with a 95% O_2–5% CO_2 gas mixture. Each preparation was allowed to equilibrate for at least 60 min prior to the initiation of experimental procedures, and during this period the incubation media were changed every 10 min. After this equilibration period, the ring preparation was contracted with noradrenaline (10^{-7} M) before cumulatively adding putative relaxing agents. The relaxation response was expressed as a percentage of the maximal relaxation developed by papaverine (10^{-4} M).

Statistics All values are presented as the means±S.E.M. Data were evaluated for statistical significance using the Student's *t*-test. When the variances of two groups were different, the Welch test was used. A probability of less than 0.05 was considered significant. Statistical analyses were carried out with a computer program (StatView 5.0, SAS Institute Inc., Cary, NC, U.S.A.).

RESULTS

Effect of GBE Diet on Blood Pressure and Heart Rate Figure 1 shows systolic blood pressure and heart rate in Dahl rats during 24 days' administration of control or GBE-containing diets. Systolic blood pressure in control Dahl rats significantly increased with age (after days 0, 8, 16 and 24 the systolic blood pressures were 129.2±5.1, 142.3±2.9, 158.6±2.3 and 166.3±3.6 mmHg, respectively). This increase was significantly suppressed by administration of GBE (after days 0, 8, 16 and 24 the systolic blood pressures were 132.9±4.3, 120.5±3.0, 130.6±2.8 and 145.5±3.5 mmHg, respectively). However, GBE-containing diet did not affect heart rate over the 24 d administration period.

Effect of GBE Diet on Isolated Rat Atria Figure 2 compares the effects of acetylcholine and isoproterenol on the atria isolated from Dahl rats receiving GBE-diet with those from Dahl rats receiving the control diet. The heart rate and contractile force in atria isolated from Dahl rats receiving GBE diet were not significantly different from those from control rats. Acetylcholine-induced negative chronotropic and inotropic actions in atria of GBE rats were not significantly different from those of control rats. Also, isoproterenol-induced positive chronotropic and inotropic effects in atria of GBE rats were not significantly different from those of control rats.

Effect of GBE Diet on Isolated Rat Aorta Figure 3 shows the influence of GBE-containing diet on relaxation induced by acetylcholine and sodium nitropurusside in the aortic rings pre-contracted with noradrenaline (10^{-7} M). In the aorta of Dahl rats fed GBE-containing diet for 24 d, the maximum relaxation by acetylcholine (87.0±2.4%) was significantly larger than that observed in Dahl rats fed the control diet (74.3±5.3%). Sodium nitropurusside-induced relaxation was not changed after 24 days' administration of GBE-diet. The contractile force with noradrenaline (10^{-7} M) was not significantly different after 24 d of GBE-containing diet (0.63±0.03 g) compared with control diet (0.69±0.03 g).

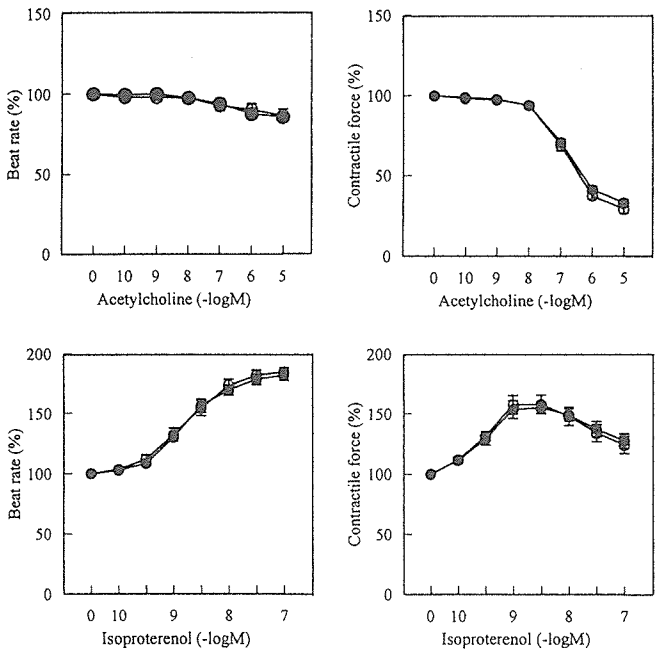

Fig. 2. The Effects of 0.5% *Ginkgo biloba* Extract (GBE) Diet on Chronotropic and Inotropic Actions Induced by Acethylcholine and Isoproterenol in the Atria Isolated from Dahl Rats

The ordinate denotes the ratio of beat rate (left) and contractile force (right), and the abscissa indicates the concentration of acethylcholine (upper) and isoproterenol (lower) after control (●) and GBE (○) diets. Each point represents the mean±S.E.M. (n=6).

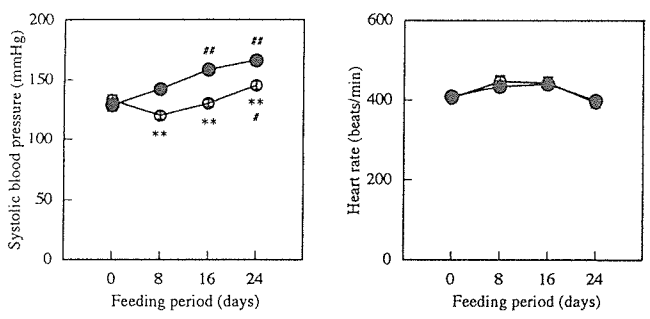

Fig. 1. Effects of 0.5% *Ginkgo biloba* Extract (GBE) Diet on Systolic Blood Pressure and Heart Rate in Dahl Rats

The ordinate denotes systolic blood pressure (left) and heart rate (right), while the abscissa indicates the time course (d) after control (●) and 0.5% GBE (○) diets. Each point represents the mean±S.E.M. (n=6). $^{**}p<0.01$ *vs.* control. $^{\#,\#\#}p<0.05$, 0.01 *vs.* each control (0 d).

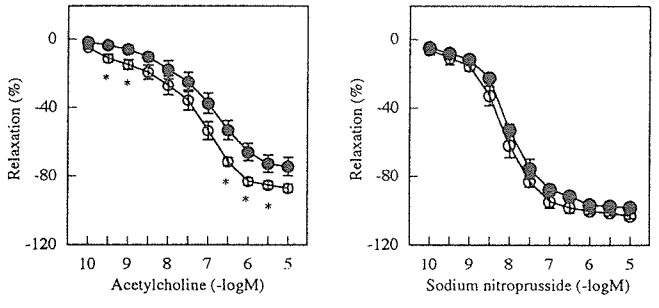

Fig. 3. The Effect of 0.5% *Ginkgo biloba* Extract (GBE) Diet on Relaxation Induced by Acethylcholine and Sodium Nitropurusside in the Aortic Rings Preconstricted with Noradrenaline (10^{-7} M) Isolated from Dahl Rats

The ordinate denotes the ratio of relaxation (%) to maximum relaxation in response to papaverine at 10^{-4} M and the abscissa indicates the concentration of acethylcholine (left) and sodium nitropurusside (right) after control (●) and GBE (○) diets. Each point represents the mean±S.E.M. (n=6). $^{*}p<0.05$ *vs.* control.

DISCUSSION

GBE contains approximately 30 kinds of flavonoids and their derivatives, plus terpenoids such as ginkgolide A, ginkgolide B, ginkgolide C and bilobalide.[10] Our previous study[11] demonstrated that GBE produced dose-dependent vasodilation *via* nitric oxide (NO) synthesis and release by increasing the intracellular calcium level in vascular endothelial cells of rats. We have also suggested that one of the principal ingredients of GBE for bringing about vasodilation is quercetin, which has already been reported to exert antihypertensive effects in spontaneously hypertensive rats (SHR) when administered orally on a long-term basis.[1,2] Sasaki *et al.*[12] have shown that the age-related increase in blood pressure observed in SHR was suppressed significantly by GBE at 60—120 mg/kg each day for 3 weeks. Umegaki *et al.*[13] have already found that GBE produced antihypertensive effects in deoxycorticosterone acetate-salt hypertensive rats. In the present study, 24 days' administration of GBE caused a significant hypotensive effect in Dahl rats. It is well known that vascular resistance is regulated by the endothelium *via* the synthesis and secretion of a variety of vasoactive substances, such as nitric oxide, prostacycline, endothelium-derived hyperpolarizing factors (EDHF) and endothelium-derived contracting factor (EDCF). The stable balance of these factors released from the endothelium is disturbed in diseases such as hypertension, atherosclerosis, and diabetes. In hypertension, the endothelium-dependent relaxation induced by a variety of vasodilator agents, such as acetylcholine, is markedly impaired, and this has been documented repeatedly by various investigators.[14—18] The overproduction of vasoconstrictor prostanoids[14,19] and super oxide anions generated in this pathologic process have been proposed as factors that contribute to the impaired relaxation of vessels to endothelium-dependent vasodilators.[15,20] Akpaffiong and Taylor[21] have also suggested that either excess production of oxidants or deficiency of antioxidant systems may contribute to high blood pressure and vascular endothelial impairment in SHR. Recently, Sasaki *et al.*[12] reported that the anti-oxidant effects of GBE were increased by measurement of urinary 8-hydroxy-2′-deoxyguanosine.

In our *in vitro* experiments, relaxation of aortae isolated from Dahl rats in response to acetylcholine was potentiated by long term administration of a GBE-containing diet. Relaxation in response to NO, *e.g.* sodium nitropurusside-induced relaxation was not affected by treatment with GBE in Dahl. In the aorta of Dahl rats fed on a high-sodium diet, endothelium-dependent relaxations in response to various vasodilators are impaired, while the relaxations in response to the endothelium-independent agonist, sodium nitropurusside, are only slightly impaired.[22,23] These findings suggest that enhanced relaxation resulting from GBE administration is due to increased or restored NO production/release from the endothelium or to greater NO bioavailability.

Flavonoids are considered important dietary antioxidants.[24] GBE also has antioxidant properties[1—3,25] and one of its major active ingredients is considered to be quercetin. Indeed, quercetin was reported to inhibit hypoxanthine-xanthine oxidase activity and scavenge super oxide, hydroxyl radicals, and peroxynitrite *in vitro*.[26] Moreover, metabolites of quercetin after oral administration have also been found to retain the antioxidant properties of the parent compound.[27] Superoxide is generally recognized to impair endothelium-dependent vasodilation *via* inactivation of synthesis and/or release of NO and consequently to elevate blood pressure.[28] Taken together, these findings suggest that the antioxidant properties of the flavonoids within GBE, such as quercetin, may participate in the effects of GBE on blood pressure and endothelium-dependent relaxation observed in the present study. However, further study will be necessary to elucidate the site of action and mechanism of GBE.

In conclusion, the present data demonstrate that GBE reduces the elevation of blood pressure and improves the dysfunction of the NO pathway in the endothelium of Dahl rats. These pharmacological activities are considered to contribute to the possible beneficial properties of GBE in clinical practice, including the regulation of hypertension.

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Effects of *Ginkgo biloba* extract on blood pressure and vascular endothelial response by acetylcholine in spontaneously hypertensive rats

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Abstract

We previously demonstrated that *Ginkgo biloba* extract (Ginkgo) produced vasodilation via the nitric oxide pathway in aortic segments isolated from Wistar rats. In this study, we have analysed the effects of daily long-term oral Ginkgo treatment on blood pressure, vascular tone, and calcium mobilization to evaluate the clinical availability. Spontaneously hypertensive rats (SHR) and Wistar Kyoto rats (WKY) were fed either a control diet or a diet containing 0.05%–0.5% Ginkgo for 30 days. Administration of Ginkgo did not change systolic blood pressure in WKY, but significantly decreased systolic blood pressure in SHR. In thoracic aortic preparations isolated from SHR, diminished relaxation in response to acetylcholine was improved by a Ginkgo-containing diet. This diet significantly decreased the EC50 value and significantly increased maximum relaxation in response to acetylcholine in SHR. In aortic segments isolated from WKY, acetylcholine-induced relaxation was not affected by a Ginkgo-containing diet. Sodium nitroprusside-induced relaxation was unchanged by a Ginkgo-containing diet in SHR and WKY. We also examined the effects of a Ginkgo-containing diet on the intracellular calcium level of aortic endothelium using a fluorescent confocal microscopic imaging system. Calcium Green 1/AM preloading indicated that acetylcholine significantly increased the endothelial intracellular calcium level. The Ginkgo-containing diet significantly enhanced this increase in the aortic endothelium of SHR, but did not change that of WKY. The results suggested that Ginkgo enhanced endothelium-dependent vasodilation and elevation of the endothelial intracellular Ca^{2+} level in SHR, resulting in hypotension. This accelerative effect of Ginkgo on Ca^{2+} mobilization seemed to be associated with restoration of impaired dilatory function induced by acetylcholine in endothelial cells.

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Introduction

Over the last decade, interest by the general public in the use of herbal dietary supplements has risen exponentially. One of the most popular herbal supplements is *Ginkgo biloba* extract (Ginkgo), which is commonly used in the treatment of early-stage Alzheimer's disease, vascular dementia, peripheral claudication, and tinnitus of vascular origin (Sierpina et al 2003). Ginkgo is marketed as a dietary supplement in the United States and Japan. However, in some European countries, it is prescribed clinically and is recognized as being particularly effective in the amelioration of peripheral vascular diseases such as intermittent claudication and cerebral insufficiency (Kleijnen & Knipschild 1992). Ginkgo exerts various pharmacological actions, including the scavenging of free radicals, improvement of the microcirculation, and antagonism of platelet-activating factor (Spinnewyn et al 1987; McKenna et al 2001; De Smet 2002; Ernst 2002). Ginkgo and its constituents, specifically terpenoids (bilobalide and ginkgolides A, B, and C) and flavonoids (quercetin and rutin), are also reported to possess vasorelaxant properties (Duarte et al 2001a; Ibarra et al 2003). This finding has led to the postulation that Ginkgo might have protective effects in cardiovascular disease. Indeed, Sasaki et al (2002) showed that Ginkgo decreased blood pressure in stroke-prone spontaneously hypertensive rats. In this study, we have analysed the effects of daily long-term oral Ginkgo treatment on blood pressure and endothelial function in spontaneously hypertensive rats (SHR) and normotensive Wistar Kyoto rats (WKY), to elucidate the mechanism of hypotension by Ginkgo.

Materials and Methods

Materials

Ginkgo powder containing 24.2% flavone glycosides, 2.7% ginkgolide A, 1.1% ginkgolide B, 1.7% ginkgolide C, 3.9% bilobalide, and less than 1 ppm ginkgolic acid was supplied by Tama Biochemical Co., Ltd (Tokyo, Japan); this mixture was similar to that of EGb 761, a preparation used in European countries. Calcium Green 1/AM was obtained from Molecular Probes, Inc. (Eugene, OR). Other reagents used were purchased from Wako Pure Chemical Co. Ltd (Osaka, Japan).

Animals and diets

Male spontaneously hypertensive rats (SHR/Izm) and Wistar Kyoto rats (WKY/Izm) were obtained from SLC (Hamamatsu, Japan). They were cared for in accordance with the procedures outlined in the Guidelines for Animal Experimentation of Mukogawa Women's University, which was compiled from the Guidelines for Animal Experimentation of the Japanese Association for Laboratory Animal Science. Rats were housed individually in stainless steel, wire-bottomed cages at a constant temperature (22–24°C) with a 12-h light–dark cycle. After adaptation to these conditions for three to five days, SHR were divided into five groups of six 6-week-old rats each and WKY were divided into two groups of six 6-week-old rats each, as follows. Group 1, control WKY rats; group 2, WKY rats treated with 0.5% Ginkgo (0.5% Ginkgo WKY); group 3, control SHR rats; group 4, SHR rats treated with 0.05% Ginkgo (0.05% Ginkgo SHR); group 5, SHR rats treated with 0.1% Ginkgo (0.1% Ginkgo SHR); group 6, SHR rats treated with 0.5% Ginkgo (0.5% Ginkgo SHR). Ginkgo was added to the commercial rodent diet (CE-2; Clea Japan Inc., Tokyo, Japan) and was given to animals in those groups other than controls. Animals had free access to drinking water. All rats were initially fed a control diet (CE-2 without Ginkgo) for seven days before receiving the experimental diet for 30 days. Blood pressure was measured by the tail-cuff method (Model MK-2000, Muromachi Kikai Co., Ltd, Tokyo, Japan) in unanaesthetized rats between 1300 and 1700 h (light on), at 23–25°C. Rats were placed in plastic restrainers. A cuff with a pneumatic pulse sensor was attached to the tail. Rats were trained to become familiar with this procedure for seven days before the experiment. The first day of blood pressure measurement was the day before the experimental diet was first administered. The final day of blood pressure measurement was the thirtieth day after the experimental diet was first administered.

Relaxation studies

After administration of the experimental diet for 30 days, rats were then anaesthetized with pentobarbital sodium (60 mg kg^{-1}, i.p.) and the thoracic aorta was rapidly removed. A part of the thoracic aorta was immediately placed in a Krebs–Henseleit solution of the following composition (mM): NaCl, 118.4; KCl, 4.7; $MgSO_4$, 1.2; $CaCl_2$, 2.5; $NaHCO_3$, 25.0; KH_2PO_4, 1.2; and glucose, 10.0. After periaortic fat and connective tissue were removed, the aorta was cut into ring segments of approximately 3 mm in length, and used for a relaxation study and intracellular calcium ion measurement. Each ring preparation was mounted vertically under a resting tension of 1 g in a 5-mL water-jacketed organ bath filled with Krebs–Henseleit solution and attached to a force-displacement transducer (Model T-7, NEC San-ei Instruments, Ltd, Tokyo, Japan). The bath solution was maintained at 37°C and bubbled with a 95% O_2/5% CO_2 gas mixture. Each preparation was allowed to equilibrate for at least 60 min before the initiation of experimental procedures, and during this period the incubation media were changed every 10 min. After this equilibration period, the ring preparation was contracted with noradrenaline (10^{-7} M) before the putative relaxing agents were cumulatively added. The relaxation response was expressed as a percentage of the maximal relaxation produced by papaverine (10^{-4} M).

Measurements of intracellular Ca^{2+} level

Using microscissors, a window of approximately 1 mm in diameter was opened in the thoracic aorta wall to observe the endothelium. The remaining aortic sections were then immediately immersed in physiological saline solution (PSS) of the following composition (mM): NaCl 140.0; KCl 4.0; $MgCl_2$ 2.0; $CaCl_2$ 2.0; N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) 10.0; and glucose 10.0, adjusted to pH 7.4 with NaOH. The aorta was loaded with 5 μM Calcium Green 1/AM for 30 min at room temperature and then rinsed three times with PSS. The aorta was then allowed to incubate in PSS for an additional 15 min at 37°C in a 95% O_2/5% CO_2 atmosphere to permit complete hydrolysis of any intact ester linkages in the intracellular Calcium Green 1/AM.

A U-shaped stainless steel wire (diam. 0.3 mm) was inserted into the aortic lumen to flatten the surface (endothelial layer). This aortic preparation was placed in a glass-bottomed chamber so that the window faced the objective lens of a microscope. The chamber was immediately filled with PSS and placed on a microscope stage (ECLIPSE TE 300, Nikon, Tokyo, Japan) coupled to a Nipkow disk confocal scanner (CSU10, Yokogawa Electric Corporation, Tokyo, Japan). An excitation wavelength of 488 nm was provided by an argon-krypton laser (Omnichrom Inc., Chino, CA, USA) and the emitted light was collected with a 510-nm long-pass diachronic reflector and a 515-nm long-pass emission filter through the planfluor objective (× 20). The fluorescence images were acquired with an ICCD camera (Hamamatsu Photonics, Hamamatsu, Japan) and captured on a personal computer using the ARGUS-50 program (Hamamatsu Photonics, Hamamatsu, Japan). To quantify the Ca^{2+} responses in endothelial cells, images constructed from 195 × 130 pixels were collected every 5 s. Calcium Green 1 fluorescence intensity was directly indicated from the resulting values.

Statistics

All values are reported as mean ± s.e.m. Statistical analysis of data for the groups was carried out using analysis of variance followed by post-hoc tests. A probability of less than 0.05 was considered significant. Statistical analyses were carried

out with a computer program (StatView 4.5, Abacus Concepts, Cupertino, CA, USA).

Results

Body weight and food intake during Ginkgo administration

The effects of 30 days of feeding with a Ginkgo-containing diet on the body weight and food intake in WKY and SHR are shown in Table 1. The dose of Ginkgo was also calculated. Addition of Ginkgo (0.05%–0.5%) to the diet of WKY and SHR did not significantly influence body weight or food intake.

Effect of a Ginkgo-containing diet on blood pressure

Figure 1 shows the systolic blood pressure of WKY and SHR after 15 and 30 days on the 0.5% Ginkgo diet. Systolic blood pressure in SHR increased with age and was significantly higher than that in WKY. This increase was significantly suppressed by the 30-day administration of Ginkgo. A Ginkgo-containing diet did not affect systolic blood pressure in WKY over the 30-day administration period.

Effect of a Ginkgo-containing diet on vasorelaxation

Figure 2 shows the influence of 30 days of a Ginkgo-containing diet (0.05–0.5%) on relaxation induced by acetylcholine (ACh) in WKY and SHR aortic rings precontracted with noradrenaline (10^{-7} M). Ginkgo significantly enhanced the maximum relaxation induced by ACh in a dose-related manner. The EC50 values of relaxation for ACh in SHR aortic rings were significantly decreased by administration of a 0.5% Ginkgo diet, as shown in Table 2. In contrast, 30 days of a 0.5% Ginkgo diet did not affect ACh-induced relaxation in WKY (Figure 2, left panel). In the control diet groups of SHR, ACh-induced maximum relaxation (88.8±1.2%) was significantly lower than that observed in WKY (94.6±1.5%). In the aortas of SHR fed 0.1% or 0.5% Ginkgo for 30 days, maximum relaxation induced by ACh was significantly increased relative to that in the control diet groups of SHR (Figure 2, right panel). In other words, Gingko completely restored the relaxation of SHR aortas to that of WKY aortas. Sodium nitroprusside-induced relaxation was not changed after 30-day administration of a Ginkgo-containing diet in either SHR or WKY (Figure 3).

Effects of a Ginkgo-containing diet on the ACh-induced increase in intracellular Ca^{2+} level

Figure 4 shows typical changes in intracellular calcium level induced by ACh in the endothelial layer of aorta isolated from SHR. The left panel is a phase-contrast microscopic image of the endothelial layer in the absence of drugs. Parts of the stainless steel wire inserted to the arterial lumen are observed in the top and bottom of the image. As shown in the middle panel, the distance between the wires was shortened by contraction induced by noradrenaline. This distance was lengthened by the vasorelaxant action of ACh (right panel). Furthermore, the intensity of Calcium Green fluorescence was increased in the presence of ACh. This ACh-induced increase in intracellular calcium ion level was enhanced in the endothelial layer of SHR fed with a 0.5% Ginkgo diet for 30 days (Figure 5).

Discussion

Ginkgo is a well-defined plant extract. Its main constituents are terpenoids (such as bilobalide and ginkgolides A, B, and C) and 30 types of flavonoids (such as quercetin and rutin) (Kleijnen & Knipschild 1992). Our previous study (Kubota et al 2001) demonstrated that Ginkgo produced dose-dependent vasodilation via the nitric oxide pathway in the isolated Wistar rat aorta and suggested that one of the principal ingredients of Ginkgo responsible for this vasodilation was quercetin. This evidence suggested that Ginkgo may produce a hypotensive effect. Sasaki et al (2002) have shown that the age-related increase in blood pressure observed in SHR was suppressed significantly by Ginkgo at 60–120 mg kg^{-1} each day for three weeks. Umegaki et al (2000) have already found that Ginkgo produced antihypertensive effects in deoxycorticosterone acetate-salt hypertensive rats. However, this hypotensive action was observed on systolic blood pressures of 120–140 mmHg, but was not observed on systolic blood pressures more than 140 mmHg. Thus, Ginkgo seemed to improve mild hypertension. Indeed, Jezova et al (2002) suggested that

Table 1 Effects of 30-day treatment with *Ginkgo biloba* extract (Ginkgo) on body weight, food intake, and dose of Ginkgo in Wistar Kyoto rats (WKY) and spontaneously hypertensive rats (SHR)

	WKY		SHR			
	Control	0.5% Ginkgo	Control	0.05% Ginkgo	0.1% Ginkgo	0.5% Ginkgo
Body weight (g)[a]	260.5±2.6*	254.5±4.8*	278.4±3.3	276.0±3.7	276.6±5.8	281.1±1.6
Intake of diet (g/day)[b]	14.3±0.2*	14.8±0.3*	17.3±0.3	17.0±0.2	17.3±0.2	17.2±0.3
Dose of Ginkgo (mg/day)[c]	0	73.7±1.3	0	8.5±0.1	17.3±0.2	86.3±1.4

[a]The average body weight after 30 days. [b]The average intake of each diet per day for 30 days. [c]The average dose of Ginkgo per day for 30 days. Each value is the mean±s.e.m. for six rats. *$P<0.05$ vs each control.