Establishment of an *in Vitro* Peyer's Patch Cell Culture System Correlative to *in Vivo* Study Using Intestine and Screening of Lactic Acid Bacteria Enhancing Intestinal Immunity

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Received September 7, 2009; accepted November 9, 2009; published online November 11, 2009

Some lactic acid bacteria (LAB) are known as representative of probiotics. To screen LAB effective to enhance intestinal immunity, in the present study, we developed an accurate and convenient *in vitro* evaluation system using Peyer's patch cells (PP-cells) isolated from the mice intestine. We observed that the amount of immunoglobulin A (IgA) produced by PP-cells co-cultured with LAB was well correlative to that in PP-cells, intestine and feces isolated from live mice after oral administration of LAB [correlation coefficient (r)=0.888, 0.883, and 0.920, respectively]. In addition, using this *in vitro* system, we suggest that the IgA level of PP-cells co-culturing with plant-derived LAB might be more enhanced than with animal-derived LAB.

Key words lactic acid bacterium; intestinal immunity; immunoglobulin A; probiotic

Some lactic acid bacteria (LAB) function as probiotics. The beneficial functions brought by LAB on gastrointestinal conditions, such as constipation, diarrhea, inflammatory bowel disease, *Helicobacter pylori* infection, lactose intolerance, and colon cancer, have been reported.[1,2] LAB have also been found to modulate systemic and/or intestinal immune responses, including allergies,[3—10] although their application in medical treatment is still far from possible. It is important to accumulate information about numerous LAB strains to screen the most appropriate and effective LAB for each purpose.

According to the circumstances of their isolated sources, LAB can be roughly classified into two groups: one is from animal sources, such as raw milk, cheese, or intestine, and the other is from plant sources, such as grasses, vegetables, or fruits. Animal-derived LAB have been traditionally used for fermented foods, such as yogurt and cheese. On the other hand, plant-derived LAB are used for Japanese traditional fermented dishes and miso. *Lactococcus* (*Lc.*) *lactis*, *Lactobacillus* (*Lb.*) *bulgaricus*, and *Streptococcus salivarius* subsp. *thermophilus* are typical animal-derived LAB. *Lb. plantarum* and *Lb. brevis* are representatives of plant-derived LAB. But, it is also true that *Lc. lactis* was isolated from many kinds of flowers (unpublished data). We insist that plant-derived LAB, in general, must be more resistant to rigorous environments than animal-derived LAB. Indeed, the plant-derived LAB strains newly isolated by our group, *Lb. plantarum* SN13T, SN35N, and *Lb. brevis* 925A, were found to be more viable in artificial gastric fluid and bile than animal-derived LAB, like *Lc. lactis*, *Lb. bulgaricus*, and *Lb. acidophilus* (unpublished data). Our previous clinical study has demonstrated that the yogurt made by *Lb. plantarum* SN13T improves constipation, serum lipid levels, and liver function more efficiently than the yogurt made by animal-derived LAB.[11] It is known that even the same species of LAB show different probiotic properties. Considering the advantage of plant-derived LAB resistant to digestive fluids in gastrointestinal tract, it will be significant to screen various plant-derived LAB strains which show excellent probiotic activities.

Gastrointestinal mucosa is the primary site of antigen invasions. The gut-associated lymphoid tissue (GALT) is composed of Peyer's patches, peripheral lymphoid tissues, and the appendix. The GALT plays an important role in the immune system of the gastrointestinal tract.[12,13] Immunoglobulin A (IgA) is the most abundant in the body of all immunoglobulin isotypes as well as the predominant immunoglobulin isotype in the mucosal surface of the gastrointestinal tract.[12,14] Peyer's patch, where IgA is produced by plasma cells, is considered as the most important defense site in intestinal immunity to prevent microbial invasion.[15—18] The effect of probiotics on intestinal immunity has been investigated using laboratory animals; IgA assessments were conducted using isolated intestine, feces, or a culture of *ex vivo* Peyer's patch cells (PP-cells) after oral administration of LAB.[5,19,20] This method is, however, not suitable for the screening of hundreds of LAB strains at once and consumes an excessive number of laboratory animals. Therefore, a system in which PP-cells are co-cultured with LAB may be a convenient and useful tool for estimating the influence of LAB on intestinal immunity.

To evaluate how accurate an *in vitro* PP-cells culture system is by comparing with the *in vivo* experiments using animals, in this study, we investigated the correlation between *in vitro* PP-cells culture system and *in vivo* oral administration in terms of the change of IgA production under the LAB influences.

MATERIALS AND METHODS

Animals Female BALB/cA (SPF) mice, 6 to 7 weeks of age at commencement, were purchased from Japan CLEA (Shizuoka, Japan) and housed in plastic cages under 12 h light/dark cycles. Animal experiments were conducted in accordance with the "Guidelines for the Care and Use of Laboratory Animals" established by Hiroshima University. Mice were sacrificed by ether anesthetization.

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Table 1. LAB Strains Used in This Study

LAB species	Isolated source	Abbreviation
Pediococcus pentosaceus LP28	Plant (longan, fruit)	LP28
Enterococcus avium G-15	Plant (carrot's leaves)	G-15
Enterococcus mundtii 15-1A	Plant (mibuna, vegetable)	15-1A
Lactobacillus plantarum SN13T[a)]	Plant (Nahm)	SN13T
Lactobacillus brevis 925A	Plant (kimchi)	925A
Lactococcus lactis 527	Animal (yogurt)	527
Lactobacillus bulgaricus B-5b	Animal (yogurt)	B-5b
Streptococcus salivarius subsp. *thermophilus* 510	Animal (yogurt)	510

Streptococcus lactis 527, *Lactobacillus bulgaricus* B-5b, and *Streptococcus salivarius* subsp. *thermophilus* 510 have been used as a starter for yogurt production by a dairy company (Nomura Dairy Co., Japan). These strains have been purchased from the foundation of the Japan Dairy Technical Association. *Streptococcus lactis* is now taxonomically classified into *Lactococcus lactis*. In our laboratory, *Lactococcus lactis* 527, *Lactobacillus bulgaricus* B-5b, and *Streptococcus salivarius* subsp. *thermophilus* 510 have been published as *Lactococcus lactis* A6, *Lactobacillus bulgaricus* C6, and *Streptococcus thermophilus* 510, respectively.[11) a)] SN13T was isolated from Nahm, the fermented sausage made in Thailand. To make Nahm, the pig minced meat is wrapped and fermented with a leaf of the tropical vegetation, like banana.

Bacterial Cell Preparation The LAB strains used in the current study are summarized in Table 1. Five plant-derived LAB strains, *Pediococcus* (*P.*) *pentosaceus* LP28 (LP28), *Enterococcus* (*E.*) *avium* G-15 (G-15), *E. mundtii* 15-1A (15-1A), *Lb. plantarum* SN13T (SN13T), and *Lb. brevis* 925A (925A), were newly isolated in our laboratory. Three typical animal-derived LAB strains, *Lc. lactis* 527 (527), *Lb. bulgaricus* B-5b (B-5b), and *Streptococcus salivarius* subsp. *thermophilus* 510 (510), were kindly provided from Nomura Dairy Products Co., Ltd., Japan. All LAB strains were cultured in De Man-Rogosa-Sharpe (MRS) broth (Merck KGaA, Darmstadt, Germany) and lyophilized for preservation. Heat-killed LAB were prepared by autoclaving at 120 °C for 20 min followed by washing three times with phosphate-buffered saline (PBS).

PP-Cell Preparation Preparation of primary cultures of PP-cells was performed as described previously[21)] with the following slight modifications. BALB/cA mice were sacrificed by ether, and their small intestines were placed in a petri dish filled with PBS containing penicillin (100 U/ml; Invitrogen, Carlsbad, CA, U.S.A.) and streptomycin (100 μg/ml; Invitrogen) on ice. Visible Peyer's patches were carefully isolated from the small intestinal wall and placed in an ice-cold complete medium, an RPMI-1640 medium (Invitrogen) containing 5% fetal bovine serum (FBS, Invitrogen), 50 μM 2-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin. To obtain a single PP-cell suspension, the Peyer's patches were digested with type 1 collagenase (70 U/ml; Wako Pure Chemical Industries, Ltd., Tokyo, Japan) dissolved in the same complete medium and incubated for 60 min at 37 °C. After filtration through a 100 μm nylon mesh (Cell Strainers, BD Biosciences, Bedford, MA, U.S.A.), the PP-cells were washed three times with a complete RPMI-1640 medium. Cell viability was assessed by trypan blue exclusion.

Co-culture of PP-Cells with LAB A total of 5.0×10^6 PP-cells in 100 μl of the complete medium supplemented with 5 μg/ml of concanavalin A (Sigma, St. Louis, MO, U.S.A.) and 50 μg/ml of lipopolysaccharide (LPS, Sigma) were added to each well of a 96-well tissue culture plate (BD Biosciences), and then each LAB strain suspended in 100 μl of the same medium was added at final concentrations of 2.5×10^4, 2.5×10^5, and 2.5×10^6 colony forming unit (cfu)/ml. PP-cells and LAB were co-cultured for 16 h or 24 h at 37 °C in a humidified atmosphere with 5% CO_2, and the IgA levels of each supernatant were then assessed using an enzyme-linked immunosorbent assay (ELISA) kit for IgA according to the manufacturer's instructions (Bethyl Laboratories, Inc., Montgomery, TX, U.S.A.). To determine the effect of secretions from LAB, each LAB strain was incubated at 2.5×10^6 cfu/ml in a complete medium at 37 °C for 24 h, and the supernatants were then prepared by 0.22 μm-filter sterilization. The LAB supernatants were added to the PP-cells at 1 : 1 ratio, and IgA production was measured after 16 h of incubation at 37 °C. For the experiment of heat-killed LAB, PP-cells were co-cultured with them instead of with living LAB.

In Vivo* Analyses of the Intestinal IgA Productivity Using Mice** Mice were orally administered each LAB strain for 7 d with their drinking water, which contained LAB at a concentration of 1.0×10^8 cfu/ml. Control mice received only autoclaved distilled water instead of the LAB suspension. The drinking water containing LAB was changed twice a day to keep it fresh. Fresh feces were collected by isolating mice in an individual cage for defecation on day 3, day 5, and day 7. The feces were stirred in PBS containing 0.1 mg/ml trypsin inhibitor and 50 mM ethylenediaminetetraacetate (EDTA) at 4 °C overnight and centrifuged at 9300 ***g for 10 min. The resulting supernatant was used to measure the quantity of IgA contained in fecal extracts. After feces were collected on day 7, mice were sacrificed. Peyer's patches and whole intestines were obtained from the mice. After both organs were dispersed into PBS and centrifuged, each supernatant fluid was used to assess the IgA level.

RESULTS

PP-cells and LAB were alive in an RPMI-1640 medium containing 5 μg/ml concanavalin A and 50 μg/ml LPS at least for 24 h at 37 °C. Under this condition, the live LAB did not affect the viability of PP-cells (data not shown). The IgA level produced by PP-cells was significantly increased when co-cultured with the plant-derived LP28, G-15, 15-1A, or SN13T strain in a dose-dependent manner (Fig. 1). In contrast, all animal-derived LAB, such as 527, B-5b, and 510, and a plant-derived strain, 925A, did not enhance or even decreased the IgA production by PP-cells.

After oral administration of each LAB strain to mice every day for 7 d, we obtained the feces, Peyer's patches, and the whole intestine excluding Peyer's patches, and then used them for the determination of IgA productivity. Ingestion of LAB *via* drinking water was $4.0\pm0.5\times10^8$ cfu/day/mouse. Secretion and excretion of IgA into feces were remarkably elevated by the consumption of LP28, G-15, 15-1A, and SN13T, when compared with the others tested in the present study. In fact, these four LAB strains enhanced the IgA production of PP-cells in *in vitro*. The IgA levels were continuously increased until day 7 (Fig. 2A). Similarly, these four LAB strains significantly enhanced the IgA level in PP-cells and the whole intestine after oral administration, whereas the other strains, 925A, 527, B-5b, and 510, did not (Figs. 2B, C). Thus, the amounts of IgA produced by PP-cells co-cul-

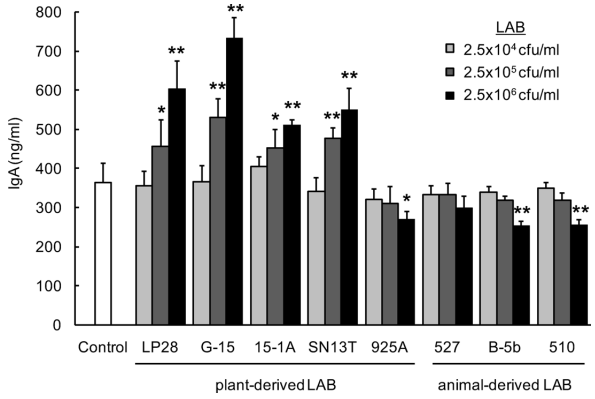

Fig. 1. Effects of Various LAB Strains on IgA Production by Co-culture with PP-Cells

Each LAB strain or medium alone was mixed with PP-cells (2.5×10^7 cells/ml) in a complete RPMI-1640 medium containing 5% FBS, 50 μM 2-mercaptoethanol, 100 U penicillin/ml, 100 μg streptomycin/ml, 5 μg concanavalin A/ml, and 50 μg LPS/ml. LAB cells were added at 2.5×10^4, 2.5×10^5, and 2.5×10^6 cfu/ml. They were incubated for 16 h at 37 °C and analyzed to determine the IgA level contained in supernatants. Each bar represents the mean±S.D. (n=4). $*p<0.05$, $**p<0.01$ (Dunnett's multiple comparison test).

tured with LAB are correlative to those produced in feces, *in vivo* PP-cells, and *in vivo* whole intestine, after oral consumption of LAB. The correlation coefficients between *in vitro* and *in vivo* studies using feces, Peyer's patch, and intestine were 0.920, 0.888, and 0.883, respectively (Fig. 3), suggesting that this simple and convenient *in vitro* system is accurate and useful for screening any LAB effect on the intestinal immune response.

To analyze a phenomenon in which the level of IgA produced by PP-cells is enhanced by co-culturing with the plant-derived LAB, we made an attempt to determine whether some LAB secrete the stimulating substances. When each LAB-culture filtrate, which was prepared through a membrane filter (pore size: 0.22 μm), was cultured with PP-cells, the filtrates from the G-15 and SN13T cultures enhanced the IgA production by Peyer's patch (Fig. 4A). This indicates that only the G-15 and SN13T strains in eight LAB strains used may release some substances effective to intestinal immunity. Although a plant-derived 925A strain and animal-derived LAB (527, B-5b, and 510) were ineffective, this observation is agreeable with the result obtained by the *in vitro* direct co-culture of LAB and PP-cells. We also assessed whether an increment in IgA production was observed when each of various LAB sterilized for 20 min at 120 °C was added to the PP-cell culture. Figure 4B shows that the increment of IgA production was observed only in the case of heat-killed G-15 and SN13T cells. On the other hand, PP-cells stimulated IgA production only when co-cultured with the LP28 and 15-1A strains in the living state. Thus, the mechanism to enhance the level of IgA produced by PP-cells varies by species of plant-derived LAB. In addition, in the present study, it was observed that the enhancements of IgA production by co-culture with plant-derived LAB were completely abolished when $CD4^+$ T cells were depleted from the total PP-cells (data not shown).

DISCUSSION

Some LAB strains, which are effective on intestinal immu-

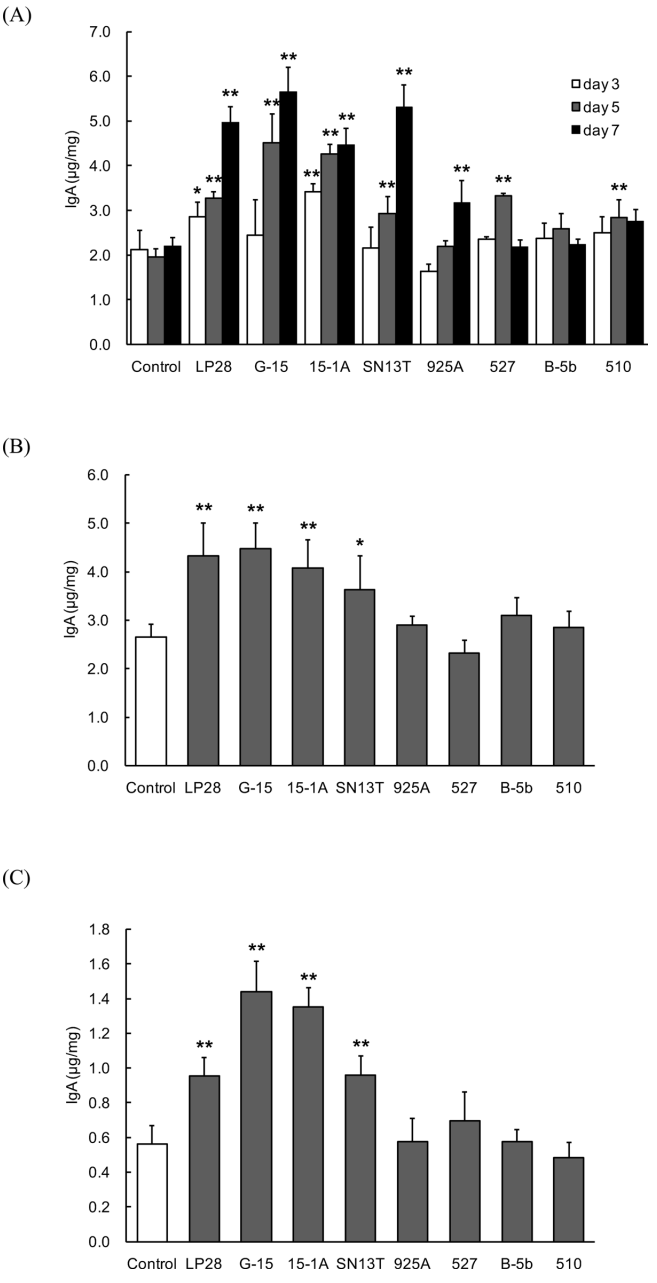

Fig. 2. Amounts of IgA Produced in Feces at Days 3, 5 and 7 (A), PP-Cells at Day 7 (B), and Intestine at Day 7 (C) after Oral Administration of Each LAB Strain or Vehicle Alone as a Control

Each bar represents the mean±S.D. of five mice. $*p<0.05$, $**p<0.01$ (Dunnett's multiple comparison test).

nity, are recognized as representative probiotics. PP-cells are the most important defense mechanism providing intestinal immunity. In the present study, we established a system which rapidly and accurately detects the probiotic effect of LAB on the intestinal immune response by using PP-cells co-cultured with LAB. This method will be a substitutive method for the time-consuming *in vivo* studies which require the sacrifice of a number of experimental animals. The amount of IgA produced by PP-cells co-cultured with LAB was well correlated with that by PP-cells, intestine, and feces isolated from live mice after oral administration of LAB.

In this study, we evaluated the effect of LAB on intestinal immunity using five plant-derived LAB and three animal-

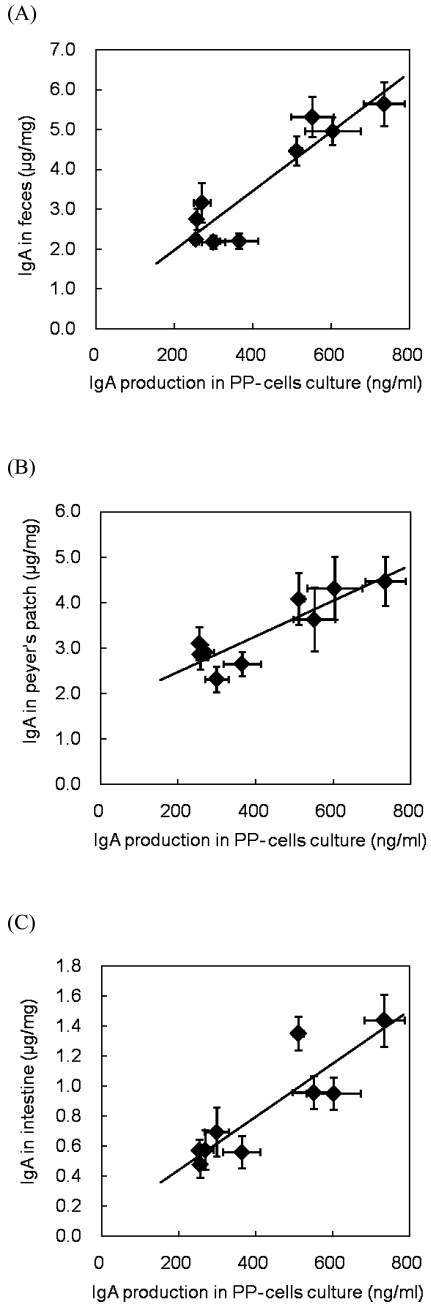

Fig. 3. Correlations between *in Vitro* and *in Vivo* Results Regarding the Change of IgA Production

(A) *In vitro* PP-cell culture *versus* feces; (B) *in vitro* PP-cell culture *versus in vivo* PP-cells isolated from mice after oral administration of LAB; (C) *in vitro* PP-cell culture *versus* intestine. The correlation coefficients were 0.920 (A), 0.888 (B), and 0.883 (C). Each point represents the mean±S.D.

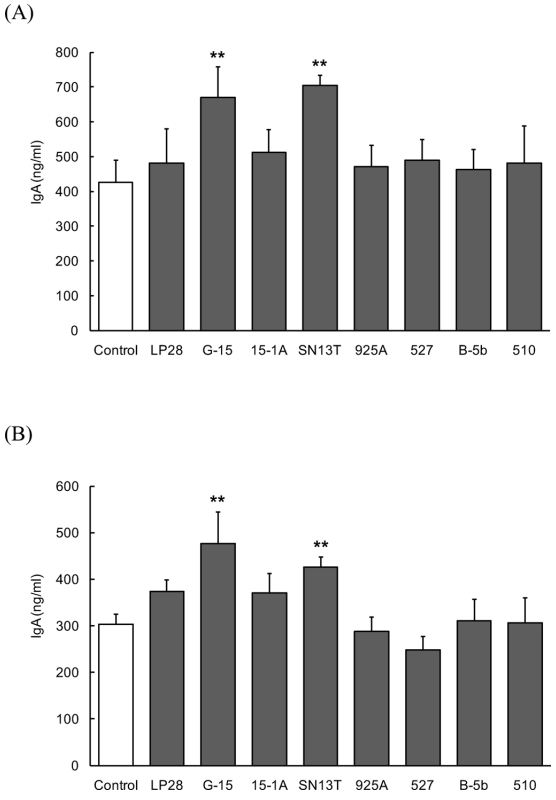

Fig. 4. Effects of Various LAB Strains under Different Conditions on IgA Production by the PP-Cell Culture

(A) PP-cells were incubated with the LAB culture filtrate after eliminating the cell mass through a 0.22 μm pore size filter. (B) PP-cells were incubated with heat-killed LAB. They were incubated in a complete RPMI-1640 medium containing 5% FBS, 50 μM 2-mercaptoethanol, 100 U penicillin/ml, 100 μg streptomycin/ml, 5 μg concanavalin A/ml, and 50 μg LPS/ml for 16 h, and the IgA levels of the supernatants were measured. Each bar represents the mean±S.D. (n=4). ** p<0.01 (Dunnett's multiple comparison test).

derived LAB strains. As a result, we observed that plant-derived LAB are more effective on intestinal immuno-stimulation than animal-derived LAB strains. To study how a certain LAB strain can stimulate IgA production by PP-cells, we compared the direct and indirect effect of LAB on PP-cells. Some substances as a cell wall component of LAB may function as immune stimulants in many cases, as shown in reports according to which heat-killed LAB are able to stimulate the immune response.[3,9,10] It has also been reported that the immunomodulatory activity of *Bifidobacterium bifidum* was not due to components secreted from bacteria.[5] Gram-positive bacteria, such as LAB, contain lipoteichoic acids as a component of the cell walls, which function as major immunostimulatory factors and agonists of the toll-like receptor 2.[22—24] In this study, we showed that the the G-15 and SN13T culture filtrates enhanced the IgA level produced by PP-cells. This suggests that some substances enhancing IgA production by PP-cells may be secreted from the G-15 and SN13T cells. Furthermore, the heat-killed LAB, G-15 and SN13T, also exhibited the IgA-enhancing effect, suggesting that the result is consistent with those reported previously.[3,9,10] Interestingly, the supernatant fluids from LP28 and 15-1A cultures and the heat-killed LP28 and 15-1A cell mass were ineffective for enhancing IgA production. The heat-unstable substances such as proteins, which are contained in these LAB, might play a role in enhancing the IgA level produced by PP-cells. Judging from these observations, these plant-derived LAB may have multiple mechanisms to stimulate intestinal immunity. Antigen-specific IgA has been reported to be produced from the plasma cells located in PP-cells in both T cell-dependent and -independent manners.[25] The increment of IgA-producing cells brought by LAB is not always correlative with that of $CD4^+$ T cells.[6,7] Under the presence of SN13T or G-15, the expression levels of interleukin-6 (IL-6), IL-10, and/or tumor growth factor-β (TGF-β) in PP-cells were enhanced until 2—3 times. In addition, when $CD4^+$ T cells were depleted from PP-cells, the IgA increments by co-cultured with the plant-derive LAB was completely disap-

peared (data not shown), suggesting that the enhancements of intestinal immunity may be T cell-dependent.

In conclusion, this *in vitro* co-culturing system of PP-cells with LAB is convenient and accurate to evaluate probiotic influence on intestinal immunity, suggesting that this system may be a useful tool to screen the IgA-enhancing LAB at one time. Using this *in vitro* system, we found that *Lb. plantarum* SN13T and *E. avium* G-15 are useful as probiotics to enhance intestinal immunity.

Acknowledgements Experiments were carried out in part at the Research Center for Molecular Medicine and the Analysis Center of Life Science at Hiroshima University. This work was supported by Hiroshima Biocluster (Cooperative Link of Unique Science and Technology for Economy Revitalization), Japan (M. S.).

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