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A novel *in vitro* co-culture model comprised of Caco-2/RBL-2H3 cells to evaluate anti-allergic effects of food factors through the intestine

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ABSTRACT

The prevalence of type I allergic diseases such as food allergy and allergic rhinitis has increased. Therefore, many studies have focused on food factors with anti-allergic activities in recent years. In order to investigate the effect of food factors on mast cell activation, a RBL-2H3 cell monoculture system has been widely used, in which various food factors have been reported to inhibit degranulation of RBL-2H3 cells. However, some orally administered food factors do not interact directly with immune cells but do so indirectly through intestinal epithelial cells. In this report, we established a novel *in vitro* co-culture model to evaluate anti-allergic effects of orally administered food factors. The co-culture system, comprised of Caco-2 cells (apical component) and RBL-2H3 cells (basolateral component), was able to evaluate the effects of two flavonoids that are known to have the inhibitory effects on mast cell degranulation. Moreover, we evaluated the anti-allergic effects of *Enterococcus faecalis* strains that are not absorbed through the intestine. We identified two strains of lactic acid bacteria that had inhibitory effects on mast cell degranulation using this co-culture system and possessed anti-allergic properties in a passive cutaneous anaphylaxis model mouse. This novel *in vitro* co-culture model was applicable for finding food factors with anti-allergic effects and might be useful for examining its anti-allergic mechanisms.

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1. Introduction

There are four types of allergic diseases, and type I allergic disease is a major global public health concern. Type I allergic disease, including food allergy, allergic rhinitis, asthma, and atopic dermatitis, is induced by certain allergens (antigens) such as food, dust, and pollen (Galli et al., 2008). However, the fundamental approaches of therapy for type I allergic diseases have not been established.

The cascade of type I allergy is as follows. First, allergens are captured by antigen-presenting cells (APCs), especially dendritic cells (DCs), and internalized by APCs via phagocytosis, pinocytosis, or endocytosis (Gould and Sutton, 2008). The degraded allergens are presented by major histocompatibility complex class II (MHC II) to T cell receptors (TCRs) on naïve CD4⁺ T cells. The activated CD4⁺ T cells are divided into two T helper cells (Th1 or Th2), which are classified by the type of cytokines they produce (Mosmann et al., 1986; Mosmann and Sad, 1996). Allergen-specific Th2 cells secrete Th2 cytokines such as interleukin (IL)-4 and IL-13, which enhance class switching of immunoglobulin from IgM to allergen-specific IgE in B cells (Takhar et al., 2005). B cells produce many allergen-specific IgE antibodies, which bind to the high affinity IgE receptor I (FcεRI) on the surface of mast cells (Galli and

Tsai, 2013). Upon second exposure to an allergen recognized by allergen-specific IgE on FcεRI, cross-linking of IgE-FcεRI with the allergen induces the activation of mast cells, leading to degranulation and the release of inflammatory mediators including histamine, β-hexosaminidase, lipid mediators, Th2 cytokines, and chemokines (Izawa et al., 2012). It is considered that allergic symptoms are mitigated by inhibiting mast cell activation.

Recent studies have demonstrated a relationship between gut microbiota and allergic disease (Björkstén et al., 1999). Lactic acid bacteria (LAB) such as *Enterococcus*, *Lactobacillus*, and *Lactococcus* are a group of Gram-positive, anaerobic bacteria that are an important component of the indigenous microbiota. Recently, many reports have revealed that LAB mitigate allergic symptoms. Indeed, it has been reported that several *Lactobacillus* strains, including *L. brevis* SBC8803, *L. casei* Shirota, and *L. plantarum* K37, could inhibit the serum levels of antigen-specific IgE in mouse models (Segawa et al., 2008; Shida et al., 2002; Liu et al., 2014). Furthermore, studies on *Enterococcus faecalis* FK-23 have shown that it could inhibit allergen-induced peritoneal accumulation of eosinophils and active cutaneous anaphylaxis in a mouse model (Shimada et al., 2003, 2004). Many clinical trials revealed that several *Lactobacillus* strains, including *L. rhamnosus* GG, *L. acidophilus* L-92, and *L. casei* Shirota, were effective in the prevention of early atopic disease in children and of allergic symptoms in patients sensitive to Japanese cedar pollen (Kalliomäki et al., 2001; Ishida et al., 2005; Tamura et al., 2007). Therefore, many studies have focused on the

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therapeutic and preventive effects of LAB in allergic diseases. However, the exact mechanisms of the interaction of LAB and allergic diseases are not yet well understood.

In order to investigate the effect of food factors on the activation of mast cells, especially degranulation, an IgE-dependent passive cutaneous anaphylaxis (PCA) mouse model has been used as a type I allergy model (Bouike et al., 2011). However, *in vivo* experiments take a long time to produce results and need a large amount of test samples and animals. Hence, a rat basophilic leukemia cell line, RBL-2H3 cell monoculture system has been widely used as a good tool for investigating the anti-allergic effect of food factors (Yamada et al., 2007). RBL-2H3 cells display phenotypic characteristics of mucosal-type mast cells and express several hundred thousand FcεRI on their surface. After stimulation with an antigen, RBL-2H3 cells release β-hexosaminidase, which is used as a marker of mast cell degranulation. Recently, various food factors have been reported to inhibit degranulation of RBL-2H3 cells (Han et al., 2013), but almost all of them were applied directly to RBL-2H3 cells.

Bouike et al. demonstrated that oral administration of *Agaricus blazei* Murill (ABME) suppressed allergic responses in an allergy model mouse, and the active compound contained in ABME indirectly inhibited allergic responses through a human intestinal epithelial cell line, Caco-2 cells, *in vitro* (Bouike et al., 2011). These results suggested that intestinal epithelial cells (IECs) stimulated by ABME might affect immune cells. IECs play an important role in antigen invasion through the body orally, and in the regulation of the intestinal immune responses (Lu and Walker, 2001). However, there are few *in vitro* experimental methods that evaluate the anti-allergic effects of food factors via IECs.

Flavonoids are the plant secondary metabolites found abundantly in plants and they have a variety of biological effects, including anti-allergic effects (Singh et al., 2011; Lee et al., 2010; Trinh et al., 2010). We have established *in vitro* model using Caco-2 and RAW264.7 cells (Tanoue et al., 2008). We demonstrated that lentinan which is a polysaccharides containing *Lentinus edodes* possessed anti-inflammatory effects using this model (Nishitani et al., 2013a). In this report, it was made clear the mechanism which lentinan affected Caco-2 cells to cause endocytosis to decrease TNFR1 contents in basolateral sides. Thus, it was suggested that co-culture system is very useful to investigate the crosstalk between different cells. However, no co-culture model exists to measure food factors possessing anti-allergy activity on orally administration. In this study, an *in vitro* co-culture model to evaluate anti-allergic effects of food factors was established, in which human IECs, Caco-2 cells, were placed in a Transwell on the apical side and rat basophilic leukemia cell line, RBL-2H3 cells, were placed on the basolateral side. When RBL-2H3 cells were stimulated with anti-dinitrophenyl (DNP) IgE and DNP-albumin, β-hexosaminidase, which is an index of allergy activity, was released from them. We investigated whether treatment with luteolin or kaempferol, which are absorbed through the small intestine, suppressed β-hexosaminidase release by degranulation of RBL-2H3 cells in this co-culture system. Moreover, this study examined the effects of three strains of LAB that are not absorbed by the small intestine in this *in vitro* co-culture model and a conventional *in vivo* model.

2. Materials and methods

2.1. Reagents

Eagle's Minimum Essential Medium (MEM) was purchased from Nissui Pharmaceutical (Tokyo, Japan). Dulbecco's Modified Eagle's Medium (DMEM, High Glucose) with glutamine, streptomycin, and Evans blue were purchased from Wako Pure Chemical Industries (Osaka, Japan). Anti-dinitrophenyl (DNP) IgE, DNP-albumin, and p-nitrophenyl N-acetyl-β-D-glucosaminide were purchased from Sigma (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from Biological

Industries (Beit, Israel). Trypsin and MEM non-essential amino acids (NEAA) were purchased from Gibco BRL (Grand Island, NY, USA). DNP-bovine serum albumin (DNP-BSA) was purchased from Cosmo Bio (Tokyo, Japan). Penicillin was purchased from MP Biomedicals (Aurora, OH, USA). Luteolin was purchased from Sarsynthese (Merignac, France). Kaempferol was purchased from Extrasynthese (Genay, France). Anti-Toll-like receptor (TLR) 1, anti-TLR2, and anti-TLR6 antibodies were purchased from Hycult Biotech (NL, Netherlands). IgG1 Isotype Control was purchased from Bay bioscience (Hyogo, Japan).

2.2. Bacterial strain

E. faecalis IC-1 and FK-23 were purchased from Chichiyasu Pharmaceutical Co., Ltd. (Hiroshima, Japan) and Nichinich Pharmaceutical Co., Ltd. (Iga, Japan), respectively as a commercial product which was killed by heat. A commercial product of a heat killed preparation of *E. faecalis* EC-12 (Combi Corporation, Saitama, Japan) was used.

2.3. Mice

Female 7-week-old BALB/c mice were purchased from Japan SLC (Shizuoka, Japan). The mice were housed in an air-conditioned animal room at 25 ± 1 °C and acclimated for 7 days before experiments. The mice were maintained in filter-top cages in specific pathogen-free conditions with free access to laboratory chow and water. All animal experiments were approved and carried out in accordance with the Animal Experiment Ethics Committee of Kobe University.

2.4. Cell culture

Rat basophilic leukemia cell line, RBL-2H3 cells, were cultured in dishes in Eagle's MEM supplemented with 10% (v/v) heat-inactivated FBS (57 °C, 30 min), 100 μg/ml streptomycin, 100 U/ml penicillin, and 2 mM L-glutamine. Cell cultures were incubated at 37 °C in a 5% CO₂ incubator. Passage numbers 14–32 were used. Human intestinal epithelial cell line, Caco-2 cells, were cultured in a 75 cm² plastic flask in DMEM (high glucose) supplemented with 10% FBS, 1% MEM-NEAA, 100 μg/ml streptomycin, and 100 U/ml penicillin, and incubated at 37 °C in a 5% CO₂ incubator. Passage numbers 48–64 were used. When either cell line reached 80% confluence, cells were recovered from the culture dish or flask by trypsin digestion after washing with phosphate-buffered saline (PBS). The cells were replated in a new dish or flask.

2.5. Transepithelial electrical resistance measurement

The integrity of the Caco-2 monolayer was evaluated by measuring the transepithelial electrical resistance (TER) value. Tight junctions serve as barriers to paracellular diffusion, and TER reflects the tightness of the junctions between epithelial cells (Hidalgo et al., 1989). The monolayer cells were gently rinsed with Hank's Balanced Salts Solution (HBSS: 137 mM NaCl, 5.36 mM KCl, 1.26 mM CaCl₂, 0.55 mM MgSO₄, 0.44 mM KH₂PO₄, 0.34 mM Na₂HPO₄, 2.92 mM NaH₂PO₄) for 30 min in a CO₂ incubator. The integrity of the cell monolayers was evaluated by measuring TER using Millicell-ERS equipment (Millipore, Eschborn, Germany).

2.6. β-Hexosaminidase assay

To evaluate anti-allergy effects, an *in vitro* assay using the RBL-2H3 mono-culture system was performed in accordance with a previous study (Yamada et al., 2007). RBL-2H3 cells (4.0 × 10⁵ cells/ml) were plated at 500 μl/well in a 24-well tissue culture plate in Eagle's MEM and were sensitized overnight with 1 μg/ml anti-DNP IgE in a 5% CO₂ incubator at 37 °C. The cells were washed three times with Siraganian buffer (SB; 119 mM NaCl, 5 mM KCl, 0.4 mM MgCl₂, 1 mM CaCl₂,

40 mM NaOH, 25 mM PIPES, 5.6 mM glucose, 0.1% BSA, pH 7.2) and exposed to 200 μ l of test sample solutions for 6 h at 37 °C. After incubation, the cells were challenged with 10 ng/ml DNP-albumin for 10 min at 37 °C. The plate was cooled in an ice bath for 10 min to stop degranulation responses. The supernatant (50 μ l) was transferred to a 96-well plate, and incubated with an equal volume of substrate solution (5 mM *p*-nitrophenyl-*N*-acetyl- β -D-glucosaminide in 0.2 M citrate buffer at pH 4.5) for 1 h at 37 °C. After adding 100 μ l/well of stop buffer (0.2 M Tris, pH 8.0), the absorbance at 405 nm was measured using a microplate reader. The percentage of β -hexosaminidase released into the supernatants was calculated as a percentage of the degranulation group.

2.7. *Caco-2*/RBL-2H3 cells co-culture system

Caco-2 cells were seeded at the concentration of 0.6×10^5 cells/well onto 24-well Transwell insert plates (0.33 cm², 0.4 μ m pore size, Corning Costar Corp., Cambridge, MA). Cell culture medium was changed every 3 day until the cells were fully differentiated (TER value >300 Ω ·cm²). RBL-2H3 cells were seeded at 2.0×10^5 cells/500 μ l/well onto 24-well tissue culture plates in Eagle's MEM and incubated overnight with 1 μ g/ml at a final concentration of anti-DNP IgE. After replacing all media with SB, the Transwell inserts on which *Caco-2* cells had been cultured were added into the plate wells preloaded with RBL-2H3 cells as shown in Fig. 1. In an experiment to evaluate the anti-allergic effect of test samples, 0.2 ml of SB or test sample solution was applied into the apical side. After incubation for 6 h, the cells were challenged with 10 ng/ml final concentration of DNP-albumin for 10 min at 37 °C. The plate was cooled in an ice bath for 10 min to stop degranulation responses. A β -hexosaminidase assay was performed as described above.

For assessing the influence of TLR1, TLR2, or TLR6 neutralization on *E. faecalis* IC-1 activity, 5 μ g/ml of each anti-TLR antibody or IgG1 Isotype Control were used to treat *Caco-2* cells at 37 °C in a CO₂ incubator. After 30 min, *Caco-2* cells were washed three times with PBS to remove the excess antibodies prior to test sample treatment.

2.8. PCA reaction in mice

An IgE-dependent PCA reaction was performed in accordance with a previous study (Trinh et al., 2010). Female 8-week old BALB/c mice were administered with 100 μ l of test sample orally for 10 days. Anti-DNP IgE (0.1 μ g) was injected subcutaneously into both ears of each mouse under anesthesia on day 10. After 24 h, the mice were injected intravenously with 0.2 mg of DNP-BSA in 100 μ l PBS containing Evans Blue (10 mg/ml), via tail vein. Thirty minutes after the DNP-BSA injection, mice were sacrificed and both ears were collected for measurement of the pigmented area. The ears were mixed in 250 μ l of 3 N KOH at 37 °C overnight, and 225 μ l of a mixture of acetone and phosphoric acid (13:5) was then added. The samples were centrifuged at 3000 rpm for 15 min and supernatants were used for measurement of absorbance at 620 nm.

2.9. Statistical analysis

Each result was expressed as the mean $\pm$ standard error (M $\pm$ SE). Statistical significance between any two groups was analyzed using Student's *t*-test. Statistical significance between more than two groups was analyzed by one-way ANOVA and Tukey–Kramer test. Statistical significance was defined as *P* < 0.05.

3. Results

3.1. Establishment of a type I allergy model using a *Caco-2*/RBL-2H3 co-culture system

The immunological response of IECs to various food factors is a key factor to promote or mitigate type I allergic responses (de Kivit et al., 2014; Li et al., 2013; De Matos et al., 2012). It has been reported that activated mast cells stimulated with an allergen release large amounts of pro-inflammatory chemical mediators such as β -hexosaminidase (Izawa et al., 2012). As shown in Fig. 1A, the co-culture model constructed with *Caco-2* cells in the apical compartment and RBL-2H3

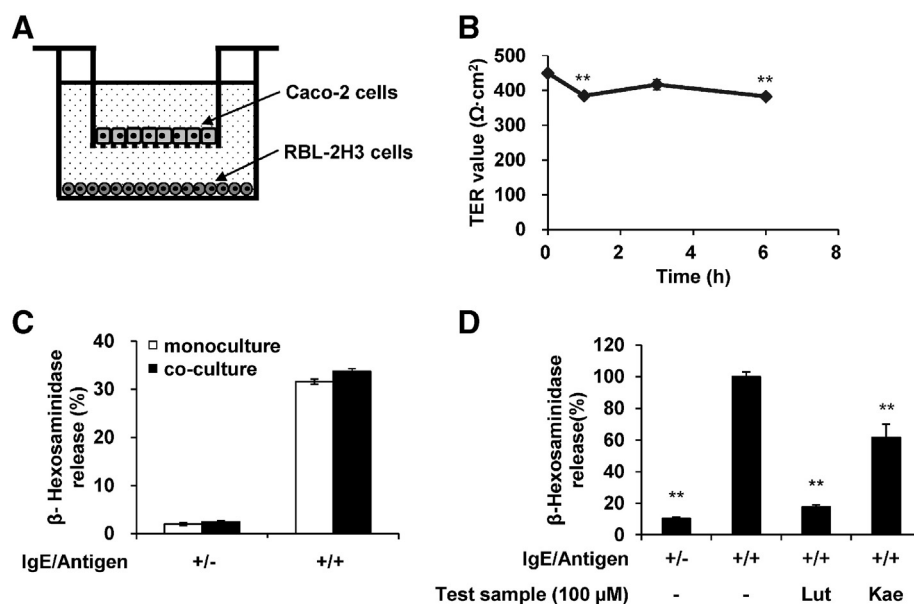


Fig. 1. An *in vitro* co-culture system comprised of *Caco-2* cells and RBL-2H3 cells to evaluate the anti-allergic effects of food factors. (A) Transwell inserts on which *Caco-2* cells (intestinal epithelial cells) had been cultured were added into the plate wells preloaded with RBL-2H3 cells (mast cells). (B) The transepithelial electrical resistance (TER) value was measured at the indicated time points. (C) Degranulation of RBL-2H3 cells was induced by anti-dinitrophenyl (DNP) IgE/DNP-albumin stimulation in the RBL-2H3 cells monoculture system (white bar) or the *Caco-2*/RBL-2H3 cells co-culture system (black bar). (D) Luteolin and kaempferol (100 μ M) were added to the apical compartment of the co-culture system and incubated for 6 h at 37 °C. Values represent means $\pm$ SE (*n* = 3). ***P* < 0.01 versus the initial point (B) or the degranulation group (D).

cells in the basolateral compartment was established as an *in vitro* intestinal model. First, we investigated whether or not SB in the β -hexosaminidase assay affected Caco-2 cells. The TER value was measured to assess the integrity of the Caco-2 monolayer. The Caco-2 monolayer with a TER value of $>300 \Omega \text{ cm}^2$ was used in experiments. The TER value of Caco-2 cells replacing all media with SB was kept higher than $300 \Omega \text{ cm}^2$ for 6 h (Fig. 1B). In order to investigate whether the Caco-2 cells in the co-culture system affected degranulation of RBL-2H3 cells, anti-DNP IgE and DNP-albumin were applied to the basolateral compartment to imitate degranulation. β -Hexosaminidase assays were performed using a RBL-2H3 cells monoculture system (Fig. 1C; white bar) and the Caco-2/RBL-2H3 cells co-culture system (Fig. 1C; black bar). There were no significant differences between the monoculture and co-culture systems.

Recent studies have shown that flavonoids such as luteolin and kaempferol could suppress IgE-mediated degranulation of RBL-2H3 cells (Lee et al., 2010; Trinh et al., 2010). The anti-allergic effects of luteolin and kaempferol were investigated in the present Caco-2/RBL-2H3 cells co-culture system. Luteolin and kaempferol were reported to be absorbed by Caco-2 cells (Nishitani et al., 2013b; DuPont et al., 2004). In agreement with previous studies, luteolin and kaempferol significantly inhibited degranulation of RBL-2H3 cells (Fig. 1D). The utility of this co-culture system was demonstrated by evaluating their inhibitory effects. Luteolin was used as a positive control in the following experiments because luteolin had higher inhibitory effects than kaempferol.

3.2. Anti-allergic effects of *E. faecalis* IC-1 and FK-23 on degranulation of RBL-2H3 cells in the Caco-2/RBL-2H3 cells co-culture system

It was assumed that this Caco-2/RBL-2H3 cells co-culture system could be used to search for food factors inhibiting mast cell degranulation through IECs. To test this hypothesis, food factors that are not

absorbed in small intestine (e.g. β -glucan, LAB, ceramide) were applied to the co-culture system. As shown in Fig. 2A, *E. faecalis* IC-1 (1.0×10^8 cfu/ml) and *E. faecalis* FK-23 (1.0×10^8 cfu/ml) inhibited IgE-mediated degranulation of RBL-2H3 cells via Caco-2 cells by 81% and 86%, respectively, whereas *E. faecalis* EC-12 (1.0×10^8 cfu/ml) in three strains did not. However, direct treatment with all three strains to RBL-2H3 cells did not inhibit degranulation (Fig. 2B). These results suggested that *E. faecalis* IC-1 and FK-23 exerted anti-allergic effects indirectly through IECs and the Caco-2/RBL-2H3 cells co-culture system, making the system beneficial for screening for food factors inhibiting mast cell degranulation through IECs.

3.3. Anti-allergic effects of *E. faecalis* IC-1 and FK-23 on PCA reaction in mice

As shown in Figs. 2A and 2B, it was demonstrated in the co-culture system that *E. faecalis* IC-1 and FK-23 might inhibit mast cell degranulation through IECs. In order to evaluate the inhibitory effects of *E. faecalis* IC-1 in an *in vivo* experiment, a PCA reaction experiment was performed in mice (Trinh et al., 2010). Oral administration of test samples did not cause any significant difference in weight or drinking amount compared with the control group throughout the experiment (data not shown). It was ascertained that oral administration of *E. faecalis* IC-1 (1.0×10^8 cfu/mouse) for 10 days significantly inhibited IgE-mediated PCA reaction (Fig. 3A). In addition, the inhibitory effects of the two other strains were investigated in a PCA reaction experiment. As shown in Fig. 3B, it was demonstrated that oral administration of *E. faecalis* FK-23 (1.0×10^8 cfu/mouse) also inhibited PCA reaction, whereas *E. faecalis*

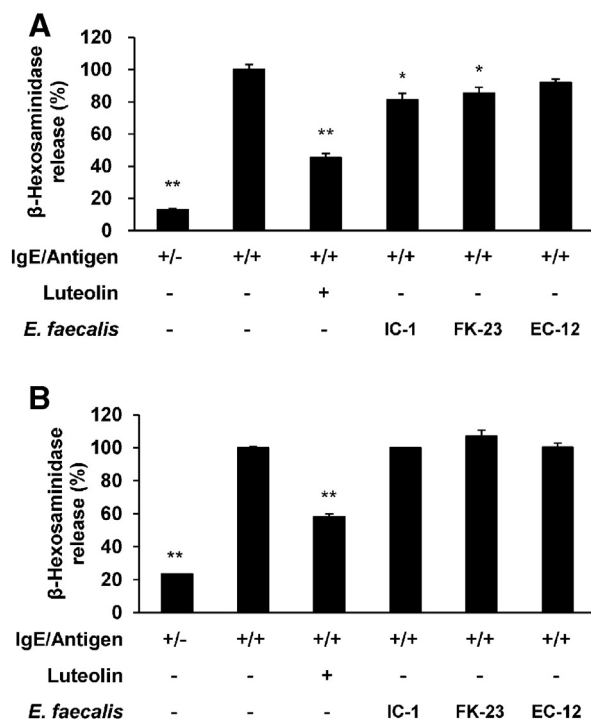


Fig. 2. The inhibitory effect of *E. faecalis* on degranulation of RBL-2H3 cells through an indirect mechanism. *E. faecalis* IC-1, FK-23, or EC-12 (1.0×10^8 cfu/ml, respectively) was added to the apical compartment of the Caco-2/RBL-2H3 cells co-culture system (A) or the RBL-2H3 cells monoculture system (B), and incubated for 6 h at 37 °C. Values represent means $\pm$ SE ($n = 3$). * $P < 0.05$, ** $P < 0.01$ versus the degranulation group.

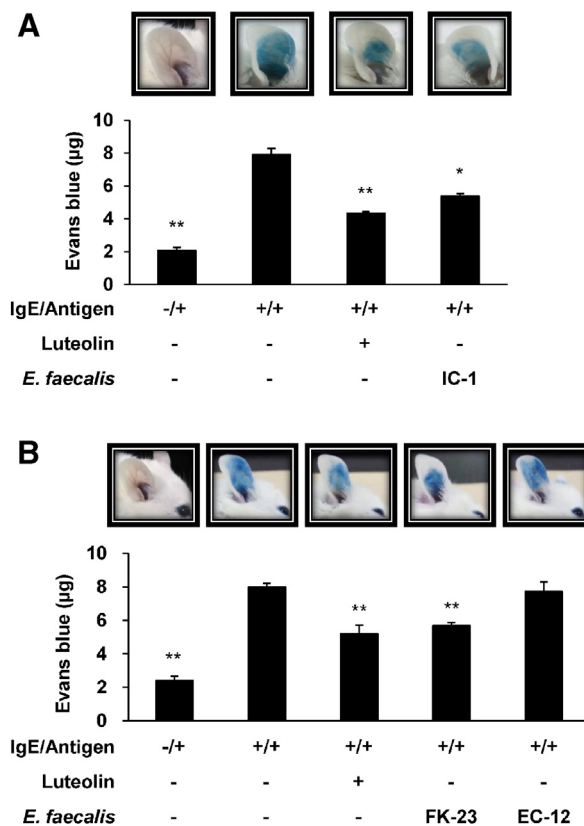


Fig. 3. Inhibitory effect of *E. faecalis* on passive cutaneous anaphylaxis reaction in mice. *E. faecalis* IC-1 (1.0×10^8 cfu/mouse/day; A), FK-23, EC-12 (1.0×10^8 cfu/mouse/day, respectively; B) or luteolin (400 μg /mouse/day) were administered orally to mice for 10 days before anti-dinitrophenyl (DNP) IgE sensitization. After 24 h, DNP-bovine serum albumin (BSA) and Evans blue were injected via the tail vein as an antigen challenge. Evans blue stained ears were photographed. The ear-absorbed dye was extracted with acetone and phosphoric acid (13:5), and absorbance at 620 nm was measured. Values represent means $\pm$ SE ($n = 4$). * $P < 0.05$, ** $P < 0.01$ versus the degranulation group.

EC-12 (1.0×10^8 cfu/mouse) did not. Thus, the *in vivo* results were completely consistent with the *in vitro* results. These results suggested that the Caco-2/RBL-2H3 cells co-culture system was applicable for the screening of anti-allergic food factors against type I allergy.

3.4. Recognition of *E. faecalis* IC-1 by TLR2 expressed on Caco-2 cells

It is well-known that LAB affects the immune system through interaction with the pattern recognition receptors such as TLRs (Wells, 2011). TLR2 plays a major role in Gram-positive bacterial recognition, such as LAB (Chapot-Chartier and Kulakauskas, 2014; Kawahara, 2010) and generally forms heterodimers with TLR1 or TLR6 (Abreu, 2010). It was reported that TLR1, TLR2, and TLR6 expressed on IECs in human, mouse, and also in the Caco-2 cell line (Abreu, 2010). Therefore, we investigated whether or not *E. faecalis* IC-1 exerted anti-allergic effects through TLR1, TLR2 and TLR6 expressed on Caco-2 cells. Each TLR on the apical side of the Caco-2 layer was blocked using anti-TLR1, anti-TLR2, or anti-TLR6 antibodies. The treatment with anti-TLR2 antibody just abolished the inhibition of degranulation of RBL-2H3 cells by *E. faecalis* IC-1 (Fig. 4). This result suggested that *E. faecalis* IC-1 was recognized by Caco-2 cells through TLR2, resulting in the inhibition of degranulation of RBL-2H3 cells.

4. Discussion

The number of patients suffering type I allergy, such as food allergy, allergic rhinitis, asthma, and atopic dermatitis, has increased dramatically in developed countries, and a variety of factors (e.g. food, environment, genetics) are known to impact on its development (Galli et al., 2008). Thus, the factors causing this increase in the prevalence of type I allergy are a key concern (Galli et al., 2008; Devereux and Seaton, 2005). In addition, many studies on factors with anti-allergic activities have been reported, and various factors in food have been examined as potential resources for anti-allergic therapeutics (Singh et al., 2011; Trinh et al., 2010). In order to investigate the inhibitory effect of food factors on the activation of mast cells, especially degranulation, a RBL-2H3 cell monoculture system has been widely used as a good tool for investigating the effects (Yamada et al., 2007). Although many food factors have been recently reported to inhibit mast cell degranulation, almost all of them were applied directly to RBL-2H3 cells (Han et al., 2013; Trinh et al., 2010). However, orally administered food factors can not interact directly with immune cells including mast cells but do so through the intestine. The intestinal epithelium is the first barrier

against antigens and continuously exposed to antigens in the form of food, dust, and pollen (Abreu, 2010). Therefore, we established a novel *in vitro* co-culture model, constructed by Caco-2 cells in the apical component and RBL-2H3 cells in the basolateral component (Fig. 1A).

Flavonoid aglycons such as luteolin and kaempferol are widely distributed in plants and are known to have a variety of biological activities, including anti-allergic effects (Singh et al., 2011) by the inhibition of mast cell degranulation (Lee et al., 2010; Trinh et al., 2010). The utility of the Caco-2/RBL-2H3 cells co-culture system was demonstrated by evaluating the effects of luteolin and kaempferol (Fig. 1D). Although most of the flavonoid aglycons are conjugated with glucuronic acid or sulfuric acid by IECs during intestinal absorption, only a few percent of intact aglycons are absorbed in the small intestine (Murota et al., 2002). Therefore, it was considered that luteolin and kaempferol added to the apical compartment were transported as their aglycons through the Caco-2 monolayer and acted directly on RBL-2H3 cells. Furthermore, we evaluated the anti-allergic effects of food factors that are not absorbed through the intestine, such as LAB, using this co-culture system. Recently, a variety of LAB have been reported to possess anti-allergic effects in mice and humans. This study demonstrated that *E. faecalis* IC-1 and FK-23 inhibited mast cell degranulation in the *in vitro* co-culture system, whereas they did not show any activities during direct treatment to RBL-2H3 cells (Fig. 2A and 2B). In accordance with the *in vitro* results, it was demonstrated that *E. faecalis* IC-1 and FK-23 also exerted anti-allergic effects in an *in vivo* PCA model (Fig. 3A and 3B). *E. faecalis* EC-12 did not inhibit *in vitro* and *in vivo* (Figs. 2A and 3B). We discovered food factors possessing anti-allergic effects as well as in an *in vitro* co-culture system. We demonstrated that this novel co-culture system was able to evaluate the anti-allergic effects of various food factors even if they did not show any anti-allergic activities in direct treatment with conventional monoculture. Moreover, it was assumed that many new food factors with anti-allergic properties may be discovered using this co-culture system.

Although common structures of LAB are conserved among Gram-positive bacteria, many studies on LAB have revealed the structural diversity existing among bacterial species and strains, resulting in strain-dependent differences in the intestinal immune responses of hosts (Chapot-Chartier and Kulakauskas, 2014; Abreu, 2010). LAB stimulate the immune system through interaction with TLRs (Kawai and Akira, 2010). Previous studies have shown that various active components of LAB, such as lipoteichoic acid, peptidoglycan, and lipopeptides, act as ligands of TLR2 on the surface of epithelial cells (Wells, 2011; Chapot-Chartier and Kulakauskas, 2014; Kawahara, 2010). Although TLR2 is the main recognition receptor for LAB, TLR1 and TLR6 can form functionally active heterodimers with TLR2 (Lee et al., 2010). It was revealed that neutralization only with an anti-TLR2 antibody on the Caco-2 cell-surface abolished the anti-allergic effect of *E. faecalis* IC-1 (Fig. 4). This indicated that *E. faecalis* IC-1 recognized by TLR2 on IECs inhibited mast cell degranulation. TLR1 and TLR6 are required for recognition of tri- and di-acylated lipopeptides, respectively (Alexopoulou et al., 2002; Buwitt-Beckmann et al., 2006). Therefore, it has been commonly assumed that TLR2 complexes with TLR1 or TLR6 are involved in the recognition of bacterial lipoproteins (Akira and Takeda, 2004). However, in this study, it was demonstrated that TLR1 and TLR6 were not involved in the recognition of anti-allergic components of *E. faecalis* IC-1. Receptor gene knockdown cells or knockout mice have been widely used (Takeuchi et al., 1999). However, these methods have high costs and require long wait times. This co-culture system may be a good tool for preliminary identification of the receptors for food factors.

In this study, we demonstrated the possibility that a novel *in vitro* co-culture model, comprised of Caco-2 and RBL-2H3 cells, was applicable for searching for food factors with anti-allergic effects and might be useful to examine the anti-allergic mechanisms involved. However, it will be necessary to investigate more food factors on proving the usefulness of this model.

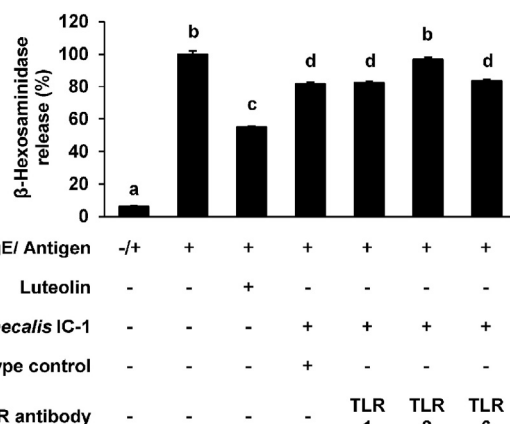


Fig. 4. Recognition of *E. faecalis* IC-1 by Caco-2 cells via Toll-like receptor 2. Each anti-Toll-like receptor (TLR) antibody was applied to the apical compartment of the Caco-2/RBL-2H3 cells co-culture system 30 min prior to *E. faecalis* IC-1 treatment. Values represent means $\pm$ SE ($n = 3$). Different superscript letters after values indicate statistical significance between groups ($P < 0.05$).

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