

Therapeutic Effect of Kakkonto in a Mouse Model of Food Allergy with Gastrointestinal Symptoms

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Key Words

Kakkonto • Herbal medicine • Food allergy • Diarrhea • Intestinal mucosal immunity • Mucosal mast cells • Cytokines

Abstract

Background: The number of patients with food allergy has increased dramatically over the last several decades. However, there is no effective drug for food allergies. In the present study, we evaluated the effects of kakkonto, a traditional Japanese herbal medicine, in a mouse model of food allergy with gastrointestinal symptoms. **Methods:** BALB/c mice were systemically sensitized twice with ovalbumin (OVA) and then were repeatedly given OVA by oral intubation (OVA mice). Kakkonto was administered orally before the OVA challenges. **Results:** The OVA mice developed allergic diarrhea ($91.8 \pm 3.8\%$ after 6 OVA challenges), and myeloperoxidase (MPO) activity was dramatically elevated in the colons of the OVA mice. Kakkonto significantly suppressed the occurrence of allergic diarrhea and MPO activity in the OVA mice. Furthermore, the number of mucosal mast cells was greatly increased in the proximal colons of the OVA

mice, and this was also suppressed by kakkonto. Interestingly, mRNA expression of helper T cell type 1 (Th1) cytokines (IFN- γ) and Th2 cytokines (IL-4, IL-5 and IL-10) were significantly upregulated in the proximal colons of the OVA mice, an effect which was also reduced by kakkonto. Transcriptome analysis detected increased mRNA expression of suppressor of cytokine signaling-3 in the proximal colons of OVA mice, which was decreased by kakkonto administration. **Conclusion:** Kakkonto has immunosuppressive effects and interferes with the infiltration of mucosal mast cells in the colons of mice with induced food allergy, leading to improvement of allergic symptoms. Kakkonto has potential as a therapeutic drug for treatment of allergic symptoms induced by the disruption of intestinal mucosal immunity.

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Introduction

Food allergies are adverse immunological responses to proteins in food. It was recently reported that up to 6% of young children and up to 4% of adults are affected by food allergies [1]. Food allergies have become increasing-

ly prevalent during the past few decades. Severe cases of food allergy can result in anaphylactic shock and even lead to death. At present, no therapeutic drugs for food allergies have been developed; the only clinical treatment is the use of preventative elimination diets to avoid food antigens. The development of effective drugs based on experimental pharmacology is therefore of great importance.

However, the underlying pathogenic mechanisms of food allergy remain poorly understood. Several animal models of food allergy have been established to investigate the underlying mechanisms [2, 3]. In 1999, Li et al. [4] reported the development of systemic anaphylaxis accompanied by vascular leakage and significantly increased plasma histamine in a mouse model of food allergy. Regarding the influence of food allergy on the intestine, the functions of motility [5] and ion transport [6, 7] in the intestine of mice with food allergy have been shown to be affected in *in vitro* studies. However, intestinal allergic symptoms such as diarrhea failed to develop in all these animal models of food allergy. In 2000, Kweon et al. [8] reported that systemically primed mice develop severe diarrhea after repeated oral challenge with ovalbumin (OVA). Furthermore, we subsequently modified this protocol [8] and demonstrated one of the pathogenic mechanisms of allergic diarrhea in our established mouse model [9].

Mammal immunity is composed of mucosal and systemic immune systems, but the nature of the crosstalk between these two immune systems is not yet clear. In systemic immunity, allergens generally induce a shift in the helper T cell type 1 (Th1)/Th2 balance toward Th2-dominant immunity [2], and this pathological Th2 environment causes various allergic responses [10]. On the other hand, mucosal immunity appears to play an important role in the progression of several immune diseases, and the manipulation of mucosal immunity is thought to therapeutically inhibit local and systemic inflammation [11]. It is suggested that intestinal mucosal immunity participates in the development of food allergy symptoms [12–16]. However, the precise pathophysiological role and the detailed immune profiles of mucosal immunity are not fully understood, particularly in the pathogenesis of food allergy.

In the pathological Th2 environment, mast cells can function as immunoregulatory cells that influence both innate and adaptive immunity [17, 18], and they are best known as primary responders in allergic reactions such as anaphylaxis and asthma [6]. The functions of mast cells are enhanced by interaction with immune regula-

tory factors. When mast cells are activated by antigens and antigen-specific IgE antibodies, they release various mediators from their characteristic granules and cause various allergic reactions.

Several herbal medicines have been used in the treatment of various immune diseases [19] and intestinal diseases [20, 21]. In particular, some traditional Japanese herbal medicines markedly ameliorate the symptoms of Th2-type diseases such as animal models of skin allergy [22, 23] and airway inflammation [24], but these herbal medicines had no therapeutic effect on allergic diarrhea in our food allergy model (data not shown). Kakkonto is a traditional Japanese herbal medicine that originated in China (Ge gen Tang) and is one of the herbal medicines most frequently used in Japan. Kakkonto is used not only most frequently for treatment of the common cold [25], but also for treatment of allergic rhinitis [26]. Kakkonto is composed of 7 species of medicinal plants: *Puerariae radix*, *Cinnamomi cortex*, *Zizyphi fructus*, *Paeoniae radix*, *Ephedrae herba*, *Zingiberis rhizoma* and *Glycyrrhizae radix*. Furthermore, kakkonto is also sometimes used to ameliorate some kinds of diarrhea in traditional Japanese and Chinese herbal medicine practice. Taken together, these reports led us to the working hypothesis that kakkonto has therapeutic potential for food-allergic diarrhea. In the present study, we examined the therapeutic effect of kakkonto on food allergies using an experimental mouse model.

Materials and Methods

Animals and Induction of Intestinal Anaphylaxis

Male BALB/c mice (5 weeks old) were purchased from Japan SLC Inc. (Shizuoka, Japan). All mice were housed in the experimental animal facility of the University of Toyama. The experimental methods employed were modified from previous reports [8, 9, 27]. Mice were sensitized twice at 2-week intervals by intraperitoneal injection of 50 µg of OVA (fraction V; Sigma, St. Louis, Mo., USA) in the presence of 1.3 mg of aluminum hydroxide gel (Sigma) as an adjuvant. Two weeks after systemic priming, mice were repeatedly given 50 mg of OVA dissolved in 0.3 ml of water with intragastric feeding needles 3 times per week. Diarrhea was assessed by visually monitoring mice for up to 1 h following intragastric challenge. Mice showing profuse liquid stool were recorded as having diarrhea. All experiments were approved by the Animal Experiments Committee of the University of Toyama.

Drug Treatment

Kakkonto (TJ-1) was purchased from Tsumura Co. (Tokyo, Japan) and was evaluated as a therapeutic drug for allergic symptoms. Kakkonto (500 mg/kg) suspended in 0.5% methylcellulose solution (Wako, Osaka, Japan) was administered orally 1 h before OVA oral challenges.

Myeloperoxidase Analysis

Myeloperoxidase (MPO) activity, an index of leukocyte adhesion and accumulation, indicates the level of inflammation. One hour after the 6th oral OVA challenge, the colons of the mice were excised and flushed with ice-cold saline. Each colon was weighed and cut into small pieces. The colons were homogenized on ice in 1 ml of 0.5% hexadecyltrimethylammonium bromide in 50 mM potassium phosphate buffer (pH 6.0) using a Polytron homogenizer (Kinematica, Littau, Switzerland) and then fortified with additional hexadecyltrimethylammonium bromide buffer to 1 ml/100 mg wet weight of tissue. The homogenates were frozen and thawed 3 times and then vortexed. The suspensions were centrifuged at 15,000 g for 20 min at 4°C. MPO activity of the resulting supernatants was measured. The supernatants (100 µl) were mixed with 2.87 ml of 0.5 mM *o*-dianisidine in 50 mM potassium phosphate buffer (pH 6.0) and 30 µl of 0.05% H₂O₂. The mixtures were measured spectrophotometrically at 460 nm during 4 min of incubation. MPO activity was expressed as units per wet weight of colon tissue in grams (g wwt).

Expression of Cytokine mRNA

One hour after the 6th oral OVA challenge, 2 cm of the proximal colon was excised. The proximal colon was flushed with ice-cold saline and frozen in liquid nitrogen. Total RNA was extracted from the proximal colon by Sepasol Super (Nacalai Tesque, Kyoto, Japan) according to the manufacturer's instructions. Reverse transcription was performed using the Exscript RT reagent kit (Takara Bio, Shiga, Japan) and random primers, followed by real-time PCR. Real-time PCR amplification of IL-4, IL-5, IL-10, IFN-γ and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed using SYBR Premix Ex Taq (Takara Bio). The following primer pairs were used: IL-4, forward 5'-GGTCTCAACCCCAAGTAT-3' and reverse 5'-GCCGATGATCTCTCTCAAGTAT-3'; IL-5, forward 5'-GAAGTGTGGCGAGGAGAGAC-3' and reverse 5'-GCACAGTTTTGTGGGGTTTT-3'; IL-10, forward 5'-GGCCCTTTGCTATGGTGTCC-3' and reverse 5'-AAGCGGCTGGGGGATGAC-3'; IFN-γ, forward 5'-CGGCACAGTCATTGAAAGCCTA-3' and reverse 5'-GTTGCTGATGGCCTGATTGTC-3'; GAPDH, forward 5'-TGACCA-CAGTCCATGCCATC-3' and reverse 5'-GACGGACACATTGGGGGTAG-3'. Real-time PCR was carried out using the Mx3000p system (Stratagene, La Jolla, Calif., USA). The PCR reaction conditions were 10 s at 95°C, followed by 40 cycles of 5 s at 95°C and 20 s at 63°C. Target mRNA was normalized to GAPDH mRNA as an internal control in each sample. Results were expressed as the ratio relative to the naïve group average.

Microarray Analyses

Total RNA was extracted from the proximal colon. mRNA was purified from the mixture of total RNA using an RNeasy Mini Kit (Qiagen, Crawley, UK), and mRNAs from at least 5 mice from each group were mixed. Microarray analysis was performed as described previously [28], using Genechip Mouse Genome 430 2.0 Array (Affymetrix, Santa Clara, Calif., USA). Data were analyzed using High-Resolution Scanning Genechip Operating Software version 1.3.

Immunohistochemistry

One hour after the 6th oral OVA challenge, the proximal colon was excised and flushed with ice-cold saline. The proximal colon

was fixed by immersion in 4% paraformaldehyde (w/v) in 0.1 M sodium phosphate buffer (pH 7.3) at 4°C for 12–18 h. The tissue was washed with 0.01 M phosphate-buffered saline (PBS; pH 7.3), cryoprotected by infiltration with 30% sucrose in 0.01 M PBS and then embedded in OCT compound. Frozen sections (50 µm) were cut at –20°C using a cryostat microtome (Leica Microsystems, Nussloch, Germany). The sections were permeabilized for 12–18 h in 0.01 M PBS containing 0.3% Triton X-100, incubated for 30 min with normal donkey serum (1:10; Jackson Immuno-research Laboratories, West Grove, Pa., USA) to block nonspecific binding of antisera and then washed in 0.01 M PBS. The sections were exposed for 12–18 h to antiserum against mouse mast cell protease (mMCP)-1, a marker of mouse mucosal mast cells (1:5,000; Moredun Scientific, Scotland, UK), washed with 0.01 M PBS, then incubated for 2 h with Cy3-conjugated sheep antidonkey IgG (1:200; Jackson Immuno-research Laboratories). Finally, the sections were washed in 0.01 M PBS and mounted in sodium carbonate-buffered glycerol (pH 8.6). All incubations were carried out at room temperature. The immunostained sections were examined using a fluorescence microscope (IX71 system; Olympus, Tokyo, Japan) with a U-MWIG3 filter set (Olympus) and photographed using an Olympus digital camera (DP70; Olympus). The brightness and contrast of the images were modified using Adobe Photoshop Elements 2.0 (Adobe Systems, San Jose, Calif., USA).

OVA-Specific IgE and mMCP-1 Levels in Plasma

The mMCP-1 level in the plasma was assayed using an ELISA kit purchased from Moredun Scientific. The level of OVA-specific IgE in the plasma was measured using an ELISA kit purchased from DS Pharma Biomedical (Osaka, Japan). Measurements were performed according to the manufacturers' instructions.

Statistical Analyses

Data are expressed as the means ± SE. Statistical comparisons were made with Student's unpaired t test, and a significant difference was defined as $p < 0.05$.

Results

Induction of Intestinal Allergic Symptoms

Following induction of intestinal anaphylaxis, the stool of OVA-challenged mice (OVA mice) shifted from normal (fig. 1a) to a profuse liquid stool (fig. 1b). In OVA mice, allergic diarrhea began to occur about 30 min after the 3rd oral OVA challenge (fig. 1c). The incidence of diarrhea increased with repeated oral OVA challenges and reached $91.8 \pm 3.8\%$ (6 independent experiments, total $n = 58$ –65) after the 6th OVA challenge.

Effect of Kakkonto on Intestinal Anaphylaxis

We examined the effect of kakkonto on allergic diarrhea. As shown in figure 1c, the administration of kakkonto significantly reduced the incidence of OVA-induced diarrhea. After the 6th OVA challenge, the inci-

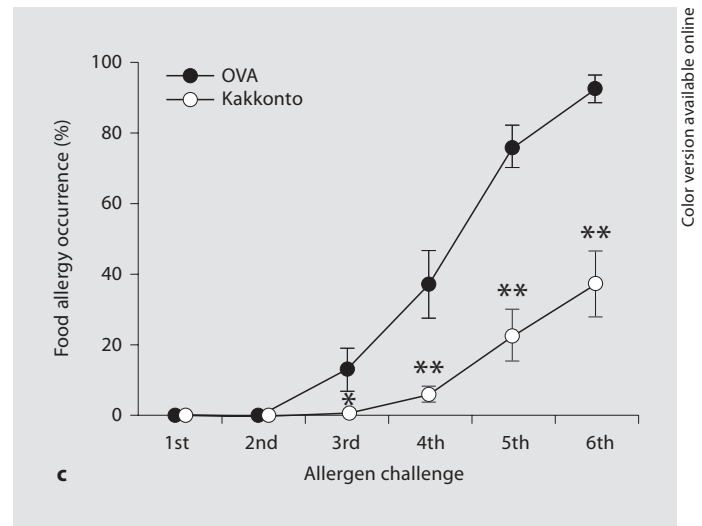
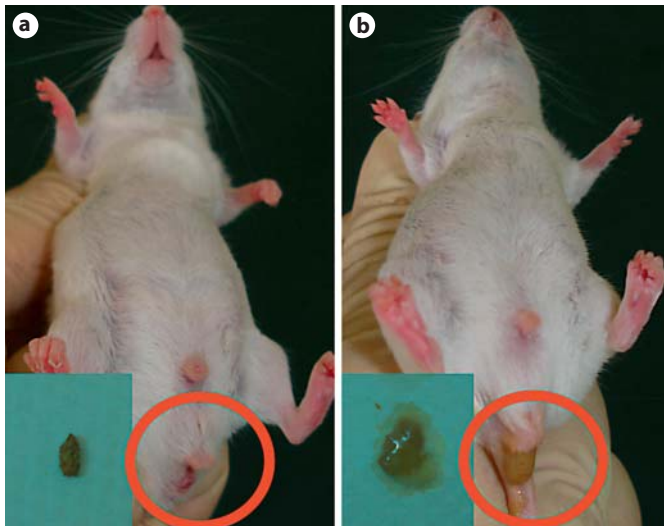


Fig. 1. Induction of allergic diarrhea and the effects of kakkonto. **a, b** Typical photographs are shown of a mouse that had no diarrhea (**a**; inset: stool) and a mouse that had severe allergic diarrhea (**b**; inset: stool). **c** Repeated oral OVA (50 mg) challenges resulted in allergic diarrhea. The occurrence of OVA-induced diarrhea was significantly suppressed by preadministration of kakkonto. Data shown are means $\pm$ SE (6 independent experiments, total $n = 54-66$). * $p < 0.05$, ** $p < 0.01$.

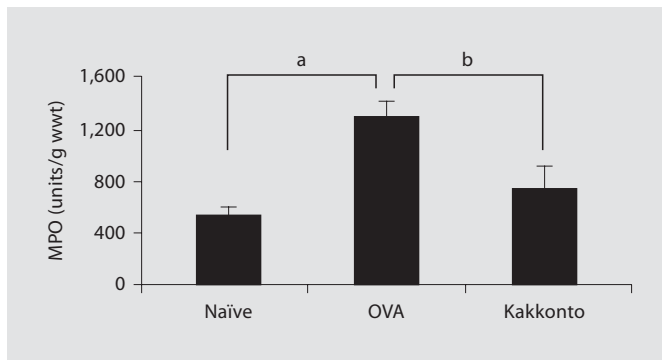


Fig. 2. Effect of kakkonto on MPO activity in the colon. OVA mice showed significantly increased MPO activity compared to naïve mice. Kakkonto significantly reduced food allergy-enhanced MPO activity. ^a $p < 0.01$, ^b $p < 0.05$ ($n = 4-9$).

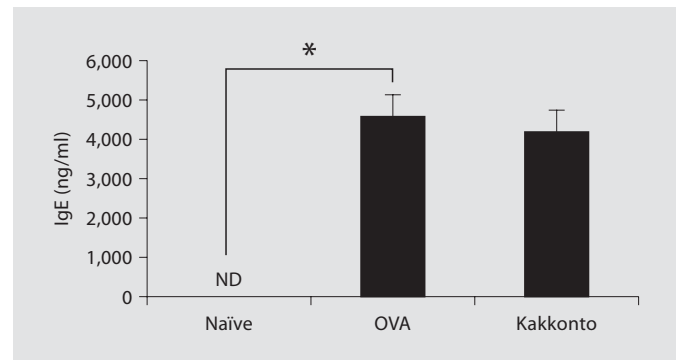


Fig. 3. Effect of kakkonto on the plasma levels of OVA-specific IgE. The IgE level of OVA mice was significantly increased when compared to naïve mice. However, kakkonto had no effect on OVA-specific IgE levels in OVA mice. ND = Not detectable. * $p < 0.01$ ($n = 5$).

dence of diarrhea was markedly decreased from 91.8 ± 3.8 to $36.9 \pm 9.4\%$ (6 independent experiments in each group, total $n = 54-66$). As shown in figure 2, MPO activity was significantly higher in the colon of OVA mice ($1,286 \pm 124$ units/g ww; $n = 9$) compared to naïve mice (535 ± 56 units/g ww; $n = 3$), and kakkonto treatment markedly suppressed the increase in MPO activity in OVA mice (738 ± 175 units/g ww; $n = 4$).

OVA-Specific IgE in the Plasma

The level of OVA-specific IgE in the plasma was measured after the 6th oral OVA oral challenge (fig. 3). OVA-specific IgE was not detected in naïve mice, whereas repeated OVA administration markedly elevated OVA-specific IgE ($4,568 \pm 548$ ng/ml; $n = 5$). Kakkonto treatment did not alter the increased level of OVA-specific IgE ($4,192 \pm 546$ ng/ml; $n = 5$).

Table 1. List of genes selected by microarray analysis

Probe	Accession No.	Annotation	Fold change log ratio	
			OVA/ naïve	kakkonto/ OVA
1416077_at	NM_009627	adrenomedullin	2.7	-1.8
1421134_at	NM_009704	amphiregulin	3.6	-1.9
1434350_at	BG070296	AXIN1 up-regulated 1	2.8	-1.8
1420582_at	AF373412	Cd209e antigen	7.2	-3.8
1421688_a_at	NM_011329	chemokine (C-C motif) ligand 1	5.7	-3.2
1419282_at	U50712	chemokine (C-C motif) ligand 12	2.8	-1.8
1420380_at	AF065933	chemokine (C-C motif) ligand 2	3.4	-2.5
1450488_at	AF281075	chemokine (C-C motif) ligand 24	4.8	-2.3
1419561_at	NM_011337	chemokine (C-C motif) ligand 3	6.7	-3.1
1421578_at	AF128218	chemokine (C-C motif) ligand 4	3.2	-2.8
1421228_at	AF128193	chemokine (C-C motif) ligand 7	4.1	-3.1
1419684_at	NM_021443	chemokine (C-C motif) ligand 8	2.2	-1.4
1419209_at	NM_008176	chemokine (C-X-C motif) ligand 1	4.4	-2.9
1449984_at	NM_009140	chemokine (C-X-C motif) ligand 2	5.6	-4.2
1419728_at	NM_009141	chemokine (C-X-C motif) ligand 5	6.8	-2.3
1419764_at	NM_009892	chitinase 3-like 3	3.1	-0.4
1449227_at	NM_009890	cholesterol 25-hydroxylase	3.6	-2.1
1416953_at	NM_010217	connective tissue growth factor	2.1	-1.8
1416039_x_at	NM_010516	cysteine rich protein 61	3.0	-1.3
1419334_at	NM_009843	cytotoxic T-lymphocyte-associated protein 4	2.8	-3.6
1417065_at	NM_007913	early growth response 1	2.6	-0.8
1427682_a_at	X06746	early growth response 2	3.9	-1.6
1436329_at	AV346607	early growth response 3	3.9	-2.0
1419431_at	NM_007950	epiregulin	2.7	-0.9
1417487_at	U34245	fos-like antigen 1	2.8	-2.9
1438439_at	BB229616	G protein-coupled receptor 171	2.5	-1.2
1450333_a_at	NM_008090	GATA binding protein 2	2.1	-1.5
1419060_at	NM_013542	granzyme B	2.6	-2.7
1453851_a_at	AK007410	growth arrest and DNA-damage-inducible 45 gamma	2.5	-2.0
1426063_a_at	U10551	GTP binding protein	2.1	-1.1
1451796_s_at	AF109137	histidine decarboxylase	4.9	-4.7
1418678_at	NM_008216	hyaluronan synthase 2	5.2	-1.9
1417171_at	NM_010583	IL2-inducible T-cell kinase	2.1	-1.3
1419647_a_at	NM_133662	immediate early response 3	2.2	-1.4
1425247_a_at	BC008237	immunoglobulin heavy chain 4	5.3	-1.3
1442014_at	BB531645	Interferon-related developmental regulator 1	2.6	-1.3
1449399_a_at	BC011437	interleukin 1 beta	3.2	-1.6
1422317_a_at	NM_010743	interleukin 1 receptor-like 1	2.4	-1.4
1450330_at	NM_010548	interleukin 10	2.3	-2.8
1420802_at	NM_008355	interleukin 13	4.7	-2.5
1449864_at	NM_021283	interleukin 4	2.4	-2.1
1450297_at	NM_031168	interleukin 6	8.8	-4.4
1459841_x_at	BB264849	lysosomal-associated protein transmembrane 5	3.4	-0.6
1419605_at	NM_010796	macrophage galactose N-acetyl-galactosamine specific lectin 1	2.8	-1.2
1422352_at	NM_008570	mast cell protease 1	8.0	-0.9
1449989_at	NM_008571	mast cell protease 2	8.6	-1.0
1425967_a_at	BC026198	mast cell protease 4	8.3	-1.9
1449456_a_at	NM_010780	mast cell protease 5	3.0	-1.6
1422180_a_at	NM_010781	mast cell protease 6	2.1	-2.2
1417256_at	NM_008607	matrix metalloproteinase 13	3.1	-2.4
1430838_x_at	AK007371	methyl-CpG binding domain protein 1	3.8	-1.7
1438767_at	BB237825	oncostatin M	2.1	-1.5
1418666_at	NM_008987	pentaxin related gene	2.9	-3.4
1417523_at	AF181829	pleckstrin	2.3	-1.2
1450290_at	NM_021396	programmed cell death 1 ligand 2	2.1	-1.7
1417262_at	M94967	prostaglandin-endoperoxide synthase 2	2.4	-0.8
1436058_at	BB132493	radical S-adenosyl methionine domain containing 2	2.4	-0.5
1419394_s_at	NM_013650	S100 calcium binding protein A8	5.8	-2.1
1448756_at	NM_009114	S100 calcium binding protein A9	7.2	-2.2

Table 1 (continued)

Probe	Accession No.	Annotation	Fold change log ratio	
			OVA/ naïve	kakkonto/ OVA
1441887_x_at	BB548587	SH3-binding domain glutamic acid-rich protein like	3.6	-1.7
1416576_at	NM_007707	suppressor of cytokine signaling 3	3.8	-1.7
1460302_at	AI385532	thrombospondin 1	2.4	-2.4
1455034_at	BB703394	transcribed locus	2.3	-1.4
1418424_at	NM_009398	tumor necrosis factor alpha induced protein 6	2.8	-2.3

Fold change log ratio means the natural logarithm of the ratio of the expression level in OVA mice to that in naïve mice or the ratio of the expression level in kakkonto mice to that in OVA mice.

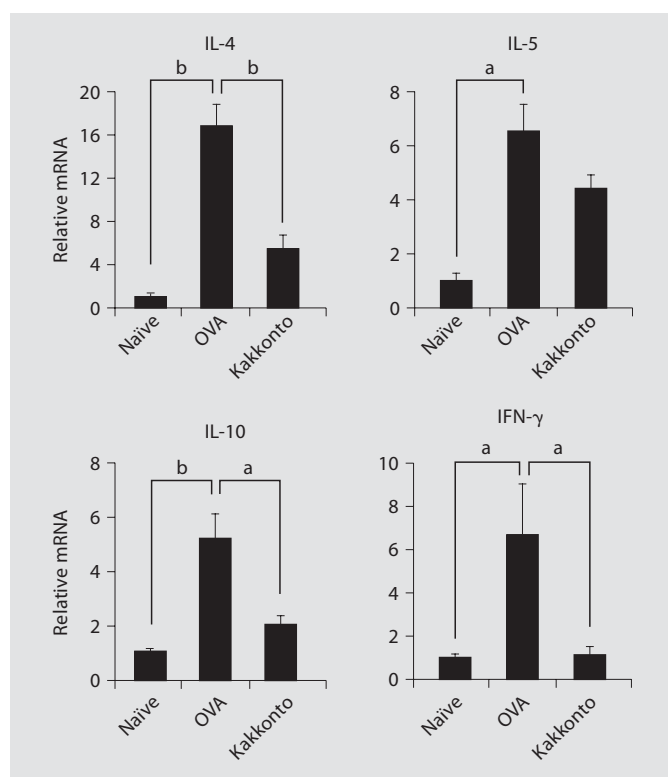


Fig. 4. Effect of kakkonto on the expression of cytokine mRNAs in the proximal colon. mRNAs of Th1 (IFN- γ) and Th2 (IL-4, IL-5 and IL-10) cytokines were significantly upregulated in OVA mice. Kakkonto reduced the enhanced expression of all of these cytokines except for IL-5. ^a $p < 0.05$, ^b $p < 0.01$ ($n = 5-8$).

Differentially Expressed Genes Revealed by Microarray Analysis

In order to investigate the mechanism underlying the therapeutic effect of kakkonto on intestinal anaphylaxis, we examined global mRNA expression patterns in the

proximal colons of naïve mice, OVA mice and kakkonto-administered mice (kakkonto mice) using the Affymetrix Genechip Mouse Genome 430 2.0 Array (table 1). We found 64 genes with expression patterns that were associated with kakkonto treatment. Expression levels of these genes were elevated more than 7-fold in OVA mice as compared with naïve mice, and this elevation was reduced by kakkonto administration. The associated genes included genes for Th2 cytokines (IL-4, IL-10 and IL-13) and 11 kinds of chemokine ligands, indicating that Th2 responses were largely augmented by oral OVA administration and reduced by kakkonto. Furthermore, expression of the suppressor of cytokine signaling (SOCS)-3 gene, which plays an important role in regulating the onset and maintenance of Th2-related allergic immune diseases [29], was also associated with OVA and kakkonto treatment. Among the 64 genes, the greatest changes were observed in the mRNA expression of mMCP-1 (3,000-fold increase in OVA mice) and mMCP-2 (5,500-fold increase in OVA mice), which are associated with mucosal mast cells. Other mast cell proteases associated with connective tissue mast cells (mMCP-4, -5 and -6) were also included in the set of 64 genes, but changes in their expression were much lower than those of mMCP-1 and -2. There were no genes whose expression levels were suppressed by more than 85% of the normal level in OVA mice and whose expression was then increased in kakkonto mice.

Transcription of Cytokines in the Proximal Colon

To further examine the detailed immunological profile in the proximal colon, the mRNA expression of Th1 and Th2 cytokines was measured by real-time PCR. The mRNA expression levels of Th2 cytokines (IL-4, IL-5 and IL-10) and Th1 cytokines (IFN- γ) in OVA mice were more than 5 times higher than those in naïve mice (fig. 4).

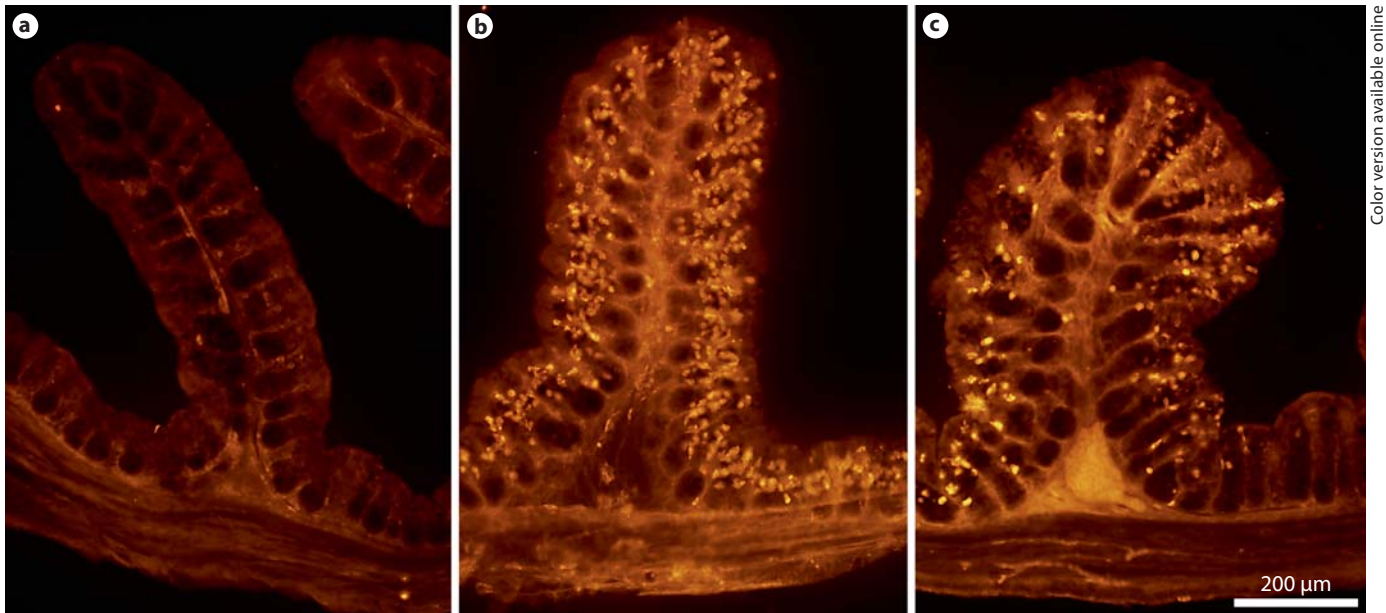


Fig. 5. Effect of kakkonto on mMCP-1-positive mucosal mast cells in the proximal colon. Naïve mice had a few mMCP-1-positive mucosal mast cells (**a**). The number of these cells was increased in OVA mice with allergic diarrhea (**b**) and was partially decreased in kakkonto mice (**c**).

We evaluated the effect of kakkonto on the upregulated expression of the cytokine mRNAs in OVA mice. The administration of kakkonto reduced the transcription of both Th1 and Th2 cytokines in the proximal colon (fig. 4).

Changes in Mucosal Mast Cell Infiltration

We were next interested in determining whether OVA-induced diarrhea was associated with mucosal mast cell infiltration. To assess mucosal mast cell infiltration, we detected mucosal mast cells in the proximal colon by immunohistochemistry and measured the concentration of plasma mMCP-1 by ELISA. As shown in figure 5, immunohistochemical staining with the mMCP-1 antibody revealed mucosal mast cells infiltrating into the mucosal layer of the proximal colon. Mucosal mast cells were dramatically increased in the proximal colon of OVA mice (fig. 5b), but the number of infiltrating mucosal mast cells was decreased in the proximal colon of kakkonto mice (fig. 5c). These results suggest that the significant amelioration of allergic symptoms by the administration of kakkonto is attributable to suppression of the number of mucosal mast cells in the proximal colon.

To investigate the degranulation of mucosal mast cells, we measured the concentration of mMCP-1 in the plasma

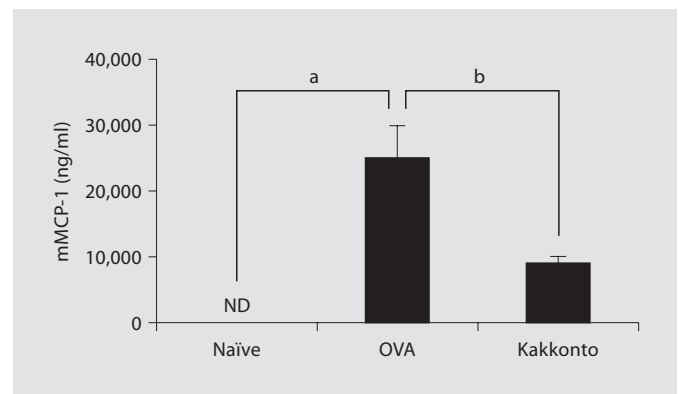


Fig. 6. Effect of kakkonto on plasma mMCP-1 levels. mMCP-1 was not detected in plasma from naïve mice. mMCP-1 levels were significantly decreased by kakkonto treatment. ND = Not detectable. ^a $p < 0.01$, ^b $p < 0.05$ ($n = 5$).

(fig. 6). There was no detectable mMCP-1 in samples from the naïve mice. The repeated OVA administrations resulted in a significant increase in plasma mMCP-1 ($21,082 \pm 4,552$ ng/ml; $n = 5$). In comparison with OVA mice, the levels of plasma mMCP-1 were suppressed in kakkonto mice ($8,498 \pm 759$ ng/ml; $n = 5$).

Discussion

Our results indicate that kakkonto has therapeutic effects on the development of allergic diarrhea in a food allergy model. In OVA mice, the OVA-specific IgE level was significantly increased in the plasma, and class switching to IgE was promoted in the spleen but not the colon (data not shown). Kakkonto treatment did not alter the increased level of OVA-specific IgE. Furthermore, intestinal mucosal immunity showed the enhancement of both Th1- and Th2-type responses, and the number of mucosal mast cells was greatly elevated in the colon. Kakkonto exerted an immunosuppressive effect on Th1- and Th2-type hyperresponses to food antigens and reduced the number of mucosal mast cells in the colon, resulting in the suppression of allergic symptoms. These findings indicate that kakkonto has therapeutic potential for food allergies through its inhibitory effects on the intestinal mucosal immune system.

It has been established that disodium cromoglycate can improve allergic asthma, whereas it failed to improve allergic diarrhea in our experimental model [30]. In addition, some traditional Japanese herbal medicines that markedly ameliorate the symptoms of Th2-type diseases such as animal models of skin allergy [22, 23] and airway inflammation [24] also had no therapeutic effect on allergic diarrhea in our animal model (data not shown). In contrast, kakkonto suppressed enhanced intestinal mucosal immunity in OVA mice and ameliorated allergic symptoms, suggesting that the therapeutic effects of kakkonto result from the control of intestinal mucosal immunity. Previously, it has been reported that a Chinese herbal medicine, food allergy herbal formula-1, ameliorates systemic anaphylaxis in a mouse food allergy model [31]. However, the medicinal herbs and their composition in kakkonto differ from those in food allergy herbal formula-1. Moreover, we demonstrated the therapeutic effects of kakkonto on the mucosal immune system of the colon, but not the systemic immune system, suggesting that the therapeutic mechanisms of these two herbal medicines are not the same. Thus, we think that herbal medicines have therapeutic potential for some types of food allergy.

Kakkonto contains many bioactive components, as it is extracted from 7 medicinal herbs, including *Puerariae radix*, *Zizyphi fructus*, *Glycyrrhizae radix*, *Paeoniae radix* and *Zingiberis rhizoma*. These medicinal herbs have anti-inflammatory effects, and their components [daidzein (*Puerariae radix*), fructofuranoside (*Zizyphi fructus*), glycyrrhizin (*Glycyrrhizae radix*), paeoniflorin

(*Paeoniae radix*) and gingerol (*Zingiberis rhizoma*)] are considered to contribute to their pharmacological effects. It is important to individually evaluate the effect of each anti-inflammatory component in kakkonto on allergic diarrhea. However, the traditional herbal medicines, including kakkonto, are well documented to exert pharmacological effects due to integrated and/or synergistic actions of the component medicinal herbs. Therefore, first of all we investigated the effects of kakkonto in detail.

There have been some reports about intestinal mucosal immunity in mouse food allergy models with allergic diarrhea. Using ELISA, Kweon et al. [8] demonstrated increases in IL-4, IL-5 and IL-13, but little or no production of IFN- γ , in colonic lymphocytes of mice with allergic diarrhea. Similarly, Brandt et al. [27] reported increases in IL-4, IL-10 and IL-13 mRNA but no changes in IFN- γ mRNA in the mouse jejunum concomitant with the development of allergic symptoms. Thus, in contrast to our findings, changes in IFN- γ were not observed in previous studies. This discrepancy is probably due to differences in immunization methods, cytokine measurement methods and the segments of the mouse intestine that were examined. In our model, we observed increased MPO activity and pathological changes (such as edema and hypertrophy; data not shown) in the colon of OVA mice, but not in the jejunum, suggesting that the primary tissue for allergic diarrhea in our model is the colon. Moreover, in general, cytokine levels are more sensitively detected by real-time PCR than ELISA. Therefore, enhancement of the Th1-type response is considered to contribute to the pathogenesis of allergic diarrhea.

Nevertheless, our results as well as the other reports indicate that enhancement of Th2-type responses is important for the induction of allergic gastrointestinal symptoms. In the present study, the upregulation of mRNA expression of IL-4 and IL-10 (Th2 cytokines) was significantly reduced by kakkonto administration in the proximal colon. In addition, our microarray analysis data demonstrated that the OVA-induced upregulation of Th2 cytokines (IL-4, IL-10 and IL-13) and various chemokines, including Th2-regulated chemokines, was suppressed in kakkonto mice. Therefore, it is suggested that suppression of Th2 cytokine responses in intestinal mucosal immunity is one of the major mechanisms by which kakkonto ameliorates allergic diarrhea. However, IL-5 upregulation was not significantly suppressed by kakkonto in our food allergy model. Brandt et al. [27] examined food allergies using IL-5-knockout mice and IL-5-transgenic mice and demonstrated that IL-5 and eosinophils are not involved in the development of diarrhea

because oral allergen-induced diarrhea occurred at similar levels among IL-5-knockout mice, IL-5-transgenic mice and normal mice. Taken together, these findings indicate that the immunosuppressive effect of kakkonto on Th1-type as well as Th2-type hyperresponses to food antigens is the main mechanism underlying the therapeutic effect of kakkonto on allergic symptoms.

Microarray analysis detected increased SOCS-3 mRNA expression in the proximal colons of OVA mice, which was decreased by kakkonto treatment. SOCS is a negative feedback inhibitor of cytokine signaling transduction that blocks the Jak-STAT pathway. Moreover, it has been reported that SOCS-3 participates in the pathogenesis of the allergic symptoms of atopic dermatitis [32], asthma [29] and Th2-dominant inflammation in the intestinal tract [33–35]. The production of Th2 cytokines such as IL-4 is higher in T cells from SOCS-3-transgenic mice than in control T cells, indicating that constitutive SOCS-3 expression enhances Th2 development [29]. SOCS-3 suppresses IL-12 signaling and thus inhibits the differentiation of naïve T cells into Th1 cells [29]. Induction of IL-12 prevents and treats intestinal allergic diarrhea [36, 37]. Taken together, these findings suggest that SOCS-3 is involved in the mechanism underlying the regulation of cytokines by kakkonto in intestinal mucosal immunity.

We were next interested in determining the effect of kakkonto on the infiltration of mucosal mast cells into the lamina propria of the colon in OVA mice. There are 2 types of mast cells, mucosal mast cells and connective tissue mast cells, although their phenotypic and functional differences are poorly understood. Nonetheless, numerous studies have already demonstrated the importance of mast cells in the induction of allergic diseases and Th2-mediated immune responses [38, 39]. Notably, plasma levels of mMCP-1 are significantly higher in both food allergy mice [27, 40] and parasite-infected mice [41, 42], whereas there are few precise studies about the function of mucosal mast cells in the intestine of each model. Mast cells are important cellular sources of proteolytic enzymes and are found in almost all tissues [43]. Five subtypes of mast cell proteases have been identified in mice: mMCP-1 and -2 are stored in mucosal mast cells and mMCP-4, -5 and -6 are stored in connective tissue mast cells [43]. In our mice, the number of mMCP-1-positive mucosal mast cells in the colon and the concentration of mMCP-1 in the plasma were greatly increased upon the induction of allergic diarrhea. In a previous study to investigate the role of mucosal mast cells in the pathogenesis and development of food allergy, we used phosphati-

dylinositol-3 kinase-deficient mice (PI3K^{-/-} mice) selectively lacking gastrointestinal mast cells [44]. PI3K^{-/-} mice fail to develop allergic diarrhea. Furthermore, there are only a few mMCP-1-positive mucosal mast cells in the colons of OVA-challenged PI3K^{-/-} mice in our model, and mMCP-1 levels in plasma of OVA-challenged PI3K^{-/-} mice are much lower than those in OVA mice [45], indicating that mucosal mast cells in the intestinal mucosa play a pivotal role in the pathogenesis and development of allergic diarrhea. In the present study, kakkonto treatment decreased the number of mucosal mast cells and the plasma level of mMCP-1. In addition, kakkonto treatment decreased the upregulated expression levels of mMCP-1 and -2 mRNA after the induction of allergic diarrhea. Taken together, these findings show that kakkonto has an inhibitory effect on the proliferation and/or differentiation of mucosal mast cells in the colon and thus ameliorates allergic diarrhea.

To date, no therapeutic drugs for human food allergy have been developed. In this study, we have revealed that the oral administration of kakkonto before administration of the food antigen suppresses the occurrence of allergic diarrhea in a mouse model of food allergy. These results support the feasibility and potential of kakkonto for the therapy of human intestinal allergic symptoms provoked by ingested food allergens, indicating that taking kakkonto before a meal can ameliorate the food allergy symptoms.

The pathogenesis of allergic diarrhea is not fully understood. Our results demonstrate that allergic diarrhea results from the enhancement of Th1 as well as Th2 intestinal immune responses and the infiltration of mucosal mast cells. We also show that kakkonto is a potential therapeutic medicine for food allergy with unique and notable profiles and that kakkonto acts selectively at the site of colonic anaphylactic mucosal immunity.

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