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Suppression of ovalbumin-induced allergic diarrhea by diminished intestinal peristalsis in RAMP1-deficient mice

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ABSTRACT

Recent studies have revealed that various neurotransmitters regulate the immune system via their receptors expressed on the immune cells. Calcitonin gene-related peptide (CGRP), a sensory nerve C-fiber neuropeptide, is also known to have the ability to modulate the functions of immune cells *in vitro*. However, the contribution of CGRP to the immune regulation *in vivo* remains to be fully elucidated. Here we report that mice deficient in receptor activity-modifying protein 1 (RAMP1), which is a subunit of the CGRP receptor, showed a significantly lower incidence of diarrhea compared with wild-type (WT) mice in the ovalbumin (OVA)-induced food allergic model. Serum OVA-specific IgE levels and the differentiation of T helper cells was comparable in WT mice and RAMP1-deficient mice. Moreover, there were no significant differences between recruitment and degranulation of mast cells in the small intestine of these mice. In contrast, significantly diminished intestinal peristalsis was observed by the allergy induction in RAMP1-deficient mice compared with WT mice. These results suggest that this suppression of allergic diarrhea is due to the diminished intestinal peristalsis in RAMP1-deficient mice.

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1. Introduction

Food allergy is defined as an adverse immune response to responsible food allergens. The prevalence of food allergy has increased over the past few decades [1] like other allergic diseases. In Japan, 12.8% of infants and 5.1% of 3-year-olds are affected with food allergies [2]. Patients with food allergies develop various symptoms such as skin symptoms (e.g. rash, pruritus and angioedema), gastrointestinal symptoms (e.g. nausea, vomiting, and diarrhea), respiratory mucosal symptoms (e.g. sneeze, nasal obstruction, and wheeze), and life-threatening systemic symptoms (e.g. anaphylactic shock) [3]. At present, there are no curative treatments for food allergies, therefore food allergen avoidance is a general countermeasure. In just the past decade, several animal models of intestinal anaphylaxis were developed [4–7], and contributed to clarifying the pathogenic mechanism on the mucosal surface at molecular and cellular levels. The intestinal allergic model established by Kweon et al. [4] was dependent on the signal

transducers and activators of transcription (STAT6) signaling in CD4⁺ Th2 cells in the large intestine. On the other hand, Brandt et al. [5] showed that exposure of OVA/alum-sensitized mice to repeated administration of intragastric OVA induces allergic inflammation of the small intestine characterized by acute diarrhea. IgE and mast cells seems to play important roles in the development of this allergy.

Recent accumulating evidences suggest the existence of cross-talk regulation between the nervous system and the immune system via common ligands and receptors [8]. It is well recognized that the breakdown in this communication network causes the pathogenesis and the exacerbation of immune-mediated diseases. Therefore, elucidation of the communication mechanism between the nervous system and the immune system is of great importance not only to understand how to maintain physiological homeostasis but also to develop a new treatment strategy for immune-mediated diseases. CGRP is a 37-amino acid neuropeptide widely distributed in the central and peripheral nervous systems, mainly in C-type sensory nerves, and participates in nociceptive transmission [9]. CGRP is also known as a potent vasodilator [10] and is previously shown to promote Th2 differentiation in the presence of a CD3/CD28 co-stimulation signal [11], suppress the cytokine production from macrophages [12], and regulate B cell differentiation [13]. There are two isoforms of CGRP, α CGRP and β CGRP, in human and rodents. α CGRP is a product from the primary transcript of the

Abbreviations: Th, T helper; STAT6, the signal transducers and activators of transcription; OVA, ovalbumin; PBS, phosphate buffered saline; m MCP-1, mouse mast cell protease-1; LNs, lymph nodes.

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calcitonin/ α CGRP gene by the tissue-specific alternative splicing [14], and is synthesized almost exclusively in neuronal tissues. In contrast, β CGRP is encoded by a different gene that does not produce calcitonin [15]. β CGRP is not predominant in sensory nerves, but appears to be the major isoform in enteric neurons [16]. So far, no obvious difference in pharmacological function was not elucidated between two isoforms. The CGRP receptor is composed of a heterodimer of a 7-transmembrane G protein-coupled receptor called the calcitonin receptor-like receptor (CLR) and a single transmembrane receptor called RAMP1 [17,18], of which RAMP1 decides specificity to CGRP. Therefore we previously generated mice deficient in RAMP1 by a conditional gene-targeting technique, and demonstrated that the RAMP1-deficient mice exhibited high blood pressure, with no changes in heart rate [19]. Furthermore, we showed that CGRP took part in the regulation of the innate immune response *in vitro* and *in vivo*. However, the involvement of CGRP in the acquired immune responses *in vivo* remained to be elucidated. In this study, we aimed to assess the contribution of CGRP to intestinal anaphylaxis as a representative example of type 2 acquired immune responses using RAMP1-deficient mice.

2. Materials and methods

2.1. Mice

RAMP1-deficient mice were generated by intercrossing RAMP1^{+/-} mice backcrossed with BALB/c mice for at least eight generations. The knockout mice were housed and bred at our animal facility in Osaka University. BALB/c mice were purchased from Charles River Japan. All the mice used were 8–12 week-old. All animal experiments were performed with the consent of the Animal Care and Use Committee of Osaka University.

2.2. Experimental intestinal anaphylaxis model

Mice were sensitized and challenged as previously described by Brandt et al. [5]. Briefly, mice were sensitized twice intraperitoneally, 2 weeks apart, with 25 μ g of OVA (grade V; A5503; Sigma–Aldrich) in the presence of 2 mg of aluminum hydroxide gel (Sigma–Aldrich) in sterile phosphate buffered saline (PBS). Two weeks later, mice were orally administered 50 mg of OVA dissolved in 250 μ L of PBS every other day for a total of four times. Before each intragastric challenge, mice were deprived of food for 3–4 h with the aim of limiting antigen degradation in the stomach. Challenges were performed with intragastric feeding needles (Natsume seisakujo). Diarrhea was assessed by visually monitoring mice for up to 1 h following intragastric challenge. Mice demonstrating profuse liquid stool were recorded as diarrhea-positive animals.

2.3. Determination of OVA-specific IgE and mouse MCP-1 in sera

Mouse OVA-specific IgE and mouse mast cell protease-1 (mMCP-1) levels in sera were determined according to the manufacturer's instructions of mouse OVA-IgE ELISA kit (AKRIE-030; Shibayagi) and mMCP-1 ELISA Ready-Set-Go (eBioscience), respectively.

2.4. Intestinal mast cell quantification

The jejunum tissue was collected 7–10 cm distal to the stomach. All samples were fixed in 10% formalin and processed by standard histological techniques. The 5 μ m tissue sections were stained for mucosal mast cell with chloroacetate esterase activity by using a AS-D staining kit (Muto pure chemicals). Stained mast cells per

square millimeter were quantitated by counting the number of chloroacetate-positive cells from 10 fields of view.

2.5. T cell isolation and stimulation

Spleens or lymph nodes (LNs) from WT and RAMP1-deficient mice were washed once in PBS. The CD4⁺ cellular fraction of the splenocytes or mesenteric lymphocyte preparation was purified with magnetically labeled beads specific for mouse CD4 (BD Biosciences) according to the manufacturer's protocol. FACS analysis showed the purified cells to be >98% CD4⁺ T cells. The CD4⁺ T cells were cultured in a 48-well plate at 10⁶ cells/ml, and stimulated with plate-coated anti-CD3 mAb (2C11) and anti-CD28 mAb (PV-1) (both antibodies were produced and purified "in-house") at 37 °C in 5% CO₂ for 48 h. For determination of cytokine levels in cell-free supernatants, in-house ELISA systems [11] for both IFN- γ and IL-4 were used.

2.6. Gastrointestinal transit

Mice were sensitized and orally administered with OVA as mentioned above. Twenty minutes after the third oral OVA administration, mice were given a 0.25 ml suspension of charcoal meal (5% activated carbon (Sigma) in 10% gum arabic (Sigma)) with intragastric feeding needles and were killed 5 min later. The abdomen was opened and the intestine was dissected out from the pyloric end up to the ileocaecal junction. Gastrointestinal transit was expressed as a percentage of the distance traveled by the charcoal for the total length of the small intestine.

2.7. Statistics

The statistical significance of differences was determined by Student's *t*-test, and *P* < 0.05 was considered significant.

3. Results

3.1. Suppression of diarrhea induced by repeated oral challenge with OVA in RAMP1-deficient mice

Accumulating body of evidence indicates that CGRP pharmacologically affects the proliferation, differentiation and cytokine production of immune cells. However, the physiological function of CGRP remained to be fully elucidated. To clarify the involvement of CGRP in the acquired immunity *in vivo*, we used an OVA-induced

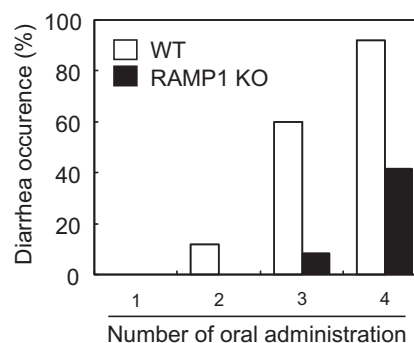


Fig. 1. OVA-induced allergic diarrhea in WT mice and RAMP1-deficient mice. Diarrhea occurrence in WT mice and RAMP1-deficient mice sensitized with twice OVA/alum intraperitoneal injections and subsequently challenged with intragastric OVA administration four times. Data are represented as a percentage of diarrhea occurrence for the number of OVA oral administration. *n* = 25, WT mice; *n* = 24, RAMP1-deficient mice.

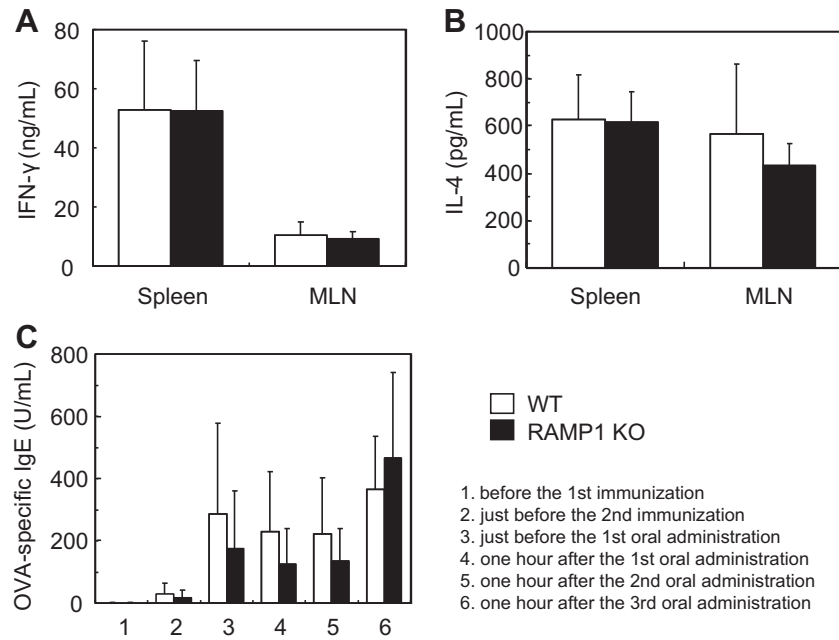


Fig. 2. No changes in Th cell cytokine and serum IgE levels in RAMP1-deficient mice induced allergic diarrhea. WT mice (white bar) and RAMP1-deficient mice (black bar) were sensitized with twice OVA/alum intraperitoneal injections and subsequently challenged with intragastric OVA administration three times. The concentrations of IFN- γ (A) and IL-4 (B) in the culture supernatant of CD3/CD28-stimulated CD4⁺ T cells purified from MLNs and spleen of the mice were determined by ELISA. Values are means $\pm$ SD ($n = 3-4$). (C) Serum OVA-specific IgE levels in the mice were determined by ELISA. Values are means $\pm$ SD ($n = 6-8$).

food allergy model. In this model, repeated oral challenge with OVA in systemically OVA/alum-primed WT mice led severe diarrhea depending on a frequency of OVA oral administration, resulting that 23 of 25 (92%) mice induced diarrhea after four times of the intragastric OVA challenge (Fig. 1). In contrast, the incidence of diarrhea was attenuated to <50% in RAMP1-mice even after the fourth challenge.

3.2. Immunological evaluation of allergic diarrhea in WT mice and RAMP1-deficient mice

We have previously reported that CGRP suppresses IFN- γ and promotes IL-4 production from Th cells *in vitro* [11]. Then, we first quantitated the cytokine levels produced by CD3/CD28-stimulated Th cells in the spleen and mesenteric lymph nodes (MLN) from WT mice and RAMP1-deficient mice that were primed and challenged three times with OVA. There is no difference in IFN- γ and IL-4 productions from the Th cells between WT mice and RAMP1-deficient mice (Fig. 2A and B). Next, we determined the serum levels of OVA-specific IgE, which is responsible for the development of this intestinal anaphylaxis. As shown in Fig. 2C, serum IgE levels are also comparable in WT mice and RAMP1-deficient mice at any time of serum collection. Mast cells play an important role by producing serotonin and platelet-activating factor in the development of allergic diarrhea [5]. Therefore, we compared the number of mast cells infiltrated into the small intestines of the diarrhea-induced mice. However, no significant difference in mast cell number was also observed between WT mice and RAMP1-deficient mice (Fig. 3A). Moreover, we could detect no changes in serum IL-9, which is implicated in the regulation of mast cell recruitment and effector function (data not shown). A previous *in vitro* study reported promotion of degranulation from mast cells by CGRP [20]. Intestinal mucosal mast cells have low contents of serotonin and histamine [21]. On the other hand, the plasma levels of mMCP-1 that is associated with the degranulation of mucosal mast cells increase approximately 5000-fold after the induction of intestinal anaphylaxis [5]. Therefore, the measurement of the mMCP-1 level

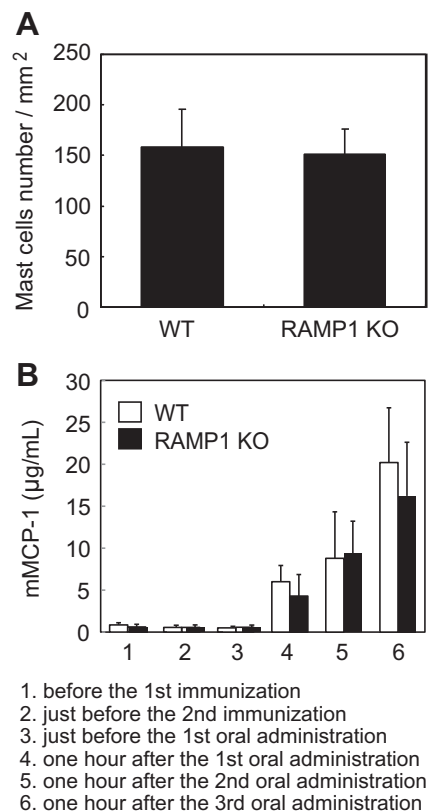


Fig. 3. No changes in recruitment and degranulation of mast cells in the jejunum of allergic diarrhea-induced RAMP1-deficient mice. (A) Number of mucosal mast cells in the jejunum from WT mice and RAMP1-deficient mice that were sensitized with twice OVA/alum intraperitoneal injections and subsequently challenged with intragastric OVA administration three times. The mast cell number was counted after staining with chloroacetate esterase. Values are means $\pm$ SD ($n = 3$). (B) Serum mMCP-1 levels in WT and RAMP1-deficient mice sensitized with OVA/alum and subsequently treated with intragastric OVA challenges were determined by ELISA. Values are means $\pm$ SD ($n = 6-8$).

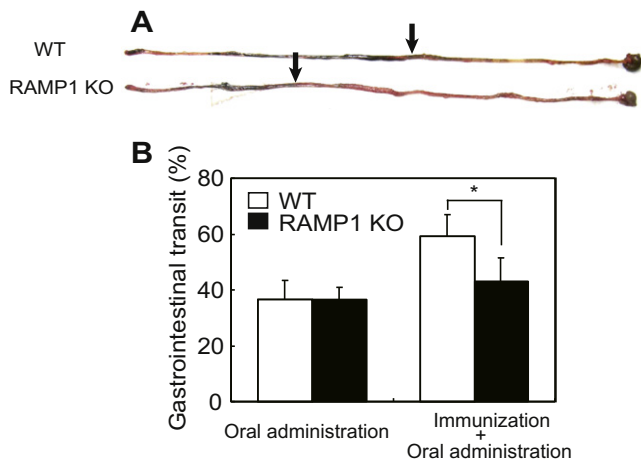


Fig. 4. Diminished intestinal peristalsis in RAMP1-deficient mice with OVA-induced allergy. (A) A representative photograph of the small intestine and cecum of WT mice and RAMP1-deficient mice administered a charcoal meal after the induction of intestinal anaphylaxis. (B) Intestinal transit of a charcoal meal in WT mice and RAMP1-deficient mice 20 min after third intragastric administration of OVA. Mice, which were not sensitized (left) or were sensitized (right) with OVA/alum, were challenged with intragastric OVA administration three times. Values are means $\pm$ SD ($n = 5-7$). * $P < 0.05$.

in the sera is generally considered as an index of mast cell degranulation in an intestinal anaphylaxis model [5,22]. To evaluate the extent of mast cell degranulation, therefore, we measured the serum levels of mMCP-1. As shown in Fig. 3B, the serum mMCP-1 levels are comparable in WT mice and RAMP1-deficient mice at any time examined.

3.3. Diminished intestinal peristalsis in RAMP1-deficient mice

We could not recognize significant differences in the immunological parameters between diarrhea-induced WT mice and RAMP1-deficient mice. Stimulation of the small bowel by excessively rapid entry of chyme or by chemical irritation induces diarrhea, and the suppression of gastrointestinal motility causes constipation [23]. Then, we compared the intestinal peristalsis between WT mice and RAMP1-deficient mice by measuring intestinal transit of a charcoal meal. As shown in Fig. 4A, obvious difference in intestinal transit of a charcoal meal was observed in the RAMP1-deficient mice compared with WT mice after the induction of intestinal anaphylaxis. Statistical analyses indicate a significantly decrease in the intestinal transit in the RAMP1-deficient mice immunized and orally administered with OVA, although the intestinal transit in WT mice and RAMP1-deficient mice without systemically priming with OVA/alum was comparable (Fig. 4B). These results indicate that the lower incidence of allergic diarrhea in RAMP1-deficient mice causes the diminished peristalsis.

4. Discussion

Sensory nerves innervate the skin and intestinal mucosa and their fibers contain some neuropeptides such as Substance P (SP) and CGRP. Growing body of evidence indicates that both SP and CGRP play important immune-regulatory functions. For example, SP enhances IL-12 production by macrophages [24] and IL-2 production by T cells [25]. Furthermore, SP is shown to cause bronchoconstriction [26]. On the other hand, CGRP also regulates cytokine production from Th cells [11] as well as dendritic cells [19] *in vitro*, and pharmacologically suppresses the type IV allergy, contact hypersensitivity [27] and delayed-type hypersensitivity [28] *in vivo*. However, the physiological function of CGRP remained to be

fully elucidated. Here we demonstrated that RAMP1-deficient mice showed a lower incidence of diarrhea compared with WT mice in the intestinal anaphylaxis model. Previously, we found that CGRP augmented IL-4 production by Th cells to skew the immune system to Th2 *in vitro* [11]. However, no difference in cytokine production was observed between Th cells from WT mice and RAMP1-deficient mice in this study. This may be because CGRP does not regulate Th cell differentiation in this intestinal anaphylaxis model. Mast cells are known to play an important role in the development of allergic diarrhea. It is reported that CGRP induces the degranulation of murine mast cells *in vitro* [20]. We detected the mRNA expression of both subunits of CGRP receptor, RAMP1 and CLR, in mouse bone marrow-derived mast cells (data not shown). Then, we examined the recruitment and degranulation of mast cells, but were not able to find a difference in their functions between WT mice and RAMP1-deficient mice. However, there may be a room for investigating the effect of CGRP on mast cell functions.

In the effect of CGRP on gastrointestinal response, there are conflicting results, that is stimulation and inhibition; Grider showed that CGRP antagonists inhibited the responses of contraction and relaxation elicited by muscle stretch or mucosal stimulation in the three-compartment flat-sheet colonic preparation system [29]. On the other hand, there is a report that administration of CGRP into the systemic circulation or into cerebrospinal fluid inhibits gastric emptying in rats [30]. Our results using RAMP1-deficient mice in the OVA-induced allergic model indicate that CGRP promotes intestinal peristalsis in physiological conditions. In the intestinal anaphylaxis model, serotonin is shown to be a major factor to induce diarrhea [5]. Furthermore, serotonin initiates peristalsis by activating serotonin receptors on sensory CGRP neurons, and exogenous serotonin induces a concentration-dependent release of CGRP [31]. These findings suggest the existence of a fine regulatory mechanism of serotonin-CGRP axis in the intestinal peristalsis.

It remains to be clarified which CGRP isoforms are more important and what cells are the main source of CGRP production in this intestinal anaphylaxis model. The principal source of CGRP in the gastrointestinal system is thought to be extrinsic primary afferent nerve fibers and intrinsic enteric neurons [32]. It is reported that a main isoform of CGRP in extrinsic afferents is α CGRP, whereas only β CGRP is produced in enteric neurons. Apart from neurons, CGRP is also found in endocrine cells of the gastrointestinal mucosa in human [33] and in blood-derived or resident immune cells within the lamina propria of the gastric mucosa in rats [34]. So far, three groups have already generated α CGRP-deficient mice [35–37], while the generation of β CGRP-deficient mice has not been reported. Recently, we could generate β CGRP-deficient mice. In near future, by using α CGRP-deficient mice, β CGRP-deficient mice and cell-specific RAMP1 knockout mice, we will be able to make a new avenue for clarifying the different functions of each CGRP isoform in the food allergic model.

In conclusion, the present findings indicate for the first time that CGRP physiologically induces the development of diarrhea by promoting the intestinal peristalsis in the intestinal anaphylaxis. We expect that this research leads to the development of a new therapeutic agent for food allergy.

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