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Ingestion of partially hydrolyzed whey protein suppresses epicutaneous sensitization to β -lactoglobulin in mice

Takeshi Matsubara  | Hiroshi Iwamoto | Yuki Nakazato | Tomoyuki Okamoto |
Tatsuya Ehara | Hirohisa Izumi | Yasuhiro Takeda

Wellness & Nutrition Science Institute,
Morinaga Milk Industry Co., Ltd., Zama,
Japan

Correspondence

Takeshi Matsubara, Wellness & Nutrition
Science Institute, Morinaga Milk Industry
Co., Ltd., Zama, Japan.
Email: ta-matsubara@morinagamilk.co.jp

Abstract

Background: Epicutaneous sensitization to food allergens can occur through defective skin barriers. However, the relationship between oral tolerance and epicutaneous sensitization remains to be elucidated. We aimed to determine whether prior oral exposure to whey proteins or their hydrolysates prevents epicutaneous sensitization and subsequent food-allergic reaction to the whey protein, β -lactoglobulin (β -LG), and investigated the underlying mechanisms.

Methods: BALB/c mice were given whey protein concentrate (WPC), two kinds of partial whey protein hydrolysate (PWH1 or PWH2), or extensive whey protein hydrolysate (EWH) in drinking water for 21 days. The mice were then epicutaneously sensitized with β -LG on tape-stripped skin. Sensitization was assessed by basophil activation tests and by measuring the level of serum β -LG-specific antibodies and cytokines secreted from β -LG-restimulated spleen and mesenteric lymph node (MLN) cells. Development of an allergic reaction was assessed by monitoring body temperature and by measuring mast cell protease-1 level in plasma after the β -LG oral challenge. Activated T-cell population among β -LG-restimulated MLN cells was also analyzed.

Results: In mice fed with WPC, PWH1, or PWH2, sensitization and the development of an allergic reaction were totally reduced. The acceleration of cytokine release from the spleen and MLN cells or T-cell activation was not evident after β -LG restimulation. In EWH-fed mice, a suppressive effect, though milder than that in WPC-, PWH1-, or PWH2-fed mice, was observed during the development of the allergic reaction.

Conclusions: Prior oral exposure to partially hydrolyzed whey protein prevents epicutaneous sensitization and subsequent allergic response to β -LG in mice.

KEYWORDS

β -lactoglobulin, anaphylaxis, cow's milk allergy, epicutaneous sensitization, extensively hydrolyzed whey protein, food allergy, mouse model, oral tolerance, partially hydrolyzed whey protein, protein hydrolysates

1 | INTRODUCTION

The past several decades have experienced an increase in the prevalence of food allergy on a global scale. Based on oral food challenges, the reported prevalence in preschool children is 2–10% in developed countries and 1–7% in rapidly developing countries.¹

Food allergy is thought to develop due to a failure in oral tolerance, the process that suppresses the immune responses to orally ingested innocuous proteins.² However, some children developed food allergy symptoms after the first oral ingestion even though neither they nor their mothers had consumed the allergen previously, suggesting that tissues other than the gut also present significant routes of allergen sensitization.^{3,4} A recent systematic review confirmed that atopic dermatitis is a risk factor for the development of food allergy,⁵ and other studies revealed that defective skin barriers contribute to food allergy development through epicutaneous sensitization,^{6,7} indicating the skin as an important site for allergen sensitization.

In animal models, epicutaneous exposure to allergens provokes IgE responses and subsequently induces systemic anaphylaxis when challenged orally with the allergen.^{6,8} Epicutaneous sensitization in these models could be elicited by repeated exposure to the allergen via skin disrupted by tape stripping or by pre-treatment with sodium dodecyl sulfate (SDS) or the vitamin D analog MC903.^{7–9} Although the models reflect primal sensitization followed by food allergy symptoms and might be more physiological in respect that the sensitization is induced without adjuvants, few animal studies demonstrate the association between oral tolerance and epicutaneous sensitization.^{9,10}

Oral tolerance is an antigen-dependent process. Not only intact proteins but also their hydrolysates are well documented to induce oral tolerance. For instance, the ingestion of partially hydrolyzed whey protein and fragmented egg proteins were shown to prevent developing allergies to cow's milk and eggs, respectively.^{11,12} However, most studies used adjuvants for sensitization to the allergens. The sensitization was induced by oral administration with cholera toxins or by intraperitoneal or subcutaneous injection with aluminum hydroxide.^{11,13,14} To the best of our knowledge, no study has demonstrated whether oral tolerance induced by hydrolyzed proteins prevents epicutaneous sensitization.

In this study, using an established model of epicutaneous sensitization through tape stripping, we evaluated whether oral exposure to partially or extensively hydrolyzed whey proteins prevents epicutaneous sensitization to a cow's milk whey protein, β -lactoglobulin (β -LG).

2 | METHODS

2.1 | Cow's milk proteins and hydrolysates

β -LG was purified from raw cow's milk as described previously¹⁵ and used for epicutaneous sensitization. The whey protein hydrolysates (extensive whey protein hydrolysate, EWH; partial whey protein hydrolysate 1, PWH1; and partial whey protein hydrolysate 2, PWH2) were obtained from Morinaga Milk Industry, Tokyo, Japan, and the whey protein concentrate (WPC) was obtained from Milei GmbH, Leutkirch, Germany. The data regarding the hydrolysates and concentrate are presented in Table 1. The amount of β -LG and molecular weight distribution was determined using a β -LG ELISA Kit (FASPEK II, Morinaga Institute of Biological Science, Yokohama, Japan) and HPLC, respectively, as described previously.¹⁶

2.2 | Mice

BALB/c mice were used in this study similar to the models of epicutaneous sensitization in previous studies.^{6–10,17} Four-week-old female BALB/c mice were obtained from CLEA Japan (Tokyo, Japan) and maintained on milk-free chow under specific pathogen-free conditions. At the onset of the experiment, the mice were 5 weeks old. All animal experiments were approved by the Institutional Animal Care and Use Committee of Morinaga Milk Industry Co., Ltd.

2.3 | Oral tolerance induction, epicutaneous sensitization, and oral challenge

Experimental schedules are shown in Figure 1. From day 1 to day 21, six groups ($n = 8$ /group) were given different 6% whey protein/

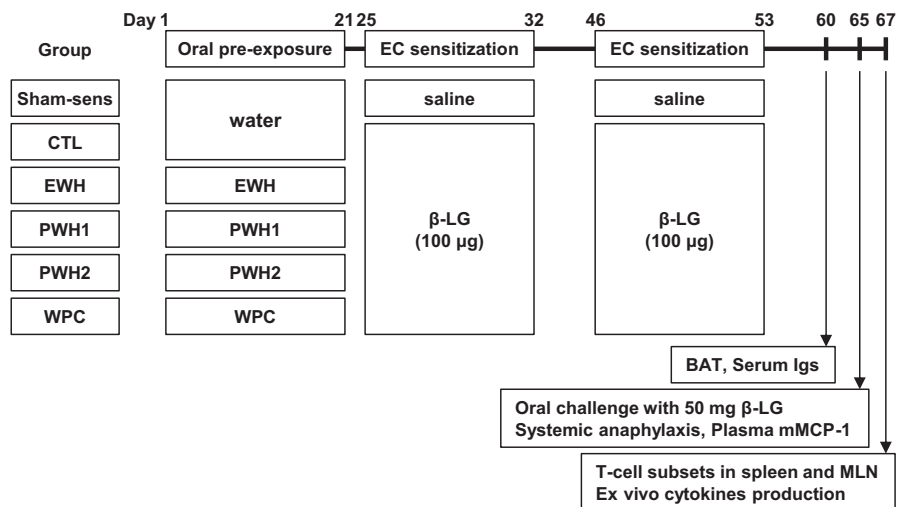
TABLE 1 Characteristics of whey proteins and their hydrolysates used in this study

Name	Classification	Molecular weight distribution ^a							Detected β -LG ^b [ppm]
		<200	200–500	500–1000	1000–2000	2000–5000	5000–10 000	10 000<	
EWH	Extensive whey protein hydrolysate	9	44	27	18	2	0	0	<0.5
PWH1	Partial whey protein hydrolysate	6	34	23	21	12	2	1	2.5×10^3
PWH2	Partial whey protein hydrolysate	7	35	21	22	11	2	1	2.1×10^3
WPC	Whey protein concentrate	0	4	0	0	1	3	92	3.3×10^5

^aData are expressed as the percentage of total protein or peptide.

^bData are expressed as part per million of antigenically equivalent β -LG in powders.

FIGURE 1 A schematic overview of pre-oral exposure and epicutaneous sensitization. Mice were given only water or EWH, PWH1, PWH2, or WPC in the water to induce oral tolerance followed by epicutaneous sensitization. Parameters were analyzed as described in the diagram. Sham-sens, sham-sensitized; CTL, control; EWH, extensive whey protein hydrolysate; PWH, partial whey protein hydrolysate; WPC, whey protein concentrate



whey protein hydrolysates solutions: WPC, PWH1, PWH2, or EWH, or water alone ad libitum in their drinking bottles and a solid milk-free chow. From day 25, all mice were epicutaneously exposed to 100 μg of β-LG or saline alone, as described previously.¹⁷ Each mouse was exposed twice in total. On day 60, blood samples were obtained from tail veins for the basophil activation test (BAT) and measurement of β-LG-specific antibodies. On day 65, the mice were orally challenged with 50 mg of β-LG (Sigma-Aldrich, St. Louis, MO). Systemic anaphylaxis was evaluated by hypothermia. Body temperature was measured before challenge, and at 5, 10, 20, 30, 40, 50, and 60 minute after challenge, using a rectal probe coupled to a digital thermometer (TD-300; Natsume Seisakusho Co., Tokyo, Japan). Data are shown as rectal temperature changes from the temperature before challenge. Subsequently, 1 hour after oral challenge, plasma samples were collected for measurement of mouse mast cell protease-1 (mMCP-1). On day 67, the mice were sacrificed by sevoflurane inhalation, and mesenteric lymph nodes (MLNs) and spleens were removed. One mouse in the control (CTL) group died on day 66. Additionally, the number of MLN cells collected from one mouse in the sham-sensitized (Sham-sens) group was low, and the population of inducible T-cell costimulator (ICOS)⁺ cells after incubation with or without β-LG could not be analyzed.

2.4 | Basophil Activation Test

Heparinized whole blood samples were mixed with 100 ng/ml β-LG in PBS or buffer alone and incubated for 2 hour at 37°C. The markers on basophils were labeled and detected as described previously.^{16,18}

2.5 | Measurement of antigen-specific antibodies and mMCP-1

β-LG-specific serum IgE and IgG1 were measured by ELISA, using Biotin rat anti-mouse IgE and IgG1 (BD Biosciences, San Jose, CA), respectively. Serum samples were diluted to 1:30 and 1:20 000 for

IgE and IgG1, respectively. Plasma concentrations of mMCP-1 were determined using an ELISA kit (eBioscience, San Diego, CA), per the manufacturer's protocol.

2.6 | Flow cytometric analysis of Treg cells and activated T cells

MLNs and spleens were dilacerated through 70-μm cell strainers (BD Biosciences). Additionally, hemolysis was performed for spleen cells. Following 2 washes, the cells were suspended in RPMI 1640 medium containing 10% horse serum and labeled with FITC anti-mouse CD4 (clone RM4-5), PE anti-mouse CD25 (clone PC61) (both from BD Biosciences), and PerCP/Cy5.5 anti-human/mouse/rat ICOS (Biolegend, San Diego, CA). Foxp3 staining was performed with APC anti-Foxp3 (FJK-16) (eBioscience), per the manufacturer's protocol. The stained cells were analyzed using a FACSCanto flow cytometer and FACSDiva software. For the T-cell activation test, MLN cells were incubated with or without 1 mg/ml β-LG for 24 h, followed by staining and flow cytometric analysis as described.

2.7 | Cytokine release

MLN and spleen cells (4×10^5 /well) were stimulated with or without 1 mg/ml β-LG for 60 h. Supernatants were collected and analyzed for IL-2, IL-5, IL-10, and IL-13 by Magnetic Luminex Assay (R&D Systems, Minneapolis, MN). The concentrations of IL-4 and IL-17A were measured using ELISA kits (Biolegend and eBioscience, respectively).

2.8 | Statistical analysis

Data were analyzed by one-way ANOVA, followed by Tukey-Kramer multiple comparisons test using statistical software (JMP™ version 5.1; SAS Institute Inc., Cary, NC). Data are expressed as the mean ± standard error of the mean (SEM), and *P* values < .05 were considered significant.

3 | RESULTS

3.1 | Oral ingestion of whey proteins and their partial hydrolysates prior to epicutaneous sensitization reduces basophil activation response and allergen-specific antibody production

To study the capability of whey protein hydrolysates to induce oral tolerance, mice were fed water with or without whey proteins or hydrolysates of whey proteins and subsequently sensitized

epicutaneously to β -LG. After the 2nd epicutaneous sensitization, the level of sensitization of mice to β -LG was evaluated, as determined by basophil activation response and the production of allergen-specific antibodies. In mouse BAT, the upregulation of CD200R1 and downregulation of CD200R3 on basophils after antigen stimulation are induced in an IgE- and IgG1-dependent manner, respectively.^{18,19} In allergic CTL mice, IgE- and IgG1-dependent basophil activations in response to β -LG and the production of allergen-specific IgE and IgG1 were significantly higher compared to that in Sham-sens mice (Figure 2A and B). Similar results were shown in the EWH-fed mice. By contrast, the mice fed PWH1, PWH2, or WPC did not show induced basophil activation or increased production of allergen-specific antibodies (Figure 2A and B). Regarding the basophil population among white blood cells in peripheral blood, no significant difference was observed between groups (data not shown).

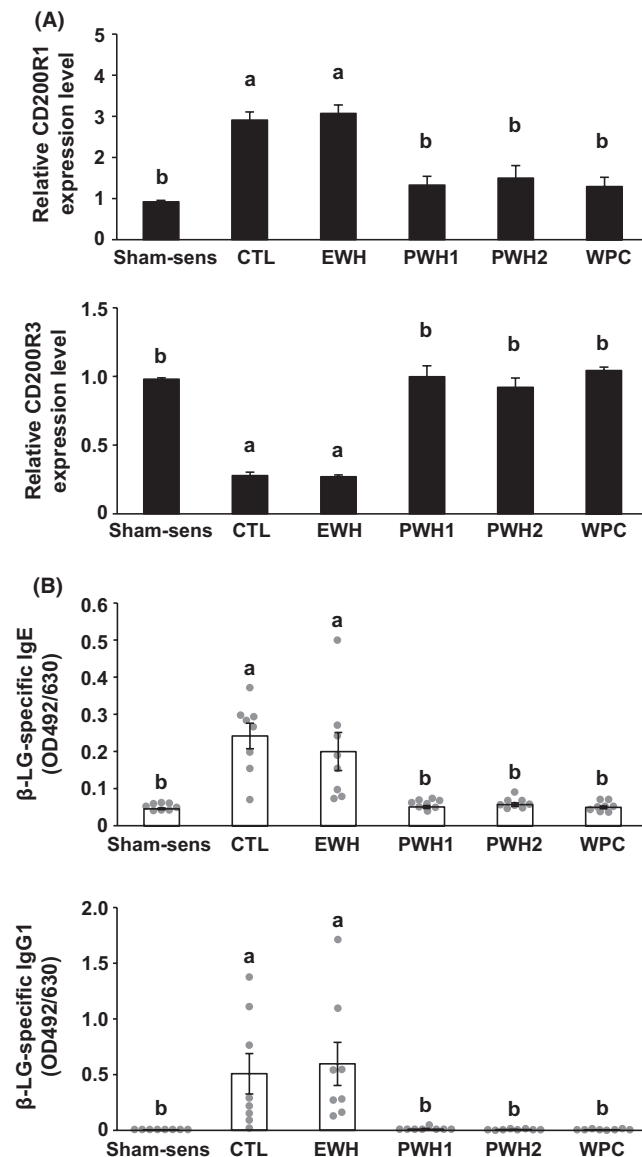


FIGURE 2 Effect of EWH, PWH1, PWH2, or WPC feeding prior to epicutaneous sensitization on basophil activation response and specific antibodies. (A) One-week after 2nd epicutaneous sensitization with β -LG, on day 60, peripheral blood was collected and incubated with or without β -LG. The expression of CD200R1 and CD200R3 on basophils was analyzed by flow cytometry and is presented as relative expression levels. (B) The levels of IgE and IgG1 specific for β -LG were measured by ELISA. Data are shown as the mean $\pm$ SEM ($n = 8$). Each dot represents an individual mouse. Means without a common letter differ significantly; $P < .05$

3.2 | Pre-treatment with whey proteins and their hydrolysates reduces systemic anaphylaxis and the degranulation of intestinal mucosal mast cells

To evaluate oral tolerance against epicutaneous sensitization at the in vivo effector level, anaphylactic reactions after the β -LG oral challenge were determined by measuring the body temperature. From 10 to 60 minute after oral challenge, mice in the CTL group had significantly lower body temperatures than those in the Sham-sens group. By contrast, mice in the groups treated with EWH, PWH1, PWH2, or WPC did not have a decreased body temperature. Although there was no significant difference between the mice in the Sham-sens and EWH groups, mice in the EWH group showed a mild decrease in body temperature at 10 and 20 minute after oral challenge (Figure 3A). To determine whether the systemic anaphylaxis corresponded to degranulation of intestinal mast cells, plasma concentrations of mMCP-1 were measured. The mMCP-1 levels were significantly higher in the CTL group than those in the other groups. Consistent with the anaphylaxis results, the mean of mMCP-1 levels in the EWH group was higher than that in the Sham-sens group; however, this was not significant (Figure 3B).

3.3 | Pre-treatment with whey proteins and their hydrolysates does not affect the population of Treg cells and activated T cells in MLN and spleen cells

To investigate whether oral tolerance induction affected the T-cell populations, the populations of Foxp3⁺ Treg cells and ICOS⁺ activated T cells were measured in the MLN and spleen 2 days after oral challenge. No difference in the population of Treg cells or activated T cells among CD4⁺ cells was observed between the groups (Figure 1).

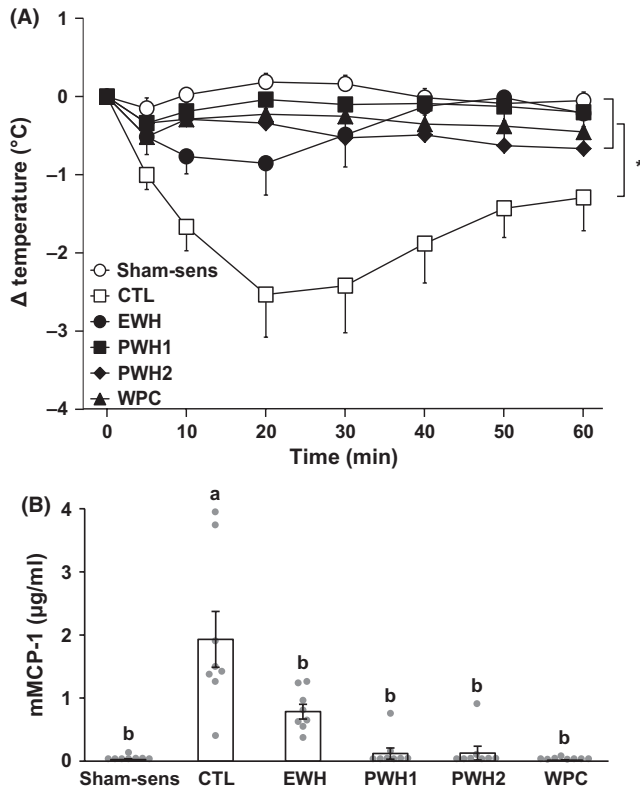


FIGURE 3 Effect of EWH, PWH1, PWH2, or WPC feeding prior to epicutaneous sensitization on allergic response to β -LG after oral challenge. (A) Rectal temperature changes in mice after challenge with 50 mg of β -LG. The asterisk shows the statistical difference compared with CTL group in each time point between 10 and 40 min. (B) The concentrations of mMCP-1 in plasma 1 hour after oral challenge. Data are represented as the mean $\pm$ SEM ($n = 8$). Each dot represents an individual mouse. Means without a common letter differ significantly; $P < .05$

3.4 | Prior oral ingestion of whey proteins and their partial hydrolysates reduces cytokine production and T-cell activation of MLN cells after allergen stimulation

To investigate the effect of pre-treatment with whey proteins and their hydrolysates on the sensitization state of lymphocytes, cytokine production by MLN and spleen cells and the activation of T and Treg cells among MLN cells after ex vivo antigen stimulation were determined. The levels of IL-2, IL-4, IL-5, IL-10, and IL-13 in the supernatant of MLN cells were significantly higher in the CTL and EWH groups than in the other groups (Figure 4A). IL-17 levels in all MLN cell supernatants were lower than the quantitation limit (data not shown). Similarly, the level of IL-2, IL-4, IL-5, IL-10, IL-13, and IL-17, secreted from spleen cells, was significantly higher in the CTL and EWH groups (Figure 2). Consistent with the cytokine release results, the population of ICOS⁺ CD4⁺ T cells among CD4⁺ T cells of MLN cells was significantly increased by β -LG stimulation in the CTL and EWH groups (Figure 4B). Additionally, significant activation of Treg cells following antigen stimulation was observed in the two groups (Figure 4B).

4 | DISCUSSION

Sensitization with food allergens via a disrupted skin barrier is thought to be a major route for the development of food allergy. However, it remains to be elucidated whether oral intake of allergens prevents subsequent epicutaneous sensitization. Here, we demonstrated that prior oral ingestion of whey protein and partial whey protein hydrolysates reduces epicutaneous sensitization and systemic anaphylaxis to β -LG. Distinctive mechanisms of suppressive effects include T-cell silencing to β -LG.

Only two laboratories have reported that prior oral intake of allergen prevents epicutaneous sensitization using ovalbumin.^{9,10} These studies demonstrated that the ingestion of ovalbumin protects allergen-specific IgE production and allergic skin inflammation and increases the Treg cell population in the MLNs of mice sensitized through tape-stripped skin¹⁰ and that oral administration of the allergen before epicutaneous sensitization, using SDS to disrupt the skin barrier, inhibits specific IgE production and subsequent development of food allergy accompanied with upregulating *Foxp3* expression in the inguinal lymph nodes.⁹ Consistent with these results, we showed that the oral ingestion of whey protein prevents epicutaneous sensitization to β -LG and oral challenge-induced anaphylaxis. Furthermore, pre-treatment with PWHs was demonstrated to have a preventive effect. However, no effect on Treg frequency was observed in the MLN and spleen. Discrepancies in the Treg population results might be attributable to differences in the methods used to disrupt the skin barriers and in the periods between the termination of skin exposure and the dissection.^{9,10}

Baek et al.¹⁰ demonstrated that oral tolerance suppresses mRNA expressions of *IL-5* and *IL-13* and eosinophil infiltration in the skin of epicutaneously sensitized mice using skin tissues harvested the day after sensitization. Additionally, epicutaneous sensitization reportedly induces Th2-type cytokine production and the infiltration of basophils and eosinophils in the skin,^{9,10,20} in which they reach to the peak the day after epicutaneous sensitization and return to basal levels 2 days later.⁹ In this study, we investigated the immunological sensitization state mainly at the systemic level by analyzing basophil activation, antibody production, and anaphylactic response more than 1 week after epicutaneous sensitization. To more precisely clarify the preventive effect of oral tolerance on epicutaneous sensitization, potential changes of the immune microenvironment in the skin immediately after epicutaneous sensitization and oral challenge need to be evaluated in the future.

Oral tolerance to whey proteins induced by exposure to partial whey protein hydrolysates has been studied in animals for more than 20 years using systemic and oral sensitization models. The partial hydrolysates are reportedly less efficient in inducing oral tolerance than intact whey proteins,^{11,14} while the standard infant formula consisting of intact milk proteins and the partially hydrolyzed formula were shown to have the same tolerizing effect in a dose-dependent manner.²¹ In this study, PWH1 and PWH2 were demonstrated to have the same accelerative effect on oral tolerance induction as WPC. However, we did not examine their dose dependency. Further

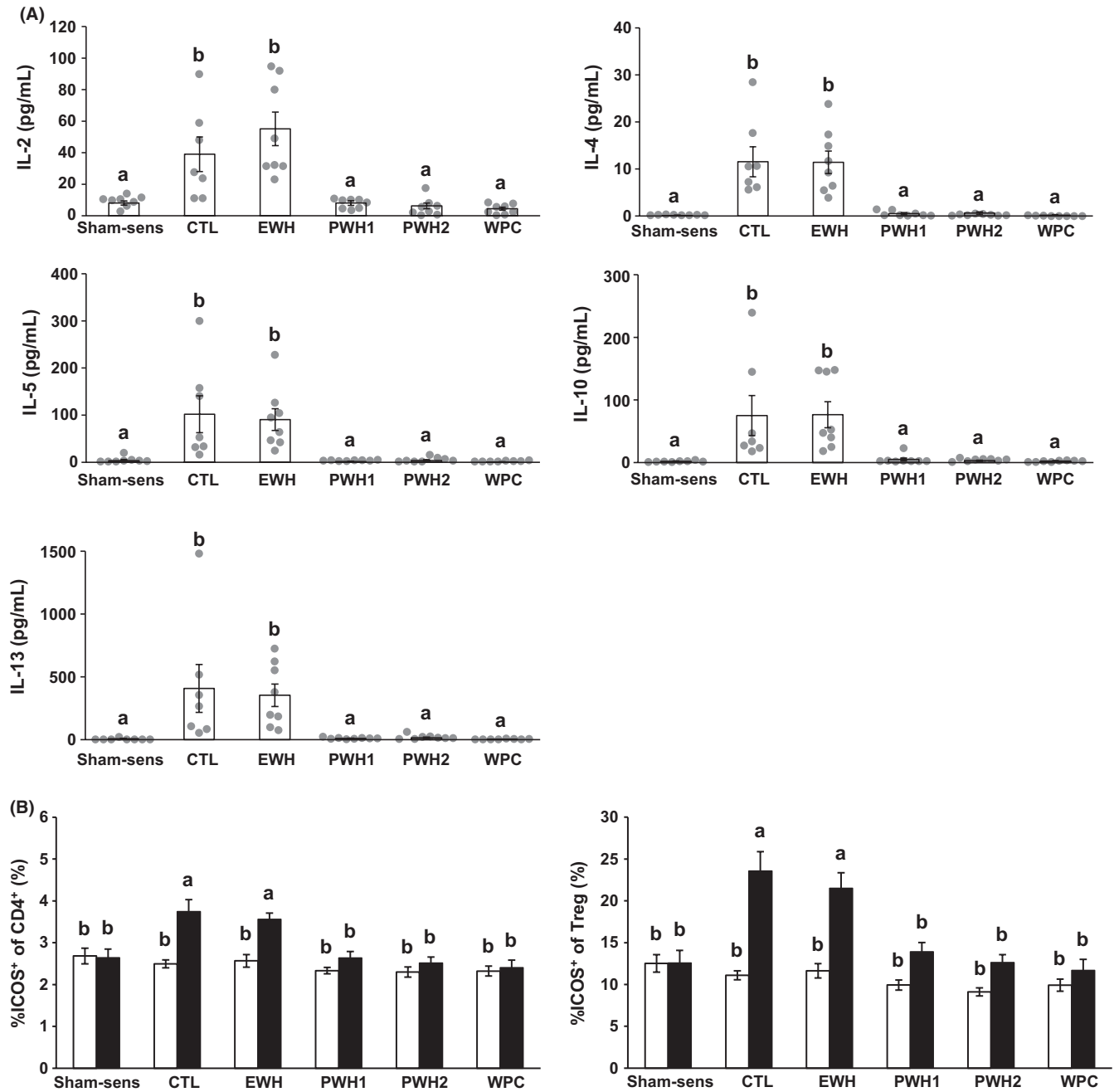


FIGURE 4 Impact of ex vivo stimulation of MLN cells with β -LG on cytokine release and T-cell activation. MLN cells were incubated with or without β -LG for 60 hour (A) or 24 hour (B). (A) After subtracting the background cytokine levels, data are shown as the mean $\pm$ SEM of $n = 7$ in CTL group and $n = 8$ in the other groups. Each dot represents an individual mouse. (B) The percentages of ICOS⁺ cells in both CD4⁺ lymphocytes (left) and CD4⁺CD25⁺Foxp3⁺ Treg cells (right) incubated with (black bar) or without (white bar) β -LG were determined. Data are shown as the mean $\pm$ SEM of $n = 7$ in Sham-sens and CTL groups and $n = 8$ in the other groups. Means without a common letter differ significantly; $P < .05$

studies investigating the factors such as the dose and ingestion periods should compare the efficiency in oral tolerance induction between whey proteins and the PWHs.

A previous study reported that epicutaneous sensitization, even without an antigen, results in mast cell expansion in the submucosa, but not the mucosa, of mouse small intestines.⁶ By contrast, two oral-antigen challenges induce significant accumulation of mast cells in the intestines of mice sensitized epicutaneously with the

antigen.^{7,22} Because anaphylaxis and mMCP-1 levels were evaluated after single oral challenge in this study, it might be likely that there was no difference in the number of intestinal mast cells between groups at the same time point. Therefore, we speculated that the difference in mMCP-1 levels observed between groups was attributable to the progression of intestinal mucosal mast cell degranulation. Further studies investigating whether repetitive oral-antigen challenge following epicutaneous sensitization in orally tolerized mice

affects the expansion of intestinal mast cells, leading to modification of food allergy symptoms, are required.

In the EWH group, preventive effects were observed only at the in vivo effector level. Additionally, suppressive effects were lower, though statistically insignificant, than in the WPC, PWH1, and PWH2 groups. Similarly, previous studies demonstrated that partial whey protein hydrolysates and β -LG fragments prevented milk allergy symptoms but not allergen-specific IgE production.^{11,23} However, they modulated the cytokine production and Treg cell population,^{11,23} while EWH in this study did not. Therefore, the mechanism associated with the preventive effect in the EWH group remains unclear. Mast cells play an important role in IgE-mediated anaphylaxis,²⁴ with previous studies reporting that IgE is necessary but not sufficient for food anaphylaxis,⁶ and that some cytokines, such as tumor growth factor (TGF)- β , directly inhibit mast cell activation.²⁵ Therefore, these cytokines might be the candidates contributing to suppression of anaphylaxis in the EWH group. Further in-depth investigation is necessary to clarify the mechanism.

As for primary effector mechanisms of oral tolerance, low doses of ingested allergens favor active suppression with the generation of Treg cells, whereas high doses favor clonal anergy/deletion, where antigen-specific T cells, including Th1, Th2, and Treg cells, would be silenced and/or deleted.^{26,27} However, the mechanisms in the two forms of tolerance are not mutually exclusive.²⁷ Previous studies, using oral sensitizing models, demonstrated that a low dose of whey protein hydrolysates or β -LG-derived peptides induces active suppression.^{11,14,23} In this study, oral exposure to a high dose of WPC, PWH1, or PWH2 suppressed cytokine production and activation of T cells, including Treg cells, among MLN cells upon β -LG restimulation, indicating that T-cell anergy/deletion was induced. Therefore, the difference in the mechanisms of oral tolerance between the previous studies and present study may have been caused by oral dosages. In this study, we analyzed the antigen-specific cytokine production of MLN and spleen cells to assess local and systemic immune responses according to previous reports evaluating oral tolerance.^{10,13} However, epicutaneous sensitization also induces Th2 differentiation of skin-draining lymph node cells.²⁸ Further studies evaluating the cytokine production in these cells would likely elucidate the preventive effect of prior PWH ingestion on epicutaneous sensitization. In addition to the oral dosages, the difference might also be attributable to the sensitization routes. Previous studies using BALB/c mice demonstrated that epicutaneous but not oral sensitization of mice resulted in systemic anaphylaxis, evidenced by a decrease in body temperature after oral challenge.^{6,9,29,30} Moreover, epicutaneous sensitization relies on the skin-to-gut crosstalk mediated by factors such as Langerhans cells, basophils, and thymic stromal lymphopoietin.^{7,22,31,32} These findings suggest that the main targets suppressed in oral tolerance are not always the same between the two models. Therefore, the doses and the ingestion periods of proteins or hydrolysates sufficient for inducing oral tolerance seem to be different between these models. Future studies need

to confirm the feeding protocol of whey protein hydrolysates for preventing epicutaneous sensitization.

In conclusion, the present study demonstrated that oral exposure to partial whey protein hydrolysates induced oral tolerance and suppressed epicutaneous sensitization and subsequent development of systemic anaphylaxis to β -LG. The sensitizing capacity of partial whey protein hydrolysates is lower than that of whey proteins in systemic and oral sensitizing models,^{33,34} although there are no reports comparing the capacities using the epicutaneous sensitizing model. Based on a growing body of evidence, the concept that early cutaneous exposure to food allergens through disrupted skin barriers leads to allergic sensitization, while early oral exposure to the allergens induces oral tolerance, has been proposed.³⁵ Partial whey protein hydrolysates, which have lower sensitizing capacity and are comparable to intact whey proteins in the ability to induce oral tolerance and suppress epicutaneous sensitization, might have the potential to prevent cow's milk allergy. The potential of infant formulas containing the hydrolysates in human babies with high-risk of atopic dermatitis needs to be investigated in the future.

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CONFLICT OF INTEREST

All authors are employees of Morinaga Milk Industry Co., Ltd., and declare no conflict of interest.

ORCID

Takeshi Matsubara  <http://orcid.org/0000-0001-7407-2451>

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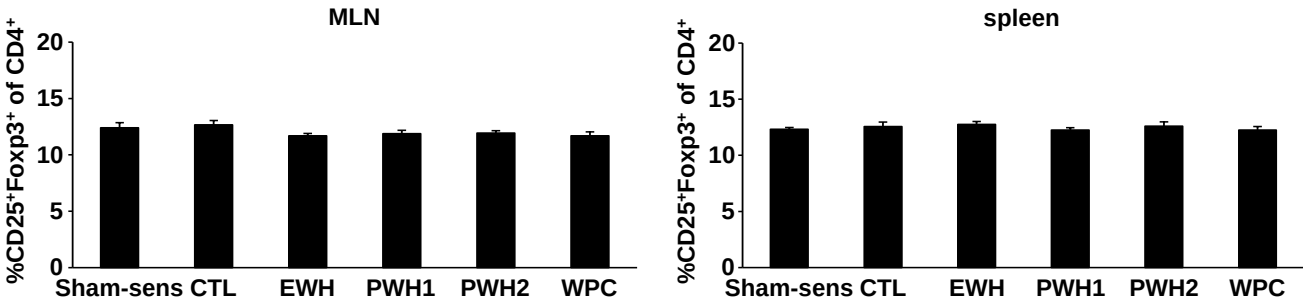
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SUPPORTING INFORMATION

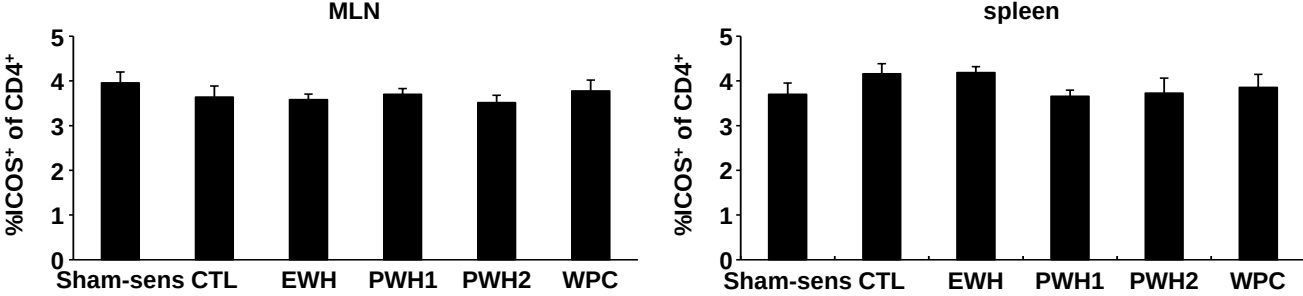
Additional Supporting Information may be found online in the supporting information tab for this article.

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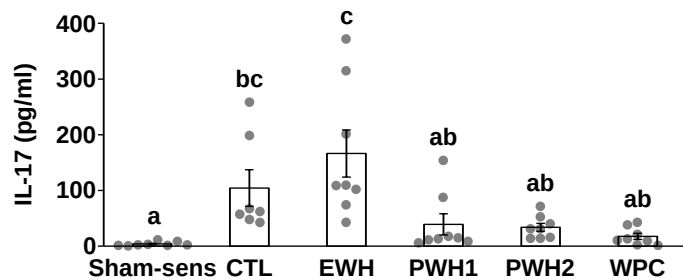
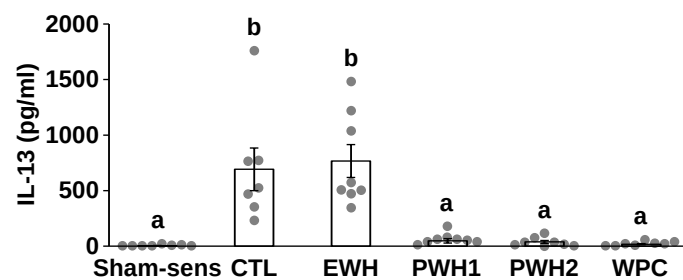
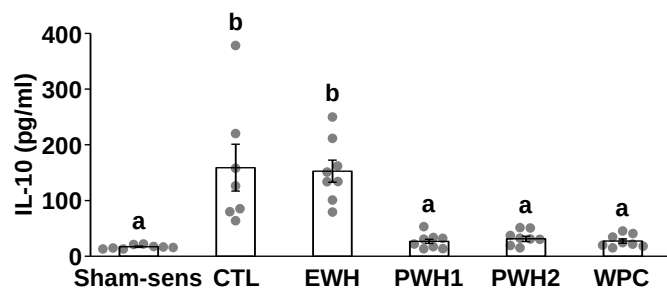
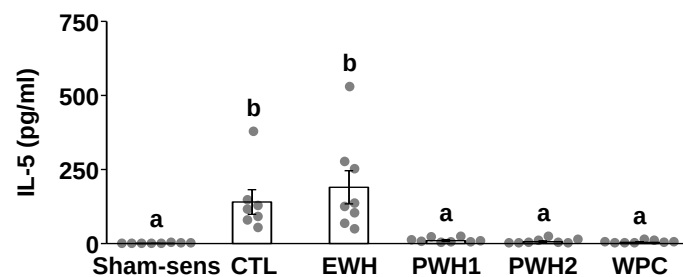
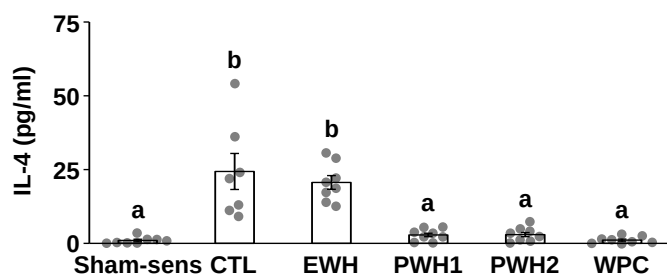
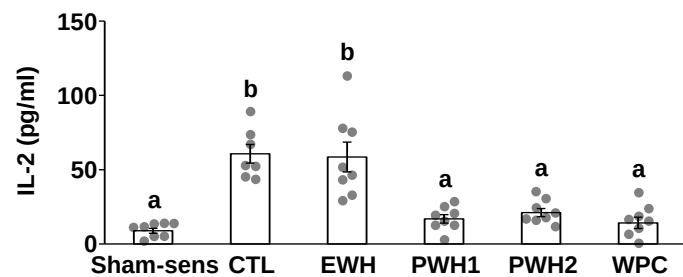
(a)



(b)



Supplementary Fig. 1 Matsubara *et al.*



Supplementary Fig. 2 Matsubara *et al.*