

Induction of Oral Tolerance by Pepsin-Digested Gliadin Retaining T Cell Reactivity in a Mouse Model of Wheat Allergy

Xuyang Li^a Takuya Miyakawa^a Tomohiro Takano^b Haruyo Nakajima-Adachi^b
Masaru Tanokura^a Satoshi Hachimura^b

^aLaboratory of Basic Science on Healthy Longevity, Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences, University of Tokyo, Tokyo, Japan; ^bResearch Center for Food Safety, Graduate School of Agricultural and Life Sciences, University of Tokyo, Tokyo, Japan

Keywords

Wheat allergy · T cell reactivity · Gliadin · Pepsin-digested gliadin · Oral tolerance

Abstract

Background: Wheat is known as the most widely consumed food all over the world. Although many types of wheat allergy have been recognized, their treatment still has a long way to go due to the complex pathogenesis. Oral immunotherapy (OIT) is under investigation for the treatment of wheat allergies. Previous studies have demonstrated that OIT using intact wheat allergens can induce tolerance, but is accompanied by a high risk of anaphylactic reactions. **Objectives:** Our objective was to prepare modified wheat allergens with hypoallergenic and tolerance-inducing properties to reduce adverse effects during immunotherapy. **Methods:** Wheat gliadin was degraded by hydrolysis with pepsin and trypsin, and then the hydrolysate was deamidated with hydrochloric acid. The IgE-binding capacity and T cell reactivity of the degraded gliadins were evaluated in vitro. Pepsin-di-

gested gliadin (peptic-GLI) was applied in a mouse model to investigate whether it would induce oral tolerance. **Results:** Degradation with pepsin decreased IgE-binding capacity and maintained T cell reactivity. Oral administration of peptic-GLI to mice before sensitization and challenge with gliadin could significantly suppress the production of IgE, IgG1, and type 2 T helper cytokines. Moreover, the development of anaphylactic reactions and allergic responses of the small intestine induced by gliadin challenge were inhibited by oral administration of peptic-GLI. **Conclusions:** The findings of this study indicate that peptic-GLI with low allergenicity and potential for tolerance induction may become useful in wheat immunotherapy with less adverse effects.

© 2020 S. Karger AG, Basel

Introduction

The immediate hypersensitivity reaction to wheat is triggered by wheat proteins. For patients with wheat allergy, a wheat-free diet is recommended by physicians.

Edited by: H.-U. Simon, Bern.

karger@karger.com
www.karger.com/iaa

© 2020 S. Karger AG, Basel

Karger

Dr. Satoshi Hachimura
Research Center for Food Safety, Graduate School of Agricultural and Life Sciences
University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku
Tokyo 113-8657 (Japan)
ahachi@mail.ecc.u-tokyo.ac.jp

Prof. Masaru Tanokura
Laboratory of Basic Science on Healthy Longevity, Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences, University of Tokyo
1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657 (Japan)
amtanok@mail.ecc.u-tokyo.ac.jp

However, unintentional ingestion of foods containing wheat always happen in patients who try to avoid wheat allergens [1]. With the increased understanding of the mechanisms involved in tolerance, the focus of treatment and prevention has shifted to induce tolerance [2]. Oral immunotherapy (OIT) involves the regular and gradual administration of small amounts of allergen via oral ingestion to first induce desensitization to the allergen and then tolerance over long-term treatment [3]. OIT has been anticipated in some studies to be an approach for the treatment of patients with wheat allergy. Regarding the use of OIT for wheat-induced anaphylaxis, OIT has been shown to increase the threshold dose of symptoms and to achieve tolerance in approximately 60% of subjects [4, 5]. However, oral administration of intact allergens can cause adverse effects and even lead to a severe allergic reaction. Anaphylaxis could be induced during the escalation phase of wheat OIT [6]. Thus, modified allergens characterized by hypoallergenicity and potential for tolerance induction have become desirable for the treatment of wheat allergy.

Gluten is the central component of wheat proteins and is composed of gliadin and glutenin [7]. Wheat gliadin extracted from gluten with 70% ethanol is one of the major allergens responsible for wheat allergies [8, 9]. Wheat gliadin is limited in its utilization because of its insolubility. A few studies have reported that enzymatic hydrolysis and deamidation of gliadin could increase its solubility in water and reduce its allergenicity in regard to the IgE level [10]. Deamidation of gliadin using cation-exchange resins could induce oral tolerance, regardless of whether deamidated gliadin was applied before or after gliadin sensitization [10–12]. Enzymatic hydrolysis combined with acid deamidation has been shown to increase the solubility, foaming, and emulsifying properties of gluten [13]. However, the effects of enzymatic hydrolysis alone or the combination of enzymatic hydrolysis and acid deamidation of gliadin on allergenicity and tolerance induction are not fully understood. It has been reported that oral administration of peptides containing T cell epitopes or peptides with T cell reactivity could induce T cell unresponsiveness and tolerance [14–16]. Hong et al. [17] succeeded in reducing the IgE-binding capacity while maintaining the T cell reactivity of peanuts through pepsin hydrolysis, suggesting that pepsin-digested peanuts may become effective in peanut immunotherapy. Peptic-digested fragments of ragweed pollen retained the T cell epitopes but no B cell epitopes recognized by IgE, leading to fewer side effects than the original allergen in patients [18]. In this study, gliadin was hydrolyzed by digestive

enzyme and hydrolyzed gliadin was deamidated by an acid to further reduce its allergenicity. Gliadin was first digested with pepsin to produce pepsin-digested gliadin (peptic-GLI), and then peptic-GLI was digested with trypsin to produce peptic-tryptic gliadin (PT-GLI). Acid deamidation was carried out by dispersing PT-GLI in hydrochloric acid (HCl) to produce HCl-PT-GLI. The effects of hydrolysis and deamidation on IgE binding and CD4+ T cell reactivity were examined in vitro. The degraded gliadin with lower IgE-binding capacity but higher CD4+ T cell proliferation responses was chosen as the most effective candidate and used in a mouse model for inducing oral tolerance.

Materials and Methods

Enzymatic Hydrolysis and Acid Deamidation of Gliadin

Gliadin was extracted from soft wheat flour (Nisshin Flour Milling Inc., Japan) as described previously [19, 20]. The extracted gliadin was dialyzed against 0.1% acetic acid for 24 h and then lyophilized. Gliadin was hydrolyzed by digestive enzymes. Pepsin from porcine stomach (Wako Pure Chemical Industries Ltd., Japan) and trypsin from bovine pancreas (Wako Pure Chemical Industries Ltd.) were used as digestive enzymes. An enzyme:protein ratio of 1:100 was used for hydrolysis. Gliadin was mixed with pepsin at a concentration of 10 mg/mL in 0.01 N HCl. After incubation at 37 °C for 4 h, hydrolysis was stopped by heating at 100 °C for 15 min. Hydrolysate was dialyzed against 0.1% acetic acid for 12 h and then lyophilized to produce peptic-GLI. The above solution of peptic hydrolysate was adjusted to pH 8.0 by NaOH and incubated with trypsin at 37 °C for 4 h. Then, hydrolysis was stopped by heating at 100 °C for 15 min. Hydrolysate was dialyzed against 0.1% acetic acid for 12 h and lyophilized to produce PT-GLI.

Deamidation of PT-GLI was carried out as described previously [13, 21]. PT-GLI (20 mg/mL) was dispersed in 0.1 N HCl at 80 °C for 3 h. The reaction was stopped by neutralization with NaOH to pH 7.0. The mixture was dialyzed against 0.1% acetic acid for 12 h and then lyophilized to produce HCl-PT-GLI. Dialysis was performed to maintain the consistency of the solution of gliadin and degraded gliadins. Thus, a membrane with a small molecular weight cutoff from 100 to 500 was used to dialyze the degraded gliadins and retain as many of the small-molecular-weight peptides as possible.

Animals

Two-week-old female BALB/c mice as well as their mothers (Oriental Yeast Co. Ltd., Japan) were fed a casein diet (gluten-free diet) and housed in a temperature-controlled room (20 ± 2 °C) with a 12-h light-dark cycle. The mice that became 6 weeks old were used for all experiments.

Gliadin-Induced Wheat Allergy Model

For the evaluation of IgE-binding capacity and CD4+ T cell proliferation of degraded gliadins in vitro, the gliadin-induced wheat allergy model was used as described previously with some

modifications [19]. Intraperitoneal injections of mice (6 weeks old) with 50 µg of gliadin absorbed onto alum were performed once every 10 days for four times as sensitized mice. The mice received the alum alone as unsensitized mice (Fig. 1a). Sera were collected on day 37 after the initial injection, and the IgE-binding capacity of native and degraded gliadins was evaluated by enzyme-linked immunosorbent assay (ELISA). On day 40, sensitized mice were orally challenged with 1 mg of gliadin in 0.2 mL of 1 mM acetic acid as the gliadin group. Unsensitized mice were administered vehicle alone as the control group. All mice were forced to run on a treadmill (Muromachi Kikai Co. Ltd., Japan) at 15 m/min for 30 min. Mice were sacrificed 12 h after challenge to isolate CD4⁺ T cells from the spleen and mesenteric lymph nodes (MLNs). The CD4⁺ T cell proliferation responses activated by native or degraded gliadins were measured by the ³H-thymidine incorporation assay.

Oral Tolerance Model

Mice (6 weeks old) were orally administered 10 mg of gliadin or peptic-GLI in 0.2 mL of 5 mM acetic acid via an intragastric feeding needle every other day for 3 weeks to give a gliadin group and a peptic-GLI group, respectively. Mice in the control group were administered 0.2 mL of 5 mM acetic acid. Three weeks after the oral treatment, all mice were intraperitoneally immunized with 50 µg of gliadin absorbed onto alum every 10 days twice. All mice were orally challenged with 20 mg of gliadin in 0.2 mL of 10 mM acetic acid on day 39 (Fig. 2a). Rectal temperatures were measured before and after the oral challenge (15 min) with a digital microprobe thermometer (Physitemp Instruments, USA). The levels of gliadin-specific IgE or IgG1 in mouse serum on day 21 (after oral treatment), day 39 (after injection), and day 40 (after challenge) were evaluated by ELISA. Mice were sacrificed on day 40. CD4⁺ T cells from the spleen and MLNs were cultured with or without gliadin to measure cytokine production and cell proliferation. Jejunum was also collected to evaluate the immunological responses in the small intestinal tissue.

Analysis of the IgE-Binding Capacity of Native and Degraded Gliadins

The IgE-binding capacity was evaluated by ELISA as described previously with some modifications [22, 23]. Gliadin and degraded gliadins in 50% ethanol at 1 mg/mL were slowly diluted in 50 mM carbonate buffer (pH 9.6) to 10 µg/mL. Microtiter plates were pre-coated with 100 µL of the respective diluted solutions and incubated overnight at 4 °C. Nonspecific binding was blocked with 100 µL of 3% BSA/PBST at 25 °C for 2 h. Then, 50 µL of each serum sample diluted 20 times with 1% BSA/PBST was added to the wells at 25 °C for 2 h. A 50-µL aliquot of biotin-labeled anti-mouse IgE (Biolegend, Japan) diluted 2,000 times with 1% BSA/PBST was added to the wells at 25 °C for 1 h. Then, 50 µL of streptavidin-alkaline phosphatase (BD Pharmingen, Japan) diluted 5,000 times with 1% BSA/PBST was added for 30 min. The wells were washed five times with PBST at every step. As a substrate for alkaline phosphatase, 50 µL of disodium 4-nitrophenyl phosphate hexahydrate (Tokyo Chemical Industry Co. Ltd., Japan) at 1 mg/mL in diethanolamine buffer was added to the wells for 1 h at 37 °C, and color development was measured at 405/490 nm with a microplate reader model 680 (Bio-Rad, Japan).

Assessment of Anti-Gliadin-Specific IgE and IgG1

To measure anti-gliadin-specific IgE and IgG1, microtiter plates were precoated with gliadin and blocked as described above.

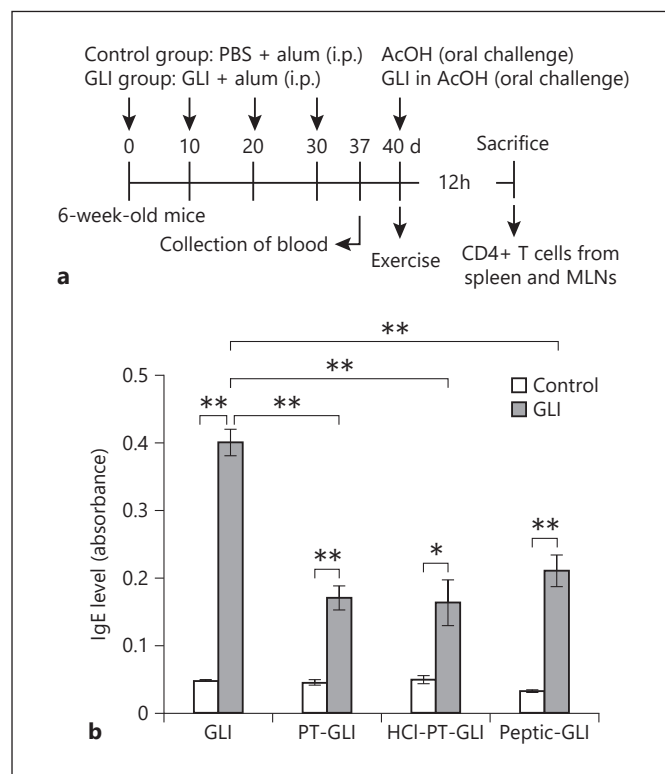


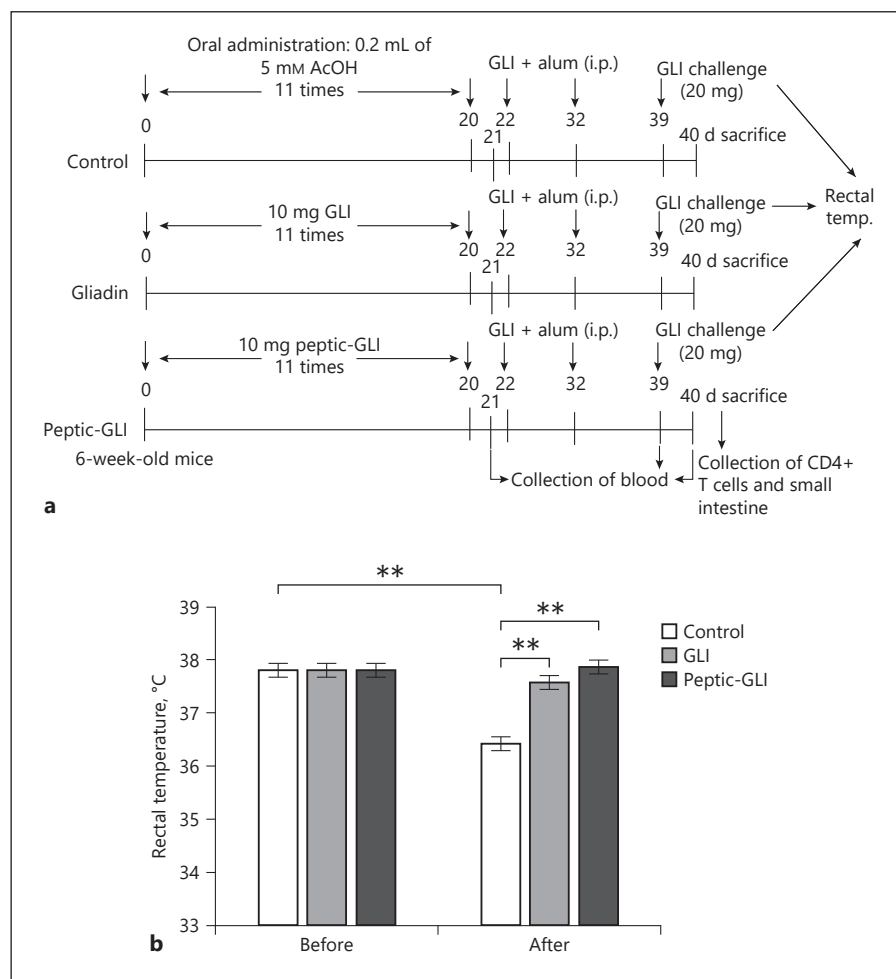
Fig. 1. Experimental protocols for the gliadin-induced wheat allergy model (**a**) and IgE-binding capacity of degraded gliadins (**b**) in sera from unsensitized (control, white bars) or sensitized (gliadin, gray bars) mice. Mice were intraperitoneally injected with gliadin once every 10 days for four times as sensitized mice. Unsensitized mice were treated with vehicle alone. Serum was collected on day 37. The data are representative of two independent experiments and are presented as mean $\pm$ standard error of the mean ($n = 3$). * $p < 0.05$, ** $p < 0.01$. AcOH, acetic acid; GLI, gliadin; HCl, hydrochloric acid; MLNs, mesenteric lymph nodes; peptic-GLI, pepsin-digested gliadin; PT-GLI, peptic-tryptic gliadin.

Serum samples (diluted 90 times for IgE and 5,000 times for IgG1) were added to the wells at 25 °C for 2 h. A 50-µL aliquot of an alkaline phosphatase-conjugated rabbit anti-mouse IgG1 monoclonal antibody (Zymed, USA) diluted 1,000 times with 1% BSA/PBST was added to the wells at 25 °C for 1 h before adding 4-nitrophenyl phosphate hexahydrate. A biotin-labeled anti-mouse IgE antibody and streptavidin-alkaline phosphatase were added as described above. Absorbance at 405/490 nm was measured.

Isolation and Treatment of CD4⁺ T Cells from Spleen and MLNs

Mice were sacrificed via cervical dislocation. The spleen was removed and rinsed in Roswell Park Memorial Institute (RPMI) medium. Then, the spleen was disrupted with the plunger of a syringe and the connective tissue was filtered. Cells from the MLNs were obtained by treatment of the MLNs with collagenase (Wako Pure Chemical Industries Ltd.) and DNase I (Sigma Aldrich, Germany) in RPMI medium with magnetic stirring to produce cell

Fig. 2. Experimental protocols for the oral tolerance model (a) and the effect of the oral challenge with gliadin on rectal temperature (b). Mice were orally administered 10 mg of gliadin or peptic-GLI for 11 times. Control mice received vehicle alone. All mice were intraperitoneally immunized and challenged with gliadin. Rectal temperature was measured before and after challenge (at 15 min). The data are representative of two independent experiments and are presented as mean $\pm$ standard error of the mean ($n = 5$). $**p < 0.01$. AcOH, acetic acid; GLI, gliadin; peptic-GLI, pepsin-digested gliadin.



suspensions that were filtered through a cell strainer. CD4⁺ T cells from the spleen and MLNs were sorted using the magnetic activated cell sorting separation method (Miltenyi Biotec, Germany). Separated CD4⁺ T cells (4×10^5 cells/well) and antigen-presenting cells (APCs) (8×10^5 cells/well) were seeded in 96-well microtiter plates in the absence or presence of gliadin or degraded gliadins at 37°C for 3 days. Gliadin or degraded gliadins (5 mg/mL) were dissolved in 10% DMSO/PBS buffer. The above solution was diluted ten times with RPMI medium when the corresponding cell culture was performed. A solution without gliadin or degraded gliadins was used as a control. APCs from the spleen of BALB/c mice were incubated with mitomycin C for 30 min at 37°C. After 3 days, the supernatant from cultured CD4⁺ T cells was transferred to new plates and stored at -20°C until further analysis. The cultured plates containing cells and supernatant were used for T cell proliferation assay.

Measurement of Cytokines from Cell Culture

Cytokines in the culture supernatants were assayed by ELISA. Interleukin-4 (IL-4) and IL-5 were quantified with corresponding purified rat anti-mouse antibodies and biotin-labeled monoclonal rat anti-mouse antibodies as previously described

[24]. All of the above antibodies were purchased from BD Pharmingen. Absorbance was measured at wavelengths of 405/490 nm.

T Cell Proliferation Assay by Using ³H-Thymidine Incorporation

Isolated CD4⁺ T cells (4×10^5 cells) were incubated with 8×10^5 APCs in the absence or presence of gliadin or degraded gliadins in 96-well microtiter plates at 37°C for 3 days as cell culture. After 3 days, cells were labeled with ³H-thymidine (1 mCi/mL; Moravsek Inc., USA) at 0.5 μ Ci/well (18.5 kBq) for 24 h. After labeling, cells were harvested onto 96-format glass-fiber filter mats using a micro96 semi-automatic cell harvester (Skatron Instruments, USA), and then the filter mats were dried. MeltiLex solid scintillator (PerkinElmer Co. Ltd., Japan) was melted into the filter mats, and the incorporation of ³H was counted in a MicroBeta counter, with the results expressed as counts/min.

Quantitative RT-PCR Analysis

Total RNA from the jejunum (3 cm) was extracted with an SV total RNA isolation system (Promega, Japan). cDNA was prepared from RNA using an iScript cDNA synthesis kit (Bio-Rad). Quan-

titative RT-PCR was performed using SYBR® Premix Ex Taq™ II (Takara Bio Inc., Japan). Expression was normalized to 18S ribosomal RNA. The following amplification reaction was employed: 95 °C for 30 s, 44 cycles of 5 s at 95 °C, and 30 s at 60 °C. The sequences of the primers were as follows: ribosomal RNA 18S, forward 5'-CTT AGA GGG ACA AGT G-3' and reverse 5'-ACG CTG AGC CAG TCA G-3'; mouse mast cell protease-1 (mMCP-1), forward 5'-GAG GAC AGA TGT GGT GGG TTT-3' and reverse 5'-AGG AGT CAA CTC AGC TTT CTC TT-3'.

Evaluation of the Total Histamine Level

Three centimeters of jejunum tissue was collected in 1 mL of 0.9% NaCl solution (saline). The tissue was homogenized in saline after being weighed. Then, 400 µL of 12% perchloric acid was added to the above solution, and the tissue sample was homogenized again and subsequently centrifuged for 10 min at 5,000 rpm (4 °C). The supernatant was collected and stored at -20 °C until further analysis. The concentration of histamine in supernatants was measured as described previously [11, 25]. The histamine level is presented as (concentration × 1.4) / weight.

Statistical Analysis

Data are presented as mean ± standard error of the mean. Differences between two groups were evaluated using Student's *t* test. Comparisons of more than two groups were performed by one-way ANOVA followed by Tukey's HSD test. A two-tailed *p* value <0.05 was defined as a significant difference.

Results

IgE-Binding Capacity of Native and Degraded Gliadins

Figure 1a displays the experimental protocols for the gliadin-induced wheat allergy model. IgE binding to degraded gliadins was measured using sera from unsensitized and sensitized mice. Sensitized mice had a notable increase in the IgE-binding capacity of both gliadin and degraded gliadins (peptic-GLI, PT-GLI, and HCl-PT-GLI) compared to unsensitized mice. The IgE-binding capacity of peptic-GLI, PT-GLI, and HCl-PT-GLI showed a significant decrease compared to that of gliadin in the sera of sensitized mice (Fig. 1b).

CD4+ T Cell Activation against Native or Degraded Gliadins

CD4+ T cells from the spleen and MLNs of gliadin-sensitized and -challenged mice (gliadin group) or unsensitized mice (control group) were stimulated by gliadin and degraded gliadins at 0.5 mg/mL. The level of proliferation of CD4+ T cells was significantly higher in the gliadin group than in the control group regardless of the activation conditions. The gliadin group showed significantly higher proliferation of CD4+ T

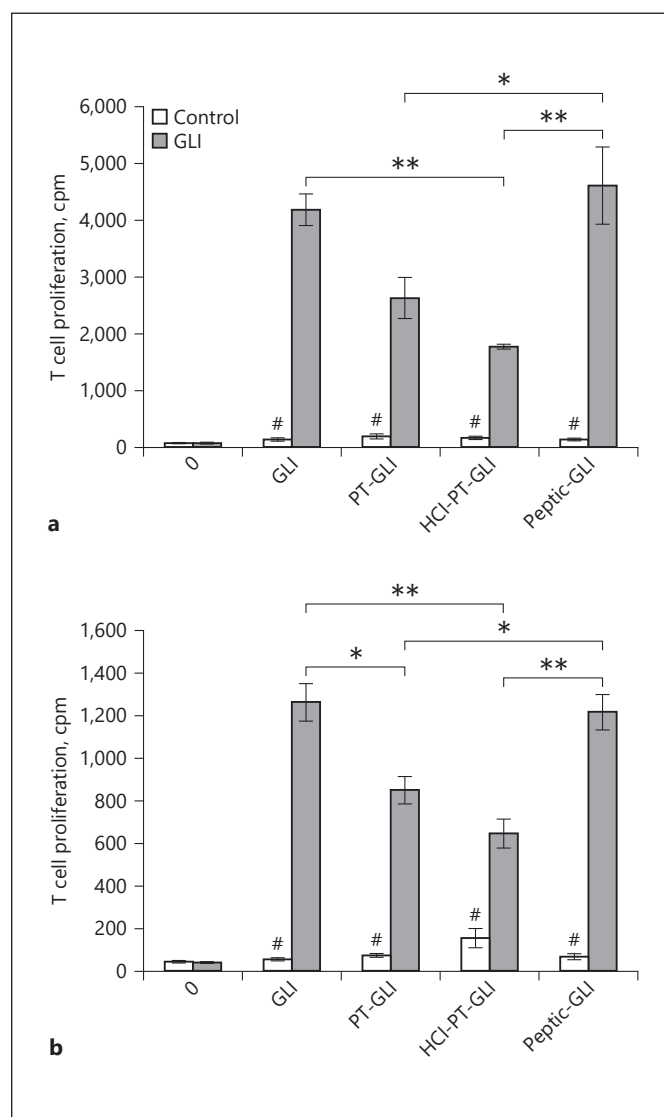


Fig. 3. Proliferation of spleen (a) and MLN (b) CD4+ T cells stimulated by native or degraded gliadins in the gliadin-induced wheat allergy model. The data are representative of two independent experiments and are presented as mean ± standard error of the mean (*n* = 3). * *p* < 0.05, ** *p* < 0.01, # *p* < 0.01 compared to the gliadin group under the corresponding activation conditions. cpm, counts/min; GLI, gliadin; HCl, hydrochloric acid; MLN, mesenteric lymph node; peptic-GLI, pepsin-digested gliadin; PT-GLI, peptic-tryptic gliadin.

cells in response to activation with gliadin or peptic-GLI compared to activation with PT-GLI or HCl-PT-GLI. There was no difference between gliadin and peptic-GLI stimulation in regard to T cell proliferation in the gliadin group (Fig. 3).

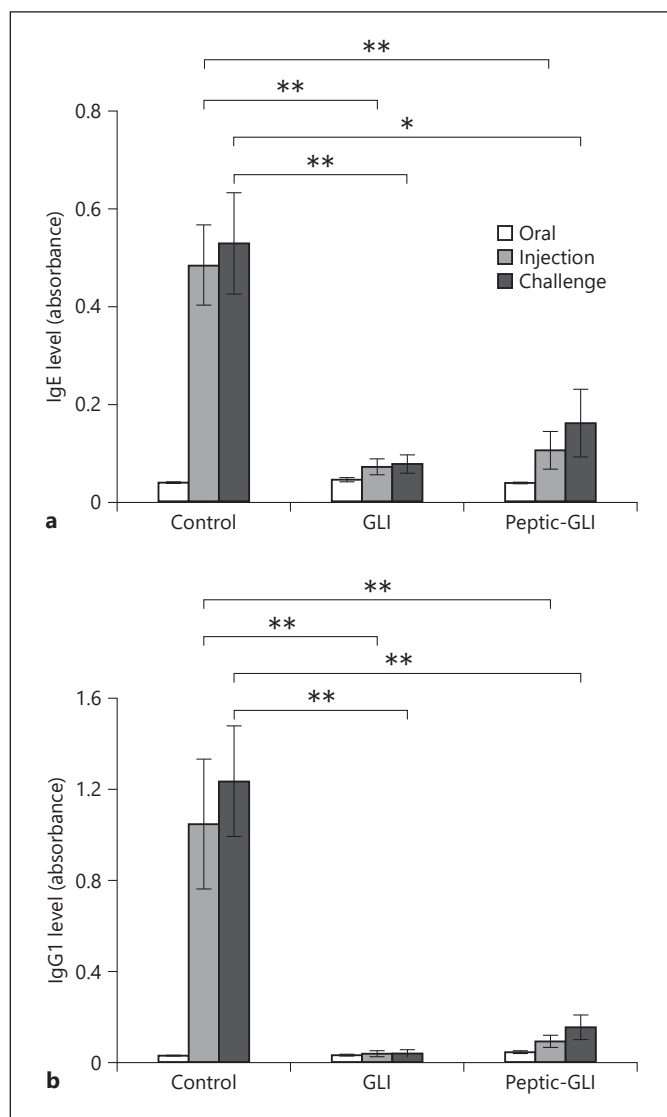


Fig. 4. IgE (a) and IgG1 (b) levels specific to gliadin in the sera of mice from the oral tolerance model. Mice sera were collected after the oral administration on day 21 (Oral), after the intraperitoneal injection on day 39 (Injection), and after the oral challenge on day 40 (Challenge). ELISA was used for the detection of IgE and IgG1. The data are representative of two independent experiments and are presented as mean $\pm$ standard error of the mean ($n = 5$). * $p < 0.05$, ** $p < 0.01$. ELISA, enzyme-linked immunosorbent assay; GLI, gliadin; peptic-GLI, pepsin-digested gliadin.

Influence of Oral Administration of Gliadin or Peptic-GLI on Rectal Temperature after Challenge in the Oral Tolerance Model

Peptic-GLI with a low IgE-binding capacity but high T cell reactivity seems to be a better candidate for inducing tolerance compared with the other degraded gliadins. To

examine the effect of peptic-GLI on the development of the clinical symptoms of anaphylaxis, mice were orally administered gliadin or peptic-GLI before gliadin sensitization and challenge in the oral tolerance model (Fig. 2a). The rectal temperature of the control group significantly decreased after challenge with gliadin for 15 min, while the rectal temperature of the gliadin group and peptic-GLI group did not show any decrease. The rectal temperature of the control group was also remarkably lower than that of the other two groups after challenge (Fig. 2b).

Anti-Gliadin-Specific IgE and IgG1 Change in the Oral Tolerance Model

Gliadin-specific IgE (Fig. 4a) and IgG1 (Fig. 4b) were measured after oral administration, injection, and challenge, respectively. Oral administration did not lead to any change in the IgE and IgG1 levels among groups (white bars). The upregulation of gliadin-specific IgE and IgG1 caused by immunization with gliadin (control group) was suppressed when gliadin or peptic-GLI was orally administered before gliadin sensitization (light gray bars). Similar results were observed after challenge (dark gray bars).

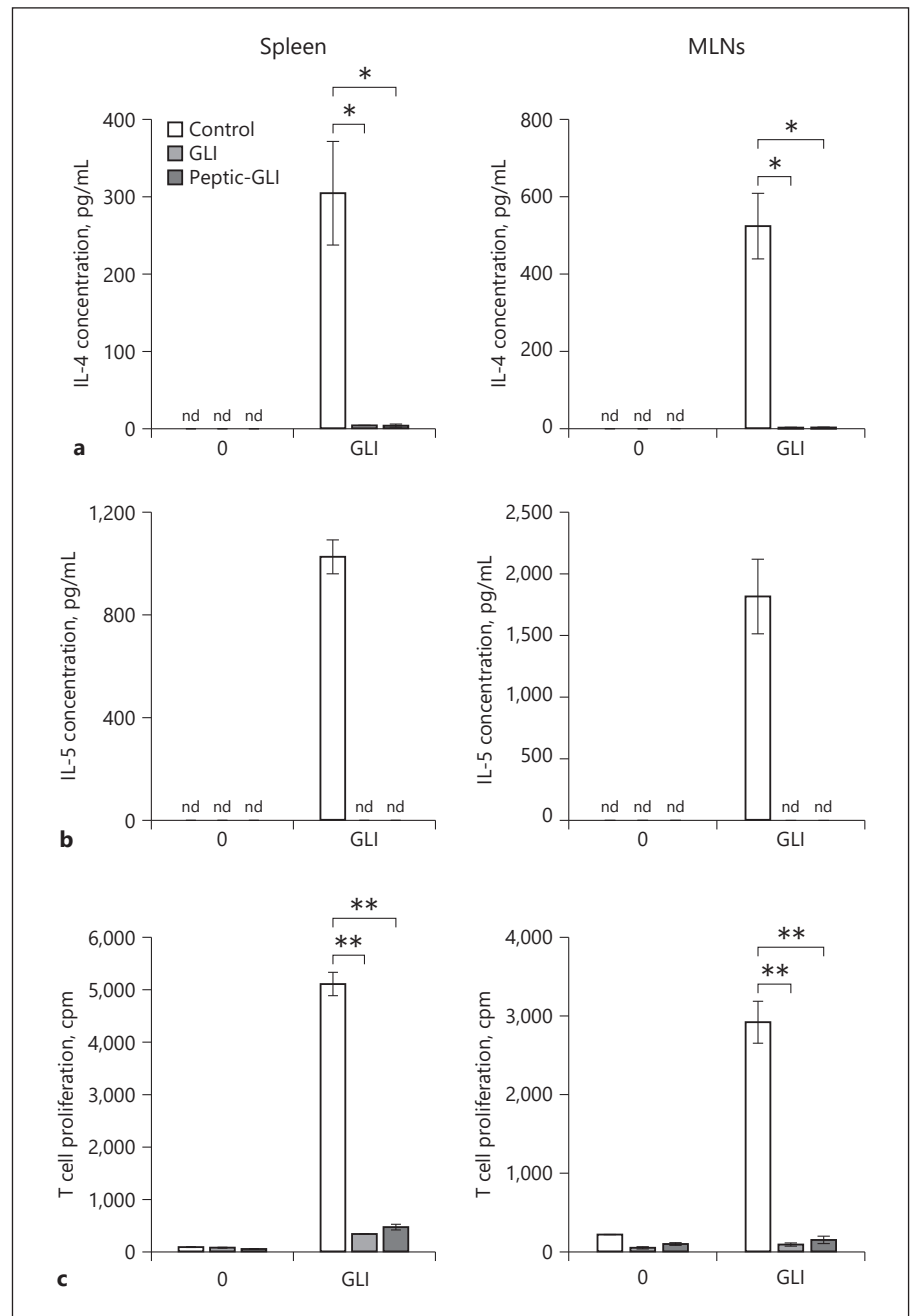
Type 2 T Helper Responses and Proliferation of CD4⁺ T Cells from Spleen and MLNs in the Oral Tolerance Model

In order to examine the type 2 T helper (Th2) responses after challenge, IL-4 and IL-5 were measured as representative Th2-type cytokines. Spleen and MLN CD4⁺ T cells from mice in the control group secreted substantial levels of IL-4 (Fig. 5a) and IL-5 (Fig. 5b) in response to gliadin (0.5 mg/mL) stimulation. However, the secretion of IL-4 and IL-5 was significantly suppressed by oral administration of gliadin or peptic-GLI before gliadin sensitization. Figure 5c shows that the proliferation of spleen and MLN CD4⁺ T cells stimulated with gliadin was remarkably increased in the control group compared to the other two groups.

Immunological Responses of Small Intestinal Tissue in the Oral Tolerance Model

After gliadin challenge, significantly higher expression of mMCP-1 in the jejunum was observed in the control group compared to the gliadin and peptic-GLI groups (Fig. 6a). As shown in Figure 6b, total histamine in the jejunum was examined. The histamine production in the control group was significantly higher than that in the other two groups, whereas no significant difference was observed between the gliadin and peptic-GLI groups.

Fig. 5. Effects of the oral administration of gliadin or peptic-GLI on IL-4 (**a**) and IL-5 (**b**) production as well as T cell proliferation (**c**) of spleen and MLN CD4+ T cells activated by gliadin. After the challenge, CD4+ T cells from spleen and MLNs were cultured with 0.5 mg/mL gliadin and the supernatants were collected after 3 days. The IL-4 and IL-5 levels in supernatants were measured by ELISA. After 3 days of cell culture, T cell proliferation was measured by the ^3H -thymidine incorporation assay. The data are representative of two independent experiments and are presented as mean $\pm$ standard error of the mean ($n = 5$). * $p < 0.05$, ** $p < 0.01$. cpm, counts/min; ELISA, enzyme-linked immunosorbent assay; GLI, gliadin; MLN(s), mesenteric lymph node(s); nd, not detected; peptic-GLI, pepsin-digested gliadin.



Discussion

Wheat has been recognized to be a common cause of food-induced anaphylaxis around the world. It is difficult for patients to avoid wheat ingestion due to its wide use in various types of foods consumed in our daily life. Therefore, wheat OIT is considered a promising treatment for anaphylactic patients in whom wheat avoidance is not suf-

ficient to avert anaphylaxis [26]. However, adverse reactions are commonly caused by intact proteins during OIT and can occur at any phase of the protocol. Watanabe et al. [27, 28] prepared hypoallergenic flour by hydrolyzing wheat flour with cellulase and actinase. Consumption of hypoallergenic flour by rats after sensitization with gluten could suppress the allergic reactions caused by the subsequent elicitation with gluten and probably induce oral tol-

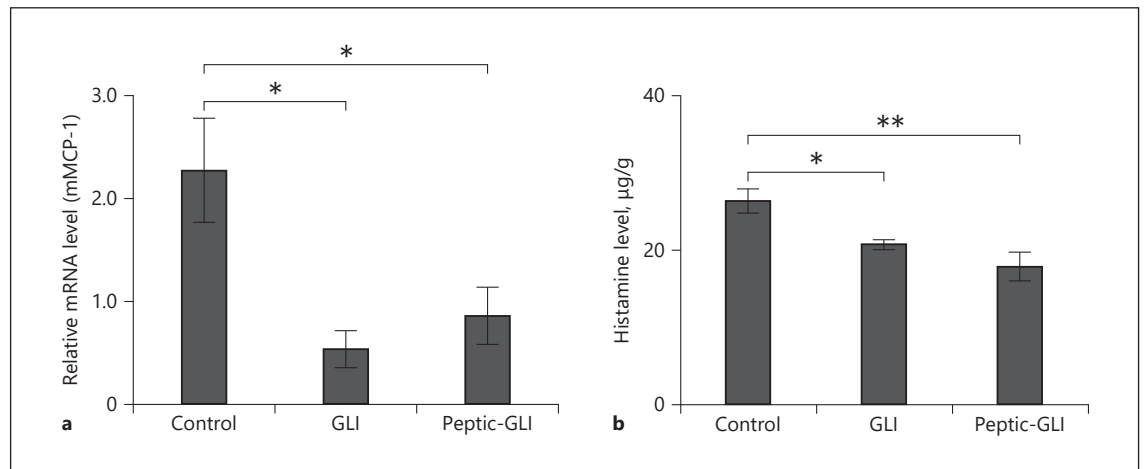


Fig. 6. Influence of the oral administration of gliadin or peptic-GLI on mMCP-1 expression (a) and the histamine levels (b) in small intestinal tissue. Quantitative RT-PCR was applied to measure the relative expression of mMCP-1. Expression was normalized to 18S ribosomal RNA. The total histamine level in the jejunum was determined by fluorometric assay. The data are representative of two independent experiments and are presented as mean $\pm$ standard error of the mean ($n = 5$). * $p < 0.05$, ** $p < 0.01$. GLI, gliadin; mMCP-1, mouse mast cell protease-1; peptic-GLI, pepsin-digested gliadin.

erance [28]. In the present study, gliadin was hydrolyzed by digestive enzymes (pepsin and trypsin) and deamidated by HCl to obtain hypoallergenic peptides.

The majority of wheat hypersensitivities are considered to be IgE-mediated allergy [29]. The cross-linking of IgE receptors by subsequent allergen exposure on the surface of mast cells and basophils causes immediate reactions, provoking immediate symptoms of allergy [30, 31]. The reactivity of IgE to peptic-GLI, PT-GLI, and HCl-PT-GLI was remarkably decreased compared with that to native gliadin (Fig. 1b). Therefore, the cross-linking of IgE bounded to mast cells might be weakened by peptic-GLI, PT-GLI, and HCl-PT-GLI. Peptide immunotherapy involves the use of peptides containing T cell epitopes [32, 33]. As for T cell activation (Fig. 3) in vitro, stimulation of CD4⁺ T cells with PT-GLI or HCl-PT-GLI showed a significant decrease in proliferation compared to stimulation with native gliadin. However, there was no difference in regard to the proliferation of CD4⁺ T cells between peptic-GLI and native gliadin activations. These results suggest that the epitopes of gliadin recognized by T cells might be partially degraded by hydrolysis and deamidation in the case of PT-GLI and HCl-PT-GLI, while T cell epitopes might be maintained after pepsin hydrolysis. Therefore, the characteristics of peptic-GLI in regard to the activation of mast cells and T cells may increase its potential for inducing oral tolerance.

It has been reported that mice receiving gliadin or a gluten-containing diet before gliadin sensitization could suppress both cellular and humoral immune responses to gliadin, and it has been demonstrated that gliadin is an effective oral tolerogen rather than an oral immunogen [34]. In order to investigate whether peptic-GLI has a similar effect on tolerance induction, gliadin and peptic-GLI were applied in such an oral tolerance model. Allergen-specific IgE antibodies play a critical role in wheat allergic responses [8, 35]. Gliadin-specific IgE levels after sensitization or challenge were significantly decreased by oral administration of gliadin or peptic-GLI (Fig. 4a). Th2 cells are excellent helpers for B cell antibody secretion (IgE and IgG1) [36]. The production of gliadin-specific IgG1 antibodies was also inhibited by oral administration of gliadin or peptic-GLI (Fig. 4b). In late and chronic reactions of IgE-mediated hypersensitivity, the presentation of allergens to T cells causes activation of T cells, leading to the release of Th2-type cytokines such as IL-4, IL-5, and IL-13 [31]. In this study, the production of IL-4 and IL-5 in CD4⁺ T cells from spleen and MLNs stimulated by gliadin was significantly suppressed by oral administration of gliadin or peptic-GLI (Fig. 5a, b). Oral treatment with gliadin or peptic-GLI could decrease CD4⁺ T cell responsiveness (Fig. 5c). These findings demonstrate that oral administration with peptic-GLI is as effective

as gliadin for suppressing gliadin-specific IgE and Th2-type cytokine responses.

As mentioned above, the immediate reactions caused by the degranulation of mast cells lead to the release of biological mediators (histamine) and provoke immediate symptoms [37]. mMCP-1 has been used as a mast cell activation marker in food-induced anaphylaxis [38]. The measurement of histamine level and mMCP-1 expression was to reflect the situation of mast cell degranulation in the small intestine. In the oral tolerance model, we found that oral administration of gliadin or peptic-GLI inhibited the development of the anaphylactic symptoms induced by the gliadin challenge (Fig. 2b). The release of histamine and the expression of mMCP-1 in the small intestinal tissue were dramatically reduced in mice orally administered gliadin or peptic-GLI compared to the control group (Fig. 6). It has been reported that the expansion of intestinal mast cells elicited by mechanical skin injury promotes oral anaphylaxis [39]. Therefore, our results suggest that the degranulation of intestinal mast cells could be weakened by oral administration of gliadin or peptic-GLI, leading to the suppression of anaphylactic responses.

In conclusion, we investigated the allergenicity and potential for tolerance induction of degraded gliadins. Among the degraded gliadins, peptic-GLI with high T cell reactivity but low IgE-binding capacity was used in the oral tolerance model. Inhibition of gliadin-specific IgE, Th2-type cytokines, and allergic symptoms was obtained by oral administration of peptic-GLI, which was also observed in gliadin administration. Therefore, peptic-GLI instead of gliadin may become a good candidate for immunotherapy of wheat allergy with less side effects. Fur-

ther studies are required to investigate the effect of peptic-GLI on tolerance induction in a mouse model that mimics the clinical OIT protocol of patients.

Statement of Ethics

All procedures were conducted according to the Animal Experiment Guidelines of the Faculty of Agriculture and the Graduate School of Agricultural and Life Sciences of the University of Tokyo.

Disclosure Statement

The authors have no conflicts of interest to declare.

Funding Sources

This project was financially supported by a grant from the Tojuro Iijima Foundation for Food Science and Technology and by the Japan Society for the Promotion of Science (Grant-in-Aid for Scientific Research [S], grant number 23228003).

Author Contributions

X. Li designed the study, carried out the experiments, analyzed all the data, and wrote the manuscript. T. Miyakawa provided critical advice regarding the experiments and revised the manuscript. T. Takano provided support regarding the mouse experiments. H. Nakajima-Adachi provided critical advice regarding the experiments and revised the manuscript. M. Tanokura designed the study and revised the manuscript. S. Hachimura designed the study, interpreted the data, and revised the manuscript.

References

- 1 Keet CA, Matsui EC, Dhillon G, Lenehan P, Paterakis M, Wood RA. The natural history of wheat allergy. *Ann Allergy Asthma Immunol*. 2009 May;102(5):410–5.
- 2 Burks AW, Laubach S, Jones SM. Oral tolerance, food allergy, and immunotherapy: implications for future treatment. *J Allergy Clin Immunol*. 2008 Jun;121(6):1344–50.
- 3 Mousallem T, Burks AW. Immunology in the Clinic Review Series; focus on allergies: immunotherapy for food allergy. *Clin Exp Immunol*. 2012 Jan;167(1):26–31.
- 4 Utsunomiya T, Imai T, Ogura K, Goto M, Iikura K, Koike Y, et al. Rush oral immunotherapy for wheat induced anaphylaxis in Japan. *J Allergy Clin Immunol*. 2012 Feb;129(2):AB26.
- 5 Sato S, Utsunomiya T, Imai T, Yanagida N, Asaumi T, Ogura K, et al. Wheat oral immunotherapy for wheat-induced anaphylaxis. *J Allergy Clin Immunol*. 2015 Oct;136(4):1131–3.e7.
- 6 Pacharn P, Siripattanamongkol N, Veskitkul J, Jirapongsananuruk O, Visitsunthorn N, Vichyanond P. Successful wheat-specific oral immunotherapy in highly sensitive individuals with a novel multirush/maintenance regimen. *Asia Pac Allergy*. 2014 Jul;4(3):180–3.
- 7 Tatham AS, Shewry PR. The conformation of wheat gluten proteins. The secondary structures and thermal stabilities of α -, β -, γ - and ω -gliadins. *J Cereal Sci*. 1985 Apr;3(2):103–13.
- 8 Battais F, Mothes T, Moneret-Vautrin DA, Pineau F, Kanny G, Popineau Y, et al. Identification of IgE-binding epitopes on gliadins for patients with food allergy to wheat. *Allergy*. 2005 Jun;60(6):815–21.
- 9 Inomata N. Wheat allergy. *Curr Opin Allergy Clin Immunol*. 2009 Jun;9(3):238–43.
- 10 Kumagai H, Suda A, Sakurai H, Kumagai H, Arai S, Inomata N, et al. Improvement of digestibility, reduction in allergenicity, and induction of oral tolerance of wheat gliadin by deamidation. *Biosci Biotechnol Biochem*. 2007 Apr;71(4):977–85.

- 11 Abe R, Shimizu S, Yasuda K, Sugai M, Okada Y, Chiba K, et al. Evaluation of reduced allergenicity of deamidated gliadin in a mouse model of wheat-gliadin allergy using an antibody prepared by a peptide containing three epitopes. *J Agric Food Chem*. 2014 Apr; 62(13):2845–52.
- 12 Abe R, Matsukaze N, Yamaguchi Y, Akao M, Kumagai H, Kumagai H. Wheat gliadin deamidated by cation-exchange resins induces oral tolerance in a mouse model of wheat allergy. *J Food Bioact*. 2018 Jun;2(2):119–28.
- 13 Mimouni B, Raymond J, Merle-Desnoyers AM, Azanza JL, Ducastaing A. Combined acid deamidation and enzymic hydrolysis for improvement of the functional properties of wheat gluten. *J Cereal Sci*. 1994 Sep;20(2):153–65.
- 14 Hoyne GF, Callow MG, Kuo MC, Thomas WR. Inhibition of T-cell responses by feeding peptides containing major and cryptic epitopes: studies with the Der p I allergen. *Immunology*. 1994 Oct;83(2):190–5.
- 15 Takagi H, Hiroi T, Yang L, Tada Y, Yuki Y, Takamura K, et al. A rice-based edible vaccine expressing multiple T cell epitopes induces oral tolerance for inhibition of Th2-mediated IgE responses. *Proc Natl Acad Sci U S A*. 2005 Nov;102(48):17525–30.
- 16 Hacini-Rachinel F, Vissers YM, Doucet-Ladevéze R, Blanchard C, Demont A, Perrot M, et al. Low-allergenic hydrolyzed egg induces oral tolerance in mice. *Int Arch Allergy Immunol*. 2014;164(1):64–73.
- 17 Hong SJ, Michael JG, Fehringer A, Leung DY. Pepsin-digested peanut contains T-cell epitopes but no IgE epitopes. *J Allergy Clin Immunol*. 1999 Aug;104(2 Pt 1):473–7.
- 18 Litwin A, Pesce AJ, Fischer T, Michael M, Michael JG. Regulation of the human immune response to ragweed pollen by immunotherapy. A controlled trial comparing the effect of immunosuppressive peptic fragments of short ragweed with standard treatment. *Clin Exp Allergy*. 1991 Jul;21(4):457–65.
- 19 Tanaka M, Nagano T, Yano H, Haruma K, Kato Y. Exercise-independent wheat-induced anaphylaxis caused by ω -5 gliadin in mice. *Int Arch Allergy Immunol*. 2011;156(4):434–42.
- 20 Tanaka M, Nagano T, Yano H, Matsuda T, Ikeda TM, Haruma K, et al. Impact of ω -5 gliadin on wheat-dependent exercise-induced anaphylaxis in mice. *Biosci Biotechnol Biochem*. 2011;75(2):313–7.
- 21 Gourbeyre P, Denery-Papini S, Larré C, Gaudin JC, Brossard C, Bodinier M. Wheat gliadins modified by deamidation are more efficient than native gliadins in inducing a Th2 response in Balb/c mice experimentally sensitized to wheat allergens. *Mol Nutr Food Res*. 2012 Feb;56(2):336–44.
- 22 Adel-Patient K, Créminon C, Bernard H, Clément G, Négroni L, Frobert Y, et al. Evaluation of a high IgE-responder mouse model of allergy to bovine beta-lactoglobulin (BLG): development of sandwich immunoassays for total and allergen-specific IgE, IgG1 and IgG2a in BLG-sensitized mice. *J Immunol Methods*. 2000 Feb;235(1–2):21–32.
- 23 Bodinier M, Leroy M, Ah-Leung S, Blanc F, Tranquet O, Denery-Papini S, et al. Sensitization and elicitation of an allergic reaction to wheat gliadins in mice. *J Agric Food Chem*. 2009 Feb;57(4):1219–25.
- 24 Sato A, Hashiguchi M, Toda E, Iwasaki A, Hachimura S, Kaminogawa S. CD11b⁺ Peyer's patch dendritic cells secrete IL-6 and induce IgA secretion from naive B cells. *J Immunol*. 2003 Oct;171(7):3684–90.
- 25 Komatsu M. Studies on histamine in bronchial asthma. *Allergy*. 1978 Feb;27(2):67–74. Japanese.
- 26 Pacharn P, Vichyanond P. Immunotherapy for IgE-mediated wheat allergy. *Hum Vaccin Immunother*. 2017 Oct;13(10):2462–6.
- 27 Watanabe M, Suzuki T, Ikezawa Z, Arai S. Controlled enzymatic treatment of wheat proteins for production of hypoallergenic flour. *Biosci Biotechnol Biochem*. 1994 Feb; 58(2):388–90.
- 28 Watanabe J, Tanabe S, Watanabe M, Kasai T, Sonoyama K. Consumption of hypoallergenic flour prevents gluten-induced airway inflammation in Brown Norway rats. *Biosci Biotechnol Biochem*. 2001 Aug;65(8):1729–35.
- 29 Palosuo K. Update on wheat hypersensitivity. *Curr Opin Allergy Clin Immunol*. 2003 Jun; 3(3):205–9.
- 30 Wedemeyer J, Tsai M, Galli SJ. Roles of mast cells and basophils in innate and acquired immunity. *Curr Opin Immunol*. 2000 Dec; 12(6):624–31.
- 31 Valenta R, Ball T, Focke M, Linhart B, Mothes N, Niederberger V, et al. Immunotherapy of allergic disease. *Adv Immunol*. 2004;82:105–53.
- 32 Haselden BM, Kay AB, Larché M. Peptide-mediated immune responses in specific immunotherapy. *Int Arch Allergy Immunol*. 2000 Aug;122(4):229–37.
- 33 Verhoef A, Alexander C, Kay AB, Larché M. T cell epitope immunotherapy induces a CD4⁺ T cell population with regulatory activity. *PLoS Med*. 2005 Mar;2(3):e78.
- 34 Troncone R, Ferguson A. Gliadin presented via the gut induces oral tolerance in mice. *Clin Exp Immunol*. 1988 May;72(2):284–7.
- 35 Matsuo H, Dahlström J, Tanaka A, Kohno K, Takahashi H, Furumura M, et al. Sensitivity and specificity of recombinant omega-5 gliadin-specific IgE measurement for the diagnosis of wheat-dependent exercise-induced anaphylaxis. *Allergy*. 2008 Feb;63(2):233–6.
- 36 Romagnani S. Type 1 T helper and type 2 T helper cells: functions, regulation and role in protection and disease. *Int J Clin Lab Res*. 1991;21(2):152–8.
- 37 Kinet JP. The high-affinity IgE receptor (Fc epsilon RI): from physiology to pathology. *Annu Rev Immunol*. 1999;17(1):931–72.
- 38 Ahrens R, Osterfeld H, Wu D, Chen CY, Arumugam M, Groschwitz K, et al. Intestinal mast cell levels control severity of oral antigen-induced anaphylaxis in mice. *Am J Pathol*. 2012 Apr;180(4):1535–46.
- 39 Leyva-Castillo JM, Galand C, Kam C, Burton O, Gurish M, Musser MA, et al. Mechanical skin injury promotes food anaphylaxis by driving intestinal mast cell expansion. *Immunity*. 2019 May;50(5):1262–1275.e4.