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Artificial sweeteners and mixture of food additives cause to break oral tolerance and induce food allergy in murine oral tolerance model for food allergy

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Inhibition of oral tolerance by sweeteners and mixture of food additives

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Summary

Background

Processed foods are part of daily life. Almost all processed foods contain food additives such as sweeteners, preservatives and colourants. From childhood, it is difficult to avoid consuming food additives. It is thought that oral tolerance for food antigens is acquired during early life. If tolerance fails, adverse immune responses to food proteins may occur.

Objective

We hypothesised that food additives prevent acquisition of oral tolerance and aimed to verify the safety of food additives.

Methods

We induced experimental oral tolerance in mice for ovalbumin (OVA), a food antigen, by previous oral treatment with OVA before sensitisation with OVA injections. Food additives were administered at the induction of oral tolerance, and food allergy was induced by repeated administration of OVA. Symptoms of food allergy were defined as a change in body

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temperature and allergic diarrhoea.

Results

Saccharin sodium and a mixture of food additives inhibited acquisition of oral tolerance.

Hypothermia and allergic diarrhoea with elevation of OVA-specific IgE were induced in the murine model of oral tolerance. Analyses of antigen presenting cells in mesenteric lymph nodes showed that food additives affected their manner of migration. Additionally, food additives decreased the proportion of CD25^{hi} regulatory T cells among CD4⁺ T cells in the mesenteric lymph nodes.

Conclusions and Clinical Relevance

A large amount of food additives may prevent acquisition of oral tolerance. Intake of food additives in early life may increase the risk of food allergies.

Introduction

Eating a meal is a basic vital activity. However, for some people, unfavourable outcomes are associated with some foods. Adverse reactions to foods include toxic and nontoxic responses. A toxic response is food poisoning, whereas a nontoxic response is an immune-mediated food allergy [1]. In those with food allergies, oral intake of causal food proteins may evoke reactions such as eczema, itching, diarrhoea, wheezing and hypothermia [2]. Generally, healthy people acquire an immune tolerance for the oral intake of food

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proteins, but allergic responses to those proteins may occur in patients with food allergies.

Food allergies often show spontaneous remission with growth; however, in some cases, they do not resolve with age and are suffered by adults. Many genetic and environmental factors have been associated with the development and prolongation of food allergies [3]. For example, single nucleotide polymorphism has been related to food allergies [4], as have environmental factors such as the duration of breastfeeding [5], nutrient intake [6] and sun exposure [7]. However, the range of factors associated with the development of food allergies has not been clarified.

Food additives may be contained in food that a child is exposed to in early life. Currently, food additives are regarded as essential in modern food. Sweeteners, preservatives, colourings and flavourings are added to foods during processing, and almost all processed foods contain some food additives. Most countries or regions have established safety criteria for food additives. However, some reports have indicated that food additives are associated with increased risks for immune diseases such as cancer (nitrates and nitrites) [8] and allergy to artificial sweeteners [9]. Japanese food additives are categorised as specified or conventional food additives. Specified additives are artificial or natural compounds containing some sweeteners, preservatives or colourings designated by the Japanese Ministry of Health. Conventional food additives are natural products commonly found in foods such as natural flavourings, natural pigments, fruit juices and compounds extracted from foods (e.g.

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caffeine from coffee beans). Japanese criteria for the use of food additives were determined based on the Acceptable Daily Intake (ADI). ADI is calculated from the results of acute and chronic toxicity tests using animals, and long-term observation of adverse effects by intake. However, as people have diverse lifestyles and meal choices, these safety tests cannot cover all dietary situations.

In our previous reported model, allergic diarrhoea and a decrease in rectum temperature were evoked by elicitation of ovalbumin (OVA) [10]. In the model, experimental oral tolerance was induced by oral treatment with OVA before a sensitisation with the antigen. In this study, we evaluated risks for food additives using the murine model of oral tolerance for food allergy. Food additives were administered simultaneously at pre-treatment with OVA inducing oral tolerance. We showed that saccharin sodium and a mixture of some food additives broke down the acquisition of oral tolerance. High doses of food additives also changed the migration manner of antigen presenting cells (APCs) followed by induction of regulatory T cells.

Materials and Methods

Animals

Female BALB/c mice (SLC, Inc., Hamamatsu, Japan) were used for the allergy model. Mice were housed in an air-conditioned animal room with a temperature of $23 \pm 1^{\circ}\text{C}$ and a humidity of $50 \pm 10\%$. Experiments were undertaken following the guidelines of the Animal Experiment Committee of Gifu Pharmaceutical University.

Food allergy and oral tolerance models using mice

The female BALB/c mice were sensitised intraperitoneally with OVA (Sigma-Aldrich Co., St. Louis, MO) at a dose of $1\text{ }\mu\text{g}$, or phosphate buffered saline (PBS) mixed with Inject Alum (Thermo Scientific, Rockford, IL) every other week. All mice were administered OVA at a dose of 30 mg/mouse dissolved in PBS orally three times per week to induce food allergy 1 week after the last sensitisation with OVA injection. For induction of oral tolerance, mice were treated orally with OVA at a dose of 1 mg/mouse for 5 successive days, 1 week before the first sensitisation with OVA injection.

Evaluation of food allergy

Severity of food allergy was estimated using previously reported methods [10]. Briefly, food allergy was evaluated by a decrease in rectum temperature and allergic

diarrhoea induced by OVA intake. The decrease in body temperature was measured as a change at 1 h from each oral administration of OVA at doses of 30 mg/mouse, using a thermometer (KN-91, Natsume, Tokyo, Japan). Allergic diarrhoea was evaluated by observation of the faeces for 1 h after OVA administration. Severity of diarrhoea was assessed by a score from 0 to 3: 0 = solid state; 1 = semi-solid form (solid containing a little liquid); 2 = slurry and 3 = watery state.

Administration of additives

Food additives were administered at induction of oral tolerance. Saccharin sodium, acesulfame potassium, aspartame, silicon dioxide, xylitol and meso-erythritol were purchased from Sigma-Aldrich. Annatto pigment, tartrazine, benzoic acid, and sodium benzoate were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), or Kishida Chemical Co., Ltd. (Osaka, Japan). Sucrose and glucose were purchased from Nacalai Tesque (Kyoto, Japan). In the food allergy model, food additives were dissolved or suspended at depicted doses in PBS. Mixture A contained 3 mg acesulfame potassium, 1 mg sodium benzoate and 1.5 mg tartrazine. Mixture B contained 30 mg acesulfame potassium, 10 mg sodium benzoate and 15 mg tartrazine.

ELISA measurements

Blood OVA-specific IgE and IgG₁ were measured using ELISA, based on modifications of previously described reports [11, 12]. OVA-specific IgE or IgG₁ was caught on an immune plate (Nunc, Roskilde, Denmark) coated with anti-mouse IgE antibody (MAC419, clone LO-ME-3; Serotec Ltd., Oxford, UK) or anti-mouse IgG₁ antibody (STAR81, Serotec Ltd.). We detected enzyme reaction after incubation with biotinylated antigen and then with horseradish peroxidase-conjugated streptavidin (Dako, Glostrup, Denmark). After the enzyme reaction, absorbance at 492 nm (reference 690 nm) was measured.

Mucosal mast cell protease-1 (MMCP-1) as a marker of degranulation of mucosal mast cells was measured in blood collected 1 h after the last OVA administration to use Mouse mMCP-1 ELISA Ready-Set-Go (eBioscience Inc., San Diego, CA) according to a manual.

Flow cytometer analysis

The antibodies used in this study were: PE-Cy7-conjugated anti- CD11c (clone HL3, BD Bioscience, San Jose, CA); APC-Cy7-conjugated anti-CD11b (clone M1/70, BD Bioscience); APC-conjugated anti-CD103 (Clone 2E7, eBioscience Inc.) and PerCP-Cy5.5-conjugated anti-CX3CR1 (clone SA011F11, Bio Legend Inc., San Diego, CA);

PE-conjugated anti-IA/IE (clone M5/114.15.2, BD Bioscience); PE-Cy7-conjugated anti-CD3 (clone 145-3C11, eBioscience Inc.); APC-Cy7-conjugated anti-CD4 (clone GK1.5, BD Bioscience); PE-conjugated anti-CD25 (eBioscience Inc.). Fixability Viability Dye eFluor520 (eBioscience Inc.) was used for elimination of dead cells.

Mesenteric lymph nodes (MLNs) were homogenised in Hanks balanced salt solution containing 1% bovine serum albumin. The cells were isolated from the homogenate in Iscove's modified Dulbecco's medium containing 5% foetal bovine serum, 10 mM HEPES, Liberase TM (100 µg/mL, Roche, Indianapolis, IN) and DNase I (10 µg/mL, Roche) for 40 min at 37°C. Flow cytometric analyses were performed using FACSVerse (BD Bioscience). Data were analysed with Cytobank (Cytobank, Inc., Mountain View, CA).

Detection of mRNA expressions by real-time PCR

Expressions of mRNA in MLNs were measured by quantitative real-time reverse transcriptase-PCR (qPCR). Total RNA was extracted from excised MLNs using TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH). Complementary DNA was prepared from the RNA by PrimeScript reverse transcriptase (Takara Bio Inc., Shiga, Japan). Interleukin (IL)-4, interferon gamma (IFN-γ), transforming growth factor beta (TGF-β) and glyceraldehyde-3-phosphate dehydrogenase (GADPH) genes were amplified using: 5'-TCTCGAATGTACCAGGAGCCATATC-3' and

5'-AGCACCTTGGAAGCCCTACAGA-3' for IL-4;

5'-CGGCACAGTCATTGAAAGCCTA-3' and 5'-GTTGCTGATGGCCTGATTGTC-3' for

IFN- γ ; 5'-GTGTGGAGCAACATGTGGAACCTCTA-3' and 5'-

TTGGTTCAGCCACTGCCGTA-3' for TGF- β and 5'-TGTGTCCGTCGTGGATCTGA-3'

and 5'-TTGCTGTTGAAGTCGCAGGAG-3' for GAPDH.

The qPCRs were performed on a Thermal Cycler Dice system (Takara Bio Inc.) with SYBR Premix Ex TaqII (Takara Bio Inc.). The qPCR conditions included a pre-incubation step at 95°C for 30 s, 40 amplification cycles of 95°C for 5 s, annealing at 60°C for 30 s and a melting curve. Expression of cytokines was normalised by each GAPDH expression.

Analysing antigen presenting cells (APCs) migration

Saccharin sodium at a dose of 100 mg or the Mixture B containing 30 mg acesulfame potassium, 10 mg sodium benzoate and 15 mg tartrazine was administered with 1 mg OVA of inducing oral tolerance for 5 successive days. For analysis of migrating APCs by flow cytometry, MLNs were collected at indicated time after the first administering the additives or the fifth administering. 0 h in figures means just before the administration.

Statistical analyses

Data were expressed as mean values with standard error. The statistical significance of the difference values was evaluated by Student's or Welch's *t*-tests, or Dunnett's test. Each diarrhoea score for a mouse was indicated as a dot, and an average of the score shown as a bar. The statistical significance in diarrhoea scores was evaluated by Mann-Whitney *U* tests or Steel's tests. *P* values of less than 0.05 were considered to be statistically significant.

Results

Screening of food additives for development of food allergy

The effects of food additives on allergy were evaluated using a procedure of oral tolerance for antigens. Food additives and 1 mg OVA were administrated orally and simultaneously (Fig. 1a). First, we evaluated silicon dioxide (30 mg/mouse), benzoic acid (20 mg/mouse), saccharin sodium (10 mg/mouse) and annatto pigment (100 mg/mouse) as a risk for a frailer of oral tolerance (Fig. 1b). Silicon dioxide is used as a moisture absorber in pulverulent foods. Silica crystal has been reported to activate inflammasome in macrophages [13–15]; therefore, we presumed that silica had an immune adjuvant effect in oral treatment. It has also been reported that benzoic acid, saccharin sodium and annatto pigment induce hypersensitivity [16–18]. Next, as sweeteners have been associated with risks for allergy [19–21], we screened acesulfame potassium (3 mg/mouse), aspartame (100 mg/mouse),

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xylitol (100 mg/mouse) and meso-erythritol (100 mg/mouse) (Fig. 1c, d). In addition, we evaluated natural sugars, glucose and sucrose (each 100 mg/mouse) (Fig. 1e). As the serial treatments of OVA inhibited the elevation of OVA-specific IgE in our murine oral tolerance model, we could judge whether or not tolerance failed. Some mice treated with saccharin showed robustly broken oral tolerance and elevated OVA-specific IgE. Specific IgE was detected in a mouse treated with acesulfame, erythritol, glucose or sucrose.

Evaluation of food allergy by saccharin

Our murine food allergy model confirmed a dose-dependency of saccharin in failure to acquire oral tolerance (Fig. 2a). Saccharin at doses of 10 or 100 mg/mouse was administered with 1 mg OVA for the induction of oral tolerance. The mice were sensitised by OVA/alum and administered 30 mg OVA to induce anaphylaxis. OVA-specific IgE and IgG₁ increased in a dose-dependent manner before and after 30 mg OVA challenges (Fig. 2b, c). A decrease in body temperature was observed from the fourth challenge in the food allergy (FA) group, but not in the oral tolerance (OT) group (Fig. 2d). Significant decreases in body temperature in the FA group were induced in the eighth challenge, compared with the OT group (Fig. 2e). In the group treated with 100 mg of saccharin, hypothermia was detected at the eighth challenge. Allergic diarrhoea was observed in a similar pattern to body temperature changes (Fig. 2f). Intake of saccharin inhibited acquisition of oral tolerance and induced

significant diarrhoea (Fig. 2g). MLNs were collected after the last challenges, and expressions of cytokine-related allergy and tolerance were measured (Fig. 3a–c). Expression of IL-4 in the OT group decreased in comparison with the FA group and was up-regulated by saccharin treatment. Expressions of IFN- γ and TGF- β were down-regulated in the saccharin-treated group compared with the OT group.

We thought that the change of gene expressions in OT group and saccharin -treated group was related with an induction of regulatory T cells. To certify the hypothesis, we measured a proportion of CD4⁺CD25^{hi}T cells in MLNs by flow cytometry. Mice were treated with saccharin sodium at 100 mg/mouse with OVA for 5 days and then sacrificed 24 h after the last saccharin treatment. MLNs were collected and homogenised for flow cytometry. A gating strategy was indicated in Fig. S1. Live cells gated as Fixable Viability Dye eFluor-negative cells were stained fluorescent for CD3, CD4 and CD25. We defined regulatory T cells as CD3⁺CD4⁺CD25^{hi} cells [22]. The proportion of CD4⁺CD25^{hi}T cells in MLNs of saccharin-treated mice was suppressed than OT group (Fig. 3d). We analysed a proportion of CD69⁺CD4⁺CD25⁻ T cells, which were known as early induced regulatory T cells [23], but there were no differences between OT group and saccharin-treated group (data not shown).

Effect of saccharin on antigen presenting cells (APCs)

To analyse the failure of oral tolerance and the decreasing in proportion of regulatory T cells in the saccharin-treated group, we measured the migration of APCs. The mice were treated with saccharin sodium at 100 mg/mouse with OVA for 5 days and then sacrificed at the indicated times. A gating strategy was indicated in Fig. S2. Live cells were stained fluorescent for CD11c, CX3CR1, CD11b and CD103. CD11c^{hi}CX3CR1⁻ (Fig. 4a–c) or CD11c^{hi}CX3CR1⁺ cells (Fig. 4d–f) were quartered by fluorointensities of CD11b and CD103. Proportions of CD11b⁺CD103⁻ cells in CD11c^{hi}CX3CR1⁻ cells in the saccharin-treated group were higher at 0 h (before the fifth saccharin treatment), and 1, 3, and 6 h after the fifth saccharin treatment (Fig. 4a). There were no differences in CX3CR1⁻CD11b⁻CD103⁺ and CX3CR1⁻CD11b⁺CD103⁺ cells in the time-course analyses (Fig. 4b, c). In CX3CR1⁺ cells, the proportion of CD11b⁺CD103⁻ cells was higher in the group treated with saccharin at 0, 3 and 6 h (Fig. 4d). Proportions of CD11b⁺CD103⁺ in CD11c^{hi}CX3CR1⁺ cells were higher in the OT group without saccharin (Fig. 4f). We think that the changing proportion of APCs and regulatory T cells doesn't depend on cytotoxicity caused by food additives, because there were no differences of percentages of live cells in APCs and regulatory T cells regardless of saccharin treatment in flow cytometry (data not shown).

Risk for food allergy by intake of plural food additives

As saccharin is rarely used as a sweetener in processed foods and low calorie beverages in Japan, risk for food allergy was estimated using intake of plural food additives. Mixture A (MixA) contained 3 mg acesulfame potassium, 1 mg sodium benzoate and 1.5 mg tartrazine, and mixture B (MixB) contained 30 mg acesulfame potassium, 10 mg sodium benzoate and 15 mg tartrazine. MixA was nearly equal to the ADI of the Food Safety Commission in Japan or the Joint FAO/WHO Expert Committee on Food Additives. MixB was 10 times the ADI dose.

Single food additive treatment of 30 mg acesulfame potassium, 10 mg sodium benzoate or 15 mg tartrazine was effective in breaking oral tolerance and resulted in elevated OVA-specific IgE levels (Fig. 5a). However, there were no differences in decreased body temperature and allergic diarrhoea for anaphylaxis between the OT group and the single treated groups (Fig. 5b, c). In the MixB group, we observed a significant up-regulation of IgE levels as well as decreased body temperature and diarrhoea. Because levels of mucosal mast cell protease-1 (MMCP-1) in blood collected 1 h after the last OVA-treatment were elevated in both saccharin and MixB treated group (Fig. 5d), food additives would break oral tolerance and induce anaphylaxis depended on mucosal mast cells in the mice. Also, proportions of regulatory T cells in the MLNs were reduced in not only saccharin but also MixB (Fig. 5e).

Analysis for migrated APCs by food additives treatments

Next, we analysed detail situation of migrating APCs by administering saccharin and the mixture. A proportion of migrating CD11b⁺CD103⁺ or CD11b⁺CD103⁻ cells gated by CD11c⁺CX3CR1⁺ in MLNs was shown in Fig. 6. The migrating APCs were classified at fluorointensity of MHC class II according to a depicted strategy in Fig. S3. MHC-II positive cells (high expression, MHCII^{hi} and intermediate expression, MHCII^{int}) were decided by data of fluorointensity minus one (FMO).

Panels a and b demonstrate the situation migrating APCs 3 and 12 h after the first administration of OVA with 100 mg saccharin or the mixture of food additives. Panels c and d show the situation of the fifth administration (0 h is data just before the fifth administration). The first administering saccharin or the mixture reduced proportions of MHCII^{hi}CD11b⁺CD103⁺ APCs at 3 and 12 h (Fig. 6a), and augmented MHCII^{int}CD11b⁺CD103⁻ APCs at 12 h (Fig. 6b). In the fifth administering the additives, decreasing in MHCII^{hi}CD11b⁺CD103⁺ APCs in 0 and 3 h (Fig. 6c) and increasing in MHCII^{int}CD11b⁺CD103⁻ APCs in 0 h were observed (Fig. 6d).

Discussion

Food allergy is defined as ‘a phenomenon in which adverse reactions are caused through antigen-specific immunological mechanisms after exposure to given food’ [2]. Oral tolerance is a state of local and systemic immune unresponsiveness to innocuous antigens such as food proteins. Almost all cases of food allergies develop in infancy, and it is thought that failure to acquire oral tolerance is established at the first/first few contacts with food antigens. Previous studies have reported risks of allergy from food additives [17]. Therefore, intake of processed foods and confectionary containing some food additives in childhood is one of several possible explanations for failure to develop tolerance.

We previously reported murine food allergy and oral tolerance models [10]. Experimental oral tolerance for allergy was induced by oral intake of the food antigen OVA. In our oral tolerance model, serial OVA intake inhibited production of immunoglobulins. Our murine food allergy model (in which OVA/alum was injected intraperitoneally) was dependent on IgE, and the inhibition of IgE production in the oral tolerance model suppressed the development of food allergy. In this study, we screened food additives by whether or not OVA-specific IgE was detected in blood. Sweeteners, preservatives and colourings were tested for inhibition of immune tolerance, and we detected OVA-specific IgE in mice treated with saccharin sodium. Low levels of specific IgE, which could not induce anaphylaxis, detected in a few mice treated with acesulfame potassium and natural sugars. As saccharin

sodium strongly induced the breakdown of oral tolerance, we checked dose-dependency and induction of food allergy using a modified food allergy model, because a stronger load than used in the previously reported model was necessary to estimate the breakdown of immune tolerance. Saccharin treatment induced dose-dependent OVA-specific IgE and IgG₁.

Anaphylaxis developed following multiple treatments of 30 mg OVA in mice treated with 100 mg saccharin. After multiple OVA treatments, MLNs of mice given 100 mg saccharin were collected for evaluation of expression of cytokines. Saccharin up-regulated IL-4 as a Th2 cytokine and down-regulated IFN- γ as a Th1 cytokine and TGF- β as a regulatory cytokine. We confirmed that the saccharin-administration inhibited inducing regulatory T cells in oral tolerance. Saccharin can therefore break oral tolerance and lead to food allergy.

A dose of 100 mg saccharin sodium per mouse corresponded to 100 times the ADI dose. As this dose was unrealistic, we estimated the effects of mixtures of food additives at lower doses on loss of oral tolerance. MixA contained acesulfame potassium, sodium benzoate and tartrazine doses nearly equal to ADI, and MixB contained 10 times the ADI doses. Solo treatment of 10 times the dose of acesulfame potassium and sodium benzoate or treatment with MixB resulted in breakdown of oral tolerance and elevated OVA-specific IgE. Mixture of 0.1 times the ADI doses of the additives could not induce to produce OVA-specific IgE (data not shown). Specifically, MixB induced strong anaphylaxis depended on degranulation of mucosal mast cells by multiple administrations of 30 mg OVA. These

results suggested that intake of plural additives amplified the risk of developing food allergies.

Solo treatment might therefore provide a weaker load for immune system tolerance than a mixture of additives. In the study, we estimated the administration with the mixture of 3 additives for 5 days. Realistically, we eat and drink some proceed foods and beverages containing more varieties of colourants and sweeteners for longer term; therefore, mixtures of smaller amount of additives might inhibit acquisition of oral tolerance in dairy life.

Several food antigens have resistance to gastric digestion, and acid resistance is an important factor in food allergies [24, 25]. In addition, gastric pH and digestion time are related to the antigenicity of food proteins [26]. Using medicines that are blockers for histamine receptor 2 (H₂) and proton pump inhibitors is a risk factor for food allergy [27, 28]. H₂-blockers and antacids were oral immune Th2-adjuvant in the murine allergy model [29, 30]. We suspected that saccharin sodium has an antacid effect and measured gastric pH in mice administered saccharin sodium and acesulfame potassium (data not shown). We found the gastric pH in mice treated with sweeteners was elevated in a similar way to mice treated with omeprazole. However, omeprazole did not affect oral tolerance in the murine model (data not shown). Next, we checked a change of OVA concentrations in serum by administration with or without 100 mg saccharin by ELISA because we thought that saccharin effected absorption of OVA. As a result, absorption of OVA was faster by simultaneous uptake of saccharin (data not shown). However, it is unclear whether the

changes of pH in stomach and absorption of OVA affect immune system.

Luminal antigens are delivered to integrin CD103⁺ dendritic cells (DCs) through goblet cell-associated antigen passages [31–33]. CD103⁺DC in lamina propria can migrate to the MLN in a CCR7-dependent manner [34, 35] and promote regulatory T cells via TGF- β and retinoic acid [36–38]. CX3CR1 is a known marker of mucosal macrophage. CX3CR1⁺ macrophage in the lamina propria can catch and transfer antigens to tolerogenic DCs [39, 40]. It was reported that CD11c⁺CX3CR1⁺ DCs contributed to oral tolerance for dietary antigen, but not CX3CR1⁺ macrophage [41]. CD11b⁺DCs were associated with allergic response in the mucosal immune system [42]. We hypothesised that food additives have an effect on the proportion of DCs and change the manner of migration to the lymph node. Serial treatment of saccharin sodium and the mixture of additives therefore induced CD11b⁺CD103⁻DCs, and inducing oral tolerance temporarily raised the proportion of CD11c⁺CX3CR1⁺CD11b⁺CD103⁺ DCs. The food additives facilitated the increasing in allergic CD11b⁺ DCs and attenuated tolerogenic CD103⁺ DCs.

MHCII^{hi} DCs have been defined migratory DCs and MHCII^{int} DCs have been resident DCs [41, 43]. The first administration of OVA with the food additives prevented migrating tolerogenic CD11b⁺CD103⁺ DCs and increasing in proportion of allergic CD11b⁺CD103⁻ DCs in the MLNs. Each administration of the additives would suppress migration of tolerogenic DCs in induction of tolerance, and the repeated administrations

constituted a high percentage of MHCII^{int} allergic CD11b⁺CD103⁻ DCs in MLNs. As results, multiple treatment of saccharin and the mixture prevented the induction of CD4⁺CD25^{hi}T cells and inhibited the acquirement of oral tolerance. A balance between migratory tolerogenic CD11b⁺CD103⁺DCs and allergic resident CD11b⁺CD103⁻ DCs might decide that local immune condition had a predisposition to tolerance or allergy.

In our murine model, a failure of oral tolerance occurred in mice treated with saccharin as well as in those treated with a mixture of food additives, suggesting the change in immune condition was independent of a specific chemical substance. This means that risks of failure of oral tolerance are associated with an amount of chemical substances rather than a specific substance. To elucidate the mechanism of failure of oral tolerance through food additives, more detailed analyses of chemical receptors in immune cells are needed.

Taken together, food additives influenced the failed acquisition of oral tolerance with changes of DCs migrations. The risks of food allergy from food additives depended on the dose and number of intakes. Our data indicated that food additives might reduce toxic response by microorganisms in foods, but might increase risks for nontoxic responses such as allergies.

Acknowledgement

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Author contributions

SM and YS screened and estimated the food additives to use mice. HM performed the experiment of gastric pH and analyses for flow cytometer. TM, HT and NI participated in the design of the study and helped to draft the manuscript. HY conceived the study, participated in its design and drafted the manuscript. All authors read and approved the final manuscript.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Figure legends

Fig. 1. Evaluation of food additives and natural sugars in an experimental oral tolerance model.

Mice were administered OVA with food additives for 5 sequential days. The mice were sensitised by OVA/alum intraperitoneally 1 week after the last administration. Blood was collected for ELISA of OVA-specific IgE on day 24 (a). OVA-specific IgE levels were

measured in the blood of mice treated with silicon dioxide (Sil), benzoic acid (BA), saccharin sodium (Sac), annatto pigment (Ann), acesulfame potassium (Ace), aspartame (Asp), xylitol (Xyl), meso-erythritol (Ery), glucose (Glu) and sucrose (Suc) at the time of acquisition of oral tolerance (OT). A positive control (CTRL) was sensitised by OVA/alum without induction of oral tolerance. Each value represents a mean $\pm$ standard error of the mean (5 or 6 mice). N.D.: not detected.

Fig. 2. Induction of oral tolerance in a murine food allergy model.

Saccharin sodium (10 or 100 mg/mouse) was administered with 1 mg OVA for 5 days before sensitisation of OVA/alum (a). Blood was collected before and after the 30 mg OVA administrations for measurement of OVA-specific IgE and IgG₁ by ELISA (b, c). Anaphylaxis was induced by repeated 30 mg OVA administrations. Food allergy was evaluated by decreases in body temperature (d, e) and allergic diarrhoea (f, g) over time (left panels) or at the last challenge (right panels). Each value represents a mean $\pm$ standard error of the mean of 5 to 7 mice. Diarrhoea scores are depicted as individual datum (dot) and mean (bar) on the last challenge. N.D.: not detected. * P < 0.05 vs. OT group.

Fig. 3. Gene expressions of inflammatory cytokines and inducing CD4⁺CD25^{hi} regulatory T cells in mesenteric lymph nodes.

Mesenteric lymph nodes (MLNs) in the food allergy group (FA), oral tolerance group (OT) and group treated with 100 mg saccharin (Sac) were collected after the last challenge of 30 mg OVA. IL-4 (a), IFN- γ (b) and TGF- β (c) mRNA levels were evaluated by qPCR. A proportion of CD4⁺CD25^{hi} T cells in MLNs collected 24 h after the fifth saccharin administration was measured by flow cytometry (d). Naive is negative control treated without OVA and saccharin. * P <0.05, ** P <0.01, and *** P <0.001 vs. OT group.

Fig. 4. Time-dependent analysis of migration of antigen presenting cells.

Mesenteric lymph nodes were collected just before the fifth OVA administration without and with 100 mg saccharin treatment (OVA and OVA+Sac, 0 h) and at the indicated time after the fifth administration. Antigen presenting cells (APCs) were distinguished as expressions of cell-surface markers of CD11c, CD11b, CD103 and CX3CR1. Panels a–c show proportions of positive or negative CD11b and CD103 in CD11c^{hi}CX3CR1⁺ APCs. Panels d–f show the proportions of the cells in CD11c^{hi}CX3CR1⁺ APCs. Lines with short dashes represent levels of naïve mice in this study. Each value represents a mean $\pm$ standard error of the mean of 4 mice (* P <0.05, ** P <0.01, and *** P <0.001).

Fig. 5. Evaluation of mixtures of food additives at the time of acquisition of oral tolerance.

Single food additives (100 mg saccharin sodium, Sac; 30 mg acesulfame potassium, Ace; 10 mg sodium benzoate, Ben and 15 mg tartrazine, Ter) and mixtures of these additives were evaluated in the food allergy model. Mixture A (MixA) contained 3 mg acesulfame potassium, 1 mg sodium benzoate and 1.5 mg tartrazine, and mixture B (MixB) contained 30 mg acesulfame potassium and 10 mg sodium benzoate and 15 mg tartrazine. Food allergy was estimated by IgE levels after the last challenge (a), change in body temperature (b) and allergic diarrhoea (c). As a marker of degranulation of mucosal mast cells, mucosal mast cells protease-1 (MMCP-1) was measured in serum collected 1 h after the last 30 mg OVA challenge by ELISA (d). A proportion of CD4⁺CD25^{hi} T cells in mesenteric lymph nodes collected 24 h after the fifth administration with saccharin and Mix B was measured by flow cytometry (d). Each value represents a mean $\pm$ standard error of the mean of 4 to 6 mice. Diarrhoea score is depicted as individual datum (dot) and mean (bar) on the last challenge. FA: food allergy; OT: oral tolerance; N.D.: not detected. * $P < 0.05$ and ** $P < 0.01$ vs. OT group.

Fig. 6. MHC-II expressing on antigen presenting cells (APCs) in mesenteric lymph nodes (MLNs) caused by the first or fifth administration of food additives.

MLNs were collected at the first (a, b) or fifth (c, d) administration of saccharin and the

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mixture. Data indicate that proportions of CD11b⁺CD103⁺ and CD11b⁺CD103⁻ cells gated on MHCII^{hi}CX3CR1⁺CD11c⁺ cells (a, c) or MHCII^{int}CX3CR1⁺CD11c⁺ cells (b, d) at the indicated time (0 h means just before the treatment). Each value represents a mean $\pm$ standard error of the mean of 5 mice (**P* < 0.05 and ***P* < 0.01). Sac: saccharin; Mix: mixture of the food additives.

References

1. Valenta R, Hochwallner H, Linhart B, Pahr S. Food allergies: the basics. *Gastroenterology* 2015; **148**:1120-1131 e1124.
2. Urisu A, Ebisawa M, Ito K *et al.* Japanese Guideline for Food Allergy 2014. *Allergol Int* 2014; **63**:399-419.
3. Lack G. Update on risk factors for food allergy. *J Allergy Clin Immunol* 2012; **129**:1187-1197.
4. Hong X, Hao K, Ladd-Acosta C *et al.* Genome-wide association study identifies peanut allergy-specific loci and evidence of epigenetic mediation in US children. *Nat Commun* 2015; **6**:6304.
5. Luccioli S, Zhang Y, Verrill L, Ramos-Valle M, Kwegyir-Afful E. Infant feeding practices and reported food allergies at 6 years of age. *Pediatrics* 2014; **134**:S21-28.
6. Allen KJ, Koplin JJ, Ponsonby AL *et al.* Vitamin D insufficiency is associated with challenge-proven food allergy in infants. *J Allergy Clin Immunol* 2013; **131**:1109-1116.
7. Matsui T, Tanaka K, Nakagawa T *et al.* Sun exposure inversely related to food sensitization during infancy. *Pediatr Allergy Immunol* 2015; **26**:628-633.
8. Song P, Wu L, Guan W. Dietary Nitrates, Nitrites, and Nitrosamines Intake and the Risk of Gastric Cancer: A Meta-Analysis. *Nutrients* 2015; **7**:9872-9895.
9. Maslova E, Strom M, Olsen SF, Halldorsson TI. Consumption of

artificially-sweetened soft drinks in pregnancy and risk of child asthma and allergic rhinitis.

PLoS One 2013; **8**:e57261.

10. Yamashita H, Takahashi K, Tanaka H, Nagai H, Inagaki N. Overcoming food allergy through acquired tolerance conferred by transfer of Tregs in a murine model. *Allergy* 2012; **67**:201-209.

11. Nagai H, Maeda Y, Tanaka H. The effect of anti-IL-4 monoclonal antibody, rapamycin and interferon-gamma on airway hyperreactivity to acetylcholine in mice. *Clin Exp Allergy* 1997; **27**:218-224.

12. Tanaka H, Masuda T, Tokuoka S *et al.* The effect of allergen-induced airway inflammation on airway remodeling in a murine model of allergic asthma. *Inflamm Res* 2001; **50**:616-624.

13. Cassel SL, Eisenbarth SC, Iyer SS *et al.* The Nalp3 inflammasome is essential for the development of silicosis. *Proc Natl Acad Sci U S A* 2008; **105**:9035-9040.

14. Dostert C, Pétrilli V, Van Bruggen R, Steele C, Mossman BT, Tschopp J. Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica. *Science* 2008; **320**:674-677.

15. Kuroda E, Ishii KJ, Uematsu S *et al.* Silica Crystals and Aluminum Salts Regulate the Production of Prostaglandin in Macrophages via NALP3 Inflammasome-Independent Mechanisms. *Immunity* 2011; **34**:514-526.

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16. Ehlers I, Niggemann B, Binder C, Zuberbier T. Role of nonallergic hypersensitivity reactions in children with chronic urticaria. *Allergy* 1998; **53**:1074-1077.
 17. Hannuksela M, Haahtela T. Hypersensitivity reactions to food additives. *Allergy* 1987; **42**:561-575.
 18. Auttachoat W, Germolec DR, Smith MJ, White KL, Jr., Guo TL. Contact sensitizing potential of annatto extract and its two primary color components, cis-bixin and norbixin, in female BALB/c mice. *Food Chem Toxicol* 2011; **49**:2638-2644.
 19. Tarlo SM, Sussman GL. Asthma and anaphylactoid reactions to food additives. *Can Fam Physician* 1993; **39**:1119-1123.
 20. Stohs SJ, Miller MJ. A case study involving allergic reactions to sulfur-containing compounds including, sulfite, taurine, acesulfame potassium and sulfonamides. *Food Chem Toxicol* 2014; **63**:240-243.
 21. Shirao K, Inoue M, Tokuda R *et al.* "Bitter sweet": a child case of erythritol-induced anaphylaxis. *Allergol Int* 2013; **62**:269-271.
 22. Huang H, Dawicki W, Zhang X, Town J, Gordon JR. Tolerogenic dendritic cells Induce CD4+CD25hiFoxp3+ regulatory T cell differentiation from CD4+CD25-/loFoxp3- effector T cells. *J immunol* 2010; **185**:5003-5010.
 23. Han Y, Guo Q, Zhang M, Chen Z, Cao X, CD69+ CD4+ CD25- T cells, a new subset of regulatory T cells, suppress T cell proliferation through membrane-bound TGF-beta 1. *J*

immunol 2009; **182**:111-120.

24. Sen M, Kopper R, Pons L, Abraham EC, Burks AW, Bannon GA. Protein structure plays a critical role in peanut allergen stability and may determine immunodominant IgE-binding epitopes. *J Immunol* 2002; **169**:882-887.
25. Takagi K, Teshima R, Okunuki H *et al.* Kinetic analysis of pepsin digestion of chicken egg white ovomucoid and allergenic potential of pepsin fragments. *Int Arch Allergy Immunol* 2005; **136**:23-32.
26. Untersmayr E, Jensen-Jarolim E. The role of protein digestibility and antacids on food allergy outcomes. *J Allergy Clin Immunol* 2008; **121**:1301-1308.
27. Pali-Scholl I, Jensen-Jarolim E. Anti-acid medication as a risk factor for food allergy. *Allergy* 2011; **66**:469-477.
28. Trikha A, Baillargeon JG, Kuo YF *et al.* Development of food allergies in patients with gastroesophageal reflux disease treated with gastric acid suppressive medications. *Pediatr Allergy Immunol* 2013; **24**:582-588.
29. Untersmayr E, Scholl I, Swoboda I *et al.* Antacid medication inhibits digestion of dietary proteins and causes food allergy: a fish allergy model in BALB/c mice. *J Allergy Clin Immunol* 2003; **112**:616-623.
30. Brunner R, Wallmann J, Szalai K *et al.* Aluminium per se and in the anti-acid drug sucralfate promotes sensitization via the oral route. *Allergy* 2009; **64**:890-897.

31. McDole JR, Wheeler LW, McDonald KG *et al.* Goblet cells deliver luminal antigen to CD103+ dendritic cells in the small intestine. *Nature* 2012; **483**:345-349.
32. Miller MJ, Knoop KA, Newberry RD. Mind the GAPS: insights into intestinal epithelial barrier maintenance and luminal antigen delivery. *Mucosal Immunol* 2014; **7**:452-454.
33. Knoop KA, McDonald KG, McCrate S, McDole JR, Newberry RD. Microbial sensing by goblet cells controls immune surveillance of luminal antigens in the colon. *Mucosal Immunol* 2015; **8**:198-210.
34. Johansson-Lindbom B, Svensson M, Pabst O *et al.* Functional specialization of gut CD103+ dendritic cells in the regulation of tissue-selective T cell homing. *J Eex Med* 2005; **202**:1063-1073.
35. Jang MH, Sougawa N, Tanaka T *et al.* CCR7 is critically important for migration of dendritic cells in intestinal lamina propria to mesenteric lymph nodes. *J Immunol* 2006; **176**:803-810.
36. Annacker O, Coombes JL, Malmstrom V *et al.* Essential role for CD103 in the T cell-mediated regulation of experimental colitis. *J Exp Med* 2005; **202**:1051-1061.
37. Coombes JL, Siddiqui KR, Arancibia-Carcamo CV *et al.* A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. *J Exp Med* 2007; **204**:1757-1764.

38. Scott CL, Aumeunier AM, Mowat AM. Intestinal CD103+ dendritic cells: master regulators of tolerance? *Trends Immunol* 2011; **32**:412-419.
39. Schulz O, Jaensson E, Persson EK *et al.* Intestinal CD103+, but not CX3CR1+, antigen sampling cells migrate in lymph and serve classical dendritic cell functions. *J Exp Med* 2009; **206**:3101-3114.
40. Mazzini E, Massimiliano L, Penna G, Rescigno M. Oral tolerance can be established via gap junction transfer of fed antigens from CX3CR1(+) macrophages to CD103(+) dendritic cells. *Immunity* 2014; **40**:248-261.
41. Esterházy D, Loschko J, London M, Jove V, Oliveira TY, Mucida D, Classical dendritic cells are required for dietary antigen-mediated induction of peripheral Treg cells and tolerance. *Nat Immunol* 2016; **17**:545-555.
42. Khandelwal P, Blanco-Mezquita T, Emami P *et al.* Ocular mucosal CD11b+ and CD103+ mouse dendritic cells under normal conditions and in allergic immune responses. *PloS one* 2013; **8**:e64193.
43. Jiao Z, Bedoui S, Brady JL *et al.* The closely related CD103+ dendritic cells (DCs) and lymphoid-resident CD8+ DCs differ in their inflammatory functions. *PloS one* 2014; **9**:e91126.









