

Ingestion of Casein Hydrolysate Induces Oral Tolerance and Suppresses Subsequent Epicutaneous Sensitization and Development of Anaphylaxis Reaction to Casein in Mice

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Keywords

Casein · Casein hydrolysate · Cow's milk allergy ·
Oral tolerance · Epicutaneous sensitization

Abstract

Background: Casein is the most dominant causal allergen in cow's milk allergy (CMA). Casein hydrolysates are frequently applied in infant formulas for children with a risk or history of CMA. However, there is limited information on the oral tolerance-inducing ability of casein hydrolysates. **Objectives:** The aim of this study was to investigate whether the ingestion of casein hydrolysate induces tolerance to casein, ultimately preventing subsequent epicutaneous sensitization and development of an anaphylaxis reaction. **Methods:** BALB/c mice were orally administered casein or a casein hydrolysate (CNH) via the drinking water and were then epicutaneously sensitized by repeated exposure of casein on tape-stripped skin. Sensitization was assessed by basophil activation tests, the serum levels of casein-specific antibodies, and cytokine production from casein-stimulated spleen and mesenteric lymph node (MLN) cells. Occurrence of an anaphylaxis reaction was evaluated by measuring rectal temperature and the plasma level of mouse mast cell protease-1 (mMCP-1) after oral casein challenges. The T cell population in the spleen and MLN was assessed by flow cytome-

try. Intestinal mast cells and basophils were analyzed histologically. **Results:** Sensitization and anaphylaxis reaction to casein were significantly suppressed in casein- or CNH-fed mice compared to controls. Prior ingestion of casein or CNH had no effect on the population of regulatory T cells and activated T cells in lymphoid tissues. Intestinal basophils increased by the epicutaneous sensitization of casein, which was suppressed in casein- or CNH-fed mice. Although the increase in the plasma level of mMCP-1 after oral challenge was suppressed in casein- or CNH-fed mice, there was no change in the number of intestinal mast cells. **Conclusion:** Prior ingestion of casein or CNH induced oral tolerance and suppressed subsequent epicutaneous sensitization and development of systemic anaphylaxis to casein.

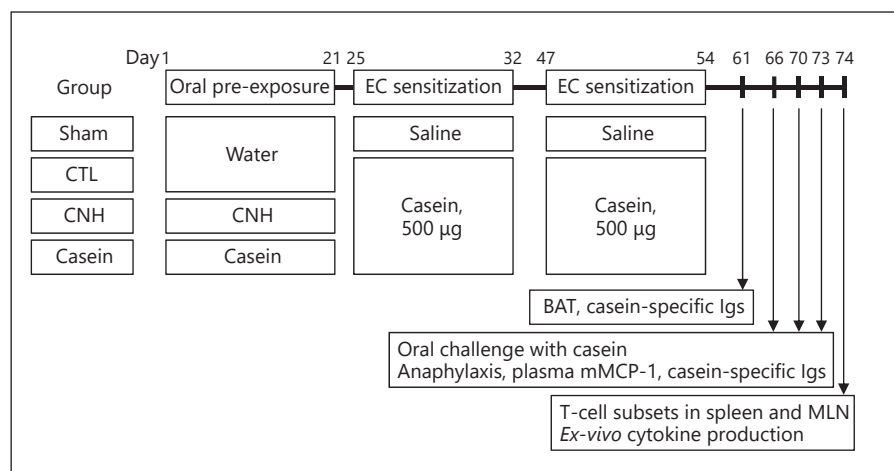
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Introduction

Cow's milk allergy (CMA), characterized by an adverse immunological reaction to the protein present in cow's milk, is one of the most frequent food allergies in infants and young children, with a prevalence of approximately 2–3% in developed countries [1]. Cow's milk con-

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Fig. 1. Schematic representation of the experimental design. Mice were given water alone, or CNH or casein in the drinking water for 3 weeks. EC sensitization was performed twice at 2-week interval, followed by analysis of the indicated parameters. CNH, casein hydrolysate; EC, epicutaneous; BAT, basophil activation test; mMCP-1, mouse mast cell protease-1.



tains several proteins that could elicit an allergic reaction in a sensitized individual, including α -lactalbumin (Bos d 4), β -lactoglobulin (Bos d 5; β -LG), serum albumin (Bos d 6), immunoglobulins (Bos d 7), and casein (Bos d 8). β -LG, the dominant whey protein in cow's milk without a human ortholog, is considered to be the major allergen and is thus used as a model allergen for immunological investigations. There are many studies aiming to reduce the allergenicity of β -LG by using enzymatic hydrolysis [2, 3] or chemical modification techniques [4, 5]. In addition, many researchers have focused on β -LG with regard to oral tolerance induction [6–9]. However, in clinical practice, it is reported that casein, rather than β -LG, is the more dominant causal allergen in CMA [10, 11]. Casein is contained in various processed foods such as infant formulas; therefore, it is important to investigate the immunological properties of casein in these foodstuffs. Indeed, several studies have investigated the antigenicity of casein and its processed materials such as casein hydrolysates or infant formulas containing casein hydrolysates [12–14].

Moreover, studies regarding the oral tolerance induced by casein and its hydrolysates demonstrated suppression of the sensitization to casein in mice [15–17]. The oral tolerance-inducing ability of casein hydrolysate was first reported in the 1990s [16]. These early studies used an adjuvant combined intraperitoneal immunization model. However, recent studies revealed that food allergen sensitization occurs through the inflamed skin [18, 19]. Accordingly, some epicutaneous sensitization animal models have been developed and are now widely utilized for investigations of food allergy [20–23]. We also recently demonstrated that prior oral ingestion of par-

tially hydrolyzed whey protein could prevent β -LG sensitization and an anaphylaxis reaction using a tape-stripping epicutaneous sensitization model [24]. These epicutaneous sensitization models are considered to be more physiologically accurate in terms of the adjuvant-free design and realistic sensitization route.

Therefore, the aim of this study was to test whether the ingestion of casein hydrolysate induces tolerance for casein, using a tape-stripping epicutaneous sensitization mouse model. In addition, we investigated the contribution of immune cells such as mast cells, basophils, and Regulatory T (Treg) cells to epicutaneous sensitization and induction of oral tolerance in this model.

Materials and Methods

Oral Tolerance Induction and Epicutaneous Sensitization

Four-week-old female BALB/c mice were purchased from CLEA Japan (Tokyo, Japan) and maintained on cow's milk-free chow under specific pathogen-free conditions for 1 week for acclimation. All animal experiments were approved by the Institutional Animal Care and Use Committee of Morinaga Milk Industry Co., Ltd.

To investigate whether the ingestion of casein hydrolysate induces oral tolerance, mice were orally administered casein or casein hydrolysate followed by epicutaneous sensitization. The schedule of the animal experiment is shown in Figure 1. From day 1 (at 5 weeks old) to day 21, the mice were fed a 6% (w/v) solution of the test material, casein or a casein hydrolysate (CNH; Morinaga Milk Industry Co., Ltd., Tokyo, Japan), or water alone (sham/CTL) *ad libitum* along with solid milk-free chow. CNH was prepared as the ingredient of partially hydrolyzed infant formula. The molecular weight profile of CNH, determined by high-pressure liquid chromatography, was as follows: 14% <200 Da, 48% 200–500 Da, 20% 500–1,000 Da, 13% 1,000–2,000 Da, 5% 2,000–5,000

Da, 0% >5,000 Da. The residual antigenicity detected by a commercial enzyme-linked immunosorbent assay (ELISA) kit (Morigawa Institute of Biological Science, Kanagawa, Japan) in CNH was 0.52 mg/g powder. As an antigen for sensitization and provocation, whole casein was isolated from fresh cow's milk by acid precipitation as described previously [25]. For the prior ingestion, sodium caseinate (Tatua Co. Dairy Company Ltd., Morrinsville, NZ), the ingredient of infant formula, was used.

The epicutaneous sensitization procedure was conducted according to a previous report [24]. Under isoflurane (Pfizer Japan Inc., Tokyo, Japan) anesthesia, the back skin of each mouse was shaved and tape stripped 6 times. Casein (500 µg) in 200 µL saline was placed on two square (1 × 1 cm) gauze patches. The patches were then fixed with a transparent dressing (Tegaderm™; 3M, MN, USA) for 1 week. Mice in the sham group were exposed to saline without casein in the same manner. The epicutaneous sensitization procedure was repeated twice at 2-week interval.

Measurement of Antigen-Specific Antibodies

Blood samples were obtained from the tail veins. Casein-specific serum IgE (1:30) and IgG1 (1:5,000) levels were measured by ELISA, using biotin rat anti-mouse IgE and IgG1 (BD Bioscience, San Jose, CA, USA), respectively.

Basophil Activation Test

Basophil Activation Test was performed according to previous reports [26, 27]. In brief, heparinized whole blood samples were incubated with or without 300 ng/mL casein at 37 °C for 2 h. The cells were blocked with 15% horse serum (GE Healthcare, Chicago, IL, USA) and labeled, followed by hemolysis. The surface expression of CD200R1 and CD200R3 on CD49b⁺/IgE⁺ cells was analyzed using a FACS Canto flow cytometer equipped with FACS-Diva software (BD Biosciences).

Induction and Evaluation of Anaphylactic Reactions

The mice were orally challenged with casein 3 times at 3- to 4-day intervals. The dosage of the first challenge was 25 mg and that of subsequent challenges was 50 mg. Systemic anaphylaxis was evaluated by successive measurement of rectal temperature using a digital thermometer (TD-300, Natsume Seisakusyo Co., Tokyo, Japan). At 60 min after each challenge, blood samples were obtained from the tail veins for measurement of plasma mouse mast cell protease-1 (mMCP-1) using a commercial ELISA kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

Assessment of Treg Cells and Activated T Cells in Lymphatic Tissues

The mesenteric lymph nodes (MLNs) and spleens were collected from sacrificed mice. Single-cell suspensions were prepared and labeled with FITC anti-mouse CD4 (clone RM4-5), PE anti-mouse CD25 (clone PC61; both from BD Bioscience), and PerCP/Cy5.5 anti-human/mouse/rat ICOS (clone C398.4A, Biolegend, San Diego, CA, USA) in RPMI1640 medium containing 10% horse serum. The cells were fixed and labeled with APC anti-Foxp3 (clone FJK-16, BD Bioscience) using a Foxp3 staining buffer set (Thermo Fisher Scientific). Flow cytometric analysis was performed as described above.

Evaluation of Lymphatic Cytokine Production

MLN and spleen cells (4×10^5 cells/well) were stimulated with or without 5 mg/mL casein in complete medium (RPMI1640, 10% horse serum, 2 mM L-glutamine, 100 IU/mL penicillin, and 100 µg/mL streptomycin). After 96 h of stimulation, the supernatants were collected, and the levels of IL-2, IL-4, IL-5, IL-10, IL-13, IL-17A, and IFN-γ were measured using Milliplex™ map Mouse Cytokine/Chemokine Magnetic Bead Panel (Merck KGaA, Darmstadt, Germany). The levels of IL-2, IL-17A, and IFN-γ in MLN cell supernatants were lower than the detectable limits of multiplex assays, and thus commercial ELISA kits (Thermo Fisher Scientific, respectively) were used for these cytokines.

Histological Analysis

The small intestines (jejunums) were fixed in 10% buffered formalin, paraffin-embedded, processed, and stained with hematoxylin. Basophils were stained with anti-mMCP-8 (BioLegend) and probed with anti-rat IgG labeled with peroxidase (Histofine Simple Stain MAX-PO, Nichirei, Tokyo, Japan). A commercial esterase-stain kit (Naphthol AS-D chloroacetate Esterase, Muto Pure Chemical, Tokyo, Japan) was used for detection of mast cells. Basophils and mast cells were counted in 10 high-power fields at 400× magnification.

Statistical Analyses

All data are expressed as mean ± SEM. Comparisons to CTL mice were analyzed by Dunnett's test. All calculations were carried out using JMP version 13.0 (SAS Institute, Cary, NC, USA). Values of $p < 0.05$ were considered statistically significant.

Results

Prior Ingestion of CNH Prevents Epicutaneous Sensitization to Casein in Mice

To investigate whether the ingestion of CNH induces oral tolerance and prevent subsequent sensitization, casein-specific antibody production and basophil response to antigen were determined. Compared to CTL mice, the level of serum casein-specific IgE was significantly lower in the casein- and CNH-fed groups; however, no significant difference was observed for the IgG1 levels (Fig. 2a). The increase of CD200R1 and decrease of CD200R3 expression levels on basophils are markers of IgE- and IgG1-dependent cell activation, respectively [26]. The increasing level of CD200R1 in casein-fed mice was significantly lower than that of CTL mice. By contrast, the decreasing level of CD200R3 in CNH-fed mice was significantly lower than that of CTL mice (Fig. 2b).

Prior Ingestion of CNH Ameliorates Anaphylaxis Reactions in Casein-Sensitized Mice

To evaluate the effect of prior ingestion of CNH on the development of anaphylaxis, casein-sensitized mice

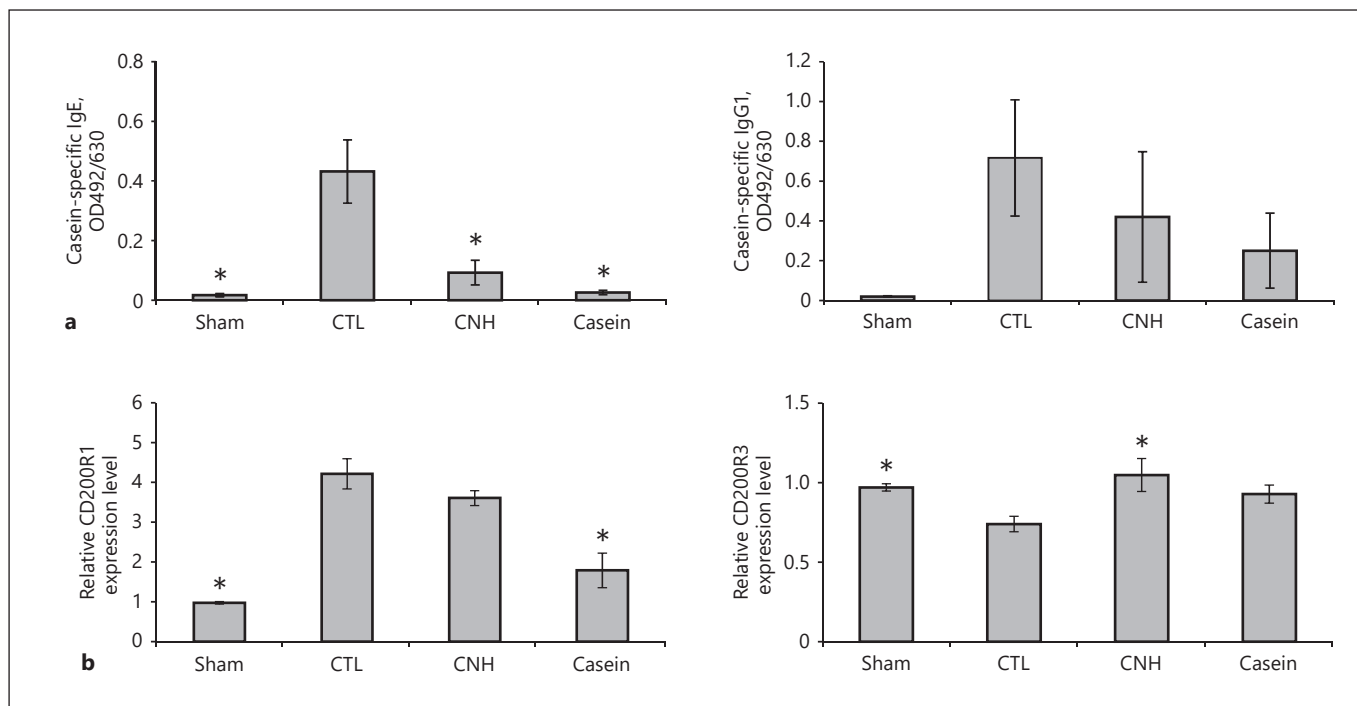


Fig. 2. Effects of CNH and casein ingestion prior to epicutaneous sensitization on the production of specific antibodies and basophil activation response. **a** Serum levels of casein-specific IgE and IgG1 measured by ELISA from blood samples obtained 1 week after the second sensitization. **b** Surface expression of CD200R1 and

CD200R3 on basophils from heparinized whole blood incubated with or without casein analyzed by flow cytometry and presented as relative expression levels. All data are represented as mean $\pm$ SEM ($n = 8$). * $p < 0.05$. CNH, casein hydrolysate.

were orally challenged with the antigen. At the first challenge with 25 mg casein, the drop in rectal temperature was significantly suppressed in casein- and CNH-fed mice compared to that of CTL mice (Fig. 3a). Although a significant temperature drop occurred in CTL mice, it was rather small. Therefore, the mice were re-challenged with a double dose of casein (50 mg/mouse) on days 70 and 73. At the second challenge, a huge drop in rectal temperature was observed in the CTL mice, and the significant suppression of this decrease in casein- and CNH-fed mice was notable (Fig. 3b). Although the change of rectal temperature at the third casein challenge showed a similar tendency to that of the second provocation, the range of decrease was smaller, and there was no longer a significant difference between CTL mice and CNH-fed mice (Fig. 3c). Plasma mMCP-1 levels in CTL mice increased significantly at all 3 casein challenges. Except for CNH-fed mice after the first challenge, the levels of plasma mMCP-1 in casein- and CNH-fed mice were significantly lower compared to those of CTL mice (Fig. 3d). Since oral antigen challenge is known to influence the specific antibody

levels in epicutaneously sensitized mice [21, 28], we also measured casein-specific IgE and IgG1 levels in plasma samples obtained after every oral casein challenge. The increase in the level of casein-specific IgE in CTL mice was significantly suppressed in casein- and CNH-fed mice after every oral challenge (Fig. 3e). In addition, the levels of casein-specific IgG1 after the second and third oral challenges in casein- and CNH-fed mice were significantly lower compared to those of the CTL mice (Fig. 3f). No diarrhea was observed at any oral casein challenge for any group.

Prior Ingestion of CNH Has No Effect on the Population of Treg Cells and Activated T Cells in Casein-Sensitized Murine Lymphoid Tissues

To investigate the effects of ingestion of CNH prior to epicutaneous sensitization on T cell populations in lymphoid tissues, flow cytometric analysis was performed on MLN and spleen cells. There was no significant difference in the population of Foxp3⁺ Treg cells and ICOS⁺ activated T cells among CD4⁺ cells in the casein- and CNH-fed mice compared to those of the CTL group (online

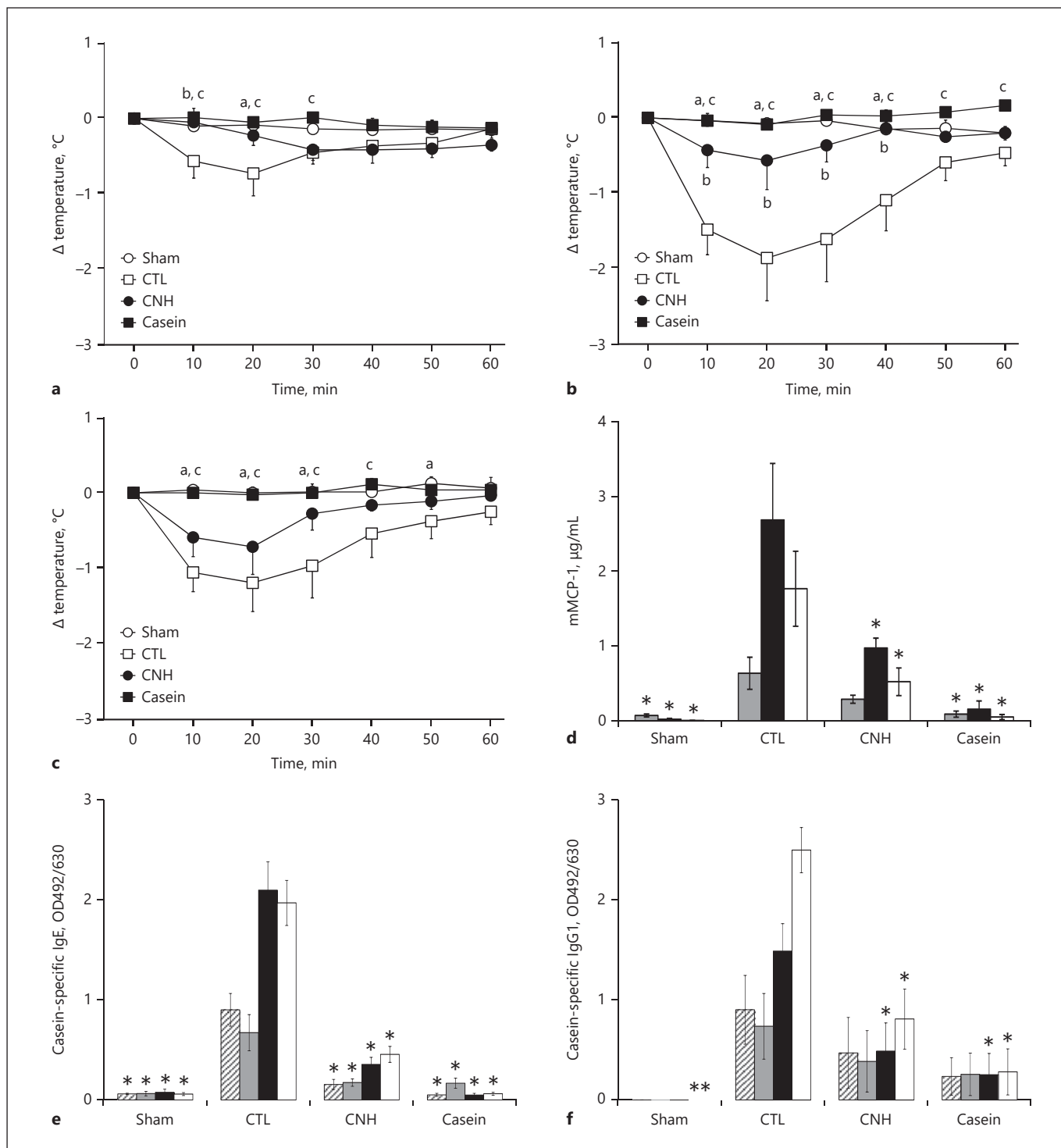


Fig. 3. Effects of CNH and casein ingestion prior to epicutaneous sensitization on the allergic response to casein after oral challenge. **a** Rectal temperature changes in mice after the first challenge with 25 mg casein. **b, c** Rectal temperature changes after the second and third challenges with 50 mg casein, respectively. Letters indicate significant differences ($p < 0.05$): **a**, sham vs. CTL; **b**, CNH vs. CTL; **c**, CN vs. CTL. **d-f** Plasma concentration of mMCP-1 (**d**), and the

levels of casein-specific IgE (**e**) and IgG1 (**f**) at 60 min after the first (gray bars), second (black bars), and third (white bars) oral challenges. Hatched bars in (**e**) and (**f**) indicate serum levels of casein-specific IgE and IgG1, respectively. The serum samples are the same as those in Figure 2a and were reanalyzed with the plasma samples. Data are represented as mean $\pm$ SEM ($n = 8$). * $p < 0.05$. CNH, casein hydrolysate; mMCP-1, mouse mast cell protease-1.

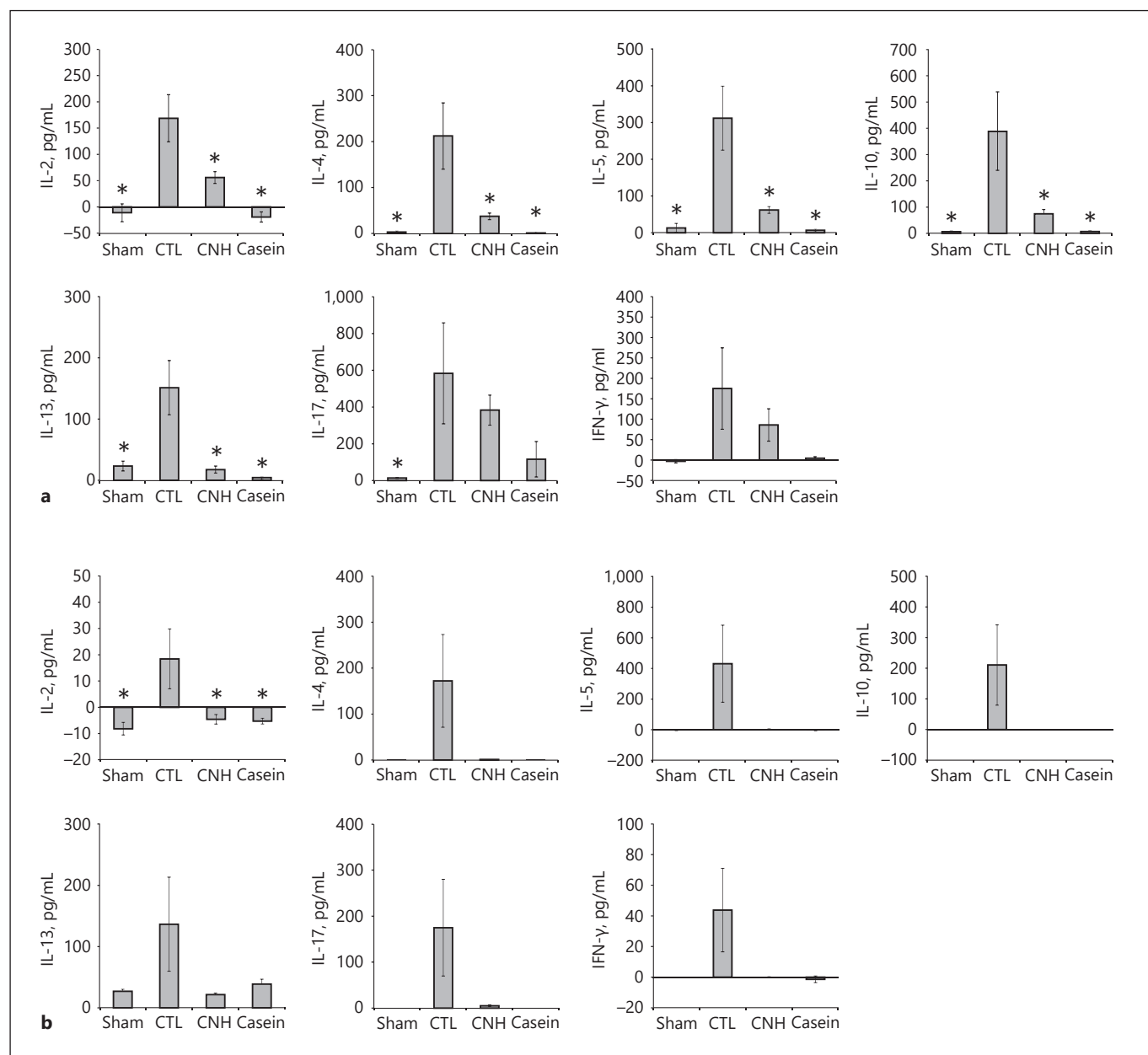


Fig. 4. Impact of CNH and casein ingestion prior to epicutaneous sensitization on ex vivo cytokine production. Spleen (a) and MLN (b) cells were stimulated with or without 5 mg/mL casein for 96 h. Levels of cytokines in the supernatants were measured. After

subtracting the background cytokine levels, data represented as mean $\pm$ SEM ($n = 8$). * $p < 0.05$. MLN, mesenteric lymph node; CNH, casein hydrolysates.

suppl. Fig. 1a, b; for all online suppl. material, see www.karger.com/doi/10.1159/000497410).

Prior Ingestion of CNH Reduces Cytokine Production in Casein-Sensitized Murine Lymphoid Tissues

To investigate the effects of prior ingestion of CNH on the sensitization states of lymphoid tissues in casein-sen-

sitized mice, the cytokine production from spleen and MLN cells were assessed. In the supernatant of cultured spleen cells, the levels of IL-2, IL-4, IL-5, IL-10, and IL-13 were significantly suppressed in casein- and CNH-fed mice compared to those of CTL mice. Although the levels of IL-17 and IFN- γ also showed a decreasing tendency in the casein-fed mice compared to the CTL group, the dif-

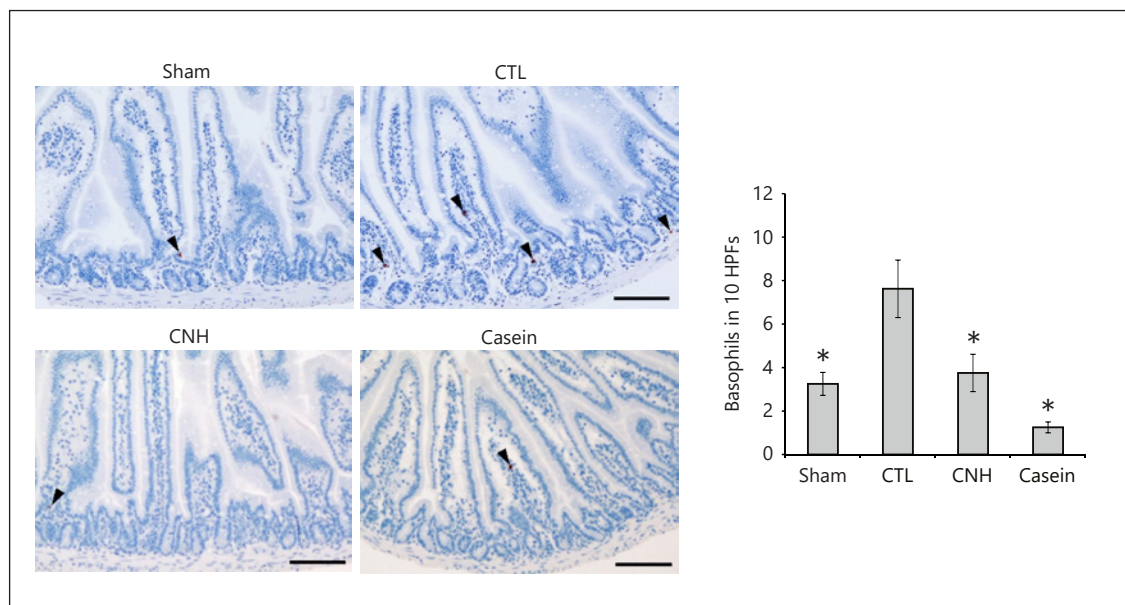


Fig. 5. Increased number of basophils in the small intestine of epicutaneously sensitized mice, and the impact of prior CNH and casein ingestion. Basophils were stained with anti-mMCP-8 and

counted in 10 HPFs at 400× magnification. Scale bar = 100 μ m. Data are represented as mean $\pm$ SEM ($n = 8$). * $p < 0.05$. CNH, casein hydrolysate; HPF, high-power field.

ferences were not statistically significant ($p = 0.093$ and $p = 0.082$, respectively), and there was no change observed in the CNH-fed mice (Fig. 4a). In addition, the level of IL-2 was significantly lower in the supernatants of cultured MLN cells from casein- and CNH-fed mice than that of CTL mice. The levels of other cytokines except for IL-13 also had a non-significant tendency for reduction ($p = 0.054$ – 0.079) in casein- and CNH-fed mice compared to those of CTL mice (Fig. 4b).

Prior Ingestion of CNH Suppressed the Increase of Intestinal Basophils in Casein-Sensitized Mice

Several studies have demonstrated that the accumulation of immune cells in intestine was observed in epicutaneously sensitized mouse models [22, 28–30]. To determine whether immune cells accumulation could be observed in our model, histological analysis was performed. Increased numbers of basophils in the small intestine were observed in CTL mice after casein challenge, which was significantly suppressed in casein- and CNH-fed mice (Fig. 5). In contrast, even in CTL mice, there was no increase in the number of intestinal mast cells, and there was no significant difference from CN- and CNH-fed mice (online suppl. Fig. 2a, b).

Discussion/Conclusion

In this study, we used a mouse model of epicutaneous sensitization with casein as the allergen. To the best of our knowledge, this is the first report of casein allergic model mice sensitized via the skin route.

The clear suppression of the production of specific antibodies, basophil response, anaphylaxis reaction, and cytokine release from the spleen cells in casein-fed mice indicates that oral tolerance was induced by ingestion of casein prior to epicutaneous sensitization. A similar pattern was observed in CNH-fed mice. Therefore, prior ingestion of not only casein but also CNH appears to effectively prevent cutaneous sensitization and the subsequent allergic response to casein.

Based on the extent of the rectal temperature decrease and plasma mMCP-1 levels after oral challenges, and increasing levels of CD200R1 in basophil activation, the ability of CNH to induce oral tolerance was considered to be weaker than that of casein. This is in line with a previous study showing that the ability of trypsin-digested casein to induce an oral tolerance to casein was weaker than that of whole casein provided at the same dosage, and was similar to that provided at one-tenth the dosage [16]. One of the limitations of our study design is the inability to discuss a potential dose-dependent effect.

Two different mechanisms of oral tolerance have been proposed: active suppression and anergy. Baek et al. [31] reported that the prior ingestion of ovalbumin (OVA) induced Foxp3⁺ Treg cells in an OVA-epicutaneously sensitized mouse model. Likewise, van Esch et al. [32] reported that the numbers of Foxp3⁺ Treg cells in the MLN were increased in whey protein hydrolysate-induced tolerized mice, and transfer of MLN cells from these tolerized mice protected the recipient mice from developing an allergic response. These investigations suggest the contribution of active suppression in oral tolerance. By contrast, in the present study, prior ingestion of casein or CNH had no effect on the population of Foxp3⁺ Treg cells in total MLN and spleen cells. However, the production of both Th1 and Th2 type cytokines, and IL-10 from tolerized murine lymphoid cells was suppressed, suggesting the induction of T cell anergy. The dosage of the ingested antigen for inducing oral tolerance in this study was comparatively higher to that used in the previous studies mentioned above [31, 32]. Low doses of antigen ingestion are known to favor the induction of active suppression, whereas high doses favor the induction of anergy [33]. Therefore, it is considered that the induction of anergy was dominant in our model. One potential explanation for the lack of a significant suppressive effect on cytokines production in MLN cells is the lower overall production levels of cytokines in the MLN cell culture supernatant than those from splenocytes in all groups, including the CTL group. Cytokine production of the lymph nodes is dependent on the exposure route for antigen sensitization. It was reported that Th2 differentiation in the MLN was effectively induced via an intraperitoneal route, but not via an epicutaneous route [34].

In the present study, the degree of rectal temperature drop and change in plasma mMCP-1 levels were different in each oral challenge. Previous studies also indicated that the extent of rectal temperature drop in an anaphylactic reaction is influenced by the number [28] and dosage [30] of the challenge agent.

Several studies have demonstrated that immune cells accumulate in the murine small intestine following epicutaneous sensitization. Two independent groups [22, 28] reported that the number of mast cells in the small intestine increased after oral antigen challenge in tape-stripping epicutaneously sensitized mice. By contrast, another group [30] reported an increased number of basophils, but not mast cells, in the jejunum after oral OVA challenge in OVA-epicutaneously sensitized mice. Such discrepancy in the behavior of intestinal immune cells might be related to the difference in experimental designs

among studies, such as the frequency of sensitizations and the timing of histological observation. In this study, although the increase in the plasma level of mMCP-1 indicates the contribution of mast cell degranulation, there was no increase in the number of intestinal mast cells. This result suggests that intestinal mast cells were activated by epicutaneous sensitization, but that this activation was suppressed in tolerance-induced mice. Furthermore, an increase of basophils was observed in the small intestine of epicutaneously sensitized mice, which was also suppressed by oral tolerance induction. Basophils are known to play an important role in the anaphylaxis reaction as effector cells in epicutaneously sensitized mice [30]. Thus, the suppression of basophilic accumulation in the small intestine of casein- and CNH-fed mice might have contributed to the observed alleviation of their anaphylaxis reaction.

The immunomodulatory effects of bovine casein or their hydrolysis peptides have been reported previously [35–37]. Moreover, a recent study indicated that ingestion of an extensively hydrolyzed casein formula reduced the level of specific IgE production and the anaphylaxis reaction in β -LG-sensitized mice [38]. Thus, our results also likely reflect this type of antigen nonspecific immunomodulatory effect. However, in contrast to previous studies [36, 38], we evaluated the serum antibodies and anaphylaxis reaction at over 40 days after the end of ingestion periods. Therefore, the suppression of immunoreaction observed in our study was considered to be largely due to antigen-specific oral tolerance.

Casein hydrolysates are widely used as ingredients in infant formula products, especially in extensively and partially hydrolyzed formulas. Extensively hydrolyzed formulas are not only considered to be hypoallergenic formulas for allergen avoidance but have also been reported to facilitate tolerance induction in children with CMA [39]. Although partially hydrolyzed formulas are not hypoallergenic, a certain portion of CMA patients can ingest partially hydrolyzed formula [40]. For such cases of CMA, partially hydrolyzed formulas may offer a therapeutic effect by inducing oral tolerance in a safe manner. In fact, oral immunotherapy using a partially hydrolyzed formula has been reported [41]. Therefore, investigations on the allergenicity or oral tolerance-inducing ability of casein hydrolysates are essential using a physiologically accurate epicutaneous sensitization model so as to more effectively translate these findings to clinical practice.

In conclusion, we demonstrated that prior ingestion of casein or its hydrolysate can induce oral tolerance and

further suppress the epicutaneous sensitization and subsequent development of systemic anaphylaxis to casein. Since casein hydrolysates are frequently applied in infant formulas given to children with a risk or history of CMA, our results may contribute to further progress in the strategies for prevention and treatment in CMA, such as oral tolerance induction in the safe and effective manner using these formulas.

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Statement of Ethics

In this study, all animal experiments were approved by the Institutional Animal Care and Use Committee of Morinaga Milk Industry Co., Ltd. certificated by Japan Health Science Foundation.

Disclosure Statement

All authors are employed by Morinaga Milk Industry Co., Ltd. and declare no conflicts of interest.

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Author Contributions

H.I., T.M., and Y.T.: were responsible for study conception and design. H.I., T.M., T.O., T.M., and M.Y.: performed experiments and analyzed data. H.I. and T.M.: drafted the manuscript. All authors read and approved the final manuscript.

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