

ORIGINAL ARTICLE

Effect of heat treatment and enzymatic digestion on the B cell epitopes of cow's milk proteins

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Clinical & Experimental Allergy

Summary

Background Processing milk leads to changes in clinical allergenicity. However, the mechanism by which heat treatment affects the allergenicity of milk proteins is not fully understood.

Objective We investigated the effect of heat treatment and enzymatic digestion on the allergenicity of B cell epitopes of milk proteins using a histamine release assay.

Methods Human basophils were passively sensitized using sera from 10 patients with allergies to cow's milk. All the patients experienced symptoms immediately after ingesting milk. The human basophils were obtained from umbilical cord blood mononuclear cells after culturing the mononuclear cells for 3–4 weeks in the presence of IL-3. After sensitization with 10% patient sera for 48 h, the cells were stimulated with untreated, heat-treated, or heat-treated and pepsin-and-trypsin-digested β -lactoglobulin or α -casein for 1 h. The histamine concentrations in the supernatants were then measured by radioimmunoassay.

Results Heat treatment alone did not alter the molecular weight of β -lactoglobulin or α -casein. Heat treatment of β -lactoglobulin significantly increased its susceptibility to enzymatic digestion in a time- and temperature-dependent manner and reduced its ability to induce histamine release from sensitized basophils. Similar findings were not observed for α -casein. The combination of heat treatment and enzymatic digestion reduced the abilities of both β -lactoglobulin and α -casein to induce histamine release from passively sensitized basophils.

Conclusions Heat treatment reduced the allergenicity of β -lactoglobulin by inducing conformational changes and by increasing its susceptibility to enzymatic digestion, both of which disrupted B cell epitopes, whereas heat treatment alone did not alter the allergenicity of α -casein.

Keywords α -casein, β -lactoglobulin, cow's milk, disulphide bond, epitope, heat treatment, histamine release, pepsin, trypsin

Submitted 13 October 2007; revised 18 November 2008; accepted 12 December 2008

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Introduction

Cow's milk allergy (CMA) affects 2.5% of children under 2 years of age, and most of their symptoms develop immediately after the ingestion of milk [1, 2] and are mediated by IgE [3]. About 80–85% of children with CMA outgrow their allergy by 3 years of age, but the remaining 15–20% do not [1, 4].

Several protein components of milk have been well characterized [5]. β -lactoglobulin occurs naturally in the

form of a 36 kDa dimer of 162-residue polypeptides, each of which contains two disulphide bonds. The four casein fractions of milk, α s1-casein, α s2-casein, β -casein, and κ -casein, comprise 32%, 10%, 28%, and 10%, respectively, of the total protein content of milk; these four proteins have minimal structural homology [5]. α s1-Casein, the major α s-casein component, contains no disulphide bonds, and has a mostly unordered structure [6]. α s-Casein has chaperone-like properties that prevent the *in vitro* thermal aggregation of both itself and other

proteins [7]. Patients with IgE antibody against casein are reported to be less likely to outgrow CMA [8–11].

Allergenic/antigenic epitopes usually consist of sequences of 8–11 amino acids [12]. Several T cell epitopes have been identified in milk proteins using synthetic peptide-induced T cell clone proliferation or cytokine production [13–16], but immediate-type allergic reactions depend on B cell epitopes [17]. Several B cell epitopes of milk proteins have been identified by ELISA inhibition [18] or Western/immunoblotting with sera from CMA patients [8–10, 19–21]. However, Western/immunoblotting using overlapping peptides cannot detect conformational B cell epitopes. Biological assays, including the basophil histamine release assay, on the other hand, can detect both conformational and linear B cell epitopes.

Processing milk leads to changes in clinical allergenicity [22–24]. During heat treatment, intermolecular-disulphide bonds are formed and also sulphydryl/disulphide bond exchange reactions occur, which are generally denoted as denaturation and aggregation [25, 26]. Such structural changes impair some conformational epitopes [27]. The effects of heat treatment on the allergenicity of milk proteins are usually determined by direct intraperitoneal injection of the proteins into experimental animals [28] or by IgE-immunoblotting with serum from CMA patients. However, because the clinical manifestations of food allergy appear after oral ingestion of the food, several processes, such as digestion, absorption [29] and mucosal immune responses, occur before the food induces an allergic reaction. No previous studies examining the influence of heat treatment on the digestion of milk proteins in association with their allergenicity of the whole B cell epitopes have been published [30].

In the present study we investigated the effect of heat treatment on the B cell epitopes of milk proteins, both before and after enzymatic digestion, by an *in vitro* histamine release assay using passively sensitized cultured human basophils.

Methods

Subjects

A total of 10 CMA patients (seven boys and three girls, age range 0–13 years old) who developed symptoms immediately (within 2 h) after ingesting cow's milk were enrolled in this study (Table 1). In our preliminary experiments using histamine release assay from passively sensitized basophils, sera from seven subjects (Patients 1–7) were reacted with β -lactoglobulin and sera from six subjects (Patients 3 and 6–10) were reacted with α -casein, including three sera (Patients 3, 6, and 7) that were reacted with both β -lactoglobulin and α -casein. Reactivity was judged as positive if the histamine release (%) exceeded the mean + 2 SD of the histamine release (%) induced by the sera from 11 control subjects who did not develop any symptoms after ingesting milk and whose RAST scores for milk were ≤ 2 . The titres of antigen-specific IgE antibody to cow's milk proteins were measured using phadebas RAST (Pharmacia Biotech, Uppsala, Sweden). Phadebas RAST has been reported to be slightly less sensitive than the Pharmacia CAP System [31], but every patient enrolled in this study had a very high titre of specific IgE against milk proteins [32]. This study was approved by the regional Ethics Committees.

Milk proteins

β -lactoglobulin from bovine milk (L3908) and α -casein from bovine milk (C6780) were purchased from Sigma (St. Louis, MO, USA). This α -casein preparation contains both α s1- and α s2-casein.

Untreated β -lactoglobulin and α -casein solutions were prepared by suspending the proteins in distilled water at a concentration of 1 mg/mL. Heat treated β -lactoglobulin and α -casein solutions were prepared by placing the same solutions in 15 mL conical tubes with lids to eliminate evaporation and placing them in a hot water bath at 80 °C

Table 1. Clinical characteristics of the CMA patients

Patient, ID#	Sex	Age (years)	Total-IgE (IU/mL)	Anti-milk IgE (RAST) (UA/mL)	Score	Clinical symptoms	Allergen*	
							β -LG	α -Casein
1.	M	2	700	12.1	3	Urticaria, erythema	Yes	No
2.	M	1	1940	> 100	6	Erythema	Yes	No
3.	F	0	906	52.1	5	Urticaria	Yes	Yes
4.	M	0	839	> 100	6	Erythema	Yes	No
5.	M	3	727	10.2	3	Urticaria, erythema	Yes	No
6.	F	13	3580	> 100	6	Vomiting	Yes	Yes
7.	M	0	1249	35.9	4	Erythema	Yes	Yes
8.	M	4	3980	> 100	6	Urticaria	No	Yes
9.	M	3	1087	35.9	4	Urticaria, erythema	No	Yes
10.	F	10	3180	> 100	6	Wheezing, erythema, anaphylaxis	No	Yes

*Serum reactivity to untreated β -lactoglobulin (β -LG) or α -casein was determined by a histamine release assay with passively sensitized basophils (see text).

or 100 °C for 15, 60, or 120 min. Under these experimental conditions, no obvious aggregation was observed in any of the samples, even after placing them in a hot water bath at 100 °C for 120 min.

Pepsin digestion was performed using the method described by Kato et al. [33] with slight modification. In brief, after heat treatment, the solutions were adjusted to pH 3.0 with 10 M HCl (Nakarai Tesque, Tokyo, Japan). The α -casein solution was pre-incubated at 37.0 °C for 10 min, and the β -lactoglobulin solution was pre-incubated at 37.0 °C for 30 min before pepsin digestion. α -casein was treated with 1% (v/v) pepsin (Pepsin A; Sigma) by gentle stirring at 37.0 °C for 30 min, and β -lactoglobulin was treated with 1% (v/v) pepsin (Pepsin A; Sigma) by gentle stirring at 37.0 °C for 90 min. The solutions were then adjusted to pH 8.0 with 10 M NaOH (Nakarai Tesque) to stop the reaction.

The pepsin-digested protein solutions were further digested with 1% (v/v) trypsin (Trypsin type III; Sigma) for 1 h at 37.0 °C.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

SDS-PAGE was performed using an automatic SDS-PAGE system (Phast System; Amersham Bioscience Corp. Piscataway, NJ, USA). The concentrations of the stacking gel and the gradient running gels (8–25% SDS gradient gel; Pharmacia Biotech) were 6% and 8–25%, respectively. Before performing the electrophoretic run, the protein solutions were suspended in sample buffer [2% SDS, 2% 2-mercaptoethanol, 20% glycerol, 31 mM Tris-HCl (pH 6.8), and 0.001% (wt/vol) bromophenol blue] and heated for 10 min at 100 °C. After the electrophoretic run, the gels were dyed using a silver staining kit (Phast System, Pharmacia Biotech). The densities of the protein bands were analyzed using NIH Image software after scanning with a film scanner (Quick Scan 35, Minolta, Tokyo, Japan). The same experiments were performed two or three times, and the average densities were calculated.

Human cord blood-derived basophils

Human umbilical cord blood-derived basophils were obtained as described by Ebisawa et al. [34] with slight modifications as follows. Cord blood was collected after normal full-term deliveries, and mononuclear cells were obtained from the cord blood cells by density centrifugation with lymphocyte separating medium (LSM; Organon Teknika Corp., Durham, NC, USA); the cells were then suspended at a cell density of 5×10^5 cells/mL in Media-1 (IBL, Gunma, Japan; a modified DMEM component containing 10 μ g/mL insulin, 10 μ g/mL transferin, 5×10^{-5} M 2-mercaptoethanol, 25 mM HEPES, and 2.6 ng/mL NaSeO₃) supplemented with 10% FCS (Biowhittaker,

Walkersville, MD, USA), 500 U/mL penicillin (Meiji Seika, Tokyo Japan), and 50 mg/mL streptomycin. The cells were incubated in the presence of 5 ng/mL human recombinant IL-3 (Kirin, Tokyo, Japan) for 4 weeks. The purity and viability of the human cord blood-derived basophils were assessed using May-Grunwald Giemsa staining and the trypan blue dye exclusion test and were 30–40% and >99%, respectively.

Histamine release assay

The histamine release assay was performed according to the protocol described by Weyer et al. [35] with some modifications as follows. For passive sensitization, cultured basophils were washed with phosphate buffer saline (PBS, Nissui, Tokyo, Japan), and suspended in culture medium containing 10% serum from a CMA patient and incubated for 48 h. After washing with PBS, the sensitized basophils were suspended in Tyrode's solution (pH 7.4) containing 124 mM NaCl, 4 mM KCl, 0.64 mM NaH₂PO₄, 1 mM CaCl₂, 0.6 mM MgCl₂, 10 mM HEPES, and 0.03% human serum albumin (Sigma). The cells were then pre-warmed for 15 min at 37.0 °C and challenged with 0.1, 1, 10, or 100 μ g/mL of untreated, heat treated, or heat treated and digested β -lactoglobulin or α -casein solution for 1 h.

The histamine concentration in the supernatant was measured using a radioimmunoassay (RIA) kit (Histamine kit, Eiken, Tokyo, Japan). The following formula was used to calculate histamine release (%):

$$\text{Histamine release (\%)} = \frac{(\text{actual release} - \text{spontaneous release})}{\times 100 / \text{total histamine content}}$$

where total histamine content was determined by freezing and thawing the cell suspension three times. All the experiments were performed in duplicate and the mean values are shown in the figures.

Heat/digestion was judged to have a positive effect if the histamine release (%) was reduced to <50% of the histamine release (%) in response to untreated milk proteins.

Statistics

All data are presented as the mean $\pm$ SEM, unless otherwise noted. Differences between groups were analyzed using the Mann-Whitney *U*-test, and the results were considered significant if *P* was <0.05.

Results

SDS-PAGE analysis of milk proteins after heat treatment and digestion

The milk protein solutions were subjected to SDS-PAGE analysis after heat treatment and digestion. Regardless of the incubation time or the incubation temperature, heat

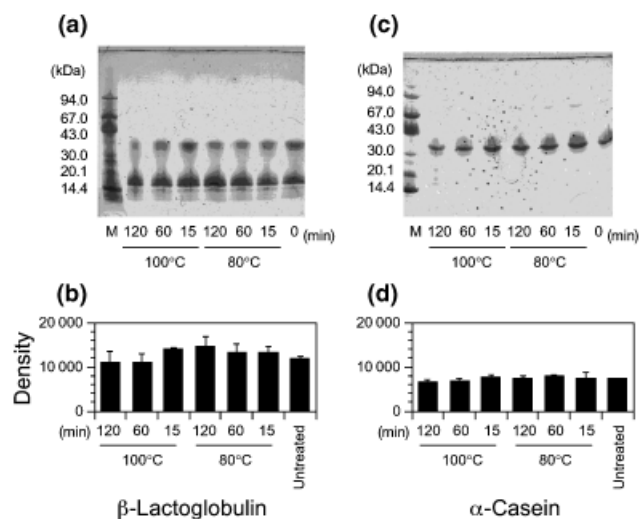


Fig. 1. Effect of heat treatment on β -lactoglobulin and α -casein. (a) SDS-PAGE analysis of β -lactoglobulin treated at 80 °C or 100 °C for 15, 60, or 120 min. The figure shows a representative result of three separate experiments with similar results. M, molecular weight marker. (b) Densitometric analysis of the density of each protein band at 18 kDa shown in (a). The data shown are the mean $\pm$ SEM of three separate experiments. (c) SDS-PAGE analysis of α -casein treated at 80 °C or 100 °C for 15, 60, or 120 min. The figure shows a representative result of three separate experiments with similar results. (d) Densitometric analysis of the density of each protein band at 34 kDa shown in (c). The data shown are the mean $\pm$ SEM of three separate experiments.

treatment alone did not alter the density or molecular weight of the protein bands of either β -lactoglobulin or α -casein (Fig. 1). Incubation of the protein solutions at 37.0 °C for 10 or 30 min at pH 3.0 also did not alter the density of the protein bands (data not shown).

Non-heat-treated β -lactoglobulin was resistant to enzymatic digestion. Before heat treatment of β -lactoglobulin significantly reduced the density of enzymatically digested-protein bands in a time- and temperature-dependent manner (Figs 2a and b, $P < 0.001$). In contrast, α -casein was completely digested by pepsin in a shorter time period (30 min), even when the solution had not been heat treated (Figs 2c and d).

The additional treatment of β -lactoglobulin with trypsin for up to 2 h after heat treatment and pepsin digestion did not digest the protein completely unless heat treatment had been performed before the pepsin digestion (Fig. 3). The density of the protein bands after trypsin digestion correlated with that of the protein bands after heat treatment and pepsin digestion (data not shown).

Histamine release assay

First, we determined the histamine release from passively sensitized cultured human basophils using four different concentrations (0.1, 1, 10, and 100 μ g/mL) of untreated

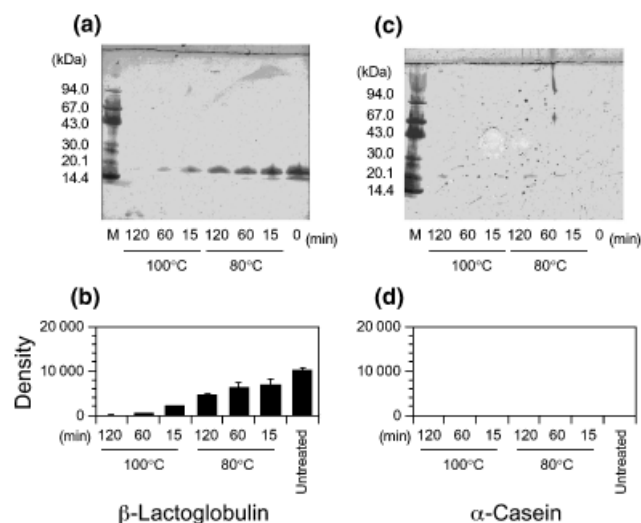


Fig. 2. Effect of heat treatment on the susceptibility of β -lactoglobulin and α -casein to digestion by pepsin. (a) SDS-PAGE analysis of β -lactoglobulin digested with pepsin for 90 min after heat treatment at 80 °C or 100 °C for 15, 60, or 120 min. The figure shows a representative result of three separate experiments with similar results. M, molecular weight marker. (b) Densitometric analysis of the density of each protein band at 18 kDa shown in (a). The data shown are the mean $\pm$ SEM of three separate experiments. $P < 0.001$. (c) SDS-PAGE analysis of α -casein digested with pepsin for 30 min after heat treatment at 80 °C or 100 °C for 15, 60, or 120 min. The figure shows a representative result of three separate experiments with similar results. (d) Densitometric analysis of the density of each protein band at 34 kDa shown in (c). The data shown are the mean $\pm$ SEM of three separate experiments.

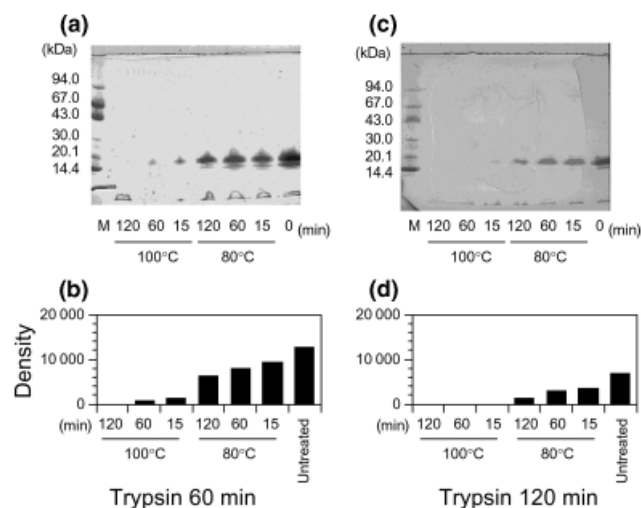


Fig. 3. Effect of heat treatment on the susceptibility of β -lactoglobulin to digestion by trypsin. SDS-PAGE analysis of β -lactoglobulin digested with trypsin for 60 min (a and b) or 120 min (c and d) after heat treatment at 80 °C or 100 °C for 15, 60, or 120 min followed by pepsin digestion for 90 min. (a and b) The figures show representative results of two separate experiments with similar results. M, molecular weight marker. (b and d) Densitometric analysis of the density of each protein band at 18 kDa shown in (a) or (c). The data shown are from one of two separate experiments with similar results.

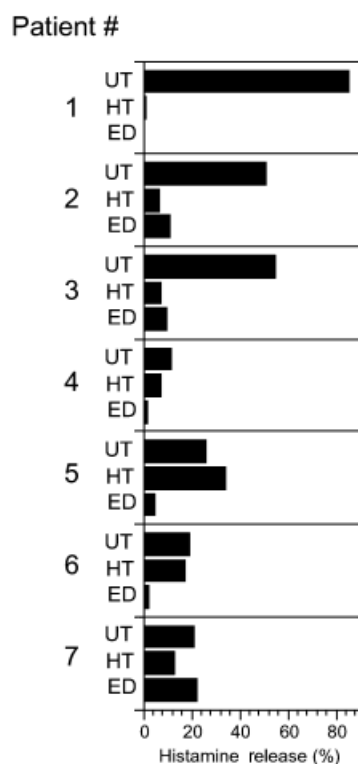


Fig. 4. Histamine release from passively sensitized cultured basophils after stimulation with β -lactoglobulin. The numbers (1–7) are the patient ID numbers shown in Table 1. Human cord blood-derived basophils were sensitized with patient sera for 48 h in the presence of 5 ng/mL of IL-3 and were then stimulated with untreated (UT), heat-treated (HT), or heat-treated and enzymatically digested (ED) β -lactoglobulin for 1 h. The histamine concentration in the supernatant was measured by RIA. The histamine release assay was performed in duplicate, and the mean values are shown.

β -lactoglobulin or α -casein and measuring the histamine concentration in the supernatant. The results showed that the concentration of the milk proteins that induced the greatest histamine release from basophils passively sensitized with the serum of an individual subject varied from subject to subject. We therefore selected one of the four concentrations at which the untreated milk proteins induced the greatest histamine release, and determined the effect of heat treatment and digestion using the results generated from that single concentration of milk proteins. The averages of histamine release (%) in duplicate experiments are shown in Figs 4 and 5.

A range of 11–85% of the total histamine content of the basophils was released from passively sensitized basophils after stimulation with the optimal concentration of untreated β -lactoglobulin (Fig. 4). Heat treatment of β -lactoglobulin reduced histamine release from basophils sensitized with the sera of three of seven patients with β -lactoglobulin allergy (Fig. 4, Patients 1, 2, and 3). Enzymatic digestion of β -lactoglobulin after heat treatment reduced histamine release from basophils sensitized with the sera of three of the remaining four patients with

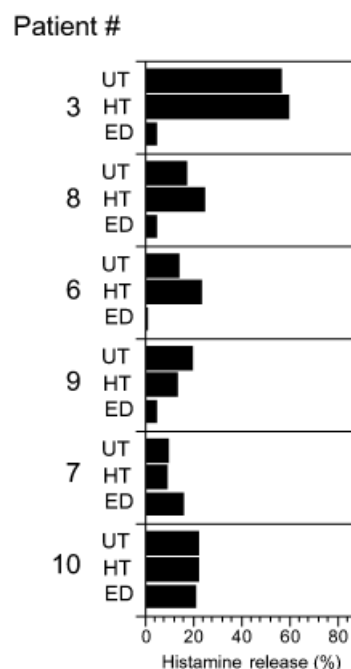


Fig. 5. Histamine release from passively sensitized cultured basophils after stimulation with α -casein. The numbers (3–10) are the patient ID numbers shown in Table 1. Human cord blood-derived basophils were sensitized with patient sera for 48 h in the presence of 5 ng/mL of IL-3 and were then stimulated with untreated (UT), heat-treated (HT), or heat-treated and enzymatically digested (ED) α -casein for 1 h. The histamine concentration in the supernatant was measured by RIA. The histamine release assay was performed in duplicate and the mean values are shown.

β -lactoglobulin allergy (Fig. 4, Patients 4, 5, and 6). Neither heat treatment nor a combination of heat treatment and enzymatic digestion of β -lactoglobulin reduced histamine release from basophils passively sensitized with serum from the remaining patient (Fig. 4, Patient 7).

A range of 9–57% of the total histamine content of the basophils was released from passively sensitized basophils after stimulation with the optimal concentration of untreated α -casein (Fig. 5). In contrast to β -lactoglobulin, heat treatment of α -casein *per se* did not reduce histamine release from basophils passively sensitized with sera from any of the patients with α -casein allergy. Enzymatic digestion of α -casein after heat treatment reduced histamine release from basophils passively sensitized with sera from four of six patients with α -casein allergy (Fig. 5, Patients 3, 8, 6, and 9). Neither heat treatment nor a combination of heat treatment and enzymatic digestion of α -casein reduced histamine release from basophils passively sensitized with sera from the remaining two patients.

Discussion

When food is ingested, intestinal enzymes cleave proteins or polypeptides to enable absorption by the gut. At the

same time, this enzymatic cleavage disrupts B cell epitopes and decreases their allergenicity [36, 37]. Among several *in vitro* assays investigating B cell epitopes, immunoblotting analyses of overlapping peptides provide information about linear B cell epitopes but not about conformational B cell epitopes; histamine release assays using passively sensitized basophils, on the other hand, provide information about both conformational and linear B cell epitopes.

In the present study, we investigated the effect of heat treatment and enzymatic digestion on the B cell epitopes of milk proteins by a histamine release assay using passively sensitized cultured human basophils. We did not use the subjects' own peripheral blood basophils for two reasons. First, the histamine release assay requires a large amount of blood (at least 0.5 mL/assay), which is not always available, especially if the subjects are small children; and second, peripheral blood basophils are often primed *in vivo*, making the assay susceptible to influence by the clinical conditions. To standardize the assay and ensure better reproducibility, we chose to use cultured basophils to assess the allergenicity of the milk proteins.

According to the results of the SDS-PAGE analysis, heat treatment alone did not alter the molecular weight of β -lactoglobulin (Figs 1a and b). However, basophils passively sensitized with sera from some CMA patients (Fig. 4) released less histamine in response to heat-treated β -lactoglobulin than in response to untreated β -lactoglobulin. Because heat treatment is known to form intermolecular-disulphide bonds and sulphhydryl/disulphide bond exchanges in β -lactoglobulin [25, 26], heat treatment is assumed to induce conformational changes in the B cell epitopes of β -lactoglobulin, reducing its allergenicity for some patients.

In the subsequent digestion experiments, β -lactoglobulin was found to be relatively resistant to digestion by pepsin in our experimental system unless heat treatment had been performed before pepsin digestion (Figs 2a and b), confirming the results of previous studies [38–40]. Before heat treatment increased the susceptibility of β -lactoglobulin to digestion in a time- and temperature-dependent manner (Figs 2a and b). The increased susceptibility was also presumably caused by intermolecular and intramolecular conformational changes in β -lactoglobulin [25, 26]. In addition, basophils passively sensitized with sera from some CMA patients (Fig. 4) released less histamine in response to heat-treated and enzymatically digested β -lactoglobulin than in response to untreated β -lactoglobulin. However, the allergenicity of β -lactoglobulin for one patient persisted even after heat treatment and digestion with pepsin and trypsin (Fig. 4). Our study showed that before heat treatment was essential for enzymatic digestion to take place (Figs 3a–d).

The results of the histamine release assays and SDS-PAGE analysis suggested that heat treatment impairs B

cell epitopes of β -lactoglobulin by at least two distinct mechanisms: by directly causing intermolecular and intramolecular conformational changes [25, 26] that disrupt the conformational B cell epitopes, and by increasing susceptibility to digestion and facilitating the enzymatic cleavage of protein sequences that disrupts linear B cell epitopes. Our findings well explain the clinical observation that the heat treatment of milk or dairy products reduces the clinical incidence of food-induced allergic reactions in some but not all CMA patients [22, 23].

Heat treatment did not alter the molecular weight of α -casein (Figs 1c and d). In sharp contrast to β -lactoglobulin, heat treatment of α -casein alone did not influence histamine release from basophils passively sensitized with sera from any of the patients with α -casein allergy (Fig. 5). Heat treatment is known to cause intermolecular and intramolecular exchanges in β -lactoglobulin [25, 26], but α s1-casein – the major component of the α s-casein preparation we used – lacks disulphide bonds [7]. Therefore, heat treatment alone did not induce conformational changes in α -casein, and this is presumably at least partly why the heat treatment did not reduce the allergenicity of α -casein in any of the patients. On the other hand, α -casein was highly susceptible to enzymatic digestion by pepsin even before heat treatment. The SDS-PAGE analysis showed that incubation with pepsin for only 30 min was sufficient to digest all the protein bands of both untreated and heat treated α -casein (Figs 2c and d), thereby confirming the digestion pattern of milk proteins *in vivo* [41]. In addition, we did not detect any increase in the susceptibility of α -casein to enzymatic digestion as a result of heat treatment because α -casein was already highly susceptible to enzymatic digestion *in vitro*.

However, basophils passively sensitized with serum from some CMA patients (Fig. 5) released less histamine in response to heat-treated and enzymatically digested α -casein than in response to untreated α -casein. These results confirmed previous reports that most IgE antibodies against α s1-casein bind to linear epitopes and that only a minority are directed at conformational epitopes [14, 19]. Therefore, the presence of disulphide bonds in the protein may be a critical factor governing the susceptibility to heat and digestion.

Skin tests or RAST provide important information for predicting the development of clinical manifestations upon the ingestion of a food [32]; however, the results do not always correlate well with the clinical manifestations after food ingestion [42–44]. Our findings suggest that new solid phase immunosolvent assays or *in vitro* histamine release assays using heat treated and/or digested food proteins may provide further information on food hypersensitivity, especially in children with IgE specific to food proteins. In fact, the use of enzyme-digested egg proteins in patients with egg allergy is useful for predicting clinical symptoms or their resolution [45, 46]. Using

heat treatment in addition to enzymatic digestion may improve the diagnostic value of this kind of assay.

The presence of anti-casein IgE antibody in the serum, but not of anti- β -lactoglobulin IgE antibody, reportedly predicts the persistence of overt symptoms [8–11]. In addition, the presence of IgE antibody against linear epitopes in the serum has been reported to be a marker of the persistence of overt symptoms in CMA patients [21]. Our findings imply the possible involvement of disulphide bonds in the allergen (i.e. susceptibility to enzymatic digestion) in the persistence of overt symptoms. The testing of sera from both patients with persistent allergy and patients who have outgrown their allergy is needed to determine whether disulphide bonds in the allergen are involved or not.

Commercially available milk preparations are usually pasteurized. A previous study showed that pasteurized milk retained its ability to induce an anaphylactic reaction in a mouse model of food hypersensitivity [47]. However, several different protocols for pasteurizing milk exist, including protocols that involve the use of low or very high temperatures with or without high pressure. Whether any of these protocols alters susceptibility to enzymatic digestion, leading to a reduction in the allergenicity of β -lactoglobulin should be investigated.

Most foods contain many different protein components. Cow's milk is known to contain β -lactoglobulin, four caseins, lactoferrin, and lactalbumin [48]; some of these proteins have been reported to interact with each other [7, 49], and such an interaction affects the heat treatment-induced intermolecular and intramolecular changes [50]. In addition, heat treatment may generate new antigenic epitopes in some of these proteins [51], and a previous study indicated the existence of a complicated heterogeneity among milk proteins that is regulated by genetic polymorphisms [48]. Thus, the heat treatment of whole milk preparations should be further tested for the effect of heat treatment and digestion on the allergenicity of milk proteins in the future.

Acknowledgements

We thank Dr Jonathan Batchlor (the Division of Allergy of the National Center for Child Health and Development) for critical review of the manuscript. We also thank Dr Kazuko Tanaka (the Department of Allergy and Immunology of the National Research Institute for Child Health and Development) and Dr Akira Akasawa (the Department of Interdisciplinary Medicine of the National Center for Child Health and Development) for their helpful suggestions. This work was supported in part by grants from the Morinagahoushi-kai and National Institute of Biomedical Innovation (ID05-24), and by a Grant-in-Aid for Young Scientist to Y. M. from the Ministry of Education, Culture, Sports, Science and Technology (MEXT).

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