

Original article

Pasteurization of milk proteins promotes allergic sensitization by enhancing uptake through Peyer's patches

Background: The underlying mechanisms responsible for allergic sensitization to food proteins remain elusive. To investigate the intrinsic properties (as well as the effect of pasteurization) of the milk proteins α -lactalbumin, β -lactoglobulin and casein that promote the induction of milk allergy.

Methods: Alteration of structure and immune-reactivity of native and pasteurized proteins was assessed by gel filtration and ELISA. Uptake of these proteins was compared *in vitro* and *in vivo*. The biological effect was assessed by orally sensitizing C3H/HeJ mice with milk proteins followed by a graded oral challenge. Required dose to induce anaphylaxis, symptoms and mean body temperature was recorded. Antigen-specific antibodies and cytokine production by splenocytes were analyzed.

Results: Soluble β -lactoglobulin and α -lactalbumin but not insoluble casein were readily transcytosed through enterocytes *in vitro* and *in vivo*. Pasteurization caused aggregation of β -lactoglobulin and α -lactalbumin inhibiting uptake by intestinal epithelial cells *in vitro* and *in vivo*. Furthermore, aggregation redirected uptake to Peyer's patches, which promoted significantly higher Th2-associated antibody and cytokine production in mice than their native counterparts. Despite this only the soluble forms of β -lactoglobulin and α -lactalbumin elicited anaphylaxis (following priming) when allergens were administered orally. Aggregated β -lactoglobulin and α -lactalbumin as well as casein required systemic administration to induce anaphylaxis.

Conclusions: These results indicate that triggering of an anaphylactic response requires two phases (1) sensitization by aggregates through Peyer's patches and (2) efficient transfer of soluble protein across the epithelial barrier. As the majority of common food allergens tend to form aggregates, this may be of clinical importance.

F. Roth-Walter¹, M. C. Berin²,
P. Arnaboldi¹, C. R. Escalante³,
S. Dahan¹, J. Rauch¹, E. Jensen-
Jarolim⁴, L. Mayer¹

¹Center of Immunobiology; ²Division of Pediatric Allergy and Immunology; ³Structural Biology Program, Department of Physiology and Biophysics, Mount Sinai School of Medicine, New York, NY, USA; ⁴Center of Physiology and Pathophysiology, Medical University of Vienna, Vienna, Austria

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Lloyd Mayer, MD
Center of Immunobiology
Mount Sinai School of Medicine
One Gustave L. Levy Place
New York, NY 10029
USA

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The fundamental question 'what makes an antigen a food allergen' remains largely unresolved, even though food allergies are increasing at an alarming rate in the industrialized world affecting 4–8% of children and about 1–2% of the adult population (1, 2).

Noteworthy, only a minority of the over 10 000 ingested proteins are able to elicit allergy (3) and usually share common features like their stability to heat, acid and protease digestion. Most of them are glycosylated and bind to lipids and fatty acids. Still not every protein with these characteristics is an allergen (3–5). It is still uncertain, which shared structural, functional or biochemical features explain their allergenic potential, although a large number of allergens have been characterized and considerable efforts have been made using bioinformatics(6).

However, the major drawback in using bioinformatics is that its information relies heavily on the crystallization of native proteins. Given the fact that most food proteins

undergo some form of processing before consumption and later in the degradative environment of the gut, it is unlikely that the native structure of food proteins is maintained.

Indeed, dry roasting had already been shown to increase the allergenicity of peanuts (7) by reducing the solubility of the peanut proteins (7) and an increased fat content in food has been associated with increased allergenicity (8).

Our study sought to define the general characteristics of food allergens that are important for both the sensitization process and the elicitation of an allergic response. We studied the well-characterized milk allergens, α -lactalbumin (ALA), β -lactoglobulin (BLG) and casein (CAS) in a murine model of anaphylaxis.

Furthermore, to assess these processes in the context of Ag-sampling in the gut we changed the physical properties of the Ags by pasteurization. Pasteurization leads to aggregation of both whey proteins, ALA and BLG and

already has been extensively reported in the literature (9–12). Indeed, up to 90% of BLG has been shown to be aggregated in processed milk (9) and ALA denatures readily at temperatures of around 64°C (12). In contrast, CAS naturally forms micelles, and exists therefore as an aggregate. Heat treatment does not alter this.

As milk and its products are mainly consumed in a pasteurized form, we evaluated the impact of pasteurization in milk allergy.

Methods

Mice

Three to four-week-old female C3H/HeJ (TLR4 signaling deficient) mice were purchased from the National Cancer Institute (Frederick, MD). Animals were kept under specific pathogen-free conditions in filter-top cages and were placed on a special milk-free diet. All procedures were approved by the Mount Sinai School of Medicine Institutional Animal Care and Use Committee, following the guidelines outlined in the 'Guide for the Care and Use of Laboratory Animals'.

Pasteurization of milk proteins

According to standardized guidelines, food is considered pasteurized when heated for more than 30 min at 63°C or for more than 17 s at 72°C (13, 14). To ensure thorough pasteurization the milk proteins ALA, BLG and casein (10 mg/ml; Sigma, St. Louis, MO, USA) were pasteurized by incubation for 33 min at 63°C and 2 min at 72°C under constant agitation. As control, proteins were also 'cooked' at 95°C for more than 30 min. Antigenicity of treated/untreated allergens were tested by ELISA. Briefly, allergens were coated on to micro-plates, blocked, incubated with serum from ALA, BLG or CAS i.p.-sensitized mice and detected with horse-radish-peroxidase-labeled rabbit anti-mouse IgG1 and TMB as substrate. Optical density was measured at 450 nm.

Sephadex G200

Two hundred microlitres of 10 mg/ml of native or pasteurized milk proteins (ALA, BLG and CAS) in PBS was applied onto a Sephadex G-200 (Pharmacia, Sweden) column and eluted samples monitored at 280 nm.

FITC-labeling of milk proteins

FITC was added under constant stirring to ALA, BLG or CAS according to the manufacturer's instruction (Sigma, St. Louis, MO, USA). Labeled proteins were purified from unconjugated FITC by gel-filtration and subsequent overnight dialysis. Aliquots of FITC-labeled milk proteins were further pasteurized.

Transcytosis studies

For transcytosis studies, Caco-2 cells (American Type Culture Collection, Rockville, MD, USA) were grown on 6.5-mm transwell filter inserts for 14–21 days. Only transwells with a transepithelial resistance $>1000 \Omega \text{ cm}^2$ were used for further studies. After washing with PBS, 200 μg native or pasteurized FITC-labeled ALA,

BLG or CAS was added on the apical side and incubated for 90 min at 37°C/5% CO_2 . Subsequently, aliquots of basal side of filters were collected and measured fluorometrically at 485/535 nm. Transcytosed proteins were quantified using standard curves of native/pasteurized FITC-labeled ALA, BLG and CAS, respectively.

In vivo uptake studies

Three to five-week-old C3H/HeJ mice were fed with 400 μl of native or pasteurized FITC-labeled protein (ALA, BLG, or CAS, 10 mg/ml). After 15 min, mice were anaesthetized and killed by cervical dislocation. The mid-small intestine containing Peyer's patches (PPs) was removed, washed, embedded in OCT (VWR, West Chester, PA, USA) and frozen with isopentane. Then 8- μm sections were fixed, counterstained with Hoechst dye (Molecular Probes, Carlsbad, CA, USA), and mounted with Mounting medium (Vector, Burlingame, CA, USA). Subsequently, images were obtained using a Zeiss Axioplan 2 fluorescence microscope.

Sensitization and oral challenge of mice

Three-week-old C3H/HeJ mice were sensitized orally with 1 mg ALA, BLG, or CAS in 0.2 M bicarbonate buffer plus 10 μg cholera toxin (CT; List Biologicals, Campbell, CA, USA) on days 0, 7, 14, 21, 28 and 35 by gavage. On day 42, a graded challenge study was performed. Mice were fed sequentially higher doses of allergens (5, 10, 20, and 50 mg without CT) 20 min apart, followed by 50 μg of allergen intraperitoneally (i.p.). Challenges were stopped after the first dose that elicited symptoms of anaphylaxis.

Anaphylaxis score

Symptoms were scored as follows: 0, no symptoms; 1, scratching around nose and head; 2, puffiness around eyes and mouth; 3, wheezing, labored respiration, cyanosis around mouth and tail; 4, no activity after prodding, or tremor and convulsion (13). Rectal temperature was measured as a further assessment of anaphylaxis.

Measurement of antigen-specific immunoglobulins

ALA, BLG, and CAS-specific IgG1, IgG2a, IgE, and IgA were measured by ELISA. Briefly, pasteurized or native milk proteins (ALA, BLG, or CAS, 10 μg /well) or serial dilutions of mouse IgG1, IgG2a, IgE, and IgA standards were coated, blocked with 10% FCS in PBS, and incubated with diluted sera (1 : 25 000 for IgG1; 1 : 5000 for IgG2a; 1 : 100 for IgA and 1 : 10 for IgE). Detection was performed by peroxidase-labeled polyclonal rabbit anti-mouse IgG1 or IgG2a, monoclonal rat anti-mouse IgE followed by polyclonal peroxidase-labeled goat anti-rat IgG and polyclonal rabbit anti-mouse IgA followed by polyclonal peroxidase-labeled goat anti-rabbit IgG, respectively (all antibodies from Zymed, South San Francisco, CA, USA). Tetramethylbenzidine (BD Biosciences, San Jose, CA, USA) was used as substrate. The reaction was stopped with 1.8 M sulfuric acid and detected at 450 nm.

In vitro cytokine responses

Splenocytes were plated at a density of 5×10^6 cells/ml in 48-well cell culture plates (Nalge Nunc, Naperville, IL, USA) and cultured with 50 μg /ml of ALA, BLG, and CAS proteins, respectively, 2.5 μg /ml concanavalin A (Sigma), or medium alone for 72 h at

37°C/5% CO₂. Subsequently, cytokines of cultured supernatants were measured by ELISA (eBiosciences, San Diego, CA, USA) according to the manufacturer's instructions.

Statistics

Differences between native and pasteurized groups were analyzed either by non-parametric Mann-Whitney *U*-test or independent *t*-test when appropriate. The data were expressed as mean $\pm$ SEM. A value of $P < 0.05$ was considered significant.

Results

Soluble ALA and BLG induce anaphylaxis

Mice were sensitized orally to milk proteins ALA, BLG, and CAS with CT as described in the *Methods* sections. Following oral challenge with the soluble whey proteins ALA and BLG, the majority of ALA- and BLG-sensitized mice developed significant anaphylactic responses after the 20 mg dose. In contrast, CAS-sensitized mice did not manifest anaphylaxis until the allergen was administered systemically (i.p.). In all sensitized mice, symptoms were severe and accompanied by a significant drop in body temperature. The dose required eliciting anaphylaxis, symptom scores, and body temperature are summarized in Fig. 1. Interestingly, the induction of antibody as well as cytokines did not correlate with the ability of the milk proteins to induce anaphylaxis as summarized in Table 1. Mice orally sensitized to any of the three native milk allergens generated a significant IgE and IgG1 response in the serum, and a detectable level of secreted antigen-specific IgE and IgA in intestinal lavage samples. Despite the fact that the soluble whey proteins (ALA and BLG) induced the strongest anaphylactic responses, CAS was the most immunogenic of the native milk proteins, inducing significantly higher antibody responses than the whey proteins.

Uptake of ALA and BLG occurs mainly through enterocytes, whereas the main entrance for CAS is Peyer's patches

In order to determine the underlying mechanisms responsible for the dichotomy between the strength of the IgE response and the ability to elicit an anaphylactic response, we contrasted the intestinal transepithelial uptake of the soluble whey proteins, ALA and BLG, with the micelle-forming CAS *in vitro* and *in vivo*. In polarized Caco-2 cells BLG (7.4 ± 1.1 μ g/ml) transcytosed more efficiently than the other proteins (3.4 ± 0.5 μ g/ml ALA and 1.2 ± 0.3 μ g/ml CAS) showing that uptake differed significantly between allergens, despite their similar molecular weight (Fig. 2A). As expected, transcytosis of CAS (present as micelles) across Caco-2-monolayers was the lowest of the milk proteins.

Uptake of FITC-labeled milk proteins was also evaluated *in vivo*. ALA and BLG were readily detected in the

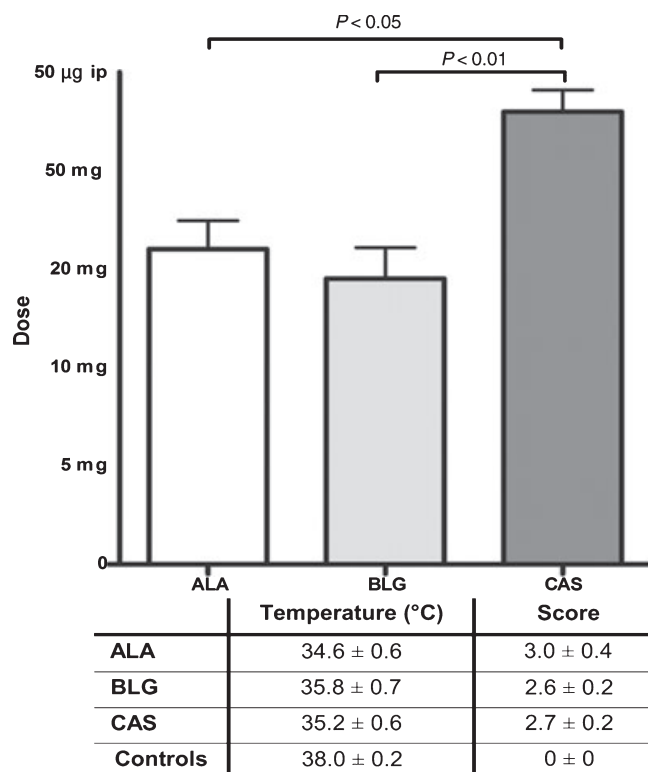


Figure 1. Dose, temperature and anaphylaxis score in orally sensitized mice challenged with native ALA, BLG or CAS. Mice were fed sequentially higher doses of allergens (5, 10, 20 and 50 mg) 20 min apart, followed by 50 μ g allergen i.p. Challenges were stopped at the first dose that elicited symptoms. Symptoms and body temperature were taken as described in *Methods*.

lamina propria 15 min after ingestion. Similar to the *in vitro* findings, BLG exhibited the greatest degree of transcytosis, while CAS uptake was rarely observed through absorptive epithelial cells after feeding (Fig. 2B).

Given the robust induction of an IgE response by CAS we looked for other sites of uptake. As shown in Fig. 2C, all three pasteurized milk proteins were detected cell associated in Peyer's patches (PPs) 15 min post-feeding. However, the main difference between the soluble whey proteins, ALA and BLG, and the micelle-forming CAS was that the vast majority of the soluble whey proteins were taken up by absorptive epithelial cells. In contrast, the formation of micelles in CAS promoted uptake through PPs.

Impact of pasteurization on milk allergens

The data described above suggest that the form of the Ag could dictate the site of uptake and alter the nature of the immune response. We therefore elected to change the physical properties of the soluble whey proteins, ALA and BLG, without altering their allergenic potential, to investigate the impact of aggregation in their potential to

Table 1. Antibody and cytokine levels of mice orally sensitized to native or pasteurized ALA, BLG or CAS

	ALA	ALA past	BLG	BLG past	CAS	CAS past
Serum ($\mu\text{g/ml}$)						
IgG1	1084 $\pm$ 244	1818 $\pm$ 133*	633 $\pm$ 176	2196 $\pm$ 212***	1231 $\pm$ 293	1337 $\pm$ 320
IgG2a	62 $\pm$ 18	183 $\pm$ 68	18 $\pm$ 6	840 $\pm$ 360*	137 $\pm$ 59	98 $\pm$ 37
IgE	40 $\pm$ 4	60 $\pm$ 6*	25 $\pm$ 4	61 $\pm$ 5***	51 $\pm$ 6	54 $\pm$ 8
IgA	8 $\pm$ 11	9 $\pm$ 1	4 $\pm$ 1	18 $\pm$ 2	12 $\pm$ 3	9 $\pm$ 2
Intestinal lavage (ng/ml)						
IgE	20 $\pm$ 4	34 $\pm$ 4*	10 $\pm$ 3	98 $\pm$ 16***	71 $\pm$ 22	85 $\pm$ 27
IgA	111 $\pm$ 14	165 $\pm$ 39	63 $\pm$ 18	350 $\pm$ 48***	230 $\pm$ 46	176 $\pm$ 35
Splenocytes (pg/ml)						
IL5	8 $\pm$ 3	20 $\pm$ 7	4 $\pm$ 3	34 $\pm$ 11*	58 $\pm$ 22	64 $\pm$ 15
IL13	116 $\pm$ 73	707 $\pm$ 286	45 $\pm$ 19	376 $\pm$ 118*	773 $\pm$ 164	838 $\pm$ 104
IL10	140 $\pm$ 54	268 $\pm$ 88	135 $\pm$ 58	455 $\pm$ 148	583 $\pm$ 144	550 $\pm$ 92
INF- γ	123 $\pm$ 56	627 $\pm$ 151**	124 $\pm$ 48	349 $\pm$ 86*	1801 $\pm$ 137	2372 $\pm$ 292

($n = 10/\text{group}$) $\pm$ SEM; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.0001$ compared with native counterparts. ALA, α -lactalbumin; BLG, β -lactoglobulin; CAS, casein.

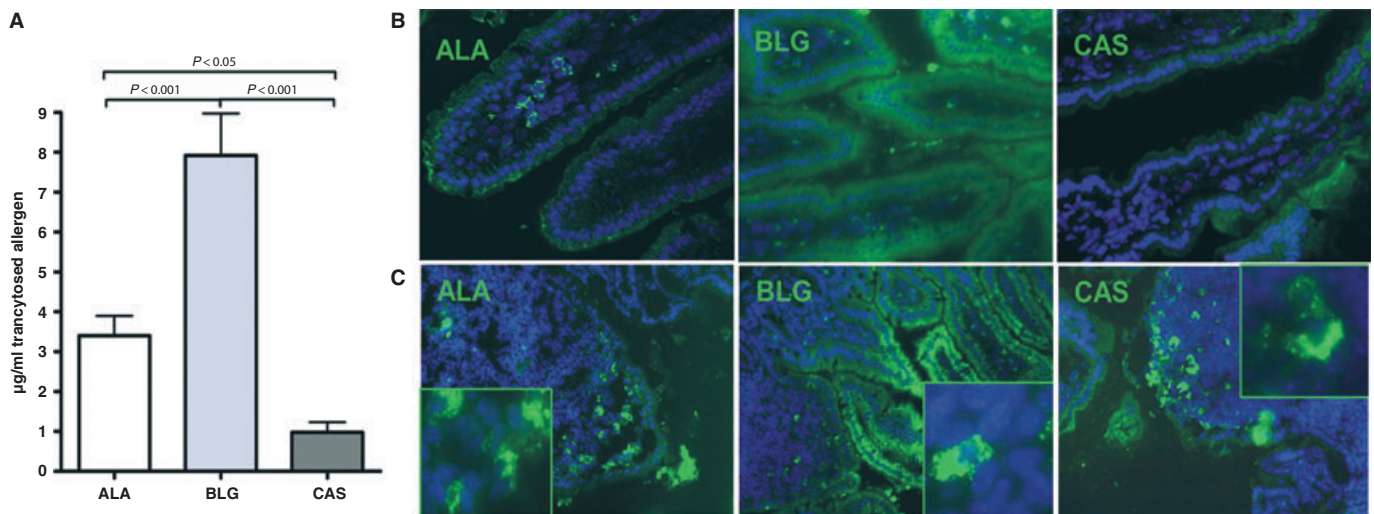


Figure 2. Transcytosis of native milk allergens *in vitro* and *in vivo*. (A) Transport of FITC-labeled ALA, BLG and CAS across polarized Caco-2 monolayers. Data are mean $\pm$ SEM of three independent experiments. (B) Transepithelial antigen-uptake in mouse jejunum. (C) Antigen-uptake through Peyer's patches. Sections were obtained 15 min after feeding of FITC-labeled ALA, BLG or CAS and counterstained with DAPI. ($n = 3/\text{allergen}$)

sensitize vs. elicit anaphylaxis. We chose pasteurization because of its potential clinical relevance. Heat aggregation of BLG and ALA already has been extensively investigated in the literature (9–12). We confirmed the promotion of aggregation of the whey proteins ALA and BLG upon pasteurization using a Sephadex-G200 column (Fig. 3A). Upon pasteurization the peak representing monomeric ALA clearly decreased with additional peaks appearing representing the formation of dimers and multimers with a molecular weight of over 670 kDa. In contrast, native BLG was found to exist predominantly in a dimeric (80%) with 20% as monomers. Pasteurization completely abolished the monomeric form and caused huge aggregates of over 670 kDa. Unpasteurized CAS naturally existed as two peaks around 180 kDa and over 670 kDa, representing the formation to micelles. Pasteurization of CAS did not alter these forms.

We next analyzed the impact of pasteurization on the antigenicity of the individual milk proteins. For this, the antigenicity of native, pasteurized and 'cooked' ALA, BLG, and CAS were tested with the serum of mice previously sensitized (*i.p.*) to the individual native milk proteins. Indeed, pasteurization did not reduce the antigenicity of the whey proteins, ALA or BLG at all. In contrast, thorough cooking of the whey proteins clearly decreased their antigenicity. The antigenicity of CAS was not affected by any treatment.

Pasteurized ALA and BLG seems to be protective in their ability to trigger anaphylaxis

The data above suggest that aggregation promotes sensitization. Given the fact that the more immunogenic CAS (micelle-forming) appeared to be less capable of

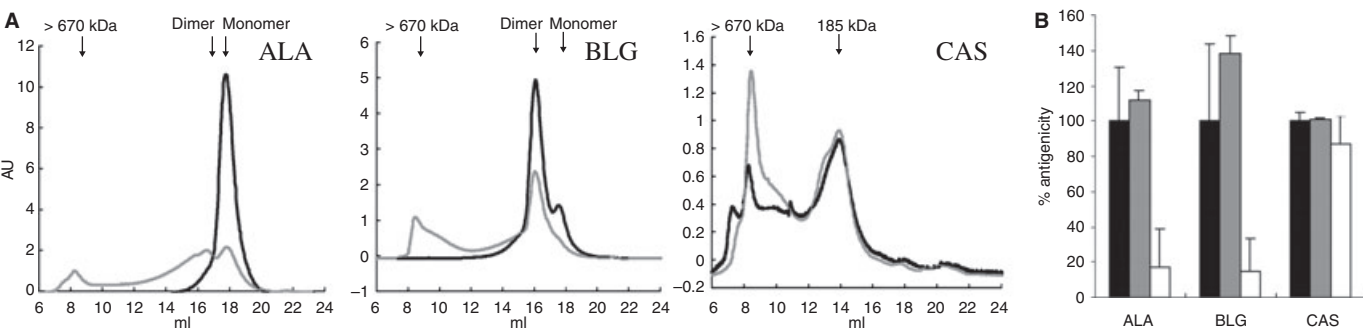


Figure 3. Effect of pasteurization on milk allergens. Allergens were pasteurized or cooked as described in *Methods*. (A) Native (black line) and pasteurized milk proteins (grey line) were run through a Sephadex-G200 column and monitored at 280 nm. (B) Antigenicity of ALA, BLG and CAS. Native (black bars), pasteurized (grey bars) and cooked (white bars) allergens were coated on to ELISA plates and detected with serum from ALA, BLG or CAS i.p.-sensitized mice using peroxidase-labeled rabbit anti-mouse IgG1 and TMB as substrate. Optical density was measured at 450 nm and values of untreated allergens were set as 100%. Pasteurized or heated ALA, BLG or CAS were compared ($n = 3$).

eliciting an anaphylactic response, we asked whether pasteurization of the whey proteins would reduce their anaphylactogenic potential. Intriguingly, comparable to CAS, pasteurization of the whey proteins ALA and BLG significantly inhibited their ability to trigger anaphylactic reactions by the oral route in sensitized mice. Systemic administration of the pasteurized whey proteins was required to induce anaphylaxis (Fig. 4), which was in sharp contrast to the capacity of soluble ALA and BLG (Fig. 1). Pasteurization of CAS did not significantly influence its ability to trigger anaphylactic reactions, as native CAS (in a naturally aggregated form) already required systemic administration to induce anaphylaxis. In all sensitized mice, i.p. administration of Ag triggered severe symptoms, which were accompanied by a significant drop in body temperature.

In summary, pasteurization of the whey proteins protected against an allergic response, since uptake through epithelial cells was impaired due to aggregation. Thus, these data emphasize the important role in antigen uptake of absorptive epithelial cells for the induction of anaphylaxis.

Uptake of ALA and BLG through enterocytes is impaired by pasteurization and occurs mainly in PPs

Given the alteration in the ability of pasteurized Ags to elicit anaphylaxis, we assessed the impact of pasteurization on antigen trafficking *in vitro* and *in vivo*. Consistent with the effect on protein-aggregation, pasteurization significantly reduced transcytosis of ALA and BLG across Caco-2 monolayers ($1.6 \pm 0.3 \mu\text{g/ml}$ pasteurized ALA, $1.4 \pm 0.2 \mu\text{g/ml}$ pasteurized BLG) to levels comparable to those seen with CAS (Fig. 5A).

Also *in vivo* pasteurization dramatically impaired uptake of BLG through absorptive epithelial cells and, to a smaller extent, of ALA. These pasteurized Ags were observed in the lumen but were not readily detectable in the lamina propria (Fig. 5B).

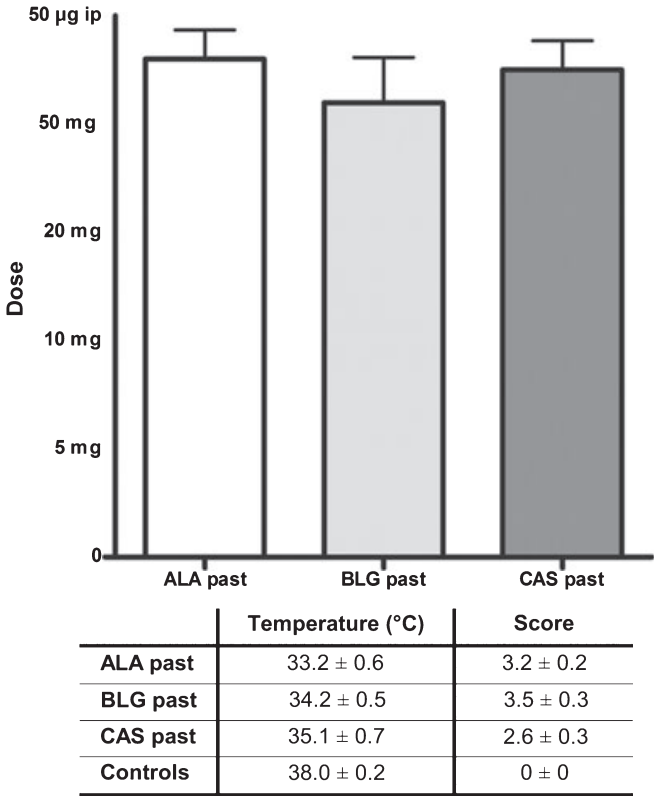


Figure 4. Dose, temperature and anaphylaxis score in sensitized mice challenged with pasteurized ALA, BLG or CAS. Allergic reactions occurred mainly when pasteurized allergens were introduced intraperitoneally (i.p.). Similar to the experimental protocol used in the experiments detailed in Fig. 1, a graded food challenge was performed.

However, all three pasteurized milk proteins were detected in a cell-associated form in PPs 15 min post-feeding (Fig. 5C). Thus, pasteurization promoted uptake through PPs while impairing uptake through absorptive epithelial cells.

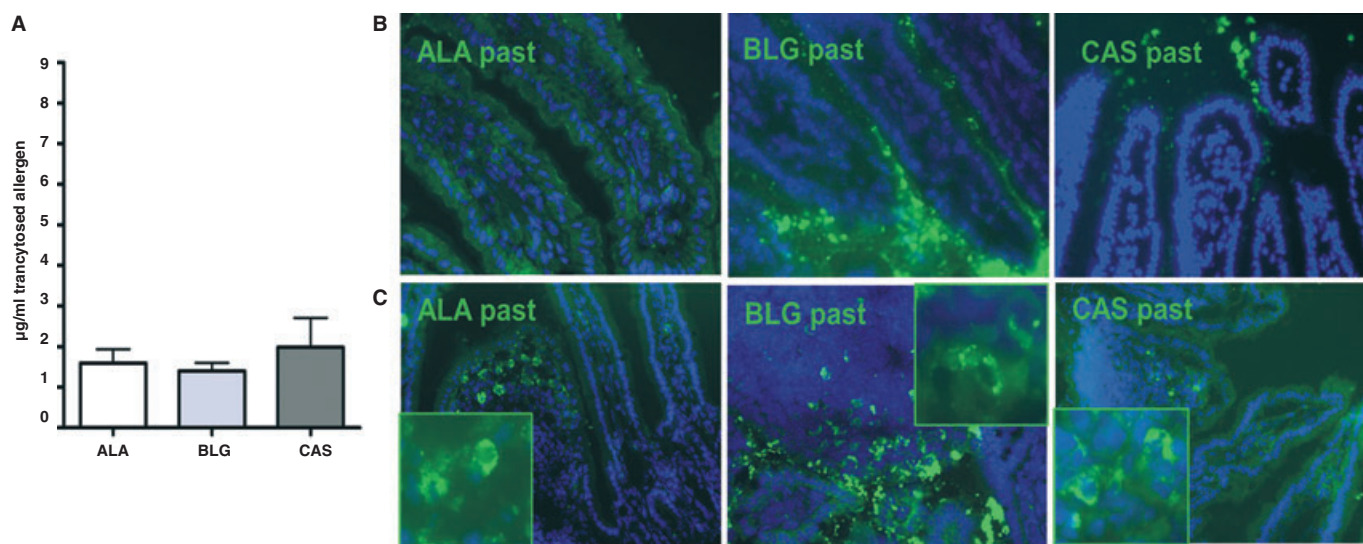


Figure 5. Transcytosis of pasteurized milk allergens. (A) Transport of FITC-labeled pasteurized ALA, BLG and CAS across polarized Caco-2 monolayers. Data are mean $\pm$ SEM of three independent experiments. (B) Transepithelial antigen-uptake in mouse jejunum. (C) Uptake of allergen through murine Peyer's patches. Sections were obtained 15 min after feeding of pasteurized FITC-labeled ALA, BLG or CAS. ($n = 3$ /pasteurized allergen)

Sensitization to pasteurized ALA and BLG results in enhanced antibody and cytokine responses

As depicted in Table 1, oral administration of pasteurized ALA and BLG resulted interestingly in significantly higher levels of the Th2-associated antibodies IgE and IgG1 compared to mice sensitized with the soluble whey proteins. No difference in the antibody response could be observed in mice orally sensitized with pasteurized CAS compared with native CAS. Also, IgA and IgG2a-levels were significantly elevated in mice sensitized with the pasteurized BLG compared to the native form although this was not seen in mice sensitized with pasteurized ALA.

Similarly to the antibody response, stimulation of splenocytes (following oral priming) with pasteurized BLG generated significantly more Th2-related cytokines IL-5 and IL-13 as well as IFN- γ compared to the soluble BLG (Table 1). Also the cytokine response to pasteurized ALA was elevated compared to native ALA; however, differences were only significant for IFN- γ -secretion. Antigen-stimulation of splenocytes from naive mice hardly induced any cytokine secretion. Similar findings were observed upon stimulation of mesenteric lymph node cells in the different groups of mice (data not shown). We further tested whether the observed increase in the antigenicity of pasteurized BLG was due to a qualitative and/or quantitative difference in the ability of these different forms of the same Ag to stimulate T cells. CD4 + T cells were isolated from mice sensitized to native BLG (and naïve controls) and cultured with isolated DCs from TLR4 deficient mice in the presence

or absence of native or pasteurized BLG. There were no significance differences seen in the amount or type of cytokines induced by native vs. aggregated BLG (data not shown). Thus, the differences seen in the type of immune response is not solely a reflection of a different response to the form of antigen administered.

These findings suggest that aggregation is a characteristic associated with the enhanced immunogenicity of food proteins following oral administration and may alter the antibody isotypes elicited. Moreover, the data support the concept that altering the route of antigen uptake affects both the antigenicity and allergenicity of food proteins.

Discussion

In general, Ags administered via the oral route will elicit tolerance as opposed to active immunity. Such a phenomenon is clearly important, since the gut is exposed to the greatest numbers of Ags. Ag solubility, the presence of adjuvants as well as the nature of the Ag clearly affect the induction of tolerance (15). Moreover, loss of oral tolerance is associated to inflammatory diseases of the bowel as well as food reactive disorders such as celiac sprue and food allergies (16, 17).

Cow milk can be divided into caseins comprising α _S1-, α _S2-, β -, and κ -casein and whey fractions, which mainly consist of BLG, ALA and BSA.

In this study, we wanted to ask the question, what makes an antigen an allergen when exposure occurs through the oral route and, in the process, to understand

whether there were gut-specific properties that enable proteins to become allergens. Our findings suggest that different aspects of ingested antigens are important for the initial sensitization step and the subsequent elicitation of an allergic response. Using the milk proteins, ALA, BLG, and CAS as model allergens, we showed that pasteurization induces aggregation of the whey proteins ALA and BLG (but not CAS that already exists in a micellar form). Pasteurization/aggregation of the whey proteins promoted entry into PPs (CAS main port is already PPs) by inhibiting uptake through enterocytes and as a consequence an increase in the immunogenicity (cytokine and antibodies) of ALA and BLG was observed.

Moreover, we observed a disparity between sensitization to individual milk allergens as measured by serum immunoglobulins (including IgE) and their ability to trigger an anaphylactic reaction following oral challenge. The disparity between the degree of sensitization and anaphylactic response appears to be correlated with the ability of the allergen to pass through the absorptive epithelium at the time of challenge. Native ALA and BLG readily transcytose the epithelial barrier and trigger a strong anaphylactic response, whereas CAS, which forms micelles and does not efficiently cross the absorptive epithelial barrier, was not able to trigger anaphylaxis when delivered orally, despite the fact that mice had been highly sensitized to it. This pattern was reproduced when comparing pasteurized proteins to their native counterparts. The ability to trigger anaphylaxis by the oral route in sensitized mice was greatly impaired following challenge with pasteurized ALA and BLG, even though they generated higher antigen-specific IgE levels.

Casein is generally considered the major allergen in milk-allergic patients, followed by the whey proteins BLG and ALA, since antibodies to casein, but not consistently to ALA and BLG are readily detected in nearly all milk-allergic individuals (18, 19).

However, studies by Sicherer et al. showed that serum from children allergic to milk contained IgE antibody levels against whey that were similar to those against casein or whole milk, even though paradoxically a significant number of children did not have detectable IgE against the individual whey proteins ALA, BLG, or BSA (20). Furthermore, previous reports failed to identify a single major milk allergen in children by serum testing, skin testing, or oral challenge (21–23). This is in agreement with our data, where we demonstrate that the soluble whey proteins were similarly capable of eliciting anaphylaxis in mice, challenging the view that antigens capable of inducing strong immune responses like CAS are the 'better' allergens than antigens inducing lower levels of antibody and cytokine secretion.

In contrast to pasteurization (9, 24), cooking clearly diminished the antigenicity of ALA and BLG and may explain the fact that cooked milk is better tolerated by many patients (25).

Surprisingly few if any studies investigated the impact of pasteurization *in vivo*, even though the formation of aggregates of the whey proteins BLG (9, 10, 26, 27) and ALA (11, 12) has been extensively analyzed.

Indeed, increased allergenicity in milk-allergic mice challenged with homogenized and pasteurized milk compared with untreated bovine milk has already been observed and associated with the homogenization procedure (28, 29). However, our findings indicate that a higher degree of sensitization is due to pasteurization/aggregation of the whey proteins and enhanced uptake by Peyer's patches.

It is to note that, so far, all known major food allergens tend to form pseudo-lipophilic protein-bodies (30), micelles (31, 32) or aggregates (6) and consequently their ability to enter the body through absorptive intestinal epithelial cells is predicted to be diminished. In pasteurized milk, CAS and aggregated whey proteins might set the stage for an anaphylactic response but it may be the soluble whey proteins, still present in pasteurized milk, that actually result in the allergic event.

The probability of clinical reactivity to a food protein might thus be a combination of the level of sensitization to the food proteins and efficiency in antigen uptake across the mucosal barrier. The fact that aggregated antigens sensitize for food allergies, but soluble antigens are required for provocation may in part be attributed to the fact that soluble Ag can transcytose across the epithelium more easily to come in contact with sensitized lamina propria mast cells. As a consequence of this, the permeability of epithelial barrier may increase (33–35) and a greater amount of antigen may gain access to the host systemically (33). In contrast, aggregated antigens that only pass through Peyer's patches might be transported to draining MLNs, bypassing the lamina propria and limiting their ability to contact tissue mast cells.

Moreover, factors such as interference with gastric acid (36, 37) contribute to the sensitization process, whereas IgE-mediated transcytosis (38) or incomplete digestion of antigens may enhance the subsequent response.

In conclusion, our results suggest that pasteurization leads to aggregation of whey proteins but not CAS. The formation of aggregates changes the path of antigen-uptake, away from absorptive enterocytes to Peyer's patches. As a consequence, pasteurized BLG leads to enhanced IgE as well as Th2 cytokine responses in the initial sensitization step, suggesting that the formation of aggregates is one important feature of food allergens. In contrast, only soluble milk proteins trigger anaphylaxis readily, since transepithelial uptake across the small intestinal epithelium is not impaired. Since milk proteins are usually consumed in a pasteurized form, this may be clinically relevant. These findings support the hypothesis that food processing may contribute to the increasing prevalence of sensitization to food proteins in westernized countries.

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