

Mechanisms underlying differential food allergy response to heated egg

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Background: Egg white proteins are usually subjected to heating, making them edible for the majority of children with egg allergy.

Objective: We sought to investigate the underlying mechanisms responsible for the reduced allergenicity displayed by heat-treated egg white allergens.

Methods: C3H/HeJ mice were orally sensitized with ovalbumin (OVA) or ovomucoid and challenged with native or heated proteins to evaluate their allergenicity. Immunoreactivity was assessed by immunoblotting using sera from children with egg allergy. *In vitro* gastrointestinal digestion of native and heated OVA and ovomucoid was studied by SDS-PAGE and liquid chromatography. Intestinal uptake of intact native and heated OVA and ovomucoid by human intestinal epithelial (Caco-2) cells was investigated. Rat basophil leukemia cells passively sensitized with mouse serum and human basophils passively sensitized with serum from children with egg allergy were used to assess the effector cell activation by heated, digested, and transported OVA and ovomucoid.

Results: Heated OVA and ovomucoid did not induce symptoms of anaphylaxis in sensitized mice when administered orally. Heating did not completely destroy IgE-binding capacity of OVA or ovomucoid but enhanced *in vitro* digestibility of OVA. Digestion of both OVA and ovomucoid diminished mediator release in rat basophil leukemia assay and basophil activation. Heating of allergens prevented transport across human intestinal epithelial cells in a form capable of triggering basophil activation or T-cell activation.

Conclusion: Heat treatment reduces allergenicity of OVA and ovomucoid. This is partially a result of the enhanced gastrointestinal digestibility of heated OVA and the inability of heated OVA or ovomucoid to be absorbed in a form capable of triggering basophils. (*J Allergy Clin Immunol* 2011;127:990-7.)

Key words: Egg allergy, ovalbumin, ovomucoid, heat treatment, heating, gastrointestinal digestion, antigen absorption, mice oral sensitization, anaphylaxis, basophil activation, passive sensitization

Food processing and gastrointestinal degradation are fundamentally important for food protein allergenicity. Numerous reports¹⁻⁶ have addressed the effect of thermal and nonthermal processing on the final food allergenicity, which can be either enhanced or reduced depending on the particular allergen. Moreover, structural stability under the extreme degradative environment found within the gastrointestinal tract is often a requisite for a protein to elicit an allergic response.^{7,8}

Food processing is of particular relevance for egg white allergens, because egg proteins are usually subjected to heat treatment such as boiling or baking and are likely to undergo important structural changes affecting their secondary and tertiary structure. It has been reported that approximately 70% of children with egg allergy tolerated baked egg ingestion.⁹⁻¹²

It is usually argued that heating induces protein denaturation, leading to the loss of conformational epitopes, suggesting that heated egg-tolerant children would present IgE antibodies mostly against conformational epitopes.^{11,13} Heat-resistant proteins like ovomucoid, the dominant egg white allergen, can retain both linear and conformational epitopes on heating. However, ovomucoid-specific IgE levels were found to be poorly predictive of heated egg reactivity in a study enrolling 117 subjects with egg allergy.¹² Heat-induced aggregation of milk allergens was shown to prevent their absorption through enterocytes and subsequent onset of allergic symptoms in mice,¹⁴ pointing at an additional explanation for tolerance to heated allergens.

These collective data suggest that extensive heating diminishes the allergenicity of egg white proteins, although the underlying mechanisms remain elusive. We sought to investigate the factors behind the reduced allergenicity displayed by the 2 major egg white allergens, ovalbumin (OVA) and ovomucoid, when they are subjected to heat treatment. We used *in vivo* and *in vitro* methods to compare digestion resistance, intestinal transport, and effector cell-triggering capacity of native and heated egg white proteins.

METHODS

Heating of OVA and ovomucoid

Ovalbumin (grade VI, 99% purity; Sigma, St Louis, Mo) and ovomucoid (trypsin inhibitor from chicken egg white, type III-O, free of ovomucoid;

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Abbreviations used

Bis-Tris: Bis(2-hydroxyethyl)-amino-tris(hydroxymethyl)-methane
DIG: Digoxigenin-3-0-succinyl- ϵ -aminocaproic acid-N-hydroxy-succinimide ester
kU_A/L: Kilo units of antibody per liter
MLN: Mesenteric lymph node
NHR: β -N-acetylhexosaminidase release
OVA: Ovalbumin
PP: Peyer patch
RBL: Rat basophil leukemia

Sigma) were dissolved as required for the different assays and heated in a boiling water bath for 30 minutes.

In vitro digestion of OVA and ovomucoid

Gastric digestion. OVA and ovomucoid were dissolved in simulated gastric fluid (35 mmol/L NaCl) at pH 2, preheated for 15 minutes at 37°C, and subjected to an *in vitro* gastric digestion with porcine pepsin (Enzyme Commission number 3.4.23.1, 3440 U/mg; Sigma) at an enzyme:substrate ratio of 1:20, w/wt (172 U/mg). The reaction was stopped after 60 minutes with 1 mol/L NaHCO₃, for a final protein concentration of 5 mg/mL and pH 7.

Duodenal digestion. The starting material were gastric digests adjusted to pH 7 by adding 1 mol/L CaCl₂, 0.25 mol/L Bis(2-hydroxyethyl)-amino-tris(hydroxymethyl)-methane (Bis-Tris), pH 6.5, and a 0.125-mol/L bile salt mixture containing equimolar quantities of sodium taurocholate (Sigma) and sodium glycodeoxycholate (Sigma). After preheating at 37°C for 15 minutes, porcine pancreatic lipase (EC 232-619-9; Sigma), colipase (EC 259-490-1; Sigma) and a commercial pancreatic mix, Corolase PP (AB Enzymes GmbH, Darmstadt, Germany) prepared in 35 mmol/L NaCl adjusted to pH 7, were added to the duodenal mix. The final composition of the mixture was 4.15 mg/mL OVA/ovomucoid, 6.15 mmol/L each bile salt, 20.3 mmol/L Bis-Tris, and 7.6 mmol/L CaCl₂, and the enzymes referred to the quantity of protein were 28.9 U/mg lipase, Corolase PP (enzyme:substrate ratio of 1:25, wt/wt) and colipase (enzyme:substrate ratio 1:895 wt/wt).

Digoxigenin labeling of egg white proteins

Proteins were incubated with digoxigenin-3-0-succinyl- ϵ -aminocaproic acid-N-hydroxy-succinimide ester (DIG; Roche Diagnostics, Indianapolis, Ind) for 2 hours at room temperature under constant shaking. Free DIG was eluted with PBS through a Sephadex PD-10 Column (Amersham Biosciences, Uppsala, Sweden).

RP-HPLC

Proteins and the corresponding hydrolysates at 4.15 mg/mL were separated in a Hi-Pore RP-318 (250 \times 4.6 mm internal diameter) column (Bio-Rad, Richmond, Calif) in a Waters 600 HPLC (Waters Corp, Milford, Mass). The samples were eluted with 0.37% (vol/vol) trifluoroacetic acid in double-distilled water as solvent A and 0.27% (vol/vol) trifluoroacetic acid in acetonitrile as solvent B at 1 mL/min and 220 nm. Data were processed with Empower 2 Software (Waters Corp).

SDS-PAGE

Proteins were separated by SDS-PAGE (NuPAGE 4% to 12%, 15 wells; Invitrogen, Carlsbad, Calif) per the manufacturer's instructions; 6 μ g protein was loaded per well. Proteins were transferred onto Immobilon-P PVDF membranes (Millipore, Bedford, Mass) and probed with sera from children with egg allergy.

Serum samples

A serum pool was made of equal parts of serum from 8 heated egg-reactive children with egg allergy as documented with an oral challenge. Levels of

specific IgE antibodies were measured with UniCAP (Phadia US, Portage, Mich), lower limit of detection, 0.35, and upper limit of detection, 100 kilo units of antibody per liter (kU_A/L). Pool specific IgE levels were as follows: egg white, 12.8; OVA, 14.0; and ovomucoid, 13.9 kU_A/L.

Immunoblotting

Immunoblots for detection of IgE binding were performed with native and heated OVA and ovomucoid. Membranes were incubated with an egg-allergic serum pool 1:10 dilution in PBS containing 0.05% Tween 20, 1% BSA, and 10% normal goat serum for 60 minutes. PBS-rinsed membranes were incubated with ¹²⁵I-goat antihuman IgE (DiaMed, Windham, Me) for 1 hour, washed, and exposed to Kodak BioMax MS Film (Carestream Health Inc, Rochester, NY) for 1 to 12 days. As a negative control, serum from a non-atopic adult was used.

Sensitization and oral challenge of mice

Five-week-old female C3H/HeJ mice (NCI, Fredrick, Md) were sensitized orally with 1 mg native OVA (n = 15) or ovomucoid (n = 24) in 0.2 mol/L bicarbonate buffer plus 10 μ g cholera toxin (List Biologicals, Campbell, Calif) per week for 6 weeks. On week 7, all sensitized mice were orally challenged with either native or heated OVA and ovomucoid. Five OVA-sensitized mice were challenged 1 week apart with both heated and unheated OVA. Total doses of 30 and 42 mg OVA and ovomucoid, respectively, were administered in 2 increments, 15 minutes apart. If no symptoms were observed, the mice were then challenged with 100 μ g allergen intraperitoneally. Animal studies were approved by the Institutional Animal Care and Use Committee of Mount Sinai School of Medicine.

Anaphylaxis assessment

Symptoms were scored as previously published.¹⁴ Rectal temperature (World Precision Instruments, Sarasota, Fla) was measured as a further assessment of anaphylaxis severity.

Measurement of antigen-specific IgE

Mouse OVA-specific and ovomucoid-specific IgE was quantified by ELISA. A 96-well plate was coated overnight at 4°C with rat antimouse IgE antibody (BD Biosciences, San Jose, Calif), then blocked with 10% normal mouse serum, 1% BSA in PBS 0.05% Tween. After incubation with serum from sensitized mice, DIG-conjugated OVA or ovomucoid was added. Finally, horseradish peroxidase-labeled anti-DIG antibody (fragment antigen-binding fragments; Roche Diagnostics) was incubated with tetramethylbenzidine (BD Biosciences) as a substrate. The reaction was stopped with 1.2 mol/L sulfuric acid, and absorbance was measured at 450 nm.

In vitro cytokine responses

Splenocytes were plated at a density of 5 \times 10⁶ cells/mL in 24-well cell tissue culture plates (Nalge Nunc, Naperville, Ill) with 50 μ g/mL OVA and ovomucoid proteins, respectively, or medium alone (RPMI 1640) in 10% FCS for 72 hours at 37°C in 5% CO₂. Cytokines in culture supernatants were measured by ELISA (eBiosciences, San Diego, Calif).

Mediator release assay

Rat basophil leukemia (RBL) cells (RBL-2H3; kind gift of Dr Stefan Vieths) were cultured in Eagle minimal essential medium with 10% FCS, and the assay was performed as published.¹⁵ Briefly, RBL cells (at 3 \times 10⁶ cells/mL) were incubated with serum at a final dilution of 1:60 at 37°C in 5% CO₂ overnight in 96-well tissue culture plates (BD Falcon; BD, Bedford, Mass). Both OVA-sensitized and ovomucoid-sensitized mouse pool sera were used. Sensitized cells were stimulated with 100 μ L per well of the dilutions of allergens. Rat antimouse IgE (Pharmingen) was used as a positive control for IgE-mediated degranulation; RBL cells were lysed with 1% Triton X-100 (Sigma) for total release. β -N-acetylhexosaminidase release (NHR) on

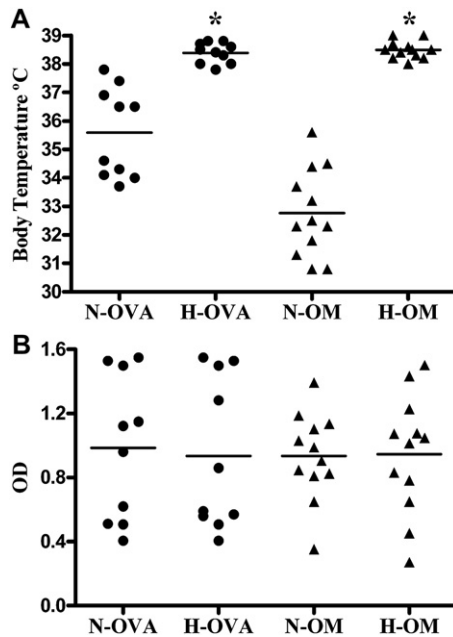


FIG 1. **A**, Mice body temperature on oral challenge with native (*N*) or heated (*H*) OVA or ovomucoid (*OM*). **B**, Serum OVA-specific and OM-specific IgE levels of the different mice groups before challenge. OD values correspond to 1/100 serum dilution. *Statistically significant differences between native and heated challenged groups; $P < .001$.

stimulation with allergen was determined. Spontaneous release (stimulation with buffer only) was subtracted from the allergen-induced NHR at every dilution point. Values were expressed as percentage of the total release, and the protein concentration that gave 50% of the maximum NHR (inhibitory concentration₅₀) was determined.^{16,17}

Transcytosis studies

Caco-2 cells (clone C2Bbe1; American Type Culture Collection, Rockville, Md) were seeded on 0.4- μ m Transwell filter (Cole-Parmer, Vernon Hills, Ill) inserts at a concentration of 5×10^5 cells/mL. After 1 week, transepithelial resistance was checked by ohmmeter (World Precision Instruments) and monolayers used if resistance was greater than 300 Ω . Then fluorescein isothiocyanate-labeled native and heated OVA and ovomucoid were added in triplicate to the apical side (0.5 mL at 0.5 mg/mL) and incubated at 37°C/5% CO₂ for 20 hours. Samples from the basolateral side were collected after the incubation period.

OVA-specific T-cell activation

CD4⁺ T cells were isolated from spleens and lymph nodes of DO11.10 mice by negative selection (StemCell, Vancouver, British Columbia, Canada) and labeled with carboxyfluorescein diacetate succinimidyl ester (Invitrogen). Cells 3 to 5×10^6 were injected into naive BALB/c recipients intravenously. The next day, mice were fed 25 mg native or heated OVA in a total volume of 1 mL, given as 2 doses 1 hour apart. After 72 hours, mice were euthanized, and mesenteric lymph node (MLN) and Peyer patch (PP) cells were isolated. Cells were stained with antibodies against CD4 and the DO11 TCR (KJ1-26), and dead cells identified and excluded with a violet live/dead staining kit (Invitrogen). Cells were acquired on a LSRII flow cytometer (BD Biosciences) and analyzed by using FlowJo software (Treestar, Ashland, Ore).

Stripping and passive sensitization of basophils from donors without allergy

PBMCs were isolated by using Ficoll from egg-tolerant adult donors. For stripping of bound IgE,¹⁸ the PBMC pellet was resuspended in 3 mL lactic

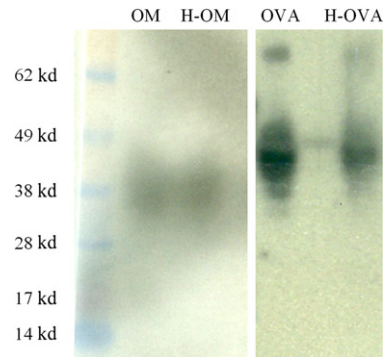


FIG 2. Immunoblotting of unheated or heated (*H*) ovomucoid (*OM*) and OVA by using pooled sera from heated egg-reactive children.

acid solution (13.4 mmol/L lactate, 140 mmol/L NaCl, 5 mmol/L KCl, pH 3.9) and incubated on ice for 5 minutes. Human serum albumin 0.5%, 7 mL (Sigma), in RPMI medium with glutamine (Fisher Scientific, Pittsburgh, Pa) was added, and the solution was neutralized with 15 μ L 12% TRIS. After centrifuging and washing the pellet with human serum albumin/RPMI, it was resuspended in 1/100 of the original blood volume. Stripped PBMCs were placed in a 96-well U-bottom plate and incubated for 1 hour at 37°C with 1:2 diluted serum (20 μ L PBMCs + 20 μ L diluted serum) from a pool of 3 children with egg allergy (OVA-specific and ovomucoid-specific IgE levels were 63.4 and 29.8 kU_A/L, respectively).

Basophil activation assay

PBMCs sensitized as described were incubated with basophil stimulation buffer RPMI with IL-3 (25 μ g/mL; R&D Systems, Minneapolis, Minn) as a negative control or with anti-IgE antibody (25 μ g/mL; Bethyl Laboratories, Montgomery, Tex) as a positive control. Samples (4 μ g/mL) were incubated with native, heated, and/or digested OVA and ovomucoid or with basolateral supernatants from transcytosis experiments at a 1:5 dilution. The reaction was stopped after 30 minutes with EDTA in cold PBS. Cells were stained for CD63, CD123, HLA-DR (BD Biosciences), and CD203c (Beckman Coulter), and fixed with Fluorescence-Activated Cell Sorting Lysing Solution (BD Biosciences). Cells were acquired as described.

Statistical analysis

Differences between mice groups were analyzed by independent *t* test. Donors' basophil stimulation percentages for heated and digested samples were compared by ratio-paired *t* test. *P* values below .05 were considered significant. Statistical analysis was performed by using GraphPad Prism software (La Jolla, Calif).

RESULTS

Heated OVA and ovomucoid do not induce anaphylaxis in an animal model

Mice were sensitized to native OVA ($n = 15$) or ovomucoid ($n = 24$) with cholera toxin. On oral challenge with the native proteins, ovomucoid-sensitized mice developed higher anaphylaxis scores and lower body temperature than OVA-sensitized mice ($P < .001$), confirming that ovomucoid is a stronger allergen. After oral challenge with native ovomucoid and native OVA, all mice presented symptoms of anaphylaxis. In contrast, mice challenged with the heated allergens did not develop anaphylaxis, as assessed by body temperature measurement (Fig 1) and symptom score (a median score of 4 and 2 for native ovomucoid and OVA, respectively, compared with a median score of 0 for both heated

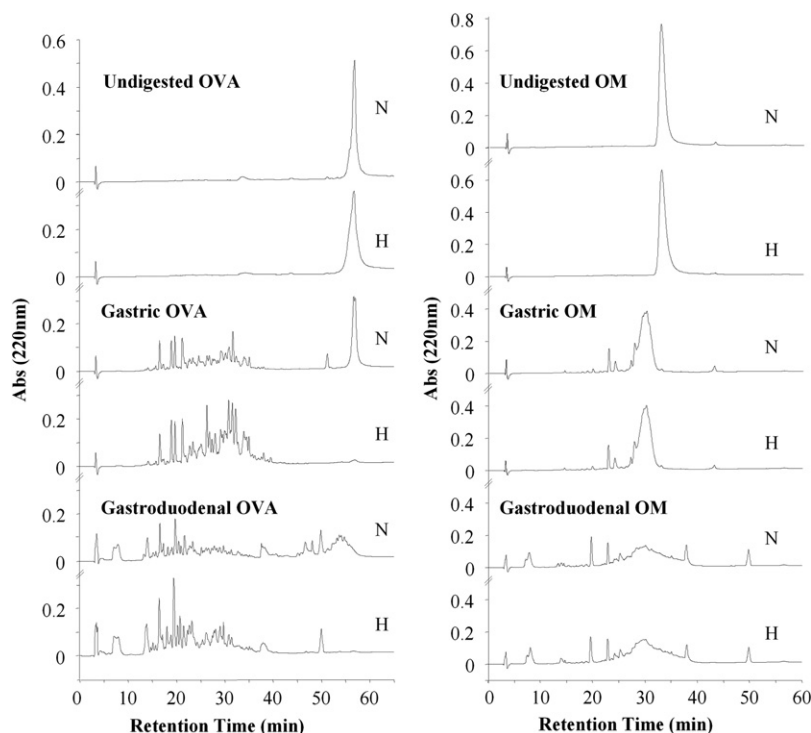


FIG 3. RP-HPLC chromatograms corresponding to native (N) and heated (H) OVA and ovomucoid (OM) undigested and subjected to gastric and gastroduodenal digestion.

ovomucoid and OVA; $P < .01$). However, when mice were subsequently systemically (intraperitoneally) challenged with the heated allergens, the majority of them (3/5 for heated OVA and 6/7 for heated ovomucoid) showed mild symptoms and a drop in body temperature. Serum specific IgE levels were similar in all mice groups (Fig 1, B), as were OVA and ovomucoid-induced IL-13 and IFN- γ production from spleen cells (see this article's Fig E1 in the Online Repository at www.jacionline.org).

Heat treatment does not completely destroy OVA and ovomucoid IgE-binding epitopes

Native and heated OVA and ovomucoid were immunolabeled with a serum pool from extensively heated egg-reactive children to assess the binding of serum IgE. There was no appreciable decrease in binding to heated ovomucoid and some decrease in binding to heated OVA. Both OVA and ovomucoid showed strong binding regardless of heat treatment (Fig 2), suggesting the persistence of linear epitopes recognized by IgE.

Heat treatment makes OVA more susceptible to digestion, whereas it does not affect ovomucoid

We used an *in vitro* digestion model to evaluate the effect of heating on the final outcome of OVA and ovomucoid. Samples were collected after 60 minutes (gastric phase) and 120 minutes (duodenal phase) and analyzed by RP-HPLC (Fig 3). Native OVA was poorly hydrolyzed by pepsin. However, heated OVA became completely hydrolyzed after 60 minutes. In contrast, heat treatment did not alter ovomucoid susceptibility to either pepsin or duodenal enzymes, producing similar digestion profiles where

ovomucoid was quickly digested, giving rise to fragments of lower molecular weight.

Heat treatment and digestion significantly reduce the capacity of OVA and ovomucoid to trigger basophil activation and degranulation in the RBL-based mediator release assay

We aimed to determine whether heated proteins or the peptides generated after digestion were able to activate basophils comparably to native proteins. We isolated basophils from 3 egg-tolerant adult donors and passively sensitized them with pooled sera from children with allergy. Passively sensitized basophils were stimulated with either native or heated OVA and ovomucoid, as well as the corresponding gastroduodenally digested proteins. The population of activated basophils analyzed by flow cytometry is shown in Fig 4. Challenge of basophils with native OVA or ovomucoid induced basophil activation as measured by upregulation of CD63. OVA exhibited a significant reduction in basophil activation only when it was both heated and digested. Ovomucoid stimulation was reduced after digestion regardless of heating, whereas heating alone did not reduce basophil activation.

We verified these results by using RBL cells passively sensitized with serum from ovomucoid-sensitized mice (Fig 5). Digested ovomucoid gave reduced NHR compared with undigested ovomucoid, regardless of heating status. The IC_{50} of ovomucoid was approximately 100-fold higher on digestion, 10 μ g/mL for digested ovomucoid versus 0.1 μ g/mL for undigested ovomucoid. These data suggest that heating does not affect ovomucoid basophil activation capacity and mediator release from RBLs, but proteolysis by gastroduodenal enzymes greatly decreases it.

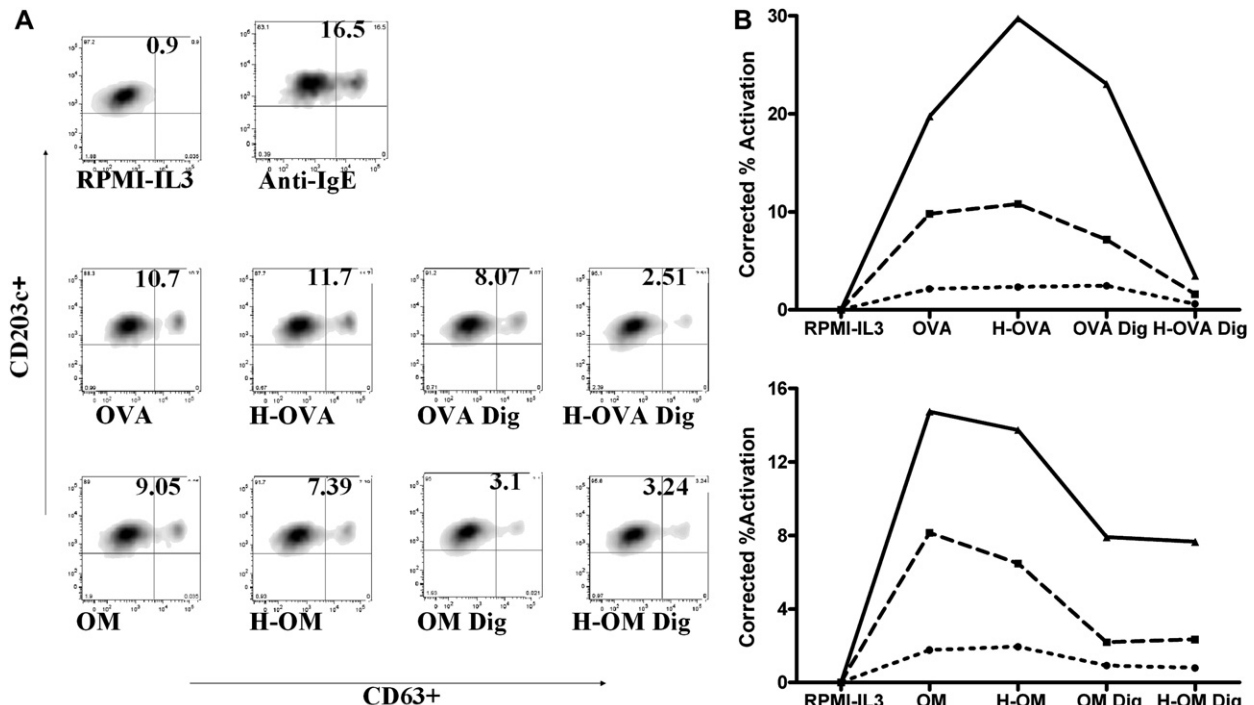


FIG 4. Percentage of activated basophils (CD63⁺CD123⁺CD203c⁺HLA-DR^{low}) on stimulation with native, heated (H), and/or digested (Dig) OVA or ovomucoid (OM). **A**, Detailed activated basophil population from 1 donor. **B**, Activation percentages from 3 donors.

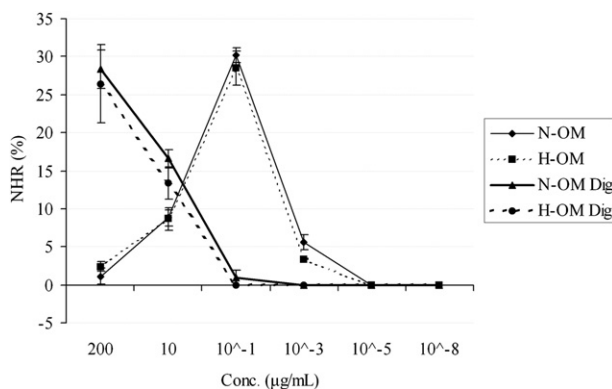


FIG 5. NHR of RBL cells passively sensitized with ovomucoid (OM)-reactive mice's sera and stimulated with different concentrations (Conc.) of undigested/digested native (N) and heated (H) OM. NHR release by negative controls (digestive enzymes, buffer) was not significantly different from spontaneous release, which was subtracted in the final graph. Positive control, anti-IgE stimulation, was 60%.

Heating prevents transcytosis of intact allergen across intestinal epithelial cells and PPs

Food allergens must cross the intestinal epithelial barrier before activating allergic effector cells. To model this, native and heated OVA and ovomucoid were added to the apical side of a polarized monolayer of Caco-2 human intestinal epithelial cells grown in Transwells. Supernatants were collected from the basolateral compartment after an overnight culture, and we then evaluated the basophil activation by the transcytosed proteins (Fig 6). Native OVA and ovomucoid were readily transported across Caco-2 monolayers and triggered significant basophil activation.

In contrast, heated OVA and heated ovomucoid triggered markedly decreased basophil activation.

To assess the impact of heating on gastrointestinal uptake of antigen *in vivo*, we assessed antigen-specific T-cell activation in the gastrointestinal-associated lymphoid tissue by using an adoptive transfer model. CFSE-labeled OVA-specific DO11.10 T cells were injected into naive BALB/c mice before oral feeding with native or heated OVA. Proliferation of DO11.10 T cells was assessed in the MLN and PP (Fig 7). Mice fed with native OVA presented extensive proliferation in both the MLN and the PP compared with unfed mice. In contrast, mice fed with heated OVA had minimal T-cell proliferation in either the MLN or the PP. Thus, in addition to facilitating digestion of OVA, heating of OVA and ovomucoid abrogates their intestinal absorption in an intact form capable of triggering effector cells and T cells.

DISCUSSION

The *in vivo* murine model of egg-induced anaphylaxis used here reproduces the observation that the majority (over 70%) of children with egg allergy can tolerate extensively heated egg.^{9,10,12,19} Mice were completely tolerant to heated egg delivered by the oral, but not the systemic, route. This suggests that heating may influence the handling of egg allergens in the gastrointestinal tract in addition to altering the conformation of IgE-binding epitopes. We showed that heating did not completely destroy epitopes recognized by IgE antibodies from children with egg allergy. This was true for IgE binding and for activation of basophils passively sensitized with serum from children with egg allergy or mice. Therefore, our goal was to determine why ingestion of heated OVA and ovomucoid did not cause symptoms despite the persistence of IgE-binding epitopes.

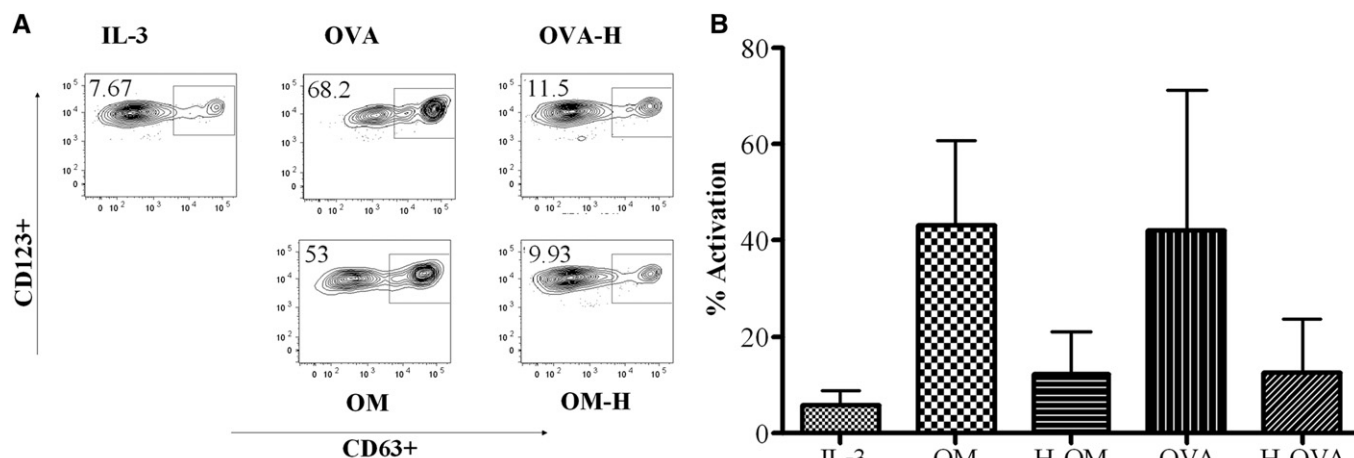


FIG 6. Percentage of activated basophils ($CD63^+CD123^+CD203c^+HLA-DR^{low}$) on stimulation with native or heated (*H*) OVA or ovomucoid (*OM*) transcytosed by Caco-2 cells. **A**, Detailed activated basophil population from 1 experiment. **B**, Mean activation percentages from 3 replicates. Error bars represent SD.

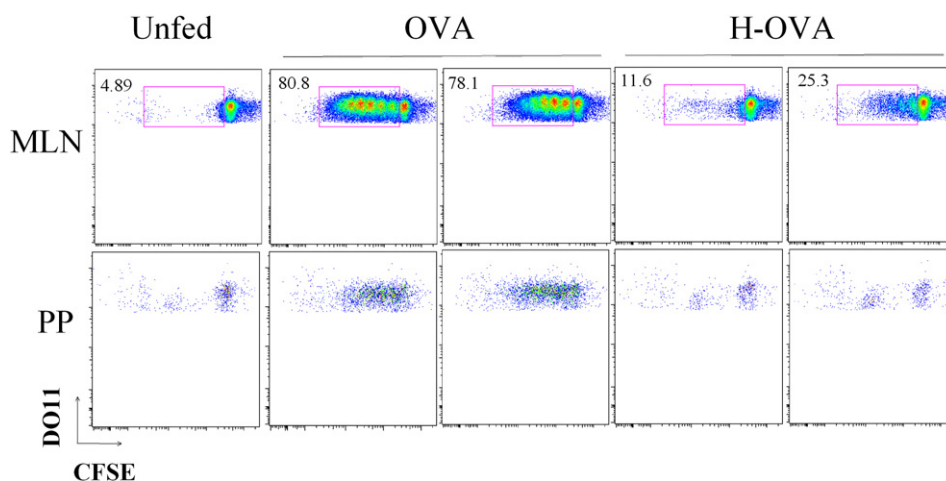


FIG 7. OVA-specific T-cell activation in the gastrointestinal lymphoid tissue. CFSE-labeled DO11.10 T cells were transferred to naive BALB/c mice before gavage feeding with 25 mg OVA or heated (*H*) OVA. After 72 hours, proliferation of DO11.10 T cells was assessed by flow cytometry by using cells isolated from MLN or PP. Data are shown from 2 individual OVA-fed or H-OVA-fed mice.

We confirmed that OVA changes its structure on heating and forms high-molecular-weight aggregates (observed by inability to enter SDS-PAGE gels; see this article's Fig E2 in the Online Repository at www.jacionline.org). In contrast, ovomucoid remains largely unaffected in the gel after heating, although there is strong evidence of an irreversibly heat-denatured form of ovomucoid with newly appearing specific IgE epitopes.²⁰

For food allergens to trigger anaphylaxis, they must escape digestion and be absorbed in a sufficiently intact or immunologically active form across the epithelial barrier.²¹⁻²³ We examined how heating of egg white proteins altered each of these factors. In globular proteins like OVA, heating is known to induce a partially folded molten globule structure responsible for their gelling and emulsifying properties.²⁴ This structure is also favored at the low pH of the gastric phase,²⁵ and it is differentiated from the native state by the absence of close packing throughout the molecule.²⁶ It has been reported that OVA's high resistance to pepsin²⁷ is lost when heated.²⁸ Heat treatment would therefore facilitate the access

of gastrointestinal proteases to potential cleavage sites, resulting in a complete degradation of OVA. In contrast, the ovomucoid structure made up of 9 disulfide bonds and 25% carbohydrate content is likely responsible for its high thermal stability and limited denaturation.²⁹ This could explain the unchanged enzymatic susceptibility of ovomucoid observed under gastrointestinal conditions.

Resistance to digestion is a common characteristic shared by many allergens. Sedimentation coefficient equal to approximately 2 albumins from mustard,³⁰ Brazil nuts,³¹ or sesame seeds³² and lipid transfer proteins from grape³³ or cherry³⁴ are only some examples of highly stable allergens. In addition, there are allergens that, despite being rapidly digested, give rise to stable fragments which retain allergenicity, as in the case of Ara h 1,³⁵⁻³⁸ the major peanut allergen. Impaired gastrointestinal function entails an increased risk of systemic absorption of food antigens. Hindered degradation of codfish allergens occurring at increased gastric pH conditions results in the maintenance of biological activity even after 2 hours of digestion, as evidenced by their histamine-

releasing capacity.⁷ Incomplete digestion of kiwifruit at an increased gastric pH has also been reported,³⁹ and the use of antacid medication has been related to sensitization to dietary proteins because of incomplete enzymatic degradation.⁴⁰ Hence, food processing such as heat treatment, which facilitates OVA degradation within the gastrointestinal tract, would have a beneficial effect on preventing adverse reactions in individuals with allergy.

We next assessed the effect of heating on transcytosis of intact egg white allergens across human intestinal epithelium. Heating of OVA and ovomucoid completely abrogated the delivery of immunologically intact forms of allergen across the epithelial monolayer. Heat treatment of milk proteins has been shown to cause aggregation of the whey proteins, redirecting antigen uptake away from absorptive enterocytes to PPs. The immediate consequence of this pathway switch was the abrogation of anaphylactic response in mice, probably because aggregated antigens transported into PP bypass lamina propria mast cells or fail to reach the systemic circulation.¹⁴ Here, however, the lack of proliferating OVA-specific CD4 T cells in the MLN or PP argues that OVA is not getting absorbed into the gut mucosa, and unlike the heated milk proteins does not seem to be getting taken up into the PP either. The aggregation of OVA was likely responsible for this blockade of absorption, whereas ovomucoid does not form aggregates when heated.¹¹ Moreover, gastrointestinal digestibility of ovomucoid was unaffected by heat treatment. However, the finding that heated ovomucoid was not transported across epithelial cells in a form capable of triggering basophils could indicate enhanced intracellular degradation as it crosses the monolayer of cells. In *ex vivo* studies of animal and human intestinal mucosa, only small amounts of intact food antigens were transcytosed (~0.1% of luminal concentration) by intestinal epithelial cells.⁴¹ Large proteins taken up by intestinal epithelial cells were released in their basal pole either as immunogenic peptides (~40%) or fully degraded into amino acids (~50%) with only a minor fraction crossing the epithelium in their intact form.⁴² We have shown that ovomucoid degradation by enzymes diminished its basophil activation capacity to a great extent, yet we did not address the additional impact of intracellular enzymes in enterocytes. In the case of ovomucoid, we hypothesize that heating could render it more susceptible to enterocytic intracellular enzymes generating nonallergenic peptides.

Another factor that might affect both the integrity of IgE-binding epitopes and gastrointestinal digestibility and absorption is interaction between heated egg white proteins and a complex food matrix.^{6,43} A study by Kato et al⁴⁴ demonstrated a marked decrease in the solubility of ovomucoid when egg white was mixed with wheat flour and wheat gluten and then heated at 180°C for 10 minutes, mimicking the process of bread-making. Immunoblotting suggested that ovomucoid polymerized and formed high-molecular-weight complexes with gluten leading to aggregation and insolubilization of ovomucoid. This phenomenon might further decrease the accessibility to digestion and slow the absorption of intact allergenic particles, relevant to human studies.¹²

The use of heated proteins arises as an attractive strategy for oral immunotherapy. Our data confirm the relative safety of heated egg white proteins. However, it is important to remark that despite their diminished capacity to trigger effector cells, alteration of the allergen structure may either enhance sensitizing potential or abrogate tolerogenic capacity. Heat treatment of milk

proteins was shown to promote sensitization in C3H/HeJ mice.¹⁴ Boiling of egg white proteins abrogated the suppression of T_H2 responses in BALB/c mice receiving the antigens before sensitization.⁴⁵ Furthermore, glycated OVA as a result of the Maillard reaction during thermal processing was shown to induce enhanced activation of OVA-specific CD4⁺ T cells on coculture with myeloid dendritic cells compared with native OVA and OVA processed without glucose.⁴⁶

Our findings emphasize that food processing can fundamentally alter the ability of food protein allergens to trigger reactions not solely by interfering with their IgE binding, but by altering their degradation and absorption within the gastrointestinal tract.

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Clinical implications: Reduced allergenicity of heated egg white proteins partially resulting from altered digestion and absorption in the gastrointestinal tract may explain the clinical tolerance of extensively heated egg in the majority of children with egg allergy.

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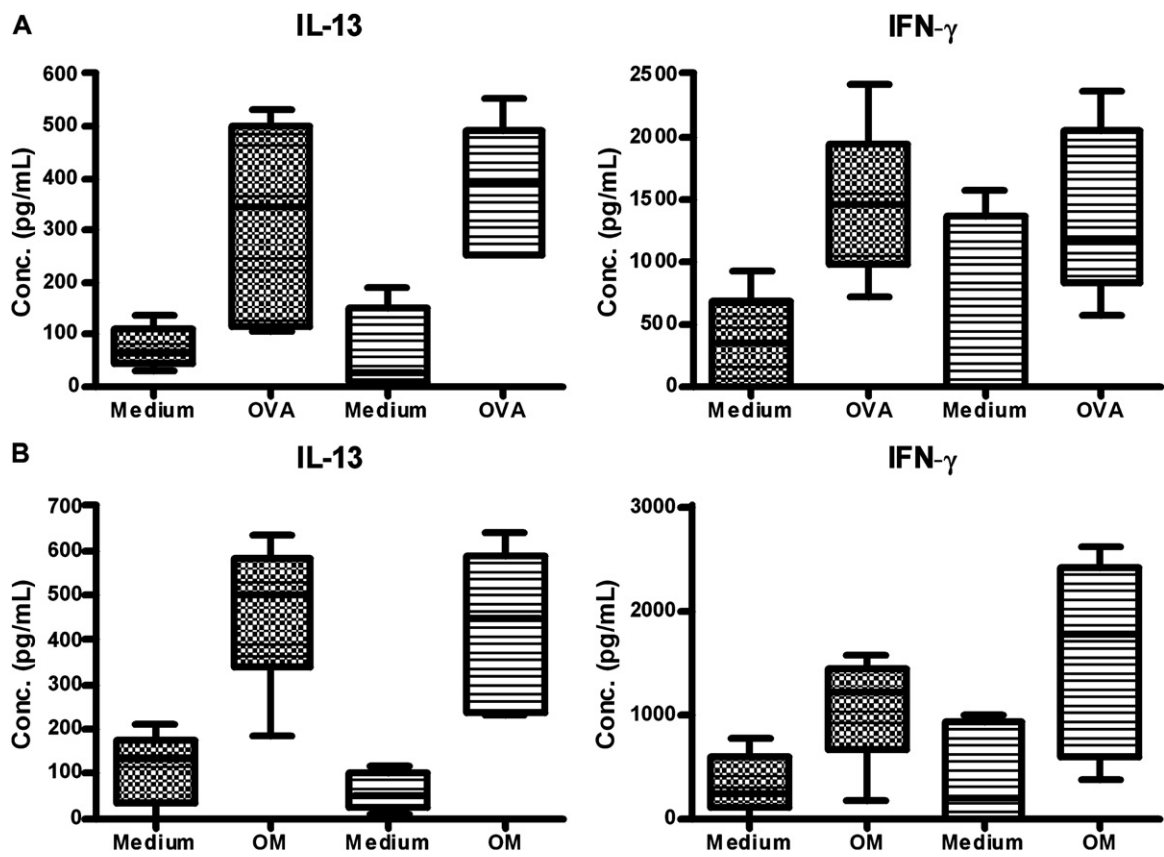


FIG E1. *In vitro* IL-13 and IFN- γ induction on stimulation with OVA or ovomucoid (OM) in cultured spleen cells from OVA-sensitized (A) or OM-sensitized (B) mice challenged with native (dotted bars) or heated (striped bars) OVA or OM. Conc., Concentration.

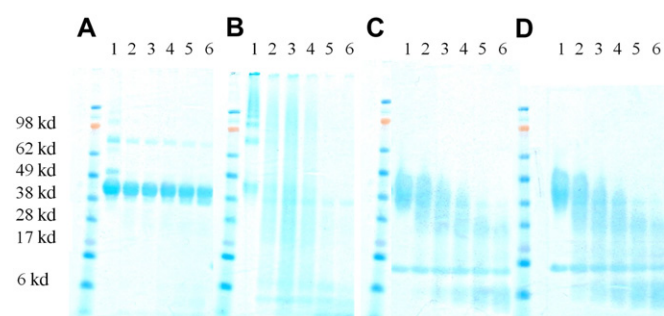


FIG E2. SDS-PAGE of unheated (**A**) and heated (**B**) OVA and of unheated (**C**) and heated (**D**) ovomucoid, subjected to pepsin digestion for 0, 1, 5, 10, 30, and 60 minutes (*lanes 1-6*, respectively).