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 reports[1-6] 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 ovoinhibitor;

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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, wt/wt (172 U/mg). The reaction was stopped after 60 minutes with 1 mol/L $NaHCO_3$, 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 $CaCl_2$, 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 $CaCl_2$, 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 × 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 μg 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 k_AU/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 ^{125}I-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 μg 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 μg 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 5×10^6 cells/mL in 24-well cell tissue culture plates (Nalge Nunc, Naperville, Ill) with 50 μg/mL OVA and ovomucoid proteins, respectively, or medium alone (RPMI 1640) in 10% FCS for 72 hours at 37°C in 5% CO_2. 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 3×10^6 cells/mL) were incubated with serum at a final dilution of 1:60 at 37°C in 5% CO_2 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 μL 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