

Immunomodulatory Effects of Heated Ovomucoid-Depleted Egg White in a BALB/c Mouse Model of Egg Allergy

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ABSTRACT: Oral immunotherapy (OIT) is a promising therapeutic approach for treating food allergy. The treatment with heated ovomucoid-depleted egg white (HOMEW) in egg-allergic patients is noteworthy; however, OIT protocols are still experimental, and a better knowledge of the underlying mechanism is required. The objective of this work was to investigate the immunomodulatory effects of HOMEW and characterize the underlying mechanism in a BALB/c mouse model of egg allergy. Mice were sensitized with EW and treated with HOMEW. Post treatment, mice were challenged with EW and euthanized for collecting blood and spleen. Markers of allergic clinical outcomes were measured as histamine concentration, serum antibody activity, and cytokine production from cultured splenocytes. Digestibility of HOMEW was assessed mimicking physiological conditions *in vitro*. The HOMEW demonstrated high digestibility. The treatment induced a marked increase of the Th1/Th2 ratio in the high-dose treatment group. Treated mice had significantly less histamine, EW-specific IgE, and IL-4 and more IFN- γ and IL-10. This study confirms the mechanisms involved in successful tolerance induction with OIT using HOMEW and allows understanding of the vital role of surrogate allergy markers involved in immune modulation.

KEYWORDS: food allergy, egg white, ovomucoid, BALB/c mice, oral immunotherapy

INTRODUCTION

Food allergies are recognized as a global medical problem that affects >25% of the population in industrialized countries¹ and accounts for one-third to a half of anaphylaxis cases worldwide.² Prevalence of food allergies is on the rise, and a concerning increase of 18% has been reported in the United States from 1997 to 2007.³ Recently it has been reported that estimates are about 5% in children and 3–4% among adults.⁴ Egg allergy is the second most common cause of food allergies in children,⁵ and four major allergens in the egg white [ovomucoid (OM or Gal d 1), ovalbumin (OVA or Gal d 2), ovotransferrin (OVT or Gal d 3), and lysozyme (LYS or Gal d 4)] contribute to induction of allergy.⁶ Among the four, OM is considered to be immunodominant⁷ due to its stability to heat treatment⁸ and enzymatic digestion⁹ and its ability to retain IgE binding epitopes after *in vitro* digestion.¹⁰

At present, the main treatment for egg-allergic patients is based on food avoidance; however, this poses a challenge due to the omnipresence of eggs in a wide range of foods.¹¹ Furthermore, it has been shown that avoidance could lead to a lower reactivity threshold in human subjects.¹² For these reasons, a therapeutic approach seems to be more appropriate for the treatment of egg allergies. Oral immunotherapy (OIT) is one of the most studied therapeutic approaches, and encouraging results have been recently reported both in mice¹³ and in humans.¹⁴ Nevertheless, oral tolerance induction protocols to food allergens are ongoing and are only experimental because there are remaining questions that need to be answered prior to the exploration of OIT as a treatment module such as the identification of the severity and type of food allergy response to treatment, whether OIT leads to desensitization or oral tolerance, whether oral tolerance occurs naturally or is induced by treatment, the optimal dose

and duration, the degree of protection, establishment of an adequate dose and whether a maintenance dose is necessary, etc. Hence, appropriate conditions for allergen preparation, treatment protocols, and outcomes for OIT need to be standardized. Although few common facts related to successful OIT have been described such as reduction in specific IgE activity, induction of IgG4/IgG2a, altered T-cell cytokine balance (shift of Th2 to Th1 type response), and T-cell anergy,¹⁵ the overall mechanism is not completely understood, for example, the role of specific IgA exerted at mucosal level¹⁶ or the controversial role of specific IgG and its respective isotype subclasses.¹⁷

With regard to egg white (EW) OIT, the use of heated EW formulas has always been a common choice and is frequently reported.¹⁸ Treatment with heated ovomucoid-depleted egg white (HOMEW) is especially notable. HOMEW can be used in approximately 95% of egg-allergic patients because of its hypoallergenic character and has been demonstrated to be safe.¹⁹ Furthermore, the effectiveness of HOMEW in EW-allergic patients has been proven recently,¹⁸ but the underlying mechanism remains unclear. Reliable protocols for OIT are underway, and more light is being shed on the mechanisms involved in OIT.^{14,20–22}

With this background, our objective was to study the immunomodulatory effect of HOMEW in a BALB/c mouse model of egg allergy to reach a better understanding of the mechanisms involved by which HOMEW desensitizes and may induce oral tolerance.

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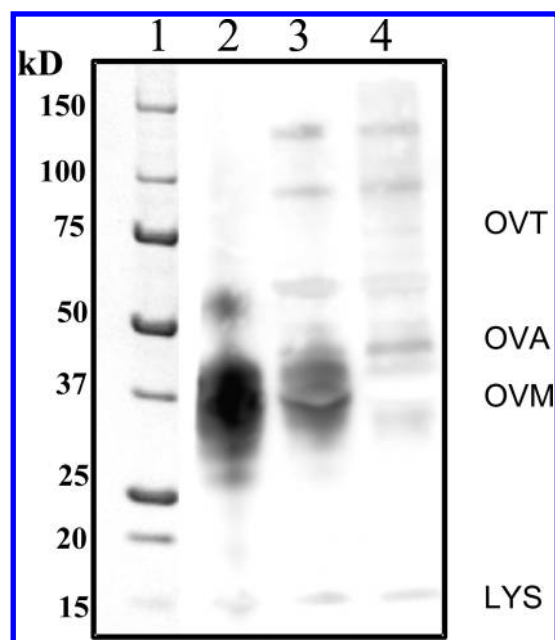


Figure 1. Immunoblot analysis of native OM, egg white (EW), and heated ovomucoid-depleted egg white (HOMEW) (20 μ g/well) by using rabbit anti-OM IgG-HRP. Lanes: 1, molecular marker; 2, pure OM; 3, EW; 4, HOMEW.

MATERIALS AND METHODS

Preparation of HOMEW. HOMEW was prepared as previously described¹⁹ with slight variations. In brief, egg white was separated and diluted (1:10, v/v) with Milli-Q water and sieved by a metallic strainer, and the pH was adjusted to 5. The sample was heated at 95 °C for 30 min. After heat treatment, the sample was centrifuged at 7000g at room temperature for 30 min. Because OM does not coagulate by heating, the OM was retained in the supernatant, whereas the precipitate contained the HOMEW. The precipitate was confirmed by Western blotting (Figure 1) to ensure that the sample was composed of HOMEW. The sample thus obtained was freeze-dried and stored at −30 °C for further use.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE). SDS-PAGE was performed according to the method of Laemmli.²³ Samples (40 μ g/well) were dissolved in sample buffer in the presence of 5% (v/v) β -mercaptoethanol, heated for 5 min at 95 °C, and run on 4–12% Criterion XT gels (Bio-Rad Laboratories, Hercules, CA). Gels were stained using Coomassie G-250 (Bio-Rad) and destained using a 40% methanol–7% acetic acid solution.

Reverse Phase High-Performance Liquid Chromatography (RP-HPLC). The RP-HPLC analysis was performed using a Waters 600 HPLC instrument (Waters, Milford, MA) and a 250 mm $\times$ 4.6 mm Widepore C18 column (Bio-Rad, Richmond, CA). Operating conditions were as follows: column at room temperature; flow rate at 1 mL/min; injection volume at 60 μ L; solvent A (0.37 mL/L TFA in Milli-Q water); and solvent B (0.27 mL/L TFA in HPLC grade acetonitrile). A linear gradient of solvent B in A, from 0 to 60% in 60 min, followed by 60% B for 30 min, was used. Absorbance was recorded at 220 nm with a Waters 2487 λ dual detector. The software Empower 2000 system data (Waters) was used.

Western Blotting. Following SDS-PAGE, the proteins (20 μ g/well) were transferred onto a 0.45 μ m nitrocellulose membrane (Bio-Rad) using a semidry transfer cell (Bio-Rad). The membrane was blocked at room temperature for 2 h with 1% casein dissolved in 1 $\times$ TBS (25 mM Tris-HCl, 0.15 M NaCl, pH 7.4; blocking buffer) and then incubated at

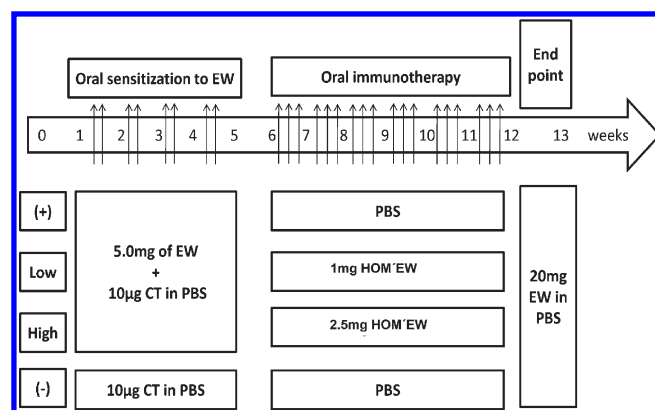


Figure 2. Groups of BALB/c mice ($n = 40$) were orally sensitized twice/week for 4 weeks with 5 mg of egg white and 10 μ g of cholera toxin and desensitized with two different doses (1 and 2.5 mg) of heated-ovomucoid depleted egg white three times/week for 6 weeks. Mice were challenged with 20 mg of intact egg white at the end, and blood and tissue samples were collected for analysis of various parameters.

4 °C overnight with rabbit anti-OM IgG antibody with horseradish peroxidase (HRP) (Immune Systems Ltd., ISL, Paignton, U.K.) diluted 1:25000 in blocking buffer. Following overnight incubation, the membrane was washed (6 $\times$ 5 min) in 1 $\times$ TBS with 0.05% Tween-20. Eventually the blots were visualized using the ECL prime Western blotting detection reagent (GE Healthcare, Buckinghamshire, U.K.).

Gastric and Duodenal Digestion. Digestibility of EW and HOMEW was demonstrated using an in vitro model system in two steps, which mimics gastric and duodenal digestion in vivo.²⁴ Both EW and HOMEW were subjected to in vitro gastric digestion at 5.7 mg/mL final concentration. In brief, the digestions were performed in simulated gastric fluid (SGF, 35 mM NaCl) at pH 2.0, for 60 min at 37 °C, with porcine pepsin (EC 3.4.23.1, 3210 U/mg protein, Sigma-Aldrich) at an enzyme/substrate ratio (E:S) of 1:20, w/w (172 U/mg). Aliquots were taken at 0, 30, and 60 min of incubation, and adjusting the pH to 7 with 1 M NaHCO₃ stopped the reaction. Duodenal digestions were performed by using the 60 min gastric digests adjusted to pH 7, as described above, with the addition of 1 M CaCl₂, 0.25 M Bis-Tris, pH 6.5, and a 0.125 M bile salt mixture containing equimolar quantities of sodium cholate and sodium deoxycholate (Sigma-Aldrich). After incubation at 37 °C for 15 min, pancreatin (Sigma-Aldrich) was added at an enzyme/substrate ratio of 1:25, w/w. The final composition of the mixture was 4.27 mg/mL EW or HOMEW, 6.15 mM of each bile salt, 20.3 mM Bis-Tris, 7.6 mM CaCl₂, and pancreatin (enzyme/substrate ratio = 1:25, w/w). Aliquots were taken after 60 min of gastric digestion and 30 min of duodenal digestion. Duplicate digestions were conducted for each condition.

Animal Sensitization and Challenge. Female BALB/c mice ($n = 40$) were purchased from Charles River Laboratories (Montreal, QC, Canada) at 6–8 weeks of age and randomly divided into four groups ($n = 10$ /group). All animals were housed in the campus animal facility at the University of Guelph under an egg-free diet [Teklad global diet, 14% protein (wheat and corn) and 3.5% fat] in a 12 h lighting cycle. Food and water were available ad libitum. All procedures were performed in accordance with the guidelines established by the Canadian Council of Animal Care (CCAC) and approved by the Animal Care Committee at the University of Guelph. As shown in Figure 2, following a 1 week acclimatization period, positive and treatment groups were sensitized with EW (5 mg/mouse) and 10 μ g of cholera toxin (CT) (List Biologicals, Denver, CO) by oral gavage twice a week for a duration of 4 weeks. After the sensitization phase, EW-specific IgE activity was determined by ELISA to ensure that the positive and treatment groups were sensitized. After the sensitization period, HOMEW was orally

administered to the treatment groups three times a week for 6 weeks in two different doses: 2.5 mg (high-dose group) and 1.0 mg (low-dose group). The positive and negative groups received 0.01 M sodium phosphate-buffered saline solution at pH 7.4 (PBS). All mice were challenged on week 13 with 20 mg of EW diluted in PBS and euthanized for collection of blood and tissue samples.

Serum Histamine Release. Following the final oral challenge, whole blood was collected by cardiac puncture, and two serum samples were pooled in equal volumes within each group ($n = 5$) due to the limited volume in individual mouse serum for performing ELISAs. Histamine concentrations were assayed using a commercial ELISA kit (Labor Diagnostika Nord, Nordhon, Germany) following the manufacturer's instructions.

Total and Specific IgE and IgG in Serum. Concentrations of total IgE and IgG in mouse serum were determined by ELISA. In brief, flat-bottom 96-well microtiter plates (Corning, Costar Corp., MA) were coated with 100 μ L of anti-mouse IgE monoclonal antibody (BD Biosciences, San Diego, CA) or goat anti-mouse IgG (Calbiochem, La Jolla, CA) at 1 μ g/mL final concentration. After overnight incubation at 4 °C, the plates were washed with PBS with 0.05% (w/v) Tween-20 (PBST) three times and blocked with 200 μ L of 1% (w/v) bovine serum albumin (BSA) in PBS for 2 h at 37 °C. The plates were washed with PBST three times, and dilutions of murine IgE (BD) (0.625–20 ng/mL) or IgG standards (AbD Serotec, Raleigh, NC) (0.625–40 ng/mL) and serum samples (1:50 for IgE and 1:40000 for IgG) in 1% BSA in PBST were added (100 μ L) to the wells in duplicate and incubated for 2 h at 37 °C. The plates were washed further with PBST four times, and 100 μ L of biotinylated anti-IgE monoclonal antibody (1:1000) (Caltag, Carlsbad, CA) or alkaline phosphate-conjugated goat anti-mouse IgG (1:2000) (Sigma-Aldrich) were added to the wells and incubated for 1 h at 37 °C. After washing four times, 100 μ L of avidin–horseradish peroxidase (BD) or extravidin conjugated to alkaline phosphatase (1:3000) (Sigma-Aldrich) was added onto the plates and incubated for 30 min at 37 °C. The reaction product was visualized by adding 100 μ L of 3,3',5,5'-tetramethylbenzidine (TMB, Sigma-Aldrich) or *p*-nitrophenol phosphatase (1 mg/mL) (Sigma-Aldrich). The reaction was incubated for 30 min followed by the addition of 25 μ L of 0.5 M H₂SO₄ or 3 N NaOH (stop solution). The optical density absorbance readings (450 or 415 nm) were taken using a microplate reader (iMark Microplate reader, Bio-Rad), and readings obtained from the individual serum samples were converted to concentrations of IgE and IgG per milliliter for each assay from the values obtained from the standard curve.

Specific IgE and IgG levels were measured by coating the plates with 50 μ g/mL of the intact EW, OM, or OVA, and a procedure similar to that described above was followed. Murine serum samples were diluted 1:5 for specific IgE and 1:1000 for specific IgG.

Measurement of Specific IgG1 and IgG2a Activity. Measurements of EW, OM, and OVA specific IgG1 and IgG2a in mouse serum samples were performed by an indirect ELISA. In brief, 96-well microplates (Corning) were coated with 100 μ L of EW or OM (50 μ g/mL) and incubated overnight at 4 °C. Plates were washed three times using PBST and blocked with 200 μ L of 1% (w/v) BSA for 1 h at 37 °C. Diluted serum samples (1:10000 dilutions for specific IgG1 and 1:1000 for specific IgG2a) were added to each well and incubated for 1 h at 37 °C. After three washings with PBST, biotinylated monoclonal rat antibodies were added for IgG1 and IgG2a (100 μ L/well; 1 μ g/mL) (BD), and the plates were incubated for 1 h at 37 °C. The plates were washed three times and incubated further with 100 μ L of avidin–HRP peroxidase-conjugated (BD) (1:2000) for 30 min at 37 °C before detection. The plate was washed six times with PBST, and the reaction was visualized using 50 μ L of TMB (Sigma-Aldrich) and incubated for 30 min. The reaction was terminated by adding 25 μ L of 0.5 M H₂SO₄, and absorbance was read at 450 nm using a microplate reader (iMark Microplate reader, Bio-Rad).

Measurement of EW-Specific IgA in Fecal Samples. In an effort to further elucidate the underlying mechanism occurring locally at the intestinal level, mouse fecal pellets were freshly collected on a weekly basis from each mouse group cage and were submitted to the following extraction procedure. In brief, fecal pellets were freeze-dried, diluted 1:7 (w/w) in PBS, and homogenized by using a vortex. Samples were subsequently centrifuged at 1600g for 15 min at 4 °C to remove large fibrous particles. Supernatants were carefully collected and centrifuged again at 9500g for 10 min at 4 °C. Concentrations of EW-specific IgA were determined using a sandwich ELISA procedure. In brief, flat-bottom 96-well ELISA plates (Corning) were incubated with 100 μ L/well of EW (50 μ g/well) in 100 mM NaHCO₃ (pH 9.6) and stored overnight at 4 °C. Plates were subsequently washed four times with PBST and blocked with 200 μ L/well of 1% BSA in PBS and incubated for 1 h at 37 °C. An additional four washings were performed, and 100 μ L/well of fecal supernatants was added onto the plate in triplicate wells and incubated overnight at 4 °C. The plates were washed four times with PBST and incubated with 100 μ L/well of biotinylated monoclonal anti-mouse IgA (1:500; BD) diluted in 1% BSA in PBST for 1 h at 37 °C. The wells were further washed four times with PBST, and 100 μ L/well of avidin–HRP conjugate (1:2000; BD) was applied onto the plate for 30 min of incubation at 37 °C. After a final four-wash cycle with PBST, EW-specific IgA binding activity was revealed by the addition of 50 μ L/well of TMB (Sigma-Aldrich). The reaction was terminated after 30 min by the addition of 25 μ L/well of 0.5 M H₂SO₄, and absorbance values were determined at 450 nm using an ELISA microplate reader (iMark Microplate reader, Bio-Rad).

Mouse Spleen Cell Cultures and Determination of Cytokine Secretion. At the experimental end point, post oral challenge, spleens from individual mice were aseptically removed into ice-cold RPMI-1640 medium (Gibco Invitrogen, New York, NY), containing NaHCO₃ (1.5 g/L), glucose (4.5 g/L), L-glutamine (2 mM), sodium pyruvate (1 mM), penicillin (50 U/mL), and streptomycin (50 mg/mL), and two whole spleens were pooled within each group ($n = 5$ /group). The cell suspensions were passed through a 100 μ m nylon membrane cell strainer, transferred to 15 mL conical centrifuge tubes, and centrifuged for 10 min at 500g at 4 °C. Erythrocytes in spleen cell preparations were lysed with 2 mL of red blood cell lysing buffer (Sigma-Aldrich), and 10 mL of RPMI medium was added to stop the lysis. The splenocytes were washed twice with 10 mL of RPMI by centrifugation. Splenocytes were resuspended in 10 mL of medium [RPMI 1640 supplemented with 8% fetal bovine serum (FBS)] (Hyclone, Fisher, Canada), and cell viability was assessed by trypan blue exclusion. Cells were cultured in 24-well plates (Corning) at a density of 2.5×10^6 /mL in the absence (negative control wells) or presence of purified EW (100 μ g/mL) in triplicates. Supernatants were collected after 72 h of incubation in a 5% CO₂ humidified incubator and assayed for the presence of cytokines. Concentrations of IFN- γ , IL-4, TGF- β , and IL-10 secreted in murine splenocyte culture supernatants were assayed by ELISA. Briefly, 96-well plates (Corning) were coated with 100 μ L of the capture antibodies, rat anti-mouse IL-4 and INF- γ (BD) (1:250) or rat anti-mouse IL-4 (BD) (1:250), and the plates were incubated at 4 °C overnight. The plates were washed three times with 200 μ L of PBST and blocked with 200 μ L of 1% BSA in PBS at 37 °C for 1 h. The plates were further washed three times with PBST and 100 μ L of standard cytokines (BD) at concentrations of 31.250–1000 pg/mL (IFN- γ) or 15.625–5000 pg/mL (IL-4) diluted in 1% (w/v) BSA in PBST, and the culture supernatant samples were added at different dilutions, 1:10 (IFN- γ) or 1:2 (IL-4), and incubated at 37 °C for 2 h. After four washings, 100 μ L of detection antibodies was added, biotinylated rat anti-mouse INF- γ (BD) (1:2000) or biotinylated rat anti-mouse IL-4 (BD) (1:2000), and incubated for 1 h at 37 °C. The plates were washed four times, and bound antibodies were detected using 100 μ L of HRP-conjugated avidin (BD) at 1:2000 dilution; plates

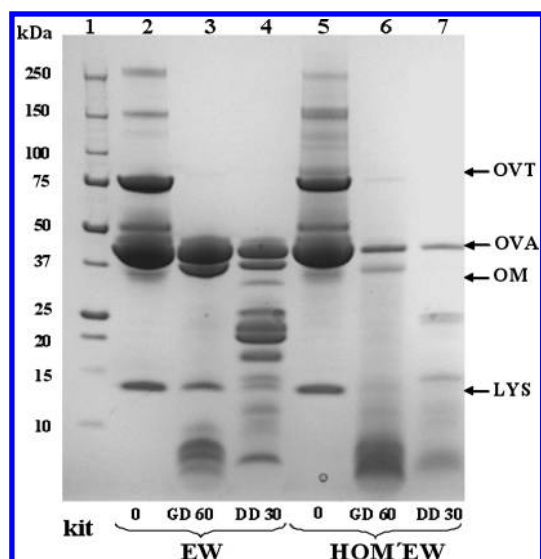


Figure 3. SDS-PAGE gel of in vitro gastric digestion at pH 2 (GD) and duodenal digestion (DD) of egg white (EW) and heated ovomucoid-depleted egg white (HOMEW) (40 μ g/well). Lanes: 1, molecular marker; 2 and 3, GD of EW at 0 and 60 min, respectively; 4, DD of EW at 30 min; 5 and 6, GD of HOMEW at 0 and 60 min, respectively; 7, DD of HOMEW at 30 min.

were then incubated for 30 min at 37 °C and washed six times with PBST. Then 50 μ L of TMB (Sigma-Aldrich) was used as a substrate, the plate was incubated for 20 min in the dark at 37 °C, and 25 μ L of stop solution (0.5 M H_2SO_4) was added. The optical density was measured by an ELISA reader (iMark Microplate reader, Bio-Rad) with a 450 nm filter. Production of TGF- β and IL-10 in the spleen cell culture supernatants was determined using the ready to use commercial kits TGF-B Ready-Set-Go and IL-10 Ready-Set-Go (eBiosciences Inc., San Diego, CA) following the manufacturer's instructions. Standard curves for each cytokine (15.625–1000 pg/mL TGF- β ; 15.625–1000 pg/mL IL-10) were used to quantify the levels of cytokines present in the culture supernatant samples.

Statistical Analysis. Histamine, immunoglobulin, and cytokine concentrations measured by ELISAs were subjected to ANOVA analyses followed by post hoc multiple-comparison using Tukey's test. In all cases, P values of ≤ 0.05 were considered to be statistically significant. Statistical calculations were performed using the GraphPad Prism package (Graphpad, San Diego, CA).

RESULTS AND DISCUSSION

High in Vitro Digestibility of HOMEW. Digestibility of EW and HOMEW was performed to assess digestibility by using an in vitro system in two steps, which mimicked digestion in the stomach (gastric) and duodenum.²⁴ HOMEW was more susceptible to digestion than intact EW as shown in Figure 3 by SDS-PAGE. Looking at the 60 min gastric digests of EW and HOMEW (lanes 3 and 6), the band of OVA (44 kDa), the most abundant allergen in the albumen, is mainly intact in the EW, whereas is almost fully digested in the HOMEW, which presented a wide band of digestion products with molecular masses of <10 kDa. OVT (76 kDa) was fully digested in the gastric phase in both EW and HOMEW, whereas LYS (14.4 kDa) resisted the peptic action as part of the EW but not in the HOMEW. The higher digestibility of HOMEW was also kept during duodenal digestion (lanes 4 and 7), where the profile of HOMEW is clearer

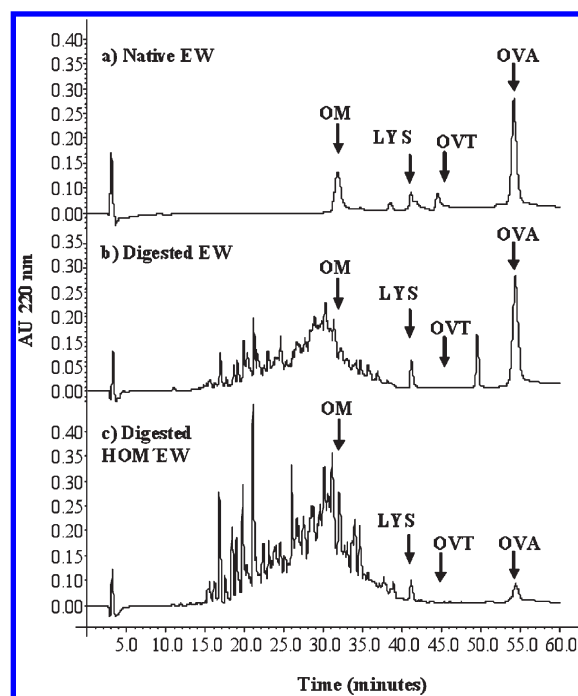


Figure 4. RP-HPLC analyses of nondigested egg white (EW) (a) and 60 min gastric digests of EW (b) and heated ovomucoid-depleted egg white (HOMEW) (c).

than that of EW because most of the protein content was completely hydrolyzed. Nondigested EW and 60 min gastric digests of EW and HOMEW were further analyzed by RP-HPLC (Figure 4), and the profiles were in agreement with SDS-PAGE outcomes. In the RP-HPLC profile of nondigested EW, the main EW allergens were identified as OVA that elutes at 55 min and OM after 33 min,²⁵ OVT at 46 min, and LYS at 42 min²⁶ (Figure 4A). A considerable amount of OVA was present in EW (Figure 4B), whereas it was almost undetectable in the 60 min gastric digest of HOMEW (Figure 4C). Upon peptic digestion the OM profile displayed a wide nonresolved mix of peaks that were difficult to identify within the complex matrix of digested EW (Figure 4B). It is interesting to note in the profile of the HOMEW gastric digest (Figure 4C) the increased amount of peptides from minutes 15–32 compared to that of the EW gastric digest (Figure 4B), which indicates the higher degree of hydrolysis in HOMEW. This increase in susceptibility to digestion of HOMEW can be attributed to the heat treatment because OVA is thermolabile and its digestibility increases by heat treatment.^{25,27} LYS and OVT have also been reported to be unstable to heat treatment.¹⁸ Furthermore, OM is reported to be digested into three fragments, and two of them resist duodenal digestion and are able to retain IgE binding activity;^{10,25} hence, the low concentration of OM in HOMEW made it more susceptible to digestion. A high stability through digestion is usually accepted as a characteristic nature of a food allergen, which helps to keep the epitopes intact;²⁸ thus, the high digestibility of HOMEW may be related to the safety of OIT performed in this experiment. The gut-associated lymphoid tissues are widespread throughout the digestive tract in which the intestinal lamina propria contains a complex population of cells including activated CD4⁺ T lymphocytes and B lymphocytes, macrophages, dendritic cells, eosinophils, and mast cells. Also, there

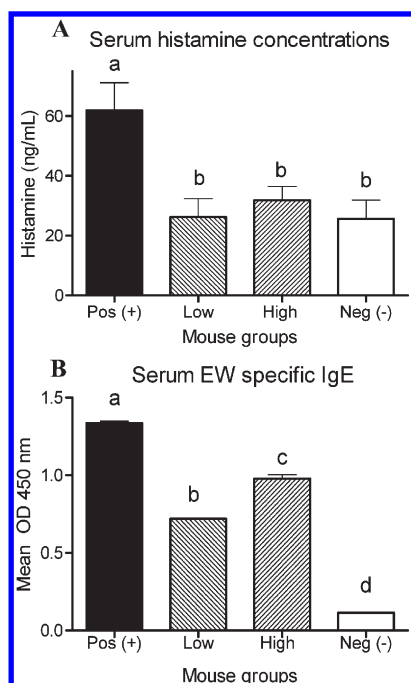


Figure 5. Serum histamine concentration and egg white-specific IgE activity in mice sera after oral challenge represented as mean standard deviation ($n = 5$) pooled sera. Different letters indicate statistically significant differences ($P < 0.05$) between groups of mice.

are organized lymphoid tissues, Peyer's patches being the most prominent, that likely have a pivotal role in triggering immune responses to digested antigens. Interestingly, it was recently reported that heat treatment of OVA and OM prevented transport across human intestinal epithelial cells in a form capable of triggering basophil activation or T-cell activation²⁹ and, together with the advanced degree of digestion of HOMEW at intestinal level, compared to intact EW, might be critical in the use of HOMEW for successful OIT. We recently reported oral administration of EW hydrolysates with peptic fragments of <1.4 kDa led to a specific immune hyporesponsiveness in EW-primed BALB/c mice.³⁰ It was also shown that the higher the digestibility, the lower the antibody binding, and heat treatment showed a significant influence on the potential allergenicity of the main egg white proteins that could be related to their resistance to denaturation and digestive enzymes.²⁵ Taken together, these data strongly support and augment our study in which HOMEW was able to induce successful tolerance to EW-sensitized mice, which may due to tolerogenic peptides present in HOMEW.

Low Histamine and EW-Specific IgE Activity after Oral Challenge. Histamine concentration was checked in mice sera after oral challenge with EW (Figure 5A). Both treated mouse groups had significantly less histamine than the positive group and were similar to the negative group, which confirmed the success of OIT. Also, EW-specific IgE antibody activity post oral challenge was less in both treatment groups than the positive group but significantly higher than negative group. Interestingly, the high-dose group showed higher EW-specific IgE activity than the low-dose group (Figure 5B).

Total and Allergen-Specific IgG and IgG Subclasses. Attempting to find other mechanisms that may have contributed to a lower histamine concentration, we studied in depth sera IgG activity. Total IgG and EW-specific IgG levels were similar in

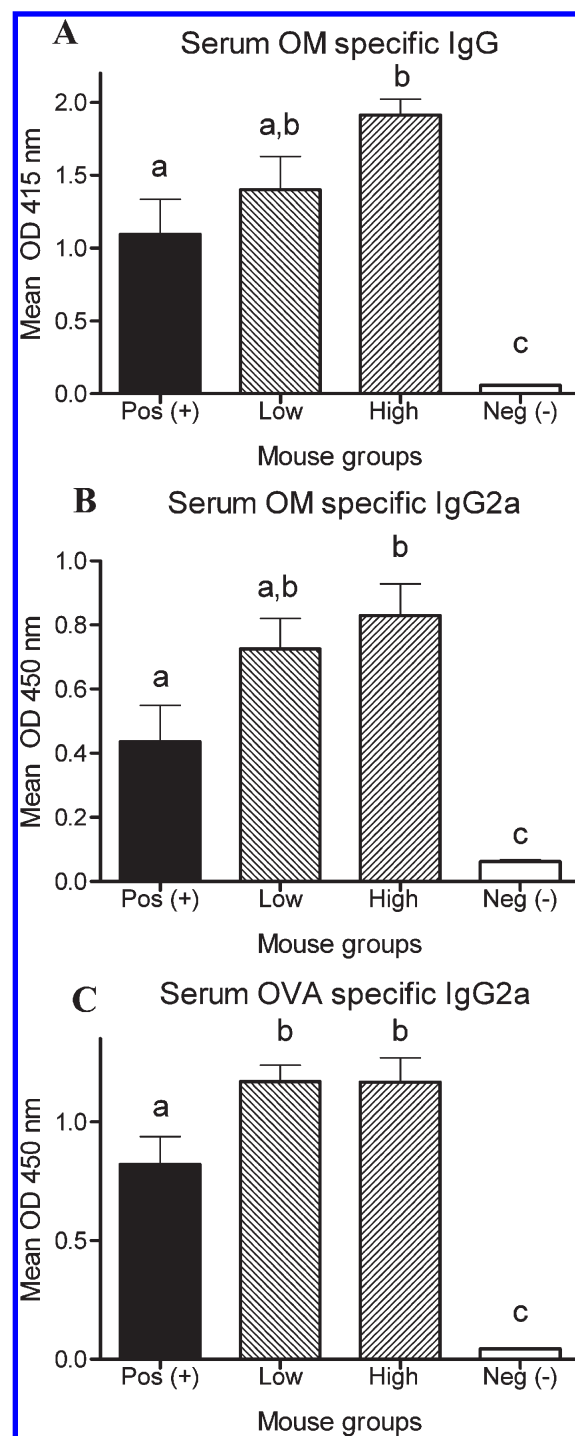


Figure 6. OM-specific IgG and IgG2a and OVA-specific IgG2a activities in mice sera post oral challenge. Data are represented as mean $\pm$ standard deviation ($n = 5$ pooled sera). Different letters indicate statistically significant differences ($P < 0.05$) between groups of mice.

sensitized mouse groups (data not shown). However, treated mice showed high OM specific IgG levels, and this increase was significant in the high-dose group (Figure 6A). Antibodies to specific IgG1 of EW, OVA, and OM were also analyzed without significant differences between groups; in contrast, EW-specific IgG2a was enhanced (data not shown) and OM- and OVA-specific IgG2a were significantly higher in the treated groups (Figure 6B,C).

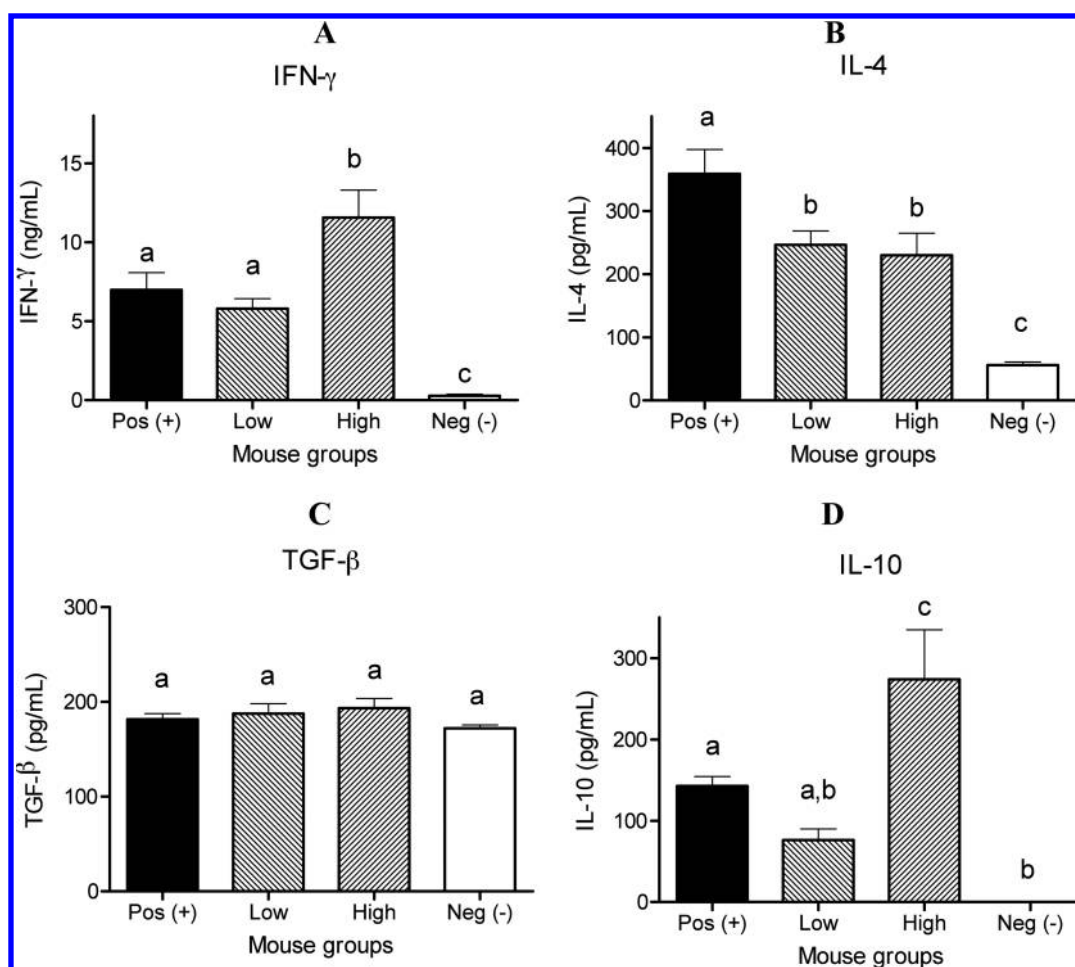


Figure 7. Determination of cytokine concentrations of IFN- γ , IL-4, TGF- β , and IL-10 following in vitro stimulation of spleen cell cultures with EW. Data are represented as mean $\pm$ standard deviation ($n = 5$ pooled spleens). Different letters indicate statistically significant differences ($P < 0.05$) between groups of mice.

The functions of allergen-specific IgG have been largely studied, but the roles in immunotherapy are still being investigated and are a point of discussion. One of the mechanisms related to allergen-specific immunotherapy is the induction of allergen-specific IgG antibodies, and it is suggested that these antibodies may compete with specific IgE to bind the allergen at the mucosal surface and block the allergic response. On the one hand, this is questioned because serum concentrations of allergen-specific IgG correlate with clinical improvement in some studies but not in others;³¹ in addition, many mast cells are on the mucosal surfaces and could meet allergens before antibodies can interpose themselves.³² On the other hand, it has been shown in a series of interesting experiments how allergen-specific IgG can counteract allergen-specific IgE activity by direct competition by binding epitopes or via inhibitory FcRIIB-dependent signals.¹⁷ On this note, in the current study the increase of OVA- and OM-specific IgG2a and the increase of OM-specific IgG might be effective mechanisms induced during OIT that contribute to a lower histamine release.

Increased Th1/Th2 Ratio. The role of Th1 and Th2 cell mediated type responses is well established in mouse models, and recent research on T-regulatory cells sheds more light on the balance of Th1 and Th2 cell mediated response. In the present study, analyses of culture supernatants stimulated in vitro with

purified EW indicated that the levels of IFN- γ , the type 1 hallmark cytokine, were significantly higher in the high-dose treated mice (Figure 7A), and no significant difference was observed in the low-dose treatment group as compared to the positive control. With regard to IL-4 concentration, the Th2 hallmark cytokine, the treatment groups (both high and low) had significantly less concentration as compared to the positive control group (Figure 7B). Also, concentrations of TGF- β (Th3) and IL-10 (Tr1) were analyzed, and no significant difference between groups was observed with TGF- β (Figure 7C); however, a significant increase of IL-10 concentration was found in the high-dose treatment group (Figure 7D), indicating a significant role of regulatory T cells in immune modulation caused by OIT with HOMEW.

It was of interest to note that the treatment effects were dose dependent and that the high-dose group experienced a marked increase in Th1/Th2 ratio, as a result of a significant increase of IFN- γ and a significant decrease of IL-4. The switch from Th2 to Th1 response could be attributed to a significant role of the regulatory cytokine IL-10. It has been reported earlier that in peanut-allergic patients undergoing OIT there is an increase in IL-10 secretion from peripheral mononuclear cells during the first months of OIT, which eventually tends to go down and likely depends on the stage of OIT: escalation, buildup, and maintenance.³³

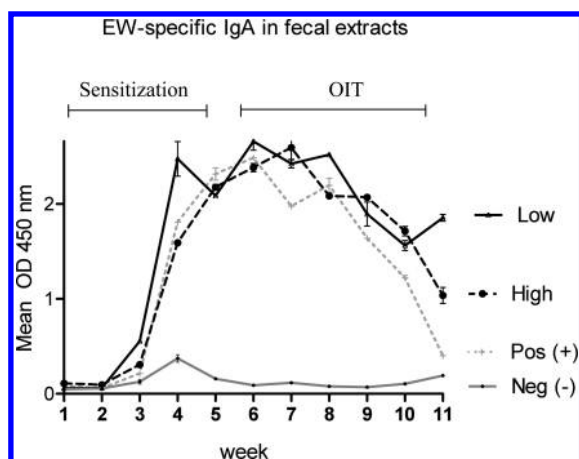


Figure 8. Time course monitoring of egg white (EW)-specific IgA levels in mouse fecal extracts before and during oral immunotherapy with heated ovomucoid depleted egg white. Data are represented as mean $\pm$ SD ($n = 3$).

This finding could be attributed to the low-dose group in which dose factor may play a significant role in OIT. In fact, it has been shown that IL-10 can modulate allergic reactions by different mechanisms: suppression of allergen-specific IgE, induction of allergen-specific IgG4/IgG2a, suppression of both allergen-specific Th1 and Th2 cells, and reduction of the release of pro-inflammatory cytokines by mast cells.^{34–36} The suppression of antigen-specific immune responses by IL-10 is essential in peripheral tolerance to allergens. In mice it was shown to be the pivotal role of IL-10 in the establishment of peripheral T-cell tolerance by administration of IL-10, which drove mice to antigen-specific T-cell unresponsiveness.^{37,38} Also, Enrique et al.³⁹ reported high levels of IL-10 in sera after sublingual immunotherapy in hazelnut-allergic patients. On the basis of our results, it may be postulated that IL-10 has exerted a critical role in immune modulation of the high-dose treatment group that might be indicative of a role of T-regulatory cells for induction of oral tolerance.

EW-Specific IgA in Fecal Samples. EW-specific IgA activity was determined in mouse fecal sample supernatants (Figure 8) in an effort to further elucidate the mechanisms occurring locally at the intestinal level. Both the allergic and treatment groups had an increase of EW-specific IgA throughout the sensitization phase. Once the sensitization phase had finished, the positive group experienced a decrease of EW-specific IgA, whereas treatment groups had an increase of EW-specific IgA at the beginning of OIT. Then specific EW–IgA activity decreased slowly but was always higher than the positive group activity. At the end of OIT, treatment groups showed higher activity of EW-specific IgA than the positive control group. The negative group had low activity of EW-specific IgA throughout the study. Secretory IgA has an important role in the immune homeostasis of the gut,¹⁶ but its role in food allergy is still unclear. It has been earlier reported that low levels of allergen-specific IgA in the gut were associated with development of food allergies.⁴⁰ Although specific IgA was elevated during the sensitization phase, at the end of OIT, just before the end point, we can clearly see how the treated groups had a high activity of allergen-specific IgA, whereas the negative and positive groups had low activities of EW-specific IgA, suggesting a susceptible state to develop allergic disease, which supports previous studies^{13,16,40} and reinforces the fact that a

higher production of EW-specific IgA at the mucosal level may have contributed to the allergy-suppressive effect of HOMEW.

In conclusion, the present study establishes that the success of HOMEW can be related to its high digestibility. OIT with a high dose of HOMEW induced desensitization through a switch from Th2 to Th1 response marked by an increase in IL-10 concentration. Also, less histamine and EW-specific IgE and more specific IgG and IgG2a could have contributed to suppression of allergic response, and high amounts of EW-specific IgA in fecal samples suggest an important role at the mucosal level that might be contributing to the therapeutic effect of HOMEW.

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ABBREVIATIONS USED

HOMEW, heated ovomucoid-depleted egg white; OIT, oral immunotherapy; OVA, ovalbumin; OM, ovomucoid; LYS, lysozyme; OVT, ovotransferin; SGF, simulated gastric fluid; IL, interleukin; IFN- γ , interferon gamma; TGF- β , transforming growth factor beta; Th1, T-helper cell response type 1; Th2, T-helper cell response type 2; T-reg, T regulatory cell response.

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