

Allergenic Properties of Ovomucoid in Man¹

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Ovomucoid, the dominant allergen in hen's egg, is a highly glycosylated protein comprising 186 amino acids arranged in three tandem domains (*Gal d* 1.1, 1.2, and 1.3). The purpose of this study was to evaluate the allergenic properties of ovomucoid. The three ovomucoid domains were isolated and evaluated with sera from egg allergic patients to determine B cell domain specificity, B cell epitopes, and the relative importance of linear and conformational structures and carbohydrate chains to B cell epitopes. Peripheral blood T cells from egg allergic patients were used to evaluate T-dominant domains and reactivity to reduced and oxidized ovomucoid. There was significantly more IgE activity to the second ovomucoid domain (median percentage of ovomucoid-specific IgE: *Gal d* 1.2, 40%; *Gal d* 1.1, 23%; *Gal d* 1.3, 26%). Quantities of patient IgG Ab were comparable for all three domains. Five IgE and seven IgG binding regions were identified. IgE Ab binding to reduced ovomucoid and IgG binding to oxidized ovomucoid were significantly reduced compared with that to native ovomucoid (28 and 69%, respectively). Peripheral blood T cells of 21 of 33 patients reacted to *Gal d* 1.3, 18 of 33 reacted to *Gal d* 1.2, and 18 of 33 reacted to *Gal d* 1.1. T cell proliferation in vitro in response to reduced and oxidized ovomucoid were significantly greater than that in response to the native protein. These results indicate a dichotomy between T and B cell domain dominance, and the presence of both unique and common IgE and IgG epitopes. Furthermore, the results suggest that conformational B cell epitopes play a more significant role in ovomucoid allergenicity than previously appreciated, and that carbohydrate moieties have a minor effect on allergenicity. *The Journal of Immunology*, 1997, 159: 2026–2032.

Chicken egg is one of the most common causes of food allergic reactions in children (1–3). In our study of 470 children and adolescents referred for evaluation of atopic dermatitis and possible food hypersensitivity, 80% were found to develop symptoms during double-blind placebo-controlled food challenges (DBPCFC)³ (4). Approximately two-thirds of children diagnosed with food allergies were reactive to egg. Egg white protein contains 23 different glycoproteins, most of which have been purified and their amino acid sequences determined (5). Ovomucoid (*Gal d* 1), OVA (*Gal d* 2), ovotransferin (*Gal d* 3), and lysozyme (*Gal d* 4) have been identified as major allergens (6–8), but ovomucoid has been shown to be the dominant allergen (9), even though ovomucoid comprises only 10% of total egg white protein, and OVA comprises >50%.

Chicken ovomucoid has a m.w. of 28,000 and an isoelectric point of 4.1. *Gal d* 1 is comprised of 186 amino acids arranged in three tandem domains (*Gal d* 1.1, *Gal d* 1.2, and *Gal d* 1.3), each about 60 amino acids in length, and is highly homologous to pancreatic secretory trypsin inhibitor, although its trypsin inhibitory activity is confined to the second domain (10). Each domain is cross-linked by three intradomain disulfide bonds, but there are no

interdomain disulfide bridges. *Gal d* 1.1 and *Gal d* 1.2 contain four carbohydrate chains, and about one-half of *Gal d* 1.3 contain one carbohydrate chain (11). The purpose of this study was to evaluate the allergenic properties of chicken ovomucoid using sera and PBMC from children with egg hypersensitivity diagnosed by DBPCFC.

Materials and Methods

Preparation and purification of ovomucoid domains

The three domains of ovomucoid were prepared by cleaving whole ovomucoid (Sigma Chemical Co., St. Louis, MO) with cyanogen bromide in the presence of 70% formic acid (ovomucoid domain 1; *Gal d* 1.1) or with Spase V8 (Sigma Chemical Co.; ovomucoid domains 2 and 3; *Gal d* 1.2 and *Gal d* 1.3, respectively) and chromatographically purified, as described by Kato et al. (10).

The purity of all isolated domains was determined by ELISA using domain-specific mAbs developed in our laboratory. Monoclonal Abs to ovomucoid domains were generated using a modification of the method of Searce et al. (12). The specificities of the mAbs were established using purified ovomucoid domains provided by Dr. Michael Laskowski, Jr. (Department of Chemistry, Purdue University, West Lafayette, IN).

Patient population

Sera and PBMCs from children referred for evaluation of atopic dermatitis and food hypersensitivity with positive DBPCFC to egg were used in this study (13). Serum egg-specific IgE concentrations were determined using the CAP-RAST FEIA system (Pharmacia Diagnostics, Uppsala, Sweden).

ELISA to determine the domain specificity of patient IgE and IgG

The extent to which egg allergic patients recognize each of the three ovomucoid domains was determined by ELISA. Prick skin test titrations with whole ovomucoid were previously shown to correlate with serum ovomucoid-specific IgE Ab levels in egg allergic patients (9). Domains and whole ovomucoid were bound to microtiter plate wells (Immulon II, Dynatech, Chantilly, VA) in 0.1 M sodium carbonate buffer (pH 9.6) at a concentration of 5 µg/ml. Wells were blocked with PBS containing 0.05% Tween-20 (PBST) and 1% human serum albumin. Sera from egg allergic patients were diluted 1/50 for IgE detection and 1/500 for IgG detection in PBST with 2% BSA (fraction V, Sigma Chemical Co.) and added to 96-well plates. Plates were washed five times with PBST, and patient IgE

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³ Abbreviations used in this paper: DBPCFC, double-blind, placebo-controlled food challenge; PBST, phosphate-buffered saline containing 0.05% Tween-20; BCIP, 5-bromo-4-chloro-3-indolyl-phosphate; NBT, 4-nitro blue tetrazolium chloride; kUA/l, Ag-specific kilo units IgE/liter.

anti-domain was detected using goat anti-human IgE Ab (unlabeled Ab purchased from Kirkegaard and Perry Laboratories, Gaithersburg, MD; adsorbed extensively with human IgG and biotinylated) followed by avidin/alkaline phosphatase (Jackson ImmunoResearch Laboratories, West Grove, PA). Patient IgG was detected using alkaline phosphatase-conjugated goat anti-human IgG (Kirkegaard and Perry Laboratories). Plates were developed with *p*-nitrophenylphosphate (SigmaFAST, Sigma Chemical Co.). ODs were read at a wavelength of 405 with a reference wavelength of 490 on a Vmax kinetic microplate reader (Molecular Devices, Menlo Park, CA). IgE and IgG domain-specific binding was expressed as a percentage of IgE or IgG binding to whole ovomucoid.

Preparation of oxidized, reduced and alkylated, and deglycosylated ovomucoid

Oxidized ovomucoid was prepared by dissolving whole ovomucoid in 70% performic acid at a concentration of 10 mg/ml. The reaction was allowed to proceed for 15 min at room temperature and then quenched with an excess of water. Oxidized ovomucoid was lyophilized and stored at 4°C until used.

Reduced and alkylated ovomucoid was prepared by dissolving whole ovomucoid in PBS at a concentration of 50 mg/ml. Solid dithiothreitol was added to a final concentration of 0.11 M, and the mixture was boiled for 2 min. The solution was then saturated with nitrogen and capped, and the reaction was allowed to proceed for 3 h at room temperature. Following this incubation period, a sample was removed to check the efficiency of the reduction reaction, and iodoacetamide (final concentration, 0.25 M) was added to the remaining sample. The pH of the solution was adjusted to 8.5 with 1 N NaOH, and the alkylation was allowed to proceed at room temperature for 30 min. 2-ME was added in a two- to fourfold molar excess with respect to ovomucoid, and the sample was dialyzed against PBS and stored at -20°C until used. The efficiency of the reduction was monitored using 5,5'-dithio-bis-(2-nitrobenzoic acid) (Ellman's reagent, Pierce Chemical Co., Rockford, IL).

Deglycosylated ovomucoid was prepared by enzymatic removal of the carbohydrate moieties from the reduced and alkylated ovomucoid preparation. Reduced and alkylated ovomucoid was adjusted to a concentration of 1 mg/ml with PBS. *N*-glycosidase F (recombinant protein; Boehringer Mannheim, Indianapolis, IN) was added to a final concentration of 4 U/ml, and the protein was deglycosylated for 24 h at room temperature. An additional 4 U/ml of enzyme was added and incubated at room temperature for another 24 h. The efficiency of the deglycosylation was monitored by the DIG glycan ELISA kit (Boehringer Mannheim).

SDS-PAGE

Proteins were separated by SDS-PAGE as previously described (14). Briefly, protein samples were resolved on discontinuous 13.5% acrylamide gel with a 3% acrylamide stacking gel; both gels contained 0.1% SDS. The protein sample concentrations were optimized to give equivalent signal when stained by amido black and analyzed by laser densitometry. The samples were boiled for 10 min before being applied to the gels, and electrophoresis was conducted at constant voltage, 80 V for 1 h through the stacking gel and 160 V for 3 h through the resolving gel. The resolved proteins were subsequently transferred to nitrocellulose overnight in 50 mM Tris-glycine containing 20% methanol at 0.15 mA. The resulting nitrocellulose was then stained with amido black to look for total protein transfer or blocked with PBST with 0.5% porcine gelatin for probing with patient serum.

Probing immunoblots with patient serum

Patient sera were diluted 1/10 in PBST plus gelatin and incubated with immunoblots for 2 h with gentle agitation at room temperature. Blots were then washed five times for 5 min in PBST and then incubated with goat anti-human IgE (Kirkegaard and Perry Laboratories; adsorbed against human IgG and biotinylated) or anti-human IgG for 2 h at room temperature. Immunoblots were washed and incubated with avidin-alkaline phosphatase (Jackson ImmunoResearch Laboratories) for 0.5 h at room temperature. Immunoblots were again washed and then developed with 5-bromo-4-chloro-3-indolyl-phosphate (BCIP)/4-nitro blue tetrazolium chloride (NBT; SigmaFAST, Sigma Chemical Co.). Probing immunoblots with non-egg-allergic sera revealed no IgE binding, i.e., no nonspecific binding (data not shown).

Inhibition ELISA

The extent to which conformation influenced IgE and IgG Ab binding to ovomucoid was determined by evaluating the ability of the altered ovomucoids to inhibit binding of egg allergic patients' Abs to native ovomucoid.

Microtiter plates were coated with whole ovomucoid at a concentration of 5 µg/ml in 0.1 M sodium carbonate buffer (pH 9.6). Patient sera (1/100) were combined with varying dilutions of whole ovomucoid, oxidized ovomucoid, or reduced and alkylated ovomucoid in 12 × 75-cm tubes and incubated at 37°C for 2 h. The serum/Ag mixtures were added to the wells of the ovomucoid-coated microtiter plates and incubated for 2 h at room temperature. Plates were washed five times with PBST, and then detection Abs were added. To detect IgE binding, biotinylated goat anti-human IgE was added, followed by avidin-conjugated alkaline phosphatase. Plates were developed with *p*-nitrophenyl phosphate. To detect patient IgG binding, horseradish peroxidase-labeled goat anti-human IgG (Dako, Santa Barbara, CA) was added, and plates were developed with *o*-phenylene diamine.

Preparation of SPOTs membrane of overlapping peptides

A membrane containing dodecapeptides overlapping by 10 amino acids was prepared using the SPOTs kit available from Genosys (Genosys, The Woodlands, TX). Briefly, using company-supplied computer software, a synthesis schedule was generated based upon the protein sequence, the desired peptide length, and the amount of overlap required. The peptides were generated by adding F-moc amino acids to the membrane in the order specified by the schedule.

Probing the SPOTs membrane with patient serum

After blocking the SPOTs membrane with PBST containing 0.5% porcine gelatin (Sigma Chemical Co.) and 1% human serum (from a nonallergic control with no detectable IgE Abs to egg proteins), sera from egg allergic patients were incubated with the SPOTs membrane to detect IgE epitopes. The blot was then incubated with a 1/5 dilution of pooled sera obtained from seven egg allergic patients. After washing five times in PBST and gelatin, the membrane was incubated with biotin-conjugated goat anti-human IgE followed by avidin-horseradish peroxidase (Kirkegaard and Perry Laboratories). The membrane was developed with the ECL chemiluminescent detection system (Amersham, Arlington Heights, IL); ECL reagent was added to the membrane and incubated for 1 min with gentle agitation. The membrane was exposed to film for 5 min and developed.

For detection of IgG epitopes, the membrane was incubated in a 1/100 dilution of a pooled sera from seven egg allergic patients, then incubated in goat anti-human IgG alkaline phosphatase conjugate and developed using BCIP/NBT.

When the membrane was developed with the ECL system, it could be re-used after stripping bound Abs by exposure to a high concentration of urea. The membrane was thoroughly rinsed in distilled, deionized water (three times, 10 min), then incubated in 8 M urea containing 35 mM SDS and 0.1% BME (three times, 10 min, for IgE stripping; three times, 30 min, for stripping IgG). The membrane was then washed three times for 5 min in 50% ethanol/10% acetic acid and rinsed twice 10 min in methanol before blocking and probing again.

T cell proliferation

PBMCs were separated from whole heparinized blood by Ficoll-Hypaque density gradient. Cells were washed in RPMI and resuspended in RPMI containing 10% autologous plasma (saved from the plasma layer of the Ficoll-Hypaque gradient). Cells were added to microtiter plates at a density of 2×10^5 , and Ags were added at the following concentrations: whole, oxidized, and reduced and alkylated ovomucoid at 0.2 mg/well and *Gal d* 1.1, 1.2, and 1.3 at 2 µg/well. Plates were incubated for 5 days at 37°C in a 5% CO₂ environment. On the fifth day plates were pulsed with [³H]thymidine (1 µCi/well) and harvested after 6 h. Data are represented as stimulation indexes, which are the counts per minute in response to the Ag divided by the counts per minute of the medium control (PBMC, RPMI, and 10% autologous plasma).

Statistics

Statistical analysis of the data was performed using the nonparametric Wilcoxon signed rank test for paired data and the Mann-Whitney tests.

Results

Each isolated ovomucoid domain was evaluated by ELISA with four domain-specific mAbs to confirm their purity. M1 is a mAb that is specific for *Gal d* 1.1, M2 is specific for *Gal d* 1.2, and M3 and M4 are specific for different epitopes on *Gal d* 1.3. Each of the isolated domains was free of other contaminating ovomucoid fractions, as depicted in Figure 1.

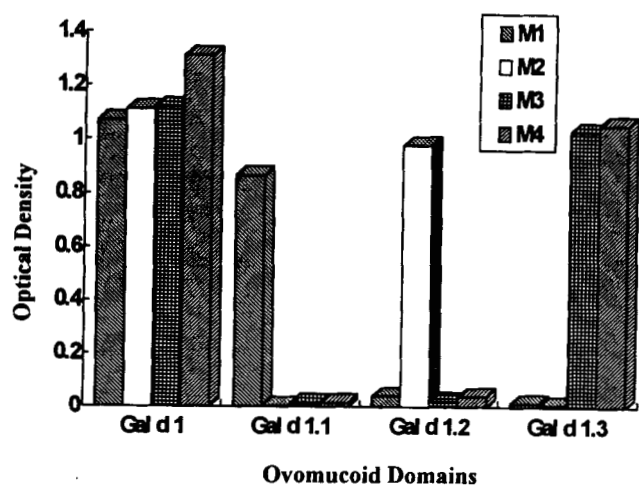


FIGURE 1. Ovomucoid domain-specific mAbs demonstrating the purity of isolated, purified domains. M1 is specific to *Gal d 1.1*, M2 is specific to *Gal d 1.2*, and M3 and M4 are specific to *Gal d 1.3*.

Serum samples from 45 patients with elevated levels of IgE Abs to hen's egg (IgE >20 kU_A/l) and egg allergy confirmed by DBPCFC were tested by direct ELISA to determine the percentage of patient ovomucoid-specific IgE reactive with each of the ovomucoid domains. Patients ranged in age from 0.5 to 19.5 yr (median, 3.5 yr). The median egg-specific IgE of the patient group was 65.3 kU_A/l with a range of 21 to >100 kU_A/L. Of the 45 patients tested, 42 had IgE Abs specific to all three domains; three patients had no detectable IgE Ab to *Gal d 1.1*. Although most patients had IgE Abs to all three domains, the percentage of IgE Abs directed to *Gal d 1.2* was significantly greater ($p < 0.05$) than that to either *Gal d 1.1* or 1.3 (Fig. 2A). The median percentage of ovomucoid-specific IgE Ab binding to *Gal d 1.2* was 48% (range 12–88%) compared with 33% (range, 2–65%) for *Gal d 1.1* and 28% (range, 8–97%) for *Gal d 1.3*. When the same 45 patients were tested for ovomucoid-specific IgG Ab binding to the three ovomucoid domains, there was no significant difference in the percentage of binding to the three domains (Fig. 2B). The median percentage of ovomucoid-specific IgG binding to *Gal d 1.1* was 45% (range, 14–99%), that to *Gal d 1.2* was 55% (range, 18–91%), and that to *Gal d 1.3* was 56% (range, 19–90%).

Sera from seven egg allergic patients (all with ovomucoid-specific IgE >80 kU_A/l) were used in inhibition ELISA studies. To investigate the importance of the conformational structure to allergenicity and antigenicity, two forms of altered ovomucoid (performed acid-oxidized, and reduced and alkylated) were compared with native ovomucoid in the inhibition of patient Ab (IgG or IgE) binding to native ovomucoid. The oxidized ovomucoid was similar to native ovomucoid in its ability to inhibit IgE Ab binding to ovomucoid (median, 93% of native ovomucoid inhibition), whereas the ability of the reduced and alkylated ovomucoid to block binding to native ovomucoid was slightly reduced (median, 72% of native ovomucoid inhibition; Fig. 3A). When the same sera were assayed for inhibition of IgG binding, it was found that the acid-oxidized ovomucoid induced significantly less inhibition of ovomucoid (median, 39% of native ovomucoid inhibition; $p < 0.05$), whereas the reduced and alkylated ovomucoid had a minimal loss of its ability to inhibit native ovomucoid (median, 82% of native ovomucoid inhibition; Fig. 3B). Sera from nine egg allergic patients were used to evaluate the contribution of the carbohydrate moieties to the allergenicity and antigenicity of ovomucoid. Overall, removal of the carbohydrate from reduced ovomucoid had no

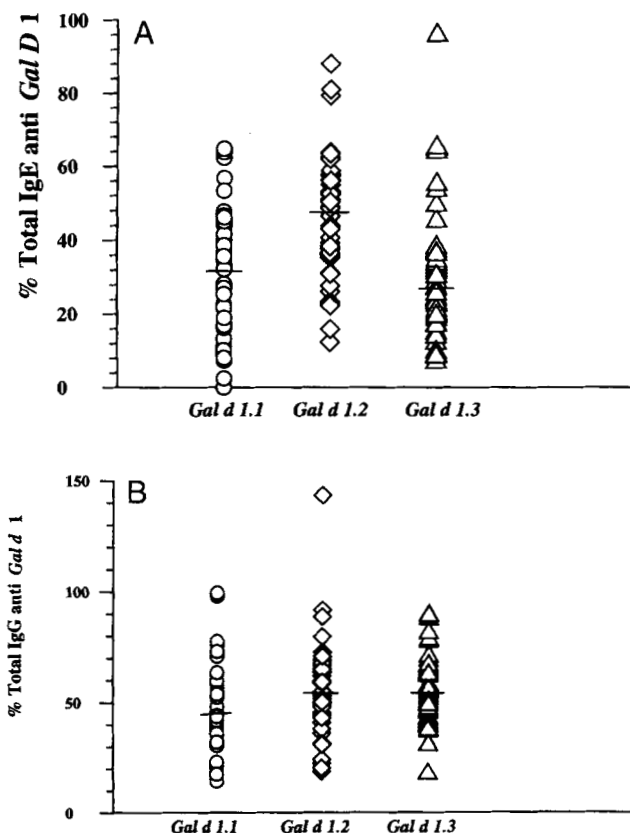


FIGURE 2. Sera from 45 egg-allergic patients were evaluated for the specificity of ovomucoid-specific IgE and IgG Abs binding to each of the three ovomucoid domains. A, Medians of IgE Abs: *Gal d 1.1*, 48%; *Gal d 1.2*, 33%; and *Gal d 1.3*, 28%. The group had significantly more ovomucoid-specific IgE to the second domain than to the first and third domains. B, Medians of IgG Abs: *Gal d 1.1*, 45%; *Gal d 1.2*, 55%; and *Gal d 1.3*, 56%. Patients had comparable ovomucoid-specific IgG to all three domains.

significant effect on IgE or IgG binding. However, as represented in Figure 4, ovomucoid-specific IgE Abs from different patients not only varied in their ability to recognize reduced ovomucoid domains, but some patients (e.g., patient 1) appeared to have a further decrease in Ab binding to the deglycosylated protein.

Sera from the same seven egg-allergic patients used for the inhibition studies were pooled to map linear IgE and IgG binding regions (epitopes) on ovomucoid using the overlapping dodecapeptides on the Genosys SPOTs membrane. A representative SPOTs membrane is shown in Figure 5. As depicted in Figure 6, A and B, pooled patient sera identified two IgE binding regions on *Gal d 1.1* (amino acids 1–20 and 49–56), two on *Gal d 1.2* (amino acids 85–96 and 115–122), and one on *Gal d 1.3* (amino acids 175–186). *Gal d 1.1* had two IgG binding regions (amino acids 3–11 and 23–56), *Gal d 1.2* had three IgG binding regions (amino acids 69–74, 87–104, and 119–124), and *Gal d 1.3* had two (amino acids 138–149 and 175–186). Although there is considerable overlap of the IgE and IgG binding regions, there appear to be four amino acid sequences recognized only by IgG Abs (two in the second domain and one each in the first and third domains) and one region recognized only by IgE Abs in the first domain.

PBMC isolated from 33 egg allergic patients were studied for their ability to proliferate in the presence of the three ovomucoid domains. A stimulation index of 5 or greater was considered a significant PBMC response to Ag. Of the 33 patients tested, 21 had

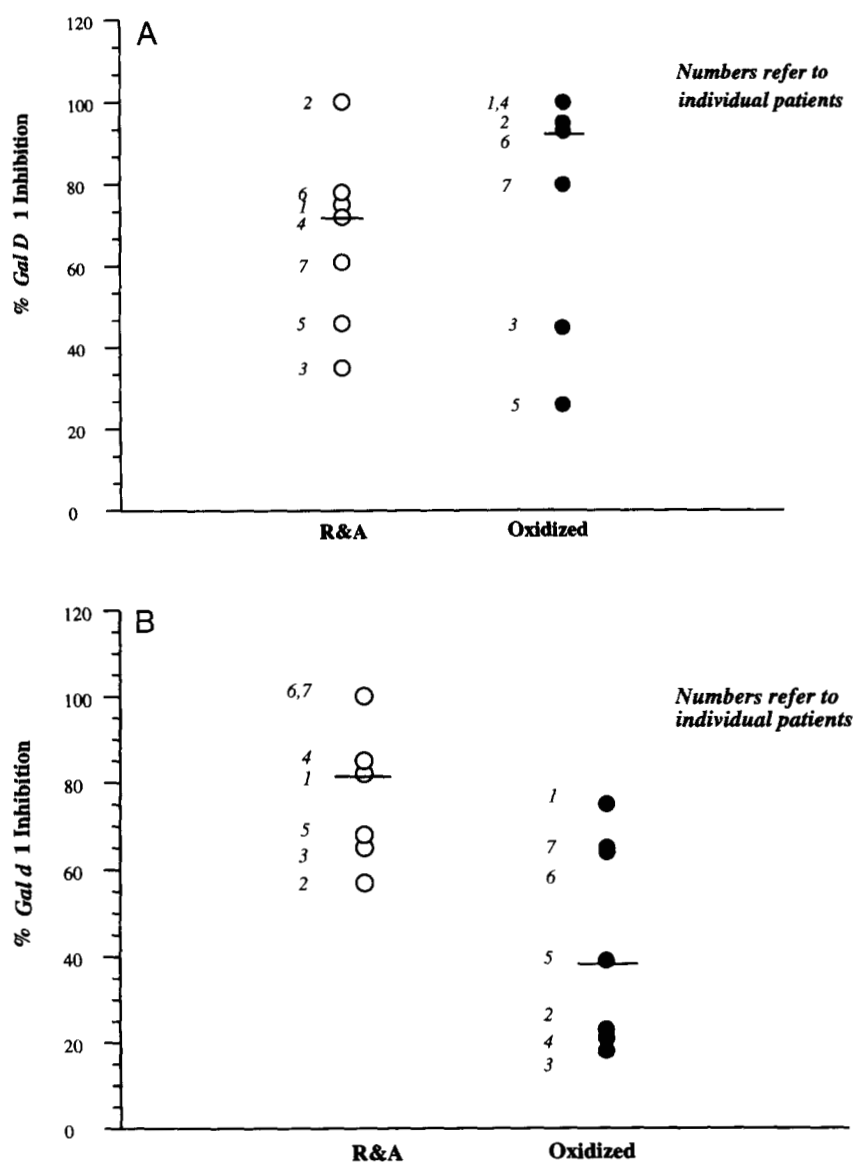


FIGURE 3. A and B, Sera from seven egg-allergic patients with varying histories of egg hypersensitivity were used to evaluate the relative roles of linear and conformational epitopes in binding of ovomucoid-specific IgE and IgG Abs. The destruction of conformational epitopes had a variable effect on ovomucoid-specific Ab among the patients studied. Only IgG binding to performic acid-oxidized ovomucoid was significantly affected in the patient group studied (B).

a significant response to *Gal d* 1.3, 18 had a significant response to *Gal d* 1.2, and 8 of 29 patients tested had significant proliferation in response to *Gal d* 1.1. When comparing the proliferative response of PBMCs to whole ovomucoid with that of each of the individual domains (Fig. 7), only *Gal d* 1.1 showed a significant decrease in proliferative response (Ag-stimulated counts per minute minus medium control background counts per minute: median ovomucoid, 7876 cpm; median *Gal d* 1.1, 2254 cpm; $p < 0.01$). Although the proliferative response to *Gal d* 1.2 (median, 4328 cpm) was lower than that to whole ovomucoid, this difference was not significant ($p > 0.1$). The proliferative response to *Gal d* 1.3 (median, 8106 cpm) was similar to that to native ovomucoid.

PBMCs from 11 egg allergic patients were tested for proliferation to the two altered forms of ovomucoid, performic acid-oxidized or reduced and alkylated. Both forms of "linearized" ovomucoid induced a significant increase in PBMC proliferation compared with native ovomucoid ($p < 0.05$). As shown in Figure 8 the median counts per minute for reduced and alkylated ovomucoid (12,105 cpm) were greater than that for the oxidized form (6,894 cpm), but this difference did not reach statistical significance.

Discussion

Allergy to hen's egg is one of the most common food hypersensitivities in the pediatric population (1–3), and ovomucoid has been shown to be the dominant allergen (9). Ovomucoid (*Gal d* 1) is a unique egg protein consisting of three tandem domains, each possessing some anti-trypsin activity but with its major trypsin reactive site in the second domain (10). The amino acid sequences of the first two domains, *Gal d* 1.1 and 1.2, are 50% homologous, whereas <30% of *Gal d* 1.3 is homologous to *Gal d* 1.1 and 1.2 (15). Up to 25% of ovomucoid is comprised of carbohydrate, with two carbohydrate chains on each of the first and second domains and one chain present on about 50% of the third domain (16). Like many other major food allergens, ovomucoid largely retains its allergenicity and antigenicity following extensive heating (e.g., 100°C for 1 h) and protease digestion (17–19). In this study, we sought to delineate allergenic and antigenic properties of ovomucoid and its domains using sera and PBMCs of patients with DBPCFC-confirmed egg hypersensitivity.

Using the method of Kato et al. (10), ovomucoid domains were isolated and purified to homogeneity, as demonstrated by domain-specific mAbs (Fig. 1). Forty-two of forty-five egg allergic patients

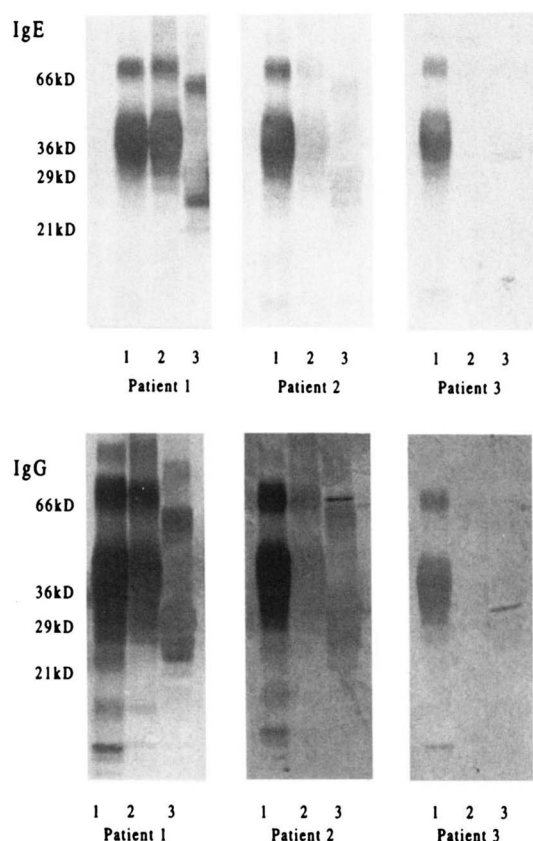


FIGURE 4. IgE and IgG immunoblots. Lane 1, native ovomucoid (12.5 μ g/lane); lane 2, reduced and alkylated ovomucoid (12.5 μ g/lane); lane 3, deglycosylated ovomucoid (25 μ g/lane). An increased amount of deglycosylated ovomucoid was added to the third lanes to provide similar optical density readings for all lanes following amido black staining. Patient 1 is representative of older patients (>8 yr old) with long-standing egg hypersensitivity, patient 3 is representative of younger patients (<3 yr old) with egg hypersensitivity, and patient 2 is representative of an intermediate group.

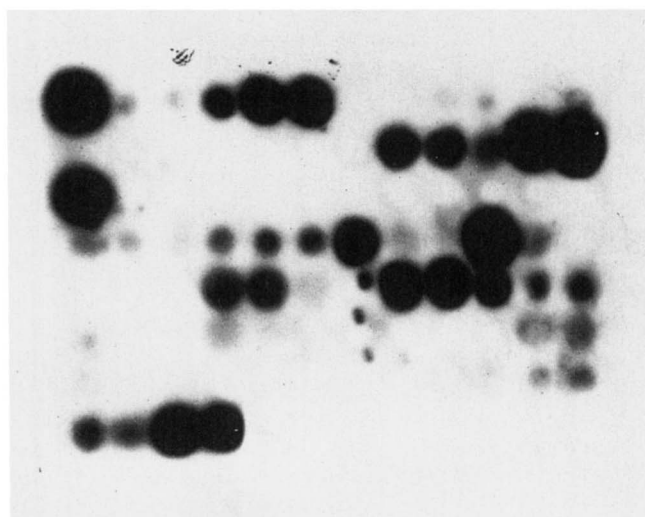


FIGURE 5. SPOTs membrane with overlapping ovomucoid peptides incubated with pooled egg allergic patient sera and developed for IgE-specific Abs.

were found to possess IgE and IgG Abs to all three isolated domains, but three patients lacked IgE Ab to Gal d 1.1. The percentage of ovomucoid-specific IgE Ab to Gal d 1.2 was significantly

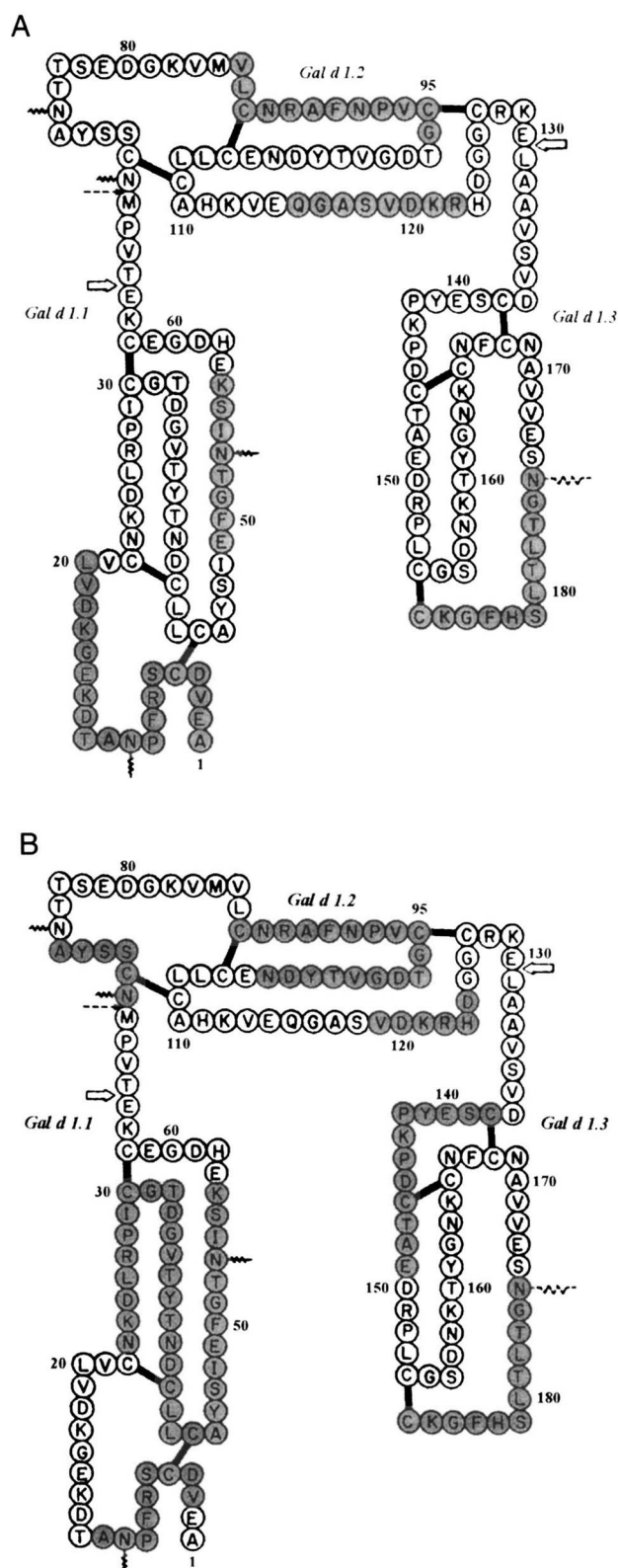


FIGURE 6. Schematic representation of Gal d 1. Broad arrows, $\Rightarrow$, represent the division between each domain; $\sim$, represent carbohydrate side chains; —, disulfide bonds. The shaded amino acid sequences in A depict ovomucoid-specific IgE Ab binding sites, and those in B depict ovomucoid-specific IgG Ab binding sites. Common and unique Ab binding sites are seen for both ovomucoid-specific IgE and IgG Abs.

greater than the percentage of ovomucoid-specific IgE Abs to the first and third domains. This would suggest that a large portion of the anti-Gal d 1.2 Ab is directed against the portion of the second

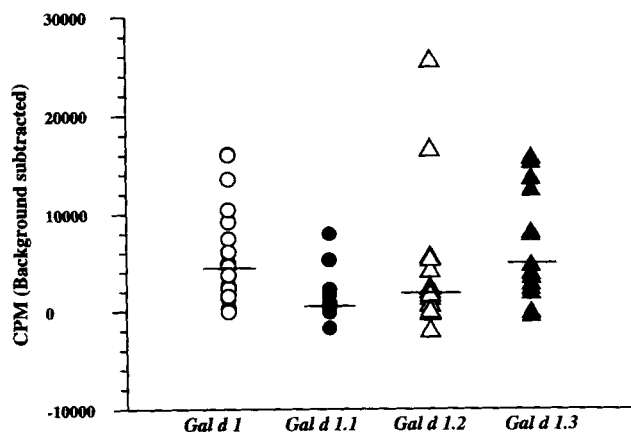


FIGURE 7. PBMCs from 33 egg allergic patients were evaluated for T cell proliferation in response to the three ovomucoid domains. T cell proliferation in response to the first ovomucoid domain was significantly less than that in response to whole ovomucoid and the second and third domains.

domain that is not homologous with the first domain. Ovomucoid-specific IgG Abs were equally distributed among the three domains. Although Matsuda et al. reported less Ab binding to the third domain (20), we saw no significant difference in the amount of IgE or IgG binding to the first and third domains (Figs. 2 and 3).

Previous studies have suggested that the allergenicity and antigenicity of ovomucoid are quite stable to heat and denaturation (17–19), suggesting that a major portion of Ab binding is directed at linear epitopes. To evaluate the degree to which ovomucoid-specific IgE Abs bind linear and conformational epitopes, two methods were employed to linearize ovomucoid by breaking disulfide bonds and disrupting its tertiary structure. Ovomucoid was treated with performic acid, which breaks sulfide bonds by oxidizing cysteinyl and cystinyl residues to residues of the corresponding sulfonic acid, cysteic acid. In addition, it oxidizes methionyl residues to the corresponding sulfone. Dithiothreitol was also used, which provides a more gentle method of breaking disulfide bonds without disrupting sulfide-containing amino acids. Using sera from seven egg allergic patients, inhibition-ELISAs were performed to compare the allergenicity (IgE binding) and antigenicity (IgG binding) of conformationally altered ovomucoid to those of native ovomucoid. As shown in Figure 3A, reduction and alkylation had a minimal effect, and oxidation had virtually no effect on median IgE binding to the altered protein. However, ovomucoid-specific IgE Abs from some patients (i.e., no. 3 in Fig. 4) had markedly reduced activity against the altered proteins, suggesting that conformational epitopes play a significant role in some patients. Interestingly, oxidation of ovomucoid markedly reduced median ovomucoid-specific IgG Ab binding, whereas reduction and alkylation had only a minimal effect (Fig. 3b). As seen with IgE Abs, there was considerable variability among patients in their ovomucoid-specific IgG binding to the altered proteins, again indicating that conformational epitopes may play a more significant role in some patients. This variability in IgE binding to linear epitopes compared with conformational epitopes may reflect differences in the hypersensitivity state of individual patients, i.e., linear epitopes may be more common in older patients with established hypersensitivity, whereas conformational epitopes may be more common in younger patients with transient hypersensitivity. Further studies are under way to address this hypothesis. When carbohydrate moieties were stripped from the reduced and alkylated ovomucoid, there was a minimal effect on IgE and IgG bind-

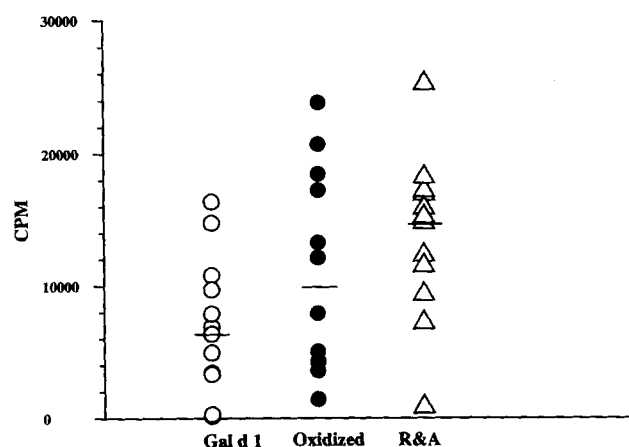


FIGURE 8. PBMCs from 11 egg allergic patients were evaluated for T cell proliferation to oxidized or reduced and alkylated (linearized) ovomucoid. T cell proliferation was significantly greater in response to both forms of linearized ovomucoid than in response to the native form.

ing. As depicted in Figure 4, however, IgE and IgG Abs from patients with longer term (established) egg hypersensitivity, e.g., patient 1, appeared to be most affected by deglycosylation, suggesting some role for the carbohydrate backbone in the B cell epitope of these patients.

Using the Genosys SPOTs membrane system with synthetic decapeptides that overlapped by 10 amino acids, ovomucoid IgE and IgG binding regions were mapped with pooled sera from seven egg allergic patients. Peptides generated with this system do not have the carbohydrate components of the native protein, but our studies suggest that the carbohydrate moiety plays a very minor role in Ab binding in most egg-sensitive patients. Ovomucoid-specific IgE Abs bind sites on all three domains: two regions in the first domain (amino acids 1–20 and 49–56), two segments in the second domain (amino acids 85–96 and 115–122), and one region in the third domain (amino acids 175–186). Ovomucoid-specific IgG binding was more extensive, recognizing broad sequences on all domains: two regions in the first domain (amino acids 3–11 and 23–56), three segments in the second domain (amino acids 69–74, 87–104, and 119–124), and two segments in the third domain (amino acids 138–149 and 175–186). The first and second ovomucoid domains are 50% homologous, with near-sequence identity of amino acids 27–54 in *Gal d 1.1* and amino acids 92–110 in *Gal d 1.2* (15). While ovomucoid-specific IgE Abs bind to these highly homologous regions, they are directed at different regions of the sequence, suggesting differences in access and/or processing of these domains by Ag-processing cells. On the other hand, ovomucoid-specific IgG Abs bind extensively to these homologous regions. Ovomucoid-specific IgE and IgG Abs recognized common regions on all three domains, but unique IgG binding sites were seen on all three ovomucoid domains, whereas unique IgE binding sites were seen only on the first and second domains. In patients with high IgG titers to *Gal d 1.3*, the absence of a unique IgE binding site could account for reports indicating an absence of IgE binding activity to the third domain, where IgG would “out compete” IgE for the binding sites.

To mount an IgE response, APCs must process Ag and present it to TCRs in the context of MHC class II Ags and the appropriate second signal to activate T cells (21, 22). These Ag-specific T cells (primarily Th2⁺ cells) are then capable of providing B cells with the appropriate signals (via secreted cytokines) to produce Ag-specific IgE Abs as well as activating an inflammatory response. In

most nonallergic individuals, activation of a Th1⁺ cell population predominates, which is associated with normal tolerance induction. Ag-induced proliferation of T cells with tritiated thymidine pulsing in vitro is a convenient method to establish the presence of Ag-specific T cells. All patients with egg hypersensitivity as well as most normal individuals exposed to egg possess T cells that proliferate when exposed to ovomucoid (9). In this study, all egg allergic patients and eight normal controls (data not shown) were found to have proliferative responses to ovomucoid. Most patients' PBMCs showed some proliferative response to each ovomucoid domain, but the proliferative responses to the first domain were significantly less than those to the other two domains. Interestingly, when whole ovomucoid was linearized by oxidation or reduction and alkylation, the proliferative responses of patient PBMCs were significantly enhanced, suggesting that APCs may have some difficulty processing the native molecule compared with denatured forms.

Ovomucoid, Gal d 1, has been shown to be the dominant allergen in hen's egg (9). In summary, ovomucoid is a well-characterized, major allergen that has multiple IgE and IgG epitopes throughout its three-domain structure. Each domain bears unique and common IgE and IgG binding sites (epitopes) that are recognized by peripheral blood T cells from egg allergic patients. These studies demonstrate considerable variability among various egg allergic patients with respect to the sites of binding of ovomucoid-specific IgE and IgG Abs and the significant role of conformational epitopes in some patients. Although further studies are needed, this study suggests that differences in IgE responses to conformational vs linear epitopes may be associated with the persistence of egg hypersensitivity. Further characterization of B cell and T cell epitopes should provide insight into the immunologic mechanisms of egg hypersensitivity.

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