JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Comparative Studies on Antigenicity and Allergenicity of Native and Denatured Egg White Proteins

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The binding activities of IgG and IgE antibodies from egg-allergic patients to physically or chemically treated egg white proteins were examined and compared with those of rabbit anti-egg white IgG antibodies. The sera from eight patients and four rabbit antibodies were used in this study. The binding activities of human IgG antibody to partially denatured ovotransferrin (Tf), ovalbumin (OA), and lysozyme (Lys) forms were increased, whereas carboxymethylation (RCM) and heat treatment caused a dramatic decrease in the antigenicity of Tf and ovomucoid (OVM). Tf and OVM were major immunogenic antigens for the rabbit IgG response. Urea also caused Tf to exhibit greater rabbit IgG binding activity. In contrast, human and rabbit antibodies did not react with ovomucin. Partially denatured Tf and Lys also induced strong IgE binding activities. The allergenicity of Tf, OVM, and Lys was decreased by RCM, whereas OA retained its binding capacity. These results suggested that anti-OA IgE recognizes more sequential epitopes and that anti-OVM and Lys antibodies recognize both conformational and sequential epitopes. Tf and OVM were dominant allergens for the IgE antibodies of anaphylaxis patients, whereas IgE from atopic patients bound more strongly with OA and OVM.

KEYWORDS: Egg allergy; egg white proteins; specific IgG and IgE antibodies; ELISA; antigenicity; allergenicity; heating; chemical modification; urea; rabbit; human sera

INTRODUCTION

Food allergies are caused by abnormal immunological responses to certain foods, usually proteins. The most common types of food allergies are mediated by immunoglobulin E (IgE). IgE-mediated reactions (type I) are known as immediate hypersensitivity reactions or anaphylaxis because symptoms occur from <1 min to a few hours after the ingestion of the offending foods. Examples of symptoms include asthma, atopic dermatitis, rhinitis, and urticaria. It is estimated that IgE-mediated food allergies afflict ∼1−2% of adults and 5−8% of infants (*1*, *2*). Type II or antibody-dependent cytotoxic hypersensitivity is both IgG and IgM mediated. The antibody binds to cell-bound antigen, leading to phagocytosis. The type III hypersensitivity is mainly IgG mediated. The antibody−antigen complexes are formed in large quantities, leading to tissue injuries. Type IV hypersensitivity or delayed hypersensitivity is not mediated by antibodies but primarily by T-cell lymphocytes and macrophages, causing a number of inflammatory responses after a long delay (>8 h after ingestion of the offending foods) (*3*). The relatively high prevalence of food allergies in infants is due to an immature gastrointestinal epithelial membrane barrier, which allows more proteins through the barrier and into the circulatory system (*4*). More than 160 food allergens have been identified. In general, they are water-soluble, heat and acid stable, and relatively resistant to proteolytic digestion (*5*).

Hen eggs are one of the most frequent causes of adverse reactions to food. Elucidation of allergic reactions has shown that they are more frequently caused by egg white proteins than egg yolk (*7*). Approximately two-thirds of children diagnosed with food allergies are reactive to egg white (*8*). The predominant protein in egg white is ovalbumin (OA), comprising 54% of the protein content; other major proteins in egg white are ovomucoid (OVM) (11%), ovotransferrin (Tf) (12%), ovomucin (OM) (3.5%), and lysozyme (Lys) (3.5%) (*9*). Many previous studies have reported on the antigenicity and allergenicity of egg white proteins; however, although OVM, OA, Tf, and Lys have been clearly identified as egg white allergens, the identification of the most important of these allergens remains a source of some scientific confusion and debate. Miller and Campbell (*10*) found, using skin tests, that the frequency of allergenic reaction to egg white had the following order: Lys > OM > OA > OVM. Bleumink and Young (*11*) concluded that OVM was the dominant allergen and was more reactive than OA using the same method. Other groups reported that OA, OVM, and Tf were the major allergens, whereas Lys was a weak allergen (*12*−*14*) or that OA was the major allergen (*15*−*18*) using various electrophoretic and immunochemical techniques. Large amounts of IgE antibodies have also been detected in sera pooled from patients with egg allergy (*15*). Recently, OVM has been reported to be the immunodominant

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10.1021/jf0112264 CCC: $22.00
Published on Web 03/27/2002

protein of egg white in egg-allergic children (*19*−*23*) using sera from egg allergic patients.

Despite a large number of studies into egg white allergy, no clear consensus has been reached as to relative antigenicity and allergenicity of egg white proteins. Egg white consists of 24 different protein fractions and a number of minor proteins (*24*, *25*). The contradictory results on egg white allergenicity may, therefore, be attributable to several aspects: (a) differing degrees of purity of individual protein fractions and antibody sources, (b) using sera from either humans with egg allergy or antibodies raised from experimental animals, or (c) different routes of administration (injection or oral administration). For example, commercially pure OA, which is generally accepted as the most allergenic protein and extensively studied as a model allergen, has been found to contain OVM as an impurity, which led to an overestimation of the dominance of OA as the major egg white allergen (*19*, *26*). Furthermore, many researchers have used rabbit or mouse antibodies for studies of egg protein allergenicity and antigenicity (*27*−*29*). These antibodies were raised in the laboratory through immunization of the animals by the injection of emulsified antigen, whereas specific antibodies from allergic patients were sensitized by antigens absorbed from the gastrointestinal tract. It is unclear whether antibody specificity is influenced by the different administration routes used for antigen sensitization. The immunodominant epitopes for IgG responses in animals from injection may differ from IgE responses in humans from ingestion of egg.

In this study, we prepared very pure egg white proteins by means of high-performance liquid chromatography (HPLC). We compared their relative antigenicity and allergenicity in different states (using the intact and modified forms of each protein) and the specificity between human sera from egg-allergic patients and rabbit antibodies raised in the laboratory.

MATERIALS AND METHODS

Egg White Proteins. Hen egg white proteins OA (type VII), OVM (type III), Tf (type I), and Lys were purchased from Sigma (St. Louis, MO). OM was prepared as described by Ito et al. (*30*). OA, OVM, Tf, and Lys were further purified using HPLC ion-exchange chromatography. Ten milligrams of each sample in 1 mL of 20 mM sodium acetate buffer, pH 4.0, was applied to a Bio-Scale S5 column (Bio-Rad Laboratories, Hercules, CA) equilibrated with the same buffer. The column was eluted with a linear gradient of 0−1.0 M NaCl in 20 mM sodium acetate buffer, pH 4.0, at a flow rate of 1.0 mL/min using a Bio-Rad Biologic HPLC system. The purity of each egg white sample was analyzed with a 491 protein sequencer (PE Applied Biosystems, Foster City, CA), after which the protein was transferred to a polyvinylidene fluoride transfer membrane (ProSorb; PE Applied Biosystems). The other chemicals used were also purchased from Sigma. The denatured forms of each protein were prepared as follows: each sample of 1 mg/mL in 20 mM phosphate buffer at pH 7.2 was heated for 15 min at 95 °C (heat-treated protein) or exposed to 6 M urea (Sigma, ACS grade) for 12 h at 37 °C (urea-treated protein). The urea-treated samples were then dialyzed against 20 mM phosphate buffer, pH 7.2, for 48 h.

Human Serum. Human serum was collected from eight patients and had a radio-allergosorbent test (RAST) score of 1−15 PRU/mL for egg. All of the patients were allergic to egg white, and the clinical history of each patient is summerized in **Table 1**. All serum samples were kindly provided by Dr. A. Urisu (Department of Pediatrics, Fujita Health University, Japan) and Dr. H. Morikawa (School of Medicine, Gunma University, Japan) and stored at −80 °C until use.

Protein Assay. The protein content was determined using the DC protein assay kit from Bio-Rad using bovine serum albumin (BSA) as a standard protein.

Enzyme-Linked Immunosorbent Assay (ELISA). The antigenicity and allergenicity of each protein were determined by an indirect ELISA, according to a modification of the method of Engvall and Perlmann (*31*). Medical grade, high-binding polystyrene, 96-well microplates (Corning, Cambridge, MA) were precoated with various native or denatured egg white proteins (1 μg/well in 100 mM carbonate buffer, pH 9.6) and incubated overnight at 4 °C. The plate was washed three times with phosphate-buffered saline (0.01 M phosphate, 0.15 M NaCl, pH 7.2; PBS) containing 0.05% Tween 20 (PBST), pH 7.2, using a Bio-Rad Immunowash microplate washer. The plate was blocked with 2.5% BSA in PBS for 2 h at 37 °C, washed with PBST, and incubated with human serum (diluted 1:25 in PBS containing 1% BSA for IgE assay, diluted 1:250 for IgG assay) or rabbit anti-egg-white antibody (diluted 1:500) overnight at 4 °C. The plate was again washed and incubated with alkaline phosphatase-conjugated anti-human IgE (diluted 1:1000 in PBS), anti-human IgG (diluted 1:5000), or anti-rabbit IgG (diluted 1:10000) overnight at 4 °C. The plate was washed and developed with *p*-nitrophenol phosphate (1 mg/mL) in 0.1 M diethanolamine buffer, pH 9.8, for 60 min at room temperature. The reaction was terminated by the addition of 25 μL of 3 N NaOH to each well, and the plate was read at $\lambda = 405$ nm using a Bio-Rad microplate reader. The IgE- and IgG-specific binding activities against the different forms of purified egg white component were expressed as a percentage of IgE or IgG binding to whole egg white.

Table 1. Clinical History of Egg-Allergic Patients' Sera

serum	age (years)	sex	hypersensitivity reaction	IgE RAST, PRU/mL
1	4	M	anaphylaxis	14.1
2	3	F	anaphylaxis	10.7
3	5	M	anaphylaxis	>15.0
4	4	F	anaphylaxis	>15.0
5	3	F	atopic dermatitis, wheezing	1.1
6	5	F	atopic dermatitis, bronchial asthma	2.2
7	3	M	erythema, eczema, vomiting, itching	1.0
8	5	M	urticaria, wheezing, vomiting	4.7

Circular Dichroism (CD). The far-UV CD spectra of native and chemically or physically treated egg white proteins were determined at room temperature in 20 mM phosphate buffer, pH 7.2, using a Jasco J600 spectropolarimeter (Easton, MD). Each sample was scanned four times in the range $\lambda = 200-250$ nm in a quartz cuvette (Japan Spectroscopic, Tokyo, Japan) with a 0.1-cm path length. A buffer baseline was subtracted from the spectra of each sample. The percentage of α-helix structure was estimated using a protein secondary structure estimation program supplied by JASCO, based on the method of Yang et al. (*32*).

Production of Anti-egg-white Polyclonal Antibodies. Four rabbits (Central Animal Facility, University of Guelph) were injected intramuscularly with 0.2 mg of native egg white that had been resuspended in 0.2 mL of 0.9% sterile saline and emulsified with an equal amount of Freund's complete adjuvant (FCA; Sigma). Two weeks later the rabbits were given a second intramuscular injection. Antibody level was monitored by ELISA. The collected antisera were precipitated with saturated ammonium sulfate to a final saturation of 35% (*33*). The precipitates were disolved in PBS, dialyzed against PBS for 48 h, and then stored at −80 °C until use.

Carboxymethylation. Each sample was carboxymethylated according to the method of Aitken and Learmonth (*34*). Briefly, the protein samples (5 mg) were dissolved in 5 mL of denaturation buffer (6 M guanidinium hydrochloride in 0.6 M Tris-HCl, pH 8.6) in a tube and flushed with N_2 gas. An equal volume of 4 mM 1,4-dithiothreitol (Sigma) was added to give a final concentration of 2 mM. The tube was wrapped in aluminum foil, and 400 μL of iodoacetic acid (Sigma) was added dropwise with stirring. The sample was incubated in the dark for 1 h at 37 °C followed by dialysis against 50 mM ammonium bicarbonate, pH 7.8, for 24 h.

Statistical Analysis. Data were analyzed by ANOVA (SPSS version 8.0 for Microsoft-Windows; SPSS, Chicago, IL), and means were separated by Duncan's multiple range test. Significance was defined at $P < 0.05$.

Table 2. Comparison of Specific Antibody Binding Activities of Human and Rabbit Antibodies to Native and Denatured Forms of Egg White Proteins[a–c]

form	ovomucin	ovotransferrin	ovalbumin	ovomucoid	lysozyme
human IgG					
native	3.4–15.3 (7.3)[a]	30.7–56.6 (45.6)[a]	15.0–31.0 (21.1)[a]	59.1–96.0 (77.5)[a]	34.1–110.0 (79.4)[a]
RCM	0–1.0 (0.3)[b]	4.0–14.0 (8.4)[b]	7.4–20.0 (12.1)[a]	0–42.7 (13.2)[b]	14.7–89.3 (32.4)[b]
urea	0–4.1 (1.2)[b]	69.0–148.0 (104.9)[c]	21.0–73.3 (36.9)[b]	33.9–121.0 (83.5)[a]	88.0–145.0 (111.4)[c]
heating	0–4.7 (0.9)[b]	2.0–44.2 (16.8)[d]	7.0–37.0 (17.0)[a]	4.4–40.4 (19.2)[b]	16.0–117.0 (47.7)[b]
human IgE					
native	3.2–16.0 (8.5)[a]	13.0–87.1 (48.3)[a]	5.9–118.0 (46.8)[a]	19.5–131.4 (85.8)[a]	24.1–63.2 (43.1)[a]
RCM	1.0–11.9 (4.3)[a]	6.6–39.4 (22.6)[b]	1.9–70.6 (22.6)[a]	12.5–40.5 (18.6)[b]	2.7–61.2 (23.8)[b]
urea	0–10.4 (3.7)[a]	22.9–114.6 (63.1)[c]	3.3–64.2 (24.8)[a]	15.2–132.0 (83.9)[a]	24.0–117.0 (51.0)[c]
heating	0–17.2 (5.2)[a]	2.9–17.8 (11.4)[d]	0–61.7 (18.2)[a]	0–75.4 (17.8)[b]	8.3–91.5 (42.5)[a]
rabbit IgG					
native	0–36.4 (1.9)[a]	48.6–61.7 (56.1)[a]	4.5–12.36 (8.5)[a]	36.9–59.0 (46.6)[a]	9.0–14.8 (11.9)[a]
RCM	0–9.5 (4.7)[a]	3.6–46.0 (17.2)[b]	4.0–11.9 (8.1)	0–6.0 (3.9)[b]	6.0–14.8 (11.0)[a]
urea	0–4.9 (1.5)[a]	77.0–116.0 (93.6)[c]	9.0–16.7 (12.6)[b]	33.9–46.6 (40.9)[a]	11.0–29.0 (20.8)[b]
heating	0–4.9 (1.2)[a]	1.4–5.0 (4.6)[d]	2.0–14.1 (9.0)[a]	3.0–7.8 (5.2)[b]	9.0–15.5 (12.5)[a]

[a] Specific antibody activities were expressed as a percent of binding activities of IgG or IgE antibodies compared to the percent of antibody binding to native, whole egg white proteins were taken as 100%. [b] Range of specific antibody activity value; mean average is expressed in parentheses. [c] Means in the same column with different letters are significantly different ($P < 0.05$)

RESULTS AND DISCUSSION

Commercially purified egg white proteins are used extensively in basic research into antigenic properties, and in many cases these proteins are used without further purification or confirmation of antigen purity. It has been demonstrated that commercially purified OA (e.g., grades V and VII, ~99% pure; according to the manufacturer's sheet) generated a number of monoclonal antibodies which appeared to be specific to both OA and its contaminant OVM. It has been reported that commercially purified OA led to an overestimation of its antigenicity and allergenicity due to the contamination by OVM (*19*). In fact, we have reported previously that commercially available OA contained ~6.7% OVM (*26*), which demonstrated the importance of using highly purified protein preparations in investigations of the allergenicity of egg white components. OA and OVM are generally considered to be the most antigenic and allergenic proteins; however, the most important antigenic and allergenic component in egg white remains a matter for further investigation. One explanation for these discrepancies is the lack of purity of the egg white protein used in the various studies. SDS-PAGE is frequently used for checking protein purity, but it is difficult to distinguish a small amount of contaminating protein using this method. In the study described here, we carefully repurified each of the commercial egg white proteins (Tf, OA, OVM, and Lys) or prepared it in our laboratory (OM) using HPLC.

Table 2 shows comparisons of the specific IgG level (antigenicity) in human sera from egg-allergic patients (orally sensitized by egg white protein/peptides absorbed from the intestine) and in rabbit sera (sensitized by egg white injection) and comparison of specific IgE levels (allergenicity) from human sera, respectively. The native egg white proteins and three different forms of modified egg white components—RCM, urea-treated, and heat-treated forms—were used as coating antigens. All forms of the proteins were soluble in 10 μg/mL of 0.1 M carbonate, pH 9.6, as ELISA coating antigens, except for the RCM OVM, which had a tendency to aggregate. As shown in **Table 2**, the binding activities of human IgG against four different forms of OM and OA were very low, except for urea-treated OA. The binding activity of native forms of Tf, OVM, and Lys (average values of 45.6, 77.5, and 79.4%, respectively) showed relatively high antigenicity generated by human sera sensitized by eggs, and the urea-treated forms of Tf and Lys (averages = 104.9 and 111.4%, respectively) exhibited significantly higher IgG binding activity than the native forms. However, RCM and heat-treated forms of Tf and Lys showed significantly less antigenicity except in the serum from one patient. Heating and chemical modification of OVM also lowered antigenicity (averages = 19.2 and 13.3%, respectively), whereas urea denaturation did not affect its binding activity (average = 83.5%). The binding of RCM OVM into a microplate was very difficult due to the aggregation of the modified protein. This might have influenced the result by producing a lower binding activity. RCM and heat-treated OA showed similar IgG binding activities (averages = 12.1 and 17.0%, respectively), whereas urea treatment significantly increased the OA binding activity (average = 36.9%). The immunological response to egg white proteins was different for rabbit sera than for human sera. Intraperitoneal immunization of rabbit gave sera for which Tf and OVM induced a major immune response. Urea treatment increased the IgG binding activity of Tf (93.6%), whereas RCM and heat treatment reduced it (averages = 17.2 and 4.6%, respectively). OM, OA, and Lys did not generate any significant IgG response when using a mixture of egg white proteins as the antigen. The rabbit IgG antibody reactivity against the native forms of OM, OA, and Lys were 1.9, 8.5, and 11.9%, respectively. In this study, the recognition of human and rabbit antibodies of egg white proteins was different, which might be attributable to species differences or the difference in administration route of the antigen. Future studies should investigate the effects of the administration route on antibody specificity.

The IgE antibody in human sera from egg-allergic patients recognized Tf, OA, OVM, and Lys, and there was a greater range of binding activity than when human IgG antibody was tested. Although the sera of most patients exhibited IgE binding activity to these four proteins, OVM was more dominant in IgE binding, followed by Tf, OA, and Lys in that order. The RCM treatment of Tf, OVM, and Lys caused a significant decrease in IgE binding activity (averages = 22.6, 18.6, and 23.8%, respectively), but it did not affect OA. The urea-treated forms of Tf and Lys showed significantly higher IgE binding activity (averages = 63.1 and 51.0%, respectively), but this treatment did not affect OA and OVM. Heating also lowered IgE binding with OVM and OA (averages = 17.8 and 18.2%, respectively) but did not significantly affect Lys. The binding activities of

Table 3. Secondary Structure of Native and Chemically or Physically Treated Forms of Egg White Proteins[a]

form	native	RCM	urea	heating
ovomucin	25.0	N.D.[b]	5.8	11.2
ovotransferrin	26.2	10.5	19.8	10.7
ovalbumin	24.4	15.0	21.1	6.3
ovomucoid	19.6	0.5	20.5	14.6
lysozyme	20.0	2.6	16.4	15.7

[a] Secondary structure is expressed as α-helix content, which was estimated from the far-UV CD spectra using a method of Yang (*32*). [b] N.D., not determined.

IgE antibody against RCM and heat-treated Tf and OVM were less than for the native forms, whereas IgE antibody in sera bound well with urea-treated Tf and Lys (averages = 63.1 and 51.0%, respectively). During sensitization, antigen-presenting cells in the human body are presented with denatured form of allergens to be processed (*35*). It is also expected that food allergens could be partially hydrolyzed or denatured before being absorbed from the intestine and following sensitization by the gut immune system. The present results strongly suggest that antibody recognition is closely related to antigen structure when sensitized.

Table 3 shows the effects of physical and chemical treatments of egg white proteins as analyzed with CD using an index of changing α-helix content. Tf and OA were very sensitive to heat treatment at 95 °C, but OVM and Lys were relatively heat resistant. Urea-treated Tf, OA, OVM, and Lys exhibited α-helix contents similar to those of intact ones. It is well-known that urea is protein denaturant; however, proteins might refold to native-like structure (molten globule state) by dialysis against Milli-Q water (*36*). It was also reported that many food proteins form a molten globule stats, and its importance to their functional properties in food systems has been discussed (*37*). Thus, urea (6 M) might covert egg white proteins (Tf, OA, OVM, and Lys) to a molten globule state, which partially denatured but retained native-like structure. The protein structure analysis showed that the human IgG and IgE from egg-allergic patients recognized partially denatured Tf and Lys strongly, and both proteins dramatically decreased their reactivity with chemical denaturation when conformational destruction of both polypeptides occurred. Heating also destroyed the secondary structure of Tf and OA but had less effect on OVM and Lys compared to RCM treatment. However, Tf lost its IgG and IgE binding capacity upon heating and chemical modification. However, the antigenic and allergenic epitopes on native and partially denatured Tf are different. This study shows that anti-OA IgE recognizes more sequential epitopes, and anti-OVM and Lys antibodies recognize both conformational and sequential epitopes. It has been reported that the antigenicity and allergenicity of OVM are quite stable to heat and denaturation, suggesting that antibody binding is directed at sequential epitopes (*29*, *38*), whereas heating of OVM at 100 °C reduced the immunogenic reactivity (*39*). Further studies revealed that no effect of reduction and alkylation of OVM on its IgE binding was observed; however, conformational epitopes play a significant role in some patients (*21*). RCM OVM domain 1-3 exhibited an IgG binding activity similar to that of the native form, whereas RCM OVM domain 3 had a significantly higher binding activity to IgE, indicating that the major epitopes in each OVM domain have a sequential structure (*22*). However, OVM also contains some conformational epitopes between interdomains (*22*). This suggests that OVM has multiple IgE and IgG epitopes throughout its unique tandem structure, and this may be the cause of the considerable variability in the

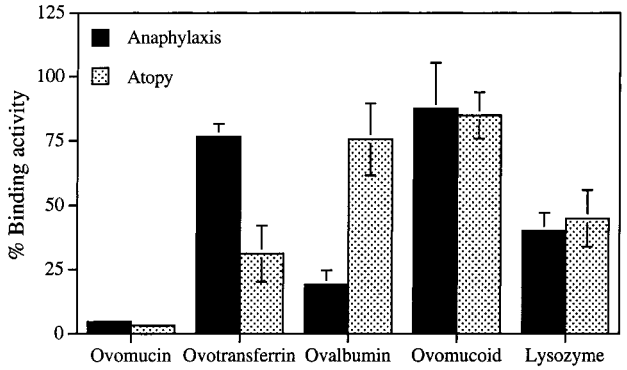

Figure 1. Binding activities of purified egg white proteins against human IgE antibodies derived from egg-allergic patients. The IgE-specific binding activities against each egg white component were expressed as a percentage of IgE binding to whole egg white.

OVM-specific IgG and IgE binding activities among the sera of the different egg allergic patients. It is believed that food allergens are resistant to proteolysis and the acidic conditions of the digestive tract and reach the intestinal mucosa, where absorption and sensitization can occur (*6*). The allergens could be partially denatured or hydrolyzed during sensitization through the gastrointestinal tract, but they may retain major allergenic epitopes, and so antigen-presenting cells in the human body could be presented with denatured pieces of allergen to be processed for subsequent display of immunogenic epitopes to T lymphocytes (*35*, *40*). It would be of great interest to determine, at the molecular level, if the nature of the allergen and the kinetics of antigen processing influence the characteristics of subsequent immune and allergic response.

We selected four sera with high RAST scores (>10 PRU/mL) with clinical symptoms of anaphylaxis (3−5-year-old patients, numbers 1−4 in **Table 1**) and compared their specific IgE binding activity to each egg component with sera from atopic allergic patients with RAST scores of <10 PRU/mL (**Figure 1**). Interestingly, Tf exhibited higher binding activity with sera from anaphylaxis allergy patients, whereas the reverse was observed in OA−IgE binding activity. OVM and Lys showed similar IgE binding activity regardless of the patient history, and OVM had the strongest specific IgE binding activity to both types of sera. The present result suggests that Tf and OVM might have important implications in the anaphylactic reaction to eggs but that OA and OVM are crucial for atopic reactions. Differences between protein structure and epitopes (conformational and sequential) may produce differences in the allergic reaction to eggs. Further confirmation with additional sera are needed before conclusions can be reached.

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Received for review September 20, 2001. Revised manuscript received January 29, 2002. Accepted January 29, 2002. This work was supported by a grant from the Ontario Egg Producers Marketing Board, Ontario, Canada.

JF0112264