

Clinical Significance of IgE-Binding Activity to Enzymatic Digests of Ovomucoid in the Diagnosis and the Prediction of the Outgrowing of Egg White Hypersensitivity

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Key Words

Egg hypersensitivity · Ovomucoid · Food challenges double-blind placebo-controlled · IgE antibodies specific · Pepsin · Trypsin · Chymotrypsin

Abstract

Background: We frequently encounter subjects without overt symptoms despite high IgE antibodies to egg white and its components. The measurements of these antibodies are not necessarily efficient for the diagnosis or the prediction of the outcome of egg allergy in children. **Methods:** Specific IgE antibodies to egg white and its components, including ovomucoid, ovalbumin, ovomucoid, lysozyme, were measured by direct RAST assays. IgE-binding activity to ovomucoid degraded by pepsin, trypsin and chymotrypsin was examined by RAST inhibition. Thirty subjects were divided into two groups with positive ($n = 18$; mean age $\pm$ SD = 42 ± 25 months) and negative ($n = 12$; mean age $\pm$ SD = 48 ± 31 months) oral challenge tests with egg white antigens. The individuals with positive results to the first challenge tests were given the second provocation tests at mean intervals of 32 months. IgE-binding activity of the sera collected on the first challenge to these ovomucoid fragments was compared between subjects with positive and negative reactions to the follow-up chal-

lenge tests. **Results:** There were no significant differences in IgE antibody titers to egg white and its components between the positive and negative groups at the first and the second challenge tests. IgE-binding activity to ovomucoid digests after treatments with pepsin ($p = 0.000008$) and trypsin ($p = 0.037$), except chymotrypsin ($p = 0.062$), were significantly higher in subjects with positive challenge tests than in those with negative results. The difference was most remarkable in the IgE-binding to pepsin digests; the average concentrations (mean – SD and mean + SD) needed for 50% RAST inhibition in the positive group and in the negative group were $2.6 \mu\text{g/ml}$ (0.3 and 25) and $94.2 \mu\text{g/ml}$ (24.7 and 358.7), respectively. A significant difference was still observed in the inhibition tests using filtrates of pepsin digests with a membrane with MW 10,000 ($p = 0.014$) and 3,000 ($p = 0.042$) of cutoff. The concentration (mean = 0.8 , mean – SD = 0.2 , mean + SD = 3.4 ; $\mu\text{g/ml}$) of pepsin-treated ovomucoid required for 50% RAST inhibition in the subjects with positive second challenge results was significantly ($p = 0.033$) lower than that (mean = 6.8 , mean – SD = 0.6 , mean + SD = 73.9) of the negative group. **Conclusion:** IgE-binding activity to pepsin-digested ovomucoid was of diagnostic value to distinguish the challenge-positive subjects from the negative subjects. Subjects with high IgE-binding activity to pepsin-treated ovomucoid are unlikely to outgrow egg white allergy.

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Introduction

Hen's eggs are one of the most common causes of food allergy in young children [1, 2]. IgE antibodies have an important role in egg hypersensitivity. Specific IgE antibodies for egg white are measured as an *in vitro* diagnostic examination for this disease in the daily practice. However, we often encounter subjects who can ingest hen's eggs without any symptoms despite high IgE antibody titers for egg white [3, 4]. The frequency of such patients was 16–43% [3, 4], although the percentage was different depending on the methods used in the reports.

According to Bock [5], eggs are one of the foods that are most likely to produce less reaction with age, approximately 40% of egg-hypersensitive patients becoming tolerant to egg ingestion. The outgrowing of hypersensitivity is observed even in individuals maintaining high IgE antibodies and positive skin response to egg white.

Egg white protein contains 23 different proteins. Ovomucoid (Gal d 1), ovalbumin (Gal d 2), ovotransferrin (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 [9–11]. The titer of IgE antibodies to ovomucoid was significantly greater in the patients with positive results to oral egg challenge tests compared to the patients with negative results. This may result from the unique characteristics of ovomucoid such as relative stability against digestion with proteinases [12] and heat [13] as well as strong allergenicity [9, 10] compared with other egg white components. However, there was a large extent of overlapping of IgE antibody titers for ovomucoid between both groups. According to Matsuda et al. [12], this allergenicity and antigenicity were still retained after fragmentation by pepsin digestion.

In this study we assessed whether the measurement of IgE reactivity to enzyme-resistant epitopes on ovomucoid is useful for more accurate diagnosis and prediction of outcome for egg-hypersensitive children with elevated IgE antibodies for ovomucoid as well as egg white.

Materials and Methods

Egg White Antigens and the Components

Freeze-dried egg white antigen for oral food challenge and direct radioallergosorbent test (RAST) was given by the Q.P. Corporation R&D Division. Ovomucoid purified with the modified method described by Waheed and Salahuddin [14] was also a gift from the Q.P. Corporation. HPLC-repurified ovalbumin was donated by former Prof. Nakamura at the Nagoya University. Ovotransferrin was purchased from Wako Pure Chemical Company (Osaka, Japan).

Lysozyme was donated by the Eisai Pharmaceutical Company. The purity of each component was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Specific IgE Antibodies to Egg White and Egg White Components

Freeze-dried egg white, ovomucoid, ovalbumin, ovotransferrin and lysozyme were conjugated to CNBr-activated paper discs with 5-mm diameter (Toyo Roshi K.K. Tokyo, Japan). After incubation of the RAST disc in 50 μ l of patient's serum for 18 h at 4°C, it was washed 3 times in phosphate-buffered saline (PBS) containing 0.5% Tween 20 (PBS-Tween). The disc was placed in 50 μ l of ^{125} I-conjugated antihuman IgE (Dainabot Co., Tokyo, Japan) for 18 h at room temperature and rinsed with PBS-Tween 3 times, followed by counting in a gamma counter. Percent binding was calculated using the formula of (bound isotope count/added total count isotope of ^{125} I-antihuman IgE) $\times 100$.

RAST Inhibition

After preincubation of 25 μ l of patient's serum and 25 μ l of serial dilutions of inhibitor including 0, 0.7, 7.0, 70 and 420 μ g/ml for 18 h at 4°C, RAST assays were performed using an intact-ovomucoid-conjugated disc as solid phase. Percent RAST inhibition was calculated as follows: [RAST counts (cpm) with inhibitor/RAST counts (cpm) without inhibitor] $\times 100$. An inhibitor concentration required for 50% RAST inhibition was calculated. The greater the concentration of an inhibitor for 50% RAST inhibition is, the lower is the IgE-binding capacity to the inhibitor. The maximum concentration of an inhibitor was used for statistical procedures when a concentration for 50% RAST inhibition was over the maximum concentration used.

Solutions of enzyme-treated and untreated ovomucoid were used as inhibitors. The enzymes were all insoluble types, conjugated with cellulose, from Sigma Chemical Co. Ovomucoid (10 mg/ml) was treated with pepsin (420 IU/ml) derived from porcine stomach mucosa in pH 2.7, 0.01 N HCl buffer for 5 h at 30°C. Then the treatment was terminated by adjustment to pH 6.0 by 0.01 N NaOH. Treatment with trypsin (78 IU/ml) or chymotrypsin (6 IU/ml), which was derived from bovine pancreas, was also done in pH 8.0, 0.1 M, Tris-HCl buffer for 5 h at 30°C. After the enzyme treatments, the insolubilized enzymes were removed by centrifugation at 400 g for 2–3 min and the supernatants were subjected to RAST inhibition assays. SDS-PAGE using WIDE-PAGE mini (16% of running gel, Tris-tricine buffer, resolution from 200,000 to 2,500 of molecular weights; Tefco, Machida, Japan) showed digests of enzyme-treated ovomucoid (data not shown). Filtrates of this enzyme-treated ovomucoid using membrane of cutoffs 10,000 and 3,000 were also used as inhibitors.

Double-Blind Placebo-Controlled Food Challenge

Double-blind placebo-controlled food challenge was performed using freeze-dried egg white with the modifications [11] of the method described by Bock et al. [15]. Briefly, oral provocations were conducted by administering in a titrated manner up to 8 g of an egg white preparation (equivalent to approximately one egg) in 50-ml of thick mixed fruit juice as a liquid vehicle. The initial dose was 0.1 ml of the preparation and then it was increased sequentially (1 ml, 10 ml and the remainder) every 30 min until objective symptoms developed or until the entire challenge dose was ingested. When reactions were observed within 2 h after final administration, immediate reactions were judged positive. The reactions occurring more than 2 h after the final challenge were considered as nonimmediate. No symptoms within 24 h of the final administration were judged to be negative.

Table 1. Characteristics of subjects

Challenge: first; second	Challenge age, months		Interval of follow-up, months
	first	second	
Negative; ND (12; 8:4)			
Mean	48		
± SD	31		
Range	24~132		
Positive; negative (10; 5:5)			
Mean	49	72	23
± SD	31	33	6
Range	19~118	39~144	13~33
Positive; positive (8; 6:2)			
Mean	33	76	43
± SD	9	23	22
Range	24~108	39~108	14~73
Positive, sum (18;11:7)			
Mean	42	74	32
± SD	25	28	18
Range	19~118	39~144	13~73
p value; first challenge (negative vs. positive)	0.594		
p value; second challenge (negative vs. positive)	0.145	0.741	0.031

Figures in parentheses represent number followed by sex ratio (m:f). Nonatopic controls (n = 12); mean age ± SD = 41.6±75.1 months. ND = Not done.

Subjects

Thirty subjects with high IgE antibodies to egg white and ovomucoid were enrolled for double-blind placebo-controlled food challenge with freeze-dried egg white (table 1). We defined percent binding of higher than 1.9% to the egg white disc as high IgE antibody titers. This cutoff titer was a mean plus 2 SD of percent bindings to the egg white disc of 12 control subjects without atopic diseases. These patients were divided into two groups based on the immediate reactions to oral provocation tests. There were no patients with isolated nonimmediate reactions. The first group (n = 12; male:female = 8:4; mean age ± SD = 48±31 months) showed a negative challenge result. The second (n = 18; male:female = 11:7; mean age ± SD = 42±25 months) consisted of subjects who showed positive reactions. In addition, the subjects with positive results to the initial challenge were divided into two subgroups based on the follow-up oral challenge with the same egg white. No significant differences were found in age at the first and the second challenges between the two groups. The subjects with positive results to the second challenge, however, showed a significantly (p = 0.031) longer interval from the first to the second provocation tests than those with negative results. This difference would have no effect on the conclusions of the experiment. If the second challenge had been done at a shorter interval in the group with persistent egg allergy, the challenge tests would have had the same results.

The sera collected from the subjects at the time of the first provocation tests were used for direct RAST and RAST inhibition assays for the prediction of the results of follow-up challenges.

Informed consent was obtained from children and/or parents. The study was approved by the ethics committee of Fujita Health University School of Medicine.

Statistical Analysis

The unpaired t test was used for specific IgE antibody values and concentrations needed for 50% RAST inhibition after logarithmic transformation.

Results

There were no significant differences in IgE antibody titers (% binding) to not only egg white but also its components such as ovomucoid, ovalbumin, ovotransferrin and lysozyme between the positive and the negative groups to the first challenge tests as shown in table 2.

Table 3 represents the concentrations of intact ovomucoid or enzyme-treated ovomucoid used as an inhibitor required for 50% RAST inhibition. There were no significant differences in the concentrations of native ovomucoid as an inhibitor needed for 50% RAST inhibition between positive and negative groups to the challenge tests. In contrast, the positive subjects showed significantly (p = 0.000008) low-

Table 2. Comparison of specific IgE antibody titers (% binding) to egg white and its components between subjects with positive and negative results to first challenge tests

First challenge	Binding, %				
	egg white	ovomucoid	ovalbumin	ovotransferrin	lysozyme
Negative (n = 12)					
Geometric mean	23.9	11.6	9.9	6.7	5.2
Mean – SD	10.2	4.5	3.6	1.6	2.1
Mean + SD	55.9	29.7	27.3	28.2	13.0
Positive (n = 18)					
Geometric mean	28.9	15.0	17.2	6.6	3.5
Mean – SD	16.5	6.1	6.2	2.2	1.5
Mean + SD	50.6	36.6	47.7	20.1	7.8
p value	0.504	0.461	0.156	0.974	0.220

Nonatopic controls (n = 12): geometric mean and mean +2 SD: egg white (1.5, 1.9%), ovomucoid (0.9, 1.2%), ovalbumin (0.9, 2.0%), ovotransferrin (0.5, 0.7%), lysozyme (0.6, 1.5%).

Table 3. Comparison of IgE-binding activity to enzyme-treated ovomucoid between subjects with negative and positive results to the first oral challenge tests

First challenge	Pepsin				Chymotrypsin				Trypsin			
	– filter (–)	+ filter (–)	+ 10,000	+ 3,000	– filter (–)	+ filter (–)	+ 10,000	+ 3,000	– filter (–)	+ filter (–)	+ 10,000	+ 3,000
Negative (n = 12)												
Geometric mean	1.0	94.2	177.9	210.0	1.0	5.5	22.0	210.0	1.3	4.3	4.8	83.0
Mean – SD	0.2	24.7	106.3	210.0	0.2	0.9	3.0	210.0	0.2	0.6	0.4	26.6
Mean + SD	5.4	358.7	300.6	210.0	5.4	33.8	158.5	210.0	8.0	30.7	64.8	259.0
Positive (n = 18)												
Geometric mean	0.4	2.6	37.6	121.1	0.5	1.5	7.2	144.8	0.5	1.0	0.9	30.6
Mean – SD	0.1	0.3	4.6	46.8	0.1	0.3	1.3	46.2	0.2	0.2	0.1	7.7
Mean + SD	1.4	25.0	307.6	313.2	1.6	7.6	40.3	453.8	1.6	3.8	6.3	121.3
p values	0.161	0.000008	0.014	0.042	0.202	0.062	0.162	0.228	0.116	0.037	0.105	0.061

IgE-binding activity was assessed by RAST inhibition using intact ovomucoid or enzyme-digested ovomucoid before and after filtration with a cutoff of 10,000 or 3,000 as inhibitors. Filter (–) means an inhibitor without filtration. Ovomucoid was digested by pepsin, trypsin and chymotrypsin. Sera were from subjects with high IgE antibodies to egg white and ovomucoid. Figures represent concentrations (μg/ml) needed for 50% RAST inhibition. The greater the concentration is, the lower is the IgE-binding activity to the inhibitor.

er concentrations (2.6 μg/ml) of pepsin-treated ovomucoid for 50% RAST inhibition than that (94.2 μg/ml) of negative subjects. This evidence means that IgE antibodies from individuals with positive results to oral provocation have greater activity binding to ovomucoid digests after treatment with pepsin. A similar significant difference was obtained in the experiments using trypsin-treated ovomucoid (1.0 vs. 4.3 μg/ml, $p = 0.037$) but not in the experiments of chymotrypsin-treated ovomucoid (1.5 vs. 5.5 μg/ml, $p = 0.062$). The difference in the IgE binding to pepsin digests

was most remarkable and still observed after filtration with the molecular weight of 10,000 and 3,000 of cutoff. However, the IgE-binding activity to the filtrates through the membrane with a cutoff of 3,000 was much smaller as compared to that to pepsin digests without filtration and after filtration with a cutoff of 10,000.

The second oral challenge tests using the same egg white antigens were performed in the subjects with positive results in the initial provocation tests. There was no significant difference in direct RAST results to untreated ovomu-

Table 4. Comparison of specific IgE antibody titers (% binding) to egg white and its components between subjects with positive and negative results to second challenge tests

Challenge; first; second	Binding, %				
	egg white	ovomucoid	ovalbumin	ovotransferrin	lysozyme
Positive; negative (n = 10)					
Geometric mean	28.0	15.2	15.1	6.1	3.8
Mean – SD	18.3	6.7	4.8	1.8	1.5
Mean + SD	42.7	34.1	47.7	20.3	9.3
Positive; positive (n = 8)					
Geometric mean	30.1	14.8	20.2	7.4	3.1
Mean – SD	14.6	5.2	8.4	2.6	1.5
Mean + SD	62.2	41.9	48.8	21.1	6.5
p value	0.800	0.957	0.548	0.713	0.624

Nonatopic controls (n = 12): geometric mean and mean +2 SD: egg white (1.5, 1.9%), ovomucoid (0.9, 1.2%), ovalbumin (0.9, 2.0%), ovotransferrin (0.5, 0.7%), lysozyme (0.6, 1.5%).

Table 5. Comparison of IgE-binding activity to enzyme-treated ovomucoid between subjects with negative and positive results to the second oral challenge tests

Challenge: first; second	Pepsin				Chymotrypsin				Trypsin			
	– filter (–)	+ filter (–)	+ 10,000	+ 3,000	– filter (–)	+ filter (–)	+ 10,000	+ 3,000	– filter (–)	+ filter (–)	+ 10,000	+ 3,000
Positive; negative												
Geometric mean	0.4	6.8	64.4	118.1	0.4	1.3	9.8	210.0	0.4	0.9	0.9	27.3
Mean – SD	0.1	0.6	13.8	39.9	0.1	0.3	2.4	210.0	0.2	0.2	0.1	7.4
Mean + SD	1.3	73.9	300.7	350.1	1.1	6.5	38.7	210.0	1.0	3.6	9.1	100.2
Positive; positive												
Geometric mean	0.5	0.8	16.7	125.8	0.6	1.9	4.5	82.9	0.6	1.0	0.9	36.2
Mean – SD	0.1	0.2	1.1	56.7	0.1	0.3	0.5	14.5	0.1	0.2	0.2	7.3
Mean + SD	1.6	3.4	248.1	279.3	2.8	10.6	41.0	472.8	3.1	4.7	3.7	179.2
p values	0.839	0.033	0.304	0.899	0.519	0.663	0.466	0.248	0.554	0.884	0.983	0.726

IgE-binding activity was assessed by RAST inhibition using intact ovomucoid or enzyme-digested ovomucoid before and after filtration with a cutoff of 10,000 or 3,000 as inhibitors. Filter (–) means an inhibitor without filtration. Ovomucoid was digested by pepsin, trypsin and chymotrypsin. Sera from subjects with high IgE antibodies to egg white and ovomucoid were collected at the time of the first challenge. Figures represent concentrations (µg/ml) needed for 50% RAST inhibition. The greater the concentration required for 50% RAST inhibition is, the lower is the IgE-binding activity to the inhibitor.

coid as well as to egg white and the other components between positive and negative subjects on the second challenge (table 4). The IgE-binding activities to enzyme digests of the sera collected on the first challenge were compared between positive and negative groups on the second challenge. As shown in table 5, IgE-binding capacity (a concentration for 50% RAST inhibition = 0.8 µg/ml) to pepsin-treated ovomucoid before filtration in the subjects with positive challenge results was significantly ($p = 0.033$) greater than that (a concentration for 50% RAST inhibition

= 6.8 µg/ml) of the negative group. The difference in IgE-binding activity to filtrates through the membrane with a cutoff of 10,000 and 3,000 between positive and negative groups showed no significant difference in IgE-binding activity to pepsin-treated ovomucoid. There was no significant difference in IgE-binding capacity to trypsin- and chymotrypsin-treated ovomucoid between positive and negative subjects.

Discussion

We performed oral challenge tests with freeze-dried egg white antigens for subjects with high IgE antibody titers to ovomucoid as well as egg white and compared IgE-binding activities to enzyme-treated ovomucoid between subjects with positive and negative results to the challenge tests. Both groups showed no significant differences in IgE antibody titers to ovalbumin, ovotransferrin and lysozyme. In contrast, the IgE-binding activity to pepsin-digested ovomucoid measured by RAST inhibition had the most useful diagnostic value to distinguish the challenge-positive subjects from the negative subjects. The statistically significant difference was also observed in the comparison of IgE-binding activity to pepsin-digested ovomucoid after filtration using a membrane with a cutoff of 10,000 and 3,000, whereas the difference in the IgE-binding activity to the filtrates through a cutoff of 3,000 was much smaller. Furthermore, the subjects with higher IgE-binding activity to pepsin-treated ovomucoid in the children with overt egg hypersensitivity were unlikely to outgrow the egg allergy.

These results indicated that ovomucoid had IgE-reactive sites with different stability against pepsin and that pepsin-stable epitopes on ovomucoid were important for clinical diagnosis and prediction of the outcome of egg allergy.

Stability to proteolytic enzymes is considered as one of the necessary conditions for dominant allergens of food [16]. Ovomucoid is relatively resistant to digestive enzymes compared to the other egg white components. Furthermore, some ovomucoid fragments produced by the peptide digestion retain their antigenicity [12]. The linear epitopes with IgE-binding capacity on ovomucoid were proved by Cooke and Sampson [17] using the overlapping peptides. Decrease in the reactivity to IgE antibodies after digestive enzymes indicated the conformational IgE epitopes on ovomucoid. However, IgE-binding sites responsible for egg hypersensitivity were retained even after degradation by enzymes, especially pepsin. Such clinically important IgE-binding sites on ovomucoid may have a linear structure of amino acid sequences. The tertiary structure is disrupted after treatment by enzymes, although the conformational structure formed by disulfide bridges is retained since ovomucoid has nine intradomain disulfide bonds. Therefore, these clinically important IgE epitopes may be present on the conformational structure associated with disulfide bonds.

The measurement of IgE-binding capacity to pepsin digests of ovomucoid was most useful to differentiate positive from negative subjects compared to other digestive enzymes such as trypsin and chymotrypsin. The reason for this evidence was unclear. It is, however, interesting that

pepsin is a proteolytic enzyme that first digests the food proteins orally ingested. Probably IgE epitopes on ovomucoid that are relatively resistant to cleavage by pepsin may be critical for the development of hypersensitive reactions by oral intake of egg white.

Bernhisel-Broadbent et al. [10] showed that egg hypersensitivity in infants with high IgE antibody titers to ovomucoid was likely to persist for a long period. This is probably due to the stability of ovomucoid against digestive enzymes compared with other egg white components such as ovalbumin, ovotransferrin and lysozyme. Our study enrolling subjects with high IgE antibody titers to ovomucoid suggested that the measurement of IgE reactivity to pepsin-resistant regions on ovomucoid was more useful to predict the outcome of egg allergy. These data may suggest the close relationship between the outgrowing of egg hypersensitivity and the maturation of production of digestive enzymes in the gut.

We frequently come across false-positive results of specific IgE to egg white. These results might lead to an unnecessarily restrictive diet. There is a strong demand to develop a reliable in vitro diagnostic method. It would be most useful for this purpose to determine the IgE-binding sites on the ovomucoid responsible for provoking egg-hypersensitive reactions. A study on the determination of the IgE-binding sites with clinical significance on pepsin digests of ovomucoid is in progress.

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