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Key Words

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Specificities of IgE, IgG and IgA Antibodies to Ovalbumin

Comparison of Binding Activities to Denatured Ovalbumin or Ovalbumin Fragments of IgE Antibodies With Those of IgG or IgA Antibodies

Abstract

We studied the binding activities of IgE, IgG and IgA antibodies in patients with allergy to hen's egg white against two different ovalbumin (OVA) preparations, which were physically or chemically denatured OVA and enzyme-digested OVA fragments. The binding activities of IgE antibodies to these OVA preparations with those of IgG or IgA antibodies were compared. It was found that the binding activities of IgE antibodies to denatured OVA by treatment with dithiothreitol, urea or hydrochloric acid were similar to those of IgG or IgA antibodies. In contrast, the binding activities of IgE antibodies to heat-denatured OVA or by treatment with sodium hydroxide at pH 11.0 were different from those of IgG or IgA antibodies to these denatured OVA. Furthermore, we found that the binding activities of anti-OVA antibodies in sera from patients with allergy to hen's egg white against fragmented OVA were different between IgE antibodies and IgG or IgA antibodies. Thus, it can be concluded that IgE antibodies to OVA in sera from patients with allergy to egg white differ from IgG or IgA antibodies in respect to binding activities against different preparations of denatured or fragmented OVA, probably due to differences in fine specificities of these antibodies against OVA.

Introduction

Food allergy is an allergic disorder based on the abnormal response to food antigens and presents various clinical symptoms, including atopic dermatitis, rhinitis and diarrhea [1]. Antigens participating in these allergic reactions, that is allergens, are mainly proteins which have numerous epitopes on the molecular surface. Different antigenic determinants may be recognized on multideterminant protein antigen by different functional subsets of specific immunocompetent cells [2]. And, recent investi-

gations of animal models for autoimmune diseases suggested that restricted epitopes of autoantigens recognized by autoantibodies and/or T cells are related to autoimmune diseases [3–5]. Thus, there is a possibility that limited epitopes on protein antigens are related to allergic disorders.

Egg white is one of the main food items that frequently induce hypersensitivity, particularly in atopic children [6]. It has been clearly shown that among 40 different egg white proteins, only 3, ovalbumin (OVA), ovomucoid and ovotransferrin, are the major allergens in patients with

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food allergy [7–10]. OVA, which is a water-soluble glycoprotein with a molecular weight of 42,693, is the most abundant of the proteins, forming 58% w/w of the whole egg white. Complete primary structure [11] and molecular structure of OVA have been defined, with the later based on X-ray crystallography and reported by Stein et al. [12]. With this background knowledge, OVA was deemed suitable as a protein for characterization of the antigenic determinants relating to food allergy and in studies on IgE response regulation.

The IgE, IgG and IgA class antibodies for OVA have been shown to exist in sera from patients with allergy [13, 14]. RAST inhibition test demonstrated that allergenic epitopes are mainly determined by primary structure and OVA peptides 1–10 and 323–339 encompass allergenic epitopes, which are recognized by IgE antibodies from patients with allergy to hen's egg white [15–17]. However, much less is known regarding the fine specificities of IgG and IgA antibodies. Therefore, it is unclear whether antigenic determinants of OVA recognized by IgE are identical or similar to those recognized by IgG or IgA antibodies.

In order to define the allergenic epitopes of OVA, we undertook identification of reactivities of IgE, IgG and IgA antibodies in patients with allergy to hen's egg white or to an OVA preparation, either physically and chemically denatured OVA or OVA fragments prepared by enzymatic digestion of OVA.

Materials and Methods

Patients

Sera were obtained from 15 Japanese patients (10 males, 5 females) with egg white allergy displaying clinical symptoms of atopic dermatitis and hypersensitivity to egg white. They were diagnosed on the basis of food elimination and challenge test [18], and RAST scores for egg white classes 2–4 were positive. They ranged in age from 1 to 10 years (mean 3.8 years). No patients had been treated with systemic corticosteroids or by desensitization therapy.

Antigens

OVA (grade V) was purchased from Sigma (St. Louis, Mo., USA).

Preparation of Rabbit Anti-OVA Antibodies and Mouse Anti-Denatured OVA Antibodies

A rabbit and mouse were used to prepare anti-OVA and anti-denatured OVA antibodies, respectively. A female, outbred NZW, rabbit and a female C3H/He mouse were purchased from Japan SLC, Inc., Hamamatsu, Japan. Rabbit anti-OVA antibodies were obtained from the NZW rabbit which was subcutaneously injected with 3 mg of OVA in Freund's complete adjuvant (CFA). The injection was repeated 3 times at 10-day intervals in CFA. The rabbit was bled 10 days after the last injection. The mouse used was an 8-week-old C3H/He

animal. It was immunized subcutaneously with heated OVA (200 µg) in CFA. Two and four weeks later it was boosted subcutaneously with an identical dose of antigens in CFA and was bled after a further 2 weeks. The sera from the mouse and rabbit were stored frozen at –80 °C until required for use.

Denaturation of OVA

Denaturation of OVA was performed by the following five procedures: (1) OVA solution (10 mg/ml of phosphate buffer saline [PBS], pH 7.4) was heated at 100 °C for 3 min. (2) OVA solution (10 mg/ml of PBS) was incubated overnight at 0.3 M NaOH at room temperature and adjusted to pH 11. (3) The disulfide bonds of OVA were reduced by the treatment with dithiothreitol (DTT) [19], according to the method used by Nissley et al. [20]. DTT dissolved in 0.5 M Tris-chloride pH 8.5 was added to 10 mg/ml OVA solution at a molar ratio (DTT to OVA disulfide groups) of 100, and the reaction was allowed to proceed for 2 h at 20 °C under argon gas. The OVA sulfhydryl groups and unreacted DTT were alkylated with iodoacetamide, added to a concentration twice that of DTT. After incubation for 30 min at 20 °C, the protein was dialyzed against PBS. (4) OVA solution (15 mg/ml of PBS, pH 7.4) was incubated for 16 h at 20 °C in the presence of 6 M urea, followed by dialysis against 2 M urea solution. (5) OVA was denatured by adjustment of pH to 3.0 by hydrochloric acid.

Preparation of OVA Fragments

OVA (60 mg/ml) dissolved in PBS, pH 7.4, was denatured overnight by incubation at 37 °C in the presence of 6 M urea. OVA was fully digested with protease when conformational structure of OVA was modified by the treatment of OVA with 6 M urea [21]. A 1:10 (w/w) dilution of *Staphylococcus aureus* strain V8 protease (molecular weight, 11,500; Sigma) was then added to OVA and incubated overnight at 37 °C. The digest was then resolved by gel filtration on either a Sephadex G-50 (Toyo Soda, Tokyo, Japan) or a Sephadex G-100 column (Toyo Soda). There were four fractions (Fr-1 to Fr-4) with proteins of molecular weights between 3,000 and 21,000. The molecular weights of the proteins in these fractions were estimated by interpolation from standards (phosphorylase b, bovine serum albumin, OVA, carbonic anhydrase, soybean trypsin inhibitor, hen egg white lysozyme) and were treated under the same conditions. Each fraction was stored at –20 °C until use. The protein concentrations were determined by a Bio-Rad protein assay taking OVA as the standard (Bio-Rad Laboratories, Richmond, Calif., USA).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) Analysis

SDS-PAGE was performed by Laemmli's method [22] with some modifications. The samples (1 µg of protein/lane) were placed on a SDS-polyacrylamide 10–15% gradient gel (PhastGel; Pharmacia LKB Biotechnology, Uppsala, Sweden) and electrophoresis was performed by PhastSystem (Pharmacia). After electrophoresis, the gel was stained with Coomassie brilliant blue R-250. Molecular weight protein standards (97.4, 66.2, 42.7, 31.0, 21.5, 14.4 kD) were purchased from Bio-Rad Laboratories.

ELISA for Anti-OVA Antibodies

IgG, IgE and IgA Antibodies to OVA in Patients with Egg Allergy. Quantitative immunoassays for IgG, IgE and IgA antibodies for OVA were performed by ELISA, using a modification of the technique described previously [23]. The binding activities of IgG, IgE and IgA antibodies to OVA reached a plateau when the plates were

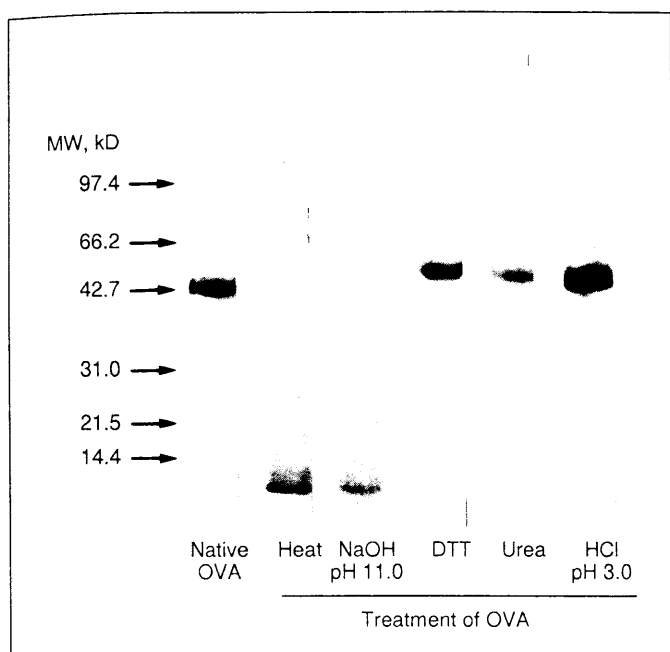


Fig. 1. SDS-PAGE analysis of native OVA and denatured OVA. Native OVA was denatured as described in Materials and Methods.

coated with more than 100 $\mu\text{g/ml}$ native OVA. Similarly, the binding activities of IgG, IgE and IgA antibodies to OVA fragments reached a plateau when the plates were coated with more than 100 $\mu\text{g/ml}$ OVA fragment Fr-1, 200 $\mu\text{g/ml}$ Fr-2, 10 $\mu\text{g/ml}$ Fr-3 or 10 $\mu\text{g/ml}$ Fr-4. Disposable polystyrene plates with a flat-bottomed well (Nunc, Roskilde, Denmark) were coated with 100 μl 100 $\mu\text{g/ml}$ native or denatured OVA, 200 $\mu\text{g/ml}$ OVA fragment Fr-1 or Fr-2, 10 $\mu\text{g/ml}$ Fr-3 or 25 $\mu\text{g/ml}$ Fr-4 in PBS for overnight incubation of the preparation at 4 $^{\circ}\text{C}$. OVA preparations used in these experiments were diluted with PBS when used for coating the plates. The final pH of OVA preparation solution for coating the plate was between 7.4 and 9.0. Unoccupied sites in the wells were saturated by 3-hour incubation with 200 μl 0.3% gelatin in PBS. The wells were washed with PBS containing 0.05% Tween-20. Sera from patients with egg allergy were tested at 1:20 dilution with PBS containing 0.1% gelatin and 0.05% Tween-20, and incubated overnight at 4 $^{\circ}\text{C}$. The wells were washed with PBS containing 0.05% Tween-20. An alkaline phosphatase-conjugated goat anti-human IgG antibody (Sigma), an alkaline phosphatase-conjugated goat anti-human IgE antibody or an alkaline phosphatase-conjugated goat anti-human IgA antibody (Kirkegaard & Perry Laboratories Inc., Md., USA) was added to each well at a final dilution of 1:1,000, 1:800 or 1:500, respectively. The plates were incubated overnight at 4 $^{\circ}\text{C}$. After additional washes, the amount of alkaline phosphatase bound to the well was determined by measuring the hydrolysis of *p*-nitrophenylphosphate (Sigma) to the yellow product, *p*-nitrophenolate, which was quantitated by absorbance at 405 nm using a Microplate Reader MTP-100 (Corona Electric Co., Ltd, Ibaraki, Ja-

pan). The background of this assay was determined using a plate coated with 0.3% gelatin instead of OVA preparations and was subtracted from all data. The binding activity of antibodies to the denatured OVA or OVA fragments is expressed as percentage of binding compared to the binding activity to native OVA. The percent binding activity was calculated when the mean absorbance of duplicate samples for native OVA was higher than 0.4; however, most cases were higher than 0.7. The background optimal density (OD) values were lower than 0.07 in all of the ELISAs.

IgG Antibodies to OVA in Mouse or Rabbit. Antibodies of mouse or rabbit to native or denatured OVA were measured by ELISA. The basic procedure was similar to that for detection of antibodies in sera from patients to OVA as described above. The plates coated with native or denatured OVA were incubated at 4 $^{\circ}\text{C}$ overnight with diluted sera (mouse 1:100, rabbit 1:10,000) from mouse or rabbit. The amount of antibodies bound to the well was detected by alkaline phosphatase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (Sigma). The binding activity of antibodies to the denatured OVA in mouse or rabbit serum is expressed as percentage of binding compared to the binding activity to native OVA. The background OD values were lower than 0.07 in mouse and lower than 0.01 in rabbit.

Statistical Analysis

Statistical evaluation of the data was performed using Student's *t* test.

Results

SDS-PAGE Analysis of Denatured OVA

The results of gel electrophoresis in figure 1, using 10–15% polyacrylamide gel (PAGE) in SDS, indicate that by heating at 100 $^{\circ}\text{C}$ for 3 min and treating with sodium hydroxide, OVA split into smaller molecules; whereas the other three treatments did not produce degradation.

Reactivities of Rabbit Anti-OVA Antibodies and Mouse Anti-Denatured OVA Antibodies against Denatured OVA Preparations

Using the serum of rabbit or mouse which were immunized with native OVA or heat-denatured OVA, respectively, the binding activities of these antibodies for OVA exposed to the five denaturing treatments were compared with those for native OVA (table 1). It was found that the binding activities of rabbit anti-OVA antibodies against heat-treated and alkalized OVA were much less (65 and 27%, respectively) as compared with those against native OVA. However, binding activities of OVA treated by the other three methods were similar to those of native OVA. And, the binding activities of mouse anti-heat-denatured OVA against each denatured OVA were all higher (168–251%) than those against native OVA. These results suggest that the denaturation procedures used caused conformational changes of OVA molecules.

Table 1. Binding activities of rabbit antibodies or mouse antibodies to denatured OVA

Treatment of OVA	Mouse anti-heated OVA Ab		Rabbit anti-OVA Ab	
	ΔOD^1 (405 nm)	% binding activities	ΔOD^1 (405 nm)	% binding activities
Native	0.331	100	1.505	100
100 °C, 3 min	0.788	238	0.972	65
0.3 M NaOH, pH 11.0	0.563	170	0.410	27
DTT	0.566	171	1.391	92
Urea	0.831	251	1.509	100
HCl, pH 3.0	0.556	168	1.881	125

Binding activities of IgG antibodies in serum to native or denatured OVA were measured by ELISA. The ELISA assay was performed as described in Material and Methods. The binding activity is expressed as a percentage of that in the presence of denatured OVA compared to that of native OVA, taken as 100%. Native OVA was denatured as described in Materials and Methods. The OD values of IgG antibodies of sera in normal mouse and rabbit were lower than 0.01.

¹ Mean absorbance at 405 nm of duplicate samples.

Binding Activities of IgE, IgG and IgA Antibodies of Patients with Egg Allergy to Denatured OVA

The binding activities of anti-OVA antibodies of IgG, IgE and IgA classes, in sera of 15 patients, to OVA denatured by the five treatments (1–5) were compared with those to native OVA (fig. 2, table 2). As for IgE antibodies (closed circles), it is clear that the binding activities of those antibodies to heat-denatured (treatment 1) and to alkalinized (treatment 2) OVA were far less (9.4 and 0.4%, respectively) than those to native OVA. In contrast, increased binding activities to OVA treated with urea (treatment 4) and with hydrochloric acid at pH 3.0 (treatment 5), and comparable binding activities to OVA obtained by DTT (treatment 3) were observed. Similar tendencies were found in the cases of IgG and IgA antibodies. The percent binding activities to heated and to alkalinized OVA showed significant differences between IgE and IgG antibodies and between IgE and IgA (table 2). However, no differences were found to denatured OVA by treatment with DTT, urea or HCl between IgE and IgG antibodies and between IgE and IgA antibodies.

Binding Activities of IgG, IgE and IgA Antibodies to OVA Fragments

Four OVA fragments, obtained by proteolysis with V8 protease followed by gel filtration, were used for binding activity estimations of anti-OVA antibodies of IgE, IgG and IgA classes in sera of patients, and compared with those to native OVA (fig. 3, table 3). The binding activity of IgE antibodies (closed circles) was below 20%, the mean value for Fr-2, Fr-3 and Fr-4, and about 50% for Fr-1.

The binding activity of IgG antibodies to each fragment (open circles) was over 30%, the mean value, compared with that of IgE antibodies. In addition, the difference between IgE and IgG antibodies was significant (table 3). The percent binding activity of IgA antibodies to each fragment was over 120% and the binding activity of IgA antibodies was different from that of IgE antibodies.

Discussion

IgE antibodies, which were evident in sera from most patients with allergy to hen's egg white but not from healthy subjects, play a major role in food hypersensitivity reactions, especially immediate type (type I) of allergic reaction within the intestinal tract [24]. In contrast, IgG and IgA antibodies to foods have been found in both food-allergic subjects [13, 14] and healthy children [25]. Evidence for involvement of IgG and IgA classes of immunoglobulin in food sensitivity reactions is much less convincing. Recently, the existence of immunodominant IgE determinants was suggested by studies of codfish Gad cI and hazel Cor aI [26, 27] which appear to elicit IgE that binds predominantly to discrete peptides. In addition, modified allergens showed reduced reactivity for IgE but retained IgG-inducing capacity [28]. Accordingly, there is a possibility that the difference in food hypersensitivity of each isotype antibody depends on its fine specificities. We therefore compared the binding activities of IgE antibodies to denatured or fragmented OVA with those of IgG or IgA antibodies.

Fig. 2. Percent binding activities of antibodies in sera from patients with allergy to hen's egg white to denaturd OVA. The binding activities of IgG (○), IgE (●) and IgA (△) antibodies to each denaturd OVA were measured by ELISA and are expressed as the percentage of antibody binding to native OVA, taken as 100%. Native OVA was denatured as described in Materials and Methods. The assay was performed in duplicate.

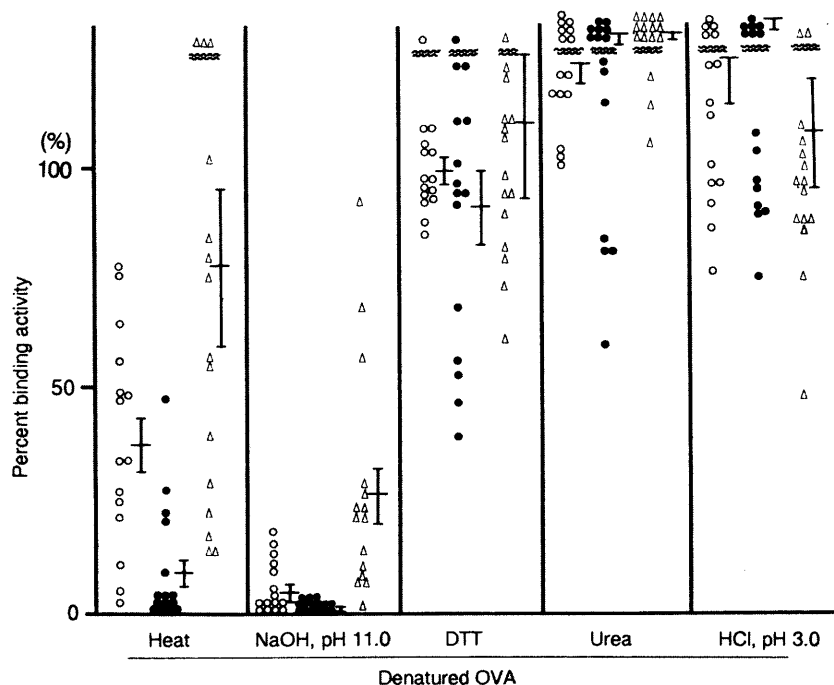


Fig. 3. Percent binding activities of antibodies in sera from patients with allergy to hen's egg white to OVA fragments. The binding activities of IgG (○), IgE (●) and IgA (△) antibodies to each OVA fragment were measured by ELISA and are expressed as the percentage of antibody binding to native OVA, taken as 100%. Native OVA was denatured as described in Materials and Methods. The assay was performed in duplicate.

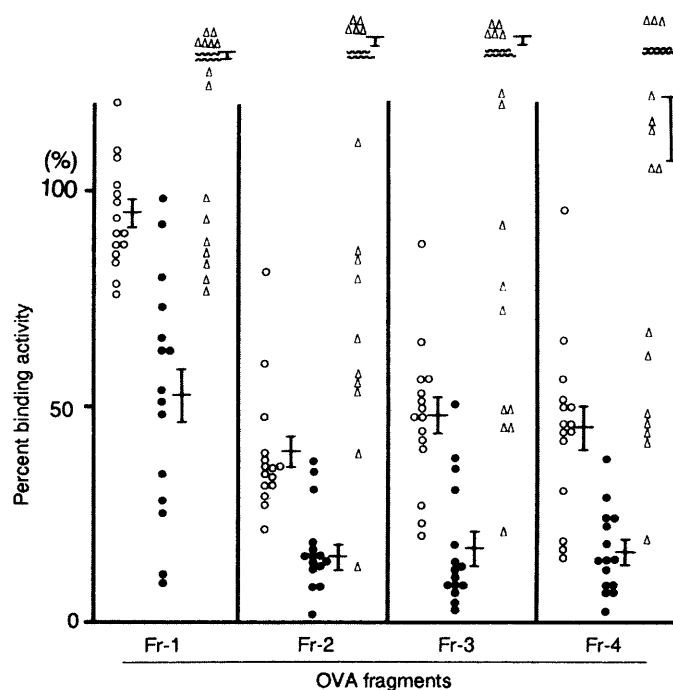


Table 2. Binding activities of IgG, IgE and IgA antibodies to denatured OVA

Treatment of OVA	Percent binding activity (mean $\pm$ SE)			Significance	
	IgG anti-OVA Ab	IgE anti-OVA Ab	IgA anti-OVA Ab	IgE vs. IgG	IgE vs. IgA
Heat	38.7 $\pm$ 6.2 ¹	9.4 $\pm$ 3.2	76.1 $\pm$ 17.2	p < 0.01 ²	p < 0.05
NaOH, pH 11.0	5.2 $\pm$ 1.7	0.4 $\pm$ 0.3	27.4 $\pm$ 6.5	p < 0.05	p < 0.01
DTT	99.6 $\pm$ 3.3	89.0 $\pm$ 8.1	110.6 $\pm$ 16.0	NS ³	NS
Urea	120.0 $\pm$ 3.6	135.7 $\pm$ 13.3	133.0 $\pm$ 5.6	NS	NS
HCl, pH 3.0	121.5 $\pm$ 10.2	156.6 $\pm$ 23.6	104.0 $\pm$ 12.2	NS	NS

The values shown are mean $\pm$ SE of the binding activity of antibodies from 15 patients with egg allergy. The binding activity is expressed as a percentage of that in the presence of denatured OVA compared to that of native OVA, taken as 100%. Native OVA was denatured as described in Materials and Methods.

¹ Taken from the results shown in figure 2.

² Significance was determined by Student's t test.

³ NS, not significant.

Table 3. Binding activities of IgG, IgE and IgA antibodies to OVA fragments

OVA fragments	Percent binding activity (mean $\pm$ SE)			Significance	
	IgG anti-OVA Ab	IgE anti-OVA Ab	IgA anti-OVA Ab	IgE vs. IgG	IgE vs. IgA
Fr-1	92.3 $\pm$ 3.0 ¹	53.1 $\pm$ 6.8	141.2 $\pm$ 26.0	p < 0.01 ²	p < 0.01
Fr-2	39.2 $\pm$ 3.6	18.8 $\pm$ 2.5	130.0 $\pm$ 33.0	p < 0.01	p < 0.01
Fr-3	48.2 $\pm$ 4.1	19.1 $\pm$ 3.7	132.6 $\pm$ 30.1	p < 0.01	p < 0.01
Fr-4	45.1 $\pm$ 5.0	18.1 $\pm$ 2.4	121.2 $\pm$ 28.6	p < 0.01	p < 0.01

The values shown are mean $\pm$ SE of the binding activity of antibodies from 15 patients with egg allergy. The binding activity is expressed as a percentage of that in the presence of fragmented OVA compared to that of native OVA, taken as 100%. Native OVA was fragmented as described in Materials and Methods.

¹ Taken from the results shown in figure 3.

² Significance was determined by Student's t test.

We studied the binding activities of antibodies to denatured and fragmented OVA by calculating the percentage of antibody binding to native OVA, taken as 100%. Points of detected absorbance on the spectrometer, the ability of antibodies represented by the linear region of the sigmoid curve, dependent on serum dilutions, were used. Similar results were obtained from the competitive inhibition study, in which the binding of antibodies to native OVA was inhibited by denatured OVA (data not shown).

The result of SDS-PAGE indicated that the primary structure of OVA was retained when OVA was treated with DTT, urea or hydrochloric acid. Meanwhile, the binding activities of heterologous rabbit or mouse anti-OVA antibodies to denatured OVA were not identical with those to native OVA (table 1), indicating that denaturation of OVA modified the conformational structure. Since

the abilities of IgE, IgG and IgA antibodies to bind to OVA treated with urea or hydrochloric acid increased as compared with those to native OVA, the abilities of OVA to react with the antibodies were altered, suggesting that part of epitopes of OVA recognized by these antibodies of patients are conformationally dependent. On the other hand, Elsayed et al. [15] described that thermal denaturation of OVA by heating to 80 °C for 10 min or treating with trypsin had no direct effect on the antigenic reactivity as shown by crossed immunoelectrophoresis (CIE). Furthermore, trypsin hydrolysis of carboxymethyl OVA did not abolish the activity of IgE antibodies in patients with egg allergy [15]. Similarly, the patients' IgE showed reactivities on IEF immunoprints for cleavage products of OVA [29]. These findings suggested that human IgE binding is associated with sequential protein structures and do not follow

our results. The difference in findings may depend on the denaturation methods of OVA used and on the estimation methods of binding activities of IgE antibodies to denatured OVA. In Elsayed's assay system, it is difficult to demonstrate the alteration of antibody abilities against denatured OVA when binding activities to denatured OVA are increased, and the specificities of IgG and IgA antibodies against OVA were not studied.

In contrast to OVA treated with DTT, urea or hydrochloric acid, alkalinized OVA and heat-denatured OVA were fragmented by these treatments. The binding activities of IgE antibodies to alkalinized or heat-denatured OVA were different from those of IgG or IgA antibodies, while no differences were found in the binding activities to OVA treated with DTT, urea or hydrochloric acid. Similarly, the binding activities of IgE antibodies to OVA fragments differed from those of IgG or IgA antibodies. These findings suggest that conformational structure associated with IgE-binding activity may be close to that associated with IgG or IgA-binding activity. In contrast, sequential antigenic determinants of OVA recognized by IgE antibodies may differ from those recognized by IgG or IgA antibodies. Elsayed et al. [15] reported that limited pepsin hydrolysis could destroy the allergenic activity of the molecule. Similarly, in our case, IgE antibodies did not bind to fragmented OVA by heating or treatment with sodium hydroxide. The decrease of binding activities of antibodies against fragmented OVA did not result from insufficiency

of coating the plates since the binding activities of mouse anti-heated OVA antibodies against fragmented OVA were higher than those against native OVA under the same condition for the antigen coating (table 1). Accordingly, the modification of OVA may destroy the antigenic determinants including those determined by the primary structure and that epitopes recognized by IgE antibodies may be constant in amino acid length.

In conclusion, differences were observed in specificities of anti-OVA antibodies, between IgE and IgG or IgA antibodies, in sera from patients with allergy to hen's egg white. This may be indicative of the fact that the abilities of a given OVA epitope significantly differ between IgE and IgG or IgE and IgA, or that epitopes of OVA recognized by IgE differ from those by IgG or IgA. The mechanisms responsible for the restriction of isotype expression of antibodies with particular specificity are not explained now. However, elucidation of epitopes on molecules recognized by IgG, IgE or IgA antibodies are important for studies concerning the pathological role of IgE, IgG and IgA antibodies in induction of allergy.

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