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New Allergens from Hen's Egg White and Egg Yolk

In vitro Study of Ovomucin, Apovitellenin I and VI, and Phosvitin

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Abstract. Three hen egg yolk proteins, apovitellenins I and VI and phosvitin, and one egg white protein, ovomucin, were purified and tested for their ability to bind IgE in the sera of patients hypersensitive to egg. All of the proteins bound IgE from the sera of egg-allergic individuals in the radioallergosorbent test, and they also inhibited binding of IgE to the parent fractions – either egg yolk (apovitellenins I and VI and phosvitin) or egg white (ovomucin). It appears that apovitellenins I and VI are major allergens for some of the individuals tested. This is the first report of the in vitro allergenicity of these proteins.

Introduction

Hen's egg white proteins that produce IgE-mediated allergic responses of the immediate (type I) reaction include ovalbumin, ovotransferrin (conalbumin), lysozyme, and ovomucoid [2, 10, 16–18]. Ovomucin, first described by Eichholz in 1898 [see ref. 23], is a complex glycoprotein which has not so far been implicated in egg allergy, although Miller and Campbell [20] reported that it gave positive cutaneous tests in a number of subjects, and Anet et al. [2] obtained preliminary indications of its allergenicity by Western blotting. Ovomucin is about 1.5% of the total egg white protein [21] and exists as two types of complex: an insoluble ovomucin, responsible for the high viscosity of egg white, and a soluble ovomucin. Both are composed of different combinations of two subunits designated alpha and beta ovomucin with M_r of 180,000 and 400,000, respectively [14, 22]. We have now examined the allergenicity in vitro of ovomucin by binding a purified ovomucin complex to nitrocellulose discs and testing sera of susceptible patients by radioallergosorbent test (RAST) [26].

Proteins from egg yolk have received little attention as allergens, although they bind specific IgE in the sera of allergic patients [2]. We have tested two proteins from the lipoprotein apoprotein fraction of the yolk for their possible allergenicity. These were

apovitellenin I and apovitellenin VI, both of which are present in the yolk in significant quantities. They each make up approximately 2% by weight of dry egg yolk [6]. Apovitellenin I is a low molecular weight protein (M_r 9,000) that is present in egg largely as a dimer with a single disulphide cross-link. At neutral pH it is a tetramer [5]. Apovitellenin VI is a large protein of M_r 170,000 [6], now known to be derived from blood apoprotein B [11]. Purified apovitellenins I and VI were tested by both RAST and RAST inhibition for their capacity to bind specific IgE.

Phosvitin, the main phosphoprotein in yolk, is notorious for its lack of antigenicity [12]. Nevertheless, earlier unpublished work in our laboratories had indicated that the phosvitins might also be allergenic. Phosvitin is 10% by weight of total yolk protein [24]. The phosphorus is monoesterified mainly to serine residues which account for over 55% of the total amino acid composition of the protein [7]. The findings of several previous workers have indicated that hen's egg yolk phosvitin is not a homogeneous protein. It has recently been found [25] that in addition to two main phosvitin subfractions several smaller phosphoproteins are present. Using the method of Wallace and Morgan [25], we prepared phosvitin containing four constituents. This mixture was used in RAST and RAST inhibition for determination of its allergenicity.

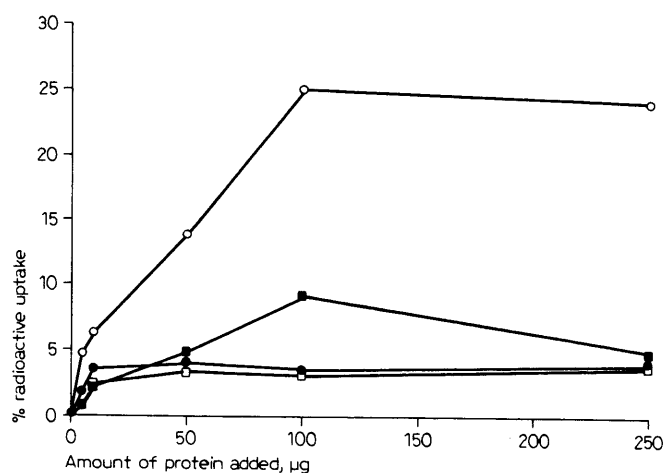


Fig. 1. IgE-binding curves for the four proteins used in the study. Each protein was coupled to discs exposed to increasing amounts of allergen, and RAST was performed using one egg white or egg yolk allergic serum. Sera used were as follows: for ovomucin (■) patient Shr, for apovitellenin I (○) patient Mey, for apovitellenin VI (□) patient Blo, and for phosvitin (●) patient Ste. The optimal allergen concentrations for coupling to the discs was determined from these graphs.

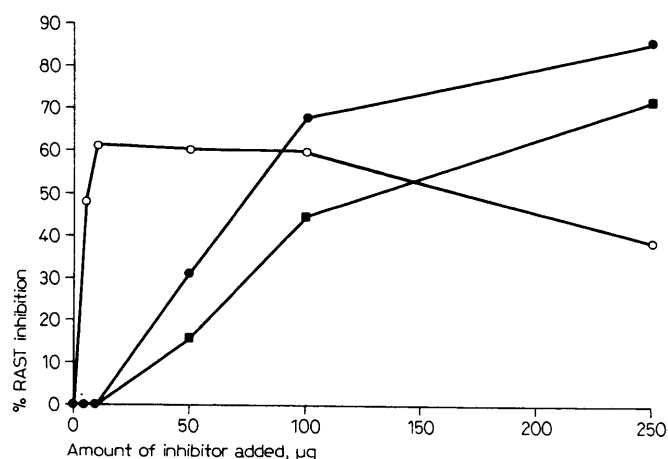


Fig. 2. RAST inhibition curves for three egg proteins. The appropriate serum was preincubated with increasing concentrations of each inhibitor, and then RAST was performed with either egg white discs (for ovomucin) or egg yolk discs (for phosvitin or apovitellenin I). Sera used were as follows: for ovomucin (■) patient Ste, for apovitellenin I (○) patient Mey, and for phosvitin (●) patient Sha. In subsequent experiments the amount of protein which gave 50% inhibition was used.

Materials and Methods

Eggs

Australorp hens kept at the Division of Animal Production, CSIRO, North Ryde, N.S.W., were the source of eggs. The hens were maintained on a commercial mash diet. Unfertilized eggs were used within 2 days of laying.

White and Yolk Protein Fractions

Ovomucin was prepared according to the method of Kato et al. [15]. Homogenized thick egg white (150 ml) was centrifuged at 59,000 g for 60 min to separate thick white into a supernatant and a gel-like precipitate. The supernatant, containing disaggregated ovomucin, was diluted fourfold with 0.05 M barbital buffer (pH 8.6) and applied to a Sepharose 4B column. Fractions were eluted with the diluent buffer. The fraction which appeared in the void volume was designated as disaggregated ovomucin. This preparation was shown to be slightly contaminated with ovalbumin by gel electrophoresis. Ovalbumin was removed by rechromatography in the same fashion. The final yield of ovomucin was 17.4 mg. Gel electrophoresis [9] of the void-volume fractions revealed four bands of high molecular weight ($M_r > 94,000$) corresponding to ovomucin. These were shown electrophoretically not to be contaminated with low molecular weight proteins including ovotransferrin, ovalbumin, ovomucoid, or lysozyme.

Apovitellenins I and VI were prepared according to the methods of Burley and Davies [5] and Burley and Sleight [6], respectively. Phosvitin was isolated by the procedure of Wallace and Morgan [25], except that an enzyme inhibitor was not added and salt was removed by dialysis against distilled water. Our preparation con-

tained no proteins that stained with Coomassie blue (R-250) after gel electrophoresis. Four bands stained with 'Stains-All' (Eastman Kodak, USA).

Preparation of RAST Discs and RAST Procedure

Filter paper discs (Whatman No. 42, 6 mm) were activated by cyanogen bromide [8]. A preliminary experiment was carried out to determine the optimal amount of allergen to which to expose the discs. The amount of phosvitin and apovitellenins I and VI exposed to the discs was 50 µg/disc. Due to the insolubility of the ovomucin complex it was difficult to freeze dry and redissolve in the 0.1 M NaHCO₃ buffer used to bind proteins to cyanogen bromide activated paper discs for RAST analysis. Therefore, ovomucin was bound to nitrocellulose discs (exposure to 52 µg/disc) according to the method of Walsh et al. [26]. The RAST procedure was performed according to the manufacturer's instructions (Kallestad Diagnostics, USA).

RAST Inhibition

The method of Gleich et al. [13], as modified by Barnett et al. [3], was used for the RAST inhibition tests. A preliminary experiment was carried out to determine the optimal amount of allergen with which to inhibit the sera. Sera (50 µl) were incubated with 50 µl RAST buffer (0.9% w/v NaCl and 0.01% w/v Tween 20) containing either 70 µg of phosvitin, 120 µg of ovomucin, or 50 µg of apovitellenin I or VI. After 3 h, the inhibition mixture was incubated with the relevant discs for 3 h. The discs were then washed in RAST wash solution (Pharmacia, Sweden) and incubated with 50 µl of ¹²⁵I-anti-IgE overnight. The discs were again washed as above, and the amount of radiolabelled anti-IgE bound to the discs was estimated by gamma counting.

Table I. Clinical symptoms of some egg-allergic patients whose sera were used in obtaining the results shown in tables II-IV

Patient code	Sex	Age years	Symptoms on ingestion of egg
Goa	M	41	laryngeal oedema, vomiting, nausea
Gow	M	3	eczema
Hor	F	?	eczema
Kas	F	5	angio-oedema, vomiting
McC	M	?	laryngeal/facial oedema, nausea
Mar	F	10	facial oedema, blotchy skin
Mey	M	3	eczema
Roo	F	52	laryngeal oedema

Results

Table I shows the clinical manifestations of egg hypersensitivity for a number of the patients whose sera were used in the testing of the various proteins. The sera used in all experiments were from patients selected on such clinical evidence.

The percent radioactive uptakes for the IgE bound to the four proteins, when increasing amounts were applied to the discs, are shown in figure 1. The data in figure 1 were obtained using sera which in preliminary tests had shown high IgE binding to the particular protein on the disc. Maximum IgE binding to each of the proteins was achieved when 100 µg of either ovomucin or apovitellenin I and 50 µg of either phosvitin or apovitellenin VI were applied to the disc. Because of the saturation encountered when nitrocellulose was exposed to 57.5 µl (containing 100 µg of protein) of ovomucin solution, all subsequent nitrocellulose discs were exposed to only 50 µg. Even though this probably did not result in maximum binding of IgE in all cases, it still gave good RAST radioactive uptakes, indicating that ovomucin does bind to IgE.

In figure 2 results are given showing the ability of increasing amounts of ovomucin to inhibit IgE binding to whole egg white and of apovitellenin I or phosvitin to inhibit IgE binding to whole egg yolk. Again, for each case a serum shown by RAST to have high IgE specific to that protein was used. Fifty percent inhibition was achieved with 120 µg of ovomucin, 70 µg of phosvitin, and 10 µg of apovitellenin I. These amounts were used in the RAST inhibition studies in-

Table II. Percent radioactive uptake in RAST for sera of 8 egg-sensitive patients and 2 controls for whole egg white and ovomucin discs; also shown is the percent inhibition of the binding of serum IgE to whole egg white discs by preincubation of 50 µl of sera with 120 µg of ovomucin

Patient code	Egg white % uptake	Ovomucin	
		% uptake	% inhibition
Gow	24.2	14.0	67.8
Hew	18.4	9.6	15.2
Mat	15.2	8.2	53.3
Rou	24.4	8.6	62.7
Ru1	19.7	7.3	41.1
Shr	19.9	5.2	49.7
Ste	27.1	12.2	62.7
Zek	7.0	5.0	71.4
Positive control ^a	0.7	1.1	not measured
Negative control ^b	0.9	0.5	not measured

^a Serum high total IgE of an individual hypersensitive to house dust mite.

^b Sample of fetal cord serum.

Table III. Percent radioactive uptakes in RAST with the sera of 9 patients for apovitellenin I discs and of 7 patients for apovitellenin VI discs; also shown are the percent radioactive uptakes for whole egg yolk and percent inhibition of the binding of serum IgE to whole egg yolk discs by preincubation with 50 µg of apovitellenin I or VI

Patient code	Egg yolk % uptake	Apovitellenin	
		% uptake	% inhibition
<i>Apovitellenin I</i>			
Bru	4.3	38.5	43.1
Bak	13.7	11.4	53.8
Gil	11.5	9.5	18.9
Hor	7.2	12.1	33.3
Kas	13.0	9.1	49.0
Mey	7.2	5.0	60.5
Nia	8.8	5.8	56.5
Tre	7.0	11.7	11.2
Too	18.3	11.2	59.7
<i>Apovitellenin VI</i>			
Bak	13.7	7.5	72.8
Bel	7.2	9.0	5.6
Blo	33.9	14.0	not measured
Ced	6.0	11.6	7.6
Rao	3.2	13.9	10.0
Tre	7.0	4.2	28.9
Ver	3.2	7.0	19.1

Table IV. Percent radioactive uptake in RAST with the sera of 7 egg-sensitive patients and 2 controls for whole egg yolk and phosvitin discs; also shown is the percent inhibition of the binding of serum IgE to whole egg yolk discs by preincubation of 50 μ l of sera with 70 μ g of phosvitin

Patient code	Egg yolk % uptake	Phosvitin	
		% uptake	% inhibition
Goa	7.7	4.8	67.5
Coo	9.7	7.9	77.3
Roo	19.1	2.1	30.9
McC	24.9	7.0	27.7
Kas	20.8	10.8	18.8
Mar	18.3	6.4	31.1
Sha	41.7	12.2	22.8
Ste	8.5	4.0	not measured
Positive control ^a	0.7	0.6	not measured
Negative control ^b	0.6	0.7	not measured

^a Serum of high total IgE of an individual hypersensitive to house dust mite.

^b Low-IgE sample of fetal cord serum.

volving whole egg white or whole egg yolk. The phenomenon of decreasing RAST inhibition with increasing amounts of apovitellenin I was observed (250- μ g point). This effect has been seen by us before, although we are unaware of a proven explanation for it. However, the effect does not occur until well above the levels of apovitellenin I used for the present RAST inhibition study.

Table II reports the RAST results for sera from egg-allergic individuals tested for ovomucin sensitivity. As can be seen the sera of 6 of the 8 patients were found to have radioactive uptakes for ovomucin of greater than 7%. The RAST results for whole egg white are also given for the sera of the same patients. They all showed high IgE binding to whole egg white discs. Ovomucin inhibited the binding of IgE to whole egg white protein in all cases, although the level of inhibition was serum dependent.

The RAST results for apovitellenins I and VI are given in table III. Both of the proteins were effective in inhibiting binding of egg yolk specific IgE. It appears that for patients Bru, Hor, and Tre apovitellenin I is a major allergen, as the purified protein binds a greater amount of specific IgE than crude egg

yolk itself. In the case of patient Bru, for example, there was a ninefold increase in radioactive uptake for apovitellenin I when compared with that for whole egg yolk. Apovitellenin VI showed similar results for patients Bel, Ced, Rao, and Ver.

Table IV shows the percent radioactive uptakes for binding of IgE specific to phosvitin and whole egg yolk. Although the RAST uptakes for phosvitin were generally lower than those for whole egg yolk or for the apovitellenins (table IV), it still bound significant levels of IgE in the sera of egg yolk allergic individuals. The phosvitin fraction was also effective in inhibiting the binding of egg yolk specific IgE. Again, the degree of inhibition varied from patient to patient.

Discussion

Study of the allergenicity of individual egg white and egg yolk proteins will increase the understanding of allergy to egg. To date only ovotransferrin [18], ovomucoid [19], and ovalbumin [10] have been examined in any detail. Any reports of new egg allergens are of interest, as many infants suffer from hypersensitivity to egg proteins. For example, 46 of 105 atopic children tested by Bock et al. [4] were allergic to egg. Since egg is included in a vast number of recipes, the basic questions of what proteins are involved in egg-allergic reactions and how these proteins are related to one another need to be answered by such studies and will assist in future diagnosis and clinical treatment of hypersensitivity.

Interassay variation is quoted by Kallestad Diagnostics to be between 3 and 8%. In our hands we have observed variations of 2–8% radioactive uptake. This is dependent upon such factors as gamma counter efficiency and the number of times a sample has been frozen and thawed. Such variations between assays resulted in 70 μ g of phosvitin inhibiting of the IgE binding to whole egg yolk in the test for patient Sha (fig. 2), but in the test result for table IV the same serum was only inhibited by 22.8%. Thus RAST and RAST inhibition should be treated as semiquantitative methods. However, the results of the tests for this paper, and our past experience, have always been reproducible within these limits for every serum sample.

The present work is an extension of earlier Western blotting experiments on whole yolk and white [2]. These experiments proved useful in indicating which

proteins bound IgE in the sera of egg-allergic individuals. There was a preliminary indication that ovomucin was an allergen which has been confirmed in the present study. From our RAST results it does not appear to be a major allergen. However, it may be that in the present study we did not couple the optimal amount of protein to the disc. We have subsequently found in a separate survey [unpubl. results] that ovomucin bound IgE in 70% of the sera of egg-allergic children.

Egg yolk can be divided into three distinct fractions: the low-density, the water-soluble, and the granule fraction. Phosvitin is from the granule fraction and apovitellenins I and VI from the low-density fraction. Each of the three yolk proteins bound substantial levels of IgE in the sera of all egg-sensitive patients tested. From our results (table III) it seems that apovitellenins I and VI could be major yolk allergens for some patients. Phosvitin on the other hand does not seem to bind IgE as strongly, nor in as many sera. This is not surprising as phosvitin prepared by older methods is not a good antigen [12]. Thus, the demonstrated allergenicity may be due to the smaller, less phosphorylated forms of the protein present in the preparation. Proteins from the water-soluble fraction of egg yolk have not been examined for allergenicity in this paper.

In conclusion, four purified proteins isolated from hen's egg have been tested separately for the first time by RAST and RAST inhibition, and all were found to bind significant levels of IgE. There is a good correlation between RAST results and the clinical incidence in most cases of egg allergy [1], so for certain patients these proteins are likely to be important allergens.

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