

ORIGINAL ARTICLES

Detection of four distinct groups of hen egg allergens binding IgE in the sera of children with egg allergy

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ABSTRACT

Background: There appears to be a lack of agreement in the literature on the allergenicity of hen egg proteins. This may be partly due to the use of impure proteins in some cases. Egg yolk proteins have also been largely ignored in such studies. We therefore set out to determine, using especially purified proteins, their relative allergenicity, and to observe whether there were any relationships between their potency and the sensitivity of patients to them.

Methods and results: The sera of 40 patients with clinically observed hen egg hypersensitivity were tested for specific IgE binding to purified egg white and egg yolk proteins using the radioallergosorbent test (RAST). Statistical treatment by correspondence analysis of the percent radioactive uptakes in the RAST to the 8 proteins demonstrated that there were four distinct groups of patients reacting in a similar way to four discrete sets of proteins.

Conclusions: The first three sets of allergens consisted of egg white proteins as follows: firstly, lysozyme and ovalbumin; secondly, ovomucoid; and thirdly, ovomucin. The fourth set contained the egg white protein ovotransferrin and the egg yolk proteins apovitellenins I and VI and phosvitin. The exis-

tence of patient groups may explain why various workers have reported different allergens to be important in egg hypersensitivity. A sufficiently large number of patients must be examined so as to give a representative distribution across each group, otherwise the results may be biased towards one allergen.

Key words: Hen egg. Food-allergy. Specific IgE. Allergens. Radioallergosorbent test. Statistical analysis.

RESUMEN

Antecedentes: Parece haber una falta de acuerdo en la literatura de la alergenicidad de las proteínas del huevo de gallina. En algunos casos esto puede deberse en parte al uso de proteínas impuras. Además, en tales estudios las proteínas de la yema del huevo han sido mayormente ignoradas. Por consiguiente nos hemos dispuesto determinar su relativa alergenicidad, usando proteínas especialmente purificadas y observar si había alguna relación entre su potencia y la susceptibilidad de los pacientes hacia ellas.

Métodos y resultados: Los sueros de 40 pacientes con clínicamente observada hipersensibilidad al huevo de gallina fueron examinados por fijación de IgE específica a proteínas purificadas de la clara y de la yema del huevo, utilizando para ello la prueba de radioalergenosorbente (RAST). El tratamiento estadístico por análisis de correspondencia del porcentaje radiactivo obtenido aplicando el RAST a las 8 proteínas demostró que habían cuatro grupos distintos de pacientes que reaccionan de manera similar a cuatro grupos separados de proteínas.

Conclusiones: Los tres primeros grupos de alérgenos consistían de proteínas de la clara del huevo,

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contenían: primero, lisozima y ovoalbúmina; segundo, ovomucoide; y tercero, ovomucina. El cuarto grupo contenía la proteína de la clara ovotransferrina y las proteínas de la yema apovitellenins I y VI y fosfovitina. La existencia de grupos de pacientes puede explicar por qué diversos investigadores han indicado que los diferentes alérgenos son importantes en la hipersensibilidad del huevo. Un número suficientemente grande de pacientes deben ser examinados para poder obtener una distribución representativa en cada grupo, de lo contrario los resultados pueden denotar parcialidad a un alérgeno.

Palabras clave: Huevo de gallina. Hipersensibilidad a los alimentos. Alérgenos. IgE y específicos. Radioalérgenosorbente. Análisis estadístico.

INTRODUCTION

As early as 1912 various proteins of egg white were implicated in egg allergy by both *in vivo* and *in vitro* investigations¹. The first report examined a child with acute egg hypersensitivity in which egg-white and ovomucoid, but not egg yolk, were reported to be active allergens by food challenge. Ovoglobulin and ovomucin were found to have only 25 % of the activity of ovomucoid¹. Miller and Campbell², on the other hand, reported that individuals sensitive to egg white reacted to ovomucoid, ovomucin, ovalbumin and lysozyme by skin test. There was no overall pattern of reaction and they suggested that sensitisation to egg white allergens is dependent upon individual variation rather than the nature of the antigen. Ovomucoid was reported to be the major skin-reactive protein in both raw and cooked egg white by Bleumink and Young³. They observed no reaction to ovalbumin, ovotransferrin and ovoglobulin. However, they did observe a reaction to lysozyme which they attributed to its irritant properties. Earlier investigations of raw and cooked egg are in conflict with this report. Ratner et al⁴ with the aid of passive transfer tests found that ovomucoid-sensitive individuals reacted to hard-boiled eggs while patients allergic to ovalbumin and ovoglobulin tolerated hard-boiled eggs. Conversely, Rhoden and Sutherland⁵ found that patients allergic to raw egg white reacted strongly to ovomucoid, while those sensitive to the heat labile portion of egg white also reacted with ovomucoid.

With the advent of improved immunological assays such as the radioallergosorbent test (RAST) the ability to define which proteins were involved in IgE-mediated egg allergy was greatly improved.

Hoffman⁶ reported that 25 out of 27 patients displaying symptoms on ingestion of egg gave positive RAST results. Three patients with strong anaphylactoid reactions to egg showed positive RAST tests to ovalbumin and ovomucoid. Both allergens may, however, have been slightly cross-contaminated. A later study by the same author⁷ using higher purity egg proteins indicated that ovalbumin, ovomucoid and ovotransferrin were important allergens, whereas lysozyme was only a weak allergen. Ovalbumin and ovomucoid and small amounts of ovotransferrin were detected in both hard- and soft-boiled eggs by radioimmuno-electrophoresis.

At this time Langeland⁸ and Langeland and Harbitz⁹ reported that for a group of egg-allergic children the most important allergens detected by crossed-radioimmuno-electrophoresis were ovomucoid, ovalbumin and ovotransferrin. Lysozyme was not detected as an allergen in this way. However, Holen and Elsayed¹⁰ found that lysozyme bound strongly to IgE in all of the sera of egg-allergic individuals that they studied, and concluded that lysozyme was one of the major allergens of egg white.

Egg yolk has largely been ignored by researchers, as early reports indicated that it was not allergenic¹¹. However Anet et al¹² recorded positive RAST scores for egg yolk in the sera of 36 egg-allergic patients. They also found that lysozyme bound specific IgE in the sera of four egg-sensitive patients. We have also recently reported IgE binding to several yolk proteins^{13, 14}. Some egg allergen fractions may be expected to give a high incidence of false positives *in vivo* due to their irritant property (e.g., lysozyme), especially for patients with atopic eczema. Often test procedures and materials were not well defined in the early literature. However these last two factors may not account for the wealth of conflicting reports of allergenicity. Therefore we have investigated binding of specific IgE in the sera of 40 egg-sensitive children to 8 purified egg white and yolk proteins by RAST in an effort to determine which of them are major allergens. The proteins were purified by high performance liquid chromatography (HPLC) where necessary to ensure purity. The results appear to explain the conflicting nature of reports on the allergenicity of egg proteins.

MATERIALS AND METHODS

Sera of egg-allergic patients

Sera were obtained from patients who were referred to the Allergy and Clinical Immunology Unit of the Royal Childrens Hospital, Melbourne, for evalua-

tion of suspected hypersensitivity reactions to egg. At the first assessment a detailed clinical history was taken. Where the reaction was not life-threatening and of doubtful significance, or had occurred more than 12 months prior to referral, a home challenge of soft-boiled egg was undertaken to confirm clinical evidence of egg hypersensitivity. Where there was unequivocal clinical evidence of persisting egg hypersensitivity, patients attended the Allergy and Clinical Immunology Unit. Here skin tests to common allergens (including egg) were performed and blood samples taken for estimation of total immunoglobulin levels and allergen-specific IgE antibodies. Patients used in this study were selected for both clinical sensitivity to egg and a 3 + or 4 + Phadebas RAST score to Pharmacia egg white discs.

Allergens

Ovalbumin was prepared by three times recrystallisation of egg white from an ammonium sulphate solution. The solution of impure ovalbumin was then chromatographed on a DEAE-Sephacel column equilibrated with 0.05 M Tris-HCl buffer (pH 8.0) containing 0.05 M sodium chloride. Ovalbumin was eluted from the column using a linear gradient of 0 to 1 M sodium chloride in 0.05 M Tris-HCl, pH 8.0. Lysozyme was prepared by the method of Alderton and Fevold¹⁵. The crystals were then further purified by chromatography on a DEAE-Sephacel column equilibrated with 0.05 M Tris-HCl buffer (pH 8.0) containing 0.05 M sodium chloride. Lysozyme was eluted from the column by a linear gradient of 0 to 2 M sodium chloride in 0.05 M Tris-HCl, pH 8.0.

Ovotransferrin (98 % pure) was purchased from Sigma Chemical Co, Missouri, USA. It was purified by ion exchange HPLC on a Brownlee X03-MP aquapore cation column. The sample, dissolved in 0.015 M NH_4HCO_3 (pH 4.7), was injected and then eluted with the same buffer until all unbound material was removed. The ovotransferrin was then eluted with a linear gradient of 0.015 (pH 4.7) to 0.35 M (pH 6.5) NH_4HCO_3 . The material thus eluted was finally purified by HPLC on a Waters Associates reversed phase C18 microbondapak column using a 30 to 70 % linear gradient of acetonitrile in water. Ovomucoid was prepared according to the method of Kurisaki et al¹⁶.

Ovomucin was prepared by the procedure of Kato et al¹⁷. Homogenised thick egg white (150 mL) was centrifuged at 59000 g for 60 minutes 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 denoted disaggregated ovomucin. This preparation was shown to be slightly contaminated with ovalbumin by gel electrophoresis. Ovalbumin was removed by rechromatography in the same fashion. Gel electrophoresis¹⁸ of the void volume fractions revealed 4 bands of high molecular mass ($M_r > 94000$) corresponding to ovomucin containing various amounts of carbohydrate. These were shown electrophoretically not to be contaminated with low molecular mass proteins including ovotransferrin, ovalbumin, ovomucoid or lysozyme.

Apovitellenins I and VI were prepared according to the methods of Burley and Davies¹⁹ and Burley and Sleight²⁰, respectively. Phosvitin was isolated by a modification of the procedure of Wallace and Morgan²¹ in which the enzyme inhibitor was omitted and salt was removed by dialysis against distilled water. The purity of each of the above allergens was determined by SDS gel electrophoresis on a 10 % T gel¹⁸. Every allergen gave a single band in this system except ovomucin, which has multiple glycosylated forms and appeared as several bands. The ovomucin preparation was shown to be free from contamination by other proteins¹⁴.

Preparation of RAST discs and RAST procedure

Filter paper discs (6 mm) were activated with cyanogens bromide by the methods of Ceska et al²². Since apovitellenin I and ovomucin are poorly soluble in the NH_4HCO_3 buffer used for coupling of allergens to discs, any experiments using these proteins were done utilising nitrocellulose as the solid phase²³. The optimal amount of each protein to be coupled was determined by constructing a binding curve in which increasing amounts of the allergen was added to CNBr-activated paper or nitrocellulose discs^{13,14}. RAST was then performed using these discs and one serum sample with high levels of IgE specific to the particular protein. Exposure to a standard amount of 50 µg of allergen/disc was employed for each of the allergens. The RAST procedure followed the manufacturer's instructions (Kallestad, USA).

Skin tests

The size of a test weal produced by an egg white allergen extract (Dome Hollister Steir, Spokane, USA, 1:20 w/v) was compared with that from histamine acid phosphate (1 mg/mL) and from a control. Skin

Table I
Nomenclature of skin test results measured relative to a histamine standard

Skin Test Result (area of reaction to egg)		Area of reaction to egg as multiple or fraction of histamine standard
> 4	equals	> 2 times the area of histamine standard
4	equals	2
3	equals	1
2	equals	0.5
1	equals	0.25 or any reaction greater than control solution

prick tests were performed by a single investigator by placing 1 drop of the extract, control solution and histamine on the patient's back. The point of a blood lance needle was passed through the drop to indent the skin but not to cause bleeding. The size of the skin weal elicited by the egg white extract was compared with that those of the histamine and the control solutions. The area of weal and pseudopodia elicited by the histamine was designated as + 3. Reactions to egg were graded as shown in table I. The weal produced in skin prick test of the manufacturer's control solution was deducted from that of the egg allergen before the score test was measured. Skin tests were carried out at the same time as blood was taken for immunological studies.

Statistical analysis

The RAST results were tabulated for the 40 patients in a random order (achieved by testing the sera in the sequence that they were received at the clinic). Since no analysis has been done in the past to establish any pattern of allergenicity for each individual protein, a correspondence analysis (Greenacre ²⁴) was performed in order to explore the possibility of any interrelationships. Correspondence analysis is a graphical technique for simultaneously representing the rows and columns of a data matrix in such a way as to identify the structural relationships between the results in the rows and columns. To analyse the data it was tabulated into rows representing the 40 children and columns representing the percent radioactive uptake in RAST to the 8 proteins.

Correspondence analysis was carried out and showed the data as two sets of points (fig. 1), one representing the patients (rows) and the other standing for the proteins (columns), on a joint map. Each point corresponding to a patient can be considered a display of the complete reactivity profile for that pa-

tient. The information has been condensed into a single point. Furthermore, the distance between the points for the patients is a measure of similarity between the RAST scores for their sera with the allergens. Thus in fig. 1, the points for the patients "caru" and "camp" are far from each other because their RAST scores for the 8 proteins are different, while the point for patient "sutt" is close to that of "slee" because their profiles are similar across the RAST scores for the 8 allergens.

The points depicting the allergens were interpreted in much the same way as correspondence analysis treats the rows and columns in a matrix. Thus each allergen point portrays the profile of RAST scores for that allergen across the set of 40 patients. So it is apparent from fig. 1 that the profile across the patients for apovitellenin VI is very different from that of lysozyme. The relative positions of the two sets of points are interpreted in a special way in correspondence analysis. Each patient point will lie more or less in the region of the allergen score in which the patient's profile is prominent. Consequently the point for child "shir" is near that of lysozyme because this patient has a relatively high RAST score for lysozyme while having comparatively low scores for the other proteins. In this way groupings of patients and allergens were determined.

RESULTS

Table II gives some clinical details for the group of patients used in this study. The symptoms observed upon ingestion of egg varied, and there is no distinct pattern of reactivity amongst the groups listed. Patient numbers 41 and 42 correspond to foetal cord serum and a housedust mite allergic serum respectively, and were used as controls for non-specific binding. For ease of comparison with figure 1, table II has been arranged according to the groups defined by the correspondence analysis. Figure 2 shows for all of the patients the percent radioactive uptakes for the purified egg white and egg yolk proteins. The results have been ordered in the groups determined by the outcomes of the correspondence analysis.

The correspondence analysis indicated, firstly, that the allergens were related in their IgE binding across the 40 patients according to the following sets: set 1, the egg white proteins (EWP) lysozyme and ovalbumin; set 2, ovomucoid (EWP); set 3, ovomucin (EWP); and set 4, ovotransferrin (EWP) and the egg yolk proteins (EYP) apovitellenins I and VI and phosvitin. Secondly, the analysis clearly divided the patients into four groups. Group A is comprised of patients numbers 1 to 9 whose sera reacted most

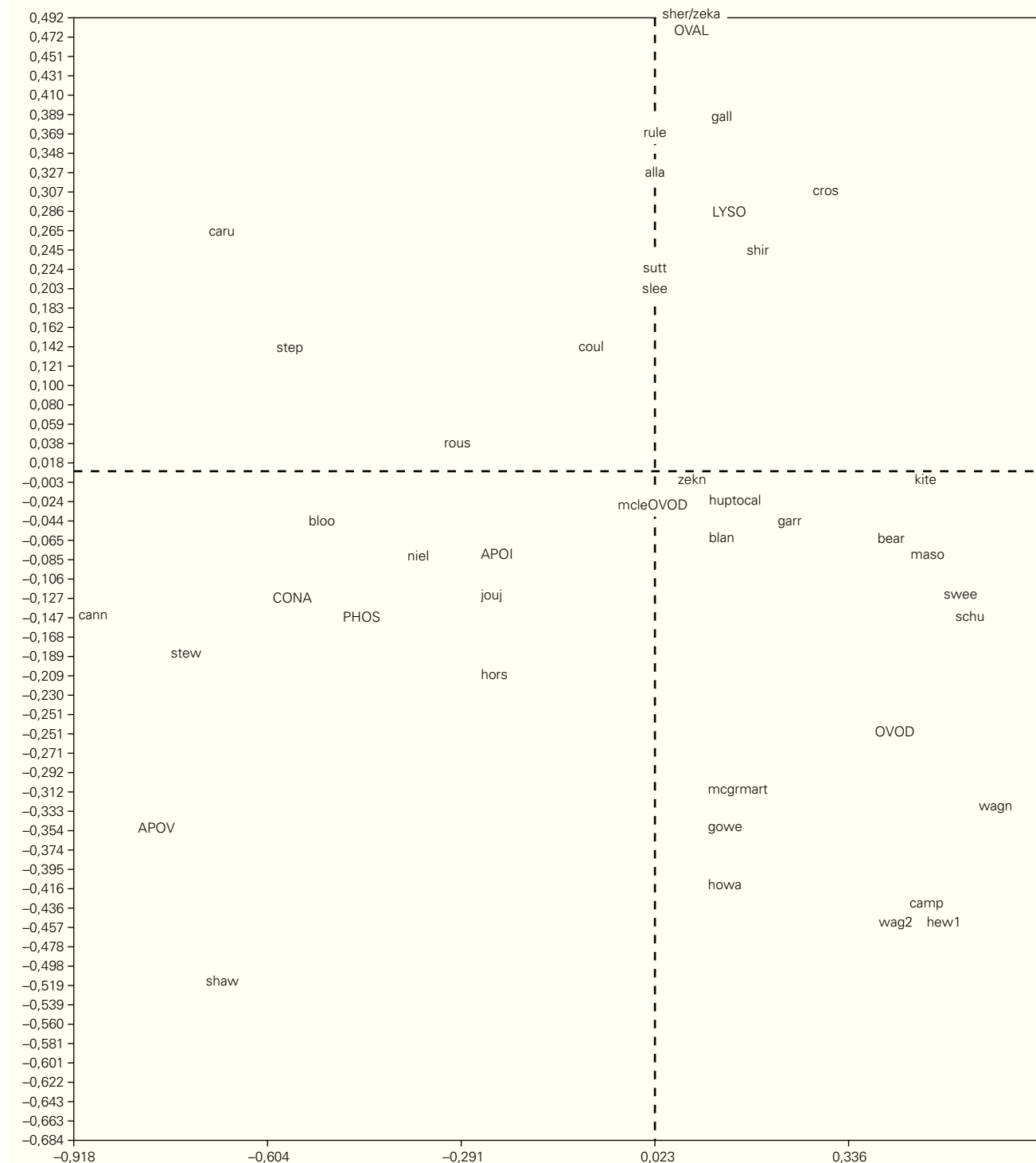


Figure 1.—Correspondence analysis diagram showing each patient point and each allergen point as a four letter code. Abbreviations are as follows: OVAL – ovalbumin, LYSO – lysozyme, OVON – ovomucin, OVOD – ovomucoid, APOI – apovitellenin I, APOV – apovitellenin VI, PHOS – phosvitin, and CONA – ovotransferrin. See Materials and Methods for explanation of diagram.

similarly to ovalbumin and lysozyme (EWP). The sera of this group generally did not display IgE binding to Set 4 allergens. Group B consists of patients 10 to 22 and their sera reacted most similarly to ovomu-

coid (EWP). Their sera generally showed little or no IgE binding to any of the other allergens.

Group C is made up of patients 23 to 30. Their sera reacted most similarly to ovomucin (EWP). Their

Table II
Clinical history of atopic patients

Patient no.	Eczema	Asthma	*Clinical symptoms upon egg ingestion	**Skin test score
Grupo A				
1	+	+	AEU	4
2	+	+	A	N.R.
3	+	+	E	4
4	+	+	N.R.	4
5	–	N.R.	VS	4
6	–	N.R.	U	4
7	–	N.R.	A	4
8	N.R.	N.R.	N.R.	N.R.
9	–	+	V	4
Grupo B				
10	–	+	AS	4
11	+	–	E	4
12	+	+	E	N.R.
13	–	+	U	4
14	+	–	E	N.R.
15	+	+	N.R.	N.R.
16	+	–	ED	4
17	+	+	E	3
18	+	–	ED	4
19	–	+	AUO	4
20	+	–	V	4
21	N.R.	N.R.	N.R.	N.R.
Grupo C				
22	+	–	E	4
23	+	+	V	4
24	N.R.	N.R.	N.R.	N.R.
25	+	+	U	4
26	+	+	UO	4
27	–	+	U	4
28	+	+	E	4
29	+	+	U	N.R.
30	+	+	U	N.R.
31	–	+	D	4
Grupo D				
32	–	+	V	4
33	–	+	V	N.R.
34	+	+	E	4
35	+	+	E	N.R.
36	N.R.	N.R.	N.R.	N.R.
37	+	+	VO	4
38	+	+	E	N.R.
39	+	+	N.R.	N.R.
40	–	+	V	4

*Asthma (A), eczema (E), urticaria (U), vomit (V), stridor (S), diarrhea (D), and angioedema (O).

**Skin test score related to histamine standard (see Materials and Methods for explanation).

+ equals positive diagnosis; – equals negative diagnosis, and N.R. equals not recorded.

sera generally showed IgE binding to most of the egg white proteins, but the sera of all members of the group had higher radioactive uptakes for ovomucin than for the other sets of allergens. The sera of this group did not show significant IgE binding to any egg yolk proteins. Group D contains patients 31 to 40. Their sera reacted most similarly to the set 4 proteins. Their sera generally gave high IgE binding to ovalbumin and ovomucoid. Also the sera of all members of the group had notably high RAST scores with ovotransferrin. This group was the only one whose sera showed significant IgE binding to yolk allergens.

DISCUSSION

The results in figure 1 indicate that ovomucoid, ovalbumin and ovotransferrin are indeed important allergens for many individuals, at least by an *in vitro* test. This is in accord with earlier findings^{3,7,8,9}. However, our results clearly show that there are a number of other allergens important for various groups of patients. Both lysozyme and ovomucin bound significant amounts of IgE in the sera of patient groups A and C. Lysozyme was statistically in a set with ovalbumin and was a significant allergen for Group A. This is consistent with the findings of Miller and Campbell² and Anet et al¹², but in disagreement with the report of Langeland and Harbitz⁹. Although the yolk allergens tested generally did not bind as much IgE as did the egg white proteins, IgE in the sera of Group D did react with the former. Interestingly, IgE in the sera of all members of this group also showed a relatively high level of binding to ovotransferrin. This agrees with the results of Langeland⁸ who found that there was allergenic cross-reaction between ovotransferrin and whole egg yolk. Whether all three of the yolk proteins tested cross-react or only one or two of them is not revealed by our results.

The allergens that are grouped together by our results, that is, lysozyme and ovalbumin (set 1) and ovotransferrin, apovitellenins I and VI and phosvitin (Set 4) may have common allergenic determinants. Alternatively, a person may be genetically predisposed to produce IgE antibodies to one group of egg proteins or another but not to all of the groups. We are led to return to the suggestion of Miller and Campbell² that sensitization to egg allergens is dependent upon variation in patients but not upon the nature of the allergens. This would explain our results leading to identification of groups of patients clearly defined by their IgE specificity, although the clinical symptoms that are displayed by members of the groups were not significantly different from each

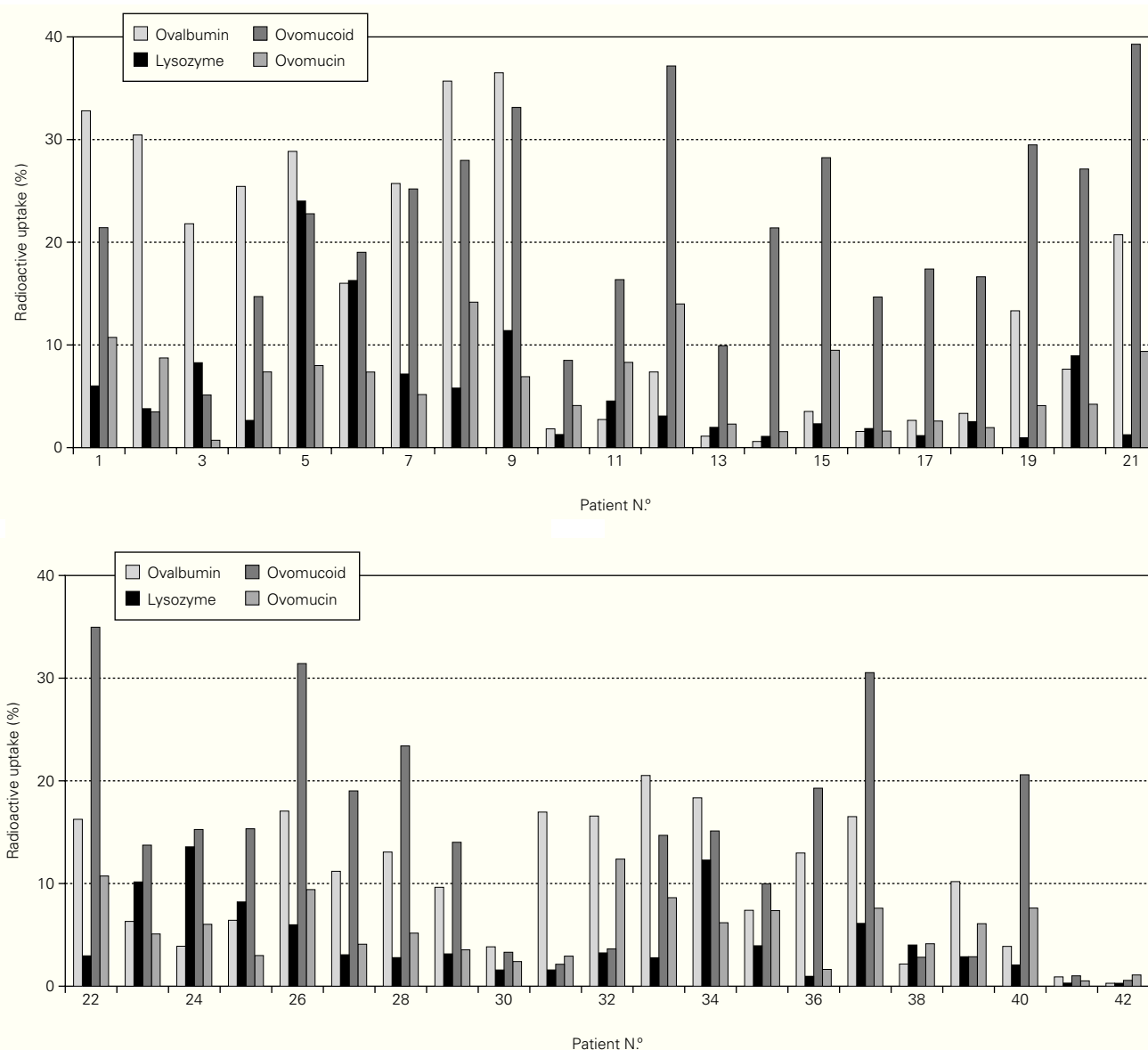


Figure 2.—RAST results, shown as percent radioactive uptake, for the egg white and yolk proteins on discs reacting with IgE in the sera of 40 egg-sensitive children. Results have been drawn according to the groups in which they were placed by the correspondence analysis (see Materials and Methods). The results for two control sera are also shown. These sera were foetal cord serum (no. 41, low IgE) and a serum with high non-egg specific IgE (no. 42).

other. It may be that some other, as yet unidentified, clinical indicator would help to differentiate between the groups. This possibility should be examined.

The existence of such patient groups may explain why various workers have reported different allergens to be important in egg hypersensitivity. For example, those examining only ovalbumin and ovomucoid would miss two groups of patients whose sera contain IgE directed against other egg proteins. Also a sufficiently large number of patients must be examined so as to provide a representative distribution across each group, otherwise the results may be bi-

ased towards one or more allergens. Finally it would appear that although some egg allergens are less stable to heat than others, in cooked egg they are still capable of generating significant levels of specific IgE.

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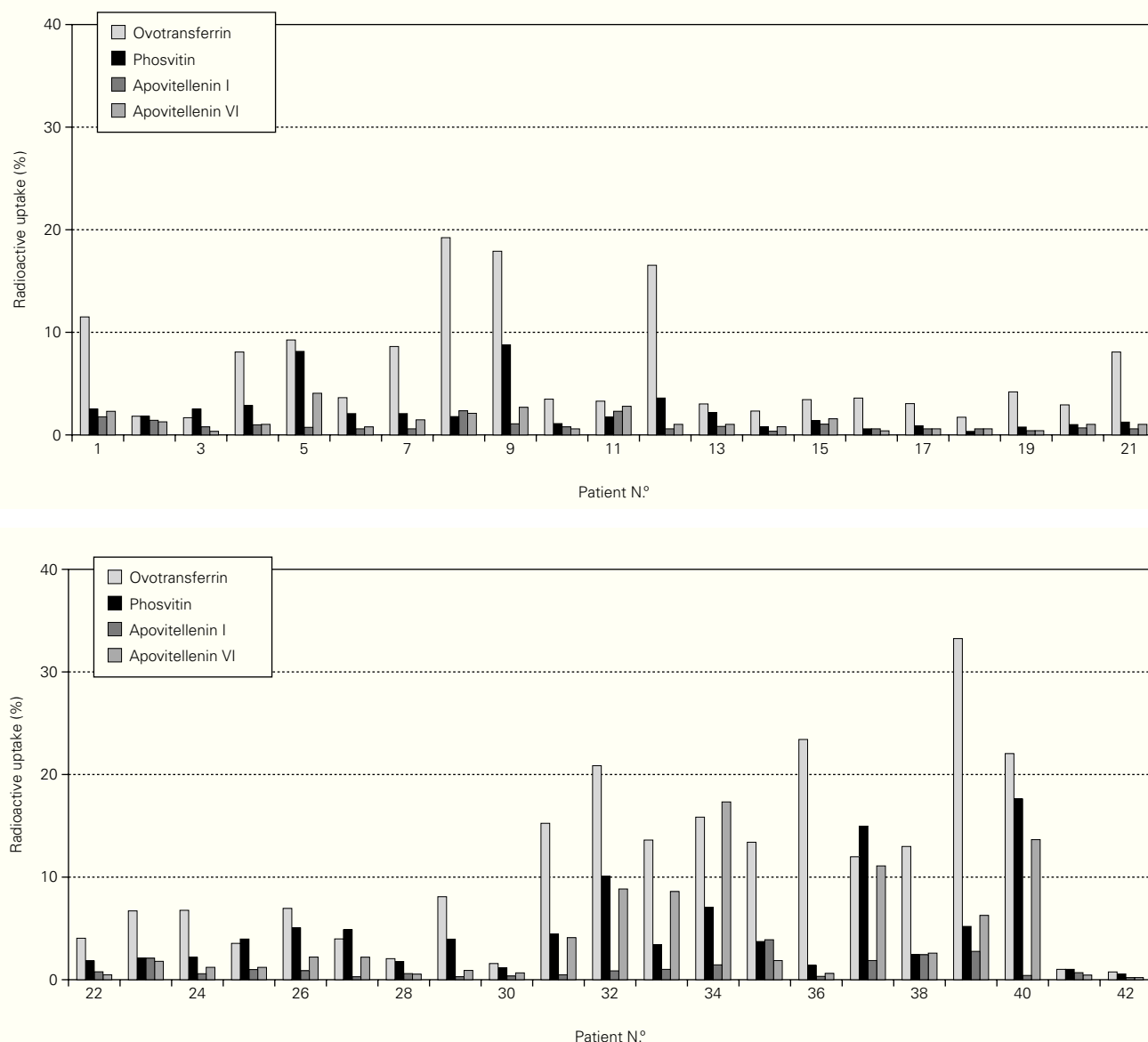


Figure 2.—2nd parts. Egg Yolk Protein (EYP).

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