

A Clinical and Immunological Study of Allergy to Hen's Egg White

III. Allergens in Hen's Egg White Studied by Crossed Radio-Immunoelectrophoresis (CRIE)

TOR LANGE LAND

Dept. of Dermatology and Children's Asthma and Allergy Institute Voksentoppen,
University Hospital, Rikshospitalet, Oslo, Norway

Allergens in hen's egg white were studied in crossed radio-immunoelectrophoresis (CRIE). Specific IgE-antibodies against different proteins in the egg white were examined in sera from 70 atopic patients with clinical hypersensitivity, and RAST ≥ 2 to egg white. Ovalbumin, ovomucoid and an unidentified protein, antigen 22, were classified as major allergens. Specific IgE-antibodies against 10 more proteins in hen's egg white were detected. IgE-antibodies against lysozyme could not be detected.

Key words: crossed radio-immunoelectrophoresis; food allergens; hen's egg white.

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Egg white is a common food allergen for atopic individuals, and may provoke various intermediate-type reactions.

Knowledge about the allergens in hen's egg white is essential for studies of egg allergy. Several authors have studied this matter before (5, 6, 7, 11, 13, 15). As early as 1913 Schloss published a pioneer work in this field, suggesting that ovomucoid was the main protein causing hypersensitivity in his egg-allergic patient (13). Bleumink & Young (5, 6), approximately 60 years later, confirmed that ovomucoid seemed to be the most important allergen, whereas ovalbumin and lysozyme appeared to be less important.

The development of quantitative immunoelectrophoretic methods, such as crossed immunoelectrophoresis (CIE) and crossed radio-immunoelectrophoresis (CRIE), has provided good tools for further studies of allergens in complex protein samples, such as egg white.

In a previous study a CIE-reference precipitation pattern for egg white was characterized (9). The present paper reports the results of

studies of the allergens in hen's egg white by means of CRIE.

MATERIAL AND METHODS

Material

Antigens. A reference hen's egg white antigen solution was prepared, previously described in more detail (9), by stirring crude egg white with an equal volume of physiological saline for 4 h. After centrifugation at 2500 r.p.m. for 30 min the supernatant was stored at -20°C in 1 ml aliquots.

Ovomucoid and lysozyme (cat. Nos. T2011 and L6875, respectively) were obtained from Sigma Chemical Co., Missouri, U.S.A. Ovalbumin was purified using ion exchange chromatography as described by Rhodes (12)*. These preparations have been characterized before

* This preparation was kindly supplied by B. Egelandstad, Norwegian Food Research Institute, Ås, Norway.

(9). For use in skin prick test the ovalbumin preparation was dialysed against physiological saline, freeze-dried and then dissolved in physiological saline to a total protein concentration of 10 mg/ml.

Antiserum. A reference anti-hen's egg white antiserum was prepared by immunizing 10 rabbits as previously described (9).

Patient sera. Sera were obtained from 70 egg-allergic patients, aged 1–15 years, and stored at -20°C until used. All patients had a history of intolerance to egg and positive RAST (≥ 2) to egg white. Reactions to egg white reported were of the immediate type with one or more of the following manifestations: exacerbation of atopic dermatitis, bronchial asthma, nasal and conjunctival reactions, urticaria and angioedema and vomiting. Most of the reactions were provoked by ingestion of egg and egg products, but in some patients egg white acted as an allergen source by inhalation as well. Further clinical details are described elsewhere (8).

Five control sera were obtained from patients with atopic dermatitis with egg tolerance and RAST = 0 to egg white. Total IgE-levels in these sera varied from < 10 to > 4000 kU/l.

RAST-paper discs coupled with egg white proteins were delivered from Pharmacia, Uppsala, Sweden.

CNBr-activated paper discs were kindly supplied by NYCO A/S, Oslo, Norway. The discs had been activated according to Yman et al. (17).

CNBr-activated Sepharose® 4B was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.

Methods

CIE was performed as described before (9), using five different gels on each glass slide (5×7 cm²), including two first dimensional gels ($1 \times 5 \times 0.16$ cm³), designated I and V as shown in Fig. 1. Ten μl of the reference antigen solution described above, diluted in physiological saline to a total protein concentration of 20

mg/ml, was applied in the well of each of the first dimensional gels. To the well of the cathodic first dimensional gel (gel V in Fig. 1b) was added 70 μg lysozyme (dissolved in 0.15 mol/l NaCl). The antibody-containing gel ($3 \times 5 \times 0.13$ cm³) (gel III in Fig. 1b) contained 13 μl reference antiserum/cm². Between the antibody-containing gel (III) and the first dimensional gels, there were intermediate gels ($1 \times 5 \times 0.15$ cm³) containing no antibodies.

The first and second dimensional electrophoreses were run for 35 min at 10 V cm^{-1} and for 19 h at 2 V cm^{-1} , respectively, both at 20°C .

After finishing the second dimensional electrophoresis the glass slides were washed in physiological saline four times for 15 min. After each washing the gels were pressed (10) and following the final pressing, dried by cold air. The CIE slides were stored at $+5^{\circ}\text{C}$ for periods varying from 0–14 days, until used in CRIE experiments.

CRIE was performed according to Weeke & Løwenstein (16) incubating CIE slides (18 h at 20°C) with single sera from egg allergic patients, and with control sera and physiological saline. The incubation was performed in plastic vessels ($7.5 \times 5.5 \times 0.5$ cm³) with 6.6 ml RAST buffer (Pharmacia, Sweden) and 0.4 ml serum or saline. After washing (7 ml RAST washing solution 4 times for 30 min) each slide was incubated with ^{125}I -anti-IgE (RAST isotope, Pharmacia, Sweden) diluted in RAST-buffer to 250,000 c.p.m. in 7 ml total volume for 18 h at 20°C . Subsequently, the slides were washed, dried and placed face down on X-ray film for autoradiography.

Autoradiography

The autoradiography was carried out six times with each glass slide, varying the duration of the exposure (from 12 h to 32 days) for semi-quantification of the radiostaining. According to the CRIE reference system, described by Aukrust & Aas (1), a standard series of paper discs containing varying, defined amounts of ^{125}I -anti-IgE were laid out for autoradiography together with the CRIE slides. After the final

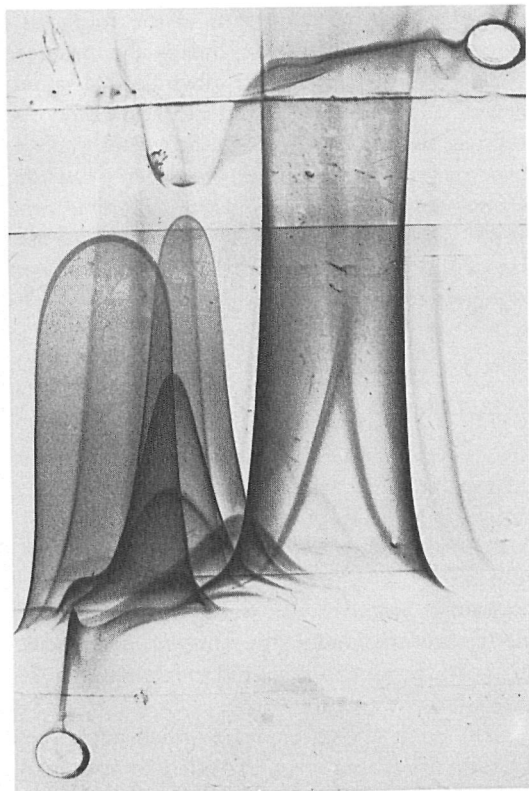


Fig. 1a. CIE reference precipitation pattern of hen's egg white.

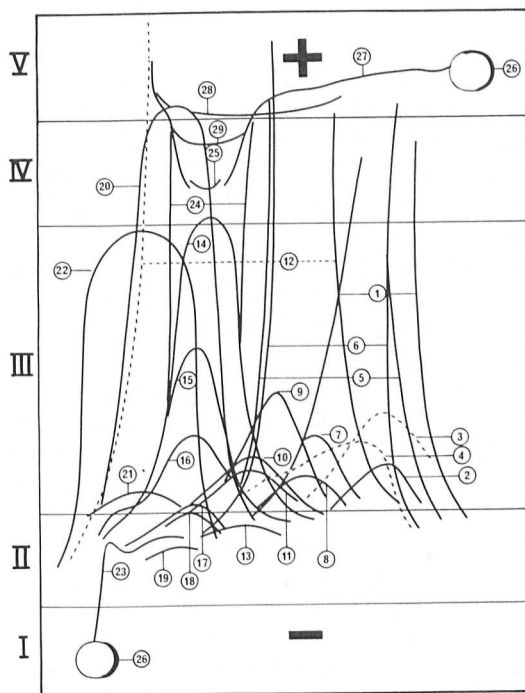


Fig. 1b. Drawing of the CIE reference precipitation pattern of hen's egg white. Precipitates drawn with dotted lines are detectable in CRIE only, using selected patient sera. Gels I and V: first dimensional gels, gels II and IV: intermediate gels, gel III: antibody-containing gel.

autoradiography period, the glass slides were stained with Coomassie brilliant blue[®], and the precipitates, appearing as radiostaining in CRIE, were identified and numbered according to the CIE reference pattern for hen's egg white (Fig. 1). Each precipitate, giving rise to radiostaining in CRIE, was assigned to a CRIE class (A–F, A being the strongest), depending on the number of paper discs visible when the precipitate was detected for the first time. As control, CRIE experiments were also performed with physiological saline replacing serum, and with control sera.

The different allergens detected in CRIE were classified as major, intermediate and minor allergens according to the following criteria, outlined by Aukrust & Borch (3). *Major*

allergens: At least 25% of the relevant patient sera must give strong IgE-binding (CRIE classes A–D), and at least 50% of all sera must show at least some specific IgE-binding (CRIE classes A–F). *Intermediate allergens*: Allergens not classified as major or minor allergens. *Minor allergens*: Less than 10% of the patients' sera must give rise to IgE-binding in CRIE classes A–F.

CRIE experiments were also performed with CIE slides lacking the ovomucoid-precipitate. In these experiments three CIE slides were incubated with three different serum pools, each consisting of equal parts of serum from four different patients, all of them giving radiostaining of precipitate No. 13 as well as No. 12 in ordinary CRIE experiments.

Paper radio-immunosorbent test (PRIST) was performed to determine the total IgE-level in the different sera by use of PRIST kits according to the instructions of the producer.

Radio-allergosorbent test (RAST). Specific IgE-antibodies against egg white in patient sera were determined with RAST using Phadebas egg white discs, F1, (Pharmacia, Sweden). Twenty sera were also analysed in RAST with egg white reference antigen solution (diluted 1:10 in 0.1 mol/l NaHCO_2) coupled to discs according to Yman et al. (17). This was to control the quality of the paper discs used in RAST to rabbit serum proteins.

All sera from egg allergic patients were analysed in RAST with rabbit serum proteins (rabbit serum diluted 1:10 in 0.1 mol/l NaHCO_3) coupled to discs (17). Sera determined to be in RAST class 2 or more to rabbit serum proteins were analysed a second time, this time also incubating discs prepared with human serum proteins (human serum with total IgE < 10 kU/l, diluted 1:10 in 0.1 mol/l NaHCO_3), to determine the amount of unspecific IgE-binding.

RAST assays were performed according to general practice (9). Results were expressed in per cent uptake of total amount isotope applied, and in RAST classes on the basis of a RAST reference kit.

Ovomucoid affinity chromatography. Ten mg of ovomucoid was coupled to 1 g of CNBr-activated Sepharose 4b according to the instructions of the producer. After washing and reacting of remaining active groups as described by the producer, 3 ml of the reference antiserum was incubated with 3 ml of the gel for 18 h at 20°C. The antiserum was then pipetted off and used as antiserum in CIE and CRIE experiments.

Skin prick test (SPT)

SPT was performed in eight patients with egg allergy, and as control, in 15 patients with atopic dermatitis and egg tolerance. The antigens used were ovalbumin and the reference antigen solution. In all cases SPT were per-

formed on the volar surface of the forearms, using histamine-HCl 1 mg/ml as the positive (+++) reference. SPT with physiological saline served as negative (-) control. The reactions were recorded as +++ when the wheals were of same size as the histamine reference, ++ if the wheals were half the size of the histamine reference, and ++++ if the wheals were twice the size of the positive reference. Reactions of ++ or more were considered positive.

RESULTS

CIE. The CIE-reference precipitation pattern is demonstrated in Fig. 1. The precipitates corresponding to ovalbumin and ovomucoid have been identified as precipitates Nos. 6 and 12, respectively, in a previous study (9). Lysozyme, migrating towards the cathode during the electrophoresis under the present conditions, has been shown to give rise to precipitate No. 24 in the same study (9).

CIE, using antiserum from which antibodies against ovomucoid were extracted by means of affinity chromatography, demonstrated the same precipitation pattern as the CIE reference pattern, the only difference being the lack of precipitate No. 12.

CRIE. Some typical examples of exposed radiographic films are shown in Fig. 3. The radiostaining corresponding to various immuno-precipitates is presented in an allergogram (Fig. 2). The degree of radiostaining of the different precipitates is recorded in CRIE classes by means of the given reference system (1).

Specific radiostaining corresponding to 13 different precipitates was detected. The different allergens were classified as major, intermediate and minor allergens (Table 1).

Radiostaining due to non-specific binding of IgE or ^{125}I -anti-IgE, was excluded, using control sera and physiological saline as substitute for allergic patient serum in the first incubation, which gave no radiostaining in CRIE class F.

CRIE with CIE slides lacking the ovomucoid precipitate (prec. No. 12), demonstrated radiostaining of precipitate No. 13 in all slides.

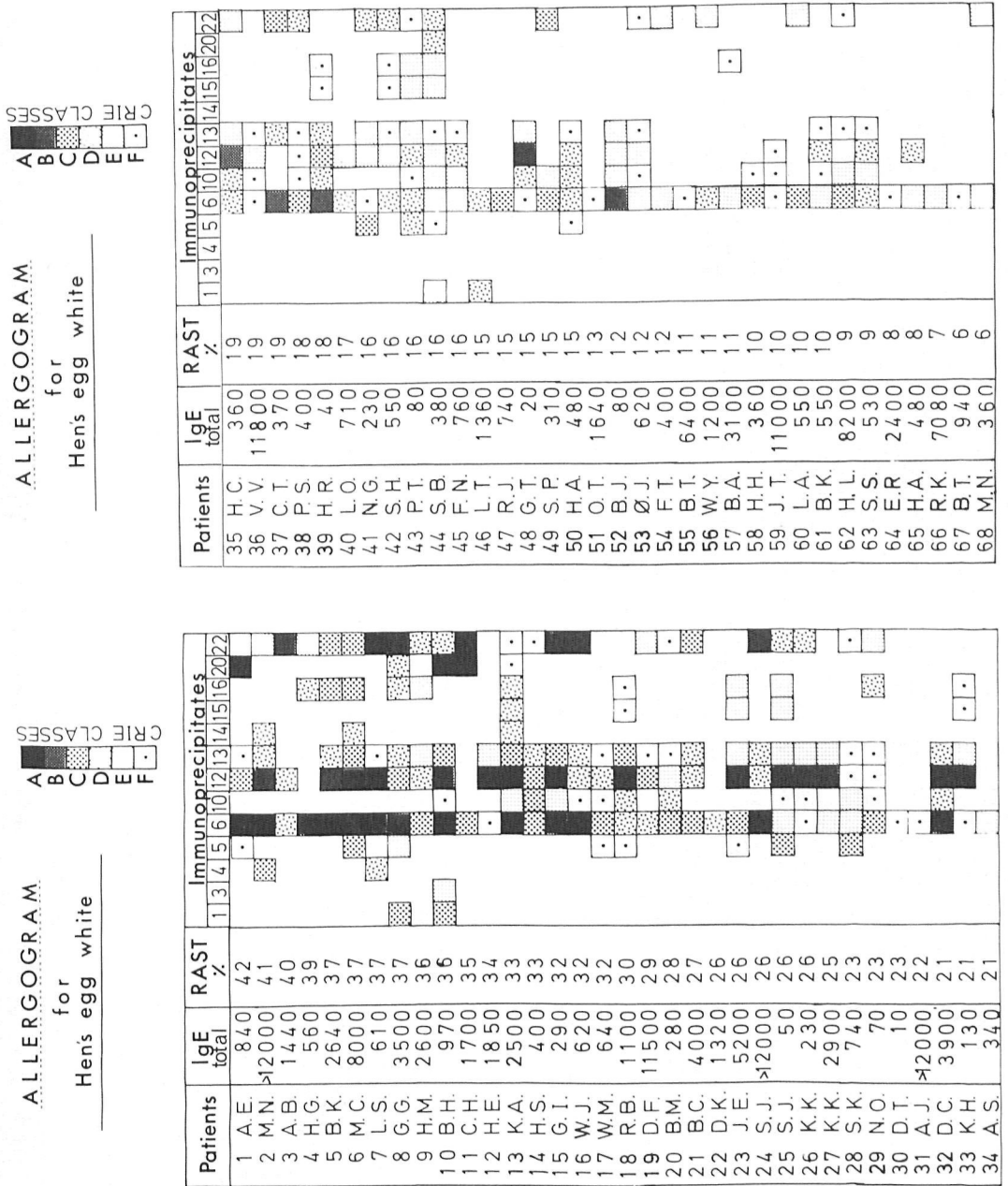


Fig. 2. Patient sera arranged according to decreasing RAST values, expressed in per cent uptake of total amount isotope applied. Total IgE-levels are expressed in kU/l. The CRIE result of each serum corresponding to various immunoprecipitates is designated by varying the darkness of the respective square from black (CRIE class A) to white with a single dot (CRIE class F).

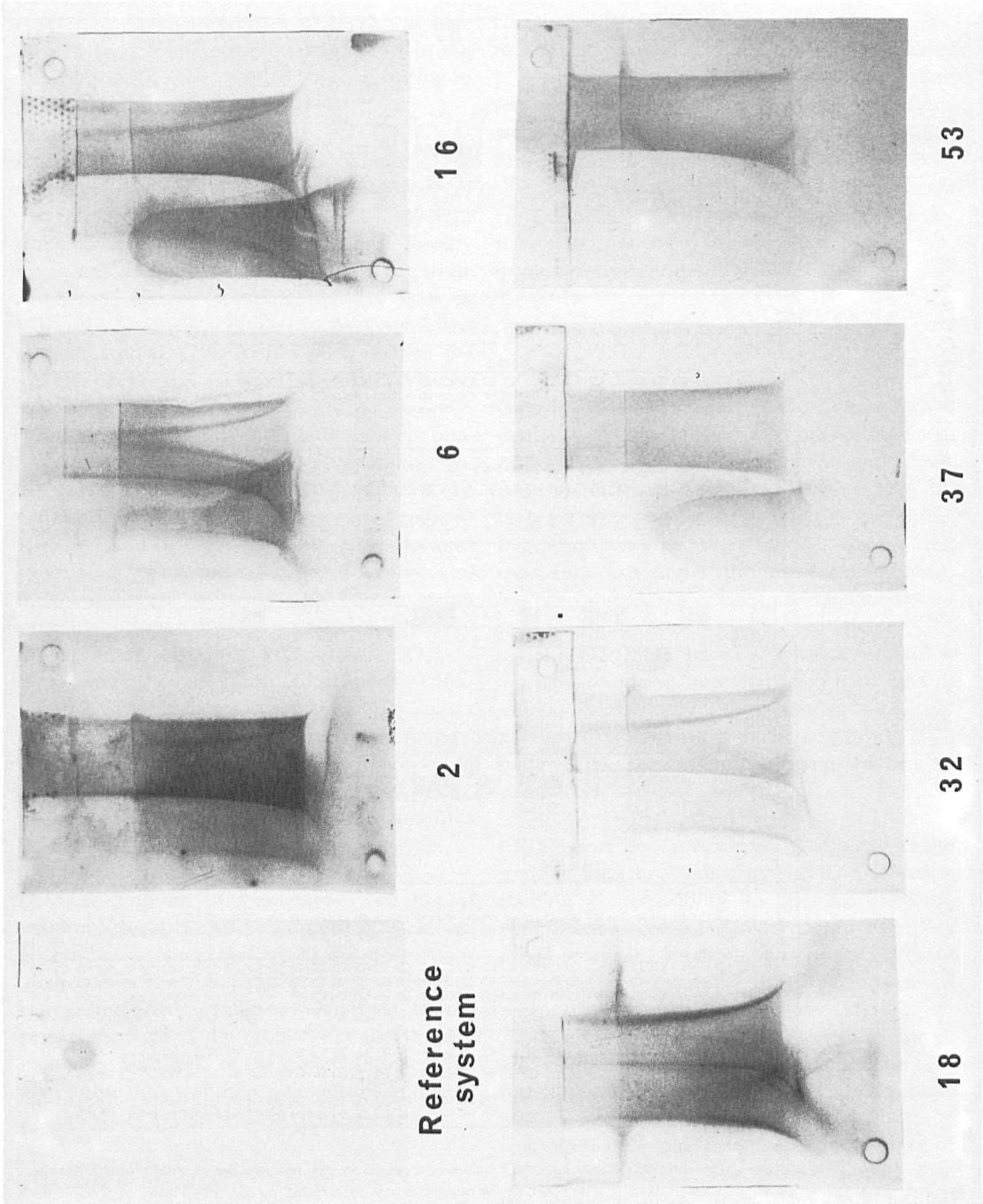


Fig. 3. CRIE results with some different sera (numbers corresponding to the allergogram), showing X-ray photographic films after autoradiography. The reference slide demonstrates radiostaining of four discs, corresponding to CRIE class E.

PRIST. Total IgE values in different sera are presented in the allergogram (Fig. 2). All values are expressed in kU/l (1 kU corresponds to 2.4×10^{-6} g IgE).

Table 1

Allergens in hen's egg white determined in CRIE

Major allergens	6 (ovalbumin), 12 (ovomucoid) and 22
Intermediate allergens	5, 10, 13, 15, 16
Minor allergens	1, 3, 4, 14, 20

Numbers correspond to precipitates in the CIE reference precipitation pattern for hen's egg white.

Table 2

RAST to hen's egg white comparing self-prepared and Pharmacia's (F1) allergosorbents

Uptake of ^{125}I -anti IgE (%) ($n = 20$)			
	Pharmacia	Self-prepared	Difference
Mean	19.6	19.1	0.5
SD	13.0	12.0	4.6
Median	17.5	17.0	1.5
Range	3-42	1-42	-13-7
<i>P</i> -value	$P = 0.30^*$		

* Two-sided Wilcoxon's signed-rank test with correction for ties.

Table 3
Results of skin prick test

Patients	Ref. antigen sol.	Ovalbumin
11. C.H.	++++	+++
19. D.F.	++++	+++
32. D.C.	++++	++++
35. H.C.	++++	-
37. C.T.	++++	++++
39. H.R.	+++	+
49. S.P.	++++	+++
S.M.	++++	+++

Patients' numbers correspond to the allergogram. Patient S.M. was not in CRIE series.

RAST. Results from RAST analyses using Pharmacia egg white discs (F1) are presented in the allergogram, all values being expressed as percentage uptake of total amount ^{125}I -anti-human-IgE applied.

Results from the RAST analyses using Pharmacia's hen's egg white discs, and discs prepared with the reference antigen solution, show that uptake of ^{125}I -anti-IgE is approximately the same for the compared allergosorbents (Table 2).

RAST results to rabbit serum proteins with correction for unspecific IgE-binding, calculated by subtracting the RAST values obtained with human serum protein discs (varying from 8 to 18% uptake of isotope), demonstrated values in RAST class 2 for two sera. These sera also gave diffuse radiostaining in CRIE, and were therefore excluded from the final CRIE series. Ten sera were found to be in RAST class 1, whereas the remaining 58 sera were in RAST class 0.

According to results obtained by RAST reference kit, the various RAST classes correspond to the following percentage uptake of isotope: RAST = 4 > 36%, RAST = 3 > 19%, RAST = 2 > 6% and RAST = 1 > 3%.

SPT

The results of SPT are presented in Table 3, demonstrating positive reactions in all egg allergic patients to the reference antigen solution, whereas six of the eight patients were positive in SPT with the ovalbumin preparation. No positive reactions were observed in the 15 control patients.

DISCUSSION

The reliability of the CRIE method is dependent upon the available antiserum and antigen solution used in CIE. The quality of these critical factors in this study has been discussed previously (9).

Unspecific binding of IgE to the CIE precipitates has been shown to be of little importance in CRIE (2). This was confirmed by the control

experiments in this CRIE study. Interference of specific IgG with the IgE binding to the precipitates is unlikely to be any problem in CRIE, as the antigens are present in great excess in the CIE precipitates (2).

Under optimal conditions it may be expected that sera with high RAST values give rise to the strongest radiostaining in CRIE (10). This tendency is demonstrated in the allergogram, as most heavy radiostaining in the upper parts of the allergogram, gradually fading when descending in the allergogram to lower RAST values. This is not appropriate for some sera (i.e. sera 4, 12, 22, 31, 32). This may be due to falsely high RAST values in sera with high total IgE levels, whereas this does not have significant influence on CRIE results (2). Falsely high RAST values may also be due to variations between different proteins in an antigen solution with respect to their ability to bind to paper discs in RAST. This may lead to overrepresentation of particular proteins on the discs, resulting in falsely high RAST values for sera reacting to these particular proteins. Another explanation for low degree of radiostaining as compared with RAST results, may be that proteins, being important allergens for some patients, have failed to precipitate in the gel in CIE. Such allergens will not be detected in CRIE.

The presence of IgE-antibodies in patient sera against rabbit serum proteins may interfere with the CRIE pattern, giving diffuse radiostaining of the slide and accentuated radiostaining corresponding to all precipitates (2). All sera were examined with respect to such antibodies by means of RAST. On the basis of these experiments two sera were excluded, having high titres of IgE-antibodies against rabbit serum proteins, and diffuse radiostaining in CRIE.

Exclusion of sera with low titres (RAST = 1) of IgE-antibodies to rabbit serum proteins was considered unnecessary as no diffuse radiostaining could be seen in CRIE with these sera.

Radiostaining corresponding to precipitate No. 13 was suspected to be due to co-precipitation of ovomucoid within this precipitate. This is because precipitate No. 13 is enclosed by the ovomucoid precipitate and always demonstrates

radiostaining together with the ovomucoid precipitate, though with a lower degree of radiostaining. Serum No. 37 is an exception from this finding, giving radiostaining of precipitate No. 13 with no simultaneous radiostaining of the ovomucoid precipitate.

To investigate this possibility, the CRIE experiments, using antiserum depleted of antibodies against ovomucoid, were carried out. Results from these experiments suggest that radiostaining of precipitate No. 13 is not due to co-precipitation of ovomucoid.

Ovalbumin and ovomucoid, both of which are well characterized (14), appeared to be the most important allergens in hen's egg white. All sera in the series reacted to the ovalbumin precipitate, whereas the control sera did not.

Antigen No. 22 is registered as a major allergen as well as ovalbumin and ovomucoid. The protein corresponding to this precipitate is not yet identified.

The finding of ovomucoid as a major allergen is in accordance with previous studies (5, 6, 13, 15). Bleumink & Young (5, 6), performing skin tests in egg-allergic patients with different egg white proteins, concluded that ovomucoid was the most important allergen in hen's egg white.

The results of the CRIE series clearly indicate the importance of ovalbumin as a major allergen in egg white. This is in conflict with the studies of Bleumink & Young (5, 6). They concluded that this protein was of little importance in provoking skin reactions in egg-allergic patients (5). If the spectrum of IgE-antibodies in serum is reflecting the spectrum of IgE-antibodies bound to mast cells in dermis, ovalbumin would be expected to elicit strong and specific skin reactions in many of the patients included in the present CRIE series. This suggestion was confirmed by SPT results in this study, which provide further evidence for the importance of this protein as an allergen in hen's egg white.

The role of lysozyme as allergen has been debated (4, 5). On the basis of skin tests Miller & Campbell in 1950 concluded that this was the most important allergen in egg white (11). This was not confirmed by later studies (5, 15). The

present work supports the concept that hen's egg white lysozyme is of little or no importance as allergen, as specific IgE-antibodies against this protein were not detected in any of the 68 sera analysed.

Miller & Campbell also suggested that ovomucin is important as allergen when performing skin tests with this protein (11). At the time of their study, crude ovomucin preparations were the only available (14). Therefore, the positive skin reactions observed by these authors may have been attributed to irritants or traces of other allergenic proteins from the egg white. In a previous study it was suggested that the non-specific precipitate No. 26 in the CIE reference pattern could be due to ovomucin (9). In the CRIE series radiostaining corresponding to precipitate No. 26 was seen with all patient sera, but not with control sera. This radiostaining seemed to be correlated to the degree of total radiostaining in each slide, indicating co-precipitation of other egg white proteins. Therefore no conclusions with regard to the participation of ovomucin as allergen can be drawn from the data presented.

The clinical significance of the data presented in this study is based upon the existence of a correlation between circulating specific IgE-antibodies and clinical symptoms provoked by exposition for the relevant allergens. This concept is well established, being the theoretical basis for the clinical use of RAST (18). Furthermore, all patients in this study were selected on the basis of clinical intolerance to egg white in addition to the presence of circulating IgE-antibodies to egg white. However, it should be emphasized that clinical symptoms of egg allergy will depend upon several factors not considered in this study, such as denaturation of proteins in the egg white during boiling, frying and digestion, as well as factors connected to the effector organ.

With these reservations in mind, the conclusion of this study is that ovalbumin, ovomucoid and antigen 22 are the most important allergens in hen's egg white, and that at least 10 more proteins may contribute to provoke immediate-type reactions in egg-allergic patients.

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Address:

Tor Langeland
Department of Dermatology
Rikshospitalet
Oslo 1
Norway

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