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Identification of IgE-Binding Egg White Proteins: Comparison of Results Obtained by Different Methods

Abstract

The binding of IgE to egg white proteins was investigated for 34 sera from adults with a positive case history and/or positive RAST towards egg, and the impact of experimental conditions on IgE binding in commonly used methods was studied. Radioimmunoblotting after SDS-PAGE of both reduced and unreduced egg white extracts showed complex reaction patterns. The results were confirmed by crossed radioimmuno-electrophoresis (CRIE). Radio dot immunobinding was used to investigate the effect of treatment of allergens for SDS-PAGE and to evaluate the other methods. As a conclusion, the use of combinations of at least two methods is recommended for the identification of IgE-binding egg white proteins. Of the 34 sera, 18 reacted with ovotransferrin, 13 with ovomucoid, 11 with ovalbumin and 5 with lysozyme. The amounts of IgE bound to ovalbumin and lysozyme were generally lower than the amounts bound to ovotransferrin and ovomucoid.

Introduction

The allergenicity of egg white proteins has been the subject of several studies [1–7], but no consensus on which of these proteins are the dominant allergens has been established. The predominant egg white proteins, ovalbumin (OA), ovotransferrin (OT), ovomucoid (OM) and lysozyme (LY) have all been found to cause production of IgE. Yet, the results from different studies are not consistent, and it is not known whether these inconsistencies are due to differences among the sera or are caused by different specificities of the techniques used [6]. The question deserves further investigation because the methods concerned are commonly used in allergen studies and because allergy towards egg is one of the more frequently reported food allergies [8]. In addition, egg white represents a suitable food allergen model system: the predominant egg white proteins are well characterized and they are very different with regard to properties that

have been associated with the probability that a food protein will act as an allergen [8, 9] i.e. size, glycosylation and resistance to denaturation. OT, OA, OM and LY constitute 12, 51, 11 and 3.5%, respectively, of the total protein weight. Their molecular masses are 78,000, 43,000, 28,000 and 14,000, respectively, and their degrees of glycosylation are 3, 1, 25 and 0%, respectively [10].

The methods used in the present study are all nonfunctional methods expected to reflect the binding of IgE to allergens: radioimmunoblotting after SDS-PAGE of reduced and unreduced extracts, crossed radioimmuno-electrophoresis (CRIE) of extracts and radio dot immunobinding of purified preparations of the four predominant proteins in egg white. The radio dot immunobinding was introduced to compare the binding of IgE to equal amounts of the egg white proteins, and to evaluate the effect of some of the treatments of proteins prior to SDS-PAGE.

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Materials and Methods

Egg White Proteins

Egg white extract was prepared from eggs from Red Rhode Island hens as described by Holen and Elsayed [11], and stored in aliquots at -20°C . The protein content was determined according to Lowry [12].

The preparations of egg white proteins were all obtained from Sigma (St. Louis, Mo., USA) and further purified by ion exchange chromatography and gel filtration according to Ebbelhøj et al. [13]. The iron complex of OT was modified to iron-free apo-OT by dialysis with 0.1 M EDTA. Quantitation was based on the following values for $A_{280}^{1\%}$ of the proteins in acid or neutral solution: OT 13.5 [14], apo-OT 11.1 [15], OA 7.5 [16], OM 5.1 [17] and LY 26.4 [18].

Chemicals

Nitrocellulose (NC), pore size 0.1 μm was from Schleicher & Schuell (Dassel, Germany), polyvinylidenedifluoride membranes (PVDF, Immobilon-P) were from Millipore (Bedford, Mass., USA). Tris was Sigma product No. T-1378, 3-cyclohexylamino-1-propanesulfonic acid (Caps) was Sigma product No. C-2632. Agarose HSA was purchased from Lixte (Glostrup, Denmark) and acrylamide was from Serva (Heidelberg, Germany), product No. 10675. Molecular-mass markers were Pharmacia Electrophoresis Calibration Kit, Low MW (Pharmacia, Uppsala, Sweden). Water was drawn from a Milli-Q System, equipped with an Organex-Q cartridge from Millipore.

Antibodies

Rabbit antiserum towards whole egg was a kind gift from ALK (Hørsholm Denmark). Polyclonal rabbit antibodies were raised against Kunitz soybean trypsin inhibitor (STI) and OM according to Bonde et al. [19]. Monoclonal antibodies were raised against OM according to Bonde et al. [20]. Visualization of immunoblots was performed with alkaline-phosphatase-coupled swine antirabbit immunoglobulins from DAKO (Glostrup, Denmark; No. D306), alkaline-phosphatase-coupled rabbit antimouse immunoglobulins (DAKO No. D314) or ^{125}I -coupled rabbit antihuman IgE immunoglobulins from Pharmacia.

Human Serum

Human serum was obtained from two sources: Sera No. 1–15 were from patients with a positive case history of egg allergy, and all but one patient (No. 9) were positive in the Phadebas RAST (Pharmacia) for egg white. These patients underwent double-blind placebo-controlled food challenges. Challenge and clinical characterization have been reported elsewhere [21]. Sera No. 100–121 were selected from the serum bank of the Allergy Unit at the National University Hospital as being class 3 or 4 for whole egg in the A1-RAST [22]. Control sera, c1 and c2, were A1-RAST class 0 towards whole egg. The total IgE level in these sera were 640 kIU/l and 6,240 kIU/l, respectively, as determined by the PRIST (Pharmacia).

SDS-PAGE and Electrophoresis

Electrophoresis was carried out on 14×16 cm slab gels in a Hoefer Electrophoresis Apparatus SE 600 (Hoefer, San Francisco, Calif., USA) at 15°C according to Laemmli [23] and Sutton et al. [24] and as modified by Anet et al. [6], but with a gel thickness of 0.75 instead of 3 mm. The buffer used for reduction contained 40 mM dithiothreitol (DTT) whereas Sutton et al. [24] used 200 mM mercaptoethanol. Egg white extract was applied in a single well of 13 cm to give 175 $\mu\text{g/cm}$. Electrophoresis to NC or PVDF was carried out in a semidry blotting

apparatus (JKA Biotech, Copenhagen, Denmark) at 0.8 mA/cm² for 1½ h [25]. The buffer used was 10 mM Caps, pH 11, 10% methanol [26]. After blotting, the membranes were blocked by soaking for 5 min in 150 mM NaCl, 0.2% Tween 20, 50 mM Tris, pH 10.3. The remaining protein in the gel was visualized by Coomassie staining [27]. Strips from NC blots were stained with colloidal gold [28]; PVDF membranes were stained with Coomassie [29].

The positions of OT, OA, OM and LY after electrophoretic separation of the proteins in egg white extracts were identified by comparison with molecular-mass standards and the purified proteins after blotting to PVDF membranes. PVDF, in contrast to NC, allows protein staining by Coomassie. The positions of OM, which stains poorly with Coomassie, were confirmed by immunostaining with monoclonal and polyclonal antibodies [30].

Dot Blotting

Solutions containing 1 and 0.1 $\mu\text{g}/\mu\text{l}$ of the purified proteins were treated as follows: native – protein dissolved in water; boiled – boiled in water for 5 min; DTT – boiled in 20 mM DTT in water for 5 min; SDS – boiled in 1% SDS in water for 5 min; DTT+SDS – boiled in 1% SDS, 20 mM DTT in water for 5 min. The protein solutions treated in this way were spotted onto NC membrane with 1 μl per dot.

The NC was blocked as described above. The binding of protein to the membrane was controlled by colloidal-gold staining of spots of the different proteins treated in these ways.

Immunoprobings

Radioimmunoblotting after SDS-PAGE and radio dot immunobinding were performed on NC membrane strips as described by Ebbelhøj et al. [13].

CRIE

The CRIE slides were prepared with 100 μg egg white extract and 35 μg LY in a cathodic gel, as described by Langeland [4]. CRIE was carried out according to Weeke and Løwenstein [31] after incubation of slides in serum for 18 h at 20°C .

Results

The presence of specific IgE towards egg white proteins in the 34 sera was evaluated by each of four methods: radio dot immunobinding, radioimmunoblotting after SDS-PAGE of reduced and unreduced samples and CRIE. The results from the four methods were compared.

Radio Dot Immunobinding

Figure 1 shows the autoradiograms of OT, OA and OM in spots with 0.1 and 1 μg submitted to various treatments of relevance to SDS-PAGE. At the exposure time for the presented autoradiogram, spots from the high-IgE control were just visible. The reactions with the native proteins are indicated in the comparative list (table 1), classified as strong reactions for serum giving visible spots with 0.1 μg purified protein, weak reactions for serum giving visible spots only with 1 μg purified protein, and no reaction for serum giving

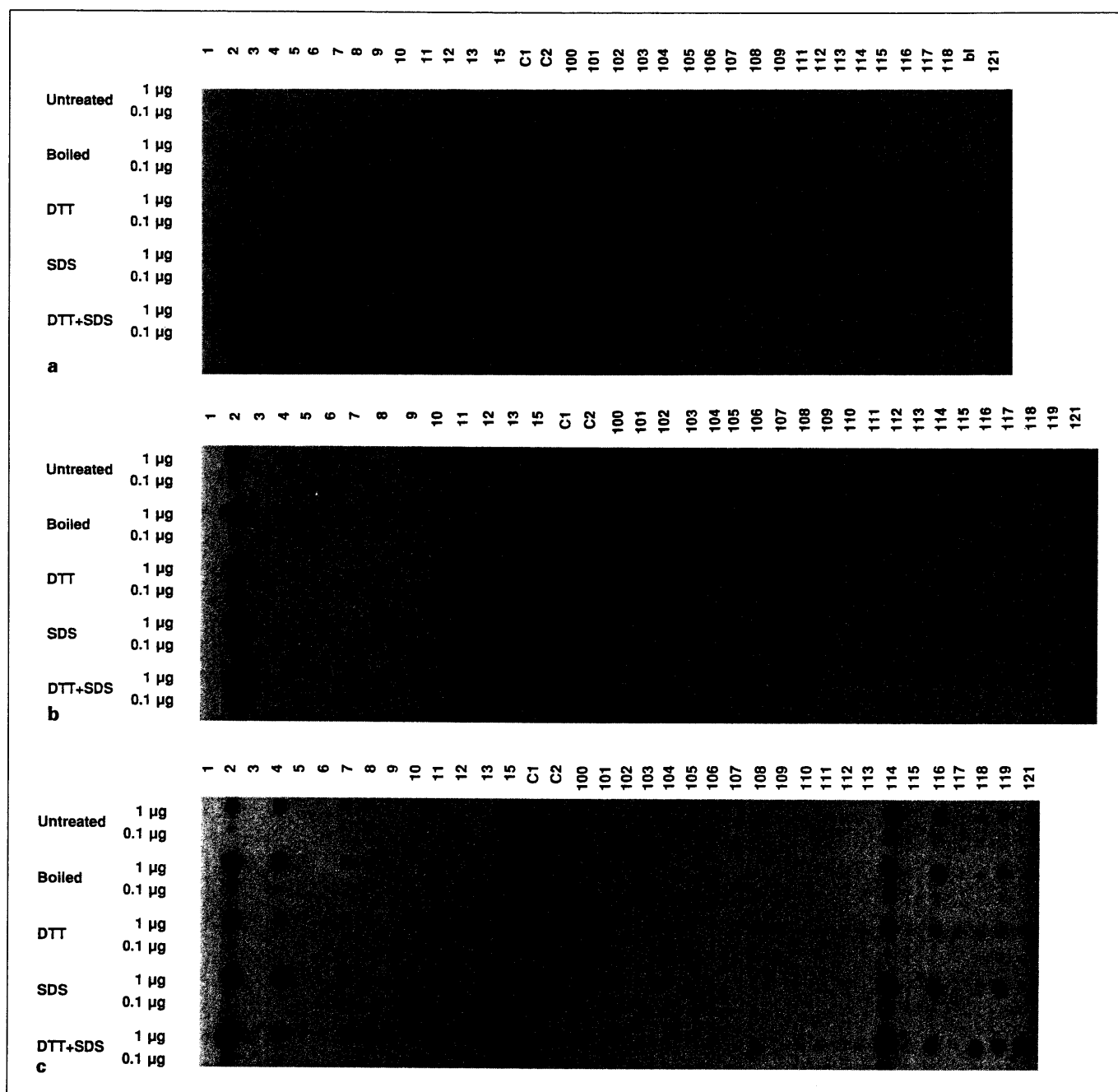


Fig. 1. Radio dot immunobinding of serum IgE to egg white proteins on nitrocellulose. Serum or control numbers are indicated above the individual lanes. **a** OT. **b** OA. **c** OM.

no spot or spots of the same intensity as one of the controls. Of the 34 sera tested, 18 reacted with OT, 7 of them strongly; 13 reacted with OM, 9 of them strongly; 11 reacted with OA, only 3 of them strongly. Of the 29 sera tested with LY, only 1 (No. 11) reacted with native LY (not presented in Fig. 1).

The effects of the various treatments were different for the four proteins. Binding of OA appeared unaffected by all treat-

ments whereas the same treatments had a dramatic effect on OT: boiling OT in water impaired the binding of IgE from about half of the 18 OT-positive sera, and only 3 of these 18 sera bound to DTT-treated OT. Addition of SDS to the reduced OT partly restored the IgE-binding capacity, resulting in 9 positive reactions, all of them among the 18 sera reacting with unmodified OT. In contrast, DTT-treated OM had higher

Table 1. Comparison of methodologies for detecting egg white proteins

Serum	RAST	OT				OM				OA				LY			
		Dot	PAGE	PAGEr	CRIE	Dot	PAGE	PAGEr	CRIE	Dot	PAGE	PAGEr	CRIE	Dot	PAGE	PAGEr	CRIE
1	4.3	(+)	(+)	(+)	(+)												
2	>52.5	+	+	+	+	+	+	+	+ ^a	+ ^a	+	+	+	(+)		+	
3	0.8								(+)					(+)			
4	2.3	(+)	(+)	(+)	(+)	+	+	+	+	(+)	(+)	(+ ^a)	(+)				
5	-					(+)	(+)	+	(+)	(+)	(+)						
6	2.4	(+)	(+)	(+)	(+)												
7	0.53	(+)	(+)		(+)	+	(+)	+	+	(+)	(+)	(+ ^a)	(+)				
8	9.8	(+)	(+)		(+)	+	(+)	+	+	(+)	(+)						
9	<0.35																
10	0.47																
11	>52.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
12	34	+	+	+	+												
13	1.3																
15	28.0	(+)	(+)		(+)	+	+	+	+	+	(+)	+ ^a	(+)				
100	32				n.d.				n.d.				n.d.				n.d.
101	28	+	+	+	+						(+)	(+)					
102	41													n.d.			
103	72				n.d.				n.d.		(+)	(+)	n.d.				n.d.
104	27	+	+	+	(+)												
105	158	+	+	+	+									(+)		(+)	
106	31				n.d.				n.d.				n.d.	n.d.			n.d.
107	43				n.d.				n.d.				n.d.	n.d.			n.d.
108	192	+	+	+	+	(+)								n.d.	(+)		
109	127											(+)	(+)		(+)		(+)
111	49	(+)							(+)			(+)	+		(+)	(+)	(+)
112	156			+													
113	30	(+)			n.d.				n.d.				n.d.				n.d.
114	128	(+)	(+)		+	+	+	+	(+)	(+)	+	(+)					
115	57										(+)						
116	141	(+)				+	+	+	+	(+)	(+)	(+)	(+)				
117	85			+													
118	74		(+)			(+)	+	+	(+)	(+)	(+)	(+)					
119	31				+	+	+		(+)		(+)			n.d.			
121	146	(+)	(+)	+	(+)	(+)	(+)	+	(+)	(+)	(+)	+	(+)	n.d.	+	+	(+)
Total positive		18	16	13		13	12	11		11	15	13		7		5	
Total strong		7	7	10		9	11	11		3	2	5		2		3	

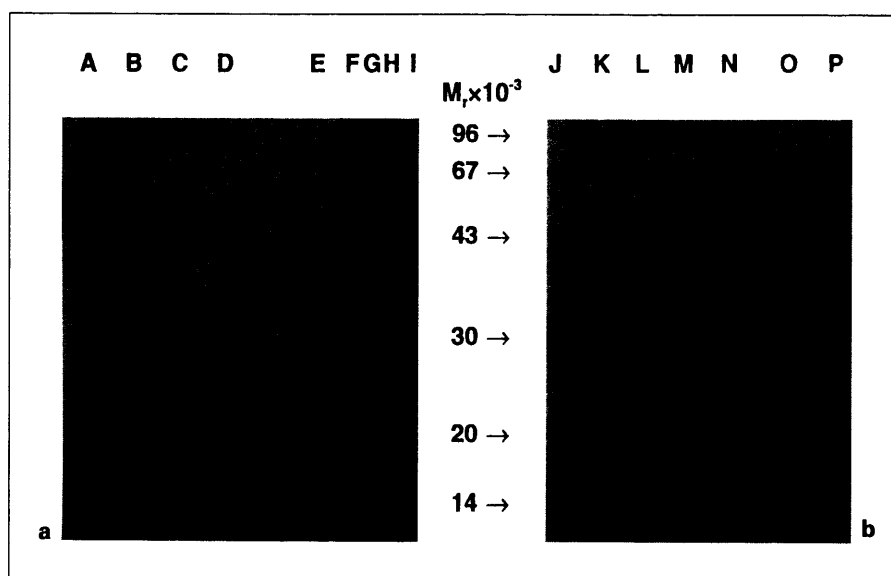
RAST = sera 1–15 in Phadebas RAST (U/ml); sera 100–121 in A1-RAST (sorbent U/ml). Dot = radio dot immunobinding; + = strong reaction, visible with 0.1 µg allergen; (+) = weak reaction, visible with no less than 1 µg allergen. PAGE and PAGEr = radioimmunoblotting of SDS-PAGE of egg white extract, nonreduced and reduced, respectively; + = strong reaction; (+) = weak reaction. In CRIE, the primary antiserum was rabbit antiserum towards egg white; + = strong reaction; (+) = weak reaction. n.d. = Not determined.

^a Analyzed after a shorter exposure time.

IgE-binding capacity than untreated OM, but at least part of this increase was caused by nonspecific binding as revealed by the controls. Combination with SDS did not change the effect of DTT on OM. The single positive reaction with LY was unaffected by boiling and SDS, but abolished by addition of DTT. Treatment of LY by DTT+SDS resulted in a faint un-specific reaction with all sera tested, including the controls.

The results presented for OT were obtained with OT containing Fe³⁺. The same experiments were carried out with apo-OT, giving results identical to those presented here (data not shown).

Fig. 2. Purified egg white proteins and egg white extract blotted onto PVDF after SDS-PAGE using unreduced (**a**) and reduced (**b**) samples. Lanes A, K = 2 µg OT; B, L = 5 µg OA; C, M = 20 µg OM; D, N = 3 µg LY; E–I = unreduced egg white extract, 75 µg/cm; O–P = reduced egg white extract, 50 µg/cm; J = M_r standard. Lanes A – E and J–O were stained with Coomassie; lanes F, G and O were developed after incubation with monoclonal antibodies 2D11, 3F5 and 6B4 respectively; lanes H and I were developed after incubation with polyclonal anti-OM and anti-STI, respectively.



Radioimmunoblotting after SDS-PAGE

Protein-stained blots of the unreduced (PAGE) and reduced (PAGER) purified proteins and egg white extract are shown in Figure 2. Purified OM gave rise to two very broad bands at higher apparent-molecular-mass positions than expected. The identification of OM was confirmed by immunostaining with polyclonal antibodies (lane H) and three different monoclonal antibodies (lanes F, G and O) towards OM. The specificity of the immunostaining was confirmed by anti-STI as a negative control (lane I).

The radioimmunoblottings are shown in figure 3, where the characteristic positions are indicated for each of the four proteins. Reactions with IgE in the serum, classified as strong, weak or no reaction [32, 33] are presented in table 1. At the exposure time for the presented autoradiogram, bands from the controls were just visible. For some sera, the reactions of many close, strong-reacting bands made the identification of IgE-binding proteins difficult. When results from these sera were analyzed at shorter exposure times, only a few ambiguities remained, as indicated in table 1.

CRIE

The positions of individual proteins in CRIE were identified by means of the purified proteins [13]. The reactions of all the tested sera, classified as the radioimmunoblottings, are presented in table 1.

Discussion

Most, but not all of the tested sera showed detectable binding of IgE to one or more egg white proteins. For sera selected by RAST towards whole egg, a negative reaction may be explained by specificity towards yolk components, but sera No. 10 and 13 were positive towards egg white proteins (0.47 and 1.3 PRU/ml, respectively) and were therefore expected to react in some of the methods used in this study.

Comparison of Methods Used in the Present Study

The reduced egg white proteins gave a complex pattern when separated in SDS-PAGE, in agreement with Anet et al. [6] and Elsayed et al. [7]. Unreduced extract gave an even more complicated pattern than reduced extract. In spite of this, the determination of the characteristic positions of the four selected proteins allows identification of the specificities of IgE in most of the tested sera, leaving uncertainty about the reaction with OM in 1 serum and with OA in 3. The scores of strong reactions with OT after SDS-PAGE of the reduced extract are higher than we would expect from the results with reduced OT in radio dot immunobinding. This, however, can be explained by the results from the radio dot immunobinding, which indicated that SDS could partly circumvent the effect of DTT on OT. The effect of SDS may be to facilitate the refolding of reduced proteins, and this effect is likely to be more efficient during electrotransfer, which is slow, than in direct blotting. It is important to emphasize that, as recommended by Tovey and Baldo [32], the reduced proteins were not alkylated, because this would probably prevent any refolding. The inclination to nonspecific bind-

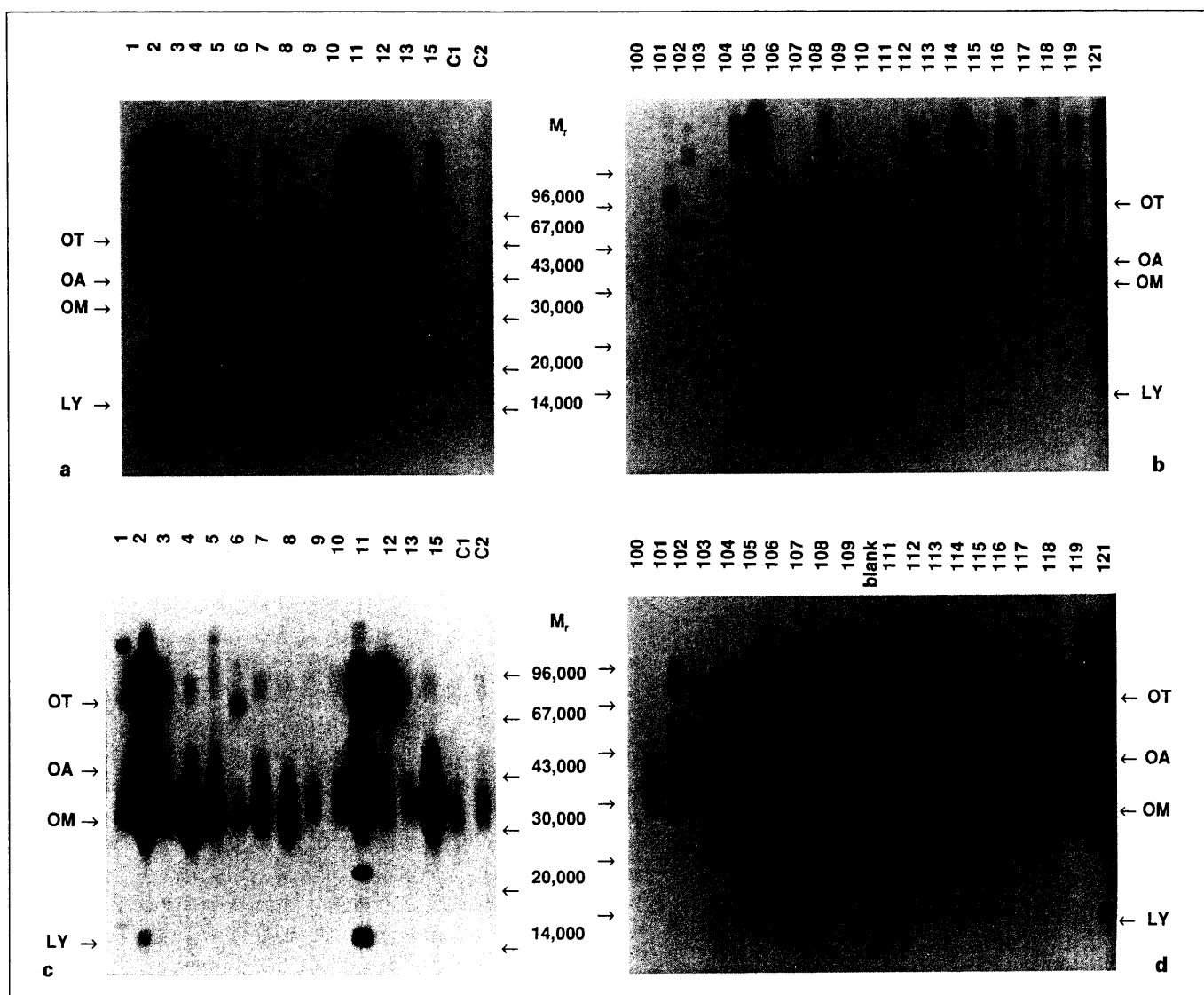


Fig. 3. IgE-positive bands on blots of SDS-PAGE of egg white extracts incubated with patient sera. Serum or control numbers are indicated above the individual lanes. **a, b** Extract not reduced prior to SDS-PAGE. **c, d** Extract reduced prior to SDS-PAGE.

ing of antibodies may be caused by exposure of hydrophobic areas of the proteins, another conceivable effect of SDS and DTT.

The possible effects of skimmed milk powder in the incubation buffer were investigated by comparison with a series of blots incubated without skimmed milk. The background blackening was more pronounced when skimmed milk powder was omitted, but only minor differences in the relative intensity of a few bands were seen.

As discussed by Tovey and Baldo [32], one protein in an unreduced extract can be found in several positions in SDS-PAGE. This seemed to be the explanation for the four bands

seen with all sera reacting with OM, e.g. serum No. 4 in figure 3a. As demonstrated in figure 2, the same pattern was seen with monoclonal anti-OM antibodies from mice and polyclonal antibodies from rabbits.

CRIE, like SDS-PAGE immunoblots, shows that IgEs towards a wide spectrum of the more than 20 egg white proteins are found in human serum. The strength of this method is that the proteins are analyzed in an undenatured state. The drawback, besides the time and amount of human serum it takes, is that the involvement of animal serum may introduce divergences among laboratories using the same method.

Radio dot immunobinding is easy to perform, takes a minimum of serum and allows semiquantitative evaluation of the binding if a series of dilutions are tested. The drawback of the method is that information is limited to the few proteins that can be tested in this way, and no information is provided about reactions with the large amount of other proteins in egg white. From the range of intensities of the spots representing 1 and 0.1 µg of allergen, we infer that the range of binding capacities identified as positive is between 2 and 3 orders of magnitude. This indicates that allergens used for this assay and for further investigations of allergen-IgE interactions must be purified to a very high degree, and further purification of commercial preparations is mandatory [11, 13]. As shown by Bernhisel-Broadbent et al. [34], it is also necessary for in vivo investigation of allergenicity.

As a whole, there is good agreement among the results obtained by the four methods, as seen from table 1. The intensities observed in the different methods cannot be compared, because the amount of individual allergens and their way of presentation are different. The quantitative considerations have therefore been limited to a simple, arbitrary classification of the intensities observed in each of the methods. There are nine examples of a positive reaction in only one of the methods. These examples are found among all of the four methods and represent all four proteins (2 OT dot, 2 OT PAGEr, 1 OT CRIE, 1 OM dot, 1 OM CRIE, 1 OA CRIE and 1 LY PAGE). As each method has its merits on its own and erroneous results are difficult to exclude on the basis of only one method, we recommend the use of two methods to evaluate the specific IgE binding. Of the methods presented here, we found dot immunobinding very useful in combination with either SDS-PAGE or CRIE.

Comparisons with Other Studies

There is general agreement among previous investigations that OM and OT are important egg white allergens. The results of the present study concur. The debate concerns the allergenicity of OA and LY. In contrast to the study of Bleumink and Young [2], the results of Hoffmann [3] and Langeland [5] indicated that OA is a major allergen with strong reactions compared to the other egg white proteins. We also found that more than half of the egg-white-positive sera reacted with OA, but only a few with the same intensity as the reactions seen with OM and OT. The trend was the same for all the methods we used and in agreement with the results of Bernhisel-Broadbent [34]. In a comparative study of the allergenicity of purified OA, OM and LY, this author found the highest amounts of specific IgE towards OM as well as the greatest diameters by skin prick test from OM. The differences between the results from CRIE presented

here and the Langeland [5] results are noteworthy as the experiments were carried out with the same procedures and the precipitation patterns seen by protein staining were so alike, that a comparison was justified. In spite of this, Langeland [5] found more sera reacting with OA than with OM, and the same number of strong reactions, whereas fewer sera reacted with OT.

Regarding LY, we observed only a few and weak reactions in agreement with Hoffmann [3] and Langeland [5] and Bernhisel-Broadbent [34]. In contrast, Holen and Elsayed [11] and Anet et al. [6] concluded that LY is an important allergen. We note especially the results of Anet et al. [6], because our methods for the SDS-PAGE and immunoblots were very similar to theirs, so that the results should be comparable. The conclusions from the present study and that of Anet et al. [6] were also based on other methods, and within each of the studies, the methods used gave consistent results. The identical reactivity of LY in reduced and unreduced extracts thus excludes the use of DTT as a reducing agent as the cause of low IgE-binding capacity. Unspecific binding of antibodies to LY has been suggested as an explanation for the demonstration of positive binding of IgE to LY [34], but we did not observe unspecific binding of polyclonal antibodies to LY (fig. 2, lanes H and I).

We conclude that some of the different results obtained in various attempts to identify dominant egg white allergens reveal real differences among the patient groups.

Egg White as a Model System

In spite of the complexity and diversity of reactions towards egg white proteins, egg white represents one of the best systems for studies on food allergens. Selection of serum for investigations with specific proteins should make use of at least two methods, one based on reactions with the potential allergens in a very pure state, one showing the diversity of reactions of the serum concerned. The latter is necessary in order to determine the extent of information excluded if further investigations are limited to a few dominating proteins, and the diversity and relative intensities of the reactions with all the proteins should be borne in mind.

As mentioned above, there is agreement among different studies that OM and OT are important allergens. Of these, OM fulfills all the common expectations of a food allergen, being a stable, highly glycosylated protein with a molecular mass below 40,000, very resistant to denaturation and enzymatic cleavage [8, 9]. OT, on the other hand, does not fit this description: it has a higher molecular mass, it is sparsely glycosylated and rather susceptible to denaturation [10]. Our expectations may still be valid, as egg is often ingested after no or very mild processing, with the proteins close to

their native state and therefore relatively resistant to proteolysis. In accordance with this, the radio dot immunobinding presented here showed that 15 out of 18 sera reactive towards OT preferentially bound native OT.

Further studies on the allergenicity and immunogenicity of egg white proteins should improve our understanding of food allergens. It would be interesting to know if resistance to proteolysis is indeed an important characteristic, if carbohydrate is important, and if so whether it is by contributing to stability or directly as part of antibody-binding areas.

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