

RAST using crude and purified anti-IgE

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Summary

The sensitivity of the RAST using anti-IgE in ^{125}I -labelled IgG fractions of sheep antiserum was compared to that using anti-IgE purified by immunosorbent techniques in tests with three allergens (grass pollens, *Aspergillus fumigatus* and the detergent enzyme 'Alcalase') on sera from 248 workers in a detergent factory. Both anti-IgE reagents measure the same antibody but the RAST procedure using the crude anti-IgE reagent is less sensitive than that using the immunosorbent-purified anti-IgE in its ability to detect circulating IgE in subjects with positive skin-prick tests. In general the agreement between positive RAST and positive skin test was improved when only skin tests equal to or greater than 3 mm were considered positive. With Alcalase, antigen non-specific binding by the crude anti-IgE reagent may give false positive results. Optimal conditions for the preparation of allergosorbents with this allergen are defined. Predictive equations relating the results of RAST and skin test show that the hitherto arbitrary definition of a positive RAST result is statistically valid.

Introduction

The radioallergosorbent test (RAST) (Wide, Bennich & Johansson, 1967) is a procedure for the detection and estimation of circulating allergen-specific IgE. In principle, IgE antibodies in a serum react with the allergen which is linked chemically to an insoluble material. The bound IgE antibodies are then detected by treating the complex with ^{125}I -labelled antibodies that combine specifically with the bound IgE. The uptake of radioactivity is directly related to the amount of IgE antibody to that particular allergen. Good agreement has been obtained between the levels of circulating allergen-specific IgE estimated by RAST and the results of *in vivo* skin and bronchial challenge tests (Wide, 1973).

The use of RAST, as described by Wide *et al.* (1967), is limited by the availability of mono or polyclonal IgE required for the purification of anti-IgE preparations which are used in the test. RAST procedures using immunoglobulin fractions from anti-IgE serum have been reported (Sarsfield & Gowland, 1973; Ozkaragoz *et al.*, 1974), but

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their sensitivity has not been compared to RAST using immunosorbent-purified anti-IgE preparations.

We now report a comparison of the RAST using immunoglobulin fractions from anti-IgE serum and the RAST using immunosorbent-purified IgE to measure IgE specific for three inhalant allergens (grass pollens, *Aspergillus fumigatus* and the proteolytic enzyme preparation 'Alcalase' ex Novo Industri A/S Copenhagen). The agreement between the results of these tests and skin prick tests to the three allergens in 248 workers in a detergent factory has been tested. In addition, factors influencing the preparation of RAST allergosorbents have been investigated.

Materials and methods

Subjects

Blood samples were obtained during routine medical examination of 248 volunteers (163 men, 85 women) who worked in a detergent factory. Immediately before the blood samples were taken, each subject was prick tested with B2 mixed grass pollens (Bencard), *A. fumigatus* (Bencard) and a standardized Alcalase skin prick test reagent (How & Cambridge, 1971) on the volar surface of the forearm using a standardized skin prick test procedure (Pepys, 1968). Skin reactions were assessed at 15–20 min and the mean weal diameter recorded.

Sixteen samples of cord blood were obtained from Bedford General Hospital.

Radioallergosorbent test (RAST)

The RAST procedure was essentially as described by Wide *et al.* (1967). The assay buffer was 0.05 M phosphate buffered saline pH 7.4 containing 0.1% sodium azide, 1% Tween 20 and 1% bovine serum albumin (BSA Grade V, Sigma Chemical Co.). The uptake of radiolabelled anti-IgE was measured in a Packard AutoGamma Spectrometer.

Crude and immunosorbent-purified anti-IgE

The crude anti-IgE was the IgG fraction isolated by batch chromatography on DEAE-cellulose (DE52, Whatman Ltd) (Stanworth, 1960) of sheep antiserum to the Fc fragment of human myeloma E which had been absorbed with immunoglobulins of the other classes. The IgG fraction was concentrated to about 10 mg protein/ml by pressure dialysis. Immunoelectrophoretic analysis using antiserum to whole sheep serum showed that the IgG fraction contained no detectable component other than IgG. The IgG fraction was labelled with 2 mCi of ^{125}I using the chloramine-T procedure of Greenwood, Hunter & Glover (1963).

Before use in RAST, aliquots (0.5 ml) of the labelled preparation were adsorbed at room temperature for 6 hr with the appropriate allergosorbent (10 mg). The adsorbed anti-IgE preparation was diluted to 60,000 ct/min/0.1 ml in 5% BSA before use in the test.

^{125}I -labelled immunosorbent-purified anti-IgE (Phadebas RAST isotope pack) was obtained from Pharmacia Ltd. This reagent is obtained by purification of an antiserum produced in rabbits.

Sera from all subjects were tested for allergen-specific IgE by the RAST using the crude anti-IgE. Sera from skin prick test positive subjects and from skin prick test

negative subjects whose sera gave doubtful or positive results in the RAST using crude anti-IgE were also tested by the RAST using immunosorbent-purified anti-IgE.

Preparation of allergosorbents

Grass pollen (B2) and *Aspergillus fumigatus* allergen extracts (3 ml) used for skin testing were dialysed for 18 hr at 4°C against 0.1 M sodium bicarbonate pH 8.0 and concentrated to 1 ml by pressure dialysis. Dialysed Alcalase (How & Cambridge, 1971) was used to prepare Alcalase allergosorbents.

Allergosorbents were prepared by reacting allergen preparations with microcrystalline cellulose (Sigmacell, Sigma Chemical Co.) which had been activated with cyanogen bromide (CNBr, Koch Light Labs Ltd) (Wide, 1969). Five batches (A to E) of CNBr-activated cellulose were prepared from the same batch of cellulose and two different batches of CNBr (one was used to prepare batches A and B; the other to prepare batches C to E). Activated cellulose batch B was prepared using a reaction time (4 min) twice as long as that reported by Wide (1969).

Various factors were investigated in the preparation of Alcalase allergosorbents and each allergosorbent was used in the RAST with sera from subjects skin prick test negative and positive to Alcalase. The proteolytic (Lin, Means & Feeney, 1968) and esterolytic (Glazer, 1967) activities of Alcalase allergosorbents prepared by reacting various concentrations of Alcalase with CNBr-activated cellulose batch A at pH 8 (0.1 M sodium bicarbonate; Wide, 1969) were measured. The protein binding capacity of CNBr-activated cellulose batches A to E at pH 8 was measured using various concentrations of Alcalase containing ^{125}I -Alcalase. Radioimmunoassay analysis of ^{125}I -Alcalase showed that all the antigenic components were labelled. Alcalase allergosorbents were prepared from batches A to E at pH 8 using the concentration ('optimal concentration') of Alcalase which had produced the best RAST allergosorbents in previous tests. CNBr-activated cellulose batch D, which had the highest protein binding capacity of the five batches at pH 8, was also used to prepare Alcalase allergosorbents by reaction with the optimal concentration of Alcalase at various pH values in the range pH 4 to 11. After the coupling reaction, unreacted iminocarbonate groups on the CNBr-activated cellulose were hydrolysed with 0.1 M-acetate buffer, pH 4, in experiments where the reaction pH was greater than pH 7.0, and with 1.0 M β -ethanolamine, pH 8.0, where the reaction pH was less than pH 7.0. The protein binding capacity of batch D in the pH range 4–11 was measured using ^{125}I -Alcalase.

Results

Preparation of Alcalase allergosorbents

The amount of bound protein and the proteolytic and esterolytic activity of Alcalase allergosorbents increased to limiting values as the quantity of Alcalase added to CNBr-activated cellulose batch A was increased. Alcalase RAST uptakes with these allergosorbents increased similarly to a limiting value. The uptake of ^{125}I -labelled anti-IgE in Alcalase RAST using control sera from Alcalase skin prick test negative subjects and Alcalase allergosorbents containing the maximum amount of bound protein was highest with allergosorbents which were prepared from excess allergen. The best differentiation between sera from subjects skin prick test positive and negative to Alcalase was obtained in the RAST using an Alcalase allergosorbent which had been prepared by reacting CNBr-activated cellulose with the minimum concentration

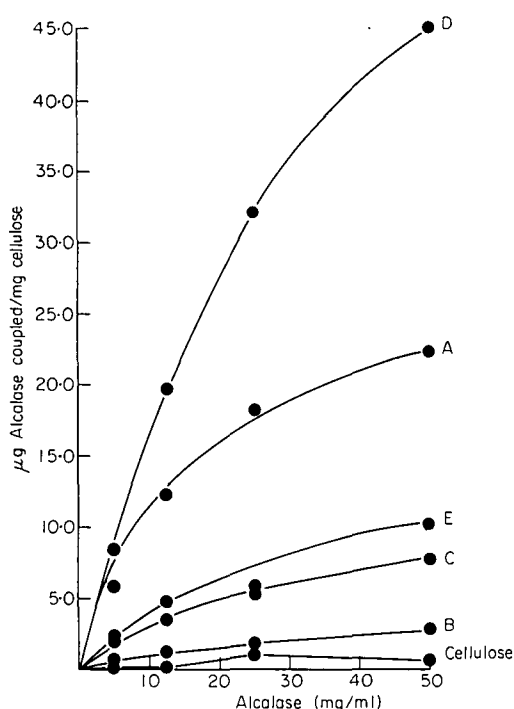


Fig. 1. Binding of ^{125}I -Alcalase to five batches (A-E) of cyanogen-bromide activated cellulose at pH 8.0.

of Alcalase required to give the maximum amount of bound protein. This concentration was defined as the optimal concentration. This Alcalase allergosorbent had 0.9% and 0.5% of the specific esterolytic and proteolytic activities (activity/mg protein) respectively, of Alcalase.

The amounts of ^{125}I -Alcalase protein which bound to the five batches (A-E) of CNBr-activated cellulose at pH 8 are summarized in Fig. 1. Batch B did not bind

Table 1. RAST results using allergosorbents prepared from five batches of activated cellulose and sera from Alcalase skin test positive subjects

Serum code	RAST result*						
	2/33	2/35	2/21	4/38	4/42	4/54	4/70
Skin prick test reaction of serum donor	1	1	2	3	5	7	8
Allergosorbent prepared from CNBr-activated cellulose at pH 8							
Batch A	1.3	1.2	1.4	1.4	2.3	3.2	4.1
Batch B	1.1	1.1	1.4	1.2	0.9	1.0	1.0
Batch C	1.4	1.4	1.7	1.9	3.0	2.5	5.2
Batch D	1.4	1.4	2.4	2.2	3.5	5.7	6.6
Batch E	1.8	1.8	1.8	1.9	3.1	3.8	5.2
Cellulose powder	1.0	1.0	0.9	1.2	1.0	0.7	1.0

* RAST result defined in terms of ratio of uptake for test serum to that for pooled serum from control skin test negative subjects. Ratio of <1.5 is negative result; $1.5-2.0$ is doubtful, ≥ 2 is positive (Wide *et al.*, 1967).

significantly greater amounts of Alcalase protein than untreated cellulose powder. This batch was prepared using an activation time twice as long as that recommended by Wide (1969). Under these conditions the cellulose became fibrous and did not readily form a suspension. The results of the RAST using sera from subjects skin prick test positive to Alcalase and allergosorbents prepared by reacting batches A to E with the optimum concentration of Alcalase at pH 8 are shown in Table 1. The same results were obtained using cord blood serum as the negative control serum.

The best differentiation between sera from subjects skin prick test negative and positive to Alcalase was obtained in the RAST using Alcalase allergosorbent prepared at pH 8 from activated cellulose batch D, the batch with the highest protein binding capacity. Tests of Alcalase allergosorbents prepared at various pH values from this batch of activated cellulose confirmed that the better allergosorbents in the RAST were those which contained the greater amount of Alcalase protein, and that the optimal pH for preparing Alcalase allergosorbent for the RAST was pH 8.

Table 2. Agreement between skin prick test result and result of RAST using crude anti-IgE for grass pollen and Alcalase in 248 subjects

Number of subjects	Grass pollen skin prick test	Number of subjects with grass pollen RAST*			% Agreement	
		Positive	Doubtful	Negative	†	‡
189	Negative	1	0	188	99	99
59	Positive	7	3	49	17	12
34	1 or 2 mm	0	0	34	0	0
25	≥ 3 mm	7	3	15	40	28
	Alcalase skin prick test	Number of subjects with Alcalase RAST*			% Agreement	
		Positive	Doubtful	Negative	†	‡
183	Negative	2	13	168	92	99
65	Positive	27	7	31	52	42
27	1 or 2 mm	3	4	20	26	11
38	≥ 3 mm	24	3	11	71	63

* RAST result as defined by Wide *et al.* (1967).

† Considering doubtful RAST results as positive.

‡ Considering doubtful RAST results as negative.

Sensitivity of RAST procedures

RAST using IgG fraction from anti-IgE serum. Repeated analyses of 248 sera for grass pollen-specific IgE and Alcalase-specific IgE showed that the RAST results were strictly reproducible. The agreements between the RAST and skin prick test results for these two allergens are summarized in Table 2. For completeness the agreements have been calculated by considering doubtful RAST results as (a) negative and (b) positive but the figures cited in the following comments refer to agreements between the results of skin prick test and RAST in which doubtful RAST results are considered as negative.

With both allergens there was a 99% agreement between negative skin test and negative RAST results. Skin reactions at 1 and 2 mm showed no agreement with the RAST for grass pollens and poor (11%) agreement for Alcalase. Only fourteen subjects gave a skin prick test reaction to *A. fumigatus*. No reaction was greater than 2 mm and none of the 248 sera gave a positive RAST for *A. fumigatus*-specific IgE. The agreement between positive RAST and positive skin test increased from 12 to 28% (pollens) and from 42 to 63% (Alcalase) for skin reactions ≥ 3 mm.

Table 3. Agreement of skin prick test and RAST results using crude and immunosorbent-purified anti-IgE for prick test positive subjects

Number of subjects	Grass pollen skin prick test	Anti-IgE preparation used in RAST	Number of subjects with grass pollen RAST*			% Agreement	
			Positive	Doubtful	Negative	†	‡
59§	Positive	Crude	7	3	49	17	12
		Immunosorbent-purified	29	7	22	62	50
34§	1 or 2 mm	Crude	0	0	34	0	0
		Immunosorbent-purified	8	5	20	41	24
25	≥ 3 mm	Crude	7	3	15	40	28
		Immunosorbent-purified	21	2	2	92	88
	Alcalase skin prick test		Number of subjects with Alcalase RAST(*)				
			Positive	Doubtful	Negative		
65	Positive	Crude	27	7	31	52	42
		Immunosorbent-purified	23	6	36	45	35
27	1 or 2 mm	Crude	3	4	20	26	11
		Immunosorbent-purified	1	2	24	11	4
38	≥ 3 mm	Crude	24	3	11	71	63
		Immunosorbent-purified	22	4	12	69	58

* RAST result as defined by Wide *et al.* (1967).

† Considering doubtful RAST results as positive.

‡ Considering doubtful RAST results as negative.

§ One serum was not analysed using the immunosorbent-purified anti-IgE.

RAST using immunosorbent-purified anti-IgE. RAST results using crude and immunosorbent-purified anti-IgE reagents for subjects with positive skin prick test reactions to grass pollens and/or Alcalase are summarized in Table 3. The strong significant relationship between the uptake of ^{125}I -anti-IgE in the RAST with the two anti-IgE reagents ($r = 0.9$ for grass pollens and 0.78 for Alcalase) showed that both reagents measure the same antibody.

The use of immunosorbent-purified anti-IgE improved the sensitivity of the RAST with the two common allergens, grass pollens and *A. fumigatus* as shown by the better

agreement between the results of skin tests and of the RAST using the purified anti-IgE.

None of the thirty-four subjects with skin prick test reactions of 1 or 2 mm to grass pollens gave positive or doubtful RAST results using the crude anti-IgE reagent. Sera from thirty-three of these thirty-four subjects were tested by the RAST using immunosorbent-purified anti-IgE. Eight gave positive and five gave doubtful RAST results with the immunosorbent-purified reagent. Thus, for these subjects the agreement between a positive RAST result and a positive skin test increased from 0 to 24% by using the purified rather than the crude anti-IgE reagent in the RAST. The agreement for all subjects with positive skin test reactions to pollens increased from 12 to 50% and for those with skin test reactions ≥ 3 mm from 28 to 88%.

Of the fourteen subjects who were skin test positive to *A. fumigatus* and negative in the RAST using the crude anti-IgE reagent, three gave doubtful and one a positive result in the RAST with the purified anti-IgE reagent. These four subjects had 1 mm skin prick test reactions to *A. fumigatus*.

For Alcalase, the agreement between positive RAST and positive skin test was reduced when the immunosorbent-purified anti-IgE reagent was used in the RAST. For the twenty-seven subjects with skin prick test reactions of 1 or 2 mm to Alcalase, the Alcalase RAST result changed from positive to negative in two subjects, from doubtful to positive in two subjects and from positive to doubtful in four subjects when the immunosorbent-purified anti-IgE reagent was used. For the thirty-eight subjects with skin prick test reactions of ≥ 3 mm to Alcalase, the Alcalase RAST result changed from positive to negative in four subjects and from negative to positive in one subject when the purified anti-IgE reagent was used.

Sera from sixteen subjects who were skin test negative to grass pollens and/or Alcalase, but gave doubtful or positive results in the RAST to the relevant allergen using the crude anti-IgE reagent, were also tested by the RAST using immunosorbent-purified anti-IgE. Serum from the one subject in this group with a positive grass pollen RAST result was positive with both RAST reagents. The fifteen sera which gave positive or doubtful Alcalase RAST results with the crude anti-IgE, however, gave negative RAST results using the purified anti-IgE reagent.

Relationship between RAST uptake and skin prick test reaction size

Analysis of the data for all 248 subjects showed that there was a strong significant relationship between uptake (ct/min) in the RAST using the crude anti-IgE reagent and the Alcalase and grass pollen skin prick test size ($r = 0.6$). The relationship could be represented by exponential equations, which could be used to predict one parameter from another. The equations could be stated precisely because of the large sample tested but prediction of one parameter from the other, for an individual subject, showed wide confidence limits because of the variation in individual data. Thus, prediction of a skin prick test weal size from the RAST titre had limits (95% confidence) of ± 3 mm. This means that the size of a skin prick test weal predicted from the RAST titre has to be greater than or equal to 3 mm if it is certain not to be negative. Similarly, the RAST titre for a skin prick test positive must be at least twice that for a prick test negative subject in order to be significant.

Discussion

Allergosorbents for use in the RAST as aids in the diagnosis of IgE-mediated allergic activity to allergens which are mixtures of components should contain all the aller-

genic components in such a form that they can combine readily with specific IgE. The importance of the quality of allergen extracts used to prepare RAST allergosorbents has already been demonstrated (Aas & Johansson, 1971; Aas & Lundkvist, 1973).

The crude enzyme preparation Alcalase, which was used to study the effect of various factors in the preparation of allergosorbents, is obtained by a fermentation process using *Bacillus subtilis* and related organisms and contains proteolytic and amylolytic enzymes, bacterial spores and cell debris and components of the fermentation medium. As with house dust mite allergen (Turner, Baldo & Anderson, 1975) and ragweed pollen allergen (Gleich & Jones, 1975) the most effective Alcalase allergosorbent in the RAST was prepared under conditions (reaction with CNBr-activated cellulose at pH 8) which bound the maximum amount of Alcalase protein.

It should be noted, however, that the allergosorbent which differentiated best between sera from Alcalase skin prick test negative and positive subjects was prepared by reacting CNBr-activated cellulose with the minimum concentration of Alcalase required to give the maximum amount of bound protein. This allergosorbent also contained the most proteolytic and esterolytic activities although these were only 0.5–1% of the values for the free enzyme. The variability of the protein binding capacity of batches of CNBr-activated cellulose stresses the importance of testing batches with a radio-labelled allergen preparation before committing valuable sera in evaluating allergosorbent preparations in the RAST.

The immunosorbent-purified anti-IgE and the crude anti-IgE reagents measure the same antibody as shown by the correlation coefficients obtained for individual sera in the RAST using these reagents.

The use of immunosorbent-purified anti-IgE in the RAST, however, improves significantly the sensitivity of the test.

Comparison of the results obtained with the two anti-IgE reagents in the Alcalase RAST suggests that some of the RAST results obtained using the IgG fraction of anti-IgE serum as the RAST reagent were non-specific. This is supported by the less significant correlation coefficient obtained between Alcalase RAST results using the two anti-IgE reagents ($r = 0.78$ cf $r = 0.9$ for grass pollens RAST using the two reagents) and by the higher incidence of Alcalase skin test negative subjects whose sera gave positive or doubtful RAST results using the crude anti-IgE reagent. Non-specific effects in dander RAST using immunosorbent-purified sheep anti-IgE reagent have been reported (Foucard *et al.*, 1975).

The reproducibility of the results of the RAST using the crude anti-IgE reagent was demonstrated for a common allergen (grass pollens) and an industrial allergen (Alcalase). For both of these allergens, the agreement between a positive skin prick test and the RAST result both with the crude and with the purified anti-IgE reagent depends on the criteria of a positive skin test reaction and improves considerably if skin reactions of 1 and 2 mm are considered as negative. A comparable study (Sarsfield & Gowland, 1973) of the agreement between positive skin test and RAST using the IgG fraction of anti-IgE as the reagent reported a 55% agreement in a study of allergy to *Dermatophagoides farinae* in 133 asthmatic children. The criterion of a positive skin reaction was a weal diameter ≥ 2 mm.

The standardization of allergens by RAST is under review (de Weck & Baer, 1975). This work emphasizes the need not only for the preparation of appropriate allergosorbents for the RAST but also the need for standardization of anti-IgE reagents.

Analysis of the results of the RAST and skin prick test for the large sample tested

show that the hitherto arbitrary definition of a positive RAST result (Wide *et al.*, 1967) is statistically valid. This increases the confidence of interpreting RAST results in subjects where skin testing is precluded or where confirmation of a skin test result is required.

Acknowledgments

The authors wish to express their grateful thanks to Dr C. P. Juniper, Environmental Medical Adviser, Unilever Medical Services, for provision of sera and skin test data; to Mr A. Kinshott for help in statistical analyses and to Dr D. R. Stanworth, Department of Experimental Pathology, The Medical School, Birmingham, for the generous gift of sheep anti-IgE.

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