

***In vivo* tests with pollen extracts previously investigated by means of direct RAST titration allergen assay**

B. S. M. STENIUS-AARNIALA, C. H. O. MALMBERG,
E. E. A. HOLOPAINEN and F. BJÖRKSTEN

*Departments of Otolaryngology and Pulmonary Diseases, and
Allergy Hospital, Helsinki University Central Hospital, Helsinki, Finland*

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Summary

The allergenic potency of different birch, Timothy and mugwort pollen extracts was determined by means of a direct RAST titration allergen assay. For birch and Timothy allergens, the results of skin and provocation tests did not confirm the results of the *in vitro* determinations of allergenicity. There was a poor correlation between the results of skin tests and the results of Phadebas RAST for determination of specific IgE to mugwort, whereas the correlation between skin tests and RAST for other allergens was excellent. It is concluded that direct RAST titration allergen assay is not adequate for all kinds of allergen preparations and that the Phadebas RAST for mugwort is less sensitive than the RAST for other allergens. The diagnostic efficacy of the different allergen preparations could not be evaluated.

Introduction

Despite the availability of *in vitro* techniques for estimating specific IgE, diagnosis of type I allergy often requires *in vivo* testing. In the evaluation of *in vivo* tests, however, the practising allergist is not infrequently faced with inconsistent and sometimes unreliable results, caused by variations in the allergen potency of available commercial allergens. It has also been demonstrated that these may contain non-specific irritants, which cause false positive reactions to provocation tests (Aas, 1975). Besides being used for the estimation of specific IgE in patient serum, the RAST may be applied to allergen assay (Yman, Ponterius & Brandt, 1975). The purpose of the present study was to investigate the reactivity *in vivo* to commercial allergens and new crude or partly purified allergen preparations, the dilutions of the extracts used being based on *in vitro* estimations of their allergen activity.

Correspondence: Dr Brita Stenius-Aarniala, Department of Pulmonary Diseases, Meilahti Hospital, 00290 Helsinki 29, Finland.

Materials and methods

Allergen extracts

Different extracts of birch (*Betula alba*), Timothy (*Phleum pratense*) and mugwort (*Artemisia vulgaris*) pollen were tested. Each pollen allergen was represented by four different preparations: a commercially available glycerinated extract for prick-testing, an aqueous extract for provocation test, both provided by Dome Laboratories Ltd, Slough, England, and one partly purified and one crude extract, provided by Pharmacia Research Laboratories, Uppsala, Sweden. The extracts are referred to in the text as 'Dome', 'purified' and 'crude'. The Pharmacia allergens were provided as freeze-dried extracts. The same batch of each allergen preparation was used throughout the study, fresh solutions being made up every week. As to the Dome glycerinated extracts for prick-testing and the aqueous solutions for provocation tests, bottles with the same lot number were used for the *in vitro* allergen assay and skin or provocation tests, respectively. The Dome extracts for prick-testing and the animal dander extracts for provocation tests contained 50% glycerol. All other solutions used for *in vivo* tests were glycerol-free. The allergen activity of all batches was determined by Pharmacia AB, using the direct RAST titration method described by Yman *et al.* (1975), and the allergens were diluted on the basis of these determinations. The allergenic activity of the Dome aqueous extract of birch pollen, as estimated by the direct RAST method, was too low to allow a comparison with the other birch extract. It was also found that a quantitative comparison was not possible between the Dome Timothy allergens on the one hand and the purified and crude Timothy allergens from Pharmacia on the other. Table 1 shows how the extracts related to each other in terms of potency, according to the *in vitro* allergen assay.

Table 1. Results of *in vitro* allergen assay. Stock solutions of extracts marked with the same capital letter were equipotent according to allergen assay by means of direct RAST titration

Extract	Birch	Timothy	Mugwort
Dome			
(a) Extract for provocation test		B	D
(b) Extract for prick test	A	B	D
Pharmacia			
Crude	A	C	D
Purified	A	C	D

Patients

Thirty-one patients with atopic (Pepys, 1975) asthma and rhinitis and nineteen patients with atopic rhinitis were selected for the study. All were, on the basis of previous investigations, known to show IgE-mediated allergy to at least one of the tested pollens.

Skin tests and test for specific IgE

Prick tests were performed with each allergen preparation in each patient. The prick

tests were evaluated as follows: if the mean weal diameter (MWD) was 3.0 mm or more, the test was regarded as positive (+), if the MWD was 1.0–2.9 mm the test was considered doubtful ($\pm$) and if the MWD was 0.9 mm or less the test was regarded as negative (–). Antihistamines were omitted 48 hr prior to skin testing.

Specific IgE for the allergens tested was determined in the Allergy Hospital, Helsinki, by the commercially available method (Phadebas® RAST, Pharmacia Diagnostics AB, Sweden). We modified the RAST score system described by the manufacturer to include the score 0.5, with which we described the specific IgE concentration in a sample giving a count rate at least twice the background, but less than that required for RAST score 1. Our 'background' is the lowest count rate given by a patient sample in a RAST series of at least fifty assays. For the present we will consider a RAST score of 1–4 as positive (+), 0.5 as borderline ($\pm$) and 0 as negative (–).

Provocation tests

Bronchial (BPT), nasal (NPT) and conjunctival (CPT) tests were performed as described elsewhere (Stenius-Aarniala *et al.*, 1978; Malmberg, Holopainen & Stenius-Aarniala, 1978).

Statistical methods

The four-fold table test and the χ^2 test were used for calculation of correlations between test methods. The difference in skin reactivity between the Dome, purified and crude extracts were analysed by means of the Friedman two-way analysis of variance.

Results

The MWD of the reactions elicited by different extracts of the same allergen are compared in Table 2. The MWD was calculated in all the cases in which a skin test with at least one of the three preparations of one allergen was positive. For birch and Timothy the purified extracts elicited smaller reactions than the crude extract ($P < 0.05$ and $P < 0.02$, respectively). The differences between the Dome extracts, on the one hand, and the purified or crude extracts, on the other, were not statistically significant. All three mugwort extracts appeared equipotent on skin testing.

Table 2. Mean diameters of weals elicited by skin reactions (MWD) obtained by three different extracts of birch, Timothy and mugwort, respectively. Cases with negative response to all three tests are excluded. Extracts marked with the same capital letter were equipotent on the basis of the direct RAST titration allergen assay

Allergen	Number of cases with positive response to at least one extract	MWD		
		Pharmacia		
		Dome	Purified	Crude
Birch	30	5.06 (A)	3.66 (A)	5.73 (A)
Timothy	35	7.43 (B)	6.7 (C)	8.6 (C)
Mugwort	46	5.36 (D)	5.38 (D)	5.17 (D)

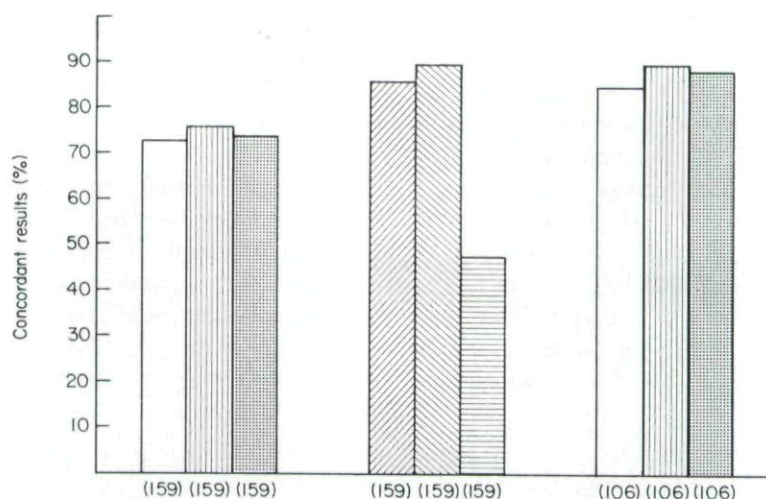


Fig. 1. Agreement between skin tests and RAST. Left-hand group: difference between Dome, Pharmacia purified and Pharmacia crude extracts, all tests included. Middle group: difference between allergens, all tests included. Right-hand group: same as left-hand group, mugwort excluded. Numbers in parentheses show numbers of tests. Extracts: (□) Dome, (▨) Pharmacia purified, (▩) Pharmacia crude. Allergens: (▧) birch, (▦) Timothy, (▥) mugwort.

The results of the skin tests are compared with the RAST in Fig. 1. Results were regarded as concordant if both skin test and RAST were positive, negative or doubtful, respectively. There were insignificant differences between the Dome, purified and crude extracts. When different allergens were analysed separately, however, mugwort differed strikingly from the two others ($P < 0.01$). For birch and Timothy, the agreement with RAST was nearly 90%, whereas skin tests with mugwort and mugwort-specific IgE in the serum determined by means of RAST were concordant in less than 50% of cases. The correlations between the extracts from different manufacturers and RAST were affected by the mugwort results, because when these were excluded, the agreement figure rose to nearly 90% for the purified and crude extracts from Pharmacia and to nearly 85% for the Dome extracts.

In Table 3 sixty-eight discordant skin test-RAST pairs with either negative or positive results are analysed. Most of the discrepant mugwort test pairs were

Table 3. Analysis of discrepant skin test-RAST results ($n = 68$)

Extract	Birch		Timothy		Mugwort	
	RAST(+) ST(-)	RAST(-) ST(+)	RAST(+) ST(-)	RAST(-) ST(+)	RAST(+) ST(-)	RAST(-) ST(+)
Dome	2	1	0	1	0	16
Pharmacia						
Purified	3	0	0	1	0	13
Crude	2	2	0	1	1	15
Total	7	3	0	3	1	54

ST = Skin test; (+) positive, (-) negative.

Table 4. Results of bronchial provocation test with three different allergen preparations in the same patient

Allergen	Allergen preparation and results of test					
	Dome		Pharmacia			
			Purified		Crude	
	Positive	Doubtful or negative	Positive	Doubtful or negative	Positive	Doubtful or negative
Birch (<i>n</i> = 5)	1	4	1	4	3	2
Timothy (<i>n</i> = 5)	2	3		5	5	
Mugwort (<i>n</i> = 6)	1	5	3	3	4	2
Total	4	12	4	12	12	4

represented by a negative RAST and positive skin test. There were another sixty-eight discrepant test pairs, each pair including a doubtful result, which are omitted from the table: twelve test pairs with birch pollen, seventeen test pairs with Timothy and thirty-nine test pairs with mugwort pollen. Two-thirds of these test pairs with mugwort were represented by a negative RAST and a doubtful skin test.

Bronchial provocation tests with all three preparations of the same allergen could be performed in one and the same patient in sixteen cases, a total of forty-eight tests (Table 4). The majority of positive reactions were elicited by the crude preparations. All the provocation tests with purified Timothy were negative, whereas the corresponding five tests with crude extract were positive. The crude preparations gave positive bronchial or conjunctival test results significantly more often than the other two preparations ($P < 0.01$ and $P < 0.05$, respectively). In the nasal reactions no such difference could be seen (Table 5).

The Phadebas RAST scale was changed to a more sensitive one after completion

Table 5. Results of nasal and conjunctival tests with three preparations of the same allergen in the same patient

Test	Allergen preparation and results of test					
	Dome		Pharmacia			
			Purified		Crude	
	Positive	Doubtful or negative	Positive	Doubtful or negative	Positive	Doubtful or negative
Nasal provocation test	29	9	30	8	33	5
Conjunctival provocation test	18	17	16	19	26	9

Table 6. Analysis of low score RAST values (<1 or 1) and corresponding skin tests

Allergen	Low grade RAST values (number of tests)	Skin tests					
		Pharmacia					
		Dome		Purified		Crude	
		(+)	(-)	(+)	(-)	(+)	(-)
Birch	5	4	1	3	2	4	1
Timothy	6	2	4	3	3	5	1
Mugwort	9	7	2*	8	1	7	2

(+) Positive (-) negative.

* One doubtful result.

of our study. It was, therefore, of interest to analyse the doubtful and score 1 RAST results in relation to the skin and provocation tests. These low score RAST results frequently corresponded to a positive skin test (Table 6). On most occasions low score RAST results corresponded to at least one positive provocation test, usually a nasal test.

Control provocation tests with crude extracts were performed in atopic patients with asthma and rhinitis, with no suspected or proven allergy to the allergen tested. Three BPTs and three NPTs with each of the crude allergens were all negative.

Discussion

A good or excellent correlation between the skin test results and amount of specific IgE in the serum has been demonstrated in many reports (Stenius *et al.*, 1971; Norman, Lichtenstein & Ishizaka, 1973; Havnen *et al.*, 1974; Bryant, Burns & Lazarus, 1975; Eriksson, Ahlstedt & Belin, 1976). The degree of this correlation, however, depends on the criteria chosen for skin test positivity. In some investigations, where skin tests have been claimed to be a poor diagnostic aid in comparison with *in vitro* methods, not enough attention has been paid to accurate evaluation of the skin reaction.

The Northern Society of Allergology recommends that skin test results should be evaluated in relation to histamine reactions (Aas & Belin, 1972). This system, however, does not determine the exact limits for positivity. In our investigation, so as to be able to compare several allergen preparations, it was necessary to express the skin reaction by means of mean weal diameter. The arbitrary limit (3.0 mm) chosen for skin test positivity was based on clinical observation and experience, with the commercial allergen solutions used as reference for the new extracts to be tested. Similar criteria of positivity were used by Bryant *et al.* (1975). Such criteria obviously vary, depending on the strength of the allergen solution used and on the technique of the prick tests, and cannot be regarded as absolute.

Extracts made equipotent on the basis of direct RAST titration *in vitro* could be expected to elicit similar reactions on skin-testing. In our investigation, however, this was so only with the three mugwort extracts. Our findings contradict those of Noster,

Schulz & Schröder (1977), who found that both the direct and the inhibition RAST-based allergen assay methods correlated excellently with *in vivo* methods. Several factors may have contributed to this discrepancy between *in vivo* and *in vitro* test results demonstrated in the present study. Timothy allergen is known to decompose easily into fragments. The freeze-drying process of an allergen after the *in vitro* potency estimations have been done may also change the allergenicity (Schröder, personal communication). After the completion of our study the purified birch extract used by us was found to differ distinctly from six other birch extracts, in that gel filtration showed that this particular extract was composed of polymerized birch pollen allergen (Belin, Lanner & Schröder, 1977). Extracts containing either aggregated or fragmented allergens, or lacking some components, will give different plateaux in the direct RAST procedure. The purified extracts of birch and Timothy used in this study contained some aggregated material, which resulted in higher plateau levels than for the crude extracts of the same allergens. The divergent result given in Tables 2 and 3 show that the direct RAST procedure was unsuitable in this case. In this context it also has to be pointed out that although the Dome aqueous birch extract was found to contain very little allergenic material as judged by the *in vitro* allergen assay, the extract elicited the same total number of positive provocation tests as did the purified birch extract. Belin *et al.* (1977) have shown that some of the difficulties encountered here can be overcome by employing the inhibition RAST procedure.

It has to be taken into consideration that the crude extracts may have elicited stronger reactions due to the amount of irritants present in the extract. Depending on the allergen, 30–50% of all skin tests with each extract were negative and negative provocation test results were obtained with the crude extracts in additional control patients. In view of this, we believe that the positive reactions obtained with the crude extracts can be regarded as reliable. In our study, however, a negative allergen provocation test control was not performed in each patient, as recommended by Aas (1975).

Our investigation in some respects reflects the situation in which the practising allergist may find himself today. The allergenicity of different batches of an allergen may vary. The allergens are provided by the manufacturers in stock solutions which cannot be concentrated by the consumer. The results of provocation tests depend on the allergen preparation used, and so does the diagnosis. As has been stressed by so many investigators before, there is an urgent need for commercial allergens standardized by means of reliable methods.

Although there were quantitative differences between skin test reactions elicited by different extracts, the differences were insignificant if consideration was given only to positive and negative skin test results, for instance in the comparison between skin test results and RAST. The overall correlation between skin tests and RAST was good. If different allergens were analysed separately, however, the RAST for mugwort-specific IgE showed a poor correlation with skin tests. Negative RASTs were frequently combined with positive skin tests, but not vice versa, which supports our clinical impression that the Phadebas RAST for mugwort is less sensitive than the RAST for other allergens.

The aim of our study was to investigate the reactivity of the different extracts, in view of the *in vitro* allergen assay. The patient material was selected and the number of provocation tests limited. Our findings indicate that extracts thought to be equipotent on the basis of a direct RAST titration allergen assay may produce unexpected

reactions *in vivo*, but the results do not allow a conclusion to be drawn about which preparation was best for diagnostic purposes. This requires further studies, which should include new allergen assays by means of indirect RAST and/or *in vivo* methods.

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