

Measurement of alum precipitated allergen extracts by RAST inhibition methods

E. PUTTONEN and J. MAASCH

*Hermal-Chemie Kurt Herrmann, Allergy Division,
Reinbek b. Hamburg, West Germany*

(Received 7 March 1980; accepted for publication 9 July 1980)

Summary

The feasibility of measuring the allergenic activities of alum precipitated pollen extracts by means of the radioallergosorbent test (RAST) was investigated. Different batches of aqueous and alum precipitated birch and mixed grass pollen extracts were used as antigens. The alum precipitates could be readily dissolved with 0.1 M EDTA (pH 9.0) without any loss in allergenicity. No significant differences in RAST activities of undissolved and dissolved alum precipitated allergen extracts could be detected in terms of ability to inhibit the binding of allergen specific IgE to solid-phase allergens.

Introduction

The radioallergosorbent test (RAST) (Wide, Bennich & Johansson, 1967; Wide *et al.*, 1971; Ceska, Eriksson & Varya, 1972; Gleich & Jones 1975a, b) is a sensitive immunoassay invaluable for the detection of allergen-specific IgE in sera of patients sensitive to allergens from various sources. Furthermore, the indirect RAST, or RAST inhibition assay (Aronsson & Wide, 1974; Gleich *et al.*, 1974) can be employed in assessing the allergenic activities of aqueous allergen preparations. In this indirect RAST the binding of allergen specific IgE to solid-phase allergens is inhibited by addition of free allergen into the test solution. The degree of inhibition can then be used to measure the biological activity exerted by the free allergen. The aim of this study was to investigate whether the indirect RAST could be applied to measure the allergenic activities of alum precipitated allergen extracts used in clinical desensitization therapy.

Materials and methods

Aqueous extracts

The purified aqueous extracts of birch pollen (*Betula verrucosa*) (PB) and mixed grass pollen (PG) were prepared by Sephadex filtration as described previously (Puttonen & Pilström, 1979). The mixture consists of equal parts of pollen from *Dactylis glomerata*,

Correspondence: Dr E. Puttonen, Hermal-Chemie Kurt Herrmann, Allergy Division, Postfach 1228/9, 2057 Reinbek b. Hamburg, West Germany

Festuca pratensis, *Holcus lanatus*, *Lolium perenne*, *Poa pratensis* and *Phleum pratense*. The purified extracts contained allergens with molecular weights (mol. wt) of 10 000 to 50 000 daltons. The whole mixed grass pollen extract (WG) was dialysed to remove the bulk of low mol. wt components.

Alum precipitated extracts

PB and PG were adsorbed on aluminium hydroxide by Hermal-Chemie, Reinbek. These alum precipitates are hereafter referred to as PBA and PGA, respectively. The alum precipitates of whole birch pollen extracts (WBA), whole mixed grass pollen extracts (WGA) and pyridine treated mixed grass pollen extracts (PTGA) were commercial products from two different manufacturers. The sources of extracts will be specified on request.

Protein and carbohydrate determinations

The protein contents of the aqueous and alum precipitated extracts were determined according to Lowry *et al.* (1951) and Bensadoun & Weinstein (1976). The aqueous and alum precipitated extracts were measured both with and without a prior treatment of 0.1 M sodium-ethylenediaminetetraacetate (EDTA), pH 9.0 (E. Merck, West Germany). Since the commercial allergen preparations contain 0.4% phenol as preservative, which interferes strongly with the applied method, the samples were precipitated with 9% trichloroacetic acid (TCA) and the precipitates were washed once with 9% TCA prior to protein determination. In preliminary experiments it was found that a second TCA-washing of the precipitates yielded no further reduction of the absorbance at 660 nm. The carbohydrate contents were determined by the anthrone method (Scott & Melvin, 1953) again with and without prior EDTA treatment of the extracts.

RAST-inhibition assay

Allergens of whole birch pollen and whole mixed grass pollen extracts were coupled on BrCN-activated paper discs (Blauband, 589/3, Schleicher & Schüll, West Germany) by the method of Ceska *et al.* (1972). Two pools of sera from birch-pollen and grass-pollen sensitive patients were used for the tests. The pools contained sera from eighteen and twenty patients, respectively. The RAST-inhibition assays (RAST-I) were performed in duplicate as follows.

(1) Allergen extracts were diluted with 0.15 M NaCl plus 0.02% NaN₃ or with 0.1 M EDTA pH 9.0 to appropriate concentrations (10 to 100 µg protein per ml). The alum precipitate-EDTA-mixtures were allowed to stay for 15 min at room temperature in order to dissolve the alum precipitates. Fifty µl of the solutions were added into 12 × 55 mm polystyrol test tubes (Pharmacia, Sweden) followed by 50 µl of pool serum, which was diluted 2:3 with 0.15 M NaCl plus 0.05% NaN₃. The tubes were incubated for 2 h at 37°C.

(2) One allergen coupled paper disc was added into each tube and the tubes were incubated overnight at room temperature.

(3) Following the overnight incubation the paper discs were washed threefold with 2.5 ml of 0.15 M NaCl plus 0.05% NaN₃ with 10 min incubations at room temperature.

(4) Fifty µl of rabbit ¹²⁵I-anti-human-IgE (Pharmacia, Sweden), corresponding to approx. 28 000 ct/min, were added into each tube followed by a second overnight incubation at room temperature.

(5) Paper discs were washed with 4 × 2.5 ml of 0.15 M NaCl plus 0.05% NaN₃ with

10 min incubations and counted by a gammacounter for bound radioactivity.

Parallel to the samples, tests were run on tubes with only paper discs and 50 μ l ^{125} I-anti-human-IgE and tubes without allergens for the determination of unspecific and 0% inhibition, respectively.

Calculations

The percentage of RAST-inhibition was calculated with the formula

$$\text{Percentage inhibition} = \left[1 - \frac{C_X - C_{\text{NSB}}}{C_O - C_{\text{NSB}}} \right] \times 100$$

C_X = ct/min bound by the sample

C_{NSB} = non-specifically bound radioactivity

C_O = ct/min bound in the absence of free allergen.

To compare the different extracts, the protein concentrations were plotted against percentage inhibition in a logit-log system (Aronsson & Wide, 1974). For the statistical evaluation, the 2-tailed *t*-test was used.

Results

Influence of EDTA and Al(OH)₃ on protein and carbohydrate determinations

The protein concentrations of the aqueous and alum precipitated extracts were determined both with and without a prior dissolving of the samples in EDTA-solution. To evaluate the effect of Al(OH)₃ alone, undissolved and by EDTA-solution dissolved Al(OH)₃ was included in the tests. All alum precipitates tested dissolved within 15 min after adding EDTA, by forming a clear, homogenous solution. The protein and carbohydrate determinations were not influenced by the presence of EDTA (Table 1). Furthermore, neither undissolved nor dissolved aluminium hydroxide interfered with the determinations.

Influence of EDTA and Al(OH)₃ on RAST-I

The treatment of aqueous and alum precipitated extracts with 0.1 M EDTA did not increase or decrease the percentage of RAST-inhibition significantly ($P < 0.05$) (Figs 1–3). Similarly, there were no significant ($P < 0.05$) differences in slopes of the regression lines of extracts before or after dissolving with EDTA, with the exception of dissolved PGA, which showed a significantly ($P < 0.05$) steeper slope after dissolving with EDTA (Fig. 3). The presence of Al(OH)₃ in test solutions did not cause any unspecific inhibition in RAST-I, as revealed by running unadsorbed Al(OH)₃ parallel to the adsorbed samples (data not shown).

Comparison of the different allergen preparations by RAST-I

The purified aqueous extracts PB and PG possessed both significantly ($P < 0.05$ and $P < 0.01$, respectively) more allergenic activity per weight protein than the corresponding alum precipitates and whole extracts (Figs 1–3). All aqueous and alum precipitated whole extracts required significantly ($P < 0.05$) higher protein concentrations than aqueous purified extracts to achieve 50% inhibition in RAST (Table 2). Interestingly, also the alum precipitated purified extracts required higher protein concentrations to achieve 50% inhibition than the aqueous purified extracts. The protein concentrations

Table 1. Protein and carbohydrate determinations of birch and mixed grass pollen extracts by the Folin phenol reagent and anthrone methods, respectively. The protein determinations and three of the carbohydrate determinations were performed both before and after treating the samples with 0.1 M EDTA pH 9.0 (+EDTA). The protein determinations were performed after precipitation of the samples with 9% TCA. The results are mean (m) results with standard deviations (s) from four double-determinations. ND=not done.

	Protein/ml (μ g)		Carbohydrate/ml (μ g)	
	m $\pm$ s	+ EDTA m $\pm$ s	m $\pm$ s	+ EDTA m $\pm$ s
Al(OH) ₃	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
PB	5880 $\pm$ 509	6160 $\pm$ 435	1771 $\pm$ 52	ND
PBA	690 $\pm$ 71	679 $\pm$ 46	11 $\pm$ 2	ND
WBA	680 $\pm$ 10	612 $\pm$ 26	58 $\pm$ 4	ND
PG	810 $\pm$ 25	ND	249 $\pm$ 14	ND
PGA	540 $\pm$ 28	600 $\pm$ 42	52 $\pm$ 7	58 $\pm$ 4
WG	528 $\pm$ 30	530 $\pm$ 35	1176 $\pm$ 26	ND
WGA	198 $\pm$ 20	185 $\pm$ 14	27 $\pm$ 6	34 $\pm$ 4
PTGA	702 $\pm$ 13	653 $\pm$ 55	39 $\pm$ 4	ND

PB = aqueous purified birch pollen extract.

PBA = alum precipitated purified birch pollen extract.

WBA = alum precipitated whole birch pollen extract.

PG = aqueous purified mixed grass pollen extracts.

PGA = alum precipitated purified mixed grass pollen extract.

WG = aqueous whole mixed grass pollen extract.

WGA = alum precipitated whole mixed grass pollen extract.

PTGA = alum precipitated pyridine treated mixed grass pollen extract.

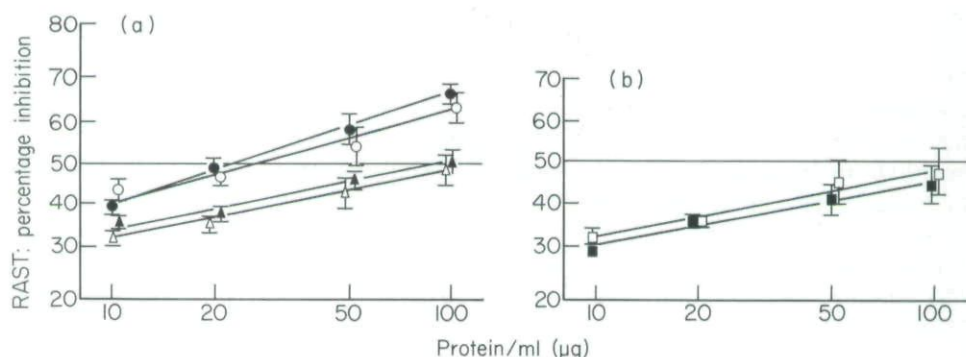


Fig. 1. RAST-inhibition assay of aqueous and alum precipitated birch pollen extracts. Assays were performed as described under Methods. (a) ●, Aqueous purified extract (PB); ○, aqueous purified extract + EDTA; ▲, alum precipitated purified extract (PBA); △, alum precipitated purified extract + EDTA. (b) ■, alum precipitated whole extract (WBA); □, alum precipitated whole extract + EDTA. The vertical bars are standard deviations of three double-determinations.

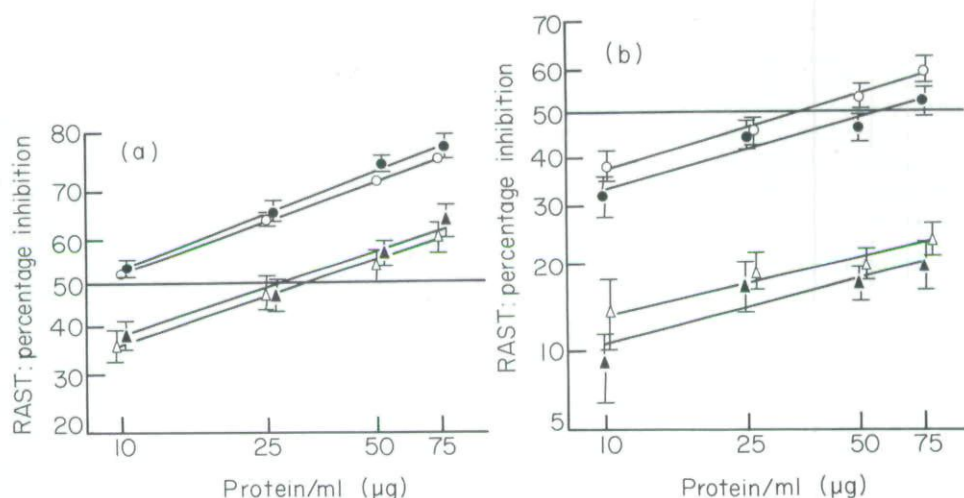


Fig. 2. RAST-inhibition assay of aqueous and alum precipitated mixed grass pollen extracts. Assays were performed as described under 'Methods'. (a) ●, Aqueous purified extract (PG); ○, aqueous purified extract + EDTA; ▲, aqueous whole extract (WG); △, aqueous whole extract + EDTA. (b) ● alum precipitated whole extract (WGA); ○ alum precipitated whole extract + EDTA; ▲ pyridine treated alum precipitated extract (PTGA); △ pyridine treated alum precipitated extract + EDTA. The vertical bars are standard deviations of 4 double-determinations.

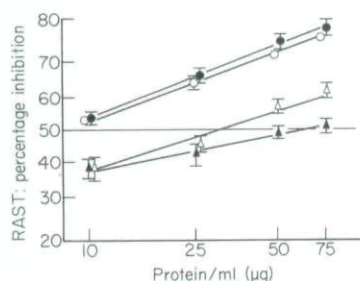


Fig. 3. RAST-inhibition assay of aqueous and alum precipitated purified mixed grass pollen extracts. Assays were performed as described under Methods. ●, aqueous purified extract (PG); ○, aqueous purified extract + EDTA; ▲, alum precipitated purified extract (PGA); △, alum precipitated purified extract + EDTA. The vertical bars are standard deviations of four double-determinations.

were comparable to those required from whole extracts. There were no significant ($P < 0.05$) differences between alum precipitated purified and whole extracts, neither treated nor untreated with EDTA prior to the assay. The only exception is PTGA (Fig. 2b). This pyridine treated grass pollen extract showed a significantly ($P < 0.02$) lower RAST-activity than all other preparations of mixed grass pollen. The tested protein concentrations of PTGA were too low to achieve 50% inhibition in RAST. The value of approx. 2000 μg protein/ml for 50% inhibition (Table 2) was estimated by extrapolation. Furthermore, the slopes of the regression lines of the undissolved and dissolved PTGA were significantly ($P < 0.05$) lower than those of all other extracts, again with the exception of the regression line slope of the undissolved PGA. No significant ($P < 0.05$) differences were found between the slopes of the regression lines of the other mixed grass pollen preparations. By comparing the slopes of the regression lines of

Table 2. Protein concentrations of different birch and mixed grass pollen extracts required for 50% inhibition by RAST-inhibition assay. The protein values for PTGA are theoretical, extrapolated concentrations. For explanations see Table 1.

Extract	Protein/ml (μ g)	Protein/ml (μ g) + EDTA
PB	25	28
PBA	90	130
WBA	190	120
PG	8	8
PGA	66	32
WG	28	32
WGA	58	34
PTGA	1700	2300

different birch pollen extracts, only the slope of PB was found to deviate significantly ($P < 0.05$) from the other slopes.

To evaluate the possible contribution of free allergen in the supernatants of the alum-precipitated extracts to the assay, samples of the different alum-precipitated extracts were centrifuged and aliquots of the undiluted supernatants were analysed. No protein or carbohydrate could be detected with the methods employed. The RAST-activities of the undiluted supernatants were in the range of unspecific inhibition, varying between 0 and 10% inhibition.

Discussion

The aim of this study was to investigate the possibility of measuring the allergenic activities of different allergen alum precipitates by RAST-inhibition assay. In addition, a simple method for dissolving the alum precipitates prior to the assay without reducing the allergenic activities of extracts was developed. The data presented indicate that dissolving of alum precipitates of pollen allergens, yields no significant improvement in percentage inhibition to RAST-I. However, the regression line of one of the dissolved alum precipitates, i.e. PGA, showed a significantly ($P < 0.05$) steeper slope than the slope of the regression line of undissolved PGA. Thus, a prior dissolving with EDTA is recommended. The aqueous purified extracts PB and PG possessed a higher allergenic activity per weight protein than the corresponding alum precipitates. The difference cannot be explained by a possible masking of allergenic determinants due to the adsorption of allergens on aluminium hydroxide, as there was no improvement in inhibition after dissolving the alum precipitates with EDTA. The fact that PG showed a significantly higher allergenic activity than WG, suggests a higher concentration of allergenic proteins in PG than in WG. On the other hand, the alum precipitates of the purified extract showed no higher allergenic activities than the alum precipitates of the whole extracts. The significantly lower RAST-I activity of PTGA, compared to the activity of all other preparations of mixed grass pollen, indicates that the pyridine treatment results in a strong reduction of RAST activity of the extract.

Acknowledgments

We gratefully acknowledge Mrs Renate Müller-Brechlin for her assistance during the study.

References

- ARONSSON, T. & WIDE, L. (1974) Studies on allergens of horse epithelium with two variants of RAST. *International Archives of Allergy* **47**, 224.
- BENSADOUN, A. & WEINSTEIN, D. (1976) Assay of proteins in the presence of interfering materials. *Analytical Biochemistry*, **70**, 241.
- CESKA, M., ERIKSSON, R. & VARGA, J.M. (1972) Radioimmunosorbent assay of allergens. *Journal of Allergy and Clinical Immunology*, **49**, 1.
- GLEICH, G.J. & JONES, R.T., (a) (1975) Measurement of IgE antibodies by the radioallergosorbent test. I. *Journal of Allergy and Clinical Immunology*, **55**, 334.
- GLEICH, G.J. & JONES, R.T. (b) (1975) Measurement of IgE antibodies by the radioallergosorbent test. II. *Journal of Allergy and Clinical Immunology*, **55**, 346.
- GLEICH, G.J., LARSON, J.B., JONES, B.S. & BAER, H. (1974) Measurement of the potency of allergy extracts by their inhibitory capacities in the radioallergosorbent test. *Journal of Allergy and Clinical Immunology*, **53**, 158.
- LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951) Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*, **193**, 265.
- PUTTONEN, E. & PILSTRÖM, L. (1980) Purification of birch pollen allergen extract by gel filtration. *International Archives of Allergy and Applied Immunology*, **61**, 299.
- SCOTT, T.A. & MELVIN, E.H. (1953) Determination of dextran with anthrone. *Analytical Chemistry* **11**, 1956.
- WIDE, L., ARONSSON, T., FAGERBERG, E. & ZETTERSTRÖM, O. (1971) Radioimmunoassay of allergen specific IgE. In: 'Proceedings of the 8th European Congress of Allergology', *Excerpta Medica*, **215**, 85.
- WIDE, L., BENNICH, H. & JOHANSSON, S.G.O. (1967) Diagnosis of allergy by an *in vitro* test for allergen antibodies. *Lancet*, **ii**, 1105.

This document is a scanned copy of a printed document. No warranty is given about the accuracy of the copy. Users should refer to the original published version of the material.