

A collaborative study on RAST inhibition using a common method

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Summary

A collaborative study was carried out to determine the reproducibility of the inhibition form of the radioallergosorbent test (RAST). Seven laboratories were provided with three coded extracts of cocksfoot (*Dactylis glomerata*) pollen. All laboratories used a common method of RAST inhibition and were supplied with all reagents required to carry out the assays. In addition, four laboratories used local methods of RAST inhibition. Potency estimates were in reasonable agreement between laboratories when the study method was used but insufficient data were available to assess reproducibility when local methods were used.

Two extracts prepared from the same source material were found to have parallel log dose-response lines but the line for the unrelated extract was steeper in all but one of the assays. This finding was an indication that the third extract differed qualitatively from the other extracts.

It was concluded that the use of a study method of RAST inhibition resulted in good agreement between potency estimates obtained by different laboratories.

Introduction

The description in 1967 of the radioallergosorbent test (RAST) for the *in vitro* detection of allergen-specific IgE antibodies completely changed the diagnosis of type-I hypersensitivity in allergic disease (Wide, Bennich & Johansson, 1967). Subsequently, the inhibition of this reaction by specific antigens was used for the assay of allergen extracts (Ceska & Lundkvist, 1972; Gleich *et al.*, 1974; Yman, Ponterius & Brandt, 1975; Gleich *et al.*, 1976; Yunginger, Jones & Gleich, 1976). Several laboratories then began to use RAST inhibition as a method for comparing commercially available allergen extracts. These studies were performed on a variety of allergens, including extracts of ragweed, June grass, timothy and birch pollen and of *Alternaria*. Potencies of extracts of the same allergen obtained from various sources, including commercial ones, were compared by RAST inhibition and a considerable range of potencies was found (e.g. Gleich *et al.*, 1976; Yunginger *et al.*, 1976).

This wide range in potencies and the growing concern about standardization and batch consistency of allergen extracts led a number of countries to begin to rationalize their systems for allergen control. In the Nordic countries a small panel of experts, under the Nordic Council on Medicines, produced the Nordic Guidelines for the Registration of Allergen Preparations (Nordic Council on Medicines, 1980), whilst in the U.S.A. the Food and Drug Administration (FDA) established a panel to review allergenic extracts which produced a comprehensive report (Food and Drug Administration, 1981). In the U.K. the Allergens Technical Working Group was set up to enable a series of meetings between the National Institute for Biological Standards and Control (NIBSC) and manufacturers to take place where agreement could be reached on, among other things, methods applicable to the potency testing of allergens. At these meetings a series of collaborative studies were planned with the aim of assessing the suitability of RAST inhibition for testing the consistency of batches of allergen extracts from individual manufacturers.

Initially, doubts were raised as to the reproducibility of this assay method in different laboratories. Accordingly, a collaborative study was set up to investigate this point. This study was to be the first of a series to investigate various aspects of RAST inhibition.

The main aim of the study was to investigate whether laboratories, using a common set of reagents and a common assay method, would obtain similar results. The secondary aims were to examine whether the dose-response curves obtained from freeze-dried extracts were comparable with those obtained from an extract stored as a glycerol/saline solution, and to compare potency estimates obtained when the study method and supplied reagents were used with those obtained when laboratories used their own methods and reagents.

Materials and methods

Allergen extracts

Extract A was the first British Standard for cocksfoot (*Dactylis glomerata*) pollen extract. The bulk material had been produced by the extraction of 600 g of cocksfoot pollen in 6 l of phenol-saline for 24 hr at neutral pH, followed by extensive dialysis against distilled H₂O at 4°C and sterile filtration. The extract thus obtained was dispensed into ampoules in volumes of 1 ml and freeze dried by the Standards Processing Section, NIBSC (Campbell, 1974); each ampoule contained 6.44 mg of the material. The ampoule contents were arbitrarily assigned 100 units of activity.

Extract B was a cocksfoot pollen extract supplied by a commercial source outside the U.K. as a 50% v/v glycerol/saline solution.

Extract C was made by reconstituting the contents of ampoules of extract A and diluting them 1 in 3 with phosphate buffered saline (PBS). The diluted extract was then freeze dried in volumes of 1 ml.

Antiserum

The antiserum was from a pool of human sera with IgE antibodies to cocksfoot pollen. It was ampouled and freeze dried at NIBSC and stored at -20°C. In the antisera pool no hepatitis B antigen had been detected when tested by radioimmunoassay.

Negative control serum

Instead of a serum which had no IgE antibodies, human serum albumin (HSA) at 5% w/v was used as the negative control in the assay. It was obtained from the Lister Institute, U.K. and freeze dried in volumes of 1 ml.

Allergen discs

Paper discs (Schleicher & Schüll No 589/3) were activated with cyanogen bromide after the method of Ceska, Eriksson & Varga (1972), and sensitized with the first British Standard for cocksfoot pollen extract. Unreacted sites were blocked with EPA 33 (0.1 M dipropylenetriamine in 1 N NaOH + 1.8 mEq of epichlorohydrin at pH 7.0).

Labelled anti-human IgE

The anti-human IgE, labelled with ^{125}I was generously donated by Pharmacia (Uppsala, Sweden).

Participants

Seven laboratories from five countries participated in this study. Throughout this report laboratories are identified by a code number. The laboratories are listed in the Appendix, but the order in which they appear does not necessarily correspond with the numerical order of the code numbers.

Design of the study

The Immunology Section, NIBSC, was the coordinating laboratory and all the materials were distributed from this laboratory. Participants were requested to assay the three allergen extracts using the study method described below and in addition they were invited to use their own preferred method of RAST inhibition if they so wished. Each participating laboratory was sent sufficient reagents (antiserum, human serum albumin, paper discs and labelled anti-IgE) to perform at least two assays. They were asked to return the data to NIBSC for statistical analysis.

Study method

The assays were performed using five doses of allergen extract in the wells of the microtitre plates. Freeze-dried allergen extracts contained in ampoules were reconstituted by the addition of 1 ml of PBS. For the top two doses, volumes of 100 and 25 μl of the reconstituted material were pipetted into the microtitre plate; for the remaining three doses, volumes of 100, 20 and 5 μl of a 1 in 20 dilution in PBS of the ampoule content were transferred to the microtitre plate. This gave five doses equivalent to 100, 25, 5, 1 and 0.25 μl of the original reconstituted material. The volume contained in each well was made up to 100 μl with PBS. An additional well containing no allergen extract but 100 μl of PBS was included to estimate zero inhibition. One hundred microlitres of the antiserum diluted 1 in 10 was then added to each well.

To assess the level of any non-specific background effects, negative controls were included. The controls were obtained by adding 100 μl of HSA (instead of the positive antiserum) prediluted 1 in 10, to wells containing doses of the individual extracts prepared as mentioned above.

The microtitre plates containing the allergen and serum mixtures were incubated at room temperature for 15 min, a disc was added to each well and the plates incubated overnight at room temperature. The serum was aspirated and the discs washed with

washing buffer (PBS containing 1% Tween 20) six times for 3 min each time. Fifty microlitres of ^{125}I anti-human IgE were added to each well and the plates left overnight at room temperature. The labelled antiserum was removed by aspiration and the discs were washed in washing buffer as before. The counts per minute on the discs were found by using a gamma or scintillation counter.

Local methods

Four laboratories carried out some assays using local methods. Laboratories 1 and 2 did not give information on how their local method differed from the study method. Laboratory 4 used its own atopic serum pool, the paper discs were sensitized with their own extract of *D. glomerata* and a different batch of anti-IgE was used. Laboratory 6 used paper discs sensitized with their own extract of *D. glomerata* but apart from this, the study method was used.

Statistical analysis

For all assays, the means of the radioactive counts obtained for the negative serum (N) in the presence of dilutions of the extracts, and by PBS with the positive serum but no extract i.e. 'zero inhibition' (Z), were each calculated. The counts (C) obtained for different doses of the extracts were then expressed as 'percentage inhibition' by the formula:

$$\text{percentage inhibition} = \frac{Z - C}{Z - N} \times 100$$

These values were then plotted on an arithmetic, log or logit scale against log dose but in no case were the relationships found to be both linear and homoscedastic over the entire range of percentage inhibition (0–100%). However, for most assays a range of percentage inhibition could be found such that the relationships between log dose and percentage inhibition were reasonably linear and three doses for each extract were included in the calculation. The values fell within the range of 15–80% inhibition. Data in this range were then analysed as parallel line assays relating percentage inhibition to log dose (Finney, 1978). Analysis of variance was used to test the significance of the departures of the relationships from linearity and from parallelism. For all the assays, potencies for preparations B and C were calculated relative to extract A.

Potency estimates were combined by taking their geometric mean. The variation between the individual potency estimates was calculated as the geometric coefficient of variation (Kirkwood, 1979).

The variation between potency estimates obtained for a single preparation can be split into two components: the assay to assay variation within individual laboratories and the variation between laboratories. Differences between laboratories can be due to different assay methods, reagents, equipment, experimental procedures and environment. In this study, a common method of RAST inhibition and common reagents were used. Analysis of variance (of log potencies) was used to assess whether the differences observed between laboratories using the study method were significant or whether they could be attributed solely to the assay to assay variation within laboratories.

Data were also examined by transforming the values for percentage inhibition to logits before analysis as parallel-line assays using weighted linear regression. This was done because some laboratories routinely analyse their data in this way. This method has the potential advantage that all the data can be included in the analysis. The

potency estimates obtained were in good agreement with the estimates obtained from the original analysis. However, there was a higher proportion of assays with significant departures from linearity. In most cases this was due to curvature at extreme values of percentage inhibition. Omitting these responses would negate the main reason for using this method of analysis. Because of this, no further analysis by this method was done and only the results of the original unweighted analysis have been presented.

Results

Seven laboratories contributed a total of eighteen assays; thirteen assays were performed using the study method and five assays using local methods. Laboratory 7 contributed data from only one assay. In the first assay of laboratory 5 and in the assays of laboratory 6, some of the extracts were prediluted before preparing the final doses used in the assays.

Apart from the local assays performed by laboratory 4, the average counts obtained for the negative serum, expressed as percentages of the counts obtained for zero inhibition, were fairly low; they ranged from 3–9% (Table 1). The ranges of responses obtained for extracts A and C were similar to each other but different from those for extract B. The second assay performed by laboratory 3 gave anomalous responses for extract C, probably due to a technical error, so this assay was analysed excluding data for this extract.

Table 1. Values obtained for the negative serum expressed as a percentage of the counts obtained for zero inhibition and the ranges of percentage inhibition obtained for extracts A, B and C

Assay method	Lab. No.	Assay No.	Negative serum (%)	% inhibition		
				A	B	C
Study	1	1	4.5	55–100	8–94	40–97
		2	2.9	26–98	7–86	26–96
		3	6.1	41–100	8–89	31–96
	2	2	6.6	46–100	11–90	–
		3	5.5	54–100	29–92	38–96
		4	5.2	23–98	0–86	6–95
	3	2	5.4	28–92	0–85	12–95
		5	5.2	54–100	22–94	50–97
	4	2	7.1	21–82	21–93	17–69
		6	8.5	0–89	0–56	0–77
		2	6.2	48–100	4–91	38–96
	5	3	6.7	17–87	16–92	22–90
		7	4.5	41–100	30–92	29–97
Local	4	1	4.6	0–86	0–68	0–85
		2	6.0	36–100	0–88	15–97
		4	11.8	17–58	15–76	9–65
		4	13.6	24–75	6–84	5–59
		6	6.6	53–100	15–95	47–100

Table 2. Potency ratios in terms of extract A obtained for extract B (based on all data) and for extract C (based on data for extracts A and C only) for individual assays

Assay method	Lab. No.	Assay No.	Potency ratio	
			B	C
Study		1	0.056*	0.30
		2	0.095*	0.56
		3	0.053*	0.28
		2	0.060	—
		3	0.058	0.18
		4	0.070*	0.35
		2	0.061*	0.28
		5	0.075*	0.57
		2	0.063	0.49
		6	0.041*	0.23
		2	0.056*	0.38
		3	0.052	0.30
		7	0.110*	0.37
Local		1	0.027	0.30
		2	0.016†	0.39
		4	0.053	0.36
		4	0.050	0.30
		6	0.048	0.30

* Deviations from parallelism significant at 1% level.

† Deviations from parallelism and from linearity both significant at the 1% level.

Statistical validity of assays and estimation of relative potencies

Extract C was a dilution of extract A, so it contained the same active constituents and had the same mode of action as extract A. It was, therefore, expected that the two extracts would give comparable dose-response curves. When data from these two extracts alone were considered satisfactory parallel line assays were obtained. Individual estimates of potency are listed in Table 2 and their frequency distribution is shown in Figure 1(a).

In addition, assay data for all three extracts were analysed as multiple parallel-line assays. The inclusion of extract B resulted in significant non-parallelism of the regression lines in ten of the eighteen assays (one assay from laboratory 2 (local method) also had significant non-linearity). Furthermore, examination of the slopes of the individual regression lines of all assays revealed that in all but one of them the slopes for the regression lines for extract B were steeper than those obtained for extracts A and C. Consequently, potency estimates from all the assays may be considered invalid, nevertheless all estimates were included in the following analysis.

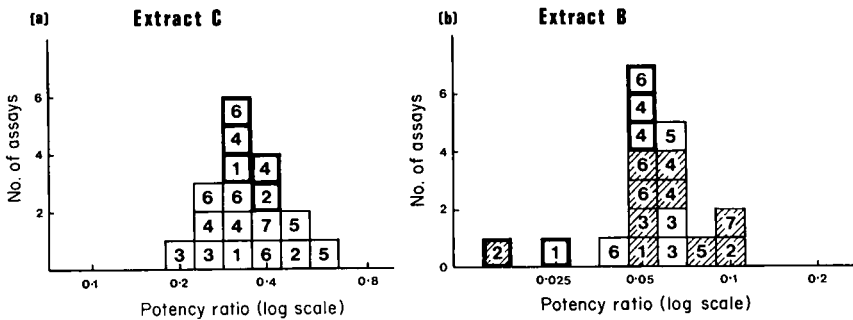


Fig. 1. Frequency distributions of potency ratios expressed in terms of extract A. Each square represents the potency estimate from one assay and the number in the square refers to the code number of the laboratory. □, study method; ▨, local method; ▩, statistically invalid assay.

For all assays the potency estimates of B in terms of A are also given in Table 2 and their frequency distribution is shown in Figure 1(b).

Comparison of potency estimates obtained by the study method

For each extract, the variation between potency estimates obtained was examined by analysis of variance. For both extracts B and C, no statistically significant differences were found between laboratories. However, the analysis was based on a limited amount of data—three of the laboratories performed only a single assay using the study method—and therefore only large differences would have been detected.

Geometric means of the potency ratios obtained from the individual assays for B and C respectively were 0.063 and 0.34 (remarkably close to the expected value of 0.33); their geometric coefficients of variation were 29% and 42%.

Comparison of potency estimates obtained using local methods

Five assays were carried out by four laboratories using local methods. Potency estimates for extract C obtained from these assays were in good agreement with those obtained from assays where the study method was used (Fig. 1(a)).

Although potency estimates for extract B obtained from the local assays of laboratories 4 and 6 were in agreement with those obtained by the other methods, the estimates obtained by laboratories 1 and 2 were lower than the others (Fig. 1(b)). Unfortunately, the latter assays had not been replicated, so it was not possible to assess the significance of this finding.

Discussion

Extract C was a dilution of extract A and, as expected, gave log dose-response relationships that were comparable with those of extract A in all assays. Potency estimates obtained from assays in which the study method was used gave good agreement between laboratories. In addition the estimates obtained from assays in which local methods were used were in good agreement with those obtained when the study method was used (Fig. 1(a)).

In all but one of the assays, the log dose-response lines obtained for extract B had a steeper slope than those of extracts A and C. This was an indication that extract B was not behaving as if it were a simple dilution of extract A, so it was not valid to express the

potency of extract B relative to extract A. Extract B differed in several respects from extract A (and C): it was in a glycerol/saline solution whereas extracts A and C were in freeze-dried form; it was prepared by a different laboratory, and it would almost certainly have been prepared from a different batch of pollen. Several participants have commented that, in their experience, the presence of a glycerol/saline solution did not have any effect on the dose-response curves of allergen extracts. Thus the lack of comparability of the coded extracts was probably due to some other qualitative differences between them.

Even though the potency estimates obtained for extract B were invalid, there was good agreement between assays in which the study method was used (Fig. 1(b)). This is not as surprising as it might at first appear since many of the possible sources of variation between laboratories had been eliminated by the use of a study method and common reagents. However, the inclusion of estimates from assays using local methods gave a small increase in variation between the potency estimates (Fig. 1(b)). In contrast, the potency estimates from local methods for extract C were in good agreement with those from the study method (Fig. 1(a)). It is a common finding that when preparations have qualitative differences, the use of different assay procedures can result in differences between potency estimates. It would appear that this might have happened, to a small extent, for the potency estimates of extract B relative to extract A.

This study was the first in a series planned to investigate the suitability of RAST inhibition to determine the potency of allergen extracts. The combination of a detailed study method and the supplying of all the reagents necessary to perform RAST inhibition resulted in close agreement between laboratories. Furthermore, the variation between potency estimates obtained from assays performed by different laboratories was no greater than between estimates obtained from replicate assays performed by individual laboratories.

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Appendix

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