

Brief communication

Brief report: Evaluation of polymerized ragweed extracts by intradermal end point titration, RAST inhibition, and parallel line bioassay

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We have evaluated six polymerized ragweed extracts on the basis of RAST inhibition, parallel line bioassay, and intradermal end point titration. The results of the parallel line bioassay and intradermal end point titration are highly correlated ($r = 0.995$, $df = 2$, $p < 0.01$). Both intradermal end point titration ($r = 0.973$, $df = 4$, $p < 0.01$) and parallel line bioassay ($r = 0.985$, $df = 2$, $p < 0.05$) are highly correlated with RAST inhibition. We have evaluated the utility of intradermal end point titration as a quantitative assay of relative allergenicity of polymerized extracts. The between assay reproducibility was derived by comparing a reference polymerized ragweed extract against itself. The 99% confidence limits of relative allergenicity were 53% to 188% when four assays were used. This compares favorably to the published 99% confidence limits of the parallel line bioassay 60% to 166% when three assays were used. Intradermal end point titration is highly reproducible, is highly correlated with RAST inhibition or parallel line bioassay, and thus is an appropriate bioassay of allergenic activity. (J ALLERGY CLIN IMMUNOL 76:123-7, 1985.)

Methods for standardization of allergenic extracts have been the subject of many articles and reviews.^{1, 2, 3} The demonstration that the quality of allergenic extracts is comparable to that of appropriate reference preparations or standards has been hampered by the lack of criteria for accepted reference standards.¹ PRW is rather unique in that two lots, each of which has been demonstrated to be efficacious and safe, are available as standards.² These standards have been characterized by chromatography, radioimmunoassay inhibition, and intradermal end point titration. These characteristics could be used to develop secondary standards.

Various methods have been proposed to standardize extracts. Among these are an in vitro method, RAST inhibition,⁴ and an in vivo method, parallel line bioas-

Abbreviations used

PRW: Polymerized ragweed
RA: Relative allergenicity

say,⁵ for standardization of allergenic extracts. These methods have been demonstrated to correlate in aqueous extract evaluation.⁴

We wished to determine the utility of intradermal end point titration and parallel line bioassay in the characterization of PRW. Specifically, we compared the reproducibility of the parallel line bioassay method to that of intradermal end point titration. We also compared those two bioassay methods in terms of correlation with an in vitro assay, RAST inhibition. Finally, we compared the two bioassay methods in terms of subject acceptance, time, and materials used.

MATERIAL AND METHODS

PRW extracts

Two batches of PRW extract, A and B, prepared at Northwestern University, have demonstrated clinical efficacy^{6, 7} and can be used as standards in comparison with other PRW extracts. Four other batches of PRW extract, C, D, E, and

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TABLE I. Intradermal end point titration of reference PRW batch B

Subject		End point titer				SD
1	0*	0	0.5	0	0	0.22
2	2.0	2.0	2.5	2.0	1.5	0.35
3	1.5	2.0	1.5	1.5	—	0.25
4	1.5	1.5	1.0	1.0	1.5	0.27
5	2.5	3.0	—	—	—	0.35
6	2.5	3.0	2.5	—	—	0.29
7	3.5	3.0	—	—	—	0.35
8	3.5	3.5	—	—	—	0
9	4.5	4.5	—	—	—	0

$\bar{x}_{SD} = 0.23$; $SD_{SD} = 0.14$; $SEM_{SD} = 0.047$.

*Log of the last dilution to result in a positive reaction.

TABLE II. Ninety-nine percent confidence limits of geometric mean of RA by intradermal end point titration method*

No. of subjects	RA lower limit (%)	RA upper limit (%)
3	34	292
4	53	188
5	61	164

*Confidence intervals were obtained by use of the t distribution.

F were made at Allergy Laboratories of Ohio (ALO, Columbus, Ohio). Methods for manufacture of PRW are delineated elsewhere.⁸ The final molecular weight fractionation of batches A and B was performed on Sephadex G-200 Superfine, whereas the fractionation of batches C, D, E, and F was performed on Sephacryl S-200.

Subjects

The subjects consisted of 18 patients with classic ragweed pollinosis and >5 mm wheal and >10 mm erythema from a prick test with 1:20 short ragweed and 1:20 giant ragweed extracts (ALO).

Patients had not received immunotherapy for at least 5 years and were recruited from our medical center.

Intradermal end point titration

Ten serial $10^{0.5}$ -fold dilutions were made of the PRW batches to be tested. The subject was titrated on the forearm with 0.05 ml intradermal injections beginning with the $10^{-2.0}$ dilution of a given PRW batch. We continued the titration with four more concentrated or diluted solutions depending on whether the $10^{-2.0}$ dilution was negative or positive. Intradermal diluent and histamine controls were also performed. A positive skin test was defined as at least a 5 mm wheal and 10 mm flare. The intradermal end point titer was the reciprocal of the log of the last dilution that produced a positive skin test. The difference between the end point titers of the reference batch and test batch was calculated. The mean of those differences in four subjects was calcu-

TABLE III. Ninety-nine percent confidence (upper) limit for SD of RA by intradermal end point titration method*

No. of subjects	SD upper limit
3	2.4
4	1.04
5	0.69

*Confidence limit was obtained by use of the chi-square distribution.

lated, and the antilog of that mean was the RA of the test extract compared to the reference extract.

Parallel line bioassay

The parallel line bioassay was performed and analyzed according to published methods.⁵

RAST inhibition

RAST inhibition assay of the PRW batches was performed by Biomedical Reference Laboratories, Burlington, N. C., according to the Bureau of Biologics RAST inhibition protocol by use of a short ragweed extract (Greer Laboratories, Inc., Lenoir, N. C.).⁴

Statistical methods for confidence limits

Data to determine confidence limits for the intradermal end point titration method were obtained by titrating nine individuals multiple times with a single batch of PRW. The SD of the titrations on each individual was determined. Subsequently, the mean, the SD, and the SEM of the titration SDs were calculated. From those values, the 99% confidence limits about the mean and SD were derived for the RA of an extract.⁹

RESULTS

Intradermal end point titration

Nine of the subjects had multiple, simultaneous intradermal end point titrations on the forearm to ref-

TABLE IV. Comparison of skin test assays and RAST inhibition

Test batch	RA of test extract compared to PRW reference batch B		RA of test extract compared to short ragweed aqueous reference
	Parallel line bioassay*	Intradermal end point titration	RAST inhibition
A	0.9	0.7	0.006
B	1.0	1.0	0.007
C	4.1	3.2	0.022
D	1.4	1.3	0.006
E		2.4	0.015
F		3.2	0.025

$r = 0.9954$
 $p < 0.01$
 $df = 2$

$r = 0.9852$
 $p < 0.05$
 $df = 2$

$r = 0.9727$
 $p < 0.01$
 $df = 4$

*Three subjects.

†Four subjects.

erence PRW batch B. End point titers of each of the subjects and the SD of those titers are listed in Table I. The sensitivity of the subjects to the reference extract varied over four logs. At the bottom of that table, the mean, SD, and SEM of the SD of those titers are illustrated. From those values the 99% confidence limits about the mean (Table II) and SD (Table III) of the RA of an extract were derived. Theoretically, the allergenicity of an extract compared to itself is 100%. If the mean and SD of a new batch of PRW fall within the 99% confidence limits for mean and SD, then the new batch does not differ statistically from the reference. Both tables illustrate limits for three, four, or five assays.

Nine of the subjects were end point titrated to the reference PRW batch B and to one or more PRW batches A to F. Only four subjects were titrated for each comparison. Those results are in Table IV.

From our experience with the intradermal end point titration method, we summarized the time, number of skin tests, and materials required (Table V). Assuming the presence of a physician, a nurse, and a technician, it would require approximately 1 hour, 12 skin tests on each of four patients, and simple computations on a calculator. Patient acceptance of this skin test method is very good.

Parallel line bioassay

We performed the parallel line bioassay on nine subjects. In each assay the reference PRW batch B was compared to one or more PRW batches A to D.

Three subjects were used in each comparison. Results are listed in Table IV. At the bottom of Table IV, there is a comparison of the RA of the extracts determined by intradermal end point and that determined by parallel line bioassay. The correlation between the two methods is excellent ($r = 0.995$, $df = 2$, $p < 0.01$).

From our experience with the parallel line bioassay, we summarized the time, materials, and number of skin tests required. Assuming the presence of one physician, one nurse, and one technician, it would require approximately 4 hours to perform the required 36 to 42 skin tests, tracing, taping, and record keeping on each of three subjects. It would require an additional 3½ hours for the required geometric constructs, measurements, diameter averaging, and analyzing the diameter averages by computer in order to obtain the relative potencies, performing log transformations on those potencies, and then completing the required calculations. Patient acceptance of this skin testing method was much less than acceptance of the end point titration.

RAST

RAST inhibition was performed on each of the six PRW batches. In each assay an aqueous short ragweed reference of 100,000 AU/ml was compared to a PRW batch. Three assays were used for each comparison. Results are listed in Table IV. At the bottom of Table IV, the correlation between RAST inhibition and each of the skin test methods is illustrated. The correlation between RAST inhibition and either method is ex-

TABLE V. Time, materials, and subject acceptance of parallel line bioassay and intradermal end point titration to compare two extracts

	Parallel line bioassay*	Cutaneous end point titration†
Time required to perform cutaneous testing on subjects	4 hr	45 min
Time for all calculations, statistics	3½ hr	15 min
Number of skin tests per two extracts on arm	12	12
Number of skin tests per two extracts on back	24 to 30	0
Materials required	Computer and software for best fit linear regression, common slope, and horizontal distance between two parallel lines; calculator for log ₃ to log ₁₀ transformations, mean, and standard deviation	Calculator for mean and SD
Patient acceptance	Nine subjects, seven refused further parallel line bioassay even if the compensation was \$50; all would prefer end point titration	All 18 patients would consent to further end point titration with no monetary compensation

*Assumes three subjects.

†Assumes five subjects.

cellent (end point $r = 0.973$, $df = 4$, $p < 0.01$) (parallel line $r = 0.985$, $df = 2$, $p < 0.05$).

DISCUSSION

A PRW extract for use in immunotherapy should be comparable to that of an appropriate reference preparation that has been designated as a standard. As stated by the Panel on Allergenic Extracts, "The requirements for purity, standardization, and potency testing must not be so strict that production is no longer feasible."¹⁰ In a previous publication we have demonstrated standardization by chromatography, intradermal end point titration, and radioimmunoassay inhibition.²

Several methods of in vivo standardization have been proposed including histamine equivalent prick units,¹¹ end point intradermal titration,² and parallel line bioassay.⁵ The statistical variability of the potency of one extract compared to itself by use of the histamine equivalent prick unit method (95% confidence limits 35-fold with 30 to 50 subjects) is much greater than the variability with the parallel line bioassay method (99% confidence limits, 2.8-fold with three subjects). We thus chose to compare intradermal end point titration to the parallel line bioassay method. It would be necessary to have five subjects in the intradermal end point titration method to obtain the pub-

lished confidence intervals with three subjects in the parallel line bioassay method. It is important, however, to distinguish between statistical significance and clinical significance. That is, although the statistical limits of the mean for the parallel line bioassay with three subjects are 2.8-fold and for the intradermal end point method with four subjects are 3.6-fold, we do not believe that this difference is clinically significant. Thus, we have used four subjects in our end point titrations. Moreover, in terms of the patients who will consent to the two in vivo methods, it is much easier to find subjects who will consent to end point intradermal titration than to parallel line bioassay. Seven of the nine subjects we have tested by both methods have refused further parallel line bioassays even if the compensation was \$50.00. All patients have emphatically stated that they would much prefer intradermal end point titration to parallel line bioassay with its four attendant threefold dilutions in triplicate on their backs for each extract tested. In terms of time, materials, and computer hardware and software required, intradermal end point titration is also much simpler than the parallel line bioassay. In short, it is much easier to perform end point titration on five subjects than to perform the parallel line bioassay on three subjects, and the statistical accuracy is comparable.

There is an additional problem with the parallel line

bioassay in that subjects to be tested must exhibit a dose-response curve over four threefold dilutions. In our experience testing three standardized aqueous extracts in 40 subjects with positive histories and prick tests, approximately one quarter of those patients have very steep dose-response curves to at least one extract. Thus, those patients do not exhibit a linear dose response over four threefold dilutions and would not be suitable for parallel line bioassay but would be able to have intradermal end point titration. In fact, we have found that four subjects are often required for the parallel line bioassay in order to have three subjects whose dose-response curves fit the statistical criterion of $r \geq 0.85$. Therefore, the number of subjects required for both methods is identical.

Intradermal end point titration and parallel line bioassay are highly correlated when they are used to compare various PRW extracts. Moreover, the result of each in vivo method is highly correlated with the RAST inhibition result of the extract. That is, as an in vitro method, RAST inhibition is highly correlated with in vivo activity by parallel line bioassay or end point cutaneous reactivity. Thus, it may be necessary to standardize PRW extracts only in terms of chromatographic profile and in vitro reactivity.

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