
Standardization of Modified Allergens Using Polymerized Ragweed as a Model System

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The appropriate methods for standardization of allergenic extracts have been the subject of several articles and reviews.¹⁻³ The demonstration that the quality of an allergenic extract is sufficiently similar to that of a reference standard has been hindered by the lack of criteria for accepted reference standards¹ and by the lack of agreement as to which tests are appropriate for standardization.³ All techniques for standardization of conventional allergenic extracts may not be appropriate for standardization of modified extracts because of unique characteristics of these extracts. An example of such a modified allergen is polymerized ragweed (PRW) which can, however, be standardized by various methods.

Polymerized ragweed is unique in that several lots, each of which is safe, efficacious, and immunogenic, are available as reference standards for other batches of PRW.² These PRW standards have been characterized by gel filtration chromatography, radioimmunoassay (RIA) inhibition, and intradermal endpoint titration.^{2,4} These methodologies are sufficient to define a batch of PRW and could be used to develop secondary standards.

Historical Review

Polymerized allergen extracts were developed to improve upon currently available, conventional allergen extracts. Although immunotherapy with conventional allergen extracts has been shown to be effective in the treatment of pollinosis,⁵ there are a number of problems associated with the use of such extracts. First, there is a significant probability that a patient will have a systemic allergic reaction at some time during a course of immunotherapy. This risk has been estimated at 7-33%.⁶ Also, immunotherapy with conventional extracts generally involves approximately 100 injections over a period of several years.⁵ The time and money expended for such therapy makes it unattractive or impossible for many patients to undergo. Because polymerized

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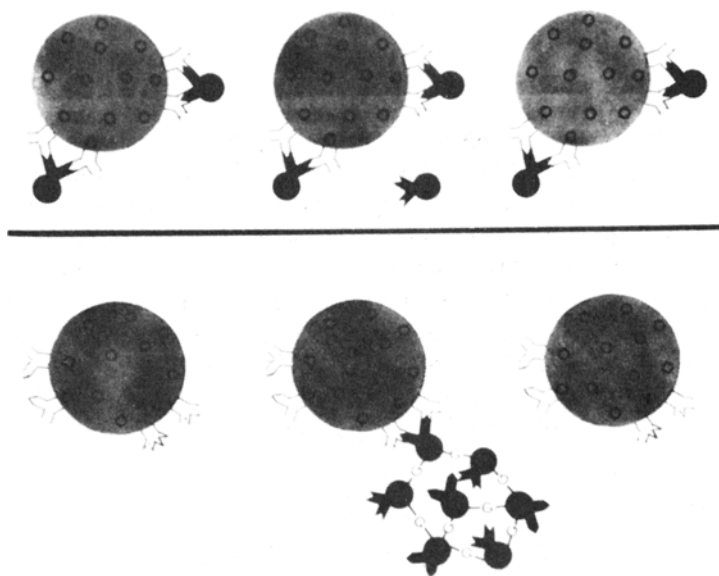
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allergen extracts can be administered with fewer injections over a shorter time period with a lower risk of systemic reactions, they represent a significant advance in allergen immunotherapy. It has been demonstrated that PRW given in 15 weekly immunotherapy injections is safe and effective therapy for ragweed pollinosis and that the incidence of systemic reactions is less than 1% per patient course.^{7,8}

Polymerized allergen extracts consist of allergen molecules that have been crosslinked by glutaraldehyde, a bifunctional aldehyde, into larger size molecular species. In our PRW clinical trials we have used molecular weight species from 200,000 to 20,000,000 daltons. We have demonstrated that the allergenicity of PRW extract is inversely proportional to its molecular weight.⁹ This is the result of 3 factors: 1) larger molecular weight polymers diffuse more slowly¹⁰; 2) larger molecular weight polymers contain more concealed antigenic determinants; 3) equal weights of larger molecular weight polymers have a lower molar concentration and thus a decreased opportunity to bridge IgE on the surface of mast cells and cause mediator release.¹¹ The reduction in allergenicity that results from the polymerization process is pictorially represented in Figure 1.

In addition to PRW we have manufactured several other polymerized pollen extracts including polymerized AgE,¹³ several different individually polymerized grass (IPG) extracts,¹⁴ and several individually polymerized tree (IPT) extracts.¹⁵ In each instance, we have demonstrated retained antigenicity

Figure 1. Hypothesis of the reduced allergenicity and retained antigenicity of polymerized allergens in comparison with monomeric allergens. (From Patterson, et al,¹² with permission)



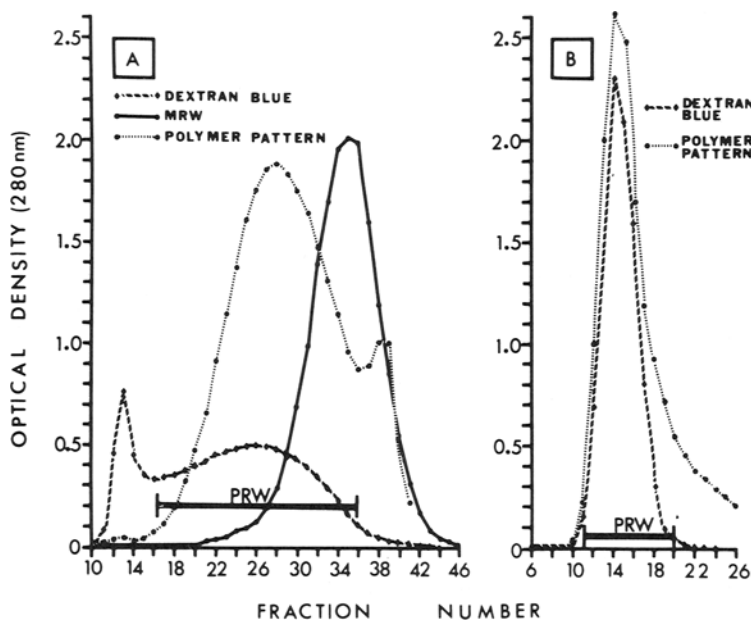
and reduced allergenicity. We have demonstrated safety, immunogenicity, and efficacy of polymerized AgE¹⁶ and IPG,¹⁷ and have demonstrated safety and immunogenicity of PRW, IPG, and IPT administered concomitantly.¹⁸ As the PRW extract has been most extensively studied, we have chosen it as the prototype for the standardization of polymerized extracts. The same standardization procedures would be equally applicable to each of the other polymerized extracts.

Production Methodology for Polymerized Ragweed

The production of a polymerized pollen extract involves several steps, which have been published in detail.⁹ First, the pollen is defatted with ether, extracted with phosphate-buffered saline (PBS), and precipitated with $(\text{NH}_4)_2\text{SO}_4$. When the precipitate is dissolved in PBS, the extract is called monomer and is essentially a conventional allergen extract. Subsequently, glutaraldehyde is added and crosslinking and polymerization of the pollen molecules occur. Next, glycine is added in excess to react with glutaraldehyde and stop the polymerization process. Gel filtration chromatography on Sepharose CL4B and then on Sephacryl S-200 is performed to obtain polymers of desired molecular weight, from 200,000 to 20,000,000 daltons.

The Sepharose CL-4B chromatographic profiles of monomer and PRW are

Figure 2.A) Sepharose CL-4B column fractionations of preparations of ragweed (RW) prepared by precipitation with ammonium sulfate for the monomeric (MRW) and the polymeric (PRW) forms produced by treatment of MRW with glutaraldehyde. B) Column fractionation of ragweed polymers through Sephadex G-200 compared with dextran blue. The excluded fractions were pooled to form the final preparation of PRW. (From Patterson, et al,⁹ with permission.)



shown in Figure 2A. The dramatic shift of PRW toward higher molecular weight species is obvious.

The final molecular weight sieving can be done on a Sephadex G-200 Superfine column and is shown in Figure 2B. The exclusion limit of Sephadex G-200 Superfine is 200,000 and thus fractionation is performed so that PRW contains only molecular weight species that are excluded from the gel. Subsequently, more efficient gels such as Sephacryl S-200 have become available with exclusion limits similar to Sephadex G-200 Superfine, and these can be used for final molecular weight sieving.

Criteria for Standardization of Polymerized Ragweed

The unique properties that characterize PRW include its reduced allergenicity, its retained antigenicity, and its molecular weight range. Polymerized ragweed must therefore be standardized by analyses that characterize its molecular weight and that demonstrate reduced allergenicity but retained antigenicity. Molecular weight can be characterized by gel filtration chromatography using a matrix with suitable exclusion limits. The availability of antigenic determinants in a PRW extract can be quantified by determining its ability to inhibit binding of a standard ragweed extract to IgG antibodies directed against the ragweed extract. Radioallergosorbent test (RAST) inhibition would be an example of such an assay. To quantify the allergenicity of a PRW extract, cutaneous testing can be performed in ragweed allergic individuals and the results can be compared with those obtained when titrating a standard conventional extract or a standard PRW extract. However, this is not necessary when the molecular weight and antigenicity of a polymer preparation is established, as is described in detail below.

Molecular Weight Characterization

As explained above, the production process of PRW involves 2 molecular weight sieving steps. Polymerized ragweed is fractionated at the exclusion limits of the gels used in the chromatographic steps and thus the manufacturing process defines the molecular weight range. It has been well demonstrated that a batch of PRW that is fractionated to contain more low molecular weight species, that is, molecules <200,000 daltons, is significantly more allergenic.^{2,9} To be certain that a batch of PRW contains only insignificant amounts of molecular weight species <200,000 daltons, Sephacryl S-200 analytical gel filtration is performed on an aliquot of PRW. We have studied 2 reference standards of PRW and several other PRW batches, each with similar allergenicity as determined by cutaneous reactivity and similar antigenicity as determined by RAST inhibition activity. In each case the analytical chromatographic profile of PRW on a Sephacryl S-200 or Sephadex G-200 is almost identical to that of dextran blue, which is totally excluded. Such profiles can be seen in Figure 3A. The width of the peak at half of the maximum optical density, W_h is a measure of peak symmetry¹⁹ and was calculated for the profile of each batch of PRW and of dextran blue. Since the desired PRW molecules

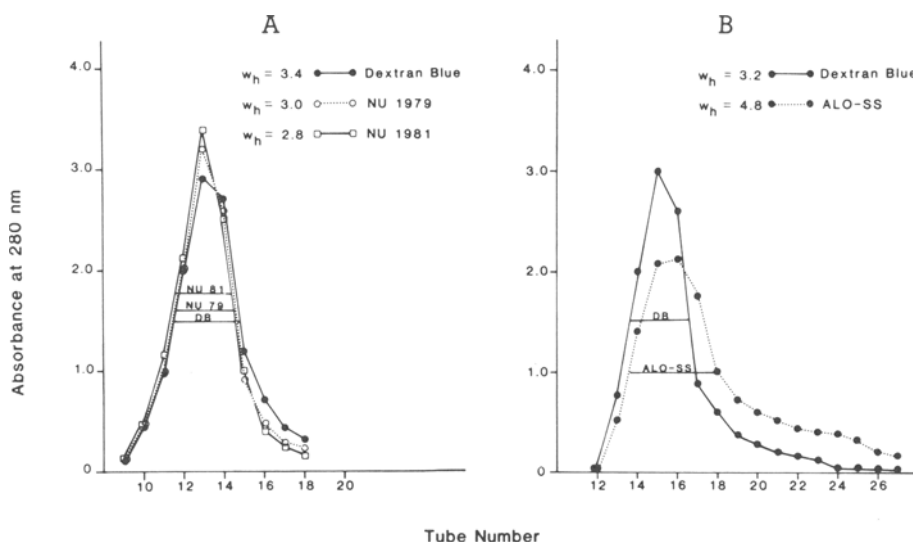


Figure 3. A) Chromatogram of dextran blue and 2 PRW standards. B) Chromatogram of dextran blue and a PRW batch fractionated to contain molecular weight species <200,000 daltons. All chromatograms are from Sephadex G-200 Superfine column. W_h is indicated for each chromatogram. (From Grammer et al,² with permission.)

have a molecular weight greater than the exclusion limit of the analytical column, the peak should be symmetric just as the dextran blue peak is symmetric, and the W_h of PRW should be very similar to the W_h of dextran blue, as shown in Figure 3A.

In contrast, if a batch of PRW contains species of molecular weight lower than the exclusion limits of the gel, those low molecular weight molecules will be eluted later. The profile will be asymmetric, appear to have a "tail," and the W_h will be wider. Figure 3B illustrates such a chromatographic profile. In short, W_h is a measure of the amount of material for which molecular size is below the exclusion limit of the gel. To compare the symmetry of a PRW profile with that of a dextran blue profile, a ratio is calculated between W_h of PRW and W_h of dextran blue. We have found that a PRW batch, such as the one illustrated in Figure 3B, with a W_h ratio > 1.3, will have unacceptably high allergenicity. In contrast, PRW batches, such as the ones illustrated in Figure 3A, with ratios of ≤ 1.3 , have acceptably low allergenicity.

Antigenicity Characterization

The availability of antigenic determinants in a PRW extract could be quantified by any of a number of immunoassays. In our publications we have cited 2 different RIAs, either of which could be performed as a measure of antigenicity.^{2,4} The first method is a modification of the ammonium sulfate precipitation method of Lidd and Farr.^{20,21} Antiserum and ¹²⁵I AgE are added to varying amounts of unlabeled AgE and then are precipitated by (NH₄)₂SO₄.

Bound counts are determined and a standard curve of cpm-bound versus log nanograms AgE is constructed. In lieu of unlabeled AgE, various volumes of PRW extract or conventional ragweed extract are added. A ratio is then determined between the amount of PRW required to reduce ^{125}I AgE binding to 35% and the amount of AgE or conventional ragweed extract needed for similar inhibition.

Another method is that of RAST inhibition, which has been described in detail.²² In simplest terms, the amount of PRW required for 50% inhibition of a RAST is compared with the amount of a standard conventional ragweed extract required for similar inhibition. We have demonstrated that PRW inhibition results using either modified Farr technique or RAST inhibition correlate well with allergenicity as measured by cutaneous reactivity.^{2,4} Several investigators have used conventional extracts and demonstrated correlation of RAST inhibition and cutaneous reactivity.²³⁻²⁵ This correlation also occurs when polymerized extracts are tested.

Table 1. Comparison of Skin Test Assays and Radioallergosorbent Test Inhibition

Test batch	Relative allergenicity of test extract compared with polymerized ragweed reference batch B		Relative allergenicity of test extract compared with conventional short ragweed reference
	Parallel line bioassay ^a	Intradermal endpoint titration ^b	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

$r = 0.9954$
 $p < .01$
 $df = 2$

$r = 0.9727$
 $p < .01$
 $df = 4$

$r = 0.9852$
 $p < .05$
 $df = 2$

df = degrees of freedom.

^a3 subjects.

^b4 subjects.

From Grammer et al,⁴ with permission.

Allergenicity Characterization

To determine allergenicity of batches of PRW, we performed intradermal endpoint titration in ragweed allergic individuals with serial $10^{0.5}$ -fold dilutions of PRW and with conventional ragweed extract.² The cutaneous endpoint titer is the reciprocal of the log of the last dilution that produces at least 5-mm wheal and 10-mm flare. The log difference is then determined between the titer of PRW and conventional ragweed.

Another skin test method for determining allergenicity is the parallel line bioassay.²⁶ This method involves intradermal skin testing on the arm to determine threshold doses and then intradermal skin testing on the back in triplicate with at least 4 dilutions of each test extract and each reference extract to be compared. The sums of erythema diameters are measured, averaged, and plotted against $\log_3$ allergen dose to produce best-fit linear regression lines. Provided that various statistical parameters are satisfied, the relative allergenicities of the test and reference extracts are related to the horizontal difference between the 2 regression lines.

We have demonstrated that the allergenicity of PRW as measured by cutaneous endpoint titration correlates with allergenicity as measured by parallel line bioassay.⁴ We also have demonstrated that either measure of allergenicity correlates with antigenicity as determined by RAST inhibition (Table 1). We have used endpoint titration (Table 2) because there are several drawbacks

Table 2. Time, Materials, and Subject Acceptance of Parallel Line Bioassay and Intradermal Endpoint Titration to Compare Two Extracts

	Parallel line bioassay ^a	Cutaneous endpoint titration ^b
Time required to perform cutaneous testing on subjects	4 hr	45 min
Time for all calculations, statistics	3½ hr	15 min
No. of skin tests per 2 extracts on arm	12	12
No. of skin tests per 2 extracts on back	24–30	0
Materials required	Computer and software for best-fit linear regression, common slope, and horizontal distance between 2 parallel lines; calculator for $\log_3$ to $\log_{10}$ transformations, mean, and SD	Calculator for mean and SD
Patient acceptance	9 subjects, 7 refused further parallel line bioassay even if the compensation was \$50; all would prefer endpoint titration	All 18 patients would consent to further endpoint titration with no monetary compensation

SD = standard deviation.

^aAssumes 3 subjects.

^bAssumes 5 subjects.

From Grammer et al,⁴ with permission.

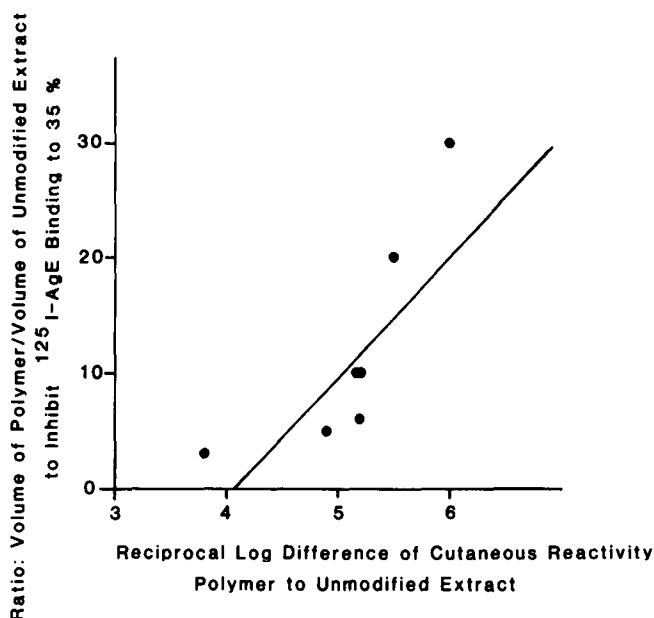


Figure 4. Correlation between the reciprocal log difference of cutaneous reactivity of polymer and unmodified extract and ratio of volume of polymer to volume of unmodified extract to inhibit $^{125}\text{I-AgE}$ binding to 35%. Pearson r is 0.80 and Spearman rank correlation coefficient is 0.97 ($p < .005$). (From Grammer et al,² with permission.)

to the parallel line bioassay method. These include the numerous intradermal injections that are required, which leads to the refusal of many patients to undergo the parallel line bioassay. Seven of 9 subjects we have tested by both methods have refused further parallel line bioassays even for significant financial compensation (\$50). In terms of time, materials, and computer hardware and software required, intradermal titration is also much simpler. Fi-

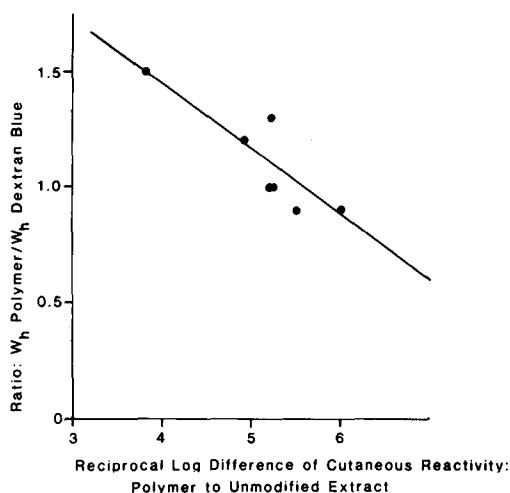


Figure 5. Correlation between the reciprocal of the log difference of cutaneous reactivity of polymer and unmodified extract and the ratio of W_h polymer/ W_h dextran blue. Pearson r is -0.87 ; Spearman rank correlation coefficient is -0.79 ($p < .05$). (From Grammer et al,² with permission.)

nally, the parallel line bioassay requires that patients exhibit a linear dose response curve over 4 3-fold dilutions. In our experience with approximately 100 patients, about a quarter of them do not meet statistical criteria for linearity.

We also have demonstrated that intradermal endpoint titration correlates with PRW inhibition of modified Farr assay described above. This correlation is graphed in Figure 4. Moreover, intradermal endpoint titration results of PRW extracts correlate with W_h ratio of PRW to dextran blue as described above. This correlation is depicted in Figure 5. Thus, because allergenicity as measured by cutaneous reactivity correlates both with chromatographic profiles, in particular W_h , and with antigenicity as measured by either of 2 RIA inhibition techniques, cutaneous testing is no longer necessary as part of standardization. Rather, PRW extracts could be standardized simply in terms of chromatographic profile and in vitro reactivity.

Conclusions

Polymerized allergens are manufactured by crosslinking allergen molecules with glutaraldehyde and then selecting a desired molecular weight range by gel filtration chromatography. Polymerized allergen extracts have retained antigenicity but reduced allergenicity when compared with conventional allergenic extracts. Thus, the important parameters to standardize in polymerized extracts are allergenicity, antigenicity, and molecular weight range. As our initial studies have demonstrated that allergenicity correlates with antigenicity and molecular weight, only the latter are necessary to measure. In short, polymerized allergen extracts could be standardized simply in terms of in vitro reactivity and chromatographic profile.

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