Brief communication

Comparison of parallel line skin test assay (PLST) and ELISA inhibition (EI) methods for determination of relative potency of polymerized grass and ragweed antigenic extracts

William K. Dolen, MD, MAJ, MC,* Brian T. Miller, DO, MAJ, MC,* Robert L. Ledoux, BS,* James M. Seltzer, MD, Matthew B. Wiener, PharmD,*** John C. Selner, MD,*** and Harold S. Nelson, MD*** *Aurora and Denver, Colo., and San Diego, Calif.*

Relative potency estimates were performed by parallel line skin test assay (PLST) and ELISA inhibition methods for three polymerized allergen extracts (Bermuda grass, orchard grass, and a copolymer of short and giant ragweed) versus four unmodified RAST standardized reference extracts (Bermuda grass, orchard grass, and giant and short ragweed) in nine subjects. One subject experienced a systemic reaction, requiring treatment at the end of the PLST assay. Another subject had a systemic reaction during limited skin testing performed approximately 72 hours after completion of PLST. Relative potency values for the polymerized extracts obtained by PLST were much lower than those obtained by ELISA inhibition, but results were significantly ($r = 0.95$; $p < 0.01$) correlated. Because polymerized allergen extracts are designed to be hypoallergenic, a skin test assay may underestimate their potency relative to an unmodified reference extract. (J ALLERGY CLIN IMMUNOL 1988;81:489-93.)

Numerous methods have been used to evaluate the potency and composition of allergen extracts. Noon[1] and Freeman[2] used conjunctival provocation to test the potency of their grass-allergen extracts in 1911. Measurement of protein nitrogen content and labeling of an extract with the weight per volume ratio used in the extraction process were later used, but neither method correlates with allergenic activity. Other in vitro methods, such as RAST inhibition[3] or quantitative immunoelectrophoresis[4] provide more clinically relevant data. An in vivo procedure using a PLST method was described in 1982.[5] A modification of this method has been recommended by the Food and Drug Administration Office of Biologics as the primary method by which allergen-extract potency is to be evaluated.[6]

In a previous study[7] using the originally described

Abbreviations used

PLST:	Parallel line skin test
EI:	ELISA inhibition
RP:	Relative potency
E:	Sum of the longest diameter of a skin test area of erythema and its midpoint orthogonal diameter
OD:	Optical density
AU:	Allergen units

From the *Allergy-Immunology Service, Fitzsimons Army Medical Center, Aurora, Colo., **Allergy and Asthma Prevention Center, Incorporated Medical Group, San Diego, Calif., and ***Allergy Respiratory Institute of Colorado, Denver, Colo.

Received for publication May 12, 1987.

Accepted for publication July 29, 1987.

Reprint requests: William K. Dolen, MD, MAJ, MC, Allergy-Immunology Service, Fitzsimons Army Medical Ctr., Aurora, CO 80045-5001.

The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of Defense. The protocol under which this study was conducted was approved by the Clinical Research and Human Use Committee of Fitzsimons Army Medical Center and the Office of the Surgeon General, United States Army.

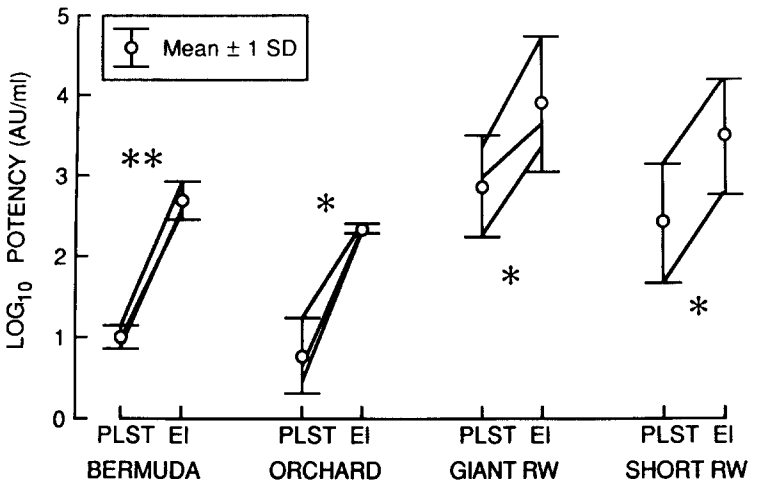

FIG. 1. Paired comparisons of potency of polymerized grass and ragweed extracts relative to unmodified standards (100,000 AU/ml); $*p < 0.05$ and $**p < 0.01$ by Student's paired t test.

methods for statistical analysis of results,[5] PLST for polymerized grass extracts yielded a wide range of estimates of RP compared to those obtained from an EI assay. To determine whether these results reflect the precision of the PLST method and to examine the relationship between RP as determined by the two methods, we performed paired PLST and EI RP determinations with polymerized grass and ragweed-allergen extracts, comparing them to the corresponding standardized aqueous extracts.

MATERIAL AND METHODS

Subjects

Nineteen subjects with positive skin tests for test allergens were selected from two allergy practices (grasses, J. M. S.; ragweed, J. C. S.). Informed consent was obtained before testing. Before PLST, each patient underwent phlebotomy for subsequent EI testing, performed in the allergy laboratory at Fitzsimons Army Medical Center.

Antigenic materials

Polymerized Bermuda grass–, orchard grass–, and ragweed- (a copolymer of giant and short ragweed material) pollen extracts (Allergy Laboratories of Ohio, Columbus, Ohio) were examined. RAST standardized (100,000 AU/ml), unmodified aqueous extracts of Bermuda, orchard, and giant and short ragweed pollens (Pharmacia Diagnostics, Piscataway, N.J.) were used for reference standards.

Parallel line skin test assay

The PLST method was performed as previously described.[5, 6] Briefly, extract dilutions just above the end point of skin reactivity were chosen by intradermal testing on the forearms, and four serial threefold dilutions of the reference extract and a test extract were injected in triplicate into areas of the back in a randomized pattern. At 15 minutes, the flare areas were outlined with a fine tip marker and transferred to paper with transparent tape.

For analysis of data, the longest diameter of the resulting area of erythema and its midpoint orthogonal diameter were measured and summed (ΣE). Linear regression analysis was performed for both the reference and test extracts, with the mean ΣE as the dependent variable and the $\log_3$ of the extract dilution as the independent variable.

For validation of an individual comparison,[5] the r value obtained from both regression analyses must be greater than an arbitrary value of 0.85. Additionally, the slopes of the two regression lines must be parallel at the $p < 0.01$ level, as determined by a standard method for slope comparison with the Student's t test. A common slope with new Y intercepts is calculated, and RP is defined as the horizontal distance between the adjusted lines.

Additional statistical criteria were suggested or implied in the original article and have been adopted in a subsequent description of the method.[6] To ensure that the regression lines are constructed on the linear portion of the dose-response curve, the mean ΣE values should, in general, not exceed a range of 0 to 120 mm. To avoid comparison of lines with flat slopes, the regression line for both the reference and test extracts should have a slope >10 mm per $\log_3$ dilution. Standard deviation criteria used for validation of a mean RP value were not used because of the paired experimental design.

ELISA inhibition

Microtiter plates (Immulon I, Dynatech, Alexandria, Va.) were coated with 50 μl per well of standardized extract diluted 1:200 (for optimal binding) in phosphate-buffered saline, pH 7.2, and incubated for 1 to 4 days at 4° C. After washing, the wells were filled with a 1% solution of normal rabbit serum in 0.9% saline with 0.05% Tween 20. After a 1-hour incubation at 37° C, plates were washed, dried, and stored at 4° C until use.

The standardized extracts were in most instances diluted in 1:25,000, 1:50,000, 1:100,000, and 1:250,000 concentrations (weight per volume). The polymerized extracts were usually diluted in 1:25, 1:50, 1:100, and 1:500 concentrations. As described later in the article, individual patient sera on occasion required use of additional dilutions of standardized or polymerized extract to provide acceptable data. Serum obtained from a nonatopic subject was used as a negative control.

Twenty-five μl aliquots of the four dilutions each of standardized and polymerized extracts were added to the wells, followed by 25 μl aliquots of negative control serum or serum from the individual subjects. Each extract dilution and serum were tested in triplicate. Plates were incubated for 3 hours at room temperature, washed, and dried. To all wells was added 50 μl of antihuman IgE alkaline-phosphatase conjugate (Kallestad Laboratories, Austin, Texas); the plates were incubated at room temperature overnight, washed, and dried. To each well was added 150 μl of disodium p-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, Mo.), 1 mg/ml, in 10% diethanolamine buffer. Plates were incubated at 37° C for 30 minutes. The reaction was terminated with 2 N of NaOH and read with a microtiter plate reader (Titertek Multiscan, Flow Laboratories, McLean, Va.) at 405 nm.

The OD of the negative control wells was subtracted from the average OD calculated from the triplicate readings. To yield percent inhibition, this corrected OD was subtracted from a positive reference OD (no inhibiting serum), and the result was divided by the positive reference OD value and multiplied by 100%. In a manner similar to that described for PLST, linear regression lines were constructed for the standardized and polymerized extracts, with the dilution as the dependent variable and the percent inhibition as the independent variable. RP was defined as the distance between the lines at the point of 50% inhibition. Extrapolation was not permitted; if the regression lines for both standard and polymer did not pass through the 50% inhibition point, the assay for that individual was repeated with additional dilutions. If the use of additional dilutions did not result in the regression line passing through the 50% inhibition point, the assay was considered to have failed.

Relative potency

For both PLST and EI, RP was defined as the antilogarithm of the horizontal distance (expressed in logarithmic units) between the regression lines for standard and polymer. The potency of an unknown extract is conventionally expressed relative to that of the standard extract that has been assigned a value of 100,000 AU/ml. Thus, the absolute potency in allergy units per milliliter is obtained by multiplying the relative potency obtained from the assay by 100,000 AU/ml.

Statistical analyses

Comparisons of potency obtained by PLST versus that obtained by EI were made by use of the paired Student's *t* test, two-tailed, at the 0.05 level. The relationship between PLST and EI potency values was determined by a linear correlation analysis, with analysis of variance to test the significance of the r value obtained. In both cases, a $\log_{10}$ transformation of the data was used.

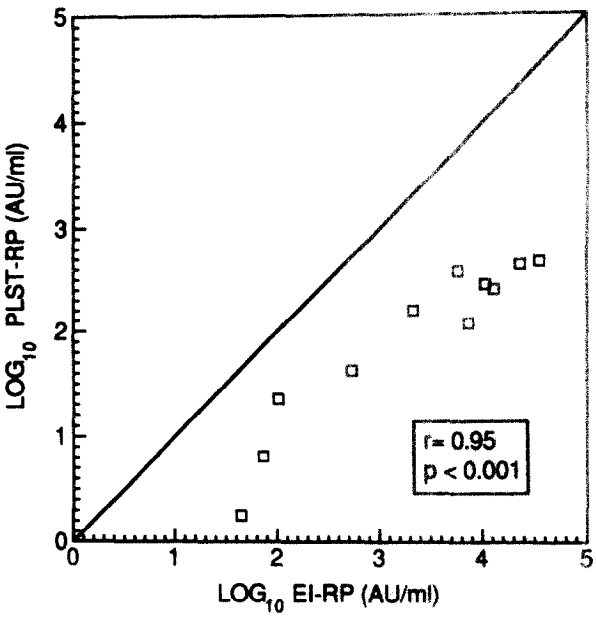

FIG. 2. Scattergram illustrating correlation of PLST with EI. The line of identity is presented.

RESULTS

Five of 21 PLST assays performed in the 19 subjects did not meet statistical criteria for validation. EI failed in five of the 16 valid PLST assays; therefore, 11 paired comparisons, meeting individual validation criteria for both PLST and EI, were available from nine subjects. Potency values for the polymerized grass and ragweed extracts, as determined by PLST and EI, are presented in Fig. 1. Potency values for these extracts as determined by EI were significantly higher than those obtained by PLST in all cases, differing from fivefold to 70-fold. As illustrated in Fig. 2, there was an excellent correlation ($r = 0.95$; $p < 0.001$) between paired EI and PLST results.

Two patients, whose data are not included in the analysis, in the group tested for ragweed (investigators J. C. S. and M. B. W.), experienced systemic reactions. One patient, a subject known to have asthma, developed flushing, dyspnea, and wheezing after administration of the fiftieth and last intradermal injection in the series. Preliminary testing, consisting of four prick skin tests and 11 intradermal injections had resulted in a maximum ΣE of 107 mm for the polymerized extract; the end point dilution used on the back produced a mean ΣE of 64.7 mm. Response to epinephrine, diphenhydramine, and corticosteroid

therapy was satisfactory. Another patient was asked to return for limited skin testing 3 days after the initial testing session to examine why there had been difficulty in achieving an appropriate end point reaction. Preliminary titrations on the arm were not repeated. The allergen dilutions used were prepared on the same data as the initial testing, had been stored at 4° C, and were one threefold dilution less potent than the weakest extract dilution used in the first session. Nine intradermal tests (six monomer and three polymer) were applied. The size of erythema produced by the extract injections was larger than expected; 30 minutes after the last injection, the patient developed facial angioedema, ocular erythema, nasal congestion, and pruritus. Symptoms resolved within 45 minutes of treatment with epinephrine and diphenhydramine.

DISCUSSION

In a study of polymerized extracts in which potency was estimated by intradermal end point titration, PLST, and a RAST-inhibition method results of the in vitro method correlated highly with the skin testing methods. Individual relative allergenicity values were not reported, but mean values obtained by RAST inhibition differed substantially from those obtained by either skin testing method. In the present study, there was a large difference between individual and mean potency values obtained by PLST and those obtained by EI for grass and ragweed extracts.

It is possible that none of these methods provides a completely accurate measure of true extract potency, that is, the ability to produce an appropriate clinical response in a course of immunotherapy. The EI and RAST-inhibition methods measure exposed allergenic determinants in a complex molecular structure. The skin test methods additionally evaluate the diffusibility of extracts in tissues and the ability to cross-link antigen-specific IgE on the surface of mast cells. Since polymerized allergen extracts are designed to be hypoallergenic while they remain immunogenic, the in vivo methods might be expected to underestimate potency relative to the in vitro methods. This possibility warrants further investigation because it suggests that PLST data should be examined with caution when it is to be used for regulatory or standardization purposes.

A study of *Aspergillus fumigatus* extracts[9] demonstrated preferential binding of certain antigenic components to plastic microtiter plates. Were a similar phenomenon to occur with pollen extracts, certain components of the aqueous extracts used to coat the plate might be underrepresented relative to the allergens present in the inhibiting extract, thus increasing the EI potency value. Although this phenomenon has not been demonstrated, it is possible that binding characteristics of allergenic extracts in an EI could result either in overestimation or underestimation of actual potency.

Potency of the polymerized extracts, as determined by either method, was lower than that of the reference standards, but the higher potency estimates obtained by EI are comparable to the observed immunologic and clinical response to the polymerized extracts.[10-12] This study did not address the question of whether extract potency is lost in the polymerization process or whether on skin testing, the complex molecular structure of the polymer masks epitopes that would otherwise be available to the unbound IgE present in the patient sera.

To our knowledge, the first patient described represents the first reported adverse rection, besides discomfort at the injection sites, related to the PLST procedure. One patient in a previous study[7] reported mild nasal congestion occurring several hours after PLST, but it is not clear whether this symptom was related to the procedure. The second patient described above experienced a reaction when nine additional intradermal tests were applied 72 hours after the initial testing. It was believed that by use of weaker extract dilutions, a true end point would be reached; the result was not an end point but extremely large reactions that produced a systemic response. This experience raises the question, not examined in this study, of whether PLST can produce an amnestic IgE response or could increase mast cell–mediator releasability and suggests that PLST should not be repeated at frequent intervals in the same individual. In addition, as with any skin testing procedure, facilities for the treatment of systemic reactions must be available.

PLST is a scientifically valid in vivo method for RP determination. In practice, however, there are several factors limiting the usefulness of the technique. It is technically difficult to perform, requiring careful attention to detail as well as constant evaluation and occasional modification of the protocol instructions during the testing procedure. PLST is uncomfortable for most patients and presents a risk, both for systemic reactions and possibly of augmentation of sensitivity, in highly sensitive subjects. Because of the complexity of the PLST method, screening more than a few subjects per session requires a large amount of space and several skilled assistants. Since each extract contains multiple major and minor antigenic determinants, some of which elicit an IgE response in only a minority of individuals, a valid assay of RP should be based on the results obtained from a large number of subjects. In the specific case of polymerized extracts, use of the PLST technique could elicit falsely low potency

estimates, since it is a skin test assay that attempts to measure allergenicity in an extract designed to have low allergenic potency. For these reasons, PLST is an inappropriate tool for evaluation and standardization of polymerized extracts.

An in vitro procedure, such as EI, RAST inhibition, or others, involves minimal patient risk and can be developed in any well-equipped immunology laboratory. Reagents are relatively inexpensive. These procedures can be automated and used to screen sera from a large number of sensitive persons so that the potency estimate obtained reflects the IgE response of a population to relevant major and minor antigenic determinants present in a given extract. In comparative studies, such as this study, the same reference extract used for skin testing can be used to coat the microtiter wells. Furthermore, serum pools can be developed, frozen, and shared by different laboratories. The major disadvantage of in vitro assays is that they are less sensitive than skin test assays; this, however, is a problem primarily with dilute rather than concentrated extracts.

The initial process of antigen standardization should include both in vivo and in vitro assay procedures; this approach has been used in the preparation of international reference standards[13-15] and has been recommended by Ingemann and Venov.[16, 17] The use of multiple procedures would ensure that the composite potency value accurately reflects the human IgE response to the allergens contained in the test extracts, but tests must be selected to obtain the maximum amount of information possible with minimal patient discomfort and risk. Subsequent extract lots could be screened for potency with a single, simple assay procedure demonstrated to be valid in the initial standardization process.

We thank Ms. Cecilia Gregg for her excellent secretarial assistance in the preparation of this manuscript.

REFERENCES

1. Noon L. Prophylactic inoculation against hay fever. Lancet 1911;1:1572.
2. Freeman J. Further observations on the treatment of hay fever by hypodermic inoculations of pollen vaccine. Lancet 1911; 2:814.
3. Gleich GJ, Larson JB, Jones RT, Bauer H. Measurement of the potency of allergy extracts by their inhibitory capacities in the radioallergosorbent test. J ALLERGY CLIN IMMUNOL 1974; 53:158.
4. Løwenstein H. Quantitative immunoelectrophoretic methods as a tool for the analysis and isolation of allergens. Prog Allergy 1978;25:1.
5. Turkeltaub PC, Rastogi SC, Bauer H, et al. A standardized quantitative skin test assay of allergen potency and stability: studies on the allergen dose-response curve and effect of wheal, erythema, and patient selection on assay results. J ALLERGY CLIN IMMUNOL 1982;70:343.
6. Turkeltaub PC, Rastogi SC, Baer H. Quantitative intradermal test procedure to determine relative potency of allergenic extracts using parallel line bioassay (confirmatory method). Washington, DC: Office of Biologics, Food and Drug Administration, 1984.
7. Miller BT, Ledoux RA, Moyer DB, et al. A comparison of standardized quantitative skin tests and enzyme allergosorbent test inhibition for assessment of extract potency [Abstract]. Ann Allergy 1985;54:356.
8. Grammar LC, Shaughnessy MA, Shaughnessy JJ, Patterson R. Brief report: evaluation of polymerized ragweed extracts by intradermal end point titration, RAST inhibition, and parallel line bioassay. J ALLERGY CLIN IMMUNOL 1985;76:123.
9. Kauffman HF, van der Heyden PJ, van der Laan S, et al. Antibody determination against *Aspergillus fumigatus* by means of the enzyme-linked immunosorbent assay. Int Arch Allergy Appl Immunol 1985;78:174.
10. Grammer LC, Shaughnessy MA, Finkle S, et al. Safety and immunogenicity of immunotherapy with polymerized tree, grass, and ragweed in patients with multiple inhalent sensitivities [Abstract]. J ALLERGY CLIN IMMUNOL 1985;75:165.
11. Grammer LC, Shaughnessy MA, Shaughnessy JJ, et al. Allergenicity, immunogenicity, and safety of immunotherapy with various molecular weight ranges of polymerized ragweed. J ALLERGY CLIN IMMUNOL 1985;76:195.
12. Miller BT, Brown JS, Moyer DB, et al. Double-blind controlled trial of polymerized grass pollens in the treatment of seasonal allergic rhinitis [Abstract]. Ann Allergy 1985;54:340.
13. Helm RM, Gauerke BA, Baer H, et al. Production and testing of an international reference standard of short ragweed pollen extract. J ALLERGY CLIN IMMUNOL 1984;73:790.
14. Gjesing B, Jäger L, Marsh DG, et al. The international collaborative study establishing the first international reference standard for timothy grass pollen allergen extract. J ALLERGY CLIN IMMUNOL 1985;75:258.
15. Ford A, Seagroatt V, Platts-Mills TAE, et al. A collaborative study on the first international standard of *Dermatophagoides pteronyssinus* (house dust mite) extract. J ALLERGY CLIN IMMUNOL 1985;75:676.
16. Ingemann L, Venov D. Experiences from 5 years of standardization of allergen extracts. Arb Paul Ehrlich Inst Georg Speyer Haus Ferdinand Blum Inst 1984;79:177.
17. Ingemann L, Venov D. Standardization of allergenic extracts—problems involved in the choice of standardization method [Abstract]. J ALLERGY CLIN IMMUNOL 1984;73:190.