

Studies on *Alternaria* allergens

II. Measurement of the relative potency of commercial *Alternaria* extracts by the direct RAST and by RAST inhibition

**John W. Yunginger, M.D., Richard T. Jones, B.S., and
Gerald J. Gleich, M.D. Rochester, Minn.**

The relative potency of 12 commercial Alternaria extracts was analyzed by end-point skin test titrations and compared to in vitro measurements of potency, including (1) the radioallergosorbent test (RAST) inhibition procedure; (2) the direct RAST procedure; and (3) the protein nitrogen unit (PNU) content. Potencies determined by skin testing 10 sensitive patients were strongly correlated among the various patients. Measurements of potency by both RAST inhibition and direct RAST assay were strongly correlated to potency as measured by skin testing. In contrast, neither the weight:volume nor the PNU content bore any relationship to allergenic potency as measured by skin testing or by either of the RAST procedures. Extracts differed by as much as 3,000-fold in allergen content by skin testing. Moreover, the extracts appeared to contain different allergenic determinants when tested by RAST inhibition. RAST inhibition offered several technical advantages over the direct RAST procedure, in that only one solid-phase RAST reagent was required, slopes of dose-response curves could be more easily compared, and a greater discrimination in allergenic potencies among extracts could be made. The RAST appears to offer an excellent method for measuring the potency of allergy extracts, pending the isolation and characterization of actual allergens.

At the present time there are no standards of potency requirements for allergenic extracts marketed for diagnosis and treatment in the United States. Extracts are labeled in terms of weight of solid per volume of solvent or in protein nitrogen content, neither of which may necessarily bear any relationship to the actual allergen content.¹ The ideal method of standardization would involve the measurement of the content of the major allergens in crude extracts; however, this goal has been reached for only a relatively few allergens, such as short ragweed,² rye grass,³ and codfish.⁴ The development of the radioallergosorbent test (RAST)⁵ has provided a powerful in vitro tool by which the naturally occurring serum IgE antibodies in sensitized persons can be utilized to identify and quantitate allergens. A direct RAST method of investigating allergenic potency involves coupling of varying quantities for each test extract to solid-phase sup-

From the Departments of Pediatrics, Internal Medicine, and Immunology, The Allergic Diseases Research Laboratory, Mayo Clinic and Mayo Foundation.

Supported in part by a contract from the Bureau of Biologics, Food and Drug Administration (FDA 223-73-1164) and by an Allergic Diseases Academic Award from the National Institute of Allergy and Infectious Diseases (AI 00107) awarded to Dr. Yunginger.

Received for publication Nov. 12, 1975.

Accepted for publication Feb. 2, 1976.

Reprint requests to: Dr. J. W. Yunginger, Allergic Diseases Research Laboratory, Mayo Clinic, Rochester, Minn. 55901.

TABLE I. Potency of commercial *Alternaria* extracts as measured in vitro by PNU and RAST procedures and in vivo by end point skin testing

Alternaria extract	W:V concentration	PNU/ml	Potency by 50% RAST inhibition (U/ml)*	Potency by direct RAST, 22 μ l/disc (% counts bound)
1 ●†	1-10	17,880	2,041	30.52
2 ○	1-20 (5%)‡	31,890	54.9	11.24
3 △	1-10‡	16,500	182	12.30
4 □	1-33 (3%)‡	8,530	417	27.47
5 X	1-20	16,500	123	16.17
6 ◇	1-10	20,740	30.3	14.89
7 ■	1-10	93,760	909	23.66
8 +	1-10	121,800	9.7	10.84
9 ◆	1-10	17,880	45.5	6.53
10 <	1-10	17,250	526	27.68
11 ▲	1-10†	16,673	303	19.36
12 *	1-10	36,200	312	28.33

*Potency by RAST inhibition is defined as the number of 50% RAST inhibition units per milliliter. For example, if 5 μ l yielded 50% inhibition, then 1 ml of the extract contains $1,000 \div 5$, or 200 RAST inhibition units.

†Symbols used to represent the various extracts in the figures.

‡Glycerinated extracts.

ports and then reacting each allergen-polymer with a standard sensitive serum pool. The potencies of birch and timothy pollen extracts⁶ and dander extracts⁷ have been studied by this approach. In addition, the RAST inhibition procedure has also been utilized to investigate the potencies of various birch and timothy pollen extracts⁸ and horse epithelium extracts.⁹ We have reported previously a 1,000-fold difference in allergenic potency of commercial short ragweed extracts¹⁰ and a 100-fold difference in potency of June grass extracts¹¹ using this technique. The successful adaptation of the RAST to measure IgE antibodies to *Alternaria*¹² has permitted the extension of these studies of potency to *Alternaria* extracts. These measurements of relative potency, including a comparison of the direct RAST and RAST inhibition procedures with skin test results, comprise the present report.

MATERIALS AND METHODS

Alternaria extracts

Extracts of *Alternaria* were purchased from 12 different United States companies; 4 of the extracts were glycerinated. The labeled weight:volume concentrations are shown in Table I.

Skin testing

In vivo assays of potency were performed by determining that dilution of each extract produced a 6 × 6 mm wheal-and-flare reaction 15 min after the intradermal injection of sufficient extract to produce an initial 3 × 3 mm wheal. All extracts were diluted initially to 1:100 concentration using phosphate-buffered saline (pH 7.4) containing 0.5% human serum albumin; serial 5-fold dilutions were then prepared for each extract. All dilutions were prepared and used within 5 wk of preparation. Between testing periods extracts were stored at 4° C, but they were brought to room temperature during the testing procedure. The skin test potency of the extracts is expressed in "skin test units." The value for each extract is the reciprocal of the dilution of the original extract necessary to produce a 6 × 6 mm wheal; a larger number indicates greater potency. Details of this calculation have been published previously.¹⁰ We

End point skin test potency (skin test units $\times 10^{-3}$)									
Patient									
J. G.	R. A.	N. K.	G. S.	G. O.	A. N.	S. D.	T. M.	J. F.	J. P.
8.0	3.3	50.6	31.2	90.4	4.5	1.5	46.7	75.5	44.5
3.1	1.2	29.7	5.5	0.9	11.3	0.5	3.1	11.3	5.9
4.2	2.8	2.1	1.7	14.0	0.05	0.2	43.1	6.2	0.8
3.9	5.3	46.9	36.8	15.9	0.6	1.9	24.6	9.3	9.4
5.3	0.2	7.0	3.1	1.4	0.9	0.6	15.6	3.1	1.1
3.3	0.3	3.6	0.7	0.4	0.05	6.2	1.5	2.8	0.3
24.5	6.2	43.1	14.0	14.0	0.3	2.8	37.9	104	24.5
3.3	0.4	3.3	0.4	1.5	0.5	1.0	6.2	31.2	0.1
7.3	0.6	1.7	1.2	0.2	0.03	0.7	4.9	6.2	0.4
349	46.7	1,168	156	199	96.4	69.9	156	781	253
2.8	1.0	10.6	11.9	2.8	0.2	0.7	39.8	39.8	5.3
26.6	20.9	113	59.5	156	31.2	31.2	322	82.1	90.4

tested 10 patients with clinical histories suggesting mold sensitivity, all of whom had significantly positive immediate wheal-and-flare skin tests to crude *Alternaria* extract, and who had not received hyposensitization injections to *Alternaria* within the preceding year. All skin testing was done during November and December, 1974, after the atmospheric *Alternaria* content had fallen for the season. Skin testing was performed on a blind basis during two separate sessions, as reported previously,¹⁰ and the order of testing of the various extracts was randomized for each patient.

RAST inhibition experiments

The RAST inhibition procedure was carried out as described previously,^{10, 11} except that the reaction volumes in each step of the RAST were 1.5 ml. The serum pool was prepared by mixing equal volumes of serum from 10 patients highly allergic to *Alternaria*. None of these patients had received immunotherapy to *Alternaria* within the previous year, and, with one exception (J. G.), were different patients than those skin-tested. The *Alternaria* solid-phase allergen was prepared in our laboratory from dry, defatted *Alternaria* powder, as described previously.¹² That this solid-phase allergen contained all relevant allergens is indicated by its ability to completely absorb skin-sensitizing antibodies to *Alternaria* from the serum pool described above, as measured by passive transfer experiments.¹² In the absence of inhibitor, the allergic serum pool yielded binding of 10% to 13% of total radioactive counts, while non-allergic human serum yielded binding of 0.3% to 0.4%. Because of this low "background" binding, negative control values were not subtracted from test results.

Direct RAST experiments

In vitro estimates of potency were also determined by a direct RAST technique. Filter paper discs (Grade 595, 6.35 mm diameter, Schleicher & Schuell, Inc., Keene, N. H.) were washed with distilled water to remove fines and activated with a 2.5% aqueous solution of cyanogen bromide for approximately 5 min at room temperature, using 10 N NaOH in an automatic titrator (Radiometer, London Co., Cleveland, Ohio) to maintain the pH at 11.0. The suspension was washed repeatedly with cold 0.1 M NaHCO₃, water, and, finally, acetone. After drying at 4° C for 2 hr, the discs were transferred to a desiccator at 4° C overnight. Samples (0.3 ml) of each commercial *Alternaria* extract were dialyzed against 0.2 M H₃BO₃-0.04 M NaOH-0.15 M NaCl (pH 8.0) (borate-saline) using 3,500 MW cutoff tubing (Dialya-Por, National Scientific Co., Cleveland, Ohio) to remove the preservatives and any low molecular weight materials. We have previously shown that crude *Alternaria* extracts do not appear to contain allergenic components with molecular weight below 5,000.¹² Dialysis casings were rinsed with additional borate-saline and volumes were all adjusted to 1.8 ml. Varying quantities of each

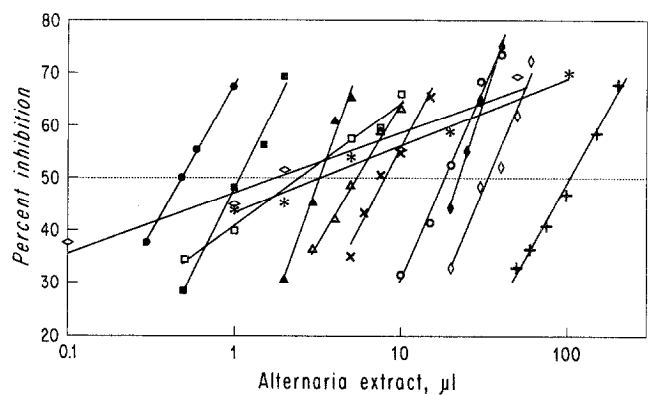


FIG. 1. Measurement of the potency of 12 *Alternaria* extracts by RAST inhibition. Correlation coefficients for regression lines varied between +0.976 and +0.999. The number of microliters of extract required to produce 50% reduction in RAST binding was selected as the end point. See Table I for symbols.

TABLE II. Relationship between the measurements of the potencies of *Alternaria* extracts by skin testing in 10 patients*

Patients									
Patients	R. A.	N. K.	G. S.	G. O.	A. N.	S. D.	T. M.	J. F.	J. P.
J. G.	0.63	0.51	0.57	0.60	0.40	0.55	0.63	0.52	0.64
J. P.	0.87	0.94	0.97	0.82	0.75	0.51	0.71	0.77	—
J. F.	0.80	0.74	0.71	0.74	0.58	0.52	0.69	—	—
T. M.	0.75	0.64	0.75	0.91	0.48	0.35	—	—	—
S. D.	0.54	0.65	0.53	0.52	0.37	—	—	—	—
A. N.	0.53	0.83	0.71	0.63	—	—	—	—	—
G. O.	0.85	0.81	0.84	—	—	—	—	—	—
G. S.	0.86	0.93	—	—	—	—	—	—	—
N. K.	0.78	—	—	—	—	—	—	—	—

For $n = 12$ and a one-tailed test, r_s values greater than +0.50 are significant at $p < 0.05$; r_s values greater than +0.67 are significant at $p < 0.01$; r_s values greater than +0.82 are indicated by boldface numbers and are significant at $p < 0.001$.¹⁵

*Twelve *Alternaria* extracts were tested in each patient, and the extract potencies between patients were compared by the Spearman Rank Correlation Test. Numbers listed are correlation coefficients, r_s ; all are positive.

dialyzed extract were added to ten activated discs and coupled by tumbling overnight at 4° C. Half of the discs were then blocked by overnight tumbling in 0.9 M ethanolamine at 4° C to assure that all activated sites were coupled. The other half were tested without ethanolamine blocking. After several washings with borate-saline, water, and finally 0.2% bovine serum albumin (BSA) RAST diluent,¹³ the discs were stored in RAST diluent. RAST assays were performed by adding 50 μ l of the same patient serum pool as used in the RAST inhibition procedure to duplicate discs, incubating overnight, and washing with 0.1 M phosphate buffer (pH 7.5) containing 1% Tween-20. Approximately 20 ng (100 μ l) of ¹³¹I-radiolabeled anti-IgE was then added, and the discs were incubated overnight, washed as before, and counted in a gamma scintillation counter. Results were expressed as the percentage of total radioactive counts bound to the discs.

Measurement of protein nitrogen content of extracts

The protein nitrogen content of *Alternaria* extracts was determined by precipitation with phosphotungstic acid and Kjeldahl analysis of the redissolved precipitate. These assays were

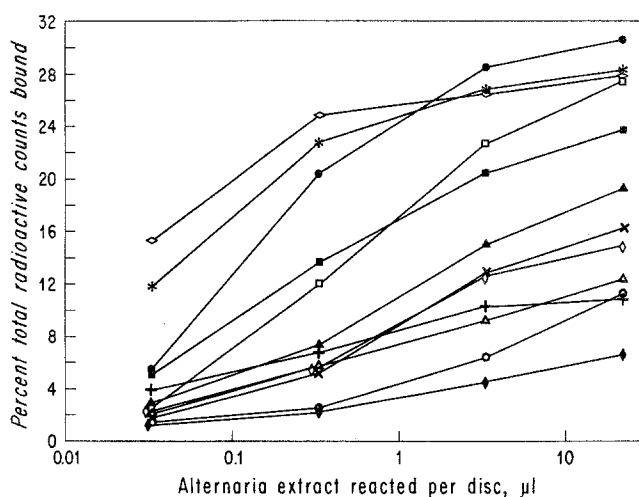


FIG. 2. Measure of the potency of *Alternaria* extracts by direct RAST. Varying quantities of dialyzed extracts were reacted with cyanogen bromide-activated filter paper discs, which were subsequently washed and then reacted with constant volumes of an *Alternaria*-sensitive serum pool in a direct RAST assay. See Table I for symbols.

performed through the courtesy of Dr. Joan C. May, Division of Control Activities, Food and Drug Administration.

Statistical tests

Results were evaluated by tests of rank correlation coefficients and by analysis of covariance.¹⁴ A programmable Hewlett-Packard 9810A calculator was used for the statistical evaluation.

RESULTS

The results of the protein nitrogen determinations and the end-point skin test titrations are listed in Table I. As seen previously with short ragweed extracts, there was as much as a 3,000-fold variation in skin test reactivity among the extracts (Patient A. N.) in spite of the fact that the extracts were comparable in weight:volume concentration. The correlation of the skin test potency measurements between various patients was calculated, and the results are shown in Table II. Forty-one of the 45 correlations were correlated at $p < 0.05$; 23 were correlated at $p < 0.01$. The results indicate that the skin test potencies in the various patients showed good agreement with one another. The poorest overall correlations were seen in Patients S. D. and A. N.; the latter patient had rather dry, eczematoid skin, and skin test reactions were occasionally difficult to measure accurately.

The potencies of the 12 *Alternaria* extracts as measured by RAST inhibition are also shown in Table I and Fig. 1. Dose-response curves were linear between 30% and 70% inhibition when plotted in a log-linear fashion; the volume of *Alternaria* extract producing 50% inhibition was selected as the end point.

The results in Fig. 1 represent the data from a single experiment; virtually identical results were obtained in another assay, and the rank order of potency

TABLE III. Correlation between individual skin test estimates of potency of *Alternaria* extracts and RAST measurements of potency*

Patient	RAST inhibition	Direct RAST		
		22 μ l/disc	3.3 μ l/disc	50% max. binding level
J. G.	0.59	0.55	0.54	0.69
R. A.	0.79	0.69	0.64	0.71
N. K.	0.78	0.89	0.87	0.63
G. S.	0.86	0.87	0.81	0.57
G. O.	0.81	0.85	0.84	0.86
A. N.	0.45	0.61	0.60	0.46
S. D.	0.39	0.62	0.68	0.70
T. M.	0.76	0.76	0.74	0.78
J. F.	0.68	0.61	0.64	0.72
J. P.	0.87	0.85	0.79	0.59

*Numbers listed are rank correlation coefficients (r_s); all are positive. See legend for Table II for significance levels of r_s .

TABLE IV. Comparison of the potencies of *Alternaria* extracts as determined by PNU, by the geometric mean skin test, by RAST inhibition, and by direct RAST assay

	Rank correlation coefficients		
	RAST inhibition	Skin test	PNU
Direct RAST (max. binding point, 22 μ l/disc)	+0.87 ($p < 0.001$)	+0.88 ($p < 0.001$)	-0.15 (NS)
Direct RAST (3.3 μ l/disc)	+0.81 ($p < 0.005$)	+0.85 ($p < 0.001$)	-0.06 (NS)
Direct RAST (50% maximum binding point)	+0.61 ($p < 0.025$)	+0.73 ($p < 0.01$)	+0.23 (NS)
PNU	-0.21 (NS)	-0.01 (NS)	—
Skin test	+0.87 ($p < 0.001$)	—	—

NS: not significant.

of the various extracts was the same in both experiments. The results showed that the extracts varied over a wide range with respect to potency, and that the dose-response curves were not parallel. Inhibition curves, in general, assumed either of two patterns; nine extracts produced rather upright curves, and the other three were relatively flat. Thus, the *Alternaria* extracts tested varied not only in allergen content per milliliter, but probably also varied in the nature of the allergenic determinants they contained.

The results of the direct RAST assay are shown in Fig. 2. Because of limiting quantities of *Alternaria* extracts, the dose-response curves could not be carried out further than the equivalent of 22 μ l of undiluted extract per disc. However, the curves were beginning to plateau at this point, and there was a change of only two positions when the rank positions at 3.3 μ l/disc and 22 μ l/disc were compared. The maximum binding level for each extract was divided by two to determine the 50% maximum binding level, which was used as an additional end-point parameter. Similar results were obtained using either discs which were blocked with ethanolamine or unblocked discs.

Correlations between in vivo and in vitro estimates of potency were calculated

for each patient skin-tested, and these results are shown in Table III. Significant correlations were noted between RAST results and skin test results in 8 of the 10 patients skin-tested; once again, the poorest correlations were noted with Patients A. N. and S. D. To further examine the overall correlation between in vivo and in vitro estimates of the potency of the 12 *Alternaria* extracts, we next calculated the geometric mean skin test potency for each extract. The comparisons between PNU, skin testing, RAST inhibition, and direct RAST are shown in Table IV. In general, there was excellent agreement between both the direct RAST and RAST inhibition and the skin test estimate of potency. No correlation was found between the PNU content of the extract and its allergenic potency, as measured by either skin testing or RAST procedures.

DISCUSSION

The slopes of the RAST inhibition curves produced by the 12 commercial extracts were not parallel, assuming either a somewhat flat (3 extracts) or more upright (9 extracts) configuration. However, even the slopes of the extracts within each of these two groups differed significantly, suggesting that the extracts did not contain qualitatively the same allergenic determinants. This may have been due to species differences between *Alternaria* strains used in preparing the extract, or to different culturing and harvesting methods used by different manufacturers. Two extracts were labeled *Alternaria tenuis*, while the other ten were labeled *Alternaria* or *Alternaria sp.* These ten manufacturers were contacted later, and seven of them stated that their materials were prepared from *Alternaria tenuis*. The other three extracts were produced from *Alternaria alternata* (No. 5), *Alternaria brassicicola* (No. 10), and from an unspciated *Alternaria* obtained as an airborne isolate (No. 3). *Alternaria alternata* is synonymous with *A. tenuis*,¹⁶ and this extract produced an inhibition curve similar in slope to that of most other *A. tenuis* extracts. *A. brassicicola* produced an inhibition curve that was much flatter than those of the other extracts. The curve of the unspciated *Alternaria* extract resembled those of most *A. tenuis* extracts. Overall, our results indicate that commercial *Alternaria* extracts are much more heterogeneous than the short ragweed or June grass pollen extracts studied previously in our laboratory.

To our knowledge, these experiments represent the first comprehensive comparison of the RAST inhibition and the direct RAST methods of measuring allergenic activity. It should be remembered that single sensitive persons may react to only one or a few of several allergens in a crude allergen mixture, a point illustrated and stressed previously by European investigators.⁹ In using the RAST to estimate the potency of allergenic extracts, this potential problem can be circumvented in part by including a large number of patient serums in the standard IgE antibody pool. The inclusion of a specified reference allergen preparation in the RAST assays would allow the potencies of various extracts to be expressed in terms of a single material. One must also be certain that all relevant allergens are contained on the solid-phase preparation, either by demonstrating complete removal of all protein and/or carbohydrate materials from the coupling super-

natants, or by demonstrating by Prausnitz-Küstner (P-K) testing the complete removal of IgE antibodies from a sensitive serum pool by repeated absorption with the solid-phase allergen.

As a primary measurement of the allergenic potency of the *Alternaria* extracts, we selected end-point skin test titration as the most convenient and quantitative in vivo test. Estimates of the potencies of the various *Alternaria* extracts by RAST inhibition were significantly correlated with in vivo estimates of potency in 8 of 10 patients skin-tested; the direct RAST estimates of potency (both 22 μ l/disc and 3.3 μ l/disc) were significantly correlated with skin test results in all 10 patients. In spite of this, we believe that the RAST inhibition method is more easily adapted to large-scale studies of allergen identification and measurement. The RAST inhibition method requires the preparation of only one solid-phase reagent, which, at least in the case of short ragweed, can be expected to remain active when stored at 4° C for several years.¹⁷ Moreover, the RAST inhibition method permitted a discrimination in allergenic potencies (approximately 200-fold) which was considerably greater than that obtainable with the direct RAST. Finally, the slopes of the dose-response curves can be more easily compared using RAST inhibition.

It should be emphasized that the labeled weight:volume designation is not an actual measurement of allergenic potency, but simply an indication of the relative amounts of solid and extracting fluid used to prepare the extract. In this study there was as much as a 3,000-fold variation in skin test potency among *Alternaria* extracts of similar weight:volume concentration. Similarly, the measurement of the PNU content of an extract includes not only phosphotungstic acid-precipitable allergens, but also nonallergenic proteins and polypeptides. Our results indicate that the present weight:volume and protein nitrogen labelings of *Alternaria* extracts are inadequate measures of allergen content.

While RAST procedures appear to be excellent in vitro methods of allergen standardization, there will still remain a future need for isolation and characterization of individual allergenic components. This would permit the production of monospecific animal antisera and the subsequent measurement of these individual allergens by radial immunodiffusion, which might be a much simpler in vitro method than either RAST procedure for the standardization of allergenic extracts.

We are indebted to the physicians of the Allergy Section of the Department of Internal Medicine for referring *Alternaria*-sensitive patients for study, and to Mrs. Pat Peterson and Mrs. Helen Hoops for performing the end-point skin test titrations.

REFERENCES

- 1 Baer, H., Godfrey, H., Maloney, C. J., Norman, P. S., and Lichtenstein, L. M.: The potency and antigen E content of commercially prepared ragweed extracts, *J. ALLERGY* 45: 347, 1970.
- 2 King, T. P., Norman, P. S., and Connell, J. T.: Isolation and characterization of allergens from ragweed pollen. II. *Biochemistry* 3: 458, 1964.
- 3 Johnson, P., and Marsh, D. G.: The isolation and characterization of allergens from the pollen of rye grass (*Lolium perenne*), *Eur. Polymer J.* 1: 63, 1965.
- 4 Aas, K., and Jebsen, J. W.: Studies of hypersensitivity to fish. Partial purification and

- crystallization of a major allergenic component of cod, *Int. Arch. Allergy Appl. Immunol.* **32**: 1, 1967.
- 5 Wide, L., Bennich, H., and Johansson, S. G. O.: Diagnosis of allergy by an in vitro test for allergen antibodies, *Lancet* **2**: 1105, 1967.
 - 6 Ceska, M., Eriksson, R., and Varga, J. M.: Radioimmunosorbent assay of allergens, *J. ALLERGY CLIN. IMMUNOL.* **49**: 1, 1972.
 - 7 Varga, J. M., and Ceska, M.: Characterization of allergen extracts by polyacrylamide gel isoelectrofocusing and radioimmunosorbent allergen assay. II. Dog and cat allergens, *Int. Arch. Allergy Appl. Immunol.* **42**: 438, 1972.
 - 8 Foucard, T., Johansson, S. G. O., Bennich, H., and Berg, T.: In vitro estimation of allergens by a radioimmune antiglobulin technique using human IgE antibodies, *Int. Arch. Allergy Appl. Immunol.* **43**: 360, 1972.
 - 9 Aronsson, T., and Wide, L.: Studies on allergens of horse epithelium with two variants of RAST, *Int. Arch. Allergy Appl. Immunol.* **47**: 224, 1974.
 - 10 Gleich, G. J., Larson, J. B., Jones, R. T., and Baer, H.: Measurement of the potency of allergy extracts by their inhibitory capacities in the radioallergosorbent test, *J. ALLERGY CLIN. IMMUNOL.* **53**: 158, 1974.
 - 11 Gleich, G. J., Leiferman, K. M., Jones, R. T., Hooton, M. L., and Baer, H.: Analysis of the potency of extracts of June grass pollen by their inhibitory capacities in the radioallergosorbent test, *J. ALLERGY CLIN. IMMUNOL.* **58**: 31, 1976.
 - 12 Yunginger, J. W., Roberts, G. D., and Gleich, G. J.: Studies on *Alternaria* allergens. I. Establishment of the radioallergosorbent test (RAST) for measurement of *Alternaria* allergens, *J. ALLERGY CLIN. IMMUNOL.* **57**: 293, 1976.
 - 13 Gleich, G. J., and Jones, R. T.: Measurement of IgE antibodies by the radioallergosorbent test. I. Technical consideration in the performance of the test, *J. ALLERGY CLIN. IMMUNOL.* **55**: 334, 1975.
 - 14 Dixon, W. J., and Massey, F. J., Jr.: *Introduction to Statistical Analysis*, New York, 1969, McGraw-Hill Book Co., pp. 222 and 349.
 - 15 Conover, W. J.: *Practical nonparametric statistics*, New York, 1961, John Wiley & Sons, Inc., p. 390.
 - 16 Simmons, E. G.: Typification of *Alternaria*, *Stemphylium*, and *Ulocladium*, *Mycologia* **59**: 67, 1967.
 - 17 Gleich, G. J., Jones, R. T., and Yunginger, J. W.: Unpublished observations.