

Studies on *Alternaria* allergens

I. Establishment of the radioallergosorbent test for measurement of *Alternaria* allergens

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The ability to covalently couple Alternaria allergens to microcrystalline cellulose particles has permitted not only the measurement of IgE antibodies to Alternaria in patient serums but also the identification of allergenic fractions from crude Alternaria extracts. Crude aqueous Alternaria extracts from 3 commercial suppliers were coupled to cellulose but failed to bind more than 5% of total radioactive counts (TRC) when reacted with serums from highly sensitive patients. Fractionation of a commercial extract through Sephadex G-25 showed that almost all allergenic activity was located in a protein- and carbohydrate-containing peak eluting at the column void volume. These fractions were pooled and coupled to cellulose to yield a RAST polymer which produced up to 20% TRC binding when tested with serums from over 100 Alternaria-sensitive patients, and only up to 1% TRC binding with 17 nonallergic serums. The study of commercial Alternaria extracts by chromatographic and RAST inhibition techniques showed that present extracts are neither qualitatively or quantitatively comparable.

Since the original paper on the radioallergosorbent test (RAST) by Wide, Bennich, and Johansson,¹ the technique has been used by several groups of investigators to measure serum IgE antibodies to various pollens,²⁻⁴ animal danders,⁵ foods,⁶ drugs,⁷ and house dust mites.⁸ There have been few reports to date, however, on the use of the RAST to identify IgE antibodies to molds.⁹ This paper describes the preparation of a RAST polymer for *Alternaria*, one of the major allergenic molds in the United States.

MATERIALS AND METHODS

Patients

Serum specimens were obtained from 166 patients receiving an allergy consultation in the adult or pediatric allergy sections of the Mayo Clinic between June, 1973, and February, 1974.

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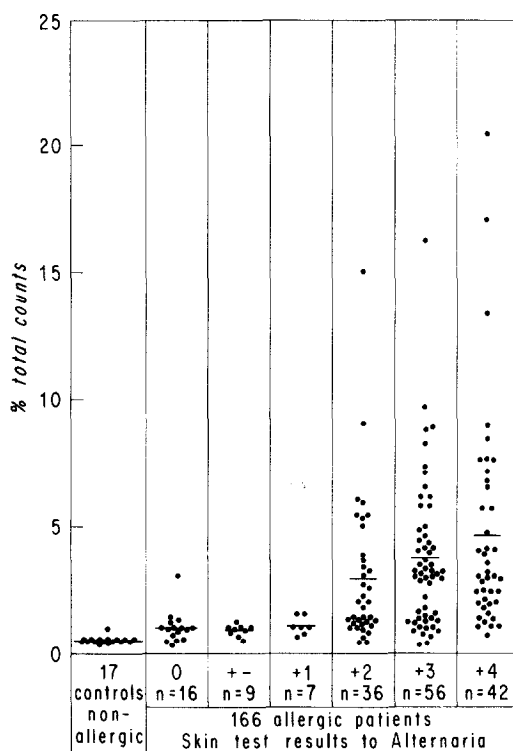


FIG. 1. RAST binding by allergic and nonallergic patient serum using the *Alternaria* polymer. Mean RAST binding by each group is indicated by the horizontal bars. See text for grading of skin test results.

Particular emphasis was placed on obtaining serums from patients whose clinical history suggested mold allergy. Serums were stored at -20° until assayed. All patients received intradermal skin tests to crude aqueous *Alternaria* extract (Center Laboratories, Inc., Port Washington, N. Y.) at 500 PNU/ml, sufficient allergen being injected to produce an initial 2 to 3 mm wheal. Those patients whose skin test wheals measured greater than 11 mm in diameter after 10 to 15 min were considered to have +4 skin reactivity. Wheal diameters of 8-11 mm were classified as +3; those of 5-8 mm, +2; 4-5 mm, +1; and below 4 mm, +-. Certain of these patients were also skin tested with chromatographic fractions of crude *Alternaria* extracts (see below).

***Alternaria* extracts**

Nonglycerinated *Alternaria* extracts (40,000 PNU/ml or 1:10 w/v) and lyophilized *Alternaria* extracts were purchased from or were gifts from Center Laboratories, Hollister-Stier Laboratories (Spokane, Wash.), or Greer Laboratories (Lenoir, N. C.). In addition, we purchased aqueous MMP (Morrow, Meyer, and Prince) *Alternaria* extract (1:100) from Hollister-Stier Laboratories and dry *Alternaria* powder from Greer Laboratories. The latter material was extracted with 0.05M Tris-HCl-0.1M NaCl (pH 7.8) (Tris-NaCl) to provide a 1:20 extract.

Three random *Alternaria* isolates were selected from colonies identified during atmospheric mold surveys in Rochester during the late summer of 1973. These isolates were inoculated into stationary cultures containing trypticase soy broth or Czapek's medium* for 3½ wk

*Czapek's medium contains the following ingredients/L: sucrose, 30 gm; sodium nitrate, 2 gm; dibasic potassium phosphate, 1 gm; magnesium sulfate, 0.5 gm; potassium chloride, 0.5 gm; and ferrous sulfate, 0.01 gm.

TABLE I. Absorption of *Alternaria*-sensitive serum pool by *Alternaria* polymer*

Serum dilution	P-K skin test wheal diameters (mm)	
	Alternaria absorbed	Bovine serum albumin absorbed
1:10	5 × 6	11 × 12
1:20	4 × 7	13 × 9
1:40	4 × 4	13 × 8
1:80	5 × 8	8 × 8
1:160	6 × 6	8 × 9
1:320	7 × 7	7 × 9
1:320	7 × 7	7 × 9
1:640	6 × 6	8 × 9
1:1280	5 × 6	6 × 6
Unsensitized site	5 × 5	

*Sensitized and unsensitized skin sites were challenged with approximately 0.004 ml of crude *Alternaria* extract containing 2.4 ml of protein and 1.1 mg of carbohydrate/ml; reactions were measured 15 min later.

before being harvested. Pellicles were isolated by paper filtration and fragmented by passage through a Waring Blendor. Following defatting with acetone, the pellicles were dried overnight in a 37° C oven and powdered by ball milling (Lortone Co., Seattle, Wash.). The final material grown from trypticase soy broth was extracted with Coca's solution to produce a 1:10 extract. The Czapek's medium produced insufficient mold growth during the incubation period to obtain a pellicle for extraction.

Czapek culture media filtrate (2,500 ml) was concentrated by passage through a UM-2 Diaflo membrane (Amicon Corporation, Lexington, Mass.), and aliquots were prepared for skin-testing by passage through 0.22 μ Millipore filters (Millipore Corporation, Bedford, Mass.). The ultrafiltration retentate was then dialyzed against Tris-NaCl (Spectrapor Membrane Tubing, M.W. cutoff app. 3,500; National Scientific Co., Cleveland, Ohio), and fractionated through superfine Sephadex G-25.

Measurement of IgE antibody to *Alternaria*

Various *Alternaria* RAST polymers were prepared with cyanogen bromide-activated microcrystalline cellulose as previously described.¹⁰ The RAST procedure itself was performed as previously described,⁴ except that approximately 20 ng of radiolabeled, affinity chromatography-purified anti-IgE was added per tube in the second step of the RAST, and reaction volumes in both stages of the RAST were increased to 1.5 ml. First-stage diluent was 0.2% bovine serum albumin (BSA) in 0.1M phosphate buffer (pH 7.4) containing 1% Tween-20 and 0.1% sodium azide; the second-stage diluent substituted 4% fetal calf serum for the BSA.

Fractionation of crude *Alternaria* extracts

Selected *Alternaria* extracts were fractionated by gel filtration through superfine Sephadex G-25 columns equilibrated with Tris-NaCl. The protein and total sugar contents of selected column fractions were monitored by the biuret¹¹ and phenol sulfuric acid tests,¹² respectively, with human serum albumin and glucose as standards.

RAST inhibition studies

Crude whole extracts and selected chromatographic fractions were also tested for allergenic potency by means of the RAST inhibition technique.¹³ Varying quantities of uncoupled extracts or column fractions were incubated with solid-phase allergen and a pool composed of serums from 10 *Alternaria*-sensitive patients in the first stage of the RAST. The addition of increasing quantities of uncoupled allergen produced a progressive reduction in binding of radioactive counts by the solid-phase allergen and thus inhibition curves were established. These curves were linear between 30 and 70% inhibition when plotted in a log-linear fashion. Inhibition results were evaluated by means of analysis of covariance¹⁴ with a programmable Hewlett-Packard 9810A calculator.

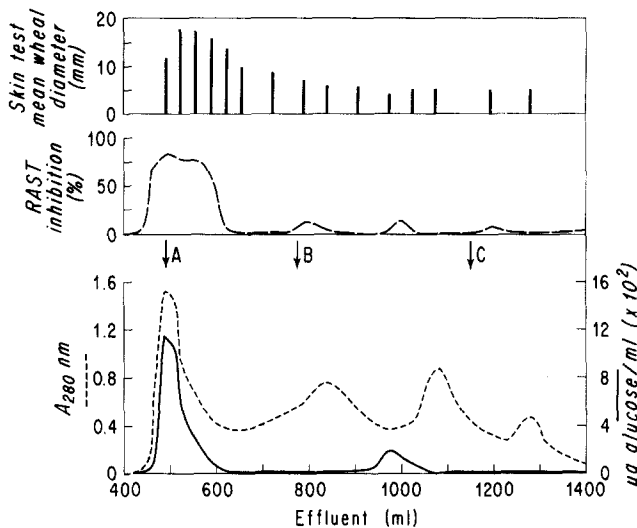


FIG. 2. Sephadex G-25 chromatogram of extract prepared from commercial dry *Alternaria* powder. Elution buffer was 0.05M Tris-HCl-0.1M NaCl (pH 7.8). Elution volumes of the markers blue dextran (MW, 1,000,000), bacitracin (MW, 1,411), and glycyl-L-tyrosine (MW, 232) are indicated by arrows **A**, **B**, and **C**, respectively. Negative control skin tests using elution buffer alone ranged between 2 and 4 mm in diameter. Skin tests of greater than 6 to 7 mm in diameter were associated with flares, and those greater than 10 mm in diameter were usually associated with pseudopod formation.

RESULTS

Preparation of *Alternaria* RAST polymers

Initial polymers were prepared by reacting 1 ml each of 3 commercial concentrated aqueous *Alternaria* extracts with 500 mg of activated microcrystalline cellulose. These polymers were tested with serums from 14 *Alternaria*-sensitive and 16 nonallergic persons; the former all had +3 or +4 intradermal skin tests to *Alternaria*. The range of binding in the allergic group was from 0.63% to 5.58% of total radioactive counts, as compared to a range of 0.34% to 1.25% in the nonallergic group. There was much overlap between the 2 groups with all 3 polymers with only 8, 5, and 2, respectively, of the 14 allergic serums exhibiting binding at least twice that of normal serum run in the same experiment. Because earlier reports suggested that *Alternaria* allergens are nondialyzable,¹⁵ 40 ml of concentrated (40,000 PNU/ml) crude *Alternaria* extract was dialyzed against Tris-NaCl to remove the phenol preservative and any lower molecular weight contaminants and then gel filtered through a 4 by 95 cm column of Sephadex G-25 equilibrated with Tris-NaCl. The void volume fractions, containing both protein and carbohydrate, were pooled, concentrated, and then coupled to microcrystalline cellulose. Retesting of the original 14 serums from *Alternaria*-sensitive patients with this new polymer now showed that 11 serums exhibited binding at least twice that of nonallergic control serum. The new polymer was therefore tested additionally with 166 allergic serums and 17 nonallergic serums and had much greater reactivity than the crude *Alternaria* polymers. Fig. 1 compares

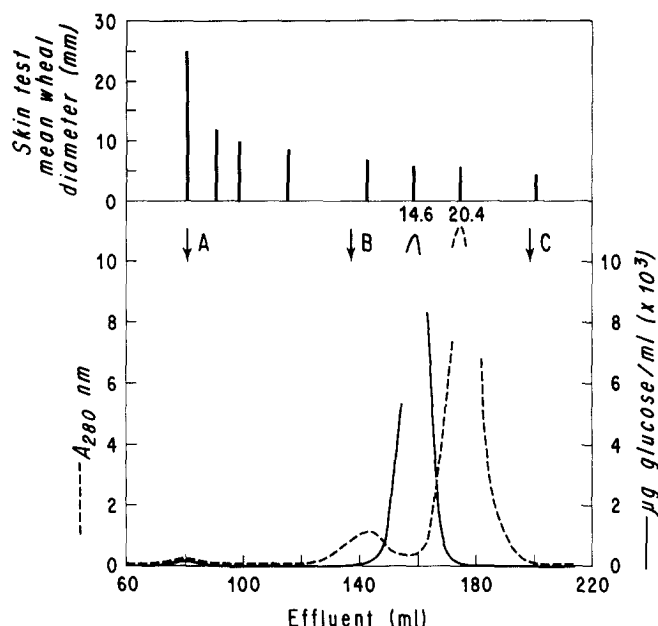


FIG. 3. Sephadex G-25 chromatogram of 180,000 PNU of commercial lyophilized *Alternaria* extract. Elution buffer, column markers, and skin test data are as listed in Fig. 2.

skin test results and RAST results from both groups with the new polymer. This polymer better differentiated *Alternaria*-sensitive patients from nonallergic patients, particularly those sensitive patients having +2 or larger skin test reactions.

In an effort to show that all major allergens were coupled to this polymer, a pool composed of serums from 10 *Alternaria*-sensitive patients was absorbed seven times with 1.5 mg aliquots of the polymer. As a control the same pool was also absorbed with a polymer of bovine serum albumin coupled to microcrystalline cellulose. The *Alternaria*-absorbed serum pool, along with the BSA-absorbed pool, was injected into a nonsensitive recipient; sites were challenged 72 hr later with crude *Alternaria* extract prepared in our laboratory from dry *Alternaria* powder. Results are listed in Table I and showed complete removal of skin-sensitizing antibody by the *Alternaria* polymer.

Fractionation of *Alternaria* extracts

Other *Alternaria* extracts were also fractionated over Sephadex G-25. Fig. 2 shows the chromatogram of a 1:20 extract of commercial powdered *Alternaria* in Tris-NaCl. Four separate absorbance peaks were observed at 280 nm along with 2 carbohydrate-containing peaks. When selected fractions were skin tested directly on four *Alternaria*-sensitive patients, however, only those peaks appearing at the void volume produced strong positive tests. These fractions were also the only ones producing significant activity in the RAST inhibition procedure. Seph-

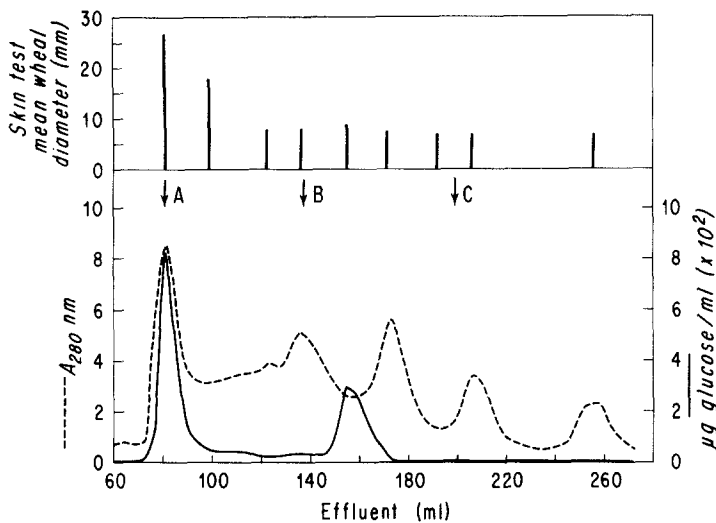


FIG. 4. Sephadex G-25 chromatogram of 101,200 PNU of a second commercial lyophilized *Alternaria* extract. See Fig. 2 for details.

adex G-25 gel filtration of the extract of atmospheric *Alternaria* isolates and the dialyzed Czapek media revealed the presence of skin test-active macromolecular substances which eluted at the column void volume in both instances.

Fig. 3 shows the chromatogram of a commercial freeze-dried *Alternaria* extract, reconstituted in Tris-NaCl and fractionated over Sephadex G-25. A very small carbohydrate-containing peak which also showed slight 280 nm absorbance was noted at the column void volume. Void volume fractions were again the only skin test active materials when tested in sensitive patients. A large second peak giving intense carbohydrate reactivity was noted, followed immediately by a large 280 nm absorbance peak which gave a negative biuret reaction. Scanning UV-spectrometry showed the latter peak to have absorbance maximums at 200, 238, and 280 nm; it appeared to be distinct from phenol, which produced absorbance peaks at 212 and 269 nm. Both the large carbohydrate-containing peak and the large 280 nm absorbance peak were nonreactive by skin test, and both peaks were removed when a fresh aliquot of lyophilized material was reconstituted in Tris-NaCl, placed in a Visking-27 casing, and dialyzed against Tris-NaCl prior to Sephadex G-25 gel filtration. A second lyophilized *Alternaria* preparation from another commercial supplier was also fractionated over Sephadex G-25; this chromatogram is illustrated in Fig. 4. There was a marked difference in elution pattern with this preparation in that a greater proportion of allergenically active materials of higher molecular weight appeared at the column void volume.

RAST inhibition studies

Fig. 5 depicts the RAST inhibition curves for 4 commercially obtained *Alternaria* extracts tested with the *Alternaria* polymer and a pool composed of

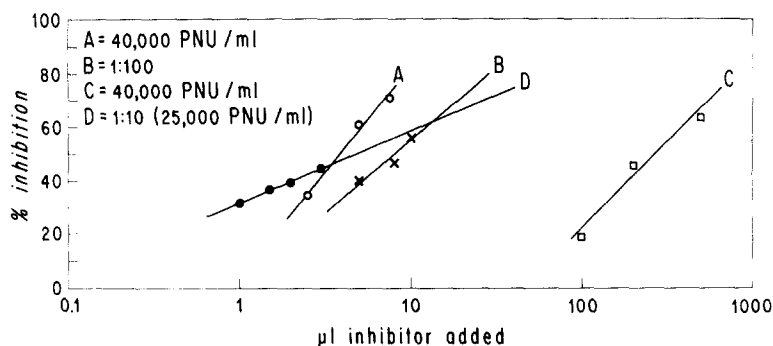


FIG. 5. Measurement of the potency of 4 commercial Alternaria extracts (A, B, C, D,) by RAST inhibition.

serum from 10 Alternaria-sensitive patients. The curves are linear between 30% and 70% inhibition when plotted in a log-linear fashion. Parallelism in slopes between various curves is taken as evidence of qualitative antigenic similarity. Extracts A, B, and C produced inhibition curves whose slopes were not significantly different, although there was almost a 100-fold difference in allergen potency as measured by 50% inhibition points. The slope of the extrapolated inhibition curve of extract D was significantly different from that of A ($p < 0.01$) or B ($p < 0.025$) and tended to be different from C ($0.1 > p > 0.05$), presumably indicating some difference in allergenic determinants in this extract when compared to the other 3. Although not shown in Fig. 5, we could measure RAST inhibition values in excess of 50% with extract D in two other experiments.

DISCUSSION

The results of skin tests with column fractions of crude Alternaria extracts and culture medium indicate that major allergenic fractions elute in the void volume of Sephadex G-25 and probably therefore have molecular weights in excess of 5,000. We have confirmed the release of allergen into the culture medium during mold growth; this observation has been made previously.¹⁵

Commercial mold allergens are prepared from the pellicle with or without the culture medium. There is not unanimity among allergists or manufacturers of allergy extracts, however, as to the superiority of one over the other. Currently, MMP mold extracts utilize both the pellicle and the culture broth as the source of allergen whereas some other commercial mold extracts utilize only the pellicle.¹⁶

The results of the RAST inhibition studies and the chromatographic patterns of commercial lyophilized Alternaria extracts indicate that presently available commercial Alternaria extracts are not comparable either qualitatively or quantitatively. They may vary over a wide range in terms of potency, as seen in Fig. 5, where extract B, a 1:100 MMP preparation, is nearly as potent as extract A, which contains 40,000 PNU/ml.

The preparation of solid-phase coupled allergens for use in the RAST in-

volves the activation of polysaccharide polymers or paper discs by cyanogen bromide, with the resultant formation of a reactive imino group. Soluble allergen may then be attached to the activated polymer by covalent bonding through an amino group on the allergen.¹⁷ Allergens which are largely protein in nature may thus be readily coupled, but allergens which are polysaccharide or glycoprotein may not be as readily coupled by this technique. LeBeau¹⁸ was able to eliminate 96% of extractable nitrogen from his crude *Alternaria* extracts by alcohol treatment of the mold pellicle and acetone precipitation of the extracts, with no subsequent loss in skin test potency. He also noted only slight loss of allergenicity in extracts boiled for 1 hr in the presence of concentrated hydrochloric acid. The latter observation was confirmed later by Bonilla-Soto, Rose, and Arbesman.¹⁹ These previous investigations cast some doubt about whether the allergens of *Alternaria* are simple proteins and they suggest that the major allergens are glycopeptides and/or glycoproteins. Our results do not resolve this question but, because IgE antibodies are reactive with solid-phase *Alternaria* allergens, they encourage belief that a significant proportion of *Alternaria* allergens contain free amino groups.

The RAST polymer described here clearly differentiated *Alternaria*-sensitive patient serums from those of nonallergic controls, particularly serums from those sensitive patients having moderate to strong skin test reactivity. In addition, RAST inhibition studies with column fractions nicely paralleled skin test results with the same column fractions. This observation is an important one, because it illustrates the value of the RAST inhibition procedure in identifying allergenically active fractions of crude allergen mixtures. RAST inhibition promises to be an important investigative tool in the isolation and characterization of purified mold allergens. Further studies are under way in our laboratory at present to accomplish this.

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