

Antigens of *Alternaria*

I. Isolation and partial characterization of a basic peptide allergen

T. W. Budd, Ph.D., C. Y. Kuo, Ph.D., T. J. Yoo, M.D., Ph.D.,
W. R. McKenna, M.D., and J. Cazin, Jr., Ph.D.

Memphis, Tenn., and Iowa City, Iowa

A basic peptide allergen has been isolated from Alternaria extracts. Flat-bed gel preparative isoelectric focusing followed by dialysis and lyophilization allowed for concentration of substantial quantities of this heretofore unrecognized allergen. Rocket immunoelectrophoresis and autoradiography (using a 2× concentrated, IgE serum fraction from Alternaria patient sera and ¹²⁵I-anti human IgE), a RAST inhibition assay, and skin testing of Alternaria-sensitive patients were used to demonstrate the allergenicity of this fraction. The allergenicity of this protein was sufficient to give 50% RAST inhibition at a concentration of 100 µg/ml using standard RAST procedures. The immunoelectrophoresis autoradiography also indicated allergenicity. Skin tests indicated that over 90% of patients hypersensitive to Alternaria crude extracts were also hypersensitive to the basic allergen fraction. (J ALLERGY CLIN IMMUNOL 71:277, 1983.)

Antigenic and allergenic differences have been documented between various sources of *Alternaria* extracts.¹⁻³ It is important to identify and characterize the major allergens of *Alternaria* (and other molds) in order to standardize the extracts used for diagnosis and immunotherapy. This type of characterization has been done with short ragweed and other inhalant allergens⁴⁻⁶ and is in the process of being done with several others. Justification for the standardization and characterization is based on the wide variability of sensitivity among mold-allergy patients.⁷

There have been several reports on the isolation of *Alternaria* allergens.^{2, 8-13} The well-characterized fraction (Alt-I) of Yunginger et al.¹¹ with a pI of about 4.5 showed six bands using Tris-borate PAGE, with each band showing allergenicity. Alt-I is a major allergen that has been assayed both immunochemically

and biologically.^{11, 12} The procedures for isolation of this and other reported fractions were quite elaborate and complex.^{10, 11} The present study reports on the isolation and partial characterization of another potent *Alternaria* allergen by use of IEF.

MATERIALS AND METHODS

Alternaria source

Three isolates designated *Alternaria* 1803, 1808, and 1809 were strains obtained from air sampling and cultured at the University of Iowa in Czapek-Dox broth supplemented with a Casamino Acids dialysate capable of passing through dialysis membrane tubing (Spectrum Medical Industries, Inc.) with a molecular weight cutoff of 6000 to 8000. This medium ensured that any peptides recovered in extraction procedures were not constituents of the original medium, but were those that had been synthesized by the mold. The mycelium was harvested by filtration, washed with acetone, air dried, and milled to a powder. The species of *Alternaria* was not determined for this study (see Discussion).

Crude extract preparation

The mycelium was extracted by homogenization in a Waring blender (high speed), using cold distilled water in a prechilled homogenizing vessel. Portions of mycelium (10 to 20 gm) were extracted in 400 ml of freshly distilled water by homogenization for 5 min. Centrifugation (8000 × g for

From the Veterans Administration Medical Center and the Department of Medicine—Division of Allergy and Immunology, University of Tennessee Center for the Health Sciences, Memphis, Tenn., Department of Medicine and Microbiology, University of Iowa, Iowa City, Iowa.

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Reprint requests to: T. W. Budd, Ph.D., Biology Department, St. Lawrence University, Canton, NY 13617.

Abbreviations used

IEF:	Isoelectric focusing
PAGE:	Polyacrylamide gel electrophoresis
RAST:	Radioallergosorbent test
¹²⁵ I-anti-HuIgE:	¹²⁵ I-Labeled antibody against human IgE
Rb anti-I-Alt:	Rabbit antibody against Iowa- <i>Alternaria</i> crude extracts
OD ₂₈₀ :	Optical density at 280 nm

15 min at 4° C) yielded an amber, usually clear, supernatant fluid with a pH of about 8.0 to 8.5. The mycelium was extracted a second and third time by rehomogenizing the pellet with 200 ml of distilled water. The resulting supernates were less amber and had a pH of about 7.0. The supernates were pooled, dialyzed against 0.05M NH₄HCO₃ (6000 to 8000 dalton cutoff), and lyophilized. The dialysis step was limited to one overnight 10-fold dilution to ensure that mineral ions would not affect the IEF of the extract sample. Preliminary experiments showed that the use of overnight extractions and buffered saline did not substantially increase the yield of crude extract.

Preparative flat-bed gel IEF

Between 0.5 to 1.0 gm of lyophilized crude extract was dissolved in 20 ml of distilled water. Insoluble material, if present, was removed by centrifugation (20,000 × g at 4° C). Six milliliters of ampholine solution (LKB 3.5 to 10 pH range; LKB, Bromma, Sweden) were added, and this mixture was combined with 120 ml of wetted gel (Ultradex-LKB) and stirred to a homogenous slurry. The slurry was poured into the bed plate and subjected to standard electrofocusing procedure. The preparation was focused for 10 to 24 hr at 4° C, with an upper limit of 500 V per plate, and gel fractions were harvested by means of a 30-fraction gel partitioner. The proteins in the gels were eluted with distilled water, and pH and OD₂₈₀ values were recorded for each fraction. The eluates of peak fractions were brought to neutrality with HCl or NaOH and tested for protein content and RAST inhibition. These values were compared with the same fractions from IEF runs by means of ampholines alone.

Concentration of the basic fraction

The eluate of fraction(s) from the cathodic end of the gel plates from several runs were combined, dialyzed against 0.05M NH₄HCO₃, and lyophilized. The resulting off-white powder was stored at -20° C until used for characterization studies. The lyophilizate dissolved readily and completely and the protein concentration of solutions was monitored with a Coomassie dye procedure¹⁴ throughout this study.

Antisera

Rb anti-I-Alt serum was obtained from two New Zealand White rabbits that were first immunized in their hind foot pads with 1 mg of a mixture of crude extracts from

I-Alternaria 1803, 1808, and 1809 emulsified in complete Freund's adjuvant. This primary immunization was followed by monthly intravenous (ear) booster immunizations with 0.5 mg of the extract mixture in saline. The animals were bled 7 days after each boosting. The sera were collected from several bleedings, pooled, precipitated with ammonium sulfate (50% saturation), redissolved in half the original volume, dialyzed against normal saline, and sterilized by membrane filtration (0.2 μm). The amount of antibody used for immunoelectrophoresis is specified as the dilution of this antisera preparation.

Human sera were obtained from *Alternaria*-sensitive patients (by skin test) and tested for anti-*Alternaria* IgE by a standard RAST procedure (Phadebas RAST; Pharmacia Diagnostics, Piscataway, N. J.). Sera that were RAST class 3 or above (13 patients) were pooled, precipitated in ammonium sulfate (50% saturation), redissolved to half the original volume, dialyzed against saline, and sterilized with a membrane filter (0.2 μm). None of the patients had undergone immunotherapy for their allergies prior to taking blood samples. All antisera were stored at -20° C until used.

Immunoelectrophoresis

The allergenicity of the fraction was determined by a modification of the radioimmunoelectrophoresis procedure of Weeke and Lowenstein.¹⁵ The radioimmunoelectrophoresis was done by means of a simple rocket immunoelectrophoresis run in 1% agarose containing 1:15 dilute (~15 μl/cm²) Rb anti-I-Alt crude extract sera. The gel was electrophoresed overnight at 2 V/cm, pressed, and washed. The pooled human serum from *Alternaria*-sensitive patients was applied to the gel surface by means of photographic lens paper saturated with the sera (250 μl) and incubated for 6 hr. The gel was then washed overnight in saline and then repressed. ¹²⁵I-Anti-HuIgE (Pharmacia RAST isotope unit, 5 μCi/0.4 gm/5 ml) was applied (250 μl via saturated lens paper) to the gel for 6 hr, and the gel was again washed overnight. The gel was then desalinated with distilled water, dried, and placed on x-ray film (Kodak X-Omat R, XR-5) for 3 wk. The film was then developed. An exposure on the x-ray film would indicate the site of a human allergen on the immunoelectrophoresis plate. Both the cathodic fraction and the crude extract were used with this procedure.

RAST inhibition

A competitive RAST inhibition assay was used to confirm the allergenicity of this basic fraction. A standard RAST procedure (Pharmacia) was used with coinubation of 50 μl of various dilutions of the fraction with the pooled human anti-*Alternaria* sera. The test was run in triplicate and the results were plotted as percent inhibition (compared with known sera controls) vs concentration.

Skin tests

The biologic significance of the basic allergen was determined by intradermal skin testing for a typical wheal and flare response. Patients were scored positive as ± with a wheal of 5 mm in diameter, + with 5 to 10 mm diameter.

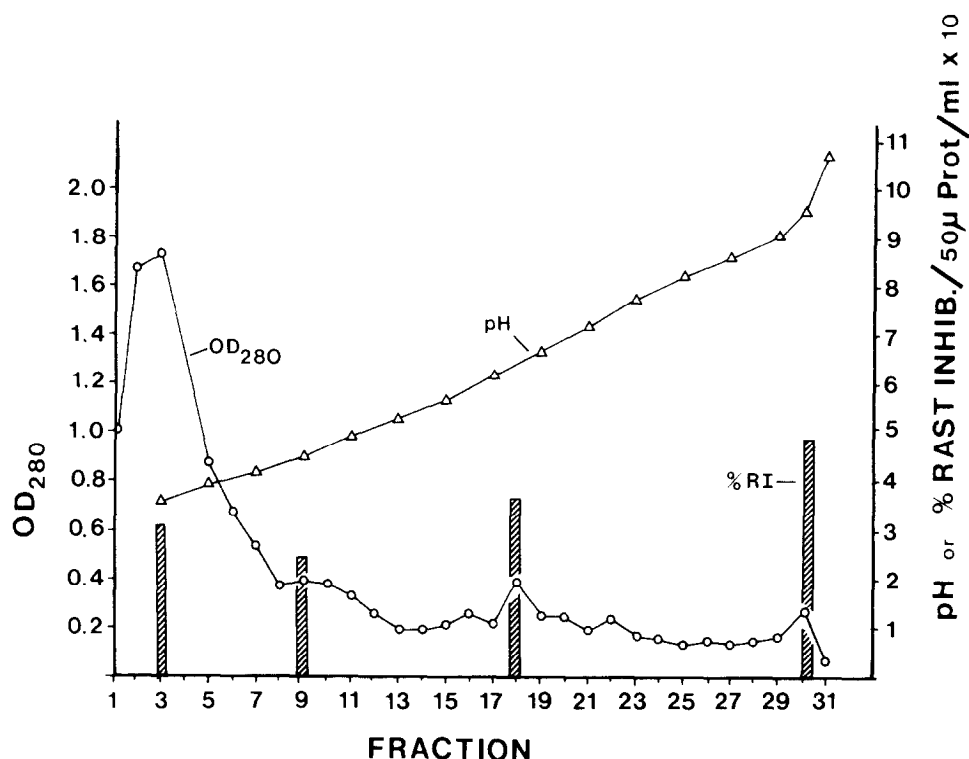


FIG. 1. Typical electrofocusing profile indicates an ultraviolet absorbing fraction at the cathodic end (pH = 9.5 to 9.8) of the gel bed separated by a wide peptide free zone. At least four bands could be seen in the gel between pH 5 and 7, while many bands grouped at the anodic end of the gel bed. The histogram bars on the graph indicate RAST inhibition (*R/I*) values calculated on an equal protein basis (per 50 μ g protein/ml).

++ with 10 to 15 mm, +++ with 15 to 20 mm, and ++++ with 15 to 20 mm wheal with pseudopod projections (10 min after injection). A group of 20 patients showing hypersensitivity (by skin testing) to a commercial *Alternaria* extract (*A. tenuis*; Greer Laboratories, Lenoir, N.C.) were tested for sensitivity to our I-Alt crude extract by intradermal challenge. Those patients giving a positive wheal and flare response were then tested intradermally with the basic allergen fraction, and the dilution giving a positive reaction (endpoint, 5 by 5 mm wheal) was recorded. The results are presented as the ratio of the number of patients hypersensitive to the I-Alt crude extract and basic allergen fraction to the total patient number. Twelve control subjects with no history of mold allergies were skin tested and all showed no reaction to *Alternaria* crude extracts or to the basic allergen fraction. The test dilutions were prepared in sterile phosphate-buffered saline and were used within 4 wk. They were stored at 4°C and warmed to room temperature before use.

RESULTS AND DISCUSSION

Fig. 1 shows the results of a preparative flat-bed gel IEF of 0.5 gm of *I-Alternaria* 1808 crude extract. Peak fractions 3, 9, 18, and 30 were tested for allergenicity using RAST inhibition. The values were represented on the graph as histogram bars as percent inhibition calculated on an equal protein basis. Table I

TABLE I. Isoelectric focusing of *Alternaria* crude extract*

	IEF fraction			
	3	9	18	30
pH	3.6	4.5	6.4	9.5-9.8
OD ₂₈₀	1.74	0.39	0.39	0.23
μ g protein/ml†	90	105	30	25
% RAST inhibition‡	57.8	49.5	22.7	24.2
% RAST inhibition/50 μ g protein/ml	32.1	23.6	37.8	48.4
% RAST inhibition§	27.5	35.6	16.9	0

*Starting material = 0.5 gm lyophilized extract.

†Coomassie dye procedure with bovine gamma globulin standard.

‡Not corrected for protein content.

§Results of duplicate control IEF runs with ampholines alone.

shows the protein content as measured by the Coomassie dye method, OD₂₈₀, and RAST inhibition values for each of these four fractions. The presence of ampholines in the eluted fractions should be taken into account. Control IEF runs without extract samples (ampholines alone) gave OD₂₈₀ values consistently below 0.05 absorbance unit. The sensitivity of

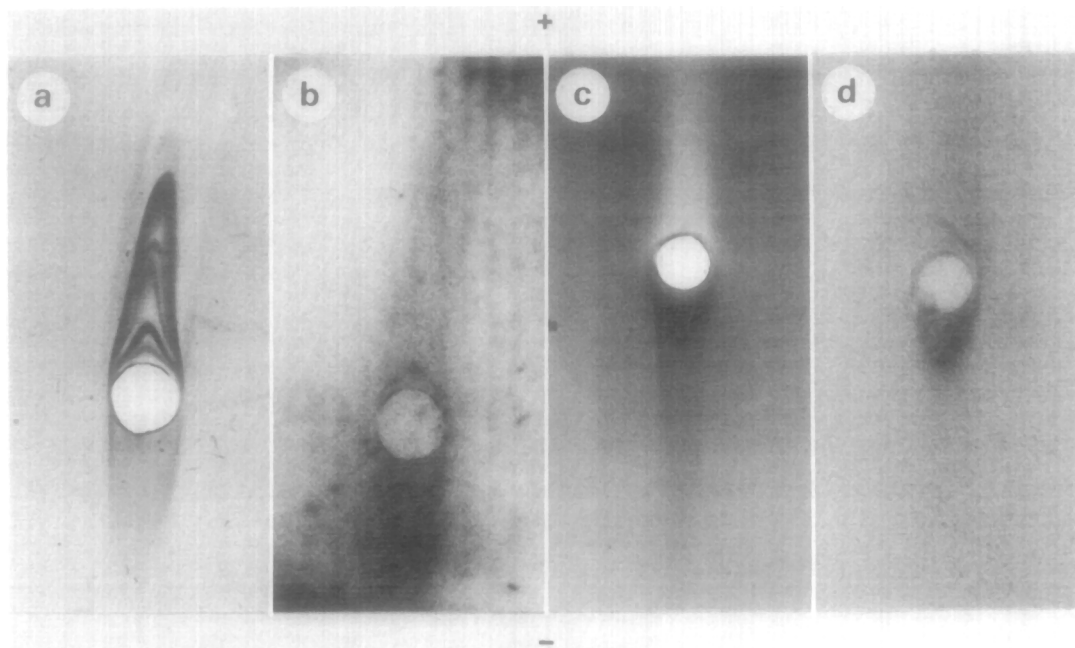


FIG. 2. Rocket immunoelectrophoresis and rocket radioimmuno-electrophoresis of *I-Alternaria* (1809) crude extract (**a** and **b**, respectively) and of the basic peptide fraction (**c** and **d**). Anode is at the top.

the Coomassie dye protein assay showed that a small portion of the fraction protein content was attributed to the ampholine peptides. For this reason, dialyzed preparations were used for the RAST inhibition dose response for the basic allergen fraction (fraction 30). RAST inhibition was used simply to screen the initial IEF peak fractions as indicated in Table I.

Fraction 9, with a pI of 4.5, showed significant RAST inhibition. The Alt-I fraction of Yunginger et al,¹¹ with a reported pI of 4.5, should be found within our fraction 9, and the RAST inhibition would be attributed to it. Fraction 3, although having a high OD₂₈₀ due to high pigment content, had less protein than fraction 9 and also showed RAST inhibition activity. It should be noted that the pigmented material has an absorption maximum of 270 to 280 nm but does not interfere significantly with the Coomassie dye protein assay. This assay measures a color reaction at 595 nm. The pigmented material absorbs very minimally at this wavelength (OD₂₈₀ of 1 gives an OD₅₉₅ of 0.07). In addition, most of the pigmented material migrated to the anodic end of the gel plate. Fraction 30 had much less protein yet showed significant RAST inhibition, indicating potent allergenicity when compared with the other fractions on an equal protein concentration basis. Fractions 1 to 13, 14 to 23, and 24 to 27 were pooled and frozen for further fractionation at a later date. The fractions containing the basic peptides (fractions 29 and 30) from several

IEF runs were pooled, dialyzed to remove ampholine, and concentrated with lyophilization.

Interestingly, some ampholines from control IEF runs (without samples) showed RAST inhibition. As shown in Table I, the ampholines from the fraction having a pH of 9.5 to 9.8 (fractions 29 and 30) did not show RAST inhibition, while those of the other fractions (3, 9, and 18) did. Two separate control runs (ampholines alone) were used for this determination and the duplicate values, which were similar, were averaged. Thus a substantial portion of the RAST inhibition of sample fractions 3, 9, and 18 were attributable to the ampholines eluted with the protein. Ampholines were removed by dialysis prior to lyophilization and subsequent characterization of the basic allergen fraction.

Radioimmuno-electrophoresis

The allergenic property of the basic protein fraction was shown by the rocket immunoelectrophoresis in Fig. 2. The binding of IgE from the pooled sera of *Alternaria*-sensitive patients was clearly demonstrated by the presence of the ¹²⁵I-anti-HuIgE on the cathode side of the electrophoretic origin. This fraction was also demonstrated in the autoradiogram of whole crude extract in the same figure. We have also observed this cathodic migrating peptide in crossed immunoelectrophoresis and crossed radioimmuno-electrophoresis (manuscript in preparation). It should be

TABLE II. Skin testing of the basic allergen fraction*

Sensitivity to:	No. positive/total†
I-Alt crude extract	19/20
Basic allergen fraction	18/20
Endpoint with basic allergen	
Dilution 1‡	10/20§
Dilution 3	1/20
Dilution 5	7/20
Negative response	2/20

*Twelve allergy-free control subjects showed no response to the crude extracts or to the basic allergen.

†Ratio of wheal and flare-positive patients to the number of patients hypersensitive to commercial (Greer) *Alternaria* extract.

‡Dilution 1, 25 µg/ml; dilution 3, 1 µg/ml; dilution 5, 0.04 µg/ml.

§Includes three patients that gave ± response to dilution 1.

noted that relatively high antibody concentrations were needed to visualize this small peptide fraction, which may be the reason why it has largely gone unnoticed. In part, this may also be attributable to the small size of the protein or to its moderate-to-low concentration in mycelial extracts; it is a potent allergen just the same. We have found that it was easily extracted with water, which may account for its low concentration in washed mycelia. It was present in high concentrations in the broth from mycelial cultures.

RAST inhibition

Fig. 3 shows the results of the RAST inhibition assay with the basic peptide fraction. The slope of the dose-response curve was not steep, and a concentration of 1.0 µg/ml gave over 20% inhibition of the standard RAST assay. The use of the commercial RAST *Alternaria* discs was justified, since (1) serum controls (no allergen present) for the inhibition assay bound a substantial portion (>17%) of the available ¹²⁵I-anti-HuIgE label, and (2) our crude extracts showed maximum inhibition values of up to 80%. This indicated that most of the allergens in our extracts were present on the commercial *Alternaria* discs. Nonallergic serum controls gave minimal background ¹²⁵I-anti-HuIgE binding (1.6%) of total counts available. The results of the RAST inhibition dose response confirmed the radioimmuno-electrophoresis demonstration that this fraction was indeed a potent allergen. It is notable that Fig. 2, c, indicated that there may be two antigenic components in the basic fraction (only one allergenic, Fig. 2, d). Thus, taking into account that at least some of the material in this fraction would not inhibit the RAST assay, it is possible that the fraction is even more potent than indicated in Fig. 3.

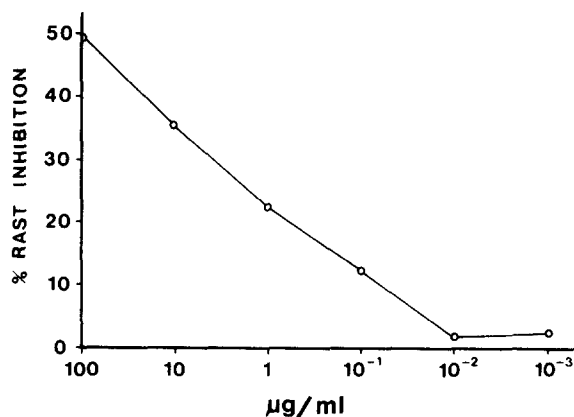


FIG. 3. RAST inhibition dose-response curve. A 13-patient serum pool containing anti-Alt IgE (RAST class 3 or 4) was fractionated with ammonium sulfate and concentrated twofold. Commercial allergen discs were incubated with 50 µl of allergen and 50 µl of the patient sera. ¹²⁵I-Anti-HuIgE was used to determine bound IgE.

Skin tests

Skin-test results indicated a high incidence (90%) of hypersensitivity to the basic allergen among those patients having hypersensitivity to commercial (Greer) *Alternaria* extract (Table II). Only two patients out of the 20 tested gave a negative wheal and flare response with both the I-Alt crude extract and the basic fraction while showing a positive (weak) response to the commercial extract. In general, a greater hypersensitivity to the basic allergen correlated with higher sensitivity to the whole extract. About one third of the patients that were hypersensitive to the basic allergen gave end-points (+wheal) at the No. 5 dilution (0.04 µg/ml). One of these patients was extremely hypersensitive, giving a +++ wheal response to the No. 5 dilution.

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This study reports the first isolation and characterization of a low-molecular-weight allergen of *Alternaria* with significant potency. By defining this allergen and noting the work of Yunginger et al.^{2, 11} and Hoffman et al.,¹³ there is good progress in profiling the major allergens of *Alternaria*. Low-molecular-weight allergens were demonstrated by Sternberger et al.⁹ in material able to permeate dialysis membranes as did Vijay et al.¹⁰ using membranes with a molecular weight cutoff of 10,000 daltons. Our preliminary studies showed allergen activity (by RAST inhibition) of crude extract material passed through an Ultra filter (Amicon-PM-10), which would presumably be 10,000 daltons or less. Our partially purified basic peptide fraction also passed through the PM-10 membrane and was largely retained within dialysis tubing, with a

cutoff of 6000 to 8000 daltons. Our studies showed some loss of allergenic material with dialysis of the basic fraction. We are not sure at this point whether this loss is due to slow permeation of the membrane due to the molecular weight being close to the membrane molecular weight cutoff value or due to degradation.

Initial sodium dodecyl sulfate-PAGE studies showed considerable smearing of the basic fraction, making molecular weight estimation difficult, but did indicate a low molecular weight. We have resolved the smearing problem with further fractionation of the basic fraction, indicating a molecular weight of about 6000 daltons (manuscript submitted). The fact that this fraction consists of such small peptides suggests the possibility that it is a cleavage product from larger allergens. Also, it cannot be ruled out that several allergens might contain a similar antigenic determinant, which, if excised, could represent a distinct fraction with unique properties (e.g., $pI = 9.5$ to 9.8). Even if this is the case, it is still reasonable that it truly represents a naturally occurring allergen of medical significance. We have detected this fraction in mycelia and broth extracts from our three isolates and in commercial extracts of *A. tenuis* (Greer and Center Laboratory, Port Washington, N. Y., manuscript in preparation); thus its occurrence is not necessarily limited to individual strains. It was not our intention, however, to define the distribution of this allergen among *Alternaria* species at this time but to simply note its presence in our samples and the presence of specific IgE against it in sera of allergic patients. It is hoped that other workers will examine their *Alternaria* extract samples for this allergen to determine its distribution.

This fraction has probably gone undetected because of its low concentration in commercial (and otherwise produced) extracts. Since it would be easily washed away when harvesting mycelial cultures and since most workers are not oriented toward looking for basic proteins, it would be easy to overlook such a fraction. Similar cathodic migrating antigens have been recorded by crossed immunoelectrophoresis from other molds and pollen extracts^{16, 17} but their allergenicity was not confirmed. Further characterization of this fraction is planned.

The demonstration of this *Alternaria* allergen will hopefully aid the standardization of extracts used for diagnosis and immunotherapy and permit the development of procedures to measure patient sensitivity more reliably and accurately.

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