

Monoclonal antibody-based assay for Alt a1, a major *Alternaria* allergen

Jay Portnoy, MD; Don Brothers, MD; Freddy Pacheco; Julie Landuyt; and Charles Barnes, PhD

Background: Allergenic materials from *Alternaria* are significant causes of human disease. One 31-kD glycoprotein, designated Alt a1, has been described previously as being a significant allergen.

Objectives: The purpose of this study is to characterize purified Alt a1 and to provide a method for measuring it in laboratory and environmental samples.

Methods: Monoclonal antibodies directed to the purified allergen were produced and used to construct a double monoclonal assay for Alt a1. The purified allergen detected by this assay was tested for IgE binding in vitro and in vivo.

Results: Purified allergen detected by this assay was shown to bind IgE from patients sensitive to *Alternaria* and gave a positive skin test in 15 of 16 patients skin test reactive to commercial *Alternaria* extracts. The assay is sensitive to Alt a1 concentrations as low as 0.2 $\mu\text{g/mL}$ and is very specific for the allergen. The three commercial *Alternaria* extracts assayed contained measurable quantities of the allergen varying from 12 to 84 $\mu\text{g/mL}$.

Conclusions: Alt a1 is a significant *Alternaria* allergen. The ability to measure this allergen may assist in standardizing *Alternaria* extracts and lead to an understanding of the significance of exposure to this allergen.

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INTRODUCTION

Fungi are ubiquitous in nature; however, to date very little progress has been made towards measuring their allergens in environmental samples. This is primarily due to a paucity of reproducible assays. Allergens from fungi of the genus *Alternaria*, for example, are known to be associated with significant and increasing human allergy¹ yet assays for their measurement are not yet widely available. As a result, standardization of *Alternaria* extracts has not occurred and information about the distribution of its allergens in the environment is poorly understood. The variability of allergen content of *Alternaria* extracts has been extensively documented.^{2–6} In addition to the dis-

crepant allergen composition between spores and mycelia,² substantial variability in biologic activity has been observed in different batches derived from the same source even when growth and extraction conditions are held constant.^{3–6}

Techniques involving purification of major allergens and production of monoclonal antibody-based assays hold great promise for measuring allergen content and standardizing allergen extracts.⁷ Work with monoclonal antibodies directed to specific allergens has produced many assays specific for individual allergens. Such assays have been reported for one allergen of *Alternaria*, GP70, two allergens from dust mite, Der p1 and Der f1, and many other major allergens.^{8–10}

Previously, two major allergens were purified from extracts of *Alternaria alternata*: GP70 and Alt a1.^{11–13} The former is a 70-kD glycoprotein that was purified by affinity chromatography using a monoclonal antibody. It caused a positive wheal and flare in 14 of 16 *Alternaria*-sensitive patients,

gave a single band on SDS-PAGE and IgE immunoblot, and was able to inhibit about 15% of the IgE activity in an *Alternaria* extract by ELISA inhibition. The later allergen is a 29 to 31-kD glycoprotein that reduces to smaller fragments with β -mercaptoethanol. It also gives a wheal-and-flare reaction in allergic patients,¹⁴ a single band on IgE immunoblot,¹⁵ and has been purified by a combination of size exclusion and DEAE ion exchange chromatography.^{16,17} Affinity purification using a monoclonal antibody has also been reported.¹⁸

In this study we further characterize purified Alt a1 and describe a double Mab-based ELISA that permits measurement of this major *Alternaria* allergen. Though an assay for measuring Alt a1 concentrations using a polyclonal antibody was described previously,¹⁹ double monoclonal antibody-based sandwich assays are generally felt to be preferable for this purpose because they tend to be more sensitive, specific, and reproducible.⁹ Such an assay was described for GP70⁸; however, Alt a1 concentrations have not yet been measured using two monoclonal antibodies. We now describe the development of a double monoclonal antibody assay that is specific for Alt a1. We have used this assay to study the differing content of this specific allergenic material in strains of ALT grown in our laboratory as well as in three commercial *Alternaria* preparations.

MATERIALS AND METHODS

Human Volunteer

All clinical studies were performed after obtaining approval from the institutional review board of the University of Missouri-Kansas City. Written consent was obtained before any experi-

Section of Allergy/Asthma/Immunology, The Children's Mercy Hospital, Kansas City, Missouri.

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mental procedure was performed. Volunteers were tested for *Alternaria* sensitivity by using three commercial extracts (Greer Laboratories, Lenoir NC; Center Laboratories, Port Washington NY; and Bayer Allergy Products, Spokane WA) and a purified preparation of Alt a1. Serum was obtained from those patients with wheals greater than or equal to 5 mm to one or more *Alternaria* extract and screened for anti-*Alternaria* IgE by direct ELISA. Samples with significant specific IgE (greater than two times the background) were pooled.

Source Materials

Strains of *Alternaria Alternata* described previously¹¹ were grown at 24 °C for 4 weeks on a chemically defined sucrose medium.²⁰ The cultures were exposed to 12-hour periods of alternating light and dark during their growth. Fungal mats produced contained both spores and mycelia by light microscopy. The fungal mats were briefly washed with distilled water, air dried for 24 hours at 37 °C, and powdered with a mortar and pestle.

Preparation of Extracts

Powdered material was extracted at 4 °C in distilled water at a concentration of 10 g of dry weight per 100 mL for 24 hours. All eluted materials were filtered through a succession of finer pore size filters and finally filtered for sterility through a 0.45- μ m pore size filter (Gelman, Ann Arbor, MI). All extracted materials were dialyzed overnight against 50 millimolar ammonium bicarbonate in a 3-kD cutoff membrane (Spectrapore, Houston, TX) at 4 °C and lyophilized. The resultant preparations were stored desiccated at -20 °C. Proteins were measured by the Lowry method using bovine serum albumin for calibration (Sigma, St Louis, MO, USA).

Electrophoresis and Immunoblotting

Sodiumdodecyl sulfate SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on vertical slab gels (BioRAD, Hercules, CA, USA) using the method of Laemmli.²¹ Prestained molecular weight markers

(BioRAD) were included with each run. Lyophilized samples were reconstituted in distilled water at a concentration of 1 mg/mL and 10 μ L of each sample was diluted in 30 μ L of TRIS-SDS (pH 6.8) and boiled for five minutes. To maintain the intact 29 to 31-kD allergen no β -mercaptoethanol was used. The SDS-PAGE gels were stained for glycoprotein using either a silver stain according to the method of Wray et al,²² or 0.1% Coomassie Blue (BioRAD). Proteins from SDS-PAGE gels were transferred electrophoretically to nitrocellulose membranes (BioRAD TransBlot) in 25 mM TRIS and 192 mM Glycine buffer, pH 8.8 containing 20% methanol at 30 V for one hour. Nitrocellulose membranes were then blocked overnight with 5% nonfat dry milk in TBS (Carnation, Nutley, NJ, USA). These blots were subsequently probed overnight with 1:10 dilution of the pooled human serum in TBS. The blots next were incubated for two hours with 1:1000 alkaline phosphatase-conjugated goat anti-human IgE and developed with alkaline phosphatase substrate until bands appeared.

Production of Monoclonal Antibodies

Monoclonal antibodies were produced in Balb/C mice using standard procedures as previously described.¹¹ Briefly, Balb/C mice were immunized IP with 50 μ g antigen in complete Freund's adjuvant three times at 2-week intervals. Mice were immunized again five days prior to sacrifice. After sacrifice, spleen cells were fused with HAT-sensitive mouse myeloma cells (653s) in the presence of 40% polyethylene glycol followed by incubation in HAT medium. Surviving colonies were screened for ALT specific antibodies using direct enzyme immunoassay with whole ALT extract in solid phase. Selected hybridomas were injected into mice for additional antibody production. Mab-containing ascites fluids were tested for biologic activity directed at ALT using direct ELISA. Strongly positive MABs were purified by ammonium sulfate precipitation. Purity was confirmed with

SDS-PAGE. Hybridomas producing anti-ALT antibodies were rescreened against chromatographically purified Alt a1. Mabs positive for Alt a1 were then used in epitope mapping experiments to identify Mabs that recognize unique epitopes. Fab and F(ab)2' fragments of monoclonal antibodies were produced with ImmunoPure Fab® and F(ab)2' preparation kits (Pierce Chemical Company, Rockford IL, USA).

Biotinylation of Antibodies

For detection, secondary antibodies were labeled with biotin. Two milligrams of purified lyophilized Mab or Fab fragment was dissolved in 200 μ L of 0.05M bicarbonate buffer (pH 7.5) and incubated at 4 °C for one hour with 3 μ L of NHS-biotin (BioRAD). At the end of this time, the biotinylated antibody was dialyzed at 4 °C overnight against 0.1 M tris buffered saline and stored at -20 °C in 50% glycerol at a protein concentration of 5 mg/mL. The activities of biotinylated antibodies were confirmed by direct ELISA using 1:1000 avidin-alkaline phosphatase (Sigma).

Sandwich ELISA

For this assay, concentrations of antibodies required to give optimal results were determined empirically. Microtiter plates were coated with the F(ab)2' fragment of Mab1 overnight and coated plates were washed and blocked with 0.5% gelatin (BioRAD) for one hour at 37 °C to reduce background. Serial dilutions of ALT or purified allergen protein dissolved in PTA were added to each well and incubated for two hours. After washing, the plates were incubated with biotinylated Fab fragment of Mab2 for two hours. The plates were again washed and incubated for one hour with 1:1000 avidin conjugated alkaline phosphatase (Sigma). The enzyme was detected as described above.

Purification of Alt a1

The Alt a1 protein was purified chromatographically by the method of Paris.¹² Briefly, *Alternaria* mycelial mats were ground with a mortar and pestle and extracted overnight at 4 °C in am-

monium bicarbonate (1 g mycelia to 10 g liquid). The ALT extract was precipitated by the addition of 4 volumes of acetone. The acetone precipitate was dissolved in tris buffered saline at a concentration of 10 mg/mL. This was applied to a DEAE ion exchange column (Memsep, Millipore, Bedford, MA) and eluted with a 0 to 1 molar salt gradient. The material that eluted between 0.3 and 0.4 M NaCl was applied to a size exclusion column (Hydrobore, Rannin, Woburn, MA) and the 29 to 31-kD fraction was collected. The presence of the 29 to 31-kD allergen was monitored by SDS-PAGE immunoblots probed with sera from Alt-sensitive individuals. Additional material was purified by affinity chromatography using Mab1 bound to cyanogen bromide-activated sepharose. Partially purified *Alternaria* extract was chromatographed on an affinity column made by binding Mab1 to cyanogen bromide-activated sepharose 6B (Sigma, St. Louis, MO). The extract that did not bind to the column was washed off with pH 7.4 TBS and the material bound to the column was eluted with pH 3.0, 0.5 molar acetic acid.

Human IgE ELISA

ELISA plates (immulon-2, Dynatech, Chantilly, VA, USA) were coated overnight with 0.5 μ g of the F(ab)2' fragment of Mab1 per well in a pH 9.6, 0.05 M bicarbonate coating buffer. Coated plates were washed and blocked with 0.5% gelatin (BioRAD) for one hour at 37 °C. *Alternaria* samples or affinity purified material dissolved in PTA was added to each column of wells in twofold dilutions starting at the rate of 1 mg dry material per well for crude extracts or 50 μ g of protein per well for affinity purified material and allowed to adsorb for two hours. After washing, an aliquot of human serum from the *Alternaria*-sensitive individuals was diluted 1:10 in PTA and placed into each well, incubated for three hours, and then washed. A biotinylated human IgE directed mouse monoclonal antibody 3A3.1D3 (Atlantic Antibodies, Stillwater, MN)

was then applied to the ELISA plate at 100 μ L/well in PTA and detected with alkaline phosphatase conjugated avidin. All plates were washed in PTA between steps. After addition of alkaline phosphatase substrate, plates were allowed to develop until an internal standard read 2.0 optical density units as measured at OD405 using a Dynatech MR7000 (Dynatech Chantilly, VA).

Standardization of the Assay

After the dry weight was measured, purified Alt a1 was dissolved in TBS and protein concentration was measured as described above. Immunoassays were performed using serial dilutions of the purified allergens to generate a standard curve. The log of the known allergen concentrations was next compared to the OD405s by linear regression. Alt a1 concentrations in simultaneously run unknown preparations were related to the purified allergens and results expressed as milligram protein (Lowry) per milliliter. To test the linearity and precision of the assay, standard material and assayed commercial material were assayed in triplicate and compared for linear correlation and coefficient of variance.

Skin Testing Methods

Human volunteers recruited either through the Allergy Clinic patient population or from the public at large were skin tested with the purified Alt a1 and each of the three available commercial extracts. Skin test subjects ranged in age from 8 to 35 years of age. Briefly, the forearm arm was washed with alcohol and dried. Both forearms were used since there was not sufficient space on a single forearm. Areas on the arm spaced 3 cm apart were numbered 1 through 21 and threefold serial dilutions of the purified Alt a1 starting at 1 mg/mL or the commercial extracts starting at 1:10 wt/vol were applied using a dermapik (Greer Laboratories, Lenoir, NC, USA). After 15 minutes, the resultant wheal-and-flare reactions were delineated on the skin using a ballpoint pen and the mark was lifted off using transparent tape and applied

to the research notebook. The investigator applying the skin test and reading the results was blinded to the contents of the individual wells. The sum of the greatest erythema diameter and the diameter perpendicular to that was measured in millimeters. A test was considered positive when the sum of erythema was at least 5 mm greater than that of the saline/glycerol control.

RESULTS

IgE Immunoblots of Alt a1

IgE immunoblots were performed for purified Alt a1, purified GP70, and whole *Alternaria* using pooled serum. The whole *Alternaria* extract was not reduced. It showed two significant *Alternaria* allergens (Fig 1): one at greater than 120 kD and another at 29 to 31 kD. These glycoproteins were purified and run both in non-reduced and reduced form. The 120 kD reduced to a 70-kD band which has previously been designated GP70. The other reduced to a band or more often a doublet in the 15 to 18-kD region, and has been previously designated as Alt a1. It was never possible to completely reduce this IgE-binding 31 kD band fully to its lower molecular weight moieties.

Evaluation of the Assay

We evaluated three possible methods for detecting Alt a1 using monoclonal and polyclonal antibodies. These were (1) coating with Mab1 and detecting with a biotinylated Mab2, (2) coating with polyclonal rabbit anti-ALT and detecting with biotinylated Mab2, and (3) coating with an Mab1 and detecting with biotinylated polyclonal mouse anti-ALT. The assay which employed coating with the F(ab)2' fragment of Mab1 and detecting with a biotinylated F(ab) fragment of Mab2 was found to be the most suitable of the methods (data not shown).

Figure 2 shows the OD405s of reciprocal dilutions of purified Alt a1 as detected by the double monoclonal antibody-based sandwich assay. As a comparison, the whole *Alternaria* extract from which the Alt a1 was purified was also assayed. Purified Alt a1

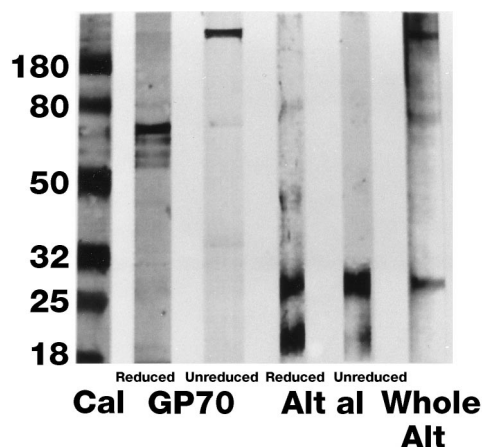


Figure 1. IgE Blots of whole ALT and purified Alt a 1 and GP70. From left to right, molecular weight markers, reduced GP70, not-reduced GP70, reduced Alt a1, not-reduced Alt a1, and not reduced Alt a1, and not reduced whole Alternaria extract.

showed a fourfold greater activity and greater than tenfold more activity on an activity per protein basis than the whole Alternaria preparation. This same extract from which Alt a1 had been removed (depleted) showed no activity at any dilution though it otherwise had twice the protein content as the purified Alt a1 as shown in Table 1. As expected, the assay signal can be completely inhibited by addition of whole Alternaria extract to the second antibody incubation step (data not shown).

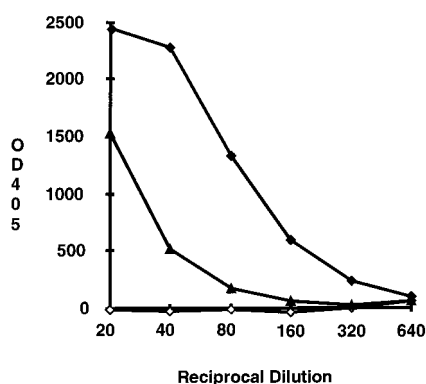


Figure 2. Sandwich ELISA using Mab1 F(ab)2' to capture and biotin-Mab2 Fab to detect for affinity chromatography purified Alt a1, whole Alternaria extract, and Alternaria extract depleted of Alt a1. ◆, Alt a1 (pure); ◇, Alt a1 (depleted); and ▲, whole Alt.

Confirmation that the Assay Captures an Allergen

We next needed to confirm that the captured Alternaria antigen was also an allergen by detecting with pooled human IgE. Figure 3 shows the IgE-binding activity of the material that had been captured by Mab1. The affinity-purified material was found to bind human IgE from Alternaria-sensitive patients at a nearly 2.5 log lower concentration than it did to the whole Alternaria extract. The Alt a1-depleted extract showed very little IgE-binding activity even though it still had allergens in it such as GP70 suggesting that those allergens were not being captured by the Mab1.

Alt a1 in Commercial Extracts

Figure 4 shows the relative Alt a1 activities of the purified standard as compared with three commercial Alternaria extracts. This figure has been standardized on the x axis using the protein content of the purified Alt a1. The assay was able to detect Alt a1 allergen in all three commercial preparations. The amount of allergen contained in these preparations ranged from 12 to 84 $\mu\text{g/mL}$ with parallel dilution curves for all of the extracts.

Skin Test Results

Prick skin test results of purified Alt a1 and the three commercial extracts were

Table 1. Protein Determinations*

Extract	Protein, $\mu\text{g/mL}$
Alt a1 (pure)	464
Alt a1 (depleted)	908
Whole Alt	1196

* 10-mg Extract Dry Weight Per ml by Lowry.

performed in 16 atopic volunteers. These results are displayed graphically in Figure 5. The values indicate the log of the highest threefold dilution of antigen that gave a positive (≥ 5 mm wheal) test. In 15 of the 16 subjects sensitive to Alternaria, the purified Alt a1 gave a positive skin test, and in 6 of the 16 subjects the Alt a1 was positive at the lowest concentration tested. The mean dilution giving a positive reaction was 9.6 for Alt a1, 9.9 for Greer, 11.7 for Center, and 11.7 for Bayer. The correlation among skin reactions listed in Table 2 was greatest for Alt a1, Greer, and Bayer. A comparison of skin prick tests using three commercial extracts and the purified Alt a1 is represented in Figure 5. The slopes of the lines generated when erythema is plotted against dilution are not the same for the four preparations since they do not have identical allergen content. Skin tests on non-Alternaria allergic individuals ($n = 5$) and non-atopic

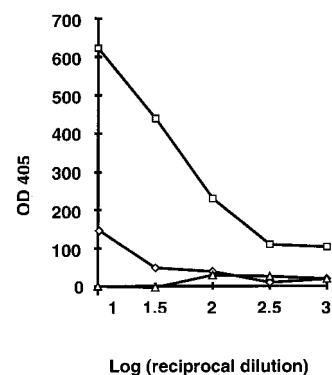


Figure 3. Validation that Mab1 bound material also binds human IgE. Wells were coated with Mab1 F(ab)2' and incubated with affinity purified allergen, allergen depleted, and crude Alternaria preparations. Bound allergens were detected with IgE containing sera from Alternaria-sensitive patients. ◇, whole Alt; □, Alt a1 (pure); and △, Alt a1 (depleted).

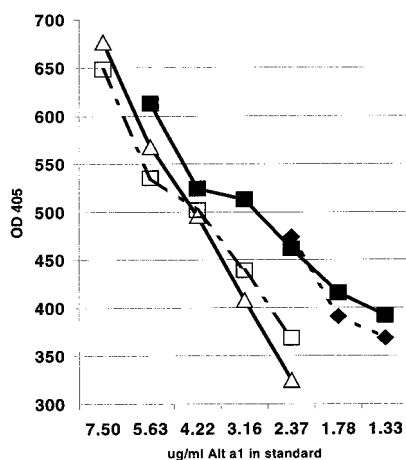


Figure 4. Measurement of Alt a1 in three commercial samples. The samples contained 12, 73, and 84 $\mu\text{g/mL}$ of Alt a1, respectively. The average CV for the calculated slopes of the four lines is 8.8 and the average correlation coefficient of the lines is 0.97. -◆-, Center; -■-, Greer; -△-, Bayer; and -□-, Alt a1 (Std).

individuals ($n = 5$) were all negative compared with positive histamine controls.

DISCUSSION

The IgE immunoblot patterns (Fig 1) are similar to patterns observed by other investigators for *Alternaria*^{2,5} although this blot does not contain the multiplicity of bands often present. The observation that the Alt a1 reduces from 31-29 kD to 12-18 kD after β -mercaptoethanol treatment has been made by other authors.^{13,16} The reduction of the high molecular weight al-

Table 2. Correlation Table Between Log of (skin endpoint dilution) for Alt a1 and the 3 Commercial *Alternaria* Extracts

	Alt a1	Greer	Center	Bayer
Alt a1	1			
Greer	0.76	1		
Center	0.50	0.64	1	
Bayer	0.75	0.76	0.83	1

lergen has also been previously reported.^{8,23} The faint band at 70 kD in the non-reduced immunoblot (Fig 1) is not an unusual observation and most likely indicates that processes other than reduction of disulfide bonds can break the allergen into fragments.

The strategy of using immunoglobulin fragments chosen for the assay was arrived at empirically. The fragments, without the crystallizable portion, have a lesser tendency to aggregate nonspecifically and therefore gave a lower background signal. The results with the affinity bound and allergen-depleted materials shown in Figure 2 indicate that the assay is quite specific for the Alt a1. The results in Figure 3 show that human IgE from Alt-sensitive patients also binds the Mab-bound material. This indicates that the detected material is indeed an allergen. Skin test results indicating that greater than 90% of *Alternaria*-allergic individuals are sensitive to Alt a1 confirm the observations of others.¹³

Tests such as the one we have developed for Alt a1 will impact the basic understanding of the development of asthma. Investigators have found that fungal allergens in homes are commonly associated with skin sensitivity in an allergy clinic population with upper and lower respiratory allergy.²⁴ The ability to test materials taken from the homes of asthma sufferers could increase understanding of these disease processes. Development of asthma later in life has been correlated with early exposure to dust mite allergen.²⁵ As standards for indoor allergen exposure are developed,²⁶ tests as the one reported here will become increasingly valuable for testing home and work environments. Tests for specific allergens will also produce improve-

ments in the areas of diagnosis and immunotherapy. This test along with another we have developed for the high molecular weight allergen from *Alternaria* make a significant contribution to the several assays which will be needed to encompass all of the significant *Alternaria*. This series of tests can then be used to standardize preparations for allergen testing and immunotherapy. These tests will also contribute to a study of possible cross-reactivity of allergens between related species of mold and to a further understanding of mold classification.

Highly sensitive and specific monoclonal antibody-based immunoassays for GP70 and Alt a1 are necessary if an understanding of the relative significance of these two glycoproteins in environmental samples is to be achieved. In addition, since they are both significant allergens, they must both be measured in unknown *Alternaria* preparations to be qualitatively and quantitatively standardized. The need to measure at least two antigens has not been as important for other allergenic extracts such as those derived from dust mite and cat because their major allergen concentrations correlate well with total biologic activity.¹⁹ For *Alternaria*, this is just not true. Significant qualitative differences have been described even when total biologic activity is constant.

As with the GP70 assay, standardization of this assay is somewhat arbitrary. For this study, we chose to measure the protein concentration of highly purified Alt a1 and use this as a primary standard. We could easily have measured the protein by Coomassie blue dye binding, or we could have used dry weight or even carbohydrate concentration as a standard measurement. Until a more fundamental standard is produced such as a primary standard with a known molarity of divalent epitopes, this arbitrary type of standard will continue to be used. Clearly, a milligram of one allergen cannot be directly related to a milligram of another one until this problem with standardization is solved.

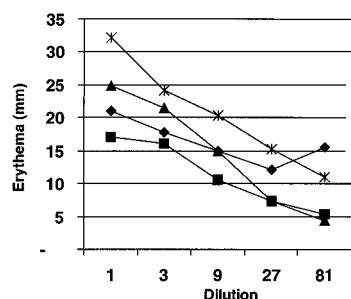


Figure 5. Prick skin test results for Alt a1 and three commercial extracts. The numbers on the x axis represent the dilution of the extract from stock and those on the y axis represent the resultant erythema. -■-, Alt a1; -▲-, Greer; -◆-, Center; and *, Bayer.

REFERENCES

1. Sneller MR, Hayes HD, Pinnaas JL. Frequency of airborne *Alternaria* spores in Tucson, Arizona over a 20-year period. *Ann Allergy* 1981;46:30-3.
2. Paris S, Fitting C, Latge JP, Herman D, et al. Comparison of conidial and mycelial allergens of *Alternaria alternata*. *Int Arch Allergy Appl Immunol* 1990;92:1-8.
3. Portnoy J, Pacheco F, Ballam Y, Barnes C. The effect of time and extraction buffers on residual protein and allergen content of extracts derived from four strains of *Alternaria*. *J Allergy Clin Immunol* 1993;91:930-8.
4. Karlsson-Borga A, Jonsson P, Rolfsen W. Specific IgE antibodies to 16 widespread mold genera in patients with suspected mold allergy. *Ann Allergy* 1989;63:521-6.
5. Kroutil LA, Bush RK. Detection of *Alternaria* allergens by Western blotting. *J Allergy Clin Immunol* 1987;80:170-6.
6. Bush RK, Voss MJ, Bashirian S. Detection of *Alternaria* allergens by crossed radioimmunoelectrophoresis. *J Allergy Clin Immunol* 1983;71:239-44.
7. Chapman MD. Purification of allergens. *Curr Opin Immunol* 1989;1:647-53.
8. Portnoy J, Pacheco F, Upadrashta B, Barnes C. A double monoclonal antibody assay for the *Alternaria* allergen GP70. *Ann Allergy* 1993;71:401-7.
9. Luczynska CM, Arruda LK, Platts-Mills TA, et al. A two-site monoclonal antibody ELISA for the quantification of the major *Dermatophagoides* spp. allergens, Der p I and Der fl. *J Immunol Methods* 1989;118:227-35.
10. Luczynska CM, Li Y, Chapman MD, Platts-Mills TA. Airborne concentrations and particle size distribution of allergen derived from domestic cats (*Felis domesticus*). Measurements using cascade impactor, liquid impinger, and a two-site monoclonal antibody assay for Fel d I. *Am Rev Respir Dis* 1990;141:361-7.
11. Portnoy J, Olson I, Pacheco F, Barnes C. Affinity purification of a major *Alternaria* allergen using a monoclonal antibody. *Ann Allergy* 1990;65:109-14.
12. Paris S, Debeaupuis JP, Prevost MC, et al. The 31 kD major allergen, Alt a I1563, of *Alternaria alternata*. *J Allergy Clin Immunol* 1991;88:902-8.
13. Vijay HM, Young NM, Curran IH, et al. A major antigen of *Alternaria alternata* with potential for safe and effective immunotherapy [editorial; comment]. *J Allergy Clin Immunol* 1993;91:826-8.
14. Kleine-Tebbe J, Worm M, Jeep S, et al. Predominance of the major allergen (Alt a I) in *Alternaria* sensitized patients. *Clin Exp Allergy* 1993;23:211-8.
15. Karlsson-Borga A, Rolfsen W. Methodological considerations when using nitrocellulose immunoblotting from polyacrylamide gels to study the mould allergens *Aspergillus fumigatus* and *Alternaria alternata* [published erratum appears in *J Immunol Methods* 1991 Apr 8; 138(1):137]. *J Immunol Methods* 1991;136:91-102.
16. Deards MJ, Montague AE. Purification and characterisation of a major allergen of *Alternaria alternata*. *Mol Immunol* 1991;28:409-15.
17. Curran IH, Young NM, Burton M, Vijay HM. Purification and characterization of Alt a-29 from *Alternaria alternata*. *Int Arch Allergy Immunol* 1993;102:267-75.
18. Landuyt J, Pacheco F, Banres C, Portnoy J. Affinity purification of the 31 kD Allergen of *Alternaria alternata* [Abstract]. *J Allergy Clin Immunol* 1993;91:191.
19. Portnoy J, Pacheco F, Barnes C, Upadrashta B, et al. Selection of representative *Alternaria* strain groups on the basis of morphology, enzyme profile, and allergen content. *J Allergy Clin Immunol* 1993;91:773-82.
20. Schaffer N, Molomut N, Center J. Studies on allergenic extracts: I. A new method for the preparation of mold extracts using a synthetic medium. *Ann Allergy* 1959;17:380-4.
21. Laemmli U. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227:680.
22. Wray W, Bouliskas T, Wray V, Hancock R. Silver staining of proteins on polyacrylamide gels. *Analyt Biochem* 1981;118:197-203.
23. Horner S, Portnoy J, Upadrashta B, et al. Measurement of a specific 70 kD *Alternaria* allergen in different fungi [Abstract]. *J Allergy Clin Immunol* 1992;89:#391.
24. Tarlo S, Franklin A, Tobin R. Skin testing with extracts of fungal species derived from the homes of allergy clinic patients in Toronto, Canada. *Clin Allergy* 1988;18:45-52.
25. Sporik R, Holgate ST, Platts-Mills TA, Cogswell JJ. Exposure to house-dust mite allergen (Der p I) and the development of asthma in childhood. A prospective study [see comments]. *N Engl J Med* 1990;323:502-7.
26. Platts-Mills TA, Chapman MD, Pollart SM, et al. Establishing health standards for indoor foreign proteins related to asthma: dust mite, cat and cockroach. *Toxicol Ind Health* 1990;6:197-208.

Request for reprints should be addressed to:
 Jay Portnoy, MD
 Section of Allergy/Immunology
 The Children's Mercy Hospital
 Kansas City, MO 64108