Quantitation of the major fungal allergens, Alt a 1 and Asp f 1, in commercial allergenic products

Lisa Vailes, MS,a Susheela Sridhara, PhD,a** Oliver Cromwell, PhD,b Bernhard Weber, PhD,b Michael Breitenbach, PhD,c and Martin Chapman, PhDa
Charlottesville, Va, Reinbek, Germany, and Salzburg, Austria

Background: Alternaria is one of the most important fungi associated with allergic disease, whereas Aspergillus fumigatus is involved in a broad spectrum of pulmonary diseases. Currently, fungal extracts used for diagnosis in the United States are unstandardized, and their allergenic content cannot be compared directly.

Objective: The goal of this study was to compare the variability of major allergen levels among US allergenic products derived from fungi: specifically, Alt a 1 levels in Alternaria alternata extracts, and Asp f 1 levels in A fumigatus extracts.

Methods: A novel 2-site monoclonal antibody ELISA was used for measuring Alt a 1 using recombinant Alt a 1 as a standard. Asp f 1 was also measured by ELISA. Allergenic products produced by 8 US manufacturers over a 2-year period were compared, as were multiple lots produced by a single company.

Results: Alt a 1 levels in Alternaria extracts from 5 companies produced in 1998 and 1999 ranged from less than 0.01 to 6.09 µg/mL (mean 1.4 ± 1.6 µg/mL, n = 15). In general, Alt a 1 levels were consistent within and between companies (1.4 ± 1.1 µg/mL, n = 27), with 21 of 32 (66%) of all extracts tested containing 0.7 to 2 µg/mL. Alt a 1. Aspergillus extracts showed much greater variability in Asp f 1 levels, with extracts from 8 companies containing from less than 0.1 to 64 µg/mL (mean 16.3 ± 23.9 µg/mL, n = 15). Overall variability was greater for Aspergillus products within and between manufacturers (22 ± 22 µg/mL). Asp f 1, n = 20).

Conclusions: ELISA-based assays for specific allergens showed greater consistency among allergenic products derived from Alternaria than from Aspergillus. These assays should facilitate improved quality control and standardization of fungal allergen extracts and lead to the development of more consistent products for clinical use. (J Allergy Clin Immunol 2001;107:641-6.)

Key words: Alternaria, Aspergillus, allergen exposure, recombinant allergens, fungi, asthma

Alternaria alternata is considered to be one of the most important fungi causing allergic disease in the United States, and sensitization to Alternaria allergens is associated with asthma.1-8 Alternaria sensitization was an independent risk factor associated with the development of wheezing and asthma in children and young adults (odds ratios 5-6.8), and skin test sensitivity to Alternaria was significantly associated with increased bronchial responsiveness.4,6-8 Alternaria was the major asthma-associated allergen in desert regions of the United States and Australia and has been reported to cause serious respiratory arrest and death in the US Midwest.9-12

Aspergillus species are important opportunistic fungi involved in a wide range of human respiratory diseases, including allergic asthma, rhinitis, sinusitis, aspergilloma, allergic bronchopulmonary aspergillosis, and systemic invasive aspergillosis of immunocompromised individuals.13,14 Aspergillus fumigatus in particular is responsible for 80% of Aspergillus infections in human beings.

Most fungal allergen extracts used for diagnosis and immunotherapy contain large amounts of nonallergenic proteins. Variability of allergen extracts is a particular problem with molds because of interstrain differences, culture methods, the origin of the source material, and differences in extraction procedures.13,15-21 Attempts to assess allergen levels in Alternaria extracts have used techniques such as immunoelectrophoresis, RAST inhibition, and RIAs. These procedures are labor intensive and use polyclonal antibodies and human serum IgE, which are in short supply.16,22,23 Currently, fungal allergen extracts manufactured in the United States are not standardized, and the allergen content of the extracts produced by different manufacturers cannot be directly compared.

Several A alternata and A fumigatus allergens have been cloned. Alt a 1 and Asp f 1 are major allergens of A alternata and A fumigatus, respectively, and more than 80% of patients sensitized to the fungi have IgE antibody to these allergens.13,14,24-28 Alt a 1 is a heat-stable dimer of 28 kd, which dissociates into 14.5-kd and 16-kd subunits under reducing conditions.26,29,30 Asp f 1 is an 18-kd protein showing homology to a family of cytotoxins and is thought to be a virulence factor for A fumigatus.14
Recently, Aden et al \(^{31}\) described the development of a novel 2-site mAb ELISA for the measurement of Alt a 1. The assay is unique in that a single mAb, which binds to a common epitope on both Alt a 1 monomers, is used as both capture and biotinylated secondary antibody. In the present study, 2-site ELISAs for Alt a 1 and Asp f 1 were used to compare Alt a 1 and Asp f 1 levels in commercially available, unstandardized \(A\) \(a\)lternaria and \(A\) \(f\)umigatus extracts, in reference extracts, and in fungal spores.

**METHODS**

**Allergen extracts**

Extracts of \(A\) \(a\)lternaria and \(A\) \(f\)umigatus were obtained from 8 manufacturers: Allergy Labs of Ohio, Allergy Labs of Oklahoma, Allermed, Bayer (now Hollister-Stier Laboratories), Center, Greer Laboratories, Meridian Biomedical Inc (ALK/Abellô), and Nelco. All extracts, purchased in 1998 and again in 1999, were prepared in 50% glycerine and labeled as 1:10 or 1:20 wt/vol. Full details of lot numbers and expiry dates are available on request. Six freeze-dried \(A\) \(l\)ternaria extracts prepared as part of an allergen standardization study in 1983 were obtained from Dr John Yunginger (Mayo Clinic, Rochester, Minn). \(^{31}\) Bayer kindly provided 12 \(A\) \(f\)umigatus and 10 \(A\) \(a\)lternata extracts for comparison.

**ELISA for Alt a 1**

mAb 121 (clone 121G 5G8) and \(A\) \(l\)ternaria extract No. 43771 containing 8.5 µg natural Alt a 1 per vial (Allergopharma, Germany) were used to establish the ELISA. \(^{31}\) In brief, Immulon II plates were coated with 1 µg/mL mAb 121 in 0.05 mol/L carbonate bicarbonate buffer (pH 9.6). mAb 121 was biotinylated as previously described and used for detection of bound allergen. \(^{32}\) Recombinant Alt a 1 (rAlt a 1, lot No. 1 28.09.96) was obtained from Dr Don Hoffman. An extract was made by incubating 28 mg of spores harvested in 1979 from Orange County, Calif, and stored in the dark at 4°C were kindly provided by Dr Linda Stetzenbach (University of Nevada, Las Vegas).

**ELISA for Asp f 1**

Asp f 1 was measured as described previously with anti-Asp f 1 mAb 4A6 for antigen capture and polyclonal rabbit anti-Asp f 1 for detection. \(^{14}\) Purified natural Asp f 1 (0.45-250 ng/mL) was used to form a standard curve.

**Spore extracts**

Dried \(A\) \(a\)lternata spores harvested in 1979 from Orange County, Calif, and stored in the dark at 4°C were kindly provided by Dr Don Hoffman. An extract was made by incubating 28 mg of spores in 0.5 mL PBS (pH 7.4) on a rotator for 30 minutes at room temperature. Spores in solution, washed from \(A\) \(a\)lternata growing on agar plates, were kindly provided by Dr Linda Stetzenbach (University of Nevada, Las Vegas).

**SDS-PAGE analysis**

Natural Alt a 1, rAlt a 1, and affinity-purified natural Asp f 1 were analyzed by silver stain on a high-density SDS-PAGE gel with the Pharmacia Phast Gel System.

**RESULTS**

**Allergen analysis**

SDS-PAGE analysis showed that natural Alt a 1 exists as a 29-kd dimer but breaks down into 15-kd and 16-kd subunits under reducing conditions. rAlt a 1 migrates as an approximately 15-kd protein under reducing conditions but tends to dimerize during storage and shows both 29-kd and 15-kd bands under nonreducing conditions. Purified Asp f 1 migrates as a single band at 18 kd (Fig 1).

**mAb ELISA for Alt a 1**

Almost identical, parallel dose-response curves were obtained with either natural Alt a 1 or rAlt a 1 in the ELISA. \(A\) \(l\)ternaria extracts from 4 different manufacturers also gave parallel dilution curves (Fig 2). Natural and recombinant allergen showed greater than 95% inhibition in both ELISA and RIA (data not shown). Because the binding of mAb 121 was the same for both natural and rAlt a 1, the ELISA was quantitated with rAlt a 1 as a standard, in control curves ranging from 0.2 to 100 ng/mL. The assay was highly specific for Alt a 1, and a panel of other fungal extracts gave negative results: \(C\) \(la\)dosporium, \(P\) \(ho\)ma, \(C\) \(anda\)ida, \(T\) \(ri\)chophyton, and \(R\) \(hiz\)opus (Greer Laboratories); \(F\) \(us\)arium, \(P\) \(en\)icillium, \(R\) \(ho\)dofarula, and \(S\) \(ta\)chytobryts (Dr Kurup, Milwaukee, Wis); and \(C\) \(ur\)vularia and \(E\) \(pi\)coccum (Centre for Biochemical Technology, Delhi, India).

**Comparison of Alt a 1 and Asp f 1 levels in allergenic products**

Alt a 1 concentrations were measured in 8 commercial \(A\) \(a\)lternata extracts, which were purchased in 1998 and 1999. As a comparison, \(A\) \(f\)umigatus extracts from the same companies were assayed for Asp f 1 content. Alt a 1 was detected in all but 1 extract, with relatively consistent levels between extracts obtained in 1998 and 1999 for each company. Alt a 1 levels ranged from 0.01 to 6.09 µg/mL, with an average of 1.4 ± 1.6 µg/mL (n = 15) (Table I). Results were quite different for \(A\) \(s\)pergillus. Four companies produced products that had low or undetectable levels of Asp f 1 (<0.06 µg/mL), whereas others produced products that contained very high levels, ranging from 4 to 64 µg/mL (average 16.3 ± 23.9 µg/mL, n = 15). Intra-assay and interassay coefficients of variation were 5% and 33%, respectively, for the Alt a 1 ELISA and 8% and 17%, respectively, for the Asp f 1 ELISA.

In the 1980s, an international collaborative study was carried out under the auspices of the WHO/IUIS Allergen Standardization Committee to develop an international reference standard of \(A\) \(l\)ternaria. \(^{33}\) The \(A\) \(l\)ternaria extracts used in that study, together with the proposed international reference, were assayed for Alt a 1 to provide a comparison of allergen levels with extracts currently in use. \(A\) \(l\)ternaria extracts prepared and stored in 1983 had remarkably similar Alt a 1 levels (~1 µg/mL) to those manufactured in 1998 and 1999, with only 1 extract having undetectable allergen (Table II). These results suggest that at least with respect to Alt a 1 con-
In tent, there has been little change in the production of *Alternaria* extracts during the past 17 years (although the results do not rule out the possibility that there may have been some degradation of the 1983 extracts over time).

Data from all the samples tested as well as multiple lots obtained from a single company are summarized in Fig 3. The mean Asp f 1 level was 22.4 ± 22.6 µg/mL (n = 20, those containing >0.2 µg/mL), with extracts

![FIG 1. SDS-PAGE analysis of *Alternaria* and *Aspergillus* allergens. Lane 1, molecular weight markers; lane 2, natural Alt a 1, nonreduced; lane 3, natural Alt a 1, reduced; lane 4, rAlt a 1, nonreduced; lane 5, rAlt a 1, reduced; lane 6, natural Asp f 1, nonreduced.](image1)

![FIG 2. Dose-response curves of natural and rAlt a 1 and commercial *Alternaria* extracts in the ELISA. ●, Natural Alt a 1; ○, rAlt a 1; ▲, Bayer; △, Allergy Labs of Ohio; ■, Allermed; □, Greer.](image2)

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<tr>
<td>Allergy Labs, Okla</td>
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<td>0.04</td>
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*Bold facing* indicates 4 companies having very high ASP f 1 levels as compared with the remaining 4 companies.

NA, Not available.

*Greer extract supplied.
showing almost a 100-fold variation in Asp f 1 content. The mean for *Alternaria* was 1.4 ± 1.1 µg/mL Alt a 1 (n = 27, those containing >0.2 µg/mL), and 21 of 32 (66%) of these extracts contained 0.7 to 2.0 µg/mL Alt a 1. Data for multiple extracts prepared by the same company closely mirrored the results found for the 8 individual companies. Intracompany results for Alt a 1 were 1.2 ± 0.3 µg/mL (n = 10, range 0.49-1.42 µg/mL) and 17.0 ± 19.8 µg/mL for Asp f 1 (n = 12, range 0.96-55.11 µg/mL).

**Spore extracts**

Because the major route of *Alternaria* exposure is through inhalation of spores, we evaluated Alt a 1 content in extracts made from both dried and actively growing *Alternaria* spores. Alt a 1 was detected at concentrations of 11 to 73 ng/mL (Table III).

**DISCUSSION**

The results show that the Alt a 1 ELISA using rAlt a 1 as a standard is a useful tool for analyzing Alt a 1 levels in natural allergenic products. The assay is specific for *Alternaria* and is highly sensitive (down to 0.2 ng/mL Alt a 1). A somewhat surprising result was that the recombinant allergen, produced as a 15-kd protein, gave equivalent ELISA results to natural Alt a 1, which exists as a 29-kd dimer. This is most likely explained by the ability of rAlt a 1 produced in bacteria to spontaneously dimerize, which has also been reported for rAlt a 1 produced in *Pichia pastoris*.³⁰ The advantage of using rAlt a 1 as a standard is that unlike natural allergen, it can be produced by bacteria (or yeast) in essentially unlimited supplies and is accurately quantitated.

Although it has generally been thought that *Alternaria* extracts varied significantly in allergen content, the data show that production methods used by different manufacturers result in extracts that contain relatively consistent Alt a 1 levels (~1 µg/mL). This is remarkable for unstandardized extracts labeled as 1:10 or 1:20 wt/vol and was consistent both between manufacturers and over time (1983, 1998, 1999). Similar batch-to-batch consistency was reported for *Alternaria* extracts from a European manufacturer (Allergopharma).³¹ The absolute values for US allergenic products reported here are 2- to 14-fold lower than those recently reported with another ELISA for Alt a 1.³³ The previous assay used 2 mAbs and reported 12 to 84 µg/mL Alt a 1 in 3 *Alternaria* extracts. The most likely explanation for these discrepancies is the use of different Alt a 1 standards in the assays. The availability of rAlt a 1 for use as a standard should resolve these differences.

In comparison with results of *Alternaria* products, results of Asp f 1 levels in the *Aspergillus* extracts were quite variable both between manufacturers and between different lots from the same company. The scale of these differences (Asp f 1 undetectable vs 4-65 µg/mL) almost certainly means that the manufacturers used different methods for producing *Aspergillus* extract. Asp f 1 is secreted at high concentrations in *A. fumigatus* culture filtrate but is present at 1000-fold lower levels in spores or disrupted hyphae.¹⁴ Conversely, Alt a 1 is present in both spores and mycelium and appears to be extracted more uniformly.³⁴ The results raise questions about whether there are significant quantitative differences between other *A. fumigatus* allergens in commercial extracts and whether these are likely to have clinical significance.

The strong association of *Alternaria* sensitivity as a risk factor for asthma in the United States, Australia, and in parts of France prompted us to investigate whether the
Alt a 1 ELISA could be used to assess environmental exposure to *Alternaria*. The fact that Alt a 1 was detected in both an extract made from 20-year-old dried spores and spore suspensions taken from actively growing *Alternaria* shows that Alt a 1 is present in spores. However, detection of Alt a 1 in the ELISA required high spore concentrations (10^5 to 10^6/mL), which is probably much greater than that likely to be found in most house dust or air samples. In fact, we assayed 1531 dust extracts from the National Cooperative Inner City Asthma Study and others in which *Alternaria* sensitivity was high, and we detected Alt a 1 in only 6 samples (data not shown). These observations suggest that although the Alt a 1 ELISA is very sensitive (0.2 ng/mL), high levels of environmental fungal exposure would be needed to be detected in this assay.

In 1997 the American Academy of Allergy, Asthma and Immunology published a position statement advocating the use of standardized allergen extracts based on skin test potency and measurement of major allergen content. It is not clear whether a concentration of 1 µg/mL Alt a 1 is optimal for diagnostic efficacy on skin testing, and further studies would be required to determine whether this level is suitable for standardization. However, Aden et al found that potency of *Alternaria* allergen correlated with skin test reactivity, and 12 of 13 patients had positive skin prick test responses to less than 1 µg/mL Alt a 1. Using Center for Biologic Evaluation and Research reference preparations or recombinant allergens, manufacturers may use ELISA testing as a means of standardization and for control of lot consistency of allergen extracts. Currently, cat, *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, ragweed, grass pollens, and some venom allergens are the only standardized allergen extracts approved by the Food and Drug Administration. The results of this study show that the specific mAb-based ELISAs for Alt a 1 and Asp f 1 will serve as useful tools in the standardization and quality control of *Alternaria* and *Aspergillus* allergenic products. In addition, because rAlt a 1 is bound equally well as natural Alt a 1 in the ELISA, it is ideal for use as a purified reference standard of known concentration.

We greatly appreciate the help of several colleagues who contributed reagents and samples for these studies: Drs Greg Plunkett, John Yunginger, and Visnavath Kurup (fungal allergen extracts); and Drs Don Hoffman and Linda Stetzenbach (*Alternaria* spores). We are also grateful to Wanda Harvey for administrative assistance.

REFERENCES