Standardization of Fungal Allergens

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Aerobiologic sampling studies show that fungal spores have a world-wide distribution, and IgE-mediated sensitivity to fungal allergens is a cause of allergic disease. The diagnosis of fungal hypersensitivity is in part predicated upon the demonstration of binding of IgE to antigens contained in extracts prepared from the fungi.

In 1980, 281 mold extracts were commercially available in the USA.¹ These fungal extracts are variable and complex in their constituents. To the clinician faced with the task of diagnosing and treating patients with fungal allergy, the lack of readily available standardized extracts is a major problem. Fortunately, the prospects for significant advances in this area appear bright. In the past few years techniques have been developed that permit adequate study of the allergenic composition and content of such extracts. The isolation and purification of allergens from fungal extracts have further aided the development of standardized allergenic extracts. Efforts are underway by the International Union of Immunologic Societies (IUIS) to develop international reference standards that meet World Health Organization (WHO) requirements. These international reference standards include fungal allergens such as *Alternaria* and *Cladosporium*. Three commercially available fungal extract preparations are now licensed as standardized extracts in the United States. Reports of the therapeutic efficacy of standard fungal extracts have begun to appear, and additional studies are in progress.

Epidemiology

For a comprehensive review of the epidemiology of fungal sensitivity, the reader is referred to several articles.²⁻⁴ In the natural environment man is exposed to more than 100 species of airborne or dust-bound microfungi.⁵ Fungal spore counts frequently exceed pollen counts in the atmosphere by 1000-fold. Several genera are considered to be universal dominants in aerosampling surveys. These include *Cladosporium*, *Alternaria*, *Fusarium*, *Drechslera* (*Helminthosporium*), and several yeasts. Indoor concentrations of fungi generally depend on the outdoor concentrations. However, certain genera, such

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as *Aspergillus* and *Penicillium*, are found indoors on a regular basis and their concentration is not dependent on the outdoor concentration.³ A larger number of fungi are recognized as aeroallergens (Table 1). In the United States, *Alternaria* is a major allergen on the east coast, in the Upper Midwest, and in southern California.⁶ *Cladosporium* is the most common airborne fungal spore in the United States. Although it does not appear to be as potent an allergen as *Alternaria*, several species are important allergens, including *C. herbarum* and *C. cladosporioides*. *Cladosporium* is found in most of the temperate zones of the world.

Allergic reactions attributable to mold sensitivity have long been recognized. In 1726, Sir John Floyer⁷ noted asthma in patients who had just visited a wine cellar. In 1873, Blackley⁸ suggested that *Chaetomium* and *Penicillium* were associated with asthma attacks. In 1924, van Leeuwen⁹ noted the relationship of climate to asthma and found a correlation between the appearance of mold spores in the atmosphere and attacks of asthma. At the same time, Cadham¹⁰ reported the first documented case of asthma due to wheat rust. Over the next 10 years, case reports appeared attributing the source of mold allergy to the home or to occupational settings. In the late 1930s, Prince et al¹¹ and Feinberg¹² reported that outdoor air was a significant source of mold

Table 1. Genera of Fungi Associated With Allergy in the Literature

<i>Absidia</i>	<i>Drechslera</i>	<i>Papularia</i>
<i>Agaricus</i> ^a	<i>Epicoccum</i> ^a	<i>Penicillium</i> ^a
<i>Alternaria</i> ^a	<i>Epidermophyton</i>	<i>Phytophthora</i>
<i>Ankistrodesmus</i>	<i>Erysiphe</i> ^a	<i>Piptoporus</i>
<i>Armillaria</i> ^a	<i>Eurotium</i>	<i>Pleurotus</i>
<i>Arthrinium</i>	<i>Fomes</i>	<i>Podaxis</i>
<i>Aspergillus</i> ^a	<i>Fugus</i>	<i>Polyporus</i>
<i>Aureobasidium</i> ^a	<i>Fuligo</i>	<i>Polystictus</i>
<i>Boletus</i>	<i>Fusarium</i> ^a	<i>Puccinia</i> ^a
<i>Botrytis</i> ^a	<i>Ganoderma</i> ^a	<i>Rhizopus</i> ^a
<i>Bracteococcus</i>	<i>Geotrichum</i>	<i>Rhodotorula</i> ^a
<i>Candida</i> ^a	<i>Gliocladium</i>	<i>Saccharomyces</i> ^a
<i>Cantharellus</i>	<i>Graphium</i>	<i>Scopulariopsis</i>
<i>Cephalosporium</i>	<i>Hormidium</i>	<i>Serpula</i> ^a
<i>Chaetomium</i> ^a	<i>Hypholoma</i> ^a	<i>Sphaerotheca</i>
<i>Chlorophyllum</i>	<i>Leptosphaera</i>	<i>Spondylocladium</i>
<i>Cladosporium</i> ^a	<i>Lycogala</i>	<i>Sporobolomyces</i> ^a
<i>Claviceps</i>	<i>Lycoperdales</i> genera	<i>Sporotrichum</i> ^a
<i>Conosporium</i>	<i>Macrosporium</i>	<i>Stemonitis</i>
<i>Coprinus</i> ^a	<i>Malessezia</i>	<i>Stemphylium</i> ^a
<i>Cryptococcus</i> ^a	<i>Microsphaera</i>	<i>Stereum</i>
<i>Cryptosporium</i>	<i>Mucor</i> ^a	<i>Tilletiopsis</i> ^a
<i>Curvularia</i>	<i>Phoma</i> ^a	<i>Trichoderma</i>
<i>Dacrymyces</i>	<i>Mycogone</i>	<i>Trichophyton</i> ^a
<i>Daldinia</i>	<i>Neochloris</i>	<i>Trichothecium</i>
<i>Debaryomyces</i>	<i>Neurospora</i> ^a	<i>Ustilago</i> ^a
<i>Dicoccum</i>	<i>Nigrospora</i>	<i>Verticillium</i>
<i>Didymella</i> ^a	<i>Paecilomyces</i> ^a	<i>Xylaria</i>

Source: Adapted from Koivikko: Patient exposure to moulds, in Foucard T, Dreborg S (eds): Mould Allergy Workshop. Uppsala, Pharmacia, 1983, p 35.

^aBoth skin test and provocation test reactions have been observed.

spores and demonstrated that many of their patients had positive skin test reactivity to mold extracts.

Initially, mold sensitivity was equated to skin test reactivity; but it was found that many patients with skin test reactivity did not experience typical symptoms at times of high levels of atmospheric fungal spore activity. This may have been due in part to irritant properties of the extracts used in the testing.

The role of mold sensitivity in a variety of allergic diseases was established in provocation studies. By exposing patients to 1 g *Alternaria* powder dispersed in a 700-cu ft room, Harris¹³ provoked asthma and rhinitis symptoms in 10 of 12 patients with positive skin tests to *Alternaria* who had a history compatible with sensitivity to the fungus. Only 1 of 10 patients with a positive skin test but less clear-cut history of sensitivity developed symptoms upon similar exposures. Pennington¹⁴ applied cotton swabs that had been rolled over mold cultures to the nasal mucosa of 61 skin test-positive individuals. Twenty-two reacted to a variety of molds to which they had skin test reactivity. Subsequently, more sophisticated challenge studies have repeatedly demonstrated reactivity to fungal extracts in patients with positive skin tests. Both immediate hypersensitivity and late phase allergic reactions have been induced by provocation studies.¹⁵ It has recently been demonstrated that inhalation of either *Alternaria* or *Penicillium* spores in quantities comparable with those encountered by natural exposure can induce both immediate and late asthma in sensitive persons.¹⁶

Despite the numerous studies implicating mold sensitivity in the pathogenesis of allergic diseases, the prevalence of mold sensitivity has not been established. In patients with asthma and rhinitis, the reported frequency of positive skin test reactions to fungal allergens varies from 4% in Scandinavian studies to more than 80% in certain studies in the United States.² Whereas skin test reactivity indicates the presence of IgE antibodies to fungal allergens, the correlation between symptoms and skin test reactivity may be only about 50%. The potency of materials used for skin testing significantly influences the results of studies of the prevalence of mold sensitivity. For example, positive skin prick test reactions in patients with histories suggestive of sensitivity to *C herbarum* varied from 12.5 to 86% in the same population, depending on the extract used for testing.¹⁷

Although fungal sensitivity is well recognized, it is not always possible to establish a correlation between mold spore counts in the atmosphere and the presence of allergic symptoms. Bruce et al¹⁸ were able to correlate autumnal asthma symptoms with *Alternaria* spore counts in only 15 of 29 subjects who had demonstrable IgE sensitivity to this fungus. In a study at the University of Wisconsin, only 1 of 20 *Alternaria*-sensitive subjects demonstrated a positive correlation between allergic symptoms and *Alternaria* spore counts.¹⁹

The introduction of aerosampling devices and techniques that can quantitate the amount of allergen in the atmosphere (as opposed to morphologic identification and quantitation of fungal spores by microscopy) may shed further light on this problem. Agarwal et al²⁰ was able to detect Alt-I (the major allergen of *Alternaria*) in the atmosphere. This correlated well with mold spore counts and with the total *Alternaria* allergen activity in the atmosphere.

Since allergenic activity may be borne on particles other than spores, such studies may enhance the measurable correlation between allergen exposure and clinical symptoms.

Environmental surveys have suggested new avenues of approach to the study of fungal allergy. Salvaggio et al²¹ reported high asthma admission rates to hospitals in New Orleans, which appeared to be associated with high mold spore counts—particularly of small spores, such as those of Basidiomycetes. Since Basidiomycetes are widely distributed and their spores are among the most common found in the atmosphere, investigations are being conducted on their role in allergic disease. Santilli et al²² demonstrated that in 32 ragweed-sensitive subjects, 65 to 75% reacted to skin testing with spore extracts of 3 Basidiomycetes species. This was further confirmed by the demonstration of positive radioallergosorbent tests (RAST) to the spore extracts, as well as IgE binding to precipitates in crossed-radioimmunoassay (CRIE) autoradiographs.

Finally, the physician needs to be aware of the possibility of sensitization to fungal materials, not only from natural outdoor exposure but also from indoor exposure. Modest numbers of undisturbed house plants contribute minimally to aeroallergen prevalence in homes.²³ However, under greenhouse conditions, where large numbers of plants are grown indoors, abundant fungal growth can occur and this can become airborne when the plants are disturbed. Cold air vaporizers also can generate high concentrations of fungal spores,²⁴ and marijuana smoking may lead to development of fungal hypersensitivity reactions.²⁵

Physicians also must consider occupational exposure as an important source of fungal sensitivity. Positive skin tests and bronchial challenges to *Alternaria* and *Aspergillus* occur in patients with baker's asthma,²⁶ and there is evidence to suggest that fungal allergens play a role in the etiology of cotton dust sensitivity.²⁷

History of Standardization of Fungal Extracts

Blackley introduced skin testing to the diagnosis of allergic disease in 1873. With the introduction of immunotherapy in 1911,²⁸ the need for a measure of allergen extract potency was recognized. Noon introduced the Noon unit,²⁸ which was equal to the amount of allergen extracted from 1 μ g pollen. In the following years, allergen extracts were defined on a weight per volume basis (wt/vol), a practice that has persisted to the present day. In the 1930s, it was recognized that most allergens were proteins or glycoproteins. This led to introduction of the protein nitrogen unit (PNU),²⁹ which is equivalent to 0.01 μ g protein. This unit also has persisted to the present time. However, studies have repeatedly demonstrated that allergen potency is not closely associated with either of these measurements.³⁰

Technical advances in the past several decades have improved our ability to quantitate the amount of allergen in various extracts. Notable advances include the discovery of IgE antibody, the ability to measure specific IgE antibodies in serum by the RAST, the RAST inhibition assay, and the isolation and purification of allergens. These techniques have now been applied to fungal allergens. Yunginger et al³⁰ used endpoint skin test titration, RAST

inhibition, and direct RAST to compare the relative in vivo and in vitro potencies of 12 commercially available *Alternaria* extracts. While all the extracts carried the same content of PNU or equivalent wt/vol units on their labels, they differed 300-fold on endpoint skin testing and 200-fold by RAST inhibition. Vijay et al³¹ studied batch-to-batch variations in *Alternaria* extracts from 2 companies. The direct RAST showed little discrimination among the extracts. However, RAST inhibition revealed a 40-fold variation in potency in batch-to-batch products from the same company and a 55-fold difference between extracts prepared by the 2 individual companies. In addition, there were marked differences in the biochemical compositions of the products from the 2 companies.

Studies with the most concentrated commercially available *C herbarum* extracts showed that their potency was insufficient for the diagnosis of most patients, but that the diagnosis could be established in patients with suspected histories when skin testing and provocation tests with highly purified, biologically standardized, freeze-dried allergen preparations of *C herbarum* were used.³² Batch-to-batch variations in allergens in *Mucor racemosus* and *Aspergillus fumigatus* preparations have been reported.³³ Agarwal et al³⁴ studied 8 commercially available *Alternaria* extracts by isoelectric focusing (IEF), crossed-immunoelectrophoresis (CIE), CRIE, RAST inhibition, and immunoassays for content of Alt-I. Since there were differences in the rank order of allergen potency as measured by the various assays, the authors suggested that the optimal method for mold allergen standardization involves a combination of techniques.

Nationwide efforts in the United States led to the standardization of short ragweed extracts in 1982. Since that time it has been required that all lots of short-ragweed extracts in the United States have a minimum antigen-E content of 135 µg/mL in a 1:10 (wt/vol) extract and that their antigen-E content appear on the label. Individual manufacturers in the United States have introduced standardization into their fungal extract products. Three preparations are available at the time of this writing (*Alternaria alternata*, 3M Diagnostics, Mountainview, CA; and *A alternata* and *Ustilago* spore extracts, Optimal Inc., Bridgeport, CT).

For the past several years, the IUIS has brought together clinicians, scientists, manufacturers, and regulatory agencies in an attempt to develop internationally recognized standards of reference for fungal allergen extracts. These references are not intended to be models of extract preparation by the manufacturers, but rather to establish a potent extract of known composition to which other extracts can be compared. In 1982, the *Alternaria* Working Group was established. Subsequently, 5 manufacturers submitted preparations for selection as the reference material. One of these products has been extensively evaluated in an international cooperative study involving 22 laboratories throughout the world. Pending the outcome of ongoing stability tests, it is anticipated that this international reference will soon be accepted by WHO. Future studies on standardized fungal extracts by the IUIS will include *Cladosporium*. Continued efforts should lead to production of quality extracts of known potency that physicians can apply to the diagnosis and treatment of patients.

Extract Preparation

Many factors contribute to the marked batch-to-batch variability of fungal extracts. Among these are accurate identification of the source material; biologic characteristics of the fungi after repeated subculture; use of mycelia, spores, or culture filtrate; conditions under which the fungi are grown and extracts are prepared (which often are similar to those used to prepare pollen extracts and may not be suitable for fungi); and stability of the extracts once they are prepared.

Source Material

Fungi may mutate, producing morphologically variant forms (Fig. 1). As fungal taxonomy is based upon microscopic appearance, particularly of the spores, disagreements may arise among mycologists.³⁵ This problem was encountered in the preparation of an international reference extract of *Alternaria*.³⁶ Six extracts were submitted to the IUIS for evaluation as an international *Alternaria* reference extract standard. Fifteen strains, independently evaluated by an

Figure 1. Selected sexual/asexual relationships in the Ascomycetes. All spore types are commonly airborne. (FSN) Adapted, with permission from Burge HA: *Fungus allergens*. *Clin Rev Allergy* 3:321, 1985.

expert in fungal taxonomy, were used to prepare these 6 extracts. Of 5 strains accessioned by the American Type Culture Collection, all were confirmed as being *Alternaria*. Of 5 strains accessioned through a European repository, 4 were *Alternaria* and 1 was identified as *Ulocladium*. Of the 5 strains not accessioned by any national repository, 3 were *Alternaria*, 1 *Ulocladium*, and 1 a mixture of *Paecilomyces* and an unidentified red yeast. One strain was found to be parasitized. Because disagreement exists even among experts, manufacturers may mislabel extracts; not through deliberate misrepresentation or carelessness, but as a reflection of changing fungal taxonomy. For example, *Helminthosporium maydis* is now considered to be a species of *Drechslera*, and *H. interseminatum* as a species of *Curvularia*.

Simply labeling extracts as *Alternaria* is insufficient. Of 10 manufacturers in the United States, 8 use *A. alternata*, 1 uses *A. brassicola*, and 1 uses an unspecified strain. Furthermore, the allergenicity of 14 strains of *A. alternata* accessioned into the American Type Culture Collection has not been established. Skin testing with *Alternaria* has demonstrated differences among species, with *A. tenuissima* being the most potent in 1 study.³⁷ As a practical and reasonable strategy in standardizing fungal extracts, a reference preparation probably should contain pooled extracts obtained from a number of similar strains grown independently.

Once a particular organism has been chosen, there is the question of whether spores, mycelia, or culture filtrate should be used in preparing the extract. Currently, most commercial extracts are from mycelia and contain little or no spore material.⁶ Several studies indicate that spores differ allergenically from mycelia.^{38–40} Spore preparations may be more active by skin testing and RAST inhibition,⁴¹ but some investigators⁴² have found that higher spore content did not increase the activity of Alt-I; the major antigen of *Alternaria*. While spores are the most important source of allergen exposure for most individuals, it is difficult to obtain pure spore preparations at a reasonable cost for commercial extract preparation. It also should be remembered that patients inhale constituents other than spores, such as mycelial fragments that would not be recognizable microscopically and particulate matter to which allergens may be bound. Aukrust³⁵ found that Alt-I activity was 3 times higher in mycelial than in spore preparations of *Alternaria*. Spore preparations were more heterogeneous and contained more allergens than preparations from mycelia. It is therefore suggested that reference materials and commercial extracts contain both spore and mycelial constituents. While culture filtrates do contain allergenic materials, they often lack sufficient quantities of allergens, and the presence of various enzymes in the culture filtrate may lead to degradation of allergens. Therefore, culture filtrates are probably not a suitable source for preparation of standardized extracts.

Culture Medium

Many variables affect the production of allergens by fungi. Factors such as glucose supply, oxygen, light and dark cycles, temperature, and pH may be important in sporulation and thus may affect allergenic composition. Several

studies suggest that chemically defined media will support the growth of fungi while preserving allergenic activity.^{43,44} Therefore it is recommended that synthetic media be used whenever possible to culture fungi to provide standardized growth conditions and to prevent the introduction of potential allergens of nonfungal origin.

Growth Time

The optimal length of time fungi should be grown in culture before allergens are harvested remains to be clarified. Kaufman et al⁴⁵ studied the release of IgE-binding antigens of *Aspergillus* into culture filtrates. There is an early growth phase lasting approximately 10 days, during which IgE-binding antigens appear in the culture filtrate. This is followed by a drop in IgE binding during the second phase, days 10 to 20, and an increase in the third phase when cells are lysed (approximately 20 to 40 days). It also was noted that different IgE-binding antigens appeared not only during the first and third phases, but also during the same phase when *Aspergillus* was grown under the same conditions of different occasions. Kroutil et al⁴⁶ examined the appearance of IgE-binding allergens in culture filtrates and mycelial extracts of *Alternaria* when cultures were harvested after 1, 2, and 3 weeks. They found that the total protein content of the mycelia increased from week to week and the fewest number of proteins were identified by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) at 1 week. However, the greatest IgE binding occurred in extracts prepared from mycelia harvested at 1 week. Furthermore, more allergens were identified by CRIE in the 1- and 2-week mycelial extracts. Culture filtrates, conversely, showed an inverse relationship in allergenic activity, with more allergens observed in the 2- and 3-week culture filtrates than in the 1-week filtrate material. Detailed studies of various fungi will be necessary to establish the ideal culture time for each fungus.

Extraction Process

Several variables enter into the extraction process. Excessively long extraction times at room temperature or above can lead to destruction of allergens by hydrolytic enzymes in the extract.⁶ Studies on the effect of extraction time on allergen release from *Aspergillus* spp⁴⁷ indicated that RAST-binding compounds are released within the first 20 minutes of the extraction process. Similar results were obtained with *Alternaria*.⁴⁸ Powdered mycelia extracted at pH 8.0 at 4°C for 15 minutes, 60 minutes, 6 hours, and 24 hours showed increasing IgE binding up to 6 hours by direct RAST. Similarly, the allergens identified by CRIE were maximal at 6 hours and diminished by 24 hours. The authors concluded that allergens are rapidly eluted from mycelia of *Alternaria*, and extraction times beyond 6 hours may alter the allergenic composition.

The pH of the extracting buffer also may affect the composition of the allergenic extract. As the pH of nasal secretions in active rhinitis is 8.0 to 8.5, extracting the raw material with buffers in this pH range may produce the most clinically relevant allergens.

Assessment of Allergenic Activity

No single available immunochemical assay will properly evaluate the allergenicity of extracts prepared even under standardized conditions. Li et al⁴⁹ evaluated 6 *Aspergillus* preparations by a variety of immunochemical and physiochemical methods. Like Agarwal et al,³⁴ they found that the rank order of antigen and allergen content differed considerably with the method used. Therefore it is suggested that a variety of immunochemical assays are required to effectively evaluate fungal extracts, and comparison of these extracts with a reference material may provide a basis for their standardization.

Stability of Extracts

No systematic studies evaluating the stability of fungal extracts have been conducted. However, lyophilized *Alternaria* and *Cladosporium* extracts that had been stored at room temperature for 15 years compared favorably with freshly prepared extracts when evaluated by skin prick testing.⁵⁰ The addition of 50% glycerine will help stabilize extracts for skin testing. The stability of extracts used for immunotherapy has yet to be established.

Characterization of Allergens

Because of the great variability of fungal extracts, their ultimate standardization will depend upon isolation and purification of the individual allergens they contain. Several investigators have isolated and characterized fungal allergens (Table 2).

Currently, the best characterized allergen is Alt-I, derived from *Alternaria alternata*.⁵¹ Crude *A. alternata* extract was fractionated by ammonium sulfate precipitation, anion exchange chromatography, flat-bed preparative IEF, and gel filtration. The isolated material contained appreciable amounts of carbohydrate, in keeping with studies showing that removal of 96% of extractable nitrogen from *Alternaria* extracts could be achieved without loss of skin test reactivity.⁵² This material had an apparent molecular weight of between 25,000 and 50,000 and an isoelectric point between 4.0 and 4.5. Further evaluation

Table 2. Purified Fungal Allergen Fractions

Species	Allergenic fraction	Approximate MW	pI	Reference
<i>Alternaria tenuis</i>	Alt-I	25,000–50,000	4.0–4.5	51
<i>A. iridis</i>	Ag-I	60,000	4.0	53
<i>A. alternata</i>	Ag-8	35,000–40,000	4.3–4.65	54
<i>A. sp</i>	Basic peptide	9000	9.5–9.8	60
<i>Cladosporium herbarum</i>	Ag-32	13,000	3.7–4.4	67
	Ag-54	25,000	5.0	67
<i>Aspergillus fumigatus</i>	Ag-3	24,000	No data	70

Source: Modified from Yunginger JW: Fungal allergens, in Kerr JW, Ganderton MA (eds): Proceedings of Invited Symposia. XI International Congress of Allergology and Clinical Immunology. London, The MacMillan Press, Ltd., 1983 p 539.

MW = molecular weight; pI = the pH of a solution containing a salute at its isoelectric point.

revealed that Alt-I was heterogeneous by thin-layer IEF and polyacrylamide gel electrophoresis. Additional fractionation by preparative polyacrylamide gel electrophoresis showed 5 fractions, 3 of which had similar amino-acid compositions.

Nyholm et al⁵³ found slight biochemical differences between Alt-I and a major allergen, termed antigen 1 (Ag-1), obtained from a different strain of *A alternata*. By tandem CIE and CRIE, however, these allergens appeared to be identical. The approximate molecular weight of this antigen was 60,000, and its isoelectric point was 4.0. Radioallergosorbent test inhibition showed parallel inhibition lines, suggesting immunological identity of these components. Further investigations⁵⁴ resulted in the identification of another major antigen, Ag-8, which was partially purified by diafiltration, gel filtration, and preparative IEF. Its molecular weight was between 35,000 and 40,000, and subsequent evaluation showed it to be a variant of Alt-I. It has been estimated that Alt-I comprises only a small fraction of the total dry weight of *A alternata*. In the richest extracts examined to date, it comprises only 0.7% of the dry weight.⁵³

The biologic activity of Alt-I has been studied.⁵⁵ IgE binding was found in sera of 21 of 25 asthmatics sensitive to *Alternaria*; wheal and flare reactions occurred at levels of nanograms per milliliter in 16 of 25 patients. Bronchial challenges in 11 of 16 individuals using 0.6 to 60 µg/mL of Alt-I produced a greater than 20% drop in FEV₁, and histamine release from leukocytes obtained from sensitive individuals was demonstrated. In further studies,⁵⁶ 100 unselected patients reactive to crude *Alternaria* were skin tested to Alt-I and 83% reacted. Therefore, Alt-I is a major allergen of *Alternaria*. Monoclonal antibodies to a subfraction of Alt-I have been prepared.⁵⁷ Development of similar monoclonal antibodies will undoubtedly be of great assistance in the standardization of *Alternaria* extracts. There also is evidence that suggests Alt-I is the major allergen in *Stemphylium*⁵⁸ and *Ulocladium*.⁵⁹

Other investigators have identified additional *Alternaria* allergens. Budd et al⁶⁰ obtained an allergenic fraction from an unidentified *Alternaria* species after water extraction, dialyzation, and preparative flat-bed IEF. This material had an estimated molecular weight of 9000 and an isoelectric point between 9.5 and 9.8. It gave positive skin tests in 18 of 20 patients reactive to crude *Alternaria* extract. Other studies⁶ confirmed the existence of allergenic activity in basic fractions of *Alternaria* spore and mycelial extracts.

Hoffman et al⁶¹ examined the allergenic characteristic of spore preparations containing 90 to 96% spores and mycelium preparations containing <1% spores. By CIE they found 40 antigens in spore preparations, 8 of which were unique to the spore. A spore-specific allergen was isolated, which had an isoelectric point between 6.2 and 6.3. Alt-I activity was found in both spore and mycelial extracts. Skin testing revealed that the vast majority of patients reacted to both spore and mycelial preparations.

Vijay et al⁶² fractionated *A alternata* and *A solani* by passing dialyzed extracts over a gel-filtration column, followed by ion exchange chromatography. Allergenic activity eluted from the gel-filtration column had a molecular weight between 15,000 and 30,000. These studies indicated that the 2 species share common allergenic determinants.

In a preliminary report, Bush et al⁶³ fractionated an *A alternata* strain differing from the one reported by Yunginger et al⁵¹. Following similar separation techniques, they identified a fraction with a molecular weight of approximately 18,600 and isoelectric point of 4.2. This material had partial but not complete identity with Alt-I. It was capable of producing significant histamine release from leukocytes of 5 *Alternaria*-sensitive subjects in vitro. This may indicate some strain differences in *A alternata*.

Further studies of allergens in *Alternaria* have been conducted using CIE and CRIE. Lowenstein⁶⁴ found 18 separate allergens in a crude *Alternaria* preparation. This included the major allergen Ag-1, which was subsequently found to be identical with Alt-I. Bush et al,⁶⁵ using a different *Alternaria* strain, identified 22 antigens by CIE. Three allergens were identified in the extract, of which antigen 3 appeared to be a major allergen, since 100% of 35 patients studied showed IgE binding to it. It was not determined if this antigen corresponded to Alt-I.

Kroutil and Bush⁶⁶ separated a crude *Alternaria* allergen extract by SDS-PAGE and transferred the proteins to nitrocellulose by the Western blotting technique. Incubation of the nitrocellulose strips with human sera, followed by incubation with radiolabeled anti-IgE and autoradiography, disclosed IgE binding to 2 allergens with molecular weights >66,000, 1 at 66,000, and another at 29,000.

In addition to allergens from *Alternaria*, 2 major allergens have been isolated from *Cladosporium*.⁶⁷ Purified allergens were obtained from a *Cladosporium* preparation by diaflow filtration, gel filtration, and preparative flat-bed IEF. Previous studies using CRIE had shown antigen 32 to be a major allergen and another fraction, antigen 54, to be a potent binder of IgE. Following the fractionation procedures described above, antigen 32 was isolated and found to be a heterogeneous protein with multiple bands at isoelectric points of 3.7, 3.9, 4.1, and 4.4. The molecular weight was estimated to be 13,000. It was, however, immunologically homogeneous. The other fraction, antigen 54, has been further purified and characterized.⁶⁸ It is a glycoprotein with a molecular weight of approximately 20,000. Neither allergen was cross-reactive as determined by passive transfer skin testing. Isolates of *C herbarum*, when analyzed by CIE and CRIE, showed complex patterns. Crossed-immunoelectrophoresis revealed up to 60 precipitates in the extracts. One major, 10 intermediate, and 25 minor allergens were identified.⁶⁹ Allergen contents of 10 isolates of *C herbarum* were analyzed and found to vary from 0 to 100% relative to a reference.⁶⁹

A preliminary report of the purification of a major *Aspergillus fumigatus* allergen has appeared.⁷⁰ This allergen, Ag-3, was prepared by ammonium sulfate precipitation of an *A fumigatus* culture filtrate. This was followed by gel filtration on Sephacryl 200. The allergen was shown to be a major inhibitor of the RAST. It was heat labile, with a molecular weight of 24,000, and did not bind to concanavalin A. Wallenbeck et al⁷¹ conducted immunoelectrophoretic studies with crude *A fumigatus* extracts. Using a 10-strain mixture extract and hyperimmune antisera, they found 44 precipitates by CIE. Two antigens, antigens 10 and 40, were determined to be major allergens. There were 10 intermediate allergens and 6 minor allergens. When the strains used

in the extract were investigated individually, they were found to vary in the quantities of the 4 most important allergens. By gel filtration, Piechura et al⁷² isolated an antigen with a molecular weight of approximately 150,000 from a mycelial preparation. This gave positive immediate skin tests in approximately 50% of patients with allergic bronchopulmonary aspergillosis. Kauffman et al⁴⁰ compared the allergenic content of spore preparations, mycelial preparations, and culture filtrates of *A fumigatus*. They found that disrupted spore antigens did not cross-react with either mycelial or culture filtrate allergens. Only minimal IgE binding could be demonstrated to intact spores since the allergens were secreted into the medium spontaneously. Further investigations leading to the isolation of relevant allergens of *Aspergillus* are needed.

Finally, steps are being undertaken to identify the major allergic constituents of the Basidiomycetes. Lehrer and Salvaggio⁷³ examined 82 patients with possible mold allergy by skin testing to extracts prepared from 6 basidiomycete species. They found the range of positive skin tests to be between 9.7 and 14.6% of 82 patients. Crossed-immunoelectrophoresis and CRIE studies were conducted on 3 species, *Xylobolus frustalatus*, *Dacrymyces delequescens*, and *Pleurotus ostreatus*. All species showed several antigenic components as well as several allergens, the most reactive being *P ostreatus*, which contained 13 antigens in a combined mycelial and spore extract. Further studies by the same group⁷⁴ with a spore extract of *P ostreatus* showed 27 precipitates by CIE. The antigens were found in spore, mycelium, or cap extracts. However, unique spore antigens also were identified, of which 5 were allergens.

Despite the progress being made in the isolation and purification of fungal allergens, much work is needed to quantitate these important allergens in fungal extract preparations used in the diagnosis and treatment of patients.

Cross-Reactivity of Allergens

The term cross-reactivity refers to the antigenic (IgG) or allergenic (IgE) determinants on molecules from different fungal variants. Historically, skin testing with fungal allergens reveal broad patterns of reactivity.⁷⁵ However, other reports indicate that sensitivity may be species-specific in some instances.⁷⁶ The development of immunologic techniques for studying cross-reactivity, such as immunoprecipitation techniques and RAST inhibition, has aided our understanding of this phenomenon but has not completely clarified the situation. Because of the large number of fungal variants found in nature, their taxonomic classification into genera, species, and strains does not account for their immunologic reactivity in human beings. In some instances there is little or no cross-reactivity with IgE antibodies between different genera. Aukrust⁷⁷ found that a crude *Alternaria* extract gave only weak precipitates with a potent rabbit antiserum against *Cladosporium herbarum*; and CRIE showed no antigenic cross-reactivity between *C herbarum* and 2 basidiomycetes, *Cantharellus cibarius* and *Coprinus comatus*. Similarly, Karr et al⁷⁸ were unable to demonstrate cross-reactivity between *Alternaria* allergens and 3 species of *Aspergillus* by RAST inhibition. In other cases, there may be extensive cross-reactivity among genera. Agarwal et al⁵⁸ demonstrated that

Alternaria and *Stemphylium* extracts contain multiple shared allergenic and antigenic determinants, through the use of RAST inhibition, immunoelectrophoresis, and immunodiffusion studies with rabbit antisera. In fact, the major allergenic determinant of *Stemphylium* was Alt-I; and *Stemphylium* actually showed higher Alt-I activity by double-antibody radioimmunoassays for the antigen than did *Alternaria* extracts themselves. Similar studies conducted with *Ulocladium*⁵⁹ also demonstrated that Alt-I was the major allergenic component. Hoffman and Kozak⁷⁹ demonstrated extensive cross-reactivity among *Alternaria*, *Drechslera* (i.e., *Helminthosporium*), *Spondylocadium*, *Stemphylium*, and *Curvularia*. These cross-reactive genera contained an antigen that appears to be distinct from the Alt-I described by Yunginger. Surprisingly, *Stachybotrys* appears to cross-react with the form genera *Alternaria*, *Stemphylium*, and *Curvularia*.⁴

Among species of the same genus, allergenic similarities may exist. For example, *A. tenuis*, *A. alternata*, and *A. solani* appear to possess common allergenic determinants.⁶² Both shared and unique antigens may occur in different species of the same genus. Karr et al⁷⁸ examined the antigenic relationships among *Aspergillus fumigatus*, *A. glaucus*, and *A. flavus*, using RAST inhibition. Each species produced significant but only partial inhibition of RAST by each of the other species analyzed. The data suggested that both shared and unique antigens are present in these 3 species. Using fused rocket and immunoelectrophoresis, cross-reactivity among several strains of *Aspergillus* was demonstrated.⁸⁰ Nonetheless, in the genus *Aspergillus*, there appear to be common antigens within the *fumigatus* and *niger* groups, which are antigenically distinct from the *versicolor*, *nidulans*, and *glaucus* groups.⁴ Radioallergosorbent inhibition and skin testing revealed unique allergenic properties in 4 *Alternaria* species (*tenuissima*, *brassicae*, *alternata*, and *longissima*).³⁷ Finally, strains of fungi may show allergenic similarities as well as differences. Shumacher et al⁸¹ noted wide biochemical variation in 7 isolates of *A. alternata*; however, precipitin studies indicated extensive cross-reactivity. Isolation of major allergens from *A. alternata* (Alt-I, Ag-1, and Ag-8) that are immunologically identical suggests marked similarities among strains. However, the content of Alt-I in these strains varies widely. Similarly pronounced immunologic and biochemical differences in 10 strains of *C. herbarum* have been demonstrated.⁶⁹

In summary, while cross-reactivity appears to occur across broad taxonomic groups, further research is needed to establish the importance and degree of IgE cross-reactivity. Patients with fungal allergy often display parallel and independent multiple sensitivity to a variety of fungi.

Clinical Data From Studies Using Characterized Mold Extracts

Alternaria

There is a paucity of clinical data concerning the diagnostic and therapeutic efficacy or safety of well-characterized or standardized *Alternaria* extracts. The biologic activity of Alt-I, an important *Alternaria* allergen fraction, has been

demonstrated at nanogram permilliliter concentrations by skin testing of *Alternaria*-sensitive individuals and in vitro induction of leukocyte histamine release using cells from the same persons.⁵⁵ In an ongoing double-blind, long-term study of *Alternaria* immunotherapy conducted at Mayo Clinic, 30 *Alternaria*-sensitive persons, primarily asthmatics, were assigned to injection treatment with either purified Alt-I, a crude *Alternaria* extract with an equal Alt-I content, or a caramelized histamine placebo solution. Positive bronchial provocation tests with both *Alternaria* preparations were induced at microgram concentrations of ALT-1 in these study patients. A preliminary report of the findings has appeared.⁸² Acquisition of additional clinical data should be greatly facilitated by development of an international standard *Alternaria* extract. A candidate standard extract has been selected³⁶ and tested in an international multicenter collaborative trial.⁸³ If WHO accepts the final working group report on this standard, investigators and commercial supplies will be able to use this standard extract to assign a unitage of allergenic potency to their *Alternaria* extracts.

Cladosporium

A group of Scandinavian investigators⁸⁴ has reported a double-blind, placebo-controlled trial of *Cladosporium herbarum* immunotherapy involving 30 asthmatic children. The lyophilized immunizing extract had been partially purified by ultrafiltration and Sephadex G-100 gel filtration and standardized in Biological Units (BU) by skin prick tests in sensitive individuals. In this method of standardization, used in the Nordic countries, the size of the skin test wheal produced by the allergenic extract is compared with the size of the skin test wheal produced by a histamine chloride solution (1 mg/mL) in a population of sensitive patients.⁸⁵ Extracts that produce a mean wheal size equal to that of the histamine solution are assigned a potency of 1000 BU/mL.

The children were skin tested and underwent conjunctival and bronchial provocation testing before and 5 to 7 months after initiation of immunotherapy. They maintained symptom and medication score diaries and recorded peak expiratory flow rates twice daily during the 10-week *Cladosporium* season (August to October). The *Cladosporium* extracts were reconstituted in saline containing 0.03% human serum albumin and administered in a clustered dosage regimen, beginning with a dose of 10 BU and building toward a target maintenance dose of 100,000 BU. When the maximum tolerated or maintenance dose was achieved, the extract diluent was changed to 0.2% aluminum hydroxide and 0.4% phenol in saline to achieve a depot effect. The placebo injection material contained caramelized histamine.

Only 5 of the 16 *Cladosporium*-treated children could not achieve the targeted 100,000 BU dose. There were 45 generalized reactions (chiefly asthma, involving 81% of the 16 children) and 4 large local reactions (>10-cm diameter). No systemic or large local reactions were encountered in the placebo-treated group. The total medication and symptom scores over the 10-week scoring period did not differ significantly in the 2 groups, but the medication scores of the active treatment group were significantly lower during the 2 weeks with the highest *Cladosporium* spore counts. The daily peak expiratory flow

rates did not differ significantly in the 2 groups. Both the conjunctival and bronchial provocation tests demonstrated a significant decrease in reactivity to *Cladosporium* in the active treatment group, but no statistically significant decrease in *Cladosporium* skin test reactivity was seen. The authors speculated that the high incidence of systemic reactions encountered during therapy resulted from continual bronchial inflammation induced by sensitivity to co-seasonal or perennial allergens to which the children also were sensitive. They further speculated that the clinical efficacy of immunotherapy would have been greater had patients sensitive only to *Cladosporium* been enrolled, but such patients are rare.

In the same study, *Cladosporium*-treated patients showed statistically significant decreases in specific IgE antibody and increases in specific IgG antibody.⁸⁶ Crossed-radioimmuno-electrophoresis experiments showed that the increase in IgG antibodies was directed to several antigens, particularly Ag-12 and Ag-26, using the reference numbering system of the authors.⁸⁷ However, there was no correlation between the individual immune response and any clinical parameters showing efficacy, such as changes in bronchial and conjunctival sensitivity and medication scores.

Danish investigators⁸⁸ have used the same *Cladosporium herbarum* and placebo extracts and dosage regimen to treat 22 *Cladosporium*-sensitive asthmatic adults. A very high incidence of systemic reactions (mainly asthma) again was noted in the active treatment group, including 4 cases of anaphylaxis that required hospitalization. One year after institution of therapy, the active treatment group showed statistically significant decreases in bronchial and skin test reactivity to *Cladosporium*, as compared with the placebo-treated group. Neither symptom/medication scores nor specific IgE/IgG antibody levels were reported for these patients.

Summary

The ability of various fungi to induce human IgE-mediated disease, especially allergic asthma, has been well established. The relative clinical importance of fungal aeroallergens varies considerably in different geographic areas. The magnitude of exposure to fungal aeroallergens can be quantitated more precisely by newer immunochemical assays than by more traditional fungal cultures or quantitation of fungal spores by microscopy. Although the wide variations in biologic potency among commercial mold allergenic extracts have been appreciated for decades, only recently have laboratory technologic advances and the production of international reference extracts permitted the development of standardized fungal extracts of defined potency. Future efforts should be directed toward documenting the expected diagnostic and therapeutic advantages of such standardized fungal extracts.

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