

Allergens of the Fungi Imperfecti

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Fungi are important in many human and environmental processes. They are found as spores, fragments of mycelium, or intracellular components, and are released from dying fungi or extracellular material secreted by the living fungus in both indoor and outdoor air samples. The importance of molds as sensitizers and causes of allergic rhinitis and allergic asthma is well known. However, the clinical history is rarely conclusive for mold allergy, since seasonal variations may exist and, furthermore, this variation may coincide with variations in pollen and mite allergens. Because of the complexity of the fungi, only limited information exists about them, and relatively few fungi imperfecti have been characterized with respect to their allergen composition.

Molds are microscopic organisms, composed of long filaments, that grow on the surface of, and inside, nearly all substrates of plant or animal origin. Since 1870 it has been known that inhalation of mold spores can produce allergic respiratory symptoms, and convincing documentation was presented by several European workers in the mid-1920s. The reported incidence of mold sensitization in atopic patients ranges between 5 and 30%. Precise figures are, however, difficult to give since there still are so many unanswered questions regarding the relationship between inhaled mold particles and the production of different allergic symptoms.

Alternaria is globally the most widely distributed mold. In the United States, for example, this mold ranks third after ragweed and

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grass pollens as a cause of allergy (1). *Cladosporium* is widely distributed in the temperature zones (2). Other, less important allergenic molds are *Aspergillus*, *Penicillium*, *Candida*, *Botrytis*, and *Helminthosporium*. Most molds investigated have been shown to be allergenic to some individuals, but in general only a few molds are sufficiently widely distributed to be important on that account. Fungi are known for their ability to produce mycotoxins (toxic mold metabolites). Extracts of molds frequently contain toxins, low-molecular-weight metabolites, and macromolecules inducing a toxic reaction (3). Some mycotoxins, such as aflatoxin B1, ochratoxin, and patulin, are well characterized, and laboratory tests exist for their determination (4,5).

Aspergillus fumigatus is known to produce aflatoxin, and this carcinogenic toxin was found in four out of 12 commercial extracts of *Aspergillus fumigatus* (6). For a mold extract to be used in humans, documentation should be available indicating that the material is negative in a test for mutagenicity, such as the Ames test (7).

Another very important aspect of mold allergy is the fact that the molds are extremely variable in their composition, which makes it difficult to reproduce extracts. Different isolates of the same species are likely to represent different strains with different allergenic properties and characteristics. Individual cultivations of the same strain may fail to produce the same allergens even when the experimental conditions are kept constant (8–10). Because of the variability of allergen content, many mold extracts are questionable for the diagnosis of IgE-mediated allergy. Bahna and Yee (11) compared Phadebas RAST® *Alternaria*, *Cladosporium*, *Penicillium*, *Candida*, and *Aspergillus* reagents, and found them to be about twice as effective as other available reagents. Screening for IgE antibodies with an expanded range of mold allergens indicated that such fungi as *Botrytis*, *Helminthosporium*, and *Candida* may be more common than previously suspected (12–14). The simultaneous occurrence of IgE against many molds can be ascribed to crossreactivity, but probably more often reflects parallel and independent sensitization to several molds.

IN VITRO ASSAY METHODS

The first assay for allergen-specific antibodies was described in 1967 (15). Even if many assay procedures have been presented, relatively few except RAST have become routine methods in laborato-

ries. Optimal use of laboratory allergy tests requires a high level of knowledge regarding allergen composition, distribution, local importance and other properties that facilitate selection of adequate tests. Furthermore, it is important to point out that all relevant methods described hitherto have both advantages and disadvantages, meaning that they differ in their sensitivity and specificity.

Table 1 summarizes the most important *in vitro* assays for measuring the allergenic activity of mold allergenic extracts and specific antibodies. The potency of allergenic extracts can be determined by the radioallergosorbent test (RAST) (16), RAST inhibition (17), and histamine release (18), using blood samples from selected allergic individuals. These three methods measure the total allergenic activity and give very limited information about the changes in the ratio between the individual allergens. Furthermore, the response is strongly dependent on the quality and characteristics of the patient serum used.

The content of individual allergens in the allergenic extract can be quantified by single radioimmunodiffusion (19) or rocket immunoelectrophoresis (RIE) (20) using monospecific rabbit antibodies. A further development of RIE is radio rocket immunoelectrophoresis (21), in which the gel, after electrophoresis, is incubated with allergic-patient serum and thus the IgE-binding components can be identified and directly quantified. The use of such immunochemical gel techniques renders it possible to measure the activities of the individual allergens without previous purification.

Crossed immunoelectrophoresis (CIE)(22)/crossed radioimmunoelectrophoresis (CRIE)(23) is a powerful method combination for characterization of individual antigens and allergens. The antigenic composition of an allergenic extract is determined by CIE analysis; the allergenic composition, as well as individual allergens, can be identified and determined by CRIE using a panel of well-defined allergic-patient sera. A common requirement for the CIE/CRIE analysis is the availability of polyspecific rabbit antibodies and adequate patient sera containing mold-specific IgE antibodies.

Various enzyme-linked immunosorbent assays (ELISA) (24) and radioimmunoassays (RIA) (25,26) can be used for assaying the presence of individual allergens using either isolated allergens or monospecific monoclonal antibodies directed toward the allergen in question. Both ELISA and RIA show high sensitivity and specificity for the quantitation of allergens (24-26).

Table 1
Laboratory Methods for Assaying Mold Allergenic Extracts

Method	Application	Reference
Radioallergosorbent test (RAST)	Determination of patients IgE antibody reactivity	(16)
RAST inhibition	Determination of total allergen-extract potency	(17)
Histamine release	Determination of total allergen-extract potency	(18)
Single radioimmuno-diffusion (SRID)	Quantitation of individual antigens(monospecific animal antiserum)	(19)
Rocket immunoelectrophoresis (RIE)	Quantitation of individual antigens (monospecific animal antiserum)	(20)
Radio rocket immunoelectrophoresis (RRIE)	Identification and quantification of allergens	(21)
Crossed immunoelectrophoresis (CIE)	Characterization of allergen extract with respect to 11 antigen composition	(22)
Crossed radioimmuno-electrophoresis (CRIE)	Identification of IgE and IgG antibodies in patient sera; identification and quantification of allergens	(23)
Enzyme-linked immunosorbent assay (ELISA)	Quantification of individual antigens and allergens	(24)
Radioimmunoassay (RIA)	Quantification of individual antigens and allergens	(25) (26)
Isoelectric focusing/immunoblotting (IEF/blot)	Antigen composition; identification of IgE-binding components	(27)
Sodium dodecyl sulfate-polyacrylamide gel electrophoresis/immunoblotting (SDS-PAGE/blot)	Antigen composition; identification of IgE-binding components	(28) (29)

Nitrocellulose immunoblot/immunoprint (27–29) can be used to study the allergenic extract composition as well as to screen patient sera for specific antibodies. The allergen–protein mixture is separated by isoelectric focusing (27) or by polyacrylamide gel electrophoresis (PAGE) in the presence of detergent (28,29). The identification of the allergens can subsequently be performed by Western blotting (28), in which specific IgE antibodies from a selected panel of allergic individuals binds to the allergenic peptides and proteins on a nitrocellulose membrane. In contrast to the CIE/CRIE techniques, the immunoblot technique is not dependent on animal antibodies as reagents, which may be a limiting factor for the CRIE procedure.

SPORES AND/OR MYCELIUM

One of the classical questions in mold allergy is whether spores/ conidia or mycelium constitute the best source of allergens. Environmental spore data have been compared with challenge doses of *Alternaria* and *Penicillium* spores, and it was found that immediate-type asthma was readily induced by both whole spores and spore extracts (30). The content of mycelial fragments was negligible. Mold spores are capable of inducing sensitization and thus being responsible for obstructive respiratory disorders. By using in vivo testing and RAST, mold spores from *Alternaria tenuis*, *Aspergillus fumigatus*, *Cladosporium herbarum*, *Mucor racemosus*, and *Penicillium notatum* were studied (31). High correlation was obtained between the results from provocation tests and RAST in patients with respiratory disorder or allergic bronchial asthma. Rockwell et al. (32) compared RAST and intradermal skin testing in mold-allergic subjects, using pure spore extracts from *Alternaria alternata*, *Cladosporium cladosporioides*, *Fuligo septica*, and *Ustilago maydis*. The spore extracts were more accurate in skin testing than in RAST for the diagnosis of allergic sensitivity.

Many commercially available extracts of fungi imperfecti are made from mycelium with a relatively low spore content (1,33). Mold extracts with no spores present would be inadequate if the mycelium did not contain the same allergens as the spores. A comparison of antigenic and allergenic composition of mycelium and spores of different fungi imperfecti is important. Hoffman et al. (34) studied spores and mycelium extracts from *Alternaria* by in vivo and in vitro methods. The authors obtained 90–96%-pure spore preparation and

97%-pure mycelium preparation of *Alternaria alternata*. The spore extracts contained eight spore-specific antigens not detected in the mycelium extract, at least one of which was a strong allergen. On the other hand, the mycelium extract did not contain any antigens not found in the spore extracts. The spore-specific fraction was isolated by immunoadsorption and tested in *Alternaria*-sensitive patients by skin test and RAST in parallel with whole spores and mycelia extracts. About half of the patients tested were more reactive to spore-specific or whole-spore extracts than to the mycelia extract. This is because *Alternaria* spores and mycelium share common antigens and allergens, but spores appear to be more potent. By using RAST inhibition and quantitative immunoelectrophoretic techniques we have also compared spores and mycelia from *Alternaria alternata* (35).

The results indicated that the spore extract was representative of the mycelium extract with respect to total allergenic content. In contrast, the mycelium extract lacked, or had low concentrations of, some allergens represented in the spore preparation. Furthermore, we found that the mycelium extract contained about three times as much of Alt-1 (Alt a 1(36)) as the spore preparation. The spore extract appeared to be more heterogeneous and contained a higher number of both antigens and allergens than was present in the mycelium extract. Lehrer et al. (37) compared several Basidiomycete mycelia and spore-allergen extracts by skin testing in patients with respiratory allergy. Of the patients, 27% had positive skin reactions to the mycelia extracts, and 32% showed positive reactions to the basidiospore extracts. These results demonstrated that the spores were important fungal allergens in the New Orleans area.

It is apparent that the spores seem to be the most sensitizing part of the fungi, but that the mycelia contain most of the same allergenic molecules, as the spores are developed from the mycelium. Reference preparations as well as commercial extracts should therefore contain at least an adequate amount of spores.

STRAIN VARIABILITY

One of the most difficult problems associated with molds as source materials for allergen preparations or allergen solid phase is the selection of strains or isolates, because of strain variability. Several studies have been published indicating strong biochemical and

immunological differences in various molds: *Cladosporium* (8,38), *Alternaria* (39,40), *Aspergillus* (10,41), and *Candida* (42).

Variability of mold extracts, ranging from weak to strong reactivity against sera from patients with mold allergy, has been reported; these observations are most probably caused by antigenic variations in the preparations used. Furthermore, studies of batch variability have revealed great variations, most likely caused by a high rate of mutation (38), as there is a relatively long culture period for molds (up to several weeks). Considerable immunological differences were noted by Aukrust (43) when comparing two apparently identical *Cladosporium herbarum* extracts from two different *Cladosporium herbarum* sources. Later, these studies were extended to comprise 10 randomly chosen *Cladosporium herbarum* strains from American Type Culture Collection (ATCC) and Central Bureau Schimmel-cultures (CBS). These were grown under identical conditions, and extracts were prepared and investigated with a panel of different biochemical and immunological methods (8,44). The extracts varied considerably with respect to protein and carbohydrate content as well as the number of identifiable antigens in the CIE patterns. A variation in total allergenic activity, measured by RAST of 7–40%, was noted. However, most notable was the content of the major allergen Ag-32 (Cla h 1 (36)), which varied from 0 to 100% relative to a reference.

Biochemical and immunological variability was also documented between different strains (isolates) and different batches of *Alternaria* (39,45); five CBS and five ATCC strains were selected by Steringer et al. (39) and grown on completely synthetic culture medium under identical and standardized conditions. Significant antigenic and allergenic variations were noted between the individual strains as well as with respect to the content of the major allergen, Ag-8 (46).

A considerable variation in the content of Ag-8 was also observed for the *Alternaria alternata* strains cultivated and harvested three times under identical and standardized conditions during a period of three years. One strain varied from 0 to more than 200% relative to a reference with respect to the content of the major allergen Ag-8 (39).

Studies by Vijay et al. (47) showed similar protein patterns, but significant differences in RAST allergenic activity of four *Alternaria tenuis* isolates grown on revised tobacco medium. On the other hand, when comparing mycelial and broth extracts, the latter exhibited lower protein content and fewer protein bands in electrophoretic

analysis. Notably, RAST and passive cutaneous anaphylaxis (PCA) testing gave similar allergenic activity (40). In another study by Vijay et al. (48), three isolates of *Alternaria tenuis* were cultured on a chemically defined medium, and considerable biochemical differences were observed, but with extensive antigenic crossreactivity between the different isolates. RAST analysis showed that all isolates possessed high allergenic activity.

Strain variability has also been documented for different strains of *Aspergillus*, but in this case the variation was somewhat less pronounced than that of *Cladosporium*. Longbottom (41) studied four identically prepared batches of extracts of *Aspergillus fumigatus* and found considerable variation in both precipitation pattern and autoradiographic profiles. Malo et al. (49) used different *Aspergillus fumigatus* extracts when studying the prevalence of precipitin reaction in 200 asthmatic subjects. Because of the variation of the extracts (assessed by CIE analysis) the authors concluded that different extracts should be used to assess the presence of precipitins. Ten randomly chosen *Aspergillus fumigatus* strains from both ATCC and CBS were investigated by Wallenbeck et al. (10) using different biochemical and immunological methods. The strains were grown on completely synthetic medium and harvested under identical conditions. Protein and carbohydrate analyses indicated significant strain variations. Furthermore, the antigenic and allergenic activity studied by CIE/CRIE analysis and RAST revealed great strain variations. From these data the authors concluded that more than one isolate of *Aspergillus fumigatus* should be used to prepare a high-quality and representative extract, and this also appears to be valid for the other molds studied.

Recently the strain variability has also been studied for *Candida albicans* (42). Allergenic variation of six strains was studied by immunoblotting analysis. There was a significant difference in the content of the two dominating allergens in *Candida albicans*, the 46- and 19-kDa proteins (50). The different strains were grown on agar plates on completely synthetic medium and the studied extracts were prepared from disrupted cells (51). However, surface extracts of these six *Candida albicans* strains contained equal amounts of mannan, the main cell-wall component of the *Candida albicans*. Fingerprinting analysis of different *Candida albicans* strains by immunoblotting analysis also demonstrated distinct differences in the antigenic profiles (52).

MOLD ALLERGEN EXTRACTS

Conditions of Growth

The variable composition of mold extracts is mainly a result of the fact that the microorganisms have an enormous capacity to adapt themselves to altered conditions. The selection of strain and growth factors (temperature, oxygen supply, time, composition of the medium, and size of inoculum) are conditions that must be standardized to obtain a reproducible source material.

Mycological media commonly used for the growth of many important fungi are, e.g., malt extract agar, Sabouraud medium, potato dextrose, and rose bengal *Streptomycin* (53). Samples from stock cultures of strains of fungi imperfecti are inoculated into a suitable medium and incubated until a spore-and-mycelium mat is developed. When the preculture has developed, it is used to inoculate cultivation bottles containing sterile medium. If the agar plate, which is cultivated in parallel to the bottles, is acceptable, the cultivation is harvested and the mold mats are freeze-dried.

Several allergenically important fungi imperfecti can also be grown on completely synthetic media, which are free of macromolecules and thus eliminate the problems of separate fungal constituents from medium constituents (1). However, cultivation of *Aspergillus fumigatus* in a synthetic medium similar to Czapek-Dox results in a poor growth of *Aspergillus fumigatus* as a result of low consumption of such ingredients as glucose (54). By addition of 0.1% yeast extract an enhanced growth of the microorganism can be achieved. This indicates that the growth of *Aspergillus fumigatus* is markedly restricted when using synthetic medium without addition of essential growth factors.

Recently Van der Heide et al. (55,56) have used both a semi-synthetic and a completely synthetic medium for cultivation of four important fungi imperfecti: *Aspergillus fumigatus*, *Penicillium notatum*, *Alternaria alternata*, and *Cladosporium herbarum*. The fungi were grown on 2% malt agar and inoculated in liquid synthetic medium (Czapek-Dox broth) with or without yeast extract. Addition of yeast to the synthetic medium significantly improved the growth of all four fungi, but especially that of *Aspergillus fumigatus* and *Penicillium notatum*. Furthermore, the IgE binding capacity assayed by RAST was increased after the addition of yeast

extract. Cultures of three *Alternaria tenuis* isolates (from ATCC and Department of Agriculture Ottawa Mycology [DAOM]) were grown in flasks on synthetic medium, either revised tobacco medium or Czapek's medium (48). *Alternaria* extracts of these cultivated isolates were analyzed and differences in both biochemical and immunological properties were found. However, both synthetic media could be used for growth of selected *Alternaria tenuis* isolates, as extracts of high allergenic potency were obtained. Recently four new isolates of *Alternaria tenuis* grown on revised tobacco medium have been described, and the mycelial and broth extracts were compared (47). CRIE data showed 1–2 dominant and 3–4 minor allergens in the mycelial extracts, whereas the broth extracts had one dominant and one minor allergen. RAST inhibition gave a 100-fold higher allergenic activity in the two broth extracts, demonstrating the importance of the selection of cultivation media as well as selection of isolates.

Extraction and Techniques for Fractionation

Extracts of allergenic material routinely used in clinical allergy for purposes of diagnosis and therapy consist of crude aqueous extracts and, to a certain degree, purified extracts. Frequently the extracts are produced from insufficiently defined source materials; thus, these extracts lack well-defined allergens and, further, they have proved to show poor stability. The manufacturing of high-quality allergen extracts is dependent on the use of high-quality source materials containing all potential allergens. This is especially important with molds because of their variability and complexity. The extraction methods used should not denature the allergen or change the allergen composition. Consequently the extraction ought to be performed under physiological conditions (pH, buffer composition) and at low temperature (57). Extended extraction periods at room temperature should be avoided to minimize the destruction of allergens by proteolytic enzymes present in the mold extract. Nonallergenic low-molecular-weight substances and irrelevant components should be removed, keeping the native allergens intact. Purification can be accomplished by different biochemical methods, such as ultrafiltration, desalting, gel filtration, ion-exchange chromatography, or affinity chromatography (57). The allergenic activity of the fractionated mold extract should be checked by potency assays

(RAST inhibition and CRIE). Mold extracts are labile in aqueous solutions (especially *Cladosporium herbarum* (58)) and therefore must be stored under conditions that preserve the allergenic activity: addition of stabilizing agents, such as albumin or 50% glycerol; low temperature; freeze-drying, or combinations of these factors (57).

For the fractionation and characterization of mold allergenic extracts it is convenient to use any protein-separation method that does not change the native structure of the allergenic protein molecules (1,57). Furthermore, it is important that the separation procedures are followed by the use of assay techniques that determine the allergenic activity. Various gel-filtration media based on crosslinked dextran have been used routinely for size fractionation of allergenic extracts (21,57). Recently Rubio and Brieva (59) have described the use of high performance liquid chromatography (HPLC) (Protein Pak™ 125 column) for separation of toxic components and enzymes from crude extracts of *Alternaria tenuis*, *Aspergillus fumigatus*, and *Cladosporium herbarum*. The distribution of allergens was monitored by solid-phase radio immunoassay and RAST inhibition. The HPLC system used gave a 100% yield of allergens, but only a limited separation was obtained with *Alternaria tenuis* because of the heterogeneous distribution of the allergens in the chromatogram. One of the major allergens in *Cladosporium herbarum*, Ag-54, was purified by a combination of ultrafiltration, HPLC (Ultropac™ TSK-G3000 SW column), and isoelectric focusing (60,61). The *Cladosporium* allergens were found to be located in the molecular-weight region 10–30 kDa after the HPLC, and Ag-54 (25 kDa) could be located within this part of the chromatogram. Ion-exchange chromatography, the traditional method for separating macromolecules by charge, is useful as a second step in the purification scheme, after gel filtration (21,57,62). Reversed-phase chromatography (RPC) has not been applied before for mold studies. However, in Fig. 1, four different molds (*Penicillium notatum*, *Aspergillus fumigatus*, *Candida albicans*, and *Alternaria alternata*) have been analyzed by RPC. High resolution efficiencies are achieved using chemically bonded phases, μ RPC™ (Pharmacia LKB Biotechnology, Sweden) with the volatile trifluoroacetic acid (TFA) as ion suppressor, and a gradient of acetonitrile for desorption. The studied fungi are characterized by highly resolved patterns whose profiles (fingerprints) can be used to discriminate between different molds or for identification of unknown molds by comparison with a reference preparation.

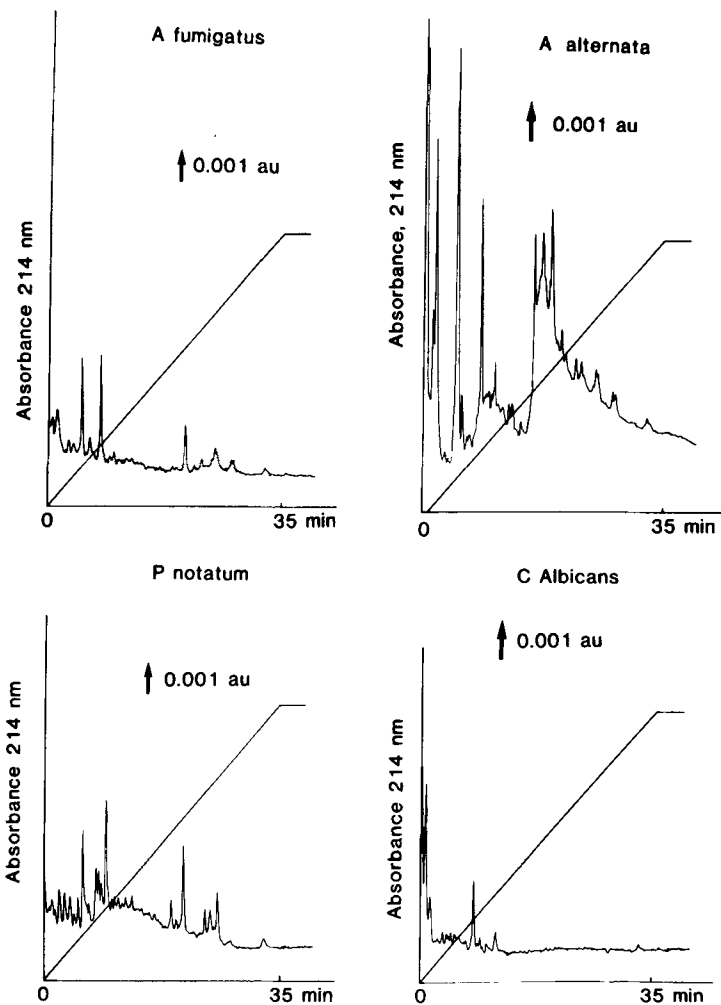


Fig 1. RPC separation of fungi; mold sample, 100 μ g in starting buffer; column, μ RPC (C_2/C_{18}) PC 3.2/3; flow rate, 0.3 mL/min; eluent A, 0.1% TFA in water, pH 2.0; eluent B, 0.1% TFA in acetonitrile–water (60/40); linear gradient from 2 to 60% B in 35 min, solid line; detection, absorbance at 214 nm.

Many other methods can be used for studying mold allergens; details of such methods (hydrophobic chromatography, metal chelate chromatography, chromatofocusing, affinity chromatography) can be found elsewhere (1,57). It is important to point out that isolation of individual allergens, as well as characterization of them, can be performed, as for protein in general, with the exemplified biochemical methods.

Standardization

Molds are extremely variable in their composition, and for that reason it is especially important to standardize mold-allergen extracts. It is a well-known fact that materials prepared for physicians to use in patients may vary greatly in both composition and potency from one supplier to another and from batch to batch. The purpose of allergen standardization is to provide reference preparations for the calibration of manufactured and experimental extracts. This allows the preparation of potent extracts with defined compositions for use for diagnosis and therapy (63). Various physicochemical methods have been applied for standardization of allergen preparations, such as wt/vol and protein nitrogen unit (PNU) determinations. However, these techniques give no information about the allergenic activity, and more relevant methods are required for standardization. Different concepts for standardization of allergenic extracts have been advanced by both allergen producers and regulatory agencies. Examples of these concepts are potency determination by skin testing using a parallel-line bioassay (64) or RAST inhibition; molecular characterization by CIE, SDS-PAGE, or IEF; semiquantitative determination of allergens by CRIE immunoblot; determination of concentrations of individual allergens in a mixture by ELISA or RIA; or any combination of these techniques.

Different regulatory agencies have taken different approaches and there are currently three systems of units: international units (IU) of the WHO; biological units (BU) of the Nordic guidelines, which are widely accepted in Europe; and the allergenic unit (AU) of the FDA (65). In order to avoid too much confusion regarding the selection of a standard preparation, the International Union of Immunological Societies (IUIS), in collaboration with allergen specialists, has prepared a number of international standards (66) to be used as calibrators, e.g., as standards for the estimation of relative potency. The development of an international standard is a two-step process that starts with a working group, after extensive studies of candidate extracts, proposing an international standard preparation. The second step is approval by WHO after an international collaborative study is performed to confirm that the proposed preparation meets a number of criteria; purity, activity, sterility, and stability. The allergen extract is assigned an arbitrary potency of 100,000 IU. The

international standards are available for several allergens (65) of which one is a mold, *Alternaria* spp. (67,68).

SPECIFIC MOLD ALLERGENS

Alternaria alternata

Alternaria alternata (*tenuis*) is considered one of the most important mold allergens in the United States (1). This mold is an extremely common species occurring on plants, soils, and food-stuffs; in house dust; and in atmospheric sampling, with an optimal growth at 25–26°C. Because of its importance, *Alternaria* is the most studied of mold allergens.

Numerous investigations have been published, wherein the complexity of the *Alternaria alternata* mycelia extracts have been discussed (69–71). Schumacher and Jeffery (72) have studied the variation between different *Alternaria* isolates; they found significant biochemical differences, but only small differences when using rabbit antibodies. In a more extensive investigation, Steringer et al. (39) have studied the variability of antigenicity and allergenicity of 10 different strains of *Alternaria alternata* by analyzing the protein and carbohydrate content and allergenic activity by RAST inhibition and CIE/CRIE. The protein content varied between 2.2 and 13% of dry wt, and carbohydrate content varied between 3.2 and 24%. Thirty-two antigens were identified in the CIE/CRIE pattern, including one major allergen, seven intermediate, and six minor allergens. The major allergen Ag-8 (partially identical to Alt-1 (73)) varied more than 200% during three separate cultivations.

The major allergen of *Alternaria alternata*, Alt-1, was first isolated by Yunginger et al. (67,74). Alt-1 is a heterogeneous glycoprotein with a mol wt of 30 kDa and mostly exists in 12 immunologically slightly different forms with isoelectric points from 4.0 to 4.5. Allergens with properties very similar to those of Alt-1 have been reported by Nyholm et al. (73) as Ag 1 and by Aukrust et al. (39,46,73) as Ag-8. Antigen 8 was studied by CIE/CRIE and fused-rocket immunoelectrophoresis, and three molecular variants could be demonstrated with isoelectric points from 4.30 to 4.65. Budd et al. (75) have described the isolation of a basic peptide allergen with a mol wt <14 kDa and an isoelectric point of 9.5–9.8. By using RAST, Hoffman (1) was able to confirm the existence of allergen activity in the basic fraction of *Alternaria* mycelial and spore extracts.

***Aspergillus* spp.**

The genus *Aspergillus* is very common in nature, and many species have been described in relation to their pathogenicity (44). Many of the *Aspergillus* species are thermotolerant and grow over a wide temperature range, 31–54°C, with an optimum between 37 and 43°C; other thermophilic forms grow best between 65 and 75°C (76–78). *Aspergillus* spp. are increasingly recognized as a cause of various pathological conditions in humans by the inhalation of conidia and mycelium. The effect of the fungus occurs mainly via growth of the fungus, which invades different organs, and by production of various mycotoxins.

Aspergillus fumigatus is the most virulent species involved in such airway diseases as allergic asthma, aspergilloma, hypersensitivity pneumonitis, invasive aspergillosis, and allergic bronchopulmonary aspergillosis (ABPA). *Aspergillus* species have also been found to cause farmer's lung, baker's asthma, and malt-worker's disease (78). Extracts of various species of *Aspergillus* have been studied in order to allow production of effective diagnostic reagents for different diseases and also to control mycotoxin production (40). Much work has previously been done to study the humoral and cellular response of patients suffering from various *Aspergilli*-induced diseases, and those studies are discussed in several papers (1,44,79).

Recently Wallenbeck et al. (10) studied the antigenic and allergenic composition of *Aspergillus fumigatus* extracts (dried mycelium containing 10% spores) by using different biochemical and immunological methods. Two clinically important allergens, Ag-10 and Ag-40, were identified by CRIE analysis of 26 individuals with suspected allergy to *Aspergillus fumigatus*. Longbottom (80,81) has isolated a major allergen from *Aspergillus fumigatus* (Ag-3, Asp f 1 (34)) and this allergen had a mol wt of 24 kDa and pI 4.5. Further, this protein molecule was extremely heat-labile and contained no sugars. Borgå (82) have recently found by CRIE analysis that Ag-10, identified by Wallenbeck et al. (10), is also a variant of Ag-3. Three other allergens (Ag-5, Ag-7, and Ag-13) have been characterized and partly purified from *Aspergillus fumigatus* (83,84). Ag-7 and Ag-13 (mol wt 150–200 and 70 kDa, respectively) were isolated from the cytoplasmic fraction by concanavalin A chromatography. Both allergens were important for patients with ABPA and aspergilloma. Ag-5 was both antigenic and allergenic with

aspergilloma patients, did not bind to concanavalin A, and has a mol wt of 35 kDa. Leung et al. (85) examined mycelium and spore extracts of *Aspergillus fumigatus* in ABPA patients by dotblot and immunoblotting. All patients showed IgE and IgG binding when probed with the mycelium extract, and the IgE binding was pronounced with a 70 kDa protein. On the other hand, very few specific antibodies against *Aspergillus fumigatus* could be detected using the spore extract. Subclass analysis revealed that 90% of ABPA sera contained IgG2 and 7% contained IgG4, whereas IgG1 and IgG3 were less frequent (85).

Immunoblot analysis was used by Baur et al. (86) for screening of patients with ABPA in different stages. They found that patients in active stage showed a strong IgE response against *Aspergillus fumigatus* components, in contrast to corticosteroid-treated patients, who demonstrated weaker IgE binding. Monoclonal antibodies against *Aspergillus fumigatus* antigens showed, in Western blot analysis, binding to low-molecular-weight components in *Aspergillus fumigatus* (87). These components were found to bind IgG and IgE antibodies in sera of ABPA patients, but not in sera from aspergilloma patients.

Cladosporium spp.

Cladosporium is one of the most frequently found airborne fungal spores in the air in temperate zones of the world (88,89). The conidia of *Cladosporium* can be detected in extremely large numbers in the air; more than 35,000 conidia/m³ were measured over Leiden (West Germany). Several species are important allergens, but only *Cladosporium herbarum* has been thoroughly studied. The allergen composition of *Cladosporium herbarum* has been studied by the use of CIE/CRIE analysis, demonstrating approx 60 antigens. By CRIE analysis using a panel of allergic patient sera, one major, 10 intermediate, and 25 minor allergens were identified (43). Aukrust and Borch (58,90) have isolated two allergens from *Cladosporium herbarum* (dried mycelium): Antigen 32 (Cla h 1 (34)) and Antigen 54 (Cla h II). The molecular weight of the major allergen Ag-32 was approx 13 kDa and the protein appeared as five immunochemically identical molecule variants with isoelectric points of 3.4–4.4. An intermediate allergen, Ag-54 with a mol wt of approx 25 kDa and an isoelectric point of 5.0, was also isolated and found to

be a glycoprotein containing 80.2% carbohydrate and 19.8% protein (61). Both allergens vary considerably from strain to strain (8). Hoffman (1) has examined the presence of Ag-32 and Ag-54 in spores and mycelium of *Cladosporium herbarum* (California isolate), *Cladosporium cladosporides*, and *Cladosporium tenuisum*. Spores from *Cladosporium cladosporides* and *Cladosporium tenuisum* contained a large amount of Ag-32, significantly more than the mycelium extracts. Only traces of Ag-54 could be found in the spore and mycelium extracts. On the other hand, in a multicenter immunotherapy trial in 30 children in Scandinavia, using a purified and standardized *Cladosporium herbarum* preparation (dried mycelium containing 1% spores), CRIE analysis demonstrated specific IgE binding against Ag-12 (partially identical to Ag-54) in all patients prior to active treatment (91).

The allergens in *Cladosporium herbarum* were shown to be extremely heat-labile, most likely because of the presence of proteolytic enzymes (58). The stability of *Cladosporium herbarum* was also tested by CIE/CRIE analysis and immunoblotting after irradiation to kill the mold, and we found that the number of IgE-binding components decreased markedly, as did the intensity of the radio staining. Another interesting study was performed by Swärd-Nordmo et al. (62), who examined the effect of oxidation of the carbohydrate epitope in relation to specific IgE-binding to a partly purified (diafiltered source material, cutoff 5 kDa) allergen preparation of *Cladosporium herbarum*. Periodate oxidation significantly decreased the IgE binding capacity.

Candida albicans

The genus *Candida* consists of at least 150 different yeast species, of which *Candida albicans* is the most important. *Candida albicans* is a dimorphic fungus, which grows well under different conditions: on agar plates; in serum; or on synthetic medium containing ammonium salts, glucose, phosphate, and biotin (92). The structure of *Candida albicans* is well studied and the cell wall contains a wide variety of proteins, mannan, glucan, and chitin polysaccharides (93,94). The mannose-specific lectin concanavalin A binds to the cell surface, indicating the presence of mannan structures (95). By using monoclonal antibodies for probing the antigenic determinants of the cell surface, up to ten different mannan structures

have been proposed (96–98). Extracts of *Candida albicans* studied in vivo by skin testing, were found to possess allergenic activity, especially fractions with high polysaccharide content (99). Mannan and protein fractions purified from *Candida albicans* elicited immediate reactions in guinea pigs in PCA testing (100); and this observation was also confirmed by Edge and Pepys (101) using RAST testing.

Other reports indicating correlation among the immediate-skin-reaction test, bronchial-challenge test, and RAST to *Candida albicans* have also been reported (102). However, problems still exist regarding the allergenicity of *Candida albicans* because of the ubiquity of this organism. By immunoblot analysis 16 allergenic components of *Candida albicans* were identified in sera from asthmatic patients with positive skin test and RAST to the microorganism (103). The IgE binding patterns were heterogeneous and the allergens identified showed mol wt ranging from 20 to 94 kDa. Studying the allergenic composition of *Candida albicans* by the use of immunoblotting analysis, Savolainen (104) has identified more than 30 IgE-binding components in *Candida albicans*. Antigens with mol wt of 46 and 29 kDa, as well as a high-mol-wt mannan–polysaccharide fraction (about 70 kDa) were found to be the most important allergens (42,105). Stability studies of *Candida albicans* allergens indicated that the 46-kDa allergen and the mannan were labile and rapidly lost their allergenicity during storage. However, this effect could be prevented by adding 10–50% glycerol to the allergen solution (106).

OTHER MOLD GENERA

Several other molds of fungi imperfecti have been observed to cause allergy. These molds have been skin-tested in patients with rhinitis and/or asthma, and positive reactions have been found. However, most of these mold extracts have not been purified or standardized, nor have their antigenic and allergenic composition been described. Because of the lack of defined extracts, there is still a great debate about which molds are the most relevant after *Alternaria*, *Cladosporium*, and *Aspergillus*. Some molds might be clinically important, as spores of *Botrytis*, *Epicoccum*, and *Helminthosporium* occur in close human environments and in such concentrations that symptoms may be provoked (107). Positive skin reactions have been

observed in mold-sensitive patients with *Bortrytis* (108), as have positive RAST (14). *Stemphylium*, together with *Alternaria* and *Cladosporium*, is one of the most important allergens in United States; this mold has also been found to cause ABPA (109). *Mucor* and *Penicillium* have, in many different clinical studies, produced positive skin-test reactions in allergic individuals, but very little is known about their composition (30,107,108). Antibodies against *Rhizopus* have been detected in patients with malt-worker's disease (110), and it is also considered an important allergen in wood-trimmer's disease (111). Crossed-line immunoelectrophoresis and RAST-inhibition studies of *Fusarium solani* demonstrated that *Fusarium solani* shares several common antigenic and allergenic determinants with both *Penicillium* and *Aspergillus* (112).

Crossreactivity of Molds

Antigenic and allergenic relationships among the fungi imperfecti are not well defined. However it is well known that mold-allergic individuals frequently demonstrate sensitivity to various mold species. This observation has led to the assumption that fungus crossreactivity is much like pollen crossreactivity. The term crossreactivity is seldom defined, but is used to express similarities of various species with respect to reactions in the human IgE system. However, crossreactivity between different fungi may also be found in IgG systems, as demonstrated with animal sera.

The degree of IgE crossreactivity between two species will depend on the number and relative importance of the individual components that crossreact. RAST-inhibition, CRIE, and immunoblot techniques are probably the best methods for studying allergenic crossreactivity. For investigation of the crossreactivity in IgG systems immunoprecipitating techniques, such as CIE and rocket immunoelectrophoresis, can be used. Even if a panel of different methods are used it may be difficult to prove that an individual mold extract is fully representative of the known clinically relevant allergens of a particular species.

Using RAST inhibition, Karr et al. (113) found some degree of crossreactivity between different species of *Aspergillus fumigatus*, *Aspergillus glaucus*, and *Aspergillus flavus*, but not between different genera, such as *Aspergillus fumigatus* and *Alternaria alternata*. Salvaggio and Aukrust (44) could identify by CIE analysis a single

antigen shared by *Alternaria alternata* and *Cladosporium herbarum*. In a similar experiment, no crossreactivity was found between the two Basidiomycetes *Cantharellus cibarius* and *Coprinus comatus*. Significant IgE crossreactivity between different genera was detected by Hoffman and Kozak (114) when studying *Alternaria*, *Stemphylium*, and *Ulocladium*. Even *Spondylocladium* and *Dresclera* showed a certain degree of allergenic crossreactivity.

Great similarities between the morphologically related genera *Ulocladium* and *Alternaria alternata* were also demonstrated by studying the presence of Alt-I in several different strains (115). By skin testing and RAST inhibition, Agarwal et al. (116) showed that *Alternaria* and *Stemphylium* contained multiple shared antigenic determinants. A double-antibody radioimmunoassay for Alt-I indicated higher Alt-I activity in *Stemphylium* than in *Alternaria* and, furthermore, double-immunodiffusion studies with rabbit anti-Alt-I antibodies gave immunoprecipitates of identity with Alt-I for both *Stemphylium* and *Alternaria* (116). Tee et al. (117) studied crossreactivity between different mold genera by RAST-inhibition and immunoblot techniques, and they found little or no crossreactivity by using *Aspergillus* RAST and *Cladosporium* or *Alternaria* extracts. However, considerable IgE crossreactivity was found between *Alternaria* and *Cladosporium* using *Cladosporium* or *Alternaria* RAST disks. The antigenic and allergenic relationship among several fungi imperfecti were further evaluated using skin-prick testing, RAST inhibition, and immunoelectrophoretic methods (112,118). These studies suggest that *Fusarium solani*, *Penicillium notatum*, and *Aspergillus glaucus* share several antigenic and allergenic determinants. Different species of the same genus may show similarities as well as differences. This has been shown by Hoffman (1) for *Cladosporium herbarum* and *Cladosporium cladosporides*, by Kim and Chaparas (119) studying several *Aspergilli*, and by Vijay et al. (120), who studied *Alternaria alternata* and *Alternaria solani*. Furthermore, various strains of each species showed similarities, but also considerable differences.

Salvaggio et al. (44) and Aukrust et al. (90) demonstrated that 10 different strains of *Cladosporium herbarum* showed pronounced immunological and biochemical differences.

Similar differences among 10 different *Alternaria alternata* isolates and *Aspergillus fumigatus* strains were shown by Steringer et al. (39) and Wallenbeck et al. (10) using a panel of biochemical and

immunological techniques. It is notable that in some cases the immunological and biochemical differences may be greater among mold strains of the same species (10,39,90) than among genera, such as *Alternaria*, *Stemphylium*, and *Ulocladium* (121). A great number of fungal variants exist in nature; some of them show extensive similarities, whereas others represent completely different allergen variants. The degree of IgE crossreactivity between them must be expected to be variable, and multiple sensitivities to several molds may occur; this phenomenon is most likely explained as multiple independent and parallel allergies.

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