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The Allergens of *Cladosporium herbarum* and *Alternaria alternata*

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The two mold species *Cladosporium herbarum* and *Alternaria alternata* are important causes of allergies. Before 1990 little was known about the relevant allergens of these two worldwide-occurring fungi.

Both molds are important sources of allergens for mold-allergic patients (perhaps after *Aspergillus fumigatus* [1]) and are not pathogens except, as reported in recent years, for a minority of immuno-compromised patients [2–4]. However, *A. alternata* can be cultured from hypersensitivity pneumonitis [5]. A question that is largely unanswered even today is: What is the most important way of sensitization of the patients? Several possibilities come to mind, among them inhalation of dried mycelia in house dust or inhalation of spores from outdoor or indoor sources. It is well known that the spores of the two molds are common in indoor and outdoor air [6]. The molds grow in soil, on decaying plant material and as plant pathogens. Recently it was found that both *C. herbarum* and *A. alternata* are also common plant endophytes. They grow within the extracellular space of plants without causing disease [H.J. Prillinger, pers. commun.].

Most of the allergens identified so far from the two molds are intracellular housekeeping proteins. Notable exceptions usually are the major allergens, which in the case of *A. alternata* and *A. fumigatus* are secreted proteins. It is an open question how the intracellular proteins are presented to the immune system. The spores of the two molds discussed here are too large to reach the alveoli of the lung, but they may still be important inhalant allergens. However, none of the allergens identified so far are spore specific.

What is further missing are longitudinal studies on children allergic to *A. alternata* and *C. herbarum*, epidemiologic studies and a sufficient number of well-documented case histories to draw conclusions about the ‘typical’ symptoms and time course of the mold-allergic patient.

Composting and mushroom-growing facilities have been suspected widely to be dangerous to the workers exposed to the emanating mold spores and 'organic dust'. There seems to be a severe health risk of developing [7–10] allergic bronchopulmonary aspergillosis (ABPA) and other forms of pulmonary complications only in asthmatic and atopic patients, but these are mainly due to thermophilic actinomycetes and *A. fumigatus*. *C. herbarum* and *A. alternata* do occur in compost, however, in much smaller numbers than the species just mentioned.

The incidence of *C. herbarum* and *A. alternata* sensitization varies in the different climatic zones of the world. In a European multicenter study [11] it was demonstrated that 3–20% of all allergic patients tested showed positive responses to *A. alternata* and/or *C. herbarum* in skin prick tests (SPT). A survey done in Israel [12] revealed that 3.3 and 12% of the allergic patients had positive SPT responses to *C. herbarum* and *A. alternata*, respectively. In Austria the number of patients with positive SPT responses to the two molds can be estimated as follows: in a large outpatient clinic, from where all sera used for the investigations of the authors [13–16] of the present review were obtained, about 15,000 new patients are diagnosed as allergics every year. About 3% of those patients usually show positive SPT and serum radioallergosorbent test (RAST) responses to a 'mold mix'. Considering the low quality of the commercial mold extracts, the number may be underestimated, as assumed for all fungal allergies [1]. About 30% of these mold allergics proved to be sensitized to *A. alternata* and/or *C. herbarum*, as shown by 'patient blots' (IgE-specific immunoblots of mold extract) performed in our laboratory. Our own mold extracts usually produced many more bands and contained more undegraded allergens than commercially available mold extracts. The patients were rarely sensitized to just one mold species but in most cases to several mold species. One reason for this might be the presence of cross-reactive phylogenetically conserved allergens (discussed later in this chapter).

Problems with Reproducibility of Mold Extracts and the Study of Mold Allergens

We will now discuss some of the reasons why mold allergens are more difficult to standardize than other aeroallergens such as pollen allergens and why some of the commercially available extracts are very poor when analyzed for the presence of major allergens. These commercial extracts were also inferior to pure recombinant allergens when compared in clinical tests. Methods developed in our laboratory for optimal production and extraction of mold allergens will be described.

It is difficult to unequivocally identify closely related species and strains by classical morphological criteria. Little is known about genetic variation below the species level in these organisms. No sexual forms (ascospores) of these molds are known, therefore identification on purely morphological criteria is difficult. The strains used by us were well-characterized strains from a major European strain collection (Institut für Gärungsgewerbe, Berlin, Germany). Identification was based on morphology and molecular criteria (rDNA sequences; see Prillinger et al. [17, this volume]). It is unknown at the moment how large intra species genetic variation is in *C. herbarum* and *A. alternata*. The Alt a 1 cDNA sequences isolated from cDNA libraries which originated from European and North American strains showed minor sequence differences at the nucleotide level but no sequence differences at the amino acid level [18] (also see ‘The major allergen of *A. alternata*, Alt a 1’ below).

The total number of relevant allergens is larger in molds than in pollens and foodstuffs. The total number of IgE-reactive allergens from *A. fumigatus* may be as high as 100 [19, this volume]; in *C. herbarum* so far at least 36 allergens have been cloned (also see ‘Other Allergens’ below). This large number of partly comigrating proteins in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) makes it nearly impossible to unequivocally identify a protein band in a so-called patient blot, particularly in the range of 30–60 kD. This difficulty can only be overcome using pure recombinant or highly purified natural allergens. However, based on data to be presented later it is likely that a small number of purified recombinant allergens of the two molds are sufficient to correctly diagnose patients sensitized to the two molds with a sensitivity and specificity superior to those obtained with commercial extracts.

Growth conditions: The presence of specific allergens (including the major allergens!) depends very much on the growth conditions. For instance, we showed that when growing the molds on petri dishes containing complex medium, allergen content was optimal on day 5, at the beginning of extensive formation of conidiospores as tested by immunoblots. Complex medium contained 1% yeast extract, 2% peptone and 2% glucose solidified with 2% agar and supported vigorous growth of *C. herbarum* and *A. alternata*. Growing the molds in liquid medium with shaking or stirring resulted in much lower yields of allergens. It is well known that for industrial purposes and for the production of commercially available mold extracts, the fungi are batch grown for different periods of time in liquid tanks. This might partly explain the poor properties of these extracts.

Protein extraction methods are critical. In previous studies various extraction procedures have been tested. Paris et al. [20] could show that after breaking *A. alternata* cells, the use of carbonate buffer supplemented with protease inhibitors (phenylmethylsulfonyl fluoride) and phenol-binding components

(polyvinylpyrrolidone) resulted in better extracts than with the use of Coca's solution containing sodium carbonate and phenol. Portnoy et al. [21] tested four different buffers and various extraction times in order to evaluate their effects on the protein composition and content of the respective *A. alternata* extracts. They could not determine an optimal extraction time valid for every allergen. With respect to the different extraction buffers they could not detect major differences between the buffers tested, except that low pH resulted in a lower yield.

In our laboratory the following method for allergen extraction was developed. The fungal 'mat' was carefully removed from the surface of the petri dish after 5 days of growth and immediately put into a mortar filled with liquid nitrogen. The frozen material was ground under liquid nitrogen and the fine powder was extracted with extraction buffer containing a mixture of protease inhibitors and centrifuged at 4 °C [13]. Aliquots of the clear supernatant were stored at -70 °C. The use of these extraction methods allowed to generate extracts showing the maximum number and intensity of IgE-binding bands in immunoblots (fig. 1, 2).

Experience with Specific Immunotherapy in the Treatment of Mold Allergies

Specific immunotherapy is defined as the administration of increasing doses of an allergen extract to an allergic patient suffering from IgE-mediated hypersensitivity. As a prerequisite the allergy has to be determined by an SPT or an in vitro test like the RAST. Several questions arise in connection with immunotherapy of mold allergy:

Which in vivo or in vitro tests yield the most reliable results in the diagnosis of fungal allergy?

How can the problem of variability of fungal extracts be overcome?

Is it possible and/or necessary to reduce the frequency of systemic side effects after specific immunotherapy in order to reduce the patients' symptoms?

In a placebo-controlled double-blind study [22] it was demonstrated that a negative SPT response to *C. herbarum* was always correlated with the absence of an allergy. In contrast a positive RAST always indicated clinical allergy; it was thus concluded that a combination of these two tests would result in 100% agreement with a positive clinical diagnosis. In this high-dose immunotherapy study 81% of the group hyposensitized with *C. herbarum* extract showed an improvement of their clinical symptoms, whereas 19% of the treated patients exhibited deterioration. In the untreated control group 73% of the patients showed aggravation of the symptoms whereas 27% improved. In a double-blind placebo-controlled study [23] 30 *Cladosporium*-allergic children suffering from asthma or

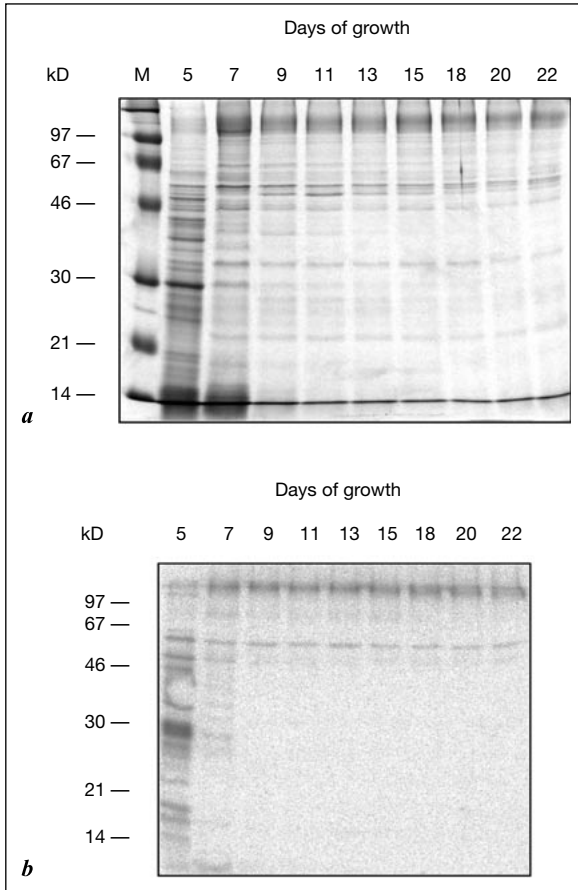


Fig. 1. Time course of allergen expression in *C. herbarum*. **a** Extracts were prepared on the days indicated after growth at 28 °C on solid YPD. Fungal proteins were separated by SDS-PAGE and Coomassie stained. Equal amounts of fungal material were used for every experiment. Protein bands between 14 and 21 kD, but also Cla h1 at 30 kD were most strongly expressed at day 5. **b** Aliquots of the same samples that were used in **a** were separated by SDS-PAGE under the same conditions, blotted onto Immobilon-P membrane and decorated with a serum pool of *C. herbarum*-allergic patients. Again it is shown that the region corresponding to Cla h1 is most strongly stained on day 5 extracts.

rhinoconjunctivitis and showing a positive SPT and RAST were studied. The authors observed an increase in tolerance to conjunctival or bronchial challenge in the 16 treated patients. Cantani et al. [24] reported the effects of *Alternaria* immunotherapy on asthma and rhinitis in 79 children in a 3-year study. They could show that immunotherapy was successful in 80% of the children sensitized

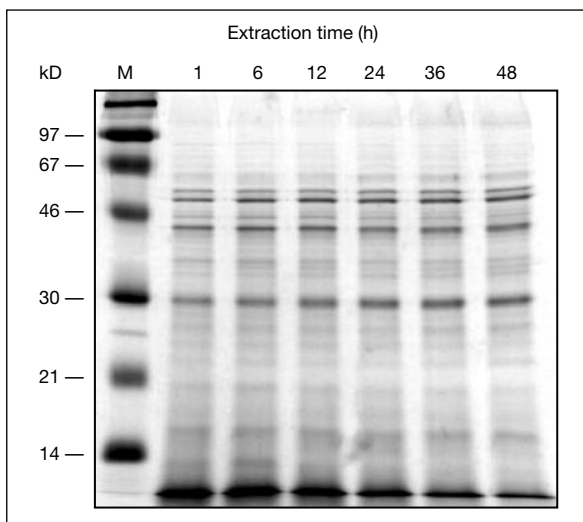


Fig. 2. Time course of protein extraction from *C. herbarum* cells. *C. herbarum* was grown as described in figure 1, harvested on day 5, treated with extraction buffer for varying times and the protein extracts were separated by SDS-PAGE. Routine extraction was done overnight.

to *Alternaria* with doses above 80,000 protein nitrogen units (PNU). In another double-blind placebo-controlled study [25] a ‘rush’ protocol was carried out with a standardized *Alternaria* extract. The patients actively treated (13 out of 24) and exclusively sensitized to *Alternaria* benefited from specific immunotherapy. The efficacy was determined by patients’ self-evaluation, global symptom-medication scores and nasal challenge tests. At the antibody level an unchanged specific IgE level and an increased specific IgG level were detected.

Immunotherapy with fungal extracts has often been associated with a high frequency of systemic side effects. It was shown that high doses of *Cladosporium* extract (top doses of 100,000 biological units) resulted in an improved therapeutic effect [22]. According to preliminary data from Norman and Lichtenstein [26], the elicitation of mild side effects was even intended and considered to indicate that this would result in increased clinical efficacy. Tuchinda and Chai [27] reported similar observations for *Alternaria*.

Although several studies about mold immunotherapy have been performed and have shown clinical efficiency [22–25], the number of patients included in the respective studies was rather small emphasizing the need for further studies.

One major problem in mold immunotherapy is the lack of reliable fungal extracts [28–30]. In the last years, molecular biology has considerably contributed to the solution of this problem. Several allergens of *C. herbarum* and *A. alternata*

have been identified by cloning techniques [13, 14, 31, 32]. Using recombinant allergens for skin tests Unger et al. [33] demonstrated that recombinant Alt a 1 and Alt a 5 (enolase) achieved a higher specificity and sensitivity than commercial extracts. Comparing specific allergen levels in mold extracts, Vailes et al. [33] used recombinant Alt a 1 (rAlt a 1) (Biomay, Vienna, Austria) as internal standard for the determination of the Alt a 1 content in the different commercial extracts. As a prerequisite Vailes et al. developed an Alt a 1-specific enzyme-linked immunosorbent assay (ELISA) with a monoclonal antibody directed to natural Alt a 1. ELISA dose response curves showed immunological equivalence of recombinant (rAlt a 1) and natural Alt a 1 (nAlt a 1). This result suggests that the biological properties of rAlt a 1 and nAlt a 1 are comparable. The rAlt a 1 used in this study may be suitable for immunotherapy. The use of recombinant allergens for immunotherapy instead of natural mold extracts is desirable as it was shown by Birkner et al. [35] that de novo sensitization after an immunotherapy with crude pollen extract can occur. An improvement could be achieved by injection of only those allergens against which the patient is sensitized. Therefore the use of recombinant proteins for the diagnosis and therapy of mold allergy seems desirable.

Importance of Molecular Cloning Techniques

As the importance of molecular cloning techniques for research on mold allergens has been reviewed several times [36–38], we will not deal with it extensively here. However, it is clear from the facts just mentioned that the importance of cloning methods can hardly be overestimated, especially in the field of mold allergy. Speaking about diagnostics, the allergogram of a patient often becomes clear only after pure recombinant allergens have been used. Immunotherapy with mold extracts has not been widely used in the past because of inherent problems and dangers related to commercially available mold extracts, but could be done with the well-characterized pure recombinant mold allergens that are available now.

Cloning, Analysis, Production and Clinical Testing of the Allergens of *Cladosporium* and *Alternaria*

Cloning Methods

In our first attempts to clone the most important allergens of the two molds, cDNA libraries were prepared in phage vectors and standard immunological