

Production profile of the major allergen Alt a 1 in *Alternaria alternata* cultures

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Background: The fungus *Alternaria* is strongly associated with asthma, but the importance of fungal allergen products is frequently underestimated. The profile of allergen release from fungal material is poorly understood.

Objective: To investigate expression of the major allergen of *Alternaria alternata*, Alt a 1, during its growth in culture conditions for allergen extract production.

Methods: Allergen expression was examined by Alt a 1-specific 2-site monoclonal antibody enzyme-linked immunosorbent assay, immunoblotting, and potency assays. The release of Alt a 1 was studied by transmission electron microscopy in conjunction with immunogold staining by using antibodies with specificity for Alt a 1.

Results: A maximum amount of Alt a 1 was obtained after 4.5 weeks of growing, and it was found predominantly in the spent culture medium. In the same way, total IgE binding activity showed 15-fold more activity in the spent culture medium than in the buffer-extractable antigen fraction. Immunogold electron microscopy provided evidence that Alt a 1 is released from spores and mycelia.

Conclusions: *Alternaria alternata* allergenic proteins were constantly released into the culture medium, where they accumulated. Alt a 1 was a good marker for checking optimal culture conditions for *A alternata* extract production intended for clinical use.

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INTRODUCTION

Fungal spores (live and dead) and fungal debris are common air components indoors and outdoors and are recognized as an important cause of respiratory allergies. Many fungi are capable of causing IgE-mediated sensitization in humans. However, the most predominant fungi implicated in allergy belong to the genera *Aspergillus*, *Cladosporium*, *Penicillium*, and *Alternaria*.¹

Allergens from fungi cause sensitization in up to 80% of asthmatic patients in the United States² and in approximately 20% of Spanish allergic patients.³ *Alternaria alternata* is considered to be one of the most important fungi to cause fungal allergic disease in the United States, and sensitization to *Alternaria* allergens has been proven to be associated with asthma.^{4,5} Alt a 1 is the major allergen in *A alternata* extracts, reacting with serum IgE in 82% to 100% of *A alternata*-sensitized patients.^{6,7} The allergen is a dimer with a molecular mass of 29 kDa that dissociates into 14.5- and 16-kDa subunits under reducing conditions.⁶ The presence of Alt a 1 has been described in fungal mats and culture filtrates,⁸ and its release has been shown to increase during spore germination.⁹

Alternaria is a complex species that exhibits significant interstrain differences in morphologic features and allergen content when it grows under various conditions. Of the numerous causes of variability, the strain, the source material

(spores or mycelia), and the extraction procedures are important in the preparation of fungal allergens.¹⁰ In pollens, allergens are present in the wall and cytoplasm and are instantly released on contact with the mucosa or with the extraction buffer.¹¹ In the case of *Alternaria*, there are no clear data about the location of allergens in the cell and the optimal extraction procedure. Identifying when and where *Alternaria* allergens are produced may lead to further understanding of how patients are sensitized to *Alternaria* allergens and thus may help develop more control strategies for their mitigation. Herein, we studied the expression of Alt a 1 in extracts obtained from different culture fractions during the growing of *A alternata*.

MATERIALS AND METHODS

Production of A alternata Cultures

Alternaria alternata (strain CBS 306.78) was grown on Czapek broth media at 25° C for 1, 2, 3, 4.5, or 5.5 weeks in static culture. Fungal extracts were obtained according to the methods of Martínez et al.¹² Briefly, the spent culture medium filtrate (CF) was separated by filtration through an AP filter (Millipore, Bedford, MA). A buffer-extractable antigen (BEA) fraction was obtained from the mycelium after 24 hours of extraction with 0.125 M bicarbonate buffer (pH 7.5) at 4° C. The mycelium was collected by centrifugation at 12,000g for 30 minutes and was homogenized in water using a Virtis-Tempest 1Q (VirTis Inc, Gardiner, NY), followed by sonication. After centrifugation for removing cell debris, the supernatant was named cellular antigen (CA). All fractions were dialyzed through a 7,000-Da cutoff membrane and lyophilized. Protein content was determined by using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA).

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Quantification of Alt a 1 by a 2-Site Sandwich Enzyme-Linked Immunosorbent Assay

Quantification of Alt a 1 was performed as described by Asturias et al.¹³ using natural Alt a 1 as a reference. Briefly, microtiter plates (Greiner, Frickenhausen, Germany) were coated with monoclonal antibody 1D6 (5 µg/mL in phosphate-buffered saline) overnight at room temperature. After blocking, they were incubated with the sample for 1 hour at 37° C. After washing, biotin-labeled rabbit anti-Alt a 1 serum was added, and the plates were incubated for 1 hour at 37° C. After further washes, bound antibodies were detected by incubation with streptavidin-peroxidase for 1 hour at 37° C. Plates were developed with freshly prepared *o*-phenylenediamine (Sigma-Aldrich Corp, St. Louis, MO), and after 30 minutes, optical density was measured at 492 nm.

Electrophoresis and Immunoblotting

Alternaria alternata extracts were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis under nonreducing conditions using the method of Laemmli.¹⁴ Proteins were transferred electrophoretically to polyvinylene difluoride membranes (Hybond P; Amersham Biosciences, Uppsala, Sweden). After blocking, membranes were incubated for 1 hour at 37° C with rabbit anti-Alt a 1 serum (1:100,000 dilution) or a serum pool of *Alternaria*-allergic patients (1:10 dilution); then, washed and bound antibodies were detected with 1:10,000 horseradish peroxidase–conjugated anti-rabbit immunoglobulins or anti-human IgE (Dako, Copenhagen, Denmark). Afterward, blots were washed and developed using the chemiluminescence Western blotting detection system (Amersham Biosciences).

Enzyme Allergosorbent Test Inhibition

The allergenic activity of *A alternata* culture fractions were measured by EAST inhibition using cyanogen bromide–activated paper disks coupled to a biologically standardized *A alternata* reference extract following the procedure described by Ceska et al.¹⁵ Bound IgE was determined using the HyTec specific IgE enzyme immunoassay test (Hycor Biomedical Inc, Kassel, Germany) as described by the manufacturer. Allergenic activity, in arbitrary units, was calculated on the basis of the 50% inhibition values.

Immunocytochemical Localization of Alt a 1

For transmission electron microscopy, freezing and conventional protocols with 2 resins, Spurr and Lowicryl K4M, were used. Fungal mats were previously inspected by light microscopy to ensure the presence of spores and mycelia in various developmental stages. Spores and mycelia were chemically fixed overnight at 4° C with a mixture of 4% *p*-formaldehyde and 0.2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3). For the freezing procedure, after washing with 0.1 M cacodylate buffer (pH 7.3) containing 50 mmol/L glycine, cells were embedded in 10% gelatin and infused in 2.3 M sucrose in 0.1 M cacodylate buffer (pH 7.3).¹⁶ Mounted gelatin blocks were frozen in liquid propane (Leica EM CPC, Vienna, Austria) and stored in liquid nitrogen. Thin cryosections were prepared in an ultracryomicrotome (Leica) and collected with 2% methylcellu-

lose, 2.3 M sucrose in 0.1 M cacodylate buffer.¹⁷ For the conventional procedure, the samples were washed in 0.1 M cacodylate buffer (pH 7.3), followed by dehydration in a methanol series and embedding in Lowicryl K4M and Spurr resins. At the end, samples were sectioned using an ultramicrotome (Ultracut E; Reichert-Jung, Heidelberg, Germany), and sections were mounted on gold grids. Both cryosections and conventional sections were immunogold labeled with Alt a 1 monoclonal antibody (2F9) and protein A coupled to 10 nm of gold. Protein A–gold conjugates were purchased from J. W. Slot, PhD (Department of Cell Biology, Utrecht University Medical School, Utrecht, the Netherlands). Then, sections were contrasted and embedded in a mixture of methylcellulose and uranyl acetate and observed under a 301 Philips transmission electron microscope (Philips Electron Optics, Eindhoven, the Netherlands). As a control for nonspecific binding, the primary monoclonal antibody was omitted.

RESULTS

Anti-Alt a 1 Western blots of different fractions showed that Alt a 1 is present predominantly in the CF and BEA fractions (Fig 1). The small amount of Alt a 1 present in the CA fraction was only detectable after 4 weeks, although the Alt a 1 assay used is highly sensitive (0.5 ng/mL of Alt a 1).¹³

The total amount of Alt a 1 quantified by enzyme-linked immunosorbent assay (ELISA) in the CF at 4.5 weeks was 6-fold higher than the amount obtained from BEA plus CA fractions (Fig 2A). Dry extract yield reached the maximum after 3 weeks, and Alt a 1 was 1.6% of the total protein content (data not shown). The IgE immunoblot of CF and BEA fractions from the 4.5-week culture incubated in a pool of *Alternaria*-allergic patients showed the same IgE binding band profile, with molecular masses of 105, 78, 62, and 30 kDa (Fig 1). The stronger band of 30 kDa in nonreduced samples corresponded to Alt a 1. In the same way, total IgE binding activity calculated by EAST inhibition of *A alternata* fractions showed 15-fold more activity in CF than in BEA plus CA fractions (Fig 2B). There was a good correlation ($r = 0.98$) between IgE binding activity values, expressed in allergenic units and determined by EAST inhibition of the CF fractions, and their Alt a 1 content, measured by the 2-site ELISA at different times of growth (data not shown).

Immunogold-labeled sections of *A alternata* using 2F9 monoclonal antibody showed that Alt a 1 seemed to be located in spores and mycelia and released outside. Alt a 1 is detected in the cytoplasm and cell wall, but without a clear association to any subcellular structure. In young spores, labeling was spread through the cytoplasm and the cell wall (Fig 3A). Allergen diffusion could be observed during hyphae growing; in young hyphae, Alt a 1 was detected mainly in the cell wall (Fig 3B), whereas in mature hyphae and spores, the labeling was found predominantly in the wall and outside surroundings (Fig 3C-E). No significant labeling was observed in the controls (Fig 3F).

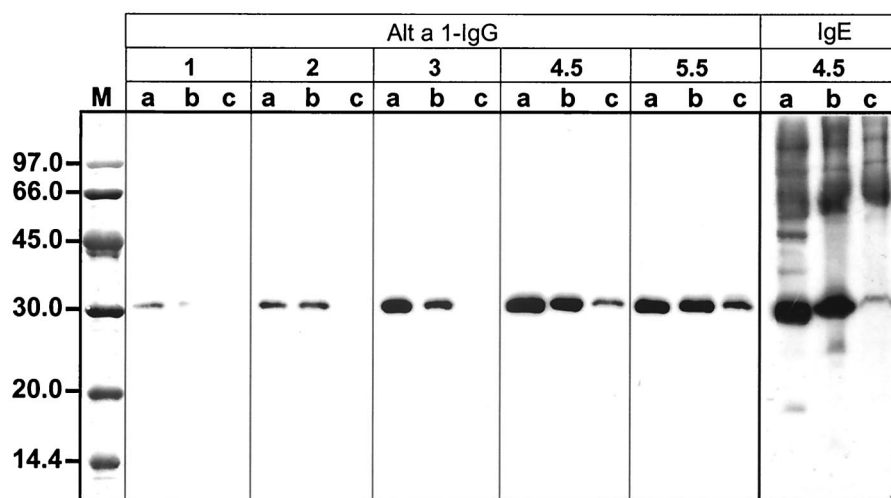


Figure 1. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis immunoblots of nonreduced *Alternaria alternata* fraction extracts: culture medium filtrate (a), buffer-extractable antigen (b), and cellular antigen (c) at different times of growth (1, 2, 3, 4.5, and 5.5 weeks) incubated with a polyclonal anti-Alt a 1 antiserum (IgG) or a human serum pool of *Alternaria*-allergic patients (IgE) at 4.5 weeks of growth.

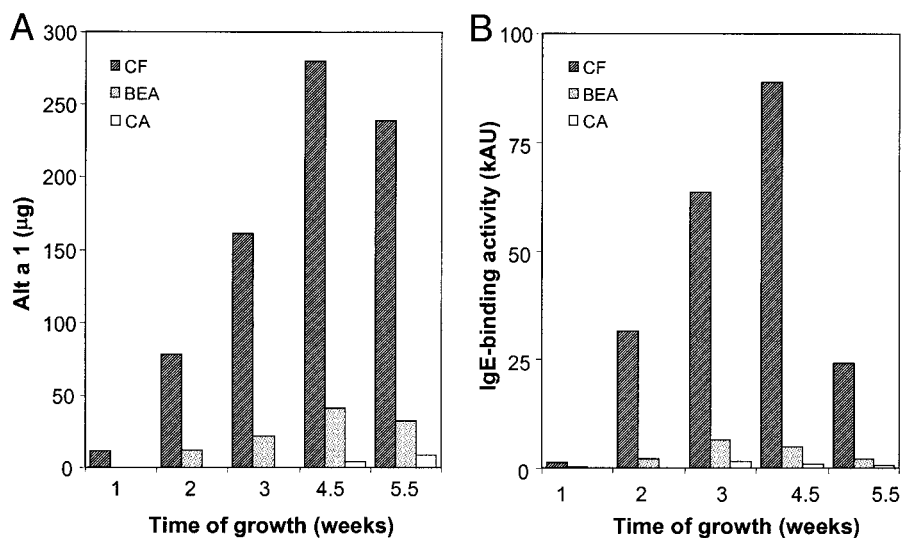


Figure 2. Quantification of total Alt a 1 by specific 2-site enzyme-linked immunosorbent assay (A) and total IgE binding activity by EAST inhibition (B) in *Alternaria alternata* fraction extracts: culture medium filtrate (CF), buffer-extractable antigen (BEA), and cellular antigen (CA) at different times of growth (1, 2, 3, 4.5, and 5.5 weeks).

DISCUSSION

Healthy individuals are continuously exposed to fungal biomass, which includes live and dead spores and fungal debris entrapped in the airways. Human exposure could be due to allergens eluted from inhaled spores or from hyphae that germinate after deposition in the respiratory tract.⁹ Alt a 1 reacted with more than 80% of *A. alternata*-sensitive patients.^{6,7} In addition, Alt a 1 inhibited, by up to 70%, the total IgE binding activity of the *A. alternata* extract,¹³ which suggests that allergens not related to Alt a 1 are remarkably less important from a clinical point of view, although they could be recognized by 61% of *Alternaria*-allergic patients.¹⁸

Standardizing fungal allergen products is an important task that has been impeded by the limited knowledge of relevant allergens and the lack of generally accepted reference reagents and potency assays.¹⁹ In this study, we used Alt a 1 as a marker of the production of *A. alternata* extracts for diagnostic and immunotherapy. Alt a 1 was found predominantly in the CF fraction when mold was grown in protein-poor media. Similar results using low-nutrient, synthetically defined medium were found by Portnoy et al.,⁸ whereas *A. alternata* grown in protein-rich medium contained more Alt a 1 in CA. Allergenic patterns of the different fractions were examined by immunoblotting in a pool of *Alternaria*-allergic

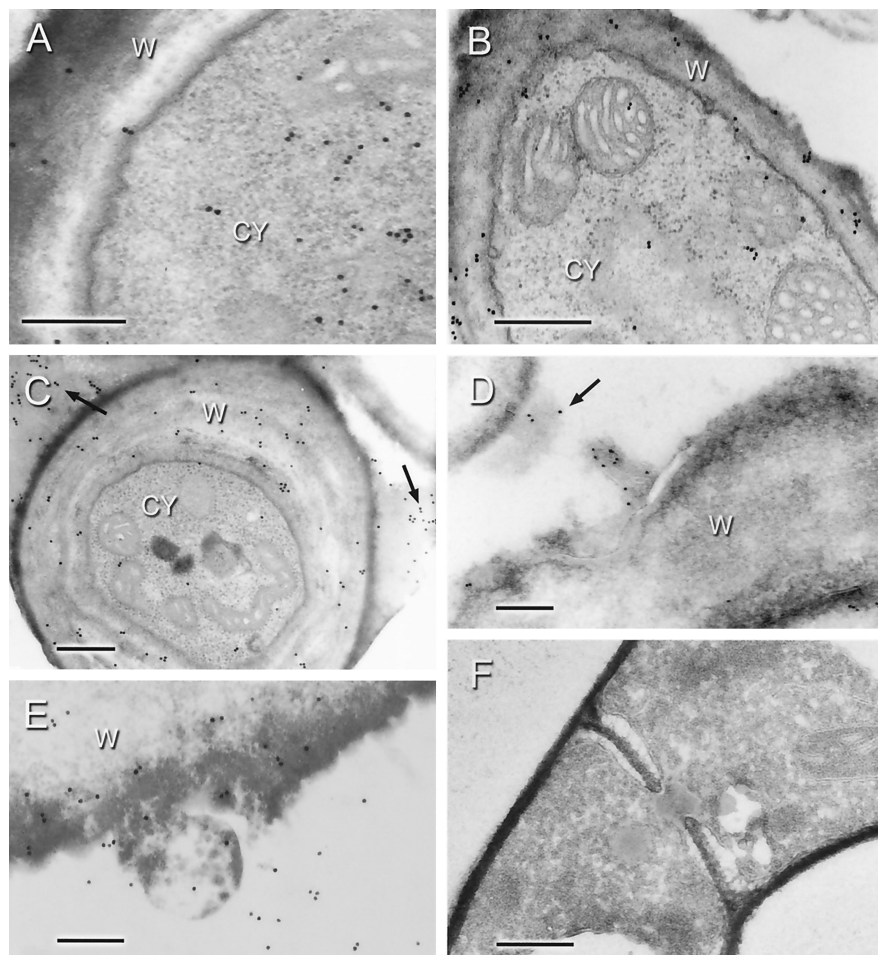


Figure 3. Transmission electron micrographs of spores/conidia and mycelia after immunogold labeling for Alt a 1 at different developmental stages. A, Young spore. B, Young hypha. C, Mature hypha. D and E, The diffusion of allergen through the spore walls. Alt a 1 allergen is indicated by gold particles (black dots and arrows). F, Control experiment with rabbit preimmune antibodies. Samples were prepared using Spurr resin (A and B), Lowicryl resin (C), and freezing (D-F). CY indicates cytoplasm; W, wall. Bar = 0.25 μ m.

patients. The CF and BEA fractions showed the same IgE binding profile of allergens, whereas the CA fraction showed fewer bands with lower intensity. The good correlation obtained between the allergen content, determined by the Alt a 1 2-site binding assay, and the total IgE binding activity, determined by EAST inhibition, for CF extracts of *A. alternata* supports that Alt a 1 is the most important allergen and contributes in a high percentage to the total allergenic activity of *A. alternata* extracts, as described previously.¹³ In our preparations, we found a constant increase in both Alt a 1 contents, determined by ELISA and IgE binding activity up to 1 month of growth. Analyses of the Alt a 1-encoding gene showed an N-terminal 28-amino acid region with an highly hydrophobic and strongly α -helical domain that seems to serve as a cleaved signal peptide that directs Alt a 1 for export from the cell.⁶ Up to 70% of total protein, 94% of total IgE binding activity, and 86% of Alt a 1 content were found in the culture filtrate fraction, which indicates the importance of

spent culture medium in the preparation of mold extracts used for diagnosis and immunotherapy. The use of spent culture medium as a source of allergenic material avoids extraction and disruption procedures, thus obtaining allergenic extracts with less contamination of nonallergenic components.

Immunocytochemical analysis with monoclonal antibodies showed a regular distribution of labeling through the cytoplasm and cell wall in spores and mycelia. Previous studies have described a cytoplasmic localization of Alt a 1, whereas the cell wall was devoid of the allergen.²⁰ Alt a 1 amino acid sequence contains a leader sequence of 28 residues that could be involved in secretion,⁶ and the allergen has been found in the culture filtrate.⁸ It has been hypothesized that Alt a 1 is secreted or released from germinating tubes,^{9,21} as has been reported for *Aspergillus*,²² and, to our knowledge, this is the first time that immunocytochemical experiments clearly show the release to the culture medium of Alt a 1 from mycelia and spores (Fig 3D-E). Because *Alternaria* spores are too large to reach the

alveoli of the lung, spore and mycelium fragments dispersed in the atmosphere would provoke allergic reactions in contact with the mucosa of sensitized patients by liberation of high amounts of Alt a 1, as has been demonstrated herein. In a similar way, abortive pollen germination has been demonstrated as a characteristic mechanism for the release of major tree pollen allergens.²³ Recently, Alt b 1, an Alt a 1 homolog, has been described in the necrotrophic fungus *Alternaria brassicicola*.²⁴ This homolog is preferentially expressed in spores that germinate on the leaf of *Arabidopsis* but not in spores that germinate in water, and it may play a role in the necrotrophic fungal pathogenesis of plants.²⁴ These results suggest that signals present on the plant leaf surface may trigger an increase in Alt b 1 gene expression. This signal is also present in nutrient-rich media, and, thus, a nutrient-rich environment (such as the nasal passage) conducive to fungal growth may be the only requirement for Alt b 1 or Alt a 1 production. The presence of Alt a 1 mainly in the spent culture medium seems to indicate that this protein plays an extracellular role, which is unknown at present. Identification of the biological function of Alt a 1 will certainly provide new ideas for potential control of this important allergen and may help answer the question of how patients are sensitized to *Alternaria* allergens.

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