

Allergens as proteases: An *Aspergillus fumigatus* proteinase directly induces human epithelial cell detachment

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Allergic bronchopulmonary aspergillosis (ABPA) is characterized by pulmonary and systemic allergic and inflammatory processes triggered by fungal antigens. Airway damage is a feature of this disorder, and although *Aspergillus*-derived proteinases have been described, the capacity of *Aspergillus*, however, to directly induce damage to human epithelium has not previously been studied. We therefore cultured *Aspergillus fumigatus* from two patients with ABPA, extracted mycelial products by sonication and filtration, and then evaluated their capacity to induce epithelial cell (EC) desquamation from basement membrane using an in vitro model that uses intact human amniotic EC and native basement membrane. *A. fumigatus* extracts induced detachment of EC in a dose-dependent fashion, producing up to $34\% \pm 6\%$ detachment ($p < 0.05$ compared to medium alone). Enzyme analysis of *A. fumigatus* extract using synthetic substrates revealed the presence of a number of different enzymes; therefore, studies with specific proteinase inhibitors were undertaken to identify the proteinase(s) responsible for detachment. *A. fumigatus*-induced desquamation was partially inhibited by phenylmethylsulfonyl fluoride and substantially inhibited by glutathione and N-acetylcysteine, but not by α_1 -antitrypsin, 1,10 phenanthroline, ethylenediaminetetraacetic acid, aprotinin, or soybean trypsin inhibitor at concentrations that inhibit other serine- or metalloproteinases. Gel filtration of the extract with a Sepharose 6B column revealed that the major epithelium-detaching activity appeared in the 20 to 35 kd fraction. Comparison with proteinase standards suggested a role for a chymotrypsin-like proteinase. Thus, *A. fumigatus* releases a proteinase that is directly able to induce EC detachment. This proteinase has some characteristics of a serine-proteinase, but its inhibitor profile is different from that of elastase, trypsin, cathepsin-G, and collagenase. Specific inhibitors of this proteinase could potentially be of therapeutic value in patients with ABPA. (*J ALLERGY CLIN IMMUNOL* 1990;86:726-31.)

ABPA is associated with numerous features that suggest an allergic basis, particularly elevated serum IgE, positive skin tests to *Aspergillus fumigatus* antigens, and detectable serum precipitins to these antigens.¹ Many patients have evidence of damage to airways, resulting in proximal bronchiectasis.² Although it has been suggested that the process that underlies the damage that results in proximal bronchiectasis may be mediated by immunologic mecha-

Abbreviations used

ABPA:	Allergic bronchopulmonary aspergillosis
EC:	Epithelial cell
BM:	Basement membrane
HPF:	High-power field
OD:	Optical density
PBS:	Phosphate-buffered saline
PMSF:	Phenylmethylsulfonyl fluoride
SBTI:	Soybean trypsin inhibitor
GSH:	Glutathione
NAC:	N = acetylcysteine

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nisms, particularly a type III reaction within the airway walls,³ we reasoned that some of this damage may be directly mediated by products released by the fungus in the area of the airway wall, particularly in view of the fact that the fungus grows within sputum plugs adjacent to the airway wall for prolonged periods in this disease¹ and that *A. fumigatus* is known to be

able to release several proteinases.^{4,5} The aim of this study therefore was to evaluate this hypothesis by studying the effects of extracts of *A. fumigatus* on human ECs with a model of human epithelium attached to native BM that we have previously characterized as being a useful model for evaluating the capacity of a variety of proteinases to disrupt the attachment structures binding human ECs to underlying BM.⁶ This model system has the advantages of being similar to airway epithelium, accurately quantifiable, and because it uses native BM rather than artificial BM and normal human EC rather than cell lines, it is more closely related to the *in vivo* situation.⁶

METHODS

Culture of *Aspergillus* species

A. fumigatus was isolated and cultured from the sputum of a patient with documented *A. fumigatus* chest disease and grown in wide-mouth McCartney bottles on Sabouraud's dextrose agar, incubated at 25° C for 5 to 7 days, and harvested while the fungus was still actively growing. The fungal cultures were harvested by adding 2 ml of chilled sodium chloride, 0.9%, to each of the bottles and abrading the surface with a sterile flattened instrument to release surface spores and mycelia.

Evaluation of EC detachment

EC detachment was evaluated with our previously described model.⁴ Briefly, term placentae were obtained fresh from King Edward Memorial Hospital for Women within 4 hours of normal delivery, and the amnion was separated from the chorion. Mucous and blood clots were removed from the stromal side of the amnion, and the membrane was washed extensively in PBS. The washed membrane was then stretched onto a glass microscope slide with the ECs facing upward. The slide was then installed in a microchemotaxis chamber (Neuroprobe, Inc., Cabin John, Md.) that afforded 48 wells for experimentation. The assembly was incubated for 4 hours (dose-response studies) or 18 hours (gel filtration studies) at 37° C in a humidified 5% CO₂/95% air incubator.

Assessment and quantitation of EC detachment

To quantify EC detachment from BM, the slides bearing the amnionic membranes were dried in air, fixed in methanol, and then stained with a modified Wright-Giemsa stain (Diff-Quick, Medicon, Perth, Australia). Detachment was evaluated as follows: The percentage of total attached cells was scored visually using an eye piece graticule at low magnification ($\times 60$) on a scale of 0% to 100% of the total well area in increments of 10%. All slides were evaluated in single-blind fashion by the same investigator (T. V.). This method had the advantages of speed plus accuracy, particularly in terms of quantification of detachment/attachment across the whole test well, thus including areas of patchy detachment in the quantification. This method has been

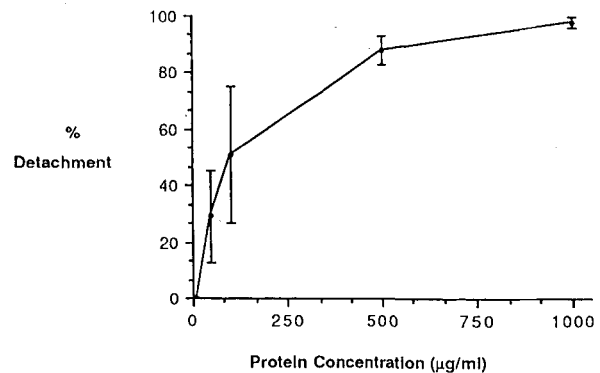


FIG. 1. Capacity of extracts from *A. fumigatus* to induce detachment of ECs from native BM. Data are expressed as percent of each test well area, from which cells have detached, expressed as mean $\pm$ SEM.

previously demonstrated to be as accurate as the direct counting of individual cells in adjacent HPFs performed by counting the number of cells in 10 adjacent HPFs randomly selected in each test and control area and the percent attachment/detachment determined with the formula: % attachment/detachment = (No. cells in 10 adjacent HPFs in test wells)/(No. cells in 10 adjacent HPFs in control wells). This latter procedure is tedious and time consuming and has an additional drawback that patchy attachment/detachment observed at low power was not always included in the quantification process.¹⁶

Quantification with a spectrophotometric method has also been compared, in which cells remaining attached to BM or plastic are stained with Diff-Quick, as described above, washed in PBS, treated with 10% Triton X (Rohm and Haas Co., Philadelphia, Pa.) in distilled water, and left for 30 minutes at 22° C to allow release of the dye from the cells. Aliquots of the supernatants are then removed, adjusted to 1 ml total volume, and transferred to 1 ml disposable spectrophotometric cuvettes (10 mm light path). The OD of the solution at 650 nm (the absorbance peak of the Diff-Quick dye) is read on a Varion 640 spectrophotometer (Varion Instrument Group, Sugar Land, Texas). Percentage attachment/detachment using the spectrophotometric method was calculated with the formula: (OD 650 nm of test sample $\times$ 100)/(OD 650 nm of negative (medium only) control - OD 650 nm of positive (Trypsin 2000 U/ml) control). This method produces identical results to the incremental scoring method described above (*r* value 0.96).⁶

Evaluation for presence of enzymes

The fungi were isolated from the cultures in NaCl (0.9%; 40 slopes per 40 ml) and sonicated on ice four times for 30 seconds at 30-second intervals (to avoid denaturing of proteins by heat). The sonicates were then further disintegrated in a polytron and centrifuged at 1000 *g* for 10 minutes. The pellet was discarded, and the supernatant was filtered through gauze and then a 0.45 µm filter (Millipore, Sydney, Australia). The final solution was stored frozen at -20° C until it was ready for use.

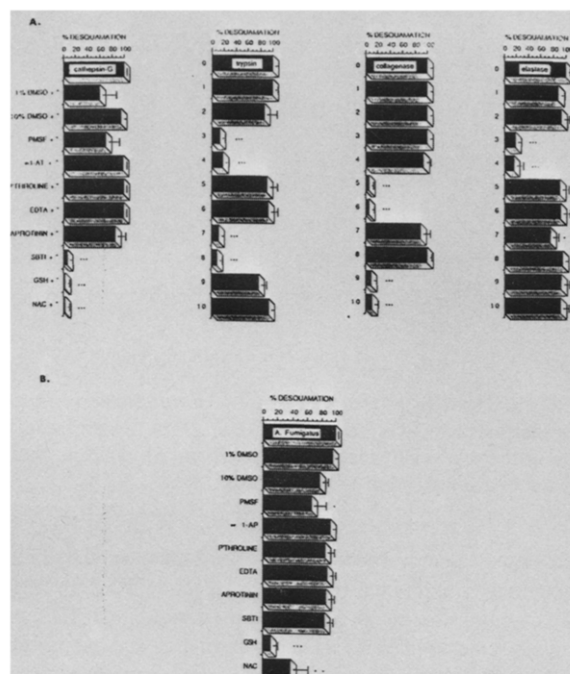


FIG. 2. Proteinase inhibitor profile for *A. fumigatus* extract. **A.** Characterization of the effects of nine inhibitors on four standard proteinases, elastase, trypsin, collagenase, and cathepsin-G. Sources of proteinases, unit definitions, and dose-response curves for detachment are listed in METHODS section. **B.** Effects of those inhibitors on extracts from *A. fumigatus* added to test wells at 500 μ g/ml. Data are expressed as in Fig. 1.

The crude supernatants were concentrated by freeze-drying, resuspended in distilled H₂O, and dialyzed overnight in 6000 dalton exclusion dialysis tubing against 0.9% NaCl. Protein concentrations were monitored at all stages and measured with a standard benzyl chloride system.

The presence of detaching agents was tested as follows: The fungal products were placed in the top wells of the microchemotaxis assembly to assay for EC detachment of amniotic EC. Dose-response curves were performed by diluting the concentrated extracts with 0.9% NaCl. The negative control was NaCl only. All tests were performed in duplicate, and desquamation was quantified as described above.

Evaluation of proteinase inhibitors

A panel of standard inhibitors were used, and each proteinase was initially characterized against four different standard proteinases. Proteinases were then assayed against *A. fumigatus*-induced EC desquamation in the detachment assay. The inhibitors and proteinases used were 1×10^3 U/ml of human sputum cathepsin-G; 2×10^3 (benzoylarginine ethyl ester) U/ml of porcine pancreatic trypsin; 2×10^2 (ninhydrin) U/ml of *Clostridium histolyticum* collagenase; and 1×10^2 (elastin) U/ml of

porcine pancreatic elastase. The final concentrations of inhibitors were as follows: dimethylsulfoxide, 1% and 10%, vol/vol; PMSF, 1 mmol/L; α_1 -antitrypsin, 50 μ /ml; 1,10-phenanthroline, 1 mmol/L; ethylenediaminetetraacetic acid, 1 mmol/L; aprotinin, 50 μ /ml; SBTI, 0.5 mg/ml; GSH, 10 mmol/L; and NAC, 10 mmol/L.

Molecular weight estimation

Fungal extracts were passed through a Sepharose 6B column (Pharmacia Fine Chemicals, Uppsala, Sweden) at 4° C and calibrated with the following markers: blue dextran (2000 kd), bovine serum albumin (fraction V, 67 kd), chymotrypsin (28 kd), cytochrome c (12.4 kd), vitamin B₁₂ (1.3 kd), and GSH (0.3 kd); 6 ml fractions were collected at 36 ml/hr in PBS buffer. Every five fractions were pooled, freeze-dried, and dialyzed, as described above. The samples were then assayed for their ability to detach ECs in the assay described above.

Statistical analysis

Results for dose dependence of fungal extracts and for enzyme inhibition were compared with a paired Student's *t* test.

RESULTS

A. fumigatus-induced detachment

A. fumigatus-induced EC detachment in a dose-dependant fashion (Fig. 1). In the unconcentrated extract, total detachment was $35\% \pm 8\%$. Increasing dilutions of this extract produced less detachment, and no detachment was observed at concentrations of 10 μ g/ml or less. When the extract was concentrated, enhancement of detachment was observed, up to $84\% \pm 9\%$ (Fig. 1), confirming the dose dependence of the detachment. No detachment was observed with the saline control.

Proteinase-inhibitor profile

A. fumigatus-induced detachment was not inhibited by dimethylsulfoxide, α_1 -antitrypsin, 1,10-phenanthroline, ethylenediaminetetraacetic acid aprotinin, or SBTI. Approximately 50% inhibition was observed with PMSF (Fig. 2) and marked inhibition (80%) was observed with GSH and NAC. Compared to the inhibitor profiles notes for four standard proteinases, trypsin, elastase, collagenase, and cathepsin-G, it was clear that the proteinase released by *A. fumigatus* had a different inhibitor profile to each of these other proteinases, although there were some similarities with cathepsin-G/chymotrypsin, particularly the inhibition by PMSF, GSH and NAC, differing only in the absence of inhibition by SBTI.

Molecular weight estimation

Column separation of the *A. fumigatus* extract revealed that the highest level of detaching activity was

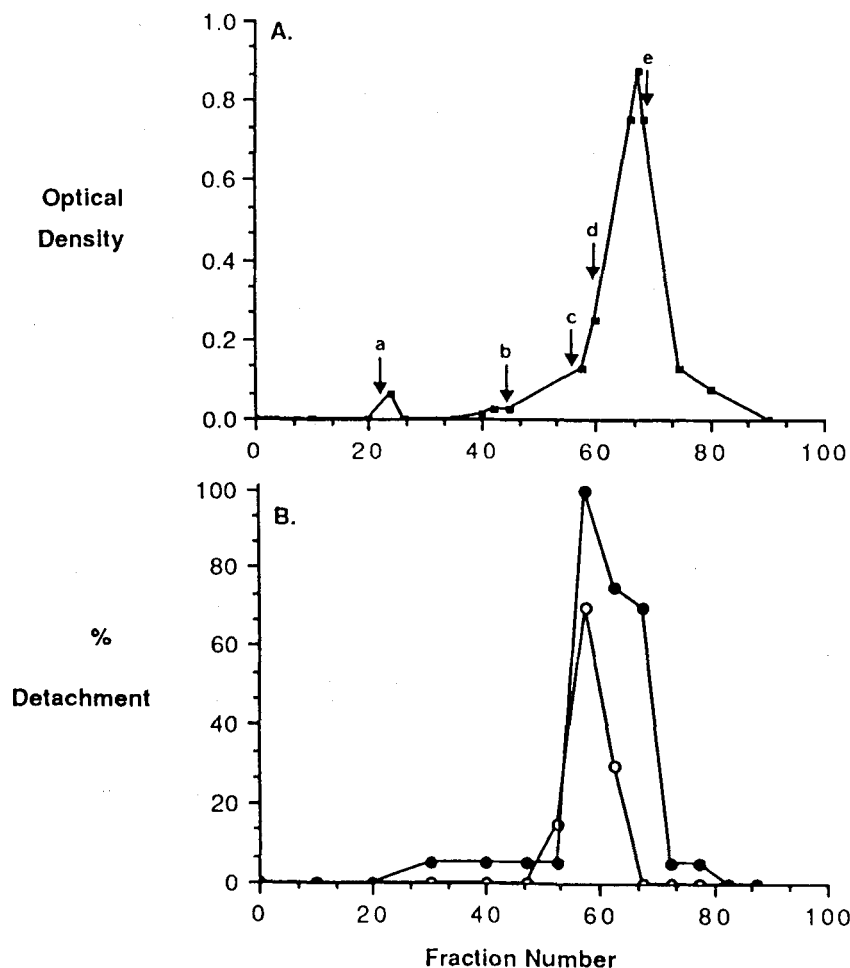


FIG. 3. Results of Sepharose 6B estimation of molecular weight of *A. fumigatus*-derived EC detaching proteinase. **A.** Data presented are molecular weight standards (*a*, blue dextran 2000 kd; *b*, bovine serum albumin 67 kd; *c*, chymotrypsin 28 kd; *d*, cytochrome 12.4 kd; *e*, vitamin B₁₂ 1.3 kd), fraction numbers, OD readings for each fraction (at 275 nm). **B.** Percent detachment for each fraction for two experiments. Control was normal saline that always produced <20% detachment and that value was subtracted from the observed value for each pool. Fractions were pooled as described in METHODS section.

noted in the 20 to 35 kd region with less epithelial detaching activity observed in the adjacent 10 to 20 kd fraction, and little significant detachment was observed at molecular weights >60 kd and <1 kd (Fig. 3).

DISCUSSION

The mechanisms underlying ABPA have not been clearly defined, particularly mechanisms that mediate the airway damage observed in this condition. In this study we used human amniotic ECs attached to natural BMs, which represents an excellent "model" for studying mechanisms associated with airway EC derangement because amniotic epithelium is very similar

to airway epithelium, exhibiting tight junction formation and production of an identical BM with type IV collagen, laminin, and fibronectin in a similar array.⁶ Also, the ECs have no proteinases or inhibitors present to affect the assay system. The assay system is simple yet accurately quantifiable and represents a useful tool to dissect the biochemical processes associated with EC damage and detachment. These data in this study demonstrate a direct effect of an *A. fumigatus*-derived proteinase on ECs, implying that the persistence of this fungus within the airways may be associated with the continued release of this proteinase and lead to chronic damage to the adjacent epithelium.

The amount of EC detachment observed in these experiments was quite marked, particularly compared to inflammatory cell-induced EC detachment.⁶ Up to 90% of the ECs were detached in these experiments within a period of 4 hours. Because the airway epithelium may be exposed to these proteinases for many years in vivo, it is clear that the potential for considerable airway damage exists.³

The extracts studied in these experiments were obtained from sonicated fungus and induced considerable detachment. Thus, it is clear that there is no requirement for an intact, viable fungus for these effects to be observed, suggesting that breakdown products of this fungus within the airway lumen may also contribute to airway damage. Since mucociliary clearance of these breakdown products is inhibited in this disease,¹ it may represent a way in which this process is maintained; that is, viable and decaying fungal elements may induce damage.

It is not known whether mycelia or spores are the major sites of release of this proteinase, but studies of industrial proteinases suggest that the major proteinase-producing area of other *Aspergillus* species is the hyphal tip of the mycelia.⁷ The concept that the mycelia may be the major contributors to the damage is consistent with the knowledge that spores of many different fungi are common within the atmosphere and are then inhaled by many individuals but that airway damage only occurs when there is colonization of the airways, usually in subjects with asthma, and growth of mycelia.¹ In fact, one of the helpful features in diagnosis of this disease is the detection of mycelia within sputum plugs.¹

Our studies of the enzyme activity of *A. fumigatus* extracts using both artificial substrates and well-characterized inhibitors demonstrated that the major EC-detaching proteinase released had some evidence of chymotrypsin-like activity. This finding is consistent with previous studies demonstrating a chymotrypsin-like enzyme in other *Aspergillus* species⁸ and is consistent with the knowledge that cathepsin-G, another chymotrypsin-like enzyme, which is released by neutrophils, is capable of mediating EC detachment.⁹ The molecular weight estimation of peak detaching activity at 20 to 30 Kd is consistent with this concept.

Other investigators have demonstrated proteinase activity in *A. fumigatus* extracts,^{4,5} and three studies have demonstrated some antigenicity of a 25 to 30 kd fraction,¹⁰⁻¹² suggesting that in ABPA, as in house dust-mite allergy, part of the immune response is directed at proteinases.¹⁸

The failure of serine- and metalloproteinase inhibitors to suppress this fungus-derived proteinase dem-

onstrates that the practice of the use of this combination of inhibitors in industrial situations in which workers are exposed to fungal products¹³ is insufficient to afford full protection to the workers from potential airway damage and that a broader spectrum of anti-proteinase activity is likely to be required to prevent occupational airway EC damage in these workers.

It was encouraging to note that two inhibitors, GSH and NAC, were able to suppress the damage induced by the *A. fumigatus*-derived proteinase. Therapy for ABPA largely relies on standard asthma therapy, often including systemic corticosteroids.¹ Attempts to destroy the fungus with inhaled or systemic antifungal agents have met with limited success.¹⁴ NAC has been used in a number of pulmonary diseases, including by inhalation in uncomplicated asthma, with evidence of benefit in some patients, although bronchospasm can occur.¹⁵⁻¹⁷ These data from this study suggest that inhaled or oral NAC in patients with allergic ABPA may be of value in suppressing the activity of damaging proteinases.

Although we have emphasized the potential role for this *A. fumigatus*-derived proteinase in mediating the airway damage/bronchiectasis observed in ABPA, it is conceivable that this proteinase may also play a role in triggering the allergic response, for example, by causing detachment of areas of epithelium resulting in increased penetration of the *A. fumigatus* antigens into the submucosa to trigger the allergic response and/or by the proteinase itself acting as one of these antigens. The potential role of allergen-associated proteinases in contributing to "allergenicity" by damaging epithelium and hence potentiating the entry of antigens into the tissue has not been well studied, but it is conceivable that other inhaled allergen-associated proteinases may induce some desquamation of ECs; for example, dust mite antigens contain proteinases that are also antigenic,¹⁸ and pollens contain proteinases that contribute to pollen germination. In that sense, some allergen-associated EC-detaching proteinases may represent "the thin end of the wedge" by creating holes for antigens to penetrate more readily epithelial surfaces to gain access to the immune system. Studies evaluating the presence of epithelium-detaching proteinases in these and other allergens are currently under investigation.

In conclusion, we have demonstrated the presence of a proteinase in *A. fumigatus* that directly induces EC detachment. This proteinase may have a direct role in ABPA. The observation enhances our understanding of the possible nonimmunologic components of ABPA and suggests that therapeutic approaches aimed at suppressing this direct effect¹⁹ may be worthy of study in ABPA.

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