

Protease-dependent activation of epithelial cells by fungal allergens leads to morphologic changes and cytokine production

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Background: Proteases in extracts of *Aspergillus fumigatus* cause epithelial cell desquamation and release of proinflammatory cytokines.

Objective: We sought to assess protease activity in *Alternaria alternata*, *Cladosporium herbarum*, and *Aspergillus fumigatus* extracts and study the ability of these extracts to cause desquamation and release of proinflammatory cytokines from epithelial cells.

Methods: Protease activities of the fungal extracts were quantified. Changes with respect to cell morphology, cell desquamation, and cytokine production (IL-6 and IL-8) were measured in the absence and presence of the fungal extracts in an airway-derived epithelial cell line (A549) and primary epithelial nasal cells.

Results: Fungal proteases differentially induced morphologic changes, cell desquamation, and production of IL-6 and IL-8 in a dose- and time-dependent fashion. *Alternaria alternata* extracts induced cell shrinking and cell desquamation and strongly enhanced the production of IL-6 and IL-8 at higher concentrations. *Aspergillus fumigatus* extracts caused cell shrinking, cell desquamation, and production of IL-6 and IL-8, even at low concentrations. The *Aspergillus fumigatus*-derived extract grown on collagen medium induced a strong dose-dependent decline in cytokine production at higher concentrations. *Cladosporium herbarum* extracts did not induce morphologic changes or cell desquamation but enhanced IL-6 and IL-8 productions at higher concentrations. The dependence of these effects on intact protease activity was shown by their abrogation by protease inhibitors.

Conclusion: Proteases present in fungal extracts interact with epithelial cells, leading to morphologic changes, cell desquamation, and induction of proinflammatory cytokines. It is proposed that these fungal proteases may activate epithelial cells through a protease-activated receptor type 2-driven mechanism. (J Allergy Clin Immunol 2000;105:1185-93.)

Key words: Fungi, proteases, epithelial cells, cytokines, desquamation, protease-activated receptor, asthma, sensitization

Abbreviations used

E-ColCF:	Culture filtrate of <i>Aspergillus fumigatus</i> grown on collagen-containing medium
PAR:	Protease-activated receptor
Vmax:	Maximum velocity

Fungi are ubiquitous saprophytes that reproduce by the formation of spores that are able to enter the respiratory tract by means of inhalation. Fungal spores rarely behave as pathogens in the airways of healthy individuals, with the exception of some fungi, such as *Aspergillus fumigatus*. However, fungi have often been associated with asthmatic reactions in atopic individuals. Fungi that have been implicated in allergic airway diseases (eg, asthma and rhinitis) include *Alternaria*, *Cladosporium*, *Botrytis*, *Penicillium*, *Aspergillus*, and *Basidiomycetes* species.¹⁻⁹ Sensitization rates to fungi vary widely in different studies, ranging from low and insignificant values to high sensitization rates comparable with those of house dust mites. We found sensitization rates of less than 5% in an adult population,⁴ whereas in subsequent studies higher sensitization rates were found in younger age groups.^{5,10,11} Possible explanations for the variation in sensitization to fungal allergens are the variable level of exposure in different countries, the lack of standardization of fungal extracts used in skin tests,^{12,13} and the age of the population being studied.^{5,11} In addition to these variables, sensitization to fungal allergens may be dependent on both the presence of these allergens and on factors that facilitate their access to the immune system of the airways.¹⁴⁻¹⁶ In this respect proteases obtained from allergenic sources (eg, house dust mites and fungi) may facilitate antigen access either by proteolytic attack, leading to cell desquamation, or by direct activation of epithelial cells.^{15,17} Indeed, proteases of house dust mite and fungal extracts are potent inducers of epithelial cell desquamation and production of proinflammatory cytokines.¹⁸⁻²⁰ Although information on fungal proteases is limited, extracts of different fungi were shown to contain larger quantities of proteases than most other inhalant allergens.²¹ Some of these allergens were characterized as alkaline proteases.^{9,22,23} Recently, the protease-activated

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TABLE I. Protein (percentage of total weight) and protease activities in fungal extracts (n = 4)

Fungal extract	Protein (%)	Total protease activity (U/mg)	Elastase activity (Vmax)	Gelatinase activity (U/mg)
<i>Alternaria alternata</i>	23.0	2.18	0	0.40
<i>Cladosporium herbarum</i>	11.5	1.50	25.0	0.14
<i>Aspergillus fumigatus</i>	33.7	1.93	17.7	0.57
E-ColCF	36.3	3.89	55.1	3.15

TABLE II. Inhibition of elastase activity in fungal extracts with serine protease inhibitors and heat treatment (n = 6)

	<i>Cladosporium herbarum</i>	<i>Aspergillus fumigatus</i>	E-ColCF
Heat treatment (%)	100	100	100
Chymostatin (%)	18.0	99.7	100
Antipain (%)	9.3	98.6	100

Alternaria alternata is not included because this extract lacks elastase activity.

receptor type 2 (PAR2) was demonstrated on human airway smooth muscle and epithelial cells.^{24,25} It is possible that fungal proteases may activate epithelial cells through this type of surface receptor, resulting in generation of cytokines, cell desquamation, and facilitation of allergic sensitization. Our present study focuses on 3 frequently sensitizing fungi: *Cladosporium herbarum*, *Alternaria alternata*, and *Aspergillus fumigatus*. Extracts from these fungi were examined for proteolytic activity, the capacity to induce epithelial cell desquamation, and the capacity to activate epithelial cells, as reflected by IL-6 and IL-8 release. The dependence of these effects on intact enzymatic activity of these proteases was studied by assessing the effect of specific protease inhibitors. Proteases have also been implicated in the pathogenicity of *A fumigatus*.^{19,26,27} Therefore we monitored the effect of two *A fumigatus* extracts on cell morphology and their capacity to cause epithelial cell desquamation in vitro. One extract was prepared similarly to extracts used for skin testing, thus favoring adequate *Aspergillus fumigatus* allergen content (ALK-Abelló), and the other was prepared from a strain isolated from a patient with aspergilloma, which is known for its capacity to produce serine proteases,^{19,23} possibly reflecting pathogenic characteristics of *Aspergillus fumigatus*.

METHODS

Fungal extracts and quantification of protease activities

Biologically standardized lyophilized fungal allergen extracts from fungal hyphae and spores obtained from stationary cultures (*Cladosporium herbarum*, *Alternaria alternata*, and *Aspergillus fumigatus*) were kindly provided by Dr Lars Jacobsen (ALK-Abelló). Crude extracts were obtained by using aqueous extraction for 24 hours at 4°C to 8°C and purified by using microdialysis with a cut-off point of 10,000 d, which is similar to that of extracts used for diagnostic purposes. Culture filtrate of *Aspergillus fumigatus*

grown on collagen-containing medium (E-ColCF) containing high proteolytic enzyme activity was prepared as described elsewhere.²³ A second *Alternaria alternata* extract (AltB) was kindly provided by Dr Robert K. Bush (Veterans Hospital, Madison, Wis). A third *Alternaria alternata* extract (AltE) containing protease activity was provided by Dr Robert Esch (Greer Laboratories, Inc). Total protease (with casein as a substrate), elastase (with N-succinyl-alanyl-alanyl-prolyl-leucine *p*-nitro-anilide as a substrate), and gelatinase (with gelatin-orange as a substrate) activities of the fungal extracts were quantified as previously described.¹⁹

Epithelial cell lines and cell activation

Cells from A549, a human alveolar type II epithelium-like cell line, were obtained from American Type Culture Collection. The epithelial cells were cultured in sterile 24-well culture dishes (Costar) in RPMI-1640 with 5% heat-inactivated FCS and 0.05% gentamicin to 80% to 90% confluence, as described previously.¹⁹ Before incubation with the fungal extracts, the cell cultures were incubated with serum-free medium for 24 hours. Stimulation (24 hours) with fungal extracts of various concentrations was performed in serum-free medium at 37°C in an atmosphere of 5% CO₂. Incubation with IL-1β (20 U/mL; Boehringer Mannheim) served as a positive control. Incubation with serum-free medium served as a negative control (basal cytokine secretion). When protease inhibitors were used, fungal extracts were incubated with the inhibitor at 37°C for 15 minutes before being added to the cells. The inhibitors used were chymostatin and antipain (10 μg/mL each; both serine protease inhibitors), leupeptin (50 μg/mL; aspartic protease inhibitor), and cystatin (10 μg/mL; cysteine protease inhibitor). All protease inhibitors were obtained from Sigma. Heat treatment of fungal extracts was done at 65°C for 30 minutes. After 24 hours of incubation with the fungal extracts, the cell supernatants were collected and stored at -20°C. Cytokine production was quantified by using commercially available ELISA kits (CLB). Cell desquamation was measured by using an inverted microscope and quantified on a 3-point scale (1 = no desquamation, 2 = visual changes in morphology characterized by shrinking of the cells, and 3 = total cell desquamation). Cell viability was quantified microscopically by using trypan blue exclusion. A549 epithelial cells were incubated for 24 hours with increasing concentrations of the PAR2 agonist (NH₂-SLIGKV-C) obtained from Eurosequence. Cell activation and quantification of IL-6 and IL-8 were performed in the same manner as described for the fungal activation studies described above. Stability of IL-6 and IL-8 in the presence of fungal extracts was studied by using supernatants of A549 cells preincubated for 24 hours with IL-1β (20 U/mL; SanverTECH B.V.). Pooled supernatants were incubated with increasing concentrations of the fungal extracts for 24 hours at 37°C. The residual quantities of IL-6 and IL-8 were measured by using an ELISA according to the manufacturer's description (CLB, Amsterdam, The Netherlands).

Primary nasal epithelial cell cultures

Epithelial cells were isolated and cultured from resected material of the inferior nasal conchae of nonatopic subjects, as described elsewhere.¹⁸ Cell activation and quantification of IL-6 and IL-8 were performed in the same manner as described for the A549 cells. Bronchial epithelial cell basal medium (Clonetics Corp) was used as serum-free medium.

Data analysis

All experiments were performed at least 6 times. Statistical analysis was performed with the Student *t* test. Differences with *P* values of .05 or less were considered significant (n = 6).

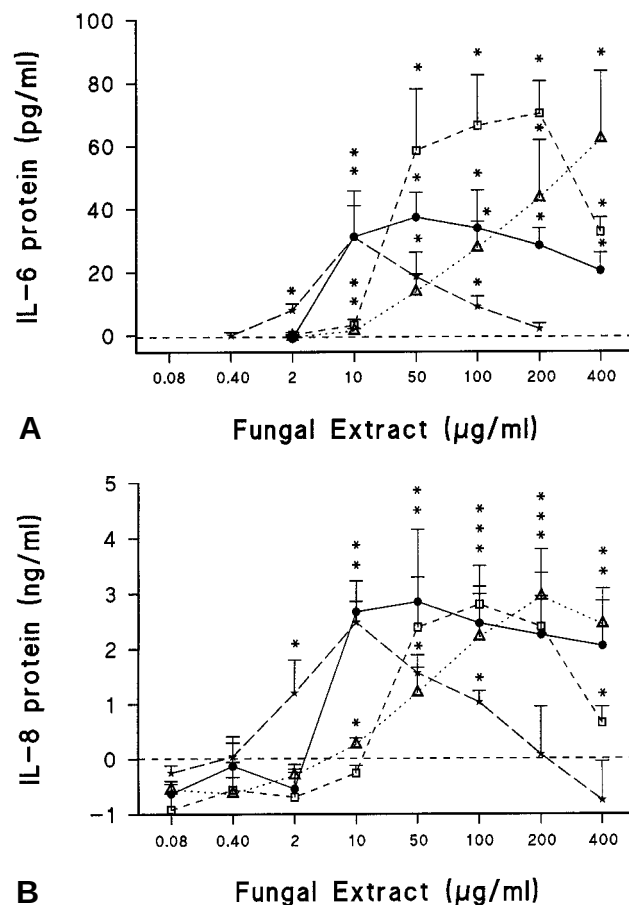


FIG 1. IL-6 (**A**) and IL-8 (**B**) production by A549 cells in the presence of various concentrations of fungal extracts: open squares, *Alternaria alternata*; open triangles, *Cladosporium herbarum*; filled circles, *Aspergillus fumigatus*; asterisks on dashed line, E-ColCF. Zero values indicate spontaneous productions in the absence of fungal extracts. Negative values indicate the protein levels below the level of spontaneous production. Asterisks not on dashed line indicate significantly increased production of cytokine.

TABLE III. Shrinking and cell desquamation of A549 cells

Fungal extract	No desquamation ($\mu\text{g/mL}$)	Cell shrinking ($\mu\text{g/mL}$)	Desquamation ($\mu\text{g/mL}$)
<i>Alternaria alternata</i>	0.08–50	100–200	400
<i>Cladosporium herbarum</i>	0.08–400	—	—
<i>Aspergillus fumigatus</i>	0.08–10	50	100–400
E-ColCF	0.08–2	10	50–400

The data represent the concentration range at which no detachment, cell shrinking, or desquamation is microscopically observed ($n = 6$).

RESULTS

Protease activity in fungal extracts

Protein content and gelatinase, elastase, and total protease activities are shown in Table I. The two *Aspergillus fumigatus* extracts contain over 30% proteins. The protein content of the *Alternaria alternata* and *Cladosporium herbarum* extracts was 23.0% and 11.5%, respectively. Total protease activity, as measured by the casein protease assay, was highest with the E-ColCF extract (3.89 U/mL) followed by the *Alternaria alternata*, *Aspergillus fumigatus*, and *Cladosporium herbarum* extracts (2.18, 1.93, 1.5 U/mg, respectively). Most extracts demonstrated gelatinase activity, ranging from low activity in the *Cladosporium*

herbarum extract to high activity in the other extracts (*Alternaria alternata* < *Aspergillus fumigatus* < E-ColCF). Elastase activity was greatest in the E-ColCF extract (maximum velocity [V_{max}], 55.1) followed by the *Cladosporium herbarum* (V_{max} , 25.0) and *Aspergillus fumigatus* (V_{max} , 17.7) extracts. In contrast, elastase activity was not found in the *Alternaria alternata* extracts. Elastase activity in the E-ColCF and *Aspergillus fumigatus* skin test extracts could be blocked by chymostatin and antipain (Table II), indicating that serine proteases are involved. The elastase activity of *Cladosporium herbarum* was inhibited only 18% by chymostatin and only 9% by antipain, respectively, indicating elastase activity unrelated to serine proteases.

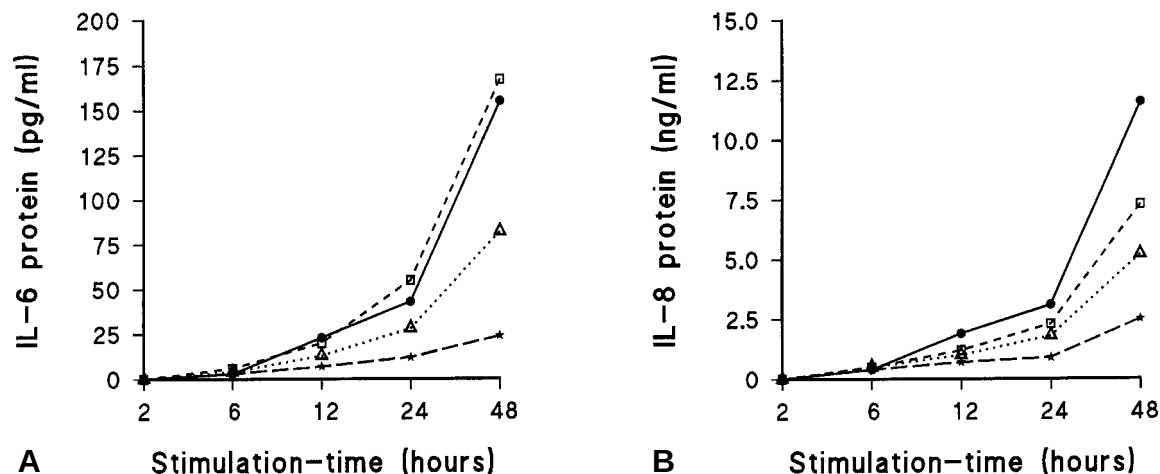


FIG 2. IL-6 (**A**) and IL-8 (**B**) production by A549 cells over time incubated with optimal concentrations of fungal extracts: open squares, *Alternaria alternata*; open triangles, *Cladosporium herbarum*; filled circles, *Aspergillus fumigatus*; asterisks on dashed line, E-ColCF.

TABLE IV. Percentage of inhibition of fungal extract-induced IL-6 and IL-8 production by protease inhibitors and heat inactivation (fungal extract = 50 μ g/mL)

Extract	Cytokine	Chymostatin	Antipain	Cystatin	Heat treatment
		Cytokine inhibition (%)			
<i>Alternaria alternata</i>	IL-6	98.0 $\pm$ 2.2	96.6 $\pm$ 2.2	81	94.6 $\pm$ 2.8
	IL-8	100 $\pm$ 0	99.2 $\pm$ 0.9	62	93.6 $\pm$ 4.0
<i>Cladosporium herbarum</i>	IL-6	96.7 $\pm$ 5.2	86.7 $\pm$ 10	35	80.1 $\pm$ 10.0
	IL-8	100 $\pm$ 0	98.4 $\pm$ 1.6	37	89.1 $\pm$ 16.8
<i>Aspergillus fumigatus</i>	IL-6	96.4 $\pm$ 0.5	92.2 $\pm$ 6.9	ND	86.8 $\pm$ 0.8
	IL-8	100.0 $\pm$ 0	93.8 $\pm$ 2.1	ND	94.1 $\pm$ 3.1
E-ColCF	IL-6	86.0 $\pm$ 3.9	71.3 $\pm$ 11.1	66	75.4 $\pm$ 16.7
	IL-8	98 $\pm$ 1.3	90.0 $\pm$ 1.1	33	77.3 $\pm$ 20.0

Data are expressed as means $\pm$ SE of 6 independent experiments. For cystatin, 3 experiments were performed. IL-6 production by 50 μ g/mL fungal extract in the absence of inhibitors is 58.0 $\pm$ 7.2, 13.4 $\pm$ 4.4, 36.0 $\pm$ 8, and 17.9 $\pm$ 8.3 pg/mL IL-6 for *Alternaria alternata*, *Cladosporium herbarum*, *Aspergillus fumigatus*, and E-ColCF, respectively. IL-8 production is 2.5 $\pm$ 0.9, 1.19 $\pm$ 0.5, 2.9 $\pm$ 0.7, and 1.5 $\pm$ 0.4 ng/mL, respectively.

ND, Not determined.

Fungal extracts induce morphologic changes and cell desquamation in A549 cultures

In the presence of fungal extracts, A549 cell cultures undergo morphologic changes (shrinking), cell desquamation, or both. As shown in Table III, the E-ColCF extract most potently affected epithelial cells. With 10 μ g/mL E-ColCF, shrinking became evident. At E-ColCF concentrations of 50 μ g/mL or greater, complete cell desquamation was observed. These morphologic changes were also observed with the ALK *Aspergillus fumigatus* extract (50 μ g/mL shrinking and 100-400 μ g/mL desquamation) and the *Alternaria alternata* extract (100-200 μ g/mL shrinking and 400 μ g/mL desquamation). Both shrinking and desquamation were protease dependent and could be blocked by more than 90% by serine protease inhibitors (both at 50 and 200 μ g/mL of fungal extract; data not shown). However, at high concentrations of the E-ColCF extract ($\geq$ 200 μ g/mL), the cells still demonstrated shrinking despite the addition of serine protease inhibitors. Desquamation induced by fungal extracts did not affect the viability of the A549 cells,

except for the E-ColCF extract, which resulted in 30% and 70% trypan blue-positive cells at 200 and 400 μ g/mL, respectively (not shown). The *Cladosporium herbarum* extract caused neither shrinking nor desquamation of the cells.

Fungal extracts induce production of proinflammatory cytokines by A549 cells

All fungal extracts had a small but significant inhibitory effect on the spontaneous production of IL-8 (medium control value) at low concentrations ($<$ 1 μ g/mL). This was not found with the spontaneous production of IL-6. As demonstrated in Fig 1, A and B, higher concentrations of the extracts ($>$ 1 μ g/mL) induced production of IL-6 and IL-8 in a dose-dependent fashion. E-ColCF significantly enhanced the production of IL-6 and IL-8 at 2 μ g/mL. Maximal cytokine production was reached with 10 μ g/mL E-ColCF. At concentrations over 10 μ g/mL, the production of IL-6 and IL-8 rapidly declined, reaching levels below baseline with concentrations over 200 μ g/mL E-ColCF. The *Aspergillus fumigatus* skin test

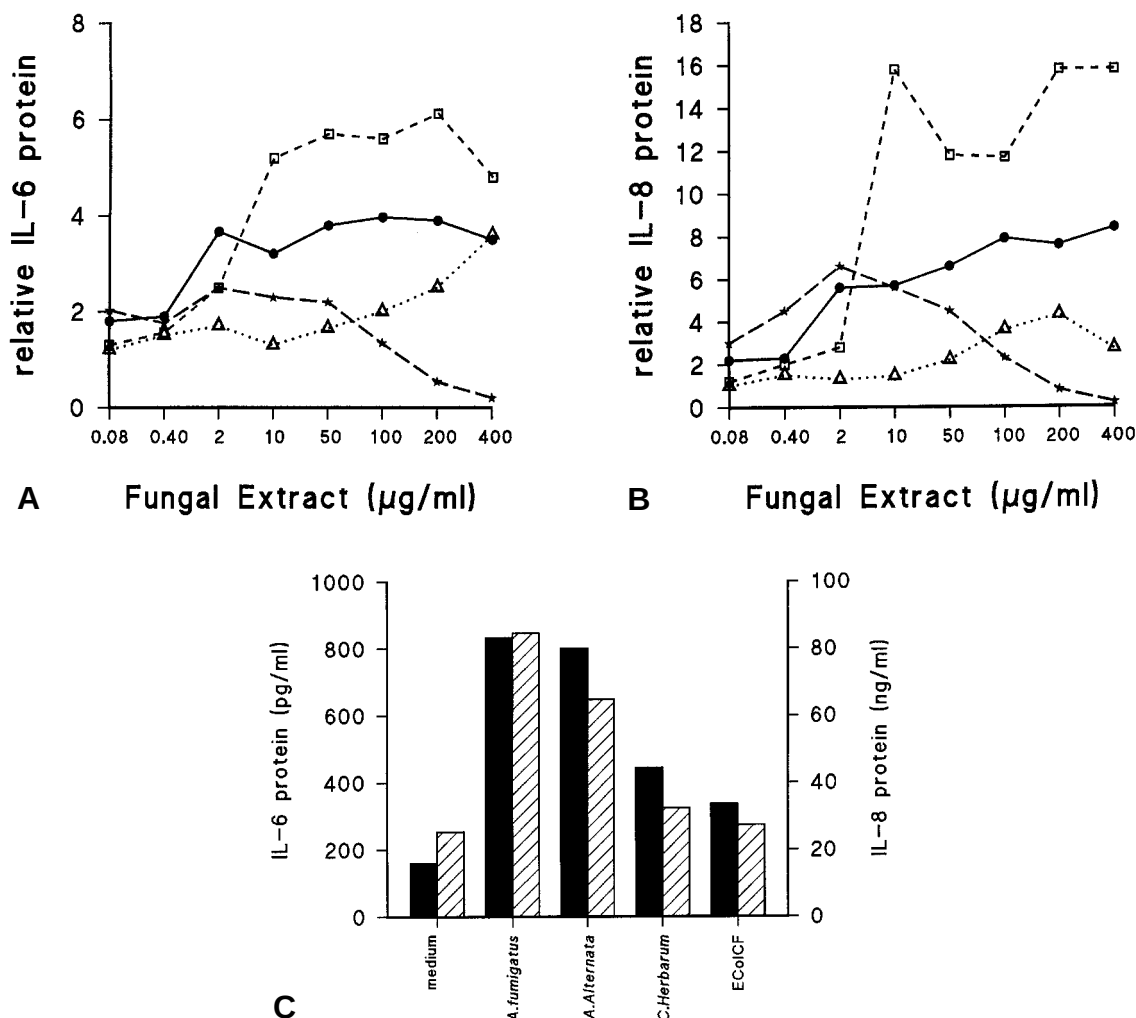


FIG 3. Relative IL-6 (**A**) and IL-8 (**B**) production by primary nasal epithelial cell cultures incubated with various concentrations of fungal extracts: open squares, *Alternaria alternata*; open triangles, *Cladosporium herbarum*; filled circles, *Aspergillus fumigatus*; asterisks, E-ColCF. Data on the y-axis are expressed as the cytokine production relative to the spontaneous production in the absence of fungal extracts. Values are expressed as arbitrary units in which 1 unit corresponds to the spontaneous production of IL-6 of 158 pg/mL and IL-8 of 52.4 ng/mL, respectively. **C** shows the baseline and maximal IL-6 and IL-8 production by primary nasal epithelial cells incubated with fungal extracts. Solid bar indicates IL-6 production (in picograms per milliliter), and hatched bar indicates IL-8 production (in nanograms per milliliter).

extract had a similar effect on cytokine production by A549 cells. At 10 μg/mL, the IL-6 and IL-8 production was significantly enhanced. With the *Aspergillus fumigatus* skin test extract, a maximal cytokine production was found at 50 μg/mL, with progressively lower values at higher concentrations (>50 μg/mL).

Alternaria alternata extracts at concentrations greater than 10 μg/mL significantly increased IL-6 and IL-8 production by A549 cells. Maximal IL-6 production was reached by using 50 and 100 μg/mL *Alternaria alternata* extract, and this produced higher levels of IL-6 than with either *Aspergillus fumigatus* extract. Maximal IL-8 levels were found with 50 and 100 μg/mL, which is comparable with the results obtained with the *Aspergillus fumigatus* extracts. As observed with the E-ColCF extract, high concentrations of the *Alternaria alternata*

extract (400 μg/mL) strongly diminished the production of IL-6 and IL-8, which coincides with complete cell desquamation. The second *Alternaria alternata* extract (AltB) induced similar IL-6 and IL-8 levels as observed with the first *Alternaria alternata* extract (ALK extracts), without causing desquamation of the A549 cells (data not shown). The third *Alternaria alternata* extract (AltE) induced both cytokine production and, at higher concentrations, cell desquamation (data not shown).

The *Cladosporium herbarum* extract induced a dose-dependent increase in IL-6 and IL-8, although the dose-response curve was less steep than that seen with the other extracts. Maximal IL-6 and IL-8 levels were observed at the highest concentration used (400 μg/mL), reaching levels similar to those observed with the *Alternaria alternata* extracts. Fig 2 shows the IL-6 and

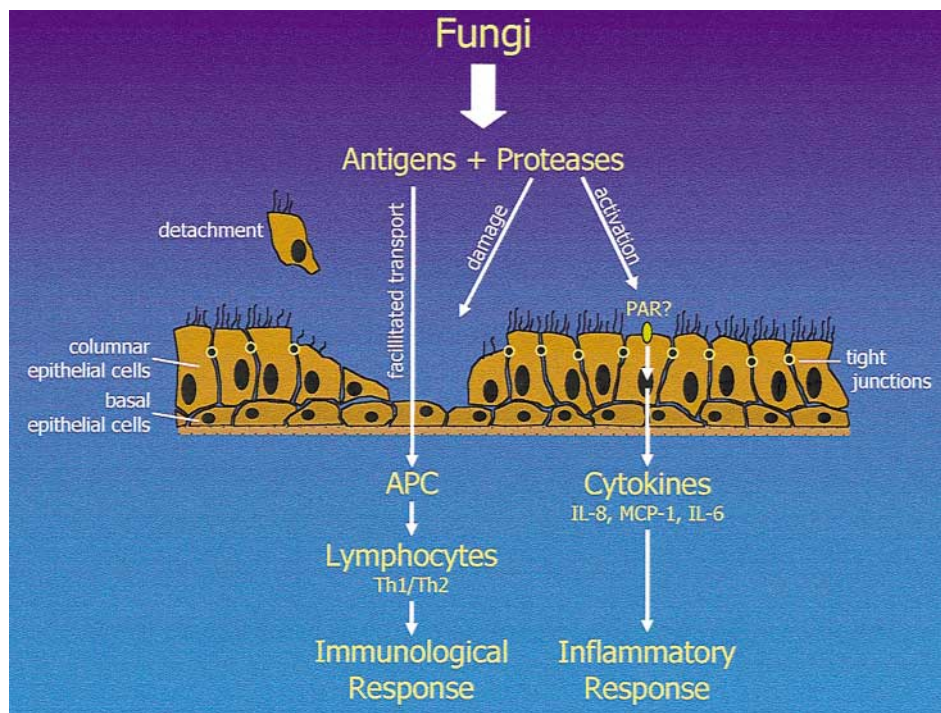


FIG 4. Interaction of fungal proteases with epithelial cells. Cell damage and cytokine release are shown concomitantly with the proposed effects on the inflammatory response and facilitation of the immune response. APC, Antigen-presenting cells; MCP-1, monocyte chemotactic protein-1.

IL-8 production over time by using optimal concentrations of the extracts for cytokine production (derived from Fig 1). As early as 2 hours after beginning incubation with the fungal extracts, IL-6 and IL-8 became detectable and continued to increase over time.

Protease inhibition studies

As shown in Table IV, the fungal extract-induced IL-6 and IL-8 production at 50 $\mu\text{g/mL}$ was strongly inhibited by chymostatin (50 $\mu\text{g/mL}$) and antipain (50 $\mu\text{g/mL}$), indicating that the cytokine production is dependent on serine protease activity in the extracts. Cystatin (50 $\mu\text{g/mL}$) also inhibited in part the production of IL-8 (62%, 37%, and 33% for *Alternaria alternata*, *Cladosporium herbarum*, and E-ColCF, respectively) and IL-6 (81%, 35%, and 66%, respectively), indicating that cysteine proteases are also involved in cytokine production, although to a smaller degree. Similar inhibitions of cytokine production were found at 200 $\mu\text{g/mL}$ fungal extracts, except for the E-ColCF extract, which showed enhanced IL-6 and IL-8 production by A549 cells in the presence of the serine protease inhibitor antipain (not shown). Furthermore, heat treatment of the fungal extracts reduced IL-6 and IL-8 production at all concentrations of fungal extract, except for high concentrations of E-ColCF. Heat treatment of 200 $\mu\text{g/mL}$ E-ColCF showed greater cytokine production than that of the untreated sample (not shown).

Because higher concentrations of E-ColCF reduce IL-6 and IL-8 levels as measured by ELISA, proteolytic

degradation of these cytokines by fungal extract was studied. As shown in Table V, high concentrations of E-ColCF (>200 $\mu\text{g/mL}$) lead to degradation of the IL-1-induced IL-6 and IL-8 protein. However, the degradation shown does not explain the decline in cytokine production seen at lower concentrations of E-ColCF, as is demonstrated in Fig 1. Furthermore, IL-6 appears to be more vulnerable to proteases because some degradation of this cytokine was also found with the ALK *Aspergillus fumigatus* extract (11.4% and 37.2% degradation with 200 and 400 $\mu\text{g/mL}$, respectively) and to a smaller extent with the *Alternaria alternata* extract (16% with 400 $\mu\text{g/mL}$).

Studies in primary nasal epithelial cells

The fungal extract-induced cytokine production by primary nasal epithelial cells is shown in Fig 3. Cultured primary nasal epithelial cells spontaneously produce high levels of IL-6 and IL-8, generally exceeding the maximum levels reached with A549 cells. Nasal epithelial cells are readily activated by both *Aspergillus fumigatus* extracts, with detectable IL-6 and IL-8 production at very low concentrations (0.4 $\mu\text{g/mL}$; Fig 3, A and B). Maximal productions easily reach the nanogram range instead of the picogram range found with A549 cells (Fig 3, C). Although these responses are similar for all fungal extracts, the responses of individual nasal isolates differ strongly with respect to absolute cytokine levels. The production of IL-6 and IL-8 in response to the *Alternaria alternata* extract was, without exception, very high. However, primary nasal cells only weakly responded to

TABLE V. Degradation of IL-6 and IL-8 protein in supernatant of IL-1–stimulated A549 cells by proteases present in several fungal extracts

Fungal extract	Cytokine	Concentration of fungal extract (μg/mL)			
		50	100	200	400
<i>Alternaria alternata</i>	IL-6	0	8	0	16
	IL-8	0	0	0	0
<i>Cladosporium herbarum</i>	IL-6	0	0	0	0
	IL-8	0	0	0	0
<i>Aspergillus fumigatus</i>	IL-6	0	0	11.4	37.2
	IL-8	0	0	0	0
E-ColCF	IL-6	15.9	22.0	48.5*	82.1*
	IL-8	0	0	51.1*	98.3*

Data are expressed as a percentage of control.

* $P < .05$ ($n = 6$).

the *Cladosporium herbarum* extract. E-ColCF induced cytokine production in primary cells similar to that of A549 cells. Cytokine production was enhanced at very low concentrations (0.4 μg/mL), reaching a maximum at 2 μg/mL, followed by a dose-dependent decline for both IL-6 and IL-8. Higher concentrations of E-ColCF (>100 μg/mL) resulted in cytokine production below baseline values (ie, in the absence of fungal protease). In contrast with the findings with A549 cells, *Alternaria alternata* and E-ColCF extracts did not induce cell shrinking or cell desquamation in primary cells.

DISCUSSION

Epithelial cells are important in innate immunity. Aside from their mechanical barrier function, they may also express surface receptors that are able to recognize microorganisms or their soluble components. Our data show that airway epithelial cells interact with proteases from allergenic fungi, resulting in IL-6 and IL-8 release. Earlier observations have shown that proteases from *Aspergillus fumigatus* induce cytokines known to be important in the recruitment of inflammatory cells (IL-6, IL-8, and monocyte chemotactic protein-1),¹⁸⁻²⁰ which may suggest that these activation mechanisms may participate in the epithelial cell defense against microbes. Furthermore, we have recently shown that transcriptional activation is involved in protease-induced epithelial cell activation.²⁰ Similar protease-dependent activation of nuclear factor κB was described for the house dust mite protease Der p 1.²⁸ Epithelial cell activation by fungal proteases may be similar to trypsin-induced PGE₂ production mediated by PAR2 expressed on airway epithelial cells, as was recently described by D'Andrea et al²⁴ and Cocks et al.²⁵ Preliminary experiments performed at our laboratory showed that incubation of A549 cells with the PAR2 agonist NH₂-SLIGKV-C increased IL-6 and IL-8 production, suggesting that functional PAR2 is expressed by A549 cells (not shown). Because antagonists of PAR2 are currently unavailable, additional studies are currently initiated to provide incontrovertible proof that fungal proteases activate epithelial cells by means of this receptor.

Epithelial cells reacted to proteases in a dose-dependent fashion, eliciting production of cytokines by low concentrations of the fungal extracts. Additionally, morphologic changes and desquamation of epithelial cells by fungal extracts were monitored as possible markers of pathogenicity. With the *Cladosporium herbarum* extract, epithelial cells produced cytokines without affecting cell morphology. In contrast, the *Alternaria alternata* and *Aspergillus fumigatus* extracts caused shrinking of A549 cells. Proteolytic degradation of cellular adhesion structures may explain the morphologic changes (shrinking of cells and cell desquamation) induced by the *Alternaria alternata* and *Aspergillus fumigatus* extracts. However, low concentrations of fungal extracts were able to induce cytokine production in A549 cells without affecting cell morphology. In primary cell cultures cytokine production was induced by fungal extracts without morphologic changes, even at high concentrations. These observations suggest that fungal extracts cause epithelial cell activation by means of a mechanism unrelated to changes in cell-matrix interactions, as was shown for BET-1A cells,²⁹ possibly through PAR2. At higher concentrations of fungal extracts, the observed morphologic changes of A549 cells and corresponding cytoskeleton rearrangement may also contribute to the production of cytokines. The fungal extracts of ALK-Abello (*Alternaria alternata* and *Aspergillus fumigatus* extracts) induced cell desquamation with A549 cells at higher concentrations, without causing cell death. Only the *Aspergillus fumigatus* extract E-ColCF was able to cause complete cell desquamation and cell death (30% and 70% cell death at 200 and 400 μg/mL, respectively), indicating destructive proteolytic attack.

Cytokine production induced by the different fungal extracts generally showed a bell-shaped dose-response curve, suggesting a first phase of activation followed by a plateau and diminished activation at higher concentrations. The lower production of cytokines at higher concentrations of fungal extracts may be due to either inactivation of the epithelial cells (eg, inactivation of PAR2) or proteolytic degradation of cytokines. Degradation of IL-1–induced IL-8 protein was found only at high concentrations of the E-ColCF extract, whereas IL-6 also

underwent degradation by the ALK *Aspergillus fumigatus* extract and to a small extent by the *Alternaria alternata* extract. Therefore the decreased production of IL-6 and IL-8 at concentrations of 5 to 100 µg/mL EColCF cannot be explained by degradation of cytokines and may be due to inactivation of the epithelial cells. The mechanism of inactivation of epithelial cells by *Aspergillus fumigatus* proteases is not known but could be due to inactivation of PAR2. Such protease-dependent PAR inactivation as has been shown for tryptase and chymase on keratinocytes and by pancreatic trypsin for PAR2 on intestinal epithelial cells.^{30,31} Because total loss of activation of cytokine production is not found with the other fungal extracts, it is tempting to speculate that this inactivation of epithelial cells by E-ColCF may be related to the pathogenic capacity of *Aspergillus fumigatus*.^{19,26,27}

Protease inhibitors and heat treatment showed almost total blockade of the cytokine production by A549 epithelial cells. This observation indicates a limited role for LPS-induced cytokine production. Although intuitively unexpected, this observation is in accordance with previous studies on *Aspergillus fumigatus*-induced cytokine production, showing almost no influence of LPS blocking agents.¹⁹

Compared with A549 cells, primary epithelial cells spontaneously produce higher levels of IL-6 and IL-8, which were further increased by low concentrations of fungal extracts. *Alternaria alternata* extract was most potent in inducing cytokine production in primary epithelial cells. None of the fungal extracts studied were able to induce shrinking or cell desquamation in the primary cultures, as was observed with the A549 cells. The reason for these differences are not known. A possible explanation could be that primary cells form stronger adhesion structures that are not easily disrupted by exogenous protease activity.

Our data demonstrate the presence of different proteolytic enzymes with serine, cysteine, and aspartic protease activities in the fungal extracts. Analysis of the protease activity in these fungal extracts, however, did not identify a causative protease or proteases for either cell desquamation or cytokine production. Studies with purified fungal proteases will be needed to determine the effect of individual proteases. Our data suggest, however, that fungal extracts with a high protease content, such as the *Aspergillus fumigatus* extracts, contain specific proteases that activate epithelial cells, whereas other proteases may be responsible for cell desquamation, inactivation of epithelial cells, or both. Damage to the epithelial cell layer may result in augmentation of mucosal permeability, as has been shown for the cysteine proteinase Der p 1 of house dust mite. Functional changes in the barrier function of bovine airway preparations by Der p 1 were shown, resulting in the facilitation of the passage of albumin over the mucosal membrane.¹⁷

Alternaria alternata extracts showed a high protease-dependent activation, especially with primary nasal epithelial cells. Furthermore, it has been reported that the germination of *Alternaria alternata* spores is much faster

than germination of most other airborne fungal spores.³² The combination of high-rate spore germination of *Alternaria alternata* and its high protease activity may contribute to the relatively high rate of sensitization found for this fungus^{3,6,7,33} and its association with severe asthma.^{34,35}

Our findings have shown that fungi- and house dust mite-derived proteases¹⁸ may cause both damage and activation of airway epithelial cells. Damage to the epithelial membrane may result in augmentation of the passage of macromolecules (eg, antigens) over the mucosal membrane, whereas activation resulting in release of cytokines may induce an inflammatory response in the airway tissue (Fig 4). These implications of protease-induced epithelial effects are important in view of recent observations by Pearce et al,³⁶ who showed that asthmatic reactions are only partly mediated by atopic mechanisms. It has recently been proposed that the bronchial epithelium may respond to a proteolytic attack by the induction of repair mechanisms. A functionally inadequate repair response observed in asthmatic subjects may result in the release of cytokines and concurrent inflammation and may provide an explanation as to why asthmatic manifestations also occur in nonatopic asthma.³⁷ We propose that proteases of fungal origin induce a similar repair response in airway epithelial cells of asthmatic subjects, thereby facilitating both the passage of allergens over the epithelium followed by sensitization and initiation of an inflammatory response (Fig 4).

In conclusion, we provide evidence that proteases present in fungal extracts interact with epithelial cells in several ways, leading to morphologic changes, cell desquamation, and production of the proinflammatory cytokines IL-6 and IL-8. These effects are dependent on the enzymic activity of these proteases because they may be abrogated by protease inhibitors. It is proposed that these fungal proteases may activate epithelial cells through a PAR2-driven mechanism, as is suggested by preliminary data on the stimulation of epithelial cells with a known agonist, showing similar effects.

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