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Dust Mite Proteolytic Allergens Induce Cytokine Release from Cultured Airway Epithelium¹

Cecile King,^{2*} Siobhan Brennan,[†] Phillip J. Thompson,[‡] and Geoffrey A. Stewart^{3*}

Endogenous proteolytic enzymes have been shown to be potential sources of airway inflammation inducing proinflammatory cytokine release from respiratory epithelial cells; however, whether any of the exogenous proteases from important allergen sources such as the house dust mite present in our environment behave in a similar fashion is unclear. To this end, we have investigated whether the mite cysteine and serine proteolytic allergens, Der p 1 and Der p 9, respectively, induced cytokine production from primary human bronchial epithelial cells and from the epithelial cell line BEAS-2B. Cells were exposed to mite proteases, and cells or supernatants were assayed for cytokine release, cytokine mRNA expression, and modulation of intracellular calcium ion concentration. Both proteases induced concentration- and time-dependent increases in the release of granulocyte-macrophage (GM)-CSF, IL-6, and IL-8 as well as an increase in the expression of IL-6 mRNA. Cytokine release and mRNA expression were first observed at 8 h and 2 h after protease exposure, respectively. The minimum concentration of each protease that was required to stimulate GM-CSF, IL-6, and IL-8 release was ~10 ng/ml. Cytokine release was initiated by 1 to 2 h of protease exposure, although maximum concentrations were detected only after a 24-h incubation. IL-6, but not IL-8 and GM-CSF, was shown to be degraded by both proteases at concentrations of >2 µg/ml. The proteases also stimulated changes in the intracellular calcium ion concentration. All mite protease-induced phenomena were inhibited using appropriate protease inhibitors. These results suggest that the proteolytic activity of an allergen may stimulate the release of proinflammatory cytokines from human bronchial epithelium. *The Journal of Immunology*, 1998, 161: 3645–3651.

Asthma is a common, chronic disease in both children and adults, with a prevalence of at least 5% worldwide. It continues to have a significant clinical and socio-economic impact on Western communities despite the introduction of a variety of pharmacologic treatments. The inflammatory response observed in asthma is complex and represents the end result of the activation of cells such as T cells, mast cells, eosinophils and, to a lesser extent, neutrophils as well as the release of a range of physiologically important products that play major roles in both the acute and chronic phases of allergic inflammation. The effects of inflammation are manifested in a number of ways, but those effects that are evident in the airway mucosa involving both injury and the activation of epithelial cells are of particular interest.

The human respiratory epithelium is a critical component of the innate immune system, acting as both a physical barrier to the environment and a modulator of local airway inflammation because of its capacity to synthesize a variety of mediators including cytokines, prostanoids, leukotrienes, nitric oxide, antioxidants, and complement components (1–3). Each of these mediators possesses the capacity to influence the activation and migration of inflam-

matory cells, modulate smooth muscle tone, or initiate mucosal repair in respiratory disease. In asthma, the respiratory epithelium releases cytokines such as IL-1, IL-6, granulocyte-macrophage (GM)-CSF,⁴ TNF-α, and TGF-β, as well as chemokines such as IL-8, macrophage inflammatory protein-1, and RANTES (2, 4, 5); such mediators are likely to be particularly important in the pathogenesis of this disease.

The precise mechanism(s) underlying the release of cytokines from respiratory epithelium is unclear, but a variety of factors have been identified. These factors include eosinophil granule proteins, ozone, isocyanates, other cytokines such as IL-1 and TNF-α, viruses, and bacteria (2, 6–8). More recently, studies have shown that endogenous proteases from neutrophils (9), mast cells (10), and cytotoxic T cells (11) also stimulate the release of cytokines from this tissue. Interestingly, the airway epithelium is also exposed to exogenous proteases in the form of allergens, particularly those originating from the house dust mites *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*. These allergens include the group 1 cysteine proteases (12), the group 3 trypsin (13), the group 6 chymotrypsins (14), and the recently identified group 9 collagenolytic serine proteases (15).

Interestingly, we have shown that these proteases possess the capacity to modulate respiratory mucosal permeability and vascular permeability (16, 17); this observation suggests that allergens possess the capacity to modulate epithelial function. On this basis, we have undertaken studies to determine whether mite allergens also initiate cytokine release from respiratory epithelium. The data show that the cysteine protease Der p 1 and the serine protease Der p 9 allergens from *D. pteronyssinus* enhance the release of cytokines from both primary cultures of human bronchial epithelium and the bronchial epithelial cell line BEAS-2B. This release was

*Department of Microbiology, University of Western Australia, Nedlands, Western Australia; [†]TVW Telethon Institute for Child Health Research, West Perth, Western Australia; and [‡]Department of Medicine, University of Western Australia, Queen Elizabeth II Medical Center, Nedlands, Western Australia

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² Current address: Department of Immunology, The Scripps Research Institute, 10666 North Torrey Pines Road, La Jolla, CA 92037.

³ Address correspondence and reprint requests to Dr. Geoffrey A. Stewart, Department of Microbiology, University of Western Australia, Nedlands, Western Australia 6907.

⁴ Abbreviations used in this paper: GM-CSF, granulocyte-macrophage CSF; SBTI, soybean trypsin inhibitor; PAR, proteinase-activated receptor; α₁-AP, α₁-antitrypsin; GAPDH, glyceraldehyde phosphate-3-dehydrogenase.

dependent upon protease activity per se and was accompanied by changes in the flux of intracellular calcium ions.

Materials and Methods

Extracts and chemicals

Extracts of *D. pteronyssinus* were prepared from fecally enriched spent growth medium as described previously (13). General chemicals were purchased from BDH (Kilsyth, Australia), and synthetic protease substrates and other reagents were purchased from Sigma (St. Louis, MO) unless otherwise stated.

Purification of mite protease allergens

Der p 1 and Der p 9 were isolated as described previously (15). In brief, Der p 1 was isolated by mAb affinity chromatography, and Der p 9 was isolated using a combination of ion exchange chromatography and soybean trypsin inhibitor (SBTI) affinity chromatography. The proteolytic activities of Der p 1 and Der p 9 were determined as described previously (15) using the insoluble collagen substrate, Azocoll (Calbiochem, Alexandria, Australia) and the synthetic substrate succinyl-alanyl-alanyl-prolyl-phenylalanyl-p-nitroanilide, respectively.

Primary epithelial cell culture

Human bronchi were obtained from thoracotomy specimens as described previously (18) and were dissected to be free of all visible blood vessels and parenchyma; primary epithelial cell cultures were derived from explants. Briefly, tissue pieces were stripped from the airway wall and cut into 2-mm² pieces that were then orientated epithelial surface uppermost on plastic culture dishes; the dishes had been previously rinsed with serum-free tissue culture medium consisting of LHC basal medium (Biofluids, Rockville, MD) supplemented with insulin, hydrocortisone, epidermal growth factor, bovine hypothalamus extract, cholera toxin, transferrin, and retinol acetate as described previously. The tissue explants were allowed to adhere onto rat type I collagen-coated (30 µg/ml) plastic culture dishes at 37°C for 16 h after which supplemented basal medium was added gently to avoid dislodging the tissue. The medium was changed at 2- to 3-day intervals until the outgrowths had reached the edge of the dish (12–20 days). The explants were removed, and cells were cultured for an additional 24 h before use in basal medium that was devoid of additives.

Characterization of epithelial cells

The purity of the primary epithelial cell cultures was confirmed by staining with anti-cytokeratin Abs. Cells were fixed in methanol for 10 min, rinsed with 0.01 M of PBS (pH 7.2), and stained with an anti-cytokeratin mAb (clone LP34; Dako, Botany, Australia) at a dilution of 1/50 in PBS for 30 min at room temperature. The slides were washed in PBS, and rabbit alkaline phosphatase-conjugated anti-mouse IgG was applied for 30 min at room temperature; the slides were subsequently rinsed, and the reaction was visualized using fast red substrate. Only epithelial cell cultures showing <3% contamination with fibroblasts were studied.

Human bronchial epithelial cell line culture (BEAS-2B)

BEAS-2B, an SV40-transformed human bronchial epithelial cell line, was kindly provided by Prof. C. C. Harris (National Institutes of Health, Bethesda, MD) and maintained at 37°C/5% CO₂ on collagen-coated plastic culture dishes in supplemented LHC medium (19). Cells were passaged by incubating the monolayer with prewarmed 0.05% (w/v) trypsin (Life Technologies, Melbourne, Australia)/0.02% (w/v) EDTA solution for 10 min at 37°C. Basal medium containing 20% (w/v) SBTI was added to the cell suspension, and the cells were pelleted by centrifugation (1200 rpm for 5 min). Following centrifugation, the cells were resuspended in 1 ml of basal medium, counted by trypan blue exclusion to determine cell number and viability, and plated at a concentration of 1×10^5 cells/ml in 200-µl volumes in 24-well plates.

Stimulation of epithelial cells in culture

Epithelial cells were washed and cultured in basal medium at 24 h before stimulation, and $\sim 5 \times 10^6$ confluent cells were incubated with either Der p 1 or Der p 9, Der p 1 or Der p 9 plus appropriate inhibitors (E-64, SBTI, and α_1 -antiprotease (α_1 -AP)), protease inhibitor alone, heat-inactivated protease (65°C for 15 min), polymyxin B, rIL-1, LPS, or basal medium. Experiments were also performed using the bovine proteases thrombin (Rhône-Poulenc Rorer, Victoria, Australia) and trypsin. All additives were prepared in prewarmed (37°C) basal medium, and inhibitors were incubated with protease for 10 min at 37°C before being added to cell

cultures. Supernatant or cells from protease-treated epithelium and non-treated controls were obtained and tested for the presence of various cytokines or cytokine mRNA as described below. The possible cytotoxic effects of various additives were assessed by trypan blue exclusion.

Cytokine assays

The presence of GM-CSF in the supernatants was determined by bioassay using the GM-CSF/IL-3-dependent cell line FDC-P1 (1×10^4 cells/well), which was kindly provided by Professor P. G. Holt (TVW Telethon Institute for Child Health). The proliferation of cells in response to the cytokine was determined by the incorporation of [³H]thymidine (Amersham, Sydney, Australia), which was added at 1 µCi/well for the last 4 h of the 3-day incubation period. Cells were harvested on fiberglass filters, and the radioactivity was measured by scintillation counting. The amount of GM-CSF was determined using rGM-CSF, which was kindly provided by Dr. A. S. McWilliam (TVW Telethon Institute for Child Health). Specificity was confirmed using a blocking goat anti-human GM-CSF (R&D Systems, Minneapolis, MN). Exposing the FDC-P1 cells to protease alone did not elicit proliferation. The concentrations of IL-6 and IL-8 were measured by a capture ELISA. For the IL-6 ELISA, the capture Ab used (1 µg/ml) was a goat anti-human IL-6 polyclonal Ab (R&D Systems), and the detection Ab used was a mouse anti-human IL-6 mAb (BioSource International, Camarillo, CA) at 1 µg/ml; the standard used was human rIL-6, which was kindly provided by Dr. R. J. Simpson (Joint Protein Structure Laboratory, Ludwig Institute for Cancer Research and the Walter and Elisa Hall Institute for Medical Research, Parkville, Australia). For the IL-8 ELISA, the capture Ab used (2 µg/ml) was a rabbit anti-human IL-8 polyclonal Ab (R&D Systems), and the detection Ab used was a mouse monoclonal anti-IL-8 at 2 µg/ml; the standard used was human rIL-8 (BioSource International). Horseradish peroxidase-conjugated sheep anti-mouse Ig (Amersham) and tetramethylbenzidine substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD) were used to detect secondary Ab binding in both assays. The enzyme reactions were stopped with 1 M sulfuric acid, and ODs were determined at 450 nm. The assays were log-linear from 0.1 to 10 ng/ml of cytokine. All data were expressed as nanograms or picograms of cytokine released per 10^6 cells, and the significance of the differences in mean responses was determined using the Student *t* test.

Northern blot analyses

RNA was extracted from mite protease-treated or nontreated control epithelial cell cultures with RNazol (Bresatec, Adelaide, Australia), electrophoresed on 1% (w/v) agarose gels containing 37% (v/v) formaldehyde before transfer onto a nylon membrane (Hybond N⁺, Amersham), and probed for IL-6 using 1000-bp *Bam*HI fragments that were kindly provided by DNAX (Palo Alto, CA). Control experiments were performed using a glyceraldehyde phosphate-3-dehydrogenase (GAPDH) probe that was kindly provided by Associate Prof. U. Kees (TVW Telethon Institute for Child Health). Each cDNA fragment was labeled with [³²P]deoxyATP by random priming, and hybridization was performed sequentially at 65°C. The intensities of the signals were determined by densitometric quantification and normalized to the responses that were obtained with the GAPDH probe.

Measurement of cytosolic free calcium (Ca²⁺)

Intracellular Ca²⁺ signaling following stimulation with dust mite proteases was determined in three separate experiments using real-time fluorescence measurements at the single-cell level. BEAS-2B cells were grown to confluence on optical grade circular glass coverslips (22-mm² and 0.14–0.16-µm thickness) and loaded intracellularly with 1 µM Indo-1/AM (Calbiochem) in loading buffer (145 mM NaCl, 5 mM KCl, 1 mM Na₂HPO₄, 2.5 mM CaCl₂, 10 mM glucose, 25 mM HEPES, and 0.5 mM MgSO₄) for 30 min at 37°C. Cells were gently washed in loading buffer, and real-time fluorescence measurements were obtained digitally using an interactive laser cytometer (ACAS 470, Bio-Rad, Regents Park, Australia). Proteases were added to the cells after a 25-s scan of a group of 10 to 20 adherent BEAS-2B cells, and scanning was continued for another 100 s. Changes in cytosolic-free calcium were expressed as the ratio of the fluorescence obtained using detector 1 (free Indo-1, 485 nm) to that obtained with detector 2 (Indo-1: Ca²⁺ complex, 405 nm).

Results

Dust mite proteases induce cytokine release from human bronchial epithelium

Initial experiments were performed to determine whether the mite proteases caused cytokine release from BEAS-2B cells. These

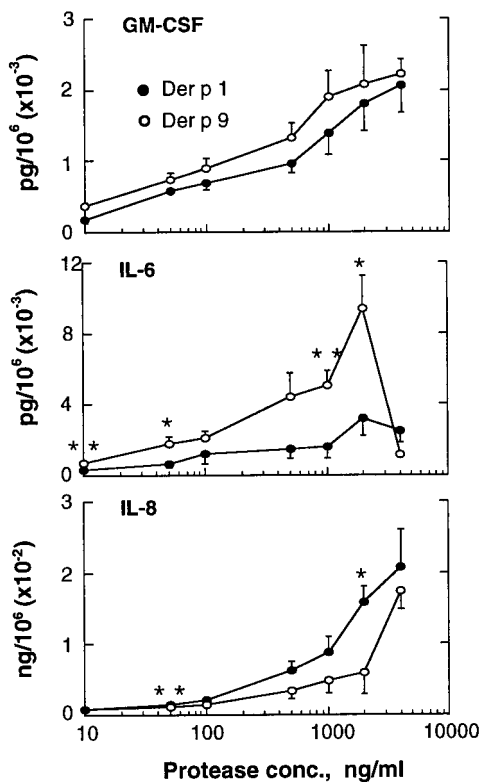


FIGURE 1. Dust mite proteases promote cytokine release from the human respiratory epithelial cell line BEAS-2B in a dose-dependent manner. Cell monolayers were cultured for 24 h with basal medium that was devoid of growth factors and then stimulated with increasing concentrations of either Der p 1 or Der p 9 for 24 h. The presence of GM-CSF in supernatants was determined by bioassay, whereas IL-6 and IL-8 were measured by ELISA. Data are expressed as the means \pm SEM per 10^6 cells from three independent experiments that were performed in duplicate. The statistical significance of the differences in means between Der p 1- and Der p 9-induced responses at various concentrations was determined by the Student *t* test; *, $p < 0.05$; **, $p < 0.02$; ***, $p < 0.01$; ****, $p < 0.001$.

studies showed (Fig. 1) that both Der p 1 and Der p 9 caused the release of GM-CSF, IL-6, and IL-8 in a dose-dependent manner after 24 h of exposure. Statistically significant cytokine responses above background were observed with 10 ng/ml of either protease for all cytokines with the exception of Der p 1-induced GM-CSF responses, which required a concentration of 50 ng/ml. For GM-CSF, maximum responses were observed at $\sim 2 \mu\text{g/ml}$, whereas maximum responses were not achieved with either IL-6 and IL-8 at the maximum concentration tested, namely 4 $\mu\text{g/ml}$. With IL-6, a diminution in cytokine response was observed after stimulation with both proteases but was most marked with Der p 9. Subsequent studies (Table I) showed that IL-6 was susceptible to proteolysis by both Der p 1 and Der p 9. There was a significant trend for higher concentrations of IL-6 to be produced by Der p 9 than by Der p 1, but this trend was reversed for IL-8 (Fig. 1). Table II shows the mean concentrations of the IL-6, IL-8, and GM-CSF released by the two mite proteases at a concentration of 100 ng/ml compared with those released using the mammalian proteases trypsin and thrombin. It is evident that, with the exception of the trypsin-induced GM-CSF release, all proteases induced significant cytokine release.

Time-course experiments were then performed with the mite proteases at a concentration of 100 ng/ml using both the BEAS-2B cell line and primary human bronchial epithelium cultures. The data obtained (Fig. 2) indicated that the earliest timepoint at which

Table I. The susceptibility of IL-6 to mite proteases^a

Protease	Concentration ($\mu\text{g/ml}$)	Percent of IL-6 Remaining After:		
		4 h	8 h	24 h
Der p 1	2	100	98	99
	3	72	74	66
	4	24	0	0
Der p 9	2	67	62	53
	3	33	0	0
	4	0	0	0

^a A total of 1 μg of rIL-6 was incubated with varying concentrations of mite protease for 4, 8, or 24 h, and the concentration of IL-6 remaining was determined by ELISA.

the statistically significant mean concentrations of the three cytokines studied could be detected for the BEAS-2B cell line was at 8 h after treatment with both proteases. Similar results were obtained for the GM-CSF and IL-6 released from primary cell cultures after treatment with Der p 1 and Der p 9; statistically significant mean differences in IL-8 production were not observed, although there was a trend for higher concentrations to be produced. There was a tendency for the primary cultures to produce greater mean GM-CSF release with both proteases, although this tendency was not significant. For the BEAS-2B cell line, significantly more IL-6 was produced after 8 h of incubation with Der p 9 than Der p 1 ($p < 0.02$). The effects of varying the period of protease exposure on the accumulated release of IL-6 and IL-8 at 24 h were subsequently determined using the BEAS-2B cell line. These studies showed (Fig. 3) that the release of both cytokines was evident after a 30-min exposure and corresponded to ~ 20 to 60% of the maximum amount released. By 1 h, the amount of IL-6 released by Der p 9 was maximal, and maximal IL-6 release was achieved after 2 h with Der p 1. Maximal IL-8 release with both proteases was achieved by a 2-h exposure. Trypan blue dye exclusion studies showed that the effects that were observed at the protease concentrations used were not related to cell death; these findings are in accord with those from previous studies using mite enzymes (16).

Dust mite proteases increase cytokine mRNA levels in human bronchial epithelial cells

Northern blot analyses demonstrated that IL-6 mRNA transcripts were undetectable in resting BEAS-2B cells (Fig. 4); however, cytokine mRNA levels increased in cells treated with mite proteases, reaching a maximum at 2 h postexposure and subsiding after 8 h. This modulation was not observed with GAPDH (Fig. 4).

Specific protease inhibitors abolish the up-regulation of cytokine production and release from human bronchial epithelium

The mechanism of cytokine induction from human bronchial epithelial cells was investigated by preincubating 100 ng/ml of each protease allergen with the appropriate inhibitors. For the serine protease Der p 9, this incubation included SBTI and α_1 -AP; for the cysteine protease Der p 1, the incubation included E-64. The effect of α_1 -AP on Der p 1 was also assessed, given recent claims that protease activity could be inhibited by this protein (20). The data obtained indicated that IL-6 and IL-8 release (Fig. 5) by both Der p 1 and Der p 9 was abolished when protease activity per se was inhibited, and that α_1 -AP had no effect on the cytokine-inducing effects of Der p 1. Similarly, IL-6 mRNA expression was ablated when both proteases were inhibited (Fig. 4). As some protease inhibitors have been shown to block the activation of the transcription factor NF- κ B as well as subsequent cytokine gene activation

Table II. Effect of various mite and non-mite proteases on cytokine release from the human bronchial epithelial cell line BEAS-2B

Protease (100 ng/ml)	Cytokine (mean pg (SEM)/10 ⁶ BEAS-2B cells)					
	GM-CSF	(<i>p</i> <) ^a	IL-6	(<i>p</i> <)	IL-8	(<i>p</i> <)
None (medium alone)	105 (19)		108 (28)		2,980 (450)	
Der p 1	712 (95)	0.001	1,108 (289)	0.02	18,900 (3,600)	0.01
Der p 9	890 (135)	0.001	1,794 (324)	0.001	17,500 (2,330)	0.001
Thrombin	734 (82)	0.001	1,626 (297)	0.001	12,900 (2,146)	0.01
Trypsin	112 (34)	NS	986 (145)	0.001	5,600 (780)	0.05

^a Differences in mean values for individual proteases compared with those obtained with medium alone were determined using the Student *t* test (*n* = 6).

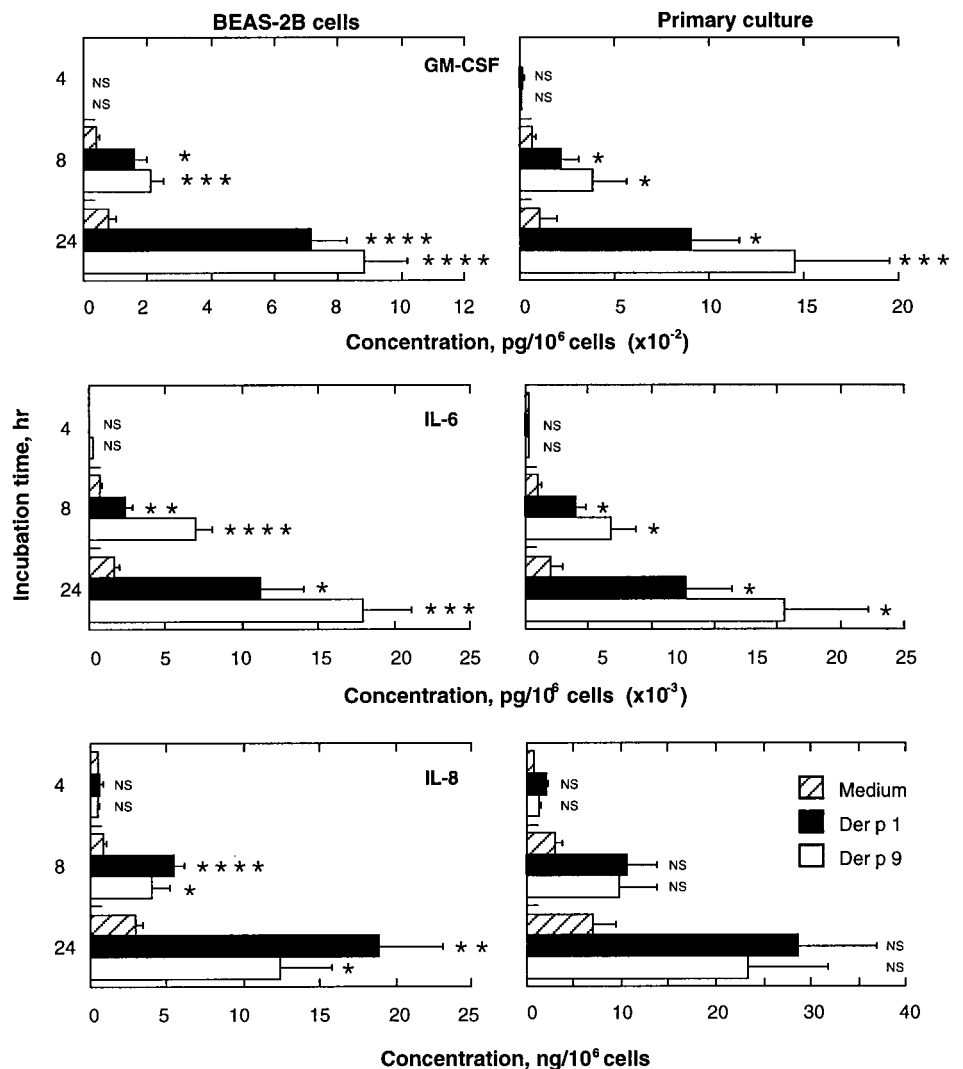
(21), the effect of SBTI on IL-1 β -induced cytokine release from BEAS-2B cells was investigated. These studies showed that this inhibitor did not affect release at the concentrations used. The cytokine-releasing effects of the mite proteases did not appear to be associated with LPS, since adding the endotoxin antagonist polymyxin B did not affect cytokine release; heat inactivation of protease activity did affect the release of cytokines however (Fig. 5).

Changes in intracellular calcium induced by dust mite proteases

The effects of Der p 1 and Der p 9 on intracellular calcium (Ca²⁺) in BEAS-2B cells that had been loaded intracellularly with the

Ca²⁺-sensitive fluorescent dye Indo-1 were determined using cell monolayers grown on glass coverslips. Both mite proteases induced changes in (Ca²⁺)_i in 90% of the cells studied. Figure 6 shows the representative data that were obtained with cells that had been treated with 1 μ g/ml of Der p 1 and Der p 9; changes occurred within 10 s of protease addition. There was a slight variation in response from cell to cell in terms of response time and the intensity of response, particularly with the cells at the edge of the coverslip, which responded more quickly. The increases in (Ca²⁺)_i that were induced by Der p 9 and Der p 1 were inhibited by SBTI and E-64, respectively. Preincubating BEAS-2B cells with Der p 1

FIGURE 2. Dust mite proteases promote cytokine release from the human respiratory epithelial cell line BEAS-2B and from primary cultures of human bronchial epithelium in a time-dependent manner. Cell monolayers were cultured for 24 h with basal medium that was devoid of growth factors and then stimulated with basal medium or 100 ng/ml of Der p 1 or Der p 9. The supernatants were harvested at 2, 4, 8, and 24 h, and the presence of cytokines was determined as described in Figure 1. Data are expressed as the means \pm SEM from four independent experiments that were performed in duplicate. Because cytokines were not detected at 2 h after protease exposure, this timepoint was excluded from the figure. The statistical significance of the differences in means between the protease-induced responses and control responses at the timepoints indicated was determined by the Student *t* test; *, *p* < 0.05; **, *p* < 0.02; ***, *p* < 0.01; ****, *p* < 0.001.



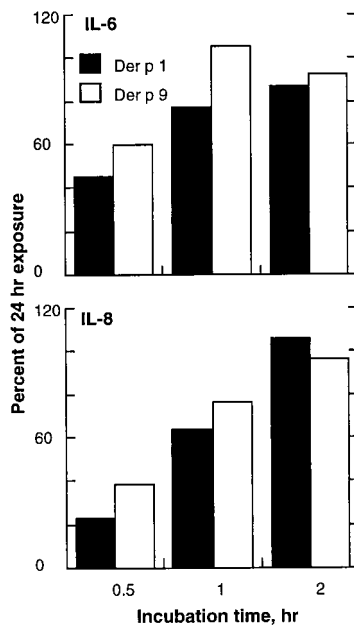


FIGURE 3. The effect of Der p 1 and Der p 9 exposure on the release of IL-6 and IL-8 from BEAS-2B cells. Cells were incubated with protease for the times indicated, and culture medium was removed. The cells were then washed and reincubated with basal medium until a combined incubation period of 24 h had elapsed. Data are expressed as a percentage of the response that was obtained after 24 h of continuous protease exposure.

had little influence on the capacity of the cells to respond to a subsequent challenge with Der p 9. However, preincubating the cells with bovine trypsin suggested a diminution in response to a subsequent challenge with Der p 9.

Discussion

This study has demonstrated that the dust mite proteolytic allergens Der p 1 and Der p 9 trigger the release of IL-6, IL-8, and GM-CSF from both BEAS-2B cells and primary cultures of human bronchial epithelium in a dose- and time-dependent manner. Our data are consistent with those showing that Der p 1 up-regulates the activity of the transcription factors that are associated with the secretion of these cytokines in bronchial epithelial cells (22). The responses obtained with the transformed cell line were similar to those obtained with freshly cultured cells, supporting the use of the

transformed cells for more detailed studies. However, there was a trend toward a greater cytokine release with primary cultures that was consistent with previously reported data (23). Cytokine release was significant at 8 h after protease exposure in both the cell line and the primary cultures, but mRNA for one of the cytokines studied, namely IL-6, was detected at 2 h. In addition, cytokine release was found to be initiated by a limited exposure (30 min) to either mite protease, and the maximum attainable response was achieved after 1 to 2 h of exposure.

The release of both IL-6 and IL-8 by Der p 1 and Der p 9 was found to be dependent upon biochemical activity, since both cytokines were not detected when the proteases were inhibited by the appropriate inhibitors. In this regard, the cytokine-releasing properties of Der p 1 were not affected by α_1 -AP. These data are consistent with those indicating that this enzyme is a true cysteine protease (12, 24, 25) rather than a cysteine-serine protease (20). Cytokine release was not related to LPS contamination and did not appear to be associated with cell death. The responses that were obtained with the cysteine protease allergen Der p 1 and the serine protease allergen Der p 9 were similar, although the latter tended to be more effective than Der p 1 at stimulating IL-6. However, the situation was reversed for IL-8 responses, suggesting that the two proteases may be acting on different targets, as their proteolytic specificities are likely to be distinct. Our data also showed that IL-6, in contrast to IL-8 and GM-CSF, was susceptible to proteolytic cleavage; this finding is in accord with previous observations (26, 27).

These studies show that enzymatically active allergens produce cytokine responses in bronchial epithelial cells in vitro, but it is more difficult to assess whether mite protease exposure in vivo evokes similar responses or whether, if similar effects occur, the concentrations of the cytokines released are of physiologic significance. However, it is clear that the cytokine concentrations observed in this study are likely to be biologically active given a variety of in vitro data (28, 29); it is also apparent that the severity of asthma as well as respiratory symptomology (coughing, wheezing, and breathlessness) correlate with Der p 1 and general protease concentrations in dust, respectively (30–32). Similarly, it is not clear whether exogenous protease activity is inhibited by one or more of the inhibitors that are known to be present in the lung given the observation that Der p 9 may be inhibited by α_1 -AP and also our unpublished observations showing that Der p 1 may be inhibited by cystatin C. In this regard, it has been shown that Der p 1 cleaves α_1 -AP in vitro (33) in a manner that is similar to that reported for papain; this finding indicates that the lung protease

FIGURE 4. Northern blot analysis showing the effects of mite proteases on IL-6 cytokine mRNA expression as determined using ³²P-labeled cDNA probes at the timepoints indicated. The left panels show the effects of the protease incubation time on expression; the right panels show the effect of pre-treating the cysteine protease Der p 1 with E-64 and the serine protease Der p 9 with SBTI. Inhibitor was added (+), and mRNA was detected after incubating cells for the times indicated. Cells were also incubated with proteases that had not been treated with inhibitor (–). Control experiments were performed using a cDNA probe for GAPDH.

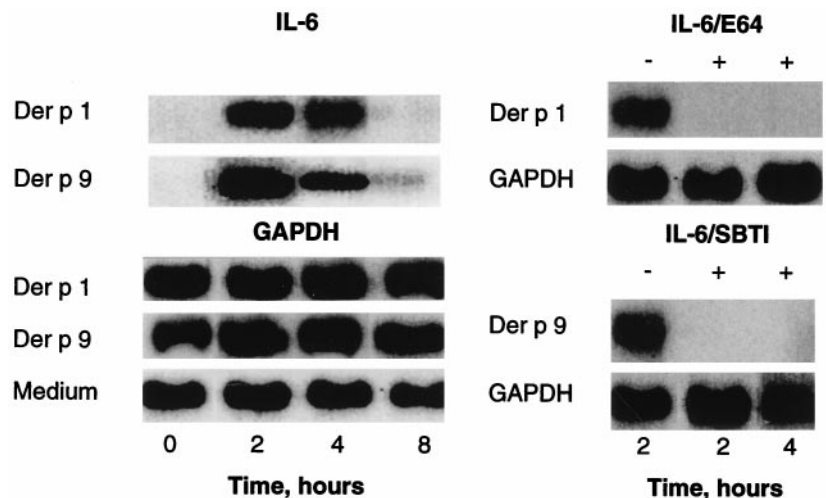
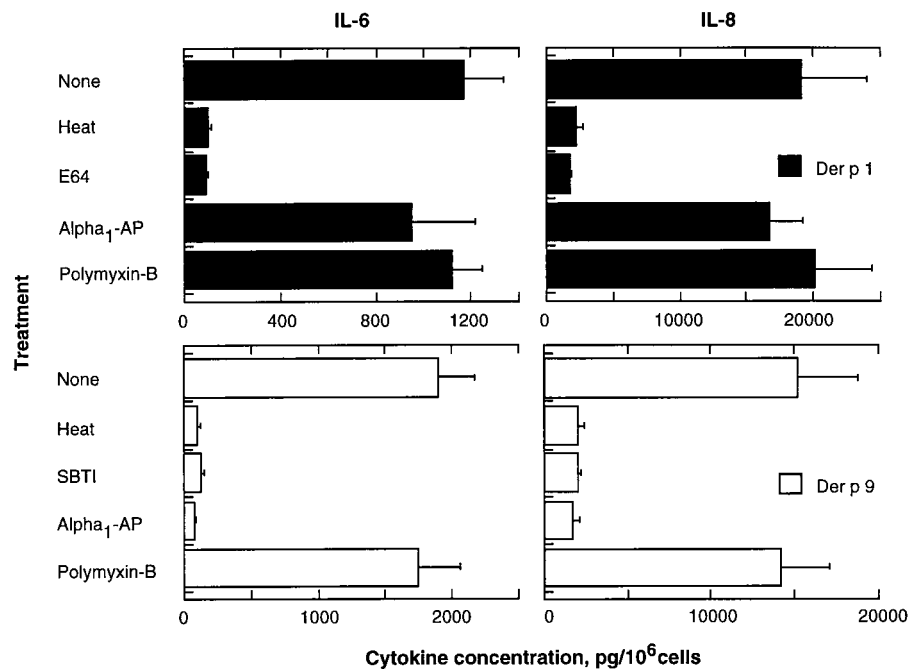


FIGURE 5. The mechanism of cytokine induction from human bronchial epithelial cells was investigated by incubating Der p 1 or Der p 9 with a variety of agents including SBTI (10 ng/ml), E-64 (10 ng/ml), α_1 -AP (100 ng/ml), and polymyxin B (50 μ g/ml) for 20 min at 37°C before their addition to BEAS-2B cultures. Cytokine release was measured by ELISA following 24 h of incubation with proteases and additives, and responses were compared with those that had been obtained with protease alone (none) and with heat-inactivated protease (heat; 60°C for 20 min). Data are expressed as the means \pm SEM of three independent experiments that were performed in triplicate.



inhibitor screen may be compromised on allergen entry. Finally, although the precise concentration of mite allergen that is deposited on respiratory epithelium has yet to be determined, studies have shown that the concentration of Der p 1 that is required to elicit cytokine release in our studies is within an order of magnitude of that demonstrated in concentrated bronchoalveolar fluid (range = <1–10 ng/ml) obtained from individuals exposed to mite allergen (34) overnight; in addition, this required concentration is much less than that which was shown to be present in mite fecal

pellets (>1 mg/ml) (35), suggesting that the consequences of mite allergen exposure that have been described are feasible.

Although the mechanism(s) underlying our findings have yet to be determined, it is unlikely that they are related to a simple proteolytic activation of cell surface-bound cytokine precursors given the up-regulation of cytokine mRNA. It is more likely that the effects observed are mediated by receptors on the epithelial cell surface. In this regard, recent studies have revealed the existence of a group of G protein-coupled receptors on a variety of cells that are activated not only by endogenous enzymes with specificities similar to those from the mite but also at similar concentrations to those reported here. This group includes receptors that are specific for thrombin and trypsin, designated proteinase-activated receptors (PARs) (36–39). Thus far, four PARs have been described (39, 40), and activation is dependent upon the cleavage of the N-terminal peptide of each receptor in each case; this cleavage allows the newly created N terminus (designated as a tethered ligand) to interact with residues within the remaining receptor, resulting in activation (41). Proteases interact with these receptors to up-regulate a range of cellular functions, including cytokine secretion, cellular proliferation, and the regulation of vascular tone. In addition, the activation of these receptors is accompanied by the mobilization of cytoplasmic Ca²⁺ from internal stores (41); in this regard, preliminary studies indicate that mite protease-induced cytokine release was shown to behave in a fashion that was similar to and consistent with a role for PARs (36–39). If receptors are involved in mite allergen-induced epithelial cell physiology, the findings that the response obtained with Der p 9 was not significantly influenced by prior exposure to Der p 1 and that a complete ablation of the response obtained with Der p 9 after treatment with trypsin was not observed suggest that there may be several types of protease receptors that are involved that warrant further study.

In conclusion, the studies described in this report suggest that the deposition of mite proteases on respiratory mucosa may induce localized inflammatory foci; these foci have the potential to influence a variety of immunologic processes in a manner similar to that postulated for agents such as viruses or airborne pollutants. For example, the release of cytokines such as GM-CSF, IL-6, and

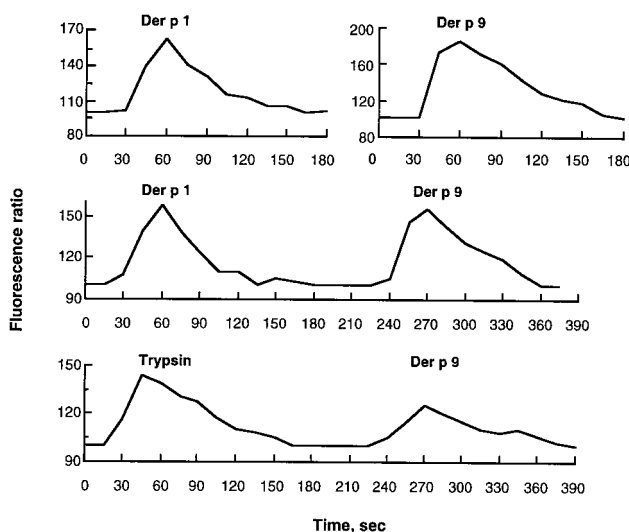


FIGURE 6. Dust mite proteases Der p 1 and Der p 9 cause a rise in the calcium ion concentration in BEAS-2B cells. Data were collected by confocal microscopy and expressed as a ratio of fluorescence at 405 nm and 490 nm ($\times 100$) using the indicator Indo-1. The top two panels show the effects of 1 μ g/ml of Der p 1 and Der p 9 on BEAS-2B cells, respectively. The middle panel shows the effects of the sequential addition of 1 μ g/ml of Der p 1 followed by Der p 9 on the calcium ion concentration, and the bottom panel shows the effect of the sequential addition of 1 μ g/ml of bovine trypsin and Der p 9 on the calcium ion concentration. Proteases were added after a 25-s initial scanning period.

IL-8 from epithelium may have an impact on immunogenicity because of the effects of these cytokines on dendritic cell growth, MHC class II expression, airway macrophage survival, B cell differentiation, and the chemoattraction of neutrophils and eosinophils; IL-6 may also help drive naive CD4 positive T cells to the effector Th2 type (42). In addition, a variety of studies indicate that the proteolytic properties of mite allergens may also influence anaphylatoxin production, activate fibrinolysin, and cleave CD23 and CD25 on B cell surfaces (16, 17, 20, 28, 43–45). Such properties raise the possibility that along with size and solubility, the enzymatic activities of allergens are important determinants of the outcome of exposure to a foreign protein.

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