

Interactions between inhalant allergen extracts and airway epithelial cells: Effect on cytokine production and cell detachment

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Background: The factors responsible for inducing or maintaining airway inflammation are poorly understood. Various studies have focussed on the mechanisms leading to allergic airway inflammation in patients with asthma and rhinitis. The observation of local airway inflammation in nonallergic patients with asthma or rhinitis, including those with nasal polyposis, suggest that *non-IgE-related* mechanisms exist that may lead to airway inflammation. Various lines of evidence suggest that epithelial cells may participate in local inflammation of the airways. **Objective:** This study focused on the interaction of airway epithelial cells with clinically relevant inhalant allergen extracts *in vitro*.

Methods: Cultures of airway epithelial cells were exposed to mite, Timothy grass pollen, and birch pollen extracts. Production of IL-8, IL-6, monocyte-chemotactic protein-1 (MCP-1), and granulocyte-macrophage colony-stimulating factor and cell detachment were monitored while protease inhibitors and chromatography techniques were applied to identify the factors responsible for these effects.

Results: With the mite extracts, cytokine production and cell detachment was largely dependent on protease activity. With the pollen extracts, cytokine production without cell detachment seemed to be independent of protease activity.

Conclusion: These findings support the view that epithelial cells may contribute to the pathogenesis of airway disease by their interaction with inhalant allergen extracts. Furthermore, allergen extracts may enhance airway inflammation by means other than their IgE-binding activity through both protease-dependent and protease-independent mechanisms. (*J Allergy Clin Immunol* 1998;102:75-85.)

Key words: Airway epithelial cells, asthma, rhinitis, mites, pollens, protease activity, *Dermatophagoides pteronyssinus*, *Lepidoglyphus destructor*, *Pleum pratensis*, *Betula verrucosa*

Many studies have focused on the cellular processes and mechanisms that lead to clinical manifestations of allergic airway disease, such as those found in asthma and rhinitis. The major concept in allergic inflammation is based on an IgE-mediated early and late phase-induced airway inflam-

Abbreviations used

APMSF:	4-aminodiphenylmethane sulfonyl fluoride
BSA:	Bovine serum albumin
EDTA:	Ethylenediamine tetraacetic acid
GM-CSF:	Granulocyte-macrophage colony-stimulating factor
HMW:	High molecular weight
LMW:	Low molecular weight
MCP-1:	Monocyte-chemotactic protein
MTT:	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide
PMA:	Phorbol myristate acetate
PMSF:	Phenylmethane sulfonyl fluoride
TLCK:	N α -p-tosyl-L-lysine chloromethyl ketone

mation, with infiltration and activation of inflammatory cells such as mast cells, lymphocytes, monocytes, and eosinophils.^{1,2} However, the factors that cause airway inflammation are as yet only partly understood.

A significant proportion of patients with asthma or rhinitis have negative skin test responses.³ These patients can be tentatively classified as having *nonallergic* asthma or rhinitis. Also, in the case of recurrent or persistent local airway inflammation, such as nasal polyposis, atopy is often not demonstrated.⁴ These clinical observations may reflect our ignorance of allergens that have yet to be identified. Alternatively, it may be possible that the interaction of resident cells in the airways with inhaled biologic substances, such as allergens, results in local airway inflammation. The epithelium composing the primary interface between the external and internal milieu of the airways is in a key position to participate in such a local event. The interaction of common inhalant allergen extracts with epithelial cells resulting in local inflammation may thus contribute to the pathogenesis of airway disease.

In addition to its function as a physical barrier, the airway epithelium is a major source of inflammatory products and can express various types of cell-adhesion molecules,⁵ and a low-affinity receptor for IgE on bronchial epithelial cells has been described.⁶ Production of cytokines and growth factors, particularly IL-6, IL-8, and granulocyte-macrophage colony-stimulating factor (GM-CSF), by airway epithelial cells has been demonstrated both *in vitro* and *in vivo* after a variety of mucosal injuries,⁷⁻⁹ whereas there are differences in cytokine release from epithelial cells cultured from nasal biopsy

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specimens obtained from various patient groups (e.g., nonatopic patients without rhinitis, atopic patients with rhinitis, and atopic patients without rhinitis).¹⁰ Products such as IL-8 and GM-CSF activate neutrophils and eosinophils and are able to direct these cells to sites of inflammation. In addition, human airway epithelial cells can produce monocyte-chemotactic protein-1 (MCP-1),¹¹ a potent chemoattractant and activator of monocytes, macrophages, and human T-lymphocytes.¹² By the release of these products, epithelial cells may modulate the local inflammatory state of the airways.

Proteolytic enzyme activity of different origins can induce the production of proinflammatory cytokines and cell detachment in epithelial cells.^{8,13,14} This study focuses on the ability of mite extracts of *Dermatophagoides pteronyssinus* and *Lepidoglyphus destructor*, both of which are known to express proteolytic enzyme activity,^{15,16} to induce proinflammatory cytokine release and cell detachment in human pulmonary epithelial cell lines and primary nasal epithelial cells from nonatopic individuals. Results were compared with those obtained with Timothy grass (*Phleum pratensis*) pollen and birch (*Betula verrucosa*) pollen extracts in which strong proteolytic activities have as yet not been reported. The findings presented here support the view that epithelial cells may play a role in the microenvironmental control of inflammation by their interaction with inhalant allergens. Furthermore, it is shown that allergens may enhance airways inflammation by means other than their IgE-binding activity through protease-dependent and protease-independent mechanisms.

METHODS

Subjects

Tissues from the concha nasalis inferior were obtained from eight subjects with no history of atopy and a negative skin test response at the time of surgery. Surgery was performed for corrective reasons, and informed consent from all patients was obtained for research purposes.

Mite and pollen extracts and their chemical composition

Standardized lyophilized extracts of *L. destructor*, *D. pteronyssinus*, Timothy grass (*P. pratensis*) pollen, and birch (*B. verrucosa*) pollen were a generous gift from Dr. Nico R. Niemeijer (ALK-Benelux, Groningen, The Netherlands). The mite extracts were obtained from both mite bodies and fecal pellets. Protein concentrations were determined by the method of Lowry, with a bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, Mo.) standard.¹⁷ Carbohydrate concentration was measured with the phenolsulfuric acid method.¹⁸

Pulmonary epithelial cell cultures

A549 cells, a human alveolar type II epithelium-like cell line derived from a lung adenocarcinoma, and NCI-H292 cells, a human pulmonary mucoepidermoid carcinoma-derived bronchial epithelial cell line, were obtained from American Type Culture Collection (Rockville, Md.) and cultured as described elsewhere.¹³

Nasal epithelial cell cultures

Tissues from the concha nasalis inferior were obtained immediately after resection and washed 10 times with cold $\text{Ca}^{2+}/\text{Mg}^{2+}$ free Hank's balanced salt solution (BioWhittaker) supplemented with gen-

tamicin sulfate (50 $\mu\text{g}/\text{ml}$) and fungizone (0.25 $\mu\text{g}/\text{ml}$; Bristol-Myers Squibb BV, Woerden, The Netherlands). After overnight incubation of tissues at 4° C in 0.1% protease solution (from *Streptomyces griseus*; Sigma) in Ham's F12 (BioWhittaker), fetal calf serum was added (up to 10% final concentration) according to the method described by Ohtoshi et al.¹⁹ Tissue-free cell suspensions were obtained by filtering through a 48 μm polyamide nylon Nitex mesh (Thompson & Co Ltd, Scarborough, Ontario, Canada). Cell suspensions in supplemented bronchial epithelial cell growth medium (Clonetics Corporation, Walkersville, Md.) were cultured at 37° C in an atmosphere of 5% CO_2 in precoated 75 cm^2 culture flasks (Costar Corp., Cambridge, Mass.). Flasks were precoated with Vitrogen 100 (30 $\mu\text{g}/\text{ml}$; Collagen Corp., Palo Alto, Calif.), fibronectin (10 $\mu\text{g}/\text{ml}$), and BSA (10 $\mu\text{g}/\text{ml}$) in Ham's F12 for 2 hours at 37° C. Semiconfluent cell layers were acetone-fixed and incubated with anti-keratin (1:50, clone: MNF116; Dako, Santa Barbara, Calif.) and anti-vimentin (1:50, clone: V9; Dako) mouse monoclonal antibodies and evaluated with a streptavidin-biotin immunoperoxidase detection kit (Universal LSAB-2/ AP kit; Dako) to confirm the epithelial cell nature of the cultured nasal cells. Human lung fibroblast (CCD8, American Type Culture Collection) and human lung epithelial cells (A549) were included as controls.

Column chromatography

Extracts of *D. pteronyssinus*, *L. destructor*, Timothy grass pollen, and birch pollen were prepared in 0.15 mol/L NH_4HCO_3 buffer (pH 8.1) (80 mg dry weight/5 ml) and submitted to Superdex 75 (FF) Hiloal 26/60 (prep grade) column chromatography (Pharmacia Fine Chemicals, Woerden, The Netherlands) by using a fast performance liquid chromatography system (Pharmacia) as previously described.²⁰ Molecular weight markers (Pharmacia) were used in a separate run to calibrate the column. Fractions were collected and tested for protein and carbohydrate content, protease activity, and the ability to induce IL-8 production in A549 cells. Samples of each fraction were submitted to heat treatment (1 hour at 65° C), and residual protease activity, and the ability to induce IL-8 production in A549 cells were quantified.

Incubation of epithelial cell cultures with mite or pollen extracts and quantification of epithelial cell detachment, cell viability, and metabolic activity

Epithelial cells were cultured in sterile 24-well culture dishes (Costar) until they reached 80% to 90% confluence. Before incubation with the extracts, cell cultures were rinsed and incubated overnight in serum-free RPMI 1640 (A549 or NCI-H292 cells) or bronchial epithelial cell basal medium (nasal epithelial cells). Incubations with mite or pollen extracts (5×10^{-4} to a concentration of 400 $\mu\text{g}/\text{ml}$) for 48 hours were performed in serum-free RPMI 1640 (lung epithelial cells) or bronchial epithelial cell basal medium (nasal epithelial cells) at 37° C in an atmosphere of 5% CO_2 . Lyophilized gel-filtration fractions were restored to their original volume in RPMI 1640 supplemented with gentamicin sulfate and fungizone, and 100 μl samples of undiluted (pollen) or 5 $\times$ or 20 $\times$ dilutions (*D. pteronyssinus* and *L. destructor*, respectively) of the restored gel-filtration fractions were added to confluent A549 cell cultures in 96-well culture dishes (Costar) and incubated at 37° C in an atmosphere of 5% CO_2 for 48 hours. Incubations with IL-1 α (20 U/ml; Boehringer, Mannheim, Germany) for A549 cells or phorbol myristate acetate (PMA) (80 nmol/L; Sigma) for NCI-H292 cells and nasal epithelial cells served as positive controls, and incubations with cell culture medium served as negative controls (i.e., spontaneous release). After 48 hours, culture supernatants were collected and stored at -20° C. ELISAs for IL-6, IL-8 (both from Central Laboratory of The Netherlands Red Cross Blood Transfusion

TABLE I. Inhibition profile of elastase, gelatinase, and total protease activities of the mite extracts with different protease inhibitors

	<i>D. pteronyssinus</i>			<i>L. destructor</i>		
	Elastase	Gelatinase	Total protease	Elastase	Gelatinase	Total protease
PMSF	96.5	51.6	50.3	88.6	39.2	60.8
APMSF	9.2	76.5	56.3	11.0	31.5	20.3
TLCK	24.4	85.2	49.0	14.4	79.3	29.1
Chymostatin	99.2	41.8	37.1	96.2	31.0	61.8
1,10 phenanthroline	73.3	67.8	64.9	40.6	70.3	69.0
EDTA	8.2	0.0	0.0	5.6	9.7	10.5
Chymostatin plus 1,10 phenanthroline	99.6	82.9	68.9	97.4	77.6	80.9
Leupeptin	13.9	85.6	62.3	10.5	82.7	29.1
Cystatin	13.1	0.0	7.3	9.7	3.6	1.4
Heat treatment	99.8	98.9	80.1	100	96.7	84.6

The data represent the percentage of inhibition compared with the untreated control (i.e., without inhibitor).

Service, Amsterdam, The Netherlands), GM-CSF, and MCP-1 (both from R&D Systems, Minneapolis, Minn.) were applied for measuring cytokine and growth factor production in cell culture supernatants. Cell detachment, cell viability, cell activation, and cell proliferation were quantified as described elsewhere.¹³

Quantification of protease activities in mite and pollen extracts and inhibition of protease activities

Elastase and gelatinase activities in allergen extracts were quantified as described elsewhere by using the substrates N-succinyl-alanyl-alanyl-prolyl-leucine *p*-nitroanilide (Sigma) and Orange Gelatine (BIOK Ltd., Vilnius, Lithuania), respectively.²⁰ Total protease activity was assayed by measuring the hydrolysis of AZO-BSA (2.5 mg/ml) (ICN Pharmaceuticals, Costa Mesa, Calif.). Incubations of 100 μ l of the mite and pollen extracts (1 mg/ml final concentration) and AZO-BSA (10 mg/ml final concentration) in prewarmed buffer (50 mmol/L Tris-HCl and 5 mmol/L Ca(CH₃COO)₂, pH 7.5) were performed for 30 minutes at 37° C. Denaturation buffer (250 μ l) (3 mol/L [NH₄]₂SO₄ and 5 mmol/L ethylenediamine tetraacetic acid [EDTA]) was added, and mixtures were cooled at room temperature and centrifuged at 10,000 *g*. Absorbance was read at 405 nm. One unit of total protease activity is the amount of protease that hydrolyzes 1 mg of AZO-BSA in 30 minutes.

When protease inhibitors were used, mite and pollen extracts were incubated with the inhibitor for 15 minutes at 37° C before the protease substrate was added. The following serine protease inhibitors were used: phenylmethane sulfonyl fluoride (PMSF; 100 μ mol/L), 4-amidinophenylmethane sulfonyl fluoride (APMSF; 200 μ mol/L), α -*p*-tosyl-l-lysine chloromethyl ketone (TLCK; 1 mmol/L), and chymostatin (10 μ g/ml). The following metalloprotease inhibitors were used: 1,10 phenanthroline (10 mmol/L) and EDTA (10 mmol/L). Finally, the aspartic protease inhibitor leupeptin (50 μ g/ml) and the cysteine protease inhibitor cystatin (10 μ g/ml) were used. All protease inhibitors were obtained from Sigma.

Statistics

All data are presented as means $\pm$ SEM. Data were tested for normal distribution with the Kolmogorov-Smirnov test. Comparisons were made by using the two-tailed Student's *t* test after recalculating cytokine productions to percentages of increase compared with the spontaneous release. Differences were considered significant if *p* values were less than 0.05. Values are from at least four different experiments performed in duplicate.

RESULTS

Characterization of the protease activities with protease inhibitors

No elastase, gelatinase, or total protease activities were found in the pollen extracts. This was in contrast to the mite extracts in which high protease activities were detectable. Gelatinase activity was high with the mite extracts, especially with *L. destructor* (data not shown). The serine protease inhibitors PMSF and chymostatin (an inhibitor of chymotrypsin-like serine proteases) largely inhibited elastase activity and partially inhibited the total protease activity in both mite extracts. The serine protease inhibitor TLCK strongly inhibited gelatinase activity in both mite extracts, whereas APMSF also inhibited this enzyme activity in *D. pteronyssinus* extracts (Table I). Substantial inhibition of most protease activities was found with the metalloprotease inhibitor 1,10 phenanthroline but not with EDTA (Table I). Elastase activity in the *L. destructor* extract was inhibited partially by 1,10 phenanthroline. The combination of serine and metalloprotease inhibitors (i.e., chymostatin and 1,10 phenanthroline) was very potent in inhibiting all three protease activities, indicating that the elastase and gelatinase activities are mediated largely by serine and metalloproteases. However, the aspartic protease inhibitor leupeptin was highly potent in inhibiting gelatinase activity in both mite extracts and total protease activity in the *D. pteronyssinus* extract. No significant inhibition of protease activity was found with the cysteine protease inhibitor cystatin. Mild heat treatment of the extracts completely abolished elastase and gelatinase activities and over 80% of the total protease activities in both extracts, indicating a low thermotolerance of the proteases involved (Table I).

Cytokine production and cell detachment in the pulmonary epithelial cell lines A549 and NCI-H292

Mite and pollen extracts caused a dose-dependent increase in IL-8 production by A549 cells, reaching statistical significance at 8 μ g/ml (mite and Timothy grass pollen) or at 40 μ g/ml (birch pollen) (Fig. 1). The

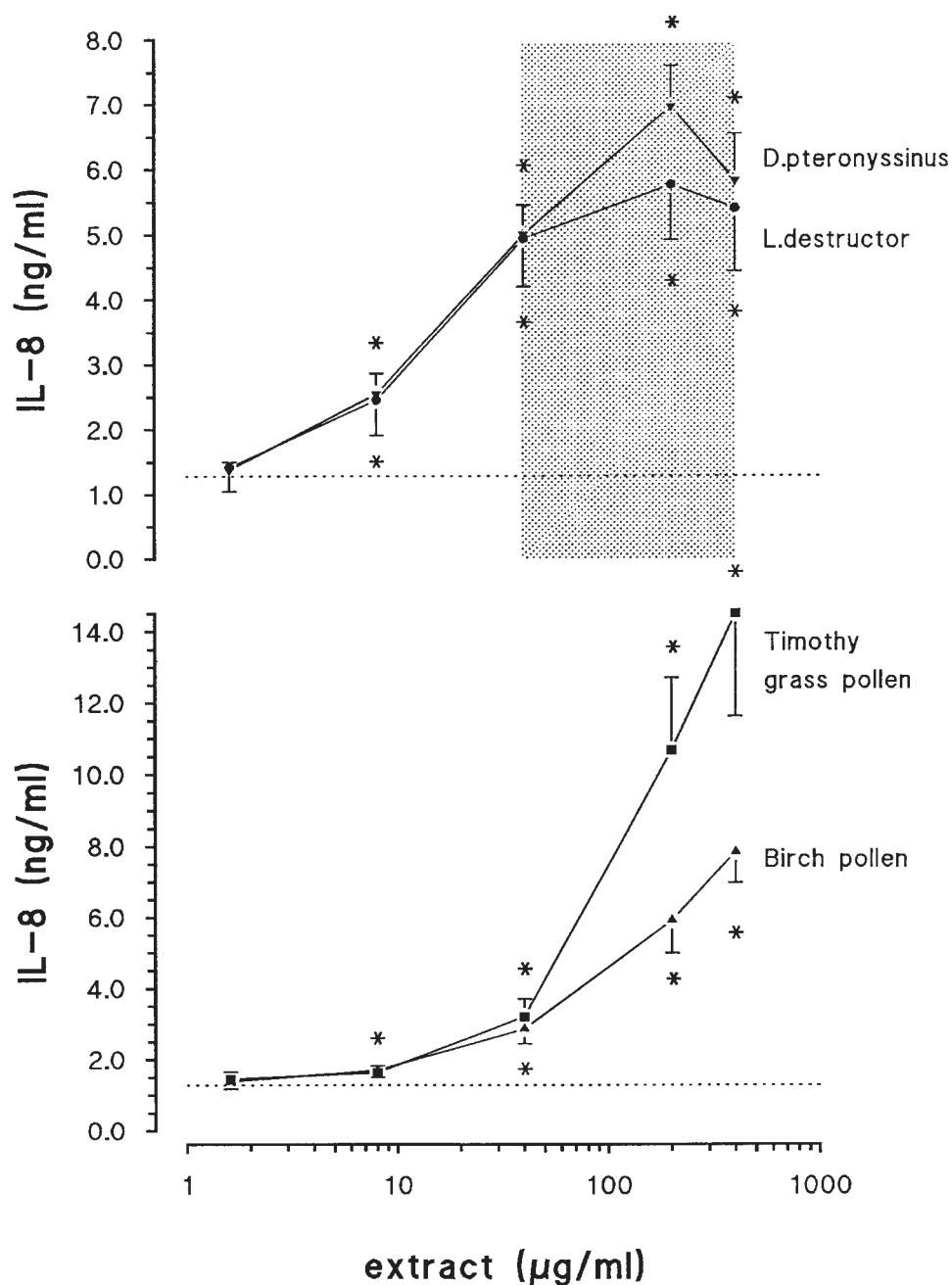


FIG. 1. Immunoreactive IL-8 in culture supernatants from alveolar epithelial cell line A549 after exposure to mite and pollen extracts. A549 cell cultures were exposed for 48 hours to different concentrations (*ordinate*) of mite (*upper panel*) and pollen (*lower panel*) extracts before IL-8 production was quantified. Comparisons were made with two-tailed Student's *t* test after recalculating IL-8 productions to percentages of increase compared with spontaneous release. Differences were considered significant if *p* values were less than 0.05. Values are from at least five different experiments performed in duplicate and are presented as means $\pm$ SEM. Asterisks indicate significantly increased IL-8 production compared with spontaneous release. *Stippled line*, Spontaneous release; *stippled area*, complete cell detachment.

dose-response curves for the mite extracts followed a comparable pattern as did the curves for the pollen extracts. Maximal IL-8 production was found at 200 μ g/ml with mite extracts and at 400 μ g/ml with pollen extracts (Fig. 1). The highest IL-8 production was found with birch pollen extract (Fig. 1, *lower panel*).

The pollen extracts did not cause cell detachment; however, with the mite extracts, cell detachment was found starting from 40 μ g/ml (Fig. 1, *upper panel*, *stippled box*). After heat treatment or in the presence of a combination of serine and aspartic or serine and metalloprotease inhibitors (i.e., chymostatin, leupeptin or

TABLE II. Maximal concentrations of immunoreactive cytokines found in culture supernatants from the alveolar epithelial cell line A549

A549 cells	IL-8 (ng/ml)	p Values	IL-6 (pg/ml)	p Values	MCP-1 (ng/ml)	p Values
IL-1 α	62.4 $\pm$ 4.8		105.4 $\pm$ 12.2		41.3 $\pm$ 2.3	
<i>L. destructor</i>	43 $\pm$ 0.7†	<0.000	9.6 $\pm$ 2.5*	<0.097 (NS)	1.0 $\pm$ 0.2*	<0.005
<i>D. pteronyssinus</i>	5.5 $\pm$ 0.7†	<0.012	18.4 $\pm$ 7.1†	<0.020	1.4 $\pm$ 0.1*	<0.007
Timothy grass pollen	13.1 $\pm$ 2.9‡	<0.017	74.5 $\pm$ 15.0‡	<0.009	4.4 $\pm$ 0.3‡	<0.002
Birch pollen	6.3 $\pm$ 0.6‡	<0.001	41.0 $\pm$ 9.3‡	<0.032	2.5 $\pm$ 0.2‡	<0.003

Cell cultures were incubated with IL-1 α (20 U/ml, positive control) or mite or pollen extracts. The data represent the mean ($\pm$ SEM) of the individual readings with inhalant allergen extracts minus the spontaneous cytokine release of five independent experiments. Spontaneous cytokine production for IL-8 is 1.4 $\pm$ 0.1 ng/ml, that for IL-6 is 0.7 $\pm$ 0.2 pg/ml, and that for MCP-1 is 1.2 $\pm$ 0.1 ng/ml. Differences between inhalant allergen extract-induced cytokine release and spontaneous cytokine release were statistically tested after recalculating cytokine productions to percentages of increase compared with spontaneous release. NS, Not significant.

*Concentration of 40 μ g/ml.

†Concentration of 200 μ g/ml.

‡Concentration of 400 μ g/ml.

chymostatin, or 1,10 phenanthroline, respectively), the mite extracts lost their ability to induce IL-8 production and cell detachment (data not shown). In Table II the IL-8, IL-6, and MCP-1 production by A549 cells are summarized, showing increased cytokine production after exposure to mite and pollen extracts.

With the mite and pollen extracts, the IL-8 production by NCI-H292 cells followed the same pattern as described for A549 cells, although the overall IL-8 production was markedly lower with the former cell line (data not shown). With the *L. destructor*, Timothy grass pollen, and birch pollen extracts, a significant increase in IL-8 production by NCI-H292 cells was reached at 40 μ g/ml, 8 μ g/ml, and 200 μ g/ml, respectively (data not shown).

The metabolic activity (MTT reduction assay) and cell viability (trypan blue exclusion) of the epithelial cells were not affected with higher concentrations (400 μ g/ml) of the mite or pollen extracts; viability was always greater than 95%. By using human serum albumin in a concentration range up to 400 μ g/ml, no increase in cytokine production was found (data not shown), indicating that the increase in cytokine production by epithelial cells was not due to changes in osmolality.

Production of IL-8 and cell detachment in A549 cells exposed to fractionated mite and pollen extracts, protease activities, and the effect of heat treatment

In Fig. 2 (*lower panel*) the protein and carbohydrate concentrations in the gel filtration fractions of the *D. pteronyssinus* extract are shown. High carbohydrate content was found in both high molecular weight (HMW) (i.e., >66 kd; Fig. 2, *lower panel*) and low molecular weight (LMW) fractions (<6.5 kd). High protein content was identified exclusively in the LMW fractions (<29 kd). Elastase activity (Fig. 2, *lower panel*) was found with fractions in the molecular weight range between 29 kd and just over 66 kd (fractions 6 to 11), with peak activities at fractions 7 and 9. Some elastase activity was

found with fractions 4 to 6 and 11 to 12 as well. Although not shown in Fig. 2, the gelatinase activity exactly paralleled the elastase activity in these fractions.

Increased IL-8 production (Fig. 2, *upper panel*) was found with gel-filtration fractions between 29 and just over 66 kd (fractions 6 to 12) corresponding with the fractions containing the protease activity and HMW fractions (fractions 2 to 5) partially associated with protease activity. Furthermore, higher IL-8 production was found with the LMW fractions (fractions 13 to 20) not associated with protease activity. Mild heat treatment resulted in loss of protease activity (data not shown) in fractions 4 to 12, as well as the ability of these fractions to induce IL-8 production in A549 cells (Fig. 2, *upper panel*). However, the IL-8 production with the LMW fraction (fractions 13 to 20) was not affected (Fig. 2, *upper panel*).

Results obtained with the gel filtration fraction of the *L. destructor* extract are shown in Fig. 3. Elastase activity was found in fractions 5 to 15, with peak activities in fraction 9 and 13, whereas gelatinase activity was found in fractions 5 to 13, with peak activities in fraction 7 and 11 (Fig. 3, *lower panel*). IL-8 production (Fig. 3, *upper panel*) was highest in the fractions with the protease activities (fractions 5 to 15), although some IL-8 production was found with HMW fractions apparently unrelated to protease activity (fractions 2 to 5). Heat treatment inhibited most of the elastase and gelatinase activities, although some residual protease activity was found with elastase at fraction 6 and gelatinase at fraction 7 (data not shown). After heat treatment, most of the IL-8-inducing capacity was lost from the fractions, but some IL-8-inducing capacity was retained by fractions 6 to 8 (Fig. 3, *upper panel*), most likely related to minor residual protease activity in these fractions. Cell detachment with both extracts was restricted to the fractions containing protease activity (data not shown).

With fraction 9 of the *D. pteronyssinus* extract (Fig. 2) and fraction 7 of the *L. destructor* extract (Fig. 3), a decrease in IL-8 production was observed in both cases, coinciding with very high protease activity in

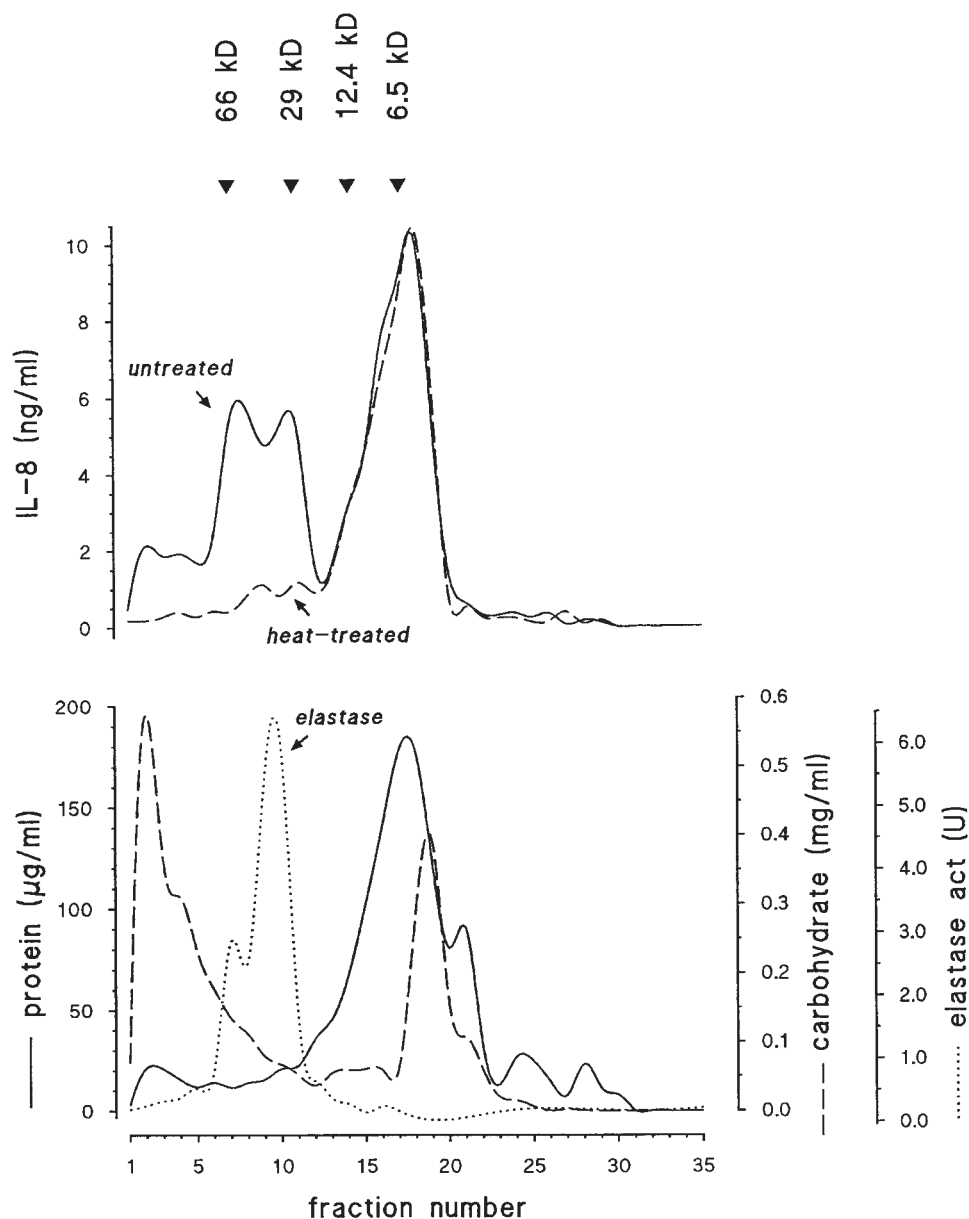


FIG. 2. Protease activity, protein and carbohydrate content, and IL-8-inducing capacity of gel filtration fractions from *D. pteronyssinus* extract. Immunoreactive IL-8 in A549 culture supernatants (solid curve, upper panel) is shown after incubation with different gel-filtration chromatography fractions (ordinate) of *D. pteronyssinus* extract. Effect of heat treatment of fractions on IL-8 production is indicated (dashed curve, upper panel). Protein concentration (solid curve, lower panel), carbohydrate concentration (dashed line, lower panel), and elastase activity (stippled line, lower panel) in fractions are shown. Gelatinase activity closely followed elastase activity in fractions (data not shown). Molecular weight markers are indicated.

the fractions. With both fractions, detachment of A549 cells was observed within 1 hour after the start of the 48-hour incubation. The lower IL-8 production observed with these fractions is therefore probably related to the immediate onset of cell detachment, resulting in a decrease in IL-8 production.

In Table III the IL-8 production induced by different gel-filtration fractions and the effect of heat treatment are summarized. The data presented in the table repre-

sent the area under the curve and are calculated for the molecular weight ranges indicated. The molecular weight range of 66 to 29 kD of the *D. pteronyssinus* extract and the molecular weight range of 66 to 6.5 kD of the *L. destructor* extract correspond to the fractions with protease activity. As can be seen in the table, heat treatment of the fractions in these molecular weight ranges inhibited their capacity to induce IL-8 production as reflected by a decrease in area under the curve

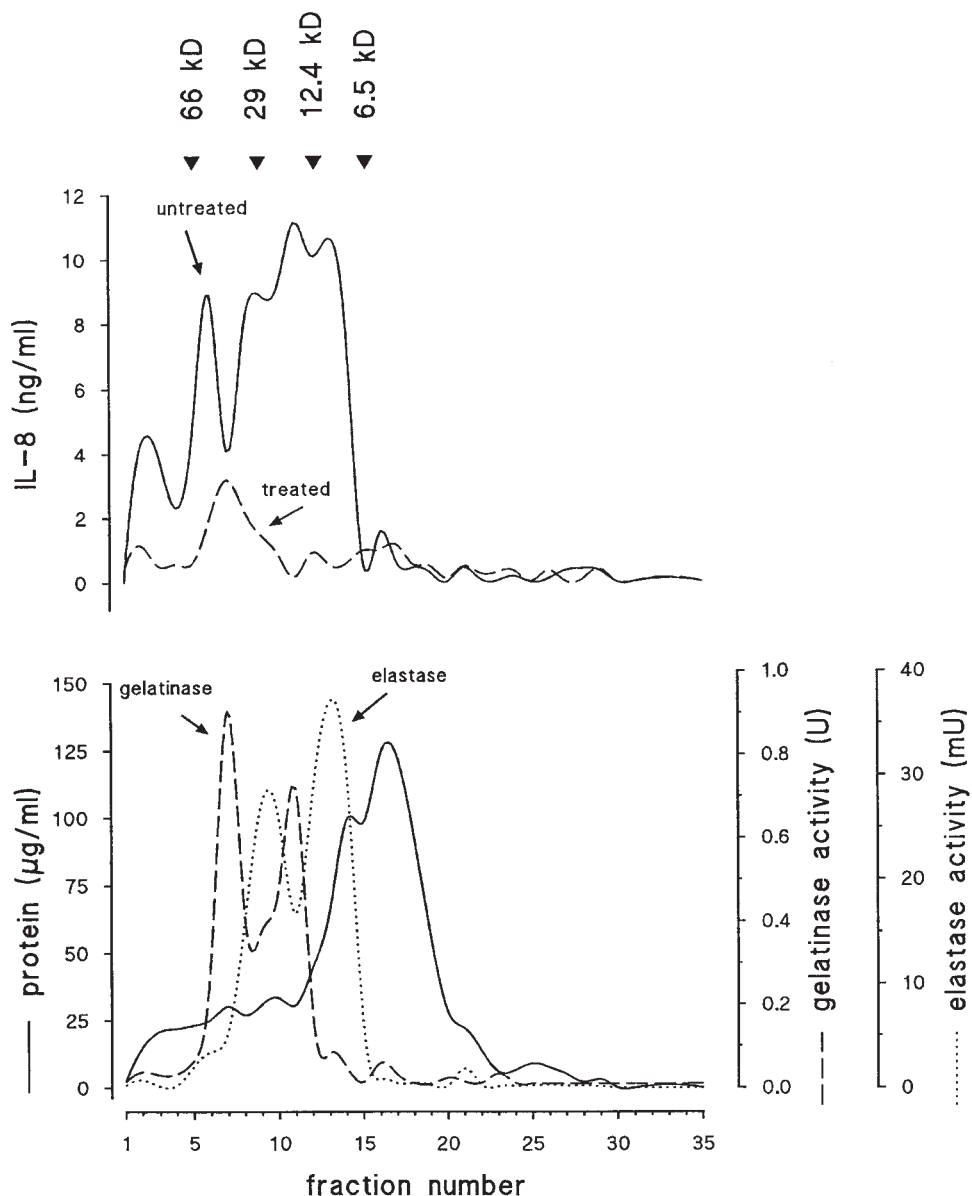


FIG. 3. Protease activity, protein content, and IL-8-inducing capacity of gel-filtration fractions from *L. destructor* extract. Immunoreactive IL-8 in A549 culture supernatants (solid curve, upper panel) are shown after incubation with different gel-filtration chromatography fractions (ordinate) of *L. destructor* extract. Effect of heat treatment of fractions on IL-8 production is indicated (dashed curve, upper panel). Protein concentration (solid curve, lower panel) and gelatinase (dashed line, lower panel) and elastase (stippled line, lower panel) activities in fractions are shown. Molecular weight markers are indicated.

value. Furthermore, the induction by the HMW fractions (>66 kd) not associated (*L. destructor*) or only partially associated (*D. pteronyssinus*) with protease activity was inhibited by the heat treatment as well. Induction by the LMW fractions (<6.5 kd) in both mite extracts and the induction by the LMW fractions (29 to 6.5 kd) of the *D. pteronyssinus* extract were unaffected. Unexpectedly, heat treatment of Timothy grass pollen and birch pollen gel-filtration fractions increased the IL-8 production for some, but not all, of the molecular weight ranges tested (Table III).

Cytokine production by cultured nasal epithelial cells from nonatopic subjects and the induction of cell detachment

The dose-response curves for the production of IL-8 by cultured nasal epithelial cells from nonatopic subjects exposed to mite and pollen extracts are shown in Fig. 4. Nasal epithelial cells produced high levels of IL-8 (maximum of 54.3 ng/ml) compared with the pulmonary cell lines tested (A549, maximum of 14.5 ng/ml; and NCI-H292, maximum of 3.8 ng/ml [data not shown]). Furthermore,

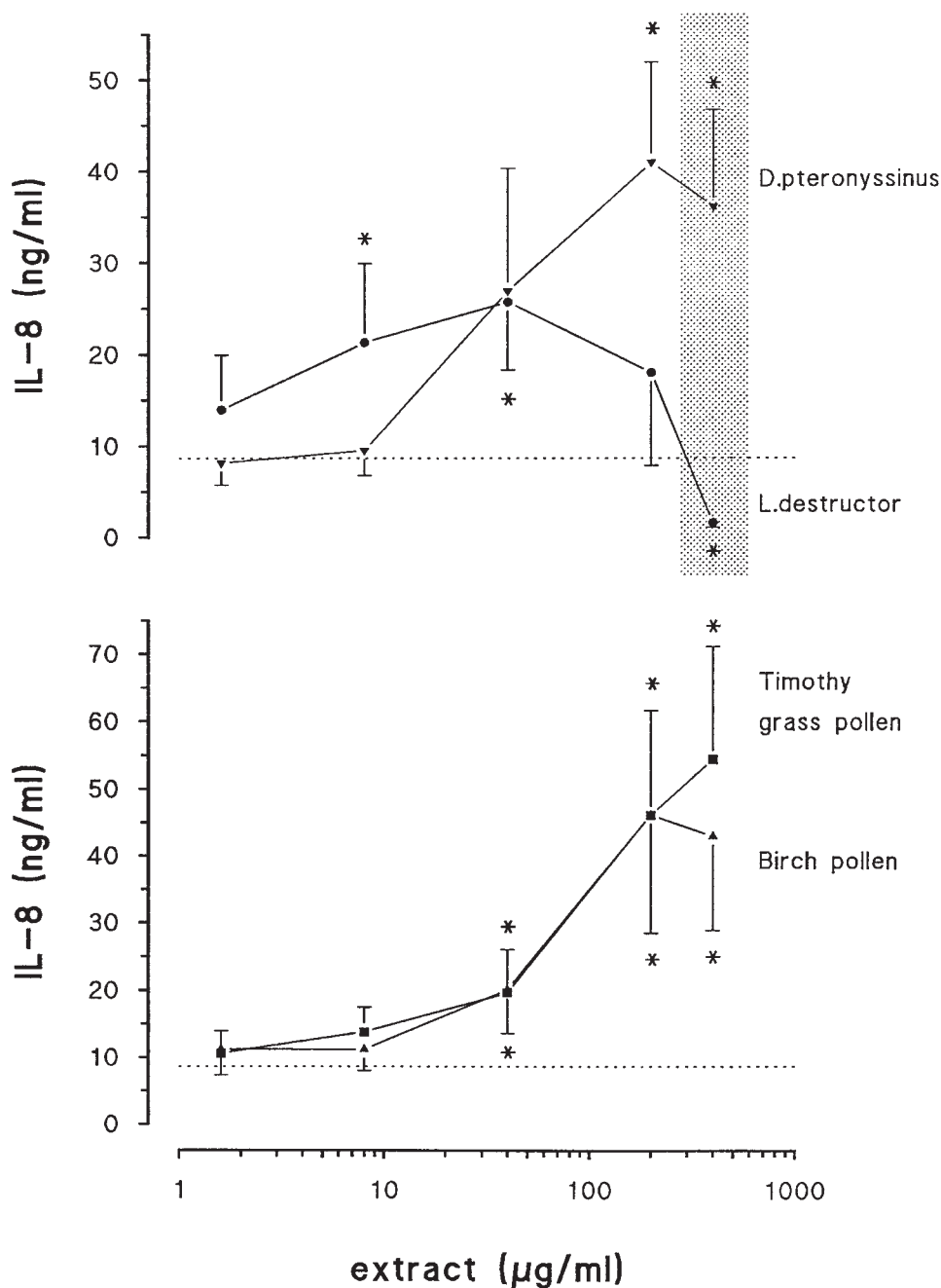


FIG. 4. Immunoreactive IL-8 in culture supernatants from primary nasal epithelial cells after exposure to mite and pollen extracts. Cell cultures were exposed for 48 hours to different concentrations (*ordinate*) of mite (*upper panel*) and pollen (*lower panel*) extracts before IL-8 production was quantified. Comparisons were made with two-tailed Student's *t* test after recalculating IL-8 productions to percentages of increase compared with spontaneous release. Differences were considered significant if *p* values were less than 0.05. Values are from eight nonatopic patients, and experiments were performed in duplicate and are presented as mean $\pm$ SEM. Asterisks indicate significantly increased IL-8 production compared with spontaneous release. *Stippled line*, Spontaneous release; *stippled area*, complete cell detachment.

large differences were found in cytokine production between the individuals tested, reflected by a high SEM (Fig. 4). A significantly increased production of IL-8, as compared with the spontaneous cytokine release (Fig. 4, *stippled lines*) was found with the *L. destructor* extract at 8 $\mu\text{g/ml}$, the Timothy grass and birch pollen extracts at 40

$\mu\text{g/ml}$, and the *D. pteronyssinus* extract at 200 $\mu\text{g/ml}$. In contrast to the observations with the pulmonary cell lines, however, a decrease in IL-8 production was found with higher concentrations of *L. destructor* extract, reaching a statistically significant decrease in IL-8 production at 400 $\mu\text{g/ml}$ (Fig. 4, *upper panel*) apparently without loss of cell

TABLE III. IL-8 production by A549 cells after incubation with the different fractions obtained by Sephadex gel filtration chromatography of mite and pollen extracts

Molecular weight (kd)	<i>D. pteronyssinus</i>		<i>L. destructor</i>		Timothy grass pollen		Birch pollen	
	Untreated	Heat treated	Untreated	Heat treated	Untreated	Heat treated	Untreated	Heat treated
>66	10.4	1.7	10.6	2.6	5.5	11.1	1.9	1.3
66-29	15.8	2.7	20.4	7.5	7.8	17.0	6.2	6.8
29-12.4	3.3	2.3	20.3	1.0	15.0	10.7	7.9	13.2
12.4-6.5	12.0	11.1	18.8	1.1	7.4	25.2	2.4	6.9
<6.5	19.9	19.9	4.5	5.3	28.4	34.4	27.1	44.0

The data represent the area under the curve and are calculated for the molecular weight ranges as indicated. The effect of heat treatment of the fractions on IL-8 production is shown. Epithelial cell cultures were incubated with undiluted (pollen), 5× (*D. pteronyssinus*), or 20× (*L. destructor*) dilutions of the restored gel filtration fractions.

TABLE IV. Maximal concentrations of immunoreactive cytokines found in culture supernatants from cultured nasal epithelial cells obtained from nonatopic subjects

Nasal epithelium	IL-8 (ng/ml)	<i>p</i> Values	GM-CSF (ng/ml)	<i>p</i> Values
PMA	29.6 ± 7.7		5.1 ± 0.8	
<i>L. destructor</i>	15.4 ± 5.2*	<0.019	2.4 ± 1.4†	<0.025
<i>D. pteronyssinus</i>	32.0 ± 9.1†	<0.005	3.7 ± 1.7†	<0.012
Timothy grass pollen	44.0 ± 14.4‡	<0.003	5.2 ± 3.0‡	<0.016
Birch pollen	37.1 ± 14.9†	<0.005	4.1 ± 2.0‡	<0.012

Cell cultures were incubated with PMA (80 nmol/L, positive control) or with optimal concentrations of mite and pollen extracts in a concentration range up to 400 µg/ml. The data represent the means (± SEM) of readings obtained with epithelial cells exposed to inhalant allergen extracts from eight subjects minus the spontaneous cytokine release. Spontaneous cytokine production for IL-8 is 9.6 ± 2.3 ng/ml, and that for GM-CSF is 1.9 ± 0.4 ng/ml. Differences between the inhalant allergen extract-induced cytokine release and the spontaneous cytokine release were statistically tested after recalculating cytokine productions to percentages of increase compared with spontaneous release.

*Concentration of 40 µg/ml.

†Concentration of 200 µg/ml.

‡Concentration of 400 µg/ml.

viability. With both mite extracts, 400 µg/ml caused complete cell detachment (Fig. 4, upper panel, stippled box). The pollen extracts did not cause cell detachment in the dose range tested. In addition to the production of IL-8, the nasal epithelial cells also produced statistically significant levels of GM-CSF and low levels of IL-6 but no MCP-1 after exposure to mite and pollen extracts. The IL-8 and GM-CSF productions by nasal epithelial cells are summarized in Table IV.

Bacterial endotoxin contamination is commonly found in commercially allergenic extracts,²¹ and endotoxins can induce a cytokine response in epithelial cells.²² To neutralize possible endotoxin contaminations in the allergenic extracts, we performed control experiments with the lipopolysaccharide-binding substance colestsin methane-sulfonate (6 ng/ml; Sigma). Addition of colestsin to the incubation media did not inhibit the cytokine production or cell detachment, indicating that the cytokine production and cell detachment were not caused by endotoxin contaminations of the extracts (data not shown).

DISCUSSION

In this study it was shown that inhalant allergen extracts can induce the production of proinflammatory cytokines in pulmonary cell lines and primary nasal epithelial cells from nonatopic individuals. Because pollen allergens are mainly deposited at the level of the nasal mucosa and only

partially enter the lower airways, we included primary nasal epithelial cells in our studies. The induction of the cytokine response was found to be either protease-dependent (HMW fractions in mite extracts) or protease-independent (pollen extracts and LMW fractions in *D. pteronyssinus*). By the production of cytokines such as IL-8, IL-6, MCP-1, and GM-CSF, epithelial cells may contribute to the accumulation of inflammatory cells (e.g., neutrophils, eosinophils, and lymphocytes) as is frequently observed after local allergen challenge of the airways in patients with asthma.^{2,23,24}

The use of cell lines as a model system has some limitations. One of the factors that may explain the observed differences in findings between individual cell lines and primary cultures of nasal epithelial cells may be due to differences in the genetic background of the cells studied. Alveolar cells, such as the A549 cells, are likely to differ from cells originally isolated from bronchial tissues (e.g., NCI-H292), whereas the use of immortalized cells, as compared with primary cell cultures, may affect the nature and magnitude of the responses when exposed to inhalant allergens.

The factors responsible for the induction of the cytokine response in the allergen extracts are unknown. Proteolytic enzymes, such as neutrophil elastase⁸ and those from *Aspergillus fumigatus*,¹³ are potent inducers of cytokine production in epithelial cells and may act by

mechanisms related to cellular detachment and deformation.²⁵ In this study it was shown that both metalloprotease and serine protease activity are responsible for the elastase, gelatinase, and total protease activities in both mite extracts, and aspartic protease activity is important in the gelatinase activities of both extracts and in the total protease activity of the *D. pteronyssinus* extract. It is well known that protease activity is abundantly present in mite extracts,^{26,27} and the mite allergens comprising groups 1 through 4 have cysteine proteinase, lysozyme, trypsin, and amylase activity, respectively.²⁸⁻³¹ In addition, it was shown more recently that Der p 6 and Der p 9 have chymotrypsin and serine protease activity, respectively.^{32,33} Inhibition of the enzyme activity by either heat treatment or a combination of a serine protease inhibitor and a metalloprotease or an aspartic protease inhibitor in our study was found to inhibit the cytokine production and cell detachment. In addition, the gel-filtration studies have shown that the IL-8-inducing activity in the mite extracts was most pronounced with the fraction expressing the protease activity. Therefore the protease activity likely is the causative factor for the larger part of the cytokine induction in mite extracts.

Cellular detachment was completely dependent on the protease activity in the mite extracts. Because the protease-dependent IL-8 production could not be completely inhibited by either one of the specific protease inhibitors exclusively, but instead, to obtain full inhibition, a combination of a serine protease and either a metalloprotease or aspartic protease inhibitor was needed, the causative protease activity is likely mediated by different proteases present in the mite extracts. The observation of cellular detachment may be of particular interest in view of recent theories on the involvement of the shedding-restitution process of damaged airway epithelium, such as that found in asthma, as a driving force for asthma-like cellular pathology and exudative pathophysiology.³⁴ Together with our findings, this may suggest that mite proteases may enhance airway inflammation directly by causing epithelial cell detachment.

Recent evidence supports the view that protease activity in mite extract plays a role in a number of other potentially significant pathophysiologic processes. Der p 1 can selectively cleave the low-affinity IgE Fc receptor (CD23) from the surface of human B lymphocytes thereby promoting and enhancing IgE immune responses by ablating an important feedback inhibitory mechanism that normally limits IgE synthesis.³⁵ In addition, mite proteases can activate kallikrein,³⁶ inactivate α 1-antitrypsin,³⁷ and degranulate mast cells by mechanisms independent of IgE cross-linkage.³⁸ Furthermore, Der p 1 can increase bronchial permeability to serum albumin and cause epithelial cell detachment.¹⁶

A LMW fraction of the mite extracts showed cytokine production that was protease-independent and resistant to heat. Similar protease-independent cytokine productions were observed for Timothy grass and birch pollen extracts. The mechanisms for these protease-independent productions are unknown. Most of the Timothy grass and

birch pollen allergens have unknown functions,²⁶ although Phl p 5 and Bet v 1 may have ribonuclease activity.^{39,40} Timothy grass and birch pollen extracts possess the protein profilin, a 14 kd protein possibly involved in actin polymerization.⁴¹ One may speculate that profilin is responsible in part for the induction of a cytokine response in our studies by the aforementioned mechanism of modulating the cellular structure, although we do not have data to substantiate this assumption. Expression of proteins that are members of the actin-depolymerizing factor protein family⁴² involved in polymerization and depolymerization has recently been found in maize pollen (*Zea mays*),⁴³ providing additional evidence that pollens at least have the potential to produce substances that can modulate cellular structure. Alternatively, the production of regulatory proteins, such as the calmodulin-related protein and major allergen from birch pollen (Bet v 3),⁴⁴ may actually affect intracellular Ca^{2+} concentrations and, consequently, cell activation. Furthermore, it cannot be excluded that innate recognition by receptors similar to that of antigen-presenting cells are involved in epithelial cell signaling.

The present data add a potential and new viewpoint to the mechanisms that may be involved in local airway inflammation. Epithelial cells may play an important role in initiating or maintaining local inflammation of the airways by the interaction with inhaled allergen extracts. Potentially, these findings may provide an explanation for airway inflammation in nonatopic patients, although the influence of this interaction in atopic inflammatory responses cannot be excluded. In addition, these data demonstrate that extracts of inhalant allergens may enhance airway inflammation by means other than their IgE-binding activity through protease-dependent and protease-independent mechanisms. However, much remains to be elucidated with regard to how such an inflammatory response is controlled.

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