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Interaction of Mite Allergens Der P3 and Der P9 with Protease-Activated Receptor-2 Expressed by Lung Epithelial Cells[1]

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The respiratory epithelium represents the first barrier encountered by airborne Ags. Two major dust mite Ags, Der p3 and Der p9, are serine proteases that may activate lung epithelial cells by interaction with the protease-activated receptor 2 (PAR-2). In this study both Der p3 and Der p9 cleaved the peptide corresponding to the N terminus of PAR-2 at the activation site. Both Ags sequentially stimulated phosphoinositide hydrolysis, transient cytosolic Ca^{2+} mobilization, and release of GM-CSF and eotaxin in human pulmonary epithelial cells. These responses were similar to those observed with trypsin and a specific PAR-2 agonist and were related to the serine protease activity of Der p3 and Der p9. Cell exposure to the Ags resulted in a refractory period, indicating that a PAR had been cleaved. Partial desensitization to Der p3 and Der p9 by the PAR-2 agonist suggested that PAR-2 was one target of the Ags. However, PAR-2 was not the only target, because the PAR-2 agonist caused less desensitization to Der p3 and Der p9 than did trypsin. A phospholipase C inhibitor prevented the cytokine-releasing effect of the PAR-2 agonist and abolished or reduced (>70%) the cytokine-releasing effects of Der p3 and Der p9. Our results suggest that Der p 3 and Der p9 may induce a nonallergic inflammatory response in the airways through the release of proinflammatory cytokines from the bronchial epithelium and that this effect is at least in part mediated by PAR-2. ***The Journal of Immunology,*** **2001, 167: 1014–1021.**

Airborne Ags represent the most common environmental factors that can induce allergic inflammatory response in the airways of susceptible individuals. High and persistent exposure to these molecules, particularly the house dust mite allergens, represents a major risk factor for the development of asthma (1, 2).

Some observations suggest that airway resident cells and their product may affect the outcome of Ag exposure (3, 4), and airway epithelial cells are the first resident cells encountered by inhaled Ags. Because the airway epithelium represents a tight and highly regulated barrier that normally prevents their access, potential allergens may cause structural and functional changes in the epithelium that facilitate their interaction with the host and direct the response toward an inflammatory reaction.

This possibility is supported by the evidence that most Ags capable of inducing an allergic response have intrinsic biochemical activity (5). At certain concentrations, those possessing proteolytic activity cause an increased permeability of epithelial cells in vitro and may potentially penetrate through the epithelial barrier in vivo by disruption of the epithelial tight junction (6). At lower concentrations, they also stimulate airway epithelial cells to produce cytokines that may affect the function of immune and structural cells in the airway mucosa (7–14).

We previously observed that the cysteine protease from the house dust mite *Dermatophagoides pteronyssinus*, Der p1, induces the expression of GM-CSF, RANTES protein, and eotaxin in bronchial epithelial cells by promoting the activation of transcriptional factor NF-κB (7, 8, 14). Such an effect depends on the allergen concentrations, time of exposure, and the ability of the cells to inactivate it (7, 8, 14). Der p1 activity is more prominent in bronchial epithelial cells of asthmatic patients, where the Der p1-induced release of cytokines occurs at much lower concentrations of the Ag and after a shorter period of exposure than in cells from nonasthmatic subjects. This is probably due to an intrinsic defect in intracellular antiprotease defenses of asthmatic epithelium (7, 14).

To evaluate further the mechanisms of interaction between airway epithelium and clinically important allergens with proteolytic activity, in the present study we examined the effect of exposure of pulmonary epithelial cells to the major house dust mite Ags, Der p3 and Der p9. Although Der p9 also possesses a collagenolytic activity, both these Ags are serine proteases (15–17) with the potential to interact with the protease-activated receptors (PARs)[3] (18, 19) expressed on human pulmonary epithelial cells (20), particularly the PAR-2 (18). There is increasing evidence that stimulation of PAR-1 and PAR-2 is associated with the release of proinflammatory cytokines and the expression of adhesion molecules on inflammatory cells (21–23). Therefore, we tested the hypothesis that Der p3 and Der p9 activate PAR-2 and induce PAR-2-mediated release of GM-CSF and eotaxin, two epithelial cell-derived cytokines involved in allergic and nonallergic airway inflammation and asthma (11, 24–26).

The experiments described in this study require a large number of epithelial cells that cannot be obtained by enzymatic digestion of surgical specimens or bronchial biopsies from living human donors as previously reported (7, 8, 10, 17). In addition, the substances to be used for effective enzymatic digestion may affect the

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[3] Abbreviations used in this paper: PAR, protease-activated receptor; AP, PAR-2-activating peptide; α_1-Ap, α_1-antiprotease; CDS, cell dissociation solution; Lp, leupeptin; PoB, polymyxin B; RP, reverse AP; SBTI, soybean trypsin inhibitor; $[Ca^{2+}]_i$, intracellular calcium concentration.

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integrity and function of PARs (18). Therefore, we used cells from the human pulmonary cell line A549 that behave similarly to cells from primary cultures of human pulmonary epithelium when exposed to proteolytic allergens (9, 13), express functional PAR-2 (27), and can be harvested from culture plates using a commercially available, nonproteolytic cell dissociation solution (CDS).

Materials and Methods

Synthetic peptides and reagents

The peptide whose sequence corresponds to the regions surrounding the activating site of human PAR-2 (residues 32–45), the human PAR-2-activating peptide (AP) SLIGKVD-NH_2 (18, 28) and the reverse AP (RP) DVKGILS-NH_2 were synthesized by solid phase methods with an automated peptide synthesizer. Peptides were purified by reversed phase HPLC, and their structure was confirmed by mass spectroscopy. CDS, thrombin, trypsin, leupeptin (Lp), soybean trypsin inhibitor (SBTI), E64, and polymyxin B (PoB) were obtained from Sigma (Poole, U.K.). Phospholipase C inhibitor U73122 and control compound U73343 were purchased from Calbiochem (San Diego, CA). Ham's F-12 medium, FCS, DMEM, BSA, and buffered solutions were obtained from Life Technologies (Paisley, U.K.). Fura-2/AM was purchased from Molecular Probes (Eugene, OR), and [^{3}H]inositol was obtained from ICN Radiochemicals (Irvine, CA).

Isolation of Der p3 and Der p9

Extracts of *D. pteronyssinus* were prepared from fecally enriched culture medium as in our previous studies (7, 8, 10). Der p3 was isolated by a combination of DEAE cellulose anion exchange chromatography, SBTI-Sepharose 4B affinity chromatography, and cation exchange chromatography, and Der p9 was isolated using a combination of ion exchange chromatography and SBTI affinity chromatography as previously described (15, 17). The serine protease activities of Der p3 and Der p9 were determined using the substrates succinyl-Ala-Ala-Pro-Leu *p*-nitroanilide, *N*-benzoyl-arginine *p*-nitroanilide, and succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide as previously described (17). The collagenolytic activity of Der p9 was tested by digestion of type III collagen from calf skin as reported previously (17).

Cleavage of human PAR-2 by Der p3 and Der p9

A peptide corresponding to residues 32–45 in the human PAR-2 sequence (29) (200 μM) was incubated with increasing concentrations of Der p3 and Der p9 in PBS for 30 min at 37°C. Control samples were also incubated with increasing concentrations of trypsin, which is known to cleave PAR-2 at the activating site, or with 20 nM thrombin, which does not cleave PAR-2 at these concentrations under the same experimental conditions (29, 30). The cleavage products were applied to a C_{18} HPLC column, eluted with a gradient from 0 to 100% acetonitrile in 0.1% trifluoroacetic acid, and analyzed by mass spectrometry according to a previously described procedure (29).

Cell cultures

Cells from the A549 cell line were purchased from American Type Culture Collection (Manassas, VA) and cultured in sterile 24-well culture plates (Costar, Cambridge, MA) in Ham's F-12 medium supplemented with 10% heat-inactivated FCS and antibiotics. Cells were passaged using CDS to detach cells from the culture plates according to the manufacturer's instruction. Before stimulation confluent cell monolayers were washed with HBSS and incubated for 24 h in DMEM without FCS and other growth factors. In the experiments involving prolonged cell exposure to proteases to detect cytokine release, possible morphological changes in the cells in culture were evaluated under an inverted microscope and scored using the following three-point scale: 0 = no morphological changes, 1 = cell shrinking, and 3 = cell detachment. Cell viability was quantified by the trypan blue exclusion method.

Inositol phosphate formation

A549 cells were incubated overnight with 4 μCi/ml [^{3}H]inositol (sp. act., 20 Ci/mmol). At the end of the incubation period cells were washed, incubated at 37°C with 20 mM LiCl for 1 h and then stimulated with trypsin, Der p3 or Der p9, and AP or RP for 45 min at 37°C. Total [^{3}H]inositol phosphates were extracted with perchloric acid, neutralized, and analyzed by ion exchange chromatography on Dowex columns after the addition of phytic acid as a carrier.

Ca^{2+} mobilization

A549 cells were detached from culture plates by exposure to CDS and washed twice with PBS containing 0.2% BSA, 0.1% glucose, and 1 mM $CaCl_2$. Cells were resuspended in PBS to a concentration of 2×10^6 cells/ml and diluted with an equal volume of 4 μM fura 2/AM in PBS. After incubation for 1 h at 37°C, free fura 2/AM was removed by repeated washing in PBS and cells were resuspended in PBS at the concentration of 2×10^6 cells/ml for stimulation. Suspensions of loaded cells were transferred to stirred quartz cuvettes and placed in a Perkin-Elmer LS-50 spectrofluorometer (Norwalk, CT) at 37°C. Fura 2 fluorescence was measured at excitation and emission wavelengths of 340 and 510 nm, respectively. After establishing a stable baseline, trypsin, Der p3 or Der p9, or AP or RP was added to the loaded cells in the cuvettes. For desensitization experiments, cells were sequentially exposed to each agent at intervals of 2–3 min, without an intervening wash. Fluorescence change was converted to intracellular calcium concentration ($[Ca^{2+}]_i$) using the dissociation equilibrium constant for the binding of Ca^{2+} to fura 2 of 224 nM (31).

GM-CSF and eotaxin release

A549 cells were exposed to increasing concentrations of Der p3, Der p9, or AP for different periods of time in serum-free DMEM. RP was used as a negative control for the PAR-2 agonist. Cells incubated in culture medium alone were used to detect any spontaneous cytokine release. The GM-CSF content in culture medium was quantified by the same ELISA used in previous studies (7, 10). Eotaxin immunoreactivity was determined by RIA as previously described (11). Culture medium (100 μl) was mixed with 100 μl PBS/azide/10% polyethylene glycol/0.5% protamine sulfate. The mixture was incubated with ^{125}I-labeled eotaxin (2300 cpm; sp. act., 1800 Ci/mM) and an anti-eotaxin antiserum (R&D Systems Europe, Abingdon, U.K.; 1/2000 dilution) diluted in 0.2 M sodium phosphate buffer containing 0.5% BSA and 10 mM EDTA/azide, pH 7.4. Separation of the Ab-bound from free eotaxin was obtained by an immunobead second Ab reagent (0.8 mg; Bio-Rad, Richmond, CA). After incubation for 6 h the mixture was centrifuged, the supernatant was discarded, and bound radioactivity in the precipitate was quantified in a gamma counter. Nonspecific binding was determined in replicate samples incubated in the absence of the antiserum or with control rabbit Ig. The eotaxin concentration was calculated by comparison with the human eotaxin standard curve. The detection limit of these assays was 50 pg/ml for GM-CSF and 15 pg/ml for eotaxin. The identity of immunoreactivity was confirmed by fast protein liquid chromatography using human recombinant GM-CSF and eotaxin (R&D Systems Europe) for calibration (10).

Inhibitory experiments

Inactivation of Der p3 and Der p9 by heat was conducted at 60°C for 20 min before their addition to cell cultures. The effects of the serine protease inhibitors, Lp, α_1-antiprotease (α_1-Ap), and SBTI, and the effect of the endotoxin inhibitor PoB were tested by incubating Der p3 and Der p9 with each agent for 20 min at 37°C before their addition to cell cultures.

Statistical analysis

Group data were compared by two-tailed unpaired Student's *t* test or one-way ANOVA with Tukey-Kramer's *t* tests for multiple comparisons, as appropriate, and are presented as the mean ± SEM. Values of $p < 0.05$ was considered significant.

Results

Der p3 and Der p9 cleavage sites on human PAR-2

PARs initiate cell signaling when activated by proteolytic enzymes. These receptors possess specific cleavage sites for thrombin (PAR-1, PAR-3, and PAR-4), trypsin (PAR-2 and PAR-4), and possibly other serine proteases within their extracellular N-terminal domains (18, 19, 32, 33). After cleavage at these specific sites, the new N terminus for each receptor functions as a tethered ligand and binds intramolecularly to the body of the receptor to affect transmembrane signaling.

Peptides corresponding to sequences within the N terminus of PARs can be effectively used for predicting potential sites of cleavage within the intact receptor (29). Therefore, we initially tested any potential interaction between the house dust mite serine proteases and PAR-2 by adding Der p3 and Der p9 to a peptide whose sequence corresponds to the region around the activating site of

PAR-2. Proteolytic fragments were then identified by HPLC and mass spectrometry.

Both Der p3 and Der p9 cleaved the peptide corresponding to residues 32–45 in the sequence of the extracellular N-terminal domain of human PAR-2 at the site (Arg^{36}-Ser^{37}), thereby exposing the receptor's tethered ligand (Table I). Trypsin, which is known to activate PAR-2, cleaved the peptide at the same site (Table I). The expected fragment, SSKGR was not detected, probably because Der p3 and Der p9 also cleaved the peptide at the site [34–35], as previously demonstrated with trypsin (30), and the fragments SSK and GR were too small to be detected by our assay (29). Thrombin, which does not activate PAR-2 under similar experimental conditions (29), did not cleave the PAR-2 peptide (Table I).

Der p3- and Der p9-induced phosphoinositide hydrolysis

For each PAR, synthetic peptides that mimic the first six amino acids of the new N terminus unmasked by receptor cleavage correspond to the tethered ligands. They function as PAR agonists that directly activate the receptors, bypassing the requirement for receptor cleavage, and are valuable agents for probing receptor function.

PAR activation results in conformation changes that allow interaction of receptors with heterotrimeric G proteins and thereby triggers many parallel mechanisms of signal transduction. Activation of phospholipase C-β results in phosphoinositide hydrolysis and formation of inositol triphosphate and diacylglycerol (18). Inositol triphosphate stimulates the release of Ca^{2+} from intracellular stores, which induces the influx of extracellular calcium, whereas diacylglycerol activates protein kinase C (18).

To detect PAR-2 activation by Der p3 and Der p9 we evaluated phosphoinositide hydrolysis in cells exposed to increasing concentrations of the two proteases. The known activator of PAR-2, trypsin and the tethered ligand agonist of human PAR-2 AP were used as positive controls. The inactive RP was used as a negative control.

The results showed that both Der p3 and Der p9 induced phosphoinositide hydrolysis (Fig. 1). The peak inositol phosphate formation was, respectively, 5.8- and 9.5-fold over the baseline, and the mean concentration causing 50% of the maximal response was 6 nM for Der p3 and 0.7 nM for Der p9. The pattern of response to Der p3 was similar to that observed with trypsin, whereas Der p9 was significantly more effective than the known PAR-2 activator ($p < 0.025$; Fig. 1). AP also induced phosphoinositide hydrolysis in a concentration-dependent manner, but was much less potent than trypsin ($p < 0.01$; Fig. 1). Peak inositol phosphate formation was 5-fold greater than baseline after stimulation with AP and 7-fold greater than baseline after stimulation with trypsin. The mean concentration causing 50% of the maximal response was 2.5 μM for AP and 4 nM for trypsin. RP did not cause any phosphoinositide hydrolysis (Fig. 1).

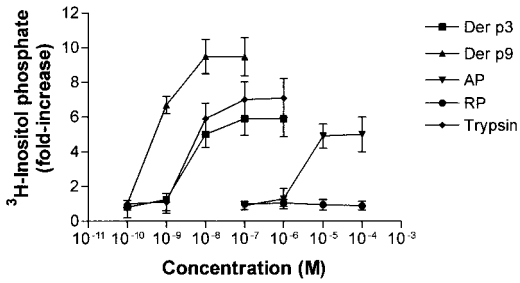

FIGURE 1. Concentration-dependent phosphoinositide hydrolysis induced in A549 cells by Der p3, Der p9, AP, and trypsin. RP was the negative control for the PAR-2 agonist. The cells were prelabeled with [^{3}H]inositol and incubated with the proteases or AP and RP for 45 min. The results are expressed as the fold increase in total [^{3}H]inositol phosphate formation over baseline and are the mean ± SEM from five experiments in which data points were determined in triplicate.

To demonstrate the requirement for active enzyme in Der p3- and Der p9-induced receptor activation, we examined the effects of some inhibitors with varying selectivity (29, 34, 35). Der p3-induced phosphoinositide hydrolysis was eliminated by heat inactivation of the protease and by adding SBTI or the multiple serine protease inhibitors Lp and α_1-Ap (Table II). The response to Der p9 was also completely abolished by heat inactivation of the protease (Table II). SBTI, Lp, and α_1-Ap significantly reduced (all $p < 0.01$), but did not eliminate, Der p9-induced phosphoinositide hydrolysis (Table II). The cysteine protease inhibitor E64 did not have any effect on the phosphoinositide hydrolysis produced by the two proteases (Table II).

Der p3- and Der p9-induced Ca^{2+} mobilization

An increase in $[Ca^{2+}]_i$ reflects phosphoinositide hydrolysis. In keeping with the results reported above, 10 nM Der p3 and 1 nM Der p9 induced Ca^{2+}mobilization in pulmonary epithelial cells. The rapid increase in $[Ca^{2+}]_i$ was followed by a slower return to near baseline level (Fig. 2, *C* and *E*). This pattern of response was similar to that observed with 10 nM trypsin and 10 μM AP (Fig. 2, *A* and *B*) and is characteristic of PAR activation (18, 36).

Der p3- and Der p9-induced desensitization upon repeated applications

The activation of PARs by proteolytic cleavage is irreversible and makes cleaved receptors resistant to further proteolytic activation (36). Thus, the cleavage of PAR-2 on cell surface by trypsin renders cells unresponsive to trypsin or other PAR-2 activators (desensitization) until plasma membrane is replenished with intact PAR-2. Resensitization usually occurs by 60–90 min. To evaluate further the specificity of activation of PAR-2 by Der p3 and Der p9, we tested their ability to induce a refractory period compared with that of trypsin or AP. RP was used as a negative control. Desensitization of PAR-2 activation was examined by sequential exposure of A549 cells to each protease or PAR-2 agonist at intervals of 2–3 min, without an intervening wash.

Similar to the effect of repeated stimulation with 10 nM trypsin (Fig. 2*A*), Der p3 and Der p9 abolished the Ca^{2+} response to a second application of these proteases (Fig. 2, *C* and *E*). Concentrations of AP capable of reducing the Ca^{2+} response to 10 nM

Table I. *Analysis of cleavage products*[a]

Protease	Concentration (nM)	PAR-2 Peptide[b]	Fragment(s) Recovered
Der p3	0	^{32}SSKGR/SLIGKVDGT^{45}	Not cleaved
	5	^{32}SSKGR/SLIGKVDGT^{45}	SLIGKVDGT
Der p9	0	^{32}SSKGR/SLIGKVDGT^{45}	Not cleaved
	5	^{32}SSKGR/SLIGKVDGT^{45}	SLIGKVDGT
Trypsin	0	^{32}SSKGR/SLIGKVDGT^{45}	Not cleaved
	5	^{32}SSKGR/SLIGKVDGT^{45}	SLIGKVDGT
Thrombin	20	^{32}SSKGR/SLIGKVDGT^{45}	Not cleaved

[a] The peptide corresponding to the human PAR-2 residues 32-45 was incubated with the indicated concentrations of Der p3, Der p9, trypsin, and thrombin for 30 min at 37°C in PBS. The cleavage products (fragments recovered) were analyzed by HPLC and mass spectrometry.

[b] The numbering refers to the sequence region of the intact receptor, and the slashes indicate the site of cleavage (Arg^{36}-Ser^{37}) that leads to exposure of the tethered ligand domain (underlined residues) in the intact receptor.

Table II. *Inhibition of the effect of Der p3 and Der p9 on phosphoinositide hydrolysis*[a]

	[^{3}H]Inositol Phosphate Fold Increase Over Baseline					
	Treatment					
	None	Heat Inactivation	+ Lp (1 μM)	+ α_1-Ap (100 nM)	+ SBTI (1 μM)	+ E64 (10 μM)
Der p3 (10 nM)	5.9 ± 1.8	1.0 ± 0.7	1.0 ± 0.6	1.2 ± 0.4	1.0 ± 0.3	6.1 ± 1.5
Der p9 (1 nM)	9.7 ± 1.2	1.1 ± 0.5	2.5 ± 0.9	1.9 ± 0.3	3.7 ± 1.1	8.9 ± 1.8

[a] Values are mean ± SEM from six experiments in which data were measured in triplicate.

trypsin (Fig. 2*B*) were also effective in reducing the Ca^{2+} response to Der p3 and Der p9 (Fig. 2, *D* and *F*), while RP had no desensitizing property (Fig. 2*H*). Differing from AP, trypsin exposure completely blocked the Ca^{2+} mobilization induced by Der p3 and Der p9 (Fig. 2*G*).

Der p3- and Der p9-induced cytokine release and relation to PAR-2 activation

To examine the ability of Der p3 and Der p9 to induce GM-CSF and eotaxin release from pulmonary epithelial cells, A549 cells were incubated for 24 h in medium devoid of growth factors and then exposed for different periods of time to increasing concentrations of the two proteases or to control medium alone. To test the hypothesis that Der p3 and Der p9 cause cytokine release by activation of PAR-2, parallel experiments were conducted with increasing concentrations of the specific PAR-2 agonist AP and with the inactive RP.

Exposure to 10 nM Der p3 and 1 nM Der p9 caused the release of both GM-CSF and eotaxin from A549 cells (Fig. 3). Appreciable amounts of immunoreactive GM-CSF could be detected in the culture medium after 1 h of Der p3 and Der p9 exposure. GM-CSF immunoreactivity peaked between 4 and 8 h (Fig. 3*A*). Eotaxin release was evident after 2 h of exposure and peaked between 8 and 12 h (Fig. 3*B*). The time course of this response was similar to that observed in cells exposed to 100 μM AP, whereas RP did not cause any detectable cytokine release (Fig. 3).

The induced release of GM-CSF and eotaxin by Der p3, Der p9, and PAR-2 agonist was concentration dependent (Fig. 4). As observed with the induction of phosphoinositide hydrolysis, Der p3 and Der p9 were more potent than AP in promoting GM-CSF and eotaxin release, and this may reflect their different modes of action. Nonetheless, the maximal (mean ± SEM) GM-CSF and eotaxin immunoreactivity at the highest concentrations of AP (469 ± 108 and 122 ± 32 pg/10^6cells) was similar to that observed with the highest concentration of Der p3 (472 ± 117 and 182 ± 47 pg/10^6

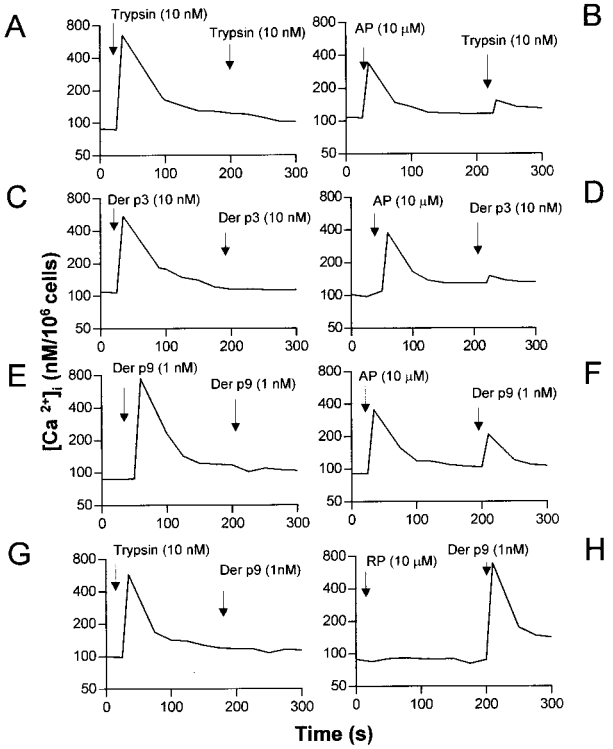

FIGURE 2. Ca^{2+} mobilization induced in A549 cells by trypsin, Der p3, Der p9, and AP and homologous (*A*, *C*, and *E*) or heterologous (*B*, *D*, *F*, and *G*) desensitization obtained by repeated application of these agents at the time indicated by the arrows. RP was the negative control for the PAR-2 agonist (*H*). Cells were sequentially exposed to each agent at intervals of 2–3 min, without an intervening wash. $[Ca^{2+}]_i$ was determined by monitoring fluorescence changes in cells loaded with fura-2. Fluorescence changes were converted to nanomolar concentrations per 10^6 cells. The results are representative traces from 8 to 10 experiments.

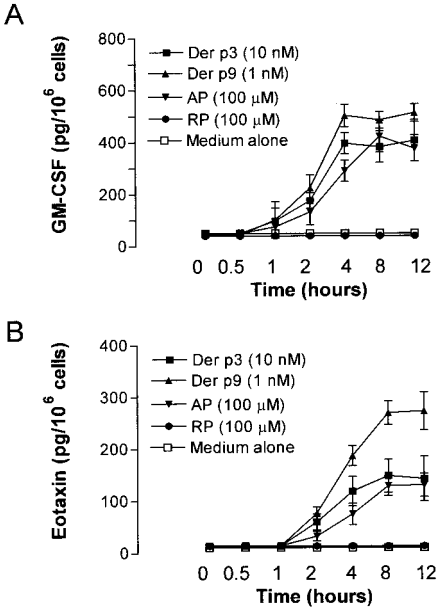

FIGURE 3. Time course of GM-CSF (*A*) and eotaxin (*B*) release induced by the indicated concentrations of Der p3, Der p9, and AP in A549 cells. RP was the negative control for the PAR-2 agonist, and cells incubated in culture medium alone were used to detect any spontaneous cytokine release. The results are the mean picograms per 10^6 cells ± SEM of immunoreactive GM-CSF or eotaxin recovered in the culture medium at the end of cell exposure in five experiments in which data points were determined in triplicate. The detection limit of the assays was 50 pg/ml for GM-CSF and 15 pg/ml for eotaxin.

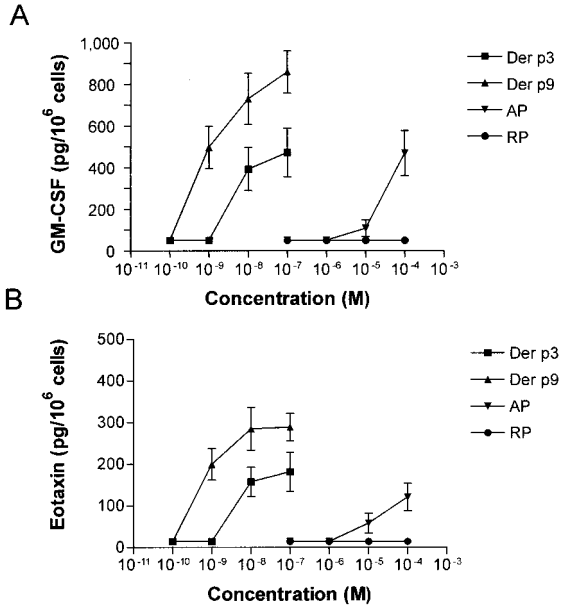

FIGURE 4. Concentration-dependent release of GM-CSF (*A*) and eotaxin (*B*) induced by exposure of A549 cells to Der p3, Der p9, and AP for 8 h. RP was the negative control for the PAR-2 agonist, and cells incubated in culture medium alone were used to detect any spontaneous cytokine release. The results are the mean picograms per 10^6 cells ± SEM of immunoreactive GM-CSF and eotaxin recovered in the culture medium at the end of cell exposure in six experiments in which data points were determined in triplicate. The detection limit of the assays was 50 pg/ml for GM-CSF and 15 pg/ml for eotaxin.

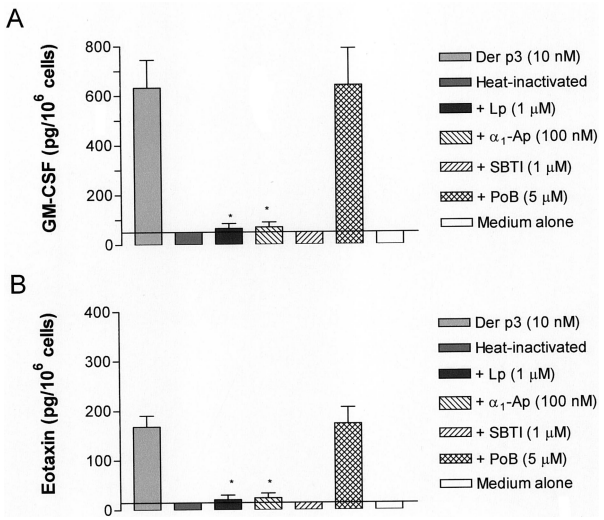

FIGURE 5. Effects of different potential inhibitors or heat inactivation on the ability of Der p3 to induce GM-CSF (*A*) and eotaxin (*B*) release in A549 cells. Heat inactivation of the protease was conducted at 60°C for 20 min. The effects of the serine protease inhibitors Lp, α_1-Ap, and SBTI and the effect of the endotoxin inhibitor PoB were tested by incubating Der p3 with each agent for 20 min at 37°C before its addition to cell cultures. Cells incubated in culture medium alone were used to detect any spontaneous cytokine release. The results are the mean picograms per 10^6 cells ± SEM of immunoreactive GM-CSF and eotaxin recovered in the culture medium after 8 h of exposure to Der p3 in five experiments in which data were measured in triplicate. The horizontal lines indicate the detection limit of the assays (50 pg/ml for GM-CSF and 15 pg/ml for eotaxin). *, $p < 0.01$ compared with cells incubated with untreated Der p3.

cells; both $p > 0.05$) and only ~2.0-fold lower than that detected with the highest concentration of Der p9 (860 ± 101 and 289 ± 33 pg/10^6cells; both $p < 0.025$; Fig. 4). However, cultures of A549 cells exposed to concentrations of Der p3 higher than 10 nM and to concentrations of Der p9 higher than 1 nM demonstrated cell shrinking and detachment. These morphologic alterations are known to induce cytokine release from epithelial cells (37); therefore, the effects of the two proteases at these concentrations may not have been specific.

Heat inactivation and pretreatment with serine protease inhibitors blocked most of the activity of concentrations of Der p3 that were unable to cause morphologic alterations in A549 cells (Fig. 5). Similar pretreatment of Der p9 also greatly reduced the ability of this protease to induce cytokine release at concentrations that did not cause cell shrinking or detachment, but did not abolish it (Fig. 6).

The cytokine-releasing effects of Der p3 and Der p9 did not appear to be related to endotoxin contamination, because the addition of the endotoxin antagonist PoB did not affect the release of GM-CSF or eotaxin associated with the exposure to the two proteases (Figs. 5 and 6).

To confirm that induction of cytokine release by Der p3 and Der p9 was indeed due to PAR activation, replicate cultures of A549 cells were pretreated with the phospholipase C inhibitor U73122 or the control compound U73343 for 1 h before the addition of Der p3, Der p9, and AP for 8 h. AP was again used as a positive control for PAR-2 specificity. Similar pretreatment of A549 cells with the phospholipase C inhibitor had completely inhibited the formation of inositol phosphates and Ca^{2+} mobilization produced by the two proteases and by AP in preliminary experiments. The results reported in Table III show that inhibition of phospholipase C also abolished both Der p3- and AP-induced release of GM-CSF and eotaxin and significantly reduced ($p < 0.01$) the release of these cytokines from cells exposed to Der p9.

Discussion

There is increasing evidence that most clinically relevant airborne allergens possess intrinsic biochemical activity and may alter the integrity and function of airway epithelium (7–14, 38). In so doing they may enhance the likelihood of allergic sensitization and activation of inflammatory reactions in the bronchial mucosa.

The major allergens implicated in the pathogenesis of allergic airway diseases and asthma are those derived from the house dust mite *D. pteronyssinus* (1, 2). Among them, Der p1, Der p2, Der p3, and Der p9 have demonstrated the highest frequency of reactivity (38). Although several studies have investigated the possible mechanisms of interaction between the cysteine protease Der p1 and bronchial epithelial cells (6–8, 10, 12), the effects of other mite Ags with known proteolytic properties (Der p3 and Der p9) are unclear. One study (12) demonstrated the release of GM-CSF, IL-6, and IL-8 from human bronchial epithelial cells and cells from a pulmonary epithelial cell line exposed to Der p9, but the mechanism of Der p9-induced cytokine release is still unknown. The hypothesis that Der p3 may also affect the function of airway epithelial cells has never previously been tested.

The results of this study indicate that both Der p3 and Der p9 can induce the release of the proinflammatory cytokines GM-CSF and eotaxin from human pulmonary epithelial cells. The concentrations of Ags tested here were close to those previously measured in the airways of asthmatic patients naturally exposed to mite allergens (39). These results are important because the production of both GM-CSF and eotaxin is increased in bronchial epithelial cells from asthmatic patients, and these cytokines play a major role in the ongoing inflammatory and structural alterations of asthma (11,

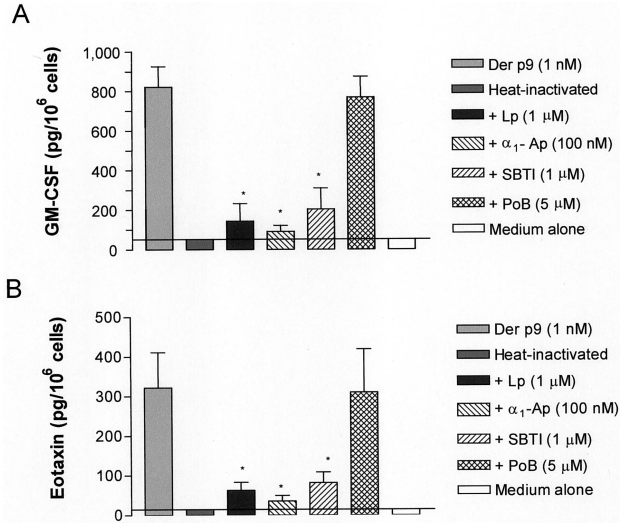

FIGURE 6. Effects of different potential inhibitors or heat inactivation on the ability of Der p9 to induce GM-CSF (*A*) and eotaxin (*B*) release in A549 cells. Heat inactivation of the protease was conducted at 60°C for 20 min. The effects of the serine protease inhibitors Lp, α_1-Ap, and SBTI and the effect of the endotoxin inhibitor PoB were tested by incubating Der p9 with each agent for 20 min at 37°C before its addition to cell cultures. Cells incubated in culture medium alone were used to detect any spontaneous cytokine release. The results are the mean picograms per 10^6 cells ± SEM of immunoreactive GM-CSF and eotaxin recovered in the culture medium after 8 h of exposure to Der p9 in five experiments in which data were measured in triplicate. The horizontal lines indicate the detection limit of the assays (50 pg/ml for GM-CSF and 15 pg/ml for eotaxin). *, $p < 0.01$ compared with cells incubated with untreated Der p9.

25, 26, 40, 41). Eotaxin and GM-CSF promote the accumulation of eosinophils in the bronchial mucosa of asthmatic patients and their local activation (24, 26, 40, 41). Epithelial cell-derived GM-CSF enhances Ag presentation by dendritic cells (3), stimulates endothelin 1-induced differentiation of airway fibroblasts into myofibroblasts after allergen exposure (10), and may contribute to direct the response to house dust mite Ags toward a Th2-mediated immune reaction (3).

The second important observation in this study concerns the mechanism by which Dep3 and Der p9 can induce cytokine release from human pulmonary epithelial cells. Because both Ags are serine proteases, they are likely to interact with the PAR-2 expressed on pulmonary epithelial cells (20). At the time of this study no anti-PAR-2 molecule suitable for inhibition experiments was available. Therefore, we planned a series of experiments addressed at providing indirect evidence that this interaction can occur and is causally related to the induced release of GM-CSF and eotaxin, on the basis of the available information concerning the mechanisms of PAR signal transduction (18).

Like trypsin, a known activator of PAR-2, both Der p3 and Der p9 cleaved the receptor at the specific site that exposes the tethered ligand, indicating that they do have the potential for activating PAR-2.

Both Der p3 and Der p9 stimulated phosphoinositide hydrolysis and transient cytosolic Ca^{2+} mobilization in A549 cells with a pattern of response similar to that showed by trypsin and the specific PAR-2 agonist. These effects were related to the serine protease activity of the two Ags because they were inhibited or markedly reduced by specific antiproteases. In addition, repeated applications of Der p3 and Der p9 abolished their ability of inducing a prompt increase in $[Ca^{2+}]_i$, and this desensitization is consistent with the irreversible proteolytic activation of PARs (18, 36). Both trypsin and AP also abolished or markedly reduced the Ca^{2+} response to a second application of Der p3 and Der p9, indicating that irreversible activation of PAR-2 by specific ligands made cells refractory to stimulation with Der p3 and Der p9 and implying that Der p3 and Der p9 cause Ca^{2+} mobilization in A549 cells by activating PAR-2. Nonetheless, AP caused less desensitization to Der p3 and Der p9 than trypsin. This difference in desensitizing properties between trypsin and the agonist of PAR-2 has been reported in a previous study (36). Because cleavage of the receptor is the only way to ensure that a single receptor molecule cannot respond again to an activator once cleaved, the larger desensitization to Der p3 and Der p9 observed with trypsin may be due to the fact that trypsin cleaves PAR-2, whereas AP does not. Alternatively, trypsin, Der p3, and Der p9 may activate an additional PAR not triggered by AP or a PAR-2 subtype that is triggered less efficiently by AP.

The experiments discussed above indicate that the exposure of human pulmonary epithelial cells to both Der p3 and Der p9 rapidly sets in motion the first steps of the PAR-2 signal transduction involving the activation of phospholipase C. This also leads to the formation of diacylglycerol and consequent activation of protein kinase C, which, in turn, activates the effector function of different enzymes resulting in increased gene transcription (18). We reasoned that if PAR-2 activation is involved in the release of GM-CSF and eotaxin induced by Der p3 and Der p9, the following events should be demonstrated: 1) the PAR-2 agonist should cause the same effect with a similar pattern of response; 2) inhibition of the proteolytic activity of Der p3 and Der p9 should abolish their ability of inducing GM-CSF and eotaxin release; and 3) inhibition of a crucial step in the PAR signal transduction pathway should abolish cytokine release induced by Der p 3 and Der p9 and that induced by AP as well. AP indeed caused GM-CSF and eotaxin release with a time course similar to those of Der p3 and Der p9. However, AP was less effective than Der p9 in terms of magnitude

Table III. *Effect of phospholipase C inhibition on the cytokine-releasing activity of Der p3, Der p9, and AP*[a]

	GM-CSF (pg/10^6 cells)			Eotaxin (pg/10^6 cells)		
	Treatment			Treatment		
	None	U73122 (10 μM)	U73343 (10 μM)	None	U73122 (10 μM)	U73343 (10 μM)
Der p3 (10 nM)	495 ± 76	<50[b]	421 ± 83	139 ± 35	<15[b]	122 ± 99
Der p9 (1 nM)	699 ± 45	184 ± 56	724 ± 101	327 ± 72	96 ± 12	451 ± 83
AP (100 μM)	324 ± 85	<50[b]	296 ± 61	115 ± 54	<15[b]	136 ± 74

[a] Values are mean ± SEM from five experiments in which data were measured in triplicate.
[b] Detection limit of the assay.

of the response. Pretreatment of Der p3 and Der p9 with inhibitors of serine proteases markedly reduced their ability to induce GM-CSF and eotaxin release, but some residual activity could still be demonstrated with Der p9. Finally, an inhibitor of phospholipase C that abolished phosphoinositide hydrolysis and Ca^{2+} mobilization by Der p3, Der p9, and AP also prevented the cytokine-releasing effect of Der p3 and AP and reduced by >70% the release of cytokines induced by Der p9. These results strongly suggest that Der p3 and Der p9 can trigger GM-CSF and eotaxin release in pulmonary epithelial cells by activation of PAR-2, but also indicate that the effect of Der p9 may be mediated by some additional mechanism. This may be related to its collagenolytic activity (17) or may involve the activation of other PARs at sites different from the trypsin site (18).

The correlative observations reported here, suggesting that PAR-2 is one of the targets of Der p3 and Der p9, are supported by the preliminary results of another study still in progress in our laboratory (M. A. Stacey, L. Mori, M. Schmidt, and S. Mattoli, unpublished observations) in which the wild-type PAR-2 and a mutant PAR-2 were expressed in *Xenopus* oocytes. The PAR-2 mutant was generated to render the cleavage site uncleavable by replacing serine with proline. Like trypsin, Der p3 and Der p9 induced a 5- to 8-fold increase in Ca^{2+} release from the cells transfected with the wild-type receptor construct. In the cells expressing the PAR-2 mutant, Der p3 and Der p9 failed to cause Ca^{2+} signaling, while the response to the tethered ligand, which bypasses the requirement for receptor cleavage, was fully detected.

In a recent study (42) activation of PAR-2 on airway epithelium caused the rapid relaxation of airway smooth muscle and inhibited induced bronchoconstriction. This effect was probably due to the release of PGE_2 from the epithelial cells and suggests a protective role for PAR-2 in the airways (43). Other studies (44, 45) have indicated that PAR-2 agonists may also promote inflammation and bronchoconstriction by neurogenic mechanisms. Our results extend those observations by demonstrating that PAR-2 activation on lung epithelial cells may directly result in the release of epithelial cell-derived substances with potent proinflammatory activity. This aspect of PAR-2 function may be particularly important in the airways of asthmatic patients, where defective antiprotease activity has been demonstrated (7, 14, 46). The persistence of enzymatically active Der p3 and Der p9 molecules that would be rapidly metabolized in normal airways may cause prolonged activation of PAR-2 despite receptor desensitization. In this case, the short-lasting protective effect of PGE_2 on bronchoconstriction and airway inflammation (43) may not be sufficient to balance the long-lasting inflammatory effects of GM-CSF and eotaxin. In addition, eosinophil activation by eotaxin and other eosinophil-activating cytokines is associated with the release of cationic proteins that affect epithelial eicosanoid metabolism, favoring the release of constricting PGs instead of PGE_2 (47).

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