

House Dust Mite Der p 1 Downregulates Defenses of the Lung by Inactivating Elastase Inhibitors

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House dust mites (HDM) are the most common source of aeroallergens and in genetic susceptible individuals can cause symptoms ranging from atopic dermatitis to bronchial asthma. Der p 1, a major target of the human immune responses to HDM, through its enzymatic properties can modulate the adaptive immune system by the cleavage of CD23 and CD25. The consequences of this would be to promote allergic inflammatory responses. Furthermore, by disrupting epithelial tight junctions Der p 1 facilitates the transport of allergen across the epithelium. Here, we report that Der p 1 has additional effects on the innate defense mechanisms of the lung, by inactivating *in vitro* and *ex vivo* the elastase inhibitors human (h) α 1-proteinase inhibitor (h-A1-Pi), mouse (m-), (but not human [h])-SLPI and h-elafin. We confirm that Der p 1 contain both cysteine and serine proteinases, and extend this finding to demonstrate for the first time that h-elafin is particularly sensitive to the biological activity of the latter. Because these elastase inhibitors have antimicrobial, as well as antielastase activity, our results suggest that inactivation of these innate components of the lung defense system by Der p 1 may increase the susceptibility of patients with allergic inflammation to infection.

Asthma is a chronic obstructive disease of the lower airways characterized by intermittent exacerbations of reversible airways obstruction, airways inflammation, and bronchial hyperreactivity (1). The chronic immune response in asthma and other atopic diseases is characterized by a predominately Th2 T cell response, associated with interleukin (IL)-4, IL-5, IL-13, and IgE production (1). Although a third of all cases of asthma can be characterized as intrinsic nonallergic, where the eliciting agent is unknown (2), it is clear that in most cases of asthma (extrinsic) the eliciting factor is of allergen origin, with a strong atopic component (3).

Prevalence of asthma varies in studies between 5 and 25% (1), and has doubled in the industrialized world over

the past 20 yr (4). Factors implicated in this increase in prevalence include altered indoor environment such as warmer housing, increased use of broad spectrum antibiotics with altered bacterial infection profiles, dietary changes, and increased aeroallergen exposure.

Exposure to a number of aeroallergens has been shown to contribute to both immediate hypersensitivity and chronic asthma, among these the common indoor allergens produced by the house dust mite (HDM). In human atopic disease, inflammatory responses to the group I antigens of *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae* (Der p 1 and Der f 1) are well documented (5–6). The Der p 1 cysteine protease is a 25-kD glycoprotein present in significant quantities in HDM fecal pellets, and is suggested to have a digestive role in the gut of the mite. A number of recent studies have demonstrated that Der p 1 is capable of cleaving human proteins with potentially immunomodulatory effects including α 1-proteinase inhibitor (A1-Pi), (7), CD23 (the human low-affinity IgE receptor) (8), CD25 (the α subunit of the human IL-2 receptor) (9), and tight junctions of bronchial epithelium, leading to increased bronchial epithelial permeability (10). In addition to A1-Pi (11–12), the lung also secretes the mucosal/alarm antiproteases secretory leukocyte proteinase inhibitor (SLPI) and elafin, which all contribute significantly to not only antielastase activity but also to activation of innate immunity (13–16). For example, using an adenovirus-over-expression strategy, we have recently reported that elafin has chemotactic activities for neutrophils and protects the lung in a *Pseudomonas aeruginosa* model of acute inflammation (17–18).

Accordingly, we sought to investigate in the present study whether or not Der p 1 was able to inactivate these important antiproteases and consequently could shift the “elastase–antielastase balance” in favor of a proinflammatory environment to facilitate both the initiation and the maintenance of the asthmatic response.

We have demonstrated here that all three antiproteases studied can potentially be degraded by Der p 1 *in vitro* and *ex vivo*. Furthermore, we have investigated in detail the enzymologic properties of Der p 1 against synthetic substrates and selective inhibitors to clarify the nature of the proteases that inactivate innate antiprotease defences. As a consequence, we have uncovered that in addition to the well-characterized cysteine proteinase present in Der p 1, a co-purifying serine protease is extremely active against human elafin.

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Abbreviations: α 1-proteinase inhibitor, A1-Pi; acute respiratory distress syndrome, ARDS; bronchoalveolar lavage fluid, BALF; dimethyl sulfoxide, DMSO; dithiothreitol, DTT; house dust mite, HDM; human neutrophil elastase, HNE; interleukin, IL; polyacrylamide gel electrophoresis, PAGE; phosphate-buffered saline, PBS; soybean trypsin inhibitor, SBTI; secretory leukocyte protease inhibitor, SLPI.

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Materials and Methods

Materials

Human (h) A1-Pi was purchased from Sigma (Poole, Dorset, UK) and human (h) synthetic elafin was manufactured by Albachem (Edinburgh, UK) as described previously (19). Recombinant human (h) SLPI was purchased from R&D Systems (Minneapolis, MN) and recombinant murine (m)-SLPI was a gift from Dr. C. Wright (Amgen, Thousand Oaks, CA). Human neutrophil elastase (HNE) was obtained from Elastin Products (Owensville, MO). E-64 (L-trans-epoxysuccinyl-leucyl-amido[4-guanidino]butane; Sigma) is an active site directed irreversible inhibitor of cysteine proteases which inhibits Der p 1 (20).

Human bronchoalveolar lavage fluid (BALF) from six patients with established acute respiratory distress syndrome (21) was a generous gift from Dr. S. Donnelly. The samples were pooled and incubated with Der p 1, as described below.

Purification of Der p 1 from HDM Fecal Pellet Extract

Sixty grams of house dust mite powder (gift from Dr. T. Wayne, University of Perth, Perth, Australia) was dissolved into 1 liter of Dulbecco's phosphate-buffered saline (PBS) (calcium and magnesium-free) containing 0.5M NaCl, 0.01% (wt/wt) sodium azide, pH 7.4. Mouse anti-Der p1 monoclonal antibody 4C1 (Indoor Biotechnologies, Clwyd, UK) was coupled to 5 g of Cyanogen-activated Sepharose 4B (Amersham Biosciences UK Limited, Polard's Wood, Buck, UK), suspended, and washed in an affinity column according to manufacturer's instructions. One hundred milliliters of Der p 1 solution was applied to the column at a flow rate of 20 ml/h. The column was washed with 400 ml PBS 0.5 M NaCl and elution performed at 20 ml/h using 5 mM glycine in 50% ethylene glycol pH 10.0. Fractions were assayed for absorbance at 280 nm, and were extensively dialyzed for 18 h against 10 liters PBS. These fractions will be denoted CS-Der p 1 for the rest of the study. Further purification was performed on half of the dialysate by applying it to an affinity column containing 100 mg of soybean trypsin inhibitor (SBTI) coupled to 5 g of Cyanogen-activated Sepharose 4B (Pharmacia Biotech), according to the manufacturer's instructions. Fractions were collected and re-assayed for absorbance at 280 nm to collect serine-protease depleted Der p 1 and re-dialysed extensively against PBS. Der p 1, which had been serine protease depleted by this process, was denoted C-Der p 1 for the rest of the study. The protein concentration of both C/CS-Der p 1 was assessed using a bicinchoninic acid plate assay (Pierce and Warriner Ltd., Chester, Cheshire, UK). Purity of the preparations was assessed using migration on 12% SDS-PAGE analysis (gels stained with using Biorad Silver Stain). Samples were stored in aliquots at -20°C .

Enzymatic Analysis of Der p 1 Fractions

7-amino-4 methylcoumarin (AMC) substrate hydrolysis. A range of synthetic fluorescent substrates (Novabiochem, Nottingham, UK) were used to analyze Der p 1 substrate selectivity, and included Cbz-Arg-Arg-AMC, H-Arg-AMC, Cbz-Phe-arg-AMC, Boc-Gln-Ala-Arg-AMC, Tosyl-Gly-Pro-Arg-AMC, H-Pro-Phe-Arg-AMC, Suc-Ala-Ala-Pro-Phe-AMC (22) and Suc-Gly-Pro-Leu-Gly-Pro-AMC (23). They were dissolved in dimethyl sulfoxide (DMSO) to generate a 10-mM stock and hydrolysis of each substrate (10 μM final) by 6 μg of CS-Der p1 was performed in triplicate in 0.5 M Tris-HCl (pH 8.0) containing 5 mM cysteine and 2% DMSO. Hydrolysis was measured by monitoring the release of AMC every 10 s for 2 min, using a Hoeffer TK 100 mini-fluorimeter (excitation wavelength of 365 nm and emission detection at 465 nm, San Francisco, CA). Substrate concentrations were kept constant by

allowing no more than 5% hydrolysis. The rate of hydrolysis was determined by linear regression analysis using the software program SIGMA PLOT, version 4.00. The concentration of AMC was determined from the regression line: $[\text{AMC}] = (y - c)/m$, where y is the fluorescent units released from hydrolysis, c is the intercept, and m is the slope of the regression line.

pH assays. The pH profile of purified CS-Der p1 against the synthetic substrates Boc-Gln-Ala-Arg-AMC and Tosyl-Gly-Pro-Arg-AMC was determined in the following buffers:

0.1 M citric acid/sodium citrate buffer (pH 3–6), 0.1M phosphate buffer (pH 6–8) and 0.5 M Tris-HCl (pH 8–9.5). All reactions were performed in the presence of 5 mM cysteine.

K_m determination. The rate constant (K_m) of CS-Der p 1 for synthetic substrates was determined by monitoring the initial rates of hydrolysis at the optimum pH for each substrate. The range of substrate concentrations used for Boc-Gln-Ala-Arg-AMC, Tosyl-Gly-pro-Arg-AMC, and Cbz-Phe-Arg-AMC were 0–600 μM , 0–10 μM , and 0–100 μM , respectively. The initial rates of hydrolysis (V_0) were plotted against the differing substrate concentrations $[S]$ to ensure that curvature was seen. The data were also represented as a straight line using the Hanes-Woolf plot; it was then applied to the Michaelis-Menten rate equation as described below using a nonlinear regression analysis program, in SIGMA PLOT version 4.00, to obtain the K_m and maximum initial velocity (V_{max}).

$$V_0 = \frac{V_{\text{max}} [S]}{K_m + [S]}$$

Differential inhibition of Der p 1. CS-Der p 1 and C-Der p 1 enzymatic activities were further characterized by differential inhibition by using E64 and APMSF, inhibitors of cysteinyl and serine proteinases, respectively. CS-Der p 1 and C-Der p 1 were preincubated with 10 μM E64 or 100 μM APMSF before assaying residual activity as described above using either Boc-Gln-Ala-Arg-AMC (10 μM final in 0.1 M phosphate buffer pH 6.0) or Tosyl-Gly-Pro-Arg-AMC (10 μM final in 0.5 M Tris-HCl pH 8.0). All buffers contained 5 mM cysteine.

SDS-PAGE and Western Blot Analysis

Protein samples were submitted to SDS-PAGE and/or analyzed by Western Blot analysis as described (24). Briefly, after electrophoretic transfer onto Hybond nitrocellulose membranes (Amersham, Bucks, UK), the membranes were probed sequentially with anti-h-elafin rabbit IgG (1:1,000 dilution, 1 h incubation at room temperature) and horseradish peroxidase-conjugated goat anti-rabbit IgG (1:2,500 dilution, 20 min at room temperature; Dako, Ely, Cambridgeshire, UK). Western blots were developed by enhanced chemiluminescence (ECL kit; Amersham).

Elastase Activity Assay

50 mM Tris 0.5 M NaCl 0.1% Triton pH 8 buffer was added to defined quantities of HNE on a 96-well ELISA plate (Linbro; Flow Laboratories, McLean, VA) to a volume of 200 μl . Fifty microliters of synthetic elastase substrate N-methoxysuccinyl-ala-ala-pro-val-p-nitroanilide (Sigma) was added to each well, and cleavage of substrate was monitored through change in absorbance (at 405 nm), measured spectrophotometrically, at defined time points, using a Dynex MRX II plate reader (Dynatech, Billingham, UK).

Anti-HNE activity of test solutions (either purified proteins or BALF) was assessed by addition of the solution to the HNE/buffer mix, maintaining a total volume of 200 μl . The plate was then incubated for 15 min at 37°C before addition of substrate as described above. It was demonstrated that Der p 1 fractions, dithiothreitol (DTT) and L-cysteine, alone or in combination, did not influence the rate of HNE cleavage of substrate (data not shown).

TABLE 1
Substrate specificity of CS-Der p 1

Substrate	Class	Specific Activity (nM of AMC released/s/mg $\pm$ SD)
Cbz-Arg-Arg-AMC	Cysteinyll	3.7 $\pm$ 0.61
H-Arg-AMC	Cysteinyll	Not detected
Cbz-Phe-Arg-AMC	Cysteinyll and serine	10.7 $\pm$ 1.75
Boc-Gln-Ala-Arg-AMC	Cysteinyll and serine	480 $\pm$ 8.8
Tosyl-Gly-Pro-Arg-AMC	"Trypsin-like" serine	100 $\pm$ 0.58
H-Pro-Phe-Arg-AMC	Serine	8.165 $\pm$ 0.88
Suc-Ala-Ala-Pro-Phe-AMC	Serine	Not detected
Suc-Gly-Pro-Leu-Gly-Pro-AMC	Metalloprotease	Not detected

Substrate specificity of CS-Der p 1 was analyzed using a number of synthetic fluorogenic substrates (10 μ M final concentration). All assays were carried out in 0.5 M Tris-HCl pH 8.0 containing 5 mM cysteine. Results are expressed as nM of 7-amino-4-methylcoumarin (AMC) released/s/mg $\pm$ SD.

Results

Enzymatic Activity of Der p 1

We first sought to establish the enzymatic specificities of our preparation of CS-Der p 1, after purification from the 4C1 affinity chromatography column. Several synthetic substrates were used as listed in Table 1. Boc-Gln-Ala-Arg-AMC, Tosyl-Gly-Pro-Arg-AMC, and Cbz-Phe-Arg-AMC were the best substrates for the CS-Der p 1 preparation, suggesting that a serine protease and a cysteine protease were the active molecules responsible for enzymatic activity. In addition, by assessing the optimal pH of enzymatic activity, we showed that CS-Der p 1 maximal enzymatic activity was observed at pH 6.0 and 8.0, against Boc-Gln-Ala-Arg-AMC and Tosyl-Gly-Pro-Arg-AMC, providing further evidence for the involvement of cysteine and serine proteases, respectively.

The affinity of these proteases was further studied in Figure 1. The mean K_m values obtained from three separate experiments for Boc-Gln-Ala-Arg-AMC, Tosyl-Gly-Pro-Arg-AMC, and Cbz-Phe-Arg-AMC were 254, 2.3, and 13 μ M, respectively, suggesting that the affinity for serine selective substrates (last two) was significantly higher than for the cysteine protease substrate (first one). The specificity of these proteases was further confirmed using the synthetic inhibitors APMSF and E-64, of serine and cysteine proteases, respectively (20). Figure 2A shows that E-64, but not APMSF, inhibited the enzymatic activity of CS-Der p 1 against the cysteine protease substrate Boc-Gln-Ala-Arg-AMC. The cysteine protease presence was further shown by the abrogation of its activity when the cysteine-activating agent L-Cysteine (L-Cys) was omitted. Conversely, APMSF, but not E64, inhibited the activity of CS-Der p 1 against the serine protease substrate Tosyl-Gly-Pro-Arg-AMC (Figure 2B).

Having established that CS-Der p 1 contained both serine protease and cysteine protease components, we segregated both activities by submitting the CS-Der p 1 fraction to SBTI-affinity chromatography.

The resulting purified C-Der p 1 was then tested on both Tosyl-Gly-Pro-Arg-AMC and Boc-Gln-Ala-Arg-AMC. We found that C-Der p 1 was active against Boc-Gln-Ala-Arg-AMC and only E-64 abolished that activity (Figure 2C).

There was no recordable activity against Tosyl-Gly-Pro-Arg-AMC (data not shown), establishing that indeed, C-Der p 1 only contained the cysteine proteinase activity.

Having demonstrated enzymatically that CS-Der p 1 contained both cysteine and serine protease activities (Table 1, and Figures 1, 2A, and 2B), whereas C-Der p 1 only contained the former (Figure 2C), the activity of both fractions on the lung elastase inhibitors h-A1-Pi, h-elafin, h-SLPI, and m-SLPI was investigated.

Interaction between Der p 1 and Human A1-Pi

The interaction of Der p 1 (CS and C) with h-A1-Pi was analyzed by SDS-PAGE: Figure 3A shows that incubation (molar ratio 2:1) for 2 h at 37°C cleaved native h-A1-Pi and generated a proteolytic fragment of 49 kD, in accordance with the literature (7). This cleavage was produced by both CS-Der p 1, containing both cysteine and serine proteases (lane 3), and C-Der p 1, containing the cysteine protease only (lane 5). In addition, the cleavage was dependent upon the activation of Der p 1 with L-Cys (not shown). Also, E-64 prevented h-A1-Pi degradation from both Der p 1 preparations (lanes 4 and 6). Incubation of h-A1-Pi alone, or with 20 mM L-cysteine, did not produce detectable degradation (lanes 1 and 2, respectively).

The same Der p 1/A1-Pi ratio was used for the functional assay where the residual anti-HNE activity of h-A1-Pi was measured (Figure 3B).

At all time points (0, 1, 2, 4 h), the results are expressed as percentage of residual HNE activity, as compared with HNE incubated with buffer alone. Mirroring the results from the SDS-PAGE analysis, we observed that both CS-Der p 1 and C-Der p 1 functionally inactivated h-A1-Pi (as determined by increased HNE activity over time). Incubation of Der p 1 with E-64 prevented h-A1-Pi inactivation because HNE activity remained minimal throughout the experiment, again demonstrating that indeed, the cysteine proteinase of Der p 1 is responsible for the activity. It was also demonstrated that Der p 1 and L-cys, alone or in combination, did not influence HNE activity (not shown).

Interaction between Der p 1 and Human Elafin

The interaction between Der p 1 and h-elafin was analyzed as described above, using SDS-PAGE and Western blot analysis (Figure 4A).

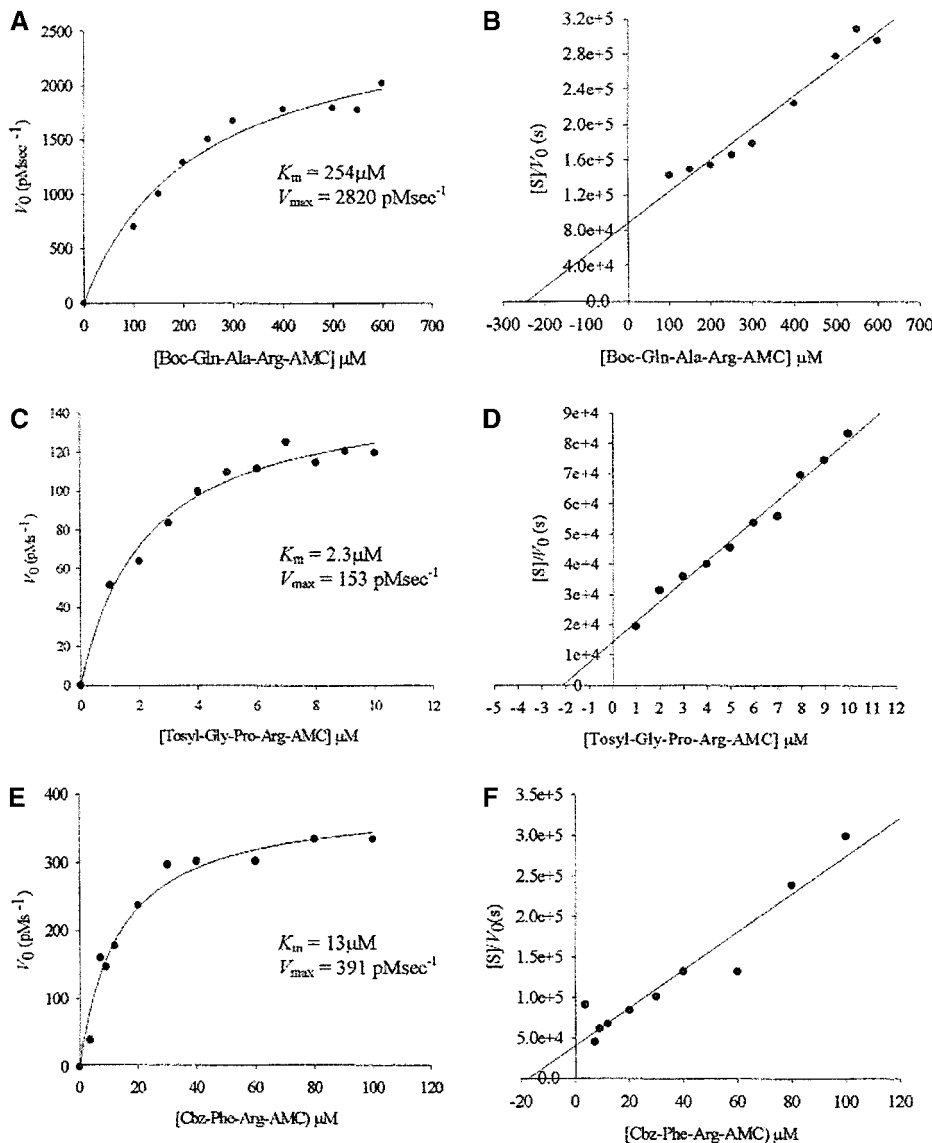


Figure 1. Michaelis-Menten and Hanes-Woolf plots for the hydrolysis of Boc-Gln-Ala-Arg-AMC, Tosyl-Gly-Pro-Arg-AMC and Cbz-Phe-Arg-AMC by CS-Der p 1. The curves in A, C, and E represent the initial rate of hydrolysis (V_0) of Boc-Gln-Ala-Arg-AMC, Tosyl-Gly-Pro-Arg-AMC, and Cbz-Phe-Arg-AMC by CS-Der p 1. The corresponding Hanes-Woolf plots are shown in B, D, and F. The K_m and V_{max} were determined by fitting the data to the hyperbolic form of the rate equation in the nonlinear regression program of SIGMAPLOT 4.00.

Cleavage of h-elafin was obtained at a CS-Der p 1/h-elafin ratio of 0.5:1 (Figure 4A, bottom, lane 4). Decreasing the ratio resulted in the gradual preservation of h-elafin integrity (Figure 4A, bottom, lanes 4–8). L-Cys alone did not influence h-elafin migration (lane 2). Very significantly, the interaction between CS-Der p 1 and h-elafin in the absence of L-Cys still induced h-elafin degradation (Figure 4A, bottom, lane 3), suggesting that the h-elafin degrading activity is at least partly due to the serine protease contained in CS-Der p 1.

Expectedly, C-Der p 1, which only has cysteine proteinase activity, needed activation with L-Cys to be able to degrade h-elafin (Figure 4A, top, lanes 4–8, compared with lane 3, without L-Cys). Taken together, the data presented here suggest that the serine and cysteine activities of Der p 1 are both able to degrade h-elafin.

For the functional assay (Figure 4B), an “intermediate” molar ratio Der p 1/h-elafin of 0.3:1 was chosen on the

basis that a ratio of 0.5:1 cleaved most of the native elafin molecule after 2 h (Figure 4A, lane 4), whereas a ratio of 0.1:1 was less efficient (Figure 4A, lane 5).

The results reveal that both CS-Der p 1 and C-Der p 1 functionally inactivated h-elafin. Incubation of CS-Der p 1 with E-64 failed to prevent h-elafin inactivation, supporting the Western blot analysis results that indeed the serine protease contributes very significantly to the h-elafin-degrading activity in the CS fraction. Nevertheless, C-Der p 1, which only has cysteine proteinase activity, lost its h-elafin-inactivating activity upon incubation with E-64, suggesting that both serine and cysteine proteases have the capacity to inactivate h-elafin.

Interaction between Der p 1 and Human SLPI

h-SLPI SDS-PAGE analysis (Figure 5) was performed on a 15% gel as described for A1-Pi and h-elafin. h-SLPI was incubated for 2 h at 37°C with C-Der p 1 and CS-Der p 1

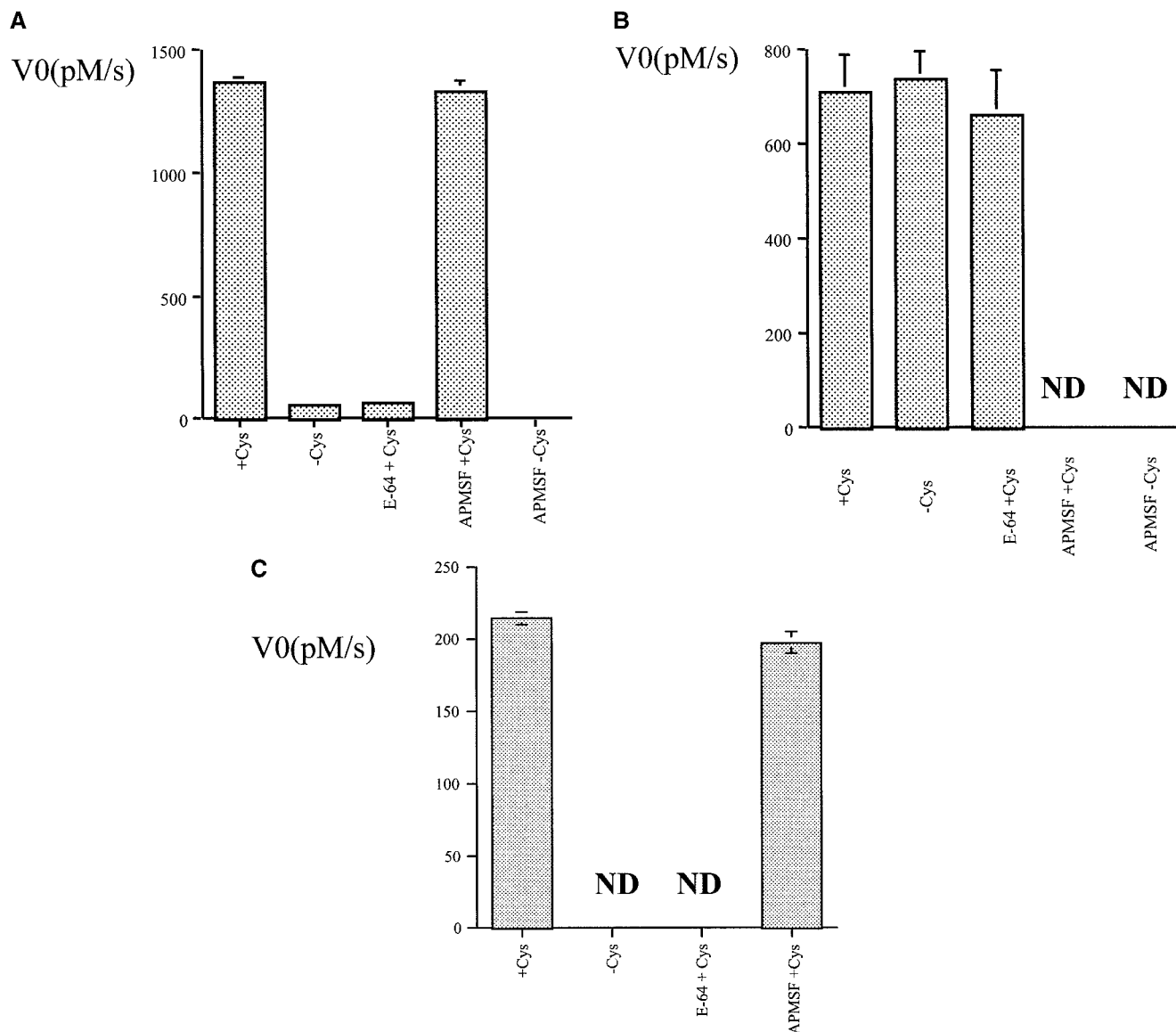


Figure 2. Inhibitory profiles of CS-Der p 1 (A, B) and C-Der p 1 (C) activities by E64 and APMSF. CS-Der p 1 and C-Der p 1 were differentially inhibited by preincubation with 10 μ M E64 and 100 μ M APMSF before assaying residual activity using either Boc-Gln-Ala-Arg-AMC (A, C) or Tosyl-Gly-Pro-Arg-AMC (B) at pH 6.0 or pH 8.0, respectively. All buffers contained 5 mM cysteine. Results are expressed as initial rates of hydrolysis (pM/s) measured over a 2-min period. ND, not detected.

(molar ratio of C/CS-Der p 1/h-SLPI varying from 0.25:1 to 1:1) in the presence or absence of L-Cys. The resistance of the h-SLPI protein to cleavage by C/CS-Der p 1 is demonstrated in Figures 5A and 5B. Because h-SLPI is a small protein containing eight disulfide bonds (13), we hypothesized that its resistance to Der p 1 could be due to the high compactness of the molecule. We therefore investigated whether reduction of the disulfide bonds of h-SLPI with DTT would favor the subsequent proteolytic activity of Der p 1 (Figure 5C).

Using a C/CS-Der p 1/SLPI molar ratio of 0.5:1, the preincubation of h-SLPI with 2 mM DTT rendered it susceptible to further cleavage by both C-Der p 1 (compare Figure 5C, *left* and *right*, lane 2) and CS-Der p 1 (compare Figure 5C, *left* and *right*, lane 4). At the dilutions of DTT

used in the experiment, we checked that DTT itself was not affecting the activity of Der p 1 (not shown). Interestingly, both C-Der p 1 (Figure 5C, *right*, lane 3) and CS-Der p 1 activities (Figure 5C, *right*, lane 5) were abolished to a similar degree with E-64, suggesting that it is the cysteine protease of Der p 1, which plays a role in the degradation of DTT-treated h-SLPI.

Interaction between Der p 1 and Murine SLPI

Contrary to h-SLPI (Figure 5), m-SLPI was very sensitive to CS-Der p 1 inactivation (Figure 6), even in the absence of denaturing agent. Significantly, in the absence of L-Cys-activating agent (lane 2), there was no inactivation of m-SLPI, suggesting that the cysteine proteinase in CS-Der p 1 plays the most important role in that process.

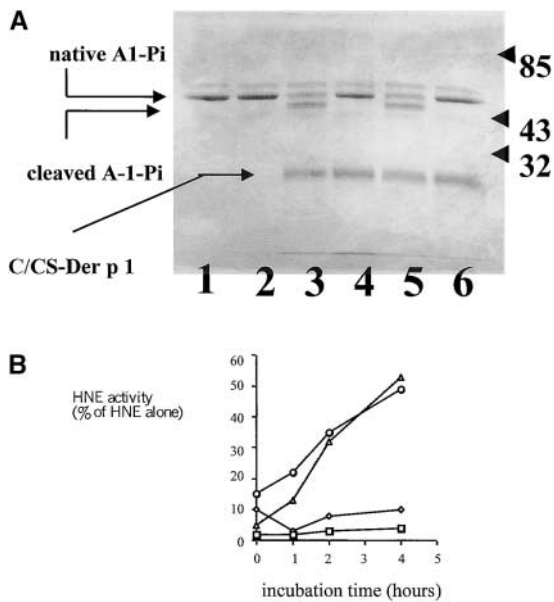


Figure 3. Inactivation of h-A1-Pi by Der p 1. (A) Degradation of h-A1-Pi by Der p 1 (SDS-PAGE analysis). h-A1-Pi (2 μ g) was incubated with C/CS-Der p 1 (2 h at 37°C), with or without L-Cys (20 mM) and E-64 (0.4 mM). A molar ratio Der p 1/h-A1-Pi of 2.0:1 was chosen after previous pilot experiments (not shown). Samples were diluted with loading buffer (SDS and 2-mercaptoethanol), and SDS-PAGE analysis was performed (12% gel). Lane 1, h-A1-Pi alone; lane 2, h-A1-Pi + L-Cys; lane 3, h-A1-Pi + CS-Der p 1 + L-Cys; lane 4, h-A1-Pi + CS-Der p 1 + L-Cys + E-64; lane 5, h-A1-Pi + C-Der p 1 + L-Cys; lane 6, h-A1-Pi + C-Der p 1 + L-Cys + E-64; standards molecular weights (kD) are indicated by arrows. (B) Functional inactivation of h-A1-Pi by Der p 1 as measured by residual HNE activity. h-A1-Pi was incubated (0, 1, 2, 4 h) at 37°C with Der p 1 (C/CS) and L-Cys (20 mM), with or without E-64 (0.4 mM). A molar ratio Der p 1/h-A1-Pi of 2.0:1 was chosen on the basis of the gel analysis (A). At the end of the incubation, the mixture was added to purified HNE (25 ng) for a further 15-min incubation at 37°C, before adding the HNE substrate N-methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilide. After 15 min, the absorbance was read at 405 nm. Results are expressed as % of HNE activity, where HNE was incubated with the control buffer alone (50 mM Tris 0.5 M NaCl, 0.1% Triton X, pH 8.0). Squares, C-Der p 1 + E-64; diamonds, CS-Der p 1 + E-64; circles, C-Der p 1; triangles, CS-Der p 1.

Interaction between Der p 1 and Human BALF

Because the three human elastase inhibitors A1-Pi, elafin, and SLPI studied above are the only three major elastase inhibitors so far identified in the lung, we were prompted to test if Der p 1 was able to inactivate the elastase-inhibitory capacity in human BALF. Because patients with established acute respiratory distress syndrome (ARDS) have a very high level of active antiproteases in their BALF (21), we chose to study a pool of six BALF samples.

As demonstrated in our previous study (21), we show here that untreated BALF had a marked anti-HNE activity over the time course of the experiment, with HNE inhibition ranging between 85 and 98%. Incubation of BALF with L-Cys alone did not change this activity. By contrast, both

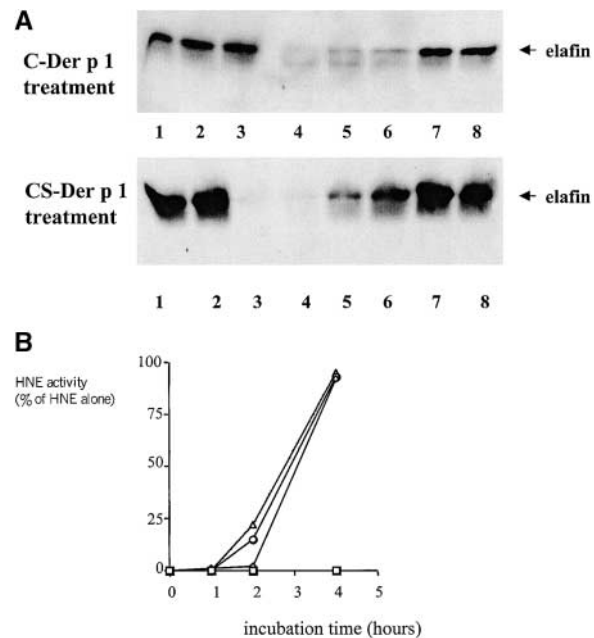


Figure 4. Inactivation of h-elafin by Der p 1. (A) Cleavage of elafin by Der p 1 (SDS-PAGE/Western Blot analysis). Top: 0.5 μ g of h-elafin was incubated with C-Der p 1 (2 h at 37°C) and L-Cys (20 mM). Different molar ratios C-Der p 1/elafin were used: 0.5:1 (lane 4), 0.1:1 (lane 5), 0.05:1 (lane 6), 0.01:1 (lane 7), and 0.005:1 (lane 8). h-Elafin was also incubated alone (lane 1), with L-Cys 20 mM (lane 2), or with C-Der p 1 in the absence of L-Cys (lane 3). Samples were diluted with loading buffer (SDS and 2-mercaptoethanol) and SDS-PAGE analysis was performed (15% gel). Following electroblotting, membranes were successively probed with IgG anti-h-elafin antibodies (1/1,000 dilution, 1 h) and goat anti-rabbit IgG (1/5,000 dilution, 20 min) coupled to horseradish peroxidase. Blots were revealed using the ECL reagent (Amersham). Bottom: As above, except that CS-Der p 1 was used in place of C-Der p 1. CS-Der p 1/elafin molar ratio used: 0.5:1 (lane 4), 0.1:1 (lane 5), 0.05:1 (lane 6), 0.01:1 (lane 7), and 0.005:1 (lane 8). Elafin was also incubated alone (lane 1), with L-Cys 20 mM (lane 2), or with CS-Der p 1 in the absence of L-Cys (lane 3, molar ratio CS-Der p 1/elafin = 1:1). (B) Functional inactivation of h-elafin by Der p 1 as measured by residual HNE activity. h-elafin was incubated (0, 1, 2, 4 h) at 37°C with Der p 1 (C/CS) and L-Cys (20 mM), with or without E-64 (0.4 mM). A molar ratio Der p 1/h-elafin of 0.3:1 was chosen on the basis of the gel analysis (A). At the end of the incubation, h-elafin residual anti-HNE activity was measured as described in Figure 3. Squares, C-Der p 1 + E-64; diamonds, CS-Der p 1 + E-64; circles, C-Der p 1; triangles, CS-Der p 1.

C-Der p 1 and CS-Der p 1, when incubated with L-Cys, degraded the anti-HNE activity of this pool of BALFs (Figure 7). CS-Der p 1 in the presence of L-cysteine resulted in the highest inactivation, suggesting that the cysteine proteinase is the most active molecule in that setting. In support of this finding, incubation with E-64 prevented the BALF anti-HNE activity from being depleted.

Discussion

Der p 1 is one of the commonest aeroallergens associated with atopic asthma (25–26). A number of recent studies

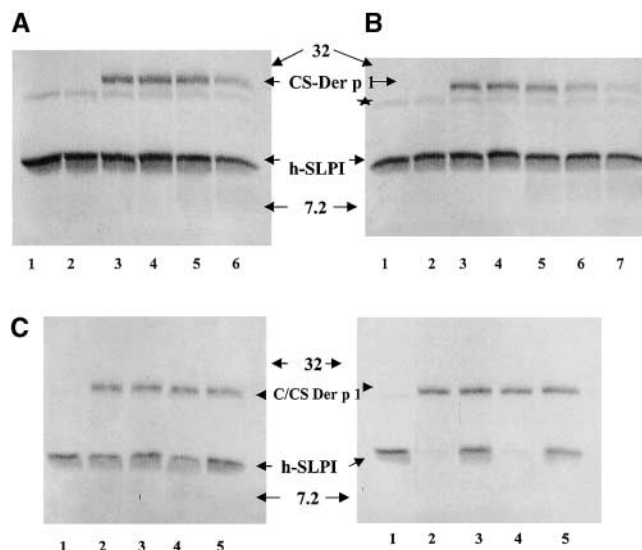


Figure 5. Der p 1/h-SLPI interactions. (A) Incubation of C-Der p 1 with native h-SLPI (SDS-PAGE analysis). Two micrograms of h-SLPI was incubated (2 h at 37°C) with C-Der p 1 and L-Cys (20 mM), with (lane 4) or without (all other lanes) E-64. Two different molar ratios C-Der p 1/h-SLPI were used: 0.5:1 (lanes 3–5) and 1 (lane 6). h-SLPI was also incubated alone (lane 1) or with L-Cys (lane 2). Samples were then analyzed by SDS-PAGE (15% gel) as explained above. (B) Incubation of CS-Der p 1 with native h-SLPI (SDS-PAGE analysis). CS-Der p 1/h-SLPI molar ratios: 0.5:1 (lanes 3–5), 1:1 (lane 6), and 0.25:1 (lane 7). Lane 1, h-SLPI alone; lane 2, h-SLPI + L-Cys; lane 3, h-SLPI + CS-Der p 1; lane 4, h-SLPI + CS-Der p 1 + E-64; lane 5, h-SLPI + CS-Der p 1 + L-Cys; lanes 6 and 7, as lane 5 (but different molar ratios, see above). N.B.: The band marked with an asterisk is a contaminant noted in some R&D h-SLPI preparations. The numbers 7.2 and 32 represent the molecular weight (kD) of standards. (C) Incubation of CS/C-Der p 1 with native or denatured h-SLPI (without or with incubation with DTT) (SDS-PAGE analysis). *Left:* no DTT preincubation: 2 µg of h-SLPI was incubated (2 h at 37°C) with C/CS-Der p 1 and L-Cys (20 mM), with or without E-64. The molar ratio C/S-Der p 1/h-SLPI was 0.5:1. Lane 1, h-SLPI alone; lane 2, h-SLPI + L-Cys + C-Der p 1; lane 3, h-SLPI + C-Der p 1 + L-Cys + E-64; lane 4, h-SLPI + CS-Der p 1 + L-Cys; lane 5, h-SLPI + CS-Der p 1 + L-Cys + E-64. Samples were then analyzed by SDS-PAGE (15% gel) as above. *Right:* DTT preincubation: 2 µg of h-SLPI was preincubated for 30 min at 37°C with 2 mM DTT. The h-SLPI mixture was then incubated (2 h at 37°C) with the same molecules as above. Lanes: as above. N.B.: The R&D contaminant in h-SLPI, although present, is less notable here. The numbers 7.2 and 32 represent, as above, molecular weight standards (kD).

have implicated the cysteine protease activity of Der p 1 in the pathogenesis of asthma (7–10). We have in the present article explored further issues relating to the enzymatic properties of Der p 1 in relation to its activity on the lung defense molecules A1-Pi, SLPI, and elafin.

The main protein present in conventionally affinity-purified Der p 1, which we have named CS-Der p 1 here, is undoubtedly a cysteine proteinase, as evidenced by silver-stained gel purity and N-terminal sequencing (7, 27). However, previous work suggested that there is a minor serine

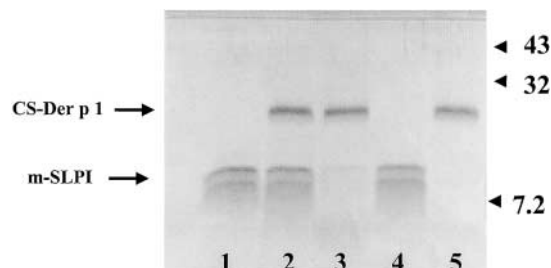


Figure 6. Degradation of native m-SLPI by Der p 1 (SDS-PAGE analysis). Two micrograms of m-SLPI was incubated (2 h at 37°C) with or without CS-Der p 1 and with or without L-Cys (20 mM). A molar ratio CS-Der p 1/m-SLPI of 0.5:1 was chosen from previous pilot experiments. Lane 1, m-SLPI alone; lane 2, m-SLPI + CS-Der p 1; lane 3, as lane 2 + L-Cys; lane 4, m-SLPI + L-Cys; lane 5, CS-Der p 1 alone. Samples were diluted and submitted to SDS-PAGE analysis (15% acrylamide) as above. N.B.: m-SLPI runs as a doublet. Standards molecular weights (kD) are indicated by arrows.

protease contaminant in that preparation, which was removed with an additional SBTI-affinity chromatography step (28–30). Our results confirm and extend these findings by showing, using a wide variety of synthetic substrates, that indeed cysteine and protease activities coexist in CS-Der p 1, and that the SBTI additional step removed the serine protease contaminant, because the remaining protein (C-Der p 1) was only able to degrade the cysteine protease substrate (Figure 2). However, we have been unable to purify and identify the serine protease component, probably because of its very high affinity for SBTI, which makes it very difficult to elute from the column (A. Brown, unpublished data).

Because of the importance of the elastase inhibitors A1-Pi, elafin, and SLPI as key molecules in the defense of the lung (13), we were prompted to determine whether or not CS-Der p 1 and C-Der p 1 were able to biochemically and functionally inactivate these molecules.

In accordance with our previous study (7), we found that CS-Der p 1 could inactivate h-A1-Pi, and further established here that it is the cysteine proteinase of Der p 1 that is responsible for the h-A1-Pi-degrading activity, because CS- and C-Der p 1 have similar activities. In contrast, for h-elafin, we found that the serine protease had the most potent inactivating activity, as illustrated by the fact that even in the presence of an inactive cysteine protease (without L-Cys) h-elafin was almost completely degraded. Furthermore, when the cysteine protease was inhibited by E-64, the activity of h-elafin was still degraded to the same extent as when no E-64 was added. These findings suggest that the serine protease, albeit probably present as a minor contaminant in the CS-Der p 1 fraction, has a much greater affinity for h-elafin than the cysteine protease.

Interestingly, h-SLPI and m-SLPI were not equally sensitive to Der p 1. Indeed, m-SLPI was much more sensitive than h-SLPI (compare Figures 5 and 6), with the latter only becoming susceptible to cleavage by Der p 1 after pretreatment with DTT, which disrupts the molecule by

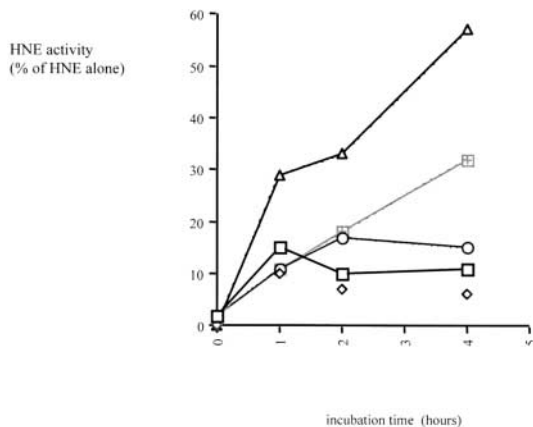


Figure 7. Inactivation of human bronchoalveolar lavage fluid (BALF) samples with Der p 1. Twenty microliters of BALF from patients with established ARDS (see MATERIALS AND METHODS) were incubated (0, 1, 2, 4 h) with 7.5 μ g (1.2 μ M) Der p 1 (C/CS) and L-Cys (20 mM), with or without E-64 (0.4 mM), in a final volume of 250 μ L. Following the incubation, 10 μ L of the incubation mixture was added to 10 ng HNE in microplate wells, and after a further 15-min incubation at 37°C, the HNE substrate was added as explained above. Squares, BALF alone; diamonds, BALF + L-Cys; circles, BALF + CS-Der p 1 + L-Cys + E-64; triangles, BALF + CS-Der p 1 + L-Cys; squares with plus signs, BALF + C-Der p 1 + L-Cys.

reduction of its internal disulfide bonds. This difference in sensitivity is notable in view of the significant homologies between m-SLPI and h-SLPI (58% overall) (31) and between human elafin (which is sensitive to Der p 1) and human SLPI (which is not, 47% homology) (13). It is interesting that the three molecules found in the present study as being sensitive to Der p 1 (h-elafin, h-A1-Pi, and m-SLPI) have an alanine at or near the antiprotease-reactive site (at positions P1, P4, and P4, respectively) whereas there is no alanine in h-SLPI. Indeed, when Der p 1 cleavage sites on h-A1-Pi were studied (7), the enzymes were shown to cleave on either side of the alanine residue. In addition, m-SLPI has an alanine-arginine motif in the P3-P4 position, also present in the cysteine proteinase synthetic substrate Boc-Gln-Ala-Arg-AMC used in this study.

Regardless of the mechanism of Der p 1 differential inhibition of lung antiproteases, our *in vitro* experiments performed on the isolated human proteins SLPI, A1-Pi, and elafin revealed that the latter two molecules were mostly susceptible to the cysteine and serine proteinases present in Der p 1, respectively, whereas the *in vitro* resistance of h-SLPI to Der p 1 would suggest that the former may not be a significant Der p 1 substrate *in vivo*.

To determine the importance of these findings *ex vivo* in human lung samples, we obtained BALF from patients with established ARDS, with the knowledge that these patients would have high levels of antiproteases (21) and would provide a good marker for lung sensitivity to Der p 1. Indeed, incubation of BALF fluid with Der p 1 of either preparation (C/CS-Der p 1) reveals that both proteases lead to a marked loss of antielastase activity in the fluid. This

finding is in keeping with our data, which show the high susceptibility of human antielastases A1-Pi and elafin to cleavage by the mite proteases. Because concentrations in excess of 3 ng/ml of Der p 1 have been detected in the BAL of allergic individuals (32) and the antiproteases studied here are also present in ng/ml in BAL (21), similar molar ratios of Der p 1:antiproteases as the ones used *in vitro* are likely to be present *in vivo*, making our findings pathophysiologically relevant. The studies presented here add to our knowledge on the biological activities of Der p 1 and the role of its enzymatic functions on the development and persistence of asthma by compromising the antiprotease defences of the lung. In addition, Der p 1 has a direct effect on some of the components of the adaptive immune system, including CD23 on B cells and CD25 on T cells, which would increase IgE synthesis by disrupting a negative feedback signal and by favoring a Th1 to Th2 shift by affecting interferon- γ responses, respectively (8, 9, 20, 27). The consequence of this would be to promote allergic inflammatory responses. Furthermore, Der p 1 has destructive activities on structural cells in that it disrupts epithelial cells junctions (10), which facilitates transport of the allergen across the epithelium.

Overall, these findings have potential implications at at least three levels. First, they suggest that following exposure of the bronchial mucosa to HDM fecal pellet, the resultant solubilized mite proteases can access the lung interstitium, promote Th2 responses, and skew the elastase/antielastase balance toward elastase and thus a proinflammatory state. Indeed, elastase, in addition of cleaving a variety of substrates within the lung (33), can upregulate the potent neutrophil chemokine IL-8 (34–35), with ensuing neutrophilic inflammation, which in extreme instances could trigger the onset of fatal asthma (36–40).

Second, the HNE inhibitor h-A1-Pi can inhibit protease-mediated airway hyperresponsiveness (AHR) in an allergic model (41). It therefore follows that inactivation of A1-Pi by Der p 1 may be deleterious by facilitating AHR.

Third, A1-Pi, SLPI, and elafin possess properties in addition to their antielastase activity. They can function as antimicrobials, either directly or indirectly (15–16, 18–19, 42) and, at least for elafin and SLPI, can “prime” the innate immune system (17, 43). Thus, inactivation of these additional innate immunity functions may contribute to the infectious exacerbations found in patients with asthma (44–45).

In addition, our report suggests for the first time that the serine protease of Der p 1 may also be an important therapeutic target and identifies elafin, a key molecule in lung defense as an important substrate for this protease (13–14, 46).

Further work will be needed to establish *in vivo* whether a dual blockade of the cysteine and serine proteases of Der p 1 will be beneficial to patients with HDM-induced allergic inflammation, by modulating type 2 responses and/or by restoring lung antimicrobial functions.

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