

Proteases as Th2 Adjuvants

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Several cysteine and serine protease allergens have been cloned from house dust mites, including Der p 1, Der p 3, Der p 6, and Der p 9. A significant body of evidence suggests that these allergens mimic helper T (Th) 2 cell adjuvants. Der p 1 cleaves CD23 from activated B cells and CD25 from T cells. Der p 1 proteolytically degrades tight junctions in lung epithelium and causes release of proinflammatory cytokines from bronchial epithelial cells, mast cells, and basophils. These synergistic effects of mite enzyme allergens may promote IgE synthesis and have direct inflammatory effects on lung epithelium, which could explain why mite allergens are associated with asthma. The crystal structures of the proenzyme and mature forms of Der p 1 have been determined, as have the structures of other indoor allergens that are not enzymes (eg, Der p 2, Fel d 1, and Bla g 2). Cockroach allergens are strongly associated with asthma in US inner cities, yet none of the cockroach allergens that have been cloned are proteolytic enzymes. Thus although mite proteases allergens may act as Th2 adjuvants, a paradoxical effect is that other allergens may elicit strong Th2 responses in the absence of enzyme activity.

biologic function. This situation changed radically in the late 1980s, when Der p 1 was the first allergen to be cloned and sequenced. The sequence information revealed structural homology between Der p 1 and the cysteine proteases, papain and actinidin, which suggested that Der p 1 was also a cysteine protease [1]. This observation initiated experiments in several laboratories to determine whether Der p 1 had cysteine protease activity, whether enzymatic activity could have helper T (Th) 2 cell adjuvant effects, or otherwise affect allergic inflammation. More broadly, the ability to rapidly search protein and DNA databases using FASTA (software for fast [FAST] protein or nucleotide [All] sequence comparison) and other homology search algorithms allowed the putative functions of many cloned allergens to be determined. Several important dust mite allergens were identified as either cysteine or serine proteases (Der p 1, Der p 3, Der p 6, Der p 9). On the other hand, important animal, cockroach, and fungal allergens belonged to protein families with diverse biologic functions. In the current era of proteomics, allergens are being defined based on their cDNA sequence, three-dimensional structure, and biologic function, as well as their intrinsic biochemical properties [2•]. Proteolytic enzyme activity is thought to enhance IgE synthesis and to contribute to allergic inflammation of the lung. Protease activity may be especially important in chronic allergic diseases, such as asthma. This presentation discusses the evidence that protease activity enhances allergenicity of mite allergens, and reviews the role and functions of allergens that belong to other protein families and are not enzymes.

Introduction

Historically, techniques of protein chemistry and immunochemistry were used to identify and purify allergens and to establish their reactivity with IgE antibodies either in vitro or in vivo. When the World Health Organization/International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature system was developed in the 1980s, all of the allergens were included at that time based on their physicochemical properties, biochemical purity, and immunologic reactivity. Little sequence information on allergens was published and there was virtually no data on allergen structure or

Protease Allergens from Dust Mites

The group 1 allergens from *Dermatophagoides* species (Der p 1 and Der f 1) are cysteine proteases with a two-domain structure. Der p 1 has an 80-amino acid pro peptide that is cleaved under acidic conditions to generate the mature active enzyme. Cysteine protease activity can be activated in vitro using reducing agents, such as β -mercaptoethanol or dithiothreitol. Both group 1 allergens were expressed as recombinant proteins in *Pichia pastoris* and were found to be hyperglycosylated. Mutation of the N-glycosylation site (N53Q) results in deglycosylated group 1 allergens with the same molecular weight as the natural allergens. The deglyco-

Table 1. Mite proteases as potential Th2 adjuvants in allergic inflammation

Study	Biologic/allergenic activity
Der p 1	
Hewitt et al. [13], Schulz et al. [14]	Cleaves CD23 from activated B cells
Shakib et al. [15], Schulz et al. [16]	Cleaves CD25 from T cells
King et al. [17], Tomee et al. [18]	Causes detachment of bronchial epithelial cells from lung segments
Wan et al. [19], Wan et al. [20]	Disrupts intercellular tight junctions through cleavage of occludin and claudin-1
Mite proteases (Der p 1, Der p 3, Der p 6, or Der p 9)	
Tomee et al. [18]	Induce pulmonary epithelial cell detachment
King et al. [17], Tomee et al. [18]	Induce production of proinflammatory cytokines (IL-8, IL-6, MCP-1, and GM-CSF) in vitro
Hewitt et al. [13], King et al. [17], Machado et al. [21]	Induce IgE-independent mast cell and basophil degranulation, and release of IL-4 and IL-13 in vitro
GM-CSF—granulocyte-macrophage colony-stimulating factor; IL—interleukin; MCP—monocyte chemoattractant protein.	

sylated, mature recombinant allergens show similar folding patterns to natural allergens and similar binding to immunoglobulin (Ig) E antibodies [3–5]. The X-ray crystal structures of proDer p 1 and fully mature Der p 1 have recently been solved to a high degree of resolution using *Pichia* expressed allergens. The rproDer p 1 structure was resolved at 1.6 Å and showed that the 80-amino acid pro-peptide contains four α -helices with no β -sheets, which appears to be unique among the C1 family of cysteine proteases [6••]. The presence of the pro-region also appeared to block some IgE binding sites on Der p 1 [5,6••]. Crystallographic studies of mature rDer p 1 (at 1.9 Å resolution) showed that Der p 1 lacking the pro-peptide forms tightly packed dimers in which the catalytic sites face each other but remain accessible to peptide ligands. This structure also revealed that Der p 1 has a magnesium ion binding site, the function of which is not known [7••]. The dimerization of Der p 1 under natural conditions in mite feces could stabilize the molecule and enable it to persist in the environment.

Neither of the crystallography studies provided information about the substrate specificity of Der p 1, though it is clear, from these and from other studies using highly purified recombinant allergens, that Der p 1 is a pure cysteine protease and does not have any serine protease activity [6••,7••,8]. However, mites also produce several serine protease allergens: group 3 allergens (trypsins), group 6 (chymotrypsins), and group 9 (collagenolytic serine proteases). These allergens have been cloned and sequenced, but are not readily available as expressed recombinant proteins. As yet, their three-dimensional structures have not been determined [9–12].

Proteolytic Enzymes as Th2 “Adjuvants” in Allergic Inflammation

In 1995, the groups of Hewitt and Shakib [13–16], working independently, carried out pivotal experiments

showing that enzymatically active Der p 1 could cleave the low-affinity IgE receptor (CD23) from activated B cells. They proposed that cleavage of CD23 would increase IgE levels by inhibiting a negative feedback signal delivered by circulating IgE, which would bind to membrane-bound CD23 and switch off IgE production. Thus, in the absence of membrane-bound CD23, IgE levels would increase [13,14]. This provided the first demonstration that the biologic function of an allergen could influence IgE responses. Subsequently, it was shown that Der p 1 also cleaved the α -subunit of the interleukin (IL)-2 receptor (CD25), which would be expected to inhibit IL-2-mediated T cell proliferation (and the production of interferon [IFN]- γ) and cause a shift favoring Th2 responses [15,16].

In parallel with these experiments, King et al. [17] showed that Der p 1 and Der p 9 caused detachment of epithelial cells from human lung segments and disrupted the architecture of bronchial epithelial cells. These responses were associated with release of the proinflammatory cytokines, IL-6, IL-8, and granulocyte-macrophage colony-stimulating factor (GM-CSF), at allergen concentrations of about 10 ng/mL [17,18]. The proteolytic activity of Der p 1 was shown to disrupt intercellular tight junctions in confluent human bronchial epithelial cell layers, an effect mediated via Der p 1 cleavage sites in occludin and claudin-1, tight junction membrane proteins [19]. Similar effects were later shown to be mediated by serine proteases in mite feces [20]. Mite proteases were also reported to cause IgE-independent release of IL-4 and IL-13 from mast cells and basophils in vitro [21].

Taken together, these studies (summarized in Table 1) provide several lines of evidence that the biologic activity of mite proteases augments allergenic activity [22,23]: 1) Der p 1 directly promotes IgE synthesis; 2) Der p 1 damages lung epithelium and increases bronchial permeability; 3) Der p 1 degrades epithelial tight junctions and

increases accessibility to dendritic antigen-presenting cells that lie beneath the epithelium; and 4) Der p 1 causes release of proinflammatory cytokines from bronchial epithelial cells, mast cells, and basophils.

More recent studies have shown that cytokine release from epithelial cells by Der p 1, Der p 3, and Der p 9 is mediated by activation of protease-activated receptor 2 (PAR-2) [24,25].

On a worldwide basis, sensitization to dust mites is arguably the strongest risk factor for the development of asthma. A large unselected prospective birth cohort that followed more than 600 children living in New Zealand (ages 9 to 26) showed that sensitization to dust mite was one of the most significant factors that predicted persistence of asthma from childhood to adulthood and relapse of asthma after remission [26]. It is tempting to speculate that the synergistic effects of mite allergens having both allergenic activity and enzymatic activity, which promotes IgE responsiveness and has inflammatory effects, could explain why mite allergy is so strongly associated with asthma. The disruptive effects of mite proteases on the bronchial epithelium and increased accessibility to antigen-presenting cells would be expected to enhance Th2 responses to other allergens. Immunization of mice with proteolytically active Der p 1 was reported to enhance total IgE levels and Der p 1-specific IgE compared to inactive Der p 1 with enzymatic activity irreversibly blocked by the cysteine protease inhibitors E-64 and iodoacetamide. It was also reported that IgE and IgG responses to a bystander antigen (ovalbumin) were increased by proteolytically active Der p 1 (but not by inactivated allergen) [27,28]. It is somewhat surprising that the bystander experiments were not carried out with other (nonenzymatic) mite allergens or other allergens associated with asthma. Indeed, a valid criticism of studies on enzyme activity is that the experiments have almost exclusively used Der p 1 (or the serine protease allergens) and have not been adequately controlled using nonenzymatic mite allergens (notably the group 2 allergens) or animal allergens (eg, Fel d 1, Can f 1). The group 2 mite allergens cause sensitization in more than 90% of mite-allergic patients and are lipid-binding proteins [29]. An argument could be made that proteolytic activity contributes to the potency of the group 2 allergens, but there are as yet no data to support this. Nonetheless, a recent report confirmed the earlier immunization experiments and showed that total IgE and specific IgE anti-Der p 1 was impaired in mice immunized with rDer p 1 that was proteolytically inactivated using E-64 [30]. These experiments indicated that proteolytic activity affected both IgE and IgG responses and the authors concluded that such activity was "crucial" for induction of antibody responses to Der p 1.

Asthma-associated Allergens Lacking Proteolytic Activity: A Paradox

"A paradox, a paradox, a most unusual paradox" (W.S. Gilbert, *The Pirates of Penzance*).

In spite of the large body of evidence supporting a role for protease allergens in chronic inflammation, it is apparent that several other allergens commonly associated with asthma are not proteases, and it has been difficult to reconcile this fact with the "enzyme hypothesis." In addition to mite group 2 allergens, the group 5, group 7, and group 10 allergens, among others, are not enzymes and most animal allergens are lipocalins (or ligand-binding proteins) [31]. The major cat allergen, Fel d 1, is structurally homologous to secretoglobulin and has an amphipathic internal cavity that binds an as-yet unknown ligand [32]. Moreover, cat allergen Fel d 3 is a cystatin (cysteine protease inhibitor), and there is evidence that dog allergen Can f 1 may serve a similar function [31]. The extent of environmental or biologic interactions between the proteases and these potential inhibitors has not been studied.

Cockroach is perhaps the allergen that is most analogous to dust mite in the etiology of asthma, sensitization, and environmental exposure. Asthma is the most common disease associated with cockroach infestation of homes in inner cities in the United States and in suburban and rural housing. Sensitization and exposure to cockroach is strongly associated with asthma morbidity, including hospitalizations, medication use, and lost school days. Environmental control procedures, including cockroach extermination, household cleaning, and patient education were reported to be effective in reducing allergen exposure and asthma symptoms in a 2-year controlled trial carried out among inner-city children in eight US cities [33,34].

Currently, between eight and 10 allergens have been cloned from German (*Blattella germanica*) and American (*Periplaneta americana*) cockroach cDNA libraries. However, none of these allergens are active proteolytic enzymes. The most well characterized *B. germanica* allergens include Bla g 1 (microvillar protein); Bla g 2 (inactive aspartic protease); Bla g 4 (male reproductive protein); Bla g 5 (glutathione transferase family protein); and Bla g 7 (tropomyosin) [2•,35,36]. The prevalence of IgE antibodies to the individual cockroach allergens ranges from 25% to 60%, but together measuring IgE to all allergens will identify about 90% to 95% of cockroach-allergic patients [37]. The highest prevalence of sensitization (60%) is to Bla g 2, which appears to be a potent allergen, causing sensitization at exposure levels that may be significantly lower than for dust mite or cat. Bla g 2 belongs to the aspartic protease family of enzymes, but has critical substitutions in the catalytic site that render the allergen enzymatically inactive. Bla g 2 is closely related to a subgroup of aspartic proteases known as pregnancy-associated glycoproteins (PAG), which are produced by ungulates, have similar substitutions at the catalytic site, and lack proteolytic activity [38,39]. Bla g 2 crystal structure was the first to be determined from PAG-related proteins; it revealed several unusual features, including substitution of conserved glycine residues in the canonical triads, a phenylalanine substitution in the flap region,

and increased distance between the catalytic aspartate residues. These features provided the structural basis for the functional lack of aspartic protease activity in Bla g 2 and other PAG [2•,38,39,40••].

Although the cockroach allergens that have been identified are not enzymes, cockroaches produce proteolytic enzymes (eg, serine proteases) which, when inhaled, may have similar nonspecific proinflammatory effects as their mite counterparts. This possibility has not yet been investigated.

Conclusions

A substantial body of evidence suggests that the biologic function of dust mite allergens, which are cysteine and serine proteases, enhances their ability to elicit IgE responses and has inflammatory effects on the lung, including direct damage to the epithelium, recognition by dendritic antigen-presenting cells, and release of proinflammatory cytokines from epithelial cells, mast cells, and basophils. Immunization experiments in mice using proteolytically active and inactive Der p 1 further support this hypothesis and have demonstrated that proteases can have bystander effects on other antigens. The synergistic effects of biologically active enzyme allergens on Th2 responses and allergic inflammation may explain why mites are the predominant allergens associated with asthma. A central paradox remains that other important asthma-associated allergens (eg, cockroach and animal allergens) are not proteolytic enzymes. It is difficult to reconcile these observations and to develop a unifying theory of allergenicity based on biologic function. Clearly, both enzymatic and nonenzymatic allergens can mediate Th2 responses, inflammation, and asthma. Whether augmentation of these responses by mite allergens is influenced by genetics or gene environment interactions may be a worthwhile area of future study.

References and Recommended Reading

Papers of particular interest, published recently, have been highlighted as:

- Of importance
- Of major importance

1. Chua KY, Stewart GA, Thomas WR, et al.: Sequence analysis of cDNA coding for a major house dust mite allergen, Der p 1. Homology with cysteine proteases. *J Exp Med* 1988, 167:175–182.
 - 2.• Chapman MD, Pomés A, Breiteneder H, Ferreira F: Nomenclature and structural biology of allergens. *J Allergy Clin Immunol* 2007, 119:414–420.
- Current review of the status of allergen nomenclature, protein families, and biologic functions of allergens.
3. Best EA, Stedman KE, Bozic CM, et al.: A recombinant group 1 house dust mite allergen, rDer f 1, with biological activities similar to those of the native allergen. *Protein Expr Purif* 2000, 20:462–471.
 4. van Oort E, de Heer PG, van Leeuwen WA, et al.: Maturation of *Pichia pastoris*-derived recombinant pro-Der p 1 induced by deglycosylation and by the natural cysteine protease Der p 1 from house dust mite. *Eur J Biochem* 2002, 269:671–679.
 5. Takai T, Kato T, Yasueda H, et al.: Analysis of the structure and allergenicity of recombinant pro- and mature Der p 1 and Der f 1: major conformational IgE epitopes blocked by prodomains. *J Allergy Clin Immunol* 2005, 115:555–563.
 - 6.•• Meno K, Thorsted PB, Ipsen H, et al.: The crystal structure of recombinant proDer p 1, a major house dust mite proteolytic allergen. *J Immunol* 2005, 175:3835–3845.
- Detailed study of the crystal structure of the pro-enzyme form of Der p 1 and its relationship to other cysteine proteases.
- 7.•• de Halleux S, Stura E, VanderElst L, et al.: Three-dimensional structure and IgE-binding properties of mature fully active Der p 1, a clinically relevant major allergen. *J Allergy Clin Immunol* 2006, 117:571–576.
- The crystal structure of mature, enzymatically active Der p 1, revealing that Der p 1 is a magnesium-binding protein and has a dimeric structure, which may explain its stability and persistence in the environment.
8. Takai T, Kato T, Sakata Y, et al.: Recombinant Der p 1 and Der f 1 exhibit cysteine protease activity but no serine protease activity. *Biochem Biophys Res Commun* 2005, 328:944–952.
 9. Nishiyama C, Yasuhara T, Yuuki T, Okumura Y: Cloning and expression in *Escherichia coli* of cDNA encoding house dust mite allergen Der f 3, serine protease from *Dermatophagoides farinae*. *FEBS Lett* 1995, 377:62–66.
 10. Bennett BJ, Thomas WR: Cloning and sequencing of the group 6 allergen of *Dermatophagoides pteronyssinus*. *Clin Exp Allergy* 1996, 26:1150–1154.
 11. King C, Simpson RJ, Moritz RL, et al.: The isolation and characterization of a novel collagenolytic serine protease allergen (Der p 9) from the dust mite *Dermatophagoides pteronyssinus*. *J Allergy Clin Immunol* 1996, 98:739–747.
 12. Flores I, Mora C, Rivera E, et al.: Cloning and molecular characterization of a cDNA from *Blomia tropicalis* homologous to dust mite group 3 allergens (trypsin-like proteases). *Int Arch Allergy Immunol* 2003, 130:12–16.
 13. Hewitt CR, Brown AP, Hart BJ, Pritchard DI: A major house dust mite allergen disrupts the immunoglobulin E network by selectively cleaving CD23: innate protection by antiproteases. *J Exp Med* 1995, 182:1537–1544.
 14. Schulz O, Laing P, Sewell HF, Shakib F: Der p I, a major allergen of the house dust mite, proteolytically cleaves the low-affinity receptor for human IgE (CD23). *Eur J Immunol* 1995, 25:3191–3194.
 15. Shakib F, Schulz O, Sewell H: A mite subversive: cleavage of CD23 and CD25 by Der p 1 enhances allergenicity. *Immunol Today* 1998, 19:313–316.
 16. Schulz O, Sewell HF, Shakib F: Proteolytic cleavage of CD25, the alpha subunit of the human T cell interleukin 2 receptor, by Der p 1, a major mite allergen with cysteine protease activity. *J Exp Med* 1998, 187:271–275.
 17. King C, Brennan S, Thompson PJ, Stewart GA: Dust mite proteolytic allergens induce cytokine release from cultured airway epithelium. *J Immunol* 1998, 161:3645–3651.
 18. Tomee JF, van Weissenbruch R, de Monchy JG, Kauffman HF: Interactions between inhaled allergen extracts and airway epithelial cells: effect on cytokine production and cell detachment. *J Allergy Clin Immunol* 1998, 102:75–85.
 19. Wan H, Winton HL, Soeller C, et al.: Der p 1 facilitates transepithelial allergen delivery by disruption of tight junctions. *J Clin Invest* 1999, 104:123–133.
 20. Wan H, Winton HL, Soeller C, et al.: The transmembrane protein occludin of epithelial tight junctions is a functional target for serine peptidases from faecal pellets of *Dermatophagoides pteronyssinus*. *Clin Exp Allergy* 2001, 31:279–294.

21. Machado DC, Horton D, Harrop R, et al.: Potential allergens stimulate the release of mediators of the allergic response from cells of mast cell lineage in the absence of sensitization with antigen-specific IgE. *Eur J Immunol* 1996, 26:2972–2980.
22. Robinson C, Kalsheker NA, Srinivasan N, et al.: On the potential significance of the enzymatic activity of mite allergens to immunogenicity. Clues to structure and function revealed by molecular characterization. *Clin Exp Allergy* 1997, 27:10–21.
23. Sharma S, Lackie PM, Holgate ST: Uneasy breather: the implications of dust mite allergens. *Clin Exp Allergy* 2003, 33:163–165.
24. Asokanathan N, Graham PT, Stewart DJ, et al.: House dust mite allergens induce proinflammatory cytokines from respiratory epithelial cells: the cysteine protease allergen, Der p 1, activates protease-activated receptor (PAR)-2 and inactivates PAR-1. *J Immunol* 2002, 169:4572–4578.
25. Sun G, Stacey MA, Schmidt M, et al.: Interaction of mite allergens Der p3 and Der p9 with protease-activated receptor-2 expressed by lung epithelial cells. *J Immunol* 2001, 167:1014–1021.
26. Sears MR, Greene JM, Willan AR, et al.: A longitudinal, population-based, cohort study of childhood asthma followed to adulthood. *N Engl J Med* 2003, 349:1414–1422.
27. Gough L, Schulz O, Sewell HF, Shakib F: The cysteine protease activity of the major dust mite allergen Der p 1 selectively enhances the immunoglobulin E antibody response. *J Exp Med* 1999, 190:1897–1902.
28. Gough L, Sewell HF, Shakib F: The proteolytic activity of the major dust mite allergen Der p 1 enhances the IgE antibody response to a bystander antigen. *Clin Exp Allergy* 2001, 31:1594–1598.
29. Gruber A, Mancek M, Wagner H, et al.: Structural model of MD-2 and functional role of its basic amino acid clusters involved in cellular lipopolysaccharide recognition. *J Biol Chem* 2004, 279:28475–28482.
30. Kikuchi Y, Takai T, Kuhara T, et al.: Crucial commitment of proteolytic activity of a purified recombinant major house dust mite allergen Der p1 to sensitization toward IgE and IgG responses. *J Immunol* 2006, 177:1609–1617.
31. Chapman MD, Smith AM, Vailes LD, et al.: Recombinant allergens for diagnosis and therapy of allergic disease. *J Allergy Clin Immunol* 2000, 106:409–418.
32. Kaiser L, Gronlund H, Sandalova T, et al.: The crystal structure of the major cat allergen Fel d 1, a member of the secretoglobulin family. *J Biol Chem* 2003, 278:37730–37735.
33. Morgan WJ, Crain EF, Gruchalla RS, et al.: Results of a home-based environmental intervention among urban children with asthma. *N Engl J Med* 2004, 351:1068–1080.
34. Gruchalla RS, Pongracic J, Plaut M, et al.: Inner City Asthma Study: relationships among sensitivity, allergen exposure, and asthma morbidity. *J Allergy Clin Immunol* 2005, 115:478–485.
35. Fan Y, Gore JC, Redding KO, et al.: Tissue localization and regulation by juvenile hormone of human allergen Bla g 4 from the German cockroach, *Blattella germanica* (L.). *Insect Mol Biol* 2005, 14:45–53.
36. Gore JC, Schal C: Cockroach allergen biology and mitigation in the indoor environment. *Annu Rev Entomol* 2007, 52:439–463.
37. Satinover SM, Reefer AJ, Pomés A, et al.: Specific IgE and IgG antibody-binding patterns to recombinant cockroach allergens. *J Allergy Clin Immunol* 2005, 115:803–809.
38. Pomés A, Chapman MD, Vailes LD, et al.: Cockroach allergen Bla g 2: structure, function, and implications for allergic sensitization. *Am J Respir Crit Care Med* 2002, 165:391–397.
39. Wünschmann S, Gustchina A, Chapman MD, Pomés A: Cockroach allergen Bla g 2: an unusual aspartic proteinase. *J Allergy Clin Immunol* 2005, 116:140–145.
- 40.●● Gustchina A, Li M, Wünschmann S, et al.: Crystal structure of cockroach allergen Bla g 2, an unusual zinc binding aspartic protease with a novel mode of self-inhibition. *J Mol Biol* 2005, 348:433–444.

First published structure of an inactive aspartic protease member of the pregnancy-associated glycoproteins, which details precisely the structural differences that affect catalytic activity.