

A Protease-Activated Pathway Underlying Th Cell Type 2 Activation and Allergic Lung Disease¹

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The respiratory allergens that induce experimental Th cell type 2-dependent allergic lung inflammation may be grouped into two functional classes. One class of allergens, in this study termed type I, requires priming with adjuvants remote from the lung to overcome airway tolerogenic mechanisms that ordinarily preclude allergic responses to inhaled Ags. In contrast, the other, or type II, allergen class requires neither remote priming nor additional adjuvants to overcome airway tolerance and elicit robust allergic lung disease. In this study, we show in an experimental model that diverse type II allergens share in common proteolytic activity that is both necessary and sufficient for overcoming airway tolerance and induction of pulmonary allergic disease. Inactivated protease and protease-free Ag fragments showed no allergenic potency, demonstrating that only active protease acting on endogenous substrates was essential. Furthermore, induction of airway tolerance could be aborted and allergic lung disease established by simply adding purified protease to a type I allergen. Thus, exogenous proteases are common to type II allergens and may be generally required to overcome the innate resistance of the airway to Th cell type 2 activation and allergic inflammation, raising concern for their potential contribution to diseases such as asthma. *The Journal of Immunology*, 2002, 169: 5904–5911.

Defining the immunopathologic basis of allergic inflammation is paramount to revealing mechanisms of allergic disease and developing improved therapies for conditions such as asthma. Central to these efforts is to understand the unique properties of respiratory allergens which initiate and sustain the allergic inflammation which underlies pathologic processes such as airway obstruction. Experimental models of respiratory Ag challenge have identified two major classes of Ag implicated in allergic lung disease. The first class, in this study termed type I, is exemplified by chicken egg OVA which, aside from airway hyperresponsiveness (1), is incapable of inducing a broad spectrum of lung allergic changes which additionally include goblet cell metaplasia, mucus oversecretion, airway eosinophilia, and peribronchovascular inflammation, if given strictly by inhalation. However, OVA is capable of inducing all of these allergic changes if given remote from the lung in a series of priming doses, typically with aluminum-based adjuvants before intrapulmonary challenge (2–11). In contrast, the other major class of allergen, in this study termed type II, is derived from the fungus *Aspergillus fumigatus* and readily induces allergic lung inflammation when given only through the airway and without the need for additional adjuvant (12–14). Although *A. fumigatus*-based allergens are derived from an organism strongly linked to human al-

lergic illness (15) and induce lung disease with a physiologically more relevant protocol, experimentally OVA is used far more often.

The distinction between allergen classes is further illustrated by comparing their immune properties. OVA given only through the airway induces a relative tolerogenic state termed IgE tolerance (16) in which murine B cells are induced to secrete only the Ig isotypes IgG1 and IgG2a (17) and Th effector responses are not observed. Allergic inflammation of the lung is not induced when OVA is given only through the airway (18), and lack of T effector responses persists even with subsequent priming with the same Ag and adjuvant (19). However, allergic reactions to other Ags are not affected in rodents tolerized to OVA (20). Thus, rather than an abnormal response with general effects on immunity, tolerance to inhaled Ag may be regarded as a highly selective, Ag-specific immune response that may have evolved as a means of protection against inappropriate immune activation in the lung.

In contrast to OVA, immunity to type II allergens derived from *A. fumigatus* is distinctly allergic, dominated by the presence of lung parenchymal and airway eosinophils and Th cells type 2 (Th2 cells),⁴ elevated serum titers of IgE, and the physiologic sequelae of this inflammation including airway hyperresponsiveness and airway mucus overproduction (13, 14, 21–23). T and/or B cell tolerance is not observed and robust inflammation is induced without the need for extrapulmonary Ag challenge or additional adjuvants. Despite these differences, the allergic lung disease induced by both type I and II allergens is highly stereotypical and pathologic airway obstruction is induced through a final common signaling pathway involving IL-13 (8, 23–28).

Therefore, respiratory allergens differ immunologically only in terms of their intrinsic ability to bypass induction of airway tolerance. Because human allergic lung disease is likely induced by inhaled allergen, it is critical to understand the adjuvant properties of type II allergens which enable them to bypass normal tolerogenic mechanisms and establish allergic disease. Pathogenic roles

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⁴ Abbreviations used in this paper: Th2 cell; Th cell type 2; BAL, bronchoalveolar lavage; NPP, nitrophenylphosphate.

have previously been suggested for proteases and amylases and other exogenous molecules common to human allergens (29–33). However, it has not been shown that exposure by inhalation to these microbial products is necessary to overcome airway tolerogenic mechanisms, enable Th2 priming, and establish allergic lung inflammation.

Using an experimental model of asthma, we have identified a variety of type II allergens and have determined that active protease represents a critical biochemical activity underlying intrinsic allergenicity. We identified three distinct type II allergens, all of which contained active protease and amylase activities. However, only active protease was required for Th2 commitment, Th2- and Ag-specific Ab isotype secretion and allergic lung inflammation. Protease-free OVA was incapable of inducing these features of asthma, but could be converted into a type II allergen simply by coadministering it intranasally with a single highly purified fungal protease. Thus, together with Ag, exogenous proteases are both necessary and sufficient to bypass tolerogenic responses to inhaled Ag and initiate Th2-dependent allergic lung inflammation. Therefore, as allergenic adjuvants, proteases are implicated as important virulence factors in diverse allergic reactions.

Materials and Methods

Mice

Female C57BL/6 mice were used between 4 and 8 wk of age and bred at the American Association for the Accreditation of Laboratory Animal Care-accredited Baylor College of Medicine (Houston, TX) vivarium. Mice were maintained under specific pathogen-free conditions and used according to institutional guidelines.

Antigens

A. fumigatus Ag was prepared from live cultures (lot no. DC980809) as previously described (14). Whole ragweed pollen extract allergen (ragweed; lot no. AK000516) was prepared by pulverizing 1 g of ragweed pollen (Sigma-Aldrich, St. Louis, MO) in 8 ml of PBS, centrifuging at 10,000 rpm for 20 min and sterile filtering the supernatant. *Aspergillus oryzae* allergen was obtained commercially (Sigma-Aldrich) and reconstituted to 10 mg/ml using sterile PBS. Chicken egg OVA (grade V; Sigma-Aldrich) was reconstituted in sterile PBS and was either given alone or was added in identical amount (25 μ g/dose) to protease-containing preparations immediately before administration. OVA fragments were prepared by incubating OVA (1 mg/ml) with *A. oryzae* allergen for 90 min at 37°C followed by centrifugation through a molecular-size exclusion column (Centricon 10). Fragment isolation and exclusion of protease were confirmed by SDS-PAGE followed by silver staining and determination of total protease activity (none detected in fragments). Mice were then administered 25 μ g protein/dose intranasally. Total protein content of allergens was determined using a bicinchoninic acid-based protein assay according to the manufacturer's instructions (Pierce, Rockford, IL).

Proteases

The purified 33-kDa serine protease from *A. fumigatus* was prepared as described (34) and inhibited >95% by repeated addition (up to three times) of PMSF (1 mM; Roche, Basel, Switzerland) for 2 h at room temperature followed by overnight dialysis against PBS. Protease activity of *A. oryzae* allergen was inhibited >95% by repeated addition of phosphoramidon (500 μ M; Roche) for 2 h at room temperature followed by overnight dialysis against PBS.

Active proteases were detected by gelatin gel zymography as described (23). Total protease and amylase activities were determined using quenched fluorescein-casein and fluorescein-starch substrates, respectively (Molecular Probes, Eugene, OR). Enzymatic release of the fluorescent signals was quantitated by a microplate fluorometer (Bio-Tek Instruments, Watford Herts, U.K.) according to the manufacturer's instructions and data were expressed as fold increase in signal above background following incubation at 37°C for 1 h (Table I). Specific activity of the *A. fumigatus* serine protease was determined to be 400 U of total protease activity per nanomole of protein (400 U/nmol).

Induction and analysis of the asthma phenotype

The Ag preparations used for these studies are listed in Table I. Doses of Ag based on protease activity we empirically determined to yield equivalent induction of the asthma phenotype following intranasal challenge (Table I and Fig. 2). Doses of protease-inhibited allergens were adjusted based on equivalent total protein content relative to protease-uninhibited allergen. For intranasal Ag challenge, mice were anesthetized with metofane vapor (Vedco, St. Joseph, MO) and allowed to inhale 50 μ l of allergen following application to the nares with a pipette every 4 days for five total challenges. Data were collected 24 h following the final Ag challenge and were expressed as means $\pm$ SEM.

Airway hyperresponsiveness by PC₂₀₀, bronchoalveolar lavage (BAL) cytology, serum Ab isotypes, lung histopathology and lung cytokine profiles by ELISPOT assay were determined as described (14). Briefly, mice were ventilated using 100% oxygen under conditions that maintained physiologic pH and arterial PCO₂. After establishing a stable baseline for respiratory system resistance as determined by continuously quantitating $\Delta Pt/V$ (where ΔPt = change in tracheal pressure and V = air flow) at 70% tidal volume, acetylcholine chloride was administered i.v. over 1 s in escalating doses. The provocative concentration of acetylcholine in microgram per gram of body weight that caused a 200% increase in respiratory system resistance, designated PC₂₀₀, was calculated by linear interpolation of appropriate dose-response curves. Significant differences (defined as $p < 0.05$) were calculated on the logarithm of PC₂₀₀ by ANOVA using reference to the specified control groups. Baseline pulmonary resistance did not differ among the various groups of mice in these studies, ensuring reliable comparison between the different experimental groups.

BAL cells were collected by serially instilling and withdrawing 2- to 1-ml aliquots of PBS (pH 7.2) from the tracheal cannula. Aliquots of 10⁵ cells were centrifuged onto glass slides, stained using modified Giemsa, and used to determine the absolute numbers of eosinophils.

Suspensions of whole lung cells were prepared by removing the lungs and dissecting away lymph node and thymic tissue. Lungs were finely minced and the fragments were pressed through a 40- μ m nylon mesh filter. RBCs were lysed in hypotonic buffer and the remaining cells were washed

Table I. *Ag used to induce the asthma phenotype in mice*

Allergen Name	Allergen Description	Protease Units/Dose ^a (% inhibitable) ^b	Amylase Units/Dose
<i>A. fumigatus</i>	Concentrated culture filtrate prepared from stationary cultures of <i>A. fumigatus</i>	6 (50)	6
Ragweed	Sterile soluble extract from the pulverized pollen of <i>Ambrosia artemisiifolia</i>	60 (60)	250
<i>A. fumigatus</i> serine protease/OVA	Purified 33-kDa serine protease from <i>A. fumigatus</i> cultures and OVA	9 (>95)	0
<i>A. oryzae</i>	Commercially obtained culture filtrate prepared from stationary cultures of <i>A. oryzae</i>	425 (>95)	30
OVA	Commercially obtained 40-kDa protein derived from chicken egg protein	0	0

^a Doses for Ags except OVA are based on protease activity and induce comparable degrees of allergic lung disease (airway hyperresponsiveness, airway eosinophilia, airway mucus production).

^b Nos. represent maximum percentage of inhibition using a variety of inhibitor mixtures.

twice, counted, and adjusted to 10^7 cells/ml in RPMI 1640 with 5% FBS and antibiotics.

For ELISPOT assays, duplicate cell samples were distributed to 96-well microtiter plates that had been precoated with mAb 11B11 anti-murine-IL-4 Ab, serial 2-fold dilutions of the cells were conducted, and the plates were incubated undisturbed for 18 h at 37°C. After washing away the cells, biotinylated secondary Ab against IL-4, BVD6-24G.2 (BD PharMingen, San Diego, CA), was added. Captured IL-4 was revealed using streptavidin-conjugated alkaline phosphatase (Jackson ImmunoResearch Laboratories, West Grove, PA) and developed using 5-bromo-4-chloro-indolyl-phosphate (Sigma-Aldrich) in 0.1 M 2-amino-2-methyl-1-propanol buffer (Sigma-Aldrich) suspended in 0.6% agarose. Individual blue spots were counted after solidification of the agar using inverted microscopy.

Serum was prepared from whole blood collected at the time of death for determination of total IgE and OVA-specific IgG1 and IgG2a. For total IgE, serum diluted 1/5 and 1/50 was added to plates precoated with anti-IgE Ab R35-72 (BD PharMingen) and developed with biotinylated Ab R35-118 (BD PharMingen) followed by streptavidin-conjugated alkaline phosphatase and nitrophenylphosphate (NPP; Sigma-Aldrich) substrate and the results compared with a standard curve prepared with monoclonal murine IgE (Sigma-Aldrich). To washed and blocked plates precoated with OVA (100 μ g/ml in PBS), serum diluted 1/5, 1/50, 1/500, and 1/5000 was added, followed by biotinylated isotype-specific Abs (IgG1: LO-MG1 (Caltag Laboratories, Burlingame, CA); IgG2a: LO-MG2a (Caltag Laboratories)). Plates were washed, developed with streptavidin-conjugated alkaline phosphatase and NPP substrate, read in a standard spectrophotometer and relative concentrations were expressed as OD₄₀₅.

Secreted airway mucin was quantitated using the mucin-binding lectin, jacalin (35), as described (23). A total of 40 μ l of BAL diluted 1/100 and 1/1000 were added to individual wells of microtiter ELISA plates (Dynatech Laboratories, Chantilly, VA) and incubated 2 h at 37°C. Plates were washed and blocked with 3% I-block (Applied Biosystems, Foster City, CA) followed by addition of 0.002% biotinylated jacalin (Calbiochem, La Jolla, CA). After incubation for 1 h at 37°C, plates were washed and developed with streptavidin-conjugated alkaline phosphatase and NPP. Results were quantitated by comparison with a mucin (Sigma-Aldrich) standard curve.

Statistics

All data are representative of at least three independent experiments with four to five mice in each in vivo experiment and are expressed as means $\pm$ SEM. Significant differences ($p \leq 0.05$) were determined using Student's *t* test (log PC₂₀₀) or Mann-Whitney *U* test (all other data).

Results

Allergenic potential of fungal and pollen-derived allergens

Allergens prepared from *A. fumigatus*, *A. oryzae* and the pollen of *Ambrosia artemisiifolia* (ragweed) were compared with chicken OVA for their ability to induce the asthma phenotype in mice when given intranasally. Allergens were administered over a broad range of protease activities (1–500 protease U/dose) and OVA was administered between 1 μ g/dose and 1 mg/dose. The data in Fig. 1 are selected to show only maximal sublethal responses as greater amounts of allergen generally resulted in unacceptable (>20%) mortality, although OVA alone induced neither allergic disease nor mortality at any dose.

Compared with a previously standardized dose of the *A. fumigatus* allergen (14), the other fungal- and pollen-derived allergens yielded comparable induction of airway hyperresponsiveness and BAL eosinophilia (Ref. 14; Fig. 1). The fungal-derived allergens further induced comparable degrees of peribronchovascular inflammation and airway goblet cell metaplasia (Fig. 2, *b* and *c*). Similar results were obtained with allergens administered intradermally or i.p. followed by intranasal challenge (Refs. 13 and 14 and data not shown). These findings document that three diverse Ag preparations are potent type II allergens, all containing an activity or property which overcomes the innate resistance of the airway to immune activation in response to Ag challenge.

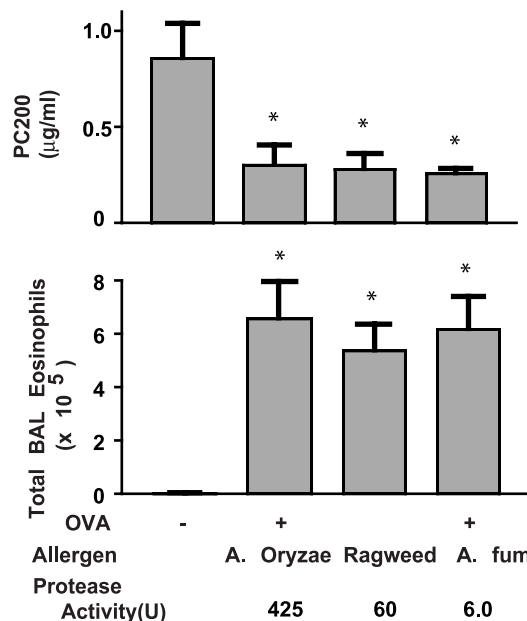


FIGURE 1. Comparison of three protease-containing allergens. Mice were intranasally sensitized with allergens derived from *A. oryzae*, ragweed pollen, and *A. fumigatus* (*A. fum*) without or with OVA (indicated by – or +, respectively) given on a schedule of every 4 days for five total challenges and compared with saline-challenged control mice (*far left bars*). Allergen doses are listed in units (U) of protease activity. *Upper panel*, Provocative concentration of acetylcholine chloride that induced a 200% rise in respiratory system resistance from baseline (PC₂₀₀). *Lower panel*, Total eosinophils influxing into BAL. Data are representative of three independent experiments simultaneously comparing the three allergens. *, $p \leq 0.05$ relative to saline-challenged mice.

Enzymatic activities of type II allergens and their requirement for allergic lung disease

The previous findings are remarkable given that they were obtained using a mouse strain that is resistant to allergic lung disease (18, 36) and other Th2-dependent phenomena (37), and were induced by delivery of an allergen through a mucosal route that normally elicits only tolerance (16, 38). Therefore, we sought to identify a factor common to these allergens that is capable of overcoming induction of tolerance and inducing strong lung allergic reactions in resistant mice. Further analyses confirmed in all three allergens the presence of protease and amylase activities (Fig. 3 and Table I) which have both been linked to allergens and allergic phenomena (39–51). Using a variety of inhibitor mixtures, we determined the maximum degree of protease inhibition achievable for subsequent in vivo studies. As shown, full inhibition of protease activity was possible only with the *A. oryzae* allergen (Table I).

To explore the allergenic potential of amylases and proteases, we performed additional experiments with the *A. oryzae* allergen prepared with OVA and administered intranasally in doses of 10-fold increasing concentration based on protease content. This protocol induced progressively severe allergic lung disease as assessed by airway hyperresponsiveness, airway eosinophilia, and secreted airway mucin equivalent at the highest dose to that elicited by a previously standardized dose of the *A. fumigatus* allergen (Fig. 4). In contrast, no induction of these allergic parameters was observed in mice challenge with vehicle (saline) or OVA alone, whereas an intermediate dose of *A. oryzae* allergen without OVA

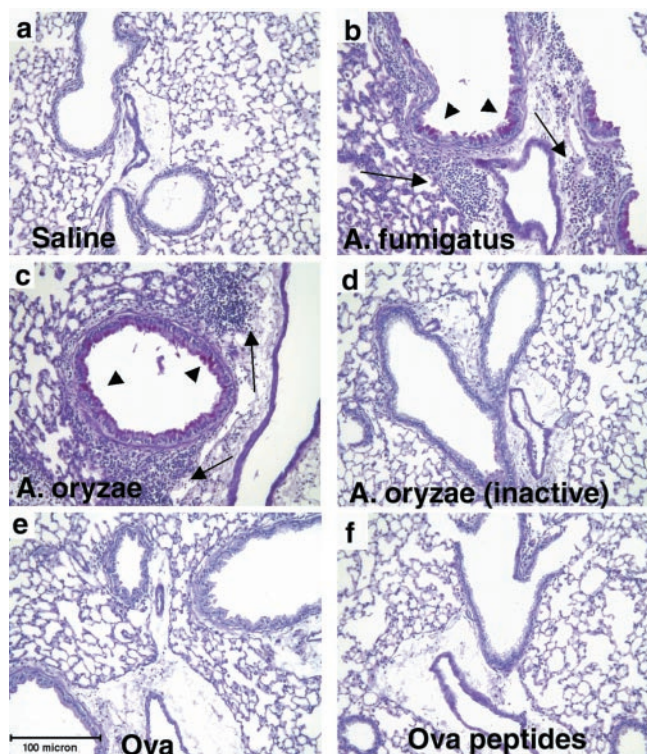


FIGURE 2. Representative periodic acid-Schiff-stained histologic sections of murine lungs. Arrowheads point to mucus-producing goblet cells within the airway epithelium, and arrows indicate significant peribronchovascular inflammation. All images include representative bronchovascular bundles at $\times 200$ magnification. *a–f*, C57BL/6 mice were intranasally challenged with saline, *A. fumigatus* allergen with OVA, *A. oryzae* allergen with OVA, protease-inactive *A. oryzae* allergen with OVA (total protease activity 6% compared with preparation used in *c*), OVA alone, or *A. oryzae*-hydrolyzed OVA (OVA peptides), respectively. Goblet cells and inflammation are seen only in mice receiving active protease (*b* and *c*).

showed no significant alteration in allergenic activity. Thus, the quantity of active protease, not OVA, determined the degree of expression of allergic disease induced by the *A. oryzae* allergen.

We conducted additional studies to understand if the protease and amylase enzymes present in the *A. oryzae*/OVA allergen were required for allergic lung disease. For these studies, the protease activity of *A. oryzae* allergen was inhibited by $>95\%$ using phosphoramidon and the dose of inactivated *A. oryzae* allergen was adjusted to contain the equivalent quantity of total protein as the protease-active allergen. This protocol, which left largely intact the amylase activity, resulted in reduced expression of all aspects of the asthma phenotype, including airway hyperresponsiveness, mucin secretion, and airway eosinophilia by 90–100% depending on the parameter (Fig. 4). Furthermore, such treatment abrogated the robust peribronchovascular inflammation and airway goblet cell metaplasia induced by the *A. oryzae* allergen (Fig. 2, *c* and *d*). To confirm that protease activity, and not exogenously derived digestion products, is allergenic we compared reactivity of *A. oryzae* allergen alone to a preparation containing only allergen-hydrolyzed OVA (OVA peptides). As expected, *A. oryzae* allergen reconstituted airway hyperresponsiveness and airway eosinophilia while the OVA peptides showed no potential to induce the asthma phenotype or elicit lung inflammation (Figs. 2*f* and 4). These studies demonstrate that the protease present in the *A. oryzae* allergen, not the amylase, was required for allergic disease.

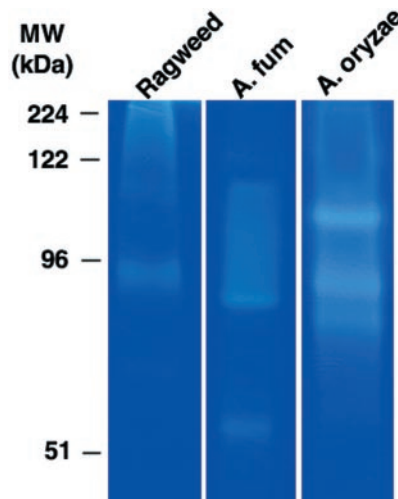


FIGURE 3. Demonstration of proteases in allergens by zymography. Allergens derived from ragweed pollen (Ragweed), *A. fumigatus* (*A. fum*) and *A. oryzae* were subjected to gelatin gel zymography and protease bands were revealed following Coomassie blue staining. All three allergens possessed significant quantities of active protease as indicated by clear bands within the gels.

Requirement of protease for Th2 commitment and Ag-specific Ab secretion

In prior studies, we showed that the Ag-induced asthma phenotype is dependent on the generation of Th2 cells and not other effector cells such as CD8⁺ T cells (52), NK-T cells (52), and B cells (14). Thus, consistent with induction of the asthma phenotype that is itself a robust indicator of Th2 effector development, we detected Th2 cells following challenge with *A. oryzae*/OVA allergen. OVA added to allergenic preparations permitted detection of Ag-specific IgG1, which in the mouse is largely Th2- and IL-4-dependent (53–56) and IgG2a, an IFN- γ -dependent isotype (57). Similar to indices of allergic lung disease, relative quantities of total IgE and OVA-specific IgG1 titrated according to the strength of *A. oryzae*/OVA allergen administered and were comparable at the highest dose to those achieved with *A. fumigatus*/OVA allergen (Fig. 5). IgE and, in contrast to prior studies (16, 17), IgG1, were largely absent from sera of mice receiving protease-inactive *A. oryzae*/OVA allergen or OVA alone, consistent with induction of Ag-specific tolerance. Furthermore, IL-4-producing cells, consisting of predominantly Th2 cells and eosinophils, were only detected in lungs of mice receiving active protease (Fig. 6). In contrast, IgG2a was expressed to the highest degree in mice receiving only OVA (16). Together, these studies demonstrate that, in addition to allergic lung disease, *A. oryzae*/OVA allergen induced an Ag-specific Th2 response dependent only on the presence of active protease.

Effect of adding protease to a type I allergen

The preceding studies establish that the protease present in the *A. oryzae* allergen is required for Th2 activation, total IgE and Ag-specific IgG1 production and allergic lung disease, whereas OVA alone is insufficient for these endpoints. However, it remains unclear if these are properties unique to the *A. oryzae* protease or if other proteases possess similar adjuvant properties. To demonstrate that these findings are applicable to other proteases and distinct allergens, we combined OVA with the secreted serine protease purified from the extracellular fluid of *A. fumigatus* cultures (34), the same serine protease contained in the *A. fumigatus* allergen. When administered intranasally to mice according to the same

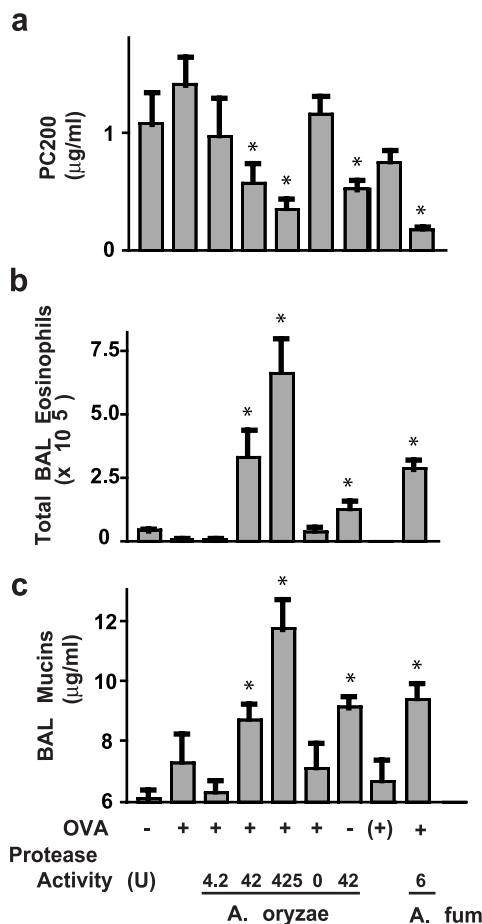


FIGURE 4. Requirement of protease activity for allergic lung responses. C57BL/6 mice were intranasally challenged with saline (–), or the indicated combinations of OVA (indicated by +), *A. oryzae* or *A. fumigatus* (*A. fum*) allergens according to the schedule as in Fig. 1. *A. oryzae* allergen was given in increasing amounts from 4.2–425 U of protease activity/dose and following phosphoramidon inactivation of an intermediate dose originally containing 42 U of protease activity (protease activity: 0). *A. oryzae*-hydrolyzed fragments of OVA (indicated by (+)) were also administered as an additional control. *a*, Airway hyperresponsiveness by PC₂₀₀. *b*, Total BAL eosinophils. *c*, BAL mucin concentration. Data are representative of three independent experiments; *, $p \leq 0.05$ relative to saline-challenged mice

protocol as used with previous allergens, *A. fumigatus* serine protease/OVA induced airway hyperresponsiveness identical with that elicited by the more complex *A. fumigatus* allergen and significant eosinophilia (Fig. 7). A 10-fold more active protease dose of *A. fumigatus* serine protease further induced airway eosinophilia comparable to levels observed in *A. fumigatus* allergen-challenged mice ($4.1 \times 10^5 \pm 1.6 \times 10^5$ vs $5.3 \times 10^5 \pm 3.6 \times 10^5$ total BAL eosinophils, respectively). We further documented recruitment of IL-4-producing cells to the lungs of *A. fumigatus* serine protease/OVA-challenged mice in numbers consistent with results obtained with other allergens (Fig. 6). In contrast, no significant induction of these allergic parameters was observed following challenge with saline, OVA alone, protease alone, or OVA and protease inactivated >95% by PMSF (a serine class protease inhibitor). These studies demonstrate that an active protease distinct from that present in the *A. oryzae* allergen is sufficient for induction of allergic lung disease. Furthermore, our findings demonstrate that active protease in combination with Ag is both necessary and sufficient to overcome the innate resistance of the airway to Th2 activation and allergic lung disease.

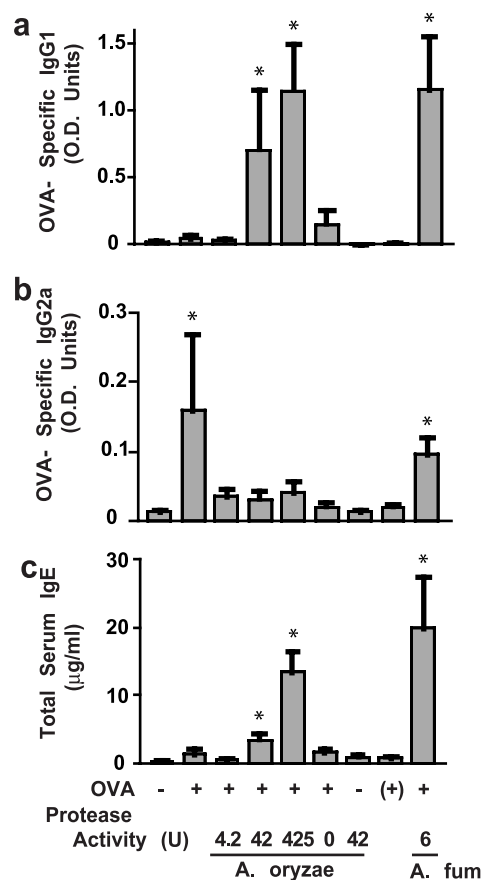


FIGURE 5. Effect of exogenous protease on Ab responses. Relative concentrations of OVA-specific serum IgG1 and IgG2a (*A* and *B*, respectively) in OD units and total serum IgE in micrograms per milliliter (*C*) were determined in the same treatment groups as in Fig. 4. Data are representative of three independent experiments; *, $p \leq 0.05$ relative to saline-challenged mice.

Discussion

Our studies demonstrate that type II allergens, which do not require additional adjuvants or immune priming remote from the lung to elicit allergic lung inflammation, are not unique to *A. fumigatus*, but rather are easily derived from diverse species relevant to human atopy. We have further shown that active protease accounts for the intrinsic allergenic activity of the *A. oryzae* allergen and that a highly purified protease derived from *A. fumigatus* is sufficient to convert the type I allergen OVA to a type II when combined with protease. Therefore, regarding the type II allergens explored in this study, active proteases are the essential adjuvant factor responsible for allergic potency.

Although absolute protease activity varied considerably in different preparations having equivalent allergenic activity, only protease activity correlated with disease induction; complete inactivation of protease in the *A. oryzae* allergen abrogated both inflammation and Th2 activation. Neither OVA (a type I allergen), a purified active protease (*A. fumigatus* serine protease), nor a protease-inactive allergen complex (*A. oryzae* allergen) was sufficient to induce the asthma phenotype if given intranasally. Remarkably however, a combination of only two molecules, a protease and type I allergen, was sufficient to induce allergic lung disease in a manner dependent only on the dose of active protease. Therefore, active protease, in combination with a type I allergen, is sufficient to bypass airway tolerance induction, stabilize Th2

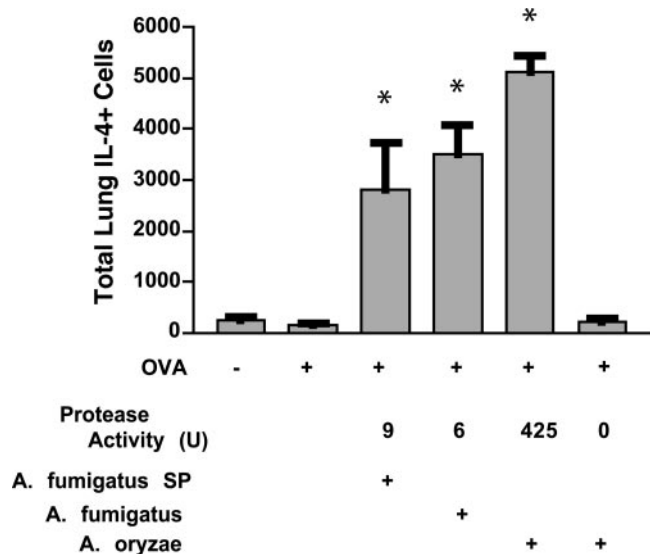


FIGURE 6. Lung IL-4 responses to allergens. C57BL/6 mice were challenged with saline (–), 25 μ g of OVA (+), or the indicated combinations of OVA and nine protease units (U) of *A. fumigatus* serine protease (*A. fumigatus* SP), 6 U of *A. fumigatus* allergen, 425 U of *A. oryzae* allergen or protease-inactive *A. oryzae* allergen (original protease activity 425 U) given every 4 days for five total challenges. Twenty-four hours after the final challenge, single-cell suspensions were prepared from lungs from which IL-4-producing cells were enumerated by ELISPOT. Fewer than 1000 IFN- γ -producing cells were detected in all mice. Data are representative of at least three independent experiments. *, $p \leq 0.05$ relative to saline-challenged mice.

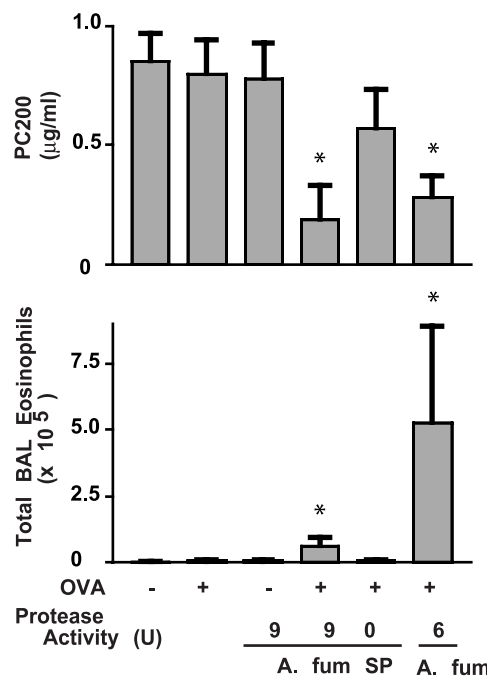


FIGURE 7. Induction of allergic lung disease by addition of exogenous protease to intranasal OVA. C57BL/6 mice were challenged intranasally with saline (–), 25 μ g of OVA (+), or the indicated combinations of OVA, nine protease units of the secreted serine protease derived from *A. fumigatus* cultures (*A. fum* SP), protease-inactive *A. fumigatus* SP, or *A. fumigatus* allergen. Each challenge was given every 4 days for five total challenges and the asthma phenotype was determined 24 h after the final challenge. *Upper panel*, Airway hyperresponsiveness by PC200. *Lower panel*, Total BAL eosinophils. Data are representative of three independent experiments. *, $p \leq 0.05$ relative to saline-challenged mice.

effector commitment to the type I allergen, and sustain allergic lung inflammation.

Proteases have previously been linked to allergic asthma and many of the Ags most frequently implicated in disease are either proteases or strongly associated with protease activity. These include Der P I (29), the Ag most commonly linked to asthma worldwide (58), Fel d I (59), the most important allergen derived from the domestic cat and many pollens (48, 60, 61). Fungi are also implicated in allergic disease, and as shown in this study and elsewhere (62, 63), are potent sources of active protease. Intriguingly, occupational outbreaks of asthma are associated with the handling of bacterial-derived proteases used in the manufacture of detergent products, although it is not clear from these investigations which of several bacterial products were etiologic (64, 65).

A pathogenic role for exogenous protease, especially Der P I, has previously been suggested through cleavage of a variety of endogenous proteins, including the low-affinity IgER (CD23; Ref. 29) and the IL-2R α chain (30), and by modifying the balance between IFN- γ and IL-4 (66, 67). Furthermore, the proteolytic activity of Der P I enhances titers of IgE specific for the protease (68) and bystander Ags such as OVA (69). However, the importance of exogenous proteases regarding Th effector development and allergic lung disease have not been investigated until now. Because proteases distinct from Der P I are both necessary and sufficient for the complete spectrum of allergic lung disease induced by the parent allergen, the data together suggest that the lung responds to exposure to divergent proteases by activating a stereotypical allergic effector response. The mechanism by which proteases accomplish this is the subject of ongoing investigation, but it is unlikely to involve Toll-like receptors, as mice deficient in all Toll-like receptor signaling have diminished Th1, but not Th2, responses (70). A more likely mechanism may involve C proteins. We have recently shown that C protein 3, which undergoes exten-

sive proteolytic modification by a variety of exogenous proteases (71–74), is necessary for Th2 commitment and robust allergic lung disease in response to a proteolytically active type II allergen (75).

Exogenous proteases have also been shown to degrade proteins comprising the tight junctions of airway epithelium. Although this facilitates Ag presentation (31), IgG2a responses and IgE tolerance, both Ag-specific processes induced by intranasal OVA, proceed in the complete absence of airway protease challenge and, thus, proteolytic disruption of the epithelium. Thus, active protease is not required for airway Ag presentation, only allergic responses to Ag. Although protease and amylase activities were common to all of our allergens and numerous additional molecules (32, 33) potentially relevant to allergic disease are doubtless present in the *A. oryzae* allergen, only protease was required for disease. Thus, while many microbial factors may contribute to expression of some features of experimental allergic disease, our data strongly suggest that only protease is necessary for the asthma phenotype.

Although our findings establish a potential link between allergenic proteases and human allergic disease, many atopic patients show serum reactivity to Ags with no known protease activity, raising questions as to the true relevance of protease to human illness (76). We demonstrated that fungal proteases are specifically capable of conferring allergenic potential to otherwise innocuous type I allergens applied to the respiratory tract. Thus, in response to inhaled allergens, Th2 activation and allergic lung disease require at least two exogenous signals: an Ag and allergenic adjuvant. Although the same molecule may represent these immune elements, we have shown that distinct molecules may just as likely express them. Interestingly, fungal proteases, some of which are excellent allergenic adjuvants as shown in this study, inexplicably

make poor Ags for some heavily exposed patients, a property which likely explains why protease alone did not induce significant allergic lung disease in this study (43). Therefore, demonstration in allergic patients of serum reactivity to nonprotease allergens fails to account for potential concomitant exposure to an allergenic adjuvant, particularly when the latter is not serologically identifiable. Although these studies alone cannot exclude the existence of protease-inactive allergenic adjuvants, our data more importantly demonstrate the possibility that Th2 commitment and allergic phenomena arising from airway allergen exposure are ultimately driven by a protease-based mechanism.

Additional studies are required to define the protease virulence factors relevant to human allergic disease. Secreted endogenous proteases are abundant in normal human lung and airway secretions, but are tightly regulated through various means. Therefore, exogenous allergenic proteases that are uniquely capable of triggering inflammatory responses likely escape the regulatory processes which govern the activity of endogenous enzymes. Additional proinflammatory properties of exogenous proteases potentially include relative stability under a wide variety of environmental conditions and insensitivity to the effects of endogenous inhibitors. Another critically important issue is the relative abundance of free protease activity in combination with defined Ags in human environments, a subject about which little is known. Our study establishes that such analyses are now feasible and that in addition to serologically defined Ags, protease activity should be correlated in future studies with the incidence, prevalence and severity of a variety of human allergic conditions. Such studies promise to provide additional pathophysiologic and therapeutic insight into common allergic ailments such as asthma.

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