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Respiratory tract allergic disease and atopy: experimental evidence for a fungal infectious etiology

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Abstract

Allergic asthma is an obstructive lung disease linked to environmental exposures that elicit allergic airway inflammation and characteristic antigen-specific immunoglobulin reactions termed atopy. Analyses of asthma pathogenesis using experimental models have shown that T helper cells, especially T helper type 2 (Th2) cells and Th2 cytokines such as interleukin 4 (IL-4) and IL-13, are critical mediators of airway obstruction following allergen challenge, but the environmental initiators of lung Th2 responses are less defined. Our studies demonstrate that fungal-derived proteinases that are commonly found in home environments are requisite immune adjuvants capable of eliciting robust Th2 responses and allergic lung disease in mice. We have further shown that common household fungi readily infect the mouse respiratory tract and induce both asthma-like disease and atopy to otherwise innocuous bystander antigens through the secretion of proteinases. These findings support the possibility that asthma and atopy represent a reaction to respiratory tract fungal infection, suggesting novel means for diagnosis and therapy of diverse allergic disorders.

Keywords

asthma; atopy; fungus

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Introduction

Allergic asthma is a respiratory disease that is induced by exposure to environmental antigens that elicit allergic inflammation and intermittent airway obstruction, the latter which is believed to underlie the characteristic symptoms of cough and dyspnea. As one of the most common of all diseases of adults and children and a major cause of decreased quality of life in societies where its prevalence is high, asthma is one of the most important of all diseases in highly industrialized societies. The chronic, often incurable nature of asthma further contributes to the economic burden it places on the health care delivery system [1,2].

A characteristic feature of asthma is atopy, as defined by the predilection to develop antibody reactions (IgE and IgG) against environmental antigens [3]. Indeed, atopy is one of the strongest risk factors for acquiring asthma and is often assessed by means of the skin prick test [3] in which an immediate wheal and flare reaction to antigen preparations injected intradermally are interpreted as positive. Such reactions are known to be immunologically mediated through the mechanism of type 1 (immediate) hypersensitivity in which antigen-mediated crosslinking of IgE that has been captured by the high affinity IgE receptor (FcεRI) leads to activation of mast cells, dendritic cells, eosinophils (in humans) and other cells [4]. The degranulation products of these IgE/antigen-activated cells, including histamine and many others, were believed to account for not just the wheal and flare reaction of the skin prick test but also the airway obstruction that underlies the major clinical manifestations of asthma [5].

Animal models of asthma, termed allergic lung disease in the experimental context, have been useful for dissecting pathogenic mechanisms with potential relevance to human disease. A characteristic feature of these models is airway obstruction, which can be assessed in terms of airway hyperreactivity, a measure of the airway contractile response to acetylcholine challenge and goblet cell metaplasia, a reaction of airway epithelial cells in which they acquire a mucus-secreting phenotype. Other features of the model include lung and airway inflammation characterized most prominently by infiltration with eosinophils and production of antigen-specific IgE and IgG (atopy). Mice with disruption of type I hypersensitivity mechanisms, including mice lacking IgE and other antibodies [6,7], B cells [6], eosinophils [8], and mast cells [9] all manifest airway obstruction that is very similar to that observed in wild type animals. These studies demonstrate that type I hypersensitivity mechanisms are not required for the essential disease feature of airway obstruction, nor are they required for robust allergic inflammation. Type I hypersensitivity mechanisms remain relevant in asthma as they most likely underlie other allergic phenomena such as upper respiratory tract allergic reactions (allergic rhinitis), hay fever and more serious processes such as systemic anaphylaxis that may contribute in distinct ways to the expression of asthma. Nonetheless, the persistence of lower respiratory tract allergic inflammation and airway obstruction (airway hyperresponsiveness, mucus hyper-secretion), the key characteristics of asthma, in mice with abrogation of type I hypersensitivity provided the first evidence for the existence of alternate requisite immune mechanisms.

Further analysis of allergic lung disease in our laboratory established that airway obstruction following allergen challenge in mice was due to a specialized subset of T helper cell termed T helper type 2 (Th2) cells that secrete a restricted repertoire of cytokines including IL-4, IL-5, and IL-13. Th2 cells elicit all major features of allergic lung disease in the complete absence of atopic (antibody) reactions, although they are also required to instruct B cells to produce antigen-specific IgE and IgG [10]. Moreover, whereas all Th2 cell cytokines contribute to the allergic lung disease phenotype, IL-13 was ultimately shown to act directly on constitutive cells of the lung, such as airway epithelium, to induce airway obstruction [11,12].

Further insights into the immune basis of airway obstruction in asthma continue to be made, including the discoveries that epithelial cytokines such as thymic stromal lymphopoietin (TSLP) and IL-25 are essential for robust Th2 cell development and allergic lung disease [13, 14]. Until recently, however, insights into the exogenous factors that induce asthma have been lacking. We review in the following sections new insights into the environmental factors underlying Th2 cell development and atopy in experimental asthma.

Traditional experimental allergens

Asthma is believed to be caused by inhalation of environmental antigens, termed allergens, which direct the development and recruitment to lung of Th2 cells. Diverse antigens have been used to elicit allergic lung inflammation in many animal species. However, the majority of experimental work has been conducted in mice and guinea pigs using the antigen ovalbumin [15]. Somewhat paradoxically, concerns with the ovalbumin-dependent mouse model began to arise as it nonetheless proved to be highly successful in elucidating the fundamental immune and physiological mechanisms of allergic lung disease. Ovalbumin is clearly capable of eliciting allergic lung disease, but inexplicably its potency in causing robust allergic lung disease is restricted to the Balb/c and A/J mouse strains [16,17]. Furthermore, ovalbumin will only elicit allergic lung disease if animals are first immunized against ovalbumin precipitated in aluminum-based adjuvants, a protocol with uncertain physiological relevance to human disease. However, once ovalbumin-dependent allergic lung disease has been established, the disease phenotype cannot be maintained for periods beyond approximately six weeks, presumably because of contravening tolerogenic mechanisms that may be unique to the lung [18]. A final concern with ovalbumin is that this antigen is not a significant cause of human allergic lung diseases and may therefore be suboptimal for exploring the relevant extrinsic causes of asthma.

The search for more relevant allergens: proteinases as allergic adjuvants

To overcome the limitations of ovalbumin, we analyzed a series of antigens prepared from organisms previously known to be highly allergenic for humans (fungi and pollens) to understand if a common biochemistry might provide the essential link to their allergenic properties. We focused initially on the presence of proteinases, which had previously been noted to be a common biochemical feature of human allergens [19] and which had already been shown to exhibit intriguing immunological properties [20–28]. Complex, crude allergens prepared from the culture filtrate of the fungus *Aspergillus fumigatus* and the

pollen of *Ambrosia artemisiifolia* (common ragweed) proved to be highly allergenic in diverse mouse strains, including C57BL/6 mice that are resistant to ovalbumin [16,29]. Moreover, both allergens contained abundant proteinase activity in addition to other enzymatic activities and potentially allergenic moieties [29].

To determine if proteinase was in fact contributing to the allergenic nature of these preparations, we conducted further mouse experiments using only highly purified fungal proteinases. These enzymes proved to have allergic potency equivalent to more complex allergens derived from whole organisms, eliciting airway hyperreactivity, goblet cell metaplasia and allergic lung inflammation in C57BL/6 mice when given only through the airway [29]. Allergenic activity of proteinases required intact enzymatic activity [29]. The technique used to inactivate proteinase activity in these studies was the addition of small molecules (phosphoramidon, PMSF) that bind to and inactivate the enzyme active site without denaturing or otherwise altering protein structure and therefore antigenicity. This suggests, therefore, that the allergenic principle of these proteinases is biochemical, and not structural (i.e., antigenic), in nature. Equally striking, we showed that whereas ovalbumin given intranasally to mice in the absence of prior immunization induces no specific immune reaction, lung inflammation or airway hyperresponsiveness, when given together with *Aspergillus oryzae* proteinase, ovalbumin elicits specific IgE and IgG1 responses [29]. Thus, the fungal proteinase acts as an adjuvant to promote Th2-dependent allergic reactions to innocuous antigens that by themselves would elicit no active inflammation if inhaled. Regardless of the allergen (ovalbumin, proteinase or complex extracts of fungi and pollens combined with ovalbumin), the minimum time from first exposure to maximal allergic lung disease is approximately 10–12 days (D. Corry, unpublished). This suggests that although the initial mechanisms by which allergic responses are initiated may differ, they all ultimately lead to a stereotypical Th2 cell-dominated response that drives airway obstruction through a common mechanism, i.e., IL-13. Our experimental studies with allergenic proteinases are important because they are the first to demonstrate that an organism-specific biochemical activity (proteinase) can induce asthma-like disease.

The ultimate source of allergenic proteinases: fungi as infectious agents

The unexpectedly powerful allergenic effect of fungal and pollen proteinases in mice prompted us to consider the potential relevance of these and related enzymes as human allergens. Analysis of house dust obtained from the homes of asthmatic children revealed that most, if not all of these households contained active dust proteinases [30]. Further analysis revealed that a major source of many house dust proteinases was fungi, especially one fungus, *A. niger*, which we have found in most households (85%). We further confirmed that proteinases from dust mites (e.g., Der p 1), which have long been linked to human asthma [31], were present in many of our house dust samples. However, a surprise from these studies was that the dust mite proteinases were not enzymatically active in the majority of samples. This finding is important because enzymatic activity is required for allergenic activity in mice, at least for fungal proteinases [29]. Our findings therefore suggested that fungal proteinases, especially from *A. niger*, were potentially more relevant than dust mite proteinases as allergenic adjuvants in human disease.

Further analyses of our data indicated that potentially allergenic fungal proteinases present in house dust likely did not exist in quantities sufficient to elicit allergic disease directly by inhalation [30]. We therefore considered alternative means by which fungi could be linked to allergic lung disease through their secreted proteinases. Many species of *Aspergillus* have previously been shown to infect the human respiratory tract and cause invasive disease, but invasive disease due to *A. niger* is rare, usually being observed only in the setting of severe immune compromise [32,33]. In contrast, as many as 32% of immunologically normal asthma subjects demonstrate serum or skin prick test-based reactivity to fungal conidia antigens [34]. However, fungal-specific IgE may be detected in up to 66% of children with asthma or allergies [35]. Thus, invasive fungal disease due to *Aspergillus* spp. is rare, but sensitization to fungi is both common and widespread in human societies, especially in asthmatic individuals. Moreover, such sensitization is currently perceived to reflect hypersensitivity to immunogenic, but not necessarily infectious, fungal antigens that are ubiquitous in human environments [36].

To distinguish between the twin possibilities of fungal infection and hypersensitivity to fungal antigens and the role that secreted proteinases may play in either process, we next developed an infectious model of allergic lung disease. For this model, mice were challenged intranasally with the conidia of an *A. niger* isolate obtained from a house dust sample. These studies showed that a minimum dose of 50×10^3 conidia given daily was necessary to induce the complete spectrum of allergic lung disease (allergic airway inflammation and airway obstruction), although much smaller doses were sufficient to induce lung eosinophilia and IL-4 responses [30]. Moreover, although conidia sterilized by irradiation induced substantial eosinophilia and airway mucus secretion, they were unable to elicit highly polarized lung IL-4 responses or airway hyperresponsiveness even if given in very high doses [30], producing instead a lung phenotype more closely resembling the distinct lung syndrome of hypersensitivity pneumonitis and not asthma [37].

These studies therefore showed that the conidia of *A. niger* produced a true infection that was required to elicit the full spectrum of allergic lung disease. We have subsequently analyzed 12 additional fungi found in households of asthmatic children and determined that all are capable of inducing allergic lung disease in mice, with *Aspergillus*, *Penicillium* and *Curvularia* spp. being most potent (P. Porter, D.B. Corry, *et al.*, submitted). To determine if proteinases secreted by *A. niger* were required for disease expression, we next challenged mice with the conidia of genetic mutants of *A. niger* lacking secreted proteinases [38]. Lack of one or more proteinases did not affect the ability of *A. niger* to grow in the mouse airway as equal or greater numbers of organisms were recovered from lung tissue after 2 weeks of challenge with conidia, irrespective of the fungus' ability to secrete proteinases [30]. However, proteinase-deficient *A. niger* mutants were unable to elicit allergic lung disease [30]. These studies therefore confirmed that secreted proteinases are essential for eliciting allergic lung disease in the setting of active airway infection due to a common household fungus.

Relatively large numbers of *A. niger* conidia (minimum of 5×10^4 /day) were required to induce all relevant aspects of the allergic lung disease phenotype, especially airway hyperreactivity. Extrapolating directly from mice to humans that are $\sim 3,000 \times$ larger, this

would translate into a daily human conidia exposure dose of 150 million. There are no reliable estimates of actual inhaled conidia exposures in humans and therefore it is not possible to assess the plausibility of such a dose. Moreover, such crude estimates fail to consider the potentially vast differences in susceptibility of different mammalian hosts to inhaled conidia. Nonetheless, to make the model as realistic as possible and to further explore the relationship of fungal airway infection to atopy we further modified it to include a bystander antigen (ovalbumin) that alone is not sufficient to induce inflammation when inhaled [39]. This modification to the model was made to simulate the more realistic human situation in which conidia are being inhaled together with other antigenic material.

Carefully performed dose titration experiments demonstrated that in the presence of established, contained airway fungal infection, the formerly innocuous ovalbumin was converted into a true allergen that elicited both antigen-specific Th2 cell and IgG1 responses. In other words, the fungal infection induced atopy to a bystander antigen. The effect of having lung Th2 responses directed against both the fungus and ovalbumin was synergistic, such that as few as 2000 conidia/day were sufficient, when combined with ovalbumin, to induce the full spectrum of allergic lung disease [30]. Moreover, at these relative low doses of conidia, we could not detect serum fungal-specific IgE or IgG responses. These experiments thus demonstrated that low-grade airway infection with *A. niger* induces atopy to a bystander antigen, leading to synergistic allergic inflammation and an antibody response that reveals the bystander antigen but, remarkably, fails to reveal the underlying etiology, i.e., the fungal infection. These findings are important because they reveal the insensitivity of antibody-based immunodiagnostic approaches that are universally used now to diagnose fungal infection. They further suggest that alternate approaches, i.e., those based on T cell reactivity, may be superior to diagnosing especially low-grade fungal infections that may underlie human allergic lung diseases.

Finally, we considered the possibility that the asthma-like response to active fungal infection was protective and not maladaptive, as asthma is typically perceived [36]. Two additional experiments demonstrated that IL-13 was required in the mouse for optimal clearance of *A. niger* from the lungs after a single large conidia challenge and that eosinophils are directly fungicidal when incubated with the conidia of either *A. niger* or *A. fumigatus*. These studies thus indicated that the allergic response is protective in the setting of fungal airway infection and that, rather than aberrant, may well have evolved as an effective anti-fungal response mechanism.

Mechanism of proteinase-dependent allergic lung disease

The importance of proteinases to the pathophysiology of allergic lung disease raises important questions as to their mechanism of action. The general role of pathogen associated molecular pattern (PAMP) receptors such as Toll like receptors (TLR) for pathogen recognition and induction of requisite innate and adaptive immune mechanisms is now well established [40,41]. However, the specific role of PAMPs in proteinase-dependent allergic responses is controversial. We previously published that mice deficient in MyD88 (that are deficient in most TLR responses [42]) show enhanced innate allergic inflammation in response to allergenic proteinase challenge [39]. This does not rule out the possibility that

pattern recognition receptors might yet contribute to adaptive immune Th2 and IgE responses. Indeed, TLR4 has been implicated as being essential for the allergenic activity of the dust mite proteinase Der p I, although it is not clear if proteinase activity was also involved or if this observation is relevant to other allergens [43]. Much further study is required to fully resolve the requirement of PAMPs in proteinase-dependent allergic inflammation.

Regardless of the involvement of pattern recognition receptors, we speculate that a likely general mechanism underlying the activity of allergic proteinases is the cleavage of an endogenous, most likely ubiquitous substrate that releases an active fragment that subsequently engages an endogenous receptor that ultimately initiates the allergic inflammatory cascade. There is certainly experimental precedent for this potential mechanism: contact of complement protein C3 with fungi leads to the proteolytic generation of the C3a anaphylatoxin that is required for Th2 cell development and allergic lung disease [44,45]. Proteinase activated receptor 2 (PAR2) is another potential substrate for exogenous allergenic proteinases. A potentiating role for PAR2 in allergic lung disease has been shown for proteinase-inactive ovalbumin [46,47], whereas an inhibitory role has been shown in models using alternative allergens such as pollens [48]. Our own unpublished findings indicate that PAR2 has no role in mediating proteinase-dependent allergic lung inflammation (D. Corry, unpublished). Clearly, an important task for the field is to identify the endogenous substrates and molecular pathways by which proteinases elicit allergic lung disease.

Conclusions and future directions

Our findings in mice provide firm experimental support for the possibility that asthma may represent a specific response against fungal airway infection that limits the organism to the epithelial surface and prevents potentially lethal invasion into the lung parenchyma. The concept that asthma may represent a fungal infectious disease is further supported by several clinical trials in which anti-fungal antibiotics were shown to be of benefit in patients with allergic bronchopulmonary aspergillosis and severe asthma with fungal sensitization [49–51]. In contrast, currently accepted asthma therapy is centered on broad-based immunosuppression using topical or systemic corticosteroids. This approach is clearly effective at inhibiting the inflammation that leads to airway obstruction. Unfortunately, immunosuppression may simultaneously lead to fungal overgrowth and persistent infection – potentially masking without eradicating the ultimate infectious cause of disease. Future studies are therefore needed to clarify the relationship between common environmental fungi and asthma so that therapeutic trials comparing immunosuppressants, anti-fungal antibiotics or both can be optimally designed and interpreted.

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