

Structural Biology of Allergens

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Major allergens may have special aerobiological properties and allergenic structures. It would also be instructive to consider the properties of nonallergens and nonallergenic responses. In some cases, nonallergenic responses appear to result from a lack of antigenicity and in others from regulation. Proteolytic activity has been proposed as an adjuvant for allergenicity, but lipid binding is far more common and is found for more than 50% of the major allergens. Such structures can enhance allergenicity via Toll-like receptor (TLR) or CD1 pathways. TLR signaling can enhance both Th1 and Th2 responses and be induced by peptides as well as nonproteinaceous ligands.

Introduction

There is now a wealth of information on the structure of allergens, determined either directly by x-ray crystallography or nuclear magnetic spin resonance or from molecular models based on solved structures of sequence homologues. They constitute a diverse range of molecules with representatives of all the common polypeptide folds, with a possible under-representation of Rossmann folds [1••], although fungal aldehyde dehydrogenase and heat-shock protein allergens have such structures. Allergens vary from the small, single immunoglobulin fold of the group 2 house dust mite allergens, Der p 2 and Der f 2, through to the coils of tropomyosin and paramyosin and large, multi-domain proteins, such as cockroach arylphorin and mite apolipophorins. There, thus, appears to be few constraints on structure. The heterogeneity is also evident for function. House dust mite allergens provide an example [2]. There are several proteolytic enzymes that include the group 1 cysteine protease allergen and the group 3, 6, and 9 serine protease allergens. Other allergenic house dust mite enzymes are alpha amylase (group 4), chitinase or chitinase-like (groups 15 and 18), and the newly designated group 20, arginine kinase allergen. Structural proteins can be important allergens, as shown by paramyosin (group 11), and so are molecules that mediate molecular interac-

tion, namely tropomyosin (group 11), EF hand calcium binding protein (group 17), and gelsolin (group 16). There are two lipid transport molecules represented by the group 2 allergens, which are homologues of the Nieman Pick type C-2 (NPC-2) protein and the group 14 allergens, which are apolipophorin or vitellogenin-like proteins. By analogy with its function in other organisms, the NPC-2-like protein is important for the vesicular transport of cholesterol [3], whereas the apolipophorin/vitellogenin has an energy storage and transport function.

Major Allergens

The properties of major allergens, defined loosely for these purposes as the most important specificities from potent allergen sources, should be explored as a special category. Not only do these molecules induce the highest allergic response but also it is likely that this response creates an inflammatory milieu that stimulates responses to molecules presented at the same time. For most common allergen sources, the major specificities induce 50% to 80% of the immunoglobulin E (IgE) antibodies. It could be instructive to compare the properties and immunologic responses induced by the major allergens with those of the less important specificities.

Pollen Allergens

Some of the major pollen allergens have few counterparts from sources outside the pollen of closely related species whereas some are molecules with biochemical homologues found in many species and in diverse tissues [4]. The grass pollen allergens are essentially molecules only found in grass pollen. The group 1 allergens are β -expansins, which function to loosen noncovalent matrix protein interactions. Other plants have related expansins but they are expressed in the fruit and leaves. The other major allergen is the group 5. Their function is unknown, and they are only found in the pollen of species of Pooideae. Even the pollen of Bermuda grass from subfamily Chloridoideae does not produce this type of allergen. Allergens related to the major birch pollen allergen Bet v 1 are, in contrast, widely distributed. They are pathogenesis-related PR-10 proteins that probably function to transport lipid hormones within the plant [5]. Although universally found in plant tissues, PR-10 pollen allergens are restricted to Fagales trees. As a consequence, PR-10 proteins are widely distributed food allergens, but are Fagales-specific

pollen allergens. Disparate species do, however, have homologous pollen allergens as found for pectate lyases. The major Amb a 1 and Amb a 2 allergens of ragweed pollen are pectate lyases. These are found in other weeds, as well as being the group 1 conifer allergens. The possible interaction of homologous allergen presented in food and pollen is of interest, particularly considering that PR-10 allergens [6] and pectate lyase allergens [7] from food sources can stimulate T-cell responses to pollen allergens. The major allergens of Laminales plants, Ole e 1 from olive [8] and the English plantain, Pla l 1, are biochemically homologous to the major allergen of the goosefoot weed, although the function of these proteins is unknown. Olive also has two other major pollen allergens. One Ole 7 is a lipid transfer protein, and this is shared with the two major *Paritaria* weed allergens as well as with Ar t v 3 from the weed mugwort [9]. These lipid transfer proteins are widely found in fruit and are prominent food allergens. The function of the other major olive allergen Ole e 4 is unknown. The major allergen of the mugwort is a defensin-like protein and not a pectate lyase, similar to the related weed ragweed. Mugwort does produce a pectate lyase, but it is only a minor allergen in this species [9].

Mammalian Allergens

The major allergens from mammalian sources of dog, cow, and horse are typically from the lipocalin super family, which are small proteins with a characteristic β barrel and C-terminal alpha helix. These often form dimeric structures as shown for Equ c 1. Curiously, a lipocalin allergen had not been identified for the cat, which is the most common source of mammalian allergens. A cat salivary lipocalin, Fel d 4, has now been described, and it not only has very frequent IgE binding but subjects with low IgE titers of anti-Fel d 1 antibodies often have more anti-Fel d 4 antibody. The lipocalin-like allergens are not restricted to mammals. The Bla g 4 of cockroach is a calycin [10].

The recently solved structure of Fel d 1 [11•] shows the allergen consists of a heterodimer of two chains, which pack together to form a bundle of eight helices. The heterodimers then dimerize to form a tetramer. A key result from the structural study was to establish that Fel d 1 is indeed a uteroglobin. Although the uteroglobin nature of chain 1 had been readily recognized by amino acid sequence similarity, the similarity of chain 2 with uteroglobin was only 13%. The structural studies show that despite the divergent sequence both the chains are from a canonical uteroglobin fold with the same dimerization. Both Fel d 1 and other uteroglobins are steroid-induced, reinforcing the functional similarity. A key feature of the uteroglobins is that they bind small medium lipids in a cavity produced between the polypeptides of the dimer. However, the lipid-binding cavity of Fel d 1 is different from the other uteroglobins (of known structure), suggesting a diversity of function. There are similar molecules in the saliva or in the

pelt of other mammals and, indeed, the androgen-binding protein of the mouse is now known to be very similar to Fel d 1 [12], showing similar conserved uteroglobin residues. The reason that the murine androgen-binding protein is not allergenic could point to specific properties that enhance the allergenicity, or it is possible that mouse allergens have not been sufficiently characterized.

Arthropod Allergens

Cockroaches produce many potent allergens [10]. The function and structure of the group 1 allergens is unknown, but the respective functions of the group 2, 3, 4, 5, 6, and 7 are an aspartate protease-like ligand, the hemocyanin-like arylphorin, calycin, type 2 glutathione-S-transferase, tropomyosin, and tropomyosin. These represent a diverse variation of structure and function. Tropomyosin allergens also appear to be important mite allergens, at least in some regions, and are universally important food allergens, especially for shellfish. The sequences of tropomyosins are very conserved between disparate species so at least some of the reported sensitivities are cross-reactivities or, more interestingly, there may be an immunologic interaction in the induction or regulation of the response. The Bla g 2 allergen is not a classic aspartate protease, but its structure shows that the cleft that forms an active site for such enzymes could have alternative ligand-binding properties.

The major mite allergens are the group 1 cysteine proteases and the group 2 NPC-2-like lipid-transporter proteins [2]. An emerging source of mite allergen is the glycyphagid *Blomia tropicalis*, particularly in tropical regions. Many of the homologous allergens found in the pyroglyphid *Dermatophagoides* mites have been found for *B. tropicalis*. Cysteine proteases similar to the group 1 allergens have recently been identified, and they have shown high allergenicity and little cross-reactivity with the *Dermatophagoides* homologues [13,14]. Consistent with this, and the evolutionary pattern of cysteine proteases, the *Blomia* allergens only have 30% to 35% identity with the cysteine proteases of other mites. The two *Blomia* allergens, however, are not the same, but show only 65% identity. The sequence of Cheong aligns best with cathepsin L proteins, as does Der p 1, and, therefore, they may be from the same family. This raises the issue: Because cysteine proteases constitute a large family of proteins, are there other allergenic cysteine proteases in the pyroglyphid mites? Recently Holt *et al.* [15] found that of 92 out of 3168 expressed sequence tag (EST) clones from *Dermatophagoides pteronyssinus* cyclic DNA (cDNA) were Der p 1 homologues, and all of the clones were minor variants of Der p 1. Thomas *et al.* [2] also demonstrated that there was only one gene for Der p 1-like proteins. Although, as discussed later, the cysteine proteases appear to have some inherent property that promotes allergenicity, group 1 homologues have not been described for other mites, such as *Lepidoglyphus destructor* and *Tyrophagus putrescentiae*. The

data of Holt *et al.* [15] also demonstrated that 2.9% of the messenger RNA transcripts from *D. pteronyssinus* were for Der p 1, indicative of an extremely high abundance of this protein. The group 2 allergens have clearly been established as major allergens in the pyroglyphid mites and in *Lepidoglyphus*, *Glycyphagus*, and *Tyrophagus*, but curiously have remained unidentified in *B. tropicalis*.

Non-allergenic Responses

In contrast with the many studies on allergens, there is little information on proteins that are inhaled but are not allergenic. It is therefore difficult to compare allergenic and nonallergenic responses. The immunologic responses of people who do not become allergic also differ for different types of allergen. For the most part, people who do not become allergic to house dust mites do not make immunoglobulin G (IgG) antibody to mite allergens [16]. Only allergic subjects make IgG, of both IgG4 and other IgG subclasses. However, at least one report has been made to the contrary. Tame *et al.* [17] described IgG antibody to Der p 2 in sera from nonallergic subjects. The study used a recombinant Der p 2 with the maltose-binding protein of *Escherichia coli* and made no mention of absorbing out or allowing for the reactivity to the fusion partner. In an unpublished study by the authors, similar techniques were used to find that not only were the IgG antibodies only found in the sera of allergic subjects but that they were directed to Der p 1 and Der p 2, and not to the less allergenic proteins Der p 3, 4, 5, 7, 8, and 10. Similarly, Western blotting studies with mite extract have only revealed occasional IgG antibodies from nonallergic sera reacting with high molecular weight bands [18]. Thus, for mites, there is an association of allergenicity and immunogenicity, both with respect to allergic status and the relative allergenicity of the proteins. Platts-Mills *et al.* [19] have reported similar data for the ragweed allergen Amb a 1. Cats, on the other hand, induce IgG anti-Fel d 1 antibody in both allergic and nonallergic subjects, with higher IgG titers being associated with protection from allergy and perhaps higher exposure to cats [20]. Nonallergic subjects also produce IgG antibodies to birch pollen but mainly to the minor allergens and, to a lesser extent, the Bet v 1 allergen [21]. It thus appears that for mites and ragweed antigenicity and allergenicity are closely linked, but this is not always the case as described for cat.

A study by Epton *et al.* [22] undertook to search for nonallergenic antigen of the house dust mite and to examine the immune response to the antigen. A cDNA clone encoding the highly antigenic ferritin heavy chain was isolated by screening with a rabbit antiserum. It did not bind IgE antibody, and, when used for in vitro T-cell stimulation assays of human peripheral blood mononuclear cells, induced a mixture of Th1 and Th2 cytokines in a pattern that was the same for cells from allergic or nonallergic subjects. Interestingly, when compared with the

response to ferritin, the Th2 response induced by the major Der p 2 allergen was not only significantly higher in allergic subjects, but was also significantly lower in nonallergic subjects. The cytokine responses to the allergen were thus independently regulated above and below the response to the nonallergen. The response of nonallergic subjects to the major allergen, but not to the nonallergen, was apparently actively regulated. The IgG titers to ferritin were similar in allergic and nonallergic subjects, but they were not quantitated, and were probably very low. Ferritin forms a very large, 24-mer particle, which may have a very different presentation than the considerably smaller allergens.

Proteolytic Activity

The ability of proteolytic activity to enhance allergenicity was proposed for the Der p 1 cysteine protease of house dust mite. Mite allergens also include the three serine proteases—trypsin, chymotrypsin, and a collagenolytic serine protease. There has been some uncertainty about the substrate specificity of Der p 1 and whether or not it has an additional serine protease activity. A recent analysis with recombinant and highly purified proteins shows excellent evidence that the effects can be due to trace contaminants [23]. This may have influenced some experiments, but it is unlikely to affect the conclusions using the standard E64 cysteine protease inhibitor. Der p 1 has been shown to cleave immunologically important receptors such as CD23 and CD25 and could also affect epithelial integrity. As shown for the serine proteases, it could also cleave complement to anaphylatoxins [24]. The E64 inhibitor has been shown to reduce the ability of Der p 1 to induce IgE antibody and eosinophilic responses in mice, showing in vivo activity [25,26]. A Th2 adjuvant activity has been demonstrated by showing the ability of the allergen to enhance the responses of mice to co-administered antigens [25]. The experiments of Gough *et al.* [25], which use alum immunization, are hard to interpret because alum promotes very high IgE responses to nonproteolytic antigens. The experiments studying the ability of nonadjuvant Der p 1 to induce pulmonary eosinophilia were more convincing. Other cysteine proteases, such as papain [27] and *Leishmania mexicana* CPB2.8 protease [28], induce potent Th2 responses, and papain is a potent occupational allergen. However, the proteolytic activity is not an overriding factor, because the major allergen for house dust mite allergic dogs is chitinase, although dogs do produce IgE to Der p 1 [29]. Serine proteases have been shown to activate protease-activated receptors [30] and to cleave complement components [24], but the ability of these enzymes to induce sensitization in vivo has not been examined.

Lipid Binding

Lipid binding is a very common characteristic of the major allergens. Lipids have traditionally been thought of

as adjuvants for Th1 responses, such as delayed hypersensitivity, and, therefore, it is a curious common thread to find for allergens. The Th1-stimulating activity of lipids can be attributed to the activation of immune responses by natural killer target (NKT) cells and CD1 presentation as found for parasites [31], or by the activation of CD4 T-cell responses via the Toll-like receptor (TLR)-4 pathway that traditionally activates antigen-presenting cells (APCs) to produce the Th1 cytokine interleukin (IL)-12 [32]. The effect of TLR4 signaling must be considered in the context of the presence of other signals, such as from thymic stromal lymphopoietin (TSLP) produced from the mucosal epithelium, the activation state of the T-cells, and dose-response relationships. It has convincingly been shown that lipopolysaccharide and TLR4 are required to induce Th2-type hypersensitivity in mice. Indeed, the same MyD88 signal (from TLR4) that signals for dendritic cells to induce Th1 responses has now been shown to initiate the signaling pathway for pulmonary dendritic cells to induce Th2 responses [33]. The phosphorylcholine-glycan ES-62 product of nematodes induces Th2 responses by activating cells in a TLR4-dependent and MyD88-dependent pathway [34], probably via the phosphorylcholine group. Another mechanism by which lipid can enhance T-cell responses is to direct antigen into endosomal vesicles for processing and presentation. Antigen encased in large lipid vesicles (560 nm) is directed to the early endosomes, where it undergoes very efficient processing and presentation [35]. Antigen presented in smaller lipid vesicles (155 nm) is directed to the late endosomal vesicles, where the processing is not as efficient. Brewer has previously shown that the smaller vesicles also induce Th2 responses, perhaps related to the efficiency of presentation. The particle sizes in these experiments are much smaller than the size of inhaled particles that lodge in the mucosa, but could be presented as lipid complexes released after inhalation.

Pathogen-associated Molecular Patterns

Pathogen-associated molecular patterns (PAMPs) are the molecular signatures recognized by the innate immune system. They include the secreted proteins of the complement systems, and the collectins, such as surfactants, as well as cell receptors. The cellular receptors include scavenger and mannose receptors and TLRs. TLRs are a family of molecularly homologous ligands that initiate common and interacting signaling pathways. As discussed earlier, interactions with TLRs activate APCs to deliver instructive signals to T cells during antigen presentation. TLR1 binds lipopeptides, TLR2 peptidoglycans, TLR3 double-stranded RNA, TLR4 lipid, TLR5 flagellin, TLR6 zymosan, TLR 7 and 8 single-stranded RNA, and TLR9 immunostimulatory oligonucleotides, and TLR11 is specific for unknown determinants produced by uropathogenic bacteria. The ligand for TLR10 is unknown, although, interestingly, a polymor-

phism of the gene has been associated with susceptibility to asthma [36]. Similarly, a polymorphism of TLR6 has been associated with the protection from allergy [37]. The most obvious interaction of TLR signaling with allergy would, from the standpoint of our current knowledge, be via interactions with microorganisms that influence the activation of dendritic cells. Stimulation of the TLR2 receptors with peptidoglycan bacterial products in the presence of allergen, for example, enhances Th2 responses [38,39], and, as discussed earlier, the engagement of TLR4 by LPS enhances Th2 responses to ovalbumin [33].

Although the activation of TLR by microorganisms is likely to be important in allergic disease, the direct interaction of allergens and TLR remains to be investigated. The many lipid-binding allergens would be candidates to consider. The stimulation of TLR is also not limited to nonproteinaceous molecules. TLR2 is activated by fimbrial proteins and a derivative synthetic peptide ALTTE [40]. It is also activated by the polypeptide of the major porin of *Haemophilus influenzae* [41] and by a peptide from the C-terminal of exoenzyme S from *Pseudomonas aeruginosa* [42]. The exoenzyme S can also signal through TLR4, but with a sequence from the N terminal. TLR5 is classically stimulated by peptides of flagellin, which induce MD-88-dependent Th2-type responses [43]. There is, therefore, considerable scope for allergens to stimulate Th2 responses through the interaction of PAMPs.

Immunologic Subversion

To end on a speculative note, allergens could stimulate immune responses by a process of molecular subversion. This is well established in viral infections, whereby viruses acquire genes that code for immunoregulatory molecules, such as cytokines, and use them to suppress protective immunity. Similarly, allergens may be selected for potency by an innate ability to interact with immune or inflammatory responses. Recent studies have identified two candidates. The major cat allergen Fel d 1 is now known to be a bona fide uteroglobin. Uteroglobins have potent pro-inflammatory and anti-inflammatory effects, both mediated by its ability to block the action of prostaglandin D2 and its D prostanoid (DP) receptor [44]. The anti-inflammatory effect has been observed in the knock-out mice that show decreased lung inflammation. The effect of PGD2 and DP-receptor signaling, however, depends on the stage of the allergic response. At the initiation of sensitization, the DP signaling is, in fact, inhibitory for the migration of dendritic cells from the lung to the lymph node [45] and hence blocks the presentation of allergen. Recent information also raises the possibility that the group 2 house dust mite allergens have a subversive function. Signaling of the TLR4 by LPS is entirely dependent on the binding of the LPS to the myeloid differentiation protein MD-2 protein and then the docking of this molecule to TLR4. CD14 can assist the loading of MD-2; soluble MD-2 complexed with

LPS directly stimulates TLR4, and the response of the respiratory epithelium to bacterial products is limited by the amount of MD-2 [46]. The closest structural homologues of MD-2 are the group 2 house dust mite allergens, and they appear to retain some critical residues for binding to LPS and the TLR receptor [47]. There are, however, important differences, especially in the size of the two small cysteine-constrained loops that are characteristic of this family. Signaling, if it does occur, may therefore be different from MD-2. Experiments to determine the possible interaction of these allergens with human receptors are needed. Although these concepts remain to be investigated, the increasing knowledge of the structures of immunologic receptors and allergens points to the possibility that the biological activity of highly allergenic proteins could augment their allergenicity.

Conclusions

Knowledge of the structure of the major allergens and an understanding of the reason for their potency could be important for the development of allergen immunotherapy. The dominance of a few potent specificities in the most common sources suggests that their structural or functional properties may be important. Although the issue is yet to be resolved, and the properties of allergens from different sources are frequently diverse, recent knowledge of allergen structure and function points to some intriguing but varying possibilities.

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Describes the structure of the TLR4 co-receptor modeled on the group 2 house dust mite allergen.