

Review article

Intrinsic properties of allergens and environmental exposure as determinants of allergenicity*

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Accepted for publication 25 March 2002

Allergens are some of the best studied proteins related to human disease, and yet, little is known about why these proteins are associated with the production of specific IgE antibodies in susceptible individuals, and the mechanisms involved in causing sensitization. Most IgE responses are elicited by environmental exposure to inhaled allergens. Chronic exposure to low levels of indoor allergens (1–10 µg/year) leads to specific IgE antibody production and is linked to the development of allergic asthma. Cockroach allergen exposure and sensitization, followed by dust mite and cat allergens, are strongly associated with asthma morbidity among inner-city children with asthma (1). In the past 5 years, several indoor allergens from cockroach, cat and dog were selected to study their molecular structure and function, by molecular cloning and/or molecular modeling based on the known three-dimensional structure of homologous proteins. Several hypotheses for allergenicity involving intrinsic properties of the allergen have been developed from these studies. These properties include integrity of the allergenic protein, resistance to degradation, binding capacity, proteolytic function and mimicry of the allergen with endogenous proteins. Key determinants for allergenicity involve dose and route of environmental exposure and genetic predisposition of the individual towards developing a Th2 response to specific allergens.

Structural and functional diversity of allergens

Given the importance of cockroach allergens in the development of allergic disease, the structure of several cockroach allergens was studied, including *Bla g 1*, the homologous American cockroach allergen *Per a 1*, and *Bla g 2*. The German cockroach (*Blattella germanica*) is the most common in the U.S.A., and the prevalence of IgE antibodies to *Bla g 1* and *Bla g 2* is high (30–50% and 60%, respectively). *Bla g 2* is a very potent allergen, inducing IgE antibody responses at very low doses of exposure (0.33 µg/g) (2). Another cockroach allergen, *Bla g 4*, belongs to the same structural family of proteins, the lipocalins, as the dog allergens *Can f 1* and *Can f 2*. Structural studies of *Can f 1* and *Can f 2* revealed common features with the cat cystatin *Fel d 3*, whose tertiary structure was modelled on cysteine proteinase inhibitors.

Relationship between integrity of the protein and allergenicity

The molecular cloning of the Group 1 cockroach allergens (*Bla g 1* and *Per a 1*) revealed that these allergens are constituted by a novel structure containing several tandem 100 amino acid repeats (3–6). This structure was visualized as parallel lines to a diagonal in a dotplot matrix analysis (3). Interestingly, several *Bla g 1* molecular forms expressed in *Escherichia coli* and *Pichia pastoris* contained different number of repeats and bound IgE as the natural allergen, indicating the existence of repeated IgE binding units in the molecule. Protein sequencing demonstrated that the natural allergen is also constituted by several fragments starting after trypsin cleavage sites, very similar to the molecular

*This review is based on the application that was awarded the Pharmacia Allergy Research Foundation Award for 2002. The award was presented at the American Academy of Allergy Asthma and Immunology, New York, March 2002.

forms obtained in the recombinant allergen. This suggested that *Bla g 1* could be trypsin cleaved in the cockroach digestive tract where the allergen is produced, in which case, once excreted to the environment, the different *Bla g 1* molecular forms would be inhaled by atopic individuals during the process of sensitization. Therefore, in the case of the Group 1 cockroach allergens, digested fragments of the protein may be sufficient to produce sensitization.

The opposite argument can be applied to explain allergenicity of other proteins, especially food allergens. When the structure of the allergen is very resistant to degradation, conformational epitopes would persist in the body and stimulate the immune system for prolonged periods. For example, β -lactoglobulin is able to resist acidity and remain intact after passing through the stomach (7). Similarly, the peanut allergen *Ara h 1* has a trimeric structure that may protect some epitopes from degradation by gastrointestinal enzymes and low pH (8). Therefore, the relationship between integrity of a protein and allergenicity depends on the protein structure and the route of exposure to the protein.

The cockroach allergen *Bla g 2* is an inactive aspartic proteinase

The potent cockroach allergen *Bla g 2* was modelled using the X-ray crystal structures of the homologous aspartic proteinases porcine pepsin and bovine chymosin ($\sim 26\%$ identity with *Bla g 2*) (9,10). The *Bla g 2* model has a bilobal structure typical of aspartic proteinases (Fig. 1A). Aspartic proteinases are widely distributed enzymes whose proteolytic mechanism involves the participation of two coplanar aspartic acid residues in the catalytic triads interacting with a water molecule (11). The catalytic site of these enzymes is located in the bottom of the binding pocket or cleft, and is formed by two loops, each containing the triad aspartate-threonine-glycine (DTG) (Fig. 1).

The possibility that allergenicity of *Bla g 2* was promoted by its putative proteolytic activity was investigated. Several features of the *Bla g 2* binding pockets differ from the active site of catalytically active aspartic proteinases and suggested that *Bla g 2* is an inactive aspartic proteinase. Firstly, the catalytic triads that are absolutely conserved in all active aspartic proteinases have important amino acid substitutions in *Bla g 2*: residues DTG 32–34 and DTG 215–217 (using pepsin numbering) are substituted by DST and DTS, respectively (Fig. 1B). Secondly, the coplanar aspartates are inaccessible to water because the bulkier residue threonine 34 interferes with aspartate 215 (as shown in Fig. 1B). Finally, residue tyrosine 75, which is a highly conserved residue among active aspartic proteinases, is substituted by phenylalanine in *Bla g 2*. Tyrosine 75 is in the ‘flap’ region that comprises the residues 72–81 and forms a β -hairpin that partially

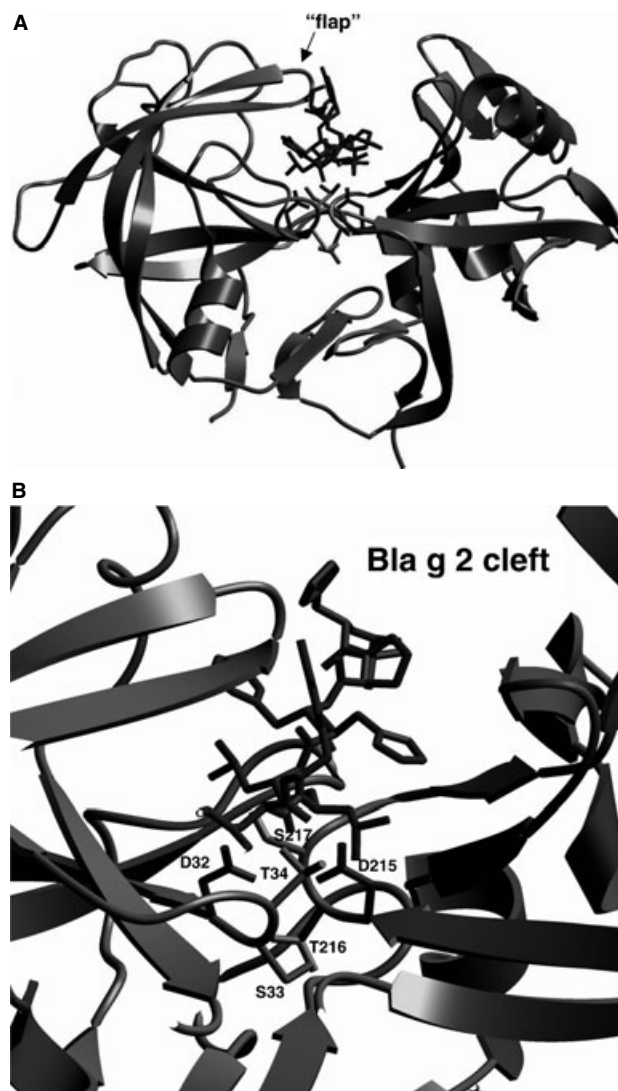


Figure 1. *Bla g 2* molecular model and area corresponding to the catalytic site in aspartic proteinases. The substrate is a renin inhibitor (CH-66) modelled into the *Bla g 2* cleft.

covers the active site cleft (Fig. 1A). This critical substitution has been reported to impair enzymatic activity. The lack of enzymatic activity was confirmed by milk clotting assay (9).

The specificity pocket of *Bla g 2* was also analysed by comparative studies using peptidomimetic inhibitors, and showed that the binding cleft is able to accommodate a wide range of peptides, suggesting that *Bla g 2* could be a binding protein (9). Surprisingly, *Bla g 2* is related to a family of mammalian glycoproteins called pregnancy associated glycoproteins (PAGs) that are thought to be binding proteins. PAGs are secreted by the outer layer (chorion) of the placentas of various ungulate species (12). They belong to the aspartic proteinases protein family, but most of them have critical amino acid substitutions similar to *Bla g 2*, that make them catalytically inactive (13).

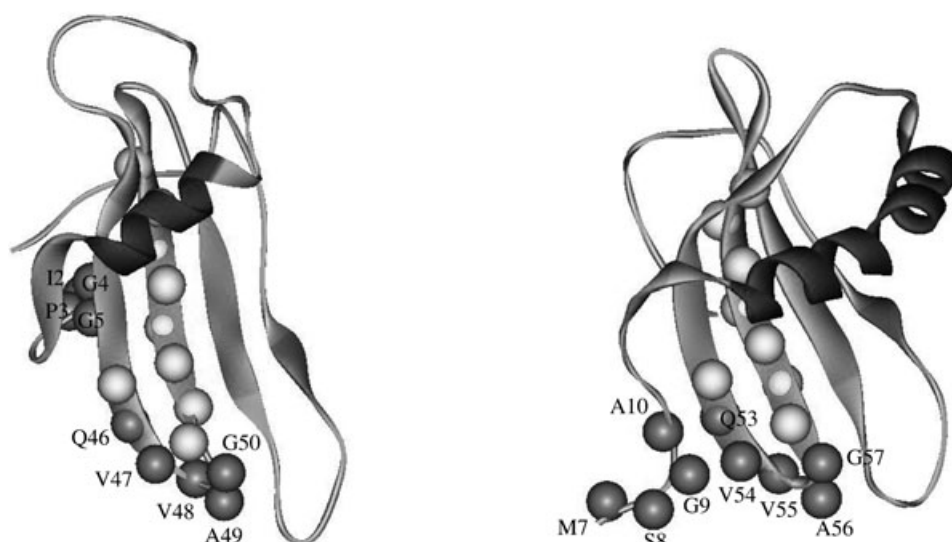


Figure 2. Cat cystatin (left) modeled on the crystal structure of three human cystatins including human stefin B (or cystatin B, right). Ribbon representation of the molecules showing the short α -helix, five anti-parallel β sheets and the hairpin loops. Spheres indicate the cysteine protease inhibitor motif in the first hairpin loop. Darker spheres (labelled with amino acid residue numbers) represent amino acids that are directly involved in binding to the cysteine protease.

Most known mammalian allergens are ligand binding proteins

The dog allergens *Can f*1, *Can f*2 and the recently cloned cat allergen *Fel d*3 were studied by molecular modeling (Fig. 2) (14,15). *Can f*1 and *Can f*2 belong to a family of animal allergens called lipocalins (14,16), including other major and minor allergens from different species: cockroach (*Bla g* 4), mouse (*Mus m* 1), rat (*Rat n* 1), cow (*Bos d* 2, *Bos d* 5 which is β -lactoglobulin) and horse (*Equ c* 1 and *Equ c* 2). The crystal structures of *Mus m* 1, *Rat n* 1, *Bos d* 2, β -lactoglobulin and *Equ c* 1 have been solved and, despite an amino acid homology as low as 20%, they show the same characteristic lipocalin folding: an α -helix and a single eight-stranded (10-stranded for β -lactoglobulin) antiparallel β -barrel enclosing an internal cavity (17-21).

Interestingly, most of the known mammalian allergens are ligand-binding proteins (lipocalins or calycins) - except for albumins and the cat allergen *Fel d* 1 - and they are secreted by hair/dander and saliva. A transport function has been attributed to lipocalins because of the capacity of their hydrophobic pocket to bind small hydrophobic ligands such as pheromones, steroids, retinoids and arachidonic acid (22). For example, the rodent urinary proteins *Mus m* 1 and *Rat n* 1 bind male pheromones, and β -lactoglobulin from cow's milk, associated with food hypersensitivity, binds palmitate and retinol within its central cavity (17,20,23).

Can f 1 is produced and secreted by the tongue epithelial tissue and shows a high degree of homology (57% identity) to the human von Ebner's gland (VEG) salivary lipocalin which is a cysteine protease inhibitor (16,24). Similarly, the cystatin cat allergen, *Fel d* 3, contains a cysteine protease inhibitor motif (Fig. 2) (15). The *Fel d* 3 structure was modelled on the X-ray crystal

structures of recombinant human cystatin A, human stefin A and human chain I of stefin B (15). The cat cystatin molecule includes a two and half turn α -helix close to the N-terminus, a five-stranded antiparallel β -pleated sheet interconnected by a hairpin loop and an additional carboxy terminal strand (Fig. 2). Both, *Can f* 1 and *Fel d* 3 could be functional cysteine protease inhibitors. These findings raise the possibility that these animal allergens could inhibit cysteine proteases such as *Der p* 1.

Hypotheses for allergenicity

Intrinsic properties of the allergen: structure and biological function

The broad spectrum of allergenic proteins, encompassing a wide variety of structures and functions, makes it unlikely that the overall structure alone is responsible for IgE antibody responses (25). An increasing number of studies support the idea of a contribution of enzymatic activity on allergenicity, and may explain why some allergens, such as the cysteine and serine protease allergens from house dust mite (*Der p* 1, *Der p* 3 and *Der p* 9) and phospholipase A from bee venom, are particularly potent (26-37). *Der p* 1 may directly promote IgE synthesis through cleavage of CD23 (the low affinity IgE receptor) on B cells and, indirectly, through cleavage of CD25 (the α -subunit of the IL-2 receptor) on T cells (27-31). *Der p* 1 significantly enhances IgE production in mice compared with enzymatically inactive allergen (32). Mite proteolytic allergens increase permeability in the bronchial epithelium and *Der p* 1 has been proven to disrupt tight junctions (33-35). Enzymatically active allergens, such

as mite proteases or bee venom phospholipase A₂, also cause release of pro-inflammatory cytokines from bronchial epithelial cells, mast cells and basophils (26,34,36,37). These lines of evidence suggest that having enzyme activity enhances allergenicity: the 'enzyme hypothesis' (30). The studies support this hypothesis by proposing interesting mechanisms whereby proteolytically active allergens could potentiate allergenicity, and may explain why mite allergens are so strongly associated with the development of allergic responses. However, a good number of allergens provide exceptions to the 'enzyme hypothesis'. *Bla g 2*, for example, is an inactive aspartic proteinase, yet is a potent allergen, inducing IgE responses at exposure levels that are often 1–2 orders of magnitude lower than for *Der p 1* (2). None of the other known cockroach allergens (*Bla g 4*, *Bla g 5*, *Bla g 6*, *Per a 7*) are proteolytic enzymes, and they have diverse biological functions. Mammalian allergens (*Can f 1*, *Can f 2*, *Rat n 1*, *Mus m 1*, *Bos d 2*, *Equ c 1*) are lipocalins and function as transporter proteins of small molecules that they bind (14). Cat cystatin and *Can f 1* may be cysteine protease inhibitors. *Der p 2* is homologous to moth molting protein and human epididymal protein, with no known enzymatic function, and the tertiary structure is an immunoglobulin fold (38,39). *Der p 2* is a potent allergen, causing sensitization in > 90% of mite allergic patients at exposure levels that are usually 2–10 fold lower than for *Der p 1*. This evidence confirms that enzymatic activity of the allergen is not a prerequisite for allergenicity. Allergens have diverse biological functions including enzymes, structural proteins, storage proteins, binding proteins and enzyme inhibitors (14).

Although allergens do not require to be enzymatically active in order to elicit IgE responses, the possibility that enzymes inhaled into the respiratory tract contribute to inflammation cannot be discarded. For example, some enzymes including *Der p 1* may contribute to inflammation by inactivating the $\alpha 1$ -proteinase inhibitor which is the major natural inhibitor of neutrophil enzymes (40). This could occur if allergens and enzymes are carried simultaneously by particles inhaled into the lung. In some proteins such as *Der p 1*, both characteristics may coexist.

Other hypotheses for allergenicity have been proposed suggesting that intrinsic properties of allergens modulate the immune response and stimulate IgE production. As mentioned earlier, resistance to degradation may be important for sensitization to some food allergens such as β -lactoglobulin and *Ara h 1* (7,8). In this sense, food allergens tend to be heat stable and pH resistant proteins. Interestingly, there could be a link between the allergenicity of some proteins and their binding function, such as the small hydrophobic ligand transport function of lipocalins, or the putative binding function of *Bla g 2*, *Can f 1* and *Fel d 3*.

Molecular mimicry of the allergens with endogenous proteins

Another hypothesis for allergenicity has been proposed, based on molecular mimicry between allergens and endogenous proteins, and involving mechanisms of self-tolerance. This hypothesis was initially proposed for lipocalins, because some endogenous lipocalins, such as retinol binding protein (RBP), apolipoprotein D (APD) and von Ebner's gland protein (VEG), are homologous to exogenous lipocalin allergens (41,42). Experimental support for this hypothesis is based on the fact that one of the main T-cell epitopes of *Bos d 2* overlapped with the conserved regions of lipocalins (43). However, it is noticeable that at the level of IgE epitopes, several allergens differ from homologous endogenous proteins. This is the case for the lipocalin allergen *Equ c 1*, albumins and tropomyosins (14,21). Albumins represent the most conserved family of cross-reactive mammalian allergens. Cat and dog albumins are cross-reacting allergens that share a high degree of homology. The lack of reactivity to self-human albumin, which shows 82.6% homology with dog albumin, may indicate that the IgE binding epitopes of the allergen are in nonhomologous areas of the molecules (44). The same argument can be applied to the invertebrate tropomyosin allergens that are structural proteins from animals such as shrimp, crab, lobster, snails, mite and cockroach allergens, explaining the lack of sensitization to human endogenous and other vertebrate meat tropomyosins.

Effect of allergen dose and route of environmental exposure on allergenicity

The key factors that affect production of IgE antibodies appear to be route and dose of allergen exposure and host immune response genes, all of which preferentially stimulate Th2 responses. The common feature of all the inhaled allergens is that they are secreted into the environment. Some of them are especially ubiquitous, such as *Fel d 1* which can induce sensitization even in the absence of cats at home. Under natural conditions, repeated exposure to low doses of allergen (1–10 μ g/year) without adjuvant on airborne particles of 1–40 μ m diameter is required for sensitization in genetically predisposed individuals. These conditions have been reproduced by administration of subclinical doses of allergen to atopic subjects, showing a shift to allergen-specific Th2 response *in vitro* (45). An opposite response, with IgE antibody reduction is achieved during immunotherapy by administration of increasing doses of allergen, in order to desensitize the allergic individual (46). Recent studies emphasize the critical effect of allergen dose by showing that the prevalence of allergen specific sensitization in atopic children is associated with the highest domestic concentration of *Bla g 2* (median levels of 0.33 μ g/g; range > 0.08–15 μ g/g) and Group 1

mite allergens (38 µg/g; range 24–150 µg/g), but not with *Fel d 1* (2). Low dose exposure to *Fel d 1* (0.5–5 µg/g) posed the strongest risk for sensitization, whereas high dose exposure to *Fel d 1* (> 20 µg/g up to 3840 µg/g) results in reduced prevalence of IgE antibody responses to *Fel d 1*, and is associated with increased specific IgG and IgG4 antibody levels, similarly to what happens during immunotherapy by administration of increasing doses of allergen (2,47). The results found for *Fel d 1* have recently been confirmed in a study of 2502 adults, indicating that the prevalence of sensitization to cat is decreased in the lowest and highest cat allergen exposure groups (48). *Bla g 2* and mite allergen environmental levels routinely fall in a window that represents the highest risk for sensitization (2). The possibility exists that ‘bell-shaped’ curves similar to the one described for *Fel d 1* could be seen for *Bla g 2* and Group 1 mite allergens if levels of exposure (not yet seen in a natural setting) reached the levels measured for *Fel d 1*, and the allergens could reach the lung as *Fel d 1* does. Exposure to low levels of *Bla g 1* and *Bla g 2* has been associated with wheezing among infants in the first three months of life and with increased proliferative T cell responses (49). Genetic factors are also involved in allergenicity, and recognition of low doses of allergens may be associated with certain *HLA DR* genes. For example, association of the *HLA-DRB1*0101* allele in the Hutterite population and the *HLA-DRB1*0102* allele in African Americans with sensitization to cockroach allergens has been reported (50).

Future directions

Once the tertiary structure of allergens such as *Bla g 2* is known, site-directed mutagenesis studies will allow the determination of crucial amino acids for IgE antibody binding on the surface of the molecule, as well as substitution of key cysteine residues for the correct folding of the molecule. The administration of modified allergens or peptides with low IgE binding capacity, could potentially avoid the side-effects of conventional immunotherapy (IT) by inducing specific IgG4 instead of IgE production (46).

Another approach to be considered for IT in the future is the use of recombinant allergens and adjuvant(s) to down-regulate Th2 responses. The adjuvants

that are now under study are the immunostimulatory sequences CpG nucleotides, which induce a proinflammatory Th1 response to the mammalian immune system (51). CpG sequences are added to allergen DNA and administered during IT in animal models and clinical trials. Other approaches to IT are the use of other adjuvants including allergens coupled to IL-12, IL-18, or lectins, and peptide-based therapy (52,53).

Concluding remarks

A combination of structural and functional studies shows that allergens have diverse biological functions and they include enzymes, structural proteins, binding proteins and enzyme inhibitors. Some intrinsic allergen characteristics, such as resistance to degradation, may be important to elicit IgE antibody responses, specially in the case of food allergens. Regarding allergen function, the strong capacity of *Bla g 2* to stimulate IgE production following low dose environmental exposure is unrelated to aspartic proteinase activity. Although proteolytic function may modulate allergenicity in some cases, including mite *Der p 1* or bee venom phospholipase A2, allergen enzymatic activity is not a prerequisite for allergenicity. Other factors such as dose and route of allergen exposure and genetic predisposition are important determinants for allergenicity. Knowledge of the molecular structure and function of allergens such as *Bla g 2* will provide a better understanding of the immune response to the allergen and should allow rational immuno-therapeutic strategies for treatment of allergy to be developed (54).

Acknowledgments

I would especially like to thank Dr Martin Chapman for his continuing support and all of the wonderful opportunities over the last five years. I owe a great deal to Lisa Vailes, Dr Alisa Smith and Dr Karla Arruda for everything they have taught me. Thanks to Dr Tom Platts-Mills and members of the Division of Allergy at the University of Virginia for providing a dynamic and stimulating environment for this work. I am also very grateful to my collaborators in structural biology at Cambridge University, U.K., Dr Tom Blundell and particularly the late Dr Venugopal Dhanaraj who will be sadly missed.

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