

Can knowledge of the molecular structure of allergens improve immunotherapy?

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Conventional immunotherapy may be associated with the development of adverse reactions, including anaphylaxis, due to the use of increasing doses of allergen. Standardization of extracts is necessary in order to assess the correct amount of allergen administered. In recent years, increased knowledge on the molecular structure of allergens has allowed the development of novel alternatives for immunotherapy. Initially, allergens were cloned and expressed as recombinant proteins in eukaryotic and prokaryotic systems. Crystallization of the purified proteins led to the elucidation of the tertiary structure of the allergen. Molecular biology techniques were used to construct modified allergens whose new IgE binding properties were studied. IgE antibody mapping combined with molecular modeling has allowed the recognition of IgE binding sites on the surface of the molecule. This information has been applied to the engineering of new modified allergens, with and without adjuvants, that retain immunogenicity but with reduced allergenicity. The use of these molecules for immunotherapy should allow the administration of greater doses of allergen, without the undesired side effects characteristic of conventional immunotherapy. *Curr Opin Allergy Clin Immunol* 1:549–554. © 2001 Lippincott

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Abbreviation

TCR T-cell receptor

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Introduction

Allergens are proteins that induce synthesis of specific IgE antibodies and this may result in the development of allergic diseases. Knowledge of the structure of the allergen is essential to understand the molecular mechanisms of IgE responses and to improve immunotherapy. Although specific allergen immunotherapy has been practiced for nearly 100 years, the immunologic mechanisms remain poorly understood. Immunotherapy involves administration of increasing doses of an allergen to which the patient is specifically allergic. Immunotherapy has proven to be successful for treatment of allergic rhinitis, allergic asthma, and insect sting (venom) sensitivity [1•]. Immunologic changes associated with successful immunotherapy include an increase in serum levels of allergen-specific IgG, suppression of specific IgE, and diminished T-cell activity (reviewed in [1•]). While pharmacological treatment can ameliorate the early and late phase symptoms of allergic diseases, immunotherapy is the only treatment that focuses on the etiology of the disease.

Several problems associated with immunotherapy can be improved by applying the knowledge derived from structural studies of allergens. First, natural allergenic products used for immunotherapy are a mixture of allergenic as well as non-allergenic proteins, and standardization is necessary for administration of optimal doses required for immunotherapy [2]. Substitution of these products by a cocktail of recombinant allergens and custom administration of the specific allergen(s) would be a more precise mode of treatment. Second, adverse reactions, including anaphylaxis, may be triggered during immunotherapy by administration of increasing doses of allergen. Currently, immunotherapy is recommended only to patients lacking risk factors for both fatal and non-fatal reactions such as increasing age (>50 years), decreased lung function (<70%) and severity of asthma [1•,3]. The use of modified allergens, or peptides derived from allergens that do not bind or bind weakly IgE, could reduce adverse effects and improve therapy because they would not be able to cross-link IgE binding receptors in mast cells. These modified allergens would be expected to interact poorly with IgE antibodies, but retain the T-cell epitopes capable of inducing anergy or tolerance. Molecular biology techniques allow allergens to be engineered with such characteristics. Structural modifications that affect the overall three-dimensional structure of the allergen or specific IgE binding epitopes are preferred

for immunotherapy. Initially, chemically modified allergens were used (reviewed in [4]). With the introduction of molecular biology techniques, and a better knowledge of the three-dimensional structure of allergens, specific modifications are introduced to change the overall structure or the IgE binding epitopes of the allergen. The site-specific changes are defined and can be validated for immunotherapeutic use. Over the period covered by this literature review, several allergens have been cloned, characterized and expressed *in vitro* in prokaryotic and eukaryotic systems for this purpose.

Elucidating the primary structure of allergens by molecular cloning

The most common technique for cloning allergens involves screening of cDNA libraries. Sera from allergic patients containing specific IgE, or specific monoclonal or polyclonal antibodies, are used for screening expression libraries [5]. In other cases, the cDNA library is screened with primers whose design is based on the known N-terminal and/or C-terminal sequence of the allergen [6•,7,8]. Degenerate oligonucleotides derived from sequencing of tryptic peptides or from the known N-terminal amino acid sequence of homologous proteins may be used [9•,10,11], as well as primers designed from conserved regions of homologous proteins [12]. Recently, phage display technology has been described and used to identify three new allergens from the dust mite *Lepidoglyphus destructor* [13,14]. Cloning of certain allergens may be missed by this technique: (a) if the proteins are expressed and displayed on the phage surface, but they are not correctly folded; or (b) if the allergens are secretion proteins with secretion signal sequences that act as a stop transfer signal, preventing the display of the protein on the phage surface; or (c) if the protein has more than one polypeptide chain [14]. If a full clone is not obtained after screening the library, the process is followed by the use of the RACE system to obtain the 5' or 3' portions of the cDNA [6•,7,11].

Knowledge of the primary structure of allergens allows expression of recombinant proteins and the engineering of proteins for diagnosis and therapy [15•]. Genetic modification is also being used to suppress or eliminate allergens from foods. The strategy assumes that the allergen(s) are not essential for plant growth or function (which in many cases may be unlikely). An example is the recent cloning of a 33 kDa allergen from rice, a staple food source, which the authors speculate could be deleted to produce rice variants lacking the allergen [9•]. Similar research is being carried out on other important allergens, including peanut.

Structural basis for allergenic cross-reactivity

Homology and structural studies of cloned allergens lead to a better understanding of allergenic cross-reactivity.

The presence of homologous epitopes not only explains cross-reactivity between aero-allergens, but also between aero-allergens and food proteins like the birch pollen profilin, Bet v 2, and celery profilin, or Bet v 1 and the major cherry allergen Pru av 1 [5,10,11,16]. Peptides containing cross-reactive epitopes common to different allergens could be used for IT in such cases. For example, in cedar pollinosis, allergens Jun a 2, Cry j 2 and Cha o 2 share a high degree of sequence identity [7].

Proteins with >30% sequence homology can have similar secondary and tertiary structures, but most of the sequence variations are located on their surfaces where the B-cell epitopes are located [17]. Cross-reactivity among allergens is due to common epitopes located on the surface of the allergen. Cross-reactivity is rare below 50% amino acid identity, and in most cases requires 70% identity [18•]. Recently, several interesting examples of cross-reactive allergens have been described, including the Group 2 mite allergens, fungal enolase and latex allergens [19•,20•,21,22].

Another kind of IgE cross-reactivity exists between glycoprotein allergens with unrelated protein structures, but with shared N-glycans that provide the similarity in structure [18•]. The recently cloned Cup a 1 has a pectate lyase active site that may explain cross-reactivity among different families of plant allergens [10]. The cross-reactivity is mostly due to carbohydrate moieties rather than to shared protein epitopes. Similarly, the structural basis for IgE recognition of plant N-glycans from two major pollen allergens and a major food allergen has been reported to involve both $\alpha(1,3)$ -fucose and $\beta(1,2)$ -xylose determinants [23•].

Tertiary structure of allergens by X-ray and NMR crystallographic studies

The crystal structure of several major allergens has been reported: pollen allergens Bet v 1 [24], Phl p 2 [25] and Amb t 5 [26]; a minor birch allergen profilin [27]; the homologous house dust mite allergens Der f 2 [28] and Der p 2 [29]; bovine lipocalin allergen Bos d 2 [30], and the honeybee venom allergens phospholipase A2 and melittin [31,32]. Over the past year, the structure of the major cherry allergen Pru av 1, the horse allergen Equ c 1 and a major allergen of bee venom have been resolved [16,33•,34]. Equ c 1 belongs to a family of well characterized proteins called lipocalins that include other allergens: mouse urinary protein mMUP, rat urinary alpha2-globulin, dog allergens Can f 1 and Can f 2, bovine beta-lactoglobulin (Bos d 5) and Bos d 2, and cockroach allergen Bla g 4 [33•]. The major allergen of bee venom is the first hyaluronidase whose structure is known [34]. Pru av 1 NMR structure revealed the basis for the cross-reactivity with Bet v 1 [16].

All these allergens have different structural and functional characteristics which demonstrates that allergenicity cannot be attributed to the overall folding pattern or function of the proteins. The same conclusion was obtained in a comparative analysis of the structure of an extensive number of allergens [18••]. However, another paper reports that the overall features of known allergen structures are similar. The authors mapped the IgE-binding epitopes of Jun a 3 on one surface-exposed helical/loop region of the molecule, and the location of these epitopes was consistent with that reported for other aeroallergens [35].

The crystal structure of an allergen provides clues that may explain its allergenicity and the basis for the study of the IgE binding epitopes. For example, the peanut allergen Ara h 1 has a stable, heat and digestion resistant, trimeric structure that may confer a protective effect of the IgE binding epitopes from enzymatic degradation in the stomach. This structure may explain why peanut is such a potent food allergen [36•].

B cell epitope mapping

Allergic reactions are triggered by the interaction of allergens with allergen-specific IgE bound to the high affinity FcεRI receptor on mast cell surfaces. Formation of IgE has initially been induced upon exposure of the individual to the allergen, in a process that involves antigen presentation to the T cells and stimulation of T helper type 2 cell responses. Allergen-IgE interaction on mast cell surface induces an immediate allergic reaction characterized by the release of mediators. At least two IgE-binding epitopes are necessary to cross-link IgE-FcεRI complexes in mast cells. A late response follows some hours later by infiltration of cells: eosinophils, macrophages, activated T cells and few neutrophils. Given the importance of the antigen-antibody interactions in the allergic responses, the knowledge of the points of interaction, i.e. the IgE-binding epitopes of the allergen, is of crucial importance to try to develop new ways of avoiding this interaction.

Epitope mapping can be done indirectly by disrupting the tertiary structure of the allergen and measuring the consequent changes on IgE antibody binding. This kind of study is considered functional, to distinguish it from a more direct or structural approach, that involves crystallographic or NMR studies of the antigen-antibody interactions.

One approach to functional epitope mapping involves substituting the key amino acid residues required for protein folding by site-directed mutagenesis. For example, disruption of disulfide bonds by cysteine substitutions affects IgE binding sites of the mite allergens Der f 2, Der p 2 and Lep d 2 [37,38]. Proline

mutations can also affect IgE binding and histamine release by Group 2 mite allergens [39]. Second, IgE binding epitopes can be studied by allergen gene fragmentation. This was used to map the binding site of the IgE Fab to the major timothy grass pollen allergen Phl p 5 [40•]. Third, other studies focus on the construction of deletion mutants. This approach allowed the identification of a major B-cell epitope in the N-terminus of Asp f 2 [41], but is not recommended for the identification of certain conformational epitopes because the protein obtained after deletion may refold differently than the native allergen. Proteolytic epitope mapping can be done by enzymatic cleavage of the native or recombinant allergen and analysis of IgE binding to the HPLC purified fragments [6•,35]. Small synthetic peptides that cover the whole sequence of the allergen can also be analyzed for IgE binding [22,42]. In this case, the experimental design limits the results to linear epitopes, some of which may also be missed due to lack of the structural environment provided by the rest of the molecule or because they may not include the full epitope. Aalberse has presented an interesting argument supporting the lack of dichotomy of linear versus conformational epitopes. All proteins would have conformational IgE epitopes. The shorter the fragment of the allergen studied for IgE binding, the smaller the fraction of the epitope, thus the strength of the interaction of the protein with the antibody is decreased [18••]. Analysis of IgE binding to different size fragments of an allergen expressed as fusion proteins has allowed the recognition of continuous as well as discontinuous epitopes [43••]. Similarly, glutathione-S-transferase fusion recombinant peptides and site-directed mutagenesis were used for the epitope mapping of *C. herbarum* enolase [21]. An exception to the argument may be the case of food allergens which are degraded into small fragments before being exposed to the immune system. The IgE antibodies raised against these fragments may also bind strongly the small allergen fragments, so synthesis of small peptide fragments may also be useful for epitope mapping of food allergens [44]. Finally, a novel approach using allergen hybrids has also been used to map discontinuous B-cell epitopes in insect venom allergens [17,45••].

The structural study of the antibody binding epitopes requires crystallization of the allergen-antibody complex. This work has been recently completed for the first time using birch pollen allergen Bet v 1 and Fab fragments from monoclonal murine IgG1 antibodies [46••]. Hydrogen exchange nuclear magnetic resonance spectroscopy has been applied to locate amino acids involved in mAb binding on the house mite allergen Der p 2 [47]. The use of molecular modeling techniques has also allowed the localization of the mapped B-cell epitopes in the molecular structure of the allergen [21,42].

Due to the polyclonal nature of the IgE ab and the requirement of homogeneous reagents, it is very difficult to study IgE binding epitopes by X-ray crystallography. The use of a recombinant human monoclonal IgE Fab for crystallography, as described for Bet v 1, should allow IgE allergen complexes to be crystallized [40•].

Use of modified allergens to change T-cell responses in immunotherapy

Allergens are presented as peptides by the MHC molecules to the T-cell receptors (TCR), and the interaction MHC-peptide-TCR leads to T-cell activation. The administration of modified allergens or peptides with low IgE binding capacity, could potentially avoid the side-effects of conventional IT [45•,48]. T-cell epitope derived altered peptide ligands for immunotherapy: (a) should not bind IgE, and (b) should bind efficiently class II MHC molecules. They should be 11–20 amino acids long or even longer. Promiscuous binding of peptides to more than one MHC molecule has been demonstrated recently [49,50•]. Moreover, modified allergens or modified peptides from specific allergens, may still interact with the MHC and TCR, but be unable to elicit a T-cell response, acting as antagonists or partial agonists of the TCR. The use of these altered peptide ligands has been proposed for immunotherapy to modulate immune responses to allergens [51].

A difference in antigen presentation and immune response by native and modified allergens has been proposed. The use of modified allergen variants, with low IgE-binding properties, would induce, mainly by increased interleukin-12 secretion, a T helper type 1-dominated cytokine profile leading to IgG4 production [52].

Another approach for IT is the use of recombinant allergens and adjuvants(s) to down-regulate T_H2 responses. The adjuvants that are now under study are the immunostimulatory sequences CpG nucleotides, which act as T_H1 adjuvants. The bacterial CpG motif is a signal for the mammalian immune system to induce a proinflammatory Th1 response [53]. DNA encoding for allergens, like β -galactosidase, Der p 5, Ara h 2 or Cry j 1, has been tested as a therapeutic tool in rodent models [54–57]. CpG sequences are added to allergen sequences to create a DNA preparation administered during IT in animal models and clinical trials [15•]. Recently, conjugation of immunostimulatory DNA to the short ragweed allergen Amb a 1 has been shown to enhance its immunogenicity and reduce its allergenicity in human subjects [58•]. Other approaches to IT are the use of other adjuvants including allergens coupled to interleukin-12, interleukin-18, or lectins, and peptide-based therapy [15•,59].

Conclusions

Application of molecular biology techniques has resulted in an important increase on the knowledge of the tertiary structure of allergens, and the molecular elements involved in allergen-IgE antibody interactions. From these studies, development of new strategies has arisen to substitute the allergenic extracts used in conventional immunotherapy by recombinant and/or modified allergens that keep their immunogenicity with a reduced capacity to induce allergic responses.

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