

Impact of Native, Recombinant, and Cross-Reactive Allergens on Humoral and T-Cell–Mediated Immune Responses

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Allergic and asthmatic diseases are an increasing health burden affecting large parts of the population, and the incidence of the diseases is still increasing [1,2]. T-cell responses to allergens are the key regulators of the pathogenesis of allergic disease, such as rhinitis, conjunctivitis, atopic dermatitis, and bronchial asthma, and allergic responses to insect stings and food ingestion [3,4]. The disease complex is an important cause of morbidity and, occasionally, mortality [5]. Therapeutic approaches to control allergy and asthma include symptomatic treatment with antihistamines, corticosteroids, bronchodilators (β -adrenergic receptor antagonists), and anti-IgE antibody therapy [6,7]. These drugs ameliorate allergy symptoms and concomitant inflammatory reactions, but require long-term recurrent administration [8].

Therefore, until primary prevention succeeds, immunomodulation will remain the principal practical approach to controlling allergic diseases. Allergen-specific immunotherapy (SIT), introduced in early twentieth century, is the only treatment that can cure allergic diseases [9,10]. Despite considerable success in individuals sensitized to insect venom [11] and pollen-mediated allergy [12], the clinical benefits in treating more complex allergy forms, such as those to house dust mites, mold, or food, are disappointing and largely contradictory. These limitations might be caused by factors related

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to the type of adjuvant, route of application, and duration of treatment used. However, the most important reason for SIT failure probably relates to the type of vaccine used, although confirmatory studies for this assumption are lacking.

Although insect venoms and pollen extracts are easily standardized, extracts from complex allergen sources, such as mites, molds, and foods, which contain dozens of structurally different allergen molecules [13–15], are difficult to standardize [16]. The best example is represented by commercially available mold extracts that contain major allergens in concentrations varying over three orders of magnitude [17]. Using these extracts in SIT and studies investigating the mechanisms underlying immunotherapy, which are both concentration-dependent, obviously cannot be expected to deliver reproducible results when different batches of a given extract are used. Using biochemically purified natural allergens or recombinant allergens, which are standardized in absolute terms through physicochemical methods, will contribute to a detailed understanding of the immunologic mechanisms underlying the pathology of type I hypersensitivity reactions. Progress in understanding the mechanisms governing SIT will help experts generate ideas and concepts for improving therapeutic schedules and provide a scientifically based, reproducible, and safe treatment for allergic disease.

Native and recombinant allergens

Although older literature and current allergology practice define *allergen* as an extract from a natural allergen source [18,19], molecular biologists define it as a single molecular structure able to bind IgE from sera of individuals sensitized to that particular molecule. This difference in definition has far-reaching consequences for basic experiments, diagnosis, and treatment of allergic diseases. Every experimental or clinical setup involving allergen extracts depends on the quality of the extract used, which strongly depends on the batch used [20,21] and the heterogeneous, polyclonal responses to natural exposure that elicit patient-specific IgE responses to a variable pattern of allergen structures present in the extract [22,23]. This unlucky situation does not allow patient stratification, and does not permit experts to know a priori either the number of molecular structures to which a given patient is sensitized or if the extract contains the single allergens in sufficient amounts to obtain the desired diagnostic or therapeutic effect. In fact, increasing clinical evidence shows that SIT can induce sensitization to molecular allergen structures to which the patients were not allergic before treatment [24,25].

The application of biochemical methods to purify single allergens from complex extracts [26,27] and the introduction of molecular biology techniques in allergology research [28–34] have drastically changed this picture. This fact is best exemplified by phospholipase A₂ (PLA₂), a major allergen of bee venom (Api m 1), which has been available as biochemically purified

protein from the natural source for decades and was later cloned and produced as recombinant allergen [35]. The availability of both native and recombinant PLA₂ allowed experts to show that allergens can be produced as highly pure recombinant proteins in an active form with biochemical and immunologic properties practically indistinguishable from those of their native counterparts [35]. Natural and recombinant PLA₂ have been shown to have comparable type I skin reactivity [36] and the same potency to release histamine from basophiles [37]. Moreover, the structure [38], but not the enzymatic activity [39], could be shown to be important for the immunogenicity of the protein. Recombinant PLA₂ also has been shown to be useful in increasing the specificity of diagnostic tests [40], a property that seems to be typical for recombinant allergens [15,41–44]. Recombinant allergens can easily be used in fully automated diagnostic devices [45,46]. Recombinant allergens can be produced as standardized proteins with biochemical properties practically indistinguishable from those of natural allergens and of a quality that satisfies the standards for use in humans. They will most likely replace most extracts for diagnosing allergic conditions in the near future.

The most important issue, however, is that recombinant allergens have drastically changed the understanding of allergy, opening frontiers for the development of new diagnostic and therapeutic concepts. Based on a long list of cloned allergens [47], it is now possible, at last in specialized research laboratories, to determine patient-specific sensitization profiles and thus to perform a component-resolved diagnosis of allergy [48]. The ability to stratify patients according to the molecular structures responsible for the patient-specific sensitization will allow the possibility of patient-tailored, component resolved immunotherapy in the future [49].

Purified native and recombinant allergens have strongly contributed to expanding the knowledge of the basic mechanisms modulating the immune response against normally innocuous environmental antigens and allergens in healthy and allergic individuals. Native and recombinant bee venom PLA₂ have played a pivotal role in modern allergology, and work performed at T-cell level with these substances has strongly influenced this field. Most individuals stung by a bee initially elicit low-affinity IgG1 anti-PLA₂ antibodies and develop high-affinity IgG4 antibodies to PLA₂ after repeated exposure [50]. In contrast, some individuals generate PLA₂-specific IgE antibodies and develop bee sting allergy [51]. PLA₂ displays three immunodominant T-cell epitopes (Fig. 1) equally recognized by allergic and nonallergic individuals [52], and one glycopeptide T-cell recognition site [53]. These epitopes are equally recognized by allergic and nonallergic individuals [54] and have been successfully used in specific immunotherapy of patients allergic to bee venom [55].

Cross-reactive allergens

Cross-reactive allergens represent the most interesting, but also the most challenging, molecular structures. The elucidation of hundreds of sequences

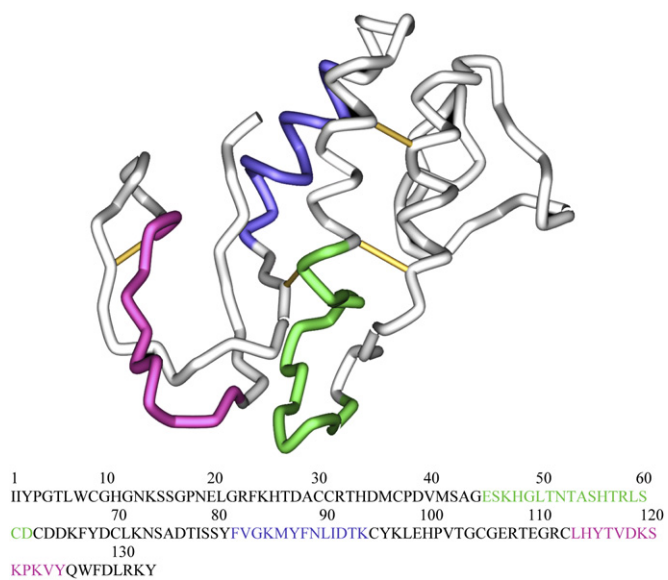


Fig. 1. Localization of the linear T-cell epitopes of PLA₂, the major bee venom allergen, on the amino acid sequence and on the structure of the enzyme. The epitopes defined in T-cell proliferation experiments with overlapping peptides [52], highlighted in green, blue, and magenta in the linear structure, are depicted in the same colors on the three-dimensional context.

encoding allergens, combined with homology searches, enabled a first in silico identification of molecular families of homologous allergens potentially involved in cross-reactivity [56]. Bioinformatic analyses based on structural motifs [57], and Basic Local Alignment Search Tool (BLAST) similarity search methods [58] involving 1,01,602 and 1,35,850 protein entries deposited in the Swissprot database, predict 4093 (4%) and 4768 (3.5%) different potential allergen structures, respectively. Therefore, the allergen repertoire involved in eliciting allergic symptoms can be assumed to involve approximately 10⁴ different molecules organized in a limited number of phylogenetically related families. The molecular basis underlying cross-reactivity is schematically represented in Fig. 2.

However, not all structures will be required for diagnostic and therapeutic applications because many allergens can be grouped in pan-allergen families sharing cross-reactive epitopes [59–63]. Cross-reactivity will help drastically reduce the number of epitopes needed for a careful diagnosis of allergy, and the rapidly increasing knowledge about primary and three-dimensional structures of allergens [56] will speed up theoretical and practical progress in defining the number of structures or epitopes needed for a component-resolved diagnosis of allergic diseases that forms the basis for the development of rational therapy approaches.

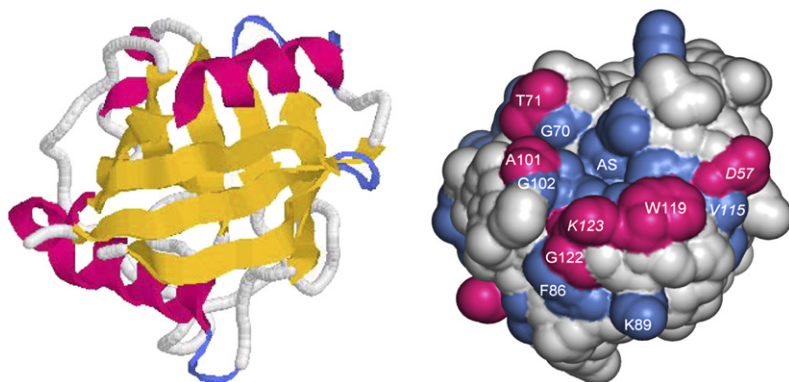


Fig. 2. Cross-reactivity derives from conserved amino acids exposed to the surface of molecules sharing extended homology at primary and tertiary structure. The left panel shows the overall fold of cyclophilin consisting of a central eight-stranded β -sheet (yellow) and two flanking α -helices (pink). The right panel shows a typical patch of the identical solvent-exposed amino acids (pink >50%, blue >30% exposed) deriving from the superposition of the *Malassezia sympodialis* and human thioredoxin crystal structures. These amino acids located in different loops are assembled on the surface forming a potential conformational IgE-binding B-cell epitope. (Adapted from Limacher A, Glaser AG, Meier C, et al. Cross-reactivity and 1.4 Å crystal structure of *Malassezia sympodialis* Thioredoxin (Mala s 13), a member of a new pan-allergen family. J Immunol 2007;178:389–96.)

Humoral and T-cell mediated immune responses to cross-reactive structures

Studies have shown that recombinant allergens can be produced as perfectly standardized molecules with characteristics practically indistinguishable from those of their natural counterparts. The best documented examples are PLA₂, the major bee venom allergen [35], and Bet v 1, the major birch pollen allergen [64]. In vitro these recombinant allergens show IgE-binding capacity [42,65], potency to release mediators from effector cells [37,65], and potency to stimulate T-cell proliferation [52,64] comparable to those of the biochemically purified allergens from the natural source. Most importantly, the skin-test reactivity in vivo is no different between recombinant and natural allergens [36,42,43], definitively showing that recombinant allergens can substitute natural allergens for the in vitro and in vivo diagnosis of allergy. The list of recombinant allergens shown to be equivalent to their natural counterparts is long [66] and still growing rapidly. Again, Bet v 1 represents the best-characterized prototype of a cross-reactive structure present in many allergen sources, such as celery [67], apple [68], hazelnut [69], cherry [70], and carrot [41,71].

A special case of cross-reactive structures are defined by proteins showing IgE-mediated autoreactivity (Table 1) [72–80]. Increasing evidence shows that self antigens can induce IgE-mediated allergic responses. Therefore, experts must distinguish between indirect evidence obtained in in vitro

Table 1
Human proteins showing cross-reactivity with environmental allergens

Human protein	Molecular weight (kd)	Acc. no.	Cross-reactive with	Source	Reference
MnSOD	24.7	P04179	Asp f 6	<i>Aspergillus fumigatus</i>	[86]
			Dro m MnSOD	Fruit fly	[86]
			Hev b10	Latex	[72]
			Mala s 11	<i>Malassezia sympodialis</i>	[73]
			Sac c MnSOD	Baker's yeast	[86]
Profilin	14.9	P07737	Ara t 8	<i>Arabidopsis thaliana</i>	[74]
Cyclophilins (A/B/C)	17.8–22.7	P62937	Bet v 2	Birch	[75]
			Asp f 11	<i>Aspergillus fumigatus</i>	[86]
		P23284	Cand a CyP	<i>Candida albicans</i>	[86]
		P45877	Mala s 6	<i>Malassezia sympodialis</i>	[86]
Thioredoxin	11.7	X77584	Sac c CyP	Baker's yeast	[86]
			Tri a 25	Wheat	[94]
			Zea m 25	Corn	[94]
			Mala s 13	<i>Malassezia sympodialis</i>	[63]
Ribosomal protein P2	11.6	M17887	Alt a 5	<i>Alternaria alternata</i>	[90]
			Asp f 8	<i>Aspergillus fumigatus</i>	[90]
			Cla h 5	<i>Cladosporium herbarum</i>	[90]
APDH	—	—	Gad c APDH	Codfish	[76]
Casein	25.4	X17070	Bos d 8	Cow's milk	[77]
Lactalbumin	16.2	J00270	Bos d 4	Cow's milk	[78]
Insulin	12.0	V00565	Bos d insulin	Bovine insulin	[79]
			Sus s insulin	Porcine insulin	
Serum albumin	69.4	V00494	Bos d 6	Bovine serum albumin	[80]
			Can f 3	Canine serum albumin	
			Fel d 2	Feline serum albumin	
			Equ c 3	Equine serum albumin	

Abbreviations: Acc., accession; APDH, aldehyde phosphate dehydrogenase; MnSOD, manganese-dependant superoxide dismutase.

experiments or ex vivo experiments using isolated cells and direct evidence obtained from testing the self antigens in patients. Structurally, experts must distinguish between self antigens that have strong sequence identity, homology, and epitope mimicry to environmental allergens, and those that do not.

Besides profilin, which was the first self allergen shown to bind IgE from birch-sensitized patients in vitro [59], arrays of self antigens able to bind IgE isolated through different screening procedures also have been identified [81–83]. The first self antigen directly shown to induce type I skin reactions in patients was human manganese-dependant superoxide dismutase (MnSOD), a phylogenetically highly conserved protein [84]. This study showed that human MnSOD shares 51.5% identity and 67.2% homology with the corresponding allergen of *Aspergillus fumigatus* [85]. The two recombinant MnSODs were recognized by IgE antibodies from patients allergic to *A. fumigatus* MnSOD, elicited specific type I reactions in these individuals, and induced specific proliferation of peripheral blood mononuclear cells from individuals allergic to *A. fumigatus* who showed specific IgE responses and reacted in skin tests to MnSOD. Further studies showed that even MnSOD of *Drosophila melanogaster*, wherein exposure in the normal population can be virtually excluded, is able to bind IgE and induce T-cell proliferation in ex vivo material of individuals sensitized to MnSOD, indicating molecular mimicry is a basic mechanism for the observed reactivity [86]. Detailed comparisons of the crystal structures of human and *A. fumigatus* MnSOD [87] and molecular modelling of additional MnSODs [86] allowed an exact definition of the potential amino acids defining cross-reactivity. Furthermore, studies have shown that human MnSOD directly applied to the skin of patients who have atopic eczema and are sensitized to *Malassezia sympodialis* in atopy patch tests is sufficient to elicit an eczematous reaction [88,89]. In concordance with the presence of MnSOD-specific IgE antibodies, determined using enzyme-linked immunosorbent assay (ELISA), the human enzyme also elicited positive skin test reactions in all patients who had positive ELISA results, and reactivity against human MnSOD strongly correlated with the severity of the disease, corroborating a pathogenic role of self-reactivity in the exacerbation of atopic eczema. However, MnSOD is not an exotic example because comparable patterns of autoreactivity also have been obtained with acidic ribosomal P₂ protein [90], cyclophilin [62,91,92], and thioredoxin [63]. Cyclophilin and thioredoxin were first identified as allergens through screening of phage-surface displayed libraries with sera of patients who had long-lasting atopic diseases [14,62]. The corresponding human enzymes were amplified from human cDNA libraries with polymerase chain reaction and used to produce the recombinant proteins [62,63]. Screening of the protein repertoires of wheat and maize with sera from patients who had positive results from a double-blind, placebo-controlled food challenge yielded, among the allergens cloned, thioredoxin as an additional allergenic structure [93]. The

recombinant maize and wheat proteins displayed enzymatic activity, and bakers who had occupational asthma and patients who had grass pollen allergy but no clinical history of cereal allergy had sensitization rates of 47% and 35%, respectively [94]. The maize and wheat thioredoxins, sharing 74% sequence identity, exhibited distinct IgE cross-reactivity and also cross-reactivity to human thioredoxin.

As a first step in investigating cross-reactivity between cyclophilins and thioredoxins, the authors solved the crystal structures of *Malassezia sympodialis* cyclophilin [92] and thioredoxin [63]. Comparing these structures with the solved structures of human cyclophilin and thioredoxin allowed an exact definition of conserved amino acids exposed to the solvent that forms structural patches, potentially defining cross-reactive B-cell epitopes [63,92], as exemplified in Fig. 2. Although the authenticity of the postulated B-cell epitopes must be confirmed through site-directed mutagenesis, this will require crystallization and structure elucidation of each single mutant to confirm that the mutations do not affect the three-dimensional structure. Studies have shown that IgE binding exquisitely depends on the three-dimensional structure of the allergen [95,96], and therefore reduction of IgE binding through site-directed mutagenesis could be caused by either direct involvement of an amino acid exchanged at IgE binding or changes in the three-dimensional structure from the exchange without contribution of the changed amino acid to IgE binding. This crucial point is often neglected in site-directed mutagenesis experiments.

However, the list of self antigens involved in IgE binding is rapidly increasing, and autoreactivity clearly plays a role in the exacerbation of long-lasting atopic diseases [86,88–90,97]. Evidence comes from the ability of self antigens to induce strong humoral and T-cell-mediated reactions *ex vivo*, and from the ability of the self antigens to induce skin- and atopy patch test reactions in sensitized individuals [88,89]. Whether self antigens themselves are promoting the onset of IgE-mediated autoimmune reactions remains unclear. For self antigens sharing extended sequence identity or homology to environmental allergens, one can assume that autoreactivity is caused by molecular epitope mimicry at the B- and T-cell level [85,88,90]. For self antigens lacking significant sequence homology to environmental allergens, one cannot exclude that homologous, currently unidentified structures could be present in the incomplete repertoire of environmental proteins able to induce IgE sensitization. The available observations support the assumption that autoreactivity is initiated or at least preceded by a sensitization against environmental allergens [97]. If a human protein is available to the immune system, it will probably induce immune tolerance. However, this tolerance can be eluded through cross-reacting IgE antibodies raised against epitopes shared between human proteins and environmental allergens [98]. Although molecular mimicry between conserved T- and B-cell epitopes seems to be the most likely explanation for the observed autoimmune reactions to self antigens, more work remains to clarify if

sensitization to self antigens can occur as a direct consequence of inflammatory processes.

Summary

Biochemical and molecular biology methods allow the production and purification of virtually pure natural and recombinant allergens. For the first time, immune responses to allergens could be dissected at the molecular level using pure reagents. Given that T-cell responses, which are the key regulators of atopic diseases, are highly dose-dependent and that doses can affect cytokine profiles, molecular characterization of natural, recombinant, and cross-reactive allergens realized an unmet experimental need. "Clean" and straightforward experimental setups using pure allergens allowed experts to clarify most of the contradictory results obtained using allergen extracts. Diagnostic procedures based on purified allergens have contributed to increasing specificity and sensitivity and determining patient-specific sensitization patterns. This stratification will allow development of patient-tailored therapeutic schedules, with defined allergen doses likely contributing to improving therapeutic effects. However, a breakthrough in specific therapy of allergy and asthma will depend on developing an understanding of the immunologic mechanisms underlying the diseases. Specific elimination of pathogenic T-cell subsets and targeted elimination of allergen-specific B cells seem the most promising approaches. Both strategies will require engineering of chimeric molecules able to cross-link receptors on the surface of targeted effector cells, and recombinant allergens will confer an absolute specificity to these novel therapeutic lead compounds.

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