

Recombinant Allergens in Structural Biology, Diagnosis, and Immunotherapy

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Recombinant allergens · Structural biology · Allergy diagnosis · Vaccine development · Allergy immunotherapy · Clinical studies

Abstract

The years 1988–1995 witnessed the beginning of allergen cloning and the generation of recombinant allergens, which opened up new avenues for the diagnosis and research of human allergic diseases. Most crystal and solution structures of allergens have been obtained using recombinant allergens. Structural information on allergens allows insights into their evolutionary biology, illustrates clinically observed cross-reactivities, and makes the design of hypoallergenic derivatives for allergy vaccines possible. Recombinant allergens are widely used in molecule-based allergy diagnosis such as protein microarrays or suspension arrays. Recombinant technologies have been used to produce well-characterized, noncontaminated vaccine components with known biological activities including a variety of allergen derivatives with reduced IgE reactivity. Such recombinant hypoallergens as well as wild-type recombinant allergens have been used successfully in several immunotherapy trials for more than a decade to treat birch and grass pollen allergy.

As a more recent application, the development of antibody repertoires directed against conformational epitopes during immunotherapy has been monitored by recombinant allergen chimeras. Although much progress has been made, the number and quality of recombinant allergens will undoubtedly increase and keep improving our knowledge in basic scientific investigations, diagnosis, and therapy of human allergic diseases.

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Introduction

The history of recombinant allergens started with the very first publications on allergen cloning. In January 1988, the cDNA sequence coding for the major house dust mite allergen Der p 1 was discovered in Australia and published as the first complete allergen sequence worldwide [1]. Interestingly, the publication of the identification of the *lgt11* clone expressing Der p 1 was only published later that year [2]. The sequence of a major allergen of the white-faced hornet *Dolichovespula maculata*, then named antigen 5 and now designated as Dol m 5, was cloned in February 1988 in New York City and published by Fang et al. [3]. The following year witnessed the dis-

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covery of the cDNA of the very first plant allergen, the major birch pollen allergen Bet v 1, in Austria, which was published in 1989 [4]. What these 3 allergens have in common is their expression in the bacterium *Escherichia coli* following the infection by λ gt11 phages. Hence, they can justifiably be regarded as the first recombinant allergens.

So the era of recombinant allergens began in 1988. Der p 1 was identified by screening the λ gt11 cDNA expression library with a rabbit anti-Der p 1 antiserum [2]. Dol m 5 was discovered by screening a λ gt11 cDNA expression library with hornet antigen 5-specific mouse sera [3]. Bet v 1 was the first recombinant allergen that was identified by screening the expression library with IgE from sera of birch pollen-allergic patients [4]. Previously, Bet v 1 had been expressed in a cell-free wheat germ system in vitro and identified using sera from patients allergic to birch pollen [5]. Again, in 1989, the second plant allergen, Dac g 2 from cocksfoot grass pollen, was identified by screening a λ gt11 expression library with sera obtained from cocksfoot grass pollen-allergic individuals [6]. The first fungal allergen, Asp f 1 from *Aspergillus fumigatus*, was published in 1990 [7], the first animal allergen, Fel d 1 from the domestic cat, in 1991 [8], and the first plant food allergen, Ara h 1 from peanut, in 1995 [9].

Recombinant Allergens in Structural Biology

Almost all crystal and solution structures of allergens have been obtained from recombinant allergens. There are 2 reasons for this: (1) most allergens occur in the allergen source as a mixture of several isoforms that are very difficult to separate and (2) some allergens are present at very low concentrations, making it impossible to obtain a sufficient quantity of the purified allergen for crystallization. Proteomic profiling or characterization by 2-dimensional electrophoresis has shown the presence of allergen isoforms in all analyzed allergen sources including birch pollen [10], Timothy grass pollen [11], *Parietaria judaica* pollen [12], peanut [13], house dust mite [14], fire ant [15], and cow's milk [16]. The recombinant production of allergens allows the deconvolution of isoform mixtures and the crystallization of individual isoforms. This approach has yielded the crystal structures of 3 Bet v 1 isoforms, Bet v 1.0101 (former designation Bet v 1a, PDB 4A88) [17], Bet v 1.0106 (Bet v 1j, 4A8U) [17], and Bet v 1.0107 (Bet v 1l, 1FM4) [18].

The Structural Database of Allergens (SDAP, <https://fermi.utmb.edu/>) is a repository of allergenic proteins



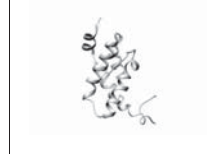








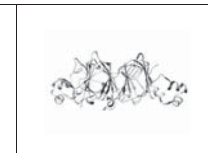





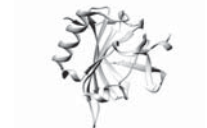




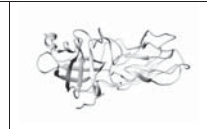

and various computational tools that can assist structural biology studies related to allergens. SDAP provides a list of and links to 92 allergen structures that are included in the Protein Data Bank (PDB, www.rcsb.org/pdb/). SDAP also provides 458 models for allergen and isoallergen structures. In their 2014 paper, Dall'Antonia et al. [19] presented 103 structures of allergens from the PDB. Structural information on allergens is helpful in reconstructing the evolutionary history of protein architectures where amino acid sequence comparisons fail to reveal sequence similarities. Thus, a large superfamily of structurally related proteins that are all based on the Bet v 1 fold could be arranged into the Bet v 1 superfamily [20]. The presence of Bet v-like molecules in Archaea such as *Aeropyrum pernix* [21] indicates that the Bet v 1 fold originated in the very early stages of life on earth. Likewise, the origin of the cupin superfamily which harbors many allergenic seed storage proteins, e.g., Ara h 1 from peanut, can be traced back to the Archaea based on available crystal structures [22]. AllFam (<http://www.meduniwien.ac.at/allfam/>), the recently updated database of allergen families, is built around the membership of allergens to protein families [23]. AllFam groups allergens according to the classification of the protein family database Pfam (<http://pfam.xfam.org/>). The September 2016 version of AllFam contains 1,018 allergens, 939 of which can be assigned to 1 of 216 Pfam families. This number corresponds to 1.3% of the 16,306 entries contained in Pfam v30.0, illustrating the highly limited distribution of allergens into protein families.

It is important to note that the vast majority of proteins in an allergen-containing family are nonallergenic. Cross-reactivity occurs almost exclusively between allergens of the same family and spans the spectrum from high (e.g., profilins, polcalcins, and parvalbumins) to medium (e.g., PR-10 and nsLTPs) and low cross-reactivity (e.g., cupins, 2S albumins, and lipocalins).

Table 1 shows ribbon representations of selected allergens from the protein superfamilies or families that contain important allergens. Proteins with a long evolutionary past are distributed over a wide range of species, e.g., the members of the Bet v 1 family, the cupin superfamily, and the lipocalin family. Other types of allergens are more restricted in their distribution, e.g., parvalbumins are restricted to fish, polcalcins to pollen, and β -expansins to grass pollen.

The available crystal and solution structures of recombinant allergens illustrate the clinically observed cross-reactivity even between distantly related allergens, as is the case for the kiwi allergen Act d 11 and also Bet v 1

Table 1. Structures of selected allergens from prominent allergen families

Prolamin superfamily: Nonspecific lipid transfer proteins and 2S albumins			
			
Peach Pru p 3 (2ALG) nsLTP	Hazelnut Cor a 8 (4XUW) nsLTP	Peanut Ara h 2 (3OB4) 2S albumin	Brazil nut Ber e 1 (2LVF) 2S albumin
EF-hand family*: Parvalbumins and polcalcins			
			
Carp Cyp c 1	Cod Gad m 1 (2MBX)	Iambsquaters pollen Che a 3 dimer (2OPO)	Timothy grass pollen Phl p 7 dimer (1K9U)
Cupin superfamily: vicilins and legumins			
			
Peanut Ara h 1 (3SMH) trimer	Soy Gly m 5 (1IPK) trimer	Peanut Ara h 3 (3C3V) subunit of hexamer	Soy Gly m 6 (1OD5) subunit of hexamer
Bet v 1 family			
			
Birch Bet v 1 (4A88)	Celery Api g 1 (2BK0)	Peanut Ara h 8 (4M9B)	Kiwi Act d 11 (4IHR)
Lipocalin family			
			
Cow Bos d 2 (1BJ7)	Horse Equ c 1 (1EW3)	Dog Can f 2 (3L4R)	Cockroach Per a 4 (3EBW)
Expansin and expansin-like family			
			
Timothy grass pollen Phl p 1 (1N10)	Timothy grass pollen Phl p 2 (1WHO), C- terminal expansin domain	Maize pollen Zea m 1 (2HCZ)	Kiwi fruit Act d 5 (4X9U), N-terminal expansin domain

Numbers in parentheses are Protein Data Bank (PDB; <http://www.rcsb.org/pdb/home/home.do>) accession numbers.

* Bound calcium ions are shown as grey spheres. The ribbon models of the allergens were created with the molecular modeling system UCSF Chimera (<http://www.cgl.ucsf.edu/chimera>).

[24]. Structural information obtained from recombinant allergens together with IgE cross-reactivity data can be combined to predict conformational epitopes in the absence of allergen-antibody complexes [25]. These predicted epitopes offer a useful starting point for introducing mutations or structural changes into known allergens in an effort to create allergen derivatives as candidate molecules for allergen-specific immunotherapy (AIT). Structural homologs of allergens can also be used as scaffolds for epitope grafting, whereby the individual surface areas of allergens are transplanted onto low or nonallergenic members of the same protein (super)family. Such chimeric molecules were produced by grafting surface areas of Bet v 1 onto either a structurally related protein from celery [26] or meadow rue [27]. The norcoclaurine synthase from meadow rue was further used as a scaffold for presenting conformational epitopes of the soybean allergen Gly m 4, in an effort to characterize its IgE epitope profile [28].

Recombinant Allergens in Diagnostic Tests

Although diagnostic assays based on purified recombinant allergens are spreading fast, extracts from natural allergen sources are still in wide use. The composition of an allergenic extract strongly affects the results of any extract-based IgE assay. Commercially available extracts may lack important allergens or may vary considerably in their allergen composition, leading to false-negative test results for certain patients. Varying allergen compositions and contents have been shown for skin prick test (SPT) solutions for the house dust mite *Dermatophagoides pteronyssinus* [29], the mould *Alternaria alternata* [30], and hazelnut [31] as well as for sublingual allergen immunotherapy products for house dust mite, storage mite, and birch pollen [32, 33]. At least for routine diagnostics, a process called spiking (i.e., the addition of recombinant allergens) has been used to improve the quality of allergenic extracts such as latex [34], hazelnut [35], and wasp venom [36]. Moreover, recombinant allergens represent an alternative to natural allergens for allergy diagnosis when the natural allergen can only be isolated in low quantities from the original source [37].

Molecule-based allergy diagnosis uses purified natural and recombinant allergens to elucidate the sensitization patterns of a patient at the molecular level [38]. This procedure can (i) increase the diagnostic accuracy, (ii) distinguish genuine sensitization from sensitization due to cross-reactivity, (iii) help to assess the risk and type of al-

lergic reaction, and (iv) facilitate the selection of eligible patients and suitable allergens for allergen-specific immunotherapy [39].

Today, singleplex (1 assay/serum sample) and multiplex (multiple assays/serum sample) platforms for measuring sIgE antibodies to allergens are available. Singleplex assays allow testing for the presence of IgE specific to only those allergens that are indicated by the patient's clinical history. In contrast, a multiplex platform allows the definition of an individual's IgE response to the whole spectrum of allergens arrayed on a chip. Thus, the multiplex microarray approach often measures the presence of specific (s)IgE to allergens not indicated by the patient's history. This results in the generation of IgE antibody data that can be inconsistent with the patient's history and difficult to explain to the patient.

Screening large populations by allergen microarrays may also produce unexpected results. Panzner et al. [40] could detect sensitizations patterns to allergen sources which were not present in the study area. The microarrayed allergen approach which facilitated the measurement of sIgE to a large number of allergenic proteins using a small volume of serum was first introduced in 2002 by Hiller et al. [41]. VBC Genomics developed the allergen microarray technology which was then advanced into the commercial product ImmunoCAP Immuno Solid-Phase Allergen Chip (ISAC) by ThermoFisher Scientific. Further development of the ISAC was funded by the European Union (EU) project MeDALL to increase the original number of allergens from 112 to 176, 127 of which are recombinant allergens [42]. The ISAC makes it possible to semiquantitatively test for sensitization to 112 allergens, providing, as is sometimes argued, too much information. Incorvaia et al. [43] caution against basing an allergy diagnosis on ISAC data alone.

In addition to ThermoFisher Scientific's ISAC, several other companies offer systems to test for the presence of allergen-specific IgE in patients' sera. These companies include Siemens with their Immulite immunoassay system and Hycor with their HYTEC 288 Plus system. The technological advancements for in vitro allergy diagnosis do not stop there [44]. Small-scale suspension arrays for a small number of purified recombinant and natural allergens have been developed on the Luminex xMAP® platform [45] and on the Becton Dickinson cytometric bead array system [46]. Indoor Biotechnology produces an IgE quantitative binding array for the simultaneous detection and quantitative determination of total and allergen-specific human IgE against 11 allergens. Kühne et al. [47] demonstrated, in a proof-of-concept study, that

Table 2. Mutational strategies to produce recombinant hypoallergens

Strategy	Definition	Allergen source	Molecule/s	Ref.	
Fragmentation	The cDNA coding for the allergen is fragmented into ≥ 2 parts; the fragments may overlap; the fragments are expressed individually	birch pollen	Bet v 1	58, 59	
		cow dander	Bos d 2	60	
		storage mite <i>L. destructor</i>	Lep d 2	61	
		Timothy grass pollen	Phl p 1	62	
		house dust mite <i>D. pteronyssinus</i>	Der p 2	63	
Oligomerization	Two or more copies of the allergen-encoding cDNA are linked by short oligonucleotide spacers with an open reading frame; the complete construct is expressed	birch pollen	Bet v 1	64	
Mosaics	The allergen-encoding cDNA is fragmented into several parts and the fragments are re-joined in an order different from the original sequence; if the sequence parts originate from >1 allergen, the resulting protein is regarded as a hybrid mosaic	Timothy grass pollen	Phl p 2	66	
			Phl p 1	67	
		birch pollen	Bet v 1	68	
		cat	Fel d 1	69	
Chimeras/allergen hybrids	Chimeric proteins or hybrid proteins are created by joining the genetic information of at least 2 different proteins; such constructs may contain parts of or the complete original proteins	house dust mite <i>D. pteronyssinus</i>	Der p 1, Der p 2	71, 72	
		Timothy grass pollen	Phl p 2, Phl p 6	73	
			Phl p 1, Phl p 2, Phl p 5, Phl p 6	74	
		yellowjacket, paper wasp	Ves v 5, Pol a 5	75	
		honey bee	Api m 1, Api m 2 Api m 3	76	
		Japanese cedar	Cry j 1, Cry j 2	77	
Point mutations	One or more nucleotide triplets coding for a specific amino acid is/are altered to replace the original amino acid at its exact position by an amino acid with different physicochemical characteristics	birch pollen	Bet v 1	79, 101	
			Bet v 4	83	
		<i>Brassica rapa</i> pollen	Bra r 1	84	
		carp	Cyp c 1	82	
		<i>P. judaica</i> pollen	Par j 1	85	
		latex <i>H. brasiliensis</i>	Hev b 6.02	86, 98	
		house dust mite <i>D. pteronyssinus</i>	Der p 2	87	
		peanut	Ara h 1, Ara h 2, Ara h 3	94–96	
		latex <i>H. brasiliensis</i>	Hev b 5	97	
		ryegrass pollen	Lol p 5	99	
		egg white	Gal d 1	100	

multi-peptide microarrays of the peanut allergen Ara h 1 and all 3 subunits of the soybean allergen Gly m 5 could be used for large-scale screening of IgE epitopes of food allergens.

In response to the need of allergologists for guidance and background information on allergen molecule-based diagnostics, the European Academy of Allergy and Clinical Immunology (EAACI) has published the Molecular Allergology User's Guide, compiled by a panel of 65 ex-

perts in the field [48]. The EAACI regards the need to learn to interpret "allergomics" data as a challenge for practicing allergologists. The list of important allergenic molecules used in the microarray format is still incomplete. Recently, Becker et al. [49] demonstrated that Der p 23 was a new important house dust mite allergen which could contribute to the clarification of perennial allergic symptoms when the known major house dust mite allergens Der p 1, Der p 2, and Der p 10 test negatively. This

importance of Der p 23 was confirmed by a recent study in Thailand, where 54% of 222 house dust mite-allergic individuals displayed Der p 23-specific IgE responses [50]. The Ani s 11-like protein, Ani s 11.0201, a recently discovered new major allergen of the fish parasite *Anisakis simplex* was shown to be recognized by 78% of the patients in the study group while 13.5% only detected the Ani s 11-like allergen [51]. It is predicted that in vitro IgE assays based on allergenic extracts or purified allergens will coexist in clinical practice for quite a while. Hence, the EAACI suggests combining the results from SPTs, IgE assays using allergenic extracts, and molecular diagnosis with the clinical history of the patient, in order to provide the best possible allergy diagnosis for the patient.

Vaccine Development

The first AIT trial was reported in 1911 by Leonard Noon [52], who injected grass pollen extracts subcutaneously into grass pollen-allergic patients and thus achieved a reduction in allergic symptoms. Since then, several clinical trials have shown that AIT is able to modify allergic diseases and can produce long-lasting effects in treated patients [53–55]. However, natural allergen extracts often show great variations in allergen content, may lack important allergens, and may be contaminated with allergens from other sources [29, 56, 57]. In contrast, recombinant technologies allow the production of well-characterized, noncontaminated vaccine components of known biologic activity. Moreover, genetic engineering allows the modification of wild-type allergens to generate allergen derivatives with reduced IgE reactivity (“hypoallergens”), a reduced risk of triggering undesirable allergic reactions during the course of AIT, and a retained immunogenic activity. Modifications that are used to destroy conformation-dependent B-cell epitopes, while conserving T-cell epitopes, include the fragmentation or fusion of molecules, point mutations and mutations, and the formation of chimeras and mosaics. Table 2 gives an overview of the most commonly applied methods of producing hypoallergens.

Recombinant Allergen Fragments

The IgE antibody response to respiratory allergens is mainly directed to conformational epitopes. Hence, the disruption of the 3-dimensional structure by separately expressing fragments of the allergen can reduce or abolish the allergen’s IgE reactivity. The production of allergen-derived fragments was first described for the major birch pollen allergen Bet v 1 by Vrtala et al. [58, 59]. Two parts

of Bet v 1, representing amino acid residues 1–74 and 75–160, were expressed in *E. coli*. The 2 recombinant fragments showed almost no IgE-binding activity and exhibited random coil conformation. Both fragments induced the proliferation of human Bet v 1-specific T cell clones. Two overlapping recombinant fragments (amino acid residues 1–131 and 81–172) of the respiratory allergen Bos d 2 from cow dander showed only a low level of residual IgE reactivity but vigorously stimulated Bos d 2-specific T-cell clones [60]. Six fragments of the major allergen Lep d 2 of the storage mite *Lepidoglyphus destructor* displayed only weak IgE binding, indicating that Lep d 2 does not possess dominant linear B-cell epitopes [61]. When fragmentation of an allergen is taken to the extreme, IgE haptens are the result. An expression cDNA library was constructed from a randomly fragmented cDNA coding for the major Timothy grass pollen allergen Phl p 1 [62]. An immunodominant 15mer peptide was identified with IgE from allergic patients; it bound IgE from 30% of grass pollen-allergic patients but did not induce basophil histamine release in most patients. Two recombinant fragments of the major house dust mite allergen Der p 2 (residues 1–53 and 54–129) displayed a >10-fold reduction in allergenic activity, shown by a basophil activation assay and an SPT [63].

Recombinant Allergen Oligomers

The concept of allergen oligomerization was realized by expressing 3 copies of the Bet v 1 cDNA linked by short oligonucleotide spacers with an open reading frame in *E. coli* [64]. The recombinant (r)Bet v 1 trimer contained Bet v 1-specific IgE and IgG as well as T-cell epitopes, but it exhibited a profoundly reduced allergenic activity. This could be explained by microaggregation, steric hindrance, and/or unfavorable charge interactions that hid some of the IgE epitopes required for effective cross-linking. The rBet v 1 trimer induced IgG antibodies in mice and rabbits that blocked human IgE binding to Bet v 1, Aln g 1, and Mal d 1. It was later found that the rBet v 1 trimer formed high-molecular-weight aggregates with an altered presentation of IgE epitopes to effector cell-bound IgE [65].

Recombinant Allergen Mosaics

Mosaic proteins created by genetic engineering are defined as artificial recombinant proteins designed from a set of peptides that are found in the reference protein(s). If the peptides originate from ≥ 2 proteins, then the resulting protein is regarded as a hybrid mosaic (see below). Mosaic allergens are constructed by reassembling the peptides derived from the sequence of the wild-type al-

Table 3. Recombinant allergens and recombinant allergen derivatives used in AIT studies

Allergen source	Vaccine component	Molecule	Intervention	Clinical study type	Patients, <i>n</i>	Year	Ref.
Birch pollen	recombinant WT protein, fragments, dimer, and trimer	rBet v 1, 2 rBet v 1 fragments, (rBet v1) ₂ , (rBet v1) ₃	SPT, intradermal injections	Phase 0	29/30	2000	107
Birch pollen	recombinant fragments and trimer	2 rBet v 1 fragments, (rBet v1) ₃	subcutaneous injection	Phase I safety and tolerability	124 in 3 groups	2004	108
Birch pollen	recombinant WT protein	rBet v 1, nBet v 1, birch pollen extract	subcutaneous injection	Phase II safety and efficacy	134 in 4 groups	2008	111
Birch pollen	recombinant WT protein	rBet v 1	sublingual tablet	Phase II safety and efficacy	485 in 4 groups	2015	112
Birch pollen	recombinant WT protein folding variant	rBet v 1-FV	subcutaneous injection	Phase I safety	37 in 4 groups	2013	114
Birch pollen	recombinant WT protein folding variant	rBet v 1-FV, birch pollen extract	subcutaneous injection	Phase II	51 in 2 groups	2015	115
Timothy grass pollen	recombinant WT protein	mixture of rPhl p 1, rPhl p 2, rPhl p 5a, rPhl p 5b, rPhl p 6	subcutaneous injection	Phase I	62	2005	116
Timothy grass pollen	recombinant WT protein	mixture of rPhl p 1, rPhl p 2, rPhl p 5a, rPhl p 5b, rPhl p 6	subcutaneous injection	Phase II	50 in 5 groups	2012	117
Timothy grass pollen	recombinant fusion proteins of HBV surface protein preS domain and allergen-derived B-cell epitope peptides	peptides derived from Phl p 1, Phl 2, Phl p 5, Phl p 6	subcutaneous injection	Phase II	71 in 4 groups	2016	118
Cat	recombinant fusion protein	rMAT-Fel d 1	intralymphatic injection	Phase I	20 in 2 groups	2012	121
Peanut	recombinant modified proteins	modified rAra h 1, 2, and 3 in <i>E. coli</i> cells	rectal administration	Phase 0	10	2013	122
Carp	recombinant hypoallergen	modified rCyp c 1	subcutaneous injection	Phase I/IIa	16	2016	124

WT, wild-type; FV, folding variant; r, recombinant; n, natural.

lergen to produce molecules that lack IgE binding due to changes in their 3-dimensional structure. The major allergen Phl p 2 from Timothy grass pollen was converted into a mosaic by reassembling its peptides in a changed order [66]. The recombinant mosaic protein had lost its IgE reactivity but induced high levels of IgG in mice and rabbits that inhibited IgE binding from allergic patients to group 2 allergens from Timothy grass and other grass species. Similarly, a rearranged mosaic of Phl p 1 displayed a reduction in IgE reactivity >85% and induced inhibitory IgG in immunized rabbits [67]. Three recombinant mosaic proteins were derived from Bet v 1 and displayed a strongly reduced IgE reactivity and allergenic activity [68]. The immunological properties of the de-

signed mosaic proteins cannot always be predicted. Only 3 out of 7 mosaic proteins derived from the major cat allergen Fel d 1 possessed the desired hypoallergenic characteristics [69].

Recombinant Allergen Chimeras/Allergen Hybrids

Chimeric proteins or hybrid proteins are created by joining the genetic information of at least 2 different proteins. Such constructs may contain parts of or the complete original proteins. Hybrid molecules are ideal for producing vaccine candidates for allergen sources that contain several important allergens, such as house dust mite, grass pollen, or insect venoms. Hybrid molecules increase the immunogenicity and reduce the number of

molecules that need to be produced and quality-checked [70]. Asturias et al. [71] produced a hybrid molecule derived from the house dust mite allergens Der p 1 and Der p 2 which had a significantly lower potency to induce reactions in SPTs than the individual allergens, and even induced higher T cell proliferation responses than an equimolar mixture of Der p 1 and Der p 2. Chen et al. [72] engineered hybrids from Der p 1 and Der p 2 and also replaced the cysteines with serines, showing that these molecules could be used safely for both tolerance and vaccination approaches. Linhart et al. [73] produced hybrid mosaic hypoallergens derived from the Timothy grass pollen allergens Phl p 2 and 6, and then, in another study [74], from Phl p 1, 2, 5, and 6. In both cases, the hybrid molecules displayed an increased immunogenicity by inducing higher allergen-specific IgG titers in immunized animals than a mixture of the wild-type allergens. The homologous venom allergens Ves v 5 from the yellow-jacket and Pol a 5 from the paper wasp have only low degrees of cross-reactivity. Hybrids containing different segments of these allergens can therefore be used to map discontinuous B-cell epitopes but have also been shown to be useful for immunotherapy due to their reduction in allergenicity [75]. Karamloo et al. [76] could show that a hybrid consisting of fragmented sequences of the 3 bee venom major allergens Api m 1, 2, and 3 had preserved T-cell epitopes, had lost the B cell epitopes of all 3 allergens, and possessed a highly reduced IgE cross-linking ability on human mast cells and basophils as well as a strongly reduced skin test reactivity. The 2 major allergens of Japanese cedar (*Cryptomeria japonica*) pollen, Cry j 1 and Cry j 2, were expressed as a fusion protein and then conjugated to polyethylene glycol to improve their solubility and to create a safer vaccine [77]. Treatment of mice and monkeys with this vaccine resulted in a significant increase of Cry j 1-specific IgG. In general, allergen fusions allow the production of multiallergen vaccine components as a single molecule, thus facilitating their manufacture, purification, and characterization [78].

Recombinant Allergens Modified by Mutations

Genetic engineering of genes coding for allergens requires the knowledge of their B- and T-cell epitopes and, ideally, also their 3-dimensional structure. Thus, mutations of an allergen sequence can be used to alter its functions and immunological properties.

Ferreira et al. [79] compared amino acid substitutions in several Bet v 1 isoallergens and homologs by an algorithm developed to predict residues important for IgE binding. A Bet v 1 6-point mutant, developed by site-

directed mutagenesis, exhibited extremely low reactivity with serum IgE from birch pollen-allergic patients. Its ability to induce reactions in SPTs was significantly lower than for wild-type Bet v 1. In addition, the mutations had not influenced T cell recognition.

The IgE recognition of allergenic members of the EF-hand superfamily of calcium-binding proteins is influenced by the presence of bound calcium, as has been shown for polcalcins [80] and the fish allergen parvalbumin [81, 82]. The EF-hand calcium-binding domains were disrupted resulting in recombinant hypoallergens for the birch pollen allergen Bet v 4 [83], the *Brassica* pollen allergen Bra r 1 [84], and the carp allergen Cyp c 1 [82]. All 3 hypoallergens displayed reduced IgE-binding activities. Cyp c 1 was analyzed in an SPT and induced significantly fewer skin reactions than wild-type Cyp c 1. Mouse IgG raised by immunization with mutated Cyp c 1 cross-reacted with parvalbumins from various fish species and inhibited the binding of the IgE in fish-allergic patients to the wild-type allergen.

Disulfide bonds stabilize the structure of several important allergen families and have therefore been a target of site-directed mutagenesis experiments. Hypoallergens displaying a decreased IgE-binding activity and a conserved T cell reactivity were generated for the nonspecific lipid transfer protein Par j 1 from *P. judaica* pollen [85] and the major latex allergen Hev b 6.02 [86]. Disulfide bonds present in the group 2 allergens of mites were also destroyed by site-directed mutagenesis to destabilize the antigenic structure and ablate IgE binding of Der p 2 [87], Der f 2 [88], and Lep d 2 [89, 90].

The introduction of point mutations in several peanut allergens was based on detailed studies of their IgE epitopes. For Ara h 1, 4 linear immunodominant IgE epitopes were identified by screening overlapping synthetic decapeptides [91]. Likewise, 10 linear IgE epitopes were identified in the Ara h 2 sequence [92], and 4 in the Ara h 3 sequence [93]. Amino acids critical to each IgE epitope of these 3 peanut allergens were changed by site-directed mutagenesis [94–96]. The resulting recombinant proteins had a lower capacity to bind patients' IgE but retained the ability to stimulate T cell proliferation. Beezhold et al. [97] produced a recombinant latex hypoallergen Hev b 5 by simultaneously replacing the critical amino acid residues in 8 IgE epitopes to achieve a significantly reduced IgE-binding activity. Karisola et al. [98] produced 29 Hev b 6.02 mutants to identify 6 IgE-interacting residues in the sequence of this latex allergen. SPT reactivity of the 6-residue mutant was completely abolished for all patients examined in the study.

Based on B- and T-cell epitope mapping studies and sequence comparison of group 5 allergens from different grasses, point mutations were introduced by site-directed mutagenesis in the highly conserved sequence domains of Lol p 5, the group 5 allergen from ryegrass [99]. The authors succeeded in producing rLol p 5 mutants with low IgE-binding capacity and reduced allergenic activity, determined by basophil histamine release and SPTs in allergic patients. Two amino acid positions in the egg-white allergen Gal d 1 have been identified as crucial for IgE binding as they contribute substantially to the structural integrity of the protein [100]. Sequence and fold analysis of members of the Bet v 1 family identified a short sequence stretch in Bet v 1, susceptible for mutations to induce an altered fold of the entire molecule [101]. The replacement of 7 consecutive amino acids of Bet v 1 by the homologous Mal d 1 sequence resulted in the loss of the Bet v 1-like fold and a drastic reduction in binding IgE in birch pollen-allergic individuals.

Recombinant Allergens in Clinical Studies

AIT is the only specific and disease-modifying treatment of allergy. The difficulty in preparing safe and effective vaccines from natural allergen extracts represents the main limitation of AIT [102]. Vaccines based on recombinant wild-type allergens or recombinant hypoallergenic allergen derivatives offer the solution to this problem and have therefore entered into clinical testing more than a decade ago [103]. Today, the structures of most of the important allergens are known and a wide array of recombinant hypoallergenic derivatives has been engineered [104]. As shown in Table 3, a number of these hypoallergenic molecules have already been tested in clinical trials [105]. However, there is still a strong need for further research in the area of recombinant allergens and allergen derivatives to increase the efficacy and safety of AIT [106].

Birch Pollen Allergy, Bet v 1

In 2000, Pauli et al. [107] compared the allergenic activity of rBet v 1 with rBet v 1 fragments, an rBet v 1 dimer, and an rBet v 1 trimer. In SPTs on 29 patients and in intradermal injections of 30 patients, the hypoallergenic derivatives of Bet v 1 showed a reduced capacity to induce immediate-type skin reactions. The results of the first double-blind, placebo-controlled AIT study in 124 birch pollen-allergic patients were published by Niederberger et al. [108] in 2004. One preseasonal treatment course

with the 2 rBet v 1 fragments or the rBet v 1 trimer induced protective allergen-specific IgG antibodies and resulted in the reduction of cutaneous sensitivity as well as an improvement of symptoms in actively treated patients. A significant reduction in the rise of allergen-specific IgE induced by seasonal pollen exposure was also observed. Analysis of the nasal secretions of 23/124 patients showed the induction of Bet v 1-specific IgG1, IgG2, and IgG4, and low IgA levels [109]. The observed reduced nasal sensitivity to natural Bet v 1 was significantly associated with the induced antibody levels. However, the increase in Bet v 1-specific IgG levels resulted in the improvement of birch pollen-associated OAS symptoms only in 7 of 25 patients assessed after the vaccination with either the Bet v 1 fragments or Bet v 1 trimer [110].

In 2008, Pauli et al. [111] published the comparison of a recombinant wild-type Bet v 1 vaccine, a standard birch pollen extract and natural purified Bet v 1 in a randomized, double-blind, placebo-controlled trial of 134 patients. All groups demonstrated significant and equal improvements in symptoms, medication use, and skin test reactivity in 2 pollen seasons. Clinical improvements were accompanied by marked increases in Bet v 1-specific IgG1, IgG2, and IgG4 levels, which were highest in the rBet v 1-treated group. It was clearly shown that rBet v 1 could replace the birch pollen extract in the AIT treatment of patients. Interestingly, the extract-based treatment led to de novo induction of IgE against Bet v 2, the birch pollen profilin, in 3 patients. The safety and efficacy of rBet v 1 formulated as a sublingual tablet were assessed in a multicenter, double-blind, placebo-controlled study conducted in 482 birch pollen-allergic patients [112]. The average adjusted symptom scores were significantly decreased in comparison to placebo in individuals receiving rBet v 1 tablets once daily for 5 months.

A stable monomeric and hypoallergenic folding variant of Bet v 1 (rBet v 1-FV) was produced by changing the buffers of the chromatographic steps during the purification of rBet v 1 expressed in *E. coli* [113]. Thirty adult birch pollen-allergic individuals were treated with rBet v 1-FV for 10 weeks with weekly injections of the hypoallergen [114]. Upon exposure to birch pollen in an environmental exposure chamber, a significant decrease in the total symptom score was observed. No serious adverse events occurred. A preseasonal, randomized, controlled phase II study compared the effects of subcutaneous AIT with rBet v 1-FV or an established natural birch pollen extract [115]. No statistical difference between rBet v 1-FV and the pollen extract was observed regarding the beneficial effects of the AIT. rBet v 1-FV could even be

administered in higher doses than the native protein with no increase in adverse effects.

Grass Pollen Allergy, Phl p 1, 2, 5a, 5b, and 6

A study published in 2005 by Jutel et al. [116] determined the effectiveness of a mixture of 5 recombinant wild-type Timothy grass pollen allergens, rPhl p 1, rPhl p 2, rPhl p 5a, rPhl p 5b, and rPhl p 6, in reducing symptoms and the need for symptomatic medications. This randomized, double-blind, placebo-controlled study of subcutaneous-injection immunotherapy showed a significant improvement in the symptom medication score in subjects receiving the recombinant allergens compared to the placebo group. Conjunctival provocation showed a clear trend in favor of the active treatment which was based on the pronounced development of allergen-specific IgG1 and IgG4. A randomized, double-blind, placebo-controlled, dose-ranging study was performed in 50 patients with a mixture of 5 recombinant *Phleum* pollen allergens [117]. Eight patients experienced systemic reactions grade 1 or 2, but none experienced grade 3 or 4. Hence, this first, dose-ranging safety study with Timothy grass pollen allergens showed no major side effects, even in the very high dose range.

Very recently, a randomized, double-blind, placebo-controlled AIT study of grass pollen allergy using a recombinant B-cell epitope-based vaccine was published [118]. The vaccine, called BM32, contained recombinant fusion proteins that consisted of the hepatitis B virus (HBV) surface protein preS domain as an immunogenic carrier and peptides derived from the Timothy grass pollen allergens Phl p 1, 2, 5, and 6. Sixty-eight patients completed the trial. The total nasal symptom score decreased significantly, the total ocular symptom score and SPT reactions showed a dose-dependent decrease, and only a few grade 1 systemic reactions occurred. BM32 induced highly significant allergen-specific IgG but no IgE responses. As a side effect of this grass pollen AIT, but of high interest, Cornelius et al. [119] reported, in all of the 7 recipients of the BM32 vaccine tested so far, the induction of HBV-neutralizing antibodies. Although the number of vaccine recipients evaluated was small and no titers were determined, this study showed that the preS domain alone could induce HBV-neutralizing antibodies [120].

Cat Allergy, Fel d 1

The recombinant major cat dander allergen Fel d 1 was linked to a cell membrane translocation sequence and to a truncated invariant chain for targeting the MHC class II pathway, creating the modular antigen transporter

(MAT)-Fel d 1 fusion protein [121]. In a randomized, double-blind, placebo-controlled trial, intralymphatic immunotherapy with rMAT-Fel d 1 was performed on 20 patients. After only 3 intralymphatic injections of rMAT-Fel d 1 within 2 months, nasal tolerance increased up to 74-fold and cat-dander-specific IgG4 was increased 5.7-fold. This first-in-human clinical study of intralymphatic immunotherapy was regarded as safe and efficient in inducing allergen tolerance.

Peanut Allergy, Ara h 1, 2, and 3

cDNAs coding for 3 recombinant peanut allergens (Ara h 1, Ara h 2, and Ara h 3) were modified to disrupt common IgE-binding sites [94, 95]. The modified recombinant allergens were separately expressed in *E. coli*, and remained inside the cells when these were subsequently killed using heat and phenol [122]. The 3 resulting cell suspensions were combined to form the rectally administered vaccine EMP-123 with the goal of inducing tolerance to the dominant peanut allergens. Ten peanut-allergic individuals received escalating weekly doses over 10 weeks, followed by 3 biweekly maximal doses. The rectal administration of EMP-123 resulted in mild rectal symptoms in 1 patient and adverse reactions in 5 patients, including 2 anaphylactic reactions. Due to the unexpected frequency and intensity of these adverse reactions, to date, no further development of the vaccine has been reported by the company Allertein.

Peach Allergy, Pru p 3

The EU-funded FAST (Food Allergy Specific Immunotherapy) project was aimed at developing a safe and effective subcutaneous immunotherapy for persistent and severe allergy to fish and peach [123]. For peach, FAST focused on its major allergen, the nonspecific lipid-transfer protein Pru p 3. Two rPru p 3 mutants (substitutions of surface-exposed amino acids important for IgE binding and the destruction of disulfide bridges), 2 chemically modified rPru p 3 molecules (reduction/alkylation and glutaraldehyde treatment) and a naturally occurring hypoallergen, rFra a 3 (from strawberry) were prepared and extensively tested. Of these 5 candidate molecules, 2 failed on hypoallergenicity, 2 on immunogenicity and stability, and 1 on solubility [124]. Subsequently, 2 trimeric molecules were designed with all 8 cysteines replaced by serines, 1 heat-treated wild-type rPru p 3 was prepared, and 2 mutants were produced with 4 (i.e., 8 in all) surface-exposed amino acids residues exchanged [124]. Hypoallergenicity could be achieved at the cost of immunogenicity and stability. From these results, the au-

thors concluded that Pru p 3 is a molecule that loses its immunogenicity when its structure is altered, and they decided to abandon its further clinical development.

Fish Allergy, Cyp c 1

For the development of immunotherapy for fish allergy, the FAST project focused on the major allergen from carp, the highly cross-reactive parvalbumin Cyp c 1 [123]. Hypoallergenic mutants of Cyp c 1 had been previously produced by mutating the 2 functional calcium-binding sites which are responsible for most of the IgE binding of the molecule [81, 82]. The FAST project selected the mutant Cyp c 1, in which both calcium-binding sites had been destroyed for the development of a hypoallergenic vaccine for fish AIT. Purified mutated rCyp c 1 behaved as a stable and folded molecule that had retained its immunogenicity and displayed, on average, a 1,000-fold reduction of IgE binding when tested with sera from 26 fish-allergic patients [125]. Mutated rCyp c 1 was tested in Denmark in 2013/2014 in a first-in-human, randomized, double-blind, placebo-controlled clinical study involving 16 fish-allergic subjects [124]. Due to the low level of side effects and the positive immunological response, a multicenter clinical trial in 9 clinical centers in 6 countries was initiated in October 2015.

Recombinant Chimeric Allergens for Monitoring Clinical Studies

The determination of conformational antibody epitopes on allergens is still a challenge, yet of high interest for monitoring the development of allergen-specific antibody repertoires during AIT. Epitope grafting from allergens onto scaffolds of low-allergenic homologs is an elegant approach to a solution for this problem. Four distinct areas of Bet v 1, representing in total around 80% of its surface, were separately grafted onto the scaffold of its celery homolog Api g 1 to yield the chimeras Api-Bet-1–4 [26]. Sera from 64 birch pollen-allergic patients were tested for the presence of chimera-specific IgE. The highly patient-specific IgE recognition profiles revealed that the IgE response to Bet v 1 is polyclonal and that the epitopes are spread across the entire Bet v 1 surface. The 4 Api-Bet chimeras were then applied to monitor the development of the Bet v 1-specific IgE, IgG1, and IgG4 repertoires in narrow time intervals during 3 years of AIT in 11 subjects [126]. In the majority of patients, Bet v 1-specific IgE levels increased during the early phase of treatment, followed by a gradual decrease. The overall IgE epitope di-

versity during AIT remained constant. IgE also showed the highest epitope diversity among the immunoglobulin classes investigated. All patients had Bet v 1-specific IgG4, and 7 patients had Bet v 1-specific IgG1. IgE, IgG1, and IgG4 recognized different epitope profiles. Notably, the epitopes represented by the chimera Api-Bet-3 (the C-terminus of Bet v 1 plus surrounding residues) played a prominent role in the recognition of the allergen by all studied immunoglobulin classes.

Conclusions

Recombinant allergens have considerably advanced our knowledge of the immune mechanisms of allergic diseases as well as vastly enriched our toolbox for diagnosis and therapy. While recombinant allergens have contributed significantly to allergen standardization, diagnosis, structural biology, and epitope mapping, it is still an open issue whether and when recombinant AIT will replace extract-based immunotherapy. There are 2 main aspects to consider, the regulatory aspects of AIT and the quality of the recombinant allergens. The European Community has not yet produced regulations for AIT with recombinant allergens that would be compelling for all member states. Bonini [127] states in his 2012 article that the guidelines produced by the European Medicines Agency, such as that on the production and quality issues of allergen products (EMA/CHMP/BWP/304831/2007), are only for orientation. Likewise, position papers published by expert panels or scientific societies, such as the EAACI, are of no regulatory value. In addition, recombinant allergens and hypoallergens will remain unavailable in many countries due to the strict regulatory criteria that need to be met. Hence, it is unlikely that novel allergenic molecules will be available in the near future for use as *in vivo* clinical diagnostic and immunotherapy reagents.

On the other hand, the recombinant production of certain allergens still needs to overcome technological hurdles. The EU-funded CREATE project, which aimed to evaluate the use of recombinant allergens as reference material, showed that some recombinant allergens only possess a mean of 50–80% of the IgE-binding capacity of their natural counterparts [128]. The absence of post-translational modifications could result in a decrease of the IgE-binding capacity, as was shown for the peanut allergen Ara h 2 [129]. A recent study on the expression of Ara h 1 and Ara h 2 in the chloroplasts of the unicellular eukaryotic green alga *Chlamydomonas reinhardtii* found that even the algal-produced recombinant allergens had

a reduced affinity for IgE compared to the native proteins [130]. Hypoallergenic variants of Ara h 1, 2, and 3 still produced frequent adverse side effects when administered to patients, resulting in the termination of any further development of a therapy for peanut allergy based on recombinant allergens [122]. Two companies have turned to natural peanut proteins in their peanut allergy drug formulations, and they have tested an oral peanut protein formulation and an epicutaneous patch that delivered peanut proteins via the skin [131]. For both drugs, desensitization was lost within weeks if patients terminated the treatment. Nevertheless, as the technology of the production of recombinant proteins is refined, they will become an increasingly attractive alternative to native allergens for allergy diagnostics and immunotherapy.

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The authors declare that they have no conflicts of interest with respect to the information presented in this article.

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