

From allergen structure to new forms of allergen-specific immunotherapy

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During the past decade, genetic information for most of the common allergens has been obtained. Using these genetic blueprints it has become possible to reconstruct, by recombinant DNA technology, almost complete repertoires of the relevant allergens and their epitopes. Recombinant allergens with the allergenic features of naturally occurring allergens have promoted allergy research and form the basis of new multiallergen tests for refined allergy diagnosis. Allergen derivatives with reduced allergenic activity have also been produced by recombinant DNA technology to increase safety and specificity of allergen-specific immunotherapy. These derivatives can be engineered to contain relevant T cell epitopes and to maintain those sequence motifs which are required for inducing protective antibody responses and therefore hold great promise for improving allergen-specific immunotherapy.

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Abbreviations

3D three-dimensional

CRD component-resolved diagnosis

Introduction

Type I allergy represents an immunoglobulin E-mediated hypersensitivity disease (e.g. allergic rhinoconjunctivitis, allergic asthma, atopic dermatitis, food allergy, anaphylaxis) affecting almost 25% of the population. The symptoms of allergy are caused by the recognition of normally harmless antigens (i.e. allergens) by IgE antibodies, which induces a consecutive cascade of cellular activation. Although several of the most common allergens have been partly characterised by proteinchemical techniques in the past, the precise molecular nature of most disease-eliciting allergens remained enigmatic until recombinant DNA technology was applied to allergen characterisation in the late eighties. Since then, our knowledge regarding the molecular nature of many important allergens has rapidly increased (reviewed in [1•]). This fact is illustrated by Figure 1, which shows the increasing number of scientific publications that could be retrieved under the terms 'recombinant and allergen' from Medline.

The first allergen-encoding DNA sequences were published between 1988–1991, as indicated in the timeline in

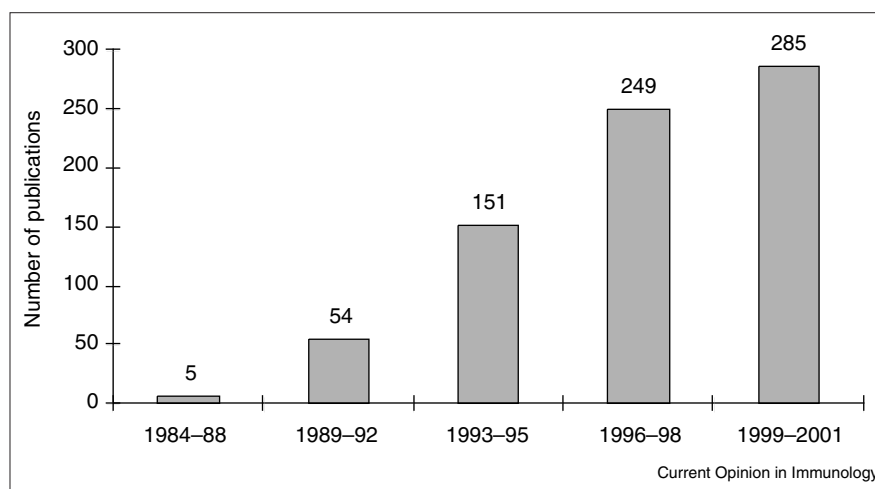
Figure 2 [2–4]. Shortly thereafter, recombinant allergens were produced by expression of allergen-encoding cDNAs mainly in prokaryotic (*Escherichia coli*) expression systems and then tested for their IgE-binding capacity [5,6], and for their ability to induce specific activation of T cells [7–10] and basophils [11–13]. Most of the recombinant allergens showed the immunological characteristics of the corresponding natural allergens and therefore could be used for *in vitro* diagnosis of allergy (reviewed in [14]). Furthermore, they represented useful tools for basic research in molecular and cellular allergology.

Subsequently, recombinant allergens were used to establish animal models for Type I allergy and to explore therapeutic strategies, based on T cell epitope-containing peptides [15–20]. Between 1994–1995 the first recombinant allergens were successfully used for *in vivo* diagnosis of Type I allergy in patients by skin testing ([21–24], reviewed in [25]). These skin test studies confirmed the previously conducted serological studies by showing that recombinant allergens exhibited specific *in vivo* reactivity comparable to natural allergens and they also allowed the determination of sensitisation profiles of patients. Although *in vivo* provocation testing is necessary to confirm clinically relevant sensitisation to certain allergen molecules it is not suitable for determining sensitivity towards a large panel of recombinant allergen molecules. For this reason multiallergen tests based on microarrayed recombinant allergens have been recently developed that allow the complex IgE reactivity profile of patients to be established using tiny amounts of serum in single determinations [26••].

The first three-dimensional (3D) allergen structures solved by X-ray crystallography and NMR were published in 1996 ([27,28], reviewed in [1•]). The structural analysis of allergens was disappointing to some degree, as it failed to unambiguously identify structural motifs or determinants that are indicative of allergenicity. However, structural analysis of allergen folds by circular dichroism explained the reduction of allergenic activity of recombinant hypoallergenic allergen derivatives as being due to either loss of fold or alteration of surface-exposed amino acids potentially involved in IgE recognition [1•]. Even before the first 3D structures of allergens became available it was demonstrated that fragmentation of allergens by recombinant DNA technology yielded hypoallergenic fragments [29]. Furthermore, the occurrence of allergen isoforms with reduced IgE-binding capacity which differed from IgE-reactive isoforms was reported [30]. Soon after this report was published, the first recombinant allergen variants with reduced allergenic activity were reported and suggested as hypoallergenic candidate molecules for safer forms of

Figure 1

Number of publications found in Medline under the terms 'recombinant and allergen'. Searched periods are displayed on the x-axis. The y-axis indicates the number of publications.



allergen-specific immunotherapy [31–37]. They represented either recombinant hypoallergenic isoforms, mutant proteins or fragments with reduced IgE binding capacity and preserved T cell stimulatory activities. Soon after the *in vitro* studies, *in vivo* provocation testing in allergic patients confirmed the reduced allergenic activity of several of the recombinant allergen derivatives [38–44] and the first clinical immunotherapy study in birch pollen allergic patients was recently initiated with recombinant hypoallergenic derivatives of the major birch pollen allergen Bet v 1.

The impact of allergen structure on basic science, standardisation, diagnosis, prevention and therapy in allergy

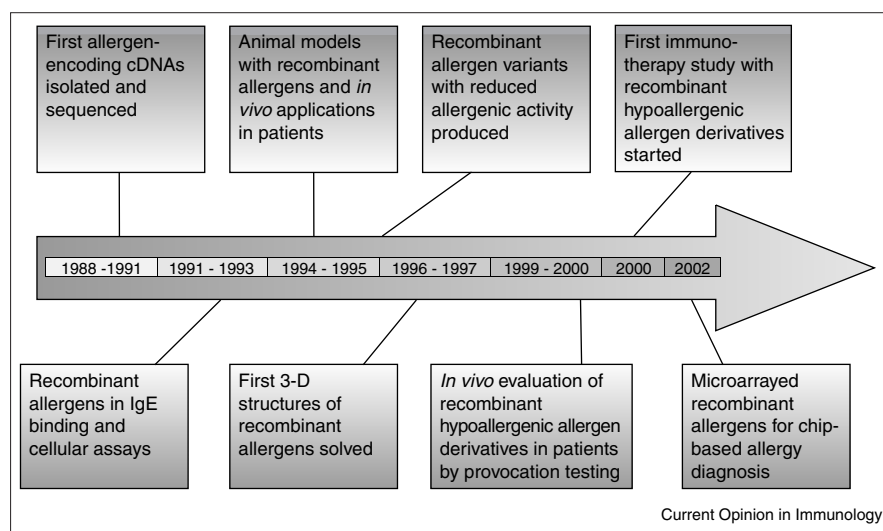
Several recently published studies continue to demonstrate the impact of detailed knowledge about allergen structure on basic and applied research in the field of allergy (Figure 3). For example, the analysis of the 3D structure of a complex between a mouse monoclonal antibody and the major birch pollen allergen, Bet v 1, visualises that surface-exposed amino acids that are conserved among the cross-reactive allergens from pollens of trees belonging to the *Fagales* (birch, alder, hazel) are critically involved in cross-reactivity [45]. Surprisingly, it was found, using the major timothy grass pollen allergen Phl p 5, that rather small (approximately 10 kDa) allergen fragments can accommodate binding sites for several distinct IgE antibodies and hence are potent triggers of basophil cell activation, as long as they fold accurately [46,47]. Another feature of the recognition of allergens by antibodies from symptomatic allergic patients seems to be the dissociation of epitopes recognised by IgE and antibodies belonging to classes (e.g. IgA) or subclasses (e.g. IgG₁₋₄) other than IgE [48]. Studies performed with recombinant pollen allergens suggest that allergen-specific IgE sensitisation against respiratory allergens is elicited or at least triggered by exposure to pollen allergens during the first years of life and appears to be a result of a non-sequential class-switch

mechanism [49,50]. Immunological cross-inhibition studies performed with recombinant pollen and plant-food allergens strongly suggest that, in many cases of plant-food allergy, pollen allergens act as the primary sensitising antigens and that associated food allergy is a result of immunological cross-reactivity with pollen allergens, rather than sensitisation via the gastrointestinal tract [51,52]. The importance of provocation testing is emphasised by a study conducted with purified recombinant allergens showing that the magnitude of clinical sensitivity as determined by skin prick and nasal provocation testing does not always correlate with levels of circulating allergen-specific serum IgE antibodies [53]. Moreover, it was demonstrated that synthetic T cell epitope-containing peptides lacking IgE binding sites prepared according to the sequence of the major cat allergen, Fel d 1, could induce MHC-class II-restricted T cell activation resulting in clinically relevant late phase symptoms (e.g. asthma; [54]).

Recombinant allergens have proven to be useful tools for establishing and calibrating assays for allergen quantification (Figure 3; [55]). These assays may be also used to identify hypoallergenic products and to develop preventive measures of allergen avoidance. In quite original approaches it has been shown that genes coding for major plant allergens can be silenced to yield hypoallergenic plants [56,57].

Perhaps even more important was the impact of recombinant allergens on diagnosis and therapy (Figure 3). Several studies have shown that recombinant allergen molecules resemble the epitope complexity of natural allergen extracts and hence may replace these extracts for diagnostic purposes [58–61]. Furthermore, it has been demonstrated that only component-resolved allergy diagnosis performed with purified allergens can identify the disease-eliciting allergens, whereas allergen extract-based diagnosis only allows identification of allergen-containing sources [61]. Detailed knowledge about the DNA, the amino acid

Figure 2



Timeline: from allergen structure to new diagnostics and therapeutics. Important steps made between 1988 and 2002 in the recombinant allergen field have been highlighted.

sequence and the 3D structure of allergens has also formed the basis of several new therapeutic approaches which have entered clinical evaluation (reviewed in [62–66]).

Component-resolved allergy diagnosis: prerequisite for improving the selection of patients for different forms of therapy

The concept of component-resolved diagnosis (CRD) is based on the use of purified, mainly recombinant allergen molecules for allergy diagnosis [61]. Using purified allergen components it is possible to delineate the reactivity profile of a given allergic patient and thus to identify the disease-causing allergen molecules. Currently used diagnostic allergen extracts consist of undefined mixtures of allergenic and non-allergenic molecules and can only be used to determine if a patient reacts to a particular allergen-containing source. Advantages of CRD are the greater precision of diagnosis and the possibility to discriminate between co-sensitisation and cross-sensitisation [61,67]. Using recombinant allergens it can be determined whether a patient is co-sensitised against allergens from different allergen sources or cross-reacts with allergens that are present in the sources.

Purified recombinant allergens have been applied to the serological diagnosis of grass pollen, venom, cat and food allergy [60,68–70], and to establish diagnostic tests measuring effector cell activation via expression of activation markers (e.g. CD203c) [71]. Furthermore, recombinant allergens have been successfully used for skin test diagnosis for mould, pollen, mite and latex allergy [21–24,72–74].

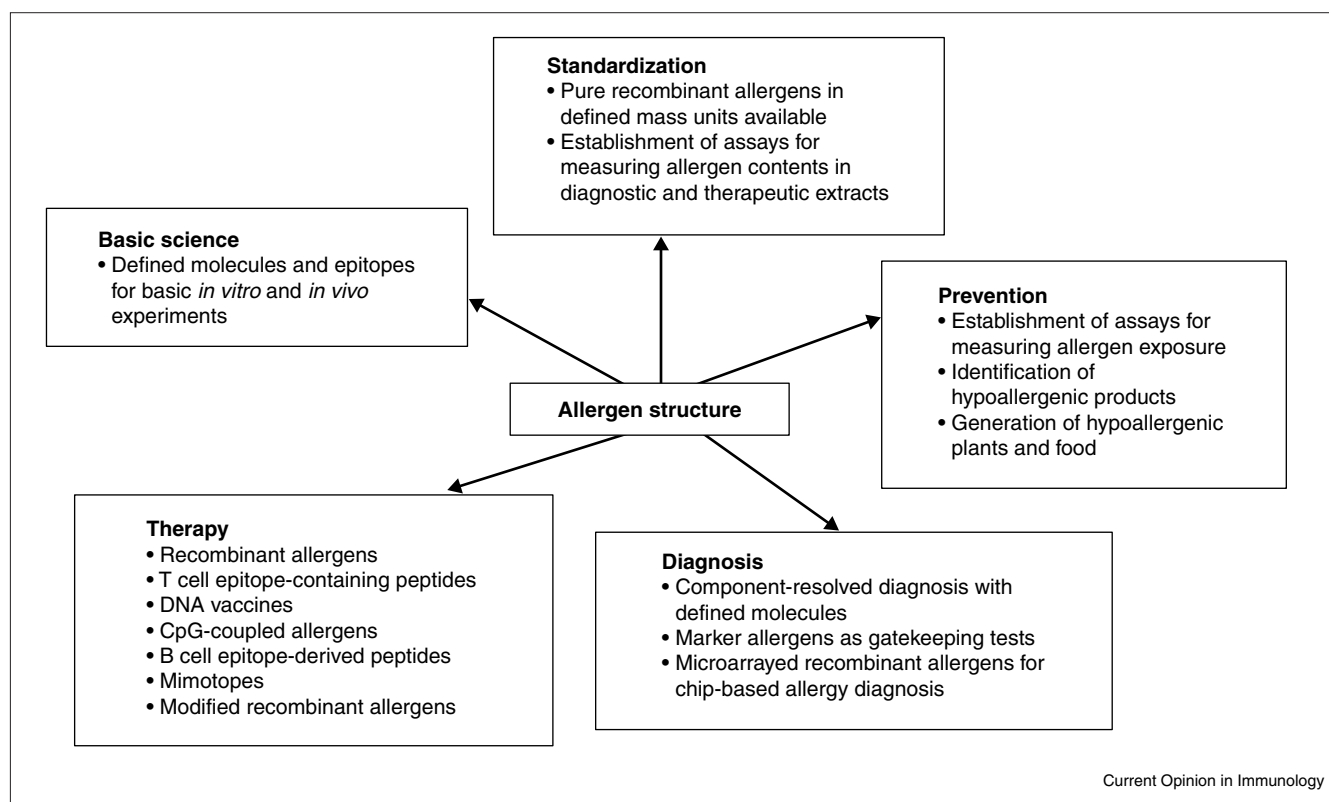
Although provocation testing is important to determine the clinical significance of IgE-mediated sensitisation towards particular allergens it is cumbersome to perform *in vivo* testing with large panels of recombinant allergen molecules.

Very recently the principle of microarray technology has been applied to facilitate CRD. It was demonstrated that a panel of close to hundred recombinant allergen molecules comprising the most common allergen sources could be immobilised by microarray technology on a chip to allow determination of complex IgE reactivity profiles by single measurements requiring only minute amounts of serum [26••].

Several recombinant marker allergens have been characterised that can be used as specific diagnostic markers for sensitisation to a particular allergen source, as markers indicative for a certain disease type, or as cross-reactive marker allergens to predict cross-sensitisation to several different allergen sources [75–89]. Using marker allergens for grass or birch pollen sensitisation it has been possible to analyse the development of allergy in early childhood and thus to reveal the importance of early postnatal contact with respiratory allergens to the sensitisation process [49•,50].

The analysis of different European populations and of an African population with recombinant allergens has demonstrated significant differences in the IgE reactivity profiles of different populations which were not apparent when allergen extracts were used for testing [90,91]. The different reactivity profiles in the various populations seemed to be caused by the characteristics of the local allergen exposure and therefore emphasises the importance of CRD in choosing correct therapeutic measures for different populations of allergic patients. For this reason, the use of diagnostic tests containing the major and rather species-specific allergens of a given allergen source together with highly cross-reactive allergen found in various allergen sources has been suggested to select a more specific immunotherapy for selected patients [92]. The analysis of IgE antibody responses in patients who had received immunotherapy

Figure 3



The impact of allergen structure on basic science, standardisation, prevention, diagnosis and therapy in the field of allergology.

with natural allergen extracts revealed new therapy-induced sensitisation's to allergens present in the administered therapeutic vaccine against which the patients had not been sensitised before the treatment [93,94*]. Although the clinical relevance of the newly induced IgE-specificities has not yet been investigated, these studies strongly suggest that immunotherapy with crude allergen extracts can induce *de novo* sensitisations that may not only be responsible for treatment failure but may also even contribute to a broadening of the patients sensitisation spectrum.

Allergen structure as a basis for various approaches towards allergy vaccination: searching for the best vaccines

Allergen-specific immunotherapy represents in principle the only specific and causative treatment of IgE-mediated allergies. Numerous clinical studies document the efficacy of this treatment but several disadvantages exist [95]. In view of the rapid development in the field of recombinant allergens it appears possible to replace the currently used, ill-defined allergen extracts by pure recombinant allergens. Several review articles demonstrate that the relevant allergens of many common allergen sources have been produced by recombinant DNA technology [96–101]. The question of whether the relevant disease-eliciting allergens have been obtained as recombinant molecules can be addressed by simple IgE-inhibition studies investigating to what extent

the recombinant allergens contain the repertoire of IgE epitopes present in natural allergen extracts [58,59]. We would advocate that the selection and reconstruction of the molecules containing the majority of the disease-eliciting epitopes must be the basis for the development of any kind of antigen-specific therapeutic strategy; an effective and specific therapeutic vaccine may only be obtained if it is based on the structure of the disease-eliciting allergen molecules. Recombinant allergens mimicking the natural allergens could be used as such for immunotherapy. They would also allow a treatment to be tailored to the sensitisation profile of the patient, which would minimise the possibility of therapy-induced *de novo* sensitisations (i.e. component-resolved immunotherapy; CRIT [61]). However, the retained allergenic activity of recombinant wild-type-like allergens may be a disadvantage leading to allergenic side effects during treatment. Therefore, several other approaches based on allergen structures have been used to try to obtain allergen derivatives with reduced allergenic activity (reviewed in [62–66]).

The attractive idea of using allergen-derived synthetic peptides containing T cell epitopes without IgE-reactivity and thus lacking immediate type reactivity for tolerance induction has been followed-up by clinical trials in patients [102–105]. Several points need attention, however. It will be necessary to identify the relevant T cell epitope-containing

Box 1. Criteria for selection and evaluation of candidate molecules for therapy or prophylactic treatment.

- Selection of molecules containing the majority of the disease-eliciting epitopes.
- Reduction of allergenic activity.
- Maintenance of immunogenicity/tolerogenicity.
- Clinical studies.

peptides of the relevant allergens recognised by the majority of allergic individuals. Furthermore, different modes of application must be considered because the administration of soluble T cell epitope-containing peptides has been shown to cause systemic late-phase reactions [54].

Another approach based on the knowledge of allergen structure is vaccination with allergen-encoding DNA [66]. It has been demonstrated that vaccination with allergen-encoding DNA can induce an allergen-specific Th1 immune response and may antagonise established allergy [106–109]. DNA vaccination circumvents the administration of allergenic proteins, but it has been found in a mouse model that, after DNA vaccination, allergen-encoding DNA is widely spread throughout the body [110].

A second DNA-based approach is the use of oligonucleotides containing CpG motifs (i.e. immunostimulatory DNA sequences comprising a central non-methylated CG oligo-nucleotide flanked by less highly conserved sequences) as Th1-driving adjuvants. These oligonucleotides may be used to reset the immune system towards a Th1 pathway in a non-specific manner, or they can be coupled to allergens and thus may function as Th1-driving adjuvants [66,111,112].

Another strategy for the generation of allergen-specific vaccines is the application of mimotope technology. Using allergen-specific antibodies, phage clones expressing random peptides (i.e. mimotopes) are isolated from a phage library. Mimotopes mimicking IgE binding sites of the allergen may be used for vaccination to focus blocking antibodies against the IgE epitopes. Mimotopes are similar to haptens; they mimic binding sites for only one species of IgE and hence lack allergenic activity [113]. To circumvent the low immunogenicity of mimotope peptides it has been suggested that monovalent coupling to carrier molecules (e.g. streptococcal albumin-binding protein) could be carried out [114].

Another possibility for the induction of protective antibody responses which antagonise IgE recognition of allergens uses synthetic peptides which are directly derived from IgE epitopes of major allergens [115]. The peptides are determined either via experimental determination of IgE epitopes of major allergens, or are selected from surface-exposed areas determined according to the three-dimensional allergen structure. These peptides can be designed to lack any IgE reactivity or allergenic activity and should assume a length sufficient to induce protective antibody responses.

A very promising approach for the production of defined allergy vaccines is the engineering of recombinant allergen derivatives with reduced allergenic activity. One important consideration is that cDNAs encoding the molecules containing the majority of disease-eliciting epitopes should be used as templates for the production of hypoallergenic allergen derivatives, otherwise the engineered vaccine will not protect against the naturally occurring variety of allergens (Box 1). Strategies which have so far been successfully applied include the production of recombinant allergen fragments [34,36,76,116,117*,118], the introduction of mutations [31,33,35,37,39,119–122,123*,124–128], the selection of hypoallergenic isoforms [32], the deletion of allergen portions [77,122,128,129], and the production of defined allergen oligomers [130**]. Furthermore, hybrid allergens consisting of different allergen molecules have been produced [131,132**].

Most of the recombinant allergen derivatives exhibit reduced IgE-binding capacity and retain the ability to stimulate T cell proliferation. In a few cases it was shown that the allergen derivatives induced less mediator release from basophils than the natural wild-type allergens [34,128,130**]. For certain modified allergens it has been demonstrated that they elicited reduced immediate type skin reactions and fewer immediate and late phase reactions in the skin and nose of allergic patients compared to those exposed to the natural wild-type allergens [32,34,37,39,40,118,125,128,130**]. The demonstration of reduced *in vivo* allergenic activity in a representative number of allergic patients is strongly recommended before an allergen-derivative is considered for evaluation in a clinical immunotherapy study.

Another important step for the characterisation of hypoallergenic derivatives is the analysis of the retained immunogenicity in experimental animals. One possibility is to determine if a given allergen derivative can induce IgG antibody responses in animals that recognise the original wild-type allergen and also if these antibodies inhibit the *in vitro* binding of allergic patients IgE antibodies to the wild-type allergen [117*,130**,132**,133]. The experimental animal model allows information to be obtained about whether the newly created immunogen will induce antibody responses that could be beneficial for allergic patients. It is also possible to study the ability of genetically-modified allergens to induce tolerance [134] or protective antibody responses in experimental animal models of Type I allergy, but it must be demonstrated that the results from such models can be extrapolated to allergic patients.

Hypoallergenic derivatives which have been evaluated according to the criteria suggested in Box 1 may then be evaluated in clinical immunotherapy trials in allergic patients.

The advantages of recombinant hypoallergenic allergen derivatives

One major advantage of recombinant hypoallergenic allergen derivatives is that they can be engineered to contain the

relevant T cell epitopes and structures necessary to induce protective antibody responses. This seems to be important because there is evidence for the necessity of both T cell- and B cell- (i.e. antibody responses) mediated effects for successful immunotherapy [135[•]]. It is also possible to produce modified recombinant allergens with reduced allergenic activity in consistent batch-to-batch quality without chemical modification steps or conjugation-procedures that are difficult to control. The consistent reduction of allergenic activity holds the promise that therapy with hypoallergenic allergen derivatives will allow the administration of high allergen doses with a reduced risk of inducing anaphylactic side effects. Furthermore, it will be easier to formulate stable adsorbates with recombinant allergen-derivatives than with small synthetic peptides which are difficult to couple to currently used adjuvants. The fact that it has been shown that both immunogenicity/tolerogenicity and immunological properties (i.e. the induction of certain cytokine profiles) can be altered by genetic engineering of the allergen molecules themselves suggests that it may be possible to circumvent the need for special adjuvants or carrier molecules for vaccine formulation with recombinant allergen derivatives [117[•], 123[•], 130^{••}, 132^{••}, 134]. Based on these advantages, the use of recombinant hypoallergenic allergen-derivatives has been suggested not only for treatment of established allergy but also for prophylactic vaccination [135[•]].

Conclusions

The development of improved vaccines for the specific treatment of allergy represents a major challenge for the future. Detailed knowledge of the structure of the disease-eliciting allergens has been greatly advanced by the application of recombinant DNA technology to allergen characterisation. Most of the common allergens have been produced as recombinant molecules and their genetic blue-prints can now be used for the development of new allergy vaccines. A very promising strategy for the production of allergy vaccines is the conversion of active wild-type allergens into recombinant allergen derivatives with reduced allergenic activity by genetic engineering. cDNAs encoding allergens for the majority of disease-eliciting epitopes can be used as templates before modification by several different strategies (e.g. fragmentation, mutation, deletion, oligomerization and production of hybrids). These derivatives can be designed to maintain T cell epitopes and structures required for the induction of protective antibody responses. Furthermore, it is possible to modulate directly the immunogenic/tolerogenic properties of these molecules by genetic modification, which in principle would even allow the use of adjuvants to be bypassed. The recombinant hypoallergenic allergen derivatives may be used for specific immunotherapy tailored to the sensitisation profile of allergic patients and, perhaps, for prophylactic vaccination.

Update

A multiallergen test based on purified arrayed allergens has been reported recently [136]. The three-dimensional structure of a highly cross-reactive pollen allergen, Phl p 7,

from timothy grass pollen has been determined. The structure of this calcium-binding allergen reveals a novel dimer assembly and provides a basis for the rational design of a hypoallergenic candidate vaccine [137]. Progress in the field of adjuvants is reported in [138]. This study exemplifies with recombinant timothy grass pollen allergen, Phl p 5, that carbohydrate-based particles represent safe and versatile adjuvants for a controlled formulation of allergy vaccines.

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