

Genetically Engineered Vaccines

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The application of recombinant DNA technology to allergen research has provided the sequence information and genetic material to produce new types of allergy vaccines. One general strategy has been to use the knowledge to produce synthetic peptides that represent selected T-cell or B-cell epitopes. The production of genetically engineered allergens provides an alternative strategy to construct hypoallergenic vaccines, which can provide a better and less selected representation of the epitopes. Many strategies have been used to produce such hypoallergens, and their ability to reduce allergenicity has been amply demonstrated by skin and nasal provocation tests. The retention of T cell-stimulating activity has also been demonstrated, and a consistent feature of the vaccines has been, despite the reduced immunoglobulin E (IgE)-binding reactivity, the ability to induce anti-allergen IgG antibody. The lead hypoallergens have been polypeptide fragments and trimeric constructs of the birch allergen Bet v I. A clinical trial with these medicaments has shown the ability to modify IgE and IgG antibody production, skin test reactivity, and symptom scores. This is the first trial of a recombinant allergy vaccine, and it has set a benchmark for further studies. A new generation of hypoallergens is now being produced based on the detailed knowledge of the tertiary structures of the allergens and of the T-cell and B-cell epitopes. The modifications have been made to change the topography of the allergens while retaining a stable, folding structure. In the case of Bet v I, tertiary structures of hypoallergens have been determined. Structurally modeled hypoallergens have been produced for pollen, venom, food, and latex allergens, with promising characteristics from preclinical studies.

Introduction

Allergens can be genetically engineered to increase the safety and efficacy of allergy vaccination. Reduced allergenicity can facilitate the administration of higher and fewer doses of allergen and, by reducing the side effects and length of treat-

ment schedules, can increase patient compliance. It can be particularly important for highly anaphylactic allergens, such as peanut. Modifications can also be made to directly increase the desensitizing ability of allergen. Reduced IgE-binding could, for example, prevent the presentation of allergen via immunoglobulin E (IgE) receptors on dendritic cells or increase allergen capture by macrophages.

Genetically engineered allergens must, on the one hand, have a reduced or altered ability to interact with the immune system, and, on the other, retain the ability to change the immune responses to the target antigenic determinants. Currently, there is no definitive evidence that therapy selectively directed entirely to either the T-cell or B-cell compartments will be effective. The time course of immunotherapy suggests that clinical symptoms are improved before IgG blocking antibodies are induced, but most of the studies have examined responses to extracts of allergen, not to the allergens. Although T cells have been a favored target, the strategy needs to be proven, and it might be a multistep process. Desensitization rapidly reduces T-cell function in most people, but usually takes 3 years to achieve lasting clinical improvement [1]. It should also be noted that subjects undergoing therapy are also exposed to the natural allergen, which could play a role in the desensitizing process.

If blocking antibodies are required, the modified allergens must retain the ability to induce anti-allergen antibodies. It is possible that this can be accomplished with a lower affinity of binding than that required to activate mediator release by the cross-linking of IgE receptors. Blocking could also be achieved by the steric hindrance of antibodies to new determinants on the allergens or antibodies and could exert regulatory effects by altering allergen presentation. The retention of the sequences for T-cell epitopes by genetically modified molecules is simpler to envisage than retaining the conformation required to induce antibody, but the processing and presentation of antigens can be markedly affected by small changes inside and outside of the T-cell epitopes. The modified vaccine, however, does not need to be a single molecule, and, therefore, mixtures can be formulated to contain the critical epitopes, but without the ability to cross-link IgE receptors.

It is critical that the modified allergens can be manufactured and stored in a consistent and stable manner, and that the structure can be verified for quality control. This is unlikely to be achieved with allergens in which the conformation is grossly distorted, although this could be possible with flexible polypeptides that do not aggregate.

Strategies for the Modification of Allergens

Mutation of disulphide bonds

The first strategy to produce a hypoallergenic allergen targeted the disulphide bonds of Der p 2 from *Dermatophagoides pteronyssinus* [2]. The disruption of the bond linking the N and C terminal, which is now known to stabilize the major fold of the molecule, reduced IgE binding 10-fold. Interestingly, mutation of the cysteines stabilizing two smaller loops had even larger effects and reduced the allergenicity 100-fold. Similar strategies for the homologous allergens from *Lepidoglyphus destructor* [3] and *D. farinae* [4] produced a loss of allergenicity and the retention of the ability to stimulate T-cell responses from peripheral blood mononuclear cells (PBMC). Further study on the Der f 2 showed that the mutants could reduce the induction of airway hyperreactivity in mice [5]. It also induced Th1 cytokine response by human PBMC [6], possibly because, as shown by circular dichroism, the mutant lacked structure and was polymerized.

Mutation has been used to disrupt the three disulphide bonds of Par j 1 from the weed *Parietaria judaica*. The modified allergen had reduced allergenicity while retaining the ability to induce mice to produce IgG and T-cell proliferative responses [7]. Removal of the C-terminal cysteine of the lipocalin allergen Bos d 2 from the cow also reduced IgE binding and skin test reactivity without affecting the ability of the allergen to stimulate T-cell clones. Interestingly, however, a mutation in the adjacent residue had even more effect [8].

Targeting predicted antigenic sequences

Smith and Chapman [9] used algorithms to predict hydrophilic and flexible regions from the amino acid sequence of Der p 2, which were then targeted for mutation. A point mutation determined by this method lost IgE binding when substituted with a proline, but this was probably caused by a major disruption of the folding because substitution with more conservative residues had no effect. Substitution of another amino acid, residue 100, affected monoclonal antibody but not IgE binding.

Proline targets

Prolines are critical residues to produce turns in protein structure and can be inserted to disrupt protein structure. Conversely, they can be mutated out. Takai *et al.* [10] mutated out the prolines of Der f 2, which are conserved amongst the group 2 allergens and located next to the disulphide bonds that stabilize the loop structures. The mutants had partially reduced IgE binding and less histamine-releasing activity with only slightly different CD spectra.

Polypeptide fragments

Genetic engineering has been used to chop a number of allergens into two large polypeptides and, thus, completely disrupt their structure. Vrtala *et al.* [11] expressed the two halves of the birch allergen Bet v 1, 1-74 and 75-160, in *Escherichia coli*. The fragments almost completely lacked

allergenicity and exhibited a random coil conformation. Both fragments were nonanaphylactic in humans [12,13] and could induce T-cell proliferation [11]. Importantly, despite their lack of structure, the fragments immunized animals to produce anti-Bet v 1 antibodies that blocked IgE binding to Bet v 1 [14,15]. Reduced allergenicity was also shown by early- and late-phase reactions to nasal challenge [16]. Studies with skin chambers showed that the fragments induced a similar number of eosinophils to the site of allergen application but they did not release the same amount of inflammatory mediators or cytokines [17]. Vaccination with the fragments has been shown to prevent airway reactivity in mice [18], as has DNA vaccination with genetic constructs encoding the fragments [19]. Pairs of overlapping polypeptide fragments representing the linear sequence of Der f 2 [20] and the grass allergen Phl p 7 [21] have shown similar reduced allergenicity.

Polymerization

Chemical polymerization of allergens has been a long-standing practice used to reduce allergenicity. The genetic equivalent was carried out by joining three Bet v 1 cDNAs together. The trimer retained the natural secondary structure of the allergen as measured by circular dichroism and did not lose T-cell reactivity [22]. It needed 100 times more allergen than natural Bet v 1 to induce skin test reactions [12,13], and induced few symptoms on intranasal provocation [16] or inflammatory mediators in skin-chamber tests [17]. The injection of alum-absorbed trimer, in a course similar to a desensitization regimen for patients, induced IgG antibody that could block the binding of human IgE to natural Bet v 1 [15]. DNA vaccination with the trimer protected mice from the development of allergic sensitization with a transient antibody response [19].

Engineering Out IgE-binding Sequences

Another strategy has been to scan allergens for IgE binding to an array of synthetic peptides that span the allergen sequence, and then to mutate these regions. This was performed for the peanut allergens Ara h 1, 2, and 3. Twenty three IgE-binding regions were found for the 63kDa Ara h 1 [23,24], 10 for the 17 kDa Ara h 2 [25], and only four for the 50 kDa Ara h 3 [26]. It was found that single amino acid substitutions in the peptides could remove most IgE-binding activity [23,24]. Modified allergens were constructed on the basis of the amino acid studies [26,27]. They had greatly reduced IgE-binding, although there was some variability from patient to patient, and they retained the ability to induce T-cell proliferation. The potential efficacy of the three modified allergens has been shown in a mouse model of peanut anaphylaxis [28].

Swoboda *et al.* [29•] performed similar studies for the Lol p 5 grass allergen. IgE-binding regions were identified

by binding to synthetic peptides, and point mutations were introduced into these regions, preferentially targeting residues conserved among the homologous allergens from other grasses. The mutants had low IgE-binding and reduced activity in histamine release assays and reduced skin test reactivity. They retained their ability to induce T-cell proliferation, and, as determined by circular dichroism, their secondary structure was similar to the natural allergen. The latter point is of interest because other approaches have avoided modifying conserved regions on the assumption that they are required for the overall structure of the molecule. The results demonstrated that a few point mutations could generate potential vaccine candidates.

Scanning Site-directed Mutagenesis

Scanning site-directed mutagenesis was used to produce modifications of the hevein chitinase latex allergen Hev b 6.02 [30••]. Twenty-nine mutations were produced across the entire 43-amino acid molecule with only glycines, cysteines, and alanines being excluded. Their small side chains were considered unlikely to significantly contribute to the antigenic structure. Instead of alanine substitution, the replacements were chosen on the basis of molecular modeling with the known structures of the family of hevein domain molecules. Substitutions were made with neutral and small residues or residues with the opposite charge, provided that they would not perturb the overall shape. A six-residue mutant was modeled so that the substitution would only introduce local changes in the tertiary structure. Its allergenicity was completely abolished.

Fusion Proteins

Genetic modifications can add useful functional groups to target receptors for antigen presentation. The production of the cat allergen Fel d 1 linked to the immunoglobulin receptor FcγR1 is an example. It retained its natural antigenicity and targeted the allergen to monocytes [31].

A DNA vaccine encoding a CD4⁺ T-cell epitope of Cry j 2 from Japanese genetically linked to the invariant chain (Ii) of the major histocompatibility complex (MHC) class II molecules for more efficient delivery of the epitope has also been examined [32]. This strategy, which has been used for other vaccines, induced a Th1 response in mice, but the response without the invariant chain was not reported.

Linhart *et al.* [33] explored a different strategy. It was reasoned that fusions of fragments from highly allergenic proteins might increase the immunogenicity of allergen vaccines. Hybrid molecules were constructed from cDNAs coding for the grass pollen allergens Phl p 1, 2, 5, and 6. The hybrids P2-P6, P6-P2, and P5-P1 contained most of the epitopes of natural grass-pollen extract and induced stronger lymphoproliferative responses in T cells of allergic patients than mixtures of the individual allergens. Immu-

nization of mice with the hybrids also yielded more antibody than immunization with the mixtures. The antibodies inhibited the IgE binding, indicating that the hybrids may be a superior vaccine.

Hybrids of Homologous Proteins

Many allergens have homologous counterparts in different species. This offers the possibility of creating hybrid molecules that retain a similar shape but with an altered surface topography. A successful hybrid has been constructed with the Ag 5 venom allergens from the yellow jacket and paper wasp, which have 59% sequence identity and low antigenic cross-reactivity. Hybrids constructed from different segments of these molecules retained much of their secondary structure [34]. Their serologic reactivity suggested that the hybrids retained some discontinuous epitopes of the natural Ag 5, but with a reduced epitope density. A 100-fold to 3000-fold reduction in allergenicity was found by basophil histamine release assay.

One of the applications of the use of homologous proteins could be in the use of the molecular breeding or shuffling technology. Here, cycles of recombination are performed by random fragmentation of related DNA sequences, followed by reassembly of the fragments in a self-priming polymerase chain reaction. The mixed recombinant proteins can provide a source of altered allergens that can be examined, preferably by high-throughput screening assays [35].

Disruption of Structural Motifs

Most pollens contain allergenic proteins from the EF-hand calcium-binding family. Mutations of Phl p 7 to block calcium binding disrupted the tertiary structure and allergenicity, whereas the mutants retained the ability to induce blocking IgG in animals [21]. A similar procedure was used for an EF-hand calcium-binding protein from turnip (*Brassica rapa*) pollen [36].

Avoiding T-cell Epitopes

Schramm *et al.* [37] used overlapping peptides to map frequently recognized T-cell epitopes of the grass pollen allergen Phl p 5b, then performed mutagenesis outside these regions. They successfully produced mutants with a poor ability to induce skin test reactions while retaining their T-cell reactivity.

The knowledge of the bee venom phospholipase A2 structure and its T-cell epitopes and MHC class II-binding peptides was similarly used [38•]. Particular attention was paid to the promiscuous human leukocyte antigen (HLA) class II-binding region 81-97. Twenty-four mutations in six different areas of the surface of the molecule were made and one deletion of a loop region. The natural amino acids were substituted with amino acids that altered the size or

charge of the side chain. Residues conserved in the phospholipase A2 were not mutated to avoid changing the folding. No immunodominant region was found such that the successive introduction of mutations led to a progressive loss of IgE. The resulting mutant was shown to induce mutant-specific antibody in mice that had weak cross reactivity with the natural allergen. T-cell reactivity was complete. Structural analyses showed that the mutant was correctly folded, but it was highly flexible compared with the natural allergen.

Multivalent Vaccines

Modified vaccines provide an opportunity to create a multivalent polypeptide for desensitization against more than one allergen.

A hybrid polypeptide comprising sequences for the seven immunodominant T-cell epitopes of Cry j 1 and Cry j 2 of Japanese cedar [39] was constructed and shown to stimulate the proliferation of T cells better than the natural allergen. It induced proliferation at a 100-fold lower concentration than a mixture of allergens. A similar strategy has been tested with DNA vaccination in mice with a fusion of five murine T-cell epitopes on Der p 1 and Der p 2 in the one polypeptide. The vaccine significantly attenuated the induction of Der p-specific hypersensitivity response in mice [40].

Sequence Matrix Prediction

A sequence matrix model of isoforms and interspecies homologues of Bet v 1 has been used to design hypoallergenic mutants of Bet v 1 [41]. The sequence of Bet v 1a and 13 sequences from isoallergens or the homologous allergens from related species were classified as having high or low IgE-binding activity. An algorithm was then adapted to predict the residues responsible for this function. It essentially compared the type of amino acid at each position with the allergenicity of each isoform. A list of the amino acid residue positions in a decreasing order of predictive power for IgE binding was established. The top predictions were used to exchange the residues of the low to the high IgE-binding isoforms. It was considered that this approach, based on differences in closely related molecules, would minimize disturbances of the protein folding. An additional algorithm of preferred amino acid exchanges to predict disturbances of the protein fold predicted that the mutations would not affect the overall structural changes to a minimum. Six amino acid exchanges produced a mutant with extremely low reactivity with skin test reactivity and normal T-cell recognition.

Structurally Designed Mutations

Spangfort *et al.* [42] have based their strategy for producing hypoallergenic Bet v 1 based on solving the tertiary structures of the allergen and allergen-antibody complexes. The structure of an immune complex of Bet v 1 with the mono-

clonal antibody BV16 was determined. This conformational epitope occupies only 10% of the surface of Bet v 1 but blocks approximately 60% of the IgE binding of most sera from allergic subjects. A single amino acid substitution abolished the binding of this monoclonal antibody and 50% of the binding of human IgE. The structure of the mutant showed that the antigenic change was due to local surface effects and not to long-range structural alterations [42,43]. Further mutations have been made to three non-overlapping regions of the surface [44••], and another mutant with nine substitutions was designed to perturb five regions of the surface. The mutations reduced the binding of IgE-induced and Bet v 1-induced histamine release from human basophils by an average factor of 10, although not in every patient. X-ray diffraction analysis and circular dichroism of the mutants showed that they retained the alpha-carbon backbone-folding pattern. The mutants stimulated unchanged proliferation of patient T cells and mouse antibodies and, when injected into mice, induced blocked IgE binding to Bet v 1.

Birch pollinosis is often accompanied by adverse reactions to food due to Bet v 1-specific IgE cross-reacting with homologous food allergens. The tertiary structure of the cherry allergen Pru av 1 is nearly identical with Bet v 1. The loop, which binds the BV16 anti-Bet v 1 monoclonal antibody discussed earlier, is conserved in birch and prune but not in other less cross-reactive homologues of Bet v 1. This was, therefore, targeted by mutagenesis [45]. The mutants had reduced IgE binding without, as shown by structural analysis, affecting the backbone conformation.

Clinical Trial

A very important development in the use of genetically engineered allergens has been the conduct of a multicenter European clinical trial for treatment of birch pollinosis [46••]. Hypoallergenic half-molecule fragments [11] and trimers of Bet v 1 [11] were absorbed onto alum. Patients were given eight subcutaneous injections of increasing doses from 1 to 80 µg at 1 to 2 weekly intervals and then 80 µg doses at 4 weekly intervals until the beginning of the pollen season. The treatments were compared with a placebo. Th1- and Th2-dependent IgG subclass antibodies were induced by the injections. The post-injection sera blocked in vitro allergen-induced basophil degranulation. The trimer induced more IgG antibody than the fragments, and subjects treated with the trimer had more blocking activity in the histamine-release assays. Perhaps the most promising aspect for the treatment was that patients did not mount the typical seasonal increase in IgE antibody and had reduced skin test reactivity to birch extract. However, the injection of the hypoallergens raised the preseasonal levels of IgE so that the differences in the overall IgE titers were not large. Clearly, the titers in succeeding years will be of paramount interest. The seasonal increase of IgE was more effectively reduced by the fragments, suggesting it might not be related to the induction

of IgG antibodies, which were raised more effectively by the trimer. Subjects receiving the treatment reported improved symptoms using a subjective assessment to compare their symptoms to those in the previous spring. Symptoms induced by the desensitizing injections were not formally presented, but the authors noted that not all patients reached the 80- μ g maintenance dose. This trial has set a benchmark for the further examination of genetically modified allergens.

Conclusions

An astonishing array of strategies has been used to genetically engineer hypoallergenic allergens. Remarkably, there are no reports on random modification, although molecular breeding has been proposed. Such techniques, combined with high throughput screening, can be very powerful. Regardless of how they are produced, if the allergens are to be used as future medicaments, they need to be produced with verifiable and stable structures. This could be achieved by structurally stable mutants, flexible non-aggregating polypeptides, and stable multimers. Mutants designed and verified by an analysis of tertiary structure will inherently be defined and reproducible. Such polypeptides have been genetically engineered for Bet v 1, Lol p 5, Hev b 6.02, Ves v 5, Pol a 5, Api m 1, and Pru av 1. They must also be effective. It is salient to note that the hypoallergens produced by all the procedures have been shown to retain the ability to induce IgG antibody to the natural allergen and the ability to stimulate T cells. It is probably important for the induction of antibodies that the engineered sequences contain sufficient surface flexibility so that the altered sequences can adopt the structure of the epitope in the presence of the B-cell receptor [47].

The first clinical trial with the injection of flexible polypeptide fragments (75 amino acid-long half allergens) and a trimer of Bet v 1 has been a major achievement. Without apparently producing better clinical outcomes than those expected from traditional desensitization, the results reported for the first year showed several promising aspects, including a modification of IgE production, reduced skin reactivity, and self-reported improvements in symptom score. More importantly, it has set a benchmark and provided precedents for using a rapid increase of allergen doses, the workup with skin and nasal provocation tests, and the production of recombinant allergens for human vaccination.

Birch allergy is probably the allergy for which the allergic response is most focused on a single major allergen. A priority is to test other types of allergy in which there are more major allergens. It is possible to induce immunoregulatory mechanisms whereby the response to one allergen can inhibit the responses to other allergens presented at the same time [48]. Genetically engineered allergens potentially allow the administration of large doses, but the appropriateness of such is yet to be established. Although it has been

shown that allergen extracts must contain an adequate dose of allergen, very high doses are not necessarily optimal. Low doses of allergen, at least in oral tolerance, induce bystander suppression [49], which would be an advantage for inhibiting responses to mixtures of allergens. There have only been a few attempts to improve the inherent immunogenicity of allergen for vaccination. However, the development of adjuvants for humans, especially immunostimulatory oligonucleotides, has advanced impressively, and such adjuvants combined with the hypoallergenic vaccines could produce very effective results.

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