

25 Experimental Forms of Allergen Immunotherapy with Modified Allergens and Adjuvants

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INTRODUCTION

Allergen-specific immunotherapy (SIT) is an effective form of treatment for allergic diseases caused by inhalant allergens (e.g., pollen, mites, animal dander) and insect venom. Despite being initially described at the turn of the 20th century, SIT remains the only "curative" approach to atopic allergic disease. In addition to the ability to modulate existing disease, SIT is capable of modifying the natural course by preventing the worsening of symptoms (from rhinitis to asthma) and sensitization against new allergens (1,2). The treatment consists of administering increasing doses of natural allergen preparations on a regular basis, and the beneficial effects for the patient depend on the amount of allergen used. Typically, between 5 and 20 μg of the major allergen is required in each monthly maintenance injection to achieve optimal clinical efficacy. Because treatment involves injecting allergens into a sensitized individual, the occurrence of typical allergic symptoms, both local and systemic reactions, is not unexpected, with risks increasing with the concentration of allergen administered per treatment. Several immunological pathways seem to be involved in the clinical improvement achieved by the common SIT schedules (3,4). These mechanisms include (i) increase in allergen-specific IgG antibodies, in particular IgG4, which is believed to exert its effect by neutralizing allergen and blocking IgE-facilitated allergen presentation to T cells (5); (ii) generation of IgE modulating CD8⁺ T cells (6); (iii) reduction in the number of mast cells and eosinophils and the release of mediators by these cells (7,8); and (iv) modulation of allergen-specific T cells by shifting the response from a Th2 to Th1 cytokine pattern. The latter results in a decrease of IL-4 and IL-5 production accompanied by an increase of IFN- γ (immune deviation) (9,10). Moreover, the induction of an anergic state in peripheral T cells (immunologic tolerance) has been reported. Tolerance may be mediated by IL-10 and has been characterized by suppressed proliferative and cytokine responses against major allergens (11).

Although this type of therapy is widely established, three major problems are still associated with SIT. First, SIT is performed with allergen extracts containing mixtures of allergens, nonallergenic proteins, and/or potentially toxic substances, including bacterial endotoxin and other macromolecules that are difficult to standardize. Indeed, new IgE reactivities to allergenic components may develop during SIT administration of complex, allergen extracts (12). Second, severe IgE-mediated side effects can occur during the treatment with anaphylaxis to fully active allergens. Third, therapeutically effective doses often cannot be achieved because of adverse events or poor standardization of extracts in the vaccines.

RECOMBINANT AND ENGINEERED ALLERGENS

Many of the problems associated with SIT could be overcome, in principle, by the use of genetically engineered allergens (13,14). Cocktails of pure and standardized recombinant allergens can be formulated to replace natural extracts, and the selected recombinant allergens can be engineered to reduce the risk of IgE-mediated side effects. Genetic engineering of allergens for SIT should aim at the production of modified molecules with reduced IgE-binding epitopes (hypoallergens) while preserving structural motifs necessary for T-cell recognition (T-cell epitopes) and for induction of IgG antibodies reactive with the natural allergen (blocking antibodies). The uptake of allergens by antigen-presenting cells (APCs) is mediated and facilitated by the interaction of the allergen with specific IgE (15,16) and leads to enhanced production of Th2 cytokines and IgE production. Engineered allergens lacking IgE binding are designed to avoid these pathways and preferentially target APCs that use phagocytosis or pinocytosis for antigen uptake (e.g., monocytes, macrophages, and dendritic cells). This in turn induces a balanced Th0 or Th1-like cytokine production by T cells and low IgE and high IgG production by B cells. The presence of intact T-cell epitopes in hypoallergens enables targeting of T cells, allowing administration of higher doses to induce tolerance of allergen-specific T cells and alteration of cytokine production towards a Th1-like pattern. In this way, recombinant allergens could replace natural extracts in vaccines and increase the efficacy and safety of SIT.

Low IgE-Binding Natural Isoforms

Complementary deoxyribonucleic acid (cDNA) cloning of allergens demonstrates that many major allergens are encoded by gene families. Within individual allergen sequences, polymorphisms occur, such as those found in ragweed Amb a 1 (17), hazel Cor a 1 (18), birch Bet v 1 (19), group 1 (20) and group 5 grass allergens (21), apple Mal d 1 (22), celery Api g 1 (23), Parietaria Par j 1 (24), olive Ole e 1 (25), group 2 dust-mite allergens (26), latex Hev b 7 (27), and cow dander Bos d 2 (28). Because of sequence variations, isoallergens may have different antigenic and/or allergenic activities. Differences in T-cell reactivity of isoforms have previously been reported for Cor a 1 (29), Bet v 1 (30), Phl p 5 (31), and for Der p 2 (32).

Investigation of the IgE-binding activity of isoallergens led to the identification of naturally occurring Bet v 1 hypoallergens (33). Isoforms Bet v 1d (Bet v 1.0401), Bet v 1g (Bet v 1.0701), and Bet v 1l (Bet v 1.1001) are highly antigenic in T-cell proliferation assays and poorly allergenic in vitro and in vivo. The crystal structure of the hypoallergenic isoform Bet v 1l (Bet v 1.1001) does (34) not differ significantly from the high IgE-binding isoform Bet v 1a (Bet v 1.0101). Thus, the low IgE-binding activity of certain isoforms is not due to problems in the recombinant production leading to unfolded proteins. The hypoallergenic nature of these isoforms was confirmed in a study using recombinant Bet v 1.0401 and Bet v 1.1001 (35). Compared with Bet v 1.0101, Bet v 1.0401 and Bet v 1.1001 isoforms were unable to induce measurable IgE levels in allergic individuals naturally exposed to birch pollen, whereas significantly higher levels of IgG4 antibodies were directed mostly to Bet v 1.0401 and Bet v 1.1001. Structural comparisons showed that most of the amino acid differences among the Bet v 1.0101, Bet v 1.0401, and Bet v 1.1001 isoforms were located on the surface of the proteins. Thus, naturally occurring hypoallergens could be an excellent source of vaccine candidates. Unfortunately, this approach could not be generally applied because naturally occurring hypoallergens have not been identified for other allergen families. Instead, genetic engineering has been widely used to generate low IgE-binding variants.

Engineered Allergens

Genetic engineering involves the targeted modification of a protein to alter its function or properties in a predictable manner. This usually requires the complete understanding of the relationship between structure and function/properties for precise and effective manipulation. Alteration of the gene includes changing specific base pairs (mutated gene), introduction of a new piece of DNA into the existing DNA molecule (chimeric or hybrid gene, head-to-tail and tail-to-head fusions), deletions (truncated gene), and fragmentation (gene fragment) (Fig. 1). With the exception of the DNA shuffling approach (described later), which bypasses the need to identify amino acid residues or motifs that are important to structure and function, engineering of allergens usually requires knowledge of B- and T-cell epitopes and, in some

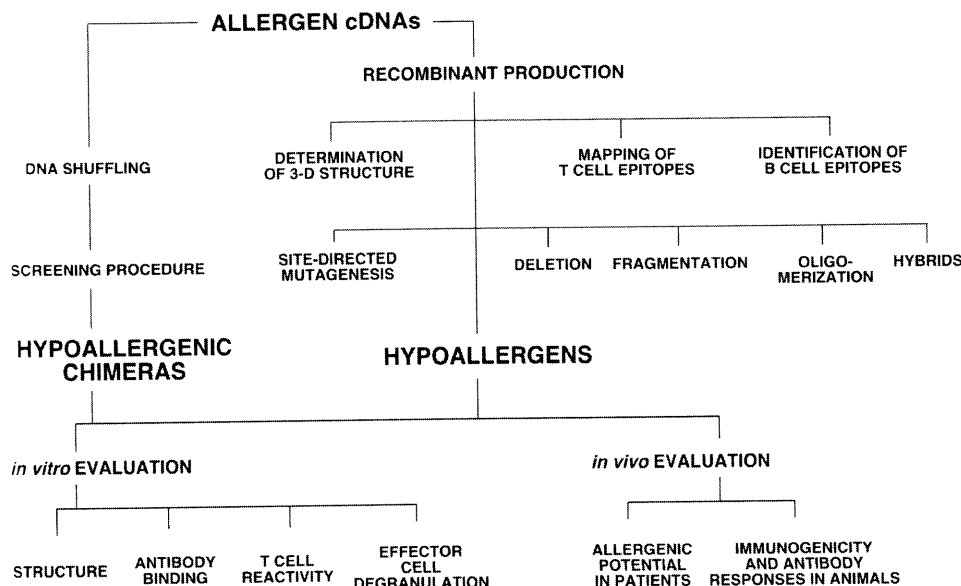


Figure 1 Task tree for genetic engineering and evaluation of hypoallergens for specific immunotherapy.

cases, knowledge of the three-dimensional structure of the allergen. No matter how allergen genes have been altered, putative hypoallergens must be subjected to a series of *in vitro* and *in vivo* evaluation procedures before being considered for therapeutic purposes (Fig. 1). To date, cDNA sequences of approximately 388 allergens (73 from pollen, 43 from mites, 15 from mammals, 76 from molds, 61 from insects, 95 from foods, 20 from other sources, e.g., latex) have been deposited in the allergen databank (<http://www.allergen.org>). In addition, the three-dimensional structures of approximately 40 allergens are defined and are accessible at the Protein Data Bank (PDB; <http://www.rcsb.org/pdb/>). Thus, the starting material and relevant information for producing new vaccines for important allergens are available.

Site-Directed Mutants

After identification of crucial amino acid residues or motifs involved in IgE recognition, dominant epitopes can be targeted using site-directed mutagenesis. The observation that Bet v 1 and other closely related tree pollen allergens consist of a mixture of closely related isoforms displaying striking differences in their ability to bind IgE (30) constituted the basis for engineering a Bet v 1 hypoallergen (36). The patterns of amino acid substitutions in tree pollen isoallergens and their IgE-binding activities were analyzed using a computer algorithm developed to predict functional residues in protein sequences (37). Using *in vitro* site-directed mutagenesis, the amino acid residues occurring in positions 10, 57, 112, 113, and 125 of Bet v 1a were substituted with those present in the same positions of low IgE-binding isoforms. Thus, a Bet v 1 mutant carrying point mutations was produced. This mutant protein displayed extremely low IgE-binding activity for all patients tested. *In vivo* (skin prick) tests indicated that the potency of the Bet v 1 mutant to induce typical wheal and flare skin reactions in allergic individuals was dramatically reduced (100–1000-fold) compared with Bet v 1a. T cell clones (TCC) established from the peripheral blood of birch pollen-allergic patients and reactive with Bet v 1a were also activated by the mutant. Other point mutations directed at molecular surfaces of Bet v 1 are described. Four- and nine-point mutants of Bet v 1 retained the pattern of the alpha-carbon backbone folding but showed significantly lower IgE-binding activity, suggesting a mutation-induced distortion of IgE-binding, B-cell epitopes (38).

IgE recognition of polcalcins, a group of EF-hand calcium-binding allergens found in pollen (39), and parvalbumin, a cross-reactive fish allergen (40), is influenced by bound calcium. This led to the idea of disruption of EF-hand calcium-binding domains for engineering hypoallergenic mutants. In this way, calcium-binding deficient mutants of Bet v 4 (41) and Bra r 1 (turnip) (42) with reduced IgE-binding activities were generated. However,

data concerning T-cell recognition of calcium-binding allergens and their engineered counterparts are not available.

The introduction of point mutations in highly conserved sequence domains of Lol p 5 (rye grass) was based on investigations of B- and T- cell epitopes and sequence comparison of group 5 allergens from different grasses. Hypoallergenic forms of Lol p 5 were produced that contained all relevant T-cell epitopes (43). Likewise, hypoallergenic variants of latex Hev b 6.02 (44) and Hev b 5 (45), egg Gal d 1 (22), apple Mal d 1 (46), and peanut Ara h 1, Ara h 2, and Ara h 3 (47,48) were also generated by site-directed mutagenesis. Modified peanut allergens retained their ability to stimulate T-cell proliferation in peripheral blood mononuclear cells (PBMCs) from allergic patients. In addition, peanut hypoallergens have been tested in a murine model of peanut anaphylaxis (48) and shown to be effective.

Conformational Variants

Akdis et al. (49) showed that bee venom phospholipase A2 (PLA, Api m 1) preparations lacking native conformation and antibody-binding activity were exclusively presented by monocytes and induced a Th1-biased cytokine profile leading to IgG4 production by B cells. In contrast, folded PLA with full antibody-binding activity was processed and presented by B cells, stimulated Th2-like cytokines, and induced IgE antibodies. Thus, the three-dimensional structure of an antigen and its recognition by different APCs are likely crucial aspects in the development of distinct T-cell cytokine patterns. These findings give further support to the use of hypoallergens in allergen immunotherapy.

The importance of the correct native fold and its stability for antibody binding was further demonstrated for Api m 1. Introduction of multiple mutations and deletions in exposed areas of Api m 1, but outside of previously identified T-cell epitope regions, led to a progressive loss of specific IgE and IgG reactivity (50). Although this Api m 1 mutant shared a similar content of secondary structure with the wild-type protein, the surface mutations dramatically affected the thermal stability of the protein. Hypoallergenic variants of the major allergen of Par j 1 (Parietaria) with altered folding were produced and characterized (51,52). Par j 1 is a member of the nonspecific lipid transfer proteins (nsLTPs) with a characteristic α - α - α - β structure, which is stabilized by four disulfide bonds. Targeting these disulfide bonds by site-directed mutagenesis resulted in molecules with altered conformation and decreased IgE-binding activity that retain their ability to stimulate T-cell proliferation. Disruption of native conformation by targeting disulfide bonds could be a generally applicable approach for engineering allergenic nsLTPs, including food-derived members. Disulfide bonds stabilizing the antigenic structure of major allergens of dust mites have also been targeted by site-directed mutagenesis. Hypoallergenic variants of Der p 2 (53), Der f 2 (54), and Lep d 2 (55) were produced and evaluated for subsequent IgE-mediated reactions and cellular responses. However, caution is warranted when targeting the conformation of allergens. Conformation changes may reduce the solubility of the final product, and denatured or unfolded proteins tend to form aggregates. Protein preparations consisting of aggregates or insoluble molecules would not be suitable candidates for therapeutic products. Thus, to avoid such problems, hypoallergenic variants of the major cat allergen Fel d 1 were generated by a rational approach using structural information and knowledge of B- and T-cell epitopes. The three-dimensional structure was systemically altered by duplication of selected T-cell epitopes (DTE) and disruption of disulphide bonds (56). When applied to the cat allergen Fel d 1, DTE accounted for reduction in IgE-binding capacity, whereas mutagenesis improved protein refolding and solubility. This novel concept may be a valuable approach for the design of new hypoallergenic derivatives of well-characterized allergens.

Deletion Mutants

Knowledge of IgE-reactive regions of allergens can be used to engineer hypoallergenic variants by deleting the corresponding DNA segment in the gene. This approach was successfully used for the timothy grass pollen allergen Phl p 5b (57). Epitope mapping was performed using overlapping recombinant fragments, and at least four continuous IgE-binding epitopes were identified. Deletions eliminating identified T-cell epitopes were then performed within these IgE-binding regions. Some of these deletion mutants showed reduced IgE-binding properties, no histamine-releasing activity, reduced skin reactivity, and no significant changes in T-cell

reactivity. A similar approach was used to engineer hypoallergens of the American cockroach, *Per a 1* (58). Based on the results obtained by proteolytic fingerprinting, a deletion mutant of rye grass, Lol p 1, was produced that displayed decreased IgE-binding activity and did not trigger histamine release up to a concentration of 10 mg/mL (59). The mutant was not tested in T-cell proliferation assays or for skin reactivity.

Allergen Oligomers

Vrtala et al. (60) constructed a Bet v 1 oligomeric form consisting of three copies of full-length Bet v 1 cDNA linked by short oligonucleotide (ODN) spacers in one open reading frame. Recombinant Bet v 1 monomer and trimer produced in *Escherichia coli* showed comparable in vitro IgE-binding activity and strongly stimulated Bet v 1-specific TCC and PBMCs from birch pollen-allergic patients. Interestingly, the Bet v 1 trimer released minimal amounts of histamine from patients' basophils and exhibited reduced skin reactivity compared with the monomer. When injected in mice and rabbits, the trimer induced IgG antibodies that inhibited human IgE binding to Bet v 1 monomer. Several explanations were proposed to explain why the IgE-binding Bet v 1 trimer showed reduced anaphylactic potential: (i) lower affinity for IgE binding, (ii) reorientation of IgE epitopes preventing efficient cross-linking of FcεRI-bound IgE antibodies, and (iii) microaggregation, steric hindrance, and/or unfavorable charge interactions causing concealment of IgE epitopes required for efficient cross-linking.

Following this line, monomers, dimmers, and trimers of wild-type and mutant Dau c 1, the major carrot allergen, were constructed and characterized. Mice were used to assess the contribution of mutations and oligomerization to IgE-binding activity and to the induction of blocking IgG antibodies (61). The results demonstrated that destruction of the native conformation by introduction of multiple mutations, rather than oligomerization of the wild-type allergen, is the appropriate strategy for the development of novel reagents for standard allergen-SIT, at least in mice.

Chimeras: Allergen Hybrids and Shuffled Molecules

King et al. (62) reported an approach to genetically modify allergens in which hybrids were prepared consisting of a small portion of the "guest" allergen of interest and a large portion of a homologous but weakly cross-reacting host protein. In this scenario, the homologous host protein serves as a scaffold to maintain the native structure of the guest allergen of interest to preserve conformation-dependent B-cell epitopes, but at a reduced density. The homologous allergens from yellow jacket venom Ves v 5 and from paper wasp Pol a 5 (59% sequence identity) show very limited cross-reactivity of antibodies from sensitized patients. Hybrids of these two molecules containing 10 to 49 residues of Ves v 5 showed a 100- to 3000-fold reduction in allergenicity in the histamine release assay with basophils from yellow jacket-sensitized patients.

Hybrids consisting of head-to-tail fusions of Phl p 5 and Phl p 1 and of Phl p 6 and Phl p 2 allergens were engineered by polymerase chain reaction (PCR) with the aim of producing combination vaccines for grass pollen immunotherapy (63). These hybrids were not hypoallergens and contained most of the IgE and T-cell epitopes of natural grass pollen extract. However, these hybrids showed higher immunogenic activity than the individual allergens when injected in mice, and thus may be useful for vaccine development.

A similar approach was reported for the major bee venom allergens. Kussebi et al. (64) fused the two major allergens of bee venom, Api m 1 and Api m 2. The hybrid molecule lost conformational B-cell epitopes but retained the important T-cell epitopes. The same could be demonstrated for hybrids of the major allergens from *Parietaria*, Par j 1 and Par j 2 (65).

The strategy of tail-to-head reassembly of hypoallergenic fragments within one molecule was demonstrated for timothy grass profilin, Phl p 12 (66). Non-IgE-reactive fragments of Phl p 12 were identified by synthetic peptide chemistry and restructured (rs) as a new molecule. Phl p 12-rs showed decreased IgE-binding activity but preserved T-cell reactivity in allergic patients. IgG antibodies induced by immunization of mice and rabbits with Phl p 12-rs cross-reacted with pollen Phl p 12 and other wild-type profilins. In addition, Phl p 12-rs inhibited profilin-induced basophil degranulation.

As discussed above, directed mutagenesis has been used to generate hypoallergens. Such a rational design method requires an understanding of the relationship between structure and

function for the target allergen. The approach has the limitation that substitutions of amino acids or segments must be appropriate for the specific position in which they are found in the protein. The effects of substitutions, even chemically conserved ones, are practically impossible to predict with the current knowledge-based system. DNA shuffling, or molecular breeding, is a novel approach that mimics natural evolution and can be performed without prior knowledge of structural or functional characteristics of the target molecules (67). It allows the generation of large and complex libraries of novel chimeric genes, from which variants with desired properties can be selected using appropriate screening methods. The features of the DNA family shuffling method would allow the generation of allergen chimeras having T-cell epitopes derived from several family members, reduced anaphylactic potential, and improved immunogenicity (68). This approach was used to generate molecules that are suitable for SIT, not only for birch pollen but also cross-reactive tree pollens like alder, hazel, hornbeam, and oak (69). The data demonstrated that it is possible to randomly recombine *in vitro* T- and B-cell epitopes of a family of related allergens and to select chimeric proteins-matching criteria presently thought to be relevant for improving SIT. The low IgE-binding activity and reduced capacity to induce release of inflammatory mediators of the shuffled chimeras combined with the high potency to react with T lymphocytes is expected to minimize IgE-mediated side effects without compromising SIT efficacy. A similar approach was successfully used to evolve two isoforms of group 2 house-dust mite allergens, Lep d 2 and Gly d 2 (70).

Allergen DNA Vaccines

An attractive alternative for immunotherapy using allergen proteins is the use of allergen genes in genetic immunization approaches. Intramuscular or intradermal injection of plasmid DNA encoding clinically relevant allergens can induce long-lasting immune responses with a Th1 bias and promote the formation of interferon (IFN)- γ -producing CD4⁺ T cells (71-73). After subcutaneous administration of plasmid DNA encoding an allergen, transcripts have been simultaneously detected in several tissues. Furthermore, immunization of mice with an allergen-cDNA cloned in a plasmid vehicle resulted in an allergen-specific IgG2a and Th1-like response, with no detectable IgE response. This is in contrast to immunization with allergen, which induced an IgE antibody response. However, despite the potential of this approach, no studies have been performed in humans to date, largely because of concerns over the introduction of "active" genetic material into man. In the mouse model, allergen-gene DNA vaccines were shown to efficiently protect from IgE expression, experimental airway inflammation, and airway hyperreactivity (AHR) by induction of Th1-biased immune reactions (74).

However, before clinical application of allergy DNA vaccines, two major concerns should be addressed: (i) anaphylactic side effects as the result of cross-linking preexisting IgE molecules by the translated native gene product and (ii) weak immunogenicity of parenterally injected conventional DNA vaccines in humans. The first problem was solved with DNA vaccines coding for hypoallergenic derivatives, such as fragmented allergen genes (75) or hypoallergens developed by mutation or gene shuffling (76). Moreover, a routine method was established for the production of hypoallergenic vaccines with any given allergy gene of interest by forced ubiquitination (77). Replicase-based DNA vaccines are immunogenic at 10,000-fold lower concentrations than conventional DNA vaccines (78), thus elegantly addressing the immunogenicity problem. Moreover, even at such ultralow doses, this vaccine type displayed highly effective protection from allergic reactions in a mouse model. Taken together, these latest DNA vaccine generations fulfill all necessary requirements for clinical trials: (i) they can be produced with high purity, (ii) they are allergen specific, (iii) they are safe with respect to anaphylactic side effects, (iv) they do not trigger new "therapy-induced" IgE antibodies, and (v) they likely can be used at nanogram doses in humans.

ALLERGEN FRAGMENTS

Large Allergen Fragments

IgE epitopes can be formed by linear sequences of amino acids (continuous epitopes) or by nonadjacent sequence elements brought together by folding (discontinuous or conformation-dependent epitopes). IgE recognition of continuous epitopes may also depend on their

conformation, which might only occur in the context of the folded allergen molecule. Thus, the disruption of the three-dimensional structure by fragmentation could be a useful approach to reduce the anaphylactic potential of allergens. The three-dimensional structure of Bet v 1 was disrupted by expressing two fragments of the cDNA, corresponding to amino acids 1-74 and 75-160, in *E. coli* (79). The fragments exhibited random coil conformation and almost no allergenicity. Together, the fragments harbored all relevant T-cell epitopes. Skin reactivity and histamine release were greatly reduced when compared with the native intact Bet v 1 allergen (80). Moreover, immunization of mice and rabbits with Bet v 1 fragments induced IgG antibodies that inhibited binding of IgE from patients allergic to wild-type Bet v 1 (81). Bet v 1 fragments were used to vaccinate 124 birch pollen-allergic patients in a double-blind, placebo-controlled study (82). Active treatment induced protective IgG antibodies that inhibited allergen-induced release of inflammatory mediators. The authors also observed a reduction of cutaneous sensitivity and an improvement of symptoms in actively treated patients. In addition, increases of allergen-specific IgE induced by seasonal birch pollen exposure were significantly reduced in vaccinated patients. This was the first clinical trial using a modified recombinant allergen preparation.

On the basis of IgE epitope mapping data of Phl p 6 from grass pollen, three allergen fragments have been produced, comprising amino acids 1-33, 1-57, and 1-110 (83). All fragments lacked a structural fold, which was accompanied by a strong reduction of IgE reactivity and allergenic activity. However, immunizing mice with fragments, adsorbed to an adjuvant allowed for human use, induced Phl p 6-specific IgG antibodies with only one of the fragments (31-110).

Nonanaphylactic fragments of the major house-dust mite allergen Der f 2 were produced by C- and N-terminal deletions. These fragments were mixed after separate refolding of the denatured fragments (84). Fragments of the calcium-binding allergens Bet v 4 (birch) (85) and Aln g 4 (alder) (86), and an N-terminal fragment of Lol p 1 from rye grass (59), also showed decreased IgE-binding activities. However, these fragments have yet to be studied with respect to T-cell reactivity and immunogenicity.

T-Cell Peptide Epitopes

Peptide immunotherapy is an approach that targets CD4⁺ T cells by using allergen-derived peptides containing short linear T-cell epitopes to induce tolerance. This approach is similar in many ways to the use of allergen fragments but employs smaller sequences specifically selected for activity as T-cell epitopes. The principle has been established in animal models, and the reduced ability of short peptide epitopes to cross-link surface-bound IgE may provide an attractive alternative to SIT in humans.

In vitro experiments have demonstrated that different concentrations of peptides can induce activation or hyporesponsiveness, depending on dose. Lamb and colleagues (87) employed high doses (50 µg) of peptide to render human T cells nonresponsive in vitro. Influenza virus hemagglutinin-specific human Th0 TCC were exposed to supraoptimal doses of peptide in the absence of APCs. Subsequent whole antigen challenge was characterized by antigen-specific T-cell hyporesponsiveness (anergy), which could be prevented or reversed by the addition of IL-2 (88). Further studies using supraoptimal doses of peptides in human CD4⁺ TCC reactive to house-dust mite reproduced clonal anergy and showed that this state was accompanied by downregulation of IL-2 and IL-4 and maintenance of IFN-γ secretion (89).

The principle that T-cell peptide epitopes may be employed to induce antigen-specific hyporesponsiveness has been extensively investigated in rodent models of autoimmune disease and allergic sensitization. Translation of the approach to human subjects was first attempted in individuals allergic to cats.

Allervax[®] CAT

Approximately 95% of individuals with a clinical history of cat allergy are sensitive to one protein, Fel d 1 (*Felis domesticus*), found in cat dander and saliva. The allergen is transported on clothing and is ubiquitous in the environment, present not only in the homes of cat owners but also in public places (90). Cloning, sequencing, epitope mapping, and preclinical studies resulted in the selection of two 27-amino acid sequences, IPC-1 and IPC-2, for evaluation in clinical trials (91).

The efficacy of Allervax[®] CAT (IPC-1/IPC-2) was evaluated by Norman and colleagues in a double-blind, placebo-controlled trial (92). Four weekly subcutaneous injections of placebo or peptides at doses of 7.5, 75, or 750 μ g were administered. Modest improvements in symptom and medication scores were observed in the highest dose group, six weeks after treatment. Clinical outcomes included nasal and lung symptoms during a 60-minute exposure in a "cat room." Treatment was associated with a significant number of early and late adverse events, including chest tightness, nasal congestion, and flushing. These occurred a few minutes to several hours after administration of the peptides. In an associated study, a decrease in IL-4 production by IPC-1/IPC-2-specific T-cell lines from subjects in the high-dose group was demonstrated. However, proliferative responses to either peptide or whole allergen remained unchanged (93).

Immunotherapy with Allervax[®] CAT was also evaluated in cat-allergic asthmatic subjects, using inhaled allergen challenge (94). The investigators measured allergen PD₂₀ [inhaled dose sufficient to reduce forced expiratory volume in 1 second (FEV₁) by 20%] before and after a variable cumulative dose of peptides. Six weeks after ending therapy, posttreatment PD₂₀ was not significantly different between the treated and placebo groups. However, in the middle- and high-dose groups, there was a significant increase in allergen tolerance between baseline and posttreatment days. In addition, IL-4 release was significantly reduced in the high-dose group. No change was observed in IFN- γ production.

In a randomized, double-blind, parallel-group study (95), 40 cat-allergic subjects received subcutaneous injections of 250 μ g of the same peptide preparation as used in the two studies described previously. Subjects received consecutively, four weekly injections. No change was seen in either early- or late-phase skin reactivity to whole cat extract up to 24 weeks after the last injection. No significant change in cat-antigen-specific cytokine production was observed. Frequent adverse events, including symptoms of asthma, rhinitis, and pruritus, were reported.

An additional study was a multicenter, randomized, double-blind, placebo-controlled study of 133 cat-allergic patients. Maguire and coworkers demonstrated modest improvements in some clinical outcome measures with peptide SIT (96). Subjects received subcutaneous injections of either 75 or 750 μ g of peptide or placebo twice weekly for two weeks in two treatment phases, four months apart (a total of 8 injections). Pulmonary function improved in the 750- μ g group. A large number of adverse events were recorded, including the requirement for systemic epinephrine in three peptide-treated patients. The majority of adverse events were associated with respiratory symptoms, occurred a few hours after injection, and declined with successive doses.

HLA-based Peptide Vaccines

In contrast to defined T-cell epitopes, other studies have focused on studies involving a large number of peptides, hence expanded recognition by several human leukocyte antigen (HLA) molecules, which are referred to as HLA-based peptide vaccines. Haselden and colleagues (97) administered intradermally a mixture of three short (16 to 17 residue-long peptides, compared to 27 residue-long peptides in Allervax[®] CAT) peptides from the cat allergen Fel d 1 to cat-allergic asthmatics. In a subset of individuals, an isolated late asthmatic reaction (LAR) was observed, characterized by a decline in FEV₁ two to four hours after peptide administration. Induction of bronchoconstriction was demonstrated to be IgE-independent and HLA-restricted, implying a T-cell-mediated reaction. Subjects receiving Fel d 1 peptides and experiencing isolated LAR subsequently displayed markedly reduced reactivity to injected peptides. In later studies, 12 overlapping peptides (16/17 residues each) were synthesized. This group of peptides spanned the majority of chains 1 and 2 of Fel d 1. Encompassing the majority of the molecules increased the number of HLA haplotypes able to bind and present peptide (98). Using this preparation of peptides, the magnitude, as well as the frequency, of isolated LARs in cat-allergic asthmatic subjects was dose dependent, with 50% of individuals developing a LAR when challenged with a single dose of 5 μ g of peptide. A second injection of peptides was associated with a marked reduction or absence in LARs, with a return to baseline values of bronchial responsiveness over a period of up to 40 weeks. Thus, peptide-induced hyporesponsiveness was long lived.

A subsequent open study employing an updosing protocol with peptides (0.1, 1.0, 5, 10, 25 μ g) injected at two-weeks' intervals demonstrated the ability to induce T-cell hyporesponsiveness in the absence of LARs. These results indicate that incremental dosing protocols,

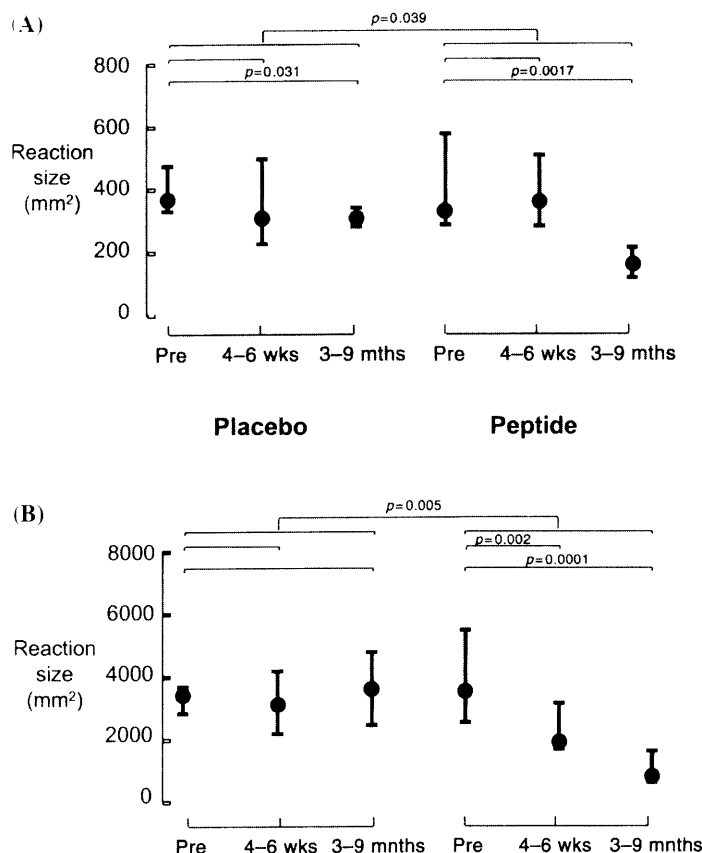


Figure 2 Peptide immunotherapy reduced both the early-phase cutaneous reaction to allergen challenge (A) and the late-phase cutaneous response to allergen challenge (B). A randomized, double-blind, placebo-controlled study of peptide immunotherapy with a mixture of 12 synthetic overlapping peptides derived from the primary sequence of the major cat allergen, Fel d 1, was performed in cat-allergic volunteers with asthma. A total of 90 μ g of each peptide was administered. Measurements of early-phase (15 minutes) and late-phase (6 hours) skin reactions to intradermal challenge with purified Fel d 1 protein were recorded before therapy, 4 to 6 weeks after therapy and 3 to 9 months after therapy. Statistically significant reductions in the magnitude of both early and late reactions were observed in the peptide-treated group.

starting at a dose that is too low to induce LARs, may allow peptide immunotherapy to be used safely in the treatment of asthma without undesirable bronchoconstriction (99).

Initial observations show that a single peptide injection reduced cutaneous late phase reactions to intradermal challenge with whole cat dander extract (98). To confirm these observations, a small, double-blind, placebo-controlled study was performed. Subjects received either placebo or peptide via an incremental dosing protocol up to a total dose of 90 μ g (100). Following peptide treatment, a significant reduction in both the early and late cutaneous reactions to allergen challenge was observed, indicating peptide-induced modulation of both the early-phase and the late-phase reactions. Changes in late cutaneous reactions were observed within weeks of completing treatment and persisted for several months (Fig. 2). Changes in early cutaneous reactions were only apparent at long-term follow-up (3-9 months). No significant differences were demonstrated in bronchial responsiveness to either methacholine or whole cat dander. However, the study was not powered to detect such outcomes. The important clinical observation was that peptide-treated subjects were better able to tolerate subsequent exposure to cats.

In vitro T-cell responses measured before and after peptide treatment demonstrated a significant decrease in peptide- and whole-allergen-induced proliferation of peripheral PBMCs and reduced production of both Th2 (IL-4, IL-13) and Th1 cytokines and IFN- γ in cultures. Furthermore, peptide treatment was associated not only with decreases in whole cat dander-induced proinflammatory cytokines by PBMCs but also with increased IL-10 production. Thus, induction of T-cell hyporesponsiveness in man may be associated with the induction and/or expansion of a population of regulatory T cells.

ADJUVANTS

Approaches that have been exploited to enhance the immunogenicity of vaccine antigens include strategies based on adjuvants, epitopes, and particulate antigens. An emerging area in the vaccinology of allergic diseases involves the use of various adjuvants to direct and redirect protective immune responses. Substantial progress has been made in some of these approaches, leading to clinical trials and licensure. However, safety concerns and economic considerations have limited their commercialization. The experimental research on improving immunotherapy has involved a combination of standardized allergens or purified/cloned allergens with an appropriate adjuvant. The latter may consist of a live or killed microorganism as a vaccine vector or molecular immunomodulators, such as those carrying cytosine-phosphoguanosine (CpG) sequences or plasmids (pDNA) encoding protective cytokines (101).

Microbial Adjuvants

To date, a large repertoire of live vaccine vectors, either avirulent or nonpathogenic organisms, capable of expressing important immunomodulatory molecules, has been investigated. These include vectors with small genome sizes, such as vaccinia virus, avipoxviruses, adenoviruses, polioviruses, salmonella, and vectors with large genomes, such as the herpes viruses and the mycobacterium bacillus calmette-guerin (BCG) (102–104). cDNAs encoding major allergens are available, which can be cloned into these nonpathogenic organisms to generate effective reagents for prophylaxis and treatment of allergies. Such organisms are expected to provide a more effective and longer-lasting adjuvant effect compared with the pDNA immunostimulants (105), presumably because of their persistence within cells of the body.

BCG

BCG is considered an excellent vaccine vector because it offers the following unique advantages (106,107): (i) BCG lends itself to the development of a *multiantigen vaccine*, as would be required for downregulation of specific allergies. (ii) Live attenuated BCG has been used for immunization of more than 2.5 billion people worldwide since 1948, with a low incidence of serious complications (case fatality rates of $0.19/10^6$). (iii) BCG has been shown to be a potent adjuvant in experimental animals and man, particularly in relation to induction of Th1 cells. (iv) BCG is heat stable and inexpensive to produce. (v) BCG can be administered by the oral route. (vi) BCG can be given at birth or at any time afterwards and is unaffected by maternal antibodies. (vii) BCG given as single inoculum sensitizes to tuberculo proteins for 5 to 50 years. (viii) Various in vitro studies of human and murine systems show that BCG-reactive CD4+ T cells are potent producers of IFN- γ (108), the principal mediator of antituberculous resistance. (ix) The evidence that BCG can be a resident within the phagosome of the long-lived macrophage for years suggests that a BCG-based vaccine may induce a potentially persistent or long-lasting allergen-specific immune response (109).

Controversial evidence in support of a role for BCG vaccination in the determination of allergic phenotype has come from the observation that Japanese children displaying a positive delayed-type hypersensitivity skin test reaction following prior BCG immunization exhibited lower prevalence of atopic allergic disease (110).

Previously, Erb et al. demonstrated that infection of mice with BCG suppressed allergen-induced airway eosinophilia (111). In order to establish if live BCG would provide an adjuvant effect similar to that of complete Freund's adjuvant, mice were vaccinated with BCG and subsequently immunized with recombinant allergen in alum. This protocol induced a decrease in total IgE and specific IgE by 5- and 10-fold, respectively. Concomitantly, IgG2a increased by 10-fold (109). Furthermore, the production of both specific and total IFN- γ was increased when cells from mice, vaccinated in vivo with BCG followed by recombinant Kentucky bluegrass allergen in alum, were stimulated in vitro with the same allergen. The result was an increase in the ratio of IFN- γ to IL-4. These findings demonstrate that live BCG as an adjuvant is capable of inducing a protective Th1 type of response.

In an effort to provide specificity to the effects of BCG, many investigators have developed recombinant BCG (rBCG) producing foreign antigens within the macrophage. Oral immunization with rBCG induces both cellular and humoral responses against foreign antigen (112). Experiments have examined the effects of rBCG vaccination on allergic responses in a

murine model (109). A BCG-*E. coli* shuttle vector was developed with the promoter and signal sequence and one of the major antigens of *Mycobacterium bovis* (A-antigen), and the vector was tested using *E. coli* β -galactosidase as the model antigen and allergen (109). This vector enabled the expression of the *E. coli* β -galactosidase (GAL) gene in BCG, which was detected in its protein extract by immunoblotting analysis. Vaccination of mice with a single dose of 10^6 rBCG generated a β -galactosidase-specific antibody response. The splenocytes of vaccinated mice, compared with controls, produced significantly higher levels of IFN- γ ($p < 0.01$) and IL-2 ($p < 0.05$) and lower levels of IL-5 ($p < 0.01$). Mice vaccinated with rBCG had significantly less ($p < 0.01$) serum IgE compared with controls. These results demonstrate that rBCG-secreting antigens or allergens may be used for the induction of a Th1-like response and the downregulation of IgE antibody response.

Cohon et al. examined the effects of BCG as an adjuvant in dust-mite allergen immunotherapy in asthmatics (113). After 12 to 16 weeks of SIT with *Dermatophagoides pteronyssinus* extract, clinical outcomes and immunologic parameters improved in asthmatic children with mite allergy. However, simultaneous BCG vaccination at the initiation of SIT provided no additional benefit, suggesting that immunomodulatory responses that might have been induced by BCG were not sufficient to translate into further clinical improvement of patients with established allergic disease. Thus, the role of BCG as an immune-redirecting adjuvant for allergy and asthma remains to be established in further studies.

Mycobacterium Vaccae

Mycobacterium vaccae has been examined for its therapeutic effect for allergy and asthma. Wang and Rook showed that *M. vaccae* injected twice after mice were sensitized with ovalbumin resulted in a reduction in IgE and allergen-specific IL-5 synthesis, suggesting a potential clinical application of this organism in the immunotherapy of allergic diseases (108). Like BCG, *M. vaccae* also evokes a strong IFN- γ response and has been suggested to be effective in enhancing antiallergic response in clinical trials. In a murine model of asthma, *M. vaccae* inhibited airway inflammation via regulating Th1/Th2 balance, suggesting that it may be beneficial in the treatment of asthma (114,115). Furthermore, studies with newborn mice show that immunization with *M. vaccae* prevents some of the chronic airway changes in asthma. Treatment of mice with SRP299 (a killed *M. vaccae* suspension) gave rise to allergen-specific CD4⁺CD45RB_(Lo)-regulatory T cells, that conferred protection against airway inflammation (116). This specific inhibition was mediated through IL-10 and transforming growth factor-beta (TGF- β), as antibodies against IL-10 and TGF- β completely reversed the inhibitory effect. Thus, regulatory T cells generated by mycobacterial treatment may have a role in restoring the balance of the immune system to prevent and treat allergic diseases.

A double-blind, randomized study investigated whether heat-killed *M. vaccae* (SRL172), a potent downregulator of Th2 cytokines, could reduce allergen-induced airway responses in patients with atopic asthma (117). A total of 24 male asthmatics participated in this study. Bronchial allergen challenge was performed along with measurement of early asthmatic responses and LARs, two weeks before and three weeks after a single intradermal injection of SRL172 or placebo. SRL vaccination prior to allergen challenge improved lung function following bronchial challenge compared with animals without SRL vaccination, but the magnitude of the effect did not reach statistical significance. SRL172 caused a trend for a reduction in serum IgE and IL-5 synthesis in vitro three weeks posttreatment ($p = 0.07$). These results suggest that multiple dosing of *M. vaccae* may be required to achieve significant benefit in asthma.

Heat-killed *Listeria monocytogenes* (HKL) potentially stimulates IFN- γ production in CD4 T lymphocytes and, when used as an adjuvant for immunotherapy, reduces IgE production and reverses established allergen-induced AHR in a murine model of asthma (118). In another study, Frick et al. (119) showed that HKL plus allergen treatment markedly improved established food allergic responses in dogs, suggesting that such an immunotherapeutic strategy in humans might improve the condition of individuals with food allergy and anaphylaxis.

These studies have identified the potential of live BCG or other microorganisms, either by themselves or in the form recombinant organisms expressing target allergens, as adjuvants for the potentiation of treatments for allergic diseases. However, these preliminary studies need to be extended in clinical trials to determine if such compounds offer real advantages to the management of human allergic diseases.

Adjuvant Effects of Toll-like Receptor Agonists

Several formulations have been developed on the basis of the concept that linking or activating specific toll-like receptors (TLRs) can modulate and bias the immune response to a preferred profile (120). Although experimental evidence indicates that lipopolysaccharide (LPS) has a kinase-like behavior on T-cell profiles, TLR-4, which binds LPS, seems to consistently support the development of a Th1 response. Thus, Pollinex QuattroTM, a cocktail of several allergens that use monophosphoryl lipid A (MPL) as adjuvant has been developed. MPL is a detoxified derivative of the LPS moiety of *Salmonella minnesota* R595. It binds to and specifically activates TLR4 and promotes the development of a Th1 response (121). MPL also acts through TLR2, which may enhance the function of CD4⁺ CD25⁺ regulatory T-cell function in a MyD88-dependent fashion (122–124).

This formulation is currently marketed in Europe as an ultrashort course of immunotherapy in patients suffering from allergic rhinitis during the pollen season. Besides the use of MPL, the grass/rye-derived allergens are treated with glutaraldehyde adsorbed onto an L-tyrosine depot, reducing the potential for adverse side effects (125). Clinical studies demonstrate that Pollinex Quattro reduces allergen-specific T-cell proliferation (126), rhinoconjunctivitis symptoms, and medication use (127–129). In addition, Pollinex Quattro blunted the elevation of IgE after allergen exposure while increasing allergen-specific IgG titers, particularly in the IgG4 subclass (128–130).

CRX-675 (aminoacyl-glucosamine 4 phosphatide), a soluble TLR4 agonist, is another promising adjuvant showing improvement in nasal symptom scores in patients suffering from allergic rhinitis. This formulation has no documented adverse side effects (131). Unfortunately, there are no indications thus far about the future marketing of this compound.

Potentially, new compounds could result from the determination of the immunomodulatory molecules in *L. monocytogenes* based on the adjuvant studies, both in an experimental, allergic-asthma mouse model and in food allergic dogs (119). Thus, a number of promising research strategies may help achieve the goal of modulating the T-cell profile through the proper stimulation of specific TLRs.

An additional, extensively studied TLR agonist is CpG-ODN. In contrast to eukaryotic DNA, the genetic material of prokaryotes is rich in sequences containing unmethylated CpG dinucleotide motifs. CpG DNA is most often coadministered with antigen in the form of synthetic oligodeoxynucleotides (CpG-ODN), which are made with a nuclease-resistant phosphorothioate backbone. Synthetic ODNs containing CpG motifs activate both innate and acquired immune responses through a signaling pathway involving TLR9. Depending on the sequence, length, number, and positions of CpG motifs in an ODN, distinct immunostimulatory profiles can be observed. These immunostimulatory profiles can be further modified and fine-tuned by appropriate chemical modifications, leading to preclinical and clinical development of CpG-ODN treatments in cancer, allergy, asthma, and infectious diseases. CpG-ODN sequences tend to induce Th1-like cytokine responses (132). The adjuvant may be injected with natural allergens or may be genetically linked with allergen cDNA. CpG-ODN, administered in conjunction with antigen, downregulates established Th2 responses. This immunomodulation is neither murine strain dependent nor model dependent. Although the effects of CpG-ODNs are associated with the induction of the Th1 cytokines IFN- γ and IL-12, neither cytokine is essential for the effect (133).

Experimental evidence indicates that CpG improves allergic airway inflammation by both inhibiting Th2 activation and preventing IgE-dependent release of Th2 cytokines (134). Specific immunostimulatory sequences containing CpG motifs have been evaluated in allergen immunotherapy (135,136). A randomized, double-blind, placebo-controlled phase 2 trial of a vaccine consisting of Amb a 1 conjugated to CpG-ODNs administered to patients allergic to ragweed shows a significant improvement in allergic rhinitis. This formulation also induces a transient increase in Amb a 1-specific IgG antibodies but suppresses the seasonal increase in Amb a 1-specific IgE antibodies. A reduction in the number of IL-4-positive basophils was also observed in formulation-treated patients. Interestingly, this treatment has long-lasting immunomodulatory effects confirmed by observations in subsequent ragweed seasons (137). A new formulation targeting allergies to house-dust mite has entered phase II clinical trials, and these results will help to determine the applicability of CpG adjuvants in a broader context (138). Treatment of rhinitis in mice sensitized to *Dermatophagoides farinae*

supports the potential use of CpG-ODNs in vaccines for the treatment of house-dust mite allergies (139). The effects observed include a reduction in IL-5 and an increase in IFN- γ in nasal lavage fluid.

Immunostimulatory ODN sequences from different sources have also been explored. ODN-BL07S from a probiotic strain, *Bifidobacterium longum*, inhibits allergen-specific IgE production and significantly reduced the levels of Th2 cytokines in an experimental mouse model. This also is accompanied with an increase in the levels of IgG2a (140).

In a mouse model sensitized to the major bee venom allergen phospholipase A2, the testing of several formulations using different TLR agonists as adjuvants shows the potential of polyriboninosinic polyribocytidylic (polyI:C) acid, a TLR3 agonist, as an adjuvant for prophylactic vaccination. This adjuvant skews the antibody response toward an IgG profile. In addition, formulations using adjuvants such as CpG, polyI:C, or peptidoglycan bias the T-cell response to a Th1 profile. In a therapeutic scenario, formulations using CpG, polyI:C, or 3M003 show excellent immunomodulatory properties (141).

Compounds such as CRX-527 (GSK), E6020 (Esai), and "Compound 1" (Biomira) are expected to be evaluated in the allergy field (104). Potentially, new compounds could result from determination of the molecules responsible for immunomodulation in *L. monocytogenes* (119). All the above indicate the promise of research to develop methods of modulating the T-cell profile through the proper stimulation of specific TLRs.

Microparticle or Nanoparticle Polymers

To improve the administration of allergy vaccines, the use of different chemistries to develop new assemblages, such as microparticles and nanoparticles, has also been explored in experimental allergic mouse models. Biodegradable polylactide-co-glycolide (PLGA) was used to prepare microparticles as a way to carry and deliver CpG-ODNs along with PLA2. This formulation was administered to mice sensitized to PLA2, resulting in an increased Th1 response and antibody profile biased to IgG2a (132). PLGA microparticles were also developed to deliver DNA vaccination against PLA2, resulting in a Th2-to-Th1 immune deviation, an increase in IL-10 production, and a reduction in IgE production (133). Use of a PLGA-based formulation could also potentially reduce both the treatment course and the amount of allergen and CpG administered in each dose, without preventing the development of strong Th1 responses (142). PLGA microparticles were also tested as carriers for allergen extracts, such as Ole e 1, in BALB/c mice previously sensitized to this allergen. This formulation also elicited a specific Th1-like response (143).

Chitosan, a biodegradable polymer, has been described as a vaccine adjuvant (144–146). Hall et al. (147) investigated whether intranasal administration of an allergen-derived peptide, either alone or adsorbed to chitosan, could prevent the induction of Th2-mediated pulmonary inflammation after sensitization and challenge of the airways with allergen. The results demonstrate that mice given peptide adsorbed to chitosan have significant reductions in airway eosinophilia, which correlate with reduced levels of IL-4 and IL-5 in the bronchoalveolar lavage fluid. There was decreased recruitment of activated CD4⁺ T cells into the airways after allergen challenge, which correlated with a loss of Der p 1-specific, T-cell cytokine responses in the periphery and localized production of IL-10 by antigen-specific T cells in bronchial lymph nodes. In another study, using a standardized mouse model of allergic asthma, Hellermann et al. showed that chitosan nanoparticles could be used to encapsulate allergen, which could then be delivered orally or as a nasal solution to reduce the sensitivity of the animals. The allergenicity of soy protein was greatly decreased by the attachment of chitosan through the Maillard reaction (148). Chew et al. (149) explored the use of chitosan-DNA nanoparticles for oral immunization to induce immune responses specific to both the left and right domains of Der p 1. Plasmid constructs encoding Der p 1 [pDer p 1; (1-222) and pDer p 1 (114-222)] were complexed with chitosan and delivered orally, followed by an intramuscular injection of pDer p 1 (1-222) 13 weeks later. Such an approach has successfully primed Th1-skewed immune responses against both domains of Der p 1. This strategy can be further optimized for more efficacious gene vaccination for full length Der p 1. These studies indicate that chitosan may not only act as a gene carrier but also as an immunostimulant in SIT.

Allergens Conjugated/Linked to Human Ig Receptors

An interesting approach is to modify allergens by conjugating them to molecules that play modulatory roles in the immune system. A chimeric fusion protein comprising human Fc γ linked to Fel d 1 was developed. This molecule binds to Fc γ RII in a fashion equivalent to that of human IgG and thereby coaggregates Fc γ RIIb with Fc ϵ RI-bound IgE. The result is inhibition of mediator release, such as histamine, in a dose-dependent fashion and inhibition of IgE-mediated Syk and extracellular signal-regulated kinase (ERK) phosphorylation in basophils from cat-allergic subjects. These results were also corroborated in cat allergen-sensitized mice (134).

In an alternate approach, Fel d 1 was linked to a single-chain fragment of the variable region (sFv) of the humanized anti-CD64 mAb H22 (H22-Fel d1). This fusion protein was administered to (i) patients allergic to cat allergen and (ii) control subjects without Fel d 1-specific IgE or IgG. It was found to induce cytokine-secreting subtypes consistent with Th0 and Th1 cells, mainly in the subset population of allergic individuals, and it did not induce a proallergic CD4+ T-cell repertoire. Although H22-Fel d 1 upregulates both an IL-5- and an IL-10-secreting T-cell population, IL-10 seems to dominate and control IL-5+ CD4+ T cells (140).

Cytokines as Adjuvants

Advances in gene transfer technology now make it possible to deliver cytokine genes to the target organ (Fig. 3). This approach will allow the evaluation of cytokines as adjuvants with antigen in vaccine or immunotherapy formulations. IL-12 and IFN- γ have been considered as potentially important adjuvants for the induction of Th1 cell-mediated, protective immunity (141). The functional effects of IL-12 in many systems are likely to be mediated through secondary production of IFN- γ . However, the Th1-inducing effect of IL-12 *in vivo* may not be accompanied by a long-lasting suppression of Th2 development, and IL-12 is toxic in some

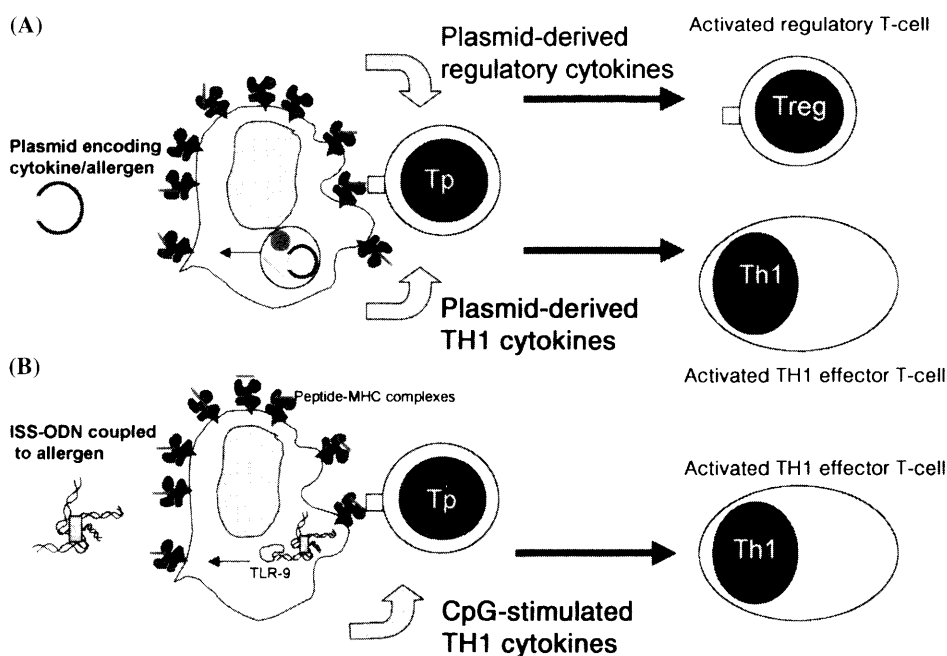


Figure 3 DNA-based approaches for the treatment of allergic diseases. **(A)** Plasmid DNA vectors encoding allergens and cytokine genes may be used to generate allergen-specific responses in an environment rich in proinflammatory, Th1 cytokines, such as IL-12 and IFN- γ , or regulatory cytokines, such as IL-10 and TGF- β . Presentation of allergen-derived peptides to Tp leads to the generation of robust Th1 or T-regulatory allergen-specific responses. **(B)** Allergens covalently coupled to ISS-ODN may be used to deliver allergen molecules to APCs together with TLR-9 activation leading to the production of Th1-stimulating cytokines such as IL-12. Presentation of allergen-derived peptides to Tp leads to the generation of robust Th1 allergen-specific responses. *Abbreviations:* DNA, deoxyribonucleic acid; ISS-ODN, immunostimulatory oligodeoxynucleotides; TLR-9, toll-like receptor-9; Tp, precursor T cells.

systems. IFN- τ , a type I IFN that lacks the toxicity associated with other type I IFNs, inhibits IgE production in a murine model of allergy and in an IgE-producing human myeloma cell line.

Administration of recombinant allergen (ovalbumin) with an IL-12 (subunit p40) fusion protein vaccine downregulates ovalbumin-specific IgE responses in vivo (104). In another model, IL-12 was delivered intranasally during allergen immunotherapy (150). In a typical protocol, DBA/2 mice were immunized intraperitoneally with GAL and then immunized parenterally with grass allergen with or without IL-12. Treatment of sensitized mice with a combination of allergen plus IL-12 shows the highest IFN- γ production and decreases the Th2-like response in GAL-stimulated splenocyte culture. IL-12 also inhibits GAL-induced IgE production and enhances GAL-specific IgG2a in GAL-presensitized mice. Intranasal delivery of IL-12 attenuates airway hyperresponsiveness and BAL eosinophilia in sensitized mice. Analysis of IL-12 receptor expression suggests a shift in the expression profile of IL-12R β 1 and β 2 in the lung tissue, consistent with the observed shift in the cytokine profile from a Th2- to a Th1-like response. These results suggest that intranasal delivery of IL-12 inhibits allergic airway inflammation in asthma via an IFN- γ -dependent pathway, involving regulation of the expression of the IL-12 receptor.

The immunomodulatory role of plasmid DNA-expressing cytokines IFN- γ (pIFN- γ) and/or IL-12 (pIL-12) as adjuvants was assessed in a murine model of Kentucky blue grass (KBG) allergy (Mohapatra, unpublished observations). Mice vaccinated with cytokine plasmid adjuvants had relatively less total serum IgE and higher levels of grass allergen-specific IgG2a when compared with the control mice injected with the empty vector plasmid. The lowest IgE and the highest IgG2a levels were found in mice vaccinated with combined pIFN- γ and pIL-12 as adjuvant. The IgG1 titers of all mice remained unchanged. The most profound decrease in airway hyperresponsiveness and pulmonary inflammation was observed in mice receiving both pIFN- γ and pIL-12 as adjuvants. These studies provide evidence that a combination of pIFN- γ and pIL-12 provides a more effective adjuvant to the grass allergen vaccine than either one of these plasmids alone, and the combination may enhance the effectiveness of allergen immunotherapy in humans.

CONCLUSION

Rapid progress is being made in the development of novel immunotherapeutics for the treatment of allergic diseases. Recombinant DNA technology has enabled the cloning and sequencing of genes encoding several hundred important aeroallergens. Knowledge of nucleotide and amino acids sequences enables the standardized production of recombinant molecules, fragments, and peptides to high degrees of purity for development as vaccine candidates. Furthermore, detailed evaluation of structural features of allergens and their isoforms has led to strategies aimed at decreasing the allergenicity of proteins while retaining the ability to induce protective immunity.

Parallel development of adjuvant technology, including the use of bacteria or their products to activate the innate immune response and also the use of Th1-stimulating plasmid-encoded cytokines, offers the potential to redirect allergic responses safely and effectively. Many of these approaches are currently being evaluated in clinical trials and hold considerable promise for future therapeutic intervention in allergic diseases.

SALIENT POINTS

- SIT has several advantages; however, current methods are not without the potential for systemic reactions.
- Recombinant allergens can be formulated to replace natural extracts.
- Allergens can be engineered to create low IgE-binding isoforms.
- Allergen DNA vaccines offer a novel form of allergen immunotherapy for the future.
- Allergen fragments, representing B- and/or T-cell epitopes, can be used for allergen immunotherapy with less side effects.

- Microbial adjuvants enhance the potency of allergen vaccines and shift the immune response away from the allergic response.
- TLR agonists and antagonists may play a role in creating adjuvant effects.
- Microparticle or nanoparticle polymers act as adjuvants for allergen vaccines and enhance vaccine efficacy in allergic animal models.
- Cytokines or other immune molecules may function as immune response modifiers for allergen vaccines.

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