

Customized Antigens for Desensitizing Allergic Patients

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I. Introduction

The most effective allergy treatment is one that specifically and with maximum safety cures clinical symptoms caused by allergens in any given patient. In this context, allergen-specific immunotherapy (SIT) offers the best possibility of accomplishing this goal. The process is highly specific because the treatment is targeted at those allergens that the patient and the physician identified as the responsible ones (Frew, 2003). In its conventional form, SIT involves repeated administration of whole allergen extracts to patients displaying immunoglobulin E(IgE)-mediated allergic symptoms and inflammatory reactions caused by natural exposure to allergens. The drawback of this approach is that extracts are complex mixtures that are difficult to standardize. In addition, systemic administration of active allergens can cause severe IgE-mediated side effects during the treatment and therapeutic effective doses often cannot be achieved because of nonstandardized extracts or side effects.

For the best performance of this tailor-made type of treatment, the first crucial step is the exact identification of the molecules causing the allergic reaction, that is, a clear diagnosis establishing the association between the disease manifestation and the IgE-mediated immune reactions. This can be accomplished with a molecule-based diagnosis. Knowledge of the pattern of IgE reactivity at a molecular level allows the formulation of vaccines containing only the allergens to which the patient is allergic, consisting, for example, of cocktails of pure and standardized recombinant allergens instead of whole extracts. Moreover, the selected recombinant allergens can be modified to reduce the risk of IgE-mediated side effects. In this way, higher doses of allergen can be administered to allergic patients increasing the efficacy of the treatment without compromising safety.

Genetic immunization is an attractive alternative for SIT using protein antigens. It has been shown that 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- γ (IFN- γ)-producing CD4⁺T cells. In addition, immunization with plasmid DNA encoding modified allergen genes would fulfill the requirements of efficacy and safety in SIT.

The increasing knowledge of the mechanisms operating in Th1 and Th2 types of immune responses opened various possibilities to modulate an allergic (Th2-type) response. The working hypothesis is that Th1-biased stimuli could prevent the development of an allergic response and might even convert established Th2 responses. Therefore, designed antigens in combination with immunomodulatory adjuvants form the basis for novel and optimized allergen vaccines.

II. Allergy Diagnosis and Immunotherapy: From Extracts to Allergen Arrays and Engineered Vaccines

So far only allergen extracts are routinely applied for diagnostic and therapeutic purposes. These extracts are difficult to standardize regarding their allergen content; several allergens might be underrepresented because of degradation, whereas other nonallergenic components are present and there might be even contamination with allergens from other sources. Thus, extract-based diagnosis goes in the direction of identifying allergenic sources and not individual allergenic molecules, which does not allow the discrimination between co-sensitization and cross-sensitization in patients showing adverse reactions to more than one allergenic source (Pauli, 2000). The development of an allergic disease always requires contact with a sensitizing agent, which does not necessarily have to be the elicitor of the allergic symptoms. Allergen cross-reactivity occurs when IgE antibodies originally raised against one allergen bind or recognize a similar protein from another source (Aalberse *et al.*, 2001). The interaction with such homologous proteins can then trigger allergic reactions or can be completely irrelevant for the patient.

The inadequacy of diagnosis with extracts in cases of cross-reactivity is clearly demonstrated by a survey of mite-allergic Orthodox Jews. Because of strict adherence to kosher dietary laws, the consumption of shellfish is prohibited in this population. Fernandes *et al.* (2003) showed that IgE antibodies of these mite-allergic individuals cross-reacted with shrimp tropomyosin Pen a 1, despite that direct contact with this aliment did not occur. When tested with single allergenic components, such tropomyosin-based cross-reactivity could be easily predicted. Furthermore, with allergen extracts there is always the risk of contamination as seen for mite allergens in animal hair dander extracts producing false-positive results on skin tests (van der Veen *et al.*, 1996). The consequences of such incorrect diagnoses can be taken to the next level, considering cases of indirect sensitization of mite-allergics patients who developed positive radioallergosorbent test (RAST) signals for shrimp and snails after receiving specific immunotherapy for dust mite allergy (van Ree *et al.*, 1996). Induction of new IgE specificities has also been reported for birch- and grass pollen-allergic individuals (Ball *et al.*, 1999b; Moverare *et al.*, 2002a).

Sixty-five percent of patients treated with birch pollen rush immunotherapy developed low levels of IgE antibodies toward additional birch pollen allergens. Among these newly recognized allergens, the highly cross-reactive Bet v 2 and Bet v 4 proteins could represent a risk of clinical consequences for the patient. All these findings demonstrate the unpredictability and shortcomings of diagnosis and immunotherapy using allergen extracts.

A. MOLECULE-BASED DIAGNOSIS: CLUSTERING OF IgE REACTIVITY PATTERNS

A question that arises is how far the allergy diagnosis can be simplified as a result of cross-reactivity phenomena. A study by [van Ree *et al.* \(1999\)](#) demonstrated that a combination of birch, hazel, and alder pollen extracts compared to birch or alder extracts alone was equally efficient in diagnosis. Also, a combination of Bet v 1 and Bet v 2 allergens covered around 94% of the tree-pollen-positive sera used. The remaining few patients were suspected of having IgE against either other minor birch pollen allergens or cross-reactive carbohydrate determinants (CCDs) ([van Ree *et al.*, 1999](#)).

Even when using purified allergens, false-positive results may occur because of CCDs present on plant and invertebrate proteins. A sensitization toward CCDs usually occurs via pollen or insect stings and results in a wide pattern of cross-reactivity to various kinds of food. Anti-carbohydrate IgE antibodies seem to have poor biological activity, and until now their clinical relevance could not be proven. Nevertheless, anti-CCD IgE antibodies frequently produce positive reactions in *in vitro* tests. To minimize these false-positive results, the use of recombinant allergens seems obligatory. However, depending on their way of production (e.g., *Escherichia coli*, *Pichia pastoris*, insect cells, and tobacco plants), recombinant allergens might also show different forms of glycosylation. All allergens produced in prokaryotic cells are not glycosylated, but because of problems regarding protein folding, eukaryotic expression systems are increasingly being employed for the production of recombinant allergens. The glycosylation of these recombinant allergens does not often correlate with their naturally occurring counterparts. In this way, incorrectly glycosylated recombinant allergens could also react with anti-carbohydrate IgE antibodies and produce false-positive results. In terms of immunotherapy with recombinant allergens, the role of glycans for protein folding and their effects on the human immune system need to be further evaluated ([Mari, 2002](#); [Mari *et al.*, 1999](#); [van Ree, 2002](#)).

The precise knowledge of cross-reactivity patterns offers the possibility to define marker allergens indicative of certain linked allergic diseases and to develop strategies for the direct treatment of allergies ([Kazemi-Shirazi *et al.*, 2002](#)). Because many allergens have become available as recombinants, few representative allergens covering most of the IgE epitopes could be selected

for diagnostic purposes. If a patient mounts IgE antibodies against Bet v 1, that individual will likely develop symptoms upon contact with other allergenic sources harboring the corresponding cross-reactive allergens either in pollen or in foods (Ebner *et al.*, 1996). This notion holds true for many cross-reactive allergens (Aalberse *et al.*, 2001; Sicherer, 2001; Vieths *et al.*, 2002). Therefore, such marker allergens could help redefine the current concept of allergy diagnosis, so they not only are useful for the diagnosis of existing allergies, but also might help predict potential sensitization to multiple allergenic sources.

For timothy grass pollen allergy, a panel of allergens, either recombinant or natural, can substitute allergen extracts used in diagnosis. A limited number of allergen molecules were sufficient to diagnose sensitization to this allergenic source in 99% of all patients tested. Moreover, a sensitization profile could be determined, identifying and quantifying the amount of IgE bound to the particular allergens (Valenta *et al.*, 1999a). A study comparing the IgE binding pattern of timothy grass pollen allergens in different populations had similar results (Laffer *et al.*, 1996).

A multipopulation study using molecule-based diagnosis was performed for the selected recombinant birch pollen allergens, rBet v 1, rBet v 2, and rBet v 4. The study revealed different population-dependent recognition patterns of these allergens among birch pollen-allergic patients (Moverare *et al.*, 2002b). With classic diagnostic measures, such patterns could not be determined, which implies that birch pollen-allergic individuals not yet sensitized to Bet v 1 could, for instance, develop such a sensitization upon SIT treatment. Another survey among allergic individuals from the urban areas of Zimbabwe was performed using recombinant timothy grass pollen and dust mite allergens. The allergen-recognition patterns were compared with the ones obtained from patients in central Europe, and striking differences were observed. This profile could be explained by a primary sensitization to Bermuda grass and not to timothy grass pollen. The unusual recognition profile toward mite allergens might also be due to different sensitization with cross-reactive allergens compared to the European population. Interesting to remark is the existence of African patients reactive to birch pollen extracts because of a sensitization to profilin (Westritschnig *et al.*, 2003).

All these findings point out the necessity of molecule-based diagnosis for allergies. Developments in this direction have already been done with the introduction of allergy chips in a microarray format. In one study, 94 purified recombinant or natural allergens were spotted robotically in triplicates on preactivated glass slides and incubated with patients' sera. The results were verified by nitrocellulose blots and skin prick tests, and most of the allergens spotted on the chips gave results comparable to RAST-based results. The advantages of such a system are obvious: Only some hundred picograms

of protein is needed per slide, the system is predestined for high throughput, and allergen-bound IgE antibodies can be quantified (Hiller *et al.*, 2002). With the array technology, it will be further possible to detect different immunoglobulin classes bound to the immobilized allergens, which could be useful to analyze the course of an allergic disease over time, enabling a clinician to monitor allergy treatment (Harwanegg *et al.*, 2003). For the establishment of molecule-based allergy diagnosis on a large scale, well-characterized allergens have to be available in large amounts, with low or no batch-to-batch variations. The use of recombinant allergens offers the best prospect for rational and accurate allergy diagnosis. Over the past few years, allergens from various allergenic sources including pollen, food, house dust mite, cockroach, animal dander, insect venom, latex, molds, and fungi have been cloned and characterized and are now available as recombinant proteins (Andersson and Lidholm, 2003; Arruda *et al.*, 2001; Chapman and Wood, 2001; Kurup *et al.*, 2002; Lorenz *et al.*, 2001; Muller, 2002; Sussman *et al.*, 2002; Thomas *et al.*, 2002). Thus, molecule-based allergy diagnosis should be gaining importance rapidly.

III. Novel Antigen Preparations to Improve SIT

Modification of allergens to improve conventional SIT should aim at the production of 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 (Maurer *et al.*, 1995; van der Heijden *et al.*, 1993) and leads to higher Th2 cytokines and IgE production (Akdis *et al.*, 1998). Modified allergens lacking IgE binding (hypoallergens) might avoid these pathways and preferentially target APCs that use phagocytosis or pinocytosis for antigen uptake (e.g., monocytes, macrophages, and dendritic cells [DCs]). 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 (Fig. 1). The presence of intact T-cell epitopes on hypoallergens would also target T cells, allowing administration of higher doses to induce tolerance of allergen-specific T cells and alteration of cytokine production toward a Th1-like pattern. In this way, vaccine preparations consisting of hypoallergens could replace natural extracts and increase the efficacy and safety of SIT. Other promising approaches include protein fusions and synthetic peptides. Synthetic peptides containing T-cell epitopes were shown in clinical studies to be safe and to induce allergen-specific T-cell hyporesponsiveness. (Fig. 2) gives an overview of designed allergens to be used in immunotherapy of allergic diseases.

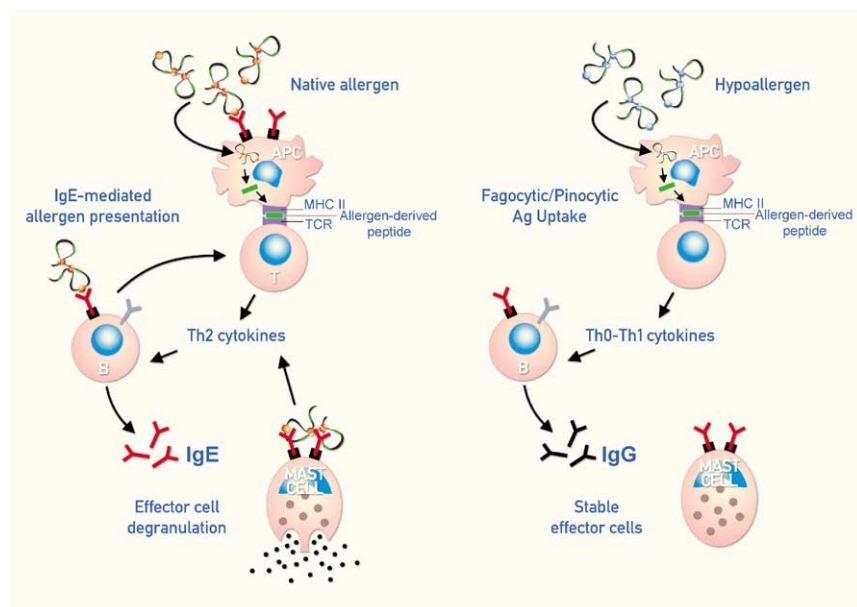


FIG 1 Possible advantages to the use of hypoallergens for specific immunotherapy. Low density of immunoglobulin E (IgE) epitopes would not trigger IgE-mediated side effects upon injection and bypass IgE-mediated allergen presentation, which leads to a Th2-biased immune response.

A. HYPOALLERGENS

The idea of modifying allergens to decrease their allergenicity (IgE binding activity) while preserving their antigenicity (T-cell reactivity) is not new. In fact, earlier studies showed that chemical modification of allergens (e.g., polyethylene glycol [PEG], glutaraldehyde, formaldehyde, etc.) could be used to abrogate IgE binding. Some of these preparations are available in clinical practice (Akdis and Blaser, 2000). More recent developments include chemical modification of allergens by conjugation with synthetic oligonucleotides containing immunostimulatory sequences from bacterial DNA (allergen-ISS conjugates). Allergen-ISS conjugates are promising therapeutics. In clinical trials, an Amb a 1-ISS preparation was shown to be safe and effective for treating ragweed-allergic patients. Recombinant DNA techniques offer the unique possibility of arbitrarily altering the nucleotide sequence of a gene to produce novel “mutant” proteins or genetically engineered proteins displaying altered properties. This is particularly interesting for the production of allergen mutants with predefined properties regarding allergenicity and antigenicity. Akdis *et al.* (1998) showed that bee venom phospholipase A2 (PLA)

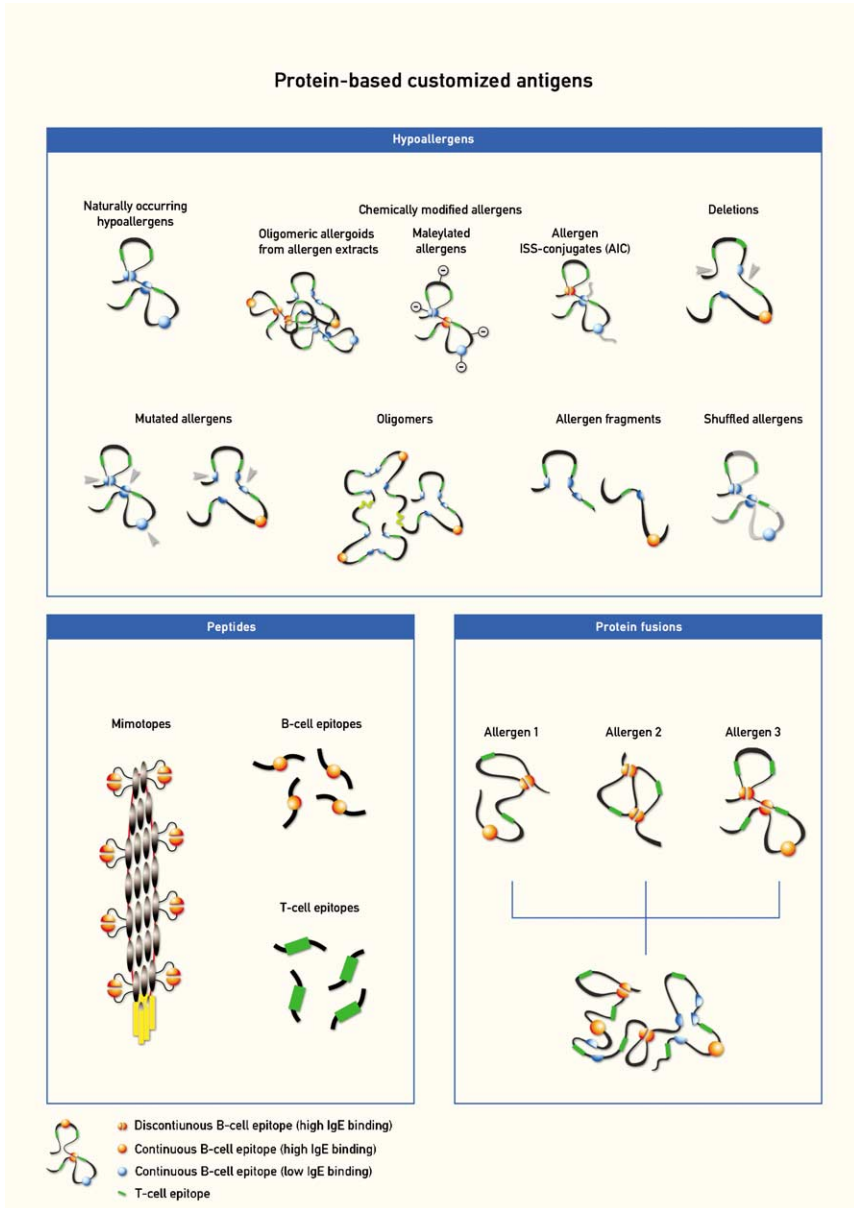


FIG 2 Cartoon representation of customized allergens to improve specific immunotherapy. Examples of naturally occurring hypoallergens are scarce and cannot be widely explored. Modification of allergens can be accomplished by chemical means and by genetic engineering approaches. Synthetic peptides include mimotopes, B-cell epitope-derived nonanaphylactic peptides, and T-cell epitope-containing peptides.

preparations lacking the 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 seem to be crucial aspects in the development of distinct patterns of T-cell cytokines. These findings give further support to the use of hypoallergens in SIT.

1. Chemically Modified Allergens

a. Allergoids. The first attempts of chemically modify allergens and allergen extracts for use in SIT were preparations of aluminium hydroxide (alum)–precipitated aeroallergens or alum-precipitated pyridine extracts with the aim to retain allergens at the injection site. Other modifications such as emulsions of aqueous allergen extracts in mineral oil were also tested but were abandoned because of lack of proof of efficacy and concerns about noxious side effects. Further, urea-denatured, PEG conjugated, and poly D-glutamic acid:D-lysine-linked allergens were investigated for the potential to suppress IgE antibody production. However, clinical trials revealed that the specific IgE levels in these patients were not greatly diminished and specific IgG antibodies raised extensively (Akdis and Blaser, 2000; Middleton *et al.*, 1993).

In other approaches, oligomers were produced by chemical modification of allergens with glutaraldehyde or paraformaldehyde. These chemical modifications seem either to destroy or to mask structural B-cell epitopes, resulting in significantly reduced IgE binding activity but retained immunogenicity. These allergoids seem to be better tolerated by patients, because higher doses of antigen can be administered during SIT and consequently, fewer injections are necessary for successful treatment (Akdis and Blaser, 2000; Middleton *et al.*, 1993). Another possibility of creating chemically modified allergens with reduced allergenic potential is carbamylation. Treating proteins with potassium cyanate at neutral pH levels leads to a transformation of the ϵ -amino group of lysines into ureido groups. Thus, the native monomeric character of the allergens is preserved. The advantage is that monomeric allergoids can be administered to the organism not only subcutaneously, but also through a mucosal pathway. These allergoids can be produced either from allergenic extracts or from purified single allergens. In animal models, the allergoids induced IgG antibodies that recognized the native allergens (Mistrello *et al.*, 1996).

Commercially available allergoids are produced by chemical modification of allergen extracts with aldehydes and are available only for a limited panel of allergens. These allergoid preparations consist of bulky macromolecules with undefined structure and sequence. Even though the standardization of

allergen extracts has improved, the batch-to-batch reproducibility still remains suboptimal. A better approach would probably rely on the use of recombinant allergens for the production of allergoids.

b. Maleylated Allergens. Many proteins are known to become ligands for scavenger receptors (SRs) when chemically modified to enhance their negative charges (Abraham *et al.*, 1995). SRs are members of the so-called *pattern-recognition receptors* (PRRs), which are expressed by APCs and are involved in the recognition of foreign ligands during early phases of the immune response (Gordon, 2002). SRs bind to various polyanionic ligands (e.g., oxidized low-density lipoprotein, maleylated proteins, polyguanylic acid, and fucoidin) and deliver them into the endolysosomal pathway. The enhancement of negative charges in proteins can be accomplished by alteration of the ϵ -amino group of lysine residues with acetic acid or maleic anhydrides. It has been shown that maleylation of protein antigens converts them into SR ligands and their delivery through SRs results in increased immunogenicity with a dominant Th1-type immune response (Abraham *et al.*, 1995; Singh *et al.*, 1998). Therefore, specific targeting of allergens to SR represents an attractive approach to modulate allergen-specific immune responses.

Maleylation of whole Orchard grass pollen proteins was reported to decrease IgE binding activity tested by immunoblots and to reduce skin reactivity (Cirkovic *et al.*, 1999). A maleylated form of shrimp tropomyosin, a major cross-reacting crustacean food allergen, displayed reduced allergenicity as compared to the native counterpart (Rajagopal *et al.*, 2000). Analysis of the cytokine profiles after stimulation of splenocytes isolated from Balb/c mice immunized with maleylated tropomyosin showed a dominant Th1 immune response. Similar results were obtained by targeting a Der p 1 peptide to macrophages via SR (Bhatia *et al.*, 2002). In addition, SR targeting of the Der p 1 peptide and elicitation of Th1-dominant responses was also accomplished by simple coadsorption of the peptide with maleylated poly-L-lysine on alum (Bhatia *et al.*, 2002). The possibility of coadsorption would be particularly interesting for the formulation of multivaccines for immunotherapy of patients allergic to several allergens.

c. Allergen-ISS Conjugates (AIC). One of the lessons from DNA immunization (see Section IV) was that certain immunostimulatory sequences (ISSs) of bacterial DNA can deviate the normally occurring Th2 response to allergens toward a nonallergic Th1 response. Taking advantage of this knowledge, oligodeoxynucleotides (ODNs) containing ISS molecules (ISS-ODN) were conjugated to allergens and led to the design of a novel type of modified allergen (allergen-ISS conjugate [AIC]) for use in SIT (Tighe *et al.*, 2000).

The immunostimulatory property of bacterial DNA was discovered nearly 20 years ago (Tokunaga *et al.*, 1984) and showed that bacterial DNA differs from that of mammals in the frequency of CpG dinucleotides, which are

avoided in mammals (*CpG suppression*) and not methylated in bacteria. Studies with synthetic ODNs have revealed a hexameric consensus sequence for immunostimulatory CpG motifs consisting of a central CpG dinucleotide flanked by two purines at the 5' end and two pyrimidines at the 3' end (5'-PuPuCGPyPy-3'). It turned out that both base-sequence and backbone chemistry influence the immunostimulatory efficacy of individual CpG motifs, which also exhibit marked species specificity (Krieg, 2002).

i. Recognition and Signal Transduction Induced by Immunostimulatory CpG DNA. The cellular events of activation of APCs by CpG DNA strikingly parallel those achieved with other microbial products. These substances are recognized by APCs via PRRs. PRRs were developed early in evolution and represent a mechanism of the innate immune system to signal “danger” based on structural surface characteristics that are shared by a variety of infectious agents but are absent on host cells. CpG-mediated intracellular signaling requires endocytosis of CpG DNA/toll-like receptor (TLR) complexes and acidification of endosomes. Signal transduction involves the myeloid differentiation factor MyD88, IRAK, and TRAF6, activation of I κ B kinase, mitogen-activated protein kinases, the stress kinases N-terminal c-Jun kinases JNK1/2, and p38, and finally results in transcriptional activation of multiple genes involving ATF-2, AP-1 and nuclear factor- κ B (NF- κ B). Many immunoregulatory genes contain NF- κ B-responsive elements in their promoter regions and inhibition of NF- κ B abrogates CpG-mediated immune cell activation (Takeda *et al.*, 2003).

ii. Cellular Targets of CpG Action. Several immune cell species have been identified that respond to CpG DNA. These include B and T cells, natural killer (NK) cells, and APCs (dendritic cells and macrophages). The direct action on APCs is probably most important for the immunostimulatory effect of CpG DNA. These cells, when encountering CpG DNA, undergo dramatic physiological changes affecting both the immunoregulatory functions of the APC themselves and via secretion of cytokines the activation status of other immune cells.

NK cells: Activated NK cells play an important role in the early phase of an immune reaction. These cells provide the majority of IFN- γ before this cytokine is supplied in relevant concentrations by activated T cells. However, purified NK cells appear not to be activated directly by CpG DNA but are potently stimulated by type 1 interferons, IL-12, and tumor necrosis factor- α (TNF- α), which are secreted by macrophages and dendritic cells (DCs) in response to CpG DNA (Ballas *et al.*, 1996).

Macrophages: Macrophages are induced by CpG DNA to secrete a panel of cytokines, in particular type 1 interferons (IFN- α/β), IL-1 β , TNF- α , IL-6, and IL-12. One of the features of interferons is to enhance the cytolytic activity of NK cells and in humans facilitate Th1 cell development by

promoting the expression of IL-12 receptor on these cells. IL-12 is a key cytokine for the development of cell-mediated immunity and the development of Th1-type immune reactions in general.

In addition to these effects, CpG DNA increases the expression of several surface molecules on macrophages, such as major histocompatibility complex class I (MHC-I), intercellular adhesion molecule 1 (ICAM-1), CD40, or CD80/86. These molecules are involved in antigen presentation and activation of lymphocytes. The alterations triggered by CpG oligonucleotides have been shown to persist for a prolonged time (Kobayashi *et al.*, 1999).

DCs: DCs are unique in their ability to activate naive T lymphocytes, and therefore, play a key role as APC for the induction of a primary immune response. Similar to macrophages, bone marrow-derived DCs respond directly to CpG DNA by secretion of TNF- α , IL-6, and IL-12 and with increased levels of CD40, CD86, and MHC-II on their surface (Sparwasser *et al.*, 1998). CpG motifs have been found that induce IFN- γ and IFN- α production in human cells, and functionally distinct types of CpG motifs have been designed to specifically address immunocompetent cells (Krieg, 2002).

T cells: The Th1-biased danger signal inherent to CpG DNA and acting on the innate immune system also has a fundamental influence on the Th1/Th2 balance. IFN- γ promotes Th1 cells by enhancing IL-12 production by macrophages and expression of IL-12 receptors on CD4⁺ T cells. IL-12, in turn, mediates the differentiation of Th1 cells by activation of STAT4. Simultaneously, IFN- γ suppresses the development of Th2 cells.

Although many processes that occur after activation with various microbial products are strikingly similar, CpG DNA may be superior over other adjuvants because of its ability to trigger the release of large amounts of IL-12 (more than lipopolysaccharide) [LPS] (Cowdery *et al.*, 1999). Thus, comparing an adjuvant's potential to induce IL-12 versus TNF- α (and IL-1) might provide a means for evaluating its applicability.

iii. Preclinical Studies with Allergen-ISS Conjugates. Soon after the discovery of the Th1-inducing capacity of ISS-containing CpG motifs, experiments with model allergens proved that systemic or mucosal application of CpG-ODN led to reduction of IL-5 and inhibition of airway hyperresponsiveness and eosinophilia of the airway, lung parenchyma, and blood. The effect on T-cell cytokine production was proven to be indirect via stimulating APCs and NK cells to generate IL-12 and interferons. A single dose of CpG application was as effective as multiple injections of corticosteroids, but only CpG treatment was able to induce allergen-specific IFN- γ production and balance a Th2-type response (Broide *et al.*, 1998; Spiegelberg *et al.*, 1998). The effect of CpG motifs was also tested in murine models using CpG-ODN coadministered with antigen. Treatment of previously sensitized animals with CpG-ODN prevented allergen-induced airway inflammation (Kline

et al., 1998). Using a protective approach with CpG-ODN coinjected with the major birch pollen allergen (Jahn-Schmid *et al.*, 1999) and ragweed allergen (Sur *et al.*, 1999), which was applied 48 hours before allergen challenge, confirmed the Th1-modulatory capacity of CpG motifs. The effects of CpG-ODN were sustained for several weeks and the reaction after boosting indicated a strong Th1-biased memory response. Although coinjection of allergens with CpG-ODN also induces Th1 responses, chemical coupling of the allergen to CpG-ODN proved superior, as demonstrated with the ragweed allergen Amb a 1 (Tighe *et al.*, 2000). Synthetic phosphorothioate (but not phosphodiester)-ODN proved more effective concerning the desired Th1-inducing capacity (Jahn-Schmid *et al.*, 1999; Parronchi *et al.*, 1999).

CpG-ODN conjugated to allergen can mask allergenic determinants, thus interfering with cross-linking of Fc IgE receptors on mast cells and basophils. Coupling the allergen to ISS-ODN also was superior to a mixture of CpG-ODN and allergen with respect to the immunogenicity profile. A possible explanation for this is that both the allergen and the CpG motifs are delivered together to the same APC for processing and induction of type 1 cytokine secretion. This proved to be the case since much less ISS-ODN in the form of an AIC was necessary to induce the same IgG2a antibody titer as compared to allergen CpG-ODN mixtures. AIC induced a Th1 response to the allergen, as shown by strong IgG2a and almost no IgE antibody production and antigen-specific IFN- γ -secreting spleen cells (in contrast to control mice, which produced IgG1 and IgE antibodies and showed elevated levels of IL-5). Sensitization with allergen and treatment with AIC induced IgG2a antibodies without significantly increasing the IgE titer, and spleen cells from AIC-treated mice showed significantly more IFN- γ secretion than spleen cells from the control groups, both pointing to a therapeutic potential of AICs (Spiegelberg *et al.*, 2002).

The effects of AIC have also been tested in a mouse model of asthma (Shirota *et al.*, 2000). Ovalbumin(OVA)-CpG-ODN conjugates were applied intratracheally and the effects on airway eosinophilia and airway hyperresponsiveness were studied. AIC reduced airway eosinophilia and hyperresponsiveness significantly more effectively than a mixture of OVA and CpG-ODN and the suppressive effect lasted for more than 2 months. In addition, the AIC induced a striking increase in IFN- γ secretion by OVA activated lymph node cells and concomitantly decreased the IL-4 and IL-5 secretion, suggesting a switch from a Th2 to a Th1 anti-OVA T-cell response. These results were confirmed in a similar model system with the clinically relevant ragweed allergen Amb a 1 (Santeliz *et al.*, 2002). Furthermore, CpG-ODN inhibited early and late phases of murine-allergic conjunctivitis after sensitization with short ragweed (Magone *et al.*, 2000).

Whereas the protective capacity of AIC seems very effective under various conditions, therapeutic protocols dealing with balancing ongoing Th2-type responses were not successful in all cases. Prevaccination with CpG-ODN coapplied with the mosquito salivary antigen Aed a 2 inhibited the development of IgE antibodies after sensitization. However, an established Th2-type response was not influenced by administration of CpG-ODN together with recombinant Aed a 2 (Peng *et al.*, 2001). The Th1-modulating capacity of CpG-ODN coupled to or mixed with the allergen obviously also depends on the antigenic and/or immunogenic nature of the allergen and the Th2-stimulating protocol (Hochreiter *et al.*, 2001), and in the case of covaccination of allergen together with CpG-ODN, prepriming with CpG motifs apparently is superior over simultaneous application (Kobayashi *et al.*, 1999). Experiments focused on the mechanisms by which AIC stimulates Th1-type responses show enhanced binding of AIC to DC, compared to native allergen, thereby improving the phagocytosis of the allergen-CpG-ODN conjugate. The enhanced binding was not sequence specific because AIC containing a non-CpG-ODN also bound better to DC than allergen without coupled ISS-DNA. However, the induction of type 1 cytokine secretion by DC occurred only with allergen-DNA conjugates that contained CpG-ODN (Shirotta *et al.*, 2001). Based on the findings that DCs, like T cells, can be subgrouped into type 1 and type 2 cytokine-secreting cells and that DCs from atopic patients secrete more type 2 cytokines than DCs from nonallergic humans (Caron *et al.*, 2001; Uchida *et al.*, 2001), it may be of interest to determine the secretion profile of DCs from atopic patients after immunotherapy in general and especially after treatment with AIC.

iv. Clinical Trials with AIC. The preclinical data obtained in mice and in *in vitro* studies with human lymphocytes, both indicating the Th1-stimulating potency of CpG motifs (Bohle *et al.*, 1999; Marshall *et al.*, 2001; Tighe *et al.*, 2000), led to the development of an early phase clinical trial with patients suffering from ragweed seasonal allergic rhinitis. AIC with an average of four ISS-ODN conjugated per molecule of Amb a 1 was tested for safety, tolerability, and immune response in comparison to a licensed ragweed allergen extract in a phase I trial (Creticos *et al.*, 2000). This clinical study with a quantitative intradermal procedure indicated that more than 100-fold higher quantities of Amb a 1 in the form of AIC were necessary to induce the same wheal and flare skin test reaction as native ragweed allergen. Preliminary data from early phase I/II clinical trials showed that the Amb a 1-specific IgE response was similar among study subjects who received subcutaneous injections of AIC and those who received placebo injections (Creticos *et al.*, 2001; Dieudonné *et al.*, 2001). In contrast, IgG anti-Amb a 1 antibody titers after six weekly injections with AIC appeared comparable to titers measured in sera from individuals who received immunotherapy for more than 1 year with licensed ragweed allergen

extracts. Additional phase II efficacy studies are underway to assess further the safety, immune response, and efficacy of AIC for immunotherapy of ragweed allergic rhinitis.

Concluding, AIC seems to display the required features of modern vaccines for an improved SIT such as a high safety profile and strong immunogenicity. In addition, and in contrast to SIT with allergen extracts, the curative principle can be clearly attributed to the balancing of the allergic Th2-type response by counteracting with Th1-inducing stimuli. Whereas the mechanisms of conventional SIT are still poorly understood and controversially discussed, SIT with AIC uses an adjuvant agent with well-known and defined functional characteristics. However, it must be stated that despite encouraging preliminary data from initial clinical trials, most of the optimistic interpretations still originate from animal experiments. Moreover, most of these data come from experiments with model molecules such as ovalbumin or *E. coli* β -galactosidase. Both antigens are highly immunogenic and differ from clinically relevant allergens in many aspects.

The main potential for harmful side effects of AIC, however, may result from the downstream events of CpG DNA, which are similar to those of other bacterial products, such as LPS. CpG DNA was able to induce septic shock symptoms, and it was found to synergize with LPS in the induction of TNF- α (Cowdery *et al.*, 1996; Sparwasser *et al.*, 1997).

With respect to clinical trials, it must be emphasized that overwhelming Th1 immunity with forced application of CPG motifs or treatment with AIC holds the risk of potential promotion of autoimmunity (Segal *et al.*, 1997). Profound knowledge of the immunogenic nature of the selected allergen, the proper choice of AIC dose, and vaccination protocols should help avoid these risks.

2. Naturally Occurring Hypoallergens

Complementary DNA (cDNA) cloning of allergens showed that many major allergens are encoded by gene families. Sequence polymorphisms have been described for ragweed Amb a 1 (Griffith *et al.*, 1991), hazel Cor a 1 (Breiteneder *et al.*, 1993; Luttkopf *et al.*, 2002), birch Bet v 1 (Swoboda *et al.*, 1995), group 1 (Au *et al.*, 2002; Chang *et al.*, 1999) and group 5 grass allergens (Muller *et al.*, 1998b; Wurtzen *et al.*, 1999), apple Mal d 1 (Helsper *et al.*, 2002; Son *et al.*, 1999), celery Api g 1 (Hoffmann-Sommergruber *et al.*, 2000), *Parietaria* Par j 1 (Duro *et al.*, 1997), olive Ole e 1 (Gonzalez *et al.*, 2002), group 1 and 2 dust mite allergens (Smith *et al.*, 2001a, 2001b), latex Hev b 7 (Sowka *et al.*, 1999), and cow dander Bos d 2 (Rautiainen *et al.*, 2001). Despite extensive characterization of isoallergens at the DNA level, very little information is available about their relevance at the level of expressed protein. Analyses at the protein level were performed for Bet v 1 and Mal d 1 isoforms (Helsper *et al.*, 2002;

Swoboda *et al.*, 1995). The possibility that isoallergen sequences result from cloning artifacts cannot be ruled out, especially those obtained by polymerase chain reaction (PCR) cloning. In fact, this was shown to be the case for one Mal d 1 isoform (Helsper *et al.*, 2002).

Because of sequence variations, isoallergens might have different antigenic and/or allergenic activities. Differences in T-cell reactivity of isoforms have been reported for Cor a 1 (Schenk *et al.*, 1994), Bet v 1 (Ferreira *et al.*, 1996), Phl p 5 (Muller *et al.*, 1998b; Wurtzen *et al.*, 1999), and Der p 2 (Hales *et al.*, 2002).

Investigation of the IgE binding activity of isoallergens led to the identification of naturally occurring Bet v 1 hypoallergens (Ferreira *et al.*, 1996). Isoforms Bet v 1d, Bet v 1g, and Bet v 1l were found to be highly antigenic in T-cell proliferation assays and low in their allergenic activities *in vitro* (Ferreira *et al.*, 1996) and *in vivo* (Arquint *et al.*, 1999). It is possible that the low IgE binding activity of these isoforms resulted from incorrect folding during the recombinant production. However, the crystal structure of the hypoallergenic isoform Bet v 1l was found (Markovic-Housley *et al.*, 2003) and has been shown not to be significantly different from the structure of the high IgE binding isoform Bet v 1a (Gajhede *et al.*, 1996). Thus, the low IgE binding activity of certain isoforms is not due to problems in the recombinant production leading to unfolded proteins. Such well-characterized molecules would be excellent candidates for specific immunotherapy. However, naturally occurring hypoallergens have not been identified for other allergen families. Instead, genetic engineering has been widely used to generate hypoallergens.

3. Engineered Hypoallergens

Genetic engineering involves the targeted modification of a protein to alter its function or properties in a predictable manner. This requires the complete understanding of the relationship between structure and function/properties for precise and effective manipulation. Genes can be altered by changing specific base pairs (mutated gene), by introducing a new piece of DNA into the existing DNA molecule (chimeric or hybrid gene), by fusions (fused genes), and by deletions (truncated gene or fragments). With the exception of the DNA shuffling approach, 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 cases, 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. 3). Presently, the allergen databank (www.allergen.org) from the Allergen Nomenclature Subcommittee of the International

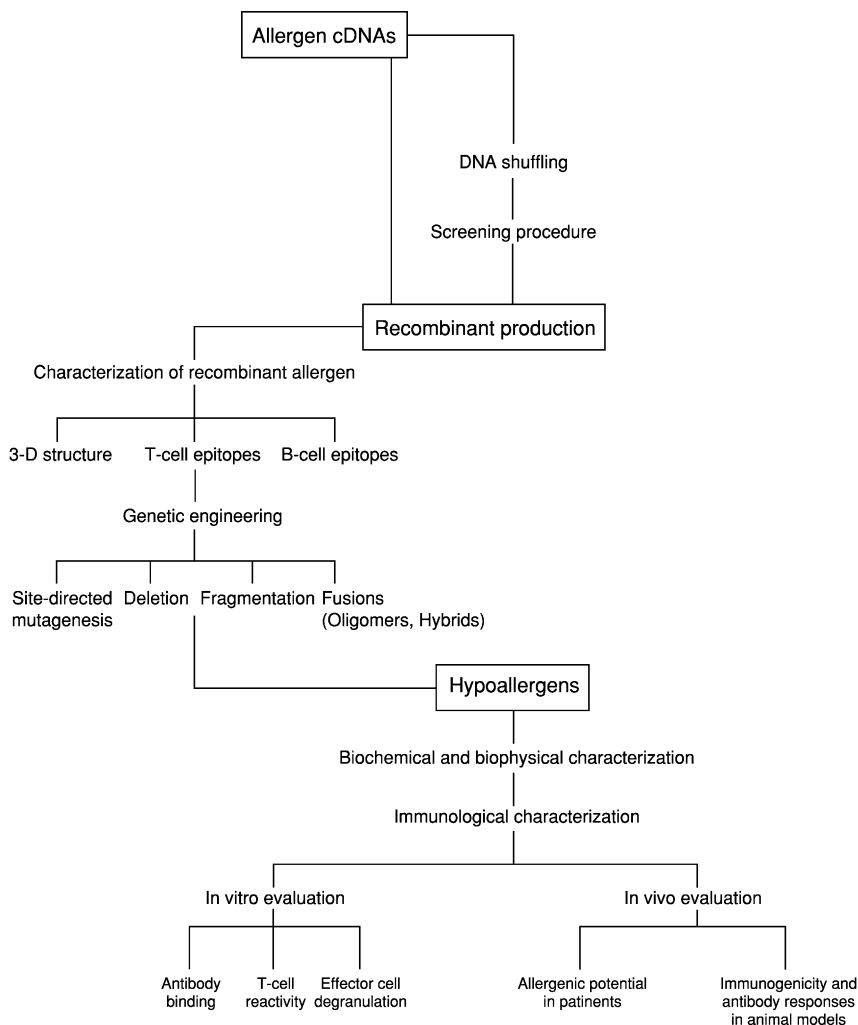


FIG 3 Task tree for genetic engineering and evaluation of hypoallergenic molecules for specific immunotherapy. The DNA shuffling approach bypasses the need to identify B- and T-cell epitopes of allergens.

Union of Immunological Societies (IUIS) contains a list with more than 400 allergens and almost 200 isoallergens. For most of these allergenic proteins originating from various sources, the complete cDNA sequences have been determined and in some cases, the three-dimensional structures. This large number of available sequences goes parallel with an impressive number of

publications dealing with B- and T-cell epitopes of allergens. In the next sections, we discuss approaches for genetic engineering and provide examples of engineered allergens and their evaluation. Fragments and oligomeric hypoallergenic forms of Bet v 1 were the most extensively characterized preparations and are now in clinical trials. Unfortunately, no clinical evaluation of wild-type recombinant Bet v 1 in comparison with extracts and engineered hypoallergenic forms has been performed.

a. Site-Directed Mutants. Knowledge of crucial amino acid residues or motifs involved in IgE recognition can be used to alter dominant epitopes by site-directed mutagenesis. Data showing 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 (Breiteneder *et al.*, 1993; Ferreira *et al.*, 1996) constitute the basis for engineering a full-length Bet v 1 hypoallergen (Ferreira *et al.*, 1998). 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. The amino acid residues occurring in positions 113, 57, 125, 112, 30, and 10 of Bet v 1a were substituted using *in vitro* site-directed mutagenesis by those present in the same positions of low IgE binding isoforms. In this way, a Bet v 1 mutant carrying six point mutations was produced, which displayed extremely low IgE binding activity for all patients tested. *In vivo* (skin prick) tests indicated that the potency of the six-point mutant to induce typical urticarial skin reactions in allergic individuals was dramatically reduced (100- to 1000-fold) compared to Bet v 1a. T-cell clones (TCCs) established from the peripheral blood of birch pollen-allergic patients and reactive with Bet v 1a were also activated by the six-point mutant.

IgE recognition of a group of calcium-binding allergens found in various pollens is influenced by bound calcium. This led to the idea of disruption of the EF-hand calcium-binding domains for engineering hypoallergenic mutants. In this way, calcium-binding-deficient mutants of Bet v 4 (Engel *et al.*, 1997) and Bra r 1 (Okada *et al.*, 1998) with reduced IgE binding activities were generated. However, data concerning T-cell recognition of calcium-binding allergens and their engineered counterparts are not available.

B- and T-cell epitope mapping and sequence comparison of group 5 allergens from different grasses provided the basic information for introducing point mutations in highly conserved sequence domains of Lol p 5. Hypoallergenic forms of Lol p 5 were produced containing all relevant T-cell epitopes (Swoboda *et al.*, 2002). Likewise, hypoallergenic variants of latex Hev b 5 (Beezhold *et al.*, 2001), apple Mal d 1 (Son *et al.*, 1999), egg Gal d 1 (Mine *et al.*, 2003), and peanut Ara h 1, Ara h 2, and Ara h 3 (Bannon *et al.*, 2001) were also generated by site-directed mutagenesis. Modified peanut allergens retained the ability to stimulate T-cell proliferation using peripheral blood

mononuclear cells (PBMC) from allergic patients. In addition, peanut hypoallergens have been tested in a murine model of peanut anaphylaxis (Li *et al.*, 2003) and shown to be effective. Hypoallergenic variants of the major allergen of Par j 1 displaying altered conformation were constructed (Bonura *et al.*, 2001). Par j 1 is a member of the nonspecific lipid transfer proteins (nsLTPs) with a characteristic α - α - α - β structure that is stabilized by four disulfide bonds (Colombo *et al.*, 1998). Targeting these disulfide bonds by site-directed mutagenesis resulted in molecules with altered conformation and decreased IgE binding activity but also activated allergen-specific T cells. Disruption of the native conformation by targeting disulfide bonds could be an approach generally used for engineering allergenic nsLTPs, including food-derived members. Disulfide bonds stabilizing the antigenic structure of major allergens of house dust mites were also targeted by site-directed mutagenesis. Hypoallergenic variants of Der p 2 (Smith and Chapman, 1996), Der f2 (Takai *et al.*, 1997), and Lep d 2 (Eriksson *et al.*, 2001; Kronqvist *et al.*, 2001) were produced and evaluated for their IgE-mediated reactions and cellular responses. One potential problem when targeting the conformation of allergens might be the solubility of the final product, because denatured or unfolded proteins tend to form aggregates. Protein preparations consisting of aggregates are not suitable candidates for vaccine development.

b. Deletion Mutants. Hypoallergenic variants can be engineered by deleting DNA segments in the gene corresponding to IgE binding epitopes. This approach was successfully used for the timothy grass pollen allergen Phl p 5b (Schramm *et al.*, 1999). Epitope mapping was performed using overlapping recombinant fragments and at least four continuous IgE binding epitopes were identified. Deletions avoiding 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 allergen (Wu *et al.*, 2002). Based on the results obtained by proteolytic fingerprinting, a deletion mutant of ryegrass Lol p 1 was produced, which displayed decreased IgE binding activity and did not trigger histamine release up to a concentration of 10 mg/ml (Tamborini *et al.*, 1997). This mutant was not tested in T-cell proliferation assays and for skin reactivity.

c. Fragments. The disruption of the three-dimensional structure by fragmentation could be a useful approach to reduce the anaphylactic potential of allergens. IgE epitopes can be formed by a stretch of amino acids in a row (continuous epitopes) or by nonadjacent sequence elements brought together by folding (discontinuous or conformation-dependent epitopes). IgE recognition of continuous epitopes might also depend on their conformation, which

might only occur in the context of the folded allergen molecule. The three-dimensional structure of Bet v 1 was disrupted by expressing in *E. coli* two fragments of the cDNA corresponding to amino acids 1–74 and 75–160 (Vrtala *et al.*, 1997). 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 to the native intact Bet v 1 allergen (Pauli *et al.*, 1999; van Hage-Hamsten *et al.*, 1999). Further, immunization of mice and rabbits with Bet v 1 fragments induced IgG antibodies that inhibited binding of IgE from allergic patients to wild-type Bet v 1 (Vrtala *et al.*, 2000). Clinical trials now show the efficacy of vaccines based on Bet v 1 fragments for immunotherapy of birch pollen-allergic patients.

Nonanaphylactic fragments of the major house dust mite allergen Der f 2 were produced by C- and N-terminal deletions and were mixed after separate refolding of the denatured fragments (Takai *et al.*, 1999). Fragments of the calcium-binding allergens Bet v 4 (Twardosz *et al.*, 1997) and Aln g 4 (Hayek *et al.*, 1998) and an N-terminal fragment of Lol p 1 from ryegrass (Tamborini *et al.*, 1997) also showed decreased IgE binding activities. However, they were not investigated concerning T-cell reactivity and immunogenicity.

d. Oligomers. Vrtala *et al.* (1999) constructed oligomeric forms of the major birch pollen allergen Bet v 1. Two or three copies of the full-length Bet v 1 molecule were linked by short spacers, expressed in *E. coli*, and purified to homogeneity. *In vitro* studies using patients sera showed comparable IgE binding for the rBet v 1 monomer, dimer, and trimer, whereas the trimer demonstrated profoundly reduced histamine release of patients' basophils compared to the monomer. This reduced anaphylactic potential was verified *in vivo* by skin testing of birch pollen-allergic patients. When compared to the Bet v 1 monomer, the Bet v 1 trimer induced similar proliferation and cytokine production upon stimulation of Bet v 1-specific TCCs. *In vivo* studies in animal models showed the ability of the Bet v 1 trimer to induce IgG blocking antibodies, which inhibit binding of human IgEs to rBet v 1 (Pauli *et al.*, 1999; van Hage-Hamsten *et al.*, 1999; Vrtala *et al.*, 2001). As for the Bet v 1 fragments, clinical trials are also underway with the Bet v 1 trimer. It will be interesting to compare the efficacy and safety of the different hypoallergenic preparations of Bet v 1.

e. Chimeras. King *et al.* (2001) reported an approach to genetically modify allergens in which hybrids are 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. The idea is that the homologous host protein serves as a scaffold to maintain the native structure of the guest allergen of interest to preserve conformational-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 10–49 residues of Ves v 5 showed a 100- to 3000-fold reduction in allergenicity determined by histamine release assays with basophils from yellow jacket-sensitized patients. Such an approach could be employed for several allergens with known homologs displaying low allergenic activity. Whether injections of such hybrids would induce novel IgE specificities remains to be established. A promising approach to generate allergen chimeras is DNA shuffling. When regions of a protein that are critical for structure and/or function are known, site-directed mutagenesis is an appropriate and well-established approach to create molecules with desired properties. Unfortunately the information required for such a rational approach in protein design cannot always be provided. Therefore, within the last decade a new DNA-based recombination technique termed *DNA shuffling* has gained prominence. DNA shuffling was demonstrated to be superior over alternative random mutagenesis techniques such as error-prone PCR or degenerate cassette mutagenesis (Patten *et al.*, 1997; Punnonen, 2000). The ambition of this *in vitro* evolution process is to mimic and profoundly accelerate natural design processes and to guide them in a certain direction accomplished by selective screening. The DNA shuffling technique itself comprises the fragmentation of two or more related genes and their random reassembly, followed by an amplification step. Because of sequence similarities of the parental genes, the gene fragments can form areas of recombination during the “shuffling” process, which results in the generation of new full-length genes. Libraries created in this way consist of highly diverse sequences and can be subjected to a diversity of screening procedures for the selection of molecules fulfilling the desired requirements (Stemmer, 1994a, 1994b).

This trend toward *in vitro* protein evolution is also reflected in the literature, where many publications describing superior molecules are becoming available. The DNA shuffling technique has been successfully applied to generate improved variants of enzymes, proteases, antibodies, viral gene therapy vehicles, and cytokines (Kurtzman *et al.*, 2001; Leong *et al.*, 2003; Raillard *et al.*, 2001; Soong *et al.*, 2000).

Because of the enormous potential of this technique, procedures for optimal shuffling have been intensively investigated. Consequently, a large panel of DNA shuffling techniques have been developed using enzyme-based approaches such as different endonucleolytic or restriction enzyme digestion protocols or primer-based methods (Kikuchi *et al.*, 1999; Kurtzman *et al.*, 2001). Following these techniques, parameters as the number of template sequences, the degree of sequence homology among the templates, and the number of crossovers per kilobase DNA can be adapted to particular needs.

DNA shuffling seems to be an ideal strategy to be exploited for generating novel allergy therapeutics and improved tools for allergy diagnosis. In terms of allergy treatment, reduced IgE binding capacity and conserved T-cell-activating properties are two critical demands. Diminished IgE reactivity could be achieved by minimizing either the number of epitopes per shuffled molecule to zero or one that would avoid cross-linking of IgE receptors on effector cells. Further, the affinity of IgE antibodies toward these epitopes could be reduced. Nevertheless, the shuffled products should be able to influence the T-cell population of an allergic individual by either shifting the typical Th2 toward a Th1 response or inducing T-cell tolerance. Considering cross-reactive allergens, the generation of allergen chimeras associating T-cell epitopes from several allergens but at the same time with extremely reduced anaphylactic potential also becomes possible by applying DNA family shuffling. Chimeric proteins obtained by family shuffling could be beneficial not only for allergy treatment but also for diagnostics, especially when considering allergens with superior IgE binding activities (Crameri *et al.*, 1998; Ferreira *et al.*, 2002; Punnonen, 2000).

In a preliminary experiment, a group of pollen allergens derived from birch, hazel, and alder pollen, all belonging to the highly cross-reactive Bet v 1 family, were selected for DNA family shuffling. The resulting chimeric allergens were shown to be composed of several epitopes derived from different parental genes. Interesting results were obtained regarding the IgE reactivity of the chimeric Bet v 1 molecules when tested with birch pollen-allergic patients' sera. In these experiments, candidate molecules showing either elevated or diminished IgE binding activity compared to the parental proteins could be identified. The antigenicity of the chimeric Bet v 1 molecules was comparable to that of wild-type Bet v 1a, as determined by *in vitro* T-cell proliferation assays using T-cell lines from Bet v 1-sensitized mice and Bet v 1-specific T-cell lines derived from birch pollen-allergic individuals (Wallner *et al.*, 2002; Ferreira, 2003, unpublished data).

A. SYNTHETIC PEPTIDES

In principle, synthetic peptides could be used in three approaches of SIT. Nonanaphylactic peptides derived from surface-exposed areas and comprising IgE binding epitopes of allergens could be used for immunization aiming at the induction of blocking IgG antibodies. The same idea is behind the concept of mimotopes: synthetic peptides mimicking IgE-binding epitopes of allergens. Some aspects could limit the use of peptides corresponding to B-cell epitopes. In general, peptides are not good antigens and induce poor antibody responses. The application of suitable carriers or multimerization could be used to improve their immunogenicity, although multimerization might also increase the risk of IgE cross-linking events. In addition, the opinion that

blocking IgG antibodies has beneficial effects could be challenged, because many mast cells are present on the mucosa, and thus, IgE would encounter the allergen before it could be captured by IgG antibodies. Very promising is the approach using peptides containing T-cell epitopes. Clinical studies have shown their applicability and effectiveness in inducing T-cell hyporesponsiveness.

1. B-cell Epitope-Derived Peptides

Fragmentation of allergenic molecules offers a possibility to convert these proteins into hypoallergenic derivatives. First, developments in this direction were done some decades ago when low-molecular-weight allergen fragments were produced by proteolytic digestion of allergen extracts. The idea was to destroy IgE epitopes and to create peptides unable to cross-link effector cell-bound IgE (Valenta *et al.*, 1999b).

In principle, one can differentiate between two types of B-cell epitopes, conformational and continuous epitopes. By epitope mapping and structural analysis, one could identify those areas responsible for IgE binding to an allergen. For the Phl p 1 allergen, five continuous IgE epitopes were identified by gene fragmentation. These epitopes represented only a portion of the Phl p 1 B cell epitopes. However, it was demonstrated that grass pollen-allergic patients mounted protective IgG antibodies against these fragments after receiving SIT *immunotherapy* with grass pollen extracts (Ball *et al.*, 1999a). In another study, nonanaphylactic peptides based on identified B-cell epitopes of Phl p 1 were synthesized. All peptides lacked secondary or tertiary structure as determined by nuclear magnetic resonance (NMR) analysis. The low allergenicity of these peptides was demonstrated by enzyme-linked immunosorbent assay (ELISA), basophil histamine release, and skin prick tests. In animal models, the synthetic peptides were able to induce IgG antibodies that recognized intact Phl p 1 and inhibited binding of human IgE antibodies to the native allergen (Focke *et al.*, 2001).

2. T-cell Epitope-Containing Peptides

Peptide-based immunotherapy uses peptide fragments containing T-cell-reactive epitopes instead of complete allergen molecules. In principle, these peptides are unable to cross-link two IgE molecules necessary to activate mast cells but can induce allergen-specific T-cell hyporesponsiveness (Briner *et al.*, 1993; Tarzi and Larche, 2003). Earlier studies with two relatively long peptides (27 amino acids) of the major cat allergen Fel d 1 showed some clinical efficacy for cat-allergic patients. However, the approach was associated with a high frequency of adverse reactions, both immediate and late-onset symptoms (Norman *et al.*, 1996). The immediate reactions very likely resulted from cross-linking of IgE. Using shorter peptides to avoid IgE cross-linking, a number of studies were performed in which peptides were administered to

allergic asthmatic subjects (Haselden *et al.*, 1999; Oldfield *et al.*, 2001, 2002). At higher peptide doses, MHC-restricted activation of allergen-specific T cells led to late asthmatic reactions. Because these peptides were not capable of inducing *in vitro* histamine release from basophils and acute bronchoconstriction, the observed reactions were assumed to be independent of IgE. Rechallenge with peptides was associated with a marked reduction of the late asthmatic reaction and in the *in vitro* proliferation/cytokine production of PBMC, suggesting the induction of a state of allergen-specific hyporesponsiveness, or tolerance. To evaluate the potential of allergen-derived peptides as a form of immunotherapy for cat allergy, a randomized, double-blind, placebo-controlled clinical trial was performed with a mixture of 12 peptides generated from the sequence of Fel d 1. The study showed statistically significant reductions in early and late-phase skin reactions to allergen and a reduction of Th1 and Th2 cytokines in PBMC after allergen stimulation. The reduction in the skin reactions to allergen was accompanied by an increase in PBMC-derived IL-10 production, suggesting a role of regulatory T cells in tolerance induced by peptides. Short peptides from phospholipase A2, the major allergen of bee venom, were also clinically evaluated (Muller *et al.*, 1998a). Successfully treated patients showed an immunomodulation toward the whole allergen with specific T-cell anergy and a decrease in specific IgE/IgG4 ratio. More recently, a double-blind, placebo-controlled phase I clinical trial in patients allergic to bee venom was conducted to evaluate long synthetic overlapping peptides (LSPs) derived from phospholipase A2 sequence (Fellrath *et al.*, 2003). The results demonstrated that LSPs were safe and induced Th1-type immune deviation, allergen-specific IL-10 production, and T-cell hyporesponsiveness.

Together, all these results indicate that allergen peptide immunotherapy might be an effective and safe alternative to conventional SIT.

3. Mimotopes

Artificial peptide structures mimicking natural antibody binding epitopes of an allergen, the so called *mimotopes*, have been described for the major birch pollen allergen Bet v 1. These peptides were obtained from phage libraries, which displayed random peptides of defined length fused to phage surface proteins. In this way, phage ligands for the monoclonal mouse anti-Bet v 1 IgG BIP 1, termed *Bet mim 1*, and for a pool of polyclonal patients' IgEs, termed *Bet mim E*, were selected (Jensen-jarolim *et al.*, 1998). Because most B-cell epitopes are conformational, these two peptide mimotopes were modeled on the three-dimensional structure of Bet v 1. The mimotope Bet mim E was found to cover an area assembled by the amino acids 9–22 and 104–113, which are found in proximity on the surface of Bet v 1. This correlates with the data obtained by Ferreira *et al.* (1998) where they identified six amino acid positions crucial for IgE binding to Bet v 1. Three out of these six amino acids are

within the area deduced for IgE binding by Bet mim E. A possible therapeutic use of such mimotopes could be the induction of IgG blocking antibodies in patients sensitized to a single allergen. This was shown by *in vitro* studies using sera from mice immunized with Bet v 1 IgE mimotopes attached to the surface of phages. These mice produced IgG antibodies, which could drastically reduce human IgE binding toward Bet v 1 (Ganglberger *et al.*, 2000, 2001b).

For the use of mimotope-based vaccines in humans, phages might not represent the ideal vector. Although phages are immunogenic vectors and the mimotopes presented on the phage surface remain in a stable conformation, phages could infect *E. coli* cells in the gut of the patient, which might have unforeseeable consequences. Further, hundreds of mimotopic peptides are presented on a single phage particle and the application of phages as immunogenic vectors might be associated with a high risk because of cross-linking of membrane-bound IgE on effector cells. Therefore, mimotopes bound to the surface of a streptococcal albumin-binding protein were tested. With this system, the mimotopes retain the same conformation as on the phage particles, protein also serves as an immunogenic vector, and the mimotopes are presented in a monovalent manner, which would reduce the risk of IgE cross-linking (Ganglberger *et al.*, 2001a).

Mimotopes have not only been identified for Bet v 1 but also for Bet v 2, Der p 1, and group 1 grass pollen allergens (Furmonaviciene *et al.*, 1999; Leitner *et al.*, 1998; Suphioglu *et al.*, 2001). Approaches using mimotopic peptides will certainly help identify more structural B-cell epitopes on allergens. Also, therapeutic applications with these molecules would seem possible.

A. PROTEIN FUSIONS

An alternative approach consisting of a fusion of two unrelated allergens from the same species was implemented in a study with the timothy grass pollen allergens Phl p 1, Phl p 2, Phl p 5, and Phl p 6 (Linhart *et al.*, 2002). Dimeric fusion molecules (rP2-P6, rP6-P2, and rP5-P1) were constructed, which showed similar secondary structures as equimolar mixes of the individual allergens, as determined by far-UV CD. IgE inhibition experiments revealed that the fusions represent the full spectrum of B-cell epitopes and all three fusions induced strong proliferative response in PBMCs from grass pollen-allergic patients. It was also observed that the immunogenicity of these hybrids was altered compared to the parental allergens. Molecules showing low immunogenicity as monomers displayed increased potential of T-cell stimulation as fusion partners. These findings offer novel possibilities in terms of SIT. Because allergen extracts used for immunotherapy bear not only the risk of de novo sensitization toward new allergens, also the different allergens administered are not equally efficient in stimulating the human immune system. The latter seems true also for equimolar combinations of

recombinant allergens. By fusing grass pollen allergens, it was possible to increase the immunogenicity of allergens displaying weak immunogenic activity, which seems ideal for combinatorial allergy vaccines.

IV. Customized Allergen DNA Vaccines

A number of preclinical studies proved the enormous potential of DNA immunization for the treatment of allergy. The antiallergic effect of DNA vaccines can be clearly attributed to the recruitment of Th1 cells and the establishment of a balancing Th1-biased cytokine milieu, and because of their unique features, DNA vaccines enable both preventive and therapeutic applications.

A major selling point for DNA vaccines is their high safety profile. The lack of viral antigens (like with viral vector vaccines) and serious side effects in animals and humans makes them attractive, especially when multiple immunizations are required. However, critics have been raising a concern of the hypothetical possibility that plasmid DNA could integrate into the genome of the transfected host cell with the risk of tumorigenesis, for example, through inactivation of tumor suppressor genes. Several studies have addressed this concern and have concluded that integration would occur at a rate that is several orders of magnitude below the rate of spontaneous mutations ([Martin et al., 1999](#)).

A. SHORT INTRODUCTION INTO DNA VACCINES

DNA vaccines, also called *genetic vaccines*, introduced a new era in vaccine research and development in the 1990s ([Tang et al., 1992](#); [Ulmer et al., 1993](#)).

The principles underlying this new generation of vaccines differ significantly from those of former generations of vaccines. Instead of an antigen itself, the genetic information for a particular protein is delivered. After injection of the gene of interest, host cells take up the DNA. The encoded gene is subsequently transcribed and translated and the newly synthesized protein is processed and presented to the immune system.

DNA vaccines not only carry the genetic information for the antigen of interest but also deliver an adjuvant effect as a result of the presence of immunostimulatory CpG motifs within the bacterial backbone (see [Section III](#)).

Considering the similarity of the cellular events to the endogenous pathways preceding antiviral immune responses, it was originally postulated that this new technology would be useful only for vaccination against viral infections ([Ulmer et al., 1993](#)). However, within a few years after the first reports of this new generation of vaccines, the number of publications describing the usefulness of DNA vaccines soared. They demonstrated their effectiveness in a wide variety of disease models including protective immunity against bacterial and

parasitic infections and offered new perspectives for the treatment of cancer, autoimmunity, and allergic diseases (Donnelly *et al.*, 1997; Gurunathan *et al.*, 2000; Leitner and Thalhamer, 2003).

Various strategies have been developed to modulate the immune response after DNA immunization, such as (1) modification of the vector by inserting or deleting various regulatory sequences and optimization of promoters, (2) codelivery of genes or adjuvant molecules with regulatory and/or stimulatory properties such as costimulatory molecules [e.g., B7-1, B7-2], adhesion molecules [ICAM-1, leukocyte function-associated antigen-3], or cytokines (e.g., granulocyte-macrophage colony-stimulating factor, IFN- γ , IL-2), and (3) employing different methods and sites of vaccine delivery. All of these possibilities, either as single approaches or in combination, led the way for new approaches to vaccine development and basic immunological research (Gurunathan *et al.*, 2000; Leitner *et al.*, 2001).

B. HISTORY AND DEVELOPMENT OF ALLERGEN DNA VACCINES

Immunotherapeutic approaches for the treatment of allergic diseases are generally aimed at either inducing tolerance or redirecting the immune response toward a Th1-type reaction. For the latter strategy, DNA immunization has already yielded promising results. In the initial proof-of-principle experiment, a DNA vaccine encoding the model antigen β -galactosidase was employed (Raz *et al.*, 1996). Intradermal DNA immunization of BALB/c mice elicited a typical Th1-biased humoral immune response with elevated IgG2a antibody levels. CD4⁺ T cells isolated from immunized animals secreted IFN- γ , but no IL-4 or IL-5. In contrast, immunization with protein stimulated a predominant Th2-type response characterized by high IgG1 levels, IgE production, and IL-4 and IL-5 expression. Injection of the DNA vaccine before protein immunization prevented IgE formation, and moreover, DNA application after protein immunization elicited a therapeutic effect.

In the same year, the protective antiallergic effect of DNA vaccines was demonstrated for the first time using a clinically relevant allergen, the dust mite protein Der p 5. This DNA construct prevented IgE synthesis in rats (Hsu *et al.*, 1996). Furthermore, preimmunization with the DNA vaccine reduced histamine release in bronchoalveolar fluids and airway hyperresponsiveness after challenge with aerosolized allergen. Transfer experiments indicated an important role not only for CD4⁺ T cells but also a supportive role for CD8⁺ T cells in mediating antiallergic effects.

A number of clinically relevant allergens have been tested in DNA vaccine studies. Several publications confirmed the protective and/or therapeutic potential of DNA vaccines with constructs encoding the allergens β -lactoglobulin (Adel-Patient *et al.*, 2001), Der p 1 and 2 (Jacquet *et al.*, 2003; Kwon *et al.*, 2001; Wolfowicz *et al.*, 2003), Der f 11 (Peng *et al.*, 2002), Bet v 1 (Hartl *et al.*,

1999a; Hochreiter *et al.*, 2003), and Art v 1 (Bauer *et al.*, 2003). Furthermore, DNA immunization has been shown to reduce anaphylaxis after oral gene delivery of chitosan-DNA nanoparticles encoding the dominant peanut allergen Ara h 2 (Roy *et al.*, 1999).

In sum, these publications demonstrate that simple DNA constructs encoding the wild-type allergen gene without further modifications fulfill the basic requirements and can be immunogenic and effective in the protection and treatment of allergies. However, there is still much room for improvement not only in terms of efficacy but also regarding the safety of such vaccines. These aspects are summarized in Fig. 4.

C. STRATEGIES TO INCREASE SAFETY OF ALLERGY DNA VACCINES

A major drawback of SIT is the common occurrence of side effects, essentially anaphylactic reactions induced by cross-linking of preexisting IgE antibodies on mast cells. Moreover, the injection of high doses of antigen can lead to

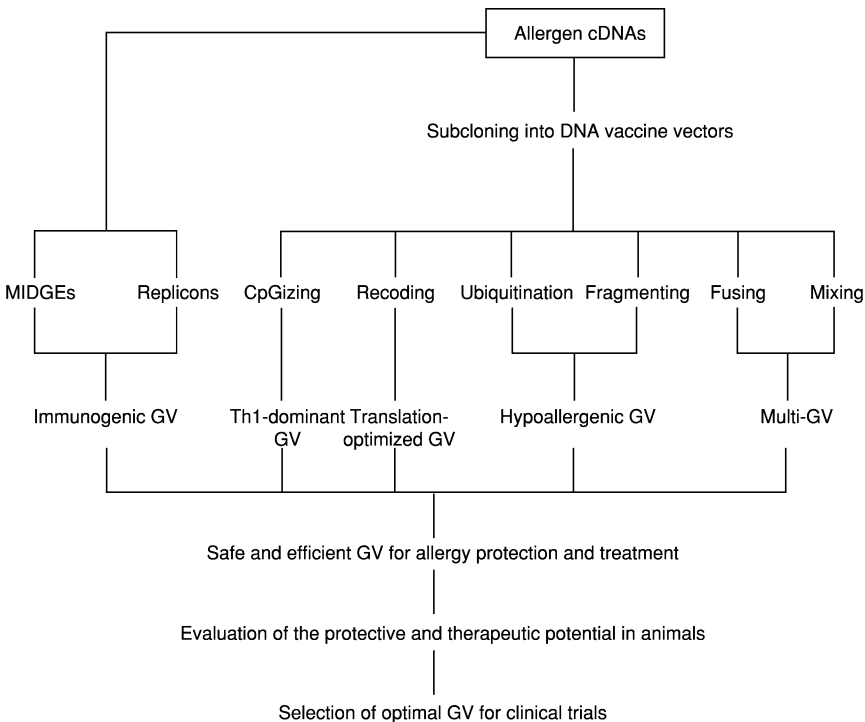


FIG 4 Task tree for the construction of gene vaccines with optimized safety and immunogenicity profiles. GV, gene vaccine; MIDGE, minimalistic immunogenic defined expression vector.

anaphylaxis during the course of treatment via stimulation of “therapy-induced” IgE antibodies.

Both reasons for these unwanted side effects are prevented by general features of DNA vaccines. The amount of translated antigen after DNA immunization in the skin comes to at least three to five orders of magnitude lower than the amount of protein injected during a typical SIT protocol. Because of the Th1-biased immune response type of intradermal or intramuscular application of DNA vaccines, *de novo* synthesis of IgE antibodies is inhibited. Furthermore, the high purity of the DNA vaccine avoids the induction of new allergic reactions via cross-reacting impurities, a possibly drastically underrated problem of SIT with allergen extracts. Nevertheless, before entering clinical trials, several concerns must be addressed: (1) the danger of systemic immune responses with side effects resulting from widespread distribution of the DNA vaccine transcript in various host tissues after immunization (Slater *et al.*, 1998), (2) induction of autoimmune responses resulting from excessive Th1 immune activation induced by DNA vaccination, and (3) anaphylactic side effects as described for SIT, which are caused by preexisting and/or newly generated IgE antibodies. To overcome these concerns, strictly allergen-specific vaccination protocols inducing mild Th1-type responses and the creation of hypoallergenic DNA vaccines are required. This necessitates the development of optimized DNA vaccines with respect to immunogenicity and safety.

1. DNA Vaccines Translating Hypoallergenic Allergen Derivatives

To increase the safety of DNA vaccines for allergies, the translation of native allergenic determinants must be avoided to prevent anaphylactic responses. These responses are caused by cross-linking of preexisting or vaccine-induced IgE antibodies on mast cells. However, to guarantee the recruitment of allergen-specific Th1 cells, T-cell epitopes on the protein must not be destroyed.

Using the major birch pollen allergen Bet v 1, we evaluated two approaches to meet these requirements: (1) cutting the allergen gene into overlapping fragments, which lack any antigenic determinant of the native allergen but display the original repertoire of T-cell epitopes, and (2) using artificial hypoallergenic derivatives. For this purpose, DNA vaccines coding for two hypoallergenic Bet v 1 fragments (Vrtala *et al.*, 2000) and for an artificial hypoallergenic Bet v 1 mutant (Ferreira *et al.*, 1998) have been constructed.

Vaccination with both types of vaccine was protected in an antigen-specific manner from IgE production and IgE-mediated cell release after repeated sensitization with recombinant allergen. Compared to the wild-type DNA vaccine that stimulates a strong humoral response, the DNA vaccine encoding the hypoallergenic mutant of Bet v 1 triggered a reduced and transient

antibody response against wild-type Bet v 1, thus indicating the altered allergenic and immunogenic properties of the mutant allergen. The vaccine encoding the two hypoallergenic fragments of Bet v 1 stimulated no antibody response against Bet v 1, confirming that the fragments do not display allergy-associated epitopes present on the entire wild-type Bet v 1 molecule. However, the fragment vaccine triggered the recruitment of Bet v 1-specific Th1 cells as indicated by IgG2a antibodies, IgE suppression, and antigen-specific IFN- γ production after sensitization with the native allergen. A therapeutic approach with sensitization followed by DNA immunization with the fragment-DNA vaccines revealed a remarkable and sustained antigen-specific reduction of IgE and IgE-mediated release thus, confirming the excellent therapeutic capacity of this type of DNA vaccine (Hochreiter *et al.*, 2003).

2. Ubiquitination: A Routine Strategy to Produce Hypoallergenic DNA Vaccines

Knowledge-based approaches to reduce anaphylactic properties of proteins by fragmentation or deliberate mutation, as described earlier, are time consuming and their suitability must be tested on a case-by-case basis. Therefore, we used ubiquitination of DNA vaccines as an alternative to develop a routine approach for destroying IgE binding epitopes on allergens in order to avoid recognition by preexisting IgE antibodies. Simultaneously, any T-cell epitope of the allergen would be preserved. This approach takes advantage of the fact that ubiquitination leads to proteasomal degradation of translated gene products. Resulting peptides are then transported by the Tap1/Tap2 heterodimer into the endoplasmic reticulum (ER), where they associate with MHC-I molecules and β_2 -microglobulin, which are in turn delivered to the cell surface for recognition by CD8⁺T cells. By still not fully understood cross-priming mechanisms, peptide epitopes can gain access to the MHC-II presentation pathway and activate CD4⁺T cells (Bristol *et al.*, 2000).

Under normal conditions, only a small percentage of plasmid-encoded proteins become ubiquitinated after DNA immunization. Stably linking ubiquitin to the antigen of interest has been demonstrated to efficiently channel the hybrid protein into the polyubiquitination pathway. As a result, the immune response is shifted toward a Th1-type, and simultaneously, antibody reactions are abolished while cytotoxic T-cell responses are enhanced (Rodriguez *et al.*, 1997). To take advantage of this mechanism, we constructed a DNA vaccine encoding a Bet v 1-ubiquitin fusion molecule. Immunization with this vector yielded no antibody production (confirming the expected degradation of the allergen) but resulted in a 100% suppression of IgE antibodies and IgE-mediated cell release in a protective experimental design. In a therapeutic setting, IgE production and IgE-mediated cell release

after sensitization markedly reduced treatment with the DNA vaccine, thus clearly demonstrating the antiallergic efficacy of hypoallergenic ubiquitin-fusion constructs (Hartl *et al.*, 2004; Leitner and Thalhamer, 2003).

3. Vaccine Dose Reduction with Self-Replicating Vaccines

Independent from these approaches, which led to an optimized safety profile of allergy gene vaccines, the high amount of plasmid DNA necessary for intramuscular or intradermal needle injection remains a weak point of DNA vaccines for allergy treatment. Using a biolistic device (such as the gene gun) enables the induction of strong immune responses with 100-fold lower quantities of plasmid DNA compared to needle injection. Unfortunately, immunization with gene gun results in a serological Th2-biased immune response with the production of high amounts of IgG1 and even IgE antibodies (Feltquate *et al.*, 1997; Hochreiter *et al.*, 2001; Weiss *et al.*, 2002). These facts make the biolistic devices useless or at least questionable for any application concerning protection and/or therapy of allergic diseases.

The principle of self-replicating RNA offered a solution of the dose problem and led to the development of a novel type of genetic vaccines. The genome of alphaviruses encodes a replicase enzyme used by the virus to efficiently replicate the viral genome inside the host cell cytoplasm. In theory, host cells only need to pick up a single molecule of this self-replicating RNA and still produce sufficient amounts of antigen to induce an immune response. It has been previously shown that antigen production in cells transfected with self-replicating RNA is rather independent of the amount of RNA or DNA used for transfection (Leitner *et al.*, 2000). Similar to the infection of cells with alphaviruses, the transfection of mammalian cells with self-replicating RNA that is based on alphaviral replicase causes host cell apoptosis (Ying *et al.*, 1999). This is believed to be a protective response of the host cell attempting to limit viral spread. Therefore, it is assumed that self-replicating RNA vaccines achieve their high effectiveness by safely mimicking a viral infection of the transfected host cells. This might trigger powerful antiviral host pathways that would enhance the immune response to the antigen encoded by the self-replicating RNA.

RNA stability still remains a problem for these vaccines during *in vitro* production. To facilitate and simplify vaccine production and handling, as well as the storage requirements for RNA, the sequence of the self-replicating RNA molecule is reverse transcribed into DNA and cloned into a conventional DNA plasmid. Now, the self-replicating RNA is produced from this plasmid by transfected cells using a conventional promoter. DNA replicons have successfully been applied in tumor models (Leitner *et al.*, 2003; Ying *et al.*, 1999) with humoral and cellular immune reactions displaying Th1-biased profiles. The Th1-type response was not induced by CpG motifs within the plasmid DNA

because the amount of plasmid DNA used for immunization with DNA replicons (10 ng to 1 μ g plasmid DNA) is far less than the effective concentration of Th1-inducing CpG motifs. The Th1-biased danger signals obviously come from the initiation of the antiviral defense pathways of mammalian cells and from activation of APCs via ISSs displayed during apoptosis (Kibler *et al.*, 1997; Restifo, 2000).

Intradermal injection of nanogram quantities of a DNA replicon vaccine encoding β -galactosidase is sufficient to trigger a Th1-biased immune response as indicated by IgG2a antibody and IFN- γ expression correlated with lacking IgE production and IL-5. Fluorescence activated cell sorting (FACS) analysis revealed both CD4⁺ and CD8⁺ as the IFN- γ -producing cells. Moreover, two injections of DNA replicons were sufficient to protect against the induction of an allergic immune response after sensitization with recombinant β -galactosidase. Vaccination induced 100% inhibition of cross-linking IgE antibodies as detected with a sensitive rat basophil leukemia cell release assay. The protective efficacy of DNA replicons is not restricted to immunodominant antigens such as β -galactosidase but could also be demonstrated with the clinically highly relevant allergen Bet v 1 (Thalhamer, 2003, unpublished data).

DNA replicons are the latest generation of DNA vaccines. Three aspects make them highly interesting candidates for effective and safety-optimized vaccine approaches in allergy: (1) The very small amounts of plasmid DNA necessary for the induction of antiallergic immune responses will increase the acceptance of DNA vaccination for allergy treatment, (2) the induction of apoptosis of cells transfected with the DNA replicon vaccine leads to “self-removal” of the vaccine after triggering the immune response, and (3) DNA replicon vaccines gain their immunogenicity via viral danger signals and induction of antiviral immune response types. The latter aspect attracted attention after clinical trials with DNA vaccines indicated poor immunogenicity of the constructs in humans in several cases (Leitner, 2001). Differences in the responsiveness of mice and primates to plasmid DNA-related danger signals via TLRs are assumed to be the major reason (Bauer *et al.*, 2001; Takeda *et al.*, 2003). TLR-independent danger signals make DNA replicon vaccines promising candidates for highly immunogenic DNA vaccines in humans.

D. IMPROVEMENT OF IMMUNOGENICITY AND TAILOR-MADE IMMUNE RESPONSES

Immunogenicity of DNA vaccines depends on various factors including the antigen expression level, the form of the antigen (intracellular, membrane bound, or secreted), and the immunostimulatory “danger” signals. Therefore, many attempts have been made to optimize these parameters by approaches such as vector modification, improving costimulation, and

co-delivery of immunomodulatory sequences and molecules. Here, some of those approaches that specifically apply to allergen DNA vaccines are discussed.

1. Harmonization of the Codon Usage

An issue that would mainly affect antigens not normally expressed by mammalian cells (such as allergens) is the codon usage that can be addressed by recoding (also called *harmonization of a protein*).

The expression level of antigens encoded on DNA vaccines is influenced predominantly by two parameters: the vector itself and the codon usage. With respect to the former, a panel of optimized vectors with sophisticated features have been developed and most of them are commercially available. In contrast, however, the aspect of codon usage has largely been ignored. Suboptimal codon usage after transfection of mammalian cells with heterologous genes (such as genes encoding plant allergens) can severely diminish protein expression. This problem was demonstrated with DNA vaccines encoding the major mite allergen precursor ProDer p 1 (Jacquet *et al.*, 2003; Massaer *et al.*, 2001) and the major mugwort allergen Art v 1 (Bauer *et al.*, 2003). Constructs encoding the plant gene Art v 1 revealed no response or only marginal immune responses. Immunogenicity of the Art v 1 DNA vaccine could not be enhanced by well-established immune modulations such as the addition of a eukaryotic leader sequence, co-injection of a GM-CSF encoding plasmid as adjuvant or cationic liposome-mediated intradermal gene delivery (Gurunathan *et al.*, 2000; Leitner *et al.*, 2001). The subsequent analysis of the gene sequence for the allergen Art v 1 revealed that the codon usage frequencies are strikingly different from those prevalent in mammals (Nakamura *et al.*, 2000). Therefore, we replaced each codon of Art v 1 with the codon displaying the highest frequency for the same amino acid in mammals (Bauer *et al.*, 2003). This resulted in a synthetic (recoded) gene encoding the amino acid sequence of Art v 1 with the mammalian codon usage. The optimized Art v 1 gene has a high GC content (71%) and a distinct codon bias for guanosine and cytosine at the third codon position (86.9% G or C at the third codon position) compared to the wild-type Art v 1 gene (53% GC and only 35.5% G or C at the third codon position). Compared to the wild-type gene, protein expression with the recoded gene was increased about 180-fold and intradermal DNA immunization elicited strong immunogenicity. The type of immune response induced by the recoded DNA vaccine fulfilled the criteria necessary for allergen DNA vaccines, for example, a Th1-biased antibody profile with high IgG2a titers, no or only marginal IgE levels, and antigen-specific IFN- γ expression. Despite its strong immunogenicity the recoded construct did not induce release-mediating IgE antibodies as measured by a rat basophil leukemia cell release assay (Bauer *et al.*, 2003).

2. *Th1/Th2 Modulation of Allergy DNA Vaccines*

An objective of DNA vaccines against allergies is the induction of a Th1-type cellular immune response, thus suppressing or converting the Th2-type response that is responsible for the high IgE production. Therefore, approaches that drive a Th1 response are potentially useful, such as the additional delivery of immunostimulatory DNA sequences (CpG motifs), Th1-stimulating cytokines, or ubiquitination of the allergen. However, it should be emphasized that the intention must not be to trigger overwhelming Th1 responses, and combinations of strong Th1 stimuli must also be seen under the light of a potential risk to induce autoaggressive reactions.

The extent of the Th1-biased reaction can be modulated by additional delivery of immunostimulatory CpG sequences. Coinjection of CpG-ODNS along with the plasmid DNA vaccine represents a simple approach to use the Th1-stimulatory capacity of CpG motifs (Hartl *et al.*, 1999a, 1999b).

CpG motifs can also be added to the gene of interest by appending a CpG-rich sequence, which codes for certain amino acids. We have attached the amino acid sequence Tyr-Asn-Asn-Val-Asn-Val-Asp-Val-Asp-Val to the carboxy-terminus of the Bet v 1a gene. This appendage is encoded by a sequence containing four CpG motifs and does not disturb the correct folding of the protein. Both approaches, appending and coinjection of CpG sequences, increased the Th1 bias of the immune response with respect to humoral and cellular responses (Hartl *et al.*, 1999a, 1999b).

Th1-directed immunomodulation was also achieved in DNA fusion vaccines encoding the gene of the model allergen ovalbumin fused to the IL-12 gene or a sequence encoding nine amino acids of IL-1 β (Maecker *et al.*, 1997). In a publication, fusion of the ovalbumin gene with the IL-18 gene, a cytokine that can indirectly act as a Th1 stimulator, demonstrated both protective and therapeutic efficiency as measured by airway hyperreactivity in a murine asthma model (Maecker *et al.*, 2001).

Another approach used the Th1-inducing capacity of viral vector vaccines. A replication-deficient adenovirus vector expressing the model allergen β -galactosidase (β -gal) was evaluated for its suitability to protect from or to treat an allergic reaction. Similar to intramuscular or intradermal plasmid DNA immunization, this vaccine elicited a Th1 type of immune response with increased titers of IgG2a antibodies and high frequency of IFN- γ -producing T cells. Furthermore, prevaccination abolished the production of IgE and modulated the Th2-biased response to a more Th1-orientated response after sensitization with β -gal protein, thus indicating its protective efficacy. However, similar to a DNA vaccine encoding bovine β -lactoglobulin (Adel-Patient *et al.*, 2001), the adenovirus vector vaccine also displayed an impaired therapeutic efficacy (Sudowe *et al.*, 2002).

Depending on the immunization schedule, the strength of nonspecific Th1-mediating signals, such as the number of immunostimulatory CpG motifs within the backbone and/or gene of interest, can have different influences on the protective and therapeutic efficacy of DNA vaccines. This may also account for the reported antigen-independent antiallergic properties of DNA vaccines (Jilek *et al.*, 2001). Within a narrow time window after DNA immunization, a strong systemic Th1 effect triggered by CpG motifs obviously can mediate a nonspecific suppression of Th2 responses. However, this antigen-independent suppressive effect is not long lasting.

E. DNA MULTIVACCINES

The approaches described are not restricted to the application of DNA vaccines encoding a single allergen. Intradermal injection of a plasmid DNA mixture of the 17-kd tree-pollen-related family (Bet v 1, Mal d 1, Cor a 1, and Aln g 1) elicited immune responses comparable to those induced by immunization with the single constructs. Both humoral and cellular responses indicated a Th1-biased reaction with increased IFN- γ and decreased IL-5 expression and various degrees of cross-reactivity between the different allergens. This cross-reaction also proved to have cross-preventive capacity; for example, DNA prevaccination with Mal d 1 revealed a similar protective efficacy against the challenge with the allergen mixture as preimmunization with the whole mixture. Similar results were found with a DNA multivaccine encoding Bet v 1 and its related food allergens (Mal d 1, Dau c 1, and Api g 1) and with a vaccine mixture coding for the profilins Art v 4, Lyc e 1, and Cap a 2 (Thalhamer, 2003, unpublished data). Complex mixtures of DNA vaccines raise the problem that upon using the aliquots of plasmid doses of single vaccines, the necessary dose of DNA vaccine would be 10-fold for a multivaccine containing 10 compounds. We, therefore, tried to circumvent this problem by creating fusion vaccines containing several allergens arranged in a string-of-bead manner, either by using the respective entire genes (gene A – gene B – gene C) or by splitting genes into fragments (gene A1 – gene B1 – gene C1 – gene A2 – gene B2 – gene C2). The latter approach additionally intends to include the hypoallergenic aspect by partially destroying the native conformation of the allergens. Preliminary experiments demonstrated the feasibility of this approach in principle. The whole-gene fusion vaccine induced antibody responses equal to the multivaccine or the respective single-gene vaccine. Fusion of gene fragments reduced the antigenicity with respect to the IgG response, indicating the assumed loss of native B-cell epitopes. Furthermore, both attempts led to allergen-specific immune responses with the desired Th1 nature pointing to the maintenance of the desired T-cell stimulatory capacity (Thalhamer, 2003, unpublished data).

Summing up, in the first decade of DNA vaccines, a huge number of publications demonstrated the usefulness of this novel vaccination approach.

Amazingly, these very simple constructs not only showed efficacy against highly immunogenic pathogens, but also appeared to be practicable for difficult conditions such as cancer and allergies. One of the advantages of DNA vaccines proved to be the “clean” immune response that is induced, that is, the absence of side effects both in animals and apparently in humans based on the limited data available. However, in the case of poorly recognized antigens, it is necessary to put strong adjuvant-type signals back into the vaccine.

Better understanding of the signals that trigger innate immune responses will allow us to design more targeted strategies. Ideally, such strategies only induce a desired innate immune response while avoiding unwanted responses such as the excessive production of inflammatory cytokines. Replicase-based DNA vaccines appear to trigger one of those innate pathways in the same way as certain viral infections, but without the problems associated with viral vectors. The triggering of PRRs (Takeda *et al.*, 2003) and immunostimulatory apoptosis (Restifo, 2000), thus stimulating the innate immune system simultaneously with DNA vaccination, may give DNA vaccines the necessary “bite” to induce optimized protective and/or therapeutic immune response.

V. Short Overview of Alternative Treatments and Supporting Adjuvants for SIT

The abnormal switch to IgE production against ubiquitous proteins is triggered by IL-4-producing Th2-type cells and leads to the establishment of specific IgE antibody responses, which are boosted by repeated allergen contact. These events can be influenced at different levels and a number of strategies have been developed that intend to suppress or mitigate allergic diseases. In terms of the progression of the “immunological disorder allergy,” the production of IgE antibodies represents a very late state of the course of the disease. Neutralization of circulating IgE antibodies prevents from binding to their high-affinity mast cell receptors and should help avoid the final release process. However, intervention at this level will be restricted to the treatment of symptoms. A number of publications, including clinical trials, demonstrated that anti-IgE antibodies display some beneficial effects with a high safety profile (D’Amato, 2003). Supporting application of anti-IgE antibodies together with standard therapy concepts may be very helpful to reduce the rescue medications and improve the quality of life.

Blocking the IL-4-dependent actions of Th2 cells with antibodies against IL-4 represents another approach using antibodies as a therapeutic tool. In the development of allergy, IL-4 acts much closer to the initial triggering processes of allergic diseases than IgE. Nevertheless, from an immunological point of view, this approach will induce neither allergen-specific curing nor long-lasting balancing of allergic responses, and moreover, IL-13 can replace several

functional activities of IL-4. In general, anti-IL-4 and IL-5 treatment has been disappointing and contradictory (Barnes, 2001).

Approaches to intervening in IgE-mediated allergic diseases also include the inhibition of allergen-specific IgE synthesis via blocking the activation or preventing the synthesis of IL-4 and IL-13. For this purpose, IL-4 mutant proteins were developed that act as antagonists of both IL-4 and IL-13 activity. Mutation led to an altered IL-4, which retained its binding capacity to the IL-4 receptor but lost the proliferative activity, thus uncoupling binding and signal transduction (Grunewald *et al.*, 1998). The mechanisms and consequences of inhibiting IL-4 and IL-13 synthesis still need to be elucidated. A 2001 study evaluated the therapeutic potential of inhaled recombinant human soluble IL-4 receptor and found clinical benefits for patients with moderate asthma who require daily inhaled corticosteroids (Borish *et al.*, 2001).

Without any doubt, allergen-specific induction of tolerance would be the most elegant approach to protect from or to cure allergic diseases. In its optimal form, it would be free of any side effects (e.g., anaphylactic reactions driven by therapy-induced IgE production) and would lack the risk of strong Th1-biased approaches (e.g., overwhelming Th1 reactions leading to autoimmunity). Animal experiments and initial clinical trials indicated the potential of approaches such as local nasal immunotherapy (Giannarini and Maggi, 1998; Hufnagl *et al.*, 2003). The role of IL-10 (and TGF- β) in the induction of antiallergic tolerance was confirmed by animal experiments (Zemann *et al.*, 2003) and by clinical studies investigating the consequences of SIT on the immune status of patients (Francis *et al.*, 2003; Jutel *et al.*, 2003). Taken together, the data indicate that nasal or oral tolerance induction may be a potential approach for an alternative SIT. However, the results also indicate that the success of these attempts was restricted to the protective application with much less efficacy concerning a downregulation of already established Th2-type responses.

With increasing insight into the mechanisms of Th1- and Th2-type immune responses, various possibilities arose to modulate an allergic Th2 immune response. Most of the attempts were based on the hypothesis that Th1-biased stimuli could prevent the development of an allergic response and even may exert the power to convert an established Th2-type response. Bacille Calmette–Guérin (BCG), as one of the candidates, fulfills the criteria of Th1 induction with an acceptable safety profile and a long clinical tradition. Animal experiments revealed that BCG, alone or in combination with allergen, induced antiallergic effects such as reduced IgG1 and IgE production, airway responsiveness, eosinophilic influx, and Th2 cytokines in bronchoalveolar lavage fluids (Herz *et al.*, 1998; Hubeau *et al.*, 2003). A 2002 clinical trial proved the therapeutic effect of BCG application in asthmatic patients (Choi and Koh, 2002). Another group of bacilli, the lactic acid bacteria (LAB), such as

lactobacilli, also proved to be antiallergic adjuvant candidates. The possible role of specific LAB strains in the prevention of allergic diseases has become more evident (Bjorksten *et al.*, 1999). A 2003 study confirmed the capacity of two LAB strains to prevent and modulate allergic immune responses (Repa *et al.*, 2003).

A practicable Th1 adjuvant potential was also demonstrated for heat-killed *Listeria monocytogenes*, an intracellular bacterium triggering strong signals to the innate immune system including Th1 danger signals, in a food allergy model (Li *et al.*, 2003). LPS represents a ubiquitous molecule of our environment and a number of important immunoregulatory processes have coevolved with this molecule. With respect to allergy and asthma, there is still the question whether LPS is beneficial or disease promoting. Animal experiments and clinical trials with LPS or lipid A as adjuvants coadministered with allergens proved Th1-inducing capacity and partly improved the efficacy of SIT without triggering harmful side effects (Drachenberg *et al.*, 2001; Ormstad *et al.*, 2003). With the availability of cytokines as recombinant molecules or expression plasmids, it became obvious to use the Th1-inducing cytokines IFN- γ , IL-12, and IL-18 as adjuvants to counteract Th2-biased allergic responses. IFN- γ causes side effects upon systemic use, but mucosal transfer of adenovirus-mediated IFN- γ was tolerated and able to reduce Th2-mediated lung parameters in animal experiments (Behera *et al.*, 2002). Indirect stimulation of IFN- γ via IL-12 and/or IL-18 also induced Th2-inhibiting effects with partly contradictory results concerning the cooperation of the two cytokines (Kim *et al.*, 1997; Lee *et al.*, 1999), indicating that any application of IL-18 must be critically assessed. In combination with IL-12, this cytokine displays double-edged features. Depending on the cytokine environment, it can trigger both inflammatory Th1-biased immune response types and allergic Th2-dominated ones (Wild *et al.*, 2000).

Recently, substances such as synthetic imidazoquinolines (Brugnolo *et al.*, 2003), microbicides (Tsuji *et al.*, 2003), and antimycotics were reported to display Th2-suppressive properties (Kanda *et al.*, 2001) and may be added to the list of future adjuvants for supporting SIT.

VI. Summary and Conclusions

Molecular cloning and recombinant production of allergens opened new possibilities for the increasing problem of allergies. The concept of molecule-based instead of allergen extract-based diagnosis was developed and is already being implemented in the field. Molecule-based diagnosis allows not only the precise identification of allergen recognition patterns of individual patients and the quantification of IgE levels to each allergen, but it might also help to predict potential sensitization to multiple allergenic sources resulting from the cross-reactivity phenomenon. Information about the sensitization profile of

individual patients forms the basis for the development of customized forms of immunotherapy based on the use of recombinant and synthetic protein antigens. For this purpose, a variety of preparations are being developed for different allergies. Major goals are to increase safety by minimizing the risk of IgE-mediated side effects and to improve efficacy of specific immunotherapy by counterbalancing the ongoing Th2-biased allergic response. Synthetic peptides in question are mimotopes, such as artificial peptide structures mimicking IgE binding epitopes, B-cell epitope-derived peptides, and T-cell epitope-containing peptides. Recombinant-based approaches are mostly focused on genetic engineering of allergens to produce molecules with reduced allergenic activity and conserved antigenicity, such as hypoallergens. An alternative to genetic engineering is the chemical modification of pure allergens with immunostimulatory DNA sequences (allergen-ISS conjugates), which mask IgE epitopes and add a desirable Th1-inducing character to the allergen molecule. Several of these customized allergen preparations have been already evaluated for their safety in clinical provocation studies. So far, clinical trials showed the efficacy and safety of immunotherapy with T-cell epitope-containing peptides and with allergen-ISS conjugates for cat-allergic and ragweed pollen-allergic patients, respectively. In addition, two preparations consisting of hypoallergenic derivatives are being evaluated for immunotherapy of birch pollen-allergic patients. In parallel, several animal studies have now demonstrated the potential of genetic immunization for allergy treatment in the future. The antiallergic effect of DNA vaccines translating wild-type allergen genes or hypoallergenic derivatives is attributed to the recruitment of Th1 cells and the establishment of a balancing Th1-biased cytokine environment.

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