

Peptide and Recombinant Immunotherapy

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KEYWORDS

- Recombinant protein • Peptide • Epitope • T-cell • Regulation
- Blocking Ab

Randomized controlled clinical trials have shown that both subcutaneous allergen immunotherapy (SCIT) and sublingual allergen immunotherapy (SLIT) are clinically efficacious and disease-modifying.^{1–6} SCIT has also been shown to be effective for treating allergic asthma,⁷ and preventing progression of allergic rhinitis to allergic asthma.⁸ However, as a result of the presence of intact B-cell epitopes, the allergenicity of treatment preparations makes treatment cumbersome because of the frequent occurrence of adverse events ranging from local reactions at the site of injection to systemic reactions that may be severe and sometimes life-threatening. Furthermore, the use of poorly defined extracts of allergen has resulted in huge variability in treatment outcomes. This variability has presented a major barrier to progress in this field through the development of molecularly characterized therapeutics that conform to modern pharmaceutical manufacturing standards and comply with regulatory requirements.

Over the past 4 decades, various technologies have been applied in an attempt to address these issues. The advent of recombinant DNA technology has enabled the molecular cloning of virtually all major allergens and has allowed the production of these molecules in recombinant form for use in both diagnosis and treatment in a “component-resolved” fashion. Recent clinical trials of recombinant allergens, described later in this article, have shown levels of efficacy comparable to existing extract-based products. Thus, a new era of allergen immunotherapy with defined products has begun. However, unmodified allergen proteins retain their allergenicity,

The author is funded by the Canada Research Chairs program, the Canadian Institutes for Health Research, AllerGen Network of Centres of Excellence, the Canadian Foundation for Innovation, the Ontario Thoracic Society, Adiga Life Sciences Inc and the McMaster University/GSK endowed Chair in Lung Immunology at St Joseph's Healthcare.

Disclosure. The author is a founder, shareholder and consultant of/to Circassia Ltd., a company developing peptide-based immunotherapy. The author is scientific founder of Adiga Life Sciences Inc, A joint venture between Circassia Ltd and McMaster University.

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Immunol Allergy Clin N Am 31 (2011) 377–389

doi:[10.1016/j.iac.2011.03.008](https://doi.org/10.1016/j.iac.2011.03.008)

immunology.theclinics.com

0889-8561/11/\$ – see front matter © 2011 Published by Elsevier Inc.

making them potentially unsafe for widespread use. Even before the advent of recombinant allergens, a variety of strategies were being developed in an attempt to reduce the allergenicity of immunotherapeutics while maintaining their ability to modify adaptive immune responses. These strategies include modification of protein surfaces with agents such as glutaraldehyde to generate “allergoids,” conjugation with synthetic bacterial DNA sequences (eg, CpG motifs), mutations in native allergen amino acid sequences (to create “hypoallergens”), display of allergen fragments on the surface of virus-like particles (VLPs), and the use of multimerized allergen, fragments of allergens (polypeptides), and peptides of various lengths. Ultimately, the future of immunotherapy may lie in a combination of molecularly defined products with reduced allergenicity.

This article focuses on recent advances in the development of recombinant allergens and synthetic peptides for the treatment of allergic diseases.

RECOMBINANT ALLERGENS FOR IMMUNOTHERAPY

Reduction in the allergenicity of allergens has been achieved in a variety of ways through the use of recombinant DNA technology. Individual point mutations at sites contributing to protein folding and conformational epitopes have resulted in molecules that retain many T-cell epitopes while displaying IgE reactivity diminished by several orders of magnitude.^{9–18} Alternative approaches include the generation of multiallergen hybrid molecules^{19–22}; the restructuring of allergens through shuffling sequence to create a new protein with reduced IgE reactivity but intact T-cell epitopes^{23,24}; the deconstruction of whole allergen sequences into polypeptide fragments^{25,26}; and the identification of short synthetic peptide sequences representing T-cell epitopes.^{27–31} Reassuringly, Mother Nature herself has provided a precedent in the form of the naturally occurring Bet v 1 hypoallergens Bet v 1d, Bet v 1g, and Bet v 1i,³² which are polymorphic variants of the major birch allergen and display markedly reduced IgE reactivity.

Genes encoding the most common allergens have been cloned and expressed, allowing the production of recombinant proteins that have been used to develop diagnostic tests or IgE reactivity (in vitro) and allergen sensitivity (in vivo).^{33,34} Recombinant proteins from aeroallergens have recently begun to be evaluated in the clinical setting. Results from trials of recombinants in patients allergic to grass and birch pollen have been described.

Recombinant Native Allergens

Jutel and colleagues³⁵ evaluated a mixture of five Timothy grass pollen allergens (Phl p1, Phl p2, Phl p5a, Phl p5b, and Phl p6) in a phase II/III trial. The study was conducted over two pollen seasons after a preseasonal up-dosing regimen followed by a maintenance dose of 40 µg of total protein. Statistically significant improvements in combined symptom and medication scores were observed after 18 months of treatment. Furthermore, significant improvements were observed in a validated rhinitis quality of life questionnaire. The allergen tolerance threshold in conjunctival provocation testing also improved although this outcome failed to achieve statistical significance. Serum allergen-specific IgG antibodies increased approximately 60-fold (IgG1) and 4000-fold (IgG4). IgG1 levels rose immediately and reached a plateau, whereas IgG4 levels increased over the course of the study. No significant changes in allergen-specific IgE concentrations were observed. Hypoallergenic variants of grass pollen allergens are currently under development for future therapy.

Pauli and colleagues³⁶ conducted a multicentre, randomized, double-blind, placebo-controlled trial that compared the safety and efficacy of recombinant Bet v 1a, purified natural Bet v 1, and standard birch pollen extract (compared with placebo) for immunotherapy of birch pollen-allergic individuals. Treatment was for a 2-year period with the maintenance dose of 15 ug of Bet v 1. All three treatment regimens led to highly significant decreases in daily mean rhinoconjunctivitis scores and daily mean rescue medication scores in both treatment years, despite very variable pollen seasons over the five centers.

Skin sensitivity to allergen in skin prick tests was significantly reduced in all three treatment regimens at follow-up in both years of treatment. Clinical effects were associated with marked increases in Bet v 1-specific IgG1, IgG2, and IgG4. All three treatment modalities had a comparable safety profile, with two patients in each arm experiencing serious adverse events. Most systemic events were not related to treatment, but one systemic anaphylactic reaction occurred in the purified natural Bet v 1 treatment arm. Immediate local reactions were reported in all groups, and delayed local reactions were reported in the three active treatment groups. A large number of local reactions, particularly swelling at the site of injection, were recorded in patients treated with the recombinant allergen. A total of 48 local reactions in 33 patients were recorded compared with 7 reactions to placebo, 10 reactions to nBet v 1, and 15 reactions to birch extract. No explanation for the increased incidence of local swelling reactions could be provided.³⁶

Engineered Hypoallergens

Valenta and colleagues³⁷ provided important insight into this issue through their work with engineered hypoallergenic constructs of the major birch pollen allergen, Bet v 1. A trimeric construct of Bet v 1 and two recombinant fragments of the same molecule³⁸ were shown to have IgE reactivity that was several orders of magnitude lower than the native allergen. Both the trimer and the fragments induced substantially reduced skin test reactivity in birch allergic subjects.³⁹ When injected into rabbits, the trimer induced IgG, which blocked the binding of allergen-specific IgE to Bet v 1.⁴⁰ Recent analysis suggests that the trimer forms defined high-molecular-weight aggregates that may be responsible for its functionally reduced allergenicity.⁴¹ Furthermore, a nasal provocation study comparing recombinant Bet v 1 with the two Bet v 1 fragments showed that in contrast to the whole allergen, the fragments did not induce an increase in allergen-specific IgE or nasal symptom scores, nor did they induce a decrease in nasal flow determined by anterior acoustic rhinometry.⁴²

The two formulations (trimer and fragments) were evaluated in a phase II multicenter clinical trial. Despite difficulties with the birch pollen season (uncharacteristically low pollen counts) in one center, investigators were able to show the induction of immunologic changes consistent with a protective response to vaccination. In particular, they showed the induction of IgG subclasses (IgG1, IgG2, and IgG4; and IgA) specific for Bet v 1, which were capable of blocking basophil activation in functional *in vitro* assays. Furthermore, serum from subjects treated with trimer and fragments was also able to inhibit CD23-dependent facilitated allergen binding (FAB).⁴³ This assay assesses the ability of IgG to functionally interfere with the ability of allergen-IgE immune complexes to bind to B cells via CD23. A statistically significant reduction in FAB was observed in trimer-treated subjects, but in the fragment-treated subjects the decrease failed to achieve significance, perhaps because of the small number of subjects analyzed.

In a further study, increases in serum antibody concentrations were reflected in nasal secretions, and Bet v 1-specific IgG4 was significantly associated with reduced

nasal sensitivity to Bet v 1 on nasal allergen challenge.⁴⁴ Increases in IgG antibodies specific for food allergens and associated with the birch oral allergy syndrome were also observed, likely because of cross-reactivity between allergens.^{25,45} Enzyme-linked immunosorbent spot (ELISPOT) analysis of T-cell cytokine responses indicated reductions in the frequency of interleukin (IL)-5- and IL-13-secreting cells, together with the trend for reduced IL-4 and increased IL-12.⁴⁶ Furthermore, skin prick test sensitivity to birch was reduced after treatment, indicating an *in vivo* clinical response that could be associated with clinical efficacy.⁴⁶ However, decreases in skin test responses were observed within the fragment and trimer group but not between groups.⁴⁷ Similarly statistically significant within-group reductions were observed in nasal provocation responses before and 12 months after treatment. Again, no statistically significant differences were observed between placebo and active treatment.⁴⁷ Seasonal increases in specific IgE were significantly reduced.²⁵

Based on these studies, it appears that markedly reducing the allergenicity of an allergen preparation need not necessarily reduce the ability to induce functional, allergen-specific blocking antibodies that may contribute to therapeutic efficacy. No significant differences were observed in the primary outcome (symptom and medications scores) in any of the treated groups. A post-hoc analysis of patient well-being on a 10-point visual rating scale also failed to show significant differences between the groups. A significant within-group (before treatment vs after treatment in the same individual) difference in well-being scores was observed in the trimer-treated group but not in the fragment or placebo groups.

Recently, because the two recombinant Bet v 1 fragments (described earlier) have been shown to have reduced allergenicity both *in vitro* and *in vivo* (skin prick test in allergic individuals), three recombinant hybrid proteins (Bet v 1-rs 1, Bet v 1-rs 2, and Bet v 1-mosaic) have been engineered from the primary sequence of Bet v 1 through shuffling of blocks of amino acid sequence from the native protein to create restructured Bet v 1 molecules that retain T-cell epitopes but that lack the appropriate three-dimensional structure to cross-link allergen-specific IgE on effector cells. Evaluation of IgE reactivity showed that in contrast to the native allergen, all three restructured proteins failed to bind IgE in nitrocellulose dot blot assays. The restructured proteins also showed markedly reduced ability to activate basophils (although very small numbers of individuals were analyzed), and serum from rabbits immunized with the proteins was capable of inhibiting the binding of IgE from birch-allergic subjects to native Bet v 1.²⁴ Thus, these novel engineered hypoallergens, which retain T-cell epitope sequences, may prove efficacious for immunotherapy of birch pollen-allergic individuals in future clinical studies.

VLPs

CpG oligodeoxynucleotides (ODN), with a structure stabilized by phosphorothioate bonds (type B CpG ODN), have a prolonged half-life, making them suitable for clinical applications. However type A CpG ODN with phosphodiester backbones induce stronger interferon (IFN)- γ responses, but are rapidly degraded. Kundig and colleagues⁴⁸ evaluated a novel delivery system consisting of VLPs derived from the bacteriophage Q β coat protein and containing type A CpG ODN. The CpG ODN-VLP was used as an adjuvant for house dust mite immunotherapy. Based on early work showing strong immunogenicity of the construct in a murine model, a phase I clinical trial of 24 healthy volunteers was initiated to evaluate the safety and tolerability of the VLP decorated with a Der p 1 peptide known to contain B-cell epitopes. IgG and IgM antibodies directed at both the VLP and Der p 1 increased during the first month after the initial vaccination but did not appear to be boosted by subsequent administrations.

Antibody titres gradually decreased to baseline levels after 18 months. The open-label phase I/IIa clinical study reported reduced skin reactivity and conjunctival sensitivity to house dust mite in allergic subjects. Clinical changes were associated with a progressive increase in specific IgG subclasses, particularly IgG1, IgG2, and IgG4.⁴⁹

B-Cell Epitope Vaccination

Clinically effective immunotherapy with whole allergens is associated with the induction of IgG (and to a lesser extent IgA) specific for the treatment allergen. Although no absolute correlation exists between the amount of allergen-specific IgG induced and clinical efficacy,⁵⁰ these “blocking” antibodies are widely accepted to contribute to clinical efficacy. Durham and colleagues showed the functional ability of IgG induced during immunotherapy to block the binding of IgE–allergen immune complexes to IgE Fc receptors.^{51,52} Focke and colleagues⁵³ identified nonanaphylactic surface-exposed peptides from Bet v 1. Skin prick testing of birch pollen–allergic individuals, together with in vitro histamine release assays using basophils from these patients, showed the absence of IgE cross-linking capacity. Peptides were coupled to keyhole limpet haemocyanin (KLH), a protein to which the immune system of humans is usually naive. The resulting vaccine was used in a murine model of birch pollen allergy, and serum from immunized mice was shown to inhibit activation of rat basophil cells primed with the IgE from allergic subjects.

In more recent studies, KLH was substituted with the rhinovirus protein VP1, which was coupled to a peptide from the major grass pollen allergen Phl p1 and expressed in *Escherichia coli*. The recombinant protein did not react with IgE antibodies from grass-allergic patients and was incapable of activating IgE-primed basophils in vitro. Sera from mice and rabbits immunized with the vaccine prevented Phl p1-induced basophil activation. Sera were broadly cross-reactive with other species of grass and, in an interesting twist, IgG antibodies elicited by the vaccine inhibited the infection of cultured human epithelial cells with rhinovirus.⁵⁴ The rationale for developing vaccines based on nonallergenic B-cell epitopes is that protective IgG antibodies will be elicited without the activation of IgE-dependent allergic mechanisms. Furthermore, the use of a “carrier” molecule (to provide T-cell help) from a nonallergen molecule should avoid the induction of IgE-Independent, T-cell-dependent, late-phase reactions that have been observed in the lung and skin.⁵⁵ However, until clinical studies are performed, whether the induction of allergen-specific IgG antibodies is, in the absence of modification of the T-cell compartment and the induction of regulatory T cells, sufficient to protect from allergen challenge cannot be determined. Furthermore, the fact that activation of allergen-specific memory T cells can result in symptoms of asthma and atopic dermatitis raises the possibility that reactivation of memory T cells to a carrier molecule such as VP1 (either through immunization of a previously infected person or through infection of a previously immunized person) could lead to exacerbations of asthma.

Chimeric Fc γ -Allergen Fusion Proteins

Using a chimeric construct (GE2) of Fc γ joined to Fc ϵ , Saxon and colleagues observed that cross-linking of high-affinity IgE receptors (Fc ϵ RI) and low-affinity IgG receptors (Fc γ RIIb) inhibited mast cell and basophil degranulation.^{56,57} GE2 blocked allergen-dependent basophil degranulation in vitro. Furthermore, in human Fc ϵ RI α transgenic mice, GE2 inhibited Fel d 1–dependent passive cutaneous anaphylaxis in skin sensitized with human serum from cat-allergic subjects. Rhesus monkeys allergic to dust mites were refractory to allergen skin testing after treatment with GE2.⁵⁷

The investigators went on to exploit this observation through the generation of a fusion protein consisting of Fc γ 1 joined to the cat allergen Fel d 1 (GFD).⁵⁸ The rationale was that binding of the Fc portion of IgG to Fc γ R1b receptors concomitantly with Fel d 1 binding to specific IgE on mast cells and basophils would lead to reduced degranulation and thus allergy symptoms. This chimeric protein was able to inhibit activation of Fc ϵ RI α , and significantly preserve lung function and core body temperature and prevent eosinophil recruitment in a BALB/c murine model of cat allergy.⁵⁹ Degranulation of human Fc ϵ RI-transgenic murine mast cells sensitized with human IgE specific for cat allergens was inhibited after in vivo administration of the chimeric protein. Although the construct contained Fel d 1, it did not induce mediator release, suggesting that in this form, Fel d 1 displays reduced allergenicity. Furthermore, in vitro application of the chimeric protein showed a dose-dependent inhibition of IgE-mediated histamine release from the basophils of cat allergic donors and sensitized cord blood-derived mast cells.

Further studies have shown that the chimeric protein, through coaggregation of Fc ϵ RI and Fc γ R1b, blocked degranulation and protected against allergen exposure when administered 6 hours before challenge, whereas reductions in lung, systemic, and cutaneous reactivity were apparent 2 weeks after treatment in a murine model of allergy.⁶⁰ More recently a human chimeric Fc–Fel d 1 was manufactured, and in the spring of 2011 will be undergoing a phase 0 study in which its allergenicity will be tested against equimolar amounts of Fel d 1 in graded skin testing in cat-allergic subjects, with the prediction that it will show 10-fold or less reactivity (Andrew Saxon, MD, personal communication, 2011).

T-Cell Epitope Vaccination

The first attempt to translate T-cell-directed peptide immunotherapy from murine models⁵⁶ into the clinic took the form of a vaccine comprised of two 27 amino acid polypeptides (IPC-1/IPC-2; AllervaxCat) from the major cat allergen Fel d 1. The results of four clinical trials evaluating safety and efficacy have been published to date. Cat-allergic subjects were treated with four subcutaneous injections over a 2-week period to evaluate three dose groups (7.5 μ g, 75 μ g, and 750 μ g per injection).⁶¹ Statistically significant improvements in lung and nasal symptom scores were reported at the highest dose. Treatment was also associated with reduced IL-4 production in peptide-specific T-cell lines derived from pre- and posttreatment blood samples and cultured in vitro.⁶² Treatment with AllervaxCat was associated with reduced sensitivity to inhaled allergen challenge in a study of individuals treated with variable doses of vaccine. Improvements in allergen PD₂₀ were observed in high- and medium-dose groups, although statistically all improvements occurred within-group rather than compared with placebo.⁶³

Some modest efficacy was reported in a multicenter study of a regimen consisting of eight subcutaneous injections of high-dose AllervaxCat (750 μ g). Although a significant improvement in pulmonary function was observed 3 weeks after therapy, this was found only in individuals with reduced baseline FEV₁.⁶⁴ Previous allergen injection immunotherapy failed in several of the subjects, and thus the study population might be considered somewhat more difficult than usual.

In a fourth study administering 250- μ g weekly injections of AllervaxCat, no significant changes in outcomes were observed. Similar to other AllervaxCat studies, treatment was associated with a high incidence of adverse events related to treatment; in this case delayed symptoms of rhinitis, asthma, and pruritus.⁶⁵

In general, adverse events occurred both acutely and up to several hours after subcutaneous injection of the vaccine. In addition to some acute IgE-mediated

reactions, which occasionally required epinephrine and may have been related to the size of the peptides, most adverse events were reported in the respiratory tract and consisted of symptoms of asthma with chest tightness and shortness of breath beginning several hours (~3 hours) after dosing. Subsequently, dedicated peptide challenge studies in subjects with asthma identified major histocompatibility complex (MHC)-restricted activation of allergen-specific airway mucosal T cells as being the cause of these manifestations, in the absence of any overt signs of mast cell or basophil activation.⁶⁶ Delayed symptoms of asthma (isolated late asthmatic reactions) were reduced with successive doses of peptide, suggesting the induction of immunologic tolerance.

Over the past decade, the authors have followed up those original observations with a series of clinical studies using broader mixtures of shorter peptides from Fel d 1.^{28-31,67,68} The peptides selected represent major T-cell epitopes within the protein and are spread throughout its entire sequence. In an uncontrolled, open study, one intradermal injection of a mixture of 12 peptides reduced late-phase skin reactions (LPSRs) to challenge with whole allergen. Concomitant reductions in allergen-specific proliferation and a reduction in both Th1 and Th2 cytokines were also observed.³⁰ Subsequently a double-blind, placebo-controlled clinical trial of the same peptide vaccine showed significant reductions in both the early-phase skin response (EPSR) and the LPSR to allergen extract challenge. Reductions in allergen-specific proliferative and cytokine responses of peripheral blood mononuclear cells (PBMC) were also observed, together with an increase in IL-10. Based on earlier observations, isolated late asthmatic reactions were expected at the peptide dose administered. However, the occurrence of these reactions was not related to the induction of allergen-specific tolerance.³¹ Related mechanistic studies used PBMC isolated from subjects before and after treatment to show that proliferative and cytokine responses were reduced to peptides contained within the vaccine, but also to those that were not, indicating intramolecular tolerance (linked epitope suppression).⁶⁹

The relationship between vaccine dose and the occurrence of isolated late asthmatic reactions was investigated in a open study in which biweekly doses of peptide were administered incrementally starting at 100 ng/mL (each peptide). In this small study, tolerance was achieved in the absence of late asthmatic reactions, suggesting that lower doses of peptide may lead to tolerance in the absence of T-effector cell activation, and further highlighting the distinct nature of the two phenomena (tolerance and isolated late asthmatic reaction).⁷⁰ The same study showed a significant reduction in airway hyperreactivity (measured by PC₂₀).²⁹ In contrast, no change in airway hyperresponsiveness was observed in the double-blind, placebo-controlled study described earlier.³¹ However, neither study was adequately powered for this outcome, and further studies in larger groups of asthmatics are awaited to specifically address this issue.

Most recently, a seven-peptide vaccine (Toleromune Cat) has been evaluated in a phase IIa safety and tolerability study.²⁷ Peptide components were selected in a multistep process involving (1) direct MHC-binding assays using 10 commonly expressed MHC alleles to determine which sequences displayed promiscuous binding to MHC class II, (2) proliferation, (3) cytokine production (IFN- γ , IL-10, IL-13), and (4) histamine release assays to identify peptides with unexpected intrinsic ability to induce basophil activation. The vaccine was administered to 88 cat-allergic subjects either intradermally or subcutaneously to evaluate safety and tolerability. The dose of vaccine providing the greatest inhibition of the LPSR to allergen extract challenge was used as a secondary outcome of efficacy. Cohorts of eight cat-allergic

subjects received a single injection of vaccine ($n = 6$) or placebo ($n = 2$). The vaccine was safe and well tolerated and the adverse events profile was indistinguishable from placebo. Intradermal delivery resulted in approximately 40% inhibition of the LPSR at a dose of 3 nmol of vaccine, comparable to earlier findings with a prototype vaccine.³⁰ Phase IIb clinical studies are currently underway to further evaluate the safety and tolerability of a vaccine and to begin to explore clinical efficacy.

Peptides have also been used in small open studies to treat bee venom allergy. The first of these studies identified immunodominant T-cell epitopes of the major bee venom allergen Api m 1⁷¹ and used them to treat five bee venom-allergic subjects who had documented systemic allergic reactions to bee venom. Subjects received incremental doses (cumulative dose, 397.1 μg starting at 100 ng) of an equimolar mixture of peptides at weekly intervals.⁷² All subjects tolerated a subcutaneous challenge with 10 μg of the major venom allergen Api m 1 after the last treatment injection, and a live bee sting challenge was subsequently tolerated by three of five subjects. Because of the small numbers of subjects and the unpredictable natural history of bee venom reactions, these results must be interpreted with caution.

In a later study, four immunodominant peptides of Api m 1 were identified using peptide-MHC binding assays to quantify the binding affinities of overlapping synthetic peptides spanning the Api m 1 sequence.⁷³ These peptides were subsequently evaluated in an open-label, single-blind study of subjects with mild bee venom allergy (no history of systemic reactions after bee sting).⁷⁴ The peptide vaccine was well tolerated and no treatment-related adverse events were observed. Treatment was associated with reduced proliferative and cytokine (Th1 and Th2) responses in posttreatment PBMC challenged with allergen. In agreement with findings in studies of cat peptide immunotherapy, IL-10 secretion was significantly upregulated and LPSRs to allergen were significantly reduced.

In the most recently published study, bee venom-allergic subjects were treated with three long synthetic peptides (LSP) encompassing the entire Api m 1 sequence in a RUSH desensitization protocol.²⁶ An incremental dosing protocol with dose escalation at 30-minute intervals and starting at 100 ng (in common with other venom studies) resulted in a cumulative dose of approximately 250 μg . Subsequently, variable maintenance injections of either 100 or 300 μg were administered at five further time points. Increased T-cell proliferation to the treatment peptides was observed, along with increases in IFN- γ and IL-10 levels, but not Th2 cytokines. Allergen-specific IgG₄, but not IgE, levels increased during the study. Peptide-specific IgE was induced during treatment in some subjects. No significant changes in skin reactions to intradermal allergen extract challenge were seen. No severe adverse events were reported, but two subjects experienced redness and itching of the hands.

SUMMARY

Because of the need to standardize allergen immunotherapy coupled with the desire to reduce allergic adverse events during therapy, a transition to recombinant/synthetic hypoallergenic approaches is inevitable in the near future. Mounting evidence supports the notion that effective therapy can be delivered using a limited panel of allergens or even epitopes, weakening the argument that all allergens must be present (eg, in an extract) for optimal efficacy. Moreover, standardized products will allow direct comparisons between studies and, for the first time, immunotherapy studies will be truly blinded (because of the lack of adverse events alerting study subjects and researchers to subject assignment), allowing an accurate assessment of the actual treatment effect that can be achieved with this form of intervention.

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