



Allergen Preparation in AIT, Now and in the Future

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Opinion Statement

Purpose of Review At present, *in vivo* allergy diagnosis and allergen-specific immunotherapy (AIT) are still based on allergens extracted from natural material. The inherent content and activity change of allergen source cannot be resolved, which impedes the standardization of AIT and limits doctors' desire to recommend it.

Recent Findings With the increasing use of allergen molecules in allergy diagnosis, the molecular allergens in AIT are believed to be the future trend. However, the current clinical phase III trials are not satisfying. Improvements have been made in the field of allergen extracts or molecular allergens—namely extraction methods, adjuvants, recombinant allergen fragments, and personalized medicine.

Summary In this article, we review recent progress in allergens preparation for AIT, the needs for further development and possible technical solutions available through molecular allergology.

Introduction

IgE-mediated allergy is the most common hypersensitivity disease. It affects more than 30% of the population worldwide and represents a heavy burden for the patients and health care system [1]. Symptom improvement has always been the primary goal of atopic diseases management. AIT is considered a disease-modifying treatment targeting long-term symptom

relief, even after cessation of treatment. Furthermore, AIT has a documented ability to prevent both the onset of new allergic sensitizations and the development of comorbidities of allergic rhinitis such as allergic asthma [2]. The AIT preparation plays a pivotal role in AIT. Allergens are isolated from the natural sources by biochemical methods [3••, 4]. The natural allergen extracts

have several problems, including the presence of undefined non-allergenic materials, contaminants, as well as high variabilities regarding contents and biological activity. Most AIT vaccines can only be provided on a patient-specific basis without the effectiveness and safety documentation required for the current registration of drugs.

With the introduction of molecular cloning technology, the isolation of allergen coding genes has made a great breakthrough [5•]. Thirty years ago, the gene encoding the first allergen was isolated and sequenced, and the first recombinant allergens were produced for *in vitro* allergy diagnosis [6–8]. For *in vitro* allergy diagnosis, problems related to natural allergen extracts have been largely resolved by the implementation of recombinant allergen molecules, which are about purity and bioactivity. Inspired by

this, some allergen preparations based on molecular allergens have been studied, which include the use of recombinant allergens, nucleic acid-based DNA/mRNA, T-cell peptides, recombinant hypoallergens, and B-cell epitopes based on peptide vectors. AIT with these molecular allergen preparations were evaluated. Unfortunately, only a few molecular AIT methods have successfully undergone clinical evaluation, and currently only allergen-based vaccines are available [9, 10, 11••, 12–16].

All above-mentioned efforts aimed at overcoming possible deficiencies in standardization, safety, effectiveness, compliance, duration of treatment, and associated high costs. In this paper, the current progresses of conventional antigen extracts and molecular allergens are reviewed and accordingly the future trend of allergen preparation in AIT is put forward.

Allergen extracts

In 1911, Leonard Noon published the first study showing that pollen extracts injection can improve symptoms of grass pollen allergy [17]. Since then, all the AIT vaccines available are based on allergen extracts from natural sources. However, the legal situation has changed dramatically in recent years and the allergens for AIT are required to comply with the good manufacturing practice (GMP) [18–23].

Allergen extracts are a mixture of allergenic and non-allergenic) contaminated proteins. The contents, concentrations, and the ratio of the individual allergens in allergen extracts vary greatly between different manufacturers, and even between different batches by the same manufacturer. Meanwhile, in these extracts, some main allergens could be missing, such as *Der p23* of HDM [24]. The adverse reactions induced by allergenicity and T cell, also bring the risk to AIT [25, 26]. It is reported that 75.6% of patients experienced local side effects, while 9.3% developed systemic side effects [27]. In the last decades, attempts were made to promote the efficacy and safety of AIT vaccines based on allergen extracts through greater immunogenicity and reduced allergenicity.

Modified preparation process

The allergenicity of extracts was diminished through chemical preparation processes. The modified allergens are called allergoids. In Henmar's research, 4 commercially available grass pollen allergoids and 3 intact grass pollen allergen vaccines were used in subcutaneous AIT. Two of the four allergens showed the lowest allergenicity. Meanwhile, their immunogenicity was significantly lower than that of intact allergens [28]. In another study, the hydrolyzed *Lolium perenne* grass pollen vaccine is clinically effective [29], but unfortunately the clinical results of the phase III trial were disappointing [30]. As the chemical process

lacked specificity, the allergoids showed reduced IgE-binding ability. However, the immunogenicity was destroyed as well. Recently, liposomes, niosomes, and transferosomes are investigated as novel carriers. These molecules encapsulate antigens to permeate hydrophobic cellular barriers to reach intracellular targets [31]. Liposomes and intranasal liposomal with allergens in murine models showed down-regulation of IgE responses, and the immunogenicity was preserved simultaneously [32, 33].

Different administration routes

Different routes of natural extracts administration were also researched. Some studies have proved that if allergen preparations are given through different ways, the effects will be different. (1) SLIT is superior to SCIT in safety and convenience. The clinical efficacy of SLIT approach has been demonstrated in several phase III studies [34••]. However, SLIT is less compliant in real life and has no effect for some common allergens, including cat, mold venom, and food allergy [35]; (2) intralymphatic immunotherapy (ILIT) is the ultrasound-guided procedure that injects low-dose allergens into the inguinal lymph nodes [36]. The lymph nodes are rich in immune cells. This route of administration improves the antigen presentation and shortens the duration of treatment [37]; (3) oral immunotherapy (OIT) is mainly for the digestion-resistant food allergens, such as milk, egg, peanuts, and wheat, not for the easily digested inhalant allergens. OIT could induce severe side effects and now only peanut OIT vaccines have been registered and have undergone clinical evaluation [38]; (4) epicutaneous immunotherapy (EPIT) induced less systemic side effects via non-vascularized epidermis at low dose [39]. However, local AEs at the patch site were still reported. Compared to OIT, EPIT administrations of food allergens prevented persistent GI symptoms or eosinophilic esophagitis [40].

Adjuvants and immunomodulatory compounds

Adjuvant could protect allergen-specific responses and reduce the side effects in AIT. Due to the fact that the duration of SCIT is at least 2–3 years, the usage of adjuvant should be very cautious of cumulative toxicity. The adjuvants used for other vaccines (usually only need 1 to 3 times of injections) may not be suitable for AIT. Alum as the conventional adjuvants is not used in some company product of allergen preparations to avoid long-time exposure toxic effect. At present, the research on adjuvants mainly focuses on the nontoxic or easily degradable substances to human. (1) The ligand of Toll-like receptor (TLR) could promote potent Th1-biased immune response. Pollinex Quattro, composed of specific pollen allergoids formulated with a potent detoxified TLR4 ligand called monophosphoryl lipid A (MPLA) and absorbed into microcrystalline tyrosine (MCT), is the only ultra-short (3 weeks) four injection-based SCIT recommended by EAACI guidelines for the treatment of pollen allergy [41]. However, birch pollen allergy with Pollinex Quattro failed in the phase III trial over peak birch pollen season [42]. (2) It is reported the relatively nontoxic adjuvants, such as probiotics, vitamin A and D could promote the symptoms and induce long-lasting clinical benefit for peanut and grass pollen [43–48]. (3) Monoclonal antibodies against immunological targets in Th1 and Th2 pathways could be regarded as adjuvants to AIT. Omalizumab is a recombinant humanized monoclonal antibody that prevents IgE binding to FcεRI. For the

patients with severe asthma or severe milk/peanut allergies, omalizumab facilitated rapid AIT with a reduction in the time required to achieve maximum doses and tolerability [49–54]. Meanwhile, omalizumab assisted patients who had developed anaphylactic reactions to hymenoptera to complete AIT [55, 56]. Although the long-term efficacy remains in question, we believe that omalizumab has the potential to reduce symptoms when combined with AIT, especially in the early stage of AIT and for patients with severe symptoms.

Molecular approaches

Thirty years ago, the gene encoding of the first allergen was isolated and sequenced. Since then, a great breakthrough of allergen has been made through the molecular cloning technology [5–7]. Today, molecular allergy diagnosis has already revolutionized allergy diagnosis [57, 58]. Inspired by this, molecular forms of AIT based on the DNA sequence and molecular structure of the allergens have been developed. The novel strategies include native-like recombinant allergens, recombinant hypoallergens, allergen-encoding nucleic acids, T cell epitope-containing peptides, allergens coupled to immunomodulatory compounds, VLP-coupled allergens, and carrier-bound B cell epitope-containing peptides.

Native-like recombinant allergens

After the first recombinant allergens have been sequenced, several clinically relevant components had been identified to resemble the features of the natural allergens, which were called major allergens. They account for a high proportion of epitopes/allergens in natural allergen extracts [59, 60]. The major allergens were produced by gene recombination technology to substitute the allergen extracts in AIT. Their advantages over allergen extracts are the high quality, easy quality control, and relative low cost.

Single molecule-based AIT was performed in some studies. Pauli's study is the only double-blind, placebo-controlled randomized multicenter clinical SCIT trial in which natural birch pollen extracts, purified natural *Bet v1* and *rBet v1*, were compared in two treatment years [11••]. The clinical improvement treated by natural or *rBet v1* was similar to that of the pollen extracts. Interestingly, the natural *Bet v1* and *rBet v1* treatments did not induce new sensitizations, whereas IgE responses to *Bet v2* were measured in 10% of the patients treated with birch pollen extracts. However, the degree of adverse reactions observed at local sites was similar to those induced by allergen extract-based SCIT. In another study, carrier-bound *rAlt a 1* induced *Alt a 1*-specific IgG in rabbits and inhibited allergic patients' IgE binding to *Alt a 1* in vitro. The results implied the *rAlt a 1* has the potential to be developed into a safe, non-allergenic vaccine for SIT of *Alternaria* allergy [61]. The single-major-allergen AIT is strictly restricted to the patients who are only allergy to the major allergen. A study of SCIT with a mix of the major timothy grass pollen allergens were conducted [10]. The vaccines

successfully induced allergen-specific IgG-blocking antibodies and exhibited clinical efficacy. However, no product has been registered up to now [62]. Compared to the allergen extracts, the native-like recombinant allergen has the advantage of high and stable quality; however, it still has the early side effect of IgE binding and late side effects induced by T epitopes.

Recombinant and synthetic hypoallergens

The goal of hypoallergens is to reduce IgE reactivity of allergen. The genetic engineering includes fragmentation, oligomerization, mutation, reassembly of sequences, and reduces IgE reactivity by altering or destroying the structural allergen fold [12, 14]. In a I/IIa phase double-blind, placebo-controlled clinical study with 15 fish allergic patients demonstrated that AIT with major component *Cyp c 1*, induced allergen-specific IgG and showed lower side effects [63]. In a phase II trial, hypoallergenic *Bet v 1* fragments or trimer in birch pollen were assessed in AIT for allergic patients [9]. The results did not show significant differences between the vaccine group and placebo group. Although the hypoallergenicity has the ability to induce T cell response, reduce IgE binding and block IgG antibodies, it is also clear that systemic adverse effects can be mediated by T cell [64].

Allergen-encoding nucleic acids

More than 20 years ago, two studies indicated that it was possible to vaccinate with allergen-encoding DNA. In the mouse study, they demonstrated that allergen-encoding DNA reduced allergen-specific IgE production and induced allergen-specific Th1 responses [65, 66]. However, in the following animal study, DNA vaccination exhibited the severe side effect that uncontrolled allergen transcripts were found in different tissues [67]. The mRNA encoding allergens are considered safer than DNA due to absence of the ability to integrate into the host genome [68]. However, the mRNA approach was impaired by the innate immunogenicity of mRNA and was inefficient in in vivo delivery. Recently, a study found nucleoside-modified mRNA reducing the innate immune activation and the combined usage of lipid nanoparticles (LNPs) that protected the mRNA [69].

T Cell epitopes

Theoretically, T cell epitopes of major allergens without IgE reactivity could trigger immune tolerance by different cellular mechanisms and reduce allergen-specific IgE production [70]. However, in a 1000-patient phase III trial, the T cell epitope treatment did not achieve improvement over placebo [71]. Another study with *Fel d 1*, the major cat allergen, got the same result [72, 73]. Actually, the T cell epitope-containing peptides could not induce allergen-specific protective IgG antibodies, due to the absence of B-cell epitopes. For this reason, these peptide-based AIT programs were discontinued. Later, all the potential T cell epitopes from *Bet v 1* without IgE-binding activity were prepared through the approach of contiguous overlapping peptides (COPs). This AIT approach induced allergen-specific IgG-blocking antibodies and significantly reduced symptom and medication scores compared with placebo group [9, 12, 14].

Carrier-Bound B cell epitope-containing peptides

The lack of allergen-specific T cell epitopes reduces the T cell-mediated late side effect. Meanwhile, the non-allergic carrier-specific T cells mediate the activation of allergen peptide-specific B cells. The B cell epitopes are mainly conformational. It is difficult to obtain B cell epitopes information. In addition to the studies focusing on antigens, some studies look for information about B cell epitopes through the hypervariable regions of allergens-specific antibodies. In Zhu's study, B cell mimotopes screened by a phage displayed random heptamer peptide library that was identified via mixed *Alternaria*-specific IgE in the sera pool [74•, 75]. Carrier-bound B cell epitope-containing peptides represents a promising approach for AIT [76, 77]. BM32, a grass pollen allergy vaccine, was developed using birch pollen B cell epitopes and hepatitis B virus preS protein as carrier proteins. This hypoallergen is characterized by a lack of IgE reactivity and will not cause any early side effects, but can block the IgG antibody after immunization [78••]. Allergen-specific T cell epitopes are largely replaced by carrier molecules, thus reducing the side effects mediated by advanced T cells. BM32 has been evaluated in several clinical trials. In a double-blind, placebo-controlled multicenter study, BM32 has been proved to be safe and can produce beneficial clinical effects [15, 79–81]. Unlike most other AIT vaccines, BM32 did not induce or enhance allergen-specific IgE responses, but it slowed down seasonal IgE production. In addition to the pollen vaccine, several other inhalant allergens-related peptide vector vaccines have been developed. This technology also can be used to produce AIT vaccines for venom and food allergens. BM32 has finished three phase II studies and a phase III study and is currently planned to register.

Allergens coupled to immunomodulatory compounds

Genetic engineering technology makes it possible that adjuvant molecules could directly conjugate to the allergens as a strategy to improve AIT protocols. (1) In a murine asthma model, CpG oligodeoxy nucleotides had been demonstrated to induce Th1 immune responses [82]. CpG coupled to ragweed allergen *Amb a1* enhanced the immunogenicity and reduced the allergenicity as well [83, 84]. (2) An engineering virus-like particles (VLP) approach was reported, in which allergen-encoding cDNA was fused to virus-encoding DNA or the immunomodulatory compounds, including CpG, Pro-Th2/Th2 cytokines [85, 86]. Packaging CpG into VLP did not only reduce the common side effects of free CPG but also optimized the activation of TLR9 in plasma cell-like dendritic cells (pDCs) and B cells [87, 88]. Pro-Th2 (especially IL-33)/Th2 (IL-4, IL-5, IL-13) cytokines into VLP promote the participation of downregulating Th2 response. In a preclinical study, VLP displaying *Fel d 1* did not activate mast cells but triggered IgG responses [89]. VLP fused mugwort pollen also induced proliferation and cytokine production of allergen-specific T cells in vitro [90]. There is no experience in human study until now [91]. (3) Major histocompatibility class II (MHC-II) plays a pivotal role in allergen presentation. It is reported that allergens could be fused to the Modular antigen translocating (MAT) module [92, 93]. MAT consisted with HIV-1 TAT-derived translocation peptide and intracellular domain from the invariable chain Ii could efficiently target to endosomal/lysosomal compartments. MAT module did not only increase the cat allergen *Fel d 1* uptake by PBMCs but also induced a shift from

Th2 to Th1. In the following study, MAT-*Fel d 1* showed significantly increased allergen tolerance through intralymphatic immunotherapy administration route [94, 95].

Cell-based therapy

Transferring of hematopoietic stem cells to a different MHC background mouse in early life could induce tolerance [96]. Baranyi demonstrated that mice could not be allergic to corresponding allergen after receiving allergen-expressing cells [97]. The result implied that life-long allergen-specific immunological tolerance can be achieved by a cell-based treatment. However, the cell-based technology may be potentially hazardous and is only in pilot experiment stage now.

Upgrading of the prevention

There is evidence that AIT could prevent the “allergic march” and also prevent the clinical manifestation from mild to severe. In PAT studies, it is demonstrated that early AIT could prevent the progression from rhinitis to asthma [98–100]. Preventive effects were also investigated in the LEAP study when children were fed peanut allergens in early life [101, 102]. However, the evidence of the AIT for secondary prevention is not strong because the majority of them lacked MP-manufactured vaccines.

A study provided evidence that maternal allergen-specific IgG may protect against allergic sensitization in the offspring [103•]. Children from mothers bearing high levels of allergen-specific IgG did not develop IgE sensitizations against these allergens within 5 years after birth. In experimental animal models, the approach of administration of hematopoietic stem cells expressing allergens for long-lasting prophylactic tolerance induction has been successfully demonstrated, like the hematopoietic stem cells expressing transplant antigens [104, 105]. These evidences encourage people to consider AIT approaches for primary prevention. However, they should be very cautious to perform the primary prevention, especially for the children or infants, due to the fact that tolerance to non-autogenous protein may increase the immune deficiency to some proteins, and some may have the similar sequence with fatal antigens.

Personalized medicine

Based on the clinical experience and knowledge of allergens, AIT maybe the most “personalized treatment” [106]. The personalized medicine includes distinct disease subsets, specific diagnoses, and targeted treatment options, including biologicals and small molecules [107, 108]. The development of molecular allergen-specific approaches makes personalized AIT possible. Hamburg and Collins [109] describe the path to a personalized medicine as 3 steps. The first step is identification of the molecular mechanism of the disease, the second step is diagnostic tool for the molecular mechanism, and the third step is treatment blocking the molecular mechanism. The biomarker is critical for the best responders, monitoring efficacy and safety, and even could help to accelerate the allergen development process. Although there is no validated and simple

marker for daily clinical practice predicting efficacy of AIT at present [110–117], patient-tailored AIT based on the individual sensitized peptides is possible.

We also need to pay special attention to the consistency of the molecular allergy sequences used in personalized detection and treatment methods. In 1999, Valenta et al [118] proposed that AIT preparation composition should match the patient's molecular profile of IgE sensitization. In Matricardi's study, an experimental AIT preparation containing *Phl p 1*, *Phl p 2*, *Phl p 5*, and *Phl p 6* only matched 7 of 176 patients (4%) [119]. The results implied that allergenic profiles of patients vary and there might not be "one for all" AIT preparation.

According to GMPs, safety and clinical efficacy need to be demonstrated for each molecule and its possible combination separately, which will hinder the development of the approach. Regulators have learned from oncology where single molecules are produced according to GMPs, mixed afterwards for a single patient (personalized medicine) and often applied within basket and umbrella studies [120]. A "toolbox" of molecules of reduced allergenicity and increased immunogenicity would be very interesting, if they could be mixed according to the sensitization profile of individual patients. Such approach should be based on the comprehensive information of allergen epitopes, as the absence of allergen epitopes will reduce the number of eligible patients and meanwhile, the efficacy of the approach.

Conclusion

Currently, the majority of AIT vaccines do not have documentation of efficacy and safety required for drug registration. Allergen extracts as a mixture are difficult to purify and standardize by traditional methods—a similar dilemma as traditional Chinese herb faces. Based on current research results, it appears that the use of preparation process, alternative routes, and adjuvant cannot overcome the limitations set by the quality of natural allergen extracts.

In phase I/II trials, the molecular form of AIT demonstrated its potential to replace traditional allergen extracts by reducing side effects and improving efficacy. However, the clinical endpoints of these new allergen derivatives were quite disappointing in phase III trials. Larger trials are needed to evaluate the clinical efficacy of these different molecular AIT and particularly long-term benefits. The recent updated implementation of regulations forces the pharmaceutical companies to produce GMP-grade allergen extracts and to evaluate their efficacy in clinical trials following GCP standards, which will push the pharmaceutical companies to continue the molecular AIT study.

Personalized AIT is the future of immunotherapy. The development of molecular allergen-specific approaches makes the personalized AIT possible. Meanwhile, the usage of shorter peptides or even the B cell/T cell epitopes of allergens made it impossible to find one allergen reagent to fit all patients. In the future, the molecules of allergens may work as a "toolbox." The screened out best responders for the patient will be used not only for diagnosis but also for AIT and efficacy monitor.

Declarations

Conflict of Interest

The authors declare that they have no conflicts of interest.

Human and Animal Rights and Informed Consent

This article does not contain any studies with human or animal subjects performed by any of the authors.

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