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Development of subcutaneous allergen immunotherapy (part 2): preventive aspects and innovations

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Abstract
Background Allergen immunotherapy with subcutaneous injection (SCIT) of the relevant allergen is the classic causal treatment method for IgE-mediated allergic respiratory disease and has already been successfully used for over 100 years.
Methods This publication is based on a selective literature search in PubMed and MEDLINE. Recent publications in German-language journals that are not available in literature databases were also analyzed. This literature search included original and review articles both in German and in English.
Results Primary, secondary and tertiary prevention characteristics have been demonstrated for SCIT; however, these require further evaluation. In combination with biologic agents, the safety, and in some cases the efficacy, of SCIT can be increased. Adjuvants seem to offer enormous development potential for SCIT. Aluminum salts, microcrystalline tyrosine (MCT), and monophosphoryl lipid A (MPL) are already used in commercial SCIT preparations. At the same time, other adjuvants are being researched, e.g., liposomes, microspheres, CpG motifs (C: nucleotide cytosine, p: phosphate, G: nucleotide guanine), or virus-like particles (VLPs). The therapeutic extracts themselves are also undergoing further development, for instance as recombinant allergens, hypoallergenic variants such as site-directed mutants (SDM), conformational variants, allergen fragmentation, allergen

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oligomers, deletion mutants, and hybrid allergens/mosaic antigens.
Conclusion SCIT preparations are among the most innovative treatment options in the immunotherapy of allergic diseases. Due to the numerous immunological approaches, they will make treatment safer and more effective in the future with reduced effort.

Keywords Allergic rhinitis · Insect venom · Adjuvants in allergen immunotherapy · Hypoallergenic recombinant allergoids · Biologicals

Abbreviations

AIT	Allergen immunotherapy
APC	Antigen-presenting cell
CpG-ODN	CpG oligodeoxynucleotides
DCs	Dendritic cells
EMA	European Medicines Agency
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
IL	Interleukin
LPS	Lipopolysaccharides
MCT	Microcrystalline tyrosine
MPL	Monophosphoryl lipid A
OIT	Oral immunotherapy
PAMP	Pathogen-associated molecular patterns
PEI	Paul Ehrlich Institute
PRR	Pattern recognition receptors
QoL	Quality of life
SCIT	Subcutaneous immunotherapy
SDM	Site-directed mutants
SIT	Specific immunotherapy
SLIT	Sublingual immunotherapy
TAV	Therapy Allergen Ordinance
Th1	T-helper cell 1 (cellular immune response)
Th2	T-helper cell 2 (humoral immune response)
TLR	Toll-like receptor
VLPs	Virus-like particles

Introduction

Allergen-specific immunotherapy (AIT) with subcutaneous administration of allergen extracts (subcutaneous immunotherapy; SCIT) has been the standard procedure for the treatment of inhalant and insect venom allergies for over 100 years.

In addition to its efficacy and safety in the treatment of existing symptoms [1, 2], a number of primary, secondary, and tertiary preventive properties have also been demonstrated for SCIT [3, 4]. Combining biologic agents with SCIT increases the safety of the treatment. The effect of combined treatment was stronger compared to the effect of individual medications. Compared with other treatment options, SCIT is cost-effective and can save disease-related costs in the long term [5, 6].

Allergens or allergoids in commercial extracts in Germany are usually physically adsorbed to a carrier, e.g., aluminum hydroxide ($Al(OH)_3$) or microcrystalline tyrosine (MCT) [4]. These not only provide the properties of a deposit, but also act as adjuvants. Aluminum salts, MCT, and monophosphoryl lipid A (MPL) are already used in commercial SCIT preparations [7]. Other adjuvants are considered to have enormous development potential, e.g., liposomes, microspheres, CpG motifs, and virus-like particles.

In combination with therapeutic extracts that have undergone further development, e.g., recombinant allergens, hypoallergenic variants such as site-directed mutants, conformational variants, allergen fragmentations, allergen oligomers, deletion mutants, and hybrid allergens/mosaic antigens, SCIT has enormous potential for development [8–10].

Methods

This publication is based on a selective literature search in PubMed and MEDLINE. Recent publications in German-language journals that are not available in literature databases were also analyzed. This literature search included original and review articles both in German and in English. Synonyms as well as translations into English were taken into account in the selection of suitable search terms in the German language, with Boolean operators AND, OR using truncations, and wildcards and the following selection of terms: allergic rhinitis; bronchial asthma, venom, insect venom allergy, wasp venom allergy, bee venom allergy, allergy; inhalant allergy; immunotherapy, hyposensitization; desensitization; subcutaneous immunotherapy, sublingual immunotherapy, therapeutic allergens, adjuvants, recombinant allergens, VLP.

Results

Preventive aspects of SCIT (including asthma and new sensitizations)

Prevention is a central issue in the treatment of children and adolescents in general, as well as allergic airway disease.

Controlled but open studies on individual preparations have shown that SCIT has preventive effects in addition to its efficacy on symptoms. To date, measures for secondary prevention, i.e., preventing the development of allergic rhinitis or bronchial asthma in patients already sensitized but yet asymptomatic, have only been investigated in small groups in clinical studies. For example, Szépfalusi et al. treated 31 children (aged 2–5 years) exhibiting monosensitization to mites or grasses but no previous symptoms of clinical relevance, with oral immunotherapy using the relevant allergen over 2 years, or observed them in the no-treatment placebo group over the same period. The in vitro investigations in this study population revealed significant upregulation of (blocking) allergen-

specific immunoglobulin G (IgG) antibodies and immunomodulating interleukin (IL)-10. However, after 2 years, there was no significant difference in the clinical endpoint, i.e., the number of newly acquired allergic sensitizations, between placebo and treatment group [11]. To the authors' knowledge, a clinical study is currently conducted in the UK on the primary prevention of allergic disease using specific immunotherapy (SIT); the study has not been completed yet. It is possible that nonspecific strategies to induce immunological tolerance are more promising in terms of primary prevention.

Other secondary preventive aspects of SIT are aimed at preventing the development of new sensitizations or allergic march from allergic rhinitis to bronchial asthma. Although open studies on this are already available, there are no data from double-blind placebo-controlled (DBPC) studies. It was shown for a variety of SCIT preparations that the rate of new sensitizations under SCIT is less pronounced compared to conventional treatment [12–16].

The PAT (Prevention of Allergy Treatment) study is a ground-breaking investigation that showed that allergic march from allergic rhinitis to bronchial asthma can be slowed down with SCIT [17]. As part of this study, children with grass or birch pollen allergy (no child had bronchial asthma at the time; this was an exclusion criterion) underwent immunotherapy with the relevant allergen. At 3 years, significantly more children in the symptomatically treated group had developed bronchial asthma. The SCIT group had markedly fewer asthma symptoms or asthma diagnoses at 3 years, as well as in the 5-year follow-up (2 years after treatment completion), and even at 10 years (7 years following treatment completion). Unfortunately, this study was neither blinded nor placebo-controlled, meaning that its validity in terms of methodology has remained questionable.

A large DBPC study on SLIT using a *Phleum pretense* "grass tablet" was conducted over a 5-year period. The goal was to find out to what extent SLIT can prevent allergic march in children with allergic rhinitis. To this end, 812 children at 101 study centers in 11 countries were randomized. The primary endpoint was the time—measured in days—from randomization to first asthma diagnosis (according to study protocol criteria). Secondary endpoints included the number of patients with asthma symptoms and/or medication at the end of the study, the rhinoconjunctivitis medication score in year 5 after the start of SLIT, and immunological parameters [18].

The results showed that SLIT with a "grass tablet" resulted in a significantly lower number of patients with asthma symptoms as well as used asthma medication during study duration as well as during the 3 year follow-up [18].

Although the primary endpoint was not met, the study shows that immunotherapy can prevent the development of asthma [18]. Further suitably designed studies are required to investigate whether this outcome can be similarly extrapolated to other allergens and other AIT preparations. From the authors' perspective, it is important that the secondary aspects of SIT in the prevention of asthma be evaluated in a product-specific manner. However, published works on both SCIT and SLIT give rise to speculation that effective SIT preparations can favorably affect disease course in a manner consistent with secondary prevention.

The goal of SIT's tertiary preventive properties is to slow down the progression of allergic disease and minimize treatment side effects. In this context, studies on AIT using mite extracts are interesting. In a clinical trial with children ($n = 65$ children, aged 6–17 years) on SCIT with mite preparations, Zielen et al. showed that SCIT with a mite allergoid contributes to a significant reduction in the dose of inhaled glucocorticoids needed to control asthma. The average daily inhaled dose of fluticasone went down from 330 to 151 µg in the SCIT group, and only from 290 to 206 µg in the placebo group. The authors conclude from their data that SCIT is an effective option to reduce steroid use while maintaining disease control in mite-allergic children with controlled asthma [19].

In a large DBPC study, Virchow et al. investigated the effect of a SLIT mite tablet in 834 adults aged 17–83 years with partially controlled asthma under regular treatment with inhaled corticosteroids (ICS). The primary endpoint was the number of asthma exacerbations following a reduction of the ICS dose according to protocol and subsequent discontinuation of ICS. Significantly fewer asthma exacerbations were observed under mite SLIT in both dose groups investigated compared to placebo. Thus, the study demonstrates significant therapeutic success in allergic asthma among patients with partially controlled asthma; the treatment was also safe. Consequently, the results of this highly regarded study have already led to a statement on SLIT in allergic bronchial asthma caused by mites in the recently revised GINA guideline [20]: SIT is mentioned there for the first time as a treatment option for bronchial asthma [21]. A similar statement can be anticipated in the revised version of the German national treatment guideline (*Deutsche Nationale Versorgungsleitlinie*, NVL).

Combining SCIT with biologic agents (anti-IgE: omalizumab)

Omalizumab (Xolair®, Novartis Pharma, Nürnberg, Germany) is a humanized, monoclonal anti-IgE antibody that binds circulating IgE molecules. This alters the binding of IgE antibodies on effector cells. Consequently, this prevents the release of inflammatory mediators that orchestrate the allergic inflammatory reaction. A second anti-IgE effect is that the number of IgE receptors on the effector cells decreases significantly when treated with omalizumab. Omalizumab

is approved for the treatment of severe, therapy-resistant bronchial asthma from the age of 6 years and for the treatment of chronic idiopathic urticaria from the age of 12 years.

The idea of combining SIT with anti-IgE therapy is not a new one. The first study to investigate combination therapy of this kind was a children's study in Germany. The primary question in that particular study was whether omalizumab has an additive effect to SIT [22]. To answer this question, children and adolescents with grass and birch pollen allergy were recruited. As part of the four-arm study, patients underwent SCIT with a grass pollen or birch pollen extract and additionally received either omalizumab or placebo. This study impressively demonstrated the efficacy of omalizumab on symptom severity and medication use in children with allergic rhinoconjunctivitis. Already in the first year of therapy, a significant add-on effect to SIT was shown.

The following studies addressed the question of whether pretreatment with omalizumab leads to better tolerance of SIT, especially in the dose escalation phase. In a study on adults in 2006, Casale et al. showed that omalizumab pretreatment led to significantly fewer systemic side effects of SIT with a ragweed extract [23]. The study was designed in such a way that the maintenance therapy would be achieved within 1 day using a rush dose escalation. Omalizumab pretreatment resulted in a five-fold lower risk of developing an anaphylactic response to SIT rush dose escalation (odds ratio [OR], 0.17; $p=0.026$). At the same time, it was shown that combination therapy with omalizumab and SIT significantly improved symptoms during the ragweed season compared to SIT alone (0.69 vs. 0.86; $p=0.044$).

Another study that included 131 grass-sensitized children and adults also showed that omalizumab significantly reduced symptom burden in patients with allergic rhinitis, as well as in patients with asthma comorbidity, compared to treatment with SIT alone [24]. This asthma patient group was also investigated by Massanari et al. [25]. The study enrolled $n=228$ patients with persistent asthma symptoms despite ICS. These patients underwent SIT (cat, dog, or house dust mite) following pretreatment with omalizumab or placebo. Significantly more patients achieved the SIT maintenance dose in the omalizumab group (110 [87.3%] vs. 88 [72.1%]; $p=0.004$). Patients in that group also had significantly fewer systemic allergic reactions following SIT (17/126 [13.5%] vs. 32/122 [26.2%]; $p=0.017$; 95% confidence interval [CI] 2.91–22.56%).

In recent years, besides SCIT against pollen allergens, greater focus has been put on a combination of anti-IgE and SIT in food allergies, most particularly food allergies to peanut and cow milk [26–29]. A phase 1 study pretreated 11 cow milk-allergic children aged 7–17 years with omalizumab prior to performing oral immunotherapy (OIT) with milk [26]. The aim of this clinical trial was to increase the quantity of milk to 2000 mg within 7–11 weeks. Nine of the 11 patients met the primary endpoint and tolerated the 2000 mg/day dose within the target time window and showed no symptoms in a DBPC food challenge (DBPCFC) with milk.

Another randomized study with cow milk-allergic patients confirmed these results [29]. A total of 57 patients aged between 7 and 32 years were recruited. OIT with milk was initiated after 4 months of omalizumab or placebo pretreatment. Oral provocation testing was performed after 28 months: 88.9% tolerated 10 g milk in the omalizumab group and 71.4% in the placebo group ($p=0.18$). However, there were significant differences in terms of safety and tolerance of OIT, which were significantly better in the omalizumab group [29]. Investigations in patients with peanut allergy also confirm these results: omalizumab permitted faster and safer OIT dose escalation.

Premedication with omalizumab in patients with recurrent intolerance reactions under venom immunotherapy improved tolerance in many cases [30–35]. However, uncertainty remains regarding the necessary duration and dose of omalizumab.

In summary, pretreatment with omalizumab is able to significantly enhance AIT tolerance [36]. Thus, omalizumab pretreatment in patients with uncontrolled bronchial asthma or a clinically severe food allergy offers the possibility of a causal therapy once symptoms are under control. It can be deduced from clinical trials, as well as from a number of case series, that omalizumab significantly reduces the risk of anaphylactic reactions under AIT.

The second question that arises in addition to an improved AIT safety profile relates to the extent to which omalizumab can also enhance the efficacy of SIT. This question has not been answered yet in clinical trials. To do this, investigations over a longer observation period are required. Since IgE plays an important pathophysiological role in the interaction of the allergen/antigen with antigen-presenting cells (APC) [37], immunomodulatory effects that could be additive to conventional AIT are conceivable. However, based on the current evidence, this question remains unanswered at present.

Based on the current study situation, one can assume that temporary omalizumab treatment does not affect the treatment success of continued SIT alone.

Adjuvants in SCIT

By definition, an adjuvant is an excipient that enhances the effect of a drug without having a pharmacological effect of its own [38].

Immunology and vaccines in particular are a classic field of application for adjuvants, since they can be used to increase the immune response to a vaccine in a nonspecific manner. Delayed release is often associated with an immunogenic effect, as a result of which

increased antibody production and an increased immune response are achieved.

This treatment concept is also used in allergology [38]. The goal of using adjuvant molecules is to enhance the immunological and thus also the clinical efficacy of AIT [38].

Adjuvants in commercial preparations

At present in Germany, adjuvants are only available in products for subcutaneous use. Adjuvants were originally developed as depot carriers designed to release the antigens bound to them in a delayed manner. The assumption was that they rendered vaccine and therapy allergens safer and more effective. It has now been shown that depot carriers also have immunostimulatory effects, particularly on antigen-presenting cells (APC) [39–43].

Aluminum salts have been used as depot adjuvants in vaccinations for almost a century and, for decades, were the only adjuvants approved for vaccines worldwide [44]. They are still the most commonly used depot adjuvants in allergen-specific SCIT [40], due mainly to the ease with which the commercial formulation is produced, their long-established use, and their acceptance by the regulatory authorities.

Aluminum's "adsorbent" properties and slower release of allergens from the depot is believed to result in greater tolerance [45]. It also improves uptake in a variety of immune cells [45]. A rapid increase in allergen-specific T-cells has been observed in clinical studies [46]. As yet, the discussion on a potential accumulation of aluminum resulting in possible neurological or immunological side effects has not prompted a reassessment of the safety of aluminum in prescription allergens for SCIT in Europe and Germany [47, 48]. The Paul Ehrlich Institute (PEI; Langen, Germany) assesses safety—based on a maintenance dose of eight subcutaneous injections each of 0.5 mg aluminum per year (at 6-week intervals in maintenance therapy) [49], according to a European Medical Association (EMA) recommendation—as harmless [50]. However, in 2015, the PEI set-up and financed a 3-year research program called "Develop a physiologically based toxicokinetic (PBTK) model for the risk assessment of aluminium exposition by vaccination and biomedical drugs" [51]. Understandably, the data from this program are not available yet. Lifelong immunotherapy with depot insect venom, as recommended for patients in special risk groups, is to be viewed critically.

Calcium phosphate is another mineral salt used to formulate depots for commercial SCIT products [52]. However, there are no SCIT products with calcium phosphate currently available in Germany.

Microcrystalline tyrosine (MCT) is another commercially used depot adjuvant [53].

L-tyrosine is a biodegradable amino acid able to bind antigens (allergens) and causes the delayed release of allergens following subcutaneous injection [54]. Radioactively labeled and allergen-bound MCT showed a half-life of 48 h in animal studies and was completely removed from the injection site within 7–10 days [54]. Clinical studies have shown MCT to be safe. In preclinical studies, MCT stimulated a rise in both IgG1 (Th2) and IgG2a (Th1), but not IgE (Th2). MCT is also commercially available in combination with the toll-like receptor (TLR)-4 agonist monophosphoryl lipid A (MPL) [54–57].

MPL is one of new generation adjuvants that interact with TLRs on phagocytic, endocytic, and/or APCs. Hereby, MPL stimulates TLR4, while other TLR agonists like CpG and DNA motifs stimulate TLR9 [58]. MPL is a Th1-activating, soluble adjuvant derived from lipopolysaccharides (LPS) of the gram-negative bacterium *Salmonella minnesota R595*. Phosphate cleavage in the LPS creates an MPL molecule with severely attenuated toxicity, but which retains the immunostimulatory activity of the original LPS molecule [59]. LPS stimulates the immune response via pathogen-associated molecular patterns (PAMP), which are detected by specific receptors (pattern recognition receptors, PRR) on the surface of APCs [60].

PAMPs are typically produced by micro-organisms, as well as unmethylated CpG motifs, which are characteristic of bacterial DNA. Since MPL exhibits important PAMP characteristics, it is easily recognized by TLR4. SCIT with MPL showed a good switch from Th2 to Th1 cells and, in combination with MCT, a strong Th1 response [61–64].

Adjuvants under development

Liposomes (e.g., hemagglutinin, lipids) and oil-in-water emulsions (e.g., squalene, sorbitan trioleate, Tween® 80, α-tocopherol) are adjuvants widely used in approved influenza and hepatitis A vaccinations; however, they have not been used in AIT products, yet [65].

Methylated deoxycytidine-deoxyguanosine (CpG-) oligonucleotides have been found in intracellular vesicles in phagocytes [66]. They stimulate the innate immune system towards a Th1 immune response, which is also a beneficial approach in SCIT [66]. In principle, CpG-oligonucleotides can be used not only for SCIT, but also for sublingual, nasal, and intradermal administration [67].

CpG-oligodesoxynucleotides (CpG-ODN) are a class of synthetically produced single-stranded DNA-oligonucleotides that contain a relatively high proportion of CpG motifs. CpG sequence motifs (C: nucleotide cytosine, p: phosphate, G: nucleotide guanine) are extremely rare in humans: less than 2% of dinucleotides are of the CpG type. Furthermore, cytosine occurs primarily in methylated form. In the bacterial genome and in viruses, however, CpG dinucleotides occur more frequently and the cytosine

is largely unmethylated. Therefore, the innate human immune system can recognize these CpG motifs in the DNA via the toll-like receptor 9.

CpG-ODN coupled with tumor antigens have been used, for example, to stimulate the induction of specific $CD8^+$ and $CD4^+$ T-cells in melanoma patients [68]. CpGs have also been successfully used as adjuvants in prophylactic vaccines [69].

Clinical trials with SCIT are already available, e.g., type-B CpG-ODNs were used with the major ragweed allergen Amb a1 in a phase III study [70].

Other adjuvant systems are found in vaccine preparations (e.g., AS04, GlaxoSmithKline: aluminum salt plus MPL), which are approved in a commercial vaccine against hepatitis B (Fendrix®) and human papillomaviruses (HPV; Cervarix®) [71], but not for AIT. The importance attached to vaccine adjuvants can be estimated by the fact that the US National Institutes of Health (NIH) promotes the development of new adjuvants with its own research program [72].

Virus-like particles (VLPs) are nanoparticles that contain no viral RNA or DNA and are therefore unable to replicate. VLPs are generally derived from capsid proteins of viruses or bacteriophages. Hepatitis B and human papillomavirus vaccines are based on VLPs and have been found to be well-tolerated, immunogenic and effective in prophylactic vaccination against infections. In addition, VLPs of the bacteriophage Qbeta (Qβ VLPs) were investigated preclinically and clinically as carriers for antigens for a variety of vaccines against neoplastic and chronic inflammatory diseases [73]. Qβ VLPs are made up of bacteriophage capsid proteins and are the same size and shape as the original bacteriophage [74]. VLPs naturally pack bacterial RNA [74]. The protein stabilizes these nucleic acids and protects them from enzyme degradation. These QβVLPs can be disassembled and again reassembled in the presence of oligodeoxynucleotides (ODN; see above).

The rationale for using VLPs is the extremely strong response of the human immune system to highly repetitive structures such as those found in pathogenic micro-organisms like viruses and bacteria. Therefore, viruses and viral proteins are strong activators of the human immune system. The VLPs imitate the repetitive characteristics of virus shells. They form spontaneously during the expression of shell proteins of the viruses. In contrast to complete viruses, VLPs do not carry the entire genome of the viruses and are therefore not infectious. Typically, they are well tolerated and highly immunogenic. The antigenic determinants of the VLPs are recognized by B-cell receptors and the particles are efficiently absorbed by APCs, which present VLP epitopes as T-helper cell epitopes to major histocompatibility complex (MHC) molecules [75].

Experience with VLPs and CpG motifs in the treatment of allergic diseases

Data from early studies on CpG-ODN conjugated to ragweed allergens are promising [76, 77]. Kündig et al. published a randomized phase I study with Qβ-VLPs coupled to a synthetic 16-amino acid sequence of the house dust mite allergen, Der p1 (Qβ-Der p1) [78]. A rapid increase in specific IgM and IgG (particularly IgG1 and IgG3) was detected on Der p1 peptides, but not in IgE antibodies with a good overall tolerance [78].

Creticos et al. tested Amb a1 antigens conjugated to CpG-ODN in 25 ragweed-allergic patients in a randomized DBPC phase II study [70]. There was no significant change in the study's primary endpoint (vascular permeability of nasal mucosa measured by the albumin level in nasal lavage fluid). However, symptomatically antibody response improved significantly, as did quality of life (QoL) parameters during first ragweed pollen season after treatment, already. This effect remained stable during the second ragweed season after therapy. Treatment was well tolerated.

Senti et al. investigated the safety and clinical efficacy of treatment with type A CpGs coupled to Qβ-VLPs (QbG10) [79]. This vaccination was administered together with a standard extract of a house dust mite allergen to 20 patients in an open monocentric study. The results showed excellent treatment safety, significant symptom reduction, and a significant rise in allergen-specific IgG antibodies [79].

In a randomized, multicenter SCIT-DBPC phase II study, Klimek et al. investigated the same VLPs, loaded with type A CpG G10 (CYT003-QbG10) [80]. In contrast to the previous study, however, no allergen or allergen components were administered with the vaccine. The treatment was well tolerated, safe, and showed a significant improvement in symptom and medication scores. This was the first study in which effective subcutaneous immunotherapy without allergen was performed [81, 82].

"Nonspecific" immunotherapy can also be considered a potential therapeutic option for the treatment of allergic diseases. However, the precise mechanism for the underlying immunological effect has not been elucidated, yet. Based on various animal and in vitro studies, it can be assumed that the above-mentioned induction of a strong Th1 immune response and the production of IFN-γ alleviate the Th2 immune response in the allergic patient in favor of a Th1 response. Furthermore, there could be a direct effect on mast cells, since these also express TLR9 [83–85]. CpGs might also affect the allergic immune response by altering the activity of indoleamine 2,3-dioxygenase (IDO), a potent enzyme in T-cell regulation [86]. The upregulation of IDO in specific dendritic cells (Dcs) via type I interferon receptors following CpG stimulation resulted in T-cell suppression [87]. This

mechanism might be able to partially explain the anti-inflammatory effects of CpGs in allergic diseases.

Microspheres are polymers that have already been successfully used in other medical disciplines [88]. These particles can serve as vectors for, e.g., immunologically active substances such as allergens, and release these in a controlled manner. However, they have not been used in clinical studies on humans, yet [41].

Novel active substances for SCIT

SCIT is the oldest form of AIT. Nevertheless, particularly interesting developments have recently been seen in this field: besides the adjuvants and immunomodulators mentioned above, these include for instance recombinant allergens and hypoallergenic antigen variants [89].

Native recombinant allergens

The major allergens in the most important sources of inhalant allergen are known and can be produced using recombinant technology, now. For SIT, this provides an elegant solution to the problem of the complex standardization of allergen extracts from natural substances, since proteins of this kind can be produced in large quantities with consistent quality. The dose can be precisely specified in micrograms (µg).

Recombinantly produced molecules can also be precisely defined in terms of their physical, chemical, and immunological properties, and production can be carried out in a standardized and reproducible manner—from the same master cell bank. Furthermore, the precise dosage and composition of the molecular allergen components can be specified and thus optimized. This guarantees greater purity of the preparations, which contain only the relevant proteins and are free of contamination. This would make it possible to better observe and research the mechanisms of action during use. Tailored treatment according to individual sensitization profiles would also be conceivable, even though this approach has initially been dismissed by the European Medicines Agency (EMA).

The first clinical trials involving the administration of recombinant grass pollen, birch pollen, and cat allergen proteins in the setting of SIT with subcutaneous administration have been published:

SCIT with a mixture of five recombinant grass pollen proteins (10 µg Phl p 1, 5 µg Phl p2, 10 µg Phl p 5a, 10 µg Phl p 5b, 5 µg Phl p 6) resulted in a 38.5% reduction in the symptom/medication score compared to placebo and demonstrates the principle efficacy of this protein mix in a proof-of-concept study [90].

The efficacy of SCIT with recombinant and natural Bet v 1 (15 µg each) compared to a native birch pollen extract that also contained 15 µg Bet v 1 was demonstrated. There were no significant differences in the efficacy of the three preparations [91].

Hypoallergenic variants of recombinant allergens

Further improvements could be achieved by modifying wild-type allergens to hypoallergenic variants. Thus, it is hoped that the reduction of IgE binding sites will result in lower IgE reactivity, resulting in a reduced rate of side effects. Increased immunological efficacy by means of other modifications is also conceivable.

As in the production of allergoids, the three-dimensional structure of recombinant proteins can also be altered. A modified Bet v-1 molecule (folding variant) has been tested for clinical efficacy and tolerance. The subcutaneous administration of an extremely high dose (80 µg) of this protein has been proved to be clinically effective and well tolerated [92].

The use of peptides containing the necessary T-cell epitopes also promises a treatment with low side effect, once the IgE epitopes responsible for side effects have been eliminated. This consideration has also been used in a SCIT approach to treating patients with cat allergy [93].

The challenges and disadvantages of recombinant DNA technology include the comparatively high development costs, the selection of the appropriate major allergen, as well as the central approval process via the EMA and the subsequent benefit assessment process. In addition, special production processes and quality-control evaluation methods need to be developed for each molecular allergen [94–97].

Once the appropriate (major) allergen has been selected, mRNA is isolated from the allergen source in the next step. Complementary DNA (cDNA) is synthesized using the enzyme reverse transcriptase [98]. Using phages cDNA ("complementary" DNA) banks can be created, which are selected with respect to the most biologically active isoforms on the basis of databases of patient sera. The relevant cDNA is then cloned in a plasmid vector and expressed in a suitable expression system. To this end, the cDNA is introduced into a suitable foreign organism via insertion in an appropriate plasmid vector. Prokaryotic bacteria (e.g., *Escherichia coli*) and eukaryotic yeasts (e.g., *Pichia pastoris*) are used as recipient organisms. Prior to selecting the system, one needs to decide which glycosylation status and what level of protein stability are desired and whether there are special structural requirements such as internal disulfide bridges. Both expression systems have their disadvantages. Bacterial expression systems lack posttranslational modification and altered codons need to be used. Yeasts such as *Pichia pastoris* can cause excessive hyperglycosylation.

Finally, the recombinantly produced allergen is purified using chromatography methods to remove foreign proteins and endotoxins. Quality control comprises two steps: first, the identity, purity, ho-

mogeneity, and structure of proteins are evaluated using physicochemical characterization. Immunological characteristics such as IgE reactivity, biological activity/allergenicity or T-cell reactivity are tested afterwards. A master cell bank for a specific allergen forms the basis of working cell banks to produce clinically applicable allergens.

Modifying hypoallergenic recombinant allergoids

By modifying the isolated cDNA before inclusion in a cell bank, hypoallergenically modified variants of the wild-type allergen are created. The aim of modifications is primarily to reduce IgE activity by destroying or reducing B-cell epitopes while preserving the T-cell epitopes required to induce T-cell tolerance. Enhancement of T-cell activation is also possible. The reduced IgE reactivity could open the possibility of administering higher allergen doses. There are two approaches: genetic engineering, which requires comprehensive knowledge of properties and structure (such as T-cell epitopes, B-cell epitopes, or 3D structure) and DNA shuffling/molecular breeding, which does not require such detailed knowledge [99–103].

Genetic engineering produces:

1. Site-directed mutants: strong IgE-binding B-cell epitopes are replaced with low-binding IgE-epitopes obtained from isoforms.
2. Conformational variants: this destroys conformational-dependent B-cell epitopes.
   a) Allergen fragmentations: these cause a subsequent loss of correct folding and thus of IgE binding sites.
   b) Allergen oligomers: these are produced by linking several identical copies of a gene to form homomers or linking several different genes to form heteromers.
   c) Deletion mutants: with this approach, site-directed mutagenesis/deletion of short sequences directly eliminates B-cell epitopes or indirectly affects conformation.
3. Allergen hybrids/mosaic antigens: by fusing different proteins to form a hybrid molecule, the immunogenicity of weak allergens is enhanced through covalent binding, and more stable and precisely defined allergen mixtures can be produced.

Peptide immunotherapy [104, 105] uses linear peptide sequences that represent fragments of the native allergen for SCIT. Due to their shorter amino acid sequence and three-dimensional structure loss, short peptide fragments exhibit lower allergenicity. Thus, there is less probability that mast cells will be activated by cross-linking of cell-bound IgE. By contrast, the possibility for adaptive, allergen-specific T-regulatory cells to be induced with IL-10 production remains undiminished.

Contiguous overlapping peptides (COPs) were developed with the aim of reducing IgE binding while maintaining the immunomodulatory effect. This principle has been implemented to date with COPs of Bet v1, the major birch pollen allergen [106], and already investigated in phase II studies [107]. Hereby only marginal clinical effects were observed, although they were statistically significant.

Another approach is BM32, a hypoallergenic, recombinantly produced, B-cell epitope-based fusion protein [108] of the major allergen of timothy grass. This molecule has already been investigated in a phase IIa study (dose-finding trial) in an allergen exposure chamber [109]. Although the subsequent phase IIb study found no significant superiority compared to placebo in the grass pollen season, there was a number of clinically and immunologically relevant effects [110]. For this peptide the results of (pivotal) phase III studies are also outstanding.

The intradermal administration of synthetic peptide immunoregulatory epitopes (SPIRE) was highly effective in a phase 2 study on cat allergy (assessment of treatment success in an allergen exposure chamber) [111]. However, a phase 3 study failed to confirm these results. Extremely strong effects were seen in both the treatment group and the placebo group [112], with the result that this peptide is currently not undergoing further development.

A further development, so-called adjuvant-free allergen peptide hydrolysate, also aims to achieve a high immunological effect with reduced IgE reactivity, [79], which has so far been demonstrated for a short duration of therapy of only three weeks [113]. The follow-up field study with these peptides on 554 grass pollen-allergic adults showed a significant but only moderate clinical advantage in the primary endpoint, as well as marked effects in the secondary endpoints investigated [114].

Overall, peptides are believed to have high innovative potential in SIT, as already demonstrated in numerous phase II studies. Further phase III studies are needed to investigate to what extent (and for which of these approaches) they will play a role in the future [89].

Summary

Subcutaneous allergen-specific immunotherapy is the longest established form of AIT. It has undergone numerous changes and refinements in its 100-year history. In addition to the development of allergen extracts, new adjuvants are also being evaluated. At present, AIT is the only available treatment capable of inducing targeted tolerance to individual allergens, particularly in allergies to inhalant allergens [115]. AIT is superior to purely symptomatic treatment due to its disease-modifying effects, which can be reflected in the prevention of asthma and new sensitizations [116]. This aspect is important not only from a medical perspective, but also in terms of AIT's very good cost–benefit ratio in the long term [5]. There is ex-

tensive evidence for the therapeutic efficacy of SCIT with subcutaneous allergen injections or chemically modified allergen extracts (allergoids), evidence that has been reported primarily in the relevant national and international guidelines [117–119]. When administered correctly by experienced physicians, and assuming contraindications have been considered, SCIT preparations are safe and well-tolerated. SIT is recommended in Germany from the age of 5 years. New data in children will be gathered in the coming years under the German Therapy Allergen Ordinance (*Therapieallergene-Verordnung*, TAV). Today, SCIT can quite rightly be considered the "classic allergen immunotherapy." Over the past decade, clinical developments were focused on sublingual preparations, mainly. Today, however, increasingly highly innovative drug developments with the subcutaneous route of administration take place again.

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