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A Comprehensive Overview of Allergen-specific Immunotherapy Types, Recombinant and Natural Extract Allergens in the Diagnosis and Treatment of Allergies

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ABSTRACT

Allergen-specific immunotherapy (AIT) involves administering allergen extracts. It is used to desensitize allergic patients. Herbal allergen extracts that are optimum in efficacy and fewest in side effects are still challenging to produce. To overcome these limitations, oral immunotherapy, epicutaneous immunotherapy, intralymphatic immunotherapy, and artificial recombinant allergen preparations have been evaluated. Recombinant allergens have become more popular with the development of molecular diagnostics and therapeutics. Besides food and drug allergens, pollen, fungal spores, and other allergens have been studied. Based on related clinical studies, this comprehensive overview will present the latest perspectives on AIT methods and available allergenic products, as well as discuss the challenges and opportunities for treating allergic disorders.

Keywords: Allergy; Allergen-specific immunotherapy; Immunotherapy; Recombinant allergen

INTRODUCTION

Hypersensitivities are exaggerated responses of the body's immune system against exogenous allergens.[1] Traditional allergy preventive measures, such as avoiding allergens, are not always effective against pollens and respiratory allergens.[2] Therefore, besides conventional treatments,[3] more specific diagnostic and therapeutic methods must be developed.

For many years, using natural allergens to diagnose and treat allergies without going through clinical approvals has been common practice. However, in recent years, due to advances in legal, health, and safety legislation, particularly in Europe, there have been changes in the use of natural allergens.[4] These changes were provided as clinical confirmations to identify the effects and mechanisms of natural allergens, including irritation testing, mucosal, bronchial, and nasal irritation, as well as the effects of food stimulation associated with these allergens.[5] Problems such as the

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source of allergen extraction, concentration and dose of use, side effects, and even the impact on the ozone layer have also been raised in this area.[6] These rules and regulations have led to clinical trials, standardization approvals, and the use of recombinant allergens.

Different allergens affect humans in different ways. For example, some trigger respiratory symptoms, while others result in pores, skin rashes, or gastrointestinal upset. Less frequently, they can cause life-threatening allergic reactions. Allergens can be divided into biological and non-biological types. Biological allergens include food allergens, animal and insect skin and hair, and airborne allergens (e.g., pollen, dust mites, pet dander, mold, and mildew). Non-biological allergens include medications, detergents, and metals.[7-9]

There is no detailed history of the use of allergen extracts; its origins can be traced back to 1914 when Freeman introduced immunotherapy, which was centered on boosting the immune system to reduce allergic reactions.[10] In the 1930s, antihistamines were used to modulate the body's allergic response,[11] and corticosteroids were first used in 1940.[12] It was not until 1963, with the discovery of mast cells and immunoglobulin (Ig) E, that the knowledge needed to identify allergic reactions developed.[13] Finally, in the 1970s and 1980s, efforts to standardize natural allergen extracts became comprehensive and systematic.[14] Recombinant DNA made it possible to identify the molecular nature of allergens. Finally, the first information on allergen cloning was examined in the late 1980s.[15] Since then, new allergen sequences have been discovered, and more recombinant allergens are being produced.[16]

Immunotherapy is one of the most established methods in this field and generally refers to injecting allergens from the lowest tolerable dose and gradually increasing the dose to the maintenance dose. The ultimate goal of this therapy is to change the immune system's response to the allergen and maintain it for a long time. Novel allergen-specific immunotherapies (AITs) have also incorporated alternative synthetic recombinant allergen preparations. To transcend the barriers of old AITs, modern AITs have exploited alternative access routes, such as oral immunotherapy, epicutaneous immunotherapy, and intralymphatic immunotherapy.[17]

Allergen-specific Immunotherapy

AIT is the only available treatment approach that inhibits and alters the primary mechanism of allergy by causing desensitization in various allergens. This method uses cumulative doses of allergen extract (subcutaneously or sublingually) to induce tolerance to a specific allergen.[18]

About a century ago, Leonard Noon used AIT to vaccinate against pollen toxins that caused hay fever. The toxin was, in fact, an extract of grass pollen that Noon believed could induce a state of immunity to hay fever.[19] This method was used for 70 years until several studies confirmed it was effective against other allergens. [20-22]

AIT products must be not only effective but also harmless. Unlike anti-inflammatory drug therapies, AIT targets the underlying mechanism of the disease and remains effective even when treatment is discontinued. Hence, lowering the necessary medication dose improves the patient's quality of life.[23] The use of molecular methods, such as the production of recombinant proteins, has led to advances in this field. By identifying IgE-binding epitopes and type 2 helper T cell (T_H2) receptors, the path was cleared to produce truncated and specific peptides. This method enhances treatment safety and reduces the frequency of injections.[24]

AIT Mechanisms

AIT activates several mechanisms (Figure 1). Subcutaneous immunotherapy (SCIT) decreases allergen-specific IgE production and increases specific IgG production.[25,26] AIT causes significant alterations in allergen-specific T cell subclasses and immunological aberration. Suppression of peripheral innate lymphoid cells (ILCs) might also contribute to T_H2 suppression and immunological tolerance.[25] The combination of these processes ultimately creates tolerance and prevents the progression of the immune response.[26,27] The management of AIT, the immunogenicity of related vaccines, and the safety of the allergen vaccine should include specific class and drug differences. Continuous monitoring in this field has shown that the systemic response rate is much lower for sublingual immunotherapy (SLIT) than for SCIT.[28,29,30] Also, SCIT and SLIT are both effective in reducing allergic rhinitis and allergic asthma symptoms caused by house dust mites. AIT, while blocking T lymphocyte responses, can significantly change allergen-specific subclasses of T

cells, leading to immunological aberration, specific T lymphocyte anergy, or regulatory cytokines.[31] Following that, peripheral ILC repression–particularly ILC2–may contribute to the repression of T_H2 and immunologic tolerance. Eventually, AIT reduces the T_H2 response to allergen exposure while increasing regulatory T (Treg) cell responses, which are vital to peripheral immune tolerance. These allergen-specific Treg cells secrete IL-10 and transforming growth factor-β (TGF-β), suppressing T_H2 reaction to allergens. IL-10 also enhances regulatory B (Breg) cells, which are responsible for maintaining class switching to IgG4-producing plasma cells. IgG4 is an obstructive antibody that competes with IgE for binding to mast cells, decreasing further downstream response.

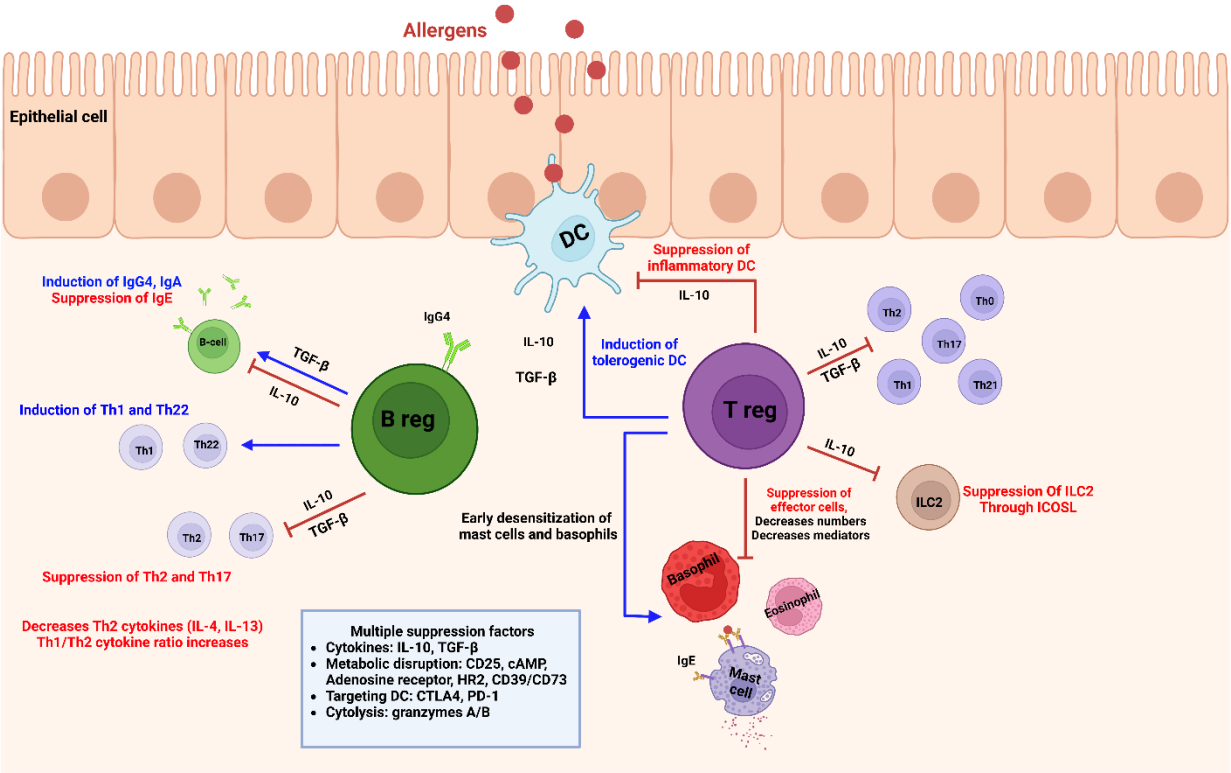

Figure 1. AIT activates several mechanisms: 1) SCIT reduces allergen-specific IgE production and increases specific IgG production. IL-10-producing Breg cells play an essential role in suppressing IgE and inducing IgG4; 2) AIT reduces the T_H2/T_H0 ratio and induces T cells that regulate cytokines such as IL-10 and TGF-β and suppresses peripheral ILCs, especially ILC2; 3) AIT reduces uptake, activation, and mediated release of inflammatory cells. AIT, allergen-specific immunotherapy; ILC, innate lymphoid cell; Treg, regulatory T cell; Breg, regulatory B cell; IL, interleukin; TGF; transforming growth factor; Ig, immunoglobulin. SCIT, subcutaneous immunotherapy; DC, dendritic cell; T_H, T helper. BioRender Confirmation of Publication and Licensing Rights, Agreement number: RF24H5X8PP

AIT Efficiency

AIT is one of the most effective strategies for the treatment of allergic diseases with the goal of reducing asthma signs and symptoms and creating immune tolerance.[32] The two main methods used in AIT are SCIT and SLIT (Figure 2). The main feature of SCIT is the improvement of asthma and rhinitis symptoms and quality of life. In contrast, SLIT is a non-invasive, tolerable, and effective treatment for respiratory allergies. However, various studies have shown that each of these methods has disadvantages.[33,34,35]

In 1986, it was reported that the risk of side- effects might be related to the type of injection method, so the SCIT injection approach was preferred to the SLIT

method, although there is still no consensus between Europe and the United States. Along with this replacement, advances such as the discovery of the helper T cell system[36] and understanding of the IgG4 role as a blocking antibody[39] have contributed to the advancement of immunotherapy technology and the development of AIT.

While the effectiveness of AIT mechanisms depends on different subclasses of immunoglobulins, its efficacy is influenced by factors such as complement activation, antibody-dependent cytotoxicity, and the formation of immune complexes.[33] Recently investigated AIT approaches include oral administration, sublingual, intralymphatic, epidermal, intradermal, and local nasal. In oral immunotherapy (OIT), regular oral doses of food allergens are given in small amounts.[38,39] SLIT is a safe and effective approach that involves daily sublingual administration of allergens. SLIT is effective in treating periodic sensitive rhinitis but not long-term rhinitis.[40]

Regarding the effectiveness of the methods, two studies presented that intralymphoid immunotherapy (ILIT) is clinically effective and that the levels of IgG antibodies in this method were not higher than in the SCIT method.[41,42] Some studies have also confirmed the effectiveness of epicutaneous immunotherapy (EPIT),[43,44] but further studies are needed to understand its mechanism. OIT is performed mainly for digestible food allergens.[45] Its effectiveness depends on the induction of specific IgG antibodies. This method cannot be used for respiratory allergies.[37,46]

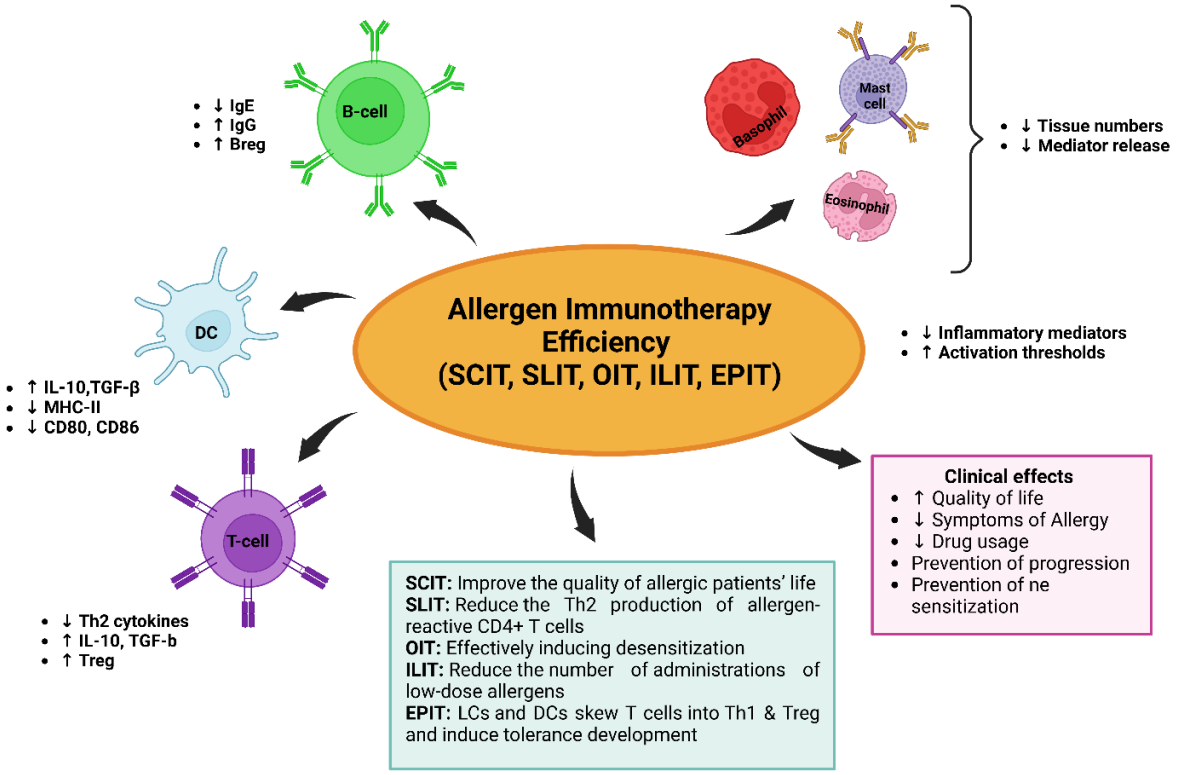

Figure 2. Clinical and experimental immune effects in different types of allergen immunotherapy. AIT is the only choice for the long-term treatment of allergic diseases. After long-term use of SCIT and SLIT, the OIT, ILIT, and EPIT routes are recently developed strategies of AIT. New advances are with reduced side effects and period. A successful AIT may result in reduced drug usage, avoidance of further sensitization, prevention of progression, decreased allergenicity, allergen responsiveness, and combined allergy indications. AIT improves the quality of life. (AIT, allergen-specific immunotherapy; DC, dendritic cells; EPIT, epicutaneous immunotherapy; ILIT, intralymphoid immunotherapy; OIT, oral immunotherapy; SCIT, subcutaneous immunotherapy; SLIT, sublingual immunotherapy.)

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Peptide Vaccines in AIT

T Cell Epitope-based Vaccines

Certain proteins are capable of eliciting an allergic reaction, such as moderate erythema, rhinitis, or even anaphylactic shock in some people. To be classified as an allergenic protein, a protein must have at least B cell peptide units and epitopes that can be detected by the immune system and bound to IgE.[38] Some other factors, such as glycosylation, resistance to proteolysis, and enzymatic action, may also affect allergenic proteins.[39]

Another AIT method uses synthetic peptides with no IgE reactivity but includes T cell epitopes within the main allergens.[40] The identification of CD4 T cell epitopes in allergens is necessary for developing T cell-targeted therapies. Flow cytometry techniques using proliferative dyes such as carboxyfluorescein diacetate succimnimidy1 ester are new methods of evaluating T cell responses to allergen peptides.[41-45] T cell epitopes and structures that induce IgG responses are of the continuous type and remain preserved. They are preserved and capable of inducing T cell tolerance without triggering an IgE-mediated rapid allergic response.[46] Their definitive mechanisms of action are yet to be resolved, but specific anergy, T_H2 cell deletion, immune deviation, and Treg inductions seem implicated.[47,48]

On the other hand, synthetic peptides in AIT have been characterized by a decrease in IgE-mediated side effects due to their decreased ability to crosslink allergen-specific IgE.[45] Their evaluation in murine models initially yielded promising results.[48] However, subsequent clinical studies using peptides from different species were either limited in the number of participants or created contradictory results.[49,50] The resulting systemic side effects were speculated to result from the activation dependent on the major histocompatibility complex (MHC) of T cells.[51] Their mechanism of action is thought to be the induction of T cell tolerance through Treg cells which secrete the regulatory cytokine IL-10.[52]

B Cell Epitope-based Vaccines

B cell epitope-based vaccines provide an improvement similar to recombinant hypoallergenic allergen derivatives engineered to decrease IgE reactivity.[49] The response of IgE antibodies to respiratory allergens is commonly directed toward conformational epitopes. Therefore, the disruption of the three-dimensional shape can minimize or abolish the allergen's IgE reactivity. Since AIT with B cell epitopes attempts to block IgG4 antibodies besides generating IgE-mediated adverse effects, the primary goal of B cell epitope vaccines is to maintain immunogenicity. The blocking antibodies must protect the patient from allergic inflammation caused by the formation of allergen-IgE immune complexes, which activate basophils, mast cells, and T cells through IgE-receptor interactions.[50] when hypoallergenic IgE reactivity was reduced or even totally abolished or when only T cell epitope-containing peptides without IgE reactivity were administered, patients developed late phase side effects due to activation of allergen-specific T cells,[55] indicating that non-IgE mediated mechanisms of allergic inflammation are present in allergic patients.[51]

In most allergic patients, late-phase allergic signs precipitated by using T cell activation and engagement of different innate inflammatory cells contribute to allergic symptoms. The clinical effects of AIT are accompanied by the production of allergen-specific IgG antibodies, which are thought to interfere with the recognition of allergens through IgE antibodies and, as a result, suppress the allergic symptoms caused by IgE-allergen immune complexes. Furthermore, a reduction of allergen-specific T cell activation and decreased IgE manufacturing boosts have been observed in AIT-treated pateints.[52,53]

Production of recombinant proteins involves cloning the appropriate gene into an expression vector under the control of an inducible promoter. The Fusion proteins can be produced in a specific way that allows the incorporation of several different allergen-derived peptides and includes a carrier protein that additionally induces an effective antiviral immune response.[54] Today, the improvement of novel vaccines often relies on the function of peptide-based vaccines. Synthetic peptide-based vaccines are an ideal solution to overcome the unfavorable consequences of conventional vaccines. The development of immunoinformatics has made it possible to predict the efficacy of peptide-based vaccines.[55] The B cell epitope-based carrier vaccine-specific BM32, which was once built for the cure of grass pollen hypersensitivity, has been evaluated in numerous medical trials and is, therefore, the most effective vaccine.[56-58]

Dermatophagoides pteronyssinus (Der P1) **Peptide Vaccine**

HDMs are the most significant triggers of allergic reactions in the house and can be found practically

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everywhere.[59] These allergens (specifically major allergens, which include Der p1, Der p2, and Der p23) have IgE identification rates of >60%. Der p1 was the primary allergen identified in HDMs and showed IgE antibody-binding rates of >80%.[60] Der p1 is generally secreted as a zymogen and is activated by removing the 18 peptide residues.[61,62] The function of this propeptide depends on a unique fold in its structure that develops independently of ERFNIN. Finally, the Der p1 propeptide is characterized by an extra fourth α-helix that replaces the unstructured C-terminal tail, commonly found in other propeptide subfamilies.[63]

Studies have shown that in acidic conditions, the peptide Der p1 changes its structure, increasing its solubility and the flexibility of residues in the spherical N-terminal domain,[64-66] which can be used for vaccines and therapeutic purposes. Under experimental conditions, spontaneous activation of pro-Der p1 at a pH of 4 leads to the creation of mediators associated with the loss of the α-helix N-terminus following a gap in -NKSY19-A20TFE- and -KYVQ40.[67,68] Production of fully active Der p1 with or lacking two extra residues (AE80) is then reached over the last cleavage at the overlap sites (-FDLN78-A79ETN- or -LNAE80-T81NAC-) positioned on the C-terminus propeptide.[68,69] It should be noted that these gaps occur in the propeptide regions, which are related to the coil exposed to the solvent that connects the different α helices, and in the sequence associated with the proteolytic property of Der p1.[70] Activation of pro-Der p1 was also shown to occur through intramolecular cleavages of the precursor by the active protease Der p1.[65] The pro-Der p1 arrangement includes two N-glycosylation positions, one within the propeptide (-N16KS-) and the other in the catalytic domain (-N132QS-).[71,72] The glycosylation of Der p1 propeptide by the yeast *Pichia pastoris* at Asn16, at the N-terminus of the cleavage site -N16KSY19-A20TFE, indicates a decrease in glycosylation rates.[73] Although the Der p1 precursor glycosylation pattern in mites is likely different, such interference in mites cannot be ruled out and may therefore be a regulatory structure for allergen development.[74]

Der p1 has cysteine protease activity, which seems to selectively enhance the IgE response, condition T cells to produce more IL-4 and less interferon-γ (IFN-γ), and can destroy the barrier function of the bronchial epithelium by disrupting the transmembrane molecules occluding and activating the protease-activated receptor PAR-2 which induces the release of pro-inflammatory cytokines.[75] These mechanisms influence the proteolytic activity of HDM allergens in the development of the allergic response, including cleavage of lung epithelium surfactant proteins by dendritic B and T cells. Proteolytic activities have been demonstrated by biochemical and structural characterization of Der p1. Der p1 acts as the activator of the precursors of Der p3 and Der p6 according to an uncommon activation cascade.

Allergen Extracts

Allergenic extracts are sterile options containing extractable elements from several organic sources, including pollens, inhalants, molds, animal epidermal, and insects. A number of advantages and disadvantages are associated with allergen extracts. Its advantages include preparation without the need for extensive purification steps, the inclusion of many allergens from the allergen source, consistent mirroring of the allergen contents of the herbal allergen sources, being on the market with long-standing authorizations, and being recognized by allergologists as regular products. Their drawbacks include the following: They may contain nonallergenic components with different properties and allergens from other sources; they may have variable contents and ratios of allergens; they may have batch-to-batch variations due to manufacturing procedures and raw materials, and they may also be unstable and degrade.[76] Currently, injectable allergen extracts, and natural substances such as molds, pollens, insects, insect venoms, animal hair, and excrements are used for both diagnostic tests and treatment. As a benchmark of intensity and strength for the allergen extract, injectable allergen extracts are recommended in standardized forms. Companies compare the strength of allergen extracts with the US reference standard. The Center for Biologics Evaluation and Research (CBER) preserves these reference standards and issues them among manufacturing companies. Currently, there are 19 identical allergen extracts in Canada.[77]

The process for extracting both standard and non-standard products is the same, despite the variation in intensity. Only the quality control procedures are different.[78] Non-standard extracts are labeled based on the amount of protein nitrogen units (PNU) or the weight of the source material extracted with a certain volume of extraction fluid, although, no studies have been reported to support the biological potency of these labeling processes.[79] However, by completing the

standardization procedure, manufacturing businesses can manage the quality, safety, and efficacy of immunotherapy.[80]

Another group of extract allergens are Culicoides extracts, which have a variety of applications, including as an allergen source for immunological studies of horses with insect bite hypersensitivity.[81-84] A disadvantage of this extract allergen is the difficulty of the standardization process. On the other hand, because this procedure requires the extraction of all its components, serological tests may sometimes have low sensitivity and efficiency.[85] However, the many benefits of these allergens, including their association with certain diseases, insect bite hypersensitivity (IBH), availability, development of specific immunotherapy, and improved diagnostic tests, have led to numerous studies on preparing effective allergens from this group.[86-88]

Allergen Extracts Challenges

Today, almost all available AIT vaccines are derived from natural allergen sources , although allergen-related molecules can be easily produced by recombinant methods. Furthermore, despite molecular advances and the emergence of recombinant allergens, some allergen extracts are still used and prescribed by physicians due to their low toxicity and high viability.[89] On the other hand, disadvantages such as changing the intrinsic content and insolubility of the allergen source activity prevent the standardization of AIT and limit physicians' willingness to recommend it. With the increasing use of recombinant allergens in diagnosing and treating allergies, it is anticipated that molecular allergens in AIT will be the next trend.[90]

On the other hand, studies of AIT vaccines related to allergen extracts show that only a tiny proportion of these vaccines can meet current efficacy and regulatory standards. Also, phase III clinical trials in this group of extracts have not been successful. Other efforts have been made to advance AIT vaccines based on allergen extracts that have not yielded compelling results.[91,92] It should be highlighted that allergen extracts cannot be used to identify the allergen molecules that cause the sickness because they are a mixture of various molecules, including allergens and sometimes non-allergens. In addition, the quality of allergen extracts may vary due to the influence of various factors in their synthesis process, including contamination, other allergens, or even manufacturer's rules or medical standards.[4,93,94]

Today, recombinant allergen derivatives covering a wide range of some of the essential sources of allergens, including plant pollens, mites, cats, dogs, and bees, have been evaluated in preclinical and experimental studies. However, recombinant AIT methods have not been widely used in therapeutic and clinical applications. One of the advantages of these recombinant allergens is that these molecules can be generated successfully in various expression systems and that there is virtually no practical barrier to their production. The availability of these molecules, despite their worldwide copyrights, is another advantage. However, most of these treatments require greater investment from pharmaceutical corporations as well as further clinical studies.[4]

Previously, prescribing allergens for therapeutic reasons relied only on the opinions of doctors and experts, with little clinical research or good clinical practice guidelines in place. However, during the last two decades, conditions have changed in many European countries, and it is possible to use allergen extracts more strictly.[94,95] Today, many countries acknowledge that the use of allergen extracts should include health and safety approvals and guarantees; this has become a main challenge of using allergen extracts. These fundamental criteria address the interaction of extracts with the ozone layer,[6,96] methods of extraction from fruits and seeds, the type of fertilizer used to grow those fruits and grains,[97] and mold-related allergens.[96]

Another disadvantage of allergen extracts is the presence of proteases that cause problems such as allergen degradation, impact on allergenic activity, immunogenicity, and immune-modulating capacity of allergen extracts. In addition, the use of protease inhibitors is ineffective in solving this problem due to their toxicity. Also, some allergens, due to their protease nature, are unable to digest different allergens and may have destructive consequences on the cells and tissues of the patient.[98-100]

The lack of a reliable method for the qualitative and quantitative analysis of these allergens is another drawback of allergen extracts. No precise approach has yet been proposed for this purpose. Some techniques, such as mass spectrometry, have been suggested for molecular analysis which are limited to identifying specific peptides and cannot recognize the immunogenic properties of allergens.[101]

Another problem with processing methods for allergen extracts is the variety of different protocols. For example, measuring protein content provides benefits, such as the ability to assess protein quality, even in denatured extracts, but is unable to detect allergen compounds. Similarly, mass spectrometry methods identify the derivatives of allergens according to their mass but cannot quantify the exact allergens.[4]

Countries and Companies

Valenta et al. list countries that use allergenic extract products for therapeutic purposes.[4] Despite the complexity of obtaining permits in this field, the leading countries include the United States, Russia, Germany, Japan, and Taiwan.[102] In the United States, standardized and non-standardized extracts of injectable allergens are marketed by various companies. However, clinical trials supporting these products are only available for sublingual tablets.[89] The situation is similar in Germany. Allergen extracts are affiliated with several companies that are available for dermatological purposes. Yet, little clinical evidence is available, and only some clinical studies are available for sublingual and subcutaneous tablets related to AIT.[103,104] In Japan, although it has strict rules for the use of allergen extracts, there are still a minimal number of them, and they are prescribed to patients by few doctors.[4] In China, few clinical studies have examined allergen extracts (e.g., licorice extract allergens for respiratory allergies).[103] HAL Allergy is one of the leading European companies in allergy immunotherapy, which has been active in the manufacture and optimization of allergen extracts in both therapeutic and diagnostic areas since 1959. This company's main activity includes the use of allergen extracts company in the form of subcutaneous and sublingual products of pollen allergens, dust mites, and insect venom. Another well-known and long-established company in this field is Laboratorio Experimental de Terapéutica Inmunógena (LETI*)* Pharma, which started in 1941 with the establishment of the *LETI* allergy service and today is one of the largest producers of allergen extracts in Europe (Table 1).

Recombinant Allergens

Today, advances in biotechnological methods have produced recombinant proteins in their purest and most precise forms. These molecular technologies have made it possible to produce allergen-derived peptides, ultimately providing modified products with reduced sensitivity that are both diagnostically and therapeutically effective.[105] The method must, however, take essential considerations into account. The first point is to choose the ideal host for the maximum output at the lowest cost.[106] Several critical advantages of recombinant allergens include: 1) production of uncontaminated proteins or peptides of defined properties and quality, 2) industrial preparation for precise engineering, 3) production in defined quantities and concentrations in a reproducible manner, 4) satisfying the monitoring requirement for medical products, innovative drugs, and vaccines, and 5) the predefined nature of their allergenic, immunogenic, and tolerogenic properties. The downside of using such products include: 1) their application demands expertise, 2) current-day knowledge of recombinant or artificial production techniques is required, 3) they require new market authorization, 4) we need to produce different components for produced recombinant allergens, 5) more clinical studies for recombinant allergens are needed.[76]

The most widely used expression host for recombinant proteins is *Escherichia coli*. It hosts the expression of variety of food allergens, including peanut allergens,[48-51,53,54,84] apples,[74] cow milk,[58] eggs,[69] soybeans, fish,[67] pistachio kernels,[76] hazelnuts.[63] The reason for choosing *E coli* as a host organism is the ease of engineering, high growth rate, easy and low maintenance, and the ability to produce proteins on a large scale.[85] *E coli* strains used for this purpose include *E coli* BL21 (DE3), *E coli* BL21 (DE3) CodonPlus-RIL, *E coli* BL21 (DE3) CodonPlus-RIPL, *E coli* BL21 Star (DE3), and *E coli* BL21 (DE3) pLysS which have been successfully used as expression systems to produce allergenic proteins. Another commonly used prokaryotic cell is the gram-positive bacterium *Lactococcus lactis*, which has been studied in food allergens such as peanuts and cow milk.[52,59,60] The main advantage of *L lactis* compared with *E coli* is the ability to secrete proteins using signaling peptides, such as SP310.[52] Other benefits of *L lactis* include safety, high growth rate, easy engineering, and the production of disulfide peptides that provide structural support for the allergen. The main disadvantage of *L lactis* is that, like *E coli*, it cannot make post-translational modifications.[52]

Table 1. products of allergen extracts in the world[4]

Type of Allergen Extracts and AIT	Type of Allergen	Number of Companies and Products
	USA	
Standardized injectable allergen extracts	Grass species, short ragweed, HDM, Cat hair and pelt, bee venom	79 manufacturers
Link: https://www.fda.gov/BiologicsBloodVaccines/Allergenics/default.htm		
Sublingual **tablets**	Grass pollen extract, grass species, HDM, pollen extract	4 manufacturers
Link: https://www.fda.gov/BiologicsBloodVaccines/Allergenics/ucm391505.htm		
	Germany	
Subcutaneous	Grass, corn, and weed pollen, tree pollen, HDM, venom	115 products
Sublingual tablets	Grass, corn, weed pollen, tree pollen, HDM	34 products
Link: https://www.pei.de/DE/arzneimittel/allergene/allergene-node.html		
	Russia	
Subcutaneous **AIT**	Tree pollen, grass pollen, HDM	1 joint company with France
Sublingual **tablet** and Sublingual **AIT**	Grass pollen, HDM, birch pollen	2 joint companies with France and 1 joint company with Italy
	Japan	
Allergen extract for Scratch test**:** ***Dermatophagoides farinae*** extract ***Dermatophagoides pteronyssinus*** extract	HDM	3 Products
Sublingual **tablets**	HDM	3 Products
Subcutaneous injection	HDM	2 Products
CEDARTOLEN SUBLINGUAL DROP Japanese	Cedar pollen	2 Products
Link: https://www.torii.co.jp/en/release/2014/20140902_1.html		
	Taiwan	
Allergen extracts are available from Allermed	Allergy solution Allergen extracts are available from Allermed (USA), now merged with Greer Co. (USA).	2 joint company with USA
	China	
Allergen extracts are available	ALK (Horsholm, Denmark), Stallergenes Greer. Co. (USA), Zhejiang Wolwo Bio-Pharmaceutical Co., Ltd (China)	1 joint company with USA and 1 Chinese company
Link: http://www.wolwobiotech.com/		

AIT, allergy immunotherapy; HDM, house dust mite.

Several cases of better performance and effectiveness of recombinant allergens against natural allergen extracts have been reported in recent years. For example, AIT, produces only partial protective immune responses against major allergens. This problem can be overcome with a recombinant hybrid allergen that contains the four main allergens of timothy pollen: Phl p 1, Phl p 52, and Phl p 6.115. Another critical point is that this hybrid molecule can be quickly produced in *E coli* with specific reproducible quality and substantial quantities.[4] The recombinant molecule is similar to grass pollen allergen and can be used to diagnose grass pollen in vivo. A clinical study also reported that Phl p 5 and Phl p 6 are very effective in the clinic.[107] In addition, some AIT approaches based on recombinant hypoallergenic molecules, recombinant allergens, and allergen-derived synthetic peptides for clinical use have been meticulously investigated. As shown in Table 2, it is clear that recombinant allergy-based approaches can be used not only for the development of AIT in respiratory allergies but also for food allergies, insect toxins, and even pet allergies.

In one study, the primary allergen of the Bet v 1 pollen, which belongs to the protein family 10 (PR-10), was generated using molecular methods to produce a recombinant allergen Bet v 1 in *Nicotiana benthamiana* leaves with IgE-binding activity in allergic patients. The allergen Bet v 1 was expressed in *N benthamiana* using an agroinfiltration-based protein expression system. Five days after infiltration, allergen concentration in *N benthamiana* leaves was 1.2 mg/mL of fresh mass, which is the maximum Bet v 1 yield reported in plants so far. This study showed that the plant expression system allows allergens' rapid and robust production while maintaining immunogenicity.[108]

In another study, Mohammadi et al. produced the recombinant form of parvalbumin from wolf-herring fish and determined its IgE reactivity. Parvalbumin cDNA was sub-cloned into pET28 and expressed in *E coli* BL-21.[115] The immunoreactivities of the recombinant and native parvalbumins were compared, and the effect of calcium binding was determined in sera from 25 fish-allergic patients. ELISA and Western blot confirmed similar IgE reactivities of the recombinant and native proteins which are highly dependent on calcium binding. The recombinant protein was 94.5% similar to carp parvalbumin (Cyp c 1). Approximately 72% of the patients reacted strongly to recombinant parvalbumin, 80% responded to the native form, and only 56% showed IgE reactivity with the crude extract. Hence, because the IgE-binding capacity of recombinant wolf-herring parvalbumin is conserved and is highly similar to Cyp c 1, the wild and hypoallergenic forms of this allergen could be used for diagnosis and immunotherapy of fish allergies, respectively.[115]

The use of recombinant allergens in food allergy-related studies has also been investigated. Akimoto et al. evaluated tropomyosin (TM) as a major shrimp allergen to monitor its allergenicity and to evaluate anaphylactic reactions in shrimp-sensitive patients. Although recombinant TM (rTM) is widely accepted in the field of allergen-specific immunotherapy, the allergenicity of rTM has not been compared to normal TM (nTM) according to in vitro digestion characteristics. In the study, IgG- and IgE-binding allergen peptides and the degranulation ability of digested samples in simulated gastric fluid, simulated intestinal fluid, and gastric fluid models from nTM and rTM were evaluated by immunoassay, proteomics, and basophil micro-assay. The results showed that the pepsin-digested and trypsin-digested samples from rTM exhibited less binding and granulation than nTM samples to IgG and IgE. More peptides from rTM-digested samples (57.8%) matched allergic shrimp epitopes than nTM samples (33.3%), providing better insight into epitope-based immunotherapy and molecular approach to shrimp allergic individuals.[114,116] In another study of food allergens, Mal d 1 was rapidly expressed and purified as the primary apple allergen in Northern Europe, with an optimized production and purification approach using plasmid conversion in *E coli* cells.[117] Novotny et al, performed a comprehensive panel experiment of recombinant *Culicoides* r-allergens on a group of horses with hypersensitivity to insect bites using allergen microarrays. The results showed that this microarray would be a powerful tool for developing improved allergen immunotherapy with a component suitable for an animal or human patient with IBH.[87] In a study, Jonsdottir et al. compared IgE levels in the serum of horses born with IBH in Iceland with other horses. It surveyed the efficacy of the recombinant *Culicoides* allergen in both. Then serum IgE binding was tested by ELISA using two recombinant allergens, Culicoides rCul n 3 and rCul n 4 produced by *E coli* and insect cells. Significantly more IgE was detected against all allergens in the serum of IBH-infected horses than in healthy

horses. Icelandic horses born with higher IgE levels against allergens and a higher area under the curve (AUC) at rCul n 4 differ from European-born horses. The recombinant allergens of *E coli* and barley produced similar functions in diagnosing IBH-infected and healthy horses.[109]

Another known recombinant allergen is fish, an essential component of food safety. The study by Huang et al. compared natural and recombinant allergens. They showed structural differences, calcium binding, IgE and IgG binding activity, micro-granulation ability, and digestion stability between normal and recombinant albumin. The results showed that the recombinant turbot parvalbumin retained its specific immunological properties. In contrast, the third incorrect folding resulted in different calcium binding, lower IgG binding activity, and stability of digestion.[118]

Table 2. Function of recombinant allergens products

Objectives	Molecules/Timeframe	Function & Clinical trial number	Conclusion	References
IgE sensitization to Culicoides recombinant allergens	Culicoides recombinant (r-) allergens-2021	Allergen microarrays No. BE 121/05 and BE 2/17	Diagnosis of about 90% of IBH horses by a combination of seven major allergens.	87
IgE levels in the serum of IBH horses	rCul n 3 & rCul n 4-2021	Binding of serum IgE to two recombinant Culicoides allergens, rCul n 3 and rCul n 4 No. 141071-051	Similar diagnosis in IBH-affected and healthy horses by barley and *E coli* allergens	109
Detection of sIgE allergens by new immunosensors	rCan f 1 2019	Enhance a Fe3O4 @ SiO2-NTA nano-capturer based on magnetic nanoparticles	Accurate and sensitive detection sIgE and in serum samples by a safety sensor	110
Recombinant LALLT allergen in brown spider venom	LALLT 2020	Express and purify the recombinant LAR protein in baculovirus-infected insect cells	Identification of allergens in the venom of Loxosceles.	111
Pollen-fruit cross-reaction on allergen Cari p 2	Cari p 2 2021	Cloning of the Cari p 2 chymopapain to synthesize recombinant allergens for patients sensitive to papaya fruit.	Introducing Cari p 2 as a new allergen in papaya-sensitive individuals.	112
ABPA detection from ASA by rAsp-specific IgE	rAsp-specific antigens 2019	A prospective study included people with ASA or ABPA who were 12 years of age or older.	IgE played the most beneficial role in separating ABPA from ASA.	113
Compared natural shrimp tropomyosin with recombinant ones	rTM 2020	Assessment of the IgG/IgE-binding and allergen peptides of the digested in nTM and rTM simulated	Less binding in samples digested with pepsin and trypsin in IgG/IgE.	114
High-yield Birch Pollen Allergen Bet v 1	Bet v 1 2020	Produce a recombinant allergen Bet v 1 in *Nicotiana benthamiana* leaves 19H0463	Both recombinant allergens had binding properties comparable to those of the IgE of allergy patients.	108

TM; tropomyosin; LALLT, *loxosceles* allergen-like toxin; sIgE, specific immunoglobulin E; PR-10, pathogen-related protein 10 ASA, *Aspergillus fumigatus*–sensitized asthma; rAsp, recombinant Aspergillus fumigatus; IBH, insect bite hypersensitivity.

In a mouse model of dog allergy, Coquet et al. evaluated the efficacy of recombinant proteins containing Can f 1, f 2, f 4, and f 6 in form of SLIT. Allergenic dog extracts induced strong TH17 cellular responses associated with airway neutrophil infiltration and increased airway overreaction more strongly than HDM allergens. T helper cells that respond to canine allergens also identified several unique clusters with TH17 cells that were identified by the expression of several receptors, including IL-17RE. T cell receptor analysis also showed the high flexibility of T helper cells in this model. Most importantly, preventive SLIT reduces airway overreaction and type 2-mediated inflammation in this model, which is one of the benefits of using recombinant allergens in immunotherapy.[119]

Natural Allergen Extracts vs. Recombinant Molecular Extracts

The pros and cons of these extracts as well as the results of clinical trials will be discussed in this section. Assuming the same quality control and safety regulations and efficacy by clinical trials, the best option is a combination of both methods. Some allergists argue that allergen extracts are less dangerous for patients due to their innate nature. This strength can be seen as a weakness from the point of view of other allergists who argue that molecular and recombinant methods are a better option to reduce the risks and increase the safety and efficacy of allergen products. In the traditional allergen approach, it is difficult to understand the allergy reaction, accurately identify the target allergen, modify the AIT version, and identify hypersensitivity or low-risk allergens.

Another significant advantage of recombinant allergens is diagnostic. This feature accurately identifies multiple allergen sources in patients with various types of allergies, thus providing an accurate diagnosis and better treatment.[4] Also, using recombinant allergens instead of natural allergen extracts can enhance the knowledge gained from the molecular properties of the allergen and help improve the quality of the final product. Controlled and easy expression in simple hosts, including *E coli*, is another significant advantage of recombinant allergens over natural allergen extracts that have led to their use over the past three decades.[85] Due to its easy preparation and cost-effectiveness, it must be noted that *E coli* is considered the most straightforward expression system of most allergens. However, disadvantages such as inadequate cell assays due to nonspecific stimulation have led some researchers to use insect hosts or barley grains for this purpose due to their low contamination and long-term storage.[120,121] Therefore, considering the advancement of molecular methods in many fields of medicine and biology, in the future, molecular experiments are expected to replace traditional methods of preparing and synthesizing allergens completely. As mentioned, due to safety and investment constraints and market conditions, it is necessary to provide clinical studies and legal licenses to prevent the removal of these allergens, especially natural allergen extracts, as is the case for extracts.

In recent years, the conditions for making traditional allergen extracts, as well as reviewing clinical trials on them, may be changing. The synthesis of allergens in the pharmaceutical industry is important to maintain. Therefore, clinical trials are essential to protect and prevent the disappearance of traditional allergens. As a result, stronger regulatory guidelines may facilitate the development of top-notch allergens for in vivo application. These strict regulatory rules may be the best approach to advance the development of allergen extracts as well as recombinant allergen-based products for clinical use. However, improvements in the diagnostic and molecular technologies for allergen extract identification have proven that the majority of the present allergen extracts violate pharmaceutical requirements and pose a concern to humans. Even with the strictest regulatory rules for their manufacture, not all quality issues caused by the limitations of allergen-based technologies can be overcome.

Therefore, efforts should be made to maintain and optimize these allergens. Also, most AIT vaccines are based on allergen extracts. Recombinant allergen molecules can be produced at low cost, with consistent quality and substantial quantities, so they easily meet the standards set for pharmaceutical products. Likewise, they can be used to formulate modern allergy vaccines. Another critical point is that various information is published yearly about different approaches to immunotherapy, both in the field of allergen extracts and recombinant allergens. With these interpretations, evaluating the quality of published studies and combining results with a focus on their meaning is essential for application in Phase III clinical trials. As clinical studies develop, pharmaceutical companies must improve clinical guidelines, standardization rules, and principles. Most current guidelines have not been very effective in application, accuracy, and stakeholder

involvement. They have not been able to fill the gaps in immunotherapy. According to the limitations of immunotherapy, it is possible to obtain a more accurate answer to some of these limitations by using recombinant allergens in the allergen immunotherapy pathway. Another suggestion to overcome these limitations is to combine several types of recombinant allergens and their simultaneous use in allergen-specific immunotherapy, which can reduce the constraints and increase the use of allergen immunotherapy. A recombinant allergen can be an effective option for treating allergies with the help of allergen immunotherapy. The other suggestion to study, to enhance the efficacy of AIT, is to use the new AIT routes, such as intradermal, epicutaneous, or intralymphatic immunotherapies with recombinant allergens.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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