Review

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Biotechnology-based allergy diagnosis and vaccination

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The diagnosis and immunotherapy currently applied to allergic diseases involve the use of crude extracts of the allergen source without defining the allergy-eliciting molecule(s). Advances in recombinant DNA technology have made identification, cloning, expression and epitope mapping of clinically significant allergens possible. Recombinant allergens that retain the immunological features of natural allergens form the basis of accurate protein-chip-based methods for diagnosing allergic conditions. The ability to produce rationally designed hypoallergenic forms of allergens is leading to the development of novel and safe forms of allergy vaccines with improved efficacy. The initial clinical tests on recombinant-allergen-based vaccine preparations have provided positive results, and ongoing developments in areas such as alternative routes of vaccine delivery will enhance patient compliance.

Introduction

Allergy can be defined as an adverse immune-mediated overreaction to otherwise innocuous environmental substances, that is, allergens. It is well established that allergic diseases (also called atopic diseases), such as hay fever, rhinoconjunctivitis, allergic bronchial asthma, allergic dermatitis, eczema, urticaria, angioedema and anaphylaxis, are global health problems affecting more than 25% of the worldwide population [1,2]. There has been a significant worldwide increase in the prevalence of asthma and allergic rhinitis since ~1960 [3]. However, some recent studies indicate that this increase has now slowed down or ceased [3]. Overall, allergic disorders remain the most common cause of chronic ill health and socio-economic burden for health-related quality of life and healthcare costs. Allergy disorders can be induced by both outdoor and indoor sources. The main contributor to outdoor allergies is pollen, whereas indoor allergies are dominated by house dust mites, animal dander, cockroaches and fungal spores [4,5]. Peanuts, tree nuts, soybean, milk, fish, egg and fruits (such as apple, celery and peach) have also been identified as sources of allergens [6]. Allergens are named according to taxonomic classification of the source, combined with the chronological order of allergen purification and characterization [5]. Both genus and species information are represented in the allergen name. The name of the allergen consists of the first three letters of the genus, followed by the first letter of the species and then a number (Arabic numeral) indicating its chronologic order, with spaces between these three items.

The defining feature of allergens from all types of sources is their ability to induce a specific immune response in the form of the production of elevated levels of specific IgE (see Glossary) antibodies (Figure 1). Although all humans are able to produce IgE antibodies, IgE is elevated only in genetically predisposed individuals exposed to an allergen. The first step in the development of allergy is the sensitization that occurs after being exposed to an allergen for the first time. Sensitization involves the development of allergen-specific IgE antibodies and their binding to surface receptors on mast cells and basophils. Once a patient is sensitized, further exposure to the allergen can trigger the allergic reaction. The route and dosage of the antigen exposure are important external factors affecting the mode of antigen presentation, whereas epitopes of the antigens interacting with T cells are the internal factors determining antigen allergenicity [7]. If epitopes of the antigen stimulate a T_H2-like response by induction and activation of T_H2 cells, this leads to the production of the antigen-specific IgE and, hence, the antigen acts as an allergen. Cytokines released by T_H2 cells, such as interleukin-4 (IL-4), IL-5, IL-9 and IL-13, are the main mediators of allergic (and asthmatic) inflammation. Allergic inflammation is characterized by enhanced IgE levels, degranulation of mast cells and eosinophil-mediated inflammation (Figure 1). Some of the external and internal factors that influence the onset of allergic conditions are summarized in Box 1.

Glossary

CpG: indicates when the nucleotide cytosine is immediately followed by the nucleotide guanine. The 'p' in 'CpG' denotes a phosphate group linking the two bases.

FcεR (Fcε receptor): cell surface receptor specific for the binding of the Fc portion of IgE.

IgE (immunoglobulin E): a class of antibody that plays an important role in allergy because of its ability to bind Fc receptors found on mast cells, basophils, eosinophils, monocytes, platelets and macrophages in humans.

IgG (immunoglobulin G): a major effector molecule of the humoral immune response in man and the most abundant form (class) of antibody in the blood plasma.

ISS (immunostimulatory DNA sequences): sequences of single-stranded DNA containing unmethylated CpG that stimulate the immune system and have therefore been proposed as adjuvants for vaccination strategies.

T_H2 (T helper 2): a class of helper-inducer lymphocytes. T_H2 cells act as helpers for B cells and are therefore essential for antibody-based immunity.

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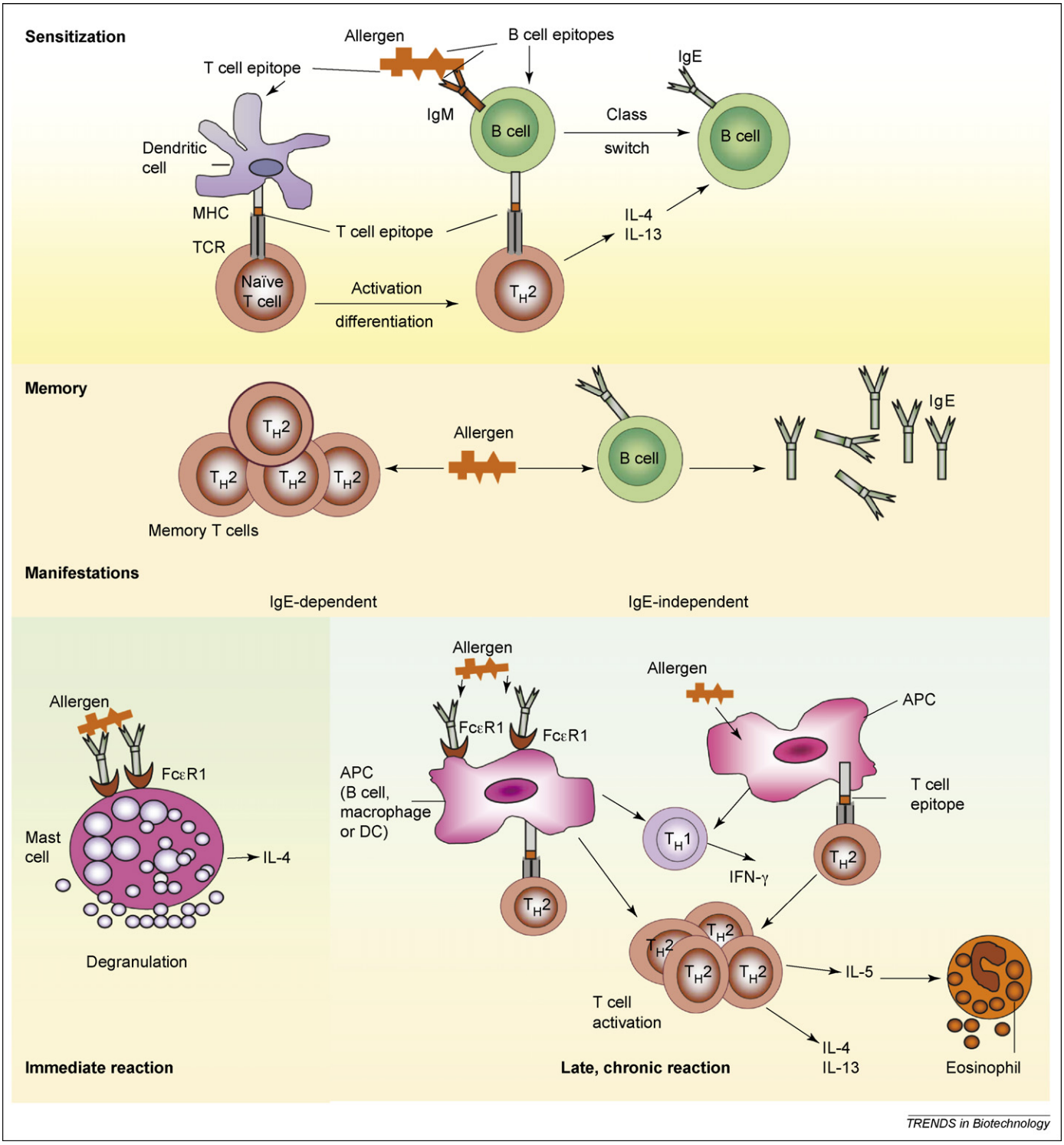

Figure 1. Mechanisms of allergy. Following entry via mucosal surfaces, particularly of the respiratory tract, the allergens are taken up by local antigen presenting cells (APCs; e.g. dendritic cells) and/or are captured by specific B cells through immunoglobulins. The allergens taken up by APCs are processed and presented to T helper 2 (T_H2) cells. In allergic individuals, T cells preferentially differentiate to the T_H2 phenotype. T_H2 cells secrete cytokines (e.g. IL-4, IL-13) that favour immunoglobulin, class switching specific B cells to immunoglobulin E (i.e. sensitization), and also resulting in clonal expansion of naïve and IgE^+ memory B-cell populations. The IgE sensitizes mast cells and basophils by binding to Fcε receptors (FcεRs) on their cell surfaces. The allergic sensitization process leads to the establishment of an allergen-specific memory T-cell pool, and the IgE memory B-cell pool, both of which can be strongly activated by contact with allergens. Immediate reactions are caused by the cross-linking of the IgE–receptor complexes on the surface of sensitized mast cells and basophils by subsequent allergen exposure, leading to their degranulation and release of mediators, such as histamine, provoking immediate symptoms of allergy. Late and chronic reactions are caused by presentation of allergens to T cells leading to their activation, proliferation and release of pro-inflammatory cytokines, such as IL-4, IL-5 and IL-13. T_H2 cytokines such as IL-5 lead to tissue eosinophilia and release of inflammatory mediators from eosinophils. Allergen presentation to T cells can occur in a highly efficient manner by IgE-dependent mechanisms using FcεR1 on APCs. Modified, with permission, from Ref. [68].

Box 1. Some key factors associated with the development and manifestations of allergic conditions

External factors

- Dosage of the antigen exposure
- Route of the antigen exposure

Internal factors

- Epitopes of the antigen interacting with T cells
- T-cell activation:
  - Affinity of the interaction between the antigen epitope(s) and the T cell
  - Concentration of the antigen
  - Type of antigen-presenting cells
- Cytokine environment of the T cell
- The subject's immune-response genes
- History of early childhood viral and bacterial infections
- Dietary habits

Challenges faced by current allergy diagnoses and treatments

Current diagnosis

Diagnosing IgE-mediated allergy usually begins with careful case history and clinical examination. Clinical examination involves *in vivo* provocation tests, such as the skin-prick test (SPT) or an oral food challenge, followed by serological testing to assess the presence of allergen-specific IgE antibodies (Box 2). Current serological and provocation tests employ crude aqueous allergen extracts prepared from various allergen sources, such as mites, pollen, food, dander, mould, cockroaches and other insects [4]. These tests, therefore, only indicate the allergen source to which the patient has been sensitized – because a total allergen extract from a given source is used, the precise identity of the disease-eliciting molecule(s) remains unknown. Other major limitations of allergen-extract-based diagnosis are as follows:

(i) Sub-standard quality of allergen extracts because of contamination from other sources, such as the presence of mites in dander extracts.
(ii) Stability problems owing to possible degradation of allergen extracts by proteolytic enzymes present therein, and incorrect representation of differentially degraded components.
(iii) Difficulty in detecting allergenic molecules present in small amounts in the total extract of the allergen source.
(iv) Inability to differentiate between whether the patient is monosensitized to only a few molecules or polysensitized to a great variety of unrelated allergens.
(v) False-positive errors owing to the presence of cross-reactive carbohydrate epitopes.

Current treatment

Specific immunotherapy is the only treatment available that addresses the underlying cause of the allergic disease. The specific immunotherapy regime involves gradually desensitizing the allergic individuals by administering increasing amounts of natural crude allergen-containing extracts to induce clinical and immunological tolerance over time [8–11]. The gradual desensitization process might involve weekly injections for 8–12 months to reach the maintenance dose [11]. In addition, the desensitization therapy needs to be administered over a longer period (ranging from 2–5 years) to achieve a long-lasting cure. However, the clinical symptoms of allergy can be rapidly controlled by pharmacotherapy, with drugs such as antihistamines and topical corticosteroids used to eliminate symptoms or suppress allergic inflammation without addressing the underlying cause. The currently practiced specific immunotherapy technique involving the subcutaneous injection of allergen-containing extracts provides a long-lasting cure from allergic diseases, but suffers from the major drawbacks outlined in Box 3. Patient safety is a paramount concern in allergy immunotherapies making it a key imperative behind engineering safer and more-effective allergy vaccines, as well as vaccine delivery (Box 4).

Box 2. Testing for allergy

The most commonly used *in vivo* allergenicity tests are skin pricks and measurements of histamine release from basophils, whereas *in vitro* tests are based on serology. Skin-prick test (SPT) and direct IgE measurement tests are based on the principle that allergic conditions are accompanied by elevated levels of IgE antibodies specific to the allergy-triggering molecule. Histamine-release tests determine the capability of an antigen to trigger degranulation of basophils isolated from sensitized individuals.

Skin-prick test (SPT)

In the SPT, a drop of dilute allergen-containing extract is placed on the skin of the forearm or back of the subject, and a small needle is then used to gently prick the skin through the drop of extract [69]. The results are then recorded after 10–15 min. The SPT is considered positive when the skin around the prick becomes red with the development of a wheal, whose diameter provides a semi-quantitative measure of the level of allergen-specific IgE.

Serological IgE measurement

Patient blood samples are used to detect and quantify allergens specific to circulating IgE antibodies [70]. These tests involve incubating the serum of an allergic patient with allergens immobilized on a solid matrix, followed by detection of the bound IgE with labelled anti-human IgE antibodies. The RAST (Radio AllergoSorbent Test) uses radiolabelled secondary antibodies, whereas the ELISA (Enzyme-Linked ImmunoSorbent Assay) test uses enzyme-labelled anti-human IgE antibodies.

Basophil histamine-release test

The histamine-release test determines the capability of an allergen to cross-link IgE molecules on the surface of basophils to trigger release of allergy mediators [13]. Either whole blood samples or washed basophils isolated from the peripheral blood of allergic patients are incubated with the antigens to be tested. After incubation, intact cells are removed by centrifugation and the histamine present in cell-free supernatants is tested by radio-immunoassay, ELISA or fluorimetry.

Towards safer allergy immunotherapy

Recombinant allergens and allergy diagnosis

The application of molecular cloning to the identification and cloning of allergy-eliciting molecules is schematically shown in Figure 2. This resulted in the availability of a wide spectrum of recombinant allergens for their potential use in various diagnostic and therapeutic applications [12–17]. Most of these recombinant allergens have been shown to possess biochemical, biological, structural and

Box 3. Drawbacks of current immunotherapy practice

- Unknown amounts of the individual allergen components in the crude extract.
- Inconsistency in the allergen extracts prepared from different sources and in the units used to measure the potency of the allergen extract.
- Variations in the relative amounts of major allergens in the extract leading to variability in allergen content and allergenic potency.
- Batch-to-batch variations in the given extract.
- Difficulty of standardizing allergen amounts for effective immunotherapy, especially for minor allergens in the extract.
- Development of sensitivity to unknown components in the crude extract.
- Safety: can lead to local, systemic and even fatal reactions.
- Extraction from unreliable sources or not from the actual causative agent.
- Unavailability of commercial extract for some allergens, such as in fungal extracts, because some fungi do not grow on artificial medium, restricting the use of immunotherapy in patients with fungal allergy.

immunological properties equivalent to natural allergens [12]. The use of recombinant molecules in specific diagnoses can reveal the antibody reactivity profile of the allergic patient and identify the disease-eliciting molecules [18,19].

The identification of the individual allergen reactivity profile has been termed 'component-resolved diagnosis' [20,21]. One advantage of this technique is that it can identify allergens that are considered minor but are actually major causes of allergic disease in individual cases. For example, a recent component-based diagnosis of house dust mites using recombinant allergens and natural purified allergens showed that including a highly cross-reactive mite allergen, Der p 10, in addition to major allergens (Der p 1 and Der p 2) might improve the diagnosis for allergen-specific immunotherapy [21]. Recombinant allergens have also been used to improve the sensitivity of allergy diagnosis through spiking of natural extracts with recombinant allergens that are present in low amounts in allergen extracts [22].

Box 4. Non-injection modes of vaccination: sublingual immunotherapy

Current allergy immunotherapy involves subcutaneous injections of the antigen. The advances in recombinant allergens for allergy vaccinations will increase the safety of the treatment, but patient compliance could be a problem (especially in paediatric patients) owing to the discomfort associated with repeated injections [71]. Therefore, identifying alternative routes for vaccine delivery has also been an active area of research since the mid-1980s [72] and has gained momentum in recent years [72]. Oral, bronchial and nasal routes of vaccine delivery are being explored. Of these, the sublingual route of delivery has been shown to be both effective and safer for respiratory disorders, and is already being routinely used for conventional immunotherapy in Europe. Sublingual immunotherapy (SLIT) involves placing antigen preparations under the tongue either in solution or in tablet form. Recent studies have concluded that SLIT is an effective treatment associated with no safety concerns and increased patient compliance. The precise mechanism underlying the induction of immune tolerance is not yet clear, although significant progress has recently been made in this direction. Therefore, it is worth exploring SLIT using recombinant allergens to increase both safety and patient compliance.

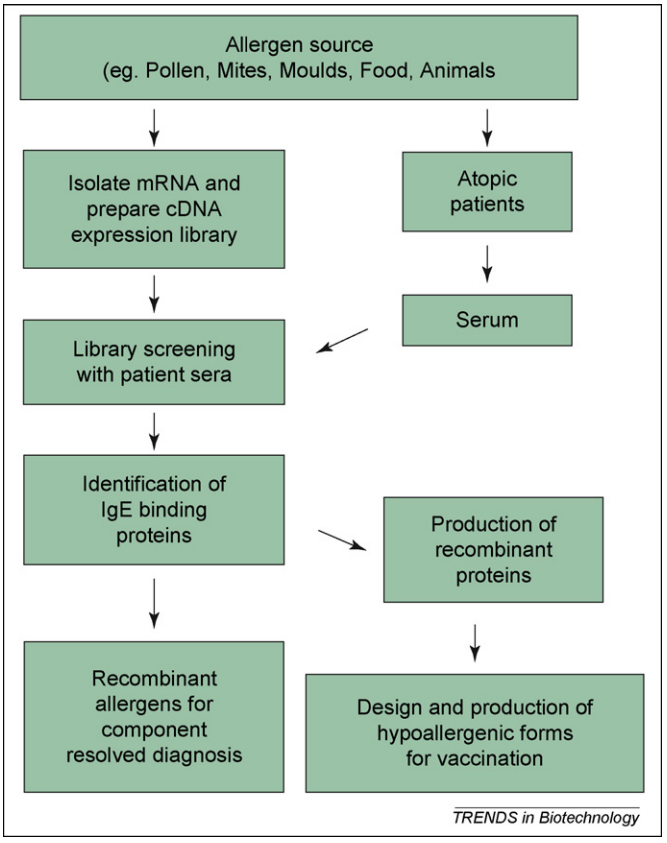

Figure 2. Schematic representation of the steps involved in the identification and development of recombinant-allergen-based allergy diagnosis and vaccination.

Protein microarrays for allergy diagnosis

In this section, we describe a recent development in component-based allergen-specific IgE detection that has made the testing of multiple allergens feasible. Currently available IgE-reactivity tests are time consuming, expensive and labour intensive, because they involve testing a single allergen, or only a few allergens, at a time using a large amount of serum. A recent development in allergy diagnostics has been the use of protein microarrays. Microarray-chip technology involves immobilizing by spotting a large number of proteins onto the surface of a glass slide, enabling the simultaneous testing of IgE reactivities against a large number of allergens from various sources [23–25].

The successful application of a microarray-based multiallergen diagnostic assay has been reported recently [25], in which 78 recombinant and 16 natural allergens representing the most important allergen sources – such as pollen (from trees, grasses and weeds), mites, dander, mould and insect-sting allergens, and various food allergens and latex – were arrayed on a glass slide. The results obtained were comparable to those obtained from various routinely used IgE-detection systems. However, a major advantage is that very small amounts of both allergens and blood samples are required for microarray-based analyses. An additional advantage of allergen-based microarray-chip technology is that the assay conditions closely mimic the *in vivo* condition in the patient, where allergens are present in very small amounts as isolated proteins, and are not available in excess to bind IgE antibodies [26].

Microarray technology is particularly suited to the diagnosis of allergies to foods, such as peanuts. IgE-mediated food allergy is usually associated with the onset of allergic symptoms after digestion [27]. The immediate resulting reactions could affect multiple organs at sites of contact, such as the lips, tongue, throat and hands. In addition, systemic symptoms on the skin and in the respiratory tract can occur after ingesting such foodstuffs. SPT and specific IgE measurements are good indicators for the presence of allergen-specific IgE but do not confirm clinical manifestations of food allergy. Thus, oral-provocation tests, which can put the patient at risk, are currently necessary for confirming or refuting food allergy [28]. Current IgE or SPT assessment of sensitization is based on use of extracts derived from peanuts. These extracts suffer from production storage and lack of standardization problems [29]. Recent evaluation of SPT reactivity of three major recombinant peanut allergens as a predictor of clinical peanut allergy showed equivalence or even superiority of recombinant forms as compared to peanut extracts. Use of synthetic peptide-based microarrays has further refined the concept of component-resolved diagnosis. A set of 213 overlapping peptide residues corresponding to three major peanut allergens were used in a microarray format.

Besides serving as a tool for diagnosis of peanut allergy, these synthetic peptides on chips enabled systematic patient-specific identification of IgE-binding sequences of these peanut allergens [30].

Recombinant technology and allergy vaccines

Recombinant allergens offer a novel approach to patient-tailored, component-resolved allergy treatments based on individual IgE-sensitization profiles [14–17]. Clinical study with recombinant timothy grass pollen allergens showed clinical efficacy of the preparation [15]. However, unmodified recombinant allergens as such are not suitable for specific immunotherapy owing to their high purity and high IgE reactivity, because they are likely to induce anaphylaxis even when administered at low doses. Therefore, it is desirable to modify the recombinant allergens to keep the general protective immune response (of T-cell reactivity and the induction of blocking antibodies) unchanged while significantly reducing (or removing) IgE reactivity [31]. These modified forms are often called 'hypoallergens'.

Allergens are also found to exist as naturally occurring isoforms displaying amino acid sequence variations that can have a profound effect on their IgE-binding activity [32]. Naturally occurring low IgE reactive forms of allergens could be considered as potential candidates for immunotherapy [32]. However, low IgE forms have only been identified in birch pollen allergen, Bet v 1 [32]. This underlines the need for using recombinant DNA methodologies to modify IgE-binding capacity of allergen molecules. Various allergens engineered to reduce IgE reactivity are summarized in Table 1. Various approaches used to convert allergens to hypoallergenic derivatives have been discussed below.

Table 1. Selected list of allergy vaccine candidates produced via biotechnological approaches

Allergen source	Allergen	Approach	Modified allergen property	Refs
Pollen				
Birch tree	Bet v 1	Break in two fragments	Nil to low IgE reactivity, retained T-cell reactivity	Vrtala *et al.* [31]
Birch tree	Bet v 1	Amino acid replacements	Low IgE reactivity, retained T-cell reactivity	Vrtala *et al.* [31]
Birch tree	Bet v 1	Trimer formation	Low IgE reactivity, retained T-cell reactivity	Vrtala *et al.* [31,34,49]
Birch tree	Bet v 1	Low IgE reactive isoform	Reduced IgE reactivity, retained T-cell reactivity	Wagner *et al.* [32]
Parietaria	Par j 1	Disulfide bond variants	Low IgE binding and SPT reactivity low to nil	Bonura [73]
Parietaria	Par j 1	Par j 1+ Par j 2 hybrid	Low IgE binding and SPT reactivity low to nil	Orlandi *et al.* [74]
Ryegrass	Lol p 1	Disulfide bond variants	Low IgE reactivity	de Weerd *et al.* [36]
Ryegrass	Lol p 5	Amino acid replacements	Low IgE reactivity and retained T-cell activity	Swoboda *et al.* [35]
Timothy grass	Phl p 5	Internal deletions	Reduced IgE binding and SPT reactivity	Schramm *et al.* [33]
Timothy grass	Phl p 5	Internal deletions	Low IgE reactivity and retained T-cell activity	Valenta and Niederberger [16]
Timothy grass	Phl p 6	Internal deletions	Reduced IgE binding and SPT reactivity	Vrtala *et al.* [38]
Timothy grass	Phl p 7	Synthetic fragments	Abolished IgE binding and SPT reactivity	Westritschnig *et al.* [37]
House dust mite				
House dust mite	Der f 2	Amino acid replacements	Reduced IgE reactivity with no SPT reactivity	Takai *et al.* [39]
Dust mite	Lep d 2	DNA shuffling with Gly d 2[a]	Low IgE reactivity and retained T-cell activity	Gafvelin *et al.* [54]
Rubber latex				
Rubber latex	Hev b 6.02	Amino acid replacements	Reduced IgE reactivity with no SPT reactivity	Karisola *et al.* [43]
Rubber latex	Hev b 6.01	Disruption of disulfide bonds	Ablated IgE reactivity and retained T-cell activity	Drew *et al.* [44]
Animals				
Cat dander	Fel d 1	Disruption of disulfide bonds	444–900 times lower IgE reactivity than rFel d 1	Saarne *et al.* [41]
Insects				
Bee venom	Api m 1	Chimeric Api m ([1/2/3] protein)	IgE reactivity abolished, 100–1000 times SPT	Karamloo *et al.* [51]
Food materials				
Apple	Mal d 1	Point mutations in B cell epitopes	10- to 200-fold reduced SPT reactivity	Bolhaar *et al.* [75]
Carrot	Dau c 1	Production of mutant dimers	Reduced IgE reactivity with retained antigenicity	Reese *et al.* [52]
Fish (Carp)	Cyp c 1.01	Mutations in Ca-binding domain	95% reduction in IgE reactivity	Swoboda *et al.* [47]
Peanut	Ara h 2	Site-directed mutagenesis	Drastic reduction in IgE reactivity	King *et al.* [76]

[a]Allergen from *Glycyphagus domesticus*, a different species of house dust mite.

Allergen fragments and mutant forms

Allergen molecules, in general, contain several IgE-binding regions (epitopes). IgE epitopes can be defined by a continuous stretch of amino acids (continuous epitopes) or by conformational structures (discontinuous IgE epitopes). These IgE-binding epitopes have been modified to abolish or reduce the IgE-binding capacity of allergen molecules.

Site-directed mutagenesis of individual, or groups of, amino acids within linear IgE epitopes has been effective in reducing IgE reactivity of grass and tree pollen allergens. The deletion and site-directed mutagenesis [33] of the group 5 allergen of timothy grass (*Phleum pratense*), Phl p 5, resulted in the generation of variants that exhibited reduced IgE reactivity and histamine-release capacity [33]. Mutagenesis by altering six amino acids at various positions within the functional residue domain of the major birch pollen allergen, Bet v 1 [34], resulted in significantly reduced IgE binding. However, site-directed mutagenesis exchanging few amino acids within the IgE-reactive domains [35] of Lol p 5, the group 5 allergen of ryegrass pollen, reduced IgE recognition and the allergenic activity of the mutants [35] demonstrating that the mutagenesis approach can be successful in producing engineered recombinant allergens for pollen immunotherapy.

The disruption of conformational epitopes approach has been successful in producing low IgE binding forms of pollen, house dust mite, cat, latex and food allergens. Conformational changes were achieved by either expressing allergen molecules as fragments or disrupting conserved cysteine residues. Expression of Bet v1 as two separate fragments led to complete ablation of IgE reactivity [17]. Conformational changes in rye grass pollen allergen, Lol p 1, introduced by replacement of conserved cysteine residues reduced IgE reactivity by up to 40% [36]. Similarly, disruption of disulphide bonds of Par j 1 (a major allergen from *Parietaria* pollen) resulted in the loss of IgE reactivity [14]. Three-dimensional structure information from X-ray crystallography of grass pollen allergen Phl p 7, formed a basis to design a vaccine candidate protein that showed disruption of conformation Phl p 7 with concomitant loss of IgE reactivity [37]; whereas three-dimensional NMR structure of Phl p 6 formed the basis of rational vaccine design [38].

Modified forms of the major house dust mite allergen Der p 2, carrying mutations at cysteine residues, showed nearly 100-fold reductions in IgE reactivity and a significant reduction in skin reactivity [39]. Similar modification of the three-dimensional structure of the major cat allergen Fel d 1 [40] was achieved by duplicating selected T-cell epitopes and disrupting disulphide bonds [41], resulting in 400–900-fold reduction in IgE-binding capacity than that of recombinant cat allergen.

Natural rubber latex is a well known source of contact allergens [42]. Mutating six amino acids important to the conformational epitope of Hev b 6.02l, major latex allergen, led to the production of its hypoallergenic form with complete removal of the skin-prick reactivity [43]. In addition, hypoallergenic forms of Hev b 6.01 were also obtained by site-directed mutagenesis of selected cysteine residues [44]. Thus, hevein forms engineered to disrupt their three-dimensional conformation make them ideal candidate vaccine molecules for latex allergy immunotherapy.

Immunotherapy has also been proposed as a modality for treatment of food allergy [45]. Fish and shellfish are important causes of food allergy [46]. The major allergens shared among various species of fish are parvalbumins. Parvalbumin is a calcium-binding protein with its calcium-binding domains playing a key role in maintaining IgE reactive sites. Mutations in the calcium-binding domains of Cyp c 1 (carp parvalbumin) resulted in conformational changes with an associated 95% reduction in the IgE reactivity and allergenicity [47]. The mutated form of parvalbumin has been proposed as a potential therapeutic for fish allergy.

Oligomeric and hybrid allergen molecules

An alternative strategy of reducing the IgE reactivity of recombinant allergens is to generate oligomeric or hybrid forms that combine multiple allergens in a single molecule. Oligomers carry multiple copies of the same allergen in a single molecule, whereas hybrid forms could be molecules comprising two or more allergens or allergen derivatives [17,48,49]. Tandem fusions of the cDNAs of major birch pollen allergen Bet v 1 led to the expression of hypoallergenic oligomers [49]. Oligomers generated showed a 100-fold reduction in allergenic activity as measured by histamine-release and SPT. The development of hybrid vaccines that comprise recombinant forms of different grass allergen molecules has also been explored [50]. These hybrid molecules induced antibodies capable of blocking the binding of the IgE of allergic patients to grass pollen allergen. A hybrid protein comprising three major allergens of bee venom (Api m 1, Api m 2 and Api m 3) was shown to have 100- to 1000-fold reduction in SPT reactivity [51]. Comparison of immunological features of oligomeric forms of wild type versus mutant forms revealed that destruction of native conformation is a superior strategy for reducing the allergenicity of carrot major allergen, Dau c 1 [52].

Hypoallergens generated via DNA shuffling

DNA shuffling is a technology that enables directed molecular evolution via generation of a large number of gene variants by reassembling random fragments of related genes [53]. This directed evolution approach has been used to create shuffled forms of dust mite [54] and birch pollen [55] allergens. Multi-gene recombination of dust mite allergen resulted in shuffled forms that showed 80-fold reduction in IgE binding with retained T-cell reactivity. Multi-vaccine forms of birch pollen allergen were generated by shuffling fragments of Bet v 1 with homologous allergens, Cor a 1 [major allergen of hazelnut (*Corylus avellana*) pollen] and Aln g 1 [major allergen of alder (*Alnus glutinosa*) pollen], resulting in chimeric proteins showing low IgE-binding capacity [55].

Allergen-immunostimulatory DNA conjugates

A key requirement for molecular design of effective vaccine is that while candidate molecules should not show IgE cross-linking activity (allergenicity), they should retain their ability to induce strong immune response

(i.e. immunogenicity) [11]. Immunostimulatory DNA sequences (ISS) containing CpG motifs have been evaluated as potential adjuvants for enhancing immunogenicity while minimizing the allergenicity [56]. In this study, conjugation with immunostimulatory DNA sequences (allergen–ISS conjugates) masked IgE epitopes and added a desirable Th1-inducing property to the allergen molecule. Furthermore, a conjugate of short ragweed allergen, Amb a 1, with 22-mer ISS oligonucleotide demonstrated the efficacy and safety of immunotherapy for ragweed pollen-allergic patients [57]. In addition, several animal studies have highlighted the potential of immunization with allergen–ISS conjugates for allergy treatment [58].

Synthetic peptides for immunotherapy

The failure to cross-link adjacent IgE molecules on mast cells and basophils makes short linear peptides suitable for immunotherapy. Recent developments include use of synthetic peptides carrying T-cell stimulatory epitopes but lacking B-cell epitopes [59,60]. Such peptide vaccines have the potential to induce protective antibody responses. Alternatively, short peptides based on hypoallergenic B-cell epitopes could be tested as candidate vaccines for allergic diseases. A recent study using peptides has shown reduced late-phase cutaneous reaction to bee venom in vaccinated patients [61]. Clinical trials with cat allergen peptides showed reduced skin reactivity, reduced airway hyper-reactivity in asthmatic subjects and reduced nasal symptoms in individuals suffering from allergic rhinitis [60].

DNA-based allergy therapeutics

DNA vaccines for allergy-specific immunotherapy offer a promising approach for inducing a strong and long-lasting protective immune response [62]. This therapy is based on direct immunization with DNA encoding the allergen protein. DNA vaccines offer ease of design and production [63]. Moreover, genetic immunization with plasmid DNA addresses concerns with protein-based immunization, such as cross-linking of pre-existing IgE on mast cells and basophils and induction of new sensitivities. DNA immunization facilitates rapid adoption of hypoallergenic forms. The large dose of DNA required for vaccination is one of the limitations of this approach. Using DNA vaccine based on viral replicases has addressed this requirement. These replicon DNA vaccines are required in extremely low doses because replicase essentially replicates viral genome inside the host cell [64,65]. Moreover, replicase-based DNA vaccine has the distinguishing features of being both extremely immunogenic and exhibiting short-term expression of the plasmid, because transfected cells are removed via apoptosis.

Clinical trials with recombinant allergens

The pipeline of modified recombinant allergens that are potential candidate vaccines for specific immunotherapy is represented in Table 1. Out of the listed candidates, recombinant vaccines based on birch and grass pollen allergens were the first to enter clinical trials. Both wild-type recombinant allergens and their hypoallergenic forms are currently being evaluated. The first seminal study with genetically engineered hypoallergens of the tree pollen allergen Bet v 1 demonstrated the effectiveness of the recombinant vaccine [66]. A multi-centre, double-blind, placebo-controlled and randomized trial involved 124 patients allergic to birch pollen who received a single preseasonal injection with either two recombinant fragments or Bet v 1 trimer (as described above). Patients were not only able to tolerate high doses (cumulative dose of 150 μg of recombinant allergen) but also showed a strong protective antibody (IgG) response against both native Bet v 1 allergen and cross-reactive allergens, such as group 1 allergens of alder and hazel nut tree pollen, and food allergens of apple, carrot and celery [67]. In addition, the symptom medication scores were significantly lower (53%) for these patients than for the patients treated with birch pollen extract. This result shows that immunotherapy with genetically modified hypoallergens is effective in targeting the underlying immunological mechanisms of allergies, and could be clinically useful for improving respiratory and pollen-associated oral syndrome induced by birch pollen allergen [67].

A second clinical trial with a mixture of five recombinant allergens from timothy-grass pollen allergens (Phl p 1, Phl p 2, Phl p 5a, Phl p 5b and Phl p 6) involving 62 patients who received a monthly injection for a period of two years showed similar results to those described above, in that the treated subjects developed strong IgG antibodies against natural grass pollen allergens and exhibited reduced clinical symptoms [29]. Clinical trials have also investigated the effectiveness of a single recombinant allergen, the birch pollen Bet v 1a, and compared it to pollen extract in 147 subjects with birch pollen allergy [67]. Similar to the timothy-grass clinical trial, a strong induction of IgG antibodies was observed. In addition, the patients showed marked reductions in skin-prick sensitivity. Thus, the clinical trials conducted so far provide evidence that recombinant vaccines could be used in the near future for vaccinating against allergic diseases.

Conclusions

Biotechnology-derived recombinant allergens have come a long way since the first allergen cloning was performed successfully less than two decades ago. Specific immunotherapy is rapidly progressing towards the use of defined biotechnology-based standardized therapeutic reagents. The combination of recombinant allergens and microarray technology might herald a new era of patient-tailored allergy diagnosis and vaccination. Data from recent clinical trials have brought safer and effective allergen vaccines closer to reality. Further advances in non-injectable methods of vaccine delivery should improve safety, convenience and compliance in the future. Biotechnological approaches offer the possibility of generating further innovative allergy therapeutics. There is already an active development pipeline of biotechnology-based recombinant allergy vaccine candidates (Table 1). Moreover, with rapid advances in our understanding of immune mechanisms, the mystery surrounding specific immunotherapy is diminishing rapidly. The commercialization of biotechnology-based allergy therapeutics requires further clinical studies to establish their efficacy, safety and reliability.

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