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# Standardized allergen extracts: past, present and future

*Linda Cox*

**Consistent high-quality allergen extracts are essential for accurate diagnosis and effective treatment of allergic disease. Allergens represent complex heterogenous mixtures of allergenic and nonallergenic proteins, glycoproteins and polysaccharides. They are derived from natural sources (i.e., collected pollen) or cultures (dust mites and fungi). The goal of allergen standardization is to produce well-characterized extracts of known biologic potency and composition. This process requires the selection of an appropriate reference extract and methods to compare the test extract with the reference. Two methods are currently used in the standardization of these complex allergen extracts: marking and functional assays. Marking assays, which include allergen-specific, qualitative monoclonal antibody assays, indicate the presence of an individual protein but do not confer information regarding the biologic action of the protein. Functional assays, such as allergen skin tests, will provide information about the biologic function, but not about the specific composition of the extract.**

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The ideal allergen extract would be of a known composition, devoid of unwanted material and customized to the allergic individual's immunoglobulin (Ig)E binding profile. Obstacles to producing the ideal allergen extract are manifold, and include allergen composition heterogeneity, variability in the allergic population's reactivity to allergens and the absence of a single assay that measures both extract composition and functional properties. Major allergens have been identified for most inhalant allergens. Major and minor allergens are defined by the percentage of the allergic population who recognize them by producing allergen-specific IgE. A protein is considered a major allergen if it is recognized by more than 50% of the allergic population, whereas minor allergens are recognized by less than 50% of the allergic population. The term minor, however, does not mean it is clinically unimportant to some allergic individuals. Major allergen content appears to correlate with the allergen extract's overall biologic potency. Many major allergens are available as recombinant proteins. Recombinant allergens can be consistently produced in high purity and quantity with defined concentrations of protein content, which makes them attractive candidates for allergen standardization reference material. The European Union's (EU) multidisciplinary program CREATE (Development Of Certified Reference Materials For Allergenic Products And The Validation Of Methods For Their Quantification) was established to promote standardization of allergenic products. It is currently investigating several recombinant allergens as possible references for allergen standardization.

## Historic overview

Specific allergen immunotherapy (SIT) has been used in the management of allergic rhinitis and asthma for approximately 100 years. William Dunbar (1863–1922), a US physician working in Germany, was convinced a pollen toxin was the cause of allergic rhinitis [1]. Dunbar unsuccessfully attempted passive immunization by injecting what he believed was an antitoxin referred to as pollatin, which was derived from the serum of immunized rabbits and horses. Attempts at active immunization with pollen extracts were also unsuccessful. The excessive high pollen doses led to undesirable systemic reactions, including potentially life-threatening anaphylaxis. Work with active

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immunization was continued by two English physicians, Noon and Freeman, in the early 1900s. This group reported their success in treating hay fever patients with inoculations of pollen vaccine in a series of articles published in *The Lancet* [2–4]. Noon discovered a relationship between immunotherapy efficacy, safety and the allergen dose, which he summarized in the following statement: "an overdose can induce a severe attack of hay fever lasting 24 h" and "sensibility of hay fever patients may be decreased by properly directed dosage, at least a 100-fold, while excessive or too frequent inoculations only serve to increase the sensibility" [2]. Noon also made the first attempts to standardize allergen extracts. To express the strengths of pollen extracts, Noon arbitrarily defined a unit of pollen as the "quantity of pollen that can be extracted from a thousandth part of a milligrame of Phleum pollen", and this became known as the Noon Unit [2]. The strength of the pollen extract was given in terms of the number of units in a cubic centimeter of extract. Other pollens were standardized by comparative tests on the eyes of hay fever patients (conjunctival challenge). Noon's work formed the basis for immunotherapy as it is still practiced today. In the ensuing years, efficacy of SIT in allergic rhinitis and asthma has been proven in numerous double-blind placebo-controlled studies [5–8]. Clinical research has provided greater insight into the mechanisms of SIT and the factors that are necessary for its efficacy, confirming Noon's observation that immunotherapy efficacy and safety correlate with the allergen dose. Unfortunately, until recently, there has been very little advancement in allergen extract standardization and most commercially available allergen extracts are not standardized for biologic potency. Nonstandardized extracts are currently labeled on the basis of protein nitrogen units (PNUs) or on the weight of source material extracted with a given extracting volume (w/v). Neither method confers any direct or comparative information about the extract's biologic potency, which is ironic considering the early Noon unit provided some estimate of extract potency with the comparative *in vivo* test (conjunctival challenge). This review outlines the allergen extract standardization process, focusing on the pitfalls and progresses in the development of well-characterized allergen extracts of a known potency. It concludes with a review of possible future strategies to improve the allergen extract standardization process.

### Allergen extracts: source & definitions

The term allergen is used to describe any substance stimulating the production of IgE. Most allergens are proteins ranging from 5 to 100 kD. Allergen extracts from natural sources are heterogenous products containing multiple allergens, nonallergenic proteins and macromolecules. Source material for pollen is derived from plants and is collected either in the field or greenhouse. Animal allergenic extracts are derived from several source materials, including hair, dander, saliva and urine. House dust mite and fungi source materials are produced by laboratory cultures.

The number of allergenic proteins from any source can vary substantially. In general, animal dander extracts appear to be the most simply composed, with between ten and 20 antigens [9]. Dust mite extracts may contain 20–40 antigens and pollen extracts appear to be more complicated, containing between 30 and 50 detectable antigens [9]. Sensitized (allergic) individuals often recognize more than one allergen in a given source. An allergen that induces production of specific IgE by more than 50% of allergic individuals is referred to as a major allergen [9]. Allergens recognized by less than 50% of allergic individuals are considered minor allergens. However, the term minor allergen can be misleading, since allergens that are considered minor on a population basis may be clinically important to some individuals. An ideal allergen extract would provide all of the relevant components for each allergic individual. Most allergen sources contain multiple major and minor allergens which, when combined with the variability of the allergic population's allergen-specific IgE responses to these allergens, makes standardization difficult.

### Nonstandardized allergen extracts

Most commercial allergen extracts are not standardized. The extraction process for nonstandardized and standardized products are essentially the same. The primary difference is the quality control measures. Nonstandardized extracts, with their designated labels of extraction ratio (w/v) or PNU, do not convey information about their allergenic potency or composition. There may be considerable lot-to-lot variability in composition and biologic potency in allergen extracts with the same labeled potency. In the absence of standardization, several measures can enhance product consistency. The inherent nature of the raw material may affect the lot-to-lot consistency, with pollen and pure mite extracts having greater lot-to-lot consistency than mold and insect extracts [10].

Manufacturers can also enhance consistency by utilizing uniform collection, storage and processing techniques. However, consistency cannot be assumed without some measure of potency for each lot of extract.

### Pitfalls with nonstandarized extracts: fungal allergens

Fungal allergen extracts exemplify the pitfalls and potential benefits of allergen extract standardization. They are particularly problematic in terms of the difficulties they pose for standardization. Source materials for fungal allergenic extracts are derived from stock cultures maintained by the manufacturer. The morphologic, biochemical and antigenic characteristics of a given fungal strain are known to change with variations in the culture medium nutritional composition. Fungi rely on a high rate of somatic mutation and this genetic instability can alter the stock culture's production of allergenic substances [11]. This inherent genetic volatility is compounded by the diversity of strains employed by the different manufacturers. This may lead to the antigenic heterogeneity commonly observed amongst commercial fungal allergen extracts.

This commercial extract heterogeneity was confirmed in a recent study that analyzed the compositional profiles of nine samples of *Alternaria alternata* from seven extract manufacturers. Analysis with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) revealed that the two companies yielded comparable banding profiles [illegible]. Significant differences were observed in the SDS-PAGE profiles of one extract manufacturer between aqueous and glycerinated *A. alternata* extracts. No significant correlation [illegible] was observed between the Alt 1 concentrations and the respective protein, carbohydrate and relative potency (RP) values.

Another study investigating the variability of major allergen levels amongst [illegible] allergenic fungal extracts found, in general, that *A. alternata* extracts were consistent within and between companies with Alt a 1 levels ranging from less than [illegible] to [illegible] µg/ml (mean Alt a 1 [illegible] µg/ml), whereas *Aspergillus fumigatus* extracts showed much greater variability with Asp f 1 levels ranging from less than [illegible] to [illegible] µg/ml (mean Asp f 1 [illegible] µg/ml) [illegible].

## Standardization of allergen extracts: why it is important

The World Health Organization (WHO) position paper on allergen immunotherapy states "successful immunotherapy is dependent on the use of high-quality allergen vaccines that are properly standardized and can be manufactured with consistency" [illegible].

Allergen extract composition variability has several potential consequences for the allergic patient. Allergy diagnosis and allergen immunotherapy efficacy are dependent on the quality of the allergen extracts. Numerous studies have demonstrated that efficacy of allergen immunotherapy is dependent on the administered dose of major allergen [illegible].

Immunotherapy safety is also affected by allergen extract variability. According to the WHO position paper, systemic, fatal immunotherapy risk is improved from [illegible] prepared allergen vaccines [illegible]. The presumed reason for this is that the composition of the extracts in [illegible] prepared allergen vaccines may vary significantly from the previous vaccine. One study that aimed to establish a clinical basis for maximum limits for the allergen vaccines, [illegible] and [illegible] were able to determine that the therapeutic equivalence is achieved over a tenfold range of allergen concentration [illegible]. Safety operational data, on the other hand, indicated that a fourfold increase in allergen concentration would be associated with a [illegible] increase in adverse reactions [illegible].

The WHO position paper, the American Academy of Allergy, Asthma and Immunology (AAAAI) and the European Academy of Allergy and Clinical Immunology (EAACI) all recommend that all allergenic vaccines be standardized for total allergen potency, biologic activity and major allergen measured in mass units [illegible].

## Methods of allergen standardization assaying extract potency

Allergen standardization requires a reference extract and assays or procedures to compare with the manufactured extract [illegible]. Allergen standardization is based on in vitro and in vivo determination of allergen-specific IgE. Ideally each allergen in the extract would be purified and quantified during the standardization process. However, this may be difficult

considering that allergen extracts can contain between ten and 50 antigens [illegible]. Overall allergenicity, which is assessed by allergy skin testing, may be a better predictor of the extract's biologic potency.

Two methods of allergy skin testing are currently used in allergen extract standardization. Titrated intradermal testing, referred to as the ID₅₀EAL method, is used in the USA. Titrated prick skin testing, referred to as the Nordic Council method, is used in Europe. Both methods are based on the skin test results of known allergic subjects and depend on the availability of allergic patients and the criteria used to select them. Both techniques use the results of the titrated skin tests to create a reference extract that will be used to compare the potency of manufactured extracts. Comparison of a manufactured extract of an unknown potency with the reference extract can be performed with a quantitative *in vitro* assay, such as competition enzyme-linked immunosorbent assay (ELISA). The competition ELISA protocol involves adding a mixture of the reference pool serum and the extract of unknown potency to wells of a microtiter plate coated with the reference allergen [illegible]. The reference pool serum binds the allergens in the test extract, which results in fewer free IgE antibodies to bind the immobilized allergen in the microtiter wells. The manufactured extract can then be assigned units based on the RP compared with the reference extract. The optimal RP range should provide minimal variability in extract lots, which will increase the safety of administering a new extract lot for patients currently on immunotherapy, although the range should not be so narrow that it results in the rejection of a significant percentage of potentially effective lots [illegible].

In the USA, standardized dust mite and grass extracts are required to have RP limits that fall within [illegible] and [illegible], in order to be approved for release [illegible].

There is good correlation between the relative potencies assigned by titration skin testing and those determined by the competition ELISA [illegible].

While the inhibition ELISA can provide information about overall extract allergenic potency, it also renders information about the quantity of the different allergenic proteins in the extract. Serious adverse immunotherapy reactions may occur if an allergenic protein is over-represented in a particular extract.

Measurement of major allergen content has been shown to correlate with biologic potency [illegible]. Allergen immunotherapy efficacy has also been demonstrated to correlate with major allergen content [illegible]. Controlled studies have established an effective therapeutic dose based on major allergen content per maintenance dose for several allergens with the optimal maintenance dose in the range of [illegible] µg of major allergen.

Other *in vitro* methods that may be used in allergen extract standardization include SDS-PAGE, IgE immunoblotting and cross-radioimmunoelectrophoresis. Isoelectric focusing (IEF) can also be used to help determine the composition of the allergen extract.

Table 1. Recommended allergen-immunotherapy maintenance dose based on major allergen content.

| Allergen | Study design | Results | Recommended dose of major allergen/comments | Ref. |
|---|---|---|---|---|
| Cat | DBPC 28 patients rhinoconjunctivitis (ARC) and asthma | ↓ M-S $p < 0.001$<br>↓ tSPT $p < 0.001$<br>↓ CPT $p < 0.001$<br>↓ Allergen BHR $p < 0.05$<br>MC : No significant difference<br>SR: 0.41% per injection | 13.2 µg of Fel d 1 | [39] |
| | DBPC 21 patients asthma | ↓ Allergen BHR ($p < 0.001$)<br>↓ Histamine BHR ($p < 0.01$)<br>↓ tSPT ($p < 0.01$)<br>SR: 23% patients – mild | 17 µg of Fel d 1 | [40] |
| | DBPC 24 patients asthma | ↓ Allergen BHR $p < 0.01$<br>↓ tSIT $p < 0.025$<br>No change in MC | 18.24 µg of Fel d 1 | [41] |
| | DBPC dose response study of 28 (AR) patients ± asthma 0.6 µg 3 µg 15 µg Fel d 1 | Dose response seen:<br>↓ tSPT and ↓ cat-specific IgG4 in the 3 and 15 µg groups<br>↓ $CD4^+/IL\text{-}4^+$ peripheral blood mononuclear cells in 15 µg group | 15 µg of Fel d 1 | [42] |
| | Similar design as above, 28 patients, three doses Fel d 1 and placebo, examined response at 5 weeks and 1 year | After 5 weeks and 1 year, significant and dose-dependent differences in:<br>Nasal challenge symptoms $p < 0.0001$<br>tSPT 5 weeks ($p = 0.014$) and 1 year $p < .0001$<br>Cat-specific IgG4 at 5 weeks ($p = 0.004$) and 1 year ($p = 0.003$) | Titrated nasal allergen challenge, tSPT, and allergen-specific IgG4 at 5 weeks is predictive of the response at 1 year | [43] |
| Grass | DBPC 30 AR Alutard SQ grass | Statistical difference in: tSPT, CPT, BHR and M–S scores | 15 µg of Dac q5 plus Lol p 5 major grass allergen<br>Progressive improvement over the 3-year period in ocular, nasal and pulmonary symptoms | [44] |
| | DBPC study 40 patients AR grass | ↓ M–S scores ($p < 0.002$ and $p < 0.001$, respectively)<br>↓ CPT ($p = < 0.001$) | 18.6 µg of Ph p 5 | [45] |
| | DBPC study of 44 points AR/36 chest symptoms | Significant reduction in:<br>M–S scores %; ($p < 0.007$ and $p < 0.01$, respectively), QOL ($p = 0.02$)<br>MC sensitivity in season in tx ($p = 0.5$) vs. ↑ MC in pl ($p = .01$ between group difference) | 20 µg of Ph p 5 | [46] |
| Short ragweed | Nasal challenge to evaluate dose response in 13 AR patients treated with the following doses: 0.6, 12.4 and 24.8 µg Amb a 1 | 0.6 µg: no change in mediator release<br>12.4 and 24.8 µg of Amb 1 similar reduction in tosyl-l-arginine methyl ester (TAME) and histamine | 12 µg Amb a 1 | [47] |
| | Compared low-dose rinkel method (0.5 ml of endpoint titration; median dose 0.0018 µg Amb a 1) vs highest tolerated (median dose 11 µg Amb a 1) | Rinkel method not significantly different from placebo<br>High dose: significant ↓ in M–S scores, ↑ specific IgG, ↓ seasonal IgE rise | µg Amb a 1<br>Study confirmed perception that low dose immunotherapy is not effective | [48] |
| Dust mite | DBPC 31 patients asthma | Decreased symptom scores and medication<br>↑ FEV1 $p < 0.01$<br>No change in home peak flow measurements | 7 µg Der p 1 and 10 µg Der f 1 | [49] |
| | DBPC study of 74 asthma asthmatic Three doses of Der p 1: 0.7, 7.0 and 2 µg of Der p 1 | Found a dose response in decrease in allergen BHR and SR | Recommended 7.0 µg of Der p 1 | [50] |

AR: Allergic rhinitis; ARC: Allergic rhinoconjunctivitis; BHR: Bronchial hyper-responsiveness; CPT: Conjunctival provocation test; DBPC: Double-blind placebo-controlled; Der p 1: *Dermatophagoides pteronyssinus* 1; FEV1: Forced expiratory volume in 1 second; Ig: Immunoglobulin; IL: Interleukin; MC: Methacholine; M–S: Medication–symptom score; PBMC: Peripheral blood mononuclear cells; pl: Placebo; QOL: Quality of life; SR: Systemic reaction; tSIT: Titrated skin intradermal test; tSPT: Titrated skin prick test; tx: Treatment group.

### Allergen extract standardization in the USA

In the USA, there are currently 19 standardized allergen extracts, 13 of which are inhalant allergens. The licensing of allergenic products for clinical use is regulated by the US Food and Drug Administration (FDA), Center for Biologics Evaluation and Research (CBER; Division of Allergenic Products and Parasitology). Allergen standardization is one the features of the CBER regulatory program. Part 680.3 (e) of Title 21 of the Code of Federal Regulations states that when a potency test exists for specific allergenic products, extract manufacturers are required to determine the potency of each lot of the product prior to release [10]. As discussed previously, allergen standardization in the USA is based on a quantitative intradermal skin test method referred to as the $ID_{50}$EAL system for intradermal dilution for 50 mm sum of erythema [101]. The $ID_{50}$EAL method entails preparing a series of threefold dilutions of a reference extract and injecting 0.05 ml intradermally to 15–20 highly sensitive allergic subjects. The dilution that results in an erythema with the sum of the longest diameter and midpoint (orthogonal) diameter equaling 50 mm is considered the endpoint ($D_{50}$). The mean $D_{50}$ is calculated, and the potency of the extract is reported in bioequivalent allergy units (BAUs).

In addition to skin testing, US standardized extracts may be evaluated with other *in vitro* tests. These tests include quantification of total protein, measurement of major allergen content or competition ELISA. Once the BAUs have been established by CBER for the reference extract, manufacturers assign potency to their standardized lot by using *in vitro* estimates of RP (e.g., competition ELISA) [10].

Manufacturers are expected to confirm the identity of certain extracts with IEF, which separates the proteins according to their isoelectric point [10]. The extract's profile is then compared with the CBER reference. CBER also requires a validated protein assay on each lot of material.

### Additional lot release tests

#### Ragweed

Short ragweed potency units were originally based on the major allergen content *Ambrosia artemisiifolia* (Amb a) 1. Ragweed extract potency is reported in FDA units and BAUs, with one FDA unit of Amb a 1 being equal to 1 µg of Amb a 1, and 350 units of Amb 1/ml being equivalent to 100,000 BAUs [10].

#### Cat

*Felis domesticus* (Fel d) 1 is the major allergen in cat extracts. Cat extracts were originally standardized based on major allergen content with 100,000 arbitrary units (AUs)/ml containing between 10 and 19.9 FDA units of Fel d 1 per ml (1 FDA unit of Fel d 1 = ~4 mg of Fel d 1) [15]. Subsequently, $ID_{50}$EAL testing suggested that 100,000 AU/ml was equal to 10,000 BAU/ml [10]. Approximately 22% of cat-allergic individuals have specific IgE antibodies to cat albumin [21]. Cat pelt extracts have a greater quantity of albumin than cat hair extracts and are required to identify a cat albumin band on IEF [10].

#### Dust mites

Dust mites were originally standardized in AU/ml by inhibition radioimmunoassay. However, subsequent $ID_{50}$EAL testing indicated the AUs were bioequivalent to BAU/ml and the original AU/ml nomenclature was maintained. Although US allergen extracts are standardized in BAUs based on skin testing, information on major allergen content can be obtained from some extract manufacturers. Of note, Bermuda grass was assigned the potency of 10,000 BAUs based on $ID_{50}$EAL testing, which is tenfold less than the other standardized grasses. However, the major allergen content of Bermuda, according to the Certificate of Analysis form from Alk-Abello, Inc. (TABLE 2), was 348 µg/ml of *Cynodon dactylon* (Cyn d) 1 with a range of 141 to 422 µg/ml. This is similar to the major allergen content of the other standardized grasses. Examples of major allergen content in commercial allergen extracts are listed in TABLE 3 [22].

It has been speculated that the apparent discrepancy in assigned potency to Bermuda extract was the result of standardization ($ID_{50}$EAL testing) undertaken in a nonendemic area for Bermuda grass. The presumption with this explanation is that the test subjects may not have been highly allergic, which is the requirement for the $ID_{50}$EAL method. This highlights one of the potential problems with utilizing an *in vivo* test such as the titrated skin test to define extract potency.

### Allergen extract standardization in Europe

The Nordic skin test method is one of the assays used in Europe to determine extract potency. It is similar to the $ID_{50}$EAL method as it employs the results of titrated skin tests in an allergic population to establish a reference. The two methods differ in testing techniques and the end points that are evaluated. The Nordic method utilizes the prick/percutaneous technique and compares the wheal size with a histamine dose–response curve, whereas the $ID_{50}$EAL method utilizes the intradermal technique and measures the erythema response.

Allergen standardization is regulated by the different member states in Europe and differs from the USA since manufacturers can use in-house references (IHRs) to standardize their extracts. Each manufacturer has their own IHRs based on their own standardization methods [23]. The process is similar to the US method described previously. The potency of the IHR is determined by serial skin testing of allergic individuals or a positive control such as histamine. Subsequently, manufactured extracts are compared with the IHR through an *in vitro* assay such as the ELISA. As stated previously, this will provide information about overall extract allergenicity but not about the individual components of the extracts. The IHR standardization method provides for lot-to-lot consistency within a company but does not permit comparison of extracts from different companies. There are a number of different terms for these IHR units, and TABLE 4 is a summary of these IHRs and their equivalence in major allergen content if this information was available [24].

Table 2. Example of a Certificate of Analysis for a US standardized extract required by the Center for Biologics Evaluation and Research.

| Analysis | Result | Specification acceptance criteria | Method |
|---|---|---|---|
| *Required testing* | | | |
| Immunoglobulin E binding | 0.940 RP | 0.699–1.431 relative potency (n = 3) | Competition ELISA |
| Preservative | 0.41% | 0.36–0.48% phenol | High-performance liquid chromatography |
| Stabilizer | 51.4% | 50–55% glycerin | Trimetric |
| Protein | 5.21 mg/ml | To be measured | Ninhydrin |
| pH | 7.6 | 6.0–8.5 | Potentiometric |
| Toxicity | Pass | To pass test | 21 CFR 610.11 |
| Sterility | Pass | To pass test | USP |
| *Additional ALK-Abello, Inc. quality assurance testing* | | | |
| Major allergen-binding ELISA | 348 µg/ml | 141–422 µg/ml Cyn d 1 | Direct |
| Protein profile | Pass identity | To pass test or pattern similar to reference pattern, not substantially | Sodium dodecyl sulfate polyacrylamide gel electrophoresis different from reference |
| Proteolytic activity digestion | Negative | For information only | Casein |

The certificate provides additional information, including major allergen content, which is not a requirement for US standardized extracts [52].

Certificate of analysis: ALK-Abello, Inc. Allergenic Extract: Std. Bermuda grass. Lot number: 4M00012. Scientific Name: *Cynodon dactylon*. Product Type (labeled as): 10,000 BAU/ml.

BAU: Bioequivalent allergy unit; CFR: Code of federal regulations; ELISA: Enzyme-linked immunosorbent assay; USP: Ultraspiracle.

## Allergen extract standardization: looking towards the future

### *Recombinant allergens*

Most allergen sources contain major and minor allergens and considerable difficulties exist in attempting to standardize this mixture of proteins. The current allergen-standardization protocols that measure potency based on reactivity with pooled allergic sera do not reveal differences in extract composition or quantify the major allergen content. Consequently, extracts with similar potency based on inhibition assays may have completely different compositions. The major allergens for most inhalant allergens have been identified, and recombinant forms of the allergen are available for many major allergens. The key advantages of recombinant allergens are that they can be consistently produced in high purity and quantity with defined

Table 3. Major allergen content of US standardized extracts [24].

| Allergen extract (number of extracts tested) | Expressed concentration | Major allergen | Mean content of major allergen | SD | Max. content of major allergen | Min. content of major allergen |
|---|---|---|---|---|---|---|
| Timothy (n = 12) | 100,000 BAU/ml | Phl p 5 | 743 µg/ml | ±294 | 1336 µg | 354 g |
| Short ragweed (n = 13) | 1:10 wt/vol | Amb a 1 | 268 µg/ml | ±109 | 458 µg | 87 µg |
| Mixed ragweed (n = 10) | 1:10 wt/vol | Amb a 1 | 174 µg/ml | ±96 | 402 µg | 56 µg |
| Dermatophagoides pteronyssinus (n = 28) | 10,000 AU/ml | Der p 1 | 172 µg/ml | ±74 | 385 µg | 68 µg |
| Dermatophagoides farinae (n = 18) | 10,000 AU/ml | Der f 1 | 44 µg/ml | ±12 | 72 µg | 30 µg |
| Cat hair (n = 12) | 10,000 BAU/ml | Fel d 1 | 40 µg/ml | ±7.2 | 52 µg | 26 µg |
| Dog hair (n = 4) | 1:10 wt/vol | Can f 1 | 5.4 µg/ml | ±2.7 | 7.2 µg | 2.7 µg |

*Values were provided by ALK-Abello, Inc, Wallingford, CT, USA.

Sources of extracts are the US Food and Drug Administration, ALK-Abello and other extract laboratories.

BAU: Bioequivalent allergy unit; SD: Standard deviation; wt/vol: Weight of source material extracted with a given extracting volume.

Table 4. Example of European allergen extract unitage and basis for in-house standardization.

| Extract manufacturer | Unitage/standardization technique | Estimated potency based on major allergen content |
|---|---|---|
| Stallergenes, France | Skin prick testing on 30 allergic patients with three tenfold dilutions<br>100 IR = concentration that elicts a wheal of 38.5 mm$^2$ or 7 mm in diameter | Major allergen content in 100 IR<br>Dust mite (2004)<br>2.7 Der p 1,<br>3.1 µg Der f 1<br>Parietaria: 70 µg Par j 1<br>Olive: 1998: 13.5 µg Ole e 1<br>Ragweed (2004) 116 µg Amb a 1<br>Timothy (1999) 8.5 µg Phl p 5<br>Lolium (2002) 3.92 µg Lol p 1 |
| ALK-Abelló (Neo-Abellò), Italy | 100 BU = SPT on 30 patients sensitized to the allergen under consideration produces a mean wheal of 70 mm$^2$<br>Old studies refer to STU<br>Mites: 100 STU = 1 BU<br>Pollen: 40 STU = 1 BU | Parietaria (1994) 10 BU = 0.6 µg of Par J 1<br>Mugword (2003) 10 BU = 18 µg Art v 1<br>Birch (2001) 10 BU = 6.6 µg of Bet v 1<br>Olive (2003) 10 BU = 15 µg Ole e 1<br>Mites (2003) 1000 STU = 10BU 4 µg Der p 1. 2 µg Der p 2<br>Alternaria (2003) 10 BU = 2.5 Vg Alt a 1 |

Other allergen extract unitage: Allergopharma, Germany, 1000 SBU (1997) = 0.54 µg of Der p 1; Leti-CBF, Spain, 1 HEP equivalent/ml 0.25 mcg/ml Fel d 1; Information provided by Désirée Larenas, MD (26), mites: 100 STU = 1 BU, but for polen: 40 STU = 1 BU.

BU: Biologic Unit; IR: Index of reactivity; SBU: Standardized biologic unit; STU: Standard treatment unit.

concentrations of protein content [25]. In comparison, natural allergenic extracts can have a variable content of specific allergens even if they have been standardized. Recombinant allergens are comparable with their natural counterparts in IgE antibody-binding capacity and show excellent reactivity on skin testing and in vitro diagnostic testing.

Recombinant allergens may enhance allergen immunotherapy safety and efficacy through engineered hypoallergens that reduce binding to IgE but retain T-cell epitopes.

Several studies utilizing recombinant Birch pollen allergen have demonstrated reduced allergenicity while maintaining immunomodulatory capacity [26,27].

Recombinant allergens have also been utilized in efforts to standardize *Alternaria alternaria* [29–31].

In a study of 43 patients allergic to *A. alternaria*, no significant difference was found in the IgE binding between natural and recombinant Alt a 1 in immunoblotting and inhibition assays or skin prick testing [31].

Recently, the EU has established a multidisciplinary consortium of extract manufacturers, biotech companies, regulatory bodies and clinicians to form a partnership aimed at promoting standardization of allergenic products. The EU consortium is referred to as the acronym CREATE (the full title is Development Of Certified Reference Materials For Allergenic Products And The Validation Of Methods For Their Quantification) [23,32]. CREATE recognizes that standardization based on mass-units of major allergens requires certified references and, therefore, the main objectives of CREATE are [23]:

- To develop candidate-certified reference material consisting of purified natural or recombinant major allergens
- The validation of existing sandwich ELISA for measurement of major allergens

CREATE is currently investigating the following recombinant allergens: Birch (Bet v 1), grass pollen (Phl p 1 and Phl p 5) olive pollen (Ole e 1) and house dust mite (group 1 and 2 allergens).

CREATE was a 3-year project that began in March of 2002 and ended in February of 2005. The first phase entailed collection of natural and recombinant allergens and serum samples of well-documented allergic patients. In the second phase, a detailed comparison of the natural and recombinant allergens with respect to their immunoreactivity and physical and chemical properties was performed. Natural and recombinant allergens were purified to homogeneity and analyzed for structural composition, antigenic and allergic activity with amino acid analysis, circular dichroism (CD) spectrum and mass spectrometry, monoclonal ELISA, immunoblotting and *in vitro* assays [33].

The first phase was successfully completed. Sufficient quantities of the natural and recombinant allergens were produced to perform the detailed composition analysis in the second phase of the project. At present, the data from the second phase are being collected. The CREATE project anticipates that these allergen reagents will be used as reference materials for standardization of allergen extracts in mass units [23].

## Expert commentary & five-year view

Allergen immunotherapy is an effective immunomodulatory treatment for hymenoptera sting anaphylaxis, allergic asthma and rhinitis. Its efficacy is highly dependent on the quality and composition of the administered allergen extracts.

The ideal allergen would be of known composition in terms of the allergenic proteins and be devoid of unwanted material. It should also be customized based on the allergic individual's IgE-binding profile. Obstacles to producing such an ideal allergen extract are manifold and include allergen-composition heterogeneity, variability in the allergic population's reactivity to allergens and the absence of an ideal assay and reference to measure and compare allergen extract potency.

Allergen extracts standardized based on *in vivo* tests, such as the titrated prick skin test, do not provide information about the specific composition of the extract. Also, allergens standardized through measurement of major allergen content potentially convey little or no information about the other allergenic proteins in the extract.

Fortunately, major allergen content appears to correlate with biologic potency and it is the basis of the EU's CREATE project to standardize allergen extracts. At present, this large, collaborative, multicountry, multidisiciplinary project is probably the most promising effort at achieving widescale allergen extract standardization. Development of certified reference materials consisting of natural or recombinant major allergens and validating ELISAs to measure allergen content are the major objectives of CREATE. The advantage of recombinant allergens is that they can be consistently produced in high quantity and consistency. In addition to serving as a reference for allergen extract standardization, recombinant allergens may play a significant role in improving immunotherapy treatment with modifications that decrease the extract's allergenicity while maintaining its immunomodulatory effect.

Despite more than 20 years of efforts directed towards standardizing allergen extracts, considerably more nonstandardized allergen extracts than standardized extracts exist on the commercial market, and it is likely there will be no significant change in the near future. This may be the rationale for investigating other means to enhance the safety and efficacy of allergen immunotherapy extracts. Modified allergens (T-cell epitope peptides) and adjuvants (CpG motifs) show some promise in fulfilling both goals, however, there have only been a few allergens investigated with these methods to date [34–38]. In addition, the technical requirements and related costs of producing these modified allergen extracts is unknown, however, they are likely to be relatively high. This could potentially limit the widespread use of these novel allergen extract formulations in the near future.

## Key issues

- Allergen extract quality affects both accuracy of allergy diagnosis and effectiveness of allergen immunotherapy.
- Allergen extracts are currently derived from natural sources (pollen) or cultures (dust mite and fungi) and the specific composition of extracts can vary considerably from lot to lot.
- Extract variability can be affected by culture medium conditions and the manufacturer's extraction methods.
- Pollen and mites have greater lot-to-lot consistency than fungi. Fungi have a high degree of somatic mutation and are known to change with variations in the culture medium nutritional composition.
- Most allergen sources contain multiple major and minor allergens. When combined with the individuality of an allergic individual's recognition of these allergens, standardization of these mixtures becomes difficult.
- Allergens in a source recognized by more than 50% of allergic individuals are referred to as major allergens.
- Some allergens that are considered minor on a population basis since they are recognized by less than 50% of allergic individuals may be very clinically significant to some individuals.
- The ideal allergen extract would be of a known composition, devoid of unwanted material and customized to the allergic individual's immunoglobulin E binding profile.
- Obstacles to producing the ideal allergen extract are manifold and include allergen composition heterogeneity, variability in the allergic population's reactivity to allergens, and the absence of a single assay that measures both extract composition and functional properties.
- Current extract standardization in the USA is based on skin testing of a reference population (intradermal dilution for 50 mm sum of erythema method) and does not confer information about the particular composition of the extract (i.e., major allergen content).
- European extract standardization has generally been based on in-house references that are often based on skin testing of a reference population.
- Recently, the European Union established CREATE (Development of Certified Reference Materials for Allergenic Products and the Validation of Methods for their Quantification), a multidisciplinary, multicountry program to promote standardization of allergenic products.
- Major allergen content appears to correlate with biologic potency and many are available as recombinant proteins.
- The main objectives of CREATE are to evaluate recombinant major allergens as candidate-certified reference materials for allergen extract standardization and to validate sandwich enzyme-linked immunosorbent assays for their quantification.
- Recombinant allergens can be consistently produced in high purity and quantity with defined compositions. They also show promise as references for allergen extract standardization and safer immunotherapy vaccines.

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