

Allergens

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Molecular characterization and standardization of allergenic extracts represent a major improvement in allergy testing with immunotherapy. Patients are benefiting from therapy with optimal doses of selected major allergens along with additional safety in the administration of extracts. Improvements in manufacturing and quality control of several important extracts should allow better patient treatment in the future. Increased knowledge about T-cell epitopes on extracts may soon lead to the design of new extracts for epitope-specific immunotherapy.

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Allergens that cause IgE-mediated allergy are defined as molecules that specifically bind IgE in at least one serum from a group of humans with clinical symptoms of allergy toward the specific allergen source. All antigenic components may be regarded as potential allergens. However, all allergens do not have the same allergenicity. Regular exposure of experimental animals to antigen leads to increased titers of IgE and tolerance, not to a persistent allergic state. What stimulates the allergenicity is not known; perhaps a combination of the physical and chemical composition of the extract, genetic factors, the time and degree of contact, and the route of immunization.

In general, potential allergens are proteins extracted by aqueous solution at physiologic pH and ionic strength. Naturally occurring allergens are proteins or glycoproteins with molecular weights (MW) between approximately 5000 and 100,000 D. A 70,000-D MW appears to be the upper limit for airborne allergens. Immunochemical studies have shown that most allergen sources contain multiple protein allergens; however, often it is possible to define one or two "major" allergens that elicit specific IgE antibody responses in most patients. Aukrust and Aas [1] and Aukrust [2] define a major allergen as one that shows some specific IgE binding in more than 50% of the population allergic to the allergen in question and strong IgE binding in at least 25%. Minor allergens are those that show IgE binding in less than 10% of the patients.

Preparation of allergens

Most extractable proteins are not allergens. In the United States most extracts are labeled in terms of weight per volume, *eg*, 1:20 weight per volume as pro-

posed by Noon [3] or protein nitrogen units as devised by Cooke and Stull [4]. Weight per volume refers to the initial weight of source material extracted in a given volume of extracting fluid. Protein nitrogen units are a measure of phosphotungstic acid-precipitable protein present in the extract. However, the biologic activity or quality of the extract cannot be estimated on such characterization because the mere weight of raw material and resulting protein content may not correlate with allergenic potency. In addition, freezing and thawing, prolonged extraction, and differences in filtration may increase the amount of nonallergenic proteins and thus dilute the extract.

The composition of an allergen extract depends on several factors, including the source material, the nature and duration of the extraction process, and the storage and handling of intermediate products. The composition is also influenced by purification and various manufacturing processes. To ensure consistency among manufacturers, instructions for selection of raw materials and production of allergen extracts are given by the Center for Biologics Evaluation and Research (CBER) and the International Union of Immunologic Societies/World Health Organization [5], in the Nordic Guidelines [6], and by the European Community Commission.

Identification and characterization of allergens

The ideal allergen extract has been described as one "that contains all the potential allergens in their native form, in the proper ratio, and with all irrelevant material removed." Allergen characterization is the first step in reaching this goal. The purpose of allergen char-

Abbreviations

AU—allergy units; BAU—bioequivalent allergy units; CBER—Center for Biologics Evaluation and Research; IS—international standards.

acterization is to identify components in the extracts that act as allergens. When identified, they are used as markers for optimization procedures and quality control in the manufacturing. Extracts are characterized by microscopic analysis, gel electrophoresis (sodium dodecylsulfate polyacrylamide gel electrophoresis [SDS-PAGE], Western blot, immunoelectrophoresis), inhibition immunoassay, protein concentration, and carbohydrate content. Furthermore, to characterize the functional ability of allergens, in vitro cellular assays such as histamine release have been used [7,8^{••},9]. Molecular characterization of allergens is becoming increasingly common. Complementary DNA cloning techniques have proved to be potent tools. By using serum IgE from individuals allergic to various pollen, domestic cats, dust mites, venoms, or other proteins, the complementary DNA encoding for the major allergens has been isolated and expressed in complementary DNA libraries [10–16]. The allergens are then expressed in *Escherichia coli* as recombinant nonfusion proteins and shown to be comparable in IgE-binding capacity to naturally derived allergens on immunoblots. Thus, the primary structures of most major allergens are known and can be used to determine the molecular structures recognized by antibodies and T cells. The rationale for using recombinant allergens for allergy diagnosis and immunotherapy is that expression systems can be used to produce one or two highly purified and homogeneous allergens, in essentially unlimited supply. Then these reagents can be used for diagnosis by skin testing or serologic assays. Thus, the diagnosis and immunotherapy would be based on one or two allergens, rather than a heterogeneous protein mixture. If this approach has clinical merit, it needs to be further documented because it relies on the fact that recombinant allergens have comparable immunologic reactivity to the natural protein. One way to examine this is by showing comparable biologic activity in in vivo or in vitro assays, eg, basophil histamine release assays. Valenta *et al.* [8^{••}], who produced recombinant birch allergens (*Bet v* I, *Bet v* II), found the recombinants to be as biologically active as the native allergens. However, similar studies with recombinant mite group I and *Fel d* I allergens showed that in *E. coli* they were not expressed in an immunoreactive form when compared with the native allergen. Thus, although these molecules can be produced, they do not necessarily react with IgE antibodies.

Standardization

Standardization refers to the methods and procedures used, both to select a “standard” or reference extract and to quantitatively compare a test extract with respect to the standard. Standardization of extracts used in immunotherapy and diagnosis of allergy is required to improve diagnosis, the precision of results, and the safety of immunotherapy. It allows comparisons of di-

agnostic procedures, individual in vivo response, and population studies [17[•]]. In the current US environment, lot-to-lot potency variation of a given allergen even from a single manufacturer may be considerable. By standardizing extracts these variations can be kept to a minimum. A standardized extract is one that has been tested and approved first by the manufacturer and then by the US Food and Drug Administration's CBER; finally the extract is assigned a potency in allergy units (AU) based on the in vitro potency of that extract relative to an approved reference preparation [18,19]. Some extracts are further biologically standardized, ie, labeled with a unit describing potency [20]. The biologically relevant unit is standardized based on skin test activity (skin prick test in Europe and intradermal testing in the United States).

Efforts by the International Union of Immunological Societies are directed toward international standards (IS) and establishment of reference preparations. Several IS extracts are available for regulatory authorities, researchers, and allergen manufacturers to establish in-house reference preparations. The IS extracts comprise *Dermatophagoides pteronyssinus* mite, *Dermatophagoides farinae* mite, cat and dog hair or dander, short ragweed pollen, timothy grass pollen, and birch pollen. Soon Bermuda grass pollen, rye grass pollen, and *Alternaria alternata* will follow. These standards have assigned potency in IU. This is an arbitrary unit and the IS extracts are only recommended for use as calibrators (standards for measurement of relative potency), not as prototypes (materials to which an extract is to be matched in all respects).

Currently available standardized pollen extracts include birch, Kentucky bluegrass, timothy, redtop, sweet vernal, meadow fescue, perennial rye and orchard grass, Russian thistle, plantain, giant ragweed, and short ragweed. These standardized extracts are available in a freeze-dried form and in 50% glycerin, and were standardized against a reference extract that was arbitrarily assigned 100,000 AU. Currently, many of these extracts are being tested by ID₅₀EAL technique [20] to determine the AU based on bioequivalent allergy units (BAU). In the United States this reference method is based on studies by Turkeltaub *et al.* [20]. Highly sensitized individuals receive several intradermal injections of dilutions (threefold increment) of the allergen extract and the flare is recorded. Both the reference and tested extract are injected. Results of the flare are compared by parallel line bioassay. The mean intradermal dilution required to produce a 50-mm sum of erythema in the allergic population tested is calculated (ID₅₀EAL method). Next, the reference and standard extracts are assigned BAU according to the mean dilution producing a 50-mm sum of erythema (Table 1). This technique is very sensitive but time-consuming. Results are given in BAU. There has been a recent change in labeling in the United States. Extracts labeled in AU/mL now refer to products that are standardized using nonbiologic methods (arbitrary units), and ex-

tracts labeled in BAU/mL are standardized using quantitative skin testing. The AU/mL and BAU/mL are not interchangeable. For example, in 1992 the CBER completed an evaluation of the potency of various commercial cat extracts using the ID₅₀EAL procedure.

Table 1. The assignment of bioequivalent allergy units

Mean dilution required to produce 50-mm sum of erythema	Allergy units per mL of standard, BAU/mL
5.0 to 6.9	10
7.0 to 8.9	100
9.0 to 10.9	1000
11.0 to 12.9	10,000
13.0 to 14.9	100,000
BAU—bioequivalent allergy units.	

Results were not consistent with the arbitrary AU/mL values that had been originally assigned to this product. ID₅₀EAL testing showed that standardized cat extracts containing 10 to 19.9 *Fel d* I units/mL should be labeled at 10,000 AU/mL rather than at 100,000 AU/mL as originally assigned, and that standardized cat extracts containing 5 to 9.9 *Fel d* I units/mL should be labeled 5000 AU/mL rather than at 50,000 AU/mL. This discrepancy between potency as determined by skin testing and the previously assigned potency units posed an obstacle against having a system that would predict biologic response. CBER decided, therefore, to change the potency labeling from AU to BAU. Future CBER standards are oak, birch, elm, box elder, English plantain, Russian thistle, and giant ragweed.

The Nordic Council has recommended the histamine equivalent potency in their guidelines for registration of allergen preparations. The method to establish bioequivalency is different from the US method in several important ways (Table 2). Besides skin testing [21•], the Nordic Council's guidelines require manufacturers to compare batches with a reference preparation for allergen composition (crossed radioimmunoelectrophoresis), protein composition (isoelectrophoresis and PAGE), and total allergenic activity (inhibition immunoassay).

Table 2. Comparison of the United States' and Nordic Council's bioequivalency protocols

Method	United States protocol	Nordic Council protocol
Skin test activity	Intradermal	Prick test
Dosage increment	Threefold	10-fold
Number of patients	15	20
Patient selection	Highly sensitive	None
Recording	Erythema	Wheal
Histamine reference	No	Yes
Calculation	Means	Geometric means
Units	Bioequivalent allergy unit	Biologic unit

It is important to realize that IS units are completely independent from biologic units, *eg*, the ratio between biologic units and IU ranges from 1 (dog) to 500 (timothy pollen). Biologic units cannot be converted yet. A comparison between biologic units and AU gave a ratio of 0.5 (dog) to 50 (timothy pollen).

Allergen extracts

Pollen allergens

Almost all pollens have potential for inducing allergy. Air sampling and skin tests have shown that there is a correlation among pollen counts, the number of sensitized subjects, and skin response. Thus, with appropriate extracts and careful interpretation it is possible to estimate which pollen is most important in any region.

Grasses

The allergens from temperate grasses may be divided into at least five groups with different physiochemical characteristics. Groups I and V are dominant. Group I allergens are already analyzed in detail and some of them are even cloned and sequenced (*Lol p* I, *Poa p* IX) [14,22].

Monoclonal antibodies provide a well-standardized tool to analyze the molecular basis of cross-reactivity between plant proteins and glycoproteins. Recently, monoclonal antibodies against group IV and V were produced and showed that these two major allergens are widespread in temperate grasses (*eg*, *poa pratensis*, orchard, plantain) [23•]. Pollen allergens from different grass species are immunochemically similar when assayed by radioallergosorbent test inhibition experiments, immunoelectrophoretic methods, and techniques including monoclonal antibody analysis.

The CBER reference preparations for eight grass pollen extracts (Bermuda, June, meadow fescue, orchard, perennial rye, redtop, sweet verbal, and timothy) have been completed. These extracts are currently being labeled in BAU/mL; soon the nonstandardized extracts will no longer be available.

Weeds

The first standardized extract in the United States (1970) was short ragweed. Today, the major ragweed allergens, *Amb a* I (antigen E), *Amb a* II, and *Amb a* V are now cloned and sequenced [11–13]. All short ragweed extracts are currently standardized based on *Amb a* I content measured by radial immunodiffusion assay. Some manufacturers are licensed to label these extracts in AU/mL, whereas others label their products as weight per volume extracts. These products will all soon be converted to BAU/mL.

Trees

In birch pollenosis, two important allergens have been cloned and sequenced: *Bet v I* and *Bet v II* (profyllin) [8•]. Substantial cross-reactivity between profyllin in birch and hazelnut has been substantiated [24]. Recently, *Aln g I*, the major allergen in pollen of alder, has been cloned and expressed. It was shown to share epitopes with *Bet v I* [25•]. A standardized tree pollen extract is not available in the United States yet.

Mammalian allergens

Previously, mammalian allergens were often contaminated and impure, resulting in false-positive test results. Now, a number of mammalian allergens have been thoroughly described and cloned [26•].

Cat (*Felis domesticus*)

Clinically, the most important cat allergen (*Fel d I*, 17,000- to 18,000-D MW in SDS-PAGE) is found in saliva and extracts of cat hair, dander, and pelt [27]. It is a glycoprotein, and deglycosylation abolishes more than 50% of the allergenic activity as measured by radioallergosorbent test inhibition. The full-length amino acid sequence has been obtained from the native protein and has been deduced from complementary DNA experiments [15]. Murine monoclonal antibodies have been developed to different epitopes on native *Fel d I*. Such antibodies have been applied to determine the level of environmental exposure to *Fel d I* [28]. In addition, at least six other allergens have been identified originating from serum, urine, or saliva. They do not fulfill the criteria of major allergen. One such allergen is serum albumin, which might be responsible for some cross-reactivity between cat and dog. It has been shown to account for 10% to 25% of the allergenic activity of hair and dander extracts.

In the United States all nonstandardized cat extracts have been removed from the market. In addition, all cat extracts are designated as either "cat hair extract" or "cat pelt extract." Both extracts are standardized based on the allergen *Fel d I* as measured by radial immunodiffusion assay. Cat hair extracts are manufactured to contain small amounts of non-*Fel d I* cat allergens, whereas cat pelt extracts do contain significant amounts of non-*Fel d I* allergens.

Dog (*Canis familiaris*)

Allergy to dogs has been shown in 14% of unselected adolescents and in 40% of children suffering from asthma. Dog allergen is present in quite high concentrations even where dogs are not present, eg, in schools [9]. Clinically, the most important allergen is *Can f I* (25,000-D MW in SDS-PAGE), and it is found at high concentrations in hair or dander and saliva. The allergen counts for at least 50% of the allergenic activity in dog hair and dander extract. In 92% of the dog-allergic patients, the allergen elicited a positive prick test result.

There is evidence that all breeds of dogs are not equally allergenic; however, these differences may be quantitative rather than qualitative, some breeds having more of some allergens than others. An IS extract is available.

Mouse (*Mus musculus*)

The major mouse allergen is *Mus m I*, which is a polypeptide with little or no glycosylation (17,000- to 21,000-D MW). The origin is liver cells and it is secreted in urine. The amino acid sequence of *Mus m I* has not been reported yet. An additional important allergen, *Mus m II*, has been identified and is found in hair follicles.

Rat (*Rattus norvegicus*)

Rat n IA (prealbumin) and *Rat n IB* (α -2-eu-globulin) are two major allergens found in urine, fur, and saliva. They show some cross-reactivity. In addition, serum albumin may also possess some allergenicity as shown by histamine release in 24% of the rat-allergic patients tested [29].

Horse (*Equus caballus*)

Three important allergens have been identified in horse hair and dander: *Equ c I*, *Equ c II*, and *Equ c III*. The amino acid sequence has been determined.

Mites

All mite extracts of the species *D. farinae* and *D. pteronyssinus* are standardized based on comparisons by radioallergosorbent test or enzyme-linked immunosorbent assay to CBER standards. They are all labeled at 10,000 AU/mL as evaluated by ID₅₀EAL testing. These products are currently being converted to BAU/mL.

Several of the most important mite allergens are cloned and sequenced. Mite allergens (*D. farinae* [*Der f*] and *D. pteronyssinus* [*Der p*]) are divided into groups I through VII [30•]. The group I mite allergens, which react with IgE in most mite-allergic patients (80% to 100%) and often account for 50% or more of the antimitic specificities, are cysteine proteases [30•]. *Der p I* is a cysteine protease homologous to papain. The group I allergen is found predominantly in feces. Evidence is accumulating that the group II allergens (*Der p II*) with IgE-binding frequency similar to *Der p I* (80% to 100%) are lysozymes. The group II is more abundant in mite bodies. The group III allergens (*Der p III*) are probably trypsin and *Der p IV* amylase. The biologic function of *Der p V* and *Der p VII* is presently not known. *Der p VI* is chymotrypsin. The groups IV to VII allergens show considerably less degree of reactivity (40%) than to *Der p I*.

Molds

Few if any well-standardized fungal allergens are currently available to the practitioner. The quality of fun-

gal extracts is generally poor. It has been shown that the incidence of positive skin tests with *Cladosporium herbarum* varied from 12% to 65% depending on the commercial extract used [31]. The same strains of a particular species grown under identical culture conditions over similar intervals can produce extracts with great variability. Additionally, fungal allergens are often rich in glycoproteins, which may be highly allergenic but difficult to show and identify.

Variability of mold extracts due to interstrain differences, culture conditions, and different methods for extracting mycelial mats and spores have resulted in major obstacles in identifying major mold allergens. Only a few molds have been studied in detail. *Alternaria* produces approximately 10 allergens. One major allergen (*Alt a* I) has been isolated, purified, and sequenced. Recently, several studies have identified similar major proteins (*Alt a* 29, *Alt a* Bd 29K) with approximately the same characteristics [32•]. It appears that they are probably the same protein and the variations of the individual antigens are within the errors of the methods or caused by strain-related differences.

The allergens of *Cladosporium* have been studied and show to number more than 60. Only four may be important. However, much variation is found when compared with a reference extract. It is still debated whether spores or mycelium are the major cause of allergy.

The following practical recommendations are given about the clinical use of molds [33••]. Limit the use of mold mixtures and always keep allergen materials cold to avoid degradation by fungal enzymes. Use dialyzed or defatted fungal allergen materials for immunotherapy and accomplish dialysis without the use of cellulose-based membranes. Generally, limit the use of mold immunotherapy to patients with symptoms that cannot be controlled by avoidance and pharmacotherapy and who show documented symptoms (rhinitis or asthma) after natural exposure during periods when high concentrations of fungal allergens are present. A standardized *Alternaria* extract will soon be available in the United States.

Venoms

Vespid (*Dol m* V) is available in recombinant form. All currently licensed venoms are standardized based on hyaluronidase activity and are labeled in micrograms of venom protein per mL. There are no plans to change to a standardization procedure incorporating BAU.

Conclusions

The advantage of using recombinant allergens is in the use of allergen extracts containing a few selected recombinant allergens. The extract does not contain nonallergenic components and allows immunotherapy

with known amounts of allergens. This approach still requires further investigation to show that diagnosis and immunotherapy with selected recombinant allergens is as effective as using the native whole extract. For diagnosis it will probably remain necessary to use a wide range of allergens. It is known that a great diversity of components in allergen extracts is recognized by IgE antibodies and it is still unclear as to what extent recombinant allergens should be in extracts used for diagnosis.

Currently, validating standards by collecting serum pools is a very large task for companies and government agencies. The advances in sequencing and cloning of allergens should lead to new highly purified standards. Monoclonal antibodies directed against certain epitopes on these purified allergens may be ideal test reagents to replace the current serum pools. Other potentially useful assay systems are T-cell proliferation assays or histamine-release assays.

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