

18 | Manufacturing and Standardizing Allergen Extracts in Europe

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INTRODUCTION

History of Standardization in Europe

Specific allergy treatment, i.e., specific immunotherapy or specific allergy vaccination, has been performed for almost a century, since it was first described by Noon in 1911 (1). The discovery in 1966 of the IgE molecule (2), and the central role of IgE in allergy facilitated a better understanding of the immunological mechanisms, led to an improvement of diagnostic tools, and consolidated the concept of specific allergy diagnosis and treatment. Scientific methods were introduced to standardize allergen extracts in the seventies and eighties (3) and, in combination with gradual improvement of the clinical procedures, established specific allergy treatment as a scientifically based, reproducible, and safe treatment for allergic diseases.

The first international initiative on allergen standardization was based on the Danish Allergen Standardization 1976 program (4) and was published as the Nordic Guidelines in 1989 (5). These guidelines established the first regulatory demands for allergen extracts. The guidelines introduced the biological unit (BU), based on skin testing, for potency measures. Each manufacturer was instructed to produce an in-house reference preparation (IHRP), adjust the potency in BU, and use the IHRP for batch-to-batch control using scientifically based laboratory testing. The significance of using the major allergen content for the biological activity was recognized in the early nineties and is now established in the WHO recommendations (6) and in the European pharmacopoeia (7). This chapter describes important issues in the control of source materials and in the preparation of extracts as part of the standardization process the way it is performed in Europe. Procedures differ from those used in the United States, as does the selection of extracts for vaccination in common allergy practice (see chap. 17).

Standardization of Allergen Extracts

Allergen extracts/vaccines are used for specific diagnosis and treatment of allergic diseases and indirectly for the detection of environmental allergens. Allergen extracts are aqueous solutions of allergenic source materials, such as pollen, animal hair and dander, dust-mite bodies or cultures, insect venoms, or mold mycelia and spore particles. Since no structural feature defining an allergen has hitherto been described, the definition of an allergen is based on the functional criterion of being able to elicit an IgE response in susceptible individuals. All allergens are proteins and they are readily soluble in water. Airborne allergens are carried by particles in the micrometer range, a characteristic that is compatible with the concept that the particle carrying the allergen is inhaled, and the allergen is deposited on the mucosal surface of the lower airways, thereby stimulating the immune system. The allergen is thus defined by the immune system of the individual patient.

By this definition, any immunogenic protein (antigen) has allergenic potential, even though most allergic patients have IgE specific for a relatively limited number of "major" allergens. Analysis of a larger number of patients leads to the identification of still more

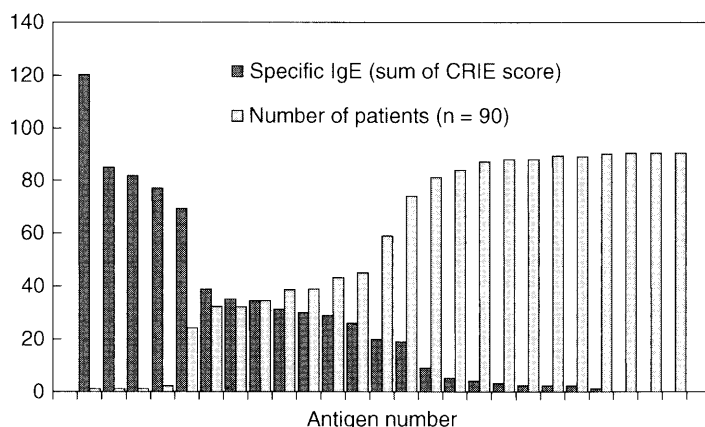


Figure 1 Complexity of patients' responses to allergen extracts. Serum samples from 90 grass-allergic patients were analyzed by crossed radio-immunoelectrophoresis (CRIE) using timothy, *Phleum pratense*, allergen extract. Labeled precipitates were assigned an arbitrary score for each patient dependent on the staining intensity of the autoradiogram. In this way, a graduated score for the specific IgE reactivity of each individual patient with each individual allergen was obtained. The scores were summed for each allergen and the antigens were arranged in ascending order and depicted on the ordinate axis. The score is depicted in the dark columns. The light column represents the cumulative number of patients having all their IgE specificities covered by the antigen in question and all other antigens to the left of the antigen in question.

Examples: A hypothetical extract containing the six most important allergens will cover all IgE specificities for 32 of the patients. Twenty-two allergens are needed to cover all IgE specificities of all 90 patients.

IgE-binding proteins (Fig. 1). Thus, the number of allergens in a given source material converge toward the total number of antigens, and any antigen has the potential to elicit an IgE response.

A major objective in the manufacture of allergen extracts, therefore, is to secure an adequate complexity reflecting the composition of water-soluble components of the allergenic source material. Another important matter of batch-to-batch control is standardization of potency, i.e., the overall IgE-binding capacity, which is a reflection of the anaphylactic potential of the preparation. The third important aspect of allergen extract manufacturing is controlling the major allergen content. The major allergens have distinct importance for the activity of allergen extracts/vaccines in diagnosis as well as treatment.

All aspects of the manufacturing procedure from selection and collection of raw materials, extract preparation and storage, to validation of assays and reagents, have impact on extract quality and are considered part of the standardization procedure.

PREPARATION OF ALLERGEN EXTRACTS

Source Materials

Inhalant allergens are present in airborne particles derived from natural allergen sources. The particles are inhaled and constitute the material to which humans are exposed. The most important allergen sources are found among the particles most frequently inhaled. Table 1 lists the most important allergen extracts in Europe and the United States.

The aim of selecting raw materials for allergen extract production is to gather materials containing the same active allergens in a manageable form. In most cases, the optimal source material is rather obvious, but in some cases, the allergen source is still debated, i.e., cat saliva/pelt/hair and dander or mouse urine/hair and dander. The source materials should be selected with attention to the need for specificity and for inclusion of all relevant allergens in sufficient amounts (8). The collection of the source materials should be performed by qualified personnel, and reasonable measures must be employed by the producer of allergen extracts to

Table 1 Most Important Allergen Extracts^a

	Europe		North America
Temperate grasses	<i>Lolium perenne</i> <i>Phleum pratense</i> <i>Poa pratensis</i> <i>Festuca pratensis</i> <i>Dactylis glomerata</i> <i>Secale cereale</i>	House-dust mites	<i>Dermatophagoides pteronyssinus</i> <i>Dermatophagoides farinae</i>
House-dust mites	<i>Dermatophagoides pteronyssinus</i> <i>Dermatophagoides farinae</i>	Temperate and subtropical grasses	<i>Lolium perenne</i> <i>Phleum pratense</i> <i>Poa pratensis</i> <i>Festuca pratensis</i> <i>Dactylis glomerata</i> <i>Cynodon dactylon</i>
Trees	<i>Alnus glutinosa</i> <i>Betula verrucosa</i> <i>Corylus avellana</i>	Ragweed	<i>Ambrosia</i> spp.
Parietaria	<i>Parietaria</i> spp.	Cat	<i>Felis domesticus</i>
Olive	<i>Olea europea</i>	Dog	<i>Canis familiaris</i>
Yellow jacket	<i>Vespa</i> spp.	Lambs quarter	<i>Chenopodium</i> spp.
Mugwort	<i>Artemisia vulgaris</i>	Mugwort	<i>Artemisia</i> spp.
Molds	<i>Alternaria</i> spp. <i>Cladosporium</i> spp. <i>Aspergillus</i> spp. <i>Penicillium</i> spp.	Pigweed	<i>Amaranthus</i> spp.
Cat	<i>Felis domesticus</i>	Plaintain	<i>Plantago</i> spp.
Honey bee	<i>Apis mellifera</i>	Molds	<i>Alternaria</i> spp. <i>Cladosporium</i> spp. <i>Aspergillus</i> spp. <i>Penicillium</i> spp.
Dog	<i>Canis familiaris</i>	Hymenoptera venoms	<i>Apis mellifera</i> <i>Vespa</i> spp.

^aThe two most important allergen sources in the world are the house-dust mites and the grass pollens. Patients often cross-react between the two important mite species, i.e., *D. pteronyssinus* and *D. farinae*, and between several species of the grasses. Commercial extracts are often based on mixtures of species within these groups. Important worldwide are also the indoor allergens from cat, dog, and molds, as well as the extracts derived from Hymenoptera venoms. In local regions other species may dominate. Examples are ragweed in large parts of the U.S., birch in Northern Europe, and *Parietaria* and olive in Southern Europe.

assure that collector qualifications and collection procedures are appropriate to verify the identity and quality of the source materials. This means that only specifically identified allergenic source materials that do not contain avoidable foreign substances should be used in the manufacture of allergen extracts. Means of identification and limits of foreign materials should meet established acceptance criteria for each source material. Where identity and purity cannot be determined by direct examination of the source materials, other appropriate methods should be applied to trace the materials from their origin. This includes complete identity labeling and certification from competent collectors. The processing and storage of source materials should be performed in a way to ensure that no unintended substances including microbial organisms are introduced into the materials. When possible, source materials should be fresh or stored in a manner that minimizes or prevents decomposition. Records should describe source materials in as much detail as possible, including the particulars of collection, pretreatment, and storage.

Pollen

The natural sources of inhalant allergens from plants are the pollen. Pollen may be obtained either by collection in nature or from cultivated fields or greenhouses. The collection may be performed by several methods, such as vacuuming or drying flower heads followed by grinding. Pollens are cleaned by dry or wet sieving and by fluid bed drying separation. Finally, pollen are dried under controlled conditions and stored in sealed containers at -20°C. The maximum level of accepted contamination with pollen from other species is 1%. It should also be devoid of flower and plant debris, with a limit of 5% to 10% by weight. Pollen may show some variation in relative composition depending on cultivar, season, and location of growth.

Established criteria for collection should be validated in order to achieve a constant composition for the production of allergen extracts.

Acarids

For the production of allergen extracts of house-dust mites, the mites are grown in pure cultures. Constituents of the culture medium should be of pharmaceutical grade and devoid of contaminating substances from other allergen sources. Source materials for the extraction are either pure mite bodies (PMB) or whole mite cultures (WMC). The advantage of the WMC extract is that it contains all the material to which a mite-allergic patient is exposed under natural conditions, whereas the advantage of the PMB extract is higher homogeneity and lot-to-lot consistency and avoidance of contamination debris from the culture medium. The WMC extract includes material from mite bodies, eggs, larvae, and fecal particles as well as mite decomposition material and contaminants from the culture medium that should not be allergenic. The PMB extract contains only material extracted from mite bodies, including eggs and fecal particles. The relative concentration of group 1 and 2 allergens in the extract is dependent on the source materials, but clinical trials comparing vaccines based on WMC and PMB show both types to have clinical efficacy in specific allergy vaccination (9).

Mammals

Allergens of mammalian origin may emanate from various sources, i.e., hair, dander, serum, saliva, or urine. The allergens to which humans are exposed depend on the normal behavior of the animal. Therefore, the optimal source of allergens from mammals cannot be generalized. Whether derived from dander or deposited from body fluids, however, most allergens are present in the fur. Source materials should be collected only from animals that are declared overtly healthy by a veterinarian at the time of collection. Established procedures should minimize the risk of inclusion of zoonoses, i.e., diseases that can be transmitted from animals to humans, and the absence of viral particles in the final extract should meet established criteria. When killed animals are used, the conditions for storing should minimize postmortem decomposition until the source materials can be collected. The optimal source materials are often close-cropped hair including dander, which should be free from visible traces of blood, serum, or other extractable materials generally of low allergenic activity.

Due to the quantitative differences in the yield of the various allergens from different dog breeds, a mixture of material from a minimum of five different breeds is recommended.

Insects

The optimal source for insect allergens is dependent on the natural route of exposure, i.e., inhalation, bite, or sting. Where whole insects or insect debris are inhaled, the whole insect body is selected as allergen source. In the case of stinging insects, venom is the ideal allergen source. With biting insects, saliva would be ideal since it contains the relevant allergens.

Fungi

Raw materials are obtained by growing the fungi under controlled conditions. The harvested raw materials should consist of mycelia and spores. Due to difficulties in maintaining a constant composition of fungal cultures, an extract should be derived from at least five independent cultures of the same species. Production of the source material should be conducted under aseptic conditions to reduce the risk of contamination by microorganisms or other fungi. The inoculum should be obtained from established fungal culture banks, i.e., American Type Culture Collection (ATCC), Manassas, Virginia (<http://www.lgcpromochem-atcc.com/>) or Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands (<http://www.cbs.knaw.nl/>). The cultivation medium should be synthetic or at least devoid of antigenic constituents, i.e., proteins. Controls performed in fungal allergen extract production must include tests for suspected toxins.

Foods

Foods constitute a diversified area, and the market for standardized allergen extracts is scarce. Foods are often derived from various cultivars and subspecies grown under a broad variety of

conditions reflecting geographical regions worldwide. In addition, foods are often cooked prior to ingestion, and cooking unpredictably affects the allergenicity of the foods. Consequently, the source of allergen exposure, qualitative as well as quantitative, is highly variable (10).

Ideally, source materials for food allergen extracts should reflect local subspecies, conditions, and habits for the cultivation, harvesting, storing, and cooking of the foods. However, ingested foods are increasingly derived from distant parts of the world. The best solution to these problems may be to combine materials from as many sources as possible to reflect variation in as many parameters as possible. The quality of the source material should be suitable for human consumption and the origin and identity of the raw material should be documented.

For some plant foods, differences in tissue distribution and solubility properties of individual allergens may prevent optimal yields in a single extraction procedure (11). In such cases, an optimal extract may be derived only by combining extracts produced using different buffers and different parts of the plant, i.e., peel and pulp for fruits, as raw material.

A further problem in food allergen extract production is the presence in many foods of natural or microbial toxins, pesticides, antibiotics, preservatives, and other additives that may be concentrated in the allergen extract manufacturing process. Raw materials containing these substances in excess should be avoided as well as overtly artificial foods based on, e.g., genetically modified foods (GMO) or farmed fish. In general, the use of organic source material should be preferred.

Aqueous Allergen Extracts

Preparation of Allergen Extracts

The production process of allergen extracts imposes a number of constraints upon both selection of source materials and the physicochemical conditions used during the extraction procedure. The process must neither denature the proteins/allergens nor significantly alter the composition, including the quantitative ratio between soluble components. The extraction should be performed under conditions resembling the physiological conditions in the human airways, i.e., pH and ionic strength, and suppressing possible proteolytic degradation and microbial growth (12). The optimal extraction time is always a compromise between yield and degradation/denaturation of the specific allergens.

Low molecular weight materials (below 5000 Da) often include irritants, such as histamine, and should be removed from the final extract. This can be accomplished by dialysis, ultrafiltration, or size exclusion chromatography. Any substance excluded from the final extract should be verified nonallergenic. The production procedure should include assessment of known toxins, viral particles, microorganisms, and free histamine, and where relevant, verifying their concentration below defined thresholds.

The final extract should be stored under conditions that impede deterioration of the allergenic activity either by lyophilizing or by storing it at low temperatures (-20°C to -80°C), possibly in the presence of stabilizing agents such as 50% glycerol or a nonallergenic protein, such as certified human serum albumin.

The most widely used extraction media are aqueous buffer systems of pH 6 to 9 and ionic strength 0.05 to 0.2. In general, nonaqueous solvents should be avoided due to the risk of protein denaturation.

Modified Allergen Extracts/Vaccines

Introduction

The efficacy of traditional immunotherapy, i.e., specific allergen vaccination, is related to the dose of vaccine administered, but the inherent allergenic properties of the vaccine imply a limitation due to the risk of inducing anaphylaxis. The risk of allergic side effects is minimized by administering repeated injections of increasing size over extended time periods. Physical or chemical modification of the extract can further reduce this risk. Physical modification involves adsorption of the allergens to inorganic gels, such as aluminum hydroxide or alum, for the purpose of attaining a depot effect characterized by a slow release of the allergens. Chemical modification includes cross-linking of the allergens by treatment with agents, such as formaldehyde, for the purpose of reducing allergenic reactivity. Modified allergen vaccines are used for allergy vaccination but are not used for diagnosis since they were intentionally modified to reduce interaction with IgE.

Physical Modification of Allergens

Physical modification of allergens involves adsorption of the allergen extract with insoluble complexes of inorganic salts, such as aluminum hydroxide or calcium phosphate. Aluminum hydroxide, $\text{Al}(\text{OH})_3$, is especially useful for vaccination purposes and is used in both human and veterinary medicine (13). Its advantages are based on two characteristics of the complexes, the depot effect and the adjuvant effect. The allergens bind firmly to the inorganic complexes, giving rise to slow release of the proteins, thereby lowering the concentration of allergen in the tissue and reducing the risk of systemic side effects. Furthermore, the depot effect reduces the number of injections needed in the course of specific allergy vaccination. Although the significance of the adjuvant effect is unclear, higher levels of IgG antibodies are observed when alum-adsorbed vaccines are used in specific allergy vaccination, as compared with aqueous vaccine (14). Compared with aqueous vaccines, patients receiving depot preparations seem to experience fewer systemic side effects (15), particularly severe early reactions. The frequency of late reactions, which seem to be milder and can be managed by the patient, is reduced to a lesser extent, especially in asthmatic patients (16).

Preparation of aluminum hydroxide-adsorbed extract. Aluminum hydroxide is available as a stable viscous homogeneous gel with a high capacity for noncovalent coupling of proteins. The adsorption is performed simply by mixing the aqueous extract and the gel. After a few minutes at room temperature, the adsorption is complete. Buffer conditions need to be controlled, as the binding capacity varies with buffer composition, ionic strength, pH, and additives (17).

Standardization of the allergen extract must be completed prior to adsorption, as the insoluble complex is difficult to analyze. Therefore, it is difficult to verify the amount of protein adsorbed. In practice, a known amount of standardized allergen extract is adsorbed, and the amount of unbound protein is determined following precipitation of the complex by centrifugation. Manufacturers must specify criteria to withdraw batches above certain thresholds, as different allergens are bound to the complex with different efficiency. Thus, if a large fraction of the allergen extract is unbound, the relative composition of the vaccine may not reflect the composition of the standardized extract. In each case the binding capacity has to be empirically determined (18).

Chemically Modified Allergens

The theory behind chemical modification of allergen extracts is based on the observation that successful allergy vaccination is accompanied by an increase in allergen-specific IgG. Thus, if the allergen could be modified in such a way as to reduce allergenic reactivity, i.e., IgE binding, while preserving immunogenicity, higher doses could be administered without the risk of systemic reactions, leading to higher levels of allergen-specific IgG and improved outcome of specific allergy vaccination (19).

Formaldehyde had been used for extract development in detoxification of bacterial toxins, when Marsh and coworkers in 1970 applied formaldehyde treatment of allergens for allergy vaccination (19). The allergens are incubated with formaldehyde yielding so-called "allergoids," high molecular weight covalently coupled allergen complexes. Compounds with similar immunological properties can be produced using glutaraldehyde instead of formaldehyde. The rationale behind the reduced allergenicity of allergoids is threefold: (i) the large polymeric structures would contain concealed antigenic determinants (epitopes) unable to react with IgE; (ii) polymeric antigens would have a lower "epitope concentration" and thus reduced ability to cross-link IgE on mast cells; and (iii) high molecular weight polymers would diffuse more slowly through tissue.

Preparation of chemically modified allergens. Several allergens are heat labile and thus not readily applicable to the standard procedure of incubation with formaldehyde at elevated temperatures. Instead, a two-step procedure has been applied (20): The first step is incubation with 2 M formaldehyde at 10°C in aqueous buffer at pH 7.5 yielding a stabilized intermediate. After 16 days, the reaction is diluted fourfold and incubated another 16 days at 32°C. The first step at low temperature results in limited inter- and intramolecular cross-linking, thus stabilizing the allergen complex. The intermediate can be cross-linked further at elevated

temperature. Residual formaldehyde is removed by dialysis, and the allergoid is distributed stabilized by addition of 50% glycerol, lyophilized, or coupled to aluminum hydroxide.

Other Modifications

Approaches have been taken to reduce the allergenicity of allergen extracts by disruption of the tertiary structure of allergen molecules using denatured or degraded antigens or peptides, however, with reduced efficacy in allergy vaccination as compared with native allergens. Such molecules do have reduced IgE-binding activity but also substantially reduced immunizing capacity leading to insufficient stimulation of a protective immune response.

The employment of structural and molecular biology has revealed molecular details to the atomic level of several important major allergens. Biotechnology and epitope engineering may facilitate the development of safer allergen molecules in the form of mutated recombinant allergens (21), which can be standardized as chemical entities, obviating the problems of current allergen standardization (22).

Standardization of Modified Allergen Extracts

Most of the techniques used to characterize and standardize aqueous allergen extracts are not applicable to modified ones. It is, therefore, recommended that standardization be completed using the intermediate allergen preparation (IMP) prior to modification, and the reproducibility of the modification process be documented by methods specific to the procedure in question. Standardization of aqueous allergen extracts is discussed elsewhere in this chapter. A brief discussion of the methods suitable for the documentation of the modification processes in aluminum hydroxide-adsorbed and formaldehyde-treated allergen extracts follows.

Protein content in itself is not a suitable standardization parameter but may be a useful measure in terms of normalization of other activities; for example, RAST inhibition capacity per Lowry unit of protein. Determination of the reduction in primary amino groups is a good indication of the degree of modification in aldehyde-treated allergen extracts, since aldehydes react preferentially with primary amino groups. This measure can also be used for stability monitoring of the allergoid, as a reversal of the coupling will lead to an increase in the number of primary amino groups.

It is essential to verify that all protein is bound for adsorbed allergen vaccines. The acceptable level of allergen in the supernatant following centrifugation should be considerably below the initial dose used in the up-dosing schedule of allergy vaccination.

Electrophoretic techniques, such as acrylamide gel electrophoresis and isoelectric focusing (IEF) possibly combined with immunoblotting, are widely used for allergen characterization. For analysis of allergens liberated from adsorbed complexes, acrylamide gel electrophoresis is preferred. However, for "allergoids," acrylamide gel electrophoresis is not useful because of the high molecular weight. As formaldehyde preferentially reacts with primary amino groups, the isoelectric point (pI) of the allergoid is more acidic relative to the allergens. The shift in pI can be monitored by IEF. Size exclusion chromatography, preferably conducted by high-performance liquid chromatography (HPLC), is suited to control for the increase in molecular weight of allergoids relative to the allergens.

Crossed (radio-) immunoelectrophoresis cannot be used to analyze modified allergen extracts. RAST inhibition or related techniques, however, are readily applicable to both alum-adsorbed allergen extracts and allergoids for the purpose of assessing the reduction in allergenicity. These methods are also suited for stability studies.

In vivo testing in patients to standardize modified allergen vaccines is theoretically attractive; however, it is not practical. First, it would not be ethically acceptable to base production of all batches of extracts on routine in vivo assays. There are also large differences in the immune responses of individual patients necessitating large patient panels for such assays. Second, in vivo tests are expensive in terms of labor, time, and money.

Comparison of Modified Extracts

Allergen extracts contain a variety of enzymatic activities, including proteolytic activities, resulting in reduced stability of aqueous extracts when stored in solution. Both chemical and physical modification enhances the stability of allergoid preparations; however, the chemical



Figure 2 Molecular structure of the major allergen from Birch, Bet v 1. The main feature of the structure is a 25 amino acid long α -helix surrounded by a seven-stranded antiparallel β -sheet. A most unusual feature of the structure is a large internal cavity with three openings to the surface. This is the first experimentally determined structure of a clinically important inhalant major allergen (52).

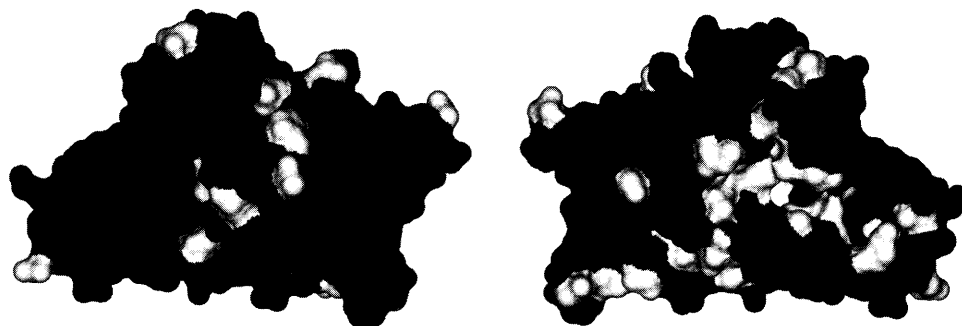


Figure 3 The molecular basis of cross-reactivity. Front and back view of the molecular structure of Bet v 1. Gray patches represent areas on the surface completely conserved among the homologous major allergens of alder, birch, and hazel. Conservative substitutions occur in dark gray areas. The conserved areas represent potential highly cross-reactive IgE epitopes on the protein surface.

modification process is slow and may permit proteolytic breakdown before completion. In addition, both physical (aluminum hydroxide) and chemical (formaldehyde) modification result in reduced allergenicity.

Acquired immune responses are driven by contact with epitopes, which are structural elements of the allergens (antigens). T-cell epitopes are linear fragments of its polypeptide chain, whereas B-cell epitopes (antibody-binding epitopes) are sections of the surface structure present only in the native conformation of the allergen (Figs. 2 and 3). Both T- and B-cell epitopes are essential for effective initiation and stimulation of immune responses; however, the repertoire of epitopes functional in any individual is highly heterogeneous (23,24).

Whereas the modification introduced by aluminum hydroxide adsorption is biologically reversible, the chemical modification of individual amino acids will irreversibly inactivate B- and T-cell epitopes. This chemical effect decreases immunogenicity explaining why higher doses of allergoid are needed to achieve clinical efficacy as compared with native allergen extract. The chemical modifications are not randomly distributed, as ϵ -amino groups on lysine residues are preferentially modified. Some epitopes are consequently more sensitive to modification than others, which may enhance the patient-to-patient variation when allergoids are used for allergy vaccination.

Contrary to expectation, however, allergoids are not safer in practical allergy vaccination compared with native allergens. This was documented in a report from the German Federal Agency for Sera and Vaccines which analyzed all reported adverse reactions to allergen vaccines over a 10-year period, 1991 to 2000, including 555 life-threatening, nonfatal events (25). Laboratory studies comparing commercial products available for birch pollen allergy vaccination in Europe demonstrated large variation among different allergoid preparations, but all allergoid products investigated showed reduced immunogenicity compared with intact allergen vaccine (26). This conclusion was confirmed by another study excluding differences based on the composition of the allergen extract, since the same extract was compared with or without chemical modification (27). In conclusion, allergoid products currently available on

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the market in Europe do not fulfill the allergoid concept as originally formulated by Marsh and coworkers (19).

STANDARDIZATION OF ALLERGEN EXTRACTS

Allergen extracts are complex mixtures of antigenic components. They are produced by extraction of naturally occurring source materials known to vary considerably in composition depending on time and place. Without intervention, this variation would be reflected in the final products.

The purpose of standardization is to minimize the variation in composition, qualitative as well as quantitative, of the final products for the purpose of obtaining a higher level of safety, efficacy, accuracy, and simplicity for allergy diagnosis and allergen vaccination. Standardization of allergen extracts can never be absolute; standardization should be progressively improved as new methodologies and technologies are developed, and the understanding of the properties of the allergens and of the immune responses of allergic patients increases. The benefits for the clinician from improved standardization of allergen vaccines include easier differentiation between allergic and nonallergic subjects, a more precise definition of the specificity and degree of allergy, and a more reliable and reproducible outcome of specific allergy vaccination.

Standardization of allergen extracts is complicated due to their complexity, the allergen molecules, and their epitopes. Allergens are complex mixtures of isoallergens and variants, differing in amino acid sequence (Fig. 4). Some allergens are composed of two or more subunits, the association and dissociation of which will affect IgE binding. In addition, partial denaturation or degradation, which may be imposed by physical or chemical conditions in the production process, is difficult to assess and has a significant effect on the IgE-binding activities of the allergens. The B-cell epitopes that bind to IgE are largely conformational by disposition, meaning that they will be missing from the extract if the allergens are irreversibly denatured.

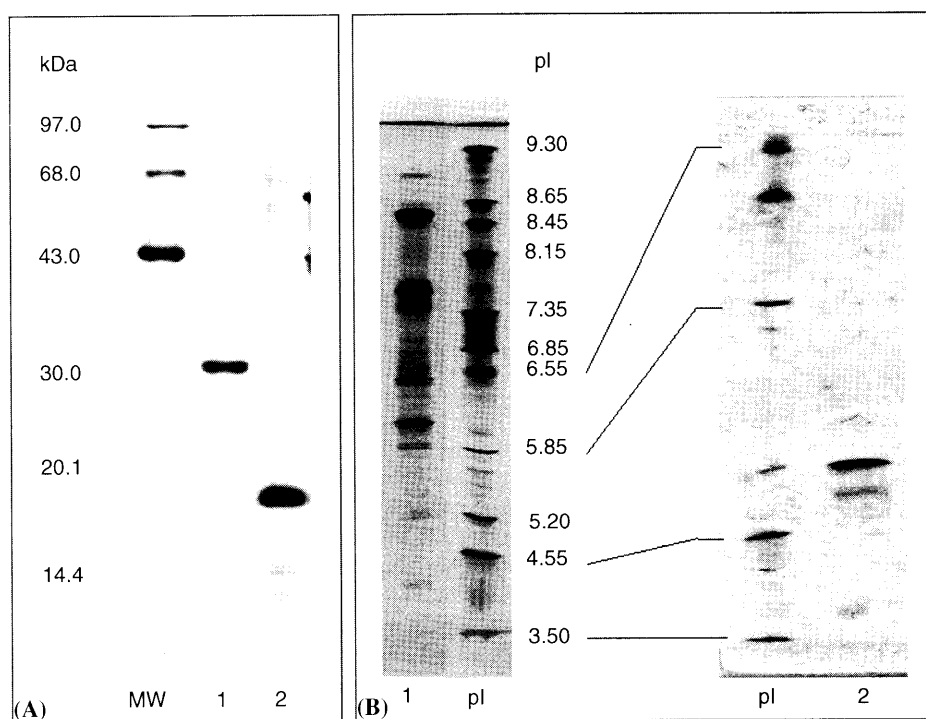


Figure 4 Isoallergenic variation. Allergens are mixtures of isoallergenic variants differing in amino acid sequence, whereas recombinant allergens are homogenous. (Panel A) A silver-stained SDS gel, lane MW: molecular weight markers, lane 1: purified natural Phl p 1, lane 2: purified recombinant Bet v 1. (Panel B) Silver-stained isoelectric focusing gels of pI markers, and the same preparations of purified allergens (lanes 1 and 2).

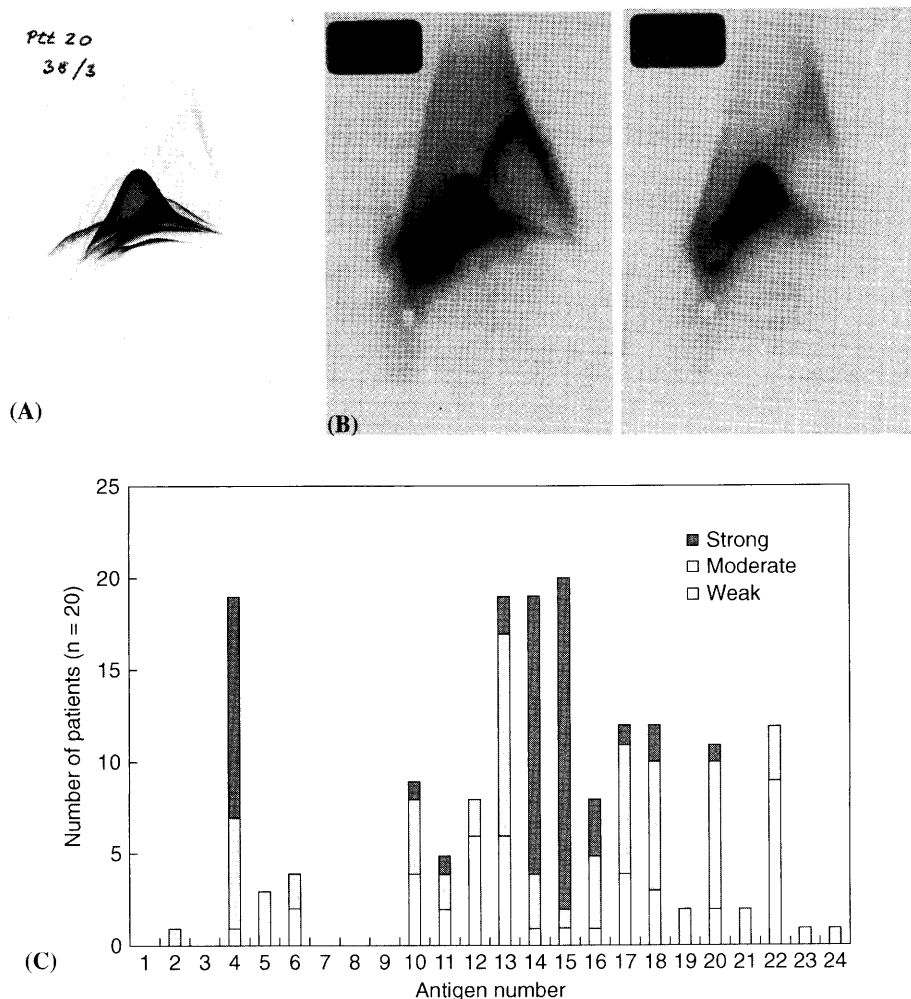


Figure 5 Complexity of allergen extracts. Crossed (radio-) immunoelectrophoresis used for the determination of important allergens. (Panel A) A crossed immunoelectrophoresis plate of a *Dermatophagoides pteronyssinus* allergen extract. Each bell-shaped precipitate represents the reaction of an antigen in the extract with the corresponding antibody present in a rabbit antiserum, raised by repeated immunization with the extract. (Panel B) An autoradiogram of similar plates after incubation with patient's serum and a radiolabeled anti-IgE antibody. Stained precipitates represent allergens. Precipitates from panel A are arbitrarily numbered, and the number of sera in a patient panel showing IgE-reactivity with each precipitate is recorded and displayed in an allergogram (Panel C). Der p 1 corresponds to antigen number 15, Der p 2 to antigen 14.

Another complicating aspect is the complexity of the immune responses of individual patients. Patients respond individually to allergen sources with respect to both specificity and potency. Allergens are proteins, and all proteins are potential allergens. A major allergen is defined as an allergen that is frequently recognized by patients' serum IgE when a large panel of patient sera is analyzed. A minor allergen binds IgE less frequently (below 50%) (28). Furthermore, patients respond individually to B- and T-cell epitopes and hence to isoallergens and variants.

A major objective of allergens' extract standardization is to ensure an adequate complexity in their composition. Knowledge of all essential allergens is a precondition for the safety of ensuring their presence in the final products (Fig. 5).

The other important aspect of standardization is the control of the total allergenic potency. The total IgE-binding activity is intimately related to the content of major allergen (29) and, for an optimal standardization procedure, control of the content of major allergen is essential.

A variety of techniques are available to assess allergen extract complexity and potency. Most techniques use antibodies as reagents, adding another level of complexity to the standardization procedure. Both human IgE and antibodies raised by immunization of animals are subject to natural variation and, in addition, may change over time.

These problems are handled by the establishment of reference and control extracts. International collaboration is necessary to ensure that manufacturers, government authorities, clinicians, and research laboratories worldwide can refer to the same preparations when comparing the results of quality control studies and potency estimates for different allergen extracts. Ideally, standards for reagents should also be established to promote and assist international collaboration.

Standards, References, and Controls

The Establishment and Use of International Standards

Guidelines for the establishment of international standards (IS) were formulated by a subcommittee under the International Union of Immunological Societies (IUIS) in 1980 to 1981. It was assumed that the collaboration and joint authority of WHO would be essential for international acceptance. In the following years, the subcommittee selected, characterized, and produced international standards from several allergenic sources. These included: *Ambrosia artemisiifolia* (short ragweed) (30), *Phleum pratense* (timothy grass) (31), *Dermatophagoides pteronyssinus* (house-dust mite) (32), *Betula verrucosa* (birch) (33), and *Canis familiaris* (dog) (34). Additional standards were planned for the *Alternaria alternata* (a mold) (35), *Cynodon dactylon* (Bermuda grass) (36) and *Lolium perenne* (rye grass) (37), *Felis domesticus* (cat), and *D. farinae* (house-dust mite). This initiative failed because of a lack of consensus and acceptance, primarily due to the differences in practical standardization between Europe and the United States (see section on "Standardization and Allergy Vaccination in Europe and in The United States").

Each of these standard reference extracts has been thoroughly investigated in collaborative studies involving laboratories and clinics worldwide. The results of the characterization and comparison of several coded extracts, which were made available by allergen manufacturers on a voluntary basis, as well as the selection of the international standards, are published and are available to all interested parties. Each international standard has been produced in 3000 to 4000 lyophilized, glass-sealed ampules, which can be obtained from the National Institute for Biological Standards and Control (NIBSC), London, United Kingdom (<http://www.nibsc.ac.uk>).

The content of each ampule is defined by the arbitrary assignment of 100,000 IU. This means that each ampule contains 100,000 IU of any included individual allergen and 100,000 IU of potency measured by any relevant method. Potency estimates will depend on methods and reagents, which must be stated, whereas IUs are independent of methods and reagents.

It is important to realize that the international standards are only recommended for use as calibrators (standards for measurement of relative potency). They are not recommended for use as prototypes, materials to which an extract is to be matched in all respects. None of the international standards have been tested in clinical trials of specific allergy vaccination, and no potency measures of their therapeutic effect are established.

Purified Allergens as International Standards

From November 2001 to April 2005, the European Commission funded an allergen standardization project called "Development of Certified Reference Materials for Allergenic Products and Validation of Methods for their Quantification" or in short the CREATE project (38). The two major objectives of the project were evaluation of the potential use of purified recombinant allergens as certified reference materials (CRM) and the evaluation of available ELISA assays for measurement of major allergens using the candidate CRM as a standard (38). The allergens included were major inhalant allergens in Europe, i.e., Bet v 1 from birch pollen, Phl p 1 and Phl p 5 from grass pollen, Ole e 1 from olive pollen, and Der p 1, Der p 2, Der f 1, and Der f 2 from house-dust mites. The result of the CREATE project was the establishment of the basis for the first two recombinant allergens as CRM: rBet v 1 and rPhl p 5.01 (previously called Phl p 5a). Both rBet v 1 and rPhl p 5.01 were found to be correctly folded molecules, and they fulfilled the necessary stability requirements and were similar to their natural counterparts with respect to

immunological characteristics (39). For each CRM, two ELISA assays were identified with potential for future use as reference methods. A follow-up study of CREATE has now been approved (BSP090) under the auspices of the European Directorate of the Quality of Medicines. The goals of this project are the establishment of rBet v1 and rPhl p 5.01 as European Pharmacopoeia reference preparations, calibrated in S.I. units, and validation of the candidate ELISA for the measurement of the two allergens in a true ring trial.

As soon as other GMP-produced recombinant major allergens with sufficient quality become available, it is expected that they will be included in the program.

The existence and availability of purified allergen references will enable the assignment of a major allergen content in mass units to the internal reference preparations (see below), which are in use in different laboratories of manufacturers, allergen research groups, or control authorities. Furthermore, the references can be used for standardizing major allergen content in bath-to-batch standardization.

The Establishment and Use of In-House Reference Preparations

Having established a production process including control of raw materials, batch-to-batch standardization is performed relative to an IHRP. The IHRP must be thoroughly characterized by in vitro laboratory methods to demonstrate an adequate complexity as well as an appropriate content of relevant major allergen(s). The potency of the IHRP must be determined by in vivo methods, such as skin testing, and ideally the content of major allergen(s) should be determined in absolute amounts. Furthermore, the IHRP should prove efficacious in clinical trials of specific allergy vaccination.

The IHRP serves as a blueprint of the allergy extract to be matched in all aspects by each and every following batch. Specific activities of the IHRPs should be compared with international standards. In this way, measures from different manufacturers can be compared and consistency in internal standardization achieved (4).

Strategy for Standardization

It is impossible to assess the clinical efficacy of each and every batch in the production of routine batches of allergen extracts. In practice, the batches are compared with the IHRP using a combination of different in vitro techniques to achieve a uniform composition, content of major allergen, and potency of extracts. The batch-to-batch standardization can be performed using the following three-step procedure:

1. Determination of allergen composition to ensure that all important allergens are present.
2. Quantification of specific allergens to ensure that essential allergens are present in constant ratios.
3. Quantification of the total allergenic activity to ensure that the overall potency of the extract is constant (in vivo and/or in vitro).

Methods for the Assessment of Allergen Extract Quality

The quality of an allergen extract is a measure of the complexity of the composition, including the concentration of each constituent. Having established careful control of raw materials and a robust production process, a relatively constant ratio between individual components can be achieved independently by quantifying only one or two components, i.e., the major allergens.

The complexity of the composition of allergen extracts can be assessed by several techniques. These techniques are standard biochemical and immunochemical separation techniques. Polyacrylamide gel electrophoresis with sodium dodecylsulfate (SDS-PAGE) (40) is a widely used high-resolution technique available in rapid and partly automated systems. The proteins are separated, but only after denaturation, according to size. Densitometric scanning has been reported, but this technique is not quantitative due to differences in staining intensities. It should only be used for a qualitative assessment of the allergen extract. In combination with electroblotting (41), the proteins can be immobilized on protein-binding membranes, such as nitrocellulose, and stained using a variety of dyes or labeled antibodies (immunoblotting), thereby considerably increasing the sensitivity. Some allergens, however, are irreversibly denatured by SDS treatment and may escape detection by IgE immunoblotting (42).

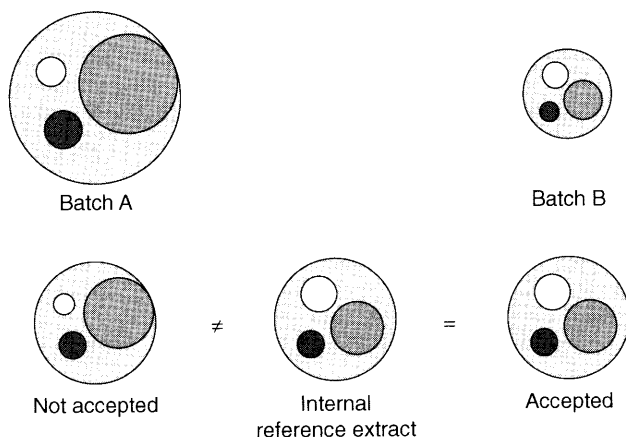


Figure 6 Standardization of allergen extracts. Complexity of allergen extracts represented by a model with three major allergens. The area of shaded circles represents the relative potency of individual components. The area of outer circles represents the total allergenic potency of the extracts. The total allergenic potency of batch A and B may be adjusted by dilution or concentration but the composition of the extracts still may vary accentuating the significance of the measurement of individual components.

IEF (43) is a qualitative electrophoretic technique that separates proteins according to charge pI. Individual allergens are difficult to identify as many proteins form several bands due to charge differences between isoallergens and variants.

Crossed immunoelectrophoresis (CIE) (44) is a technique by which individual antigens are distinguished in agarose gels in the form of bell-shaped antigen-antibody precipitates. The technique is dependent on the availability of broadly reactive polyspecific rabbit antibodies, but the method yields information on the relative concentrations of several important antigens in a single experiment. In crossed radio-immunoelectrophoresis (CRIE) (45), the plates are incubated with patient serum for the identification of allergens.

Methods based on HPLC and mass spectrometry (MS) are being evaluated for use in allergen standardization. Both methods have the potential of generating both qualitative and quantitative information on individual components in a complex mixture.

Quantification of Specific Allergens

Having determined an adequate complexity in composition, an allergen extract may still theoretically be deficient in the content of major allergen (Fig. 6). It is important to independently assess the content of major allergen(s), especially for allergen vaccines used for allergy vaccination. The maintenance dose in effective allergy vaccination contains a defined amount of major allergen (5–20 µg, regardless of vaccine), and the major allergen content is therefore a usable measure relating vaccine potency and therapeutic effect (Table 2).

The significance of controlling individual allergens in extracts is gaining more importance among government regulators and clinicians. Allergen extract manufacturers today have access to the published purification procedures of most major allergens. The purified major allergens can be used to produce antibodies for independent quantification, even in complex mixtures, such as allergen extracts. Polyspecific or monospecific polyclonal rabbit antibodies or murine monoclonal antibodies are most often used for this purpose.

Several immunoelectrophoretic techniques might be applied for the quantitative determination of individual allergens. These techniques are referred to as quantitative immunoelectrophoresis (QIE) (44), and they are convenient and reliable techniques to measure allergen concentrations relative to an in-house standard.

The area of a diffusion ring formed by the precipitated antigen in the monospecific antibody-containing gel can be correlated to the amount of antigen applied in single radial immunodiffusion (SRID), also known as the Mancini technique. The area of the precipitate, alternatively, the height of the precipitate, formed by electrophoresis of the antigen into the agarose gel containing the monospecific antibody, is proportional to the antigen concentration in rocket immunoelectrophoresis (RIE) or quantitative CIE. Both SRID and RIE are dependent on monospecific antibodies, whereas CIE is dependent on polyspecific antibodies.

The ELISA technique (46), in which the allergen is directly bound to a microtiter plate or captured using a monoclonal or polyclonal, monospecific antiserum coated to the plate, and

Table 2 Maintenance Doses in Effective Specific Allergy Vaccination^a

Allergen source	Major allergen	Major allergen in maintenance dose	Approximate equivalent FDA potency	References
Cat				53–56
<i>Felis domesticus</i>	Fel d 1	14.6 µg	2,500 BAU	
House-dust mite				9,57
<i>Dermatophagoides pteronyssinus</i>	Der p 1	9.8 µg	740 AU	
<i>Dermatophagoides farinae</i>	Der f 1	13.8 µg	2,628 AU	
Ragweed				58
<i>Ambrosia artemisiifolia</i>	Amb a 1	10.0 µg	3,000 AU	
Grasses				59–61
<i>Lolium perenne</i>	Lol p 5	12.5 µg	3,948 BAU	
<i>Phleum pratense</i>	Phl p 5	20.2 µg	5,220 BAU	
<i>Dactylis glomerata</i>	Dac g 5	12.0 µg	2,956 BAU	
<i>Festuca pratense</i>	Fes p 5	18.6 µg	12,568 BAU	

^aDiscrepancy between diagnostic and therapeutic potency illustrated by the recommended maintenance doses of various clinical studies. For the average patient the recommended maintenance dose contains 5 to 20 µg of major allergen.

subsequently detected using monoclonal or polyclonal, monospecific antiserum, is a technique offering the possibility of multisample testing and partial automation. When optimized properly, the technique is very accurate. Monoclonal antibody-based ELISA is the most widely used technique for allergen measurement in mass units (47), and a number of validated ELISA assays for major allergens from the main allergenic sources are available. Furthermore, an initiative to establish centrally available validated ELISA assays for selected major allergens in the regulation of allergen extracts in Europe is ongoing (see section on "Purified Allergens as International Standards").

The standard ELISA format is a two-site sandwich assay. An allergen-specific mAb is coated to the microtiter plate and, upon incubating the allergen vaccine, the allergen molecules are captured and subsequently detected using a second mAb or a polyclonal antiserum. An in-house reference, calibrated against a purified allergen preparation, is used as standard. The advantages of mAb-based ELISA assays are their suitability for automation, well-defined specificity and an inexhaustible reagent supply, precise quantification in mass units of allergen, detection limits in the range of 0.1 to 5 ng/mL, and good reproducibility (intra-assay coefficient of variation in the 10% to 15% range).

A potential problem of mAb-based ELISA assays is the specificity of the mAb(s) used. Allergens are heterogeneous mixtures of isoallergens and variants, and in some cases it has been shown that specific mAb reacts to individual subsets of isoallergens (48) introducing a bias in the allergen measurement. A solution to this problem is to use a cocktail of mAb's on the solid phase of the ELISA and a polyclonal antibody as the second reagent.

Allergen Extract Potency

The potency of an allergen extract is the total allergen activity, i.e., the sum of the contribution to allergenic activity from any individual IgE molecule specific for any epitope on any molecule in the allergen extract. It follows that potency measures will always depend on the serum pool or patient panel selected as well as the methodology used. The potency of an allergen extract may be expressed mathematically as shown in equation (1):

$$a = \sum_{i=1}^n f_i c_i \quad (1)$$

The potency of an allergen extract can be expressed mathematically as the sum of the activities of all individual allergens, where "a" is the total allergen activity, and "c_i" and "f_i" are the concentration and activity coefficient, respectively, of molecule number "i."

Methods used for the assessment of allergen extract potency may be divided in two techniques: in vitro or in vivo. The dominating in vitro technique for the estimation of relative

allergenic potency is RAST inhibition (49) or related methods. A standardized reference extract is coupled to a solid phase, paper discs, sepharose gels, or magnetic particles. A serum pool is added, and bound IgE is detected using labeled anti-IgE. In RAST inhibition, the binding of IgE to the solid phase is inhibited by the simultaneous addition of a dilution series of the allergen extract subject to testing. The activity is determined relative to the reference extract itself; parallel inhibition curves indicate similar composition, whereas nonparallel curves indicate that the extracts differ both qualitatively and quantitatively.

The results are dependent upon the patient panel selected. The serum pool is a critical reagent and should contain sera from 20 or more different patients with clinically established allergy to the allergen source in question. A large serum pool should be made in order to ensure continuity, and care should be taken when the control serum pool is changed. Techniques based on ELISA using microtiter plastic trays as a solid phase may be applied using the same principles.

Tests of histamine release from washed human leukocytes utilize the quantification of histamine liberated from allergic patients' leukocytes upon stimulation with allergen (50). The tests are dependent on freshly drawn blood samples from a panel of allergic individuals, thus limiting the practical applicability in routine allergen extract potency determination.

Direct skin testing of human allergic subjects is the main *in vivo* method to assess allergen extract potency (51). It is impractical to use *in vivo* testing as a routine assay for production batch release; however, production batches can be compared by suitable *in vitro* methods to internal reference extracts, the *in vivo* activity of which has been already established. Patient selection criteria for *in vivo* assays are important since all *in vivo* methods will ultimately depend on the selected patient panel.

Skin testing in humans is the principle underlying the establishment of biological units of allergen extract potency. Several units are used. In Europe, the potency unit is based on the dose of allergen, which results in a wheal comparable in size to the wheal produced by a given concentration of histamine. This unit was originally called histamine equivalent potency (HEP). The Nordic Guidelines introduced the biological unit (BU) (5). One thousand BU is the equivalent of 1 HEP.

Determination of Clinical Efficacy

The potency of allergen extracts used for specific allergy vaccination should ideally be expressed in units describing clinical efficacy, since there is no relationship between therapeutic dose and skin test potency. Approaches to relate extract potency and clinical efficacy have been performed in the United States and Europe and commented on by the WHO. For several standardized vaccines, various trials have established an optimal maintenance dose. This dose corresponds to 5 to 20 μg of major allergen (Table 2), which is a useful measure for quantification.

However, determinations of clinical efficacy are extremely laborious. They can only be performed by using highly standardized vaccines, which have been described in detail with respect to composition and *in vitro* and *in vivo* potency, so that they fulfill the criteria for internal standards.

Standardization and Allergy Vaccination in Europe and in the United States

Standardization of allergy extracts in Europe is regulated by the European pharmacopoeia. The regulation is different from the United States, where regulation is enforced by the Food and Drug Administration (FDA). Whereas allergy extract consistency in Europe is maintained primarily through the use of in-house standards and international references, this goal is achieved by the FDA by mandating detailed standardization procedures and reagents for use by all manufacturers. An advantage of the European system is that it provides options for the doctor to choose from different products and for manufacturers to continuously improve quality and incorporate new methodology in analysis and control of the extracts. The advantage of the American system is that it results in a higher degree of consistency of extracts among manufacturers. Another difference between Europe and the United States is in the formulation of the extracts used for allergy vaccination. Physicians in the United States primarily use aqueous vaccines, whereas in Europe, alum-adsorbed vaccines, either chemically modified or native, are most often used (Table 3).

Table 3 Major Differences Between United States and Europe in Allergen Vaccine Standardization and Performing of Specific Allergy Vaccination

United States	Europe
<i>Standardization of allergen vaccines</i>	
FDA select representative extract as FDA reference (FDAR)	Manufacturer select representative extract as in-house reference preparation (IHRP) according to the European Pharmacopoeia
Biological activity (in vivo and in vitro potency/total allergen activity) relative to FDAR	Biological activity (in vivo and in vitro potency/total allergen activity) relative to IHRP
Concentration of major allergen molecules (FDA optional) relative to FDA major allergen reference	Concentration of major allergen molecules (cf. WHO recommendations) relative to IHRP
Methods and reagents selected and distributed by the FDA	Methods and reagents selected and developed by manufacturer
<i>Performing specific allergy vaccination</i>	
Predominantly aqueous vaccines	Predominantly aluminum hydroxide-adsorbed vaccines
Non-modified vaccine	Non-modified or chemically modified vaccines
Vaccines are mixed for multi-allergic patients	Vaccines are predominantly injected separately

CONCLUSION

In Europe each manufacturer uses individual BUs for the measure of allergen extract potency. This may cause confusion, but is enforced by differences in allergen extract composition hampering direct comparison. Furthermore, BUs in current use are based primarily on skin reactivity measurements, which may not be relevant for therapeutic efficacy. Since there is a remarkable coherence between the content of major allergen in the optimal maintenance dose comparing various allergen sources, the content of major allergen for many allergen extracts could be used as a marker relating vaccine potency to therapeutic efficacy. The provision of certified standards and assays for convenient major allergen determination will facilitate comparison of the major allergen content of different extracts. However, major allergen content in and of itself does not completely determine potency of current allergen vaccines, since other allergens, which may vary between extracts, also contribute to their biological potency. It is, therefore, necessary to assess biological potency to avoid the misunderstanding that extracts/vaccines having equal major allergen content are interchangeable.

SALIENT POINTS

- All allergens are proteins and all water-soluble proteins are potential allergens.
- Allergen extracts are complex, biological mixtures, and standardization is essential to ensure safety and efficacy of diagnosis and treatment.
- The process of extraction is highly dependent on physicochemical conditions. Extreme conditions are likely to destroy allergen epitopes and affect activity.
- Statistically patients' IgE binds to some antigens more frequently than to others, thereby defining major allergens.
- The effective maintenance dose in specific allergy vaccination for the average patient is proportional to the content of major allergen in an allergen vaccine.
- Major allergen content alone is not a sufficient measure of extract potency.
- Chemically modified allergen extracts are deficient in specific epitopes.
- The existence and use of internal as well as external standards are essential for standardization and control of allergen extracts.
- The quality of an allergen extract is dependent on the qualitative as well as quantitative composition.
- The potency of an allergen extract is determined by the combination of the concentration of one or more major allergens and the composition, qualitative as well as quantitative, of the allergen extract.

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