

Recombinant Allergens/ Allergen Standardization

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Recombinant allergens are genetically engineered isoforms representing allergen molecules from allergen extracts. Immunologic responses of allergic patients toward allergen extracts define the major allergens. For the average allergic patient, the diagnostic sensitivity and treatment efficacy correlate with the concentration of major allergen. Standardization of allergen products (extracts or genetically engineered allergens) can therefore advantageously be performed using a selected recombinant major allergen as a standard. The standardization will furthermore require reagents for which both monospecific, monoclonal, or preferably, recombinant antibodies can be used. Due to differences in the allergenic activity of individual isoallergens and the naturally occurring mixture of isoallergens found in an allergen extract, and due to additional contribution to the allergenic activity from other molecules in the extract, a biologic potency assessment must always be performed as a supplement. This is also the case for a genetically engineered allergen product.

Introduction

Recombinant allergens/allergen standardization

Ever since the first recombinant allergen molecule was produced in *Escherichia coli* 10 years ago [1], the potential for allergen standardization has been appreciated. However, recombinant allergens are not yet in use for standardization of allergen vaccines.

Recombinant allergen would constitute an ideal standard, since homogenous preparations can be produced in large amounts. However, it may perform poorly as a representative of the complex mixture of proteins contained in an allergen source material, including isoallergens and variants of various major allergens, toward which allergic individuals react. In addition, homogenous standards would facilitate quality control of allergy vaccines due to the accuracy and simplicity by which they can be measured.

Genetically engineered products

Today, all allergen vaccines on the market that are used for specific allergy diagnosis and treatment are extracted mixtures of molecules, which in some cases may have been chemically modified. In the future, however, it is probable that most abundant major allergen molecules used for diagnoses and treatments will be genetically engineered recombinant molecules. Fulfillment of this scenario will depend on the legal requirements (GXP, etc.) being higher for therapeutic vaccines than for in vitro diagnostic reagents. The burden of fulfilling the legal requirements will certainly be the limiting factor for a real development of genetically engineered allergy products.

The following article focuses on the potential use of recombinant allergens for standardization of both current allergen vaccines produced by aqueous extraction and future genetically engineered allergen vaccines.

The Nature of Allergen Extracts

Allergen extracts are ideally complex mixtures of all water extractable molecules from the relevant source materials. Provided that the extraction is performed optimally, this mixture is expected to represent all molecules toward which allergic individuals might raise IgE antibody producing clones, thereby establishing allergic conditions. The number of different molecules in the extract may be low (*ie*, fire ant venom sac, five antigens [2]) or high (*ie*, short ragweed pollen, 51 antigens [3]), but most of them are proteins. Many of these proteins are present in various isoforms, *ie*, proteins differing in one, a few, or a substantial number of amino acids. The number of intra species isoallergen amino acid substitutions may in some cases reach the level of difference observed when comparing homologous proteins from related species [4••]. Each amino acid substitution may give rise to differences in epitope structure, resulting in a complex pattern of unique and common epitopes distributed over the isoallergens and variants. Some of these epitopes may be shared by homologous isoallergens and variants from related species, thus adding to the complexity [5].

Allergic Patients' Responses

Experiments using either electrophoretically or otherwise separated antigens from allergen extracts have clearly shown that allergic individuals develop unique IgE responses against various subsets of the molecules in the allergen extracts [6,7]. Studies performed using panels of allergic patients' sera have furthermore revealed that some antigens (major allergens [8]) have a high probability of raising IgE responses, whereas other antigens (minor allergens) have a much lower probability. It was concluded from these studies that all protein antigens were potential allergens. Since both electrophoretically separated isoallergens and variants and genetically engineered recombinant isoallergens revealed unique patterns of reactivity with allergic patients' IgE, each naturally occurring isoallergen and variant can be regarded as a potential allergen [9]. According to these experiments, each individual allergic patient has a unique response with respect to allergen-specific B and T cells, defining unique patterns of reactivity toward individual allergens, isoallergens, and variants.

Allergen Extracts As Vaccines

Specific allergy vaccination has been performed ever since 1911, when introduced by Noon [10]. Although successfully used throughout 90 years it has also been used in manners comparable with homeopathies [11], raising criticism and causing allergy treatment to develop in the direction of barely symptomatic drug treatment. Nevertheless, very recently performed clinical studies have revealed both a curing [12•] and a preventive effect. The latter was shown both in respect to asthma development [13•] as well as the development of new sensitizations. Similar effects cannot be shown with any drug developed so far, and it is questionable whether a simple drug treatment can be expected to cure allergy due to the complexity of the disease as revealed by recent genetic analyses [14].

Major Allergen Molecules As Therapeutic Vaccines

Based on the fact that a majority of IgE specificities in an allergic patient population can be accounted for by a limited number of major allergens, it has been proposed that complex vaccines be substituted with purified major allergens for specific allergy vaccination. This concept is indirectly supported by the correlation between clinical efficacy and major allergen content in the maintenance dose during specific allergy vaccination [15•]. A few double blind, controlled studies have been performed, revealing a superior effect of the multi-component extracts [16,17]. Nevertheless, for patients exclusively responding to the major allergen molecules, efficacy was similar although not better. These studies did not investigate whether patient groups treated with the purified major

allergen molecules would tolerate even higher allergen doses, which might potentially lead to improved efficacy.

For specific allergy diagnosis, purified major allergens have also been theoretically considered [18] and practical trials [19] have been performed. Undoubtedly, major allergen molecules would be quite efficient but give rise to a limited number of false negative tests [19]. On the other hand, diagnostic tests would certainly achieve a higher level of accuracy and specificity by using one or a mixture of a few major allergens instead of the currently used allergen extracts. Cross-reactivity between homologous allergens from different species due to partially identical epitope structures is a common phenomenon leading to ambiguous diagnosis. Automated equipment and IgE inhibition assays based on purified allergens have the potential to identify the true sensitization source leading to improved efficacy in specific allergy diagnosis and vaccination.

Genetically Engineered Allergy Vaccines

Biotechnology offers the possibility of producing pharmaceutical-grade allergens for which not only the active ingredient has been documented, but also the absence of unwanted impurities, especially infectious agents. Other possibilities include improving safety, since a recombinant allergen may be engineered to reduce IgE binding. Although hardly feasible, the very best vaccine would probably be a mixture of all relevant allergen molecules in engineered, safe versions. Realistically, by using one or a few major allergens for vaccination, a minority of the patients might not be optimally treated. It is still possible, however, that the majority of patients might be even more efficiently treated since higher allergen doses would be expected to be feasible. The reason for this is twofold: there would be only one or two molecules to determine the highest tolerable dose [20] and secondly, genetic engineering enables the generation of mutated recombinant allergen molecules with reduced or no IgE mediated side effects [21••]. Finally, although not shown in clinical settings, it can be hypothesized that treatment using high doses of major allergen molecules may render the allergic patient less susceptible to allergic reactions to other, minor allergen molecules.

Simplification of allergenic extracts to one, or a mixture of a few, GMP-produced, well-controlled genetically engineered recombinant allergen molecule(s) would transform allergy vaccines to pharmaceutical-grade products equal in quality to chemical allergy drugs, but preserving their unique curing and preventive abilities. Approval of recombinant allergens for allergy vaccination in addition would facilitate future developments of the active ingredient through genetic engineering aiming at higher efficacy and reduced levels of IgE mediated side effects. New delivery systems including TH1 directing adjuvants might also contribute to this development as

well as treatment facilitating use of non-injective administration routes for specific allergy vaccination.

Standardization of Allergen Vaccines.

Allergen standardization is performed in order to minimize the variation in quality and potency of allergen products. The advantage of allergen standardization is the attainment of defined limits for safety, dosage efficacy, and accuracy when the extracts are used for diagnosis and treatment of allergic diseases. Theoretically, this can be achieved by keeping the level and mutual concentrations of all potential allergen molecules under control. In practice, however, it is not possible to measure all allergen molecules. Consequently, the recommendation is to ensure the concentration(s) of one or a few selected major allergen molecules and, in addition, to perform a measurement of the total allergenic activity (extract potency) by an appropriate *in vivo* or *in vitro* method. This proposed allergen standardization procedure [22] is only in current use by one or a few allergen extract manufacturers, and unfortunately, not universally required by government agencies, including the FDA.

The first and foremost prerequisite in allergen standardization is a set of standards. Each extract manufacturer should employ an internal standard, a *prototype*, to be used according to the blueprint principle, *ie*, a standard to which all materials must be matched in all respects. Furthermore, preferably at the international level, *calibrators* should be used according to the yardstick principle, *ie*, as standards to compare concentrations of one or a few major allergen molecules and to compare extract potency.

The standard must be an allergen extract representative of potential allergenic materials extractable from the source in question. Needless to say, this is a rather vague definition that causes a lot of confusion. Nevertheless, a number of international standards have been developed and approved by the World Health Organization (WHO) [23–28] to be used as calibrators, enabling consistency in the reproduction of internal standards. The internal (in house) standards are used to control of batch-to-batch consistency.

Unfortunately, the international standards approved by the WHO have never come into use, partly for political reasons and partly due to disagreements on how they should be used technically, *ie*, as calibrators for both extract potency and concentrations of major allergen molecules. Recently, in a WHO-position paper on “allergy immunotherapy: therapeutic vaccines for allergic diseases” [15•], it was retrospectively realized that from clinical studies [29–40] using well-characterized and standardized allergy vaccines, the optimal maintenance dose was in the range of 5 to 20 µg major allergen molecule per injection; and that the therapeutic efficacy correlated with such doses. Consequently, the IUIS subcommittee on allergen

standardization [41•] has decided to develop a second generation of international standards, now in the form of purified major allergen molecules instead of the first generation of standards based on allergen extracts.

Major Allergen Molecules As Standards

The various theoretical possibilities for the selection of a standard appear in Table 1. Today, any of these standards might be made available for any allergenic source material [42•]. Provided that an allergenic activity measured by any validated quantitative assay show potency comparable with that of a preparation of a natural purified major allergen, any of these standards can, in principle, be used.

In the future, the preferred standard will probably be one selected naturally occurring recombinant isoallergen, or alternatively a mutated recombinant allergen. Purified natural major allergen would probably pose difficulties with respect to reproducibility, whereas mixed recombinant or mutated recombinant major allergens would require excessive resources. Thus, in the following discussion, it will be presumed that the selected standard is a single recombinant allergen, *ie*, genetically engineered naturally occurring isoallergen or mutated major allergen. Furthermore, it can be argued that an artificial mutant would be preferable since, when not found in nature, all potential users of the standard would be equal.

Allergen Preparations to Be Standardized

All currently available allergen preparations used for specific allergy vaccination or *in vivo* specific allergy diagnosis are based on allergen extracts produced by aqueous extraction of allergenic source materials. The standards currently used in allergen standardization are also based on aqueous extraction, which to a certain extent ensures compatibility when comparing potencies.

One or more of the genetically engineered “major allergen source,” however, might be marketed in the future. Standardization of all these concerning major allergen content would be feasible, however, accuracy would depend on the compatibility between the standard and the allergen preparation. Thus, if for example, a recombinant standard is compared to a natural mixture of isoallergens the result will depend on the reagents used in the assay.

Methods and Reagent(s) Applicable for Major Allergen Molecule Standardization

The methods used for specific determination of allergen concentration are usually variations of solid phase or liquid immuno assays. Most often used are variants of enzyme-linked immunosorbent assay (ELISA) inhibition, [43], radioallergosorbent test (RAST) inhibition [44], or in-gel electrophoretic and diffusion tests [45]. Although difficult to optimize, ELISA tests are in widespread use, and

Table 1. Standardization of allergens

Major allergen source	Dependencies in standardization process
Allergen extract	Actual source
Extracted and purified major allergen*	Actual source and purification process
Genetically engineered naturally occurring isoallergen*	Selected isoallergen
Genetically engineered mix of selected naturally occurring isoallergens*	Selected isoallergens
Genetically engineered naturally occurring isoallergens, mix of all*	Definition of limits to related species†
Genetically engineered mutated isoallergen*	Selected mutation
Genetically engineered mix of mutated allergens*	Selected mutation

*Potential standard
†No well-defined difference between homologous molecules from related species exists.

if optimized carefully, they are very precise. For this type of assay, both polyclonal, monospecific antibodies (of any animal origin), monoclonal antibodies (of any animal origin), or genetically engineered immunoglobulins can be used. The latter would be the optimal choice due to the stability of recombinant products. In any case, it is extremely important to select the optimal reagents [46••].

Standardization Procedure When Using a Recombinant Major Allergen Molecule

The performance of the necessary inhibition tests according to fulfillment of the equation:

$$\frac{\text{Extract vs. Standard} \times 100}{\text{Standard vs. Standard}}$$

and carried out according to state of the art, *eg*, parallel line curves, etc., would determine a relative content of major allergen in the analyzed extract. Theoretically, the relative major allergen content determined by antibody based methods would be precise only for extracts/preparations that solely contain the very isoallergen, which has been selected as standard. In all other cases, differences in affinities for individual isoallergens and variants would impose a source of error. The error cannot be predicted, however, it may be empirically determined. Nevertheless, it is anticipated that by careful selection of a representative isoallergen as a standard and a correspondingly carefully selected recombinant antibody as reagent, measurements should be precise within the normal uncertainty limits.

Such measurements would undoubtedly be an advantage for the standardization of allergen extracts for content of major allergen molecules which have been shown to correlate with treatment potency [29–40]. It is, however, the opinion of the authors that determination of the total allergenic activity (extract potency) would still be compulsory in order to avoid the risks of misinterpretation that different equipotent allergen preparations may replace each other.

Conclusions

This paper should not be regarded as a conventional review of scientific findings, but rather as a proposal of how future allergen products could be standardized. There is no doubt that all the proposed activities contained herein can be technically performed, provided that there is consensus among international health authorities and allergen-extract manufacturers. The allergy community—patients and doctors alike—would benefit from standardization by obtaining more predictable and thereby safer treatment opportunities.

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This recent editorial states the various assay conditions that might appear when using monoclonal antibodies as reagents in the standardization process. It emphasizes the very well-defined specificities of monoclonal antibodies and proposes the use of oligoclonal antibodies (*ie*, mixtures of monoclonal antibodies).