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ALLERGENS (RK BUSH, SECTION EDITOR)

Molecular Approaches to Allergen Standardization

Martin D. Chapman · Peter Briza

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Abstract Molecular approaches to allergen standardization include the development of purified natural or recombinant allergen standards whose structural and allergenic properties have been validated, in tandem with certified immunoassays for allergen measurement. Purified allergens can be used individually or incorporated into multiple allergen standards. Multicenter international collaborative studies are required to validate candidate allergen standards and immunoassays, as a prelude to being approved by regulatory agencies. Mass spectrometry is a sophisticated and powerful proteomics tool that is being developed for allergen analysis. Recent results using pollen allergens show that mass spectrometry can identify and measure specific allergens in a complex mixture and can provide precise information of the variability of natural allergen extracts. In future, the combined use of immunoassays and mass spectrometry will provide complete standardization of allergenic products. Molecular standardization will form the basis of new allergy diagnostics and therapeutics, as well as assessment of environmental exposure, and will improve the quality of treatment options for allergic patients.

Keywords Allergens · Immunoassays · Allergen standardization · Multiplex assays · Allergen diagnostics · Dust mites · Pollens · Mass spectrometry · Allergen immunotherapy · Allergy vaccines · Proteomics · European Pharmacopoeia · Market authorization · Molecular diagnostics

M. D. Chapman (✉)
Indoor Biotechnologies Inc,
1216 Harris Street,
Charlottesville, VA 22903, USA
e-mail: mdc@inbio.com

P. Briza
Department of Molecular Biology, University of Salzburg,
Hellbrunnerstr. 34,
5020, Salzburg, Austria

P. Briza
e-mail: peter.briza@sbg.ac.at

Introduction

The field of allergen standardization has largely moved from measurements of 'total' allergenic potency (eg, by skin test, IgE inhibition or histamine release) to measurements of specific allergens in recent years. The advent of multiplexed allergy diagnostics, coupled with new strategies for immunotherapy based on recombinant allergens, has focused attention on methods for standardization of purified allergens with well-established allergenic importance [1–5]. This is for good reason: regulatory agencies have found it hard to keep pace and develop reference materials for allergenic products using total potency estimates. The U.S. Food and Drug Administration (FDA) lists 19 standardized allergenic products (from dust mites, cat, grass pollens, and insect venoms) that are licensed by the Center for Biologics Evaluation and Research (CBER) in the U.S. (http://www.fda.gov/BiologicsBloodVaccines/Allergenics/StandardizedAllergenicExtracts/default.htm). There has been no increase in the number of CBER reference materials for over 5 years. Clinically important allergenic products remain unstandardized, eg, animal allergens (other than cat), tree pollens, and foods. The FDA recently completed a comprehensive literature review of the safety and effectiveness of 1269 non-standardized allergen extracts that are marketed in the United States. The FDA concluded that "almost all" of the non-standardized extracts were safe, based on analysis of adverse event reports, including deaths, over 23 years. Moreover, 54 % were deemed to meet provisional thresholds of effectiveness [6•]. Detailed literature reports compiled by the FDA are available online at: http://www.fda.gov/AdvisoryCommittees/CommitteesMeetingMaterials/BloodVaccinesandOtherBiologics/AllergenicProductsAdvisoryCommittee/ucm274695.htm

The limitations of standardization based on total potency estimates are well known. In an increasingly stringent environment for research on human subjects, it is hard to recruit

patients who fit skin test criteria for biologic standardization purposes or even to obtain serum samples from allergic patients with IgE antibody levels suitable for in vitro tests. Cost is also an issue and as regulatory agencies in the U.S. and Europe seek to control costs, they are moving towards high throughput, specific allergen measurements for allergen standardization purposes [7, 8••].

Knowledge of the structural biology, function and biologic activity of purified allergens has increased exponentially over the past decade, for all sources of allergens (mites, pollens, animal allergens, molds, foods, insects and insect venoms) [9–13]. Over 700 allergens have been cloned (www.allergen.org) and 75 crystal structures of allergens and allergen/antibody complexes are in the Protein Data Bank (PDB; www.rcsb.org). Panels of allergen-specific monoclonal (and polyclonal) antibodies have been developed for use in immunoassays to measure allergens in source materials and finished allergen products. Immunoassays are the essential tools of contemporary allergen standardization (Table 1). Indeed, immunoassays are currently the only tool that can deliver international standards with verifiable allergen content that can effect global harmonization of allergen measurements [14]. While the applications listed for immunoassays in Table 1 cover allergy diagnostics and therapeutic products, it is important to note that these assays are widely used for environmental detection of allergens in dust, air and biologic samples, where standardization for exposure assessment is equally important.

CREATE as Catalyst

At a meeting at the European Academy of Allergy and Clinical Immunology (EAACI) in Brussels (July 1999), the World Health Organization and International Union of Immunological Societies (WHO/IUIS) Allergen Standardization Committee overwhelmingly approved an initiative to develop purified natural or recombinant allergen standards. At subsequent committee meetings, candidate allergens were identified and criteria were developed to assess biochemical purity, IgE antibody binding and biologic activity of the proposed reference standards. The breakthrough came in 2001, when Dr. Ronald van Ree obtained funding to support the WHO/IUIS initiative from the European Union 5th Framework program to support Development of Certified Reference Materials for Allergenic Products and Validation of Methods for their Quantification (CREATE). The CREATE project involved 28 research groups, clinical and industrial partners in 9 European countries. Purified natural and recombinant forms of 8 allergens were prepared (Bet v 1, Phl p 1, Phl p5 , Ole e 1, Der p 1, Der p 2, Der f 1, Der f 2) and subjected to proteomics analyses, stability studies, IgE binding assays, histamine release assays and ELISA measurements [1–3]. The CREATE project divided recombinant allergens into 2 tiers based on their suitability as reference preparations: first tier (rBet v 1, rPhl p 5, rDer p 2), allergens which were considered identical to their natural counterparts; second tier (rDer p 1, rDer f 1, rDer f 2, rOle e 1), allergens with strong allergenic activity, but in need of further improvements to match the allergenic and structural attributes of the natural allergen. This could be achieved by genetic engineering. For example, removal of the pro-region from the cysteine protease allergen Der p 1 gave a much better correlation with IgE antibody binding to nDer p 1. Another aspect of CREATE that has, to some extent, been overlooked was the tremendous number of allergic patients recruited into the project. For each allergen source, over 250 sera were obtained from patients with a well-defined clinical history from 11 participating clinical centers across Europe. These sera had total and specific IgE measurements (ImmunoCAP) and are maintained as a serum bank for research studies at Dr. van Ree's laboratory at the Academic Medical Centre in Amsterdam, The Netherlands.

Table 1 Immunoassays: essential tools for allergen standardization

- Provide measurement of specific allergens in mass units and assays that can be certified by regulatory authorities.
- Provide accurate and precise quantification of specific allergens in allergy diagnostics and therapeutics.
- Allow regulatory agencies to develop national and international allergen references.
- Allow precise formulation and dosing of recombinant allergen vaccines and evidence-based trials of new vaccines.
- Provide a global strategy for harmonization of allergen measurements.

Beyond CREATE

European Directorate for the Quality of Medicines and Healthcare (EDQM), BSP090 Study

The CREATE study was a valuable exercise in comparing the proteomics and allergenicity of purified natural and recombinant allergens and pointed the way to subsequent allergen standardization studies using purified allergens. Since the rBet v 1 and rPhl p 5 used in CREATE were produced under GMP conditions, they were selected by the EDQM Biological Standardization Programme for further development as standards that could be included in the European Pharmacopoeia. The molecular structure of the reference materials was investigated and ELISA tests for each allergen were compared in the BSP090 study [15]. Detailed molecular analysis of the GMP grade rBet v 1.0101 by mass spectrometry, amino acid analysis, size exclusion HPLC and circular dichroism showed that the

preparation was monomeric and had identical structural features to nBet v 1. The rBet v 1.0101 had comparable allergenic activity in IgE ELISA inhibition and in a basophil activation assay (upregulation of CD203c) [16•]. Similar data were obtained using rPhl p 5a produced under GMP. These allergens were used as standards in an ELISA ring trial comparing the precision, accuracy and potency estimates of two ELISA's for rBet v 1 and one ELISA for rPhl p 5. The trial was carried out in 13 Official Medicine Control Laboratories (OMCLs) which are responsible for evaluating medicinal products across Europe and in the US. Data from this trial have been analyzed and it is anticipated that validated ELISA's for Bet v 1 and Phl p 5a will be included in the European Pharmacopoeia (Ph. Eur.) in 2013.

Multiple Allergen Standards

ELISA has been the gold standard for measurement of allergens and other proteins for the past 25 years. However, the increasing complexity of biologic systems has spurred the need to develop multiplex array technology for measuring multiple analytes simultaneously. This can be achieved using 'static' or planar arrays (eg, on a glass slide or microtiter well) or using internally dyed fluorescent microbeads in suspension arrays. Multiplex assays have received wide application in immunology eg, for detection of panels of cytokines, growth factors, or infectious agents (reviewed in [17•]). Commercial kits are available that can measure >30 cytokines at once. Our group has developed a multiplex array for indoor allergens (MARIA) which measures up to 11 allergens (including Der p 1, Der f 1, Mite Group 2, Fel d 1, Can f 1, Rat n 1, Mus m 1, and Bla g 2) [18]. This assay was standardized using a multiple "universal" allergen standard (UAS) which was formulated by mixing purified natural allergens at defined concentrations, as determined by amino acid analysis. Conversion factors were developed from comparison of dose response curves or from linear regression data which enabled values obtained using the UAS to be compared directly with previous ELISA standards [19]. The UAS was also used to determine the absolute allergen concentrations in WHO/IUIS and US FDA reference preparations. The results showed that measurements of mite Group 1 and Group 2 allergens in the FDA references were consistent [20•]. It was also possible to calculate the absolute protein value for WHO/IUIS International Units of Can f 1 (1 IU = 5 ng Can f 1) and for the FDA units of Fel d 1 (1 FDA unit = 1 ng Fel d 1). The results suggested that the FDA reference preparations could be used by US manufacturers for standardization of dust mite and cat products in terms of specific allergen content. This would enable allergists to consider major allergen content in determining optimal doses of allergens for use in immunotherapy [21, 22•].

A significant advantage of multiplex assays compared to ELISA, is that multiplex technology is inherently more standardized. ELISA uses passive absorption (mechanism unknown) to coat proteins to the solid phase, whereas in bead-based multiplex arrays, the antibody is covalently coupled to the beads via carbodiimide bonds. All of the analytes in multiplex arrays are measured in a single well (or slide) under exactly the same assay conditions (same standards, detection reagents, assay buffer, incubation times, wash cycles etc.), which reduces potential for operator error that would be inherent in preparing multiple ELISA plates. Multiplex assays are usually more sensitive than ELISA and have a broader dynamic range. In spite of these advantages, multiplex arrays have remained largely a research tool with relatively few assays cleared by the FDA for clinical use [17•].

Molecular Diagnostics

Apart from immunotherapy, the development of 'component resolved' or molecular diagnostics has been a significant driver of standardization using purified allergens. Molecular diagnostics are based on measuring IgE antibodies to multiple individual allergens as compared to using heterogeneous allergen extracts. This has dramatically raised awareness of specific allergens in general and the nature of immune responses to these allergens by different groups of allergic patients. Molecular diagnostics can resolve the basis for clinical cross-reactivity between apparently unrelated allergens. Currently, the most comprehensive and advanced molecular diagnostic is a planar array of over 130 purified allergens from inhalants, foods, venoms, and latex (marketed as the ImmunoCAP ISAC; ThermoFisher, Kalamazoo, MI). Each ISAC chip can test four sera for all 130 allergens using ~50 μL serum and the results are expressed in ISAC Standardized Units (ISU). In comparative diagnostic studies, the ISAC chip has shown a good correlation with fluorescent immunoassay (ImmunoCAP) for pollen and latex allergy and ~78 % concordance when tested against a wider panel of 15 allergens [23–25]. Proof-of-principle for using bead suspension arrays for IgE antibody and total IgE measurement on the Luminex xMAP platform has been demonstrated [26]. A similar feasibility study using a micro-bead array system for measuring IgE to food, pollen, latex, and mite allergens was recently reported [27]. Micro-bead based systems offer the advantage that the allergens can be mixed on a custom basis for research studies or targeted at specific groups of allergens, eg, indoor allergens, pollens, molds, and foods for diagnostic purposes.

New Tools for Allergen Standardization

Mass spectrometry (MS) is a sophisticated physicochemical tool that has recently been applied to allergen analysis. Unlike immunoassays, MS directly identifies the protein or peptide of interest by mass and/or sequence. Approaches to MS differ in the methodology of sample ionization, mass analysis, and detection (see Ref [28••]) for an excellent recent review). Two methods for ionization and transfer into the gas phase are almost exclusively used in proteomics: electro spray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI). In ESI, the analytes are dissolved in an aqueous solvent and ionization takes place under atmospheric conditions in an electric field. The method can easily be combined with liquid chromatography and is therefore frequently used. In MALDI, the analytes are embedded in a crystallized matrix of a low molecular weight organic compound. Transfer into the gas phase and ionization is achieved by pulses of laser light in a high vacuum. For many years, electro spray ionization quadrupole time-of-flight mass spectrometry (ESI-QTOF MS) was the method of choice for protein identification in complex samples. First, the mass of peptide ions present in the sample after a tryptic digest is determined by TOF. In a second phase, the peptides are individually selected and filtered by the quadrupole according to their mass/charge ratio and fragmented by controlled collisions with gas atoms. The resulting peptide fragments are again analyzed by TOF MS. The combined mass and fragment data can be used either for database searches or, in case of unknown proteins, de novo sequencing. Using this methodology, variants, isoforms, and post-translational modifications of proteins/allergens can be determined.

Most of the applications of MS to the allergen field have involved pollen allergens. This proteomics approach was applied to five Group 1 grass pollen allergens (Lol p 1, Phl p 1, Poa p 1, Ant o 1, and Dac g 1). Analysis of peptide digests showed molecular variability among these pollen allergens, even though they had ~90 % amino acid sequence identity. In addition, post-translational modifications involving proline hydroxylation and *N*-glycosylation also contributed to variability in the allergen structures and raised the possibility that the Group 1 allergens contain species specific determinants [29••]. ThermoFisher (Bremen, Germany) recently introduced a new type of mass analyzer, the Orbitrap, that makes high resolution and accurate MS (HRAM MS) with sensitivity to <3 ppm possible [28••]. It should be noted that Q-TOF instruments of the latest generation have a significantly improved resolution, accuracy, and sensitivity and can be used for HRAM measurements as well. Using an Orbitrap instrument with high resolving power and isotope labeled peptides, Seppälä et al were able to perform the absolute quantification of Phl p 1 and Phl p 5 in timothy pollen extracts at femtomole levels with a precision of <5 % [30••]. Advantages of this approach are the ability to measure multiple allergen sequences, variants or isoforms simultaneously, similar to multiplex immunoassays. It is clear that MS is a powerful tool for identification of allergen isoforms, which can be quite laborious using immunoassays. This has been elegantly demonstrated for birch pollen, in which the major allergen, Bet v 1, has ~50 isoforms that have been mostly identified from cDNAs. Comparison of the isoform distribution of birch pollen extracted from Swedish or Austrian pollen showed that pollen from both countries contained five isoforms (a, b, d, f, and j). These isoforms were identified by 2-dimensional gel electrophoresis, followed by determination of the intact masses of the gel-eluted proteins and MS-sequencing of the individual spots. In contrast, a commercial skin prick testing solution contained only three Bet v 1 isoforms (a, b, and d) [31••].

MS can also be used for the identification of allergens or their isoform distribution in unusual samples, such as cross-linked allergen extracts (allergoids). Due to their high molecular weight, allergoids are often insoluble. Consequently, the determination of their allergen content by conventional methods is difficult. It was shown that despite the chemical cross-links, proteolytic digests liberated diagnostic peptides that were in turn identified by MS [32].

The advantages of MS are that it is highly sensitive, can quantify the number of isoallergens in an allergen mixture, and can identify potential allergens in a complex mixture. When combined with amino acid sequencing, MS can rapidly determine peptide sequences and provide accurate measurement of their mass. In addition, MS provides precise mass and purity assessments of recombinant allergens. The limitations of MS for complex protein mixtures are variable levels of ionization of proteins in the mixture; the need to perform peptide digests and isolation procedures; the time taken for each analysis; and the high cost of the instrumentation (typically >$500,000). MS is a sophisticated procedure that requires a high level of technical expertise. Nonetheless, it is anticipated that the instrumentation costs will fall and that MS will become more widely adopted for allergen analysis over the next 5 years.

Conclusions

Molecular approaches to allergen standardization include high throughput immunoassays (ELISA or MARIA), multiplexed allergen standards and mass spectrometry. The strengths and utility of immunoassays cannot be understated (Table 1). Immunoassays have defined specificity, are accurate, precise, and robust. They are powerful tools when used in conjunction with purified allergen standards whose protein structure and

composition is established and can be independently verified. Nonetheless, considerable time, effort, and investment are needed for immunoassay development and validation. It is important that the performance parameters for each allergen are well defined and, ideally, tested for inter-laboratory performance in multicenter ring trials. Mass spectrometry is an exciting new development which obviates the need for antibodies and can directly identify allergen isoforms in complex mixtures. The attraction of having a highly sensitive physicochemical analysis, where allergen content is simply "read" in the MS, is obvious, but also premature. Current MS instruments require highly trained technical and scientific personnel, are expensive, and take up to several hours to analyze each sample. By contrast, multiplexed immunoassays can comfortably handle >500 tests per day. It can be expected that the costs and performance of MS instruments and procedures will decrease dramatically over the next 5 years and that MS will play a significant role in standardization of biologics, including allergens, in future.

The current strategy being followed in Europe is to develop allergen standards using recombinant allergens that have essentially the same structure and allergenic activity as their natural counterparts, and to develop certified immunoassays for allergen measurement that can be included in the European Pharmacopoeia. Initial results with rBet v 1 look promising [15, 16•], and the outcome of ELISA ring trials is eagerly anticipated. An important question is how quickly this program can be accelerated to produce other purified allergen standards. Both the rBet v 1 and rPhl p 5 standards were produced under GMP conditions and provided gratis by allergen manufacturers. Whether it is feasible to maintain a GMP requirement for allergen standards is arguable because regulatory agencies would be dependent on allergen manufacturers for continued supply, which would otherwise cost ~€400,000/g for GMP production. This is a stringent and, perhaps, unnecessary requirement. The WHO international biological reference preparations (which include mite, dog, and ragweed allergen extracts) do not require GMP proteins. A good analogy can be found with cytokines which are used worldwide and are measured by ELISA or multiplexed immunoassays. The WHO maintains ~100 international biological reference preparations for cytokines at the National Institute for Biological Standardization and Control in the UK (www.nibsc.ac.uk), none of which are produced under GMP. The WHO biological reference preparations are established through standard procedures (TRS 932) in multicenter studies involving WHO Coordinating Centers (http://www.who.int/immunization_standards/vaccine_reference_preparations/TRS932Annex%202_Inter%20_biol%20ef%20standards%20rev2004.pdf). The WHO approach differs from that adopted by the EDQM in that WHO assigns potency in international biological units and not in absolute mass units, which is necessary for purified allergens. Another rationale for GMP production is that it is required for biological testing in humans, but most of the applications for allergen standards are for *in vitro* use only. These observations suggest that GMP requirements should perhaps be reconsidered, if standards are to be developed for all but a few purified allergens. One solution would be to establish a hierarchy of standards that could be established with GMP produced allergens and another for non-GMP allergens of more limited clinical significance. Animal allergens provide an example. Fel d 1, as a single immunodominant allergen and target of immunotherapy, is being produced under GMP. However, other animal allergens, Can f 1, Mus m 1, Rat n 1, Bos d 2, are of clinical significance, but are unlikely to be produced under GMP. Yet purified allergen standards are needed for these allergens.

It is critical that regulatory authorities develop purified allergen standards for allergy diagnostics and therapeutics, and for accurate and consistent measurements of environmental allergen exposure. Recent European Medicines Agency (EMEA) guidelines recommend measurement of individual allergens of clinical significance by antibody-based techniques or MS, which form part of the quality assessment for marketing authorization (see Guideline on Allergen Products: Production and Quality Issues, 2009, available at: http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500003333.pdf. In addition, EMA guidelines for the clinical development of products for specific immunotherapy recommend measurement of natural allergen exposure as part of clinical studies to establish proof of efficacy [8••]. This is because of the inherent variability of allergen exposure (both outdoors and indoors) and its potential to influence symptoms. Primary allergen standards are also needed for routine environmental exposure assessments to monitor patients' exposure to allergens, provide advice and recommendations on allergen avoidance, and to develop devices for reducing allergen exposure. While it is clear that there has been substantial progress in allergen standardization over the past decade, the process of developing and producing purified allergen standards needs to be accelerated to provide reliable and consistent allergen measurements. This will facilitate development of new diagnostics, therapeutics and environmental products that will benefit allergic patients.

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