The European Union CREATE Project: A model for international standardization of allergy diagnostics and vaccines

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Allergen measurements are used extensively in the formulation of allergy diagnostics and vaccines, yet no purified international allergen standards are available for calibration purposes. The aims of the European Union CREATE project were to develop international standards with verifiable allergen content. Purified natural and recombinant allergens were analyzed by means of SDS-PAGE, mass spectrometry, circular dichroism spectra, and small-angle x-ray scattering. IgE reactivity was assessed by means of direct RAST, RAST inhibition, immunoblotting, and basophil histamine release with sera from 961 allergic patients. Three recombinant allergens, rBet v 1, rPhl p 5a, and rDer p 2, were structurally indistinguishable from their natural counterparts and showed excellent IgE reactivity suitable for use as certified reference materials. A second tier of allergens (rPhl p 5b, rOle e1, rDer p 1, rDer f 1, and rDer f 2) was identified that could provide suitable candidates for certified reference materials with minor improvements to the recombinant proteins. Only rPhl p 1 was considered unsuitable as a reference material. Quantitative ELISAs were identified that accurately measured each allergen, except for rPhl p 1. The CREATE project has provided a major step forward in allergen standardization and provides a model for the development of a comprehensive panel of international reference preparations that will harmonize allergen measurements worldwide.
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The widespread use of allergen measurements in the fields of allergy, indoor air quality, and environmental exposure assessment has created an urgent need for internationally recognized purified allergen standards and for validated and certified immunoassays. Although allergenic products used for immunotherapy are licensed based on their total potency, allergists increasingly use specific allergen measurements for dosing of immunotherapy. Maintenance doses of 5 to 20 μg of major allergen are associated with clinical improvement after immunotherapy, and natural allergenic products are being formulated in part based on specific allergen content.[1-3] Allergens and IgE responses are the immunologic targets for allergen-specific immunotherapy. Allergen concentrations should be monitored to establish dose-response relationships between allergens and treatment efficacy, to compare allergenic products from different manufacturers, and to formulate recombinant allergen diagnostics and vaccines.[3-6]

Allergen measurements have been used most extensively to assess sensitization and exposure to environmental allergens (dust mite, animal allergens, cockroach, and molds). Epidemiologic studies, population surveys, and birth cohort studies have clearly defined levels of allergen exposure in Western populations and have found strong associations between exposure and the development of asthma.[7-14] Guidelines have been developed to indicate exposure levels that are risk factors for sensitization.[7] Allergen assays are used for testing the efficacy of allergen avoidance

Abbreviations used
CD: Circular dichroism
CRM: Candidate reference material
ESI-QTOF-MS: Electrospray-ionization quadrupole time-of-flight mass spectrometry
GMP: Good manufacturing practice
SAXS: Small-angle x-ray scattering
WHO: World Health Organization

procedures and devices and for monitoring clinical trials of avoidance and the efficacy of remediation.[11,15,16] They are used in the US indoor air quality industry for evaluation of allergen exposure in homes, the workplace, and public buildings.

Although allergen measurements have become routine, few international standards are available for calibration purposes.[3] Recombinant allergens are being used to develop new diagnostics and vaccines, yet the structural and immunologic properties of the recombinant allergens had not been systematically compared with those of their natural counterparts in international collaborative studies. The World Health Organization (WHO)/International Union of Immunological Societies Allergen Standardization Sub-committee has been influential in coordinating international standardization. The committee established WHO-approved international standards for dust mite, dog hair, and birch, timothy, and short ragweed pollens and produced the WHO position paper that recommended the use of standardized allergen vaccines of defined allergen content for dosing in immunotherapy.[17,18] The approach was also endorsed by a position statement from the American Academy of Allergy, Asthma & Immunology.[19]

In 1999, the WHO/International Union of Immunological Societies Allergen Standardization Sub-committee initiated a program to develop highly purified allergens that could be used for standardization of *in vitro* assays. This provided the genesis for a European Union–funded study entitled "Development of certified reference materials for allergenic products and validation of methods for their quantification" (acronym: *CREATE*).[3,20] The aim of the European Union CREATE project was to produce international standards of purified allergens with verifiable allergen content. Such standards would enable allergen manufacturers, academic organizations, and government and regulatory agencies to use a common international standard for specific allergen measurements. A second aim was to compare the specificity, sensitivity, and reproducibility of ELISAs for allergen analysis.

Allergens were selected for the project based on the following criteria: (1) the allergen was a major allergen of well-documented clinical importance; (2) purified natural and recombinant forms of the allergen were available in greater than 20-mg amounts from academic or commercial laboratories; (3) there was strong evidence that the recombinant allergen had equivalent IgE binding to its natural counterpart and there was extensive structural data on the allergen; and (4) ELISA kits to measure the allergen were available from 1 or more laboratories.

Purified natural and recombinant allergens, as follows, were compared in the study: pollens, Bet v 1, Phl p 1, Phl p 5, and Ole e 1; mites, Der p 1, Der f 1, Der p 2, and Der f 2.

A detailed account of the aims, scope, and methods used in the CREATE project has been published elsewhere.[21] This rostrum highlights the clinical relevance of the CREATE project and discusses how the principles applied to allergen standardization in CREATE can become a model for harmonization of allergen measurements worldwide.

CREATE PROJECT OUTLINE

Participating organizations and study design

The CREATE consortium comprised 28 organizations: 9 research laboratories, 11 clinical research groups, 6 allergen manufacturers, and 2 biotech companies from 9 European countries (Austria, Denmark, France, Germany, Italy, The Netherlands, Spain, Sweden, and the United Kingdom; see Table E1 in this article's Online Repository at www.jacionline.org). The consortium included 3 laboratories from governmental institutions involved in regulatory affairs: the Paul-Ehrlich Institute (Germany), the Istituto Superiore di Sanita (Italy), and the National Institute of Biological Standards and Control (United Kingdom), a WHO-approved laboratory for international standards. The aims of the study were (1) evaluation of the suitability of purified recombinant allergens as candidate certified reference materials (CRMs) and (2) evaluation of ELISAs for measuring specific allergens using the CRM as the standard. To achieve these goals, 20 mg or more of each natural or recombinant pollen or mite allergen was purified, and the structural properties of the natural and recombinant forms were compared by using state-of-the-art proteomic analyses (Fig 1). The IgE reactivity of the purified allergens was compared by using *in vitro* assays and by means of histamine release. IgE antibodies were obtained from allergic patients by the clinical research groups, and a CREATE serum bank was established. Immunologic reactivity was further compared in several ELISAs for each allergen by using mAbs available from the CREATE partners.

Allergen purification and analysis

Natural allergens were purified from birch, timothy, or olive pollen or from *Dermatophagoides pteronyssinus* or *Dermatophagoides farinae* spent mite medium by using standard chromatographic techniques. The recombinant allergens were produced in *Escherichia coli* expression systems, with the exception of the group 1 mite allergens (rDer p 1 and rDer f 1) and rOle e 1, which were produced in *Pichia pastoris*. In all, 18 purified allergens in lots of 20 mg or more were produced (8 natural allergens and 10 recombinant allergens), and 2 allergens, rPhl p 5a and rPhl p 5b, were produced under conditions of good manufacturing practice (GMP).

Purity of the allergens was assessed by means of SDS-PAGE, and protein content was determined by using amino acid analysis, extinction coefficient at 280 nm, and colorimetric assays. Amino acid composition and partial amino acid sequencing of allergen peptides by means of tandem mass spectrometry were used to confirm the identity of the allergens.[22] The degree of homogeneity was assessed by means of analytic size-exclusion HPLC and small-angle x-ray scattering (SAXS), which determined the extent of aggregation of the proteins and whether they were monomers, dimers, or trimers.[23] Secondary structure was compared by using circular dichroism (CD) spectroscopy.[24] The isoform composition of purified natural allergens and the extent of posttranslational modifications to the proteins were evaluated by means of electrospray-ionization, quadrupole time-of-flight mass spectrometry (ESI-QTOF-MS).[22,23]

Real-time and accelerated degradation studies were performed with allergen formulated in normal saline solution containing