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EU Forum

The CREATE Project: development of certified reference materials for allergenic products and validation of methods for their quantification

Allergen extracts have been used for diagnosis and treatment of allergy for around 100 years. During the second half of 20th century, the notion increasingly gained foothold that accurate standardization of such extracts is of great importance for improvement of their quality. As a consequence, manufacturers have implemented extensive protocols for standardization and quality control. These protocols have overall IgE-binding potencies as their focus. Unfortunately, each company is using their own in-house reference materials and their own unique units to express potencies. This does not facilitate comparison of different products. During the last decades, most major allergens of relevant allergen sources have been identified and it has been established that effective immunotherapy requires certain minimum quantities of these allergens to be present in the administered maintenance dose. Therefore, the idea developed to introduce major allergens measurements into standardization protocols. Such protocols based on mass units of major allergen, quantify the active ingredients of the treatment and will at the same time allow comparison of competitor products. In 2001, an EU funded project, the CREATE project, was started to support introduction of major allergen based standardization. The aim of the project was to evaluate the use of recombinant allergens as reference materials and of ELISA assays for major allergen measurements. This paper gives an overview of the achievements of the CREATE project.

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Executive summary

Standardization of allergen extracts for diagnosis and immunotherapy of allergy is based on biological standardization, i.e. based on IgE-binding potencies. Skin tests and competitive IgE-binding assays are important elements of allergen standardization, especially from a safety perspective. However, biological standardization does not provide specific information about the major allergen content of allergen vaccines, i.e. the content of the active ingredients needed for attaining efficacy of immunotherapy. Another disadvantage of the current system is that allergen manufacturers express potencies of their products in company-specific units that do not allow product comparison and this situation is not tenable as an international system of standardization.

In the 1980s, the World Health Organization/International Union of Immunological Societies (WHO/IUIS) Allergen-standardization Subcommittee developed International Reference Preparations (IRP) of several extracts to facilitate product comparison. Unfortunately, these IRP were not adopted by the industry or by regulatory authorities. During the 1990s, most major respiratory allergens became available as recombinant molecules, and the dependence of effective immunotherapy on administration of defined quantities of major allergen had become well-accepted. That was the moment for the WHO/IUIS committee to revive the endeavor for setting up a system of allergen standardization that would allow comparison of products and at the same time give accurate information on the content of active ingredients, i.e. of the major allergens. The initiative was supported by a grant from the European Union, and the CREATE project was born.

From November 2001 to April 2005, a consortium of basic and clinical researchers, regulators, allergen manufacturers and biotech companies carried out the project that aimed at evaluating the potential of recombinant allergens to serve as future major allergen reference materials and the potential of available sandwich enzyme-linked immunosorbent assays (ELISAs) for their accurate measurement. The approach was to produce purified recombinant allergens and compare these with their natural counterparts serving as gold standard. Nine recombinant molecules representing eight major allergens were produced: rBet v 1, rPhl p 1, rPhl p 5a and rPhl p 5b, rOle e 1, rDer p 1, rDer p 2, rDer f 1 and rDer f 2. They were compared with purified natural allergens for physico-chemical (identity, purity, folding, aggregation state, solubility and stability) and immunological (IgE-binding potency, biological activity and dose-response behaviour in ELISA) characteristics. As part of these studies, panels of sera from allergic patients with seasonal or perennial rhinitis and/or asthma were collected from clinical centres in eight countries for use in comparing IgE antibody responses and in assessments of biological potency. Overall, approximately 150–350 sera with IgE antibodies to each of the allergens were obtained, comprising a bank of 961 sera.

Three recombinant allergens displayed sufficient structural and immunological similarity, and biological potency, to their natural counterparts to be selected for a follow-up project that should ultimately lead to their establishment as international reference materials: rBet v 1, rPhl p 5a and rDer p 2. In parallel with these studies,

Abbreviations: CD, circular dichroism; CRM, certified reference material; EDQM, European Directorate for the Quality of Medicines; ELISA, enzyme-linked immunosorbent assayGMP, good manufacturing practice; HPLC, high-performance liquid chromatography; IRP, International Reference Preparation; IS, International Standard; IUIS, International Union of Immunological Societies; MS, mass spectrometry; PAS, periodic acid Schiff; PDDF, pair distance distribution function; RAST, radio allergosorbent test; SAXS, small-angle X-ray scattering; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; SEC, size-exclusion chromatography.WHO, World Health Organization.

several ELISAs were evaluated for the measurement of major allergens. For most allergens one or two ELISAs were identified that showed comparable dose response curves for the recombinant and natural allergens (a requirement for measurement of major allergens in allergenic products, using a recombinant reference). This fulfilled an important aim of the CREATE project which was to develop purified allergen references together with complementary ELISA systems.

A follow-up project has recently been initiated by the CREATE partnership and the European Directorate for the Quality of Medicines (EDQM). Together, three allergen manufacturers from CREATE provided EDQM with sufficient good manufacturing practice (GMP)-grade rBet v 1 and rPhl p 5a and with two suitable ELISA kits per allergen. After physico-chemical characterization of both allergens, these reagents will be used in a ring trial together with the selected ELISAs to validate their applicability in allergen standardization based on mass units of major allergen. One of the conclusions from CREATE is that certified recombinant references will have to be linked to certified assays so that products can be directly compared on the basis of mass units of major allergen. It is expected that the rBet v 1 and rPhl p 5a allergens developed through CREATE will be established as international references by the end of 2008 and will become part of the European Pharmacopeia. The task for the future will be to apply the approach used in CREATE to produce a repertoire of purified allergens that can be used as international references for standardization purposes and to harmonize allergen measurements worldwide.

WHO/IUIS allergen-standardization initiatives

The need for a coherent strategy to develop standards for allergenic products was recognized by the WHO/IUIS Allergen-standardization Subcommittee in the 1970s. In 1981, the committee established a programme, funded by academic organizations and allergen manufacturers, to produce allergen extracts that would meet WHO specifications as International Standards (IS). The potency of candidate reference preparations was compared using *in vivo* and *in vitro* testing, including skin tests, radio allergosorbent test (RAST) inhibition, crossed radio immuno-electrophoresis and measurements of specific major allergens. Following a series of international collaborative studies, WHO-approved IS were established for birch, timothy and short ragweed pollens, dust mite (*Dermatophagoides pteronyssinus*) and dog hair (Table 1) (1–9). The IS were freeze-dried and stored in ampules at −70°C at the National Institute of Biological Standards and Controls (Potters Bar, UK), which is a WHO-approved repository. Approximately 4000 ampules of the IS were prepared and each IS was allotted a potency of 100 000 IU.

Table 1. International Standards and Reference Reagents produced by the WHO/IUIS Allergen-standardization Sub-committee (1981–1990)

WHO International Standards	Code no.	International units
Timothy pollen extract	NIBSC 82/520	100 000 (8)
Short ragweed pollen extract	NIBSC 84/581	100 000 (1)
Dermatophagoides pteronyssinus extract	NIBSC 82/518	100 000 (3, 4)
Birch pollen extract	NIBSC 82/522	100 000 (9)
Dog-hair/dander extract	NIBSC 84/685	100 000 (7)
International Reference Reagent Bermuda grass pollen extract	ATCC	(2)

WHO IS are available from NIBSC (http://www.nibsc.ac.uk). A reference serum pool from mite allergic individuals was also produced as part of these studies (NIBSC 82/528) (3, 4)

The WHO/IUIS programme to establish allergen standards was led by Drs Henning Lowenstein, Philip Norman, Thomas Platts-Mills and Alain de Weck. The aim of the programme was to develop IS that could be used a 'yardsticks' to which other allergen extracts could be compared for both total potency and specific allergen content (10, 11). The programme did much to establish the importance of allergen standardization within the academic allergy community and encouraged allergen manufacturers to increase their in-house standardization efforts. The intent was to establish standards for a large panel of allergenic products. However, this ambitious goal was compromised because regulatory agencies in Europe and in the USA did not adopt the WHO IS as national standards. There was no statutory requirement that allergen manufacturers use the IS as standards in the licensing of allergenic products and the standards were not adopted.

Nonetheless, the WHO/IUIS standards were important references for research purposes. In most cases, estimates were made of the major allergen content of the IS in absolute units μg/ampule. At that time, monoclonal and polyclonal immunoassays were being developed for important allergens (e.g. Der p 1, Der f 1, Can f 1, etc) and the WHO/IUIS standards provided an important resource of standards with known allergen content. The Der p 1 content of the *D. pteronyssinus* IS (NIBSC 82/518) was estimated at 12.5 μg/ampule. Measurements of Der p 1 over the past 25 years have been calibrated based on this standard (2, 3). The IS were also used for measuring ragweed allergen Amb a 1 and dog allergen, Can f 1 (1, 7). The use of the IS established the principle of using major allergen measurements as a key element of allergen standardization, a position which was presented at international seminars on the 'Regulatory Control and Standardization of Allergenic Extracts' held at the Paul-Ehrlich-Institut and by a Position Statement of the American Academy of Allergy, Asthma and Immunology (11–13).

Measurement of specific allergens became increasingly important in several areas of allergy and immunology. The most widespread use of allergen measurements was in

environmental studies to assess sensitization and exposure to indoor allergens (dust mite, cat, dog, cockroach, rodents) in relation to chronic allergic diseases (rhinitis, asthma and atopic dermatitis) (14). In addition to epidemiologic studies, allergen measurements were also used to assess the aerodynamic properties of allergens, their environmental distribution and the effects of allergen control procedures, products and devices. Together, assays in these research areas accounted for the largest use of major allergen measurements. The widespread use of molecular cloning in the 1990s facilitated the identification of the most important major and minor allergens and the use of purified allergens for diagnostic and therapeutic purposes. It was envisaged that measuring IgE to a panel of two to four major allergens from a given source could be used for allergy diagnosis and that standardization of purified allergens would be much less complex than for heterogeneous allergenic products (15–17). Moreover, measurements of major allergens could be used to more closely monitor the doses of allergens that were used in allergen vaccines for conventional immunotherapy. In 1997, the WHO/IUIS Allergen-standardization Sub-committee participated in a conference in Geneva, Switzerland, to discuss the use of allergen vaccines in immunotherapy. This conference was organized by the then Chair of the WHO/IUIS committee Dr Jean Bousquet, together with Drs Richard Lockey and Hans-Jorgen Malling, The WHO Position Paper that was produced from the conference recommended the use of standardized allergy vaccines of defined allergen content for immunotherapy to achieve maximal clinical efficacy. The Position Paper reviewed approximately 20 studies comparing the doses of major allergens that had been used in successful immunotherapy studies and concluded that ''There is good evidence from immunotherapy studies with ragweed, grass, mite, cat and venom allergens that a maintenance dose of 5–20 μg of major allergen per injection is associated with significant improvement in patient symptom scores' (18). Recent studies of the efficacy of immunotherapy for cat and dog allergy have confirmed these maintenance doses (19–21).

Over the past 20 years, the WHO/IUIS Allergen-standardization Sub-committee has played an influential role in promoting and coordinating international standardization efforts and in producing standards that are still being used today. In 1999, the committee, under the leadership of Drs Martin Chapman and Ronald van Ree, embarked on a new initiative to develop purified allergen standards that could be used for the standardization of *in vitro* assays. The use of specific allergen measurements for environmental studies and for assessing the allergen content of diagnostic and therapeutic products was widespread, and yet there were no approved purified allergen standards that could be used for calibration purposes. Recombinant allergens were being widely used for research and in clinical trials to produce a new generation of allergy vaccines, yet the immunological reactivity of the recombinant allergens and their natural counterparts had not been systematically compared in international collaborative studies. This background provided the genesis for the European Union CREATE project (22). The aim was to produce IS of purified natural or recombinant allergens with *verifiable* allergen content. This would enable allergen manufacturers, academic organizations, government and regulatory agencies to use a common IS as a reference for specific allergen measurements. A second aim was to compare the specificity, sensitivity and reproducibility of ELISA assays that were being used for allergen analysis.

Allergens were selected for the project based on the following criteria:

- The allergen was a major allergen of well-documented clinical importance.
- Purified natural and recombinant forms of the allergen were available in >20 mg amounts from academic or commercial laboratories.
- Strong evidence that the recombinant allergen had equivalent IgE-binding to its natural counterpart and extensive structural data on the allergen.
- ELISA kits to measure the allergen were available from one or more laboratories.

A consensus was reached and the following allergens were included in the study:

Pollens – Bet v 1, Phl p 1, Phl p 5, Ole e 1.

Mites – Der p 1, Der f 1, Der p 2, Der f 2.

It was recognized that this list excluded other important allergens, such as ragweed Amb a 1 and cat allergen, Fel d 1. However, these allergens did not fully satisfy the selection criteria and it was considered that if the study was otherwise successful, such allergens could be taken up as part of a second phase. Dr Ronald van Ree was the project co-ordinator for a grant application that was funded by the European Union under the Fifth Framework Programme in 2000.

Partnership and aims of CREATE

The full title of the CREATE project was 'Development of Certified Reference Materials for Allergenic Products and Validation of Methods for their Quantification' (22). The project partnership consisted of 28 organizations, i.e. six allergen manufacturers, two biotech companies, 11 clinical researchers and nine research laboratories (Table 2). The research laboratories included three regulatory agencies from Germany (Paul-Ehrlich-Institute), from Italy (Istituto Superiore di Sanità) and from the UK (National Institute for Biological Standards and Control). The latter organization also is a WHO-approved repository. The partnership originated from nine EU member-states: Netherlands, Spain, UK, Germany, Austria, Italy, Sweden, Denmark and France.

Table 2. Partnership of CREATE

Research Laboratories		
Sanquin Research (co-ordinator)	R. van Ree /M. Aalbers/S. Notten/ P. Ooievaar-de Heer	NL
Universität Salzburg	F. Ferreira/M. Himly/M. Wallner/G. Gadermaier	AT
Universidad Complutense Madrid	M. Villalba/R. Rodriguez	ES
Forschungszentrum Borstel	W-M. Becker	DE
Berufsgenossensch. Forschungsinstitut für Arbeitsmedizin	M. Raulf-Heimsoth	DE
Universität Wien	M. Focke/R. Valenta	AT
Research Laboratories with regulatory role		
National Institute for Biological Standards and Control	D. Bryan/C. Dolman	UK
Paul-Ehrlich-Institut	S. Vieths/K. Fötisch	DE
Istituto Superiore di Sanità	G. di Felice/C. Pini	IT
Allergen manufacturers		
Allergopharma Joachim Ganzer	O. Cromwell/H. Fiebig/B.Weber	DE
HAL Allergy	H. van Schijndel/J.W. Dorpema	NL
ASAC Pharmaceutical Int.	F.M. Marco	ES
ALK-Abelló	R. Monsalve/D. Barber	DK
CBF Leti	E. Fernandez Caldas	ES
Stallergènes	P. Moingeon/A. Didierlaurent	FR
European Allergen Manufacturers Group	A. Kroon	EU
Biotech companies		
Biomay	A. Neubauer	AT
Indoor Biotechnologies	M. Chapman/L. Vailes/A. Tsay	UK
Clinical researchers		
Imperial College (London)	S. Durham	UK
North West Lung Centre (Manchester)	A. Custovic/B. Simpson	UK
University Medical Centre (Utrecht)	A. Knulst	NL
Fundación Hospital Alcorcón (Madrid)	M. Fernandez Rivas	ES
Forschungszentrum Borstel	U. Lepp/F. Eberhardt	DE
Institut Universitari Dexeus (Barcelona)	A. Cistero Bahima/M. San Miguel Moncin	ES
Adriano Mari (as private clinician / Rome)	A. Mari	IT
University Medical School of Vienna	T. Kinaciyan	AT
Hospital Ciudad de Jaén	J. Quiralte	ES
Hôpiteaux Universitaires de Strasbourg	G. Pauli/A. Purohit	FR
Sahlgrenska University Hospital (Götenborg)	S. Rak	SE

Allergen standardization has previously been dominated by IgE-based biological standardization (23). Overall IgE-binding potencies of extracts are monitored by skin reactivity and by competitive IgE tests like RAST- or ImmunoCAP-inhibition. Potencies are expressed in company-specific units, relative to in-house reference preparations. By focusing on overall IgE-binding potencies, current allergen standardization requirements concentrate on the safety aspect of allergen products. To some extent this is understandable because IgE-mediated reactions are responsible for the immunological events that result in adverse reactions to immunotherapy. The problem with total potency measurements is that they do not include the content of major allergens. It is well accepted that the presence of sufficient major allergen is crucial for the outcome of treatment (18, 21). Major allergens are the active ingredients of allergen-specific immunotherapy and their presence should therefore be monitored. Quantification of major allergens as active ingredients of immunotherapy will not only help to further establish the dose–response relation between allergen and treatment efficacy, but it will also allow comparison of allergenic products from different companies. A system of allergen standardization based on micrograms of major allergen requires internationally recognized reference materials, as well as validated assays for their measurement. The aims of the CREATE project were formulated based on these needs:

- Evaluation of the potential of purified recombinant allergens as certified reference materials (CRM).
- Evaluation of available ELISAs for measurement of major allergens using the candidate CRM as standard.

The logo of the CREATE project (Fig. 1) symbolizes the transition from extract-based standardization to molecular standardization, in this case represented by the transformation of a mite body into the Der p 2 major allergen molecule (24). The advancement of allergen standardization based on mass units of major allergen is

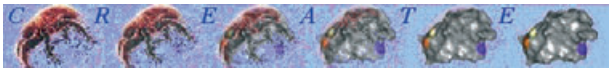

Figure 1. CREATE logo. The CREATE logo symbolizes the transition from extract-based (mite body) to molecule-based (3D-structure of Der p 2) standardization.

furthermore a logical counterpart of the development of recombinant allergen-based immunotherapy to replace extract-based approaches.

The objectives of the CREATE project included the production of purified natural and recombinant allergens (20 mg of each); detailed physico-chemical characterization and stability testing; characterization of IgE-binding potencies and biological activity; and performance evaluation as standards in ELISA in respect of such natural and recombinant allergens. An overview will be given of the results obtained for each of these areas of investigation.

Allergen production and purification

In total, 18 purified allergen preparations (eight natural allergens and 10 recombinant versions) were produced by the CREATE consortium. Table 3 lists these 18 allergen preparations, their extraction sources or host organisms used for the recombinant production, and purification strategies. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie staining showed that all proteins were >98% pure (not shown). Periodic acid Schiff (PAS) staining after SDS-PAGE and blotting onto nitrocellulose membranes revealed the presence of sugars on group 1-mite allergens (nDer p 1, rDer p 1, nDer f 1, rDer f 1), nPhl p 1 and on natural and recombinant Ole e 1 (data not shown).

Physico-chemical characterization

Physico-chemical parameters selected for evaluating the quality of purified natural and recombinant allergens (25, 26) were identity, purity, homogeneity and (secondary) structure (folding):

Identity

The allergens must display an amino acid composition and sequence that is in agreement with the known (published) primary sequence(s). Amino acid analysis and mass spectrometry (MS) were the methods selected for this task.

Purity

The allergen preparations must be at least 95% pure with respect to protein content. The method of choice was SDS-PAGE in conjunction with silver staining. Amino acid analysis provided additional information on the purity.

Homogeneity

Ideally, recombinant allergen preparations are homogeneous with respect to molecular size. One method to assess this is analytical size-exclusion chromatography (SEC) or gel filtration. The sample should then contain a single peak (>95% of the area under the curve) of the expected molecular size, or in some cases of a molecular size representing e.g. a dimer or trimer, because some proteins behave as oligomers under 'native' conditions (in solution). Multiple peaks can point towards impurities, but could also be explained by partial oligomerization/aggregation. Small angle X-ray scattering (SAXS) was used as an additional technique to monitor molecular size and aggregation. Expression of recombinant proteins in eukaryotic expression systems like *Pichia pastoris* can result in variable post-translational modifications (e.g. glycosylation). If capable of delivering well-folded protein, production of recombinant allergens in prokaryotic systems like *Escherichia coli* with a limited machinery for post-translational modifications therefore has preference. For purified natural allergens, deviations from homogeneity can be accepted if explained by the presence of multiple isoforms with significantly different molecular size, or by the existence of nonglycosylated and glycosylated versions.

Structure

Purified recombinant allergen preparations must be correctly folded proteins. Circular dichroism (CD) spectroscopy was used to evaluate folding, i.e. secondary structure (α-helix, β-sheet and random coil; Fig. 1). Several candidate molecules were correctly folded and showed typical spectra of folded proteins with peak amplitudes similar to reference spectra of natural allergen preparations (Fig. 2). Table 4 gives an overview of the methods used for physico-chemical characterization of the candidate allergens and the information that can be derived concerning identity, purity, homogeneity and secondary structure.

Amino acid analysis

Amino acid analysis is an indispensable tool in the analysis of protein pharmaceuticals. When performed on purified proteins, the technique allows the identification and quantification of the protein, providing more accurate results than colorimetric methods. The accuracy of amino acid analysis depends on the integrity of the sample and other factors including purity of the reagents used, presence of salts, metals or detergents and sample handling (27). All allergen preparations in CREATE were quantified using amino acid analysis to assure standardization of protein measurements in mass units. Identity of all allergen preparations was confirmed by close correlations found between the theoretical and experimental amino acid composition e.g. for Bet v 1 (Fig. 3A). An example revealing likely contamination was the analysis of nPhl p 1 (Fig. 3B). An increase in alanine content

Table 3. Purification of recombinant allergens and their natural counterparts

Allergen	Source	Purification method	Remarks
Bet v 1	Natural (birch pollen)	Gel filtration, anion exchange, IMAC	Method developed by ALK; Cu^{2+} used as immobilized metal ion
	Recombinant (*E. coli*)	Phosphate precipitation, hydrophobic interaction, anion exchange, RP-HPLC	Method developed by Biomay AG; soluble and inclusion bodies (solubilized in urea) fractions combined for purification
Phl p 1	Natural (timothy grass pollen)	Hydrophobic interaction, gel filtration	Method developed by Allergopharma
	Recombinant (*E. coli*)	Ammonium sulfate precipitation, anion exchange, hydroxyapatite chromatography	Method developed by Biomay AG; protein purified from inclusion bodies
Phl p 5	Natural (timothy grass pollen)	(1) hydrophobic interaction, gel filtration, (2) immuno-affinity chromatography	Method (1) developed by Forschungsinstitut Borstel; Method (2) developed by Allergopharma
	Recombinant Phl p 5a (*E. coli*)	IMAC, TEV cleavage, IMAC	Produced by Allergopharma (GMP); His-tag cleaved by His-tagged TEV; purified from soluble fraction
	Recombinant Phl p 5a (*E. coli*)	Ammonium sulphate precipitation, cation exchange, hydrophobic interaction	Method developed by Biomay AG
	Recombinant Phl p 5b (*E. coli*)	IMAC, TEV cleavage, IMAC	Produced by Allergopharma under GMP conditions; His-tag cleaved by His-tagged TEV; purified from soluble fraction
Ole e 1	Natural (olive pollen)	Two gel filtration steps	Method developed by Universidad Complutense Madrid
	Recombinant (*Pichia pastoris*)	Anion exchange, gel filtration, RP-HPLC	Method developed by Universidad Complutense Madrid; purified from culture medium
Der p 1	Natural (*D. pteronyssinus*)	Immuno-affinity chromatography, SEC-HPLC	Method developed by Indoor Biotechnologies; purified from mite bodies-free culture
	Recombinant (*Pichia pastoris*)	SEC-HPLC	Method developed by Indoor Biotechnologies; purified from culture medium
Der f 1	Natural (*D. farinae*)	Immuno-affinity chromatography, SEC-HPLC	Method developed by Indoor Biotechnologies; purified from mite bodies-free culture
	Recombinant (*Pichia pastoris*)	SEC-HPLC	Method developed by Indoor Biotechnologies; purified from culture medium
Der p 2	Natural (*D. pteronyssinus*)	Immuno-affinity chromatography, SEC-HPLC	Method developed by Indoor Biotechnologies; purified from mite bodies-free culture
	Recombinant (*E. coli*)	Immuno-affinity chromotography, SEC-HPLC	Method developed by Indoor Biotechnologies; purified from inclusion bodies
Der f 2	Natural (*D. farinae*)	Immunoaffinity chromatography, SEC-HPLC	Method developed by Indoor Biotechnologies; purified from mite bodies-free culture
	Recombinant (*E. coli*)	Immuno-affinity chromatography, SEC-HPLC	Method developed by Indoor Biotechnologies; purified from inclusion bodies

TEV, tobacco etch virus protease; IMAC, immobilized metal ion affinity chromatography; SEC, size-exclusion chromatography; HPLC, high performance liquid chromatography; RP, reversed phase; GMP, good manufacturing practice.

D. pteronyssinus and *D. farinae* culture for mite allergen purification was provided by Laboratorios Leti S.L., Madrid, Spain.

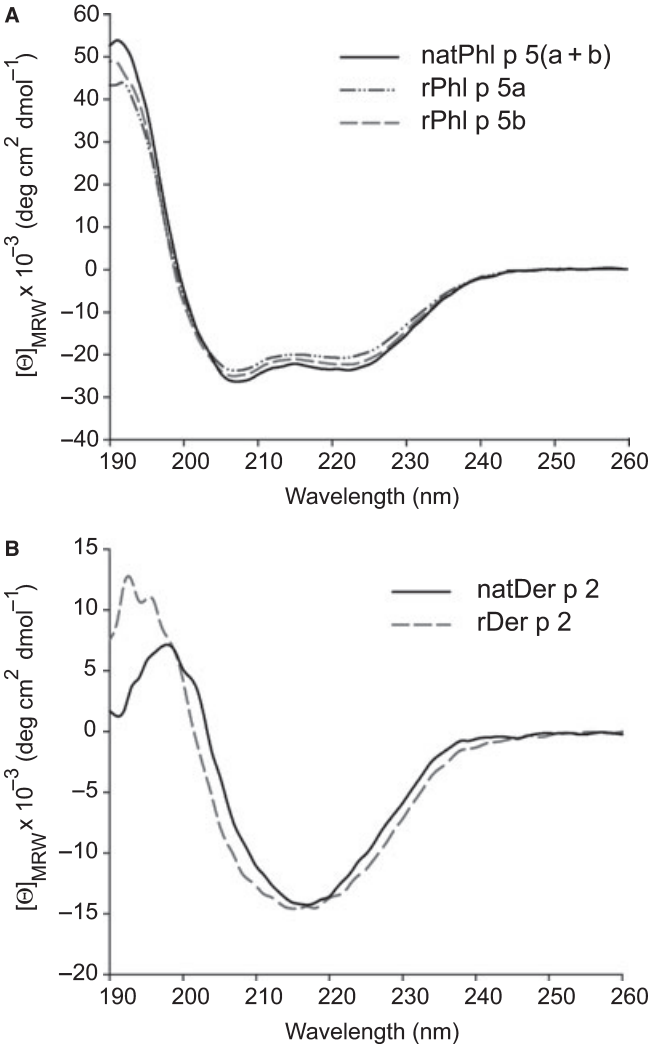

Figure 2. CD spectroscopy. (A) CD spectra of the Phl p 5 preparations evaluated in CREATE, representing an allergen dominated by α-helical structures. (B) CD spectra of the Der p 2 preparations evaluated in CREATE, representing an allergen dominated by β-sheet structures.

pointed towards contamination with Phl p 5, which has been shown to be rich in alanine (approximately 30%).

Mass spectrometry

Mass spectrometry of the allergen preparations reveals whether the expected molecular mass is the dominant component, but more reliable confirmation of identity requires digestion. A given protein with a known primary sequence can be digested with a specific protease like trypsin to give rise to a predictable set of peptides. Accurate determination of the masses of even a few peptides generated through trypsin digestion (to an accuracy of 1 Dalton) can be compared to the masses of peptides obtained through *in silico* digestion of the known protein sequence, upon which matching masses

Table 4. Parameters and selected analytical methods for the physico-chemical characterization of Candidate Reference Materials in the CREATE project

	Identity	Purity	Homogeneity	Secondary structure (folding)
Amino acid analysis	Amino acid content according to primary structure	Contamination by other proteins might change the content of individual amino acids		
Mass spectrometry (MS)/peptide mapping	Molecular mass according to primary structure. MS/MS allows confirmation of primary structure	Contamination by other proteins, glycosylation heterogeneity or nonprotein compounds gives rise to additional peaks	Post-translation modifications and chemical modifications resulting from the recombinant production can be detected and accounted for heterogeneity	
HPLC size-exclusion chromatography (HPLC-SEC)		Contamination by proteins with different molecular weight might give rise to additional peaks	Homogeneity in solution: oligomeric/monomeric forms, aggregates, and degradation products can be detected	Changes in retention times might also be attributable to denatured protein
Circular dichroism (CD)				Folded status of proteins. Quantification of secondary structure elements is possible, but usually not reliable. Thermo-stability
Small angle X-ray scattering (SAXS)			Molecular size and aggregation status of proteins in solution	Denatured proteins might show altered PDDF
SDS-PAGE/PAS staining		Contaminating proteins with different molecular weight can be detected	Post-translation modifications, aggregation, or degradation might give rise to additional bands. Sugars can be detected	

PDDF, pair distance distribution function.

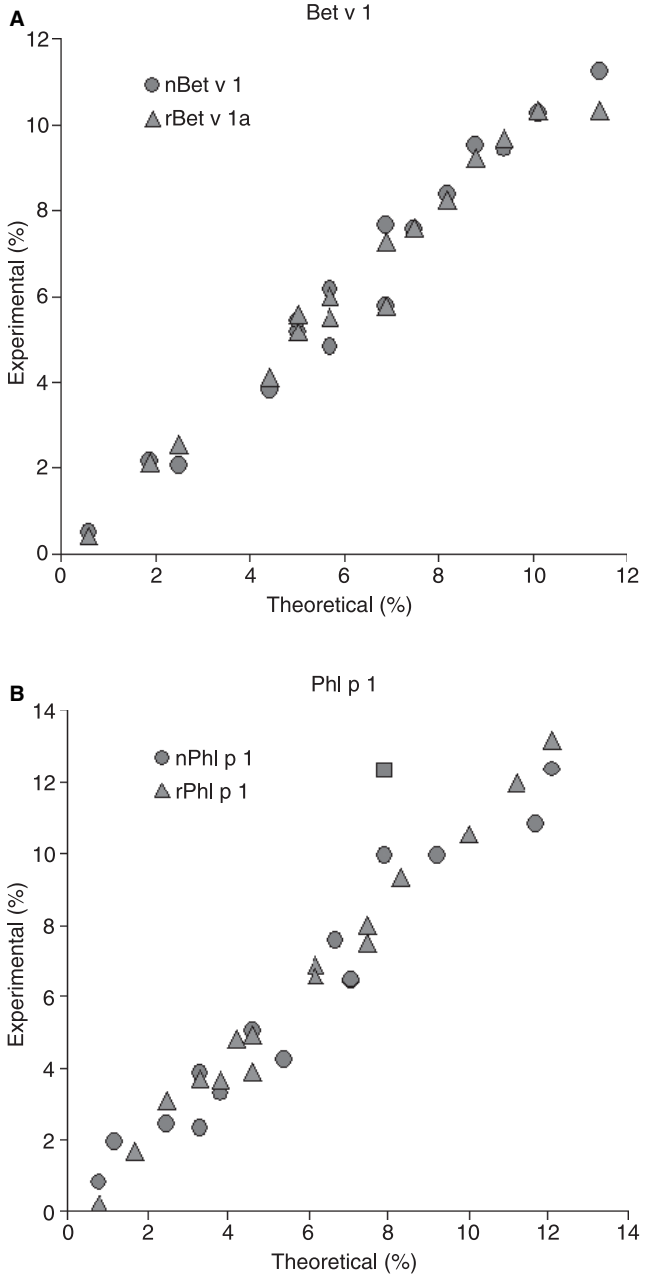

Figure 3. Amino acid composition analysis: identity and purity. Each data point represents an amino acid. There is an excellent correlation between the expected (theoretical) and observed (experimental) amino acid composition (identity). Graph A shows natural and recombinant Bet v 1, graph B natural and recombinant Phl p 1. For the natural allergens, the average of known isoforms was calculated for the theoretical value. In case of natural Phl p 1, a single amino acid (alanine) deviates from theoretical (indicated by a square) suggesting contamination with alanine rich (30%) Phl p 5.

are assigned to predicted peptides. However, the unambiguous confirmation of identity is only possible after sequencing the peptides. This requires tandem MS, which is usually denoted MS/MS (28, 29). During this process, peptides identified during the first MS are dissociated, amino acid by amino acid, by collision. The mass spectrometer determines the mass after each subsequent collision-induced removal of an amino acid. The mass differences will provide information about the nature of the amino acid cleaved off. In the CREATE project, peptides were sequenced using electrospray-ionization and collision-induced dissociation in a quadrupole time-of-flight instrument. This technique also allows the isoform composition of natural purified allergen preparations to be evaluated. Full sequencing of protein molecules using MS/MS is rarely achieved because not all peptides can effectively be ionized, which is necessary to 'fly' to the detector. For some allergens, $>95\%$ of the sequence was confirmed, e.g. for rBet v 1a, rPhl p 5a, rPhl p 5b and rDer f 2. For all allergens (except rPhl p 1), MS/MS provided convincing evidence of identity, including information about isoform-composition of natural allergens, and the presence of post-translational modifications of amino acids by e.g. glycosylation, carbamylation, deamidation and oxidation.

High-performance liquid chromatography-size-exclusion chromatography

In the CREATE project, purified natural and recombinant allergen preparations were analysed by SEC using a HP 1100 HPLC system (Agilent Technologies, Palo Alto, CA, USA) with a TSK-Gel G2000SWXL column (Tosoh Biosep, 7.8mm ID $\times$ 30.0 cm/L). All but two of the natural allergens presented as essentially homogeneous preparations, eluting at a volume indicative for a monomeric appearance. Natural Ole e 1 and nDer p 1 were heterogeneous in solution. The sensitivity of high-performance liquid chromatography (HPLC)-SEC did not allow firm conclusions to be drawn about the nature of the observed heterogeneity, but variable degrees of glycosylation and degradation are possible explanations. Heterogeneity was more common among the recombinant allergens. The elution profiles of rBet v 1, rPhl p 5a, rPhl p 5b and rDer f 2 showed a single homogeneous peak suggesting that these allergens were monomeric.

Circular dichroism spectroscopy

Circular dichroism spectroscopy is widely applied to evaluate folding of allergen molecules. Circular dichroism analysis of proteins uses the differential absorption of left and right circularly polarized light in the far UV region by chiral chromophores. In proteins, peptide bonds serve as chromophores. Absorption characteristics of peptide bonds are measurable depending on the environment and therefore on the secondary structure surrounding the chromophore. CD spectra give valuable information on

the folding of proteins (30, 31). In the CREATE project a protocol was set up to compare a series of protein pairs (natural and recombinant) in terms of their secondary structures and thermo-stability. Measurements were performed with a JASCO J-810 spectro-polarimeter fitted with a Neslab RTE-111M temperature control system. For the major allergens under investigation in CREATE, three-dimensional structures were either reported based on X-ray crystallography and/or NMR, or modeled on the basis of homology with related and structurally defined proteins. On the basis of this information, secondary structure elements of correctly folded molecules could be predicted. Bet v 1 is dominated by β-sheets with a single α-helix. The structure of Phl p 1 has not been determined, but that of Zea m 1, the homologous allergen from corn pollen, displays a two-domain structure dominated by β-sheets. Phl p 5 is a typical α-helical protein. No structure is available for Ole e 1. Group 1 allergens from house dust mites belong to the papain-like cysteine proteases and display a typical mixed α-helical and β-stranded structure, whereas the group 2 allergens have a typical immunoglobulin-like tertiary fold with two anti-parallel β-pleated sheets.

Natural and recombinant versions of Bet v 1, Phl p 5 and Der f 1 gave CD spectra in close agreement with each other and with their known tertiary structures. Ole e 1, Der p 1 and the group 2 house dust mite allergens showed the expected CD spectra based on tertiary structures, though some differences were observed in peak amplitudes. The secondary structure of Phl p 1 did not agree with its proposed structure based on the crystal structure of the homologous allergen from corn pollen (Zea m 1). The natural protein displayed some random coil structure, but no clear evidence of the expected β-sheet structure. The recombinant protein completely lacked organized secondary structure.

Upon heating to 95°C, the CD spectra of all allergen preparations with the exception of Phl p 1 changed to that of a random coil. This is a normal behaviour of polypeptides undergoing thermal unfolding. In general, the calculated values of melting temperature (Tm) were very similar for natural and recombinant preparations of the same allergen. Upon cooling to 25°C, Bet v 1, Phl p 5 and rDer f 1 showed CD spectra similar in shape and peak amplitudes to those recorded before heating suggesting that these proteins are able to correctly refold after thermal denaturation. Both natural and recombinant Ole e 1 were partially refolded after thermal denaturation. In contrast, thermal denaturation irreversibly denatured all the other natural and recombinant house dust mite groups 1 and 2 allergens.

Small-angle X-ray scattering

Small-angle X-ray scattering resembles the familiar situation when a beam of visible light is scattered by a colloidal suspension like milk or when the front lights of a car are scattered in the mist (32). Clear solutions of nonaggregated proteins, in contrast, require an electromagnetic beam of much smaller wavelength to interact with. Monochromatic X-rays are guided through the protein solution and the scattering curve (intensity × scattering vector) is recorded. Using indirect Fourier transformation, the pair distance distribution function (PPDF; distance frequency × dimension) can be calculated from the scattering curve (33). The PDDF expresses the frequency of intra-molecular distances between electrons. Thus, conclusions on the molecular size, shape and aggregation behaviour of proteins in solution can be drawn (33, 34).

For the CREATE samples, measurements were performed with the SAXSess camera (Anton Paar KG, Graz, Austria) attached to a conventional copper Kα X-ray generator (Philips, Eindhoven, Netherlands). Data evaluation was performed by indirect Fourier transformation (33) using the PCG software package (Karl-Franzens, University of Graz). Briefly, SAXS measurements showed that five of the allergen preparations (nBet v 1, nPhl p 1, nDer p 1, nDer f 1 and nDer p 2) could be assigned to monomeric or dimeric molecules. Six allergens (rBetr v 1a, rPhl p 5a, rPhl p 5b, nOle e 1, rDer p 2 and nDer f 2) displayed some tendency to oligomerize at the rather high concentrations (3.3 mg/ml) used in the SAXS measurements. A nonaggregated state (i.e. not multimerized) could be assigned to 11 of the 17 samples tested. Three preparations (rDer p 1, rDer f 1 and rDer f 2) showed moderate aggregation and rOle e 1 showed extensive aggregation. Two allergen preparations (rPhl p 1 and nPhl p 5) did not give conclusive results, probably on account of low protein concentration in the case of rPhl p 1 or a too high scattering contribution of the buffer in the case of nPhl p 5.

Immune reactivity

Another criterion to judge the quality of candidate reference molecules was their immune reactivity relative to the reactivity of their purified natural counterparts. Within CREATE, immune reactivity was essentially analysed in two ways: IgE reactivity and monoclonal antibody (mAb) reactivity in ELISA. IgE reactivity was analysed in four ways:

- Direct IgE-binding in RAST
- Direct binding in dot-blot
- RAST-inhibition
- Biological activity in basophil histamine release

RAST, RAST inhibition and dot-blot analysis

For RAST analysis, natural and recombinant versions of the eight major allergens evaluated in CREATE were coupled to Sepharose (35). For each of the four allergen

Table 5. IgE-binding potencies of recombinant allergens

Allergen	No. patients	Mean RAST (±SE) (IU/ml) natural allergen	Mean ratio rec/nat (sera >0.3 IU/ml)	*P*-value (paired *t*-test)
Bet v 1	187	17.5 (±2.6)	1.0	>0.2
Phl p 1	345	40.2 (±2.2)	0.6	<0.001
Phl p 5		24.5 (±1.3)	0.7/0.5*	<0.001
Ole e 1	166	10.9 (±1.9)	0.6	<0.001
Der p 1	280	6.4 (±0.6)	0.5	<0.001
Der p 2		10.9 (±1.3)	0.7	<0.001
Der f 1		5.1 (±0.4)	0.5	<0.001
Der f 2		10.6 (±1.4)	0.8	<0.001

* rPhl p 5a/rPhl p 5b.

sources (birch, grass and olive pollen and house dust mite), the clinical partners of CREATE collected sera of patients with a positive SPT and a convincing history of symptoms upon exposure. These sera were used in RAST analyses to compare the IgE-binding potencies of natural and recombinant allergens. Linear regression analysis showed a very good quantitative relationship between IgE antibody levels to natural and recombinant allergens for each of the allergens tested ($R_{Spearman} > 0.9$; $P < 0.01$). The tightest correlation was seen for Bet v 1, with other allergens showing broader distributions of data points, especially at low IgE levels (< 1IU/ml). Although the levels of allergen-specific IgE were highly correlated, with the exception of Bet v 1, recombinant allergens showed a mean of 50–80% of the IgE antibody binding of their natural counterparts (Table 5). Recombinant Bet v 1 showed almost identical IgE binding as the natural allergen. These results suggested that in many cases the recombinant allergens could be used for *in vitro* diagnostic purposes, but that in some cases improvements to the recombinant allergens would be needed to achieve equivalence with the natural allergens. Dot-blot analysis (Fig. 4) confirmed the similarity in IgE-binding potency of some natural and recombinant allergens (e.g. Bet v 1) but demonstrated that differences were more significant for other (e.g. Phl p 1). Radio allergosorbent test-inhibition analysis was used to quantify the differences in IgE-binding potencies. Only rBet v 1 and rDer f 2 demonstrated similar inhibitory potency as their natural counterpart. For all the other allergens, recombinants were at least 10-fold weaker in RAST-inhibition. For recombinant versions of Phl p 1 and Der p 1, the observed difference was >100-fold. This implies that the minor decrease in direct binding (≤ 2-fold) is in fact an overestimation of IgE-binding potencies. This can most likely be explained by the high allergen concentrations used in RAST (or CAP) resulting in saturating assay conditions, even for correctly folded allergen being underrepresented in some of the recombinant allergen preparations.

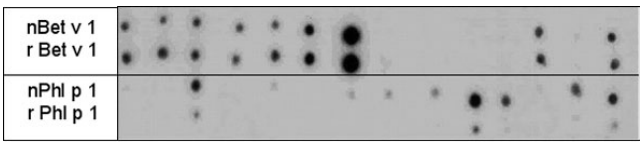

Figure 4. Dot blot analysis: natural *vs* recombinant. This dot blot illustrates that sera of birch pollen-allergic patients see no significant difference between nBet v 1 and rBet v 1a. On the other hand, grass pollen-allergic patients clearly react weaker to rPhl p 1 than to nPhl p 1.

Biological activity

Another way to evaluate the immune-reactivity of recombinant allergens is by comparing their potency to induce mediator release from basophils. In CREATE, we used the so-called stripped basophil protocol (36) in which basophils from a nonallergic donor are treated with lactic acid to remove IgE. These cells were subsequently re-sensitized with IgE from patients' sera. Using 25 different sera of allergic patients per allergen, each of the recombinant allergens of CREATE was compared to its natural counterpart with respect to their potency to induce histamine release (biological activity). In some cases, the biological activity was in agreement with the potency detected by RAST-inhibition (Bet v 1, Phl p 1, Der f 1 and Der f 2). For Phl p 5a and Phl p 5b, Ole e 1, Der p 1 and Der p 2 the biological activity was higher as was expected on the basis of RAST-inhibition. In two cases (Phl p 5 and Ole e 1), recombinant allergens were significantly more active than their natural counterparts (approximately 10-fold). A possible explanation for these observations could be that oligomerization and aggregation favour efficient histamine release by increasing the epitope valency (repetitive epitopes). Alternatively, the chosen isoforms for recombinant production could exhibit higher allergenicity.

Overall, the IgE-based analysis demonstrated that simple direct binding assays were not sufficient to get a good impression of the IgE-binding characteristics and allergenic potency of recombinant allergens. Competitive and functional assays were needed to provide a balanced evaluation.

The CREATE serum bank

The development of a serum bank from a broad spectrum of allergic patients was an essential part of the CREATE project and was co-ordinated by Drs Stephen Durham (London, UK) and Montserrat Fernández-Rivas (Madrid, Spain). The serum bank was established by recruiting allergic patients in 11 clinical centres across Europe (London, Manchester, Madrid, Jaén, Barcelona, Rome, Borstel, Utrecht, Gothenburg, Strasbourg, Vienna) by researchers listed in Table 2. Patients were recruited into the study aged 16- to 50-year old, with a skin prick test wheal of ≥5 mm diameter, a clinical history

of rhinoconjunctivitis and/or asthma upon exposure to allergen; and no previous immunotherapy for 5 years. Ethical approval was obtained from the institutional review boards of all participating centres and each patient provided written consent. In the first phase of the study, each patient who was enrolled donated approximately 20 ml blood to the project and approximately 10 ml serum was obtained for IgE antibody measurements. Serum was obtained from a total of 961 patients, as follows: birch, $n = 186$; timothy pollen, $n = 342$; olive pollen, $n = 155$; mite, $n = 278$. In a second phase of the study, candidates with a range of IgE antibody levels by RAST were selected to donate 100 ml blood to the project and the mean serum volume collected was approximately 35 ml. The 'large' serum bank contains sera from 155 patients, 34 for birch pollen, 60 for grass pollen, 20 for olive pollen and 41 for house dust mite. The CREATE serum bank is maintained by the project co-ordinator, Dr Ronald van Ree at the Academic Medical Center, Amsterdam, The Netherlands, and is available to researchers upon agreement of the CREATE partners.

Stability

Stability of allergen preparations that would in the future serve as IRP is of the utmost importance. References that rapidly decrease in quality could not be used as certified standards. CREATE evaluated the process of freeze-drying and the stability of freeze-dried allergens using accelerated degradation studies which were performed at the National Institute of Biological Standards and Control, a WHO-approved repository based in St Albans, UK.

Freeze-drying cycle conditions

Seventeen purified allergen preparations and a timothy (*Phleum pratense*) grass pollen extract (NIBSC code 02/322) were formulated in a sterile normal saline solution containing 0.1% D-(+)trehalose dehydrate and 0.2% human serum albumin. One-millilitre aliquots of the formulation containing 5 μg allergen product were dispensed into sterile, acid washed glass ampules baked at 250°C for 1 h prior to filling. A sub-sample of ampules was heat-sealed and stored at −150°C serving as frozen baseline for all future measurements. rPhl p 1 was not included in the study because of its poor solubility. The timothy grass pollen extract was used to optimize freeze-drying conditions. A freeze-drying cycle was carried out to ensure that the freezing temperature, the freeze-drying (shelf) temperature, the time profile of the vacuum, the properties of the fill solution and the glass transition temperature were all optimized.

The material was freeze-dried for over 100 h. During the freeze-drying process the shelf temperature of the freeze drier was maintained at −40°C for at least 75 h and then raised over a 20-h period to +20°C and then maintained at +20°C for 10 h.

The cycle condenser temperature was maintained at between −60 and −70°C and the vacuum between 4×10^{-2}–5×10^{-2} mbar. The ampules were fitted with a capillary plug and backfilled with dry nitrogen (O_2 level ≤ 10 ppm, $H_2O \leq 5$ ppm) and heat sealed by fusion of the glass neck. The residual moisture in each case was targeted to be $< 1\%$.

Lyophilization has no adverse effects on allergen activity

The activity of the original allergen product (by definition being 1), the formulated allergen product (frozen base line) and the formulated lyophilized allergen product was assessed. Lyophilization of the various allergen products did not significantly affect the activity of the original product as illustrated in Table 6 for one of the allergens.

Of all allergen vials filled, 1–2% were selected at random and tested for the control of variation of the fill, the oxygen level and moisture content. All met the WHO criteria for these parameters: CV of 1 ml/1g fill $< 0.25\%$, O_2-content < 40 μmoles/l and the moisture content $< 1\%$).

Study design: accelerated degradation studies

Twenty ampules of each allergen product filled were placed at each of the different storage temperatures, −150, −70, −20, +4, +20, +37, +45 and +56°C to assess the affect of temperature over a period of time on the stability of the product. Two ampules per temperature point were removed and tested at 1, 3, 6, 9, 12 and 24 months postlyophilization using an appropriate allergen-specific sandwich ELISA.

Raw data were assessed graphically to check for any anomalies and to ensure that the dose–response relation was monotonic. Additional tests for statistically significant outliers and for homogeneity have been carried out using an in-house program, SCAN (37). Row and column effects were assessed using analysis of variance; effects within plates as well as between plates were investigated. In most cases, the dose–response curves were sigmoid and could be satisfactorily described using a four parameter logistic function. For each plate in each assay, asymptotic limits for the logistic function were determined and logit-transformed responses were analysed using the methods

Table 6. Potency estimates for frozen baseline and freeze-dried Phl p 5 preparations

Allergen	Frozen baseline	Freeze-dried	Original allergen
rPhl p 5a (03/102)	0.98	1.08	1
rPhl p 5a (03/106)	1.15	1.1	1
rPhl p 5b (03/108)	1.08	1.0	1
nPhl p 5 (02/322)	0.9	1.09	1

of parallel line bioassay and an in-house program (38) to estimate potency at each temperature for each time point. The potency estimates used for calculation of degradation rates are based on geometric means of the estimates from the individual plates. Potency estimates calculated for each temperature for each time point relative to −20°C were used to predict annual loss of potency (39), assuming that the relationship between the activity and temperature is described by the Arrhenius equation. At the earlier time points, samples stored at the higher temperatures showed little or no loss of activity and reliable predictions based on these data could not be obtained. Estimates for preparations stored at 12 and 24 months postlyophilization were more likely to show a significant loss of activity and thus gave a more reliable estimate for degradation. For 88% of the allergen products tested, rates for degradation could be determined using data at these two time points. In the remaining cases, the maximum likelihood procedure used for estimation failed to converge, and no estimate of degradation rate was obtained.

Stability of lyophilized allergens at 12 and 24 months

Most allergens (nine of 17) remained stable at −20°C for 2 years, with the annual predicted potency loss being $<0.5\%$ (Table 7). For an additional six of the 17 the loss was $<0.5\%$ as well at 2 years, but a firm conclusion cannot be drawn because loss had been $>0.5\%$ in 1 year. Additional studies are therefore warranted. At +4°C, six of the 17 allergens remained stable for 2 years (consistent $<0.5\%$ loss), and six of the 17 again had discrepant results between years 1 ($>0.5\%$) and 2 ($<0.5\%$). Only for natural and recombinant Der p 1, potency loss at 1 year was 1.6% and 4.2%, respectively and 6.7% and 9.7%, respectively, at 2 years. In summary therefore, when kept at −20°C stability was good (years 1 and 2 in agreement) or promising (year 2 $<$ 0.5% but conflicting with year 1) for all other allergens when evaluated at year 2.

Table 7. Stability at 24 months postlyophilization. Question marks (?) indicate that year 2 data were <0.5% but year 1 data >0.5%

Allergen	NIBSC codes	<0.5% loss (−20°C)	<0.5% loss (+4°C)
nBet v 1	03/180	?	N
rBet v 1	03/184	Y	N
nPhl p 1	03/234	Y	Y
rPhl p 1		No data available	
nPhl p 5	02/322	Y	Y
rPhl p 5a (1)	03/106	Y	?
rPhl p5a (2)	03/108	Y	Y
rPhl p 5b	03/102	?	?
nOle e 1	03/230	?	N
rOle e 1	03/232	Y	?
nDer p 1	03/120	N	N
rDer p 1	03/122	N	N
nDer f 1	03/156	Y	Y
rDer f 1	03/154	?	?
nDer p 2	03/174	?	?
rDer p 2	03/176	?	?
nDer f 2	03/160	Y	Y
rDer f 2	03/162	Y	Y

ELISA evaluation

Incorporation of quantification of major allergens in standardization protocols not only requires availability of adequate allergen references, but is also dependent on well-validated immuno-assays for their measurement. Over the past decades numerous assays have been developed, covering most major inhalant allergens (14, 40). CREATE selected multiple sandwich ELISAs for each allergen, i.e. four different assays for Bet v 1, three for Phl p 1, four for Phl p 5, three for Ole e 1, two for Der p 1 and two for Der f 1 and three for Der p 2/Der f 2. The protocol to evaluate the performance of these assays was not set up as a true ring trial, necessary for validation according to international regulations. For practical reasons the number of participating laboratories per ELISA was too small ($n = 5$) for a true ring trial. In addition, ELISAs were not delivered as complete kits, but as sets of antibodies in some cases precoated to microtitre plates. Although uniformity of protocols was the original intention, in the end the absence of complete ready-to-use kits resulted in minor deviations between protocols used at various locations. Differences were reported in the types of microtitre plates, enzyme-conjugates and substrates used. Despite these shortcomings, the evaluation has resulted in very valuable information about the performance of the various assays, in particular with respect to differences in specificity and the consequences of those differences. A decisive criterion for the evaluation was the comparison between results obtained when using the natural allergen as standard or the recombinant. Two factors play a role here, isoform specificity and sensitivity to folding differences including aggregation state. Obtaining similar results when using either a natural or a recombinant standard would point towards a broad spectrum of reactivity with regard to isoforms and folding and aggregation status. Such a 'lack of specificity' should be seen as an advantage for the measurement of allergens that appear in several isoforms and are extracted from biological source materials using different protocols, or in the future produced in different expression systems. Five laboratories carried out two to four ELISAs for the eight allergens of CREATE, using natural and recombinant allergens as standards and freeze-dried intermediate bulk extracts from the six allergen manufacturers in the project as samples.

Recombinant *vs* natural: isoforms specificity

It has been reported elsewhere that mAb-based assays can be too specific, i.e. have preference for specific isoforms

(41, 42). Such assays are less likely to be very suitable for standardization purposes, because they will not pick up all isoforms present in an extract with similar efficacy. In extreme cases, they do not react with a specific isoform at all. Within the CREATE project two examples of assays were found where a specific (recombinant) isoform was not picked up. This was observed for an assay for measurement of Phl p 5 when using rPhl p 5b as standard, and for an assay for detection of house dust mite group 2 allergen using rDer p 2.0101 as standard. Assays based on such combinations of standard and antibodies will of course not be implemented because a standard curve cannot be produced. However, an assay with decreased but significant recognition of a relevant allergen isoform will not that easily be set aside as inappropriate, if no thorough comparison is made between purified natural and recombinant allergen as standard. A good example is one of the three assays used in CREATE for the measurement of Bet v 1. This assay was shown to be approximately fivefold less reactive with rBet v 1.0101 (Bet v 1a) than with natural Bet v 1 containing around 50% of this isoform. Theoretically, the difference could also be explained by incorrect folding of the recombinant allergen but physico-chemical analysis (e.g. CD-spectroscopy) did not provide support for this (see above). Application of this rBet v 1.0101-insensitive assay, with that particular isoform as standard, resulted in overestimation of Bet v 1 in extracts, because the absorbance per ng Bet v 1 was lower for pure rBet v 1.0101 reference than for a mix of isoforms in the sample extract. The other two assays did not distinguish significantly between rBet v 1.0101 and nBet v 1. The results obtained with these two assays closely correlated and were approximately five times lower than with the isoform-specific assay (Fig. 5). These observations clearly illustrate the importance of detailed evaluation of the fine-specificity of major allergen immuno-assays.

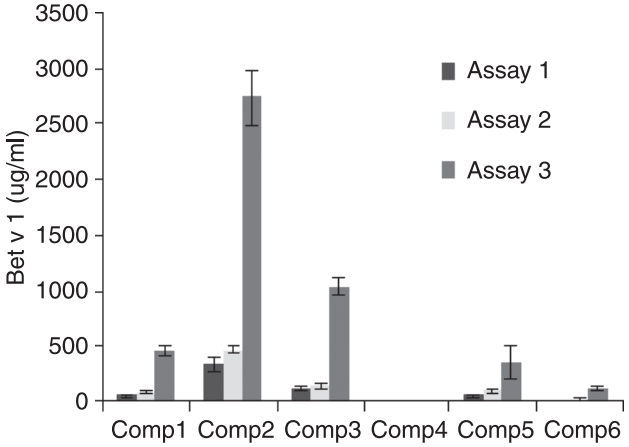

Figure 5. Role of isoform-sensitivity of Bet v 1 ELISAs. Three ELISAs were used to measure Bet v 1 in six extracts. Assay 3 was relatively insensitive for detecting Bet v 1a. Using a recombinant version of the isoform as standard results in clear over-estimation of Bet v 1 titres compared to both other assays that see no significant difference between nBet v 1 and rBet v 1a.

Folding or aggregation sensitive assays

Folding or aggregation of recombinant allergens can also influence their detection in major allergen ELISAs. Monoclonal antibodies can be directed to conformational or linear epitopes. The latter will most likely be less sensitive to incorrect folding. Oligomerization can mask one epitope and leave another available for antibody binding. A likely example of a folding/aggregation-sensitive and -insensitive (or isoform-specific) assay was observed amongst the assays used to detect Ole e 1. Using nOle e 1, both ELISAs gave very similar results (Fig. 6A). In contrast, rOle e 1 resulted in close to identical results for one assay (compared to using nOle e 1 as standard) and around 10 times lower for the other assay (Fig. 6B). Differences in sensitivity of antibodies for natural and recombinant allergens find their origin in the epitope recognized but also in the preparation used for immunization of mice for the production of mAbs. A specific isoform or an incorrectly folded or aggregated allergen used as immunogen is likely to induce different antibodies than a native mixture of well-folded isoforms. These factors should be taken into consideration when selecting immunoassays and references for standardization purposes.

Future perspectives of CREATE

Summary of achievements of CREATE

The CREATE project has delivered a wealth of data about a panel of nine candidate reference molecules representing eight of the most important major respiratory allergens. The starting point of the project was to evaluate whether recombinant molecules representing these allergens are suitable candidates to serve as CRMs for standardization purposes in the future. Moreover, CREATE aimed at evaluating whether available sandwich ELISAs are adequate tools for measuring these allergens in allergen extracts.

Recombinant allergens were evaluated using their natural counterparts as gold standard. The comparison consisted of a detailed physico-chemical (identity, purity, folding, aggregation state, solubility and stability) and immunological characterization (IgE-binding, biological activity and dose–response behaviour in ELISA). Clearly, not all recombinant allergens tested fulfilled the requirements for qualifying as a future reference material. Shortcomings included incorrect folding, significant aggregation, poor solubility, decreased IgE-binding and biological activity and insufficient stability. Obviously, these parameters are closely related and consequently

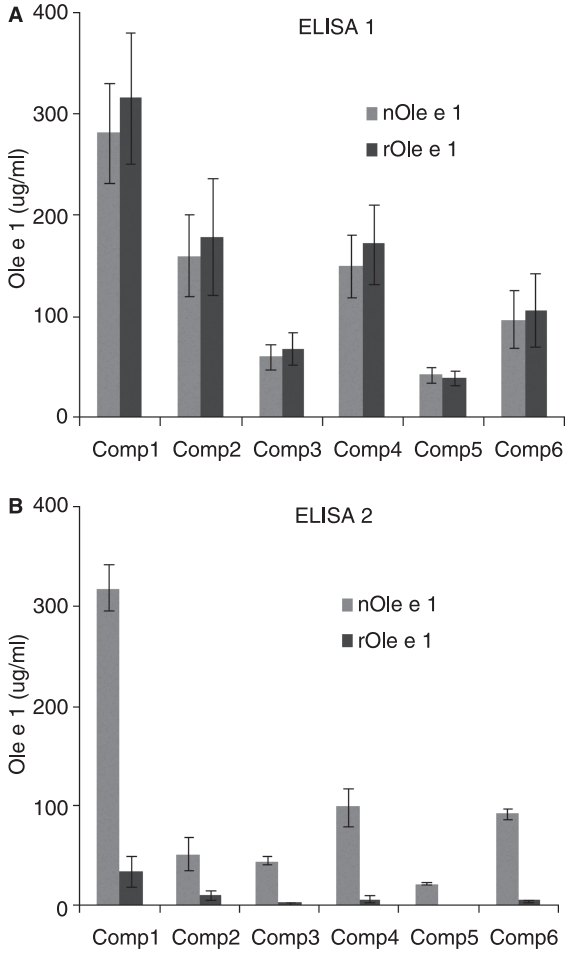

Figure 6. Effect of structural differences between n and rOle e 1 on ELISA performance. Panel A shows an ELISA that sees no significant difference between nOle e 1 and rOle e 1, resulting in similar Ole e 1 titres of six extracts using either of these molecules as standard. Panel B illustrates that an ELISA that clearly reacts differently to nOle e 1 and rOle e 1 delivers Ole e 1 titres that differ up to 10-fold.

some allergens scored poorly on a combination of them. Three allergens were characterized as well-folded, soluble and stable proteins with comparable IgE reactivity and biological activity as their natural counterparts: rBet v 1, rPhl p 5a and rDer p 2. However, this by itself did not guarantee similar dose–response curves in all mAb-based sandwich ELISAs. For both pollen allergens, two ELISAs fulfilled this requirement, for Der p 2 only one assay. In other words, application of a recombinant reference molecule in allergen-standardization protocols is not sufficient to facilitate comparability of products on the basis of mass units of major allergen. To achieve that goal, recombinant references will need to be linked to one or two validated assays that have been demonstrated to measure natural and recombinant allergen with similar performance characteristics, thus leading to the same designation of potencies in mass units of major allergen.

How to proceed?

One of the major achievements of CREATE has been the willingness of the commercial partners to collaborate and share reagents, in a joint effort to achieve the common goal of a validated system of allergen-standardization in mass units of major allergen. Two of the recombinant allergens that qualified as good reference candidates have recently been used in immunotherapy clinical trials, rBet v 1 (43) and rPhl p 5a (44). These reagents were produced under GMP conditions required for *in vivo* application. Illustrating the spirit for cooperation and progress, both companies have made available 200 mg of each of these GMP-produced allergens to facilitate a follow-up programme of CREATE aiming at establishing the first two internationally recognized certified recombinant reference materials. In addition, these companies and a third commercial partner of CREATE have agreed to provide the follow-up project with ELISA kits for both allergens that had demonstrated similar dose–response curves with natural and recombinant allergens. The follow-up project will be coordinated by the European Pharmacopoeia Commission of the EDQM under their Biological Standardization Programme. The two allergens will be freeze-dried and formulated in at least 10 000 vials. The freeze-dried product will undergo the same physico-chemical characterization as was carried out in the CREATE programme. In addition, the influence of formulation (e.g. freeze-drying, small molecule additives) on protein stability will be investigated by Fourier Transform Infrared spectroscopy. Only SAXS will be left out because the high protein concentrations needed for this technique were thought to lead to over-interpretation of the problem of aggregation. Following that analysis, a true ring trial will be set up for each of the allergens using two ELISAs per allergen. The outcome of this project is expected to be the establishment of both recombinant allergens as CRM in conjunction with at least one but preferably two certified ELISAs. The companies have agreed to allow production of allergens and mAbs also in the future for unrestricted use for standardization purposes.

The ring trial will be carried out by at least eight Official Medicines Control Laboratories. In addition, non-European laboratories like the USA's Food and Drug Administration will be invited to join. This is of great importance to ensure acceptance of the new reference materials beyond the borders of the European Union. Upon successful completion of this programme, other allergens of CREATE, and also other allergens, including Amb a 1 from ragweed and Fel d 1 from cat, will need to follow.

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