

EDITORIAL

Clinical and Experimental Allergy

New frontiers in allergen standardization

This editorial discusses the findings of the paper in this issue by Carnés et al. [3] pp. 426–34.

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In the last two decades, a large number of allergens from various sources have been characterized in detail. In some sources only few proteins or even single molecules account for allergenicity whereas for many others, a diverse and complex allergen expression pattern has been described. Despite the complexity of some allergen expression patterns, the diagnostic performance or the therapeutic effect can often be attributed to few and sometimes even to single allergens or to certain isoforms of allergens [1, 2]. Hence, measurement of individual allergens gained more importance in standardization and quality control of allergen products in recent years.

In this context, the study of Carnés et al. [3] is especially noteworthy because the authors applied a technique to identify specific allergens and isoallergens that will push forward standardization of allergen products towards new frontiers.

The first efforts towards biological standardization of allergen extracts were undertaken in the 1980s. At that time, the research on allergen standardization was focused on potency tests using IgE antibodies from allergic patients. *In vivo* testing for biological standardization was established as gold standard while standardization at the molecular level was in a relatively immature state. The European allergen manufacturers mostly used the Scandinavian System [4] for *in vivo* standardization whereas a slightly different system was used in the United States [5]. In either system, skin tests were used to determine the allergenic activity of the product. Amongst a multiplicity of biochemical methods to characterize allergen products, IgE-inhibition tests, for example, RAST inhibition, are the most frequently used assays for standardization *in vitro* and are officially demanded for control of

batch-to-batch consistency [6]. These IgE-inhibition tests are thought to closely reflect the biological *in vivo* tests as the entire diversity of the IgE-antibody response of an allergic subject is taken into consideration (reviewed in reference [7]). However, these tests allow to evaluate the batch-to-batch consistency and the overall allergenic potency, but they do not allow to draw any conclusions concerning therapeutic effects or the ability to cross-link cell-bound IgE and they do especially not give information about the content of single allergens. As the specificity of IgE antibodies present in the human serum pool is largely unknown in most cases, it is often not clear what exactly is measured by these tests. Furthermore, due to a lack of generally accepted international standards, manufacturers standardize allergen products with in house references and use different units, making a comparison of potencies of products of different manufacturers impossible.

The increasing number of identified major and minor allergens as well as the rise of available recombinant allergens during the 1990s allowed to establish approaches to measure the content of single allergens in allergen preparations. There are different systems used to measure individual allergens such as two-site ELISA, or Rocket-Immunoelectrophoresis but all assays are based on the availability of allergen-specific antibodies raised in animals. Moreover, despite the fact that allergen measurements have now widely been used for many years, no international standardized reference preparations have been available [8]. In 2001, the European Union funded an ambitious project that aimed at evaluating recombinant allergens as certified reference materials (CRM) and on evaluation of ELISA for measurement of the allergen content using the candidate CRM as standards [9]. The outcome of the CREATE project was very positive. Some promising candidate CRMs have been identified, for example, rBet v 1, rPhl p 5a and some ELISAs have been proven to be suitable for allergen measurement. But the CREATE project has also shown the limitations of recombinant allergens as reference preparations and the limitations of antibody-based test systems to

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measure the allergen content. Some recombinant allergens were found to be unsuitable to serve as a CRM due to incorrect folding, excessive aggregation, poor solubility or insufficient stability [10]. Apart from general limitations of ELISA type assays such as availability of antibodies and inter-laboratory variability, one major flaw that came up during the CREATE project was the incapability of some ELISAs to pick up all isoforms present in an extract. For example, application of an ELISA that was approximately five-fold less reactive with Bet v 1.01.01 compared with natural Bet v 1 which contains 50% of that isoform resulted in overestimation of Bet v 1 when rBet v 1.01.01 was used as a standard. In addition, it was observed that even if the same purified allergen was used as reference, different two-site ELISAs sometimes gave highly divergent results for the same allergen extract.

In summary, there are four major conclusions that can be drawn from the CREATE project.

- (a) It is possible to validate ELISA using recombinant allergens as CRM for allergen measurement, and thus the CREATE project facilitated a big step forward in allergen product standardization.
- (b) It is crucial to closely look at the composition of the natural allergen preparation with a special emphasis on isoforms of allergens for the development of ELISA as well as for selecting recombinant allergens as CRM.
- (c) There will be some allergen sources for which, in the near future, no purified recombinant allergens will be suitable as CRM.
- (d) It seems to be very unlikely that for every allergen product or allergen source, a suitable ELISA can be developed allowing the quantification of the 'true' content of major allergens. This will be mainly due to lack of antibodies with the desired specificity and lack of universally applicable reference components.

There is no doubt about the need of methods for quantification of single allergens and allergen isoforms in allergen products. Hence, the conclusion of these observations is quite evident. If possible, methods for allergen measurement should allow the quantification of all relevant allergens in all allergenic sources even on level of single isoforms [11]. Such methods ideally relied on synthetic reagents that could be produced in unlimited quantities and were available worldwide. The reference substances or standards needed to calibrate these assays should also be synthetic to be available at large quantities with a constant quality.

Carnés et al. [3] used tandem mass spectrometry to detect allergens in a therapeutic birch pollen allergen product and they have even confirmed the presence of five different isoforms of the major allergen, Bet v 1. This means they applied a physico-chemical method that is independent from allergen reference preparations and at

the same time suitable to detect in complex mixtures virtually every protein in a sequence specific manner, that is, on the level of isoforms. Mass spectrometry has of course been frequently applied on allergenic sources in recent years. But the vast majority of the studies conducted aimed at either identification of novel allergens or on the confirmation of the identity of natural or recombinant allergens [10, 12, 13]. Either purified allergens were used or in case of allergen extracts, a separation by 2D electrophoresis or chromatography preceded the mass spectrometric analysis. In contrast, the paper of Carnés et al., published in the current issue of *Clinical and Experimental Allergy* describes for the first time the application of mass spectrometry on the characterization of an allergen product [3]. The authors subjected a therapeutic allergen product based on an allergen extract preparation to enzymatic digestion and detected the resulting peptides with tandem mass spectrometry, a technique that gives information about the mass and the fragmentation pattern of peptides. The experimental masses and patterns are then matched to masses and fragmentation pattern of peptides that have been created *in silico* by processing amino acid sequences that have been already published. This approach allows confirming the presence of proteins by estimating if a match between theoretical and experimental data is statistically significant or has been obtained by chance. By applying this probabilistic approach, the authors did not specifically search for marker peptides that are characteristic for a certain allergen or isoallergen, but it is easily possible to derive such a marker peptide from MS/MS data. Synthesized as an isotopic labelled variant, such a peptide would be an ideal standard to calibrate an MS instrument for quantifying the content of the protein from which the peptide has been derived. Marker peptides and synthetic peptides as calibrators could be used to set up a method that allows the detection of several allergens and isoforms in parallel in one sample. Applying this technique on standardization of allergen products would help to overcome the limitations of antibody-based test systems for allergen measurement and would represent a true physico-chemical multiplexing system. Enzymatic digestion followed by HPLC tandem mass spectrometry can easily be applied to natural protein extracts as it has already been done in the past for different purposes. The authors, however, went even one step further. They applied this method to a chemically modified allergen preparation (allergoid) that has been intended for specific immunotherapy. For such allergen products, no methods have been described so far that would allow to measure the allergen content despite the fact that testing is demanded by new regulatory guidelines [11]. Not even IgE-inhibition as potency test can be performed as the IgE-reactivity is deliberately reduced or even abolished by modifying the allergens, for example, by polymerization with glutaraldehyde. Whereas



Fig. 1. Typical example for allergoids that do not show any individual protein bands in SDS-PAGE after silver-staining (right lane molecular weight marker).

allergoids separated in SDS-PAGE do not show any individual protein bands in the silver-stained gel (Fig. 1), strikingly Carnés et al. were able to identify distinct allergen molecules in such a sample when applying tandem mass spectrometry. Thus, the concept of Carnés et al. improves standardization for allergen products that are based on natural extracts and it will push quality control and standardization of allergoids towards new frontiers.

Standardization and quality control are of course much more than just the confirmation of identity or quantitation of individual allergens. Quality, safety and efficacy have to be shown before a marketing authorization is granted for an allergen product (reviewed in reference [14]). For ensuring quality, various methods have to be applied that give information, for example, about purity, stability and biological potency. In particular, the European Medicines Agency demands that it should be demonstrated that all relevant allergens are maintained throughout the manufacturing process [11] by using appropriate methods such as antibody-based techniques or mass spectrometry. Taking into account the well-known limitations of antibody-based tests, it would be desirable if the concept of detecting allergens and isoforms of allergens in allergen preparations by tandem mass spectrometry as demonstrated by Carnés et al. would be developed further into a quantitative application, one important step towards a comprehensive characterization of one of the most complex product categories within the area of biomedicines.

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