

Recombinant Mite Allergens: Validation Strategies and Clinical Trials

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Since the last Paul-Ehrlich-Seminar in 1999, recombinant allergens have become widely used for research purposes and purified allergens from mites, pollens, animal danders, insects, fungi, and foods are commercially available (1,2). Typically, these proteins are produced in high-level expression systems in *E. coli* or *Pichia pastoris* and purified by affinity chromatography techniques and HPLC. This presentation will review the strategies that we have developed for the validation of recombinant allergens, including dust mite, cat, and cockroach. Standards for the mite allergen Der p 1 were previously established using the WHO/IUIS International Reference Preparation (NIBSC 82/518). While this standard has been very useful in establishing uniform allergen measurements (e.g. in ELISA), it is a mite extract with a specified concentration of Der p 1, and is not a suitable standard for assessing purified natural or recombinant Der p 1. In 2000, the WHO/IUIS Allergen Standardization Subcommittee embarked on a program to develop international standards for purified allergens. The aim was to compare the purity and allergenic activity of purified natural and recombinant allergens and to develop single standards that manufacturers could use for allergen measurements *in vitro*. This project has since been funded by the EU under the acronym CREATE and is described in detail by Ronald van Ree. An integral part of the EU CREATE project is to validate the purity and allergenic activity of recombinant allergens by comparison with their natural counterparts, so that objective decisions can be made about the selection of either a natural or recombinant allergen standard. This review discusses the validation strategies that are being developed for purified allergens and the use of cocktails of mite allergens as diagnostic reagents in clinical trials.

Validation Strategies

Protein purity

Validation strategies for purified allergens are outlined in Table 1. Protein purity is primarily established by SDS-PAGE, HPLC, and N-terminal amino acid analysis, with

Table 1. Validation of Recombinant Allergens.

- ◆ Protein purity (SDS-PAGE, HPLC, amino-acid analysis and N-terminal sequencing)
- ◆ Monoclonal antibody binding (two site ELISA): confirms integrity of antigenic sites and allergen secondary and tertiary structure
- ◆ IgE Ab binding *in vitro*: quantitative comparisons of IgE binding to natural and recombinant allergens, e.g. by chimeric ELISA
- ◆ Immediate skin tests (prick or intradermal): confirm allergenic activity in humans; compare allergen formulations and diagnostic efficacy in clinical trials
- ◆ Functional assays: confirm biologic activity of allergens with known function; confirm structural integrity of recombinant allergens

secondary analyses to assess isoforms by isoelectric focusing and mass spectrometry. Purified allergens may show multiple bands on SDS-PAGE (e.g. doublets or triplets) as a result of glycosylation or post-translational modifications. Recombinant proteins expressed in *P. pastoris* (e.g. Der p 1, Der f 1) or baculovirus systems (e.g. Fel d 1) may be glycosylated and show significant differences in molecular weight compared with the natural allergen (3-7). For most companies, producing allergens that show a single band on SDS-PAGE will be an important validation tool. In addition to establishing protein purity, it is important to measure contaminants that may be derived from the source material or introduced during the purification process. These include endotoxin and other bacterial products, mouse antibodies or other ligands from affinity purification procedures, and contaminating allergens (derived from natural products). For example, most natural Der p 1 (or Der f 1) purified by mAb affinity chromatography and size exclusion HPLC contains ~0.05 - 0.1% contamination with mite Group 2 allergens in ELISA, unless procedures are used to remove the Group 2 allergen. While this level of contamination does not usually affect the intended use of the allergen, the Group 2 measurements (in this case) serve as a useful surrogate marker for other proteins derived from the source material.

Monoclonal antibody (mAb) binding

Two site mAb ELISA are simple and effective tools for assessing the reactivity of recombinant allergens and can also be used to monitor yield from purification processes. Most mAb for indoor allergens have been raised against different epitopes on natural allergens. If recombinant allergens do not retain the same tertiary structure as the natural molecule, they will not bind as effectively in mAb-based assays. Ideally, recombinant allergens should show identical binding curves to the natural product and comparable binding using different combinations of mAb. Careful assessment of mAb binding is needed for allergens that have multiple isoforms. There are >5 naturally occurring isoforms of Der p 2 and Der f 2 with 2-6 amino acid substitutions that can affect mAb binding (8-10). Isoforms Der p 2.0103 and Der f 2.0103 were selected as candidate reference preparations for the EU CREATE project because they bind to most anti-Group 2 mAb. Several mAb combinations for ELISA will be evaluated in multi-center trials as part of the CREATE study.

Assessments of IgE

Recombinant allergens can retain IgE binding activity in direct binding or indirect blotting and basophil activation assays. It is important for comparison of results with a range of IgE binding to purified allergens, inhibit binding of IgE to protein is fully intact and parallel dose response.

The 'chimeric' ELISA protocol uses recombinant allergens and allergen-specific IgE sera. Bound IgE is detected by a peroxidase. The level of binding is measured by antibody competition. Chimeric anti-Der p 1, Can f 1, Bla g 1, and IgE (Pharmacia) showed no binding to natural and recombinant allergens. et al. showed an excellent correlation between the dust mite allergen and the dust mite allergen. The ELISA results suggest that measurement of allergen in clinical and epidemiological studies is a diagnostic equipment.

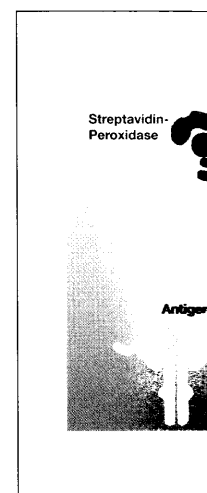


Fig. 1. Chimeric ELISA protocol. Right panel shows positive result.

Assessments of IgE binding *in vitro*

Recombinant allergens that are strongly reactive in mAb ELISA are most likely to retain IgE binding epitopes. IgE Ab binding *in vitro* can be assessed by a variety of direct binding or inhibition assays, including antigen-binding RIA, ELISA, immunoblotting and basophil histamine release. Appropriate selection of allergic sera is important for comparing natural and recombinant allergens. Typically, at least 50-100 sera with a range of IgE Ab to the source material (CAP class 2-6) should be screened for binding to purified allergens. Inhibition experiments using recombinant allergen to inhibit binding of IgE to natural allergen are useful in establishing that the recombinant protein is fully immunoreactive. The recombinant allergen should give >90% inhibition and parallel dose response curves in these assays.

The 'chimeric' ELISA is a useful tool for correlating IgE responses to natural and recombinant allergens in large panels of sera (Figure 1). Microtiter plates coated with allergen-specific mAb are incubated with allergen, followed by dilutions of patients' sera. Bound IgE Ab is detected using biotinylated anti-IgE and streptavidin peroxidase. The level of allergen-specific IgE is quantified using a control curve of a chimeric antibody comprised of the VH domains and light chains of anti-Der p 2 mAb (2B12) linked to human Fc epsilon chains (2B12-IgE) (11,12). Parallel binding curves to the chimeric anti-Der p 2 ab have been obtained using mite Group 1 allergens, Fel d 1, Can f 1, Bla g 1, and Bla g 2. This assay correlates with CAP assays for allergen-specific IgE (Pharmacia) and quantitative correlations have been obtained when comparing natural and recombinant allergens in this system (13-15, Table 2). Recently, Trombone et al. showed an excellent correlation between chimeric ELISA for Der p1 and Der p 2 and the dust mite CAP with a positive predictive value of >95% (14). These results suggest that measuring IgE to selected pure allergens can effectively demonstrate sensitization. The ELISA format may be especially useful for measuring allergen specific IgE in clinical and epidemiological studies, and in countries where sophisticated allergy diagnostic equipment is not available.

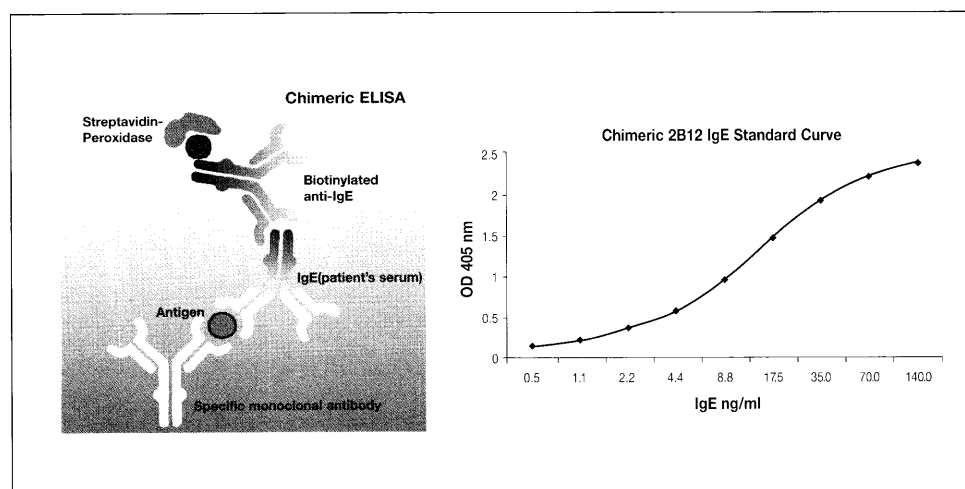


Fig. 1. Chimeric ELISA for measurement of allergen-specific IgE. Left panel: schematic diagram of assay protocol. Right panel: assay control curve using anti-Der p 2 chimeric antibody (2B12-IgE) (11-15).

Table 2. Correlations between IgE Ab binding to natural and recombinant allergens in chimeric ELISA.

Allergen	Expression System	No. of Sera tested	Correlation Coefficient	Significance (p value)
Der p 1	<i>P. pastoris</i>	33	0.93	< 0.001
	CHO cells	33	0.93	< 0.01
Fel d 1	Baculovirus	68	0.98	< 0.001
	<i>P. pastoris</i>	57	0.91	< 0.001
Bla g 1	<i>P. pastoris</i>	29	0.91	< 0.001
Bla g 2	<i>P. pastoris</i>	26	0.93	< 0.001

Clinical studies of recombinant allergens

Quantitative intradermal skin tests have been carried out with recombinant mite and cockroach allergens. The aim of these studies was to demonstrate that the recombinant allergens had biologic activity, which was compared to that of natural allergen, if available. The results showed that several recombinant allergens, e.g. Der p 2, Bla g 2, Bla g 4, gave positive intradermal skin tests at 10^{-2} – 10^{-6} µg/ml, thus confirming the potency of these proteins (16,17). Cocktails of recombinant mite Group 1 and Group 2 allergens have recently been compared with natural mite allergen extracts in skin prick test studies (15,18). The recombinant allergens, expressed in *P. pastoris* or *E. coli*, were tested individually at a concentration of 5 µg/ml or formulated in cocktails with a cumulative concentration of 20 µg/ml. The mite extracts were standardized *D. pteronyssinus* and *D. farinae* at 10,000 AU/ml. Studies on a small group of mite allergic patients in Charlottesville VA, showed that all patients with a >4 mm wheal to natural mite extracts gave a >4 mm wheal to the recombinant allergen cocktail (15). A large trial of 120 mite-allergic patients with asthma in Brazil has confirmed these studies and shown that 98% of patients with positive prick tests to mite extracts gave positive results with recombinant allergens (18). Diagnostic sensitivity was not significantly improved by including mite Group 5 allergens (Der p 5 and Blo t 5) in the cocktail. These results provide strong evidence for the efficacy of using recombinant mite allergens for diagnostic purposes.

Concluding remarks

The strategies outlined above provide a framework for validating recombinant and natural allergens based on protein purity and allergenic potency. Functional assays of allergenic proteins, e.g. enzymatic activity or ligand binding, can also be used to confirm biologic activity and provide confirmation of the integrity of the tertiary structure of recombinant allergens (Table 1). These validation strategies are being critically assessed as part of the EU CREATE study. The aims of this project are to develop objective methods for allergen standardization that can be used in laboratories world-

wide, and to prove that it is intended that the elements and specifications will be analogous to mones.

Clinical trials of recombinant allergens using techniques using recombinant allergens within minutes have already been reported. Recombinant allergens and vaccines are being tested. This is a trend that suggests that only the best can be produced under the most or more major requirements required to produce allergens.

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**Significance
(p value)**

< 0.001
< 0.01

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wide, and to produce standards that are internationally acceptable and verifiable. Thus, it is intended that the potency of these standards will be based on protein measurements and specific immunoassays, rather than *in vivo* techniques, and that the standards will be analogous to international standards for immunoglobulins, cytokines, and hormones.

Clinical trials leave little doubt that it will be possible to use selected combinations of recombinant mite and other allergens for diagnostic purposes. Novel *in vitro* techniques using recombinant allergens will enable allergy diagnosis to be carried out within minutes on blood samples. Microarrays containing over 90 purified allergens have already been tested and provide a means for greater diagnostic specificity (19). Recombinant allergens are also being used to develop new immunotherapeutic strategies and vaccine development that will require allergen production under GMP conditions. This is a time-consuming and expensive process. Economic considerations suggest that only the most clinically relevant allergens (pollens, mites, animal danders) will be produced under GMP. Nonetheless, if proof of principle can be established for one or more major allergens in immunotherapy trials, this may facilitate the investment required to produce more effective and safe allergy vaccines using recombinant allergens.

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Discussion

van Ree: I would like to ask you about the last point you raised. If you go to these vaccination strategies and talk about adding adjuvants, it is very interesting. Do you think that the reason that you get this downregulation of the IgE response is the chronicity of the exposure or is it only the level? The question is relevant because we want of course to develop strategies in which we do not have to immunize for years and years.

Chapman: One of the things with cat for example is the level of exposure you get, but it is also potentially the airborne, it is what you inhaling. This effect has been documented for cat. I do not think we are able to document that kind of an effect with dust mite e.g., and the key difference between the two allergens is one of airborne exposure. So, we would also expect to see this kind of effect potentially with dog allergen, and possibly with some of the other animal allergens that give laboratory animal exposure. The precise mechanism of that is still unclear, but I think both factors are involved.

Slater: What I am going to say is really understood by everybody in the room, but I think it is important to be very explicit about it. It is sort of highlighted by the question about half an hour ago as to whether 100-fold reduction of IgE reactivity is sufficient. I think we have to keep in mind that reduction of IgE reactivity is an irrelevant parameter looking at it in isolation. What we are really interested in is the therapeutic index of these products. If you reduce the IgE reactivity 1000-fold, we also reduce the immunotherapeutic potential for the drug 1000-fold. You have not effected any improvement

in your product, especially when immunotherapy have to keep in look at, really d look at both par

Chapman: I do will probably sa parameters that

Slater: At the n peptic potential rather rapidly w think we do need cell proliferation is. Although it i gate. But I think dies, looking fo manufacturing p and ethical way.

Chapman: I agr they did was th because there a can actually dem and then get the all should be air

de Weck: One future, based on It is clear that respect to origin enough for regu they require the are really makin component is de fragments? Becu vitro tests and so er is really much

Chapman: Let ment. Clearly, w tion: So we coul you could get th are currently us guish between t have to be biolo authorities to da