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REVIEW

Microarrays: Molecular allergology and nanotechnology for personalised medicine (I)

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Abstract
The diagnosis of antibody-mediated allergic disorders is based on the clinical findings and the detection of allergen-specific IgE based on in vitro and in vivo techniques, together with allergen provocation tests.
In vitro diagnostic techniques have progressed enormously following the introduction of the advances made in proteomics and nanotechnology – offering tools for the diagnosis and investigation of allergy at molecular level. The most advanced developments are the microarray techniques, which in genomics allowed rapid description of the human genetic code, and which now have been applied to proteomics, broadening the field for research and clinical use.
Together with these technological advances, the characterisation of most of the different proteins generating specific IgE and which conform each natural allergen, as well as their purification or genetic engineering-based synthesis, have been crucial elements – offering the possibility of identifying disease-causing allergens at molecular level, establishing a component-resolved diagnosis (CRD), using them to study the natural course of the disease, and applying them to improvements in specific immunotherapy.
Microarrays of allergic components offer results relating to hundreds of these allergenic components in a single test, and use a small amount of serum that can be obtained from capillary blood. The availability of new molecules will allow the development of panels including new allergenic components and sources, which will require evaluation for clinical use.
The present study reviews these new developments, component-resolved diagnosis, and the development of microarray techniques as a critical element for furthering our knowledge of allergic disease.
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Introduction

The scientific revolution represented by the description of the human genome was largely facilitated by the use of DNA microarray technology, which made it possible to establish the human genetic map in such a brief period of time. This novel technology was subsequently extended from molecular genetics to proteomics, allowing the start of the Human Proteome Project – an effort designed to determine protein function as an essential element in diagnosis and treatment. In application to allergic diseases, protein and gene microarray techniques have generated a new paradigm: molecular allergy – involving a pathogenic approach based on functional genetics and on the cell and signalling networks that conform these complex disorders – and allergenic component-resolved diagnosis (CRD).

Allergy at molecular level will pave the way to personalised medicine, and its enormous potential will contribute to the diagnosis, prognosis and staging of disease and to treatment selection, facilitating preventive medicine, predictive diagnosis and individualised curative therapies.

The present study reviews these new developments, component-resolved diagnosis, and the development of microarray techniques as a critical element for furthering our knowledge of allergic disease. A second part in turn reviews their application to allergology and future perspectives.

Diagnosis of type I allergy based on extracts

The diagnosis of antibody-mediated allergic disorders is based on the existence of a compatible clinical history and on the demonstration of sensitisation using in vivo and in vitro techniques for the detection of allergen-specific IgE,[1] together with allergen provocation (exposure) tests, where necessary.

These specific IgE detection techniques currently use natural allergen extracts as test allergens – a situation that gives rise to two types of problems: diagnostic accuracy problems due to the difficulty of standardising the allergens used as substrates, and their incapacity to differentiate among clinical cross-reactivity, true co-sensitisation to different allergenic sources, and immunological cross-reactivity lacking clinical relevance, in the increasingly numerous population of polysensitised patients.

Difficulty in standardising the extracts

While the extracts may have similar biological potency, they differ in terms of their allergenic content, due to the natural variability of the sources, the manufacturing process involved, and the instability of the allergenic proteins.[2]

The use of raw extracts can give rise to false-positive results due to the presence of high-cross reactivity components[3] or contamination with allergens from other sources (e.g., acarid species in extracts of animal epithelia[4] or moulds in pollen extracts), as well as to false-negative results due to the absence or degradation of certain allergenic proteins in the extract[5–8] – fundamentally those of plant origin. These considerations have led to major efforts to improve allergenic extract quality and standardisation, such as the CREATE project,[9,10] though the problems remain.

Difficulty in identifying the clinically relevant allergens

An allergic patient may be sensitised to a single allergenic source (monosensitisation), to several allergens (oligosensitisation), or to a large number of different biological sources (polysensitisation). In these cases, IgE targeted to a concrete allergenic source may be attributable to true sensitisation or to immunological cross-reactivity (not always of a clinical nature), as a result of the sharing of homologous proteins with the source in question (similar in 35% or more amino acid sequences[11]). Certain groups of these proteins may be found in several not always taxonomically related sources; they are thus referred to as panallergens, such as the prophyllins[12] or polcalcins.[13] Alternatively, other types of molecules may cause immunological cross-reactions, such as the so-called cross-reactive carbohydrate determinants (CCDs).[14,15] The determination of specific IgE against the allergenic source, but not against its individual allergenic components, does not allow differentiation between true sensitisation and immunological cross-reactivity.

The development of recombinant allergens and the purification of native allergens make it possible to resolve many of these problems.

Diagnosis based on molecular components

Development of recombinant allergens

The developments in molecular allergy over the last three decades have made it possible to characterise the main allergens and to purify them from their native sources or to produce them as recombinant proteins from recombinant DNA, thanks to advances in molecular engineering techniques. At present there is a growing number of allergenic sequences and panels of purified native and recombinant allergens that configure much of the complexity of the epitopes of the natural sources – offering the possibility of determining the reactivity pattern or profile of each individual patient by identifying the allergenic molecules that trigger disease.[16–19]

The thousands of allergens described, their sequences, and studies of interest regarding the known allergenic components, can be found in the Allergome database,[20] and their classification into different protein families and biochemical functions can be found in the AllFam database[21] – both sources being freely accessible on the Internet.

The use of these genetic engineering molecules in application to an allergy test requires careful validation,[22] since recombinant proteins can differ greatly from their natural counterparts. Recombinant allergens originate from different expression systems (*E. coli, Pichia pastoris*, tobacco or insect cells) that have different capacities to produce post-translation changes.[23] Prokaryotic cells such as *E. coli* do not produce the protein glycosylation seen in the natural protein – a fact that may affect the capacity to interact with immunoglobulins and compromise the

usefulness of such proteins if these allergens require cross-reactive carbohydrate determinants (CCDs) for adequate recognition by IgE, as occurs with bee venom hyaluronidase (Api m 2),[24] or with the major allergen of artemisia (Art v 1).[22] In addition to glycosylation, other post-translation defects such as phosphorylation or the formation of disulphide bridges can cause recombinant allergens to differ in their folding or tertiary structure, thus producing variations in their conformational epitopes – these being congregations of several amino acids often derived from different regions of the allergen,[25] and which are of great importance for antigen recognition.[26,27]

The mentioned similarity must be validated, demonstrating that the recombinant allergen has the same allergenic characteristics as the natural allergen, and with similar potency in terms of mass units of allergen.[28]

Component-resolved diagnosis

The main application of purified natural or recombinant allergenic components is the precise identification of the allergens that cause disease. These allergenic components have allowed diagnostic resolution at molecular level, in the form of component-resolved diagnosis (CRD),[29] thereby opening a new era in allergy.

With this approach it is possible to distinguish between patients who are truly allergic to a given biological source and those with cross-reactivity to molecules shared among different biological sources. In addition, it is possible to identify the molecules to which specific immunotherapy must be targeted, with a view to developing optimised treatments.[19,30,31]

Investigations based on allergenic molecules or components have been made with a broad range of aeroallergens (e.g., birch,[32] olive,[33,34] grasses,[35] chenopod species,[36] Alternaria[37] or cat epithelia[38]), food allergens (e.g., peach,[39] apple,[40] cherry,[41] hazelnut,[42,43] carrot[44] or wheat,[45,46] pollen – food plant syndromes[47]), and stinging insects.[48] These studies have revealed that component-resolved IgE recognition patterns can determine sensitisation patterns, correlating them to the presence or absence of symptoms, the severity of the latter, and the clinical phenotypes,[49] and may contribute to the analysis of geographical differences.

Uses of recombinant allergens

Diagnosis employing recombinant or purified allergens containing all or at least the most relevant epitopes of their natural counterparts can be used to develop screening tests with controlled amounts of individual allergens.[50,51] The complexity of epitopes required for precise diagnosis can be secured from a limited number of allergenic proteins, confirming that a high percentage of patients can be correctly diagnosed with the use of only a few recombinant allergens. This has made it possible for different studies to establish the panel of allergens representative of certain types of sensitisation.[52–57]

As an example, a good diagnostic yield has been demonstrated for skin tests with recombinant allergens of *Phleum* and *Betula*,[58] with a hybrid molecule of recombinant allergens of *Phleum*,[59] or with different allergenic components of *Olea*.[60] On the other hand, although dust mite allergens contain a much larger number of components, it has been demonstrated that a combined panel of recombinants can completely inhibit IgE binding to the complete allergen.[61] In a large number of paediatric patients, determination of the allergenic components of different pollens has also been shown to be useful in establishing a correct diagnosis, and important for selecting immunotherapy and follow-up.[62]

However, the usefulness of allergenic components (purified natural or recombinant) applies not only to diagnosis but also to the standardisation of extracts, by determining their contents in terms of each relevant allergenic component. In addition, allergenic components are able to contribute to research into allergens and their function, and to the development of new strategies designed to improve immunotherapy.

Allergenic component microarrays

Evolution of in vitro specific IgE diagnosis

The first in vitro diagnostic tests[63] appeared after the identification of IgE antibodies as the principal mediators of allergic disease.[64,65] These tests used raw or purified allergenic sources to detect such immunoglobulins – initially using radioimmunoassay (RIA) techniques,[66] which were later replaced by the routine use of enzyme-linked immunosorbent assay (ELISA).[67] A characteristic of these tests was that they only detected one type of allergen-specific IgE per test (i.e., single-analyte determinations).

In the late 1980s, Ekins et al.[68] described the first miniaturised multianalyte immunoassays, using microspots arranged in the form of microarrays in which miniaturised ligands were deposited, offering quantitative information and great sensitivity in application to a large number of analytes in a single determination. This advance proved possible thanks to developments in other fields such as image analytical techniques[69] and data analysis.[70]

The extensive development of this analytical tool (the microarray) took place during investigation of the human genome and its expression[71,72] – making it possible in record time to reach the principal goal of the Human Genome Project, i.e., sequencing of the human genome,[73] and defining microarrays as basic analytical tools in molecular genetics.

Posteriorly, interest focused on gene function (functional genetics), based on the Human Proteome Project, offering a new approach to the study of protein properties. To this effect, this new proteomic era also received the contribution of protein and antibody microarrays, together with the classical technologies for protein purification and characterisation, i.e., two-dimensional electrophoretic separation (2DE) and mass spectrometry (MS). This has allowed great advances in proteomics, given the capacity for large-scale analysis of the proteins encoded by the human genome,[74] though the number and complexity of the human proteins (probably close to one million, considering also the isoforms), and post-translation modifications, indicate that the objectives are still far from being reached.

At the same time, protein microarrays have been used to develop diagnostic panels and biomarkers – extending the technique to clinical use in different areas such as autoimmunity,[75] oncology,[76] infectious diseases[77] or allergy[78,79] – the latter being the field where their applications will be reviewed.

Concept and development of allergen microarrays

The term microarray or biochip refers to the distribution of small aqueous volumes of biomolecules on a surface in an orderly and highly dense manner – creating a miniaturised device that can contain hundreds or thousands of biological material probes of known characteristics and immobilised in a matrix component.

The advances made in nanotechnology make it possible to precisely deposit and immobilise a large number of protein aliquots on small surfaces, with spot sizes in the micrometers range and corresponding to amounts of between nanograms to femtograms of protein per spot. In turn, analysis and quantification of interactions with potential ligands can also be carried out.[80–83]

The above advances made it possible to develop a miniaturised IgE measurement test[84] capable of detecting the patient specific IgE profile through a single test, and to use purified natural and recombinant allergenic components from a range of biological sources to generate multianalyte panels that can be used to determine the individual IgE antibody reactivity profile.[85,86]

The antigens used in these miniaturised tests have great intrinsic complexity (e.g., size, charge, solubility, surface activity, three-dimensional structure); as a result, they must be carefully selected for the development of protein microarrays. Adjustment is also required of the antigen spotting conditions in order to secure both efficient immobilisation and to prevent negative effects such as denaturalisation of the surface, maintaining adequate accessibility to the allergen epitopes.

The test conditions must be optimised to achieve the necessary signal-to-noise ratio, and to create a sensitive detection system. In particular, and before incorporation to routine use, the relevant test parameters (e.g., sensitivity, specificity and reproducibility) must be validated to ensure the accuracy of the results afforded by protein microarrays.[87]

The microarrays developed for detecting antigen-specific antibodies are indirect solid-phase immunoassays.[88] These constructs thus correspond to specific IgE semiquantitative indirect enzymoimmunoassay (EIA) – a microELISA test involving multiple allergens.

The first of these to be described made use of raw extracts mounted on glass slides, with isothermal amplification to increase sensitivity.[89,90] These systems were followed by raw allergen microarrays on slides with nitrocellulose membranes.[84]

In 2002, Hillier et al.[86] used purified or recombinant allergens, adapting the component-resolved diagnosis (CRD) concept to the microarray format. Posteriorly, allergen microarrays have been used to determine allergen-specific antibody reactivity – with the studies published in the literature showing the sensitivity and specificity of this system to be comparable to that of conventional techniques.[84,85,91]

Description of the technique

The conventional tests use activated cellulose capsules or discs as solid phase for fixing the allergens, such as for example UniCAP® from Pharmacia,[63] while allergen microarrays generally use chemically modified glass as substrate – different surface modifiers being available that can differ greatly in terms of their chemical and physical properties.[92]

In the ImmunoCAP ISAC® system, developed by VBC-Genomics, small amounts of natural or cloned purified allergenic proteins are uniformly deposited in triplicate on the matrix,[93] to ensure the reproducibility or reliability of the test. In addition, human IgE is deposited in serial dilutions for calibration of the test and quality control.

Each glass slide contains four identical microarrays, thus allowing the evaluation of more than one serum, or several dilutions of one same serum. Each microarray in turn is surrounded by a fine Teflon® layer to prevent overflow of the sample during the test.

Before starting the test, each slide with four identical microarrays is rinsed abundantly for 120 min to remove the allergens non-covalently bound to the surface of the microarray. Subsequently, 20 μl of patient serum is deposited directly on the microarray, and after 120 min of incubation and a brief washing phase, binding of the allergen-specific IgEs is monitored with the addition of fluorescently labelled anti-human IgE antibody. After 60 min of further incubation and a second washing step, the chip is analysed using a confocal double-laser scanner, generating a fluorescent image that is analysed by specific software which calculates the IgE results semi-quantitatively for each allergenic component.

For each individual component, determination is made of the IgE concentration in arbitrary units called ISAC Standardized Units (ISUs), and these values are divided into four classes (negative, low, medium and high), corresponding to the RAST classes 0, 1–2, 3–4 and 5–6, respectively.

Development difficulties

The different microarray spotting techniques and surfaces, as well as the forms of preparing the surfaces for covalent immobilisation of the molecules, may represent a source of deficient or variable test results.

Due to their structural and physical heterogeneity, the proteins may be adversely affected by the spotting process or by drying of the microarray surface; this in turn may lead to biological inactivation of the immobilised proteins. For the microarray spots, measuring 100–200 μm in diameter, and which bind allergen amounts in the femtogram to picogram range, the above may imply a drop in sensitivity, and moreover gives rise to inter-batch variability as a result of the variability of microarray spotters. This problem has gradually been lessened as a consequence of the advances in spotting technology.

Competition between IgG and IgE antibodies for the limited amounts of allergen in turn may pose a problem

particularly for foods and in patients subjected to immunotherapy, though in the context of allergic disease IgG does not appear to have IgE-competing capacity, due to its different affinity characteristics. The strong affinity of IgE for the allergens, which may be among the strongest biologically relevant forms of binding, is in the picomolar range (10^{-10} to 10^{-11} M) versus the nanomolar range in the case of IgG (10^{-6} to 10^{-7} M).[94]

Microarray tests generally tend to produce artificial signals, even when the experiment is carefully carried out. Consequently, defects in the glass substrate, the accumulation of dust particles on the surface, or partial or complete dehumidification, may artificially increase the signal obtained. Such defects cannot be seen by the operator; as a result, strict quality control criteria must be applied in order to accept a reading as being positive.

The supposed disadvantage represented by the fact that the allergen chips do not contain excess amounts of allergen for the IgE antibodies actually appears as an advantage, since this more closely simulates the situation found in the patient, where allergens are present in small amounts. Although for many years working with an excess of antigen has been regarded as a rule for increasing the sensitivity of the in vitro tests, it has been reported that such allergen-specific IgE levels are not always correlated to clinical and biological sensitivity.[95] The assumption that a test involving a small amount of allergen does not necessarily imply a disadvantage has been reinforced by the excellent correlations observed between microarray testing and a routine standard test involving excess allergen.[91]

The current number of allergenic components tested does not cover all the relevant allergens, and some allergenic components of certain biological sources are also missing in the current biochips. This may lower test sensitivity if the patient is sensitised to components not contained in the allergen microarray.

In addition to the above, mention must be made of the fact that some recombinant allergens may not have certain post-translation modifications similar to those of their natural counterparts – thereby impeding the conformation of epitopes, and likewise reducing the sensitivity of the technique.

Thus, many critical factors must be controlled in order to optimise serum IgE analysis with biochips.[96]

Validation of allergic component microarrays

Based on the above considerations, it is seen that allergen microarray tests must be validated and reproducible. To this effect, the tests must be evaluated independently of the manufacturer, by means of carefully conducted clinical studies in order to define the allergen panels that contain a repertoire of allergens similar or identical to that found in the natural sources, and which can offer data that are reproducible between laboratories, as well as between and within tests, with a view to establishing their robustness.

In the first study[97] comparing the microarray technique in its version CRD-50 ISAC®, produced by VBC-Genomics, versus the Phadia UniCAP®, recombinant-based diagnosis was shown to be as sensitive as complete allergen-based diagnosis with the UniCAP®, in patients allergic grass species, cat epithelia or birch. In other the global correlation coefficient was 0.9.[85] Dust mite sensitivity was lower, but remained high (in the same way as specificity). Likewise, performance was seen to be lower in detecting patients sensitised to Artemisia.

A colorimetric technique applied to common allergens was also capable of detecting specific IgE levels lower than the cut-off point accepted for the conventional technique (0.35 Ku/l).[98]

Ott et al.[99] showed the allergenic components of cow's milk and egg used in microarrays to be sufficient to obtain results equivalent to the corresponding extract subjected to enzymoimmunoassay and skin testing with the natural food. The strong correlation between the IgE reactivity patterns obtained with the two in vitro techniques shows that the recombinant allergen panel is closely correlated to the respective extract-specific serum IgE level. Furthermore, these observations have been corroborated by other research groups which have characterised most of the allergenic structures used in allergen microarrays as potent antigens in previous allergenicity studies.[100]

Advantages of allergic component microarrays

By using highly purified natural or recombinant components, these proteins can be used at relatively high concentrations and thus allow optimisation of the individual conditions for spotting in a way that maximises the sensitivity of the test.

In contrast to conventional diagnosis, microarrays allow us to investigate IgE reactivity to a large number of different molecules or allergenic components with a single and rapid test. In the case of the ISAC® (VCB-Genomics), the number is presently 103 allergens per chip, derived from over 40 natural sources.[101] With the traditional methods it would be practically impossible to analyse the panel of natural and recombinant allergens ensuring the presentation of a significant number of epitopes.

Another major advantage of this technique is that the composition of the allergen panels can be expanded and improved continuously, depending on the availability of new components, with the incorporation of the most appropriate profiles defined by the clinical studies.

The technique also allows us to analyse different fluorescences. As a result, in one same test it would be possible to measure specific IgE and IgG, with a detailed study of the recognition of allergens and epitopes by antibodies that may compete with IgE, and assessing their potential protective role.[102]

The amount of patient serum consumed is far smaller than in the case of conventional immunoassaying. In effect, as little as 20 μl suffices to determine IgE for up to 400 individual allergens, while the conventional tests consume 50 μl for each allergen tested. This facilitates the use of the technique in paediatric patients, since such a minute amount of serum can be obtained from a simple capillary blood sample, with highly reliable results versus venous serum sampling (global correlation coefficient 0.92), according to the studies of Ott et al.[103]

Advances in protein and antibody microarrays

Future automatisation of the test will probably reduce variability, improve quantification by using multiple replicates, and yield results in less time. At present, a small number of workstations are available (e.g., Protein Array Workstation [Perkin Elmer] or the HS Series [Tecan]), and studies in the near future will show whether test automatisation leads to improved results. An alternative approach to automatisation of the test could be possible with the adoption of microarray spotting in microtiter plate (MTP) format.

Automated fluorescent multiplex technology, involving polystyrene microspheres measuring 5–6 μm in diameter and with internal fluorescent markers, will allow the conduction of large-scale epidemiological studies, which are particularly interesting in paediatric populations, due to the small amounts of serum required.[104,105]

An increase in IgE antibody affinity, and thus in diagnostic sensitivity and accuracy, can be achieved with the microfluid technique, which uses glass coated with a new poly [DMA-co-NAS] brush copolymer. This allows the immobilisation of allergens in their native conformation and the completion of the different incubation steps under dynamic and totally automated conditions, using a software-controlled fluid processor to apply the reagents to the surface of the array.[106]

Apparently equally promising are the microarray techniques involving microspheres bound to optic fibre.[107] Their clinical application already allows the determination of metabolites in the saliva of patients on dialysis,[108] and of cytokines in the saliva of patients with asthma or chronic obstructive pulmonary disease.[109]

Another development is a novel application of the microarray technique together with basophil activation, which is proving to be useful in allergological diagnosis.[110]

Conflicts of interest

The author declares no conflicts of interest.

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