Molecular diagnosis in Allergology: application of the microarray tecnique

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Abstract

Recombinant and purified allergens are currently available for determining specific IgE targeted to different allergenic components. In this way it is possible to diagnose the sensitization profile of each individual patient. The microarray technique makes it possible to determine specific IgE against multiple allergens simultaneously in one same patient, with a minimum amount of serum, and even allows the determination of IgG and IgM against the same allergens in one same serum sample. Microarray procedures are being developed not only for the determination of antibodies but also for cell activation tests. In addition, microarray technology will help explain cross-reactions, and will facilitate the evaluation of subjects in which skin tests cannot be performed. These techniques will allow a great step forward in the development of immunotherapy specifically targeted to the sensitizations found in each individual patient, yielding especially hypoallergenic forms of great immunogenic capacity, and thus improving the safety and efficacy of immunotherapy. Lastly, microarrays will improve our understanding of the physiopathology of allergic diseases.

Key words: Microarrays, diagnosis by components, specific IgE, immunotherapy.

Resumen

En el momento actual la disponibilidad de alérgenos recombinantes y purificados permite determinar IgE específica frente a diversos componentes alergénicos. De esta manera es posible diagnosticar el perfil de sensibilización individual de cada paciente. La técnica de las micromatrices (microarrays) permite determinar IgE específica frente a múltiples alérgenos a un tiempo en un mismo paciente con una mínima cantidad de suero e incluso permite en una misma muestra de suero determinar IgG e IgM frente a los mismos alérgenos. Ya se están desarrollando no sólo determinaciones de anticuerpos sino ensayos de activaciones celulares en micromatrices. Además ayudará a explicar reacciones cruzadas, facilitará realizar una evaluación a sujetos en los que no podemos realizar pruebas cutáneas. Va a significar un gran impulso para el desarrollo de la Inmunoterapia dirigida exactamente a las sensibilizaciones de cada paciente, consiguiendo formas especialmente hipoalergénicas con gran poder inmunogénico, mejorando la seguridad y la eficacia de la inmunoterapia. Finalmente, estas técnicas van a facilitar la comprensión de la fisiopatología de las enfermedades alérgicas.

Palabras clave: Micromatrices. Microarray. Diagnóstico por componentes. IgE específica. Inmunoterapia.

Introduction

When speaking of new diagnostic methods, it is good not to lose sight of the historical perspective. In the 1930s, Alexander Francis stated that "Skin tests are simple and fascinating; and the vaccines used to immunize against the protein found to be the cause of the symptoms became so popular as a universal treatment that the cure for all forms of asthma was believed to be at hand" [1]. Years later, the discovery of IgE generated controversy between those who defended the reliability of skin testing and those who supported in vitro diagnosis – to the point that an editorial claimed that the determination of specific IgE would "quickly replace the primitive skin tests in clinical practice" [2].

Perhaps the same will happen with molecular diagnosis. However, it is clear that the steps taken by Charles Blakely in investigating his own pollinosis by performing a pollen skin test on himself, or by Kimishige and Teruko Ishizaka on one hand and Johansson on the other in discovering IgE, constituted hallmarks in our understanding and treatment of allergic diseases.

Molecular diagnosis

In the late 1980s, when the first allergen was cloned [3], a new era was opened for the production of purified recombinant allergens of use in diagnosis and treating allergic diseases [4].

Up until that time it was possible to determine the allergenic source to which a given patient was sensitive. With the application of molecular or components diagnostic techniques it is possible to define the individual sensitization profile, i.e., we can establish what parts of the allergen are recognized by each individual patient. In recent years we have seen the characterization and production of the most relevant allergens at molecular level, and most have been generated as recombinant proteins [5, 6]. However, the different new purified recombinant allergens have steadily increased in number, and it is almost impossible to study all the recombinants of related families in one same patient.

Subsequently, effort focused (and remains centered) on the confirmation that the available recombinant allergens or natural allergens are those truly recognized by the patient IgE, and that they trigger symptoms. The aim is to establish that they possess an IgE epitope recognition profile similar to that of the allergens that had been studied to date. Furthermore, determinations are being made to establish that the allergens panel is representative of a given type of sensitization [7].

Principles of the technique

Microarrays or biochips were developed as a tool for analyzing gene expression in genomes. Since their introduction in the early 1990s, DNA microarray technology has been applied to the determination of nucleic acid, and this in turn was followed by analysis of the expression of RNA. This step was needed among other reasons because the exploration of gene expression required increasingly effective tools for studying protein expression at intracellular level. The microarray technique for proteins was thus developed, adopting the same microarray technology initially used in application to DNA.

Microarray technique is a solid phase multiple immunoassay in which the proteins (purified recombinant or natural allergens) are immobilized in a solid phase, and minimal amounts of serum are incubated with these proteins under standardized conditions. The antibodies present in the serum are captured by the different allergens, and following a washing step to eliminate the unbound substances, the antibodies are detected by means of a second fluorescent-labeled anti-isotype antibody or an enzyme that is detected by laser or chemiluminescence.

To date, specific IgE has been determined on an individual allergen-by-allergen basis. With this technique, multiple allergenic components are determined in one same serum sample, and it also allows to determine in the same serum sample not only IgE but also IgG, IgM and IgA simultaneously, and targeted to the same allergens.

As in the case of the DNA microarrays, the technique is performed on solid surfaces such as high-quality glass slides of the same kind used in light microscopy. For immobilization of the protein on the slide [8, 9], the surface is modified, for example with nitrocellulose or gel-like structures. The different types of protein (recombinant, antibodies, peptides or heptamers) are deposited in micrometric spaces using robotics (at present this allows us to deposit up to 30,000), followed by reaction with the ligand. This binding reaction in turn is detected by fluorescent-labeled antibodies, stains or combined techniques. Fluorescence is usually detected by laser. In order to calculate and analyze the results on a semiquantitative basis, software is needed to compare the fluorescence of the test allergens with a known IgE concentrations curve, used to extrapolate the test result.

In general, the entire process lasts no more than five hours, and poses no technical difficulties. The number of patients depends on the number of slides processed at the same time. In the case of specific IgE, based on the technique used by the authors of the VBC-Genomics prototype, each slide can be used to analyze up to four patients.

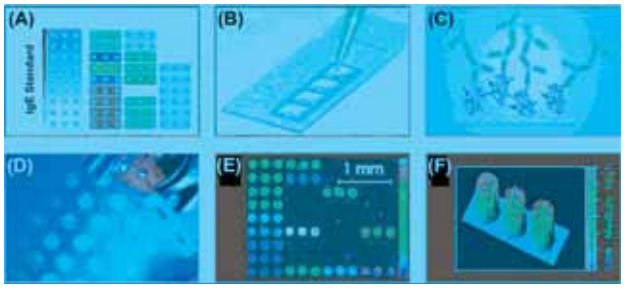

Figure. The steps of the technique comprise prior washing to eliminate all non-covalent bonds to the allergens, followed by the addition of 50 µl of serum and incubation. After a brief washing step, the fluorescent-labeled antibody is added, and the glass is laser-scanned after a second washing step.

Table 1. List of available allergens

Foods		Trees	Dust mites	Latex
Kiwi	nAct d 1 nAct d 2 nAct d 5 nAct d 8 nAct d 1 nAct d 2	Aln g 1.0101 Bet v 1.0101 Bet v 1.0401 Bet v 2.0101 Cor a 1.0103 Cup a 1 Ole e 1	Der f 1 Der f 2 Der p 1 Der p 2 Der p 10.0101 Der s 1	Hev b 10.0102 Hev b 6.02 Hev b 7.02 Hev b 8.0204 Hev b 11.0101 Hev b 1.0101 Hev b 3.0101
Celery	Api g 1.0101	Ole e 2 Pho d 2.0101	**Animal epithelia** Can f 1 Can f 3	Hev b 5.0101 Hev b 0.0101
Milk	Bos d 4 Bos d 6 Bos d 7 Bos d 8 Bos d Lactoferrin Bos d 8 alpha S1 Bos d 8 beta Bos d 8 kappa Bos d 5.0102 Bos d 5.0101 Cor a 8	Pla a 2 Pla a 1.0101 **Grasses** Cyn d 12.0101 Lol p 1 Phl p 1.0102 Phl p 5.0101 Phl p 12.0101 Phl p 2.0101 Phl p 6.0101	Fel d 1 **Fungi** Alt a 1.0101 Alt a 6.0101 Asp f 1 Cla h 8.0101 **Cockroach** Bla g 2.0101 Bla g 5.0101 Per a 7.0101	
Carrot	Dau c 1.0103	Phl p 7.0101		
Egg	Gal d 1 Gal d 2 Gal d 3 Gal d 4 Gal d 5	**Weeds** Art v 1 Hel a 2.0101	**Anisakis** Ani s 3.0101 Ani s 1.0101	
Apple	Mal d 1.0108	Mer a 1.0101		
Shrimp	Pen i 1 Pen m 1	Par j 1.0103 Par j 2.0101 Par j 3.0102	**Hymenoptera** Api m 1 Api m 4	
Peach	Pru p 1 Pru p 3			
Wheat	Tri a 18 Tri a 19 Gliadin Tri a 19.0101 Tri a aA_TI	**CCD marker*** Ana c 2		
Cashew nuts	Ana o 2.0101			
Sesame	nSe s i1			
Brazil nut	rBe re 1			
Peanut	nAra h 1 nAra h 2 nAra h 3 rAra h8			
Hazelnut	rCor a 1.0401 rCor a 8 nCor a 9			
Soya	rGly m 4 NGly m b-conglycinin nGly m glycin			

* Carbo-hydrate Cross Determinants.

The concept of a protein ligand reaction was published for the first time 15 years ago [10]. Such ligand reactions were not possible in the past because of the difficulty of immobilizing proteins in such small spaces, due to their size, charge and three-dimensional structure. It was not until 10 years later when the use of microarrays with raw allergen on conventional glass slides was first published, using signal amplification [11]. Subsequently, Kim et al. performed the technique on a nitrocellulose base chip [12]. In addition, mention must be made of the difficulty posed by possible protein denaturalization during the immobilization process, and of the fact that recognition by IgE requires preservation of the tertiary structure.

In sum, the technique constitutes a specific IgE semiquantitative indirect enzymoimmunoassay (EIA). The main advantage with respect to other methods is that we can perform multiple determinations of specific IgE targeted to a panel of allergenic components which can be expanded. The technique recognizes the patient IgE by means of a fluorescence-labeled secondary antibody. As commented above, a panel of recombinant or natural allergens is immobilized on a chip with dimensions that allow easy handling (the size of a glass slide). With the currently available technique, each allergen is bound to the slide in triplicate, to ensure reproducibility of the test. The amount of serum required is 50 ml, together with the calibrators. Each well containing the allergen is surrounded by Teflon to prevent spillage of the sample. Each well contains the same amount of allergen, in case dilutions are posteriorly to be studied. The number of allergens that can be immobilized by means of this technique is practically unlimited.

Correlation with determination of specific IgE

A number of studies have explored the correlation of the microarray technique and the different methods used to date. The first study compared the microarray technique developed by VBC-Genomics with the Phadia CAP system for determination of specific IgE for three allergens corresponding to grasses, birch and dust mites, and reported a correlation of 0.9 [13]. Lebrun [14] published the results obtained with a colorimetric technique applied to common allergens, detecting specific IgE levels lower than the cut-off point accepted for the conventional technique (0.35 Ku/l).

More recently, Wöhrl et al. [15] published their results comparing microarrays with the version CRD-50 ISAC technique, produced by VBC-Genomics, and the Phadia UniCAP. The diagnosis of recombinants was shown to be as sensitive as the diagnosis of complete allergen determination with the UniCAP for patients allergic to grasses, cats and birch. Sensitivity with dust mites was lower, but remained high (in the same way as specificity), and was likewise seen to be lower for the detection of patients sensitive to Artemisia.

Benefits

The benefits of the technique are summarized in Table 2.

The main advantage of the technique is that it allows to analyze hundreds of allergens at the same time, with a minimum amount of sample (only 50 ml of serum), and involving a single analysis. In the case of VCB-genomics, the figure is presently 103 allergens per chip (the list of allergens is shown in Table 1). The technique makes it possible to expose the greatest possible number of recognizable epitopes to IgE. The technique also allows to analyze different fluorescences; as a result, in one same test it could be possible to measure specific IgE and IgG.

Table 2. Advantages of the microarray technique

Simplicity
Minimal sample volume
Flexibility
High yield
High production capacity
Need for only scant allergen
Scalability
Automatization

A second benefit of the technique is that it facilitates component-based diagnosis [15-17]. This fact offers a greater curacy in establishing which allergen is recognized by a given patient, helping to explain cross-reactions, and resolving enigmas such as patients with positivity to multiple pollens to which they have never been exposed and which are apparently scantly related (the explanation in such cases being sensitization to panallergens). With the traditional methods it would be practically impossible to analyze the panel of recombinants and natural allergens ensuring the presentation of a significant number of epitopes. On the other hand, the molecules might not present the same immune reactivity as the complete natural allergen.

Another important benefit of this technique is that it allows the screening of individuals in whom skin tests cannot be made, such as patients with severe atopic dermatitis, dermographism or children, or cases in which the intensity of the reaction precludes skin testing.

Immunotherapy

A field in which the contribution of the microarray technique will be crucial is the development of the composition of immunotherapy. To date, immunotherapy composition consisted in allergen extracts, comprising mixtures of allergenic and non-allergenic components. These formulations are difficult to standardize and do not adapt to the sensitization profile of each individual patient. Immunotherapy currently can contain allergens to which the patient is not sensitive, or may contain insufficient doses of those allergens that are relevant to the patient. More importantly, the formulation used may lack precisely those components to which the patient is most sensitive. Lastly, immunotherapy with complete extracts possesses allergenic potential in addition to immunogenic potential, and this entails a risk of hypersensitivity reactions.

A number of studies have shown that immunotherapy targeted to recombinant allergens is both effective and safe [18-21], and moreover allows the use of especially hypoallergenic forms with great immunogenic potential. As a result, in the near future the quality, safety and efficacy of immunotherapy will be enhanced [21]. In addition, such procedures will allow to explore the mechanism of immunotherapy.

To this effect it is necessary to demonstrate that such formulations possess the same potency as the complete natural allergens, and that the panel of recombinant allergens encompasses all the epitopes recognized by the B and T cells. In the case of grasses, it has been shown that a panel of 5 recombinants (Phl p 1, Phl p 2, Phl p 5a, Phl p 5b) [22], or the mentioned panel plus Phl p 6 [23], significantly reduces the symptoms and induces a specific and very intense IgG mediated response against these allergens [24]. Birch allergens behave in a similar manner.

Although dust mite allergens yield a much greater number of recombinants, it also has been shown that by combining a panel of recombinants we can fully inhibit IgE binding to the complete allergen [25]. It is thus shown that recombinant allergens can substitute the natural extract, and that several recombinants suffice to establish the diagnosis. In patients sensitized to dust mites, the technique already allows us to determine whether they are sensitized to group 1 or two group 2 allergens, or whether in contrast the subjects are sensitized to tropomyosin – which would constitute a contraindication to immunotherapy with dust mite extracts containing group 1 and 2 allergens.

It has even been shown that recombinant hybrids from unrelated allergenic sources possess great immunogenic capacity [26].

Other applications of the microarrays technique in Allergology are commented below

An innovating application of the microarray technique in allergological diagnosis has recently been published [27]. The procedure is based on the basophil activation test, which consists of analyzing the expression of basophil activation markers such as CD63, following stimulation with different allergens [28, 29]. The authors eliminated IgE from mature human basophils and from a basophilic cell line, followed by resensitization of the cells with serum from patients with allergy to grasses. This in turn was followed by the determination of CD63 expression after incubation. The authors moreover found that the cell line responds in the same way as the adult basophils in peripheral blood. These results need to be reproduced and confirmed by other investigators, though they illustrate the potential for development of microarray technology.

Basophil immobilization was achieved with the development of microarrays for typing leukemias

This technique, in which the presence of specific IgE does not necessarily imply that it is able to bridge the IgE receptors of basophils and mast cells and thus trigger symptoms, only indicates the existence of sensitization. The technique quantifies basophil activation in response to a given allergen via binding to the specific IgE present in the serum of the studied patient – not only the presence in serum of specific IgE.

Future applications

One future application will be the identification of new patients previously classified as being non-allergic individuals, since some recombinant allergens are not present in the allergenic extracts used to date.

Regarding food allergy, the potential is particularly important, since panels could be developed that include at least the overall proteins to which we are exposed in a given diet, or to which patients from a given geographical setting are most exposed. This is being done with diets in the United Kingdom, for example [30].

Lastly, the technique may serve to establish predictive values for the symptoms severity, as is already being done for LTPs and anaphylaxis, or for the probability that food sensitization in children will be grown. Another possibility is the definition of adequate immunotherapy composition.

Ideally, and in addition to the required reproducibility and reliability of the technique, an *in vitro* diagnostic method should be minimally invasive – allowing the clinician to collect extensive and applicable information.

The technique requires validation and the conduction of further studies with large population cohorts, and correlates results with clinical symptom. We should avoid blind enthusiasm, but in the meanwhile we should seize the oportunity as Allergists.

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