

Biotechnologies in new high-throughput food allergy tests: why we need them

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Received: 11 October 2006 / Revised: 30 October 2006 / Accepted: 31 October 2006
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Abstract The increase in prevalence of food allergies generates a need for more accurate and reliable quantitative allergy testing in order to help diagnosis. In this short review, we briefly outline the history of food allergy testing and extend our comments to current multiplex techniques. Particular emphasis is given to new developments in the protein microarray area, where the use of recent advances in biotechnology has the potential to produce high-throughput devices with improved clinical significance.

Keywords Allergy testing · Basophil · Food allergy · High-throughput · Immunoglobulins · Protein microarray

Introduction

It is now clear that the prevalence of hypersensitivities in developed countries, in particular the prevalence of food allergy, is increasing (Madsen 2005). Recent studies suggest that 4% of the American population are affected by food allergies, and that the numbers are greater (6%)

within the infant population (<3-years-old). Of these children, 80% are expected to become tolerant, or lose their sensitivity, to the allergens by the time they are 5-years-old (Sampson 2004).

Contact with an allergen through body surfaces, such as skin, nasal and digestive tract mucosa in allergic individuals results in the major classical symptoms of asthma, rhinitis, conjunctivitis and sinusitis, with a range of intensities from a mild form of sneezing and coughing to potential life-threatening anaphylactic shock (Harwanegg and Hiller 2004). Whilst other forms exist, the majority of life-threatening cases of food allergy are classified under the category of hypersensitivity type I and therefore mediated by immunoglobulin E (IgE) (Sampson 2005). A plethora of drugs are now available, or are under development, for the treatment of symptoms (Sampson 2004, 2005), yet the most important advice given to patients is to avoid direct contact with the responsible allergen. Hence an accurate and reliable quantitative diagnosis is important.

Past and current allergy diagnosis

Prausnitz in the early 1920s demonstrated a transfer of sensitivity to non-atopic patients by pricking the skin with fish extracts. It took almost half-a-century since Prausnitz's initial findings to identify and characterise immunoglobulin type E (IgE) as

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the major immunoglobulin responsible for type I hypersensitivity (Blackley 1959). Due to the low levels of IgE in blood serum (Harwanegg et al. 2003), it was not until late 1960s and the development of radiolabelling techniques, that a real food-specific IgE-test became available (Ishizaka and Ishizaka 1967). This sensitive radioallergosorbent immunoassay test (RAST) format (Wide et al. 1967) is still in use today in its upgraded version using fluorescently labelled markers instead of labelled isotopes (Seltzer et al. 1984) (Fig. 1).

Early allergen detection tests lacked reproducibility, mainly due to large batch-to-batch variations in stability of the allergens following extensive extraction procedures. Hence, an important advance in allergy diagnosis testing was the introduction of standardised allergen extracts achieved in the late 1970s and early 1980s (Aas et al. 1978). The standardisation of allergens was responsible, not only for the improvement of the accuracy of skin prick tests, but also for the reliability of this test when coupled to the RAST test (Aas et al. 1978). In a subsequent step, the introduction of robust and mechanised systems such as UniCAP slowly replaced the semi-quantitative and laborious RAST test for IgE determination in large clinical labs (Lebrun et al. 2005).

During the nineties, with the availability of protein sequences, large databases and advances in protein expression, the production of large quantities of specific well-purified recombinant allergens became possible. In the past 10 years several attempts have been made to combine the availability of large amounts of purified allergens with the small quantities of test material, such as a drop of blood, in diagnostic tests. Although these are important advances, and will be discussed further in the following paragraphs, it must be

stressed that one important characteristic of food matrices is their complexity. As well as proteins, other types of molecules such as complex carbohydrates, neutral and complex lipids, phenolics, pigments, and small pharmacological active substances able to take part in the allergenic response, are always present. These factors should not be disregarded during the development of new techniques.

Allergen microarrays

As with all present-day technologies the move towards miniaturization of diagnostic testing is a popular one, not just for economic but also functional reasons. In the past 10 years the ability to produce microarrays has provided the technology for monitoring the expression of thousands of genes, under many different conditions, on a solid-based media the size of a microscope slide. Initially used for DNA, the technique has evolved to other more sophisticated applications using different attachment methods in order to determine protein functionality (MacBeath and Schreiber 2000), protein interactions (Kukar et al. 2002) and enzymatic activity amongst others (Nielsen and Geierstanger 2004).

One exciting prospect of using protein microarrays as a diagnostic tool is the ability to miniaturise a whole assortment of tests onto one small slide. Conventional protein printing robots are now able to deposit 10,000–30,000 spots on a slide of size 75 × 25 mm (Harwanegg et al. 2003). The mechanics of protein array production are now very well established and a number of comprehensive reviews are available describing this area (Predki 2004; Bertone and Snyder 2005; Merkel et al. 2005; Molloy et al. 2005).

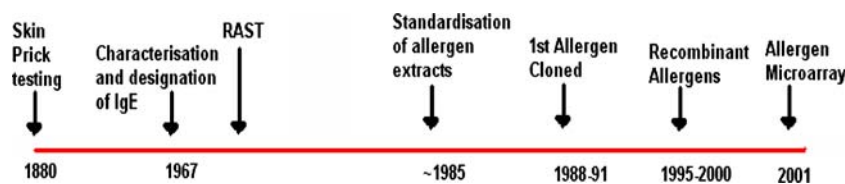


Fig. 1 Diagram adapted from Harwanegg et al. (2003) showing some important milestones on the development of food allergy testing from the 19th century to the present day

Essentially the protein microarray represents a multi-analyte solid-phase immunoassay, where proteins are immobilised onto the solid surface and micro-quantities of the serum samples are then incubated under standard conditions. Antibodies to the particular protein present in the patient's serum are captured by the immobilised protein, and after the slides are washed, the antibodies are detected with a fluorescently labelled anti-isotype antibody and a laser-based scanner (Harwanegg and Hiller 2005) (Fig. 2). The same assay format has been further developed using different and improved solid surfaces (Fall et al. 2003; Jahn-Schmid et al. 2003). Many aspects of the array technology, such as the solid support for immobilisation (Kusnezow and Hoheisel 2003), “direct” × “sandwich” format (Haab 2003), methods used for signal amplification (Haab and Lizardi 2006) and multi-analyte detection (Barry and Soloviev 2004) are reviewed elsewhere.

With the availability of a large number of well-characterised recombinant proteins, arrays of different complexity have been produced employing purified recombinant allergens (Harwanegg et al. 2003). The results obtained from these prototypes are very encouraging and a number of published examples have now shown that the sensitivity of these tests is similar to those from RAST, ELISA and immunoblot tests (Kim et al. 2002; Shreffler et al. 2005; Harwanegg et al. 2003). There are claims that the varied natural environment to which the body is exposed to on a daily basis can now be mimicked by using a large number of allergens (Hiller et al. 2002). Despite

the benefits of the use of pure proteins, the sheer numbers, complexity and logistics of producing a comprehensive assay that covers all known protein families are sometimes overlooked. One advantage of using heterogeneous protein mixtures from allergen extracts on arrays instead of purified proteins should be the detection of underrepresented proteins or protein complexes in allergenic foods (Kim et al. 2002). Some promising results have been reported in studies conducted with over 150 different crude extracts (Bacarese-Hamilton et al. 2005).

In summary, due to their simplicity, low sample volume, high-throughput capacity and flexibility, protein microarrays are becoming important tools in the determination of allergen specific-IgE, particularly in large screening programs. Thus, some recent biotechnological improvements such as solid surfaces, detection techniques, epitopes and cell based-assay in this platform will be further discussed.

Protein Microarrays—some recent improvements

One key factor when choosing the surface platform on which to immobilise the protein, is the necessity of obtaining the best possible spot morphology which in-turn leads to accurate and repeatable data output. Generally the surfaces can be classed as one-, two- or three-dimensional (1-D, 2-D or 3-D). Silanated glass for instance is considered a 1-D coating that can provide poor spot morphology and smearing. Two-dimensional coatings, such as PEG, make use of functional groups and spacer arms (Dyukova et al. 2006). Most recently, the 3-D coating surfaces, such as nitrocellulose (FAST slides) and polyacrylamide gels, are being widely used (MacBeath and Schreiber 2000). A study with different silicon structures showed that nitrocellulose surfaces give a higher signal intensity and improved performance (Steinhauer et al. 2005). The main feature of these surfaces is that they act like membranes and project the proteins from the slide surface, increasing the binding capacity. A number of good reviews on solid surfaces used in protein microarrays are now available (Kusnezow and Hoheisel 2003; Uttamchandani et al. 2005).

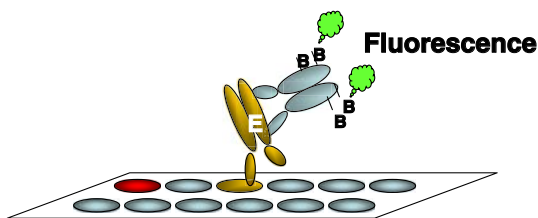


Fig. 2 Diagram showing the basic components of a standard protein array. Antibodies from the patient's serum recognise the immobilised protein molecules on the surface of the chip. Secondary antibodies and fluorescent markers allow the target molecule to be detected and quantified

With the widespread use of microarrays in the systematic analysis of complex biological samples, requirements, such as quantification, dynamic range, assay sensitivity and ability to use multi probes, have emerged. A number of labelling and detection methods have now been successfully employed. These include the simple fluorescently labelled second antibody detection system, previously mentioned, and can extend to more sophisticated rolling circle amplification (RCA) systems employing a polymerase-driven reaction initiated by DNA specific primers attached to the antibody (Table 1) (Kingsmore and Patel 2003; Nielsen and Geierstanger 2004). With different degrees of amplification and consequently different levels of background noise these new methods have the potential for recognising attomoles of a specific protein allergen or epitope.

An interesting and powerful tool to address high-throughput requirements is the ability to use multi-probes. The development of such multiplex techniques, particularly on large arrays where the specific antibody is first immobilised on the solid surface (antibody arrays) or beads, have been successfully used in many applications with high sensitivity, broad dynamic range and good reproducibility (Schweitzer and Roberts 1998; Kingsmore and Patel 2003; Nielsen and Geierstanger 2004). The prospect of using rolling circle amplification (RCA) and encoded beads (Hashmi et al.

2005) as multiple detection probes can accommodate the detection of multiple antibody types binding to each analyte. As the sensitivity increases it enables molecules that tend to exist at very low concentrations, such as cytokines, to be detected. Concentrations around the pico and femtomolar range have been commonly reported for these analytes (Schweitzer and Roberts 1998). With new amplification techniques levels of up to 3 log increase in detection could be attained but most importantly a link to the functionality of the protein of interest in biological fluid is now possible (Pavlickova et al. 2004).

The printing of protein microarray technology has traditionally relied on techniques such as micro-contact printing and spot-arraying. The spots can then be screened using common fluorescence scanning. A different approach is to look into the actual binding of molecules on the microarray slide. Studies using protein-receptor have shown that binding events cause changes in the hydrophobicity, height and shape of the two components (Lee et al. 2002). This change can therefore be measured. A new high-resolution patterning technique called dip-pen nanolithography (DPN) can create spots of 100–350 nm in diameter on a thin gold film, and the height of these spots can then be measured by atomic force microscopy (AFM). The main advantage advocated for this technique is the elimination

Table 1. Some current advances in the amplification of the signal detection currently employed for multiplexed sandwich assays

Assay	Signal generation	References
Fluorescence	Fluorescently labelled second antibody	Nielsen et al. (2003)
Fluorescence	Fluorescently labelled species-specific tertiary antibodies	Sasakura et al. (2006)
Biotin and fluorescent streptavidin	Staining of biotinylated detection antibodies with fluorescent streptavidin conjugates	Wang et al. (2002), Huang et al. (2001)
Streptavidin–phycoerythrin (SAPE)	SAPE staining amplified with biotinylated anti-SAPE antibodies and a second layer of SAPE	Wodicka et al. (1997), Saviranta et al. (2004)
Streptavidin-linked horseradish peroxidase (HRP)	HRP increases the number of biotin molecules on the antibody spot for staining with fluorescent streptavidin variants	Woodbury et al. (2002)
Streptavidin-linked HRP	HRP linked to a species-specific tertiary antibody activates chemiluminescence substrates	Moody et al. (2001), Wiese et al. (2001)
Rolling-circle amplification (RCA)	RCA of a DNA primer attached to an anti-biotin antibody combined with the hybridization of fluorescently labelled oligonucleotides	Schweitzer et al. (2002), Haab and Lizardi (2006)
Resonance light-scattering (RLS)	Resonance light-scattering colloid gold particles coated with anti-biotin antibody	Geierstanger et al. (2006), Yguerabide and Yguerabide (2001)

of non-specific binding to the background (Lee et al. 2002). A distinct advantage of using nanotechnology coupled to the arraying of allergen molecules is the abrogation of secondary antibodies. Instead, AFM can detect binding events without the need for a label (Arntz et al. 2003). One obvious disadvantage however is the inevitable cost of the initial set up.

The characterisation of the protein epitopes recognised by the IgE is fundamental in the understanding of the mechanisms leading to allergy and for the designing of safe immunotherapeutics. In its simple format, overlapping peptides immobilised in epoxy-derivatised slides with 10–20 amino acids in length have been used in the identification of specific protein epitopes for the peanut allergen Ara h2 (Shreffler et al. 2005). Sera from peanut reactive patients and a control group were analysed and nine epitope sites were identified, two of which were newly outlined. Cluster analysis has shown a high degree of correlation between patient sera and their reactivity to specific peptides (Sampson 2004; Shreffler et al. 2005).

Whilst mapping a large number of peptide epitopes can provide prognostic information about the patient and some information on protein antigenicity, it has the drawback of missing the important non-linear epitopes. In a more elaborated approach the proof-of-concept of using engineered properly folded chimeric proteins containing swapped structural domains has been established in the mapping of the 3-dimensional IgE epitope of a nut protein (Alcocer et al. 2004). With advances in the domain swapping technologies by commercial in vitro recombination systems it is expected that the knowledge accumulated will further our understanding of how the body's immune system sees and reacts to new proteins.

Future developments

Due to the minute quantities of some allergens in extracts, and the low abundance of IgE, a functional diagnostic test for type I allergic reactions will require the maximum sensitivity, dynamic range and specificity currently available. The

proof-of-concept that new developments such as the RCA could be used for diagnostic purposes has already been established. As far as the hardware is concerned, with the development of new materials, the future tendency is to depart from the quartz solid surface towards a 3-D matrix. Whether the reaction takes place on flat solid surface slides or on encoded beads or suspension arrays will greatly depend on commercial considerations rather than technical restrictions. Despite the overall tendency to produce large antibody-based assays for protein profiling, the complex nature of the allergenic extract suggests that traditional immobilisation of the protein extract would be the best way forward. With this consideration we have produced, and are currently validating, a concept of an “All-Diet” approach to the diagnosis of food allergy by creating an array of crude and purified extracts of foods found in the British diet. It is expected that by using this approach the allergenic potential of a range of food components to which a person is exposed on a daily basis could be established, rather than focusing on specific common allergenic proteins. Furthermore, overlapping of protein epitopes between different foods could allow a multiplex analysis of other immunoglobulin classes such as IgE/IgGs/IgA and IgM to be compared within large group of individuals. In this context, real-time profiling of specific immunoglobulins present in sera and milk of lactating mothers could give a further in-sight into the relationship between intake of allergenic foods and its varying effects on the newborn.

Another unique way of upgrading an allergen diagnostic test based on the type I allergic reaction is to couple the binding specificity of the IgE from patients with naïve cells of the immune system. The proof-of-concept of immobilising intact cells on the surface of the array has been elegantly established for classification of different leukemias on a microarray of antibodies to CD markers (Belov et al. 2001). Recently our group has demonstrated that isolated human/rat basophils can also bind specifically and de-granulate on a microarray format assay (manuscript in preparation). By using these technologies the cellular mechanisms of allergies could be mimicked in vitro and potentially this might provide a

biological assay which is nearer to the reality of conventional allergy testing in clinics world wide.

Summary

As we now commence the 21st century, a fourth generation of allergy tests are starting to evolve. High-throughput diagnostic tests are now possible and multiple allergen detection marks the newest tool available to produce rapid, accurate, and immunological functional allergy profiling. Coupling the diversity of an “all-diet” microarray approach with the potential functionality and biological activity of a cell-based test provides a unique insight into the relationship between immunoglobulins and effector systems in Type I hypersensitivity.

The progression from lab-based tests for specific Ig-binding to clinical practice is a long way from being realised, however new techniques, such as the ones discussed here, will provide the tools to achieve this goal.

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