SPECIALISED *IN VITRO* DIAGNOSTIC METHODS IN THE EVALUATION OF HYPERSENSITIVITY – AN OVERVIEW

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ABSTRACT

Hypersensitivity reactions can be differentiated into IgE- and non-IgE-mediated allergic and also non-allergic reactions. In this article we explore currently available tests used to distinguish non-IgE conditions. Testing involves not only estimation of the different antibody types but also cellular activation and inflammatory markers.

Allergic diseases, including reactions to foods, represent increasing health problems worldwide, and symptoms may not be easily distinguished from other disorders. The term hypersensitivity is defined as a reaction that induces reproducible symptoms and signs, initiated by exposure to a defined stimulus at a dose tolerated by normal subjects.1 Hypersensitivity can be differentiated into IgE and non-IgE allergic and non-allergic hypersensitivity, which does not involve the immune system. Therefore, different tests must be used to distinguish between these conditions. An allergic hypersensitivity is usually IgE-mediated but may involve IgG and IgA antibodies as well as other immune cells (Table I).

Most patients are sensitised to more than one allergen which might trigger clinical symptoms and often it is difficult to distinguish the major offender. In addition, the symptoms are not only dependent on IgE antibodies but also on a number of other confounding factors. These can include inflammation, presence of infection, physical and psychological stress and hormonal influences. The gold standard for food allergy or intolerance is the double-blind placebo-controlled food challenge (DBPCFC).[2,3] However, this technique does not distinguish between allergic and non-allergic hypersensitivity involving different antibody types, cellular immune mechanisms and reactions based on intolerance.

With these issues in mind *in vitro* assays need to determine the mechanisms behind the symptoms. In this article we explore currently available tests and highlight their applications and limitations.

ALLERGEN MARKERS

The ability of a test to detect specific antibodies depends on the presence of relevant allergen components in the test system. Natural allergen extracts are routinely used for *in vivo* and *in vitro* diagnostic procedures. But naturally occurring allergens vary significantly in their composition and allergenicity. To complicate the issue, most patients do not raise specific antibodies to all allergens, but only some allergenic components. This is of importance for the correct diagnosis and composition of allergen vaccines for specific immunotherapy (SIT). To address this, recombinant

Table I: Tests for the presence of allergic sensitisation and identification of offending allergens

Test aims to identify	*Principle of the test*	*Basic technology*	*Major test system*
Presence of sensitisation to specific allergen	IgE/IgA/IgG antibodies tests to allergens from one allergen source or one single allergen component	Different assays using a solid phase to bind allergen-specific antibodies and detect with anti-IgE/IgA/IgG reagents	UniCAP ELISA Immunoblot Allergen microarray
Presence of inflammation mediators from different cells	Histamine from basophils/mast cells	Solid phase with attaching antibody and labelled anti-mediator reagents	UniCAP
	Tryptase from mast cells		UniCAP
	Leukotrienes and prostaglandins		ELISA
	Eosinophil mediators such as ECP		UniCAP
	Lymphocyte mediators such as cytokines		ELISA
Cellular immune response	T-cell proliferation	Cell cultivation with specific allergen/antigen stimulation; analysis of cell proliferation	Tissue culture
	Basophil activation		CAST Flow cytometry

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allergens have recently been introduced into conventional *in vitro* testing, a strategy termed component resolved diagnosis (CRD).[4,5]

Hundreds of food allergens have been characterised and over 50 allergens have been produced as recombinant allergens (details accessible via the Internet at http://www.allergome.org/). About thirty recombinant allergens are already available in the ImmunoCAP (Phadia) system.

In addition a novel antibody detection system, the allergen microarray, has emerged as a promising approach to high-throughput large-scale profiling of allergen interactions for simultaneous monitoring of IgE and IgG antibodies directed against a variety of allergy-eliciting molecules.[6,7] The major benefit of this technology lies in its ability to screen for several hundred allergen molecules simultaneously while employing only minute amounts of the patient's serum (20 µl).

CELLULAR MARKERS

During the allergic reaction new and preformed mediators are released from cells, such as mast cells and eosinophils, into the tissue or blood and these can be quantified. These include histamine, tryptase, leukotrienes, prostaglandins and eosinophilic cationic protein.

Tryptase and histamine

Mast cells play a key role in allergic reactions and the numbers increase under inflammatory conditions. After activation, they release a range of mediators, including tryptase and histamine, which in turn can lead to allergic symptoms, including systemic anaphylaxis. Activation follows an anaphylactic reaction triggered by food, drugs or insect venom. Histamine in contrast to tryptase is a very unstable marker and degrades very fast (within minutes!). Therefore an elevated level of serum tryptase is a more reliable indicator for anaphylaxis or mastocytosis.[8] Increased levels can also be measured in nasal secretions[8] and indicate active allergic rhinitis. Values over 20 µg/l should be considered elevated and can usually be detected within 3 hours of mast cell activation.

Eosinophil cationic protein (ECP)

ECP is a highly cytotoxic protein found in eosinophil granules. Eosinophils are the main cells responsible for producing the inflammation characteristic of asthma by degranulation in the lung tissue during activation. This can increase hypersensitivity and lead to chronic inflammatory diseases of the airway.

Elevated levels of ECP can be quantified in serum, bronchial alveolar fluid and induced sputum. High levels indicate inflammation, which is a risk factor for uncontrolled asthma. The measurement of ECP in serum can be used to monitor inflammation in asthma, guide corticosteroid treatment and expose non-compliant patients.[9] Elevated ECP levels have also been observed in children with cow's milk allergy. Values over 15 µg/l should be considered elevated, but patients should act as their own control during treatment and follow-up if possible.

ANTIBODY MARKERS

Specific IgG antibodies

During an immune reaction to a foreign antigen, antibodies are produced as part of the body's complex defence mechanism. Antibodies of the IgE type are typical in type I allergic reactions; however, high titres of antigen-specific IgG and IgA antibodies are also observed. In autoimmune disorders, these antibodies are directed against self-antigens (autoantigens). The presence and level of specific IgG antibodies in serum can reflect the extent of exposure to that antigen. IgG antibodies can be quantified via the ImmunoCAP system or the micro-arrays system. The IgG antibody response can be quantified to all available ImmunoCAP allergens; however, only a few allergens have been evaluated and respective cut-off values determined (Table II).

Table II. Validated IgG ImmunoCAP tests using the UniCAP system

- Alpha-lactalbumin
- *Alternaria alternate*
- *Aspergillus fumigatus*
- Beta-lactoglobulin
- *Candida albicans*
- Casein
- *Cladosporium herbarum*
- Common wasp venom
- Common silver birch
- *Dermatophagoides pteronyssinus*
- *D. farinae*
- Egg white
- Gliadin
- Honey bee venom
- Pigeon/parrot/budgerigar
- Rice
- Thyroglobulin
- Thyroid peroxidase
- Timothy grass pollen
- Wheat protein

Measuring specific IgG antibodies may provide valuable information in different areas of allergology.

Allergic diseases

- Marker for exposure in different lung diseases, including aspergilloma, aspergillosis and allergic alveolitis to bird allergens. The latter can be regarded as positive when values exceed 30mg/l.[10]

Food allergy

- Presence of IgG is a sign of exposure (also of particular interest for cross-reactivity to foods which are not usually consumed by these individuals, e.g. kiwi, avocado)
- Diagnostic importance for certain food antigens, e.g. gliadin in coeliac disease (see below)

Immunotherapy

- Monitoring success of immunotherapy with inhalant allergens and hymenoptera venoms (increase indicates positive response to therapy)

Autoimmunity

- Elevated levels of antibodies to thyroid peroxidase (TPO) and thyroglobulin (TG)

Elevated IgG antibodies have also been detected in cow's milk allergy (CMA), which is a very complex disease with diverse clinical manifestation and allergen recognition.[11] Bovine milk contains about 3.5% protein of which casein constitutes 80% while whey proteins and minor allergens constitute 20%. Furthermore, casein can be divided into four fractions while the major whey proteins are alpha-lactalbumin and beta-lactoglobulin. The latter is acid-stable and likely to remain

intact even after passage through the stomach, which explains its role as an IgG-binding allergen in CMA.

Specific IgA antibodies

IgA antibodies are part of the mucosal immune defence system of the body and present in blood as well as in secretions such as saliva and mucus. Increased levels of specific IgA antibodies to food antigens vary considerably according to exposure and geographical area but are not directly linked to an allergic disease. However, elevated levels may indicate increased exposure as a result of damage to the intestinal mucosa, which is frequently seen in coeliac disease (CD).[12]

In comparison with antibody-mediated hypersensitivity, there are no *in vitro* diagnostic assays to predict cell-mediated hypersensitivity. The only exception is CD, which is an autoimmune disorder of the small intestine resulting from inappropriate T-cell-mediated immune responses against gliadin. Gliadin is the alcohol-soluble fraction of gluten found in nutrients such as wheat, barley, rye and oats. A special enzyme called tissue transglutaminase catalyses the transformation of gliadin, which in turn activates gliadin-specific T-cells. This suggests an active role for this enzyme in the inflammatory response to gluten-containing grains. The measurement of elevated levels of specific IgA and IgG (in IgA-deficient patients) antibodies to gliadin are highly sensitive and specific and can also be used to monitor elimination diets as gliadin specific IgA disappears. Suggested cut-off values for gliadin specific IgA and IgG antibodies are about 2.0 mg/l and 18 mg/l respectively, but should be validated against levels in normal healthy individuals in a given geographic area.

In contrast, food hypersensitivity to wheat is a different disease which is mediated via IgE antibodies, and several wheat allergens may be implicated.

IMMUNOBLOT

Sometimes patients present with a clear history of allergic sensitisation but commercially available assays do not detect elevated specific antibodies. In this situation when sensitisation to an unknown allergen source is suspected, immunoblotting (also called Western blotting) should be conducted. Protein extracts of the offending allergen source are separated by gel-electrophoresis (in an electrical field) according to molecular size, the allergens are then transferred to a membrane (blotting) and detected with serum IgE or IgG antibodies from sensitised patients.[13] This method can be very sensitive; however, the evaluation of the results requires a sound knowledge of molecular allergens and it is advisable to compare results with those in non-sensitised individuals.

BASOPHIL ACTIVATION

The purpose of this test is to mimic *in vitro* the contact between allergens and circulating basophils. The release of histamine and tryptase can be measured using the ImmunoCAP system and the release of leukotrienes (which are more stable biological markers) via the cellular antigen stimulation test (CAST). In recent years an increasing number of studies have demonstrated that flow cytometry is a reliable tool for monitoring basophil activation on allergen challenge by detecting surface expression of protein markers such as CD63 and CD203c.[14] The assay is relatively fast with results produced within 1-2 hours, and requires about 5 ml of fresh whole blood. Protein allergens or drugs can easily be tested; however, healthy control subjects have to be included and assessed for each allergen and concentration tested.

CONCLUSIONS

Confirmation of immunological hypersensitivity reactions rely in the laboratory setting on the detection of allergen specific antibodies. IgE antibodies play a pivotal role in these reactions, but allergen specific IgA and IgG have been useful markers for detecting conditions such as CD and allergic alveolitis respectively. Nevertheless, the evaluation of food allergy based on these antibody types remains questionable. The assessment of *in vivo* activation of cells and mediator release are important indicators used to confirm that allergic asthma, rhinitis and anaphylaxis have indeed taken place. Furthermore, T-cell and basophil activation can be utilised *in vitro* under controlled conditions to identify allergic reactions to food additives, clinically relevant cross-reactivity and true latex allergy. Future developments include novel sensitive and specific tests for routine allergy diagnosis such as the allergen microarray, while cellular tests are likely to remain specialised tests for the evaluation of specific clinical cases.

Declaration of conflict of interest

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