Review paper

Differential diagnostics of food allergy as based on provocation tests and laboratory diagnostic assays

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Abstract

Due to the scale of the phenomenon, food allergy constitute a significant health problem and significantly impair the quality of life of patients. Differential diagnostics, including skin tests, sIgE detection tests, basophil and mast cell activation tests as well as double-blind placebo-controlled food challenge tests, is the gold standard in the diagnosis of food allergy. Recently, increasing attention has been paid to the potential use of nasal provocation test in the diagnosis of food allergy. Allergen dose, protocol standardization, assessment of subjective complaints and objectivization of test results are important factors determining the applicability of provocation tests.

Key words: food allergy, laboratory diagnostics, double-blind, placebo-controlled, nasal provocation test.

Introduction

The prevalence of food allergy (FA) has been steadily increasing over the past few decades, posing a significant burden on public health and the quality of patients' lives [1, 2]. Data from epidemiological studies suggest that both the general prevalence of FA and the relative importance of individual food allergens are extremely heterogeneous in different world regions. In Europe, the prevalence of patient-reported FA ranges from 1.7% to 37.3% depending on the country, whereas in North America the respective values range from 3.1% to 11% [3]. The highest incidence of IgE-mediated FA is observed in infancy and early childhood due to the relatively high prevalence of egg and cow's milk allergies which often resolve later on in childhood. In contrast, peanut and tree nut allergies, also usually prevalent in infancy, are less likely to resolve with time and therefore become predominant in later years [4]. The most sensitizing food allergens include cow's milk protein, chicken egg, soy, wheat, peanuts, tree nuts, fish, seafood, and sesame [5].

The double-blind, placebo-controlled food challenge (DBPCFC) remains the gold standard in the diagnosis of FA despite being a time-consuming procedure fraught with the risk of severe reactions. Other routine tests used in the diagnostics of FA include skin patch tests and specific IgE detection tests, in particular molecular testing of allergenic components. Other promising assays remain in development and are currently limited to research settings alone; these include the basophil activation test, the mast cell activation test, and the bead-based epitope assay (BBEA) [1, 6]. In addition, increasing attention has been recently paid to the potential use of nasal provocation test (NPT) in the differential diagnostics of FA [7].

FA are caused by immune system dysregulation and tolerance loss. The mechanisms of FA have not been fully elucidated and require further clarification. Symptoms of FA can arise from 3 different pathomechanisms, including IgE-dependent (atopic), IgE-independent (cellular), and mixed (IgE-dependent and independent) reactions. It seems that the initiating event triggering the development of sensitization to FA consists in the physiological contact with food through a dysfunctional, inflamed barrier within the gastrointestinal tract, skin, or respiratory tract. Damage to the skin or the intestinal barrier may promote FA. In addition, it has been postulated that the Western lifestyle disrupts normal intestinal microflora leading to dysbiosis which leads to the induction of immune responses due to increased intestinal permeability. The genetic background of FA should also be kept in

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mind. The most repetitive genes associated with FA include the filaggrin (FLG) gene and the human leukocyte antigen (HLA) [8–10].

Manifestations of FA, often nonspecific and diverse, depend largely on the pathomechanism and the allergen responsible for sensitization. In atopic reactions, FA is manifested as urticaria accompanied by angioedema, anaphylaxis, immediate gastrointestinal symptoms (diarrhoea, vomiting, abdominal pain, colic), and oral allergy syndrome (OAS)/pollen-food allergy syndrome (PFAS). In IgE-independent reactions, observations include the food protein-induced enterocolitis syndrome (FPIES), food protein-induced allergic proctocolitis (FPIAP), and food protein-induced enteropathy (FPE). Mixed-type reactions are responsible for the development of atopic dermatitis, eosinophilic esophagitis, gastritis, enteritis, or colitis [8, 10]. As one can see, the diversity of FA symptoms is due not only to the symptomatology, but also to the manifestations from different organs and systems. In addition, it should be kept in mind that FA can be accompanied by symptoms of other allergic diseases, particularly allergic rhinitis (AR), atopic conjunctivitis, and asthma [2, 5, 8]. Epidemiological data indicate that PFAS is accompanied by concomitant AR in 20–40% of patients [2]. Notably, nearly one quarter (23.1%; 95% CI: 19.1–28.3) of adult patients reporting an asthma attack within the past year have also reported at least one of the FA symptoms [2]. The phenomenon of the "allergic march", i.e. the natural progression of various manifestations of allergy as observed with age, should also be kept in mind. In the classic course of this process, allergic disease starts with atopic dermatitis with an accompanying FA (with or without gastrointestinal symptoms) in infants and may be followed by AR and/or asthma [5, 8, 10]. The purpose of this study was to analyse the available literature on the differential diagnostics of FA within the framework of funds from the National Science Centre grant no.2021/05/X/NZ5/01099.

Double-blind, placebo-controlled food challenge

The DBPCFC remains the most important test and the gold standard in the diagnosis of FA (Table 1) [11–22].

The main indication for the challenge test is to confirm the causal relationship between the intake of a particular food and the hypersensitivity reaction. The challenge test is used to recreate and mimic the natural systemic response to the administered allergen. Patients should be thoroughly prepared for DBPCFC [12, 23]. Infection should be ruled out and the symptoms of any chronic diseases should be stabilized. Suspicious foods should be discontinued 4 weeks before the challenge test to avoid a delayed reaction. Discontinuation of medications potentially affecting the course of the test (antihistamines, glucocorticosteroids) is also recommended. The tested food is best served in its natural form; however, the test's reliability requires that it be blinded. The rule of thumb is that the volume, colour, taste, and consistency of the placebo must be the same as that of verum. Clinical presentation of FA is extremely varied and depends on the type of food as well as the age and personal predisposition of the patient. Undoubtedly, gastrointestinal symptoms are the most common manifestation and may occur within all sections of the gastrointestinal tract [12, 23, 24]. Nasal symptoms such as itching, sneezing, watery discharge and nasal blockage, were also observed in patients subjected to oral food challenge tests. During the DBPCFC, the patient's condition is monitored; this includes the measurement of the heart rate and blood pressure, assessment of skin condition, PEF measurement or spirometry, assessment of the oral cavity, throat, and upper respiratory tract. As of present, most DBPCFC studies are conducted according to the standard protocol presented in the document titled *Standardizing double-blind, placebo-controlled oral food challenges: American Academy of Allergy, Asthma & Immunology-European Academy of Allergy and Clinical Immunology PRACTALL consensus report*, published in 2012 [12]. A positive challenge test result usually determines the introduction of an elimination diet and confirms the clinical efficacy of specific oral immunotherapy. The unquestionable advantage of the DBPCFC approach consists in double blinding, which eliminates the possibility of the course of the study being affected by psychological factors on the side of the patient and/or the physician. It is important to note that any diagnostic challenge test is associated with a potential risk of arduous or dangerous symptoms. Taking into account the risk of anaphylaxis, the test should be carried out in a hospital setting. The possibility of false positive or false negative results as well as the high cost of the test must also be taken into account [11, 24].

Specific IgE determination versus epitope mapping

Allergen-specific IgE antibodies are involved in the mechanism of FA development. The binding of membrane-bound IgE antibodies to specific allergens results in the activation and degranulation of effector cells (mast cells or basophils) with the release of histamine and other inflammatory mediators, leading to the manifestation of allergic symptoms. The groups of amino acids bound by specific antibodies within individual allergenic proteins are referred to as epitopes. To date, numerous IgE-binding epitopes have been identified from various food allergens. Recent advances in the epitope mapping methods have facilitated research into better understanding of the relationship between IgE-binding epitopes and the clinical sensitivity of FA patients. Studies are currently underway to identify the so-called informative IgE-binding epitopes to be used as biomarkers of the clinical severity of FA. Two different types of IgE-binding epitopes are

Table 1. Oral food allergen challenge tests

Study	Study group (size, age)	Provocation test (allergen)	Doses used	Result
van de Vorst-van der Velde K *et al*. [13]	A retrospective study in children, 2015–2019. A total of 513 food provocation tests administered to 365 children (median age of 6.9 years) were included in the analysis.	Data on peanut and tree nut provocation tests were analyzed.	Food allergy tests were carried out according to the standardized PRACTALL schedule of 1, 3, 10, 30, 100, 300, 1000 and 3000 mg of allergen proteins in gingerbread matrix.	As many as 40% (204/513) of the food samples were positive. Fifteen children had reacted to as little as 1 mg of protein (7%), including 3 cases of grade 3 reaction. Accordingly, early responses can be expected when following the PRACTALL guidelines.
Tagliati S *et al*. [14]	A retrospective study, 2015–2019. The analysis included 184 children with positive results of patch/sIgE tests towards nuts. Patients were aged from 8 to 175 months (mean: 42 months).	Nut challenge test. Positive in 113 children, negative in 71.	Oral challenge tests were carried out according to the standardized PRACTALL schedule of 1, 3, 10, 30, 100, 300, 1000 and 3000 mg of allergen proteins.	The diagnostic usefulness and safety of the oral challenge test were confirmed. Hazelnuts were the most common causative allergen.
Brough HA *et al*. [15]	A prospective study in children (n = 122) aged 2 to 12 years. 3 sites of the Pronuts research group: London, Geneva and Valencia.	Nuts: 866 trials were carried out, including 238 (27.5%) positive.	Oral challenge tests were carried out according to the standardized PRACTALL schedule of 1, 3, 10, 30, 100, 300, 1000 and 3000 mg of allergen proteins.	The concomitance rate for the peanut, hazelnut and sesame seed allergy amounted to 60.7% (n = 74/122; 95% CI: 51.4% to 69.4%). Oral challenge tests are labour-intensive and fraught with the risk of severe allergic reactions. They are necessary for the determination of further dietary management.
Hourihane JO [16]	A prospective study in 518 children, average age: 6.8 years. The study was completed by 378 children.	Peanuts	A single oral dose of the allergen ED05 for peanuts (1.5 mg) in the novel single-dose challenge.	The single-dose oral food challenge appears to be clinically safe and acceptable to patients, facilitating identification of the most dose-sensitive population of food allergy patients.
Purington RS *et al*. [17]	A retrospective study, 2010–2016. 410 subjects aged 1 to 52 years.	Peanuts, almonds, egg, milk, sesame, pistachios, pecans, hazelnuts, cashew nuts	A total of 1054 trials were carried out according to the modified PRACTALL protocol.	A very useful method in the diagnosis of food allergies. Patients with asthma and high sIgE levels are at higher risk of systemic reactions.
Salari F *et al*. [18]	A prospective study, 2018–2019. Adults aged 18–55 years, 50 subjects, eventually 20 subjects.	Sesame seeds	Europrevall French protocol by Dano *et al*.	The challenge test using this allergen is not completely safe.
Yanagida N *et al*. [19]	A retrospective study, 2008–2012. Children above 5 years of age, average age of 8.9 years old, 393 subjects.	Milk, eggs, peanuts, wheat	Protocol according to the European Academy of Allergology and Clinical Immunology (EAACI) guidelines and Japanese Guidelines for Food Allergy 2014.	The history of anaphylaxis and patient's age are risk factors for severe post-challenge reactions. The lowest number of reactions were observed in egg challenge tests.
Dambacher WM [20]	A prospective study. Children aged 2.5 to 134 months, 124 subjects.	Milk	Proprietary protocol involving challenge doses of 18 to 1620 mg at 20-minute intervals.	The exclusion of milk allergy by the DBPCFC method has facilitated effective discontinuation of unnecessary elimination diets in most children.
van de Ven CA *et al*. [21]	A retrospective study, 2011 to 2014. Children aged 12 to 60 months, 485 subjects. Positive results in 188 subjects, negative results in 288 subjects, including 124 subjects tested at the low dose and 164 subjects tested at the high dose.	Milk	Protocol as per the European Academy of Allergology and Clinical Immunology (EAACI): 2 groups: low dose (2.2 g) and high dose (4.4 g) administration.	Successful introduction of milk following a negative challenge test did not depend on the total dose of milk proteins or the type of the milk product used in the challenge test.

Table 1. Cont.

Study	Study group (size, age)	Provocation test (allergen)	Doses used	Result
de Weger WW [22]	A literature review to examine the diagnostic accuracy of oral food challenge and interviews with 19 parents of children with confirmed or suspected food allergies regarding the design of a research trial in this area.	–	–	There is an urgent need to study the diagnostic accuracy of different oral food challenge protocols. The presented rationale and the design of the ALDORADO (ALlergy Diagnosed by Open oR DOuble-blind food challenge) study suggest the need to investigate whether the outcomes of the open food challenge are comparable to those of the DBPCFC.

known within the allergens, namely the sequential and conformational epitopes. A sequential epitope is a sequence of linearly adjacent amino acids while a conformational epitope is a sequence of amino acids that line up next to one another due to the tertiary structure of the allergen. Sequential epitopes have been suggested to be of greater importance in FA since food proteins are usually subjected to heat processing which leads to denaturation and alteration of the tertiary structure, and are digested within the gastrointestinal tract, which also leads to the breakdown of the tertiary structure prior to any reaction with the immune system. In contrast, conformational epitopes are more relevant to inhalant allergens, although patients with oral allergy syndrome (OAS), a type of pollen-related FA, may react to conformational epitopes due to the cross-reactivity between food allergens and homologous pollen-related allergens [24].

The natural history of tolerance development, and the differences between patients with different clinical courses are the reasons behind the FA being the most diagnostically challenging type of allergy. Thanks to the newly available epitope mapping methods, i.e. the SPOT membrane technology and peptide microarrays, numerous studies have been conducted to investigate the relationship between epitope-specific IgEs and the clinical symptoms of allergy, as well as to predict the possible course of food allergies. As demonstrated in all of these studies, the number of sequential IgE-binding epitopes was much higher in older patients as compared to younger patients, indicating a link between the recognition of certain epitopes and the clinical symptoms of food allergy [25, 26].

Thus, in the studies evaluating IgE-binding epitopes responsible for e.g. milk allergy, not only has the wide variety of epitopes been linked to severe cases of allergy, but also the sequential epitopes recognized by IgE antibodies in older patients with chronic allergy have been shown to differ from those in younger children who are likely to outgrow their allergy. It was also found that some epitope-specific IgE antibodies had been present at a very young age in patients in whom chronic allergy has subsequently developed with age. These epitopes were termed "informative" because the presence of IgE antibodies against at least one of these epitopes is sufficient for identification of all patients with chronic allergy to milk. Beyer *et al.* evaluated the utility of informative epitopes for the prediction of chronic milk protein allergy. This was a prospective study carried out in patients with milk protein allergy as confirmed by a challenge test, albeit with different natural histories of the allergy: patients with persistent milk protein allergy (n = 45; median age: 8 years) and patients presenting with milk allergy at an earlier (n = 15; median age: 3 years) or at a later age (n = 14; median age: 8 years). Patients with chronic allergy to milk proteins presented with significantly higher levels of IgEs against peptides containing informative epitopes than those who had outgrown their milk protein allergy, confirming that IgE antibodies directed against these informative epitopes can be used as a marker of chronic milk protein allergy [27]. Thus, epitopes labelled as informative may be useful as a biomarker of chronic food allergy and predict the natural history of the disease.

Peripheral blood eosinophilia and tissue eosinophilia

Most allergic diseases are characterized by eosinophilic inflammation. Accumulation of eosinophils can be a measure of the severity of allergic inflammation, and therefore the assessment of the presence and number of these cells can be used in the diagnostics and monitoring of allergic diseases. The absolute eosinophil counts are calculated on the basis of total leukocyte counts and the percentage of eosinophilic leukocytes as established in blood smears. The reference absolute eosinophil counts are those of > 350/µl (> 700/µl in children). Values of > 1500/µl are referred to as hypereosinophilia. The absolute number of eosinophils in peripheral blood is increased in the periods of exacerbation of allergic diseases and in patients with multiorgan allergies. However, one should remember that an increase in the absolute number of eosinophils in the peripheral blood is not exclusively characteristic of allergic diseases. The greatest increase in peripheral blood eosinophil counts is observed in parasitic infestations, eosinophilic leukemia, or hypereosinophilic syndromes, while increased absolute

peripheral blood eosinophil counts similar to those obtained in allergic diseases are found in some infections, connective tissue diseases (lupus erythematosus, scleroderma), immune deficiencies, or hypopituitarism [28–30].

Eosinophil-derived neurotoxin

The eosinophil-derived neurotoxin (EDN) protein can be found within the matrix of eosinophilic peroxidase-positive granulocytes. It is released from activated eosinophils, mainly in places where these cells are present, i.e. skin, lungs, gastrointestinal tract, and genitourinary tract. Eosinophil-derived neurotoxin presents with strong cytotoxic properties and plays an important role in antiviral prophylaxis. Determination of EDN levels facilitates the diagnosis of FA involving an immediate reaction, differentiation between FA and food intolerance, and verification of the effectiveness of clinical elimination diets. In addition, the measurement of EDN is helpful in the examination of the integrity of the intestinal mucosa when considering the possibility of inflammatory bowel disease. Determinations of EDN levels can be performed in stool, urine, serum, and plasma samples [31].

Basophil Activation Test

It is a functional test that measures the degree of basophil degranulation under allergen stimulation using flow cytometry techniques. The test is performed in a fresh blood sample collected not longer than 24 h before the test. The test is considered to facilitate a reduction in the number of challenge tests that have to be performed. The Basophil Activation Test (BAT) is a diagnostic tool that has been available for 25 years, used to assess sensitization to hymenopterous insects, food and inhalant allergens, latex, and certain medications. The usefulness of BAT in the diagnostics of FA was demonstrated by Song *et al.* who had carried out the DBPCFC tests in 67 paediatric patients using allergens such as peanuts, nuts, fish, shrimp, and sesame. Skin patch tests, sIgE, sIgG, and BAT with the sensitizing allergens had also been performed. In contrast to other diagnostic methods, the results of the BAT test were correlated with the severity of symptoms as determined using the DBPCFC [32]. In another study, Wai *et al.* examined a group of 35 subjects, 15 of whom presented with positive DBPCFC symptoms (urticaria, pruritus, OAS symptoms) following the intake of shrimp; the remaining subjects had presented with tolerance to shrimp foods. When used with the shrimp allergen extract, BAT proved significantly more effective compared to SPT and IgE determinations [33].

Mast cell Activation Test

The Mast cell Activation Test requires the availability of mast cells which are obtained from CD117+ peripheral blood progenitor cells following an initial 30-day culture followed by subsequent culturing the cells in another medium until fully mature at 6–8 weeks. Next, mast cells are passively sensitized with patient's serum and incubated *in vitro* with the test allergen. A dose-dependent increase in the expression of CD63 and CD107a was observed using flow cytometry techniques. Mast cell degranulation was confirmed by the assessment of allergen-dependent release of PGD2 in the prostaglandin level determination assays [34].

Nasal provocation test

The advantages of nasal provocation test (NPT) using food allergens have been recognized by experts in the *European Academy of Allergy and Clinical Immunology position paper on the standardization of nasal allergen challenges,* where NPT was presented as possibly helpful in predicting food allergies [7]. The first attempts at using NPT in the differential diagnosis of FA were made by French and American researchers [35, 36]. In an experiment carried out in mice (aged 8–12 weeks) subjected to the oral and intranasal challenge with peanut protein extracts, the researchers observed significant increases in IL-4, IL-17, IFN, and CCL-11 levels in the latter test. In addition, in contrast to the oral test, intranasal challenge promoted the development of neutrophilia and led to higher MAC-1 levels [35]. The study gave rise to the use of the nasal cavity, being a well-vascularized and innervated area with a large number of mast cells, in food allergen provocation tests. Evidence is available for the involvement of the upper respiratory tract in the pathomechanism of the response to oral food challenge; the response is considered to be a manifestation of a broader, systemic reaction, while on the other hand presenting with an opportunity for implementation of a novel pathway for the diagnosis of patients sensitized to food allergens. Interestingly, a large percentage of patients diagnosed with AR in the course of the oral challenge test were shown to present with an allergic response within the nasal mucosa: out of the total of 142 subjects, 29 patients presented with an upper respiratory response within 3 h after the oral challenge test, whereas another 38 patients presented with such a response within the time window of 6–24 h [36]. In a study involving a food challenge test administered to 24 children allergic to egg allergens, Clark *et al.* demonstrated the significant involvement of the nasal cavity in DBPCFC. As early as 20 min after oral provocation, a 0.8°C increase in the temperature of the test area was recorded and persisted until 60 min into the test (sensitivity of 91% with a positive predictive value (PPV) of 100% and specificity of 100% with a negative predictive value (NPV) of 93%). Antihistamines reduced all complaints and normalized the head temperature as measured by vision thermography [37]. Further, milestone attempts at using NPT in the diagnosis of FA, were con-

Table 2. Intranasal food allergens provocation tests

Study	Study group	IAPT	Doses used	Result
Clark A *et al*. [38]	16 subjects	Peanuts	Single dose: 10 µg	Vision thermography: temperature rise (18.2°C/min (range 4.7–80.8) vs. placebo 4.8°C min (0–24.7)); p = 0.0006, no adverse events
Gelis S *et al*. [39]	45 subjects	Seafood	Escalating doses: 1 : 100; 1 : 10; 1 : 1	– Positive NPT in sensitized-non allergic group 2/18 (11.1%) – Positive NPT in sensitized-allergic group 18/20 (90%) – Negative NPT in the control group, one symptomatic subject (local reaction)

ducted using chicken egg and peanut allergens (Table 2) [38, 39].

The allergens used for NPT were obtained from their natural sources and extracted under laboratory conditions. Complex procedures had resulted in products having the form of lyophilizates for subsequent dissolution in saline. In one study, a single dose of 10 µg of the allergen was used [39] whereas increasing doses of 1 : 100, 1 : 10, and 1 : 1 were used in the second study [38]. The advantage of NPT over DBPCFC consists in the possibility of test results being objectivized by means of the readily available nasal patency testing techniques: acoustic rhinometry, rhinomanometry, or peak nasal inspiratory flow (PNIF). On the other hand, a specific limitation of NPT with food allergens consists in the lack of commercially available standardized (e.g. in SBU/ml units) food lyophilizates which would undoubtedly facilitate attempts at further standardization of this diagnostic approach.

Conclusions

The differential diagnostics of FA is mainly based on skin patch tests, laboratory assays and double-blind, placebo-controlled food challenge tests. Increasing attention is being paid to expanding the indications for NPT so as to include other conditions, including FA.

Conflict of interest

The authors declare no conflict of interest.

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