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Review

In vivo and in vitro techniques to determine the biological activity of food allergens

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Abstract

Methods for determination of the biological activity of food allergens comprise both determination of the allergenic potency, i.e. the capability to elicit an allergic reaction in an already sensitized individual, and the allergenic potential, i.e. the risk for sensitizing a hitherto non-allergic individual. Several methods are discussed for determination of potency including the double-blinded placebo-controlled food challenge, skin testing, in vitro effector cell assays such as basophil histamine release, and IgE-based techniques such as RAST and RAST inhibition. No reliable methods have yet been developed which can predict the allergenic potential of a food or a food allergen. The progress in the areas of stability studies and animal models for food allergy are discussed.

Keywords: Reviews; Food allergy; Double-blind placebo-controlled food challenge; Skin prick test; Basophil histamine release; Immunoglobins

Contents

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1. Introduction

The biological activity of food allergens or mixtures thereof may be determined by various in vivo and in vitro methods that may quantitatively or semi-quantitatively express the combined effects of individual allergenic molecules in a mixture. Even in the rare case of testing an individual food allergen molecule, a response will emerge that is only declared relatively to other allergenic substances or mixtures. Thus, an important feature of testing the biological activity of mixtures is the lack of a response which can be directly linked to individual molecular entities, and this put special emphasis on the definition of both the test systems and the mixtures that are tested.

The biological activity of a substance in relation to food allergy may be understood in various ways. In the context of this review only food allergic diseases believed to be mediated by immunoglobulin E (IgE) will be considered even though adverse reactions to foods include other disease entities and even the term food allergy may comprise diseases elicited by several other mechanisms (Johannes Ring's review in this volume, p. 3). As with other IgE-mediated diseases, the pathogenesis of food allergies can be divided into an afferent-sensitization phase where the immune system develops an IgE response to one or more constituents of a food (allergens), and an efferent-eliciting phase where a clinical, allergic reaction occurs after ingestion of the food in question. Accordingly, the term allergenic may be understood both as the capacity to sensitize, i.e. induce an IgE immune response, and as the capacity to elicit an allergic reaction in an individual already sensitized. Our understanding of the process leading to sensitization of a patient is still very immature, but probably both factors intrinsic (dose, primary to quaternary structure defining what the immune system recognizes as epitopes) and extrinsic (adjuvant effect, host specific factors) to the individual molecule are involved. Only a limited amount of literature is available on models testing the allergenicity — understood as ability to sensitize humans — of molecules or mixtures. The scientific and societal gains and profits that could be derived from such models are obvious however, and these will be briefly discussed below under the heading biological methods for assessment of allergenic potential.

The other meaning of allergenic, i.e. the eliciting of an allergic reaction in an already sensitized individual, has been much more successfully investigated and as described below, numerous models exist for determination of the biological potency (Table 1). The Table illustrates the hierarchy that exist among these test systems as challenge of human patients are considered as closest to the relevant biological response, i.e. elicitation of an actual allergic response, albeit under controlled and safe circumstances. The next level in the hierarchy is to use the skin as a restricted and localized area for challenge. This system obviously involves the skin mast cells, which must be sensitized by IgE in order to respond to the offending allergen. Leaving the in vivo systems, the next step is to use the sensitized basophil granulocyte as a model for the sensitized mast cell present in the relevant organ of the patient. Moving further away from the actual patient, basophil from a non-allergic donor such as cord blood may even be used as an reagent which are then sensitized by IgE derived from an actual patient. A pure system can be obtained by immunochemical assays detecting IgE-allergen binding directly or indirectly by inhibition designs. The above-mentioned human model systems all have their animal experimental counterparts which will only briefly be touched upon. Many of the parameters of the human systems discussed below, will also apply to animal models. The basic problem with experimental animals is to actually make them allergic. Although several immunization schemes — often parenteral — are available, which will readily produce an IgE

Table 1
Hierarchy of test systems for testing of allergenic potency of pure substances or mixtures

Vivo/vitro	Target organ/system	Species	Test system	Examples
In vivo	Entire organism	Human	Challenge of allergic patients	DBPCFC, open challenges
		Experimental animals	Peroral challenge of animals	Anaphylactic response
	Skin	Human	Skin testing of allergic patients	Skin prick tests, intrademal tests
		Experimental animals	Actively or passively sensitized animals	PCA
In vitro	Basophils	Human	Actively or passively sensitized basophils + allergen	Basophil histamine release, cord blood basophil histamine release, CAST
	Basophil or mast cells	Humanized, i.e. transfected with a human IgE receptor	Passively sensitized cells	Mediator release
	Mast cells basophils	Rodents	Peritoneal mast cells, RBL cell line	Histamine or other mediator release
	IgE	Human	(Inhibition of) IgE-allergen binding in immunochemical assays	RAST or RAST-inhibition
	IgG/IgE	Experimental animal including monoclonal antibodies	Immunochemical assays	ELISAs, Dipsticks etc.

response, it is still not known whether the mere presence of IgE specific to a food allergen gives a good prediction of allergenicity.

The final line in Table 1, i.e. the production of animal antibodies to individual allergens and the use of such antibodies in ELISAs etc., forms the border between the biological assays and molecular identification of individual allergens and will not be discussed further in this review.

2. Determination of biological potency

2.1. The human challenge model: double-blinded, placebo-controlled food challenges

The ultimate determination of the biological activity of a food allergen or a mixture of these is the effect on a sensitized food allergic patient. This is normally carried out by double-blinded, placebo-controlled food challenges where a patient suspected of food allergy is challenged and observed in an allergy clinic. Allergic symptoms may vary, however, and the patient may even experience symptoms for psychological reasons when eating a food that he or she suspects may cause an reaction. In order to get an impression of the spontaneous variation of the disease, the patient receives an active (=the suspected food) on one occasion and a placebo (=the same challenge preparation but without the suspected food) on another occasion — ideally on two different days with an interval of 3–7 days. Thus, the challenge procedure becomes placebo-controlled. To further reduce the psychological factor the food is given to the patient in a double-blinded fashion, meaning that the food is disguised in a matrix, and that neither the patient nor the health care personal seeing the patient know on which day the patient receives the active and the placebo. Only when both challenges have been carried out, and it has been decided whether none, one or both challenges produced symptoms the code is broken, and it can be

decided whether there was no reaction, a positive reaction to only the active challenge or a positive reaction to the placebo challenge. In the latter case, the result of the challenge is considered inconclusive, and it may be considered to retest the patient when his or her symptoms are less frequent or serious. It follows from the above that if the patient can react to the placebo by chance, the same could happen during the active challenge. If the symptoms are severe and grossly exceeds the spontaneous variation in symptoms normally experienced by the patient, then the doctor will typically evaluate the result as truly positive, but if the symptoms on the other hand are mild and unspecific, it may be necessary to increase the number of active and placebo challenges to obtain statistical evidence that the food is the causative agent. As mentioned below, it is important to register the number of placebo reactions in a particular study since this indicates the magnitude of the problem. For both practical and ethical reasons it is obvious, however, that patients cannot be routinely challenged to allergenic preparations. Thus, the main reason for conducting controlled food challenges in patients is to verify or rule out a suspicion of food allergy, establishing a clinical diagnosis for the benefit of the patient. It is possible, however — and in my opinion also ethical acceptable — to perform clinical trials on allergic patients in order to obtain knowledge about for example cross-reactivity patterns or quantitative relationships such as determination of no-effect levels.

The first report on double-blinded placebo-controlled food challenges (DBPCFC) is probably a study of May, where asthmatic children were challenged with freeze-dried foods in capsules. Albeit the patients were highly selected based on case history and skin test only 29% reacted with objective symptoms [2]. By 1991 Bock and Atkins reviewed about 500 challenges performed at their pediatric centre, and a pattern emerged with relatively few placebo reactions and a high degree of safety [3]. Thus, DBPCFC has been said to be the gold standard of food hypersensitivity diagnosis [4], and is recommended by the European Academy for Allergy and Clinical Immunology as the only conclusive evidence of a food allergy, provided it is performed properly [1]. Two aspects of the procedure will be discussed in detail here: blinding and dose-titration. Many other parameters must be considered and controlled carefully. These parameters include:

- *Selection of the patients*. Some investigators recommend to avoid challenge of patients with a well-documented history of severe anaphylaxis. On the other hand, if a study aims at defining no-effect levels of a food allergen it is important, that all patients in the study has previously been challenge-positive, and that the most sensitive patients are not excluded, since this will produce a false impression of the no-effect level.
- *Premedication of the patient*. Too high doses of, i.e. steroids, may mask symptoms, but reducing a maintenance treatment may itself induce symptoms of asthma or atopic dermatitis.
- *Fasting of the patient*. Most protocols recommend that patients refrain from food intake from the day before challenge.
- *Safety*. It is of paramount importance to ensure the safety during the challenge sessions. The case history of previous reactions is very important in this respect. Safety issues comprise establishment of an intravenous line, level of ICU backup, selection of initial dosages, and decision on the observation period in the clinic.
- *Recording of signs and symptoms*. Objective signs or symptoms are preferable but these are often preceded by subjective symptoms, such as in the oral allergy syndrome, where itching of the mouth and palate may be the first symptom experienced by the patient. Before a study is initiated decisions should be reached on both the level of symptoms to be recorded and the recording period.
- *Placebo reactors*. The above point is closely related to the amount of positive reactions to placebo that will be recorded in a study. For evaluation of the study, it is important to report the frequency of placebo reactions, and to decide whether placebo reactors should undergo a new set of active and placebo challenges.
- *Open challenge subsequent to a negative DBPCFC-outcome*. In order to rule out flaws in the challenge procedure, some investigators have found it advisable to challenge patients openly with the fresh food, if they do not react in the DBPCFC. This may be of special importance for foods where the allergenic activity is difficult to

maintain by the blinding recipe or for which no previous blinding experience exist such as for many fruits.

For a further discussion of the DBPCFC procedure including the above points, the reader is referred to Ref. [5]. A number of international studies are currently being conducted in order to establish consensus on challenge procedures.

2.1.1. Blinding

The vehicle used for blinding should obviously mask both the taste, texture, and smell of the food. Subtle differences between active and placebo challenge preparations regarding smell or appearance may be hidden by using liquid recipes served in opaque containers and ingested by a straw to the patient who is wearing a nose clip. It is advisable to test the blinding procedure on healthy subjects. It is important to assure that the vehicle does not in itself contain substances to which the patients react, and relatively simple recipes are to prefer.

The blinding procedure may limit the maximal amount of food which can be given in a challenge. Examples of maximal amounts of fresh foods which have been hidden in different studies are given in Table 2. One technique to increase the amount of allergen that is used for challenges is to make an extract and use this for the challenge. Daul and coworkers reported that up to 128 ''shrimp equivalents'' corresponding to 500 g of shrimp could be masked by this method [6]. Whole foods [7] or allergen extracts [8] may also be freeze dried and administered to the patients via capsules. It should be emphasized that when an extraction is performed it is important to know the actual yield in terms of allergen. This is especially important if it is the intention of the study to evaluate the tolerance limit of the fresh food. The use of capsules presents another problem: since the capsule is dissolved in the stomach the mouth is bypassed by which clinical relevant information on the elicitation of oral allergy syndrome is lost.

Table 2
Amounts of fresh foods which have been blinded in studies using DBPCFC

Food	Amount (g)	Ref.
Egg	50	[11]
Milk	250	[11]
Codfish	5	[10]
Hazelnuts	20	[65]

2.1.2. Dosing

Large variations in the dose that elicit reactions have been reported. Hourihane and coworkers challenged a selected group of highly sensitive peanut allergic patients and found objective symptoms to doses as low as 2 mg of peanut protein or about 4 mg of peanut flour (about 4 and 8 mg accumulated, respectively). These symptoms had been precessed by subjective symptoms to doses of about 100 μg peanut protein [9]. In a study of fish allergic patients, Hansen and Bindslev-Jensen demonstrated reactions to accumulated doses of 6 mg of codfish in some patients and of 6.7 g of codfish in other patients [10]. Likewise, Nørgaard and Bindslev-Jensen found dose ranges of 5 mg to 50 g for egg, and 5–250 g for milk [11]. Thus, it is reasonable to expect four or even five decades of variation of sensitivity in the food allergic population, and this necessitates a titrated dose-increase. Increments between two- and ten-fold have been reported in the literature, but for a two-fold dose-increase to cover 4 decades, 13 titrations are needed and this probably exceeds what is manageable in a clinical routine. Another problem is the possibility of performing a desensitization of the patient. Desensitization occurs when suboptimal doses of allergen are given to a patient, who will then develop a transient non-responsiveness to the allergen. This principle has been used in emergency cases of penicillin allergy, where it is possible to administer therapeutically effective doses of penicillin to critically ill penicillin-allergic patients [12]. At present there are only suggestions that such phenomena may also happen during a titrated food challenge [13], but future studies will have to address the optimal compromise between safety for the patient (demanding a slow increase in concentration) and the avoidance of obtaining a false–negative response due to densitization (demanding a steep increase in the titration). Partly related to this is the unsolved question whether the reactions are related to the individual dose or to the accumulated dose.

In conclusion, the DBPCFC has become the gold standard for diagnosis of food allergy, but it is not

easily used for biological standardization of food allergens. The number of adult patients available for challenge are scarce, and several methodological aspects still needs to be optimized. In the future it will be important to obtain information on no-effect levels by a number of foods tested on allergic patients, but since the number of allergens and potential allergenic products probably exceeds the capacity of centres that can perform the challenges, it is important to establish links between DBPCFC-results and other methods, such as those described below.

2.2. Skin tests in humans

For the standardization of inhalation allergens the skin test has been the most important tool, and it is the recommended method for biological standardization of allergen extracts [14,15]. For this reason skin tests have also been used extensively for diagnosis of food allergy [1], and it seems well justified to use it for biological activity measurements of food allergens. The rationale behind skin testing is that by introducing a small volume of allergen in the skin — either intradermally or via a small puncture of the *stratum corneum* as in the skin prick test — mast cells sensitized with specific IgE are activated via allergen cross-linking of this IgE. The activation of mast cells results in release of mediators — primarily histamine — which induces a wheal and flare reaction of the skin. Within a certain concentration range there is a dynamic response, i.e. a wheal and flare with a larger area develop after application of a higher concentration of allergen. The biological response is measured by planimetry as the area of the wheal or the flare [16,17] and the result may be quantified by end-point titration, i.e. the highest concentration in a titration which produces a negative response, or by comparison with a standard, typically histamine in a concentration of 10 mg/ml. The patient must — besides being well-defined as a patient — fulfill certain conditions such as an intact skin, lack of dermographism, and abstinence from drugs such as antihistamines which will dramatically inhibit the skin reaction [18]. For ethical and safety reasons the test substance must be assured to be without infectious or toxicological potential besides its allergenic properties. A detailed outline of the technique is given in the guidelines, and will not be discussed here, but some points of special relevance for food allergens should be mentioned, however. The guidelines recommend the use of 20 patients with symptoms of moderate severity, but with the number of available patients that have undergone a DBPCFC-procedure this may present a problem in many centres, especially if — for ethical reasons — only adults or adolescents are selected. Moreover, infants and small children have a good prognosis for outgrowing their food allergy [19] early in their life, which makes them less suited for participation in standardization studies. Due to the paucity of DBPCFC+ patients and the individual responses which may be quite varying, it can be difficult to perform standardization. The Nordic Guidelines recommend that three 10-fold dilutions of the allergen extract (each in quadruplicate) are used for calculation of a dose–response curve, from which the allergen concentration corresponding to the biological response of 10 mg/ml of histamine is calculated (Fig. 1). This concentration is then designated 10 HEP (histamine equivalent prick). In a study of biological standardization of extracts of food allergens Hansen and coworkers found substantial variations in the concentration to which the patient reacted, and for some patients it was not possible to prepare allergen extracts of sufficient potency to produce positive skin tests with three 10-fold dilutions [Skamstrup Hansen et al. in this volume, p. 19].

2.3. Effector cells in vitro: experimental systems for mast cell or basophil activation

As an alternative to the skin test the basophil granulocytes which are believed to be sensitized analogously to skin mast cells have been used extensively for many immunological studies of the allergic response [20,21]. Being an in vitro method this technique has obvious advantages compared to the skin tests, since less strict requirements are posed on the test substance regarding non-toxicity and non-infectivity, albeit it should not be cytotoxic in the applied concentrations. For studies of inhalation and food allergy histamine release tests are correlating well with other measures of IgE sensitization, such as skin prick tests or determination of specific IgE in plasma [22–25], and based on these findings

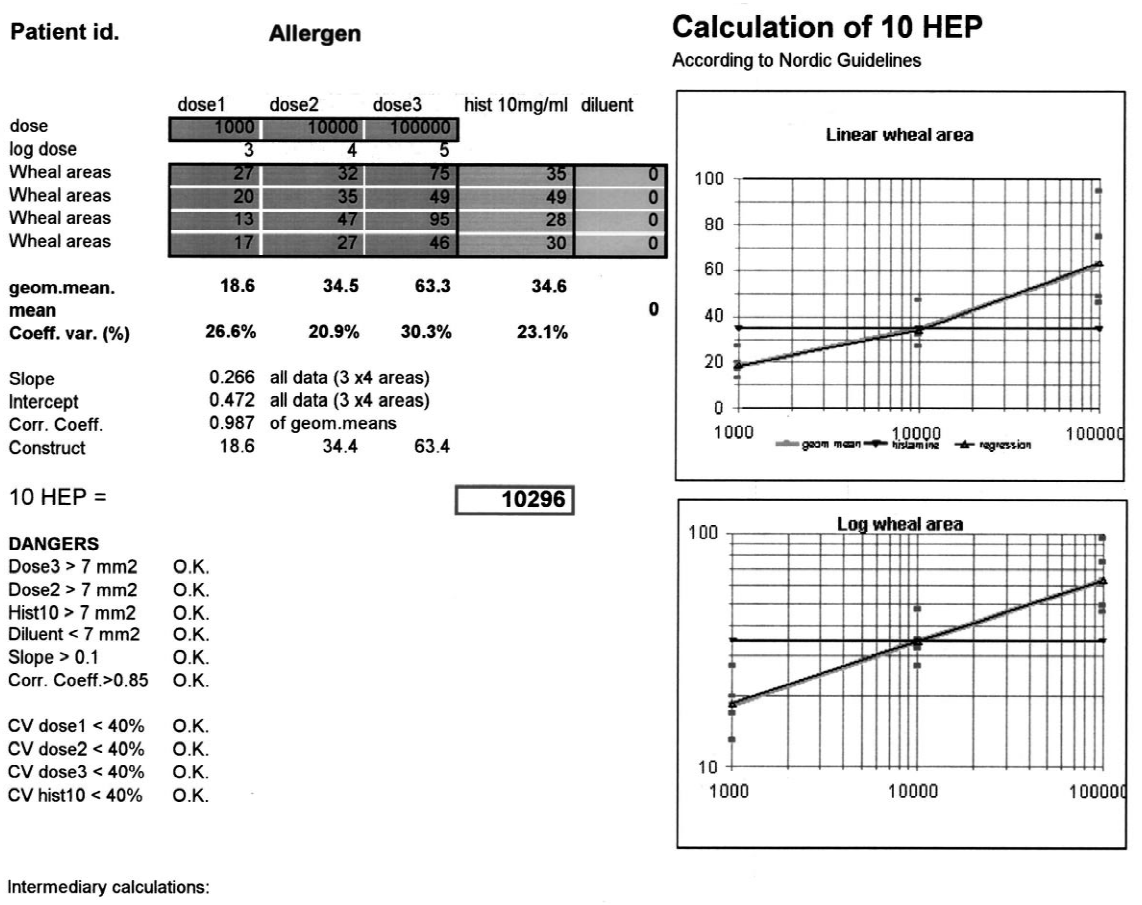

Fig. 1. Algorithms for calculation of the biological potency of an allergen extract according to Nordic Guidelines [15]. Three 10-fold dilutions of the extract are tested together with histamine 10 mg/ml and a negative control (saline). Areas are determined by planimetry and geometric means and coefficients of variation calculated. In a semi-log plot is determined the point on the dose–reponse line which corresponds to the area of the histamine response. The criteria to be met are listed as checkpoints under DANGERS: All three concentrations of extract and the histamine must be positive (i.e. >7 mm^2) and C.V.s below 40%. Likewise should the negative control in fact be negative. The line constructed from the three allergen extract dilutions should have a slope larger than 0.1 and a coefficient of correlation larger than 85%.

it has been suggested to use the technique for biological standardization of allergen extracts including food allergens.

The principle of the method is to challenge sensitized basophils with allergen which will cross-link surface-bound specific IgE causing histamine to be released from the cells. Histamine is determined and a dose–reponse curve can be constructed and be compared with an appropriate standard. In order to ensure that the basophils are responding properly the universal reagent anti-IgE is applied as a positive control, whereas the test substance is applied on basophils with no specific IgE to ensure that no un-specific histamine release, i.e. caused by cytotoxicity, takes places. Histamine can be measured fluorometrically after coupling to a fluorophore (*O*-phthaldialdehyde). Alternative methods for detection of histamine are immunochemical: the Immunotech Radioimmunoassay [26], and the automated fluorometric histamine assay [27]. The agreement between the three methods have been established [28,29].

In one application of the histamine release method

glass-fibre coated microtiter plates are used for separation of histamine from other constituents of the assay [30,31]. Heparinized washed blood may be used without further separation, but the use of whole blood or gradient-enriched basophil suspensions have also been described. Twenty-five microlitres of blood are stimulated in glass-fiber coated microtiterplates with dilutions of allergen extracts for 1 h at 37°. Three 10-fold dilutions of anti-IgE are used as positive control, and medium (PIPES buffer+ Magnesium and Calcium) are used as negative controls. All stimulations are performed in duplo, and each stimulation produces one histamine determination. Histamine is subsequently separated from the cellular suspension since it is absorbed to the glass-fibres of the microtiterplate. The plates are washed extensively, and the histamine can be released by a change in the pH and finally measured fluorometrically after coupling to a fluorophore (*O*-phthaldialdehyde). The histamine determination is calibrated by histamine standards in the medium, which are incubated in glass-fibre wells in parallel with the blood. A number of biogenic amines (spermidine, cadaverine), histamine metabolites and histidine have been tested and found not to interfere with the assay [31]. Since some allergen extracts have been reported to contain histamine [32] it is essential that the histamine content of each allergen extract is checked when a new batch of allergen extract is used in the analysis. Moreover, since new and unproven preparations of allergens or other offending substances may have cytotoxic activities, a control should always be carried out using a non-allergic basophil donor. The reproducibility of the histamine determination has been reported to be 2–3% (coefficient of variation) [29]. The variation of the cellular response has not been published.

The reference interval is class 0, i.e. that none of the used dilutions of antigen elicits a histamine release exceeding 10 ng. No age- or sex-related variations are considered. The rate of inconclusive (i.e. samples not responding to anti-IgE) has been reported to be 2.9% ($n=68$) [22]; 8.1% ($n=124$) [23]; and 24% ($n=33$) [25].

Fig. 2 illustrates two dose–response curves of allergen mixtures tested in the histamine release test. The figure illustrates several important points in relation to the test. First it is not uncommon to

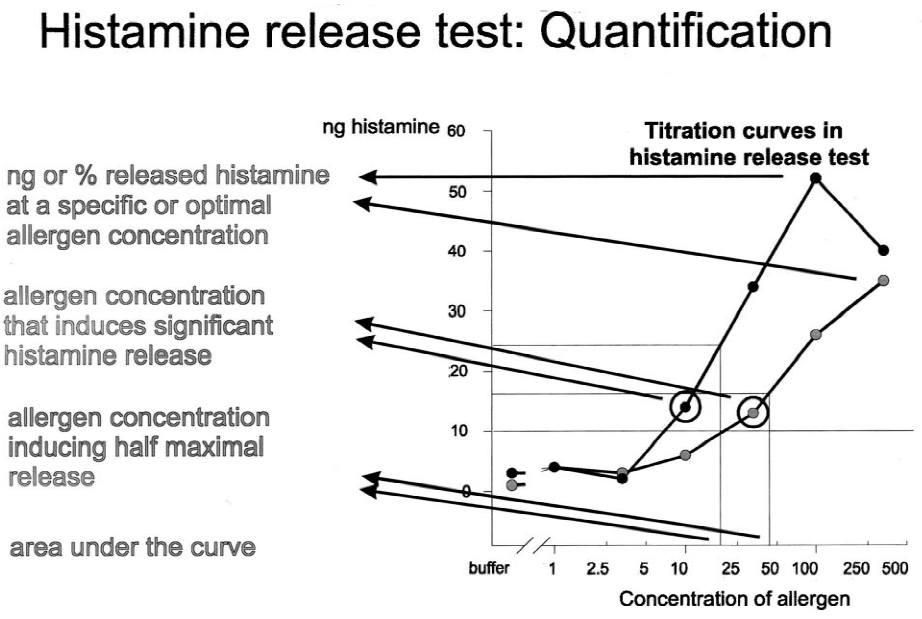

Fig. 2. Dose–response curves obtained in the histamine release test. Increasing doses of allergen are used to challenge the basophils and the secreted histamine is determined and expressed in absolute amounts or in percent of total histamine content in the cells. Possible ways to express the results are illustrated on the figure.

experience a ''hook'' effect, i.e. a decreasing response after the optimal concentration of allergen has been reached. Accordingly it is important to make full titration curve to ensure that the relevant part of the dose–response relationship has been determined. The figure also illustrates that different ways of quantitation are available, but for comparison of different titration curves it is recommended to use the concentration eliciting 50% of maximal histamine release.

2.3.1. Passive sensitization

The histamine release method described above uses blood from a sensitized patient, and this limits the practical possibilities for running the method to patient-near centres, since the whole blood must be used within 24 h after drawing of the blood. This obstacle can be overcome by combining basophils from a non-sensitized person with serum containing specific and relevant IgE-antibodies. In its original form, basophils from adult donors were stripped of their original IgE by a brief treatment with low pH, followed by a new incubation with the sensitizing serum [21,33]. More recently cord blood basophils have been used as recipient cells, which has the advantage of eliminating the low pH IgE dissociation step, which may interfere with the biological functions of the cells (Dr. Per Stahl Skov, personal communication). It is conceivable that basophil cell lines, such as the KU812 [34] or animal cell lines transfected with the the human FcεRI, i.e. the IgE high affinity receptor [35], may also be used as recipient cells for this purpose.

2.3.2. Other cellular systems

Rather than using histamine release from basophils it is also possible to monitor the synthesis of leukotrienes from basophils [36] or in animal cells

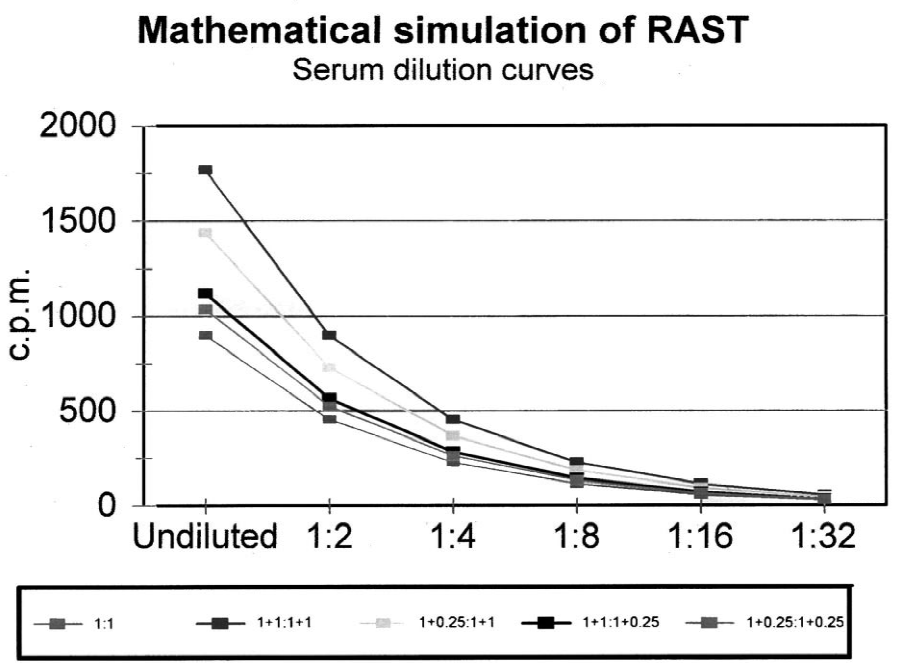

Fig. 3. Mathematical simulation of a RAST system. Assumptions were that the mass equilibrium law is acting with mean dissociation constants of allergen–IgE binding of 1 nM and of IgE–anti-IgE of 0.5 nM. A theoretical allergen with a MW of 50 kDa and a binding efficiency to the solid phase of 10% was assumed to be applied in a starting concentration of 2 μg/ml. The IgE serum was assumed to be of a concentration of 400 KIU/l (about 1 μg/ml) with 10% of the total protein being specific to the allergen. In the RAST it is used 1:10, called ''undiluted''. The anti-IgE is assumed to be used in a concentration of 100 ng/ml with an activity of 25 000 c.p.m. per sample. ''1+1'' means a single allergen–IgE system with the above mentioned properties. The double allergen–IgE systems combines this system and 25% of the second allergen, the second IgE-specificity or both.

using β-hexoseaminidase such as has been done for the RBL cell line [37,38].

2.4. In vitro studies of IgE–allergen binding: RAST and RAST-inhibition

Since the binding between the allergen and the IgE is central in eliciting of the biological function in the test systems described above, it is obvious to use a test system that measures this binding, and the RadioAllergoSorbentTest and modifications of this play an important role in allergen standardization. Since IgE was first purified and anti-IgE antisera became available [39–42] immunoassays have been designed for specific IgE using either radiolabelled allergen [43,44] or radiolabelled anti-IgE antibody as in the Radio Allergo Sorbent Test (RAST) [45]. The initial design of the RAST was based on the use of dextran-derived materials [43,45] but later solid phases have comprised the widely used paper discs [46], aluminium hydroxide gel [47], polystyrene tubes [48], cellulose polymers [49,50], and magnetic microparticles [51]. Besides varying the solid-phase, the detection principle has also been modified comprising enzyme catalyzed reactions (enzyme-linked allergosorbent assays, EAST), fluorescence, and chemiluminometric procedures. Reviews of the available technologies and a discussion of method evaluation have been given in [52,53].

Before the tests are discussed in relation to their applicability to allergen measurements, the question of multiple allergens and IgE-specificities should be mentioned. Most patients react to more than one allergen in a given allergen system, but the quantitative importance of the individual allergens in a system has never been described in relation to clinical symptoms. Fig. 3 illustrates a mathematical simulation of a serum-dilution curve run in a theoretical RAST with a single allergen–IgE specificity versus an allergen extract–serum pair with two allergens and two IgE specificities. As illustrated in the Figure, the double system produces a substantially higher response than the single system when there are equal amounts of the two allergens and IgE-specificity, but in the situation where the one allergen–IgE system is reduced to 25% of the other, there is only a marginal contribution from the second system, leaving only small room for detection of quantitative differences in the allergen content of the second allergen system. It should be noted that a reduction to 25% of the original allergen or specific IgE response may not easily be detected by semi-quantitative systems such as western blotting or Crosssed RadioImmunoElectrophoresis (CRIE) systems.

2.4.1. The inhibition RAST for measurements of allergens

In the inhibition RAST or analogous methods, serum from an allergic patient is preincubated with the allergen before being applied in the RAST. The more allergen in the sample the higher the inhibition. Normally the direct read-out, such as counts per minute, is transformed to percent inhibition according to the following formula:

$$100\% - \frac{\text{c.p.m.}_{\text{inhibited}} - \text{c.p.m.}_{\text{background}}}{\text{c.p.m.}_{\text{uninhibited}} - \text{c.p.m.}_{\text{background}}}\% \qquad (1)$$

. . . after which a diagram of allergen concentration versus percent inhibition can be constructed. If the same extract that is used for the allergosorbent is used for inhibition, it is normally possible to obtain a maximal inhibition of 90–95%. If another allergen extract is used and the maximal inhibition is less than 90% this indicates that one or more components are lacking in the inhibition extract.

In order to quantitatively compare two extracts, the basic appearance of the inhibition curves must be comparable, i.e. the same maximal inhibition and the same slope, although one curve may be right- or left-shifted compared to the other. Different slopes and/or different maximum inhibition indicate that the two extracts are qualitatively different, which makes a quantitative comparison meaningless. If the shape of two curves are identical, one extract can be expressed relatively to the other by the concentration which induces an inhibition of the test serum of e.g. 50%.

Due to different allergen-binding profiles of different sera, it may be advisable to run a comparison using different sera. Test sera used for inhibition RAST should preferably be characterized by immunoblotting against the allergen extract used for the

allergosorbent. It is an inherent property of the test system, that if an allergen is missing in the allergen extract used for the allergosorbent, or if the test serum does not react to this component, then the inhibition RAST will not be able to determine this component in the tested allergen extracts. Thus, it is an important quality control experiment to ascertain the presence of all necessary allergenic components in the allergen extract used for the allergosorbent and to confirm the IgE-reactivity against these.

The RAST inhibition or comparable methods have great advantages in not relying on the presence of the patient, and a large number of samples may be run in each setup. Due to this the method is recommended by the EAACI and the Nordic Guidelines as the reference method for batch-control allergen extracts, once an initial in-house reference has been established by skin prick test [54,15].

3. Comparison of methods for biological potency

In the discussion of the RAST-type methods it was mentioned that minor allergen–IgE systems could be difficult to detect as the RAST response may easily become dominated by the quantitatively most important IgE–allergen pair. It is not known how such differences in the relative concentration of allergen–IgE pairs contributes to the overall response in the biological assays such as skin prick test or histamine release mentioned above, but since the dynamics of these systems are far from linear, it would probably be prudent only to base any assumptions on specific experimental data.

Cross-reactivity seems to be differently distributed between the methods. In a study of allergy to legumes Bernhisel-Broadbent and coworkers found extensive reactions to more than one legume in both skin prick test and by specific IgE determinations, whereas only 2/41 patients reacted clinically to more than one legume when challenged in a DBPCFC protocol [7,55]. In relation to cross-reactivity between fish species Bernhisel-Broadbent and coworkers found a low degree of clinical cross-reactivity between different fish species, whereas a marked cross-reactivity was found in relation to specific IgE as determined by inhibition studies [8]. In contrast to this finding, Hansen and coworkers, found clinical reactivity to three other fish species in nine codfish allergic patients [13]. Several studies from this centre have confirmed the clear skin test reactivity and serological cross-reactivity in fish-allergic patients [13,56]. Several reasons for the discrepancy between the above-mentioned studies could be offered: The North American study comprised mainly pediatric patients whereas the Danish study comprised adult patients. Moreover, the selection of fish species as well as the dosages and challenge procedures were also at variance between the two centres.

The difference between the outcome of DBPCFC and of skin test or specific IgE studies have not been elucidated. It could be speculated that antibodies to the food to which the patient is originally sensitized will have a lower affinity towards homologous allergens from other species. Such low-affinity cross-reacting antibodies may be more easily determined by in vitro tests or in the skin, whereas they may play a less important role during challenges.

Further studies are needed, however, to explore this possibility. The cited studies underline the importance of knowing the clinical diagnosis of the patients that are used for biological investigations by e.g. skin tests, basophil histamine release, or specific IgE serology. Moreover, they emphasize the extreme caution that should be applied when skin test or serological studies are interpreted in relation to clinical reactivity.

4. Biological methods for assessment of allergenic potential

As described in the introduction, methods for describing the ability of an allergen to sensitize humans are far less developed than methods for biological potency determination. One major reason is that our knowledge of the natural history of food allergy is still limited. Both genetic and environmental factors are at play. It is believed that children with an atopic disposition have increased risk for becoming food allergic, and there seems to be a crude relation between frequent sensitizing foods and major food intake in different regions of the world:

Rice in Japan, codfish in Norway, crustacea in New Orleans (cajun-food) [57]. These observations does not form an adequate basis for predicting whether a protein will have sensitizing properties, however. The major food allergens so far described have been summarized as water-soluble glycoproteins having molecular weights in the range of 10–60 kDa [58], but these characteristics probably also apply to proteins in general. Comparison of the primary structure, i.e. the amino acid sequence, has not suggested specific ''allergenic epitopes'' [58], although cross-reacting allergens as expected have a high degree of homology [59,60].

4.1. Stability to digestion

It has been suggested that a common feature of food allergens is a resistance to degradation during processing and subsequent ingestion [58,61], and indeed the allergens must survive in order to reach the immune system. The major allergen of apple, Mal d1, is on the other hand one example of an extremely labile protein, which can still cause symptoms by its cross-reaction with the major allergen Bet v1 in birch pollen allergic patients. A method has been suggested that determines the stability of food allergens to ''simulated gastric fluid'', i.e. a 0.32% (w/v) pepsin solution at pH 1.2. When this method was used to assess the stability of a number of known food allergens from soy, peanut, mustard, egg, and milk most of them demonstrated a high stability, which was defined as more than 60 min resistance to degradation when subjected to the simulated gastric fluid [61]. However, two major allergens such as the peanut Ara h1 and the soy Gly m1 demonstrated low and intermediate degradability in this assay [61], suggesting that even proteins which are degraded very fast may be important allergens.

4.2. Animal models

It is tempting to use animal models for assessment the allergenic potential but in general it has been difficult to raise an IgE response in rodents by oral feeding. Li and coworkers recently described a mouse model where 3-day old mice are immunized with cow's milk together with cholera toxin as an adjuvant. When the animals are intragastrically challenged anaphylactic reactions develop with mast cell degranulation, increased plasma histamine, and increased intestinal permeability [62]. This model will be very valuable for studies of the pathophysiology of the food allergic reaction, but it is not yet known whether it can be used for other foods, or whether it can be used for predicting sensitizing potential of different antigens.

4.3. Matrix effects

From the above-mentioned study it is clear that adjuvant factors may play an important role in inducing food allergy as have been suggested for inhalation allergy [63]. An observation which may be of interest in that respect, is the fact that peanut allergy seems to be much more frequent than soy allergy [7] despite the close botanical and serological relationship between the two legumes [58,55]. A striking difference between the consumption of the two species is that peanut is often administered in conjunction with lipid (whole peanut, peanut butter), whereas soy protein is often defatted before use. Albeit this suggested mechanism remains purely speculative, the difference between closely related species such as soy and peanut, emphasizes the need for consideration of other factors apart from the allergenic proteins themselves when models for assessment of allergenic potential are being developed and evaluated.

5. Conclusion

There is a large demand for characterization of both the allergenic potency and allergenic potential of foods and food allergens. The introduction of novel foods and the more complex use of different ingredients in industrially processed and home-cooked foods calls for the determination of biological potency in order to assure the safety for food

allergic patients [64]. Moreover, there is a strong need for standardized allergen extracts for in vivo and in vitro diagnosis, and only very few attempts have so far been carried out to apply biological standardization on food allergen extracts like it has been done for inhalation allergen extracts for many years. As has been described, the methods for standardization exist, but the low number of patients who have been sufficiently clinically characterized may still present a problem. Since food allergy, especially among adults, are much less frequent than inhalation allergies, the area may benefit from international collaborations in order to perform studies with a sufficient number of patients [65].

In the area of risk assessment in relation to inducing new allergies the available methods are still not sufficient. Several attempts in the direction of: (1) homology and sequence studies; (2) degradation studies and (3) animal immunization studies have been carried out, but it is conceivable that a real breakthrough in this area may still await a better understanding of the sensitization process in humans.

6. Nomenclature

DBPCFC	Double-blinded, placebo-controlled food challenges
EAST	Enzyme-linked Allergo Sorbent Test
HR	Histamine release (from basophil granulocytes)
ICU	Intensive care unit
IgE	Immunoglobulin E
SPT	Skin prick test
RAST	Radio Allergo Sorbent Test

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