

OPINIONS IN ALLERGY

What is a food allergen?

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Clinical and Experimental Allergy

Summary

With the increasing prevalence of allergies, accurate identification of allergens is a major priority for allergists, scientists, the food industry, and food regulators. Knowledge of allergens is essential for risk assessment of novel genetically modified (GM) foods, and to develop recombinant proteins for the treatment and diagnosis of allergies. This Opinion Paper considers the lack of standardization for the clinical and scientific assessment of proteins before they are labelled as allergens. Food allergens are being reported and recorded in allergen databases, with minimal or in some cases apparently no published justification. IgE binding, rather than clinically relevant reactivity, is inappropriately used to confirm allergenicity. Using kiwifruit as an example, the lack of rigor in identifying allergenic proteins is considered.

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Introduction

Foods contain a wide variety of proteins and yet only a very small number are allergens. With the increasing prevalence of allergies, the issues of allergenicity of foods have become a major consideration for the food industry and its regulators. New foods, including genetically modified (GM) foods, need to be scrutinized before entry to the market to assess their allergenic potential. The risk assessment of GM foods is an evolving field and not all tests currently being used to assess their allergenicity have a sound scientific basis [1]. Current evaluation appropriately includes reviewing the sequence homology of the newly introduced protein to known allergens and assessing the immunochemical binding of the newly introduced protein with IgE from the serum of individuals with known allergies to the transferred genetic material. However, the role of investigations including use of non-validated animal models and targeted serum screens has been brought into question recently [1]. Perhaps more importantly, valid assessment for allergenicity requires detailed and accurate data about known allergens. The increasing use of recombinant proteins in the diagnosis and treatment of allergies also demands accurate knowledge of the allergens responsible. There is no accepted

standardization for the clinical or the scientific assessment of proteins before they are labelled allergenic. Food allergens are being reported with minimal or, in some cases, apparently no published justification.

Perhaps the most clinically useful definition of an allergen is 'an antigen that stimulates an allergic reaction' [2]. Many allergens have been designated on the basis of IgE reactivity (skin test sensitization or circulating specific IgE) with no evidence of clinical allergic reaction. Palacin et al. [3] (in this edition of *Clinical and Experimental Allergy*) have studied a large group of patients with sensitization to kiwifruit, but in common with other study teams [4, 5], only 50% of the patients reacted positively to a double-blind placebo-controlled food challenge (DBPCFC). There are various reasons why a patient may be sensitized, but show a negative food challenge. Reasons include an inadequate food challenge protocol (e.g. the allergen in question is denatured during preparation of the challenge) or clinically irrelevant cross-sensitization to the food allergens (because of IgE binding to glycoproteins or proteins that do not result in mast cell degranulation). Assuming that the former has been excluded, we argue that a protein cannot be said to be allergenic if allergen-specific IgE is found at the same frequency in both the food-tolerant but sensitized group and the truly

Table 1. Suggested requirements for designation of an allergen

1. A study population with clinical allergy to the source food [assessed by skin prick test (SPT), specific IgE and double-blind placebo-controlled food challenge (DBPCFC)]
2. A control population without clinical allergy to the source food (including atopic and non-atopic volunteers)
3. Scientific rigor and documentation of clinical investigations equivalent to that required for clinical drug trials
4. Accurate identification of the source food used for the clinical and laboratory investigations
5. Extraction and purification process reported with sufficient detail for other laboratories to validate the results
6. Data for immunoassays using the allergic and non-allergic sera to be shown
7. A protein should not automatically be labelled an allergen if sera from non-allergic patients bind at a similar frequency to sera from non-allergic controls
8. <i>In vivo</i> confirmation of the allergenicity of the protein (assessed by SPT or basophil histamine release with purified and recombinant protein)
9. Reproducibility of results demonstrated in different laboratories

Table 2. Questions for debate by the scientific community

What is the minimum study population size?
Should the protein be studied in patients from different geographical and ethnic backgrounds?
How will we administer the exchange of sera and allergens between laboratories to validate results?
What information is required for documenting ripeness, cultivars, etc.?

allergic group. Even in peanut allergy, a high percentage of schoolchildren with positive skin prick tests had a negative DBPCFC with peanut [6]. We can find no evidence that such patients regularly go on to develop clinical allergy, and this cannot therefore be considered to be a pre-clinical state. They have non-relevant sensitization, confirmed by gold standard DBPCFC. Palacin et al. [3] appropriately report their clinical investigation and food challenge outcome results to kiwifruit, enabling the reader to assess the relevance of the data, but this is not always the case.

We challenge the current methods for reporting allergens, using kiwifruit as an example. We discuss our rationale for minimum requirements for a protein to be designated an allergen (Table 1) and suggest topics that need to be considered by the scientific community (Table 2).

Allergen databases

There are several publicly accessible allergen databases, including a WHO/IUIS facility, SWISS-PROT and FARRP (reviewed in [7–9]). These databases typically contain the allergen name, accession number(s), sequence data and literature reference(s). These data provide an important source of information to support basic and applied allergy research, but it is paramount that proteins should be confirmed as allergenic before they are referenced.

There are currently no clinical requirements concerning the evidence required for a protein to be classified as an allergen. An allergen can be reported on the basis of evidence from a few patients, from one study centre and with inadequate negative control sera. There are no standards dictating how immunogenicity is confirmed,

and there is no requirement for confirmation of the findings in different study populations or laboratories. The WHO/IUIS Allergen Nomenclature Subcommittee has said 'in order to maintain the integrity of the nomenclature system, only allergens which meet one or both of the following characteristics will be included: (1) A prevalence of IgE reactivity greater than 50% and (2) a minimum of five patients show IgE reactivity' [10]. However, as this article will highlight, there are kiwifruit allergens that have been designated on the basis of two poorly characterized patients, and others for which we can access no peer-reviewed clinical data. It is alarming that there is no requirement for peer review of all available data to ensure an acceptable minimum standard of clinical and scientific evaluation. Some websites are more transparent. The FARRP database is peer-reviewed (www.allergenonline.com), with the website clearly describing its review process and criteria for labelling a protein an allergen or a putative allergen. Allergome (www.allergome.org) is a comprehensive database that references up-to-date literature.

Many allergen websites collate scientific information, but do not critique it. Published conference proceedings are referenced in the same way as peer-reviewed manuscripts. Unfortunately, once information is released, it cannot be easily retrieved from the public domain – even if subsequently proven incorrect. It should be our aim to provide information that the public (and not just the medical/science community) can have confidence in. Freedom of information to the public is appropriate, but websites managed by auspicious organizations have a responsibility to fully explain the limitations of their data.

Kiwifruit allergens

We will use kiwifruit as an example of an allergenic food. Kiwifruit allergy is an interesting case study as the fruit's availability and consumption has increased greatly since the early 1970s. Consumers are eating kiwifruit on a more regular basis and increasing numbers are becoming sensitive presumably due to increased contact [5]. The genus

Actinidia contains over 60 species, but until recently only green-fleshed kiwifruit, *Actinidia deliciosa*, has been eaten regularly in the western world. Recently, other species, including baby kiwifruit (*Actinidia arguta*) and yellow-fleshed kiwifruit (*Actinidia chinensis*), have been increasingly consumed. *A. chinensis* is very similar to *A. deliciosa* and until 20 years ago, they were classified as one species. However, there are distinct morphological differences between the two species – most notably *A. deliciosa* has long stiff hairs that are generally persistent on the fruit, while *A. chinensis* is soft-haired with smooth-skinned fruit.

Kiwifruit allergenicity has been recognized for over 20 years and reported allergy appears to be increasing [5]. In adults, the symptoms of kiwifruit allergy are typically mild, but in children, the condition can be more serious and can occasionally cause anaphylaxis [5]. There has been considerable activity in recent years to identify the responsible allergens. There are currently eight kiwifruit proteins recorded in the WHO/IUIS allergen database. We have reviewed the evidence for each of these proteins to be labelled as allergens by searching for peer-reviewed publications using various search engines including PubMed, Web of Science and Google.

The first kiwifruit allergen to be identified was actinidin. Pastorello et al. [11] reported that 100% of Italian kiwifruit-allergic patients had IgE binding on Western blotting to a 30 kDa protein. The allergic patients were diagnosed on the basis of history, skin test, specific IgE and open food challenge. The researchers did not show Western blots using non-allergic sera as controls, and the binding to this abundant protein may therefore have been non-specific, a problem that we have encountered in our own work with kiwifruit. Pastorello et al. [12] later identified the protein as actinidin by using a pool of sera from the well-characterized allergic group, and actinidin was designated Act c 1 (now Act d 1). Reactivity by individual sera to purified actinidin was not undertaken in the study and so it is possible that not every patient's serum in the pool would have recognized actinidin. Perhaps, more importantly, non-specific binding to purified actinidin by non-allergic control sera can be seen in the published figures and this has been an issue with reports from some other groups working with actinidin [13]. Our study of 76 UK patients with kiwifruit allergy failed to find any patients with IgE reactivity to actinidin [14]. We need to consider whether the huge discrepancy between the results from these studies is due to a lack of standardization of the study protocol rather than a genuine difference in immunoreactivity.

A thaumatin-like protein (Act d 2, formerly Act c 2) of 24 kDa was reported to be a major allergen in kiwifruit (*A. deliciosa*) by Gavrovic-Jankulovic et al. [15]. Sera from seven patients allergic to kiwifruit were used in this study. All patients showed oral-allergy syndrome and one of them had severe contact dermatitis and anaphylaxis when

touching the fruit. All had either a positive skin test or positive specific IgE to kiwifruit, but the authors did not report food challenges. All seven sera demonstrated IgE binding to a 24 kDa protein that was identified as a thaumatin-like protein, and a pool of sera from non-allergic patients showed no binding. The patients were skin tested with the purified protein and four showed *in vivo* reactivity. We suggest that it should be a requisite to perform an additional skin test with recombinant protein to eliminate the chance of protein contamination.

The basis for designating kiwellin (Act d 5) as an allergen was an *in vitro* study using sera from two patients with symptoms on eating kiwifruit, but one of the two had a negative skin test [16]. Kiwellin is a novel cysteine-rich protein that was isolated from kiwifruit purchased from local markets. The researchers report the species as *A. chinensis*, but it is listed in the WHO/IUIS database as an allergen from *A. deliciosa*. As green-fleshed kiwifruit is the form most commonly consumed in Italy, this is probably the correct designation. Identifying the species and cultivar of fruits can be difficult and this confusion highlights the need for clear identification of the source food, preferably by a plant geneticist or a botanist. In common with many other papers, no information on the cultivar or ripening stage of the fruit was provided, although they are known to be relevant to expression of allergens. Our greatest criticism is that this publication was used to label the protein as an allergen, although in reality the findings were primarily scientific, with paucity of clinical data to substantiate allergenicity.

We have failed to identify any peer-reviewed publications relating to (Act d 3) that appear as a direct submission to SWISS-PROT in September 2006. We have been unable to access any data that confirm this protein as clinically relevant in kiwifruit-allergic patients. Likewise, we were unable to identify any peer-reviewed documentation confirming clinical reactivity to cystatin (Act d 4). We were also unable to identify peer-reviewed publications to verify three additions to the WHO/IUIS allergen database within the last few months (Act d 6, Act d 7 and Act d 8).

Although Act d 3–Act d 8 may subsequently be confirmed as being clinically important allergens, their designation before being subject to rigorous clinical and scientific peer review is clearly premature. We suggest that papers should use GenBank/UniProt accession numbers to identify the protein sequence. This is much more accurate and allows for protein variants to be identified clearly. Only when convincing evidence is supplied to support allergenicity should a protein be given an allergen code.

Discussion

The accurate identification of allergens to inform the food industry, health professionals and allergy diagnostics is

well recognized. The current situation is unacceptable. Proteins are being designated allergens in the absence of supporting data of clinical relevance. In part, this is a consequence of proteomic groups, clinicians and plant biologists all working in isolation. It is clear that greater cooperation between these groups, especially the international exchange of samples, needs to occur in order to validate observations. The limiting factor is often the availability of patient sera; however, the exchange of purified recombinant proteins, protein extracts and antibodies for testing against other groups' sera needs to be more strongly encouraged. Full characterization of an allergen may take years, but until its allergenicity has been fully elucidated, it cannot be termed an allergen.

The first step in designing a study to identify allergens must be identification of a study group of well-characterized patients with allergic status confirmed by appropriate investigation including a DBPCFC. The food challenge protocol should be designed with the same rigour as the scientific studies, because it can be biased by the same problems as producing good protein extracts, for example allergen degradation [5]. The only exclusion to DBPCFC should be patients with a history of anaphylaxis, for whom a challenge would be inappropriate. An adequate control group of non-allergic patients is also essential due to the well-recognized phenomenon of IgE binding to proteins in the absence of clinical relevance. The discrepancy in findings surrounding actinidin as a major allergen highlights the need for analysis to occur in geographically and culturally different study populations.

Clearer guidance is required for standardization of scientific techniques used for the preparation and analysis of food samples. Protein extracts are inherently variable in the number, amount and stability of their components. Variations in extraction procedures may contribute to the conflicting and confusing results reported in the allergen field. It is essential that detailed extraction procedures and electrophoretic conditions are provided for results to be reproduced. Rigour to avoid degradation of allergens during preparation needs to be documented. All proteins are subject to degradation (enzymatic or non-enzymatic). In kiwifruit, this is a particular problem as actinidin is a protease – hence, samples are particularly susceptible to enzymatic degradation. Actinidin is also susceptible to autolysis. Standardization of extracts may not be achievable, but the exchange of protein extracts to validate protocols in different laboratories would be desirable.

A full description of food samples used in allergen studies is also essential for reproducing results. For example, fruit purchased 'from the local market' appropriately reflects what the patient is likely to consume, but will differ between countries. Accurate identification of the species any food sample is derived from is important to avoid the confusion highlighted by Act d 5. Identification should include the cultivar (Act d 1 protein is found in

some but not all yellow-fleshed kiwifruit cultivars [17]) and the country of origin as allergen levels are influenced by environmental conditions during plant growth. Levels of allergen also vary by the stage of fruit ripeness, e.g. Act d 2 protein levels increase as the fruit ripens [18], during storage and by tissue type. Inclusion of skin and seeds in the extract will provide allergens not present in the cortex alone.

Currently, protocols for the identification of food allergens make use of total protein extracts. In kiwifruit, many proteins, including some potential allergens, have molecular weights similar to the highly abundant actinidin protein, which may result in misidentification on SDS gels and LCMS. To date, three of the five allergens identified in kiwifruit (actinidin, thaumatin and kiwellin) represent highly abundant proteins expressed in the fruit. Is this a consequence of identifying the prevalent proteins rather than the allergen that might be present in minute amounts? As the consequences of misidentification are increasingly important, we suggest that *in vivo* confirmation of the allergenicity of a protein is a pre-requisite for submission to the allergen databases. Ideally, skin testing or basophil histamine release assays should be performed with recombinant proteins (produced in the absence of contaminating plant proteins), together with purified plant proteins (which retain the correct conformation and/or post-translational modifications), to confirm allergenicity. There is no question that both highly purified 'natural' and 'recombinant' proteins might be contaminated with heterologous proteins. However, it is highly unlikely that the same contaminants would be present in both plant and recombinant extracts. Testing both protein sources strengthens the reliability of calling a protein an allergen.

In summary, there is no race to name an allergen and this label should be delayed until there is a sufficient body of data to make the designation with confidence. Improved guidelines for appropriate study populations, laboratory methods and methods of validating of results are essential. Until all data regarding a new allergen have undergone vigorous peer scrutiny, designation should not occur. The problem of delayed designation will surely be outweighed by increased confidence in the currently inadequate system.

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