Critical Reviews in Toxicology

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/itxc20

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To cite this article: Nora L. Krutz , Ian Kimber , Sebastian Maurer-Stroh & G. Frank Gerberick (2020): Determination of the relative allergenic potency of proteins: hurdles and opportunities, Critical Reviews in Toxicology

To link to this article: https://doi.org/10.1080/10408444.2020.1793895

Published online: 30 Jul 2020.

CRITICAL REVIEWS IN TOXICOLOGY
https://doi.org/10.1080/10408444.2020.1793895

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REVIEW ARTICLE

Determination of the relative allergenic potency of proteins: hurdles and opportunities

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ABSTRACT

The use of proteins and protein-containing materials in a variety of industrial and commercial products is increasing, with applications in pharmaceuticals, agrochemicals and consumer and personal care products. As a consequence there is a need to ensure potential and environmental risks are understood. One important requirement is an appreciation of the ability of proteins to induce allergic sensitization and allergic disease. However, there is currently no clear guidance for determination of whether or not to accept a new protein in a product based on potential allergenicity. A key requirement for effective risk assessment in this respect is an understanding of sensitizing potency. Here we describe issues and challenges associated with measurement of allergenic potency and explore emerging opportunities and possible ways forward. Effective assessment of the risk of allergy demands not only information about the likely conditions of exposure, but also an understanding of the sensitizing potency of protein allergens. For the purposes of this article sensitizing potency can be viewed as being the ease with which, and the concentration at which, proteins will induce sensitization in a previously non-sensitized subject. The immunological bases of protein allergy are summarized, and the properties that confer on proteins the ability to induce allergic sensitization are considered prior to a detailed exploration of the issues that have to be addressed for evaluation of sensitizing potency. Included among the important considerations are: the impact of route of exposure, identification of relevant dose metrics, and the requirement for reference standards. Finally, new and emerging opportunities to evaluate the sensitizing potency of allergenic proteins are reviewed, including the use of *in silico* modeling.

ARTICLE HISTORY
Received 27 May 2020
Revised 6 July 2020
Accepted 6 July 2020

KEYWORDS
Protein allergy; sensitization potency; immunogenicity and allergenicity; exposure metrics; risk assessment

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Introduction

There is increasing interest in the use of proteins and of protein-containing materials in a number of applications, including pharmaceuticals, agrochemicals, and consumer and personal care products. Ensuring the safety in use of proteins in such applications requires an appreciation of their ability to induce allergic sensitization (Sarlo and Baccam 2007). Allergic sensitization to proteins typically results from the production of allergen-specific IgE antibodies which prime the individual to mount an allergic reaction when subsequently encountering the same protein. Allergic reactions can

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take a number of forms, including asthma, eczema, rhinitis, conjunctivitis and food allergy-related symptoms. Effective assessment of the risk of allergy demands not only information about likely conditions of exposure but also an understanding of sensitizing potency.

The purpose of this review article is to consider the factors that influence the sensitizing activity of proteins (including, in addition to native proteins, genetically modified or synthetically designed proteins), to describe problems associated with the evaluation of relative sensitizing potency, and what opportunities exist to develop new paradigms for potency assessment.

In order to frame this discussion it is necessary first to consider some fundamental aspects of allergy, general toxicological principles as they relate to protein allergenicity, and to adopt some working definitions.

Allergy, allergens and sensitizing potency

For the purposes of this exercise allergy is best defined as the adverse health effects that may result from the stimulation of an adaptive immune response. Allergy is commonly associated with immune reactions to what would normally be innocuous antigens where protective immunity is not required.

All forms of allergy develop in two phases. In the first phase, exposure of an inherently susceptible individual to the inducing antigen (allergen) triggers an adaptive immune response resulting in immunological priming and a lasting heightened responsiveness to the stimulus. In this way specific sensitization is acquired. This is termed the induction phase. If the now sensitized subject is exposed subsequently (via a relevant route) to a sufficient amount of the inducing allergen then an accelerated and more aggressive secondary immune response can be triggered resulting in a local, and in some instances a systemic, inflammatory reaction (the allergic reaction). This is the second or elicitation phase (Kimber et al. 2011; Figure 1).

The focus of this article is on the relative sensitizing potency of protein allergens, and in most instances, this is dependent upon the induction of IgE antibody responses. Some factors affecting sensitization to proteins and the development of protein allergy are summarized diagrammatically in Figure 2. In this context there are 3 major considerations: the subject, exposure and the allergen itself.

The susceptibility of the subject is driven largely by the possession of an atopic phenotype (inherited or acquired); atopy being defined as a disposition to mount IgE antibody responses. The age of the subject at time of exposure to allergens is probably also important, together with other poorly defined traits.

Exposure is a key consideration. It is clear that the development of allergic sensitization to proteins can result from dietary exposure, inhalation exposure and/or skin exposure. There is little information regarding the relative effectiveness of various routes of exposure, although differences undoubtedly exist, not least because the cellular mechanisms of antigen handling, processing and presentation will vary markedly between these tissue sites. Clearly, the route and duration of exposure will be critical determinants of the effectiveness of sensitization, and other factors may also be influential such as, for instance, barrier function in the skin, and clearance mechanisms in the respiratory and gastrointestinal tracts.

Finally, the characteristics of the protein allergen will play a decisive role. Of particular importance will be the inherent potency of the allergen itself. What drives the potency of protein allergens is the major theme of this article, but for the purposes of this introduction it is sufficient to identify the following as being important: (a) structural features and characteristics of 2 and 3 dimensional epitopes (antigenic determinants), (b) enzymatic activity (and possibly other

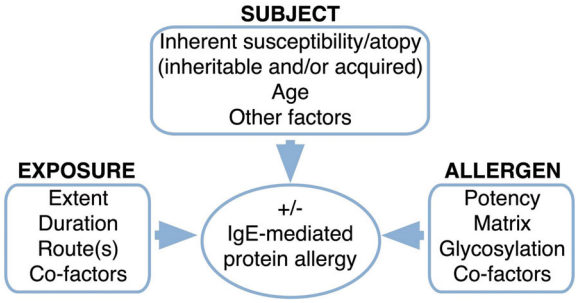

Figure 2. **A** schematic representation of the major factors that influence the acquisition of IgE-mediated allergic sensitization: (a) susceptibility of the subject, (b) conditions, duration and route of exposure, and (c) characteristics of the allergen itself.

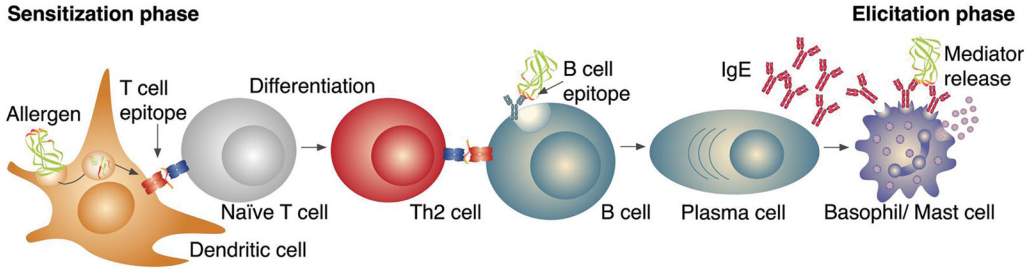

Figure 1. A simplified representation of key cellular/molecular events associated with, and required for, the stimulation of IgE antibody responses and the elicitation of an allergic reaction. Protein antigen (the inducing allergen) is internalized and processed by dendritic cells. The processed antigen is presented to responsive T lymphocytes expressing a complementary receptor for antigen. Responsive lymphocytes of Th2 phenotype interact with B lymphocytes to stimulate an IgE antibody response. IgE antibody then associates with tissue mast cells and blood basophils. This signals the acquisition of sensitization. If the sensitized subject is exposed subsequently to the same inducing allergen then an allergic reaction can be elicited. The allergen reacts with, and cross-links, membrane-bound IgE. This in turn stimulates cellular activation and the release both pre-formed and newly synthesized molecular mediators that together drive inflammation and elicitation of an allergic reaction.

functional attributes of the protein), (c) the influence of post-translational modifications (and perhaps in particular glycosylation), and (d) physico-chemical properties such as stability and resistance to proteolytic digestion. In addition, the matrix in which allergenic proteins are encountered, and other co-factors may also be influential (Sarlo et al. 1997b; Bufe 1998; Huby et al. 2000; Alberse 2001; Bredehorst and David 2001, Kimber and Dearman 2002a; Scheurer et al. 2015).

Colloquially, protein allergenicity can be considered as being the ease with which an allergenic protein is able to induce sensitization based on some or all of the features listed above. However, for the purposes of this discussion potency is best described as reflecting the level of exposure to a protein allergen that is necessary to drive the acquisition of sensitization. Sensitization is defined as the presence of allergen-specific IgE in serum and bound to tissue mast cells and blood basophils. Thus, in general terms, the more potent the allergen, the smaller will be the amount required to trigger immunological priming and sensitization.

The concept of potency in allergic sensitization is perhaps best illustrated by an understanding of allergic contact dermatitis (ACD) where topical exposure to a chemical (contact) allergen causes skin sensitization. It is known that contact allergens differ by up to five orders of magnitude in terms of their relative potency – that is their ability to induce skin sensitization (Gerberick et al. 2005; Kern et al. 2010). Clearly, therefore, an understanding of relative skin sensitizing potency plays a pivotal role in developing accurate risk assessments and for determining safe levels of exposure that will not result in the acquisition of sensitization, and ultimately ACD (Api et al. 2008).

In the case of protein allergy, there is much less certainty about the extent to which there are differences in sensitizing potency. Experience from occupational health studies, and from patterns of food allergy and hay fever, for instance (Sarlo et al. 1997a; Robinson et al. 1998; Houben et al. 2016; 2019), suggests that there are differences in potency, but certainly not of the magnitude seen with skin sensitizing chemicals. However, it is frequently difficult to distinguish between differences in inherent sensitizing potency of a single protein and exposure metrics. That is, it is often impossible to establish with any certainty whether differences in the prevalence of allergy to proteins are attributable to variable exposure, or to differences in the inherent sensitizing potency of a single protein, or both. Further, the clinical picture of allergy is very variable and allergy is commonly diagnosed with respect to the protein allergen source such as dust, animal or plant species, but rarely to a specific protein. Despite these difficulties there are available potentially helpful experimental approaches that provide some information on potency, and these will be discussed later.

Notwithstanding these uncertainties, it is important for worker and consumer safety assessment that any differences in the sensitizing potency of proteins are understood and factored into the risk assessment process. If information on relative potency is lacking, then of course the unhelpful default position is to regard all proteins as being potent sensitizers.

Aspects of protein allergenicity relevant to potency

The basis of allergenic potential

It is important to appreciate that not all foreign proteins are allergenic. Foreign proteins all have the potential to induce immune responses in exposed subjects (i.e. the proteins are immunogenic), however, only some of these have the ability to induce allergy. Thus, some, but by no means all, foreign proteins are able to induce allergic sensitization and allergic disease in a fraction of the exposed population (those subjects that are susceptible). Examples of allergenic proteins include, for instance, those derived from certain foods (such as peanut, tree nuts, cows' milk and hens' eggs), indoor allergens such as house dust mite proteins, environmental (outdoor) allergens such as proteins in pollens and molds, and occupational allergens such as detergent enzymes. Other proteins are never, or only very rarely, implicated as allergens (Krutz et al. 2019). This distinction between immunogenic proteins and allergenic proteins is important – and is the first consideration in identifying whether a protein represents an allergenicity hazard.

From an immunological perspective, one (perhaps overly simplistic) way of describing the difference between protein immunogens and protein allergens is as follows. All foreign proteins – because they are foreign – have the potential to induce immune responses in exposed subjects, but only certain proteins (those with structural allergenic properties) are able to induce the class of immune response that is necessary for the acquisition of sensitization. The key component of the immune response that drives sensitization to allergenic proteins is most commonly an IgE antibody. Protein immunogens (that fail to induce allergy) elicit IgG antibody responses. In contrast, protein allergens characteristically produce IgE antibody (as well as IgG antibody), but not necessarily in all individuals (Kimber and Dearman 2002a).

The allergic process can be summarized briefly as follows: protein allergens induce the production of specific IgE antibodies. These circulate systemically and associate via, specialized membrane receptors, with basophils in the blood, and with mast cells that are resident in virtually all vascularized tissues. At this point the subject is sensitized and primed to respond following subsequent exposure to the inducing allergenic protein. When such re-exposure occurs, via a relevant route, the protein allergen will associate with, and cross-link, membrane bound IgE antibody. This, in turn causes cellular activation and the release of preformed and newly synthesized mediators (such as vasoactive amines) that collectively trigger an inflammatory allergic reaction. These reactions can manifest clinically as urticaria, angioedema, allergic rhinitis, allergic asthma and anaphylaxis.

What properties confer on proteins allergenic potential?

An intriguing question is what characteristics of proteins are associated with allergenic potential? This question has been addressed by a number of authors (Bufe 1998; Huby et al. 2000; Aalberse 2001; Bredehorst and David 2001; Kimber and Dearman 2002a; Bannon 2004; Scheurer et al. 2015).

Those properties that appear to be important include but are not limited to: (a) the possession of linear and 3D epitopes that can engage with the adaptive immune system to elicit responses of the type that will drive allergic sensitization, (b) glycosylation status, (c) enzymatic activity, and (d) stability and resistance to proteolytic digestion. It is possible that glycosylation and enzymatic activity may each contribute toward the inherent adjuvant properties of some allergenic proteins and thereby facilitate interaction with elements of the innate immune system to promote the elicitation of allergic responses (Scheurer et al. 2015).

Irrespective of the molecular features that contribute toward allergenic activity, there is no doubt that the development of sensitization will be driven by the appropriate interplay between the innate and adaptive immune systems, and the elicitation of responses that favor and support the production of IgE antibody specific for the inducing allergenic protein (Scheurer et al. 2015). A more detailed consideration of the cellular and molecular mechanisms that drive allergic sensitization are beyond the scope of this article, but information is available elsewhere (Johnston et al. 2014; Scheurer et al. 2015; Chiang et al. 2018).

The properties listed above have a role to play in distinguishing between immunogenic and allergenic proteins, and the identification of sensitizing hazards. This is perhaps best illustrated by the deployment of methods for evaluation of whether novel proteins expressed by transgenic crop plants have the potential to cause allergic disease among consumers. Here the traditional approach has been to consider, among other factors, stability to proteolytic digestion, glycosylation, and also cross-reactivity (and structural similarity and amino acid sequence homology) with known allergenic proteins (Kimber and Dearman 2002b; Germolec et al. 2003; Goodman et al. 2005; Ladics 2019). Structural similarity with known protein allergens is used by *in silico* tools such as AllerCatPro to evaluate whether novel proteins have sequences similar to those associated with allergenic activity (Maurer-Stroh et al. 2019).

In addition, there has been a continuing interest in the use of animal models for the assessment of protein allergenic potential (Sarlo et al. 1997a; Knippels et al. 1998; Robinson et al. 1998; Kimber et al. 1999, 2003a, 2003b; Buchanan and Frick 2002; Helm et al. 2002; Ladics et al. 2010; Schülke and Albrecht 2019). Although it must be acknowledged that such animal models are not without limitations and constraints (Kimber et al. 2003b; Ladics et al. 2010), they do have the advantage of providing a holistic assessment of the interaction of proteins with an intact immune system. For instance, arguably, the most useful readout of animal models has been the ability to measure the potential of test proteins to induce IgE antibody responses. Routine evaluation of the stimulation by proteins of IgE responses *in vitro* is not (yet) feasible.

The question addressed here is whether such methods (*in vivo, in vitro* or *in silico*), and the events and properties they measure, can be used to evaluate the sensitizing potency of proteins.

Requirements for evaluation of sensitizing potency

Identification of hazards can be accomplished by the measurement – by whatever means – of one or more properties or induced events that are known to be causally associated (to a greater or lesser extent) with the development and expression of the particular hazard of interest. However, it is important to appreciate that an understanding of causal associations, although useful for the purposes of hazard identification, does not necessarily provide useful information about relative potency. An accurate assessment of potency, in this case of the sensitizing potency of protein allergens, requires an understanding of those features or events that are causally AND quantitatively associated with the acquisition of sensitization and allergic disease.

Thus far, the only such readout is probably the ability of proteins to elicit IgE antibody responses in test animals. One can speculate, for instance, that sensitizing potency might correlate with the magnitude of the IgE antibody response, or with the amount of test protein required to elicit a certain pre-determined level of IgE antibody production. However, even in this instance, there is no guarantee that the induction of IgE antibody responses by experimental animals will provide a robust and consistent read-out, and importantly, the quantitative assessment of IgE responses induced in experimental animals is associated with significant technical problems (Basketter and Kimber 2011). In addition, there is no certainty that the relative ability of proteins to induce IgE responses in rodents will provide a reliable correlate of sensitizing potency among humans.

For evaluating the potency of individual detergent enzymes to aid in setting occupational exposure guidelines, both the mouse intranasal test (MINT) and guinea pig intratracheal (GPIT) model have been used (Sarlo et al. 1997a,b; Robinson et al. 1998). The protease enzyme Alcalase (protease Subtillisin Carlsberg) has been used as a benchmark for evaluating the potency of other enzymes. Data from both the MINT and GPIT showed that, based on specific antibody titers, the bacterial amylase Termamyl and a fungal exocellulase were more potent sensitizers than Alcalase, and that a fungal alpha-amylase (Fungamyl) was less potent than Alcalase (Robinson et al. 1998). These data show that when the exposure of the protein allergens can be controlled, differences in the potency of individual proteins can be observed for the induction of respiratory sensitization.

The other relevant issue is, of course, that there has been in recent years an increasing appetite to move away from animal models for the assessment of the sensitizing properties of both proteins and chemicals. Examples of cell-based models that seek to identify respiratory sensitization potential, but not (yet) sensitizing potency include a 3D co-culture model that has been developed to investigate sensitizing properties of chemicals and, to a lesser extent, proteins and identified potential biomarkers (Chary et al. 2019). In addition, the GARDair method is an *in vitro* assay designed for the prediction of respiratory sensitization potential of chemicals based on induced changes in gene expression signatures (Forreryd et al. 2015). Also, Zeller

et al. (2018) identified potential biomarkers for protein allergenicity in a similar model. Although these *in vitro* models represent an important step forward in the development of non-animal methods for hazard identification, further work is required to identify critical biomarkers covering all protein allergens and thus allow the assessment of their sensitizing potency. Importantly, there is a need for a reliable set of reference proteins of varying sensitizing potency that can be used to evaluate and calibrate novel approaches.

In a proof of concept proposal for risk management of foods, Houben et al. (2016; 2019), and more recently Remington et al. (2020) have proposed ranking of allergenic foods according to a combination of prevalence and potency data. They used ED50 food challenge values derived from the TNO-FARRP Threshold Database for defining potency. The ED50 values, with a 95% confidence interval, ranged from 14 mg for mustard to 256 mg for fish. Thus, foods with an expected high prevalence of allergy and/or low ED50 value could be categorized as relatively potent allergenic foods (Houben et al. 2019). In this context, however, potency is seen as the ability of whole foods to elicit reactions following challenge. It is therefore difficult to infer potency for any individual protein allergens within these foods. This approach could in theory be applied to single proteins if the data were available, but it is important to appreciate that this model addresses questions of elicitation, rather than the potency with which sensitization is induced.

The relationship between exposure and potency of plant-derived proteins for respiratory allergy has been examined by Blackburn et al. (2015). The authors suggest that the varying occupational limits for plant-derived materials may give insight into the potency of the proteins associated with causing respiratory allergy. The No Effect protein Exposure Levels considered safe for plant-based materials ranged from less than 0.1 $\mu g/m^3$ (e.g. latex protein) to greater than 100 $\mu g/m^3$ (e.g. corn protein). Of course, these limits are not set for individual proteins so it is difficult to know with any certainty the relative respiratory sensitizing potency of an individual protein. Still, these data show that when the exposure of the total protein content of certain plant-based materials is controlled, differences can be observed for the induction of respiratory sensitization. This is critical for an understanding of potencies of individual proteins once the mixtures are further analyzed to identify and semi-quantify highly abundant individual proteins, as well as other constituents within the plant matrix that may also have an impact on how the exposure limits were determined. This approach is not without limitations in that it is only semi-quantitative at best, but it does give insight into the evaluation of the relative potency of proteins in humans.

Against this background, the remainder of this article will explore firstly the hurdles that need to be considered in developing reliable approaches for the measurement of the relative sensitizing potential of allergenic proteins, and secondly what new opportunities might exist to achieve this (without recourse to animal models).

Measurement of the sensitizing potency of allergenic proteins: issues to be considered

The need to distinguish between prevalence and potency

Prevalence is not equivalent to potency. One illustrative example of this is provided by consideration of skin sensitization to nickel. In the USA and Europe nickel is the most common cause of ACD. However, experience indicates that nickel is, in fact, a weak skin sensitizer. The explanation for this apparent anomaly is that exposure to nickel is ubiquitous; the result being that continued exposure – even to this weak allergen – can result in a relatively high incidence of sensitization. Nevertheless, although prevalence does not equate with potency, an appreciation of the prevalence of allergy to a particular protein can be used as part of a weight of evidence approach to assess sensitizing potency. This will be discussed in the following sub-section.

Availability of reference proteins for calibration and verification of new approaches

The development of reliable methods (*in silico, in vitro* or *in vivo)* for measurement of sensitizing potency requires the availability of reference proteins of known allergenic activity that can be used for calibration of putative assessment tools. Clearly this need presents significant challenges because, as yet, there are no methods for the categorization of potency; a case of a toxicological chicken and egg. There is, however, a recent report that has proposed a paradigm that will allow the identification of allergenic proteins that display low sensitizing potential (Krutz et al. 2019). The principle is based on the assumption that proteins to which humans are known to have significant exposure (such as proteins from spinach and corn, for example), but that are not (or only rarely) associated with allergy, can be classified as having low (or even absent) sensitizing potential (Krutz et al. 2019). These can be paired with allergenic proteins from sources that are commonly associated with food allergy, or allergy of other forms, that can be assumed to have greater sensitizing potential. This does not provide a fully comprehensive approach to the calibration of methods for the classification of protein allergens on the basis of sensitizing potency, but it does represent an important step forward. This approach will be described in greater detail later in this article.

Consideration of relevant routes of exposure

Without doubt, the almost exclusive route of exposure for the development of skin sensitization resulting in ACD is the skin itself. In the case of sensitization to low molecular weight chemical respiratory allergens both skin contact and inhalation have been identified as relevant routes of exposure (Kimber et al. 2018). In the case of protein allergy there is a general acceptance that inhalation exposure is associated with sensitization resulting in respiratory allergy, and that dietary (and sometimes inhalation; Ramirez and Bahna 2009) exposure is associated with sensitization resulting in food allergy. However, there is a growing acceptance that in some circumstances skin exposure to protein allergens can also result in sensitization (Kimber et al. 2014; Coenraads 2016;

Basketter and Kimber 2018). It is therefore important to appreciate that the route of exposure through which an allergenic protein is experienced may vary and that, in theory at least, this could impact on its sensitizing potency.

Appreciation of important exposure dose metrics

The important metrics in linking exposure with the acquisition of sensitization will almost certainly vary with the route of exposure. In the case of skin sensitization, it is now well-established that the important exposure metric is concentration of chemical experienced per unit area of skin (Kimber et al. 2011). That same metric may possibly also be relevant for the induction of sensitization to a protein allergen via skin contact, but in the absence of evidence that cannot be assumed. In the case of inhalation and dietary exposure to protein allergens there is no real understanding of what the relevant metrics are, except for certain detergent enzymes, which allows a conclusion on their relative sensitizing potency in addition to animal experiments (Sarlo et al. 1997a, 1997b; Heederik et al. 2002). Other dose–response relationships for protein respiratory allergens in humans have been studied for latex, wood dust, animal dander, etc. (Baur et al. 1998) to minimize the risk of sensitization and elicitation. The studies show high variabilities in exposure concentrations to specific proteins and various prevalence of IgE-sensitization. Due to the opportunities for various exposure routes as well as different exposure environments (bakery, hospital, farm), the results do not allow the direct assessment of metrics in linking exposure with the acquisition of sensitization and thus relative sensitizing potency of the proteins.

It is also worth pointing out that exposure to sensitizing proteins, via whatever route, almost invariably occurs in the context of a complex matrix. For example, allergenic food proteins are experienced usually as part of a complex food that contains multiple proteins. When attempting to determine exposure levels for specific protein allergens it is therefore important that there is an appreciation of their abundance within a matrix.

Relationship between sensitizing potency and elicitation thresholds

As mentioned above, contact allergens vary by up to 5 orders of magnitude in their relative skin sensitizing potency. However, it appears that the differences between contact allergens with respect to the concentration of chemical that is required to elicit ACD in a sensitized subject are much narrower. That is, thresholds for elicitation of ACD do not correlate with thresholds for the induction of skin sensitization.

It appears that this holds true also for protein allergens. Houben et al. (2016) reported ED50 values varying by less than 20-fold for 10 allergenic foods, and more recently Remington et al. (2020) found that ED01 and ED05 values for 14 allergenic foods varies between 700- and 900-fold. Although the determination of elicitation thresholds for protein allergy in sensitized subjects can be of considerable value for the purpose of establishing safe levels in products and effective risk management, there is no reason to suppose that such elicitation thresholds reflect sensitizing potency.

New opportunities to address the sensitizing potency of allergenic proteins

Quantitative risk assessment (QRA); the overall goal

The ultimate objective of a toxicological evaluation is development of a risk assessment; an assessment of the likely risks to human safety (or environmental damage) under anticipated conditions of exposure. Understanding the toxicological potency of a chemical or protein can be critical to conducting a robust quantitative risk assessment.

An illustrative example of how this can be achieved, albeit with the use of an experimental animal (mouse) model, is the local lymph node assay (LLNA) (Kimber et al. 2001). This method was developed originally for the hazard identification of skin sensitizing chemicals, based upon measurement of the ability of topically applied test chemicals to induce a proliferative response by T lymphocytes in lymph nodes draining the site of topical exposure (Basketter et al. 2000; Ryan et al. 2000; Kimber et al. 2002; Gerberick et al. 2007). It subsequently became clear that such proliferative responses, in addition to providing a read-out for hazard, also correlated closely with the sensitizing potency of contact allergens. That is, the more vigorous the proliferative response induced by a chemical, the greater the sensitizing potency (Basketter et al. 2000; Kimber et al. 2001). The use of the LLNA for assessment of potency, measured as a function of dose per unit area, combined with an appreciation of the likely conditions and extent of exposure, has provided a sound basis for development of a QRA for skin sensitization (Gerberick et al. 2001; Api et al. 2008). There is no doubt that if conducted properly, the QRA for skin sensitization provides an assessment of likely risks and thereby forms a sound basis for protection of human health. Currently, there is a need now to develop alternative (non-animal) methods for measurement of skin sensitizing potency, and work to achieve this is in progress.

The availability of a similar approach for the quantitative assessment of risks associated with exposure to proteins with sensitizing properties is of course the ultimate goal in protein allergy. However, as described above, the problem is that there are no accepted methods available for measuring the relative sensitizing potency of allergenic proteins that can provide the data necessary to fuel a QRA. Moreover, even if there was an acceptance that assessment of IgE antibody responses *in vivo* might provide the basis for potency assessment, there is now no appetite for reliance on the use of experimental animals for this purpose.

The current situation therefore requires that ingenuity is applied to the development of new, and possibly unconventional, approaches that can fill this knowledge gap. It is probably the case that viable approaches will rely, to a greater or lesser extent, upon integrating data about the known properties of protein allergens, in ways that inform their sensitizing potency.

Exploring those opportunities is the purpose of this final section of the article.

Opportunities for in silico approaches

While some characteristics that may infer sensitizing potency for allergenic proteins require *in vitro* data and/or human data, other properties of proteins can already be predicted or determined with *in silico* tools. In this section, opportunities are described that are either already available, or are in development, to better determine whether they provide information relevant for sensitizing potency. Some parameters are strongly related to allergenic activity, while other properties do not necessarily play a direct role in sensitization *per se* but may nevertheless contribute to the overall sensitizing potency of a protein allergen. None of the characteristics can alone be directly correlated with sensitizing potency. However, once data have been extracted and integrated in an appropriate way then collectively these characteristics may provide information of value in assessing the sensitizing potency of protein allergens.

For example, data on the prevalence of IgE antibodies to certain allergens are available from Allergome (www.allergome.org). These data illustrate that certain individuals have developed IgE antibodies reactive with a particular protein. These antibodies may have been induced by the protein itself, or by exposure to a protein with similar cross-reactive epitopes. Such IgE prevalence data indicate that the proteins with which the antibodies react has allergenic potential and that there has been human exposure sufficient to result in the stimulation of an IgE antibody response. However, such data must be treated carefully because high prevalence does not necessarily equate with high allergenic potency. Thus, a relatively high prevalence of subjects displaying IgE antibodies reactive with a particular protein can indicate significant sensitizing potency, or a high level of exposure among the target population, or both. In addition, high prevalence can reflect exposure to allergenic proteins of a highly cross-reactive family.

Measurement of serum IgE levels in patients with confirmed allergic disease might also provide an understanding of the inherent sensitizing potency of individual protein allergens. For example, the serum IgE titers of a Southeast Asian population were reported for a group of protein allergens, including indoor and outdoor allergens (Andiappan et al. 2014). The authors concluded that the allergic response in that region was dominated by a single allergen class, house dust mite. The IgE levels of two common species of house dust mite were more than 10-times higher than those observed for other well characterized allergenic sources such as German cockroach, cat dander, Bermuda grass, Mugwort and Aspergillus. Further investigations into other geographical regions, linked with a greater understanding of exposure, could be helpful in deciding whether the use of serum IgE level data might be of value in evaluating the sensitizing potential of allergenic proteins.

One can speculate that it might be the case that the amount of protein allergen that is required to elicit a response in a sensitized subject might correlate with the sensitizing potency of that protein. Although superficially attractive there is no evidence to suggest this is the case. It is more likely that the amount of protein required to elicit a reaction in a particular subject is a reflection of the degree to which sensitization has been acquired. Thus, a highly sensitized subject will be expected to react to lower amounts of the protein than will individuals that are less strongly sensitized. Of course, it is possible that the level of sensitization displayed may, in part, be associated with the potency of the inducing protein allergen, but it may also be a function of the frequency and levels of exposure that induced sensitization. Nevertheless, if there are data available regarding likely patterns of exposure then it is possible that the amount of protein that will elicit a response may provide some insight into sensitizing potential. There are data available on the elicitation thresholds of certain protein allergens (primarily food allergens) that can be used for this purpose (Houben et al. 2019), but again, caution is necessary.

Potentially important data regarding sensitizing potency can be generated or compiled using *in silico* models that employ relevant information. Such information includes protein sequence data intrinsic properties of proteins such as physicochemical characteristics, protein function, and the expression of 2D and 3D epitopes. Most models such as Allergen Online database from the Food Allergy Research and Resource Program (FARRP; www.allergenonline.org), Allermatch (http://www.allermatch.org/allermatch.py/form), AllerTOP v. 2.0 (http://www.ddg-pharmfac.net/AllerTOP/), etc. are based on identification of sensitizing hazard and predicting whether a protein is or is not a potential food or respiratory allergen.

In contrast, AllerCatPro provides additional information as it also categorizes predictions with high, moderate or no evidence supporting the similarity to known allergens (Maurer-Stroh et al. 2019). However, currently these *in silico* models do not provide quantitative or qualitative information on sensitizing potency. Prediction models can assign biological or biochemical roles to proteins (Lee et al. 2007), which in turn have been studied intensively with regard to cross-reactivity of certain plant-derived protein allergens (e.g. Lu et al. 2018). Protein functions and cross-reactivity may help to understand important allergenic motifs that, in turn, could be related to potency. Studies have found structural differences in tropomyosins among species; those derived from shrimp versus pig and insects (James et al. 2018; Ruethers et al. 2018). Such information may help to better understand T cell and B cell epitopes as well as adjuvant effects and their role in sensitizing potency.

Similarly, glycosylation of proteins can be predicted by models (e.g. NetOGlyc 4.0 Server). However, although glycosylation patterns are believed to influence the sensitizing properties of proteins the nature of such influences, and the possible impact on potency, is poorly understood.

It is believed also that the proteolytic potential of proteins may impact on sensitizing activity. Certainly it is the case that many well-known allergens are proteases, e.g. serine proteases such as Alcalase and major allergens in fungi (Asp f 18), bees (Api m 7, Bom p 4), and house dust mites (Der p 3, Der f 3) as well as cysteine proteases (Der p 1). Although it may be the case that the possession of enzymatic activity may, for whatever reason, facilitate sensitizing potency the relationship with potency remains uncertain.

As discussed above, the digestibility or resistance of proteins to digestion commonly forms part of the hazard identification process; the view being that stability and resistance to proteolytic digestion is associated with sensitizing activity. Certainly resistance to degradation would facilitate the maintenance of epitope expression and thereby facilitate the development of sensitization (Pekar et al. 2018). Protein digestibility can be partially predicted with information from the MEROPS database or measured *in vitro* (Foster et al. 2013). However, as with many other protein characteristics that may be associated with sensitizing properties, the impact of resistance to digestion with potency is not known.

Despite this lack of certainty regarding the drivers of potency it may well be that an integrated assessment of characteristics known to influence sensitizing activity (possession of 2D and 3D allergenic motifs, patterns of glycosylation and other post-translational modifications, enzymatic and other protein functions and resistance to digestion) may provide one way forward.

Thus, *in silico* tools that can identify and integrate effectively data such as those discussed above might be able to infer information on potency, and be used as evidence for informed decisions on potency in a qualitative, but not (yet) quantitative manner.

The current state-of-the-art is that *in silico* tools can only provide predictions based on what we already know, but not (yet) what we do not know. In the future it may be possible to enhance *in silico* tools to provide additional quantitative information to support or revise the qualitative informed decisions on potency.

Integrating data for potency assessment

An important step forward would be an approach that is able to integrate allergenicity-related information from above mentioned characteristics to allow at least a qualitative assessment of potency. Integrating data from different approaches and sources is already used actively for the quantitative assessment of skin sensitization by using *in vitro*, *in chemico* and *in silico* such as the Bayesian integrated testing strategy (Jaworska et al. 2015), or defined approaches (OECD 2016). In terms of assessing sensitizing potency of proteins in an at least qualitative manner, data can be integrated from different approaches such as clinical studies, *in vitro* or *in chemico*/proteomic analyses, to gain further insights and help improving the *in silico* model in an iterative approach.

An example how *in silico* tools can help to substantiate that some proteins can be considered with low allergenic potential, was published recently by Krutz et al. (2019). Protein sources for which there are known to be significant opportunities for human exposure were analyzed to identify and semi-quantify single proteins by label-free proteomic analysis. Subsequently, the allergenic potential of each identified proteins was predicted using AllerCatPro (https://allercatpro.bii.a-star.edu.sg/). Individual proteins with significant relative abundance, but no evidence for allergenicity were considered as having low allergenic potential. Taking this approach to another level, analyzing protein-containing sources known to have multiple (major and minor) allergens and identifying, semi-quantifying and predicting the allergenic potential of its proteins can drive better identification of proteins of lower concern. However, the relative abundance and ranking of protein allergens are limited to the source itself and thus does not include the actual individual exposure. Nevertheless, data from multiple sources can be used to further investigate, if there are any characteristics that are specific to e.g. major allergens at low relative abundances versus minor allergens or low allergenic proteins at high relative abundances.

In vitro models can provide additional understanding of the biological impact of proteins, for example, on antigen-presenting cells or epithelial barriers. However, a measured biological impact *in vitro*, which might imply allergenic potency of a protein, needs to be interpreted with care, since it may not directly represent what we know from human exposure.

Finally, we believe that as new information relevant for the allergenic potency of proteins becomes available, an integrated testing strategy will be a useful approach for moving forward in assessing sensitizing potency of protein in a quantitative manner.

Acknowledgements

Sebastian Maurer-Stroh was supported by grant BMRC-SPF APG2013/051 as part of the A*STAR IFCS programme. The authors thank Cindy Ryan as well as two anonymous reviewers selected by the editor for their helpful and constructive criticism which greatly improved the manuscript.

Declaration of interest

This review article was prepared during the normal course of the authors' affiliations or employment shown on the first page of the paper. None of these authors has participated in legal or regulatory proceedings on the subject of this paper during the last 5 years. The authors have sole responsibility for the preparation and content of this manuscript.

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