

FORUM

Approaches to Assessment of the Allergenic Potential of Novel Proteins in Food from Genetically Modified Crops

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The safety assessment of food derived from genetically modified plants continues to attract considerable attention. Among the important issues that need to be considered is whether the products of novel genes introduced into crop plants will have the potential to induce allergic sensitization or to elicit allergic disease. Hierarchical approaches to allergenicity testing have been proposed, and these incorporate evaluation of the structural and sequence homology and serological identity of novel proteins with known allergens, measurement of resistance to proteolytic digestion, and assessment of allergenic potential using animal models. Accounts of these approaches are available elsewhere, and it is not the purpose of this article to provide a detailed critique of specific methods. Our intention is rather to look more broadly at the strategy for assessment of allergenic potential, the challenges such assessments pose for the practicing toxicologist, and how some of these might best be addressed.

The safety assessment of foods derived from genetically modified crops has been the subject of considerable discussion and debate (Goldman, 2000; Hodgson, 2001; Kuiper *et al.*, 1999; Lachmann, 1999). Among the aspects that have attracted most attention is allergenicity. The concern here is that genetic modification of crop plants may in some instances be associated with a risk of either inducing allergic sensitization in susceptible (but previously nonsensitized) individuals, or with the elicitation of allergic reactions in those who are already sensitized. Specifically, the concerns are that: (a) the product of a novel gene introduced into the plant may have the ability to induce *de novo* sensitization amongst susceptible consumers, (b) that the product of a novel gene may be immunologically cross-reactive with protein allergens to which consumers are already sensitized, and will as a consequence have the potential to provoke allergic reactions, or (c) that transformation will

result in the altered levels of endogenous protein allergens already expressed by the host plant.

The requirement for allergenicity assessment of novel foods has been reviewed previously (Kimber and Dearman, 2001a; Kimber *et al.*, 1997; Metcalfe *et al.*, 1996; Taylor, 1997; Taylor and Hefle, 2001), and it is not our intention to cover the same ground here. Nor is it the purpose of this article to compare and contrast in detail the utility of specific methods that have been proposed for hazard identification and characterization. Our goal is rather to reflect on some of the more general aspects of protein allergenicity assessment, the challenges they pose, and how some of these might best be addressed.

Scientific Context

The concern that a novel gene product may have the potential to induce sensitization is legitimate: food allergy is not uncommon, the prevalence being in the order of 1–2% in adults and even higher among infants (Helm and Burks, 2000; Hourihane, 1998), and a variety of plant proteins have been implicated as food allergens (Breiteneder and Ebner, 2001; Bush and Hefle, 1996). However, it is important to appreciate that a normal diet will result in exposure to many thousands of proteins and that only a small proportion of these have been shown to display sensitizing activity. The question that arises from this is why some proteins are allergenic, whereas others are not. This is of more than academic interest—an understanding of the requirements for the initiation of sensitization will provide the foundations for future developments in approaches to the identification and characterization of allergenic proteins, and may create opportunities for designing out sensitizing properties.

In the context of food allergy, sensitization is dependent in most instances on the induction of IgE antibody responses. This antibody associates with mast cells that are found throughout vascularized tissues, including the gastrointestinal tract. Following subsequent exposure, the inducing allergen

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cross-links membrane-bound IgE antibody, resulting in degranulation and the release of a variety of inflammatory mediators, including histamine, serotonin, chemotactic factors, and prostaglandins. These factors act in concert to cause an increase in vascular permeability, the contraction of smooth muscle, and the accumulation of leukocytes that together precipitate an allergic reaction. The most frequent symptoms of food allergy include nausea and vomiting, abdominal pain, and diarrhea. However, other organ systems (the skin and respiratory tract) may be involved, and occasionally severe systemic (anaphylactic) reactions are provoked that may prove fatal.

It is generally acknowledged that a number of factors can contribute to the expression by proteins of sensitizing activity, although not all of these may be influential in each instance. Among these are the size and structure of proteins, resistance to proteolytic digestion, glycosylation status, biologic function (such as enzymatic activity), overall immunogenicity (the ability to provoke an immune response of any type), and the way in which the protein is recognized, processed, and presented to the immune system (Aalberse, 2000; Bredehorst and David, 2001; Bufe, 1998; Huby *et al.*, 2000). It is assumed that these, and possibly other as yet unidentified factors, will collectively determine the immunological outcome of exposure, and whether and to what extent an allergic response is provoked. In practice, the assumption is that protein allergens have the properties necessary to induce and sustain the quality of immune response required for IgE antibody production (a preferential type 2 response) (Stevens *et al.*, 1988) and/or to prohibit the genesis of type 1 immune responses that antagonize the elaboration of IgE (Aalberse, 2000; Huby *et al.*, 2000; Kimber and Dearman, 2001a).

Safety Assessment: Available Approaches and General Considerations

Perhaps the most important question to initially ask is what a safety assessment should have as an objective. The absence of risk in this context is not achievable, and our view is that the aim, holistically, should be to ensure that a food deriving from a genetically modified crop is as safe as its traditional counterpart. With this in mind, the objective with regard to allergenicity is to establish whether the novel food has an increased potential to induce sensitization or to elicit allergic reactions.

The first structured approach to allergy safety assessment resulted from a collaboration between the International Food Biotechnology Council (IFBC) and the International Life Sciences (ILSI) Allergy and Immunology Institute. A tiered approach was proposed in which the route taken was dictated by whether or not the protein of interest derived from a source that had previously been associated with allergic disease in humans (Metcalfe *et al.*, 1996). In such cases, the suggestion was that the identity of the protein of interest with proteins recognized by patients allergic to the source of the transferred gene should be investigated serologically (that is, reactivity of IgE antibody

from sensitized patients with the protein of interest) and possibly by challenge of sensitized subjects. For proteins derived from sources considered not to be allergenic, a different strategy was proposed. Here, the advice was to consider amino acid sequence homology with, and/or structural similarity to, known protein allergens (Gendel, 1998), and the resistance of the protein of interest to digestion in a simulated gastric fluid (Astwood *et al.*, 1996). Since the original description of this decision tree, modifications have been proposed, among these being more conservative criteria for establishing identity with known allergens based on sequence homology, and the introduction of what was termed “targeted serum screening” to investigate the immunoactivity of the novel protein with sera prepared from subjects with allergy to materials that are broadly related to the source material (FAO/WHO, 2001). More recently still, the Codex *Ad Hoc* Intergovernmental Task Force on foods derived from biotechnology has, through a working group on allergenicity, given further consideration to some of the recommendations made previously by IFBC/ILSI and by FAO/WHO.

For the purposes of this article, much of the detail is not directly relevant, and it is in any event available elsewhere (FAO/WHO, 2001; Metcalfe *et al.*, 1996). The overarching question is whether the recommendations that resulted originally from the IFBC/ILSI consultations, and/or some of the more recent suggested amendments to these, provide a suitable framework for ensuring that no new or increased risk of allergy will result from the introduction of novel foods. Our view is that with certain provisos and caveats, these paradigms form a sound, but as yet incomplete, basis for safety assessments.

One important issue is that both the initial and modified recommendations for evaluation of allergenicity were displayed, and have since been viewed, as a decision tree. Although a binary system—wherein the results of one investigation dictate the route taken for further analyses—has some attraction, this hierarchical approach does not necessarily encourage an holistic appreciation of the data available in reaching conclusions about the existence or otherwise of a potential hazard. Components of the current decision trees do not necessarily provide an unambiguous indication of the presence or absence of sensitizing hazard.

Thus, for instance, it is recognized that there are important limitations in relating sensitizing potential to a linear amino acid sequence. Moreover, the apparent association between allergenic activity and resistance to digestion by pepsin or a simulated gastric fluid is by no means absolute, and certainly not all proteins that display resistance to proteolytic digestion have the ability to induce allergic sensitization. The relationship between proteolytic stability and sensitizing potential is probably worthy of further comment, as it serves to illustrate that our understanding of the factors that influence allergenic activity is as yet far from complete. It is generally accepted that the positive correlation between resistance to proteolytic digestion (measured frequently using a simulated gastric fluid or

pepsin) and allergenicity is a reflection of the fact that to induce sensitization proteins encountered via oral exposure will have to survive in a relatively intact form for a minimum period of time to allow their interaction with the immune system. Logical as this may appear, survivability in the stomach or small intestine is not the whole story. There is evidence, then, that even labile proteins can provoke immune responses when administered by gavage. Furthermore, proteins that apparently differ in terms of their ability to cause food allergy continue to display the same variable potential to stimulate type 2 immune responses and IgE antibody production when given by a parenteral route (Dearman and Kimber, 2001; Dearman *et al.*, 2002). We speculate that the association between stability and allergenic potential may instead be related to variable antigen processing and presentation, the hypothesis being that increased resistance to intracellular cleavage by proteolytic enzymes (such as cathepsins B and D), which are responsible for the production of immunogenic peptides within antigen presenting cells, may in some way favor the stimulation of selective type 2 responses and the elaboration of specific IgE antibody. Although definitive evidence to support this view is lacking, there are some intriguing indications that the inherent stability of proteins, and the characteristics of peptide antigens displayed by antigen presenting cells, can impact in various and sometimes contradictory ways on the quality of induced immune responses (Pfeiffer *et al.*, 1995; So *et al.*, 2001). Clearly this needs further exploration. However, if indeed there are other ways in which inherent protein stability influences the effectiveness with which sensitization is induced, then there may be opportunities to reconfigure tests of proteolytic digestion to reflect more accurately the relevant biological events.

Although there is no doubt that structural and sequence homology with known allergens and protein stability provide valuable information as part of the safety assessment process, they do not necessarily supply a definitive answer. For this reason there has been a growing interest in the application of appropriate animal models that may provide a more holistic view of allergenic potential. In the original IFBC/ILSI decision tree, there was no specific mention of animal models as part of the process, although there was a view expressed that suitable methods should be developed (Metcalf *et al.*, 1996). However, in an acknowledgment of the progress that has been made in the interim, the approach proposed by the FAO/WHO includes, in the decision tree, use of appropriate animal methods (FAO/WHO, 2001). Despite this progress, it is important to emphasize that no model has yet been evaluated fully or is at the stage where a formal validation exercise could be conducted. Indeed, there is currently a vigorous and healthy debate about how best to incorporate animal models and what parameters in such methods are of greatest importance.

In examining the relevance of animal methods, it is important to distinguish between those that have been developed primarily to investigate the molecular mechanisms of food allergy (and therefore seek to model one or more aspects of

human clinical disease) and those in which the primary interest is in pursuing safety evaluation. With regard to the latter, attention to date has focused largely on methods based on assessment of induced immune responses in rats and mice (Dearman and Kimber, 2001; Kimber and Dearman 2001b; Penninks and Knippels, 2001), although there has also been some interest in the use of dogs bred to display an atopic phenotype (Ermel *et al.*, 1997).

A not infrequent criticism of animal methods is that they will be unable to mirror the great complexity of food allergy in humans or to model the heritable and environmental factors that together determine susceptibility. While this is true, the same arguments can be leveled at predictive test methods in animals that are used to evaluate the potential for other adverse health effects. One example that might be considered to have relevance for the assessment of food allergy is the identification and characterization of potential contact allergens, where for many years guinea pig and mouse models have been used with some success in the context of hazard and risk assessment (Basketter *et al.*, 1999). The important first objective is to ensure that the method selected, even if it fails to reflect all aspects of the relevant human disease, has endpoint(s) that provide acceptable levels of selectivity and sensitivity, so that, when employed and interpreted judiciously, intrinsic hazards can be identified with some confidence. If of course the endpoints used can be shown to relate quantitatively and causally to the genesis of the relevant adverse health effects, then it is possible also to consider definition of relative potency as part of the risk assessment process.

The two general approaches that appear to be most suitable for further development and refinement for the purposes of safety evaluation are methods that employ the BALB/c mouse (Dearman and Kimber, 2001) or the Brown Norway (BN) strain of rat (Penninks and Knippels, 2001). In both instances, the strains selected are considered to have an atopic-like phenotype associated with a predisposition toward the development of type 2 immune responses, and in both cases the assessment of sensitizing potential is based upon evaluation of immune responses induced following exposure to the test material. The approaches differ in the selection of route of exposure. The method developed in BN rats employs repeated oral exposure. Such exposure is achieved by gavage, since administration of protein in drinking water has proven ineffective, or less effective, at inducing IgE antibody responses (Akiyama *et al.*, 2001; Knippels *et al.*, 1998), possibly due to the development of oral tolerance (Strobel and Mowat, 1998). In contrast, studies in BALB/c strain mice have focused largely, but not exclusively, on parenteral exposure, and primarily intraperitoneal administration.

Although gavage exposure might be considered the more appropriate route, insofar as it more accurately reflects (what is believed to be) the most common route of sensitization in humans, in our hands it appears to lack the sensitivity necessary for hazard identification. Thus, in a series of comparative

investigations it was found that when administered by gavage, ovalbumin—a known food allergen—failed to elicit IgE antibody responses in BN rats and, compared with responses provoked by intraperitoneal exposure, elicited only low-grade IgE responses in BALB/c mice (Dearman *et al.*, 2001). Although others have had greater success at eliciting IgE responses following gavage exposure of rats and mice to ovalbumin (Akiyama *et al.*, 2001; Penninks and Knippels, 2001), our view remains that, for the purposes of hazard identification, oral administration lacks sufficient sensitivity. Our preferred strategy, therefore, is to administer test proteins by intraperitoneal injection, under which conditions the opportunities for the generation of vigorous immune responses are optimized.

Using this approach, the assessment of potential allergenicity is based on an evaluation of the ability of the test material to provoke an IgE antibody response (as measured by homologous passive cutaneous anaphylaxis). Importantly, however, such measurements are made within the context of the overall immunogenicity of the test protein, and for this purpose specific IgG antibody responses are also measured. The interpretation of such data is presently relatively unsophisticated, insofar as proteins with the inherent potential to induce allergic sensitization are defined as those that are able to stimulate a measurable IgE antibody response. Conversely, proteins that are clearly immunogenic in terms of eliciting an IgG antibody response, as most foreign proteins will do, but which fail under the same conditions of exposure to induce IgE antibody production, are viewed as having no (or at least a very limited) inherent potential to cause allergic sensitization.

It must be acknowledged that despite some considerable progress, animal models have yet to be fully evaluated or validated. However, work is continuing apace, and already there are opportunities to combine the results of animal experiments with other sources of data about novel proteins to inform the safety evaluation process.

Towards an Holistic Approach to Safety Assessment

The question remaining is to what extent, with the tools currently available, is it possible to make informed judgments regarding the sensitizing potential of novel proteins. We are perhaps some little way from having robust paradigms, but it is relevant to examine what may already be possible. Two hypothetical examples serve to illustrate this. Let us consider a novel protein that is found to be resistant to digestion by pepsin or in a simulated gastric fluid, that displays some homology with known human allergens, and that is able in mice or rats to provoke a vigorous IgE antibody response. Collectively, these data would indicate that this protein has a significant potential to cause sensitization. At the other end of the spectrum, a hypothetical protein may be very labile and lack any sequence or structural homology with known allergens. If, combined with this, the protein was found not to elicit the production of IgE antibody production under conditions of exposure where a

strong IgG antibody response was induced, then the conclusion might be that the protein lacked any significant inherent potential to stimulate the quality of immune response necessary for IgE production and the acquisition of sensitization.

Two points need to be made. First, the above paradigm is rather conservative, insofar as it has to be recognized that proteins identified as potential hazards, as a function of their ability to induce IgE antibody responses following intraperitoneal injection of mice, may not represent a risk of sensitization to humans following dietary exposure. That is, the protein may lack the ability to stimulate IgE responses when encountered in the gastrointestinal tract. Notwithstanding this, the current value of this approach is that proteins that fail to stimulate IgE antibody production, in circumstances where vigorous IgG responses are elicited, may be regarded as having little intrinsic hazard. Second, while interpretation of polarized responses may appear relatively straightforward, intermediate responses pose more of a challenge. Increased experience will clearly be needed to translate this into a robust paradigm that is applicable in a wide range of circumstances.

Concluding Comments

It will be apparent that much has still to be achieved. However, during the last five years there has been a growing and substantial interest in the safety assessment of novel proteins generally, and in particular in the assessment of allergenic potential. With continued investment we can anticipate accelerated progress in the coming years. Such will be facilitated by the following: (a) a detailed appreciation of the molecular and structural characteristics that confer on proteins the ability to induce allergic sensitization, (b) the further development of appropriate databases for consideration of the structural and sequence homology of novel proteins with known allergens, (c) standardization of methods for measuring the resistance of proteins to proteolytic digestion and the relationship of this characteristic with the genesis of allergic responses, and (d) the further calibration, refinement, and evaluation of appropriate animal models that, in conjunction with other data, are able to inform the safety assessment process.

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