FORUM

Toxicology of Protein Allergenicity: Prediction and Characterization

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The ability of exogenous proteins to cause respiratory and gastrointestinal allergy, and sometimes systemic anaphylactic reactions, is well known. What is not clear however, are the properties that confer on proteins the ability to induce allergic sensitization. With an expansion in the use of enzymes for industrial applications and consumer products, and a substantial and growing investment in the development of transgenic crop plants that express novel proteins introduced from other sources, the issue of protein allergenicity has assumed considerable toxicological significance. There is a need now for methods that will allow the accurate identification and characterization of potential protein allergens and for estimation of relative potency as a first step towards risk assessment. To address some of these issues, and to review progress that has been made in the toxicological investigation of respiratory and gastrointestinal allergy induced by proteins, a workshop, entitled the *Toxicology of Protein Allergenicity: Prediction and Characterization*, was convened at the 37th Annual Conference of the Society of Toxicology in Seattle, Washington (1998). The subject of protein allergenicity is considered here in the context of presentations made at that workshop.

Protein Allergenicity: Assessment of Genetically Modified Foods (S. L. Taylor)

By definition, allergy describes the adverse health effects that result from the stimulation of an immune response. In this context, it is important to distinguish true food allergy from other types of food sensitivity or food intolerance that do not require an immune pathogenesis, but which may nevertheless be associated with similar symptoms. In making this distinction it is worth drawing attention to the facts that non-immune food intolerance is not associated typically with discrete proteins (Taylor, 1997a) and that sensitive individuals can frequently tolerate ingestion of some quantity of the relevant food; this not usually being the case with true food allergy (Lemke and Taylor, 1994).

Most common food allergies are mediated by IgE antibod-

ies; this being the class of antibody associated with immediate-type hypersensitivity reactions. Following exposure via an appropriate route, the inducing allergen will provoke, in susceptible individuals, IgE antibody production. Such antibody distributes systemically and binds to discrete membrane receptors expressed by mast cell basophils. After subsequent encounter of the now sensitized individual with the same protein allergen, an immediate-type hypersensitivity response will be elicited. The allergen associates with, and cross-links, mast cell-bound IgE antibody. This in turn results in mast cell degranulation and the release of both preformed and newly-synthesized mediators, including histamine and leukotrienes, that effect the inflammatory tissue changes characteristic of an immediate hypersensitivity reaction (Garssen *et al*., 1996).

It is estimated that between 1 and 2% of the population have IgE-mediated food allergies, with the prevalence among children being up to three times higher (Bock, 1987; Sampson, 1990; Taylor *et al*., 1989). Among the better characterized allergens in common foods are naturally-occurring proteins deriving from cows' milk (including casein and β-lactoglobulin), fish and crustacea (including Pen a1 from shrimp), legumes (including peanut allergens Ara h1 and Ara h2), tree nuts, cereal grains, fruits, and eggs (including ovomucoid and ovalbumin) (Hefle *et al*., 1996; Kimber *et al*., 1997). The common gastrointestinal symptoms of food allergy are nausea and vomiting, abdominal pain, and diarrhea. However, other manifestations may include urticaria, pruritis, rhinitis, asthma, laryngeal edema, and anaphylactic shock.

Many food allergens, particularly those most commonly associated with allergic reactions, are present as major protein components of the allergen, typically comprising between 1 and 80% total protein. In general terms, food allergens are comparatively stable proteins (Taylor and Lehrer, 1996), and the theory is that this is a reflection of the fact that proteins must gain access to the immune system in an antigenically intact form to stimulate an allergic response.

Not all proteins display allergenic potential, despite being immunogenic (able to stimulate IgG antibody responses), or

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probably more accurately, proteins appear to differ markedly with respect to their ability to cause IgE-mediated allergic sensitization (Taylor, 1997b). The reasons for such differences are unclear, but it is against this background that it is necessary to evaluate whether a candidate protein for introduction into a crop plant has the ability to provoke allergic sensitization.

This issue has been addressed systematically by the International Food Biotechnology Council (IFBC) in collaboration with the International Life Sciences Institute (ILSI) (Metcalfe et al., 1996). The report resulting from this exercise sought to provide the basis for a rational approach to the evaluation of the allergic potential of genes introduced into transgenic food plants. An important step in the proposed hierarchical approach is consideration of the source of the candidate gene and whether it derives from a food or other biological source (such as pollen) known to cause allergy. In such circumstances, the recommendation is that the protein is analyzed in vitro for reactivity with sera prepared from patients known to be allergic to the relevant food or other source. Within these sera, the presence of IgE antibody specific for the candidate protein is determined, using a relevant immunoassay such as the radioallergosorbent test (RAST). Sera from a minimum of 14 subjects with confirmed allergic sensitivity to the relevant food are examined. If all fail to display serological reactivity, then the interpretation is that a very high probability exists that the protein in question does not constitute an important food allergen. If negative or equivocal results are generated, then it is proposed that confirmation is obtained using skin prick tests, again employing no fewer than 14 allergic subjects. Further confirmation can, if necessary, be derived by conduct of double-blind, placebo-controlled food challenges (DBPCFC). If, however, the protein derives from a food source that is less commonly allergenic, then it may prove impossible to identify the required number of sensitized subjects. In such instances, where negative results have been obtained using RAST or related assays, then further investigations will be required, including a consideration of protein stability.

A different route is taken if the protein derives from a source which is believed not to be associated with food allergy. Here it is proposed that homology investigations are performed and that the amino acid sequence of the candidate protein is compared with sequence data available for known human allergens. Based upon a consideration of the minimum peptide length that is able to serve as a T cell epitope (IgE antibody responses being T lymphocyte dependent) a match of eight or more contiguous amino acids between the protein of interest and known allergens is sought. If such sequence identity is absent then the conclusion is that there does not exist a shared linear epitope with established allergens. However, if this degree of identity is found then it would be necessary here also to consider the immunological reactivity for the candidate protein of sera drawn from patients sensitized to the relevant allergen. There are, of course, limitations to this approach and in the absence of sequence homology it is considered prudent to
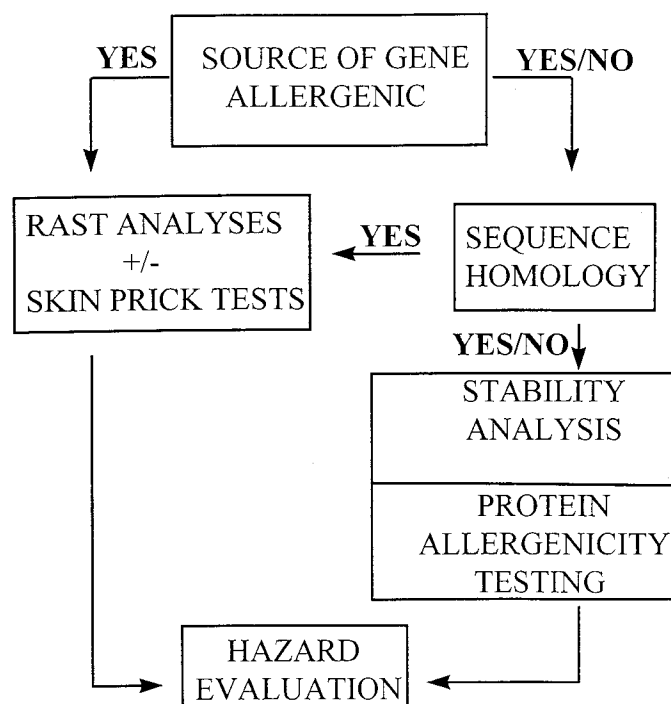

FIG. 1. Schematic illustration of a hierarchical approach to assessment of the allergenic potential of novel food proteins. Based upon and modified from the recommendations made by the IFBC in collaboration with the ILSI Allergy and Immunology Institute (Metcalfe et al., 1996).

examine the stability of the protein. The value of stability assessments in the evaluation of protein allergenicity is considered in the next section.

A schema for the assessment of novel food proteins is illustrated in Figure 1. This is based upon, and modified from, the hierarchical approach to the evaluation of allergenic potential proposed by the IFBC in association with the ILSI Allergy and Immunology Institute (Metcalfe et al., 1996). One change introduced here, to reflect the discussion at the Workshop and the objectives of some current research initiatives, is the inclusion of an additional step wherein direct assessment of allergenic potential, using appropriate experimental models, would be deployed in association with considerations of protein stability. The lack of availability of validated animal models that are able to identify, accurately and reproducibly, potential food allergens and to discriminate them from non allergens was recognized as an important limitation of the original scheme (Metcalfe et al., 1996). The difficulties involved in developing such methods are not trivial. Progress in this area is described in later sections.

Allergenicity of Bio-Engineered Foods: Relationships to Protein Digestibility and Exposure (J. D. Astwood)

Two of the important factors that contribute to the likelihood of food proteins inducing an allergic response are exposure and stability. Indeed, stability to digestion in the gastrointestinal

tract has the effect of increasing overall dietary exposure to the protein. The stability of proteins and their resistance to digestion in the gastrointestinal tract are considered to be critical elements of allergenicity assessment (Metcalfe *et al.*, 1996; Taylor, 1997b). The argument is that for a protein to stimulate an allergic response following oral ingestion it is necessary that it be sufficiently stable to reach and to cross the mucosal membrane. The most detailed and most systematic evaluation of protein digestibility has been conducted by Astwood *et al.* (1996), who examined stability *in vitro* using a simulated gastric fluid as a model of digestive processes in the stomach. A variety of proteins was examined, ranging from potent egg, milk, soybean, mustard, and peanut allergens, to common plant proteins (including examples from spinach, corn, wheat, barley, and potato), that are believed not to cause food allergy. In each instance, kinetic analyses were performed and protein stability in simulated gastric fluid defined as the last time point at which the protein or its proteolytic fragments could be detected using polyacrylamide gel electrophoresis. It was found that known food allergens are not digested rapidly. They displayed either complete stability for at least one h with no detectable proteolytic fragments, intermediate stability for the whole protein of less than one h but greater than 30 s, with fragments that were stable for additional periods, or, finally, no stability with respect to the parent protein, but with stable fragments detectable for at least 8 min. In contrast to the above, those food proteins believed to lack allergenic potential were found to have been digested fully in simulated gastric fluid within, usually, 15 s (Astwood *et al.*, 1996). The conclusion drawn from these investigations was that protein allergens are resistant to proteolysis in the stomach. On this basis the recommendation is that digestive stability represents a key parameter in the safety assessment of novel food proteins.

A second route of exposure to protein engineered into crops may be via the respiratory tract, from inhalation of particles such as pollen. While not all, and probably very few, pollen proteins are aeroallergens, it is useful to define parameters that will determine levels of exposure. A wide variety of pollen allergens has been cloned and characterized (Astwood and Hill, 1996). In general terms, it appears that the pollen proteins having the potential to cause sensitization are soluble and diffuse rapidly when in contact with the upper respiratory tract mucosa. The kinetics of pollen-protein diffusion correlates with sensitization potential (Vrtala *et al.*, 1993). Minor allergens (such as the birch pollen allergen Bet v 2) and non-allergens (such as the pollen protein HSP70) diffuse, by comparison, very slowly (Table 1). Consideration of diffusion kinetics will allow estimation of the potential of bio-engineered proteins to cause sensitization resulting from inhalation of pollen.

As indicated above, the assumption is that resistance to digestion in the gastrointestinal tract will facilitate the survival of the protein a relatively intact form, such as to enable an immune response to be provoked. While there is no reason to

TABLE 1
Release Kinetics of Soluble Pollen Proteins

Pollen protein	Protein designation	Release time (min) from pollen grain
Birch pollen allergen	Bet v1	1–10
Timothy grass pollen allergen	Phl p1	1–10
Timothy grass pollen allergen	Phl p5	1–10
Ryegrass pollen allergen	Lol p1	1–16
Ragweed pollen allergen	Amb a1	1–20
Ragweed pollen allergen	Amb a5	1–4
Birch pollen minor allergen (profilin)	Bet v2	"slower"
Pollen heat shock protein	HSP70	Not released

Note. Data based on Baraniuk *et al.,* 1988; Staff *et al.,* 1990; Vrtala *et al.,* 1993.

suspect that this is not the case, it is appropriate to speculate whether the ability of proteins to survive in hostile biological matrices and environments may also favor, in other ways, the induction of allergic responses. Presumably such stability will influence also the way in which foreign proteins are processed by antigen-presenting cells for subsequent display of immunogenic epitopes, to T lymphocytes. It will be of interest to determine if the nature and/or kinetics of antigen processing influences the characterisics of subsequent immune and allergic responses and whether inherent stability is an important parameter in this respect (Landry, 1997).

Although evaluation of protein stability (and diffusion in the case of pollen) provide very useful tools in the overall assessment of the potential for allergenicity, there is no doubt that methods for the direct examination of allergenic activity would be valuable also. In the following sections, the development of methods for measuring the respiratory allergic potential of detergent enzymes and for the evaluation in mice of the inherent allergenicity of proteins are described.

Protein Respiratory Allergy: Approaches to Risk Assessment (K. Sarlo)

Occupational respiratory disease, including asthma, resulting from allergic sensitization to enzymes used in detergent manufacture was first described nearly 30 years ago (Flindt, 1969; Pepys *et al.*, 1969). Since then, improved engineering controls, changes in formulation, and the establishment of occupational exposure guidelines have served together to reduce substantially the incidence of respiratory hypersensitivity to enzymes, and asthma is now rare (Juniper and Roberts, 1984). In order to ensure the continued safe manufacture of laundry products containing enzymes, new experimental test methods have been developed and applied. Two of these, the guinea pig intratracheal test (GPIT) and the mouse intranasal test (MINT), are considered below.

The GPIT is designed to evaluate the allergenic potential of novel enzymes, relative to the index enzyme allergen Alcalase,
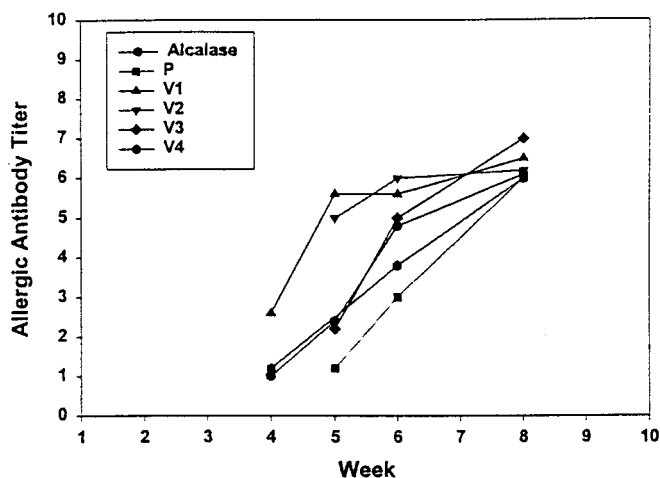
FIG. 2. Use of the guinea pig intratracheal test for comparative evaluation of the allergenic potential of enzymes. A kinetic analysis of allergic antibody responses induced following intratracheal exposure to a protease derived from *Bacillus* (parental enzyme, P), to variants of P (V1, V2, V3 and V4) or to Alcalase. Guinea pigs were exposed to enzyme solutions via intratracheal instillation once/week for 10 weeks as described previously (Ritz *et al.*, 1993). Sera were collected at selected time points and allergic antibody titer was measured using the passive cutaneous anaphylaxis test. The antibody titer was expressed as the logarithm to the base 2 of the reciprocal of the endpoint serum dilution.

as a function of IgG1-antibody production (Ritz *et al.*, 1993). By comparing the extent of allergenic antibody production induced by the new enzyme, with responses provoked by Alcalase, it has proven possible to rank proteins and to establish occupational exposure guidelines to the new material, based upon that used for Alcalase of 15ng/m^3. More recently, the GPIT has been used to determine the relative allergenic potential of enzyme variants that differ from each other by a small number of amino acids in the absence of changes to net charge, conformation, or other physicochemical characteristics. Four variants of a protease derived from *Bacillus* have been examined using the GPIT. Antibody responses were evaluated by enzyme-linked immunosorbent assay, immuno-precipitation in gel, and Western blotting. The results revealed that, despite complete immunological cross-reactivity between the parent and variant enzymes, there were clear differences with respect to allergenic activity, as measured in the GPIT as a function of induced allergic antibody titers in dose-response analyses. Compared with the parent enzyme and Alcalase, some of the variant enzymes tested provoked more vigorous antibody responses at lower exposure concentrations (Fig. 2). These results are potentially of some considerable interest, as they indicate that factors other than allergenic epitopes, *per se*, will influence the ability of proteins to stimulate antibody responses and, by implication, their ability to cause allergic sensitization. In such instances it will be instructive to consider how these proteins interact with the immune system and to determine why they exhibit differential allergenic potential in the absence of overt immunochemical variations.

More recently a new approach, the MINT, has been described. The principles of this method are similar to those of the GPIT; allergenic activity is measured (relative to Alcalase) as a function of IgG1 antibody responses, induced in BDF1-hybrid-strain mice following repeated intranasal exposure. The potential advantages of this method are that it may provide a more rapid and a more cost-effective approach to safety assessment compared with guinea pig tests (Robinson *et al.*, 1996).

A variety of detergent enzymes, derived from both bacterial and fungal sources, has now been evaluated using the GPIT and the MINT (Robinson *et al.*, 1998; Sarlo *et al.*, 1997). In both cases, novel enzymes have been compared with Alcalase to determine relative potency. Thus, for instance, in both assays the bacterial amylase Termamyl was shown, on the basis of antibody responses, to be some 3 to 10 times more potent than Alcalase. The exposure guideline for Termamyl was set to be at least 3-fold lower than that for Alcalase, and, despite the fact that exposure to the former enzyme has been lower, the overall prevalence of occupational sensitization has been similar to that recorded for Alcalase (Sarlo *et al.*, 1997).

It is clear that the methods described above provide a valuable means of assessing the hazards and risks associated with enzymes used in the detergent industry. Limited testing suggests that guinea pig models may be useful with non-enzyme proteins also. Another approach to the assessment of allergenic activity is described below.

Allergenicity and Immunogenicity of Proteins: An Experimental Approach (R. J. Dearman)

There have been many attempts to characterize the nature of immune responses to protein allergens in experimental animals. Some of these have focused on the assessment of respiratory allergic potential (such as those described above), while others have sought to develop models of food allergy (Atkinson *et al.*, 1996; Curtis *et al.*, 1990; Knippels *et al.*, 1998; Piacentini *et al.*, 1994; Turner *et al.*, 1990; van Halteren *et al.*, 1997). It can be argued that the most important marker of allergic sensitization to proteins is the induction of an IgE antibody response. However, in many experimental systems, it has been found that the stimulation of a vigorous and persistent protein-specific IgE response requires that animals be exposed to antigen in the presence of materials such as *Bordetella pertussis* or alum, which act as adjuvants for IgE antibody production. A concern is that the use of adjuvant will make it difficult to accurately evaluate the relative intrinsic potential of proteins to provoke IgE antibody responses and to cause allergic sensitization. An experimental approach to characterization of allergenicity that does not demand the use of adjuvant has been described recently, in which anti-protein antibody responses are measured in mice following intraperitoneal (or intranasal) exposure to the test material (Hilton *et al.*, 1994; 1997). In those investigations, responses induced in BALB/c-

strain mice by ovalbumin (OVA; a major allergenic component of egg protein) were compared with those stimulated by bovine serum albumin (BSA; a protein that, although immunogenic, appears to have a somewhat lesser potential than OVA to cause allergic sensitization). It was found initially that while intraperitoneal exposure to either OVA or BSA induced IgG and IgG1 antibody responses in mice, treatment with OVA only was associated with the appearance of specific IgE antibody as measured by passive cutaneous anaphylaxis. In the same series of experiments, the nature of immune responses stimulated by two other proteins known to cause respiratory allergy was examined; a lipase from *Aspergillus oryzae* and an amylase from *Bacillus subtilis*. Each of these were shown to provoke both IgG and IgE antibody responses (Hilton *et al.*, 1994). In subsequent studies, the differential ability of OVA, BSA, and the lipase to stimulate IgE responses following intraperitoneal administration was confirmed. In only one instance was BSA found to induce measurable levels of IgE antibody, and then only of low titer at the highest concentration of the protein administered. Moreover, following intranasal exposure, OVA but not BSA, induced detectable, albeit low, titer, IgE antibody (Hilton *et al.*, 1997).

Based upon the data summarized above, the working hypothesis is that the differential serological responses induced in mice by proteins of varying allergic potential are associated with, and possibly dictated by, the activity of discrete functional subpopulations of $CD4^+$ T helper (Th) lymphocytes (although there is no reason to exclude the possibility that similar subpopulations of $CD8^+$ T lymphocytes play a role also). Two main populations of Th cells, designated Th1 and Th2, have been described, which differ with respect to the cytokines they secrete (Mosmann and Coffman, 1989). Among the cytokines secreted by Th2 cells, interleukin 4 (IL-4) is a factor known to be essential for the stimulation of IgE antibody responses. Interferon γ (IFN-γ), a product of Th1-type cells, in contrast inhibits the production of IgE antibody (Finkelman *et al.*, 1988a,b). It follows that the stimulation of IgE antibody responses and the development of allergic sensitization will be favored by conditions where there is the selective development of Th2 cells. The corollary is that proteins that instead stimulate the preferential development of Th1-type cells, and where the influence of IFN-γ predominates, will fail to induce IgE responses. In the latter circumstances, immune responses to proteins would be expected to be associated with higher levels of IgG2a antibody; the production of this antibody isotype in mice is augmented by IFN-γ (Finkelman *et al.*, 1988a). Consistent with this is the result of recent experiments in which the isotype distribution of antibody responses induced in mice by OVA and BSA has been compared. It was found that BSA stimulated a substantially greater IgG2a antibody response than did OVA; a result that provides additional evidence that BSA and OVA are associated, respectively, with Th1- and Th2-type immune responses. A schema illustrating the proposal that
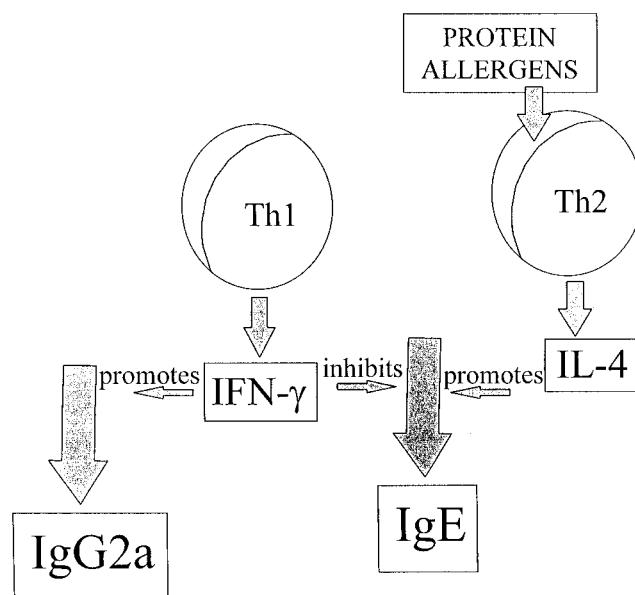
FIG. 3. A schematic illustration of the influence of Th cell subpopulations (Th1 and Th2) and their secreted products, respectively, interferon γ (IFN-γ) and interleukin 4 (IL-4), on the characteristics of humoral responses to proteins.

protein allergens induce the development of preferential Th2-type immune responses is displayed in Figure 3.

CONCLUSIONS

Allergic sensitization to proteins is a complex issue. It is clear that some considerable progress has been made towards understanding the physiochemical, physiological, and immunological bases of sensitization, and there are now strategies available for assessing the potential of proteins to cause gastrointestinal and respiratory allergy. Although much has been achieved in establishing models for protein respiratory allergy, a current limitation to full implementation of the scheme, proposed in Figure 1 for the evaluation of food allergy, is the lack of a fully validated and robust animal model that can discriminate successfully between food allergens and nonallergens. The development and thorough evaluation of appropriate animal models, and other methods for the assessment of the allergenic potential of proteins, are important priorities for toxicological research. Only then can major questions be answered. Chief among these is definition of the characteristics that confer on proteins the ability to induce sensitization and allergic reactions in susceptible individuals. If some or all of these characteristics can be identified, then the evaluation of hazards and risks associated with exposure to novel proteins will be enhanced significantly.

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