Assessment of the Inherent Allergenic Potential of Proteins in Mice

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There is considerable interest in the design of approaches that will permit the accurate identification and characterization of proteins that have the inherent potential to induce sensitization and cause food allergy. Among the methods used currently as part of such assessments are consideration of structural similarity to, or amino acid sequence homology with, known human allergens; whether there exists immunologic cross-reactivity with known allergens; and measurement of resistance to proteolytic digestion in a simulated gastric fluid. Although such approaches provide information that will contribute to a safety assessment, they do not—either individually or collectively—provide a direct evaluation of the ability of a novel protein to cause allergic sensitization. For this reason, work is in progress to design and evaluate suitable animal models that will provide a more holistic assessment of allergenic potential. In this laboratory, the approach we have taken has been to examine the characteristics of immune responses induced in mice following parenteral (intraperitoneal) exposure to test proteins. The basis of this method is to determine simultaneously the overall immunogenic potential of proteins [measured as a function of immunoglobulin (Ig) G antibody responses] and to compare this with their ability to provoke IgE antibody production, IgE being the antibody that effects allergic sensitization. Although this approach has not yet been evaluated fully, the results available to date suggest that it will be possible to distinguish proteins that have the inherent potential to induce allergic sensitization from those that do not. In this article we summarize progress to date in the context of the scientific background against which such methods are being developed.
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An increasing interest in novel foods, including those developed from genetically modified (GM) crops, has resulted in debate about the likelihood and source of potential adverse health effects and how the safety of new foods can best be assured (FAO/WHO 2000; Flamm 2001; Goldman 2000; Hodgson 2001; Kuiper et al. 1999, 2001; Lachmann 1999). In this context, a major focus has been on allergenicity and consideration of whether the product of a novel gene introduced into a crop plant will display the ability to induce or elicit allergic sensitization among consumers, or whether transformation will in some other way influence allergenic potential. The requirements for evaluation of the potential allergenic activity of novel foods have been reviewed in some detail (FAO/WHO 2001; Kimber et al. 1997, 1999; Kimber and Dearman 2001a; Metcalfe et al. 1996; Taylor 1997; Taylor and Hefle 2001). The concern is legitimate, and there is clearly a need to develop methods and strategies that will allow the accurate identification of sensitizing hazard.

The first attempt to address this issue in a systematic way was undertaken by the International Life Sciences Institute (ILSI) Allergy and Immunology Institute in collaboration with the International Food Biotechnology Council (IFBC). This initiative resulted in a report that recommended a hierarchical approach to safety assessment (Metcalfe et al. 1996). The proposal was that the route taken in assessing safety depends on whether the gene product of interest derived from a source known to be associated with allergic disease in humans. Included in the testing strategies were the following components: consideration of the serologic identity of a novel protein with proteins implicated as human allergens; assessment of the structural similarity to, or amino acid sequence homology with, allergenic proteins; and measurement of the resistance of the novel protein to proteolytic digestion in a simulated gastric fluid (Metcalfe et al. 1996). These recommendations provided for the first time a conceptual and practical framework for safety evaluations. However, it must be acknowledged that, although collectively the methods listed above are a source of valuable information about the properties of novel proteins and allow identification of proteins that are likely to cause sensitization on the basis of their structural similarity to known allergens, they do not provide a definitive assessment of inherent sensitizing potential. Consequently, there has been considerable interest in the possibility of developing appropriate animal models.

The report of the ILSI/IFBC deliberations was guarded about the possible application of animal models and considered then that no suitable methods were available. In the intervening period, however, progress has been made. In 2001 a special consultation panel was convened by the Food and Agriculture Organization and the World Health Organization to review and, if necessary, revise the recommendations made previously by ILSI/IFBC (FAO/WHO 2001). One of the conclusions reached by that consultation was that animal models might contribute valuable information regarding the likely allergenicity of foods derived from GM crops. This view has provided further impetus to the development and application of animal models, specifically those based on studies in mice, rats, and other species. In this article, we discuss progress in the evaluation of a method using BALB/c mice. However, before reviewing the details of this approach, it is necessary to consider briefly the scientific background to allergenicity assessment.

Scientific Context

Natural history of food allergy. True food allergy is an important health issue; the prevalence among adults in Europe and North America is 1–2%, with a prevalence among infants of approximately 5% (Helm and Burks 2000). In these regions, more than 80% of food allergies are thought to be associated with a limited range of produce: specifically peanuts, tree nuts, eggs, cows' milk, wheat, soybeans, fish, and shellfish (Bush and Hefle 1996; Hefle et al. 1996; Sampson 1988; Young et al. 1994). It is apparent, however, that significant geographic differences exist regarding the frequency with which certain foods are implicated as the cause of food allergy, and these differences derive primarily from variations in dietary preferences (Hourihane 1998).

Predisposition and exposure. Several factors determine interindividual differences in susceptibility to food allergy and whether or not sensitization will be acquired (Figure 1). Probably chief among these is genetic predisposition and inheritance of an atopic phenotype (Rowntree et al. 1985; Ruiz et al. 1992). It is clear also that exposure plays a pivotal role in the pathogenesis of food allergy. One reflection of this is the fact that although a predisposition to mount

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and sustain immunoglobulin (Ig) E antibody responses (atopy) is heritable, the particular proteins and foods against which allergic responses will be directed appears not to be genetically programmed. The assumption is that, against a background of increased generic susceptibility, it is the nature, route, extent, and duration of exposure and the time at which such exposure occurs that will determine the foods to which a subject may acquire sensitization.

The influence of exposure parameters is currently of considerable interest, in particular, the possible importance of exposure *in utero* and via breast-feeding. There is interest also in the extent to which sensitization to food proteins can be induced by topical or inhalation exposure to the causative allergen or to an immunologically cross-reactive protein.

Immune and allergic responses. Notwithstanding considerations of individual predisposition and the nature of exposure, the critical event in the development of sensitization is the elicitation of an immune response. By definition, "allergy" describes the adverse health effects that may result from the induction of a specific immune response. Although other (cell-mediated) immune reactions may be critical in some circumstances (e.g., celiac disease associated with gluten sensitivity), it is IgE antibody-dependent mechanisms that are most commonly implicated in food allergy. The acquisition of sensitization depends therefore on the initiation of an immune response of sufficient vigor and of the quality required to sustain IgE antibody production. Such IgE antibody will distribute systemically and associate with specific receptors expressed by mast cells and basophils. If the now-sensitized individual is exposed subsequently to the same allergen or to an immunologically cross-reactive allergen, then an allergic reaction will be provoked. Antigen will bind to, and cross-link, mast cell–associated specific IgE antibody, and this in turn will precipitate cellular activation and degranulation, resulting in the release of an array of inflammatory mediators (e.g., histamine, serotonin, leukotrienes, and prostaglandin). These mediators will act in concert to initiate inflammation and the symptoms of an allergic reaction. These symptoms commonly include nausea and vomiting, abdominal pain, flatulence, and diarrhea. However, other organ systems may also be involved, specifically, the skin (acute urticaria and angioedema, and atopic dermatitis) and the respiratory tract (allergic rhinitis and asthma). Occasionally, severe systemic (anaphylactic) reactions are induced (Sampson 1999).

The initiation and maintenance of IgE antibody production depend on the development of a selective type 2 immune response. It has been recognized for some time that the quality of adaptive immune responses reflects in large part functional heterogeneity among T lymphocytes. Such functional diversity was first characterized in $CD4^+$ T helper (Th) cell populations. Although the situation is complex, two main populations of Th lymphocytes have been identified (designated Th1 and Th2 cells) that develop from common precursors during the evolution of an immune response. These subsets differ primarily in their cytokine secretion patterns. The relevance of this for the pathogenesis of food allergy and other forms of atopic disease is that IgE antibody production depends on the availability of interleukin (IL) 4, a product of Th2 cells but not of Th1 cells. Moreover, IL-4 and other cytokine products of Th2 cells (notably, IL-10 and IL-13) favor the expression of immediate-type allergic reactions. Conversely, Th1 cells antagonize acute allergic reactions, and interferon γ, a cytokine secreted by Th1 cells, inhibits IgE antibody production. More recently, it has become apparent that there also exists heterogeneity among $CD8^+$ T cytotoxic (Tc) lymphocytes, with Tc1 and Tc2 subsets displaying cytokine expression profiles comparable with their Th1 and Th2 counterparts, respectively (Corry and Kheradmand 1999; Kimber and Dearman 1997; Mosmann et al. 1986; Mosmann and Coffman 1989; Mosmann and Sad 1996; Stevens et al. 1988).

On this basis, therefore, it is clear that the effective development of allergic sensitization to food proteins will require the stimulation of a preferential type 2 immune response and the elaboration of specific IgE antibody. The corollary is that not all immune responses will result in allergic sensitization and adverse health effects.

Oral tolerance. It is not uncommon for immune responses to dietary proteins to be viewed as having two possible outcomes: priming for allergic sensitization, or the development of tolerance resulting in immunologic unresponsiveness. On this basis, food allergy has been characterized as reflecting a breakdown in tolerance (Strobel 1997). Although this is an attractively simple paradigm, the reality is rather more complex.

Conceptually, the phenomenon of oral tolerance is well established experimentally, and at least some of the important immunologic mechanisms have been described (Brandtzaeg 1996; Mowat 1987; Strobel 1997; Strobel and Mowat 1998). There is even some rather limited, and mostly circumstantial, evidence that in humans oral exposure to antigens may down-regulate specific immune responses (Husby et al. 1994; Lowney 1973; Van Hoogstraten et al. 1991). However, oral tolerance is not an absolute phenomenon and most commonly manifests as a down-regulation in some but not all aspects of immune responsiveness. In general terms, T-lymphocyte responses and IgE antibody production are more readily down-regulated (and with lower doses of antigen) than is IgG production (Strobel 1997). It is therefore inappropriate to regard oral tolerance as immunologic unresponsiveness; the more accurate descriptors would be hyporesponsiveness, or possibly partial responsiveness.

Because immunologic tolerance is incomplete, it is not unexpected that IgG antibodies specific for food proteins are found in normal

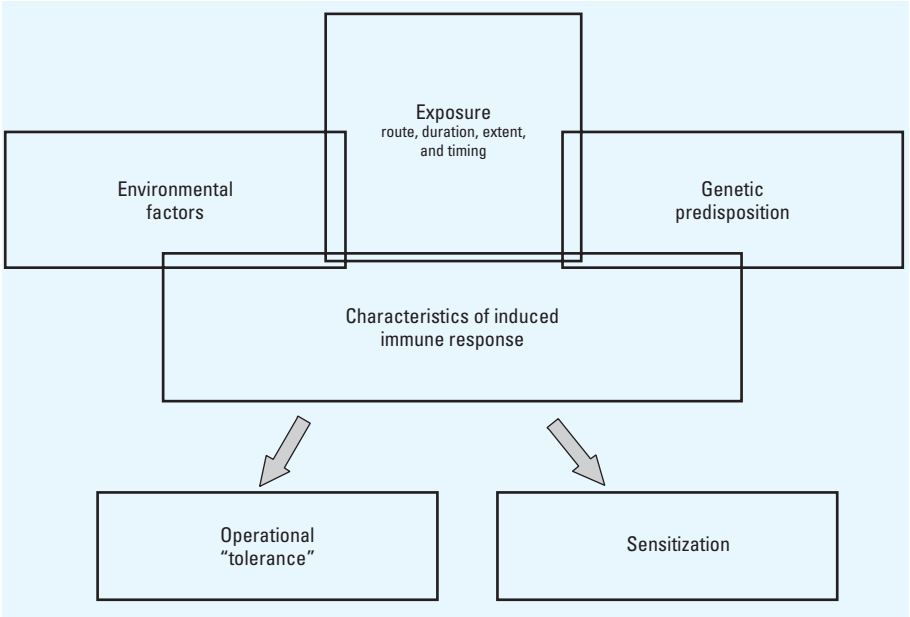

Figure 1. A diagrammatic summary of the major factors affecting susceptibility to the acquisition of food allergy.

subjects with no signs or symptoms of food allergy (Barnes 1995; Barnes et al. 1983, 1988; Johansson et al. 1984). The important point is that dietary proteins can elicit IgG responses that are apparently without ill effect, or IgE responses that (if of sufficient vigor) will induce sensitization. Indeed, one may speculate that the normal situation is for the elicitation of IgG antibody responses to dietary proteins, which will of course in almost all instances be potentially immunogenic and will be recognized as foreign by the host immune system. Indeed, IgG responses to food proteins may provide a beneficial mechanism for clearing proteins or peptides that have been absorbed inadvertently from the gastrointestinal tract.

Role of antigen. It will be apparent from the considerations summarized above that the successful acquisition of sensitization will be determined by the congenital or acquired susceptibility of the subject, the conditions and timing of exposure, and the characteristics of induced immune responses. The other key element is the nature of the food proteins themselves. An argument could be made that given appropriate levels of exposure, it might be possible to induce allergic responses to any protein, particularly if a suitable adjuvant were used. In practice, however, this is not the case, and only a minority of food proteins has been implicated as causes of food allergy. Given that such differences exist, the important question is what factors distinguish protein allergens from other proteins that, despite being inherently immunogenic, fail to cause allergic sensitization. The question is probably best posed as follows: What characteristics confer on proteins the ability to induce allergic sensitization? In fact, there is no clear answer to this question, although it would appear that among the important variables are the size of the protein, glycosylation status, biologic function (e.g., enzymatic activity), resistance to proteolytic digestion, overall immunogenicity and the way in which the protein is recognized, internalized, and processed by antigen-presenting cells, and the manner in which peptides are presented to responsive T lymphocytes (Aalberse 2000; Astwood et al. 1996; Bredehorst and David 2001; Bufe 1998; Huby et al. 2000).

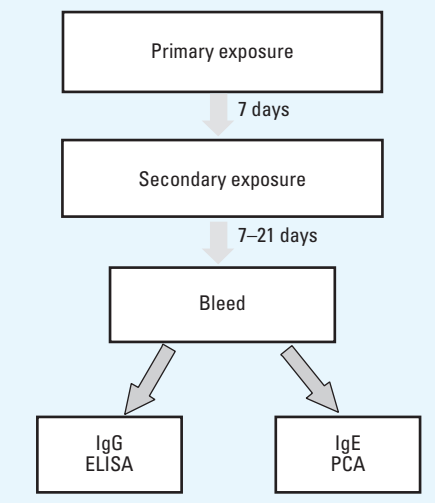

Figure 2. A schematic summary of the current approach for assessment of the inherent allergenic potential of proteins using BALB/c mice. Immunogenicity (IgG antibody production) is measured using ELISAs, and allergenicity (IgE antibody production) is measured using homologous PCA assays.

Regardless of a detailed understanding of the biologic and/or structural properties of proteins that govern their differential sensitizing activity, attempts to identify inherent allergenic hazard in the context of a safety assessment are predicated on the basis of being able to model such differences experimentally. Thus, animal methods proposed for characterization of the sensitizing potential of proteins have focused on measurement of induced IgE antibody responses and/or the elicitation of IgE antibody-dependent allergic reactions. The approach we have taken using BALB/c mice is summarized below.

Allergy Assessment Method using BALB/c Strain Mice

General considerations. Although mouse models of food allergy and food anaphylaxis are available, for example those described by Li and colleagues (Li et al. 1999, 2000, 2001), they are not necessarily suitable for the purposes of hazard identification in the context of a safety assessment. In attempting to develop an approach that will be appropriate for hazard characterization, a number of general issues should be addressed.

The first of these is the species and strain of choice. We have elected to focus on the use of mice largely because for this species there is a sophisticated appreciation of the immune response, coupled with the availability of a range of reagents. The selection of BALB/c strain mice was based on an understanding that such mice are high IgE responders, which in this context may be regarded as equivalent to an atopic phenotype.

Perhaps the most contentious issue is that of the preferred route of exposure. Although it might appear initially that oral administration represents the most appropriate route of exposure for a method designed to identify potential food allergens, this is not necessarily the case. For instance, there is reason to believe that exposure via the diet or in drinking water is more likely in rodents to cause immunologic hyporesponsiveness than sensitization. Thus, experience in rats has shown that *ad libitum* exposure to ovalbumin (OVA; a known human allergen) failed to induce IgE antibody responses in Brown Norway rats (Knippels et al. 1998b). Moreover, in our hands, at least, even gavage exposure appears to be considerably less sensitive than parenteral administration with respect to eliciting IgE antibody responses in BALB/c mice (Dearman et al. 2001). For these reasons, we have chosen to focus primarily, but not exclusively, on assessment of immune responses induced in mice after intraperitoneal administration of protein. Of course, this approach will not necessarily reflect what will happen regarding the induction of mucosal immune responses after normal dietary encounter with a food protein allergen. However, in the context of providing a method of the sensitivity required for hazard identification and characterization and for evaluating the inherent potential of a protein to induce the quantity and quality of an immune response required for the elicitation of an IgE response, this is the most appropriate approach.

A final generic issue that is worth addressing is the option for the use of adjuvant. There is no doubt that combining exposure to antigen with adjuvant administration will augment induced immune responses, and that some adjuvants (e.g., cholera toxin) are used to potentiate IgE antibody responses (Li et al. 1999, 2000, 2001). However, the danger of employing such a strategy is that increased sensitivity will be gained at the cost of a loss of selectivity. That is, some adjuvants may have the ability to confer on inherently nonallergenic proteins the potential to provoke IgE antibody production, thereby generating what are in effect false-positive responses.

Of course, other variables are worthy of consideration and may or may not affect the sensitivity, selectivity, and overall accuracy of an approach such as this. Among such considerations are the dosing regimen and the age and sex of the animals. We are currently exploring the potential impact of some of these variables.

Current approach. Against this background, our approach currently is to use young adult (8–12 week) female BALB/c mice. The basic strategy is to examine the characteristics of immune responses provoked in these animals at various periods after intraperitoneal exposure (two injections, 7 days apart) to the test protein. To this end, IgG antibody responses are measured using enzyme-linked immunosorbent assays (ELISAs), and IgE antibody responses are measured using homologous passive cutaneous anaphylaxis (PCA) assays (Figure 2). Experiences with this and similar protocols have been described and reviewed comprehensively elsewhere (Dearman et al. 2000, 2001; Dearman and Kimber 2001; Hilton et al. 1994, 1997; Kimber and Dearman 2001b), so a detailed consideration is unnecessary here.

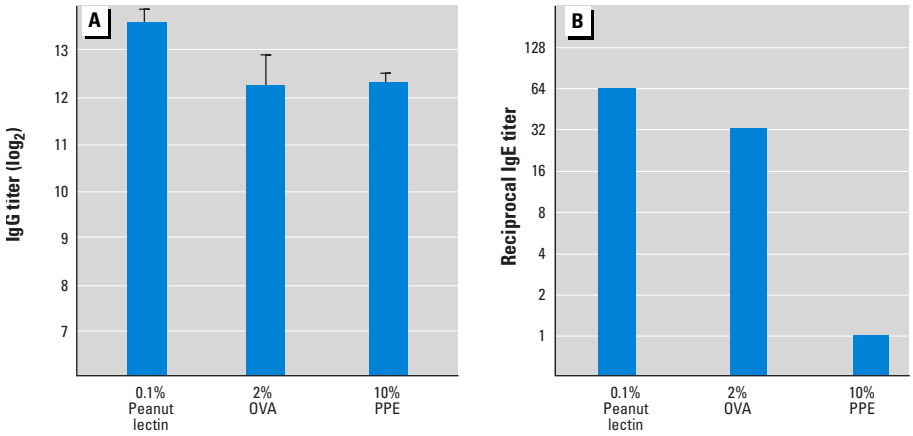

Figure 3. Groups of BALB/c mice (n = 5) received 0.25 mL of 0.1% peanut lectin, 2% OVA, or 10% PPE in phosphate-buffered saline by intraperitoneal injection on days 0 and 7. Fourteen days after the initiation of exposure, animals were exsanguinated and serum samples prepared. (*A*) Individual serum samples were tested for the presence of IgG antibody by ELISA. IgG titer is recorded as the highest dilution at which substrate conversion [optical density at 450 nm (OD450 reading)] was ≥ 0.5. For control sera derived from naive (untreated) mice, OD450 readings never exceeded this value, even at the maximum concentration tested (1 in 25) in all ELISAs. Data are expressed as mean and SE of IgG reciprocal titer (log_2) for each treatment group. Serum samples were pooled on an experimental group basis; serial doubling dilutions were prepared and used to derive IgE antibody titers by homologous PCA assay, using four naive recipient mice per sample. (*B*) In every analysis, pooled serum samples from naive (untreated) animals were tested concurrently and were uniformly negative at the maximum concentration used (data not shown). IgE antibody titer is recorded as the highest dilution of serum resulting in a positive PCA reaction in most recipient animals.

Collectively, we have been able to demonstrate that this approach can discriminate among proteins on the basis of their relative ability to provoke IgE antibody responses. Thus, under conditions of exposure where proteins were found to elicit IgG responses of comparable vigor, substantial differences were observed with respect to IgE antibody production. Such differences are even more marked when viewed in the context of the differential doses required to elaborate antibody responses. Consideration of a representative experiment serves to illustrate the point.

Groups of mice (n = 5) were exposed by intraperitoneal injection to 0.25 mL of phosphate-buffered saline containing 0.1% peanut lectin [a minor peanut allergen (Burks et al. 1994)], 2% OVA, or 10% potato protein extract [PPE; containing proteins that are considered not to possess significant sensitizing potential (Dearman et al. 2001)]. This treatment was repeated 7 days later, and in this experiment all mice were exsanguinated 2 weeks after the initiation of exposure. Serum was prepared and IgG and IgE antibody levels measured. The results of this experiment are illustrated in Figure 3. The data reveal that each of the proteins was able to induce in mice a vigorous IgG antibody response. In contrast, however, there were significant differences in IgE antibody responses. Although both peanut lectin and OVA provoked high-titer IgE antibody production, only a very low-grade response was seen with PPE.

From these and similar investigations, our view currently is that this approach allows discrimination among proteins in terms of their ability to provoke IgE responses and that, on this basis, it is possible to identify those proteins that have an inherent potential to cause allergic sensitization.

Finally, it must be emphasized that hazard identification represents only the first step in any safety evaluation or risk assessment process. It is our view that the results of experiments in which inherent sensitizing potential is measured must be incorporated into an holistic safety assessment that also includes consideration of the sites and levels of expression of the protein of interest in modified crops plants and the likely exposure of consumers to foods or food products derived from such crops. Clearly, it is important also to consider data from animal models in parallel with information relating to the structural similarity to, or sequence homology with, known allergens; resistance to digestion by simulated gastric fluid or pepsin; and the presence or absence of serologic identity with known protein allergens. A holistic approach such as this will provide a rational basis for future safety assessments. In the meantime, there is a pressing need to evaluate more extensively the sensitivity, selectivity, and overall reliability of this and other proposed animal models.

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