

Protein Allergenicity: Mouse Models

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INTRODUCTION

As described in previous articles, there is a need to identify and characterize the allergenic potential of novel proteins introduced into genetically modified crop plants. In 1996, a collaboration between the International Life Sciences Institute (ILSI) Allergy and Immunology Institute and the International Food Biotechnology Council (IFBC) outlined the first systematic approach for such allergenicity testing (1). A hierarchical strategy was proposed that incorporated consideration of the serological identity of the protein of interest with known human allergens; examination of amino acid homology with, and/or structural similarity to, allergenic proteins; and measurement of the stability (digestibility) of the protein in a simulated gastric fluid (1–3). It was recognized at the time that there were no suitable animal models that could be used in concert with the approaches summarized above. There has, however, been rapid progress in this area over the past few years, and several methods are now under development based upon measurement of serological responses induced in rats (see Knippels and Penninks, this issue) or in mice, the subject of this article. This progress has been acknowledged recently in a re-evaluation of the 1996 ILSI/IFBC decision tree reported by the joint Food and Agricultural Organization of the United Nations and the World Health Organization (FAO/WHO) Expert Consultative Committee on the Allergenicity of Foods Derived from Biotechnology (4), which concluded that there is sufficient evidence that some animal models are likely to provide valuable information regarding the potential allergenicity of foods.

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SYSTEMIC EXPOSURE

The application of murine models has found increasing favor for the characterization of the cellular and molecular mechanisms of various types of IgE-mediated allergic disease, including asthma to proteins (5) and respiratory sensitization to chemicals (6) or to protein detergent enzymes (7, 8). The mouse offers considerable advantages compared with other animal models, particularly with respect to the availability of inbred high IgE responder strains and various immunological and molecular reagents, including transgenic animals in which particular genes of interest have been overexpressed or deleted. The approach we have adopted here has been to evaluate the allergenic potential of test proteins as a function of serological responses (IgG and IgE antibody production) elicited in the high IgE responder BALB/c strain mouse following systemic (intraperitoneal) exposure. In this regimen, material is administered to animals in the absence of adjuvant, allowing the intrinsic allergenic potential of each protein to be assessed. Specific IgG (and IgG subclass) antibody production is measured by enzyme-linked immunosorbant assay (ELISA) and specific IgE antibody responses assessed by homologous passive cutaneous anaphylaxis (PCA) assay.

In our initial experiments, the vigor of specific IgE antibody responses induced in mice by ovalbumin (OVA), a potent respiratory and food allergen (9), and by bovine serum albumin (BSA), a protein considered to have a lesser allergenic potential was examined (10–12). Using this protocol, we previously have shown differences in the ability of these proteins to stimulate IgE antibody responses, with OVA consistently inducing relatively high titer IgE, whereas BSA provoked lower levels of IgE (10, 11). Indeed, analyses of the subclass distribution of the IgG antibody response revealed that BSA, but not OVA, elicited marked IgG2a antibody production under conditions of exposure where both materials were shown to induce similar total IgG antibody responses (12). In mice this immunoglobulin isotype is promoted by the T helper (Th) 1 type cytokine interferon γ (IFN- γ) and inhibited by the Th2-type cytokine interleukin 4 (IL-4), whereas the initiation and maintenance of IgE production require IL-4 and is antagonized by IFN- γ (13–15). These divergent patterns of IgG subclass and IgE induction therefore indicate that BSA and OVA are preferentially stimulating Th1- and Th2-type cells, respectively.

The kinetics of IgG and IgE antibody responses provoked by systemic exposure to OVA was then examined (16, 17). BALB/c strain mice received an intraperitoneal injection of 250 μ l of 2% OVA in phosphate buffered saline (PBS) on days 0 and 7, and animals were exsanguinated on days 14, 28, and 42. Antibody responses provoked by two additional proteins were analyzed also; peanut agglutinin (0.2%) or a crude extract of potato protein containing acid phosphatase enzymatic activity (PPE) (10%). Peanut

agglutinin is a minor peanut allergen (18, 19), whereas the potato is a relatively common foodstuff for which there is little evidence of allergenicity (20). All three proteins were immunogenic, inducing IgG antibody production in all or in the majority of exposed animals in each case (16). Responses were of maximal vigor 14 days after initiation of exposure and declined somewhat by 28 and 42 days. Exposure to peanut agglutinin and OVA each stimulated marked IgE antibody responses, which were also somewhat more vigorous 14 days after initiation of treatment. At every time point examined, intraperitoneal exposure to both proteins induced high titer (1 in 16 or greater) IgE antibody. In contrast, PPE failed to elicit high titer IgE, with detectable IgE (positive with neat serum only) induced at 28 days following the initiation of treatment (16, 17).

In subsequent experiments, serum antibody responses were measured at 14 days after intraperitoneal exposure and the differential ability of these proteins to provoke IgE antibody responses was confirmed (Figure 1). Mice ($n=5$) received intraperitoneal injections of 0.2% peanut agglutinin, 2% OVA, or 10% PPE; serum samples were isolated 14 days after the initiation of exposure and analyzed for specific IgE antibody expression. Three independent experiments were performed. In each experiment, administration of 0.2% peanut agglutinin or of 2% OVA was characterized by high titer IgE antibody production, varying from 1 in 64 to 1 in 128 and from 1 in 32 to 1 in 64, respectively. In contrast, treatment with 10% PPE either failed to induce

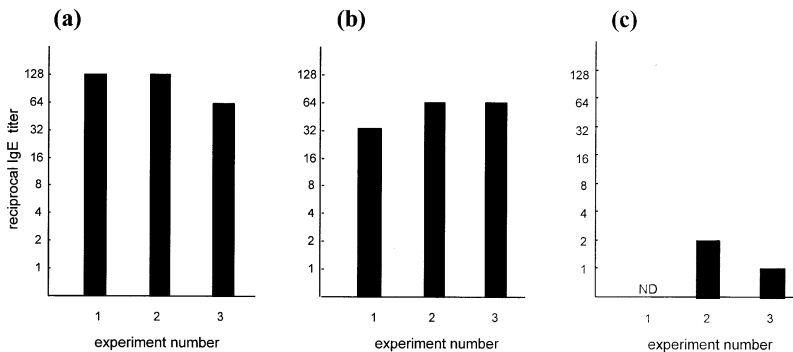


FIGURE 1 Groups of mice ($n=5$) received 250 μ l of peanut agglutinin (a), 2% OVA (b), or 10% of PPE (c) in phosphate buffered saline on days 0 and 7. Then 14 days after the initiation of exposure, mice were exsanguinated and pooled serum samples were tested for the presence of specific IgE antibody by homologous PCA assay. IgE antibody titer is recorded as the highest dilution of serum resulting in a positive PCA assay. In every PCA assay, neat serum isolated from naïve (untreated) animals failed to provoke positive PCA reactions (data not shown). Results of three independent experiments are shown. ND = not detectable.

detectable IgE antibody (experiment 1; Figure 1) or elicited low titer IgE only (1 in 2 or positive with neat serum). It appears therefore that food proteins of varying allergenic potential in humans exhibit consistent differences in their ability to provoke IgE antibody production following intraperitoneal administration to mice.

INFLUENCE OF ROUTE OF EXPOSURE

There is currently some debate regarding the most appropriate route or routes of exposure for the purposes of hazard identification and characterization. Although in theory oral administration appears the most attractive and relevant, it is well known that exposure of rodents to proteins in this way can result in immunological tolerance (21). Although parenteral (intraperitoneal) administration of protein avoids the development of oral tolerance and provides a clear indication of the inherent ability of proteins to induce IgE antibody responses, we must acknowledge that stimulation of IgE responses via this route of exposure will not necessarily translate into sensitization when the same protein is encountered following oral ingestion. Both methods have some merit, and it is possible that intraperitoneal administration and gavage exposure would each be of value in a holistic testing strategy.

We have therefore compared directly the serological responses of proteins when administered to mice intraperitoneally or by gavage (16). Groups of BALB/c strain mice ($n=6$) received a daily dose by gavage of 1 mg of protein in 250 μ l of water. Mice were exsanguinated 28 or 42 days following the initiation of exposure. Alternatively, groups of mice ($n=5$) received 250 μ l of various concentrations of protein (0.2% peanut agglutinin, 2% OVA, or 10% PPE) in PBS by intraperitoneal injection with the same treatment repeated 7 days later. Fourteen, 28, or 42 days following the start of exposure mice were exsanguinated and serum prepared and analyzed for specific IgG and IgE antibody production as described above. Gavage administration of peanut agglutinin resulted in vigorous IgG antibody responses at both time points (28 and 42 days) measured. Similar treatment of mice with either OVA or PAP resulted in weaker and more variable IgG responses (16). Marked differences were observed between proteins with respect to IgE antibody. Relatively high titer (1 in 32) IgE responses were provoked by 28 and 42 daily gavage administrations of peanut agglutinin, whereas identical exposure to OVA or PPE stimulated no detectable or only low titer (positive only with neat serum) IgE.

A different pattern of responses was recorded following intraperitoneal exposure of mice to the same proteins. All proteins induced IgG responses that were of maximal vigor 14 days following exposure and generally of higher titer than those observed after gavage administration (16). Intraperitoneal exposure to both peanut agglutinin and OVA induced high titre (1 in 16 to 1 in 128) IgE antibody at all time points investigated. In contrast, PPE

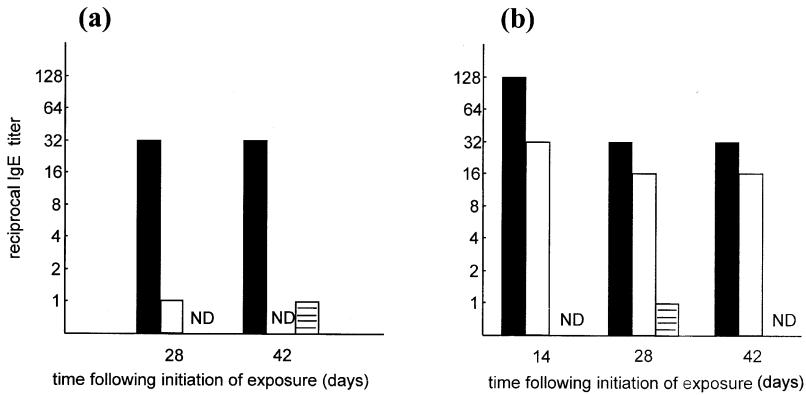


FIGURE 2 Groups of BALB/c strain mice ($n = 6$) received a daily dose by gavage of 1 mg of peanut agglutinin (■), 2% OVA (□) or 10% of PPE ▨ in 250 μ l of water (a). Mice were exsanguinated 28 or 42 days following the initiation of exposure. Groups of mice ($n = 5$) received 250 μ l of peanut agglutinin (■), 2% OVA (□), or 10% PPE (▨) in phosphate buffered saline by intraperitoneal injection on days 0 and 7 (b). At 14, 28, or 42 days after the initiation of exposure, mice were exsanguinated. Pooled serum samples were tested for the presence of specific IgE antibody by homologous PCA assay. IgE antibody titer is recorded as the highest dilution of serum resulting in a positive PCA assay. In every PCA assay, neat serum isolated from naïve (untreated) animals failed to provoke positive PCA reactions (data not shown). ND = not detectable.

failed to stimulate vigorous IgE antibody responses, with low titer antibody induced only at 28 days following initiation of exposure (Figure 2). Thus, oral exposure of BALB/c strain mice appears to result in a substantial underestimation of the allergenic potential of OVA. The lack of sensitivity of this approach suggests that administration of protein by this route of exposure to BALB/c strain mice may be of limited value for hazard identification.

INFLUENCE OF ADJUVANT

An alternative approach to the examination of the intrinsic ability of proteins to stimulate allergic (IgE) responses is to enhance the sensitivity of IgE responses by concurrent exposure to adjuvants. In the Brown Norway rat, IgE responses to a variety of proteins have been induced by intraperitoneal injection of or gavage exposure to protein combined with intraperitoneal administration of the adjuvant carrageenan (22–24). A similar approach has been used with some success to characterize immune responses induced in

mice by peanut proteins (25). C3H/HeJ strain mice were exposed orally (by intragastric gavage) to freshly ground whole peanut together with cholera toxin adjuvant. Oral challenge of sensitized mice with peanut extract stimulated anaphylactic reactions, and analysis of serum IgE revealed the presence of murine IgE antibody that recognized the same allergenic epitopes of the major peanut allergen Ara h 2 as IgE from peanut allergic human subjects. The adjuvant alum also has been utilized to augment IgE antibody responses to proteins, including IgE production to the milk allergen β -lactoglobulin (26) and to OVA (27). There is no doubt that appropriate adjuvants induce or enhance IgE antibody responses. What is not clear at present, however, is the extent to which adjuvant perturbs or modifies the inherent properties of proteins to induce IgE antibody responses. That is, the use of adjuvant may well compromise discrimination between proteins with respect to allergenic potential, possibly generating false-positives and conferring the appearance of sensitizing potential on nonallergens. This issue is currently unresolved, as to date few nonallergenic proteins have been examined using this type of approach, although some preliminary evidence suggests that intraperitoneal exposure of mice to protein and carrageenan does indeed result in some loss of selectivity of IgE responses to proteins (28).

ALTERNATIVE ENDPOINTS FOR ALLERGENICITY

Another issue that requires consideration is the nature of the endpoints used to determine allergenicity. In murine models of food allergy, the most common approach is to measure the induction of specific IgE antibody. There is usually a requirement to measure specific IgE, rather than changes in the total serum concentration of this immunoglobulin, because exposure to protein allergens does not appear to increase total serum IgE levels (10). Exposure to proteins in the presence of adjuvant does cause measurable increases in total serum IgE concentration (27); however, as described above, such elevations have yet to be shown to be selective for protein allergens. A variety of methods is available for the analysis of specific IgE antibody responses. Commonly, homologous or heterologous PCA assays are used because many investigators agree that the demonstration of biologically active IgE is important (16, 17). ELISA assays are also utilized by some investigators (26, 27, 29, 30). Because IgE antibody is present in the serum at concentrations that are orders of magnitude lower than other isotypes of immunoglobulin including IgG, precautions must be taken against the blocking effects or cross-reactivity of reagents with such antibody. Thus, steps such as the capture of total IgE using plates coated with monoclonal anti-IgE antibody followed by the detection of antigen-specific IgE utilizing biotinylated or digoxigenin labeled antigen are incorporated (26, 27, 29, 30). For example, for the detection of OVA-specific IgE antibody, plates were coated with rat antimouse IgE monoclonal antibody and incubated with

serum from OVA-exposed mice. Subsequently, bound anti-OVA IgE was detected by incubation with biotinylated-OVA and then visualized with streptavidin-alkaline phosphatase enzyme conjugate (29). This requirement for labeled antigen means that for each individual protein of interest, a separate ELISA protocol must be developed, including the preparation of a biotinylated or digoxigenin-labeled antigen complex. These requirements do not lend themselves readily to the development of a routine predictive test. Another potential method for the measurement of IgE utilizes a rat basophilic leukemic cell line (RBL) that expresses Fc receptors for IgE and, once sensitized by protein-specific IgE, can be induced to degranulate by challenge with specific antigen, releasing radiolabeled serotonin. This method has been used to measure IgE antibodies induced by intranasal exposure to respiratory sensitizing detergent enzymes (7), but it has not yet been validated for a wider range of proteins.

In addition to the measurement of IgE antibody responses, other parameters of the immediate-type hypersensitivity reaction have been measured. In some experiments, an active anaphylactic reaction has been induced by intravenous injection of protein in previously sensitized mice (29). However, the deliberate provocation of systemic anaphylactic responses raises serious animal welfare concerns and cannot be recommended as an endpoint for a routine assay. There has been some interest in the measurement of allergen-induced inflammatory reactions stimulated in the small intestine as a result of intragastric allergen provocation, with responses assessed as a function of local histamine release (27). Such changes, however, did not correlate well with allergic sensitization. The ability of lymphoid cells isolated from previously sensitized animals to elaborate type 2 cytokines has been investigated also. For example, it has been demonstrated that following oral ingestion of the allergen OVA, lymph node cells derived from the Peyer's patches of BALB/c strain mice could be restimulated *in vitro* with antigen to express the type 2 cytokines IL-4 and IL-5, although detectable IFN- γ and IL-2 production was observed also (29). Although these experiments suggest that treatment with food allergens such as OVA can provoke Th2-type responses, as yet there are insufficient data to determine whether analysis of such induced cytokine secretion profiles could provide a method to identify proteins with the potential to cause allergy.

CONCLUSIONS

The evidence summarized in this article suggests that with continuing effort, it should be possible to develop mouse models that will be of value in identifying proteins with the inherent potential to cause allergic sensitization. It must be acknowledged that the pathogenesis of food allergy is complex, particularly with regard to interindividual variations in susceptibility, and it will be difficult to define a method using experimental animals that predicts

accurately the likely prevalence, severity, and persistence of food allergy in human populations exposed to a novel protein. Accurate risk assessment of food allergy represents an important challenge for the future. There are real opportunities, however, for the development of experimental approaches that allow the first step in the safety assessment process, i.e., the identification of intrinsic hazard, that in the context of food allergy is the inherent potential of a protein to cause allergic sensitization. On the basis of the data described herein, the BALB/c strain mouse may provide an appropriate model for the identification and characterization of protein allergens. Serological responses induced by intraperitoneal administration appear to distinguish between allergenic proteins and other proteins that despite being immunogenic fail to provoke IgE antibody responses. Moreover, at least to some extent, the vigor of induced IgE responses, particularly when considered in conjunction with dose levels, reflects what is known of the relative sensitizing potential of known human allergens. Although oral administration by gavage may be reflect more accurately the relevant route of human exposure, the data summarized here indicate that this experimental approach in mice may not possess the sensitivity or reliability to provide an initial assessment of allergenic potential.

Clearly, there is a need to interrogate the sensitivity and selectivity of these approaches in greater detail using a wider range of allergenic and nonallergenic proteins. The most promising of these may then be incorporated within an integrated strategy for allergenic hazard identification and characterization, supplementing data derived from complementary analyses such as amino acid sequence homology with known allergens and/or protein stability in simulated gastric fluid.

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