



Workshop Report

Utility of rodent models for evaluating protein allergenicity

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ABSTRACT

Animal models are needed to assess novel proteins produced through biotechnology for potential dietary allergenicity. The exact characteristics that give certain foods allergenic potential are unclear, but must include both the potential to sensitize (induce IgE) as well as the capacity to avoid induction of oral tolerance (specific inhibition of IgE production). EPA has developed two complementary mouse models; one which distinguishes allergenic from non-allergenic food extracts using oral sensitization with adjuvant (cholera toxin) and another which further distinguishes highly potent allergens following oral administration without adjuvant based on the development (or not) of tolerance. For the foods tested thus far (roasted or raw peanut, Brazil nut, egg white, turkey, and spinach), the ability to sensitize and/or tolerate in these models are consistent with observed allergenicity as well as persistence and severity among allergens. Additionally, in vitro pepsin-resistance analysis of these food extracts suggests an association between sensitization capacity and proteins which are stable to gastric digestion. A subcutaneous exposure model did not distinguish allergenic from non-allergenic foods and does not appear useful for assessing potential allergenicity.

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1. Introduction

The current approach for assessing potential allergenicity of genetically engineered food is based on a weight-of-evidence as described by Codex (CAC, 2003a,b), which has replaced the hierarchical decision tree devised by the Food and Agricultural Organization (FAO) of the UN and the World Health Organization (WHO) in 2001 (FAO/WHO, 2001). This updated approach recommends the integrated use of relatively well established assessment techniques and cites the potential use of other methods which are part of the original decision tree but remain undeveloped. One prospective tool still needed for comprehensive risk analysis is an appropriate animal model(s). Also needed is a more thorough understanding of the relationship between digestibility (pepsin resistance) and allergenicity, since resistance to degradation by acid proteases is included in the recommended approach and has been used to make regulatory decisions (Bucchini and Goldman, 2002). Resistance to enzymatic digestion with pepsin is a feature common to many, but not all, allergenic food proteins (Astwood et al., 1996; Fu, 2002; Fu et al., 2002; Herman et al., 2006). The prevailing logic asserts that proteins that are able to survive passage through the stomach are more likely to be available for subsequent immune responses within or beyond the intestinal mucosa.

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Several attempts have been made toward development of an appropriate animal model (Akiyama et al., 2001; Dearman and Kimber, 2001; Dearman et al., 2003; Gaudry et al., 2004; Hilton et al., 1997; Kimber et al., 2003; Knippels et al., 1999, 1998; Navuluri et al., 2006). An ideal animal model should mimic human disease with respect to route of exposure, mechanisms underlying the disease and symptomology. Most importantly, it should readily demonstrate differences between foods known to be allergenic and those thought not to be allergenic. Because IgE is the antibody class most strongly associated with allergic reactions, the presence of food-specific antibody of this type is regularly used as an indicator of food allergic disease both in humans and animals. An equally important indicator may be failure to induce oral tolerance. Although the normal response to ingested proteins is a state of non-reactivity known as oral tolerance, patients with food allergy exhibit active immunity to food proteins (antigens), implying that immune priming or sensitization was the result instead of tolerance during antigen exposure. Indeed, IgE is the antibody type most susceptible to suppression by oral tolerance (Christensen et al., 2003; Saklayen et al., 1984). Assuming food allergy in humans is initiated and provoked through oral exposure to the offending protein(s), the oral route might be considered the most appropriate route of exposure when developing such a model. This is particularly relevant in light of the clearly established role oral tolerance plays in regulating IgE responses as well as the ambiguous relationship between allergenicity and digestibility. As in healthy humans, the predominant response in laboratory animals

to ingestion of soluble proteins is oral tolerance. There are currently two approaches available to avoid oral tolerance in adult animals: (1) use an alternate route of exposure or (2) include an adjuvant (i.e. cholera toxin) in the oral exposure.

Our laboratory has evaluated subcutaneous and oral exposures with the hypothesis that adult female C3H/HeJ mice exposed via either of these routes could be used to make distinctions between extracts from foods known to cause food allergy in humans (peanut, egg white, Brazil nut) (Borja et al., 1999; NIAID, 2006; Pastorello et al., 1998; Sicherer et al., 2003) and from foods rarely associated with food allergy (spinach, turkey meat) (Koppelman and Hefle, 2006) based on IgE antibody responses. Additionally, we have used the oral exposure model along with in vitro assays to explore aspects of digestibility. Finally, we have used the oral exposure model to assess the potential of individual food extracts to induce oral tolerance.

2. Methods

Groups of 5–8 adult female C3H/HeJ mice (6–8 weeks old, The Jackson Laboratory, Bar Harbor, ME) were used in all studies. This mouse strain produces high levels of IgE antibody and demonstrates anaphylactic symptoms upon challenge in mechanistic studies of food allergy (Li et al., 1999, 2000). Commercially prepared, acetone-defatted aqueous extracts of raw or roasted peanut, egg white, spinach, turkey and Brazil nut were used as a panel of allergenic and non-allergenic foods (Greer Laboratories, Lenoir NC). For subcutaneous exposures, mice were injected subcutaneously with 60 or 300 µg (total protein) of food extract in two 50 µl volumes of sterile Hank's balanced salt solution (HBSS) over each haunch; doses were administered twice at a three week interval. For oral sensitization, mice were gavaged two or four times at a weekly interval with 1, 2, or 5 mg (total protein) of food extract in plain HBSS or HBSS containing 0.2 M sodium bicarbonate, with or without 10 µg CT (List Biological Laboratories, Inc., Campbell, CA). For oral tolerance induction, mice were gavaged once with 1 or 2 mg (total protein) of food extract or purified protein in plain Hank's balanced salt solution (HBSS) or HBSS containing 0.2 M sodium bicarbonate. One week after oral exposure, mice were intraperitoneally (i.p.) immunized with 100 µg of the same food extract or protein in 200 µl alum adjuvant (Alhydrogel 2%, Accurate Chemical & Scientific Corp., Westbury, NY). Oral tolerance was demonstrated by suppression of IgE responses to i.p. immunization by prior oral exposure. Blood samples were collected one week after the last treatment for specific antibody analysis by ELISA. In vitro pepsin resistance assays were conducted by incubating freshly prepared test extracts in simulated gastric fluid (2 mg/ml NaCl, pH 2.0, 1:1 ratio of test protein to pepsin from Sigma–Aldrich, St. Louis, MO) at 37 °C for 15 min prior to the addition of neutralizing agent (0.2 M Na₂CO₃) and sample buffer for sodium-dodecyl sulfate polyacrylamide gel electrophoresis.

3. Subcutaneous exposure

The subcutaneous route has not been previously evaluated as a method for assessing allergenicity. However, prior studies characterizing cholera toxin have shown it is required for IgE responses to subcutaneously injected tetanus toxoid (Marinaro et al., 1995), a protein rarely associated with clinical allergic reactions, but not for allergenic proteins such as hen egg lysozyme and ovalbumin (Snider et al., 1994). Similarly, IgE is produced upon intradermal exposure to peanut agglutinin without adjuvant (Betts et al., 2004). Though these studies might collectively suggest that the subcutaneous route of exposure would yield predictive differential IgE responses to allergens and non-allergens, our results did not

demonstrate differential effects. Subcutaneous injection with 60 or 300 µg of food extract resulted in substantial IgE responses to egg white, roasted peanut and spinach, while raw peanut elicited more modest IgE production. Comparing what might be considered the most allergenic food, roasted peanut, to the least allergenic food, spinach, the increase in extract-specific IgE over naïve levels was roughly equivalent. Furthermore, subcutaneous injection of raw peanut, which should be highly allergenic, elicited low IgE responses. A multi-laboratory study evaluating intraperitoneal (i.p.) injection of five strains of mice presented findings similar to our own with regard to non-allergenic spinach antigens (Thomas et al., 2005). The investigators proposed that bacterial endotoxin contamination of the protein preparation might have enhanced the allergic antibody response. The food extracts employed in our studies contained little more endotoxin than the HBSS vehicle (less than 2 U/ml), except for the raw peanut preparation, which contained 11 U/ml. Although endotoxin contamination of the raw peanut extract was high, and must be a consideration for any material tested by injection, it should be noted that the C3H/HeJ mouse strain is regarded as “endotoxin resistant”, having a mutation in the toll-like receptor 4 gene (*Tlr4*^{Lps-d}) required for endotoxin signaling. Still, the influence of other toll-like receptor ligands or unidentified adjuvants within a specific test material cannot be ruled out, even for purified protein preparations. Regardless of the actual mechanism underlying the observed low IgE responses to raw peanut and the high responses to spinach, it is clear that under the current experimental conditions subcutaneous exposure is not a functional method of distinguishing allergenic proteins.

4. Oral exposure

Induction of allergic responses to dietary proteins administered orally using cholera toxin (CT) as an adjuvant has been reported for peanut (Adel-Patient et al., 2005; Fischer et al., 2005; Li et al., 2000; Morafo et al., 2003; van Wijk et al., 2004, 2005), cow's milk (Adel-Patient et al., 2005; Li et al., 1999; Morafo et al., 2003), shrimp (Reese et al., 2005), egg white (Kroghsbo et al., 2003; Marinaro et al., 1995; Snider et al., 1994) and soy antigens (Christensen et al., 2003; Kroghsbo et al., 2003). Using this method, we clearly demonstrated allergic antibody production after two oral exposures to 1 or 2 mg of allergenic peanut, Brazil nut and egg white, but not to non-allergenic spinach and turkey. Oral exposure to both roasted and raw peanut stimulated significant dose-dependent increases in extract-specific IgE. Egg white elicited more moderate levels of IgE with statistically significant increases observed only at the higher 2 mg dose. Although the magnitude of the IgE response to the 1 mg dose was reproducibly greater than that of the 2 mg dose, variability was considerably higher in the 1 mg dose group, reducing statistical significance. IgE responses to spinach extract were not significantly increased at either dose. Oral exposure to Brazil nut extract resulted in modest but significant elevations in IgE at both doses and as expected very little IgE was produced in response to turkey. Oral exposure to food extracts in the absence of CT did not elicit food-specific antibody responses.

Based on these observations, we hypothesized that a dosing regimen extended to four exposures instead of two might serve to amplify those differences and result in a model that more clearly distinguishes protein allergens from non-allergens. Along the same vein, we reasoned that administering a higher dose of 5 mg could potentially generate more obvious differential responses. Consistent with previous results, roasted peanut was the most potent elicitor of IgE. However, under the revised experimental conditions spinach also elicited production of allergic antibody. Spinach-specific IgE at the 5 mg dose was statistically significantly elevated, reaching levels similar to those at the same dose of egg white. A

5 mg dose of turkey elicited statistically significant increases in IgE compared to naïve levels after only two exposures, indicating less frequent or lower doses may be more effective in achieving differential effects. Thus, a short term (2 week) exposure regimen at doses of food extract lower than 5 mg appears to be optimal for distinct responses to allergenic and non-allergenic foods.

5. Digestibility

Although some studies have shown that food allergens are not necessarily more resistant to digestion than are non-allergenic proteins (Fu, 2002; Fu et al., 2002), protecting an antigen from degradation may enhance its immunogenicity by allowing it to reach the gut associated lymphoid tissues intact. For example, oral administration of enteric-coated encapsulated soluble protein appears to be one of the few methods for activating the murine gut mucosal immune system in adult animals without the use of adjuvant (Flanagan et al., 1996; Jain et al., 1996a,b). In our oral exposure studies and in vitro digestibility assays, we noted that the magnitude of IgE responses to orally administered foods correlated with their observed digestion stability. Peanut contains pepsin-resistant proteins and elicits high IgE responses orally in our study, confirming the results of others (Astwood et al., 1996; Fu, 2002; Fu et al., 2002). Egg white also contains digestion-stable proteins and induces moderate IgE levels. The major spinach protein, RuBisCO, has been shown to be so digestively labile that it degrades completely after only 30 s of in vitro digestion (Astwood et al., 1996; Fu et al., 2002). Thus it is not surprising that spinach extract elicits very little IgE (or other antibody) via the oral route. Few data were available regarding the digestibility of Brazil nut or turkey proteins, but the presence of stable fragments in digested Brazil nut and their complete absence after digestion of turkey (Bowman and Selgrade, 2008) is consistent with the respective IgE responses observed in mice following oral exposure. This association of IgE responses with foods containing digestion-resistant proteins, along with evidence that digestion stable fragments from Brazil nut contain IgE epitopes (Moreno et al., 2005), lends credence to the use of digestibility data as one criterion for allergenic hazard identification.

6. Oral tolerance

Although egg white is considered a major human allergen that contains digestion stable components, we observed only moderate allergic responses to egg white after oral exposure. It should be noted that egg white allergy is predominant among children and is frequently outgrown (AAAAI, 2005), raising the possibility that adult mice do not represent a susceptible test population for egg allergy. Additionally, the major egg white protein, ovalbumin, is the model antigen for oral tolerance induction, and because IgE is the antibody type most susceptible to suppression by oral tolerance (Saklayen et al., 1984), IgE responses may have been dampened by this process. Oral tolerance induction may also account for the consistently lower IgE levels elicited by the higher 2 mg dose, a phenomenon which appears unique to egg white. Oral tolerance induction is assessed by oral exposure to 1 mg of food extract or protein, one week prior to i.p. injection of the extract in alum adjuvant. Tolerance is evidenced by lower IgE responses in animals orally exposed prior to parenteral immunization compared to animals without prior oral exposure. Evaluation of food extracts for their ability to induce oral tolerance by our laboratory has revealed tolerance induction by egg white but not by peanut, turkey, Brazil nut or spinach at the same dose (Bowman and Selgrade, 2008). These observations indicate that the normal response to ingested proteins may not be oral tolerance induction, or at least

not equivalent induction for all food proteins. Because oral tolerance specifically inhibits IgE, a food that elicits IgE via the oral route but is unable to induce tolerance may pose a greater risk. Thus the ability of a given dietary protein to induce oral tolerance may be an important consideration in assessing allergenic potential.

7. Conclusions

Based on our observations thus far, it may be possible to distinguish allergenic foods from non-allergenic foods via a short term regimen of oral exposure to low doses (1 or 2 mg) of food extracts. In contrast, it appears that the subcutaneous route of exposure will not generate differential responses in accordance with known allergenicity since it resulted in IgE to both allergenic and non-allergenic foods. Taken together, results from our laboratory and others support oral exposure over injection as a preferred method for distinguishing allergens from non-allergens. We propose that those foods containing digestion-stable proteins are more likely to elicit IgE after oral exposure and thus are associated with greater allergenic risk (peanut, Brazil nut), which can be mitigated by a food's ability to suppress IgE through oral tolerance induction (egg white). Those foods that do not induce oral tolerance but also lack oral sensitization potential (i.e. turkey, spinach) pose minimal allergenic risk.

Food	Sensitizing	Tolerizing	
Peanut	+	—	High risk
Brazil nut	+	—	
Egg white	+	+	
Turkey	—	—	↓
Spinach	—	—	
			Low risk

The oral exposure protocol requires further development with an expanded test panel of known allergenic and non-allergenic foods and subsequent validation by independent laboratories prior to its use in assessing novel proteins of unknown allergenicity. Experiments are under way in our laboratory to evaluate other allergenic foods, such as sesame, and non-allergenic foods, such as lamb and pinto bean, for their oral sensitization potential, digestibility, and ability to induce oral tolerance as part of a weight-of-evidence approach to allergenic risk assessment.

8. Disclaimer

This report has been reviewed by the EPA's Office of Research and Development, and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the Agency nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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