Differences in Allergenic Potential of Food Extracts following Oral Exposure in Mice Reflect Differences in Digestibility: Potential Approaches to Safety Assessment

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An animal model for food allergy is needed to assess genetically modified food crops for potential allergenicity. The ideal model must produce allergic antibody (IgE) to proteins differentially according to known allergenicity before being used to accurately identify potential allergens among novel proteins. The oral route is the most relevant for exposure to food allergens, and a protein's stability to digestion is a current risk assessment tool based on this natural route. However, normal laboratory animals do not mount allergic responses to proteins administered orally due to oral tolerance, an immunologic mechanism which specifically suppresses IgE. To circumvent oral tolerance and evoke differential IgE responses to a panel of allergenic and nonallergenic food extracts, female C3H/HeJ mice were exposed subcutaneously or orally with cholera toxin as an adjuvant. All foods elicited IgE by the subcutaneous route. Oral exposure, however, resulted in IgE to allergens (peanut, Brazil nut, and egg white) but not to nonallergens (spinach and turkey), provided that the dose and exposures were limited. Additionally, in vitro digestibility assays demonstrated the presence of digestion-stable proteins in the allergenic food extracts but not in the nonallergenic foods. Our results suggest that the subcutaneous route is inadequate to distinguish allergens from nonallergens, but oral exposure under the appropriate experimental conditions will result in differential allergic responses in accordance with known allergenicity. Moreover, those foods containing digestion-resistant proteins provoke allergic responses in this model, supporting the current use of pepsin resistance in the decision tree for potential allergenicity assessment.

Key Words: allergenicity; food allergy; biotechnology.

There are mounting concerns regarding the introduction of genetically modified plants into the human food supply. Specifically, the engineering of novel genes into food allows for expression of proteins which may cause allergic reactions in susceptible individuals. Although food allergy is relatively rare, it currently affects 6–8% of children under the age of 4 and 3.7% of adults and is believed to be increasing in prevalence (Dioum et al., 2003; NIAID, 2006; Sicherer et al., 2003). The consequences of allergic reactions to food can be severe or even fatal. In the United States, food allergy causes roughly 30,000 episodes of anaphylaxis and 100–200 deaths per year (NIAID, 2006). Food allergies are antigen specific and associated with a limited number of foods; cow’s milk, egg, peanut, soy, wheat, and fish are prominent allergens in children, and adults are most often allergic to peanut, tree nuts, fish, and shellfish. Characterization of these food proteins has yet to define any feature which confers allergenic properties, making it difficult to assess potential allergenicity of novel food proteins.

The current strategy for assessing potential allergenicity of genetically engineered food is based on food and agriculture organization (FAO)/world health organization (WHO) Expert Consultation recommendations given in the 2003 Codex Alimentarius guidelines (FAO/WHO, 2003). This weight-of-evidence approach includes comparing the introduced protein with known allergens based on gene source, sequence homology, and reactivity with serum from allergic patients, as well as evaluating the protein’s stability to digestive enzymes. 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 if the route of allergic sensitization (and elicitation) in humans is oral, proteins able to survive passage through the stomach are more likely to be available for subsequent immune responses within or beyond the intestinal mucosa. However, the correlation between allergenicity and digestive stability is not absolute, and because resistance to degradation by acid proteases is used to make
regulatory decisions (Bucchini and Goldman, 2002), a more thorough understanding of this relationship is needed.

An animal model to test for potential food allergens is highly desirable and several attempts have been made toward this end (Akiyama et al., 2001; Dearman and Kimber, 2001; Dearman et al., 2003; Gaudry et al., 1999; Hilton et al., 1997; Kimber et al., 2003; Knippels et al., 1998, 1999; 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. Although the normal response to ingested proteins is a state of nonreactivity known as oral tolerance, patients with food allergy exhibit active immunity to food proteins (antigens), implying that immune priming or sensitization resulted instead of tolerance at some point 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, however, 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 (cholera toxin [CT]) in the oral exposure.

The subcutaneous route has not been previously evaluated as a method for assessing allergenicity. However, prior studies characterizing CT 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 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). Induction of allergic responses to dietary proteins administered orally using CT as an adjuvant has been reported for peanut (Adel-Patient et al., 2005; Fischer et al., 2005; Li et al., 2000; Morafó et al., 2003; van Wijk et al., 2004, 2005), cow’s milk (Adel-Patient et al., 2005; Li et al., 1999; Morafó 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). To our knowledge there are no published articles comparing allergenic and nonallergenic food proteins using oral exposure with CT. In the current study subcutaneous and oral exposures were used with the hypothesis that C3H/HeJ mice exposed either subcutaneously or orally with CT could be used to make distinctions between extracts from foods known to cause food allergy in humans (peanut, egg white, Brazil nut) and from foods rarely associated with food allergy (spinach, turkey meat) based on IgE antibody responses. The ability to make such distinctions is critical for an animal model used to test food extracts from genetically engineered crops. Additionally, we have used the oral exposure model along with in vitro assays to explore issues of digestibility. The C3H/HeJ mouse strain has a propensity for high IgE production and has previously been used for mechanistic studies of food-allergic disease (Li et al., 1999; Reese et al., 2004).

Materials and Methods

Animals

Six- to 8-week-old female C3H/HeJ mice (The Jackson Laboratory, Bar Harbor, ME) were group-housed in polycarbonate cages with hardwood chip bedding in an environmentally controlled, association for accreditation of laboratory animal care-accredited vivarium. All animal procedures were reviewed and approved by the U.S. Environmental Protection Agency, National Health and Environmental Effects Research Laboratory, Institutional Animal Care and Use Committee. Mice were maintained on a 12-h light/dark cycle and allowed access to food (Purina Rodent Lab Chow, St Louis, MO) and water ad libitum. Mice were allowed to acclimate 1 week prior to the start of the experiment, and had no known prior oral exposure to test proteins.

Food Antigens

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 nonallergenic foods (Greer Laboratories, Lenoir, NC). Food extract preparations were assayed for total protein concentration using Pierce BCA protein assay kit (Pierce, Rockford, IL) according to manufacturer’s instructions. All doses are based on the total protein content, thus 1 mg of peanut extract refers to 1 mg of total peanut proteins. Endotoxin levels in the extracts were measured using a Limulus amebocyte lysate test kit (BioWhittaker, Walkersville, MD) and all were found to contain less than 2 endotoxin units (EU)/ml except for raw peanut, which contained 11 EU/ml.

Experimental Design

Subcutaneous Exposure. 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 hindpaw; doses were administered twice at a 3-week interval and blood samples were collected 1 week after the last dose for specific antibody analysis. Control animals were injected subcutaneously with the HBSS vehicle only. Experimental groups consisted of five animals each (n = 5).

Oral Exposure with CT. 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 0.2M sodium bicarbonate, with or without 10 µg CT (List Biological Laboratories, Inc., Campbell, CA). Control animals received HBSS and CT only. Blood was collected for antibody analysis 1 week after the last oral exposure. Experimental groups consisted of six to eight animals each (n = 6–8).

Blood Collection. Mice were anesthetized with sodium pentobarbital and blood samples were collected by cardiac puncture. Whole blood was allowed to clot for 1 h at room temperature prior to serum separation by centrifugation, and serum was stored at –80°C until analysis.
Measurement of Antigen-Specific IgE, IgG1, and IgG by Enzyme-Linked Immunosorbent Assay

All reagents and incubation periods were at room temperature for one hour and all volumes added were 50 μl unless otherwise noted. Microtiter plates (Costar Corp., Cambridge, MA) were coated with 1 mg protein/ml food extract or purified protein in phosphate-buffered saline (PBS), pH 7.3, and incubated overnight at 4°C. Following blocking with 100 μl assay buffer (PBS/1% bovine serum albumin, Sigma-Aldrich, St Louis, MO), plates were incubated with serum samples diluted in assay buffer, then washed with PBS-Tween before addition of biotinylated detection antibody (rat anti-mouse IgE, IgG1, or IgG; Pharmingen, San Diego, CA). Optimal serum dilutions were empirically determined by titration of individual samples in each antibody isotype assay for a specific food extract. Serum dilutions of 1:5 for IgE, 1:20 for IgG1, and 1:80 for IgG were used to measure samples from multiple experimental groups within a given plate, thereby reducing plate-to-plate variability. Plates were washed and incubated with streptavidin horseradish peroxidase (Zymed, San Francisco, CA) prior to a final wash and addition of tetramethylbenzidine substrate (Dako Corp., Carpinteria, CA). Reactions were stopped with 1/N sulfuric acid and absorbance at 450 nm wavelength was measured using a SpectraMax 340 PC Plate Reader (Molecular Devices Corp., Sunnyvale, CA). Softmax Pro version 2.6.1 (Molecular Devices Corp.) software was used for data collection.

Antigen-Specific IgE Reactivity Assay

Rat basophil leukemia (RBL) cell β-hexosaminidase release assay was performed as an indirect measure of functional antigen-specific IgE in serum. The procedure is based on the method by Hoffman et al. (1997) with modifications by D. Leadbeater and D. A. Baskett (personal communication, Unilever Safety and Environmental Assurance Center, UK). Briefly, RBL-2H3 cells (ATCC, Rockville, MD) were maintained in a growth medium (72% [vol/vol] Eagle’s minimal essential medium [EMEM, Gibco BRL, Gaithersburg, MD], 23% [vol/vol] RPMI-1640 [Gibco BRL], 5% [vol/vol] fetal calf serum [FCS; Gibco BRL], 100 U/ml penicillin, 100 μg/ml streptomycin [Gibco BRL], and 1% [vol/vol] 0.2M L-glutamine) at 37°C in a 5% CO2 humidified incubator. At confluence, the cells were trypsinized (0.05% [wt/vol] trypsin and 0.53 mM ethylenediaminetetraacetic acid [EDTA] in HBSS) for 15 min at 37°C in a 5% CO2 humidified incubator. RBL cells were collected and washed by centrifugation, then suspended in assay medium (93% [vol/vol] EMEM, 5% [vol/vol] FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1% [vol/vol] 0.2M L-glutamine) and plated in 96-well flat bottom tissue culture plates (104 cells/100 μl/well). The cells were then incubated for 18 h at 37°C in a 5% CO2 humidified incubator. At confluence, the cells were trypsinized (0.05% [wt/vol] trypsin and 0.53 mM ethylenediaminetetraacetic acid [EDTA] in HBSS) for 15 min at 37°C in a 5% CO2 humidified incubator. RBL cells were collected and washed by centrifugation, then suspended in assay medium (93% [vol/vol] EMEM, 5% [vol/vol] FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1% [vol/vol] 0.2M L-glutamine) and plated in 96-well flat bottom tissue culture plates (104 cells/100 μl/well). The cells were then incubated for 18 h at 37°C in a 5% CO2 humidified incubator. Cells were passively sensitized for 2 h by incubation with a 1:10 dilution of individual mouse sera in assay medium. Control cells for spontaneous and total release were sensitized with naive serum from C3H/HeJ mice. Subsequently, cells were washed three times in Tyrode’s buffer (130mM NaCl, 5mM KCl, 1.4 mM CaCl2, 1mM MgCl2, 5.6mM glucose, 10mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid, and 0.1% [wt/vol] bovine serum albumin, pH 7.45). For cross-linking, cells were incubated with 100 μl of 10 μg/ml antigen in Tyrode’s buffer for 1 h at 37°C in a 5% CO2 humidified incubator. Control cells for spontaneous release and total release were incubated with Tyrode’s buffer and Tyrode’s buffer with 1% (vol/vol) Triton X-100 (Sigma-Aldrich), respectively. Cell supernatant (40 μl) from each well was transferred to a sterile 96-well plate. The β-hexosaminidase activity was measured by adding 80 μl of substrate, p-nitrophenyl N-acetyl β-d-glucosaminide (1.3 mg/ml in 0.1M citric buffer, pH 4.5) and incubating for 1 h at room temperature. The enzyme reaction was stopped by adding 100 μl of 0.2M glycine. Absorbance was measured 30 min later using SpectraMax 340 PC Plate Reader (Molecular Devices Corp.) at a wavelength of 405 nm and data collected by Softmax Pro software (version 2.6.1, Molecular Devices Corp.). Results are expressed as percent of the total release after subtraction of spontaneous release.

Digestibility Assay

Assessment of pepsin resistance was performed using a modified protocol similar to published methods (Dearman et al., 2002; Takagi et al., 2003). Simulated gastric fluid (SGF) was prepared as follows: 10 mg NaCl in 5 ml distilled water, pH adjusted to 2.0 with dilute HCl, plus 3.8 mg pepsin (Sigma-Aldrich). One thousand eight hundred and fifty microliters of SGF was preheated to 37°C for 2 min in a 5-ml tube prior to the addition of 150 μl of prewarmed 10 mg/ml test extract freshly prepared in distilled water. After 15 min, 1 ml each of neutralizing agent (0.2M Na2CO3 and 4× NuPage lithium dodecyl sulfate (LDS) sample buffer (Glycerol, 10%; Tris Base, 141mM; Tris HCl, 106mM; LDS, 2%; EDTA, 0.51mM; SERVA Blue G250, 0.22mM; Phenol Red, 0.175mM, pH 8.5) were added before transferring to ice. Samples were heated for 5 min at 95°C and cooled to room temperature before loading 13.5 μl on to a 10% Bis-Tris polyacrylamide gel for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis (NuPage reagents, Invitrogen, Carlsbad, CA).

Statistical Analyses

The data were analyzed with GraphPad Prism 4 software (GraphPad, San Diego, CA) using one-way analysis of variance followed by Tukey’s multiple comparison test. Student’s t-test was performed in some instances for direct comparison of treatment groups to naive control groups. Significance was attributed to an effect or difference if the probability (p value) was less than 0.05.

RESULTS

All Food Extracts Elicit IgE when Injected Subcutaneously

Although work by other investigators (Marinaro et al., 1995; Snider et al., 1994) collectively suggested that the subcutaneous route of exposure would yield predictive differential IgE responses to allergens and nonallergens, 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, whereas raw peanut elicited more modest IgE production (Fig. 1). Spinach was the only extract to demonstrate a clear dose-dependent response upon subcutaneous exposure, with the low (60 μg) and high (300 μg) doses eliciting 1.8- and 2.4-fold increases over naïve IgE levels (Fig. 1D). For all other extracts, exposure to the lower dose resulted in similar in slightly greater responses compared with the higher dose; 4.4- versus 3.9-fold, 2.8-versus 2.7-fold, and 0.7- versus 0.3-fold increases over naïve IgE levels for egg white, roasted peanut, and raw peanut, respectively. IgG1 and IgG antibody levels were significantly increased after subcutaneous exposure to all food extracts tested and did not vary by dose (data not shown).

Food Extracts Vary in their Ability to Elicit IgE upon Oral Exposure with CT

In contrast, oral exposure to both roasted and raw peanut (Figs. 2A and 2B) stimulated significant dose-dependent increases in extract-specific IgE. Egg white (Fig. 2C) 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 1 mg of egg white was reproductively greater the response to 2 mg, it was not
statistically significantly increased ($p = 0.194$) compared with naïve levels due to considerable variability among animals in the 1 mg dose group. IgE responses to spinach extract were not significantly increased at either dose (Fig. 2D). Oral exposure to Brazil nut extract (Fig. 2E) resulted in modest but significant elevations in IgE at both doses, but very little IgE was produced in response to turkey (Fig. 2F). Examination of IgG1 and IgG antibody responses indicated results similar to those observed for IgE (data not shown). Oral exposure to food extracts in the absence of CT did not elicit food-specific antibody responses (data not shown).

**Confirmation of IgE Functionality by RBL Cell Assay**

Cultured RBL cells were passively armed with sera from mice exposed to food extracts either subcutaneously or orally (measured by enzyme-linked immunosorbent assay [ELISA] in Figs. 1 and 2), and then incubated with food extracts for cross-linking of bound IgE antibody. For most food extracts, the overall pattern of degranulation due to antigen-specific IgE reactivity paralleled IgE levels measured by ELISA (Fig. 3). However, unlike the ELISA assays, reactivity against roasted peanut in samples from orally exposed animals was notably lower than that from mice exposed subcutaneously (Fig. 3A). Additionally, reactivity from animals exposed to egg white via either route was slightly lower than expected (Fig. 3C). As predicted by the ELISA data there was no reactivity against spinach extract except in sera from animals exposed subcutaneously (Fig. 3D), where a fairly robust IgE response was induced.

**Extended Oral Exposure Obscures Differential IgE Responses to Allergens and Nonallergens**

As shown in Figure 2, two oral exposures to food extracts in the presence of CT leads to high or moderate IgE responses to allergens (roasted or raw peanut, egg white, Brazil nut) and low IgE responses to nonallergenic foods (spinach, turkey). 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 which more clearly distinguishes allergens from nonallergens. Along the same vein, we reasoned that administering a higher dose of 5 mg could potentially generate more obvious differential responses. Female C3H/HeJ mice were gavaged four times at a weekly interval with 1, 2, or 5 mg of roasted peanut, egg white, or spinach and 10 $\mu$g CT. The resulting IgE responses are shown in Figure 4. Consistent with previous results, roasted peanut was the most potent elicitor of IgE (Fig. 4A). However, under the revised experimental conditions spinach also elicited allergic antibody (Fig. 4C). IgE levels at the 5 mg dose were statistically significantly elevated compared to naïve levels ($p = 0.0007$), almost to the same extent as those at the 5 mg dose of egg white (Fig. 4B). A 5 mg dose of turkey elicited statistically significant increases in IgE compared with naïve levels after only two exposures (not shown). Levels of IgG1 were substantially increased after exposure to all food extracts and varied little by dose or extract tested (data not shown). Although IgG responses were similar to IgE, differences between dose groups were not significant and there were no statistically relevant differences in IgG among food extracts.

![Fig. 1](https://academic.oup.com/toxsci/article-abstract/102/1/100/1630940)
tested at any dose (data not shown). No IgE or other antibody was produced in the absence of CT (IgE, Fig. 4, all panels, 5 mg no CT).

Oral Exposure Model is Modified to More Accurately Reflect Ingestion

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). If intact protein is truly required for sensitization, we would expect only digestion-stable food extracts to yield substantial IgE responses in orally exposed mice. However, initial experiments included sodium bicarbonate for oral dosing, hindering digestion, and potentially making digestible proteins available for allergic responses. In order to more closely mimic physiological sensitization conditions, we repeated the two-exposure dosing regimen without sodium bicarbonate. Results are shown in Figure 5. IgE production was elicited by roasted peanut (Fig. 5A) and Brazil nut (Fig. 5C) and to some extent by egg white (Fig. 5B), but not by turkey (Fig. 5D). In vitro digestion assays demonstrate that peanut, Brazil nut, and egg white all contain digestion-stable proteins or protein fragments after pepsin treatment, in contrast to turkey, which is completely digested after 15 min (Fig. 5E).

**DISCUSSION**

The present study suggests that it may be possible to distinguish allergenic foods from nonallergenic foods using a protocol in which food extracts are administered orally with CT in the absence of sodium bicarbonate, provided the exposure is short term (2 weeks) and the exposure dose does not exceed 2 mg. In contrast, we were unable to distinguish allergenic from nonallergenic food extracts using a subcutaneous route of exposure because most of the extracts produced comparable levels of IgE via that route. Additional food
extracts of known allergenicity will need to be tested to further develop and validate the oral exposure model as a method for assessing potential allergenicity. Our findings thus far suggest that there is good correlation between digestive lability of foods and failure to elicit an IgE response via the oral route, supporting the use of pepsin resistance in the current guidelines.

The initial mouse model for peanut hypersensitivity developed by Li et al. (2000) utilized the oral route of exposure in conjunction with CT and resulted in IgE against Ara h1 and Ara h2, the same proteins recognized by human IgE, validating the model as being relevant to human disease. A published abstract by Gaudry et al. (2004) described oral exposure of mice to allergenic (shrimp, peanut, walnut, cashew, and cod) versus nonallergenic (rice, chicken, and beef) food extracts with CT. High levels of IgE and IgG were generated in response to all allergens tested except cod, which elicited minimal IgE and lower IgG as did the nonallergens. Furthermore, they measured comparable IgE reactivity between allergen immunized mice and food-allergic patients by immunoblot, suggesting that in this model the murine reaction to food allergens is similar to that of humans.

FIG. 3. IgE functionality assessed by RBL cell assay: roasted or raw peanut (A, B), egg white (C), or spinach (D). Results are reported as a percentage of total release by Triton X treatment and are representative of two independent experiments. * Indicates statistically significant increase over naïve levels ($p < 0.05$).

FIG. 4. Extended oral exposure: extract-specific IgE to roasted peanut (A), egg white (B), or spinach (C). Results are reported as mean absorbance values + SE ($n = 7–8$). * Indicates statistically significant increase over naïve levels ($p < 0.05$).
In this study we clearly demonstrate allergic antibody production after oral exposure to allergenic peanut, Brazil nut and egg white, but not to nonallergenic spinach and turkey. We noted that the magnitude of IgE responses to these orally administered foods correlated with their observed digestion stability. Peanut contains pepsin-resistant proteins (Fig. 5E) (Astwood et al., 1996; Fu, 2002; Fu et al., 2002), and elicits high IgE responses orally in our study as well as numerous others. The functionality of this allergic antibody response has previously been demonstrated by anaphylaxis upon oral challenge of similarly sensitized mice (Li et al., 2000), although this was not necessarily reflected by reactivity in our RBL assay. Egg white also contains digestion-stable proteins and induces moderate IgE levels. The major spinach protein, ribulose-1,5-bisphosphate carboxylase/oxygenase (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, even though it clearly evokes IgE production when administered subcutaneously. Few data were available regarding the digestibility of Brazil nut or turkey proteins, but the presence of stable fragments in digested Brazil nut (Fig. 5E) and their complete absence after digestion of turkey is consistent with the respective IgE responses observed in mice following oral exposure, particularly when sodium bicarbonate is excluded from the dosing regimen. This association of IgE responses with foods containing digestion-resistant proteins, along with evidence that

FIG. 5. Oral exposure without sodium bicarbonate: extract-specific IgE to roasted peanut (A), egg white (B), Brazil nut (C), or turkey (D). Results are reported as mean absorbance values ± SE (n = 6–8). * Indicates statistically significant increase over naive levels (p < 0.05). (E) SDS-PAGE analysis of native or digested food extract proteins. Digests were performed by incubation with pepsin for 15 min at pH 2.0.
digestion-stable fragments from both peanut and Brazil nut contain IgE epitopes (Bannon et al., 2003; Moreno et al., 2005), lends credence to the use of digestibility data as criteria for allergenic hazard identification.

Although egg white is considered a major human allergen, and certainly 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 (American Academy of Allergy, 2005), raising the possibility that adult mice do not represent a susceptible test population for egg. Additionally, the major egg white protein, ovalbumin, is the model antigen for oral tolerance induction, and because IgG 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. 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, 2007). The specific inhibition of IgE responses by oral tolerance induction suggests that it should be considered in assessing allergenic potential.

Subcutaneous injection resulted in IgE to both allergenic and nonallergenic foods, indicating that this particular route of exposure will not generate differential responses in accordance with known allergenicity. 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 (Fig. 1). Furthermore, subcutaneous injection of raw peanut, which should be highly allergenic, elicited low IgE responses. Although some studies have demonstrated IgE elicitation by ip injection with peanut agglutinin (Dearman et al., 2001) and whole peanut extract adsorbed to alum (Adel-Patient et al., 2005), another study found Ara h-6-specific IgE antibody levels to be lower in animals exposed to peanut extract ip as opposed to orally with CT (van Wijk et al., 2004). Additionally, a multilaboratory study evaluating ip injection of five strains of mice with the purified peanut antigen Ara h 2 demonstrated low levels of IgE production (Thomas et al., 2005), indicating that this route may be highly sensitive to the type of preparation and specific proteins introduced. The same multilaboratory study presented findings similar to our own with regard to supposedly nonallergenic spinach antigens. Even using purified spinach protein (RUBISCO), significant IgE responses were detected by all four laboratories after ip injection without adjuvant (Thomas et al., 2005). The investigators proposed that bacterial endotoxin contamination of the protein preparation may have enhanced the allergic antibody response. The food extracts employed in the current study contained little more endotoxin than the HBSS vehicle (less than 2 units/ml), except for the raw peanut preparation, which contained 11 units/ml (data not shown). 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 (Thr469–474) 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.

The oral exposure protocol requires further development with an expanded test panel of known allergenic and nonallergenic foods and subsequent validation by independent laboratories prior to its use in assessing novel proteins of unknown allergenicity. However, based on our findings using a limited number of food extracts, we propose that those foods containing digestion-stable proteins are more likely to elicit IgE after oral exposure with adjuvant 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 which do not induce oral tolerance but also lack oral sensitization potential (spinach, turkey) pose minimal risk. Taken together, results from this study and others support oral exposure over injection as a preferred method for distinguishing allergens from nonallergens. Experiments are under way in our laboratory to evaluate allergenic foods such as sesame and nonallergenic foods such as lamb and pinto bean for their oral sensitization potential and digestibility, along with their ability to induce oral tolerance as part of a weight-of-evidence approach to allergenic risk assessment.

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