

Failure to Induce Oral Tolerance in Mice Is Predictive of Dietary Allergenic Potency among Foods with Sensitizing Capacity

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Animal models are needed to assess novel proteins produced through biotechnology for potential dietary allergenicity. Currently proposed rodent models evaluate sensitizing potential of food extracts or proteins following parenteral administration or oral administration with adjuvant. However, food allergy requires not only the potential to induce immunoglobulin (Ig) E but also the capacity to avoid induction of oral tolerance (specific inhibition of IgE production). Here we describe a mouse model that assesses the potential of food extracts to induce oral tolerance. Adult C3H/HeJ mice were exposed orally to food extracts (without adjuvant) and subsequently challenged with the extract *ip*. Reduction of antigen-specific serum IgE relative to appropriate controls was used to indicate tolerance. Foods associated with persistent, severe allergy (peanut, Brazil nut), and nonallergens (turkey, spinach) were less tolerizing than foods associated with frequently resolving allergy (egg white). Digestibility was assessed *in vitro*, and pH alterations or encapsulation were used to modify solubility or digestibility. Egg white, peanut, and Brazil nut proteins were resistant to gastric enzyme (pepsin) degradation; turkey and spinach were not. Among pepsin-resistant proteins, peanut and Brazil nut appeared more sensitive to intestinal enzyme than egg white. For the extracts tested, full gastric digestion appeared to prevent induction of tolerance. Once through the stomach, only proteins resistant to intestinal enzymes induced tolerance. Limiting gastric digestion with sodium bicarbonate enhanced tolerance to peanut and Brazil nut. This model represents a complementary method of assessing potential allergenicity. Also, the conditions under which the test protein is encountered may impact experimental outcome.

Key Words: allergenicity; oral tolerance; food allergy; biotechnology; digestibility; genetically modified food.

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The advent of genetically modified food crops has prompted concerns regarding the potential introduction of novel allergens into the food supply. This is of particular importance given that food allergy currently affects 6–8% of children under the age of 4 and 3.7% of adults, can be fatal, and is increasing in prevalence (NIAID, 2006). The need for an animal model that can be used to screen novel proteins for potential allergenicity has been frequently cited (FAO/WHO, 2001, 2003). However, this has been difficult to achieve because the usual response to ingestion of antigen is oral tolerance, a process by which the immune system actively suppresses reactions to antigens upon subsequent exposure. Oral tolerance generally prevents adverse reactions to antigens from food and commensal organisms and can include suppression of both cell-mediated and humoral immune responses, particularly immunoglobulin (Ig) E and delayed-type hypersensitivity (Christensen *et al.*, 2003; Saklayen *et al.*, 1984). Previously, we reported a mouse model that distinguishes allergenic from nonallergenic food extracts using oral sensitization with cholera toxin (Bowman and Selgrade, 2008). Here we report on a complementary model which further distinguishes highly potent allergens following oral administration without adjuvant based on the development (or not) of tolerance.

Food allergies are generally associated with a limited number of foods; cow's milk, egg, peanut, soy, wheat, and fish are prominent allergens in children, whereas adults are most often allergic to peanut, tree nuts, fish, and shellfish. The exact characteristics that give certain foods allergenic potential are unclear but must include both the capacity to sensitize (induce an IgE response) and the ability to avoid oral tolerance. Many (but not all) allergenic proteins are heat stable, glycosylated, 10–70 kDa in size, and resistant to degradation by acid and digestive enzymes. A concerted research effort has attempted to identify truly definitive characteristics shared by these proteins which might confer allergenicity. Several databases have been developed to characterize protein structures which may be associated with IgE induction (Gendel, 2004; Mari *et al.*, 2006). Much less attention has been paid to characteristics of foods which may prevent induction of oral tolerance.

Food allergic patients may lack oral tolerance; however, the specificity of allergic reactions indicates that tolerance is lacking only to a small subset of orally encountered antigens. This then begs the question of whether food allergens are somehow excluded from the oral tolerance pathway and therefore unable to dampen the allergic IgE produced against them. While the parameters of oral tolerance have been widely studied with respect to both immunologic factors and the nature of antigen delivery, the ability to induce oral tolerance in mice has only been demonstrated for select dietary antigens using highly varied experimental protocols (egg, milk, and peanut) (Carr *et al.*, 1986; Kjaer and Frokiaer, 2002; Michael, 1989; Mizumachi and Kurisaki, 2002; Peng *et al.*, 2004; Priault *et al.*, 2003; Strid *et al.*, 2004). We hypothesized that foods known to cause allergy, particularly those causing persistent allergy (peanut and Brazil nut), would be less likely to induce oral tolerance than foods not associated with allergy (turkey and spinach) or food to which patients eventually become tolerant (egg white) and that differential ability to induce oral tolerance would be a useful indicator of potential allergenicity. Additionally, we examined the roles of digestibility and solubility in oral tolerance induction through antigen encapsulation, oral treatment with sodium bicarbonate, and *in vitro* pepsin and trypsin resistance assays.

MATERIALS AND METHODS

Animals

Female C3H/HeJ mice (6–9 weeks old; The Jackson Laboratory, Bar Harbor, ME) were group housed in polycarbonate cages with hardwood chip bedding in an environmentally controlled, Association for Assessment and Accreditation of Laboratory Animal Care-accredited vivarium. All animal procedures were reviewed and approved by the 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, Prolab RMH 3000, St Louis, MO, certified free of test proteins) 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. Ovalbumin (grade V or grade III) was supplied by Sigma-Aldrich (St Louis, MO). For experiments with encapsulated ovalbumin beads, microcrystalline cellulose cores were coated with ovalbumin (grade III, 30% coating), followed by acid-resistant Eudragit L30D55 enteric polymer (The Coating Place, Inc., Verona, WI).

Experimental Design

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 0.2M sodium bicarbonate. Control animals received HBSS only. For exposures to encapsulated ovalbumin, mice

received 20 mg ovalbumin or placebo beads with 50 μ l of acidified water orally under light isoflurane anesthesia (Isoflo; Abbott Laboratories, Chicago, IL). One week after oral exposure, mice were ip immunized with 100 μ g of the same food extract or protein in 200 μ l alum adjuvant (Alhydrogel 2%; Accurate Chemical & Scientific Corp., Westbury, NY). Blood was collected for antibody analysis 1 week after the ip immunization. Experimental groups consisted of six to eight animals each ($n = 6$ –8). Each experiment was repeated on at least one occasion with similar results to ensure reproducibility.

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 ELISA

All reagents and incubation periods were at room temperature for 1 h, and all volumes added were 50 μ l 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 μ l assay buffer (PBS/1% bovine serum albumin; Sigma-Aldrich), plates were incubated with serum samples diluted in assay buffer and 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, 1:40 for IgG1, and 1:80 for IgG were used for all ELISA assays 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 1N 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 Restimulation Assay

Spleens or mesenteric lymph nodes were harvested 1 week after oral exposure and passed through 70- μ m nylon cell strainers to prepare single-cell suspensions (BD Biosciences, Bedford, MA). Cells were counted, and viability was assessed using trypan blue exclusion and adenosine triphosphate measurement with the CellTiter-Glo Luminescent Viability Assay according to manufacturer instructions (Promega, Madison, WI). Splenocytes or lymphocytes from individual animals ($n = 5$ –8) were cultured in 24-well plates containing 0.5 ml RPMI with 10% fetal calf serum (Sigma-Aldrich, St. Louis, MO) at a concentration of 5×10^5 cells/well and were stimulated with 1 μ g/ml of relevant antigen, irrelevant antigen, Concanavalin A (Sigma-Aldrich), or medium only as positive and negative controls. Cell counts for proliferation after 24 h were performed using CellTiter-Glo. Data are represented as a percent increase in cell number over matched unstimulated control wells. Peanut, but not ovalbumin, caused nonspecific proliferation of naive cells (included in the results). Endotoxin levels in ovalbumin and peanut preparations were measured using a Limulus amoebocyte lysate test kit (BioWhittaker, Walkersville, MD) and were found to contain less than two endotoxin units per milliliter. 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.

Digestibility Assay

Assessment of pepsin and trypsin resistance was performed using a modified protocol similar to published methods (Dearman *et al.*, 2002; Fu *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). SGF (1850 μ l) was preheated to 37°C for 2 min in a 5-ml tube prior to the addition of 150 μ l of prewarmed 10 mg/ml test extract freshly prepared in distilled water. After 15 min, 1 ml each of neutralizing agent (0.2M Na_2CO_3) and 4 $\times$ NuPage lithium dodecyl sulfate (LDS) sample

buffer (glycerol 10%, Tris base 141mM, Tris-HCl 106mM, LDS 2%, ethylenediaminetetraacetic acid 0.51mM, SERVA Blue G250 0.22mM, and phenol red 0.175mM, pH 8.5) were added before transferring to ice. Simulated intestinal fluid (SIF) was prepared as follows: 1 mg of trypsin (Gibco-Invitrogen, Carlsbad, CA) was added to 5 ml of 0.05M KH_2PO_4 , pH 7.45. SIF (1850 μl) 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 or 30 min, the reaction was stopped with LDS sample buffer and immediate heating. All samples were heated for 5 min at 95°C and cooled to room temperature before loading 13.5 μl onto a 10% Bis-Tris polyacrylamide gel for nonreduced sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis (NuPage reagents; Invitrogen, Carlsbad, CA).

Statistical Analyses

The data were analyzed with GraphPad Prism 4 software (GraphPad, San Diego, CA) using one-way ANOVA followed by Tukey multiple comparison test. Student *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

Food Extracts Vary in Their Ability to Induce Oral Tolerance

Consistent with other studies, prior oral exposure of C3H/HeJ mice to purified ovalbumin resulted in reduced ovalbumin-specific serum IgE responses to ip immunization compared to responses in animals receiving only ip immunization. The reduction in antigen-specific IgE indicates that oral tolerance to ovalbumin was induced (Fig. 1A). When the same experimental protocol was performed with egg white extract, of which ovalbumin is a major component, egg white-specific IgE responses were suppressed by prior oral exposure (Fig. 1B). These results demonstrate that like ovalbumin, egg white induced oral tolerance in mice under similar conditions. In contrast, IgE responses to immunizations with raw or roasted peanut and Brazil nut were not inhibited by prior oral exposure to 1 mg of food extract (Figs. 2A–C). Our results indicate that unlike ovalbumin and egg white extract, peanut and Brazil nut extracts were unable to induce oral tolerance under the same experimental conditions. Similarly, extracts of spinach and turkey failed to induce oral tolerance (Figs. 2D and 2E). Levels of antigen-specific total IgG and IgG1 were not altered by prior oral exposure (data not shown).

Digestibility of Food Proteins in Whole Extracts

Because oral tolerance requires antigen processing and presentation by the immune system either within or beyond the intestinal mucosa, a protein's ability to survive enzymatic degradation after ingestion is likely to have an effect on oral tolerance induction. To examine the digestibility of food proteins in whole extracts, *in vitro* pepsin and trypsin resistance assays were performed in SGF and SIF. As shown in Figure 3A, egg white, Brazil nut, and peanut extracts all contain proteins which resist complete gastric digestion after a 15-min incubation in SGF. Turkey and spinach, however, were completely

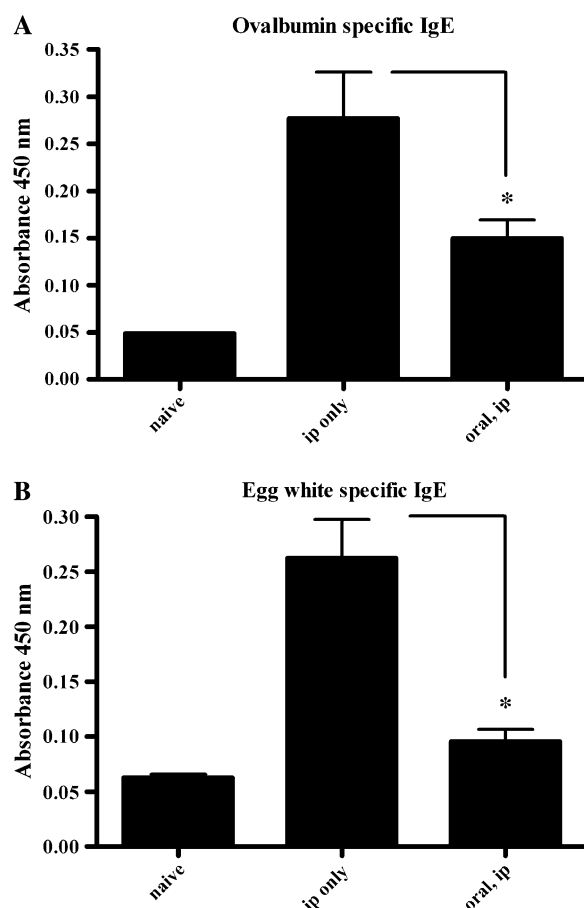


FIG. 1. Levels of serum IgE specific for ovalbumin (A) and egg white (B). Animals were orally exposed to 1 mg of total protein 1 week prior to ip immunization to assess oral tolerance induction (oral, ip). Animals in ip-only groups received no prior oral exposure. Results are reported as mean absorbance values + SE (*n* = 8). “*” Indicates statistically significant reduction compared to levels in animals receiving only the ip immunization (*p* < 0.05).

degraded in the same amount of time, indicating that these proteins would not be likely to survive passage through the stomach. SGF treatment of egg white yielded products larger than 40 kDa, whereas the pepsin-resistant proteins in peanut and Brazil nut were 17 kDa or smaller. To determine whether foods able to survive the stomach would also survive the intestinal tract, egg white, peanut, and Brazil nut were incubated in SIF for 15 or 30 min. All three foods contain trypsin-resistant proteins (Fig. 3B), but most proteins under 30 kDa were degraded after 30 min, thereby eliminating most peanut and Brazil nut proteins able to resist gastric digestion.

Altering Antigen Solubility or Digestibility Affects Oral Tolerance

Acidification (pH 3.0) of an ovalbumin solution resulted in visible aggregation and precipitation of ovalbumin protein. Oral exposure to 5 mg of ovalbumin prepared by this method failed to induce oral tolerance, as demonstrated in Figure 4A.

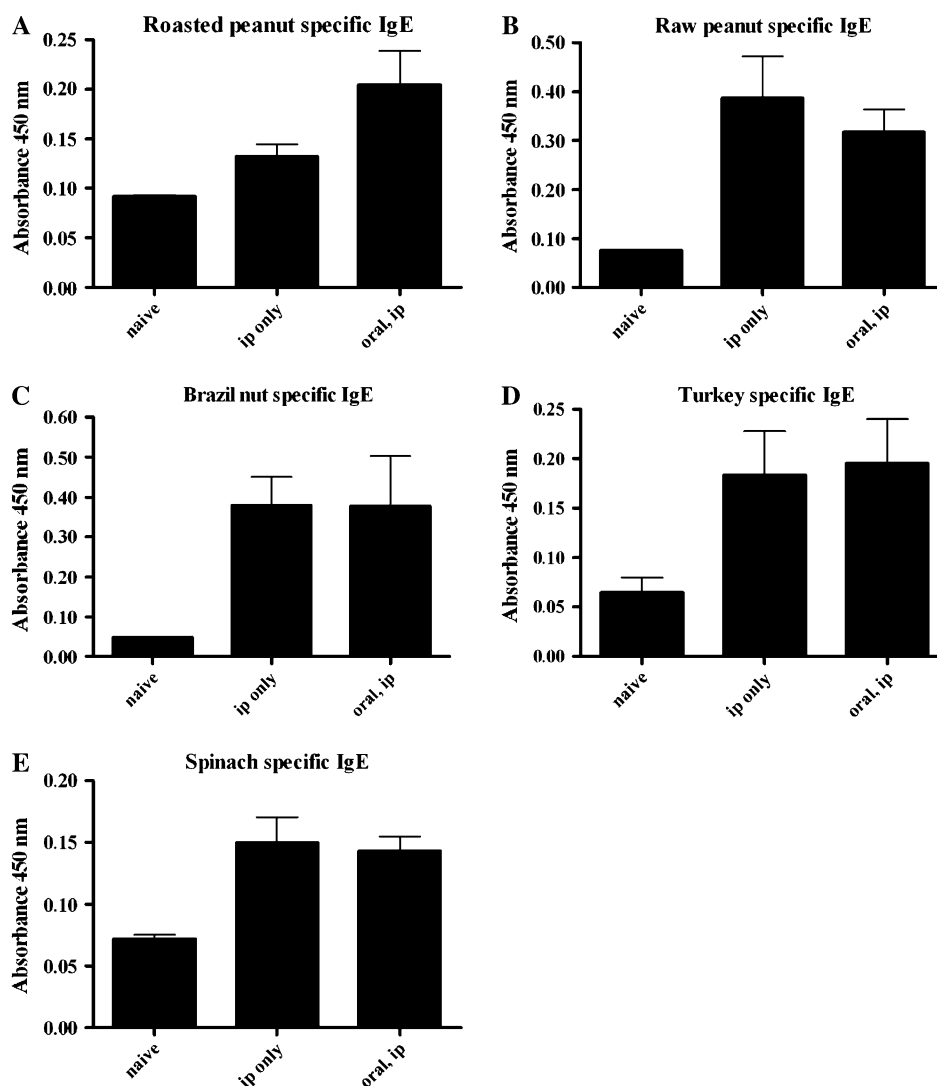


FIG. 2. Levels of serum IgE specific for roasted peanut (A), raw peanut (B), Brazil nut (C), turkey (D), and spinach (E). Animals were orally exposed to 1 mg of total protein 1 week prior to ip immunization to assess oral tolerance induction (oral, ip). Animals in ip-only groups received no prior oral exposure. Results are reported as mean absorbance values + SE ($n = 7-8$).

Encapsulation of ovalbumin in acid-resistant copolymer beads protects the protein from digestion, releasing it in the small intestine in a pH-dependent manner. As shown in Figure 4B, feeding encapsulated ovalbumin to mice did not induce oral tolerance, a result consistent with those from other studies (Jain *et al.*, 1996a, 1996b). The beads themselves are not significant inhibitors of tolerance induction since soluble ovalbumin given with placebo beads resulted in reduced IgE levels (not significantly different from naive levels or ip-only levels).

Sodium Bicarbonate Enhances Oral Tolerance to Some Foods

The addition of 0.2M sodium bicarbonate to roasted peanut extract increased the pH from 7.5 to 8.8 and reduced the opacity of the solution, indicating greater solubility. To assess the effect

of this preparation method on oral tolerance induction, mice were orally exposed to 1 or 2 mg of peanut extract with or without sodium bicarbonate. As shown in Figure 5A, oral tolerance to peanut was significantly enhanced by the addition of sodium bicarbonate at both doses. In additional experiments to directly compare inhibition of digestion by pretreatment with sodium bicarbonate versus sodium bicarbonate added directly to the dosing solution, we observed equivalent levels of tolerance induction to peanut ($p > 0.05$; data not shown). Sodium bicarbonate also increased oral tolerance to Brazil nut (Fig. 5B), although these results did not reach statistical significance. Sodium bicarbonate addition did not bring about any obvious visible changes in the solubility of Brazil nut extract. Oral tolerance to egg white was not enhanced by the addition of sodium bicarbonate (data not shown).

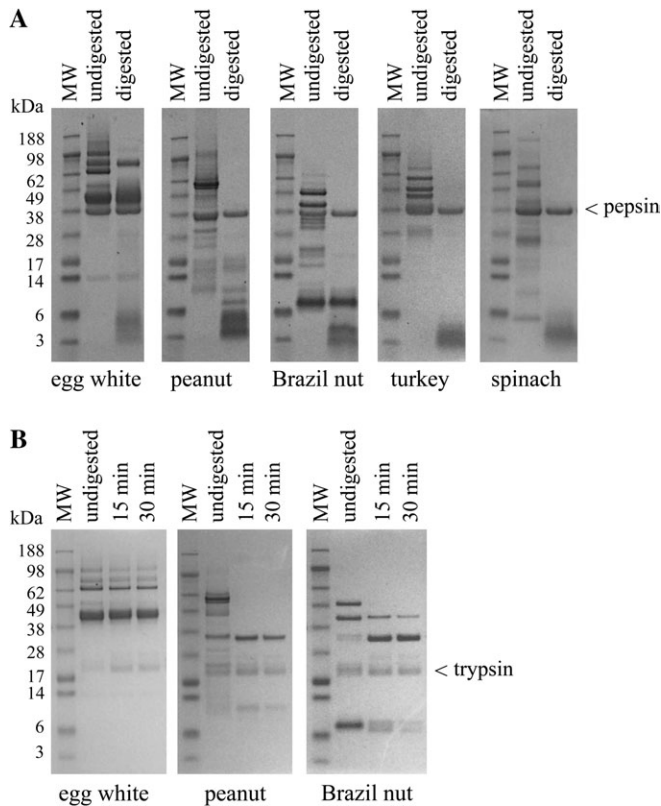


FIG. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of native or digested food extract proteins. Digests were performed by incubation with SGF (pepsin) for 15 min (A) or with SIF (trypsin) for 15 or 30 min (B).

Tolerance Demonstrated by Suppression of Proliferative Recall Responses

As another measure of tolerance induction, splenocytes or lymphocytes from animals orally exposed to ovalbumin or roasted peanut extract were stimulated *in vitro* to assess proliferative recall responses to the same antigen. Suppression of proliferation upon reexposure to antigen is a key feature of tolerance due to the generation of regulatory T cells (Battaglia *et al.*, 2004; Dubois *et al.*, 2003). In accordance with systemic tolerance demonstrated by serum IgE levels, splenocyte proliferation was suppressed in animals orally exposed to ovalbumin but not peanut (Fig. 6A). Cells from naive mice proliferated when exposed to peanut, most likely due to peanut mitogens (naive peanut control) (Novogrodsky *et al.*, 1975; Ryder *et al.*, 1992). Splenocytes from mice given peanut orally did not respond differently than those from naive mice. However, suppressed proliferation in response to peanut was observed in the mesenteric lymph nodes, indicating that some tolerance was initiated on a local level after peanut ingestion (Fig. 6B). Peanut administered with sodium bicarbonate resulted in slightly reduced proliferative responses compared to peanut alone at both sites, consistent with the enhanced tolerance demonstrated by IgE levels in Figure 5A.

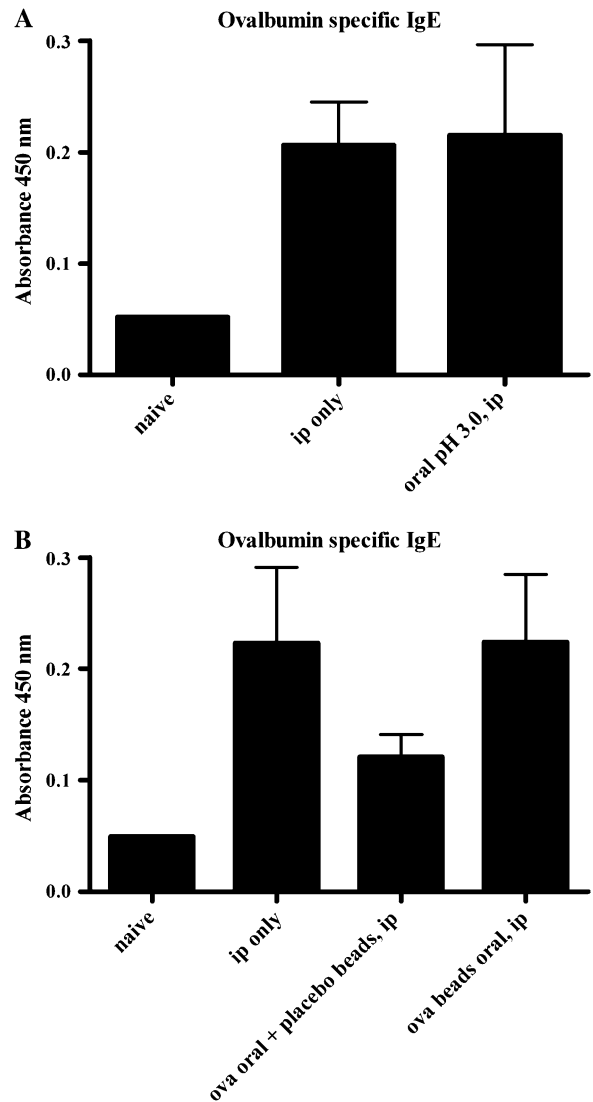


FIG. 4. Serum levels of ovalbumin-specific IgE after exposure to acidified (A) or encapsulated (B) ovalbumin. Animals were orally exposed 1 week prior to ip immunization with native ovalbumin to assess oral tolerance induction (oral, ip). Animals in ip-only groups received no prior oral exposure. Results are reported as mean absorbance values + SE ($n = 8$).

DISCUSSION

In this study, we demonstrate that food extracts vary in their capacity to induce oral tolerance. Extracts from foods known to be very potent allergens as well as extracts from nonallergenic foods failed to induce tolerance, but for different reasons. Different food extracts exhibited unique patterns of resistance to pepsin (stomach digestion) and trypsin (intestinal digestion), which appeared to be related to the ability to induce oral tolerance. Material preparation of the food extracts including pH and solubility also influenced the ability to induce tolerance. Our study suggests that a model of oral tolerance may be applied in assessing biotechnology products for

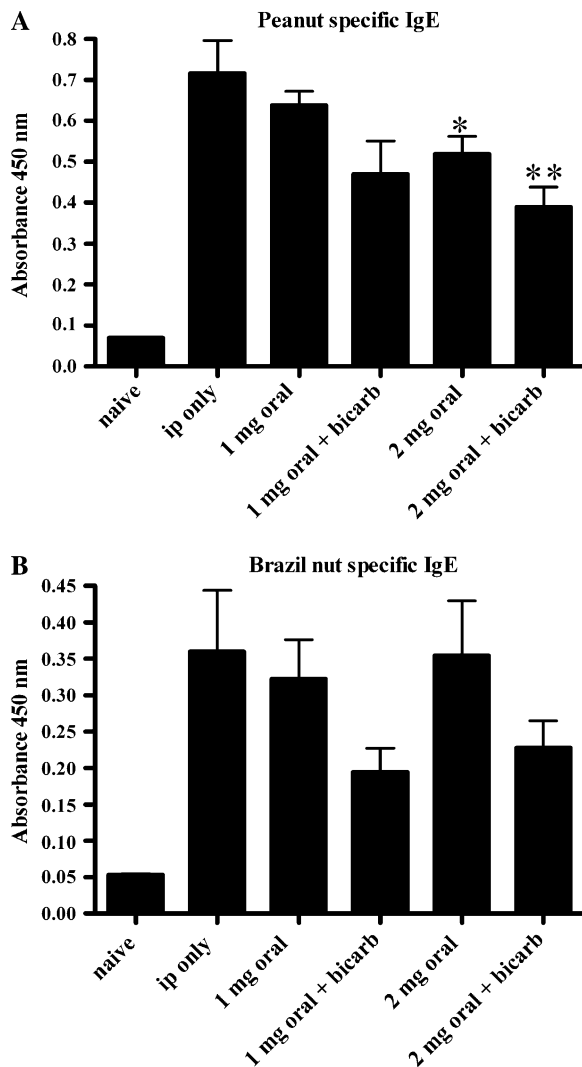


FIG. 5. Levels of serum IgE specific for roasted peanut (A) or Brazil nut (B). Animals were orally exposed to 1 or 2 mg of total protein with or without sodium bicarbonate (bicarb) 1 week prior to ip immunization to assess oral tolerance induction. Animals in ip-only groups were orally exposed to vehicle alone. Results are reported as mean absorbance values + SE ($n = 6-8$). Symbols indicate a statistically significant reduction compared to levels in animals receiving only the ip immunization (* $p < 0.05$, ** $p < 0.001$).

potential allergenicity and is complementary to other current and proposed approaches.

Ovalbumin, a model antigen for oral tolerance induction, and egg white extract, with high ovalbumin content, readily induced oral tolerance in C3H/HeJ mice. However, oral tolerance was not effectively achieved with extracts of raw or roasted peanut, Brazil nut, turkey, or spinach using the same experimental parameters. Although egg white proteins, including ovalbumin, are considered allergenic, egg white allergy is predominant in children and frequently outgrown (Boyano-Martinez *et al.*, 2002). Therefore, oral tolerance to these proteins in adult mice does not necessarily contradict their

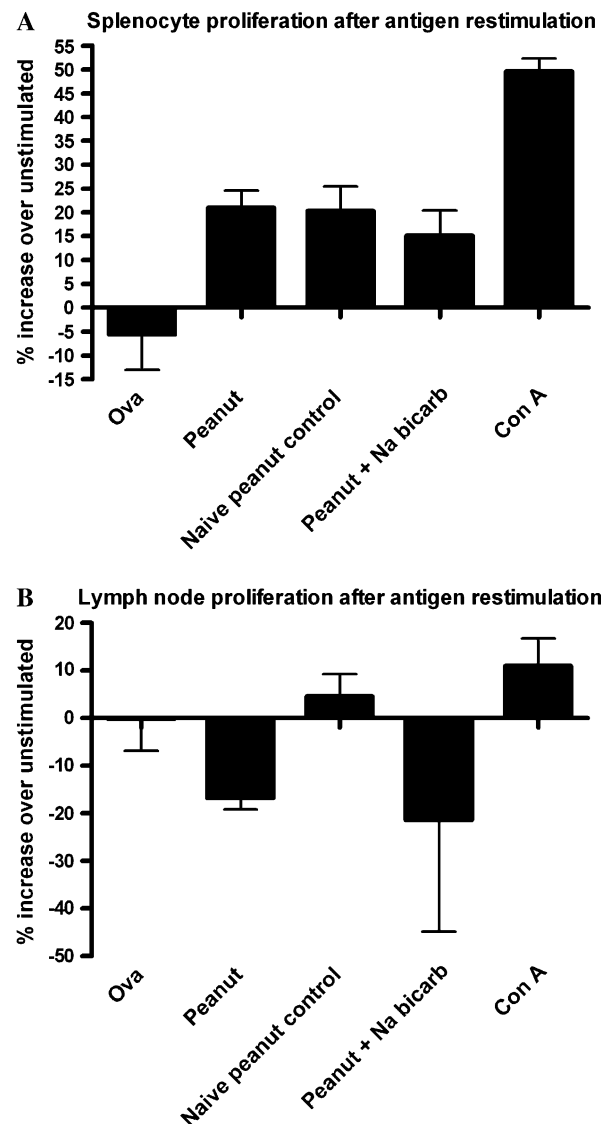


FIG. 6. Proliferative antigen recall responses in the spleen (A) or mesenteric lymph nodes (B). Animals were orally exposed to 1 mg of ovalbumin or peanut extract with or without sodium bicarbonate (Na bicarb) 1 week prior to harvesting of spleens and lymph nodes to assess cellular proliferation upon reexposure to the same antigen. Results are reported as percent increase in cell number over matched unstimulated controls + SE ($n = 6-8$). Naive peanut control demonstrates nonspecific proliferation of cells from naive animals in response to peanut in culture.

allergenic status. For example, oral tolerance to ovalbumin in neonatal BALB/c mice is defective (Strobel and Ferguson, 1984), a phenomenon we have also observed in neonatal C3H/HeJ mice (Bowman and Selgrade, unpublished study). The reduced capacity of peanut and Brazil nut to induce oral tolerance compared to egg white is consistent with the persistence and severity of allergies to these foods in humans. It should be noted, however, that this model for oral tolerance does not include the oral and sublingual exposure that would accompany normal ingestion. Defective oral tolerance to

peanut has also been observed in BALB/c mice (Strid *et al.*, 2004). We propose that the lack of oral tolerance is due to enzymatic degradation and that egg white proteins induce tolerance by resisting both stomach and intestinal enzymes.

Oral tolerance in mice is major histocompatibility complex dependent and antigen specific, indicating a requirement for available intact peptides following ingestion. Furthermore, generation of systemic tolerance requires transcytotic trafficking of antigens from the small intestine into the bloodstream to the liver (Li *et al.*, 2004; Ostman *et al.*, 2005; Yang *et al.*, 1994). Thus, an antigen must survive exposure to both pepsin in the stomach and trypsin in the small intestine to some degree before crossing the intestinal barrier to enter the hepatic portal. Ovalbumin and other egg white proteins are known to resist complete digestion (Astwood *et al.*, 1996) and therefore meet this requirement. Turkey and spinach proteins are fully degraded by SGF (Astwood *et al.*, 1996); thus, it is not surprising that they lack oral tolerance induction capabilities, given that they would fail to provide intact peptides to the intestinal mucosa after passing through the stomach. This is consistent with reports of defective tolerance induction in mice exposed to extensively hydrolyzed milk proteins (Peng *et al.*, 2004). Interestingly, turkey and spinach also failed to exhibit oral sensitizing potential in a mouse model of food allergy (Bowman and Selgrade, 2008). Their lack of IgE elicitation when administered orally with adjuvant is likely also a function of their gastric lability; when sodium bicarbonate was included to limit digestion, we observed increased oral sensitization to turkey. However, this phenomenon was not observed with egg white, peanut, or Brazil nut, which all contain pepsin-resistant proteins (Astwood *et al.*, 1996; Koppelman *et al.*, 2005; Kopper *et al.*, 2004) and are clearly able to sensitize via the oral route in humans and mouse models (Bowman and Selgrade, 2008; Li *et al.*, 2000; van Wijk *et al.*, 2004).

Egg white, peanut, and Brazil nut appear to provide sufficient targets for sensitization after gastric digestion, but in the current study, these foods differed in their ability to orally tolerize mice. This phenomenon cannot be attributed to the abundance of any single protein, as peanut protein content has been reported as 50% Ara h3, (van Wijk *et al.*, 2005), and therefore comparable to egg white in that half the content is comprised of a single protein. Additionally, although peanut and Brazil nut appear to have more individual proteins (possibly due to multiple banding for a given protein under nonreducing conditions), turkey and egg white contain similar amounts of individual proteins and still differ considerably in their tolerizing capacity. The proteins surviving SGF seen in peanut and Brazil nut (≤ 17 kDa) appeared to be degraded by SIF; those of egg white, however, were larger (> 40 kDa) and also resistant to intestinal enzyme. Thus, the peanut and Brazil nut proteins which could survive the stomach appear to be sensitive to degradation by the intestine, while egg white contains proteins which resist both stomach and intestinal enzymes. Complete degradation of peanut via the combined

effects of stomach and intestinal enzymes has been demonstrated by sequential digests of peanut extract (gastric digests followed by intestinal digests) (Vieths *et al.*, 1999). Oral tolerance to peanut and Brazil nut was enhanced by adding sodium bicarbonate to the dosing solution. Sodium bicarbonate treatment increased the pH of the solution from 7.5 to 8.8, likely inhibiting stomach pepsin activity during oral exposure (Kopper *et al.*, 2004). Limiting digestion of peanut and Brazil nut may have preserved the stomach labile proteins, some of which were resistant to intestinal enzymes, and therefore supplied intact antigens to the intestine for immune processing and subsequent tolerance induction. Increased exposure to antigens, via larger or more frequent doses, appears to enhance tolerance induction, as seen with the responses to 2 mg of peanut or Brazil nut, as well as results from studies by van Wijk *et al.* (2007) using 6 mg of peanut or repeated doses of 1 mg. However, it appears that exposure is still a function of digestive stability as a 2-mg dose of turkey is insufficient for tolerance induction unless sodium bicarbonate is added (Bowman and Selgrade, unpublished results).

Although the preservation of intact targets appears important, certain antigens require some degree of digestion to induce tolerance (Jain and Michael, 1995; Michael, 1989). A lack of digestion was likely the basis for defective oral tolerance induction to encapsulated ovalbumin. We demonstrate that this form of ovalbumin failed to suppress IgE responses, consistent with results from other studies in which encapsulated ovalbumin failed to suppress and even increased ova-specific IgG1 and IgE in BDF1 mice (Jain *et al.*, 1996a, 1996b). Another study has shown that inhibition of digestion blocks oral tolerance to ovalbumin. Cimetidine treatment of mice prior to ingestion of ovalbumin leads to decreased tolerance, while oral administration of pepsin-treated ovalbumin to cimetidine-treated mice results in immune unresponsiveness (Jain and Michael, 1995). Taken together, these findings suggest that an intermediate level of digestion is optimal for tolerance induction.

While digestion is clearly pH dependent, pH also influences protein solubility. It is known that oral tolerance is more readily induced by soluble proteins (Schleimer *et al.*, 1982; Strobel and Mowat, 1998; Thomas and Parrott, 1974), like ovalbumin. Alterations in ovalbumin solubility by chemical or heat denaturation have been shown to reduce its tolerizing capacity in mice (Peng *et al.*, 1998; Stransky *et al.*, 1998). In this study, we show that an acidified ovalbumin solution containing precipitated protein failed to induce oral tolerance, suggesting that reduced solubility (or altered digestibility due to structural changes) interferes with tolerance induction. Peanut proteins tend to be insoluble (Maleki *et al.*, 2000; van Boxtel *et al.*, 2006) and, thus, may be less likely to participate in the tolerance pathway. The addition of sodium bicarbonate to an aqueous extract of roasted peanut resulted in a marked increase in solubility. The enhanced oral tolerance induction to peanut observed when sodium bicarbonate was added to the dosing solution may therefore have

TABLE 1

Summary of Oral Tolerance Induction and Oral Sensitization in Mouse Models for Assessing Potential Allergenicity

Activity in Mouse Models			
Food	Tolerizing	Sensitizing	Allergenicity
Peanut	—	+	High risk
Brazil nut	—	+	
Egg white	+ (*N)	+	
Turkey	—	—	↓
Spinach	—	—	
			Low risk

Note. *N, not in neonates (ovalbumin).

resulted from increased solubility of peanut proteins. Whether these changes in tolerance induction resulted from altered digestibility or solubility remains unclear, but a predominant role for digestibility is suggested by the identical results obtained when digestion was inhibited with sodium bicarbonate pretreatment prior to oral dosing with peanut compared with direct addition to the peanut dosing solution.

The ability to induce oral tolerance and suppress allergic IgE responses is an important consideration in a weight of evidence approach using animal models to assess potential allergenicity, summarized in Table 1. Taken together, the ability to sensitize and/or tolerize in these models are consistent with observed allergenicity as well as persistence and severity among allergens. The oral tolerance model, notable in that the oral exposure does not utilize adjuvant, complements the oral sensitization model by identifying foods unlikely to tolerize (peanut, Brazil nut) as high risk among those with the ability to sensitize. Foods which sensitize and tolerize (egg white) may still be defined as allergens by their ability to sensitize but may exhibit more moderate or age-limited allergenicity. An inability to tolerize, however, should not be taken as evidence of allergenicity (turkey and spinach) but instead must be considered in the context of sensitizing capacity (or lack thereof). Pepsin stability is clearly required for sensitization in animal models using the oral route (Bowman and Selgrade, 2008; Untersmayr *et al.*, 2003), but resistance to both pepsin and trypsin appears to be required for oral tolerance. Further validation of both models for testing of novel proteins is needed, but the results thus far demonstrate the importance of digestibility and, therefore, the need to carefully consider test material preparation and the matrix in which the food would be normally encountered. Ultimately, the role of digestive stability (either to pepsin or trypsin) may provide a biochemical means of assessing potential allergens.

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