

A neonatal swine model for peanut allergy

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Background: Peanut allergy represents a significant health threat in the United States. The factors contributing to the severity of the allergic response and the immunopathogenic mechanisms underlying peanut allergy remain to be completely characterized. As yet, no animal model has been developed that will completely mimic the physical, immunologic, and histologic features of food allergy.

Objective: The purpose of this investigation was to develop a neonatal pig model of peanut allergy that would mimic the allergic symptoms and the immunologic and histologic profile of human peanut allergy.

Methods: Newborn piglets sensitized intraperitoneally with peanut extract and cholera toxin were orally challenged repeatedly with peanut meal. Physical symptoms, including emesis, lethargy, diarrhea, and respiratory distress, were monitored to determine the allergic response. Immunologic assessment was conducted through use of skin testing and the antigenic response to peanut proteins. Histologically, tissues derived from the esophagus, stomach, small intestine, and colon were assessed for morphologic changes after the oral challenge.

Results: Peanut-sensitized pigs responded with physical symptoms that mimicked those seen in double-blinded, placebo-controlled oral food challenges to peanuts in children and adults. Skin testing suggested an IgE-mediated response; this was confirmed by a negative passive cutaneous anaphylaxis response of heat-treated sera obtained from peanut-sensitized animals. Damage to villi of the small intestine was similar to that seen in endoscopically obtained tissue specimens from certain food-allergic individuals.

Conclusion: The neonatal pig model of peanut allergy mimics the physical and immunologic characteristics of peanut allergy in human beings. The model will be useful for determining IgE-mediated mechanisms and conducting endoscopic histo-

logic assessment of tissues and immunotherapeutic intervention strategies with repeated allergen challenges. (*J Allergy Clin Immunol* 2002;109:136-42.)

Key words: Animal model, peanut allergy, food allergy

Peanut sources represent a major food that causes severe allergic reactions.¹ Although its actual prevalence is unknown, peanut allergy represents a significant health threat in the United States.² Studies from England suggest that children are being increasingly sensitized to peanut products.³ However, factors contributing to the increased prevalence of peanut allergy and to the severity of the allergic response following ingestion of peanuts, as well as the immunopathogenic mechanisms underlying peanut allergy, are still largely unknown.

Sensitization to dietary proteins has been attributed to a variety of factors, including the amino acid sequence of the protein allergens, their carbohydrate structure, and their stability with respect to digestive enzymes in the intestinal tract. Several in vitro allergenicity assays are available to assess IgE reactivity to proteins. However, access to human tissues and immune cells to study cell-cell interaction after a food-stimulated IgE-mediated event is limited, and no fully validated animal model is available to study food allergy. Significant differences with respect to immunophysiology and a lack of specificity in allergic sensitization and extrapolation of symptomatology of allergenic challenge in small animals are considered drawbacks. However, the physiological and immunologic similarity of pigs and human beings has made the pig an important large-animal model for biomedical research.⁴

Investigations performed in pigs and calves demonstrate similar hypersensitivity reactions that are more closely aligned with human responses.⁵ Developing piglets have an anatomy and nutritional requirements, a transient neonatal porosity of the gut to dietary proteins, a distribution and maturation of intestinal enzymes, an enteric absorption of antibody,⁶ and a sensitization/tolerance to cow's milk and soy proteins⁷⁻¹² that are similar to what is seen in young children.

Accordingly, questions regarding allergen structure, food-induced allergic sensitization/tolerance, serum IgE and IgG levels, the influence of cytokines with respect to inflammation, and membrane barrier permeability can be readily studied in the pig model. A pig experimental model for allergic inflammation in the airways shows characteristics of human asthma, such as airway obstruction and eosinophilia as well as a late-phase asthmatic

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Abbreviation used
SP: Soybean product

reaction following antigen challenge.¹³⁻¹⁵ Young pigs have also been used for compliance and stability testing during bronchial inflammation.¹⁶ Investigations into the swine model are still limited with respect to T_H1/T_H2 profiles and anaphylactic antibody responses; however, the outward physical symptoms are clearly similar to those seen in human beings receiving double-blinded, placebo-controlled food challenges.

This report describes the usefulness of the pig as a peanut allergy model that is consistent with the human allergic response.

METHODS

Pigs

Pregnant Large White/Landrace sows with prior histories of 12 to 16 piglets per litter were obtained from Tyson Farms (Plumerville, Ark). Animals were maintained according to National Research Council regulations and standards of the Institutional Care and Use of Animals Committee.

Diet formulation

Diets devoid of soy and peanut proteins were provided by Dr Charles Maxwell of the University of Arkansas in Fayetteville. Gestation diets contained grain sorghum and corn gluten meal supplemented with 0.5% lysine. Lactation formulations contained 0.65% lysine, and nursery diets contained 1.4% lysine and 24.4% lactose.

Pig experimental design

The experimental design is shown in Fig 1. Briefly, newborn piglets were intraperitoneally sensitized on days 9, 10, and 11 after birth with 500 μ g crude peanut extract containing 100 μ g cholera toxin and boosted on days 18 and 25. Two groups of animals in each litter served as controls; one group received PBS and the other received PBS carrier plus 100 μ g cholera toxin. Pigs were challenged at 1-week intervals by either intragastric challenge or skin testing. Young animals (aged 1-6 weeks) were easily restrained by physical means for oral challenges; animals aged ≥ 8 weeks required sedation with Rompon (100 mg/mL, 6-8 mg/lb body weight) and reversal with antidote (yohimbine, 2 mg/mL, 0.11 mg/kg delivered intravenously). Three independent observers made the evaluation of an allergic response using the criteria shown in Table I.

Cutaneous hypersensitivity

Cutaneous skin testing was performed by intradermal injection with 0.1 mL of protein in physiological saline solution in previously shaved flank regions of individual pigs. Protein concentrations were determined by colorimetric assay (BCA protein assay kit, Pierce Chemical Co, Rockford, Ill) and adjusted to provide 50 or 100 μ g per injection site. Peanut extract, Ara h 1, and Ara h 2 were used to assess skin reactivity to peanut proteins; physiological saline solution served as the negative carrier control, rice allergen extract as a nonrelevant allergen, and histamine HCl as a positive control. Fifteen minutes after injection, wheal and flare diameters were measured and recorded.

Passive cutaneous anaphylaxis

The abdomens of naive animals were shaved 24 hours before intradermal injection of 500 μ L unheated serum or heat-treated serum

(56°C for 4 hours) to inactivate IgE. Twenty-four hours later, 1.0 mL crude peanut extract (5 mg/mL) was injected intravenously. Wheal and flare measurements were made 30 minutes later and recorded.

Swine immunoglobulin titers

Serum samples were collected from piglets on day 7 and weekly thereafter. Specific antiserum to swine IgE is not commercially available; therefore, anti-peanut IgG present in the blood sera was detected by ELISA. For ELISA, 96-well microtiter plates were coated with 100 μ L per well of solutions consisting of 5 μ g/mL of protein concentration in 0.1-mol/L $\text{NaHCO}_3/\text{Na}_2\text{CO}_3$ (pH 9.6). Plates were sealed and incubated at 37°C for 4 hours. After incubation, the plates were washed 5 times with PBS/0.5% Tween 20. Serum samples and the swine IgG standard curve were added and incubated for 1 hour at 37°C. Plates were again washed 5 times, and goat anti-pig serum horseradish peroxidase conjugate (Jackson Labs, West Grove, Pa), diluted 1:2,000, was added for an additional 1 hour at 37°C. Plates were washed 5 times; 3,3',5,5'-tetramethylbenzidine substrate (KPL, Gaithersburg, Md) was then added and allowed to develop before the plates were read at 450 nm on a Molecular Devices Emax plate reader (Packard Instruments, Downers Grove, Ill). Antibody production is expressed in micrograms per milliliter for each animal tested.

Histology

Animals were sedated with Rompon/Telazol/Ketamine (0.15 mL/10 lb), and 200 to 300 mL blood was collected. The animals were then killed through use of Euthanol (pentobarbital, 1 mL/10 lb). Tissues were fixed in 3 to 5 volumes of 10% buffered formalin for standard paraffin embedding, sectioning, and hematoxylin and eosin staining.

Statistics

Log transformations of peanut IgG were done before analysis. Paired *t* tests were done on these and on the diameters. No adjustment was done for multiple comparisons; however, the exact *P* values are given so that a Bonferroni adjustment can be done if desired. Repeated identical values appear only once each in the graph.

RESULTS

Characterization of systemic anaphylaxis after oral challenge

Preliminary experiments with intragastric or intraperitoneal sensitization were performed with 250, 500, 1000 or 2000 μ g of peanut on days 5, 6, and 7 or days 9, 10, and 11 and boosted 1 and 2 weeks later. Positive clinical symptoms in approximately 25% of the animals that were orally challenged (*n* = 6 animals per group) with peanut were observed with doses of 250, 1000 or 2000 μ g of peanut in piglets sensitized by either route. Optimal sensitization was achieved with 500 μ g of peanut extract plus 100 μ g of cholera toxin administered intraperitoneally on days 9, 10, 11, 18, and 25 (*n* = 14 animals). Oral challenges performed on days 39 and 53 with 10 g of peanut meal resulted in grade 2 symptoms in 8 of 14 pigs.

Physical appearance of respiratory distress and anaphylactic shock requiring epinephrine (grade 3 symptoms) was identified in 3 piglets 30 minutes after oral challenge. In 11 of the 14 responders, evidence of diarrhea was also noted 12 to 18 hours after the oral challenge. Three sensitized animals failed to respond. The result of repeated oral

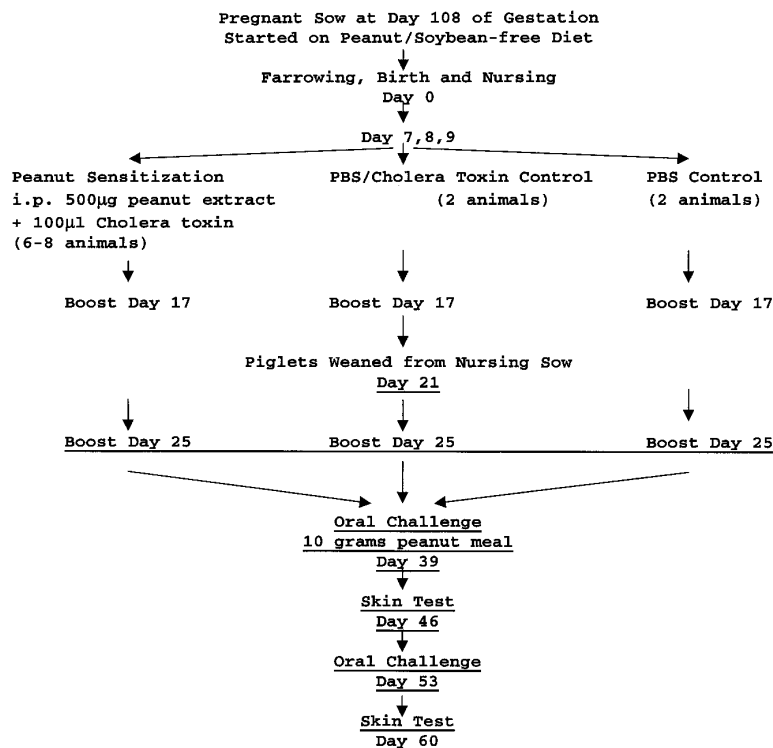


FIG 1. Flow diagram shows the experimental regime.

TABLE I. Symptomatic evaluation of allergen challenge

Grade	Symptoms
0	No evidence of any reaction or signs
1	Coughing, gagging, evidence of stomach contractions; reduced activity, tendency to lie down, easily activated to movement; minor rashes
2	Vomiting, lethargy/malaise, tremors; convulsions reduced activity that could not be activated by prodding; major areas of edematous rashes
3	Respiratory distress and symptoms requiring epinephrine; evidence of diarrhea 12-18 hours after challenge

challenges was that sensitized animals responded with increasing degrees of physical symptoms. Control animals receiving PBS or PBS/cholera toxin challenged with 10 g of peanut meal did not respond. Peanut-sensitized animals challenged with peanut/soybean-free diet failed to show any symptoms.

Cutaneous hypersensitivity and passive cutaneous anaphylaxis reactions

Skin prick test results with 50 and 100 µg of peanut extract were positive (5-15 mm with pseudopods) in peanut-sensitized animals. Positive histamine responses ranged from 6.5 to 18.5 mm with occasional pseudopods. Controls did not react to peanut or rice antigens (<3 mm). A typical skin response is shown in Fig 2, and the data from a representative litter are presented in Fig 3. Positive skin test responses have been confirmed to day 112.

One clinical nonresponder did not show evidence of skin reactivity; the remaining 2 nonresponding animals showed marginal responses (6-8 mm). Ara h 1 and Ara h 2 peanut skin tests confirmed sensitization to major peanut allergens (data not shown).

The intradermal passive transfer of normal and heat-treated sera from peanut-sensitized animals into naive animals confirmed that the clinical reactions were IgE-mediated. Intravenous challenge with 5 mg of peanut extract in 1.0 mL PBS 24 hours later demonstrated that heat treatment ablated the skin test response. Sera from control animals did not react in the passive cutaneous test. No delayed response (4-24 hours) was in evidence.

Immunoglobulin response

IgG data from a single litter, representative of all litters, is shown in Fig 4. Piglets at 3 days showed IgG values from 1 to 100 µg/mL that were considered to be cross-reactive IgG antibodies to legume proteins received in the colostrum. These values fell to levels of 0.1 to 0.5 µg/mL by day 11 of the sensitization phase. Sow colostrum IgG levels were not measured. The peanut-specific IgG reached a mean of 1318 µg/mL (range, 26-7700 µg/mL) by day 37 and tapered to a mean value of 456 µg/mL (range, 51-1516 µg/mL) at day 50. Peanut-specific IgG response varied from litter to litter and within each litter; however, the courses of the immune response were similar.

A preliminary correlation between IgG levels and clinical symptoms suggests that sensitized animals with

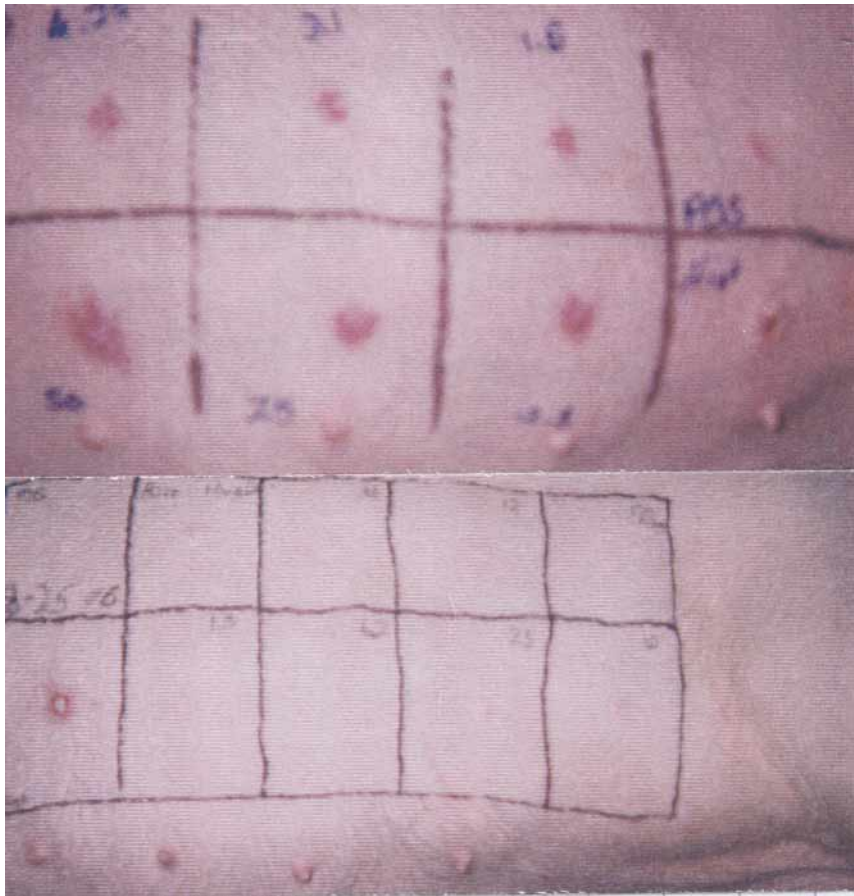


FIG 2. Representative photographs of skin test results. *Upper panel:* Skin test results 1 week after a positive oral challenge with peanut meal. *Lower panel:* Skin test results from a nonsensitized piglet.

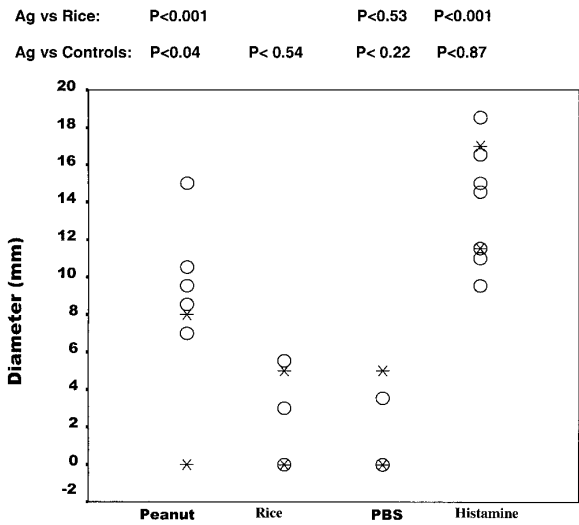


FIG 3. Intradermal skin challenge in peanut-sensitized and control swine at day 45. The mean for 7 peanut-sensitized animals (○) and the mean for 2 control animals (*) are represented. Representative P values are shown at the top of the figure. Identical values appear only once each.

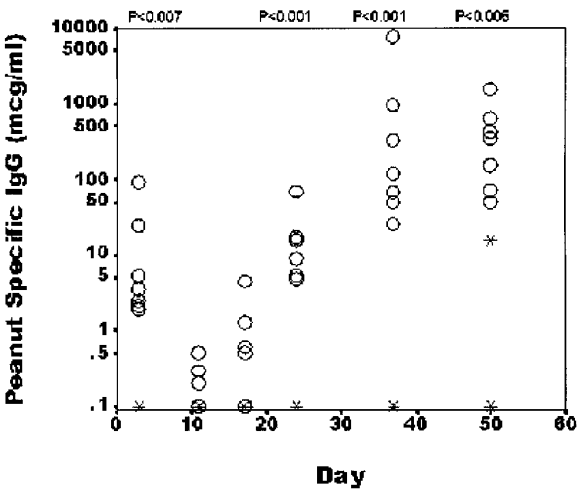


FIG 4. Peanut-specific IgG levels in peanut-sensitized and control swine. Results are expressed as the means for 7 animals (○) in the peanut-sensitized group and 2 control animals (*). Representative P values are shown at the top of the figure. Identical values appear only once each.

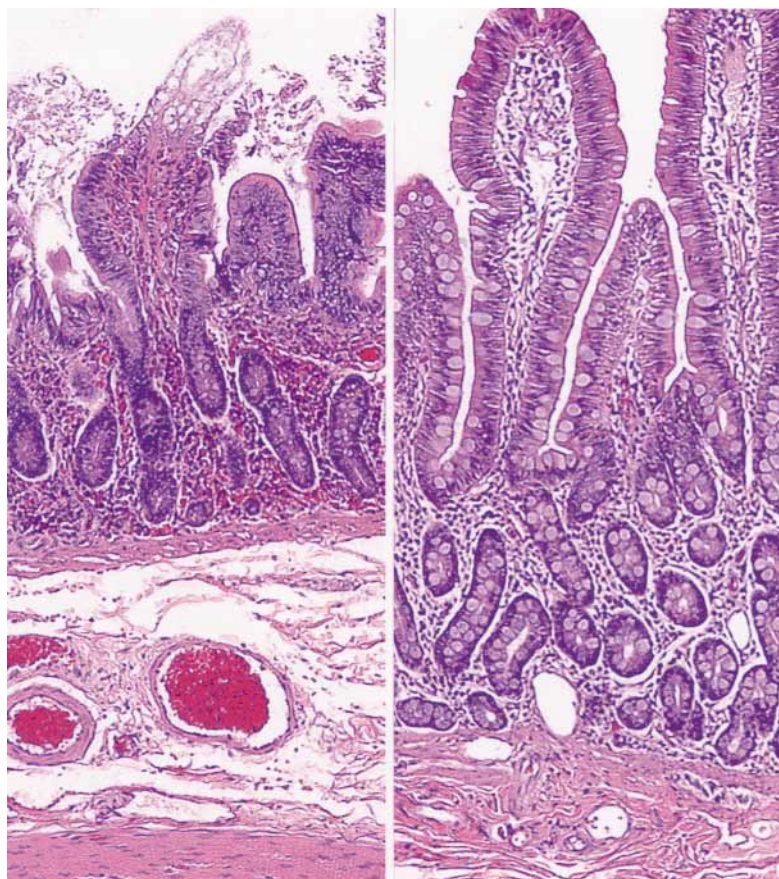


FIG 5. Histologic assessments of the small intestine in peanut-sensitized and nonsensitized control swine. **A**, Peanut-sensitized piglet after an oral challenge with peanut. **B**, Peanut-sensitized piglet that did not receive an oral challenge with peanut. Vascular congestion and edema of the mucosa and submucosa are evident in the sensitized swine sections (10 $\times$).

IgG levels greater than 500 $\mu\text{g/mL}$ receive a modest level of protection from food challenge. The significance of IgG in relation to protection will require more animals to be evaluated.

Histologic assessment

Minor amounts of peanut meal were observed in the stomachs and intestines of the peanut-sensitized animals; this compared with abundant quantities of soybean/peanut-free meal in the controls.

Gross tissue morphology and the histologic architecture of the distal esophagus, gastric antrum, duodenum, ileum, cecum, and rectosigmoid colon of 2 control and 2 peanut-sensitized piglets were assessed in 2 separate experiments. The most striking histologic feature was in the small intestine, where vascular engorgement and frank hemorrhage into the intestinal lumen were identified (Fig 5). In addition, prominent submucosal edema could be seen separating the muscularis propria from the mucosa. Focal denudation of the tips of the villi was evident, as was goblet cell mucus depletion. Within the lamina propria, an expansion of lymphocytes and macrophages was present, there being no evidence of increased neutrophils or eosinophils.

Esophageal tissue taken midway between the buccal

cavity and the entry into the stomach did not reveal any significant findings. In the stomach, the major finding was the presence of mild hemorrhage seen at the superficial surface of the glands, just below the epithelium. The colon presented with minor vascular congestion within the lamina propria; a mononuclear infiltration was observed within the mucosa.

DISCUSSION

The present study describes a neonatal swine model of peanut-specific food allergy. The physical symptoms following oral challenge were similar to those observed in human double-blinded, placebo-controlled food challenges. Skin rashes on the abdomen, back, and ears appeared within 30 to 45 minutes of oral challenge and resolved within 8 to 12 hours in at least 2 peanut-sensitized animals from each litter. Respiratory symptoms included difficulty in breathing, evidenced by panting and gasping in animals. Evidence of diarrhea was profound 12 to 18 hours after the oral challenge in approximately 3 of 14 sensitized piglets. Immunologically, repeated positive skin test results and passive cutaneous anaphylaxis suggested the presence of IgE antibody. Histologic assess-

ment revealed mucosal and submucosal edema and vascular congestion in the stomach, small intestine, and colon. In the small intestine, focal denudation of the tips of the villi, goblet cell mucus depletion, and an infiltration of lymphocytes and macrophages were evident. The physical, immunologic, and histologic evidence in this model suggests that the neonatal swine will be useful in determining mechanisms of gastrointestinal food allergy.

Gastrointestinal food allergy, a well-recognized clinical problem, is associated with a wide and variable spectrum of clinical responses, including cutaneous, respiratory, and gastrointestinal symptoms. Knowing which animal strain will respond is important in the selection of animals. For example, in allergen gene immunization protocols, AKR and BALB/c strains of mice—in contrast to the C3H strain—did not elicit any signs of anaphylaxis.¹⁷ In studies using female C3H/HeJ, high-IgE responder mice sensitized intragastrically with cow's milk¹⁸ or peanut¹⁹ plus cholera toxin exhibited the characteristics of IgE-mediated food allergy. In the murine model for peanut-specific anaphylaxis, a single episode of anaphylaxis, as well as a single animal responding with no activity after prodding or tremors and convulsions, was noted at the 3-week oral challenge. Decreasing symptom scores were associated with the following progression: wheezing with labored respiration and cyanosis about the tail and mouth; puffiness around the eyes and mouth, diarrhea, pilar erecti, and reduced activity; scratching and rubbing around the nose and head; and (in a single animal) no symptoms. At the 5-week oral challenge, the number of animals responding increased by 1 symptom score. In the swine model, symptom scores, though less descriptive, were comparable. Although no animal was allowed to proceed to death after a single oral challenge, 3 of 16 presented with significant respiratory distress and lethargy requiring epinephrine. Eight of 16 animals presented with vomiting, malaise/lethargy, tremors, convulsions, and rashes. In 3 of 16 animals, no overt symptoms were in evidence when the modified scoring system was used. On repeated challenge (either on the same day or at 2- and 4-week intervals), the symptoms increased in animals by at least 1 symptom score. Allergen-sensitizing properties of proteins by skin testing have also been performed in Balb/c mice²⁰ and atopic dogs.^{21,22}

Rodent studies offer the advantage of small animal size, large numbers, a well-defined immunologic system, and a reasonable cost; however, rodents do not vomit and their small size makes invasive techniques such as endoscopy difficult. In addition, rodent T_H1/T_H2 profiles are less ambiguous, and a second anaphylactic antibody mechanism is involved.

Although outbred animal strains are considered to be a disadvantage, Yorkshire pigs have been bred for high and low antibody response and cell-mediated immune responses.²³ Investigation into the swine model is still limited with respect to T_H1/T_H2 profiles and anaphylactic antibody responses. Other disadvantages include the cost, the limited availability of large-animal facilities, and the limited amounts of immunologic reagents. The

recent cloning, expression, and purification of pig IL-5, a key cytokine in allergic inflammation, will facilitate studies involving allergic pigs.²⁴ However, the physiological and immunologic similarity of pigs and human beings has made the pig an important large-animal model for biomedical research.⁴

In soybean allergy, both piglets and calves exhibit gut villus atrophy and gut accumulation of IgA (swine) and IgG1 (cattle) immunocytes, sustaining the view that a specific environment in ruminants is responsible for immunoglobulin production.²⁵ The altered immune response to proteins fed to piglets after neonatal exposure of the animals to antigen showed that very early oral exposure to antigen potentially affected the ability of neonatal animals to mount immune responses to specifically fed proteins while leaving the response to systemic antigen largely intact.²⁶ A transient hypersensitivity response to soybean protein, glycinin, and β -conglycinin in piglets that were fed soybeans suggested that the neonatal pig could be useful as a chronic food allergy model.²⁶ Results from sows that were fed ovalbumin as a novel protein antigen, either throughout gestation and lactation or during lactation, suggested that factors of immunologic importance could be passed from mother to offspring.²⁷ Anaphylactic reactions have been provoked in pigs by intravenous injections of chicken egg albumin given approximately 2 weeks apart after intravenous sensitization.²⁸

At present, there is no commercial or known assay available for the determination of swine IgE; however, considerable evidence is available that verifies the presence of swine IgE. A primary *Bordetella bronchiseptica* infection in *Ascaris suum*-infected pigs induced a type I hypersensitivity in the host.²⁹ Only native sera of infected pigs caused passive cutaneous anaphylaxis 24 hours after inoculation into guinea pigs. Heating the sera at 56°C for 4 hours abolished this property. When pigs were given either an antigenic soybean product (SP) or a nonantigenic SP, there was a larger erythema area in the antigenic SP group than in the nonantigenic SP group 30 minutes after intradermal injection of glycinin, but no significant difference could be detected with α - or β -conglycinin or whole soybean extracts.³⁰ In the swine model, the immediate skin test results to crude peanut extract and peanut allergens Ara h 1 and Ara h 2, combined with the loss of heat-inactivated, skin-sensitizing ability to these proteins by passive cutaneous anaphylaxis, also suggests an IgE-mediated mechanism.

Although the intraperitoneal route of sensitization is not likely to mimic oral induced sensitization, intraperitoneal sensitizations increased the number of animals responding to peanut sensitization and challenge. In the protocol for peanut sensitization, cholera toxin, which is known to stimulate a T_H2-type response in some animals,^{31,32} was used for its adjuvant effect. The advantage of using a known T_H2 adjuvant is indicated by the fact that the bacterin inoculations of *A suum* were suggested to provide an effective potentiator of heterologous IgE response.²⁹

The histologic assessment of the small intestine during the peanut sensitization study revealed denudation of the

villi, edema, and vascular congestion; these were similar to the marked small intestinal changes noted in the soybean hypersensitivity model.²⁶ Moreover, architectural abnormalities observed in the pig model for peanut allergy, including partial villous atrophy and cellular mucosal changes, have been observed in endoscopically obtained intestinal mucosa tissues of subjects with food allergy.³³

In conclusion, in comparison with other animal models, the pig has similarities to human beings with regard to digestive function that make it a good model by which to improve our knowledge of gut hypersensitivity reactions to food antigens that occur in various infant gastrointestinal pathologies. Questions regarding allergen structure, food-induced allergic sensitization/tolerance, serum IgE and IgG levels, the influence of cells and cytokines, and the effects of commensal/pathogenic microorganisms and their proteins that could influence the epithelial barrier in the gastrointestinal track can readily be studied in the pig animal model. The pig food allergy model will be useful for determining the factors contributing to the induction and mechanisms of IgE responses in young children and adults. Intervention and treatment options are being investigated to reduce the allergenic risk from hidden peanut sources.

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