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Esophageal eosinophilia accompanies food allergy to hen egg white protein in young pigs

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Abstract

Background: Esophagitis with eosinophilia, inflammation, and fibrosis represents a chronic condition in humans with food allergies.

Objective: In this investigation we asked whether esophagitis with an eosinophilic component is observed in young pigs rendered allergic to hen egg white protein (HEWP).

Methods: Food allergy was induced in young pigs using two protocols. In one protocol, sensitized pigs were challenged by gavage with a single dose of HEWP. Clinical signs were monitored for 24 h and then gastrointestinal (GI) tissues were collected for histological examination. The phenotype of circulating, ovalbumin (OVA)-specific T cells also was examined

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Conflict of interest

No conflicts were identified.

in HEWP-challenged animals. In the second protocol, sensitized animals were fed HEWP for 28 days. Animals were then examined by endoscopy and gastrointestinal tissues collected for histological examination.

Results: In pigs challenged by gavage with HEWP, clinical signs were noted in 5/6 pigs including diarrhea, emesis, and skin rash. Clinical signs were not seen in any control group. Histological analysis revealed significant levels of esophageal eosinophilic infiltration ($p < 0.05$) in 4/6 of these animals, with two also displaying eosinophilic infiltration in the stomach. Eosinophils were not increased in ileum or colon samples. Increased numbers of circulating, OVA-specific CD4⁺ T cells also were observed in pigs that received HEWP by gavage. In the group of animals fed HEWP, endoscopy revealed clinical signs of esophagitis including edema, granularity, white spots, and furrowing, while histology revealed edema, immune cell infiltration and basal zone hyperplasia.

Conclusions and Clinical Relevance: Food allergy in the pig can be associated with esophagitis based on histological and endoscopic findings, including eosinophilic infiltration. The young pig may, therefore, be a useful large animal model for the study of eosinophilic esophagitis in humans.

Keywords

eosinophil; esophagitis; food allergy; swine; animal model

1 | Introduction

Food allergies result from an inappropriate immune response against specific food proteins. In humans, the major allergenic foods identified by the Food and Drug Administration are milk, egg, fish, shellfish, tree nuts, peanuts, wheat, and soy (1). Many of the proteins in these foods that induce allergic responses have been identified; including the Ara h proteins in peanuts (2), and the proteins ovomucoid and ovalbumin in hen egg white (3). Food allergies are increasing in the human population with levels of food allergy to specific proteins reaching 10% in certain parts of the world (4). A number of reasons have been suggested for the increase in food allergy such as, low levels of sun exposure leading to reduced vitamin D production (5), changes in the microbiota (6, 7), and dietary compounds that arise from the Maillard reaction (8, 9).

Food allergies typically develop in two stages. Initially, an individual becomes sensitized to the allergen, during which time the immune response against the food protein is initiated. Sensitization has been linked to failures in the normal regulatory processes that inhibit immune responses against food proteins, including reduced levels of Treg cells and the tolerogenic cytokines IL-10 and TGF- β (10, 11). A number of additional host genes also have been implicated (12). Later, when a sensitized individual is re-exposed or “challenged” by the allergen, a vigorous T_H2 cell-type immune effector response ensues (4). T_H2- and ILC2-derived cytokines (13) such as IL-4 (14), IL-13 (15), and IL-33 (16) drive the canonical IgE-dependent allergic pathway which ultimately leads to release of mast cell mediators. A second pathophysiological pathway is activated by T_H2 derived IL-5, which drives the differentiation and proliferation of eosinophils (17). Eosinophils, in turn utilize chemokines

such as CCL26 (eotaxin-3) (18) and the chemokine receptor CCR5 (19) to accumulate in the esophagus, triggering an inflammatory disorder known as eosinophilic esophagitis (EoE) (20). Typically, EoE is chronic and progresses from inflammation to fibrosis; and it is characterized by symptoms of dysphagia and food impaction (due to esophageal strictures) in adults, and abdominal pain, heartburn, regurgitation/vomiting, and failure to thrive, in children (21). Diagnosis is made based on clinical symptoms, demonstration of marked esophageal eosinophil using upper endoscopy and biopsy, and after evaluating for other potential causes of esophageal eosinophilia (22).

Much of the available information on food allergies, including EoE has come from the mouse model. The small size, low cost, and ready availability of immunological reagents, coupled with an incredible wealth of biological information has made mice the standard model. On the other hand, there are several features of the murine anatomy and physiology, and the food allergic response in mice, which differ significantly from humans. For these reasons a number of groups are studying the food allergic response in pigs. The anatomy, physiology, life span, and the immune responses in pigs should make them an excellent alternative model to study human food allergy. The esophagus, in particular, is similar in size to the human esophagus allowing for the use of endoscopy. In addition, pigs display esophageal submucosal glands, which are not seen in rodents (23). Pigs can be sensitized to human food allergens, and pigs display many of the symptoms of human food allergy including, vomiting, diarrhea, skin rashes, and GI disorders (24–26). Pigs produce allergen-specific IgE and produce mast cell mediators that impact GI function. While a recent report showed that eosinophil levels were elevated in the stomach and skin of peanut allergic pigs (27) none have specifically examined esophageal eosinophilia in a porcine model. In this report we show that esophagitis with an eosinophilic component can develop in young, HEWP-allergic pigs.

2 | Methods

2.1 | Reagents

HEWP (Cat. # A5253), and dimethyl sulfoxide (DMSO) were purchased from Sigma Aldrich St. Louis, MO, USA. Cholera toxin (CT) (Cat. # 100 B) was obtained from List Biological Laboratories (Campbell, CA, USA). Ficoll-Plaque PLUS was obtained from GE Healthcare (Pittsburgh, PA, USA). Hank's balanced salt solution was purchased from Corning Cell Gro (Manassas, VA, USA). Buffered formalin (10%) was obtained from Fisherbrand and heat inactivated fetal bovine serum (FBS) was purchased from Gemini Bio (West Sacramento, CA, USA).

2.2 | Animal study design

Food allergy to HEWP was induced by two different protocols in this study. In the first protocol, forty-eight newborn crossbred pigs from 6 litters with 3–9 pigs per litter were used in this investigation. Pig diets were formulated to meet all nutrient requirements (28) and were devoid of egg-containing ingredients. Within 24 h of birth, each newborn piglet received 1 mL of penicillin and 2 mL of iron (100 mg mL⁻¹). All pigs were weaned on day 22 and moved to the nursery with 3 pigs per pen and maintained under normal husbandry

conditions at the North Carolina State University Field Laboratory (Swine Education Unit). Forty-two pigs were sensitized to HEWP while 6 were housed separately as negative controls. Following intradermal screening (see below), 18 with positive responses to HEWP were included in the challenge phase of these studies. In the second protocol, 3 Yorkshire pigs were obtained from the Swine Education Unit, and transported to North Carolina State University College of Veterinary Medicine (CVM) where they were housed in separate pens by Laboratory Animal Resources. On the day of endoscopy, they were held off feed 12-hours prior to transport to Central Procedures Laboratory at the CVM for general anesthesia. All animal use was approved by the Institutional Animal Care and Use Committee at North Carolina State University.

2.3 | Allergic sensitization and challenge

Pigs in the first protocol were sensitized with 500 µg HEWP and 10 µg CT *ip* on days 7, 14, and 21 of age (Fig. 1). On days 29 and 30, allergic status was screened via intradermal injections of 1 µg HEWP. Reactions to HEWP were categorized as strong, moderate, or absent. Eighteen animals with strong or moderate responses were then randomly assigned to experiment groups shown in this report. Then, on day 36, a group of 6 animals (group 1) received 10 g HEWP in 60 ml deionized water containing HEWP by oral gavage. A second group of 6 received only water while the third group remained untreated. Also, as a control for antigen exposure from the environment, a fourth group of age matched, but non-sensitized, non-challenged pigs, which were housed in an adjacent room of the same building. Gavage was performed using a 60 mL syringe attached to a 10Fr 16 in catheter directly into the stomach of the pig. Pigs were then monitored for 24 h by at least two researchers for clinical signs. Rectal temperatures were taken every 4 h and observations were made for the following symptoms; emesis, diarrhea, erythema, scratching, sneezing, lethargy and appetite. A four step grading system was used to characterize the severity of each symptom; “0” not observed, “1” mild, “2” moderate, and “3” severe. Epinephrine was kept on site in case of anaphylactic shock.

Pigs in the second protocol were sensitized with 500 µg HEWP and 10 µg CT *ip* on days 1 and 14. HEWP challenge began on day 28 with 10 g of HEWP in a mixture of strawberry jam (J. M. Smucker Co., Orrville, OH) and Karo corn syrup (ACH Food Companies, Inc., Oakbrook Terrace, IL) mixed in their feed and repeated daily for 28 days. Blood samples were taken at days 7, 14, 21, 28, 42, and 57. Endoscopy was performed on day 57. Skin testing was not performed on this set of animals.

2.4 | Peripheral blood mononuclear cell (PBMC) isolation from whole blood

Blood was diluted and layered over Ficoll-Plaque PLUS in 50 mL conical tubes and centrifuged at $400 \times g$ for 30 min at room temperature. The mononuclear cell layer was removed, re-centrifuged and red blood cell lysis buffer (155mM NH_4Cl , 12mM NaHCO_3 , 0.1 mM EDTA, doi:10.1101/pdb.rec079152, Cold Spring Harbor Protocols, 2015) was added. The remaining cells were washed and centrifuged 3X times with Hanks buffered saline solution. Buffer was decanted and freezing medium consisting of 10% dimethyl sulfoxide and 90% heat-inactivated FBS was added to each cell pellet. Cells were re-

suspended and transferred to cryogenic vials (BioExpress, Kaysville, UT, USA), stored at -80°C for 2 days and then transferred to liquid nitrogen storage.

2.5 | Euthanasia and tissue collection

Blood was collected for PBMC isolation from all animals prior to euthanasia using an AVMA-approved method of electrocution (110V, AC). Tissue samples collected post-mortem included esophagus, stomach, duodenum, jejunum, ileum, cecum, colon and mesenteric lymph nodes. Tissues were washed thoroughly with $1\times$ PBS, stored for 48 h in 10% neutral buffered formalin and then transferred into labelled cassettes containing 70% aqueous ethanol (v/v). Cassettes were then stored at room temperature for histology.

Paraffin-embedded tissues were sectioned ($5\text{ }\mu\text{m}$) and stained with hematoxylin and eosin (H & E). Histologic analysis was performed by a veterinary pathologist (LBB) who was blinded to experimental groups. Epithelial height measurements were acquired using imaging software (DP2-BSW, version 2.2, Olympus Corporation, Tokyo, Japan) operating a high-resolution digital camera (DP72, Olympus) equipped with a clinical light microscope (BX45, Olympus). Before imaging, the system was calibrated with the use of a stage micrometer. Measurements (10 per sample) were taken by using the arbitrary line tool and exported onto a spreadsheet program (Excel 2016; Microsoft) to calculate average epithelial heights. Lymphoplasmacytic infiltrate was scored from 0–4 as follows: 0= normal (minimal infiltrate); 1 = single focal mononuclear cell infiltrate in lamina propria; 2= multiple focal mononuclear cell infiltrates in lamina propria; 3= infiltrates involve a large area of mucosa and extend into the submucosa; 4 = transmural infiltrates. Eosinophils were enumerated in 10 consecutive fields at $400\times$ magnification (2.37mm^2) (29).

2.6 | Lymphocyte proliferation and phenotypic determination.

To determine OVA-specific proliferation, PBMC were stained with CellTrace™ Violet Cell Proliferation Kit (ThermoFisher Scientific, Waltham, MA, USA). After staining, 2×10^5 cells/well were cultured *in vitro* in 96-well round-bottom plates for 4 days in the absence or presence of $50\text{ }\mu\text{g/ml}$ OVA. After cultivation, cells were harvested and stained with primary antibodies against CD4 (clone 74-12-4, mouse IgG2b, BEI resources, Manassas, VA, USA), CD8 β (clone PG164A, mouse IgG2a, Kingfisher Biotech Inc., Saint Paul, MN, USA) and TCR- γ/δ (clone PGBL22A, mouse IgG1, Kingfisher Biotech Inc.) followed by secondary antibodies anti-mouse IgG1-AlexaFluor 488 (SouthernBiotech, Birmingham, AL, USA), anti-mouse IgG2a-RPE (SouthernBiotech), and anti-mouse IgG2b-AlexaFluor 647 (SouthernBiotech). PBMC were recorded on a BD LSR-II. Data analysis was performed with FlowJo version 10 (FlowJo LLC, Ashland, OR, USA).

2.7 | Graphics and Statistical analysis

Graphics and statistical analyses were performed using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). Unless otherwise indicated, statistical significance was determined using a one way ANOVA with Dunnett's multiple comparison post-test.

2.8 | Endoscopic methods

General anesthesia was induced by intramuscular injection of a combination of xylazine and ketamine (0.25mg/kg, and 11 mg/kg), followed by mask induction with isoflurane vaporized in 100% O₂. Pigs were orotracheally intubated, and maintained on isoflurane, after which, a standard upper endoscope (Olympus Q140) was introduced orally into the esophagus. The endoscope was advanced into the proximal stomach, and then the esophagus was carefully examined under direct visualization for any findings, including those related to EoE such as edema (decreased vascularity), white exudates, linear furrows, or esophageal rings. Esophageal biopsies were obtained from areas of interest using standard large capacity forceps (RJ4, Boston Scientific, Marlborough, MA, USA).

3 | Results

3.1 | Clinical signs of food allergy.

In this report we present results from two different experimental protocols for inducing food allergy in young pigs; an acute protocol where the HEWP challenge dose was delivered in a single treatment by gavage, and a chronic protocol where pigs were sensitized to HEWP slowly over a month through daily feeding and then challenged (Fig 1). Our original intent was to use the acute protocol animals to test several food additives for their ability to block acute food allergic responses, however, these additives became unavailable and we decided to refocus the experiments on a fundamental question of the food allergic response in pigs, i.e., do pigs display evidence of eosinophilic esophagitis? Based on the results from the acute protocol, we performed a follow up experiment with the chronic challenge protocol.

Our experiments with the acute protocol began originally with 56 animals of which 6 animals were housed separately and not exposed to HEWP during either sensitization or challenge to control for potential allergen exposure in the barn environment. The remaining 50 animals were injected ip with 500 µg HEWP and 10 µg CT at days 7, 14, and 21 to induce allergic sensitivity. Skin hypersensitivity testing was performed 1 week later and we noted three levels of reactivity. Twenty one animals developed a central, 1–2 mm wheal with appx. 1 cm of surrounding erythema. These animals were categorized as strongly reactive. In contrast, 20 pigs developed only erythema without the central wheal and were categorized as intermediate responders. Nine animals failed to display any noticeable response and these were labeled non-responders. Representative images of these responses, taken by cell phone camera in the barn, are shown in Supplemental Fig. 1. Due to our shift in focus we continued the experiment with 24 of these animals, 18 that were sensitized to HEWP and displayed either strong or intermediate reactivity, and the 6 control animals that were housed separately. The animals with strong or intermediate responses were pooled and divided randomly into 3 groups; one group receiving HEWP by gavage to induce an acute food allergic response, one group received water by gavage as a control, and one group that did not receive a challenge. Following challenge, the animals were monitored for 24 h for clinical signs of a response. We found that 5/6 pigs that received HEWP by gavage displayed at least one clinical sign. For example, moderate to severe diarrhea was observed in 4 animals (Fig. 2A), although because several cases of diarrhea were noted in the control groups, the diarrheal response of the challenge group did not reach statistical significance.

($p=0.06$). One of the 4 animals with diarrhea also displayed emesis (Fig. 2B) while 2 of the animals with diarrhea also displayed severe lethargy (Fig. 2C). The fifth animal in the HEWP-challenge group displayed a severe erythematic rash on its entire abdomen and chest (Fig. 2D) but not any other symptoms, while the sixth animal did not display any clinical signs. Body temperatures remained constant and similar between the groups (data not shown).

3.2 | Eosinophilic esophagitis.

Following euthanasia, GI tissues were collected, fixed, and sections stained with H&E to identify the eosinophils. We found that esophagi from 4/6 pigs challenged with HEWP displayed strong evidence of eosinophilic esophagitis as defined by >15 eosinophils/high power field (HPF) ($p<0.05$), with a mean count of 29 eosinophils/HPF (Fig. 3A). Esophageal eosinophilia was not observed in the esophagi from any of the other treatment groups. Eosinophilia was also observed in 3/6 stomach samples from challenged animals (Fig. 3B), two of which also displayed esophageal infiltration while one did not. However, the level of eosinophilic infiltration in the stomach did not reach statistical significance in these experiments ($p=0.18$). Similarly, we did not detect changes in eosinophilic content of ileum or colon samples from any animals. Fig. 4A shows a typical section through the esophagus with H&E staining, 24 h after challenge with HEWP. Eosinophilic infiltrates were confined to the lamina propria, and were often visualized perivascularly, but did not infiltrate the epithelium. Again, eosinophils were not observed infiltrating the esophagus in sensitized animals challenged with water (Fig. 4B), in animals not challenged (Fig 4C), or in animals that were not sensitized or challenged (Fig. 4D).

3.3 | General gastrointestinal inflammation.

In addition to eosinophils, we also observed additional infiltrating inflammatory cells composed mainly of lymphocytes and plasma cells (Fig 4A). Therefore the general inflammatory state of the GI tract was evaluated using an inflammation index which scores the severity of total lymphoplasmacytic infiltration into the tissue. As shown in Fig. 5A, 3 of the HEWP-challenged animals displayed elevated, general esophageal inflammation causing a significant change in the mean ($p=0.02$). Increased lymphoplasmacytic infiltrates were noted in certain animals in the stomach and colon (Figs.5B and D, respectively), but elevated scores did not correlate with sensitization or challenge regimen. Abnormal lymphoplasmacytic infiltration was not noted in any of the ileum samples.

3.4 | Circulating T cells.

Studies in mice and humans have shown a crucial role for $CD4^+$ T cells in food allergy (28). Therefore, we sought to confirm that $CD4^+$ T cells were indeed activated in these pigs following sensitization and challenge with HEWP. In addition, we sought to confirm that the responding T cells were specific for OVA, one of the target allergens in egg allergic humans (3). PBMC from sensitized and challenged pigs were restimulated with OVA *in vitro*, and the proliferative response of T-cell subsets was measured using flow cytometry. As shown in Fig. 6A, we found that the level of circulating, OVA-specific T cells was increased following sensitization and challenge. The main T-cell responders to OVA were $CD4^+$ T cells with a strong proliferative response in the presence of OVA. In contrast, the proliferative response

of cytotoxic T cells was similar in the absence or presence of OVA. Finally, among the 4 HEWP challenged animals included in these proliferation tests, we found that CD4⁺ T cells from all animals tested displayed a significant ($p=0.03$) proliferative response to OVA stimulation *in vitro* (Fig 6B).

3.5 | Chronic exposure model

In a follow up experiment we examined esophageal inflammation following a month of feeding HEWP to three sensitized animals (Fig. 1B). Endoscopy revealed a range of findings, with typically less pathology in the distal esophagus compared with the proximal esophagus. Distal esophageal findings ranged from normal to displaying mild furrows and white spots. In contrast, the proximal esophageal had edema, linear furrows, white spots and granularity. An image of the proximal esophagus from the pig with the most severe endoscopic findings is shown in Supplemental Fig. 2. Unfortunately, we did not have a mechanism to store endoscopy images digitally, and this image was obtained by cell phone from the endoscope screen. Despite its low quality the image clearly shows furrows, granularity and white spots. Representative histological images from this experiment are shown in Fig. 7. Eosinophils (arrows) were scattered throughout the epithelium or within defects resembling microabscess (*) in low numbers (A), at the interface between the epithelium and laminae propria (inset A) in higher numbers, and predominantly infiltrating laminae propria (A-B) adjacent to vessels. Higher numbers of lymphocytes were arranged in clusters at the interface between subepithelial stroma and epithelium (C), infiltrating the basal epithelial layers (D), or forming small microabscesses (E). Perivascular infiltrates included eosinophils and lymphocytes which surrounded vessels (F) and were occasionally within small vessels (G). Basal zone hyperplasia varied from trace (H) to moderate (I) with rete peg elongation (black bars). Adaptive hyperplastic changes, including thickening of stratum basale with intercellular fluid accumulation (inset I), had increased severity adjacent to areas with increased numbers of inflammatory infiltrations.

4 | Discussion

The esophagus is a resilient, highly muscular swallowing organ that typically functions well throughout life. Esophageal nerves, receiving cues from the brain and autonomic nervous system, stimulate the movement of esophageal muscles in coordinated waves of contraction propelling liquids and solids downward towards the stomach. Occasionally, in individuals with food allergies and other GI disorders, the esophagus becomes the target of a pathophysiological immune response known as EoE. Responding to cytokines and chemokine signals, eosinophils infiltrate the esophagus producing an inflammatory condition (30) which in turn damages esophageal tissues and prevents normal function. EoE is increasing in prevalence, with a substantial health care burden (31).

We are interested in modeling food allergy in pigs, and more specifically EoE, for a number of reasons. Given the size of pigs, their anatomical similarity to humans, and 10–20-year life span, a pig model would allow us to study EoE chronically, over months and years, as it occurs in humans, including endoscopic evaluation. A chronic EoE model in pigs could be used to examine lead compounds for effectiveness and successful treatment of EoE in the

pig would strongly support inclusion of that compound in human clinical trials (27). Similarly, the roadblocks encountered in the development of therapeutic monoclonal antibodies to alleviate food allergy and esophagitis (32) might be lessened by preclinical validation in the pig model. Pigs with EoE could also be used guide physicians on more efficient diagnosis of EoE through hands-on learning (30). For example, the typical endoscopic findings could be demonstrated in a controlled setting, biopsy protocols demonstrated, and in cases where strictures might develop, esophageal dilation could be additionally taught. Of course, modeling EoE in pigs will be more expensive than in mice and experiments will have to be carefully considered.

We utilized two different protocols in an attempt to induce esophagitis in sensitized young pigs. In the first protocol, we delivered a single large challenge dose of HEWP by gavage in an attempt to induce a uniform, acute gastrointestinal response. We then monitored clinical signs and gastrointestinal tissues were subsequently examined by histology. Clinically, this experiment revealed a range of responses, including; vomiting, diarrhea, lethargy, and in one animal an extensive skin rash. Histological examination revealed significant accumulation of eosinophils indicating that pigs do have the ability to develop this response. Future experiments will require greater numbers of animals, based on statistical power analyses, to allow us to conclude with certainty that this condition is associated with sensitization to food allergens in pigs. With this model, the eosinophils were found in the lamina propria but not in the epithelial layer indicating that we observed the early stages of the response. Interestingly, the 4 animals that were positive for esophagitis were among the most symptomatic. Three displayed additional symptoms of food allergy including diarrhea, emesis, or lethargy suggesting that the esophagitis we observed may be linked to the pathways that produce these clinical signs. We also noted eosinophil infiltration into the stomach (although this effect did not reach statistical significance) in agreement with Mondoulet *et al.* (27) who found enhanced eosinophil infiltration in the gastric mucosa of peanut allergic pigs. It is possible, therefore, that the esophagus and stomach share chemotactic signaling and adhesion proteins required for eosinophil extravasation.

In a second experiment, in an attempt to drive a more severe chronic response, sensitized pigs were exposed daily to HEWP, via their feed, for a one month period. These animals were examined by endoscopy and again tissues collected for histological analysis. Endoscopy revealed several abnormalities in the esophagus resembling those seen in human patients with EoE, including; granularity, white spots, edema and furrowing. Histologically, we also noted a number of pathological epithelial characteristics similar to those seen in human patients including; basal zone hyperplasia (BZH), dilated intracellular spaces (DIS), and rete peg elongation (RPE). Again, eosinophils were observed primarily in patches in the lamina propria. Infiltrating immune cells were primarily lymphocytic and we did not observe large numbers of eosinophils in the epithelia nor layering of eosinophils in this model as is seen in humans. Also, we did not observe thickening of collagen fibers (fibrosis) in the lamina propria.

There are several possible explanations for the pathology we observed in the chronic pig model and the differences from human clinical patients. First, despite a month of feeding, it is possible we observed an early stage of disease in pigs. In human EoE patients, where

severe pathology and tissue remodeling is typically observed, first endoscopy generally occurs years after initial symptoms are reported (33). Longer studies may be necessary to bring about extensive eosinophil infiltration into the epithelial layer with accompanying tissue remodeling. Additionally, in humans, impaired esophageal barrier function has also been linked to EoE, with reduced levels of adhesion molecules (34, 35) and increased conductivity (36) noted in patients with EoE. It is unlikely the pigs we tested were displaying decreased barrier function. As opposed to human clinical studies which rely on adult subjects, some with disease for years (35), the pigs we utilized were young, challenged for only 1 month, and otherwise healthy. It is possible therefore that decreased barrier function, and the resulting infiltration of allergens into the esophageal epithelium, where localized antigen processing and presentation, and T cell activation occur, are necessary for increased inflammation and the movement of large numbers of eosinophils into the epithelium. Clearly, more experiments will be necessary to fully characterize the esophageal response in food allergic pigs and determine how closely it mirrors the human response.

As with many immune responses, T cells are key to food allergic responses. During sensitization, CD4⁺ T_H2-type T cells respond to peptides presented by antigen presenting cells by producing IL-4 (14) and IL-5 (17) that stimulate eosinophil proliferation and migration. IL-17 from T_H17-type T cells is also important for the stimulation of basophils and mast cells during allergic responses (37) while IL-13 can induce esophageal remodeling (38). Later, following repeated exposures to the allergen, distinct populations of memory and effector T cells become resident in GI tissues where they coordinate vigorous secondary responses (39). Our study demonstrated readily detectable, allergen-specific CD4⁺ T cells in pigs and the high similarity of the porcine and the human immune systems presents a great opportunity to refine our understanding of the crucial T cell responses that lead to food allergy in a biologically relevant large animal model. Food-allergic pigs could be monitored for months or potentially years, under varying conditions of exposure to the allergen, to understand how food allergen uptake influences the survival, differentiation, tissue-homing and activity of allergen-specific T cell clones.

In summary, we successfully sensitized pigs to HEWP and demonstrated selective eosinophilic infiltration of the esophagus. Both pig models we utilized displayed incomplete penetrance and variations in the expression of symptoms and pathology, which is typical for an outbred population. Pigs may therefore be an effective model to study both the clinical and molecular events that lead to EoE and identify key variables which impact development of the disease. Further studies will be necessary to fully document the regulation of eosinophil infiltration in the porcine esophagus and its impact on esophageal function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Study design.

In A, pigs were sensitized by *ip* injection of 0.5 mg HEWP with 10 µg cholera toxin (CT) on days 7, 14, and 21 after birth. Skin testing was performed on days 29 and 30 with 18 responding pigs apportioned to 3 challenge groups with six animals in each group. On day 36 these pigs were challenged by gavage with 10 g HEWP in 60 mL water, water alone, or left unchallenged. Another group of control animals were housed separately without sensitization or challenge. Tissues were collected on day 37. In B, a group of 3 animals were sensitized by *ip* injection 0.5 mg HEWP with 10 µg cholera toxin (CT) on days 7 and 14. These animals received a daily challenge of 10 g HEWP in 60 mL strawberry jam/corn syrup mixture added to their feed between days 28 and 56. Endoscopy and tissue collection were performed on days 56 and 57, respectively.

Figure 2. Clinical Signs.

Sensitized animals were challenged by gavage with 10 g HEWP in water (Group 1), water alone (Group 2), or left unchallenged (Group 3). A fourth group of animals were housed separately and neither sensitized nor challenged (Group 4). Pigs were monitored continuously for 12 h after challenge for clinical signs of food allergy, including; diarrhea, emesis, erythema, and lethargy. A four step grading system was used to characterize the severity of each symptom; “0” not observed, “1” mild, “2” moderate, and “3” severe. Means $\pm$ SEM are shown for each group of six animals in addition to the individual values.

Figure 3. Eosinophil infiltration by organ in the gastrointestinal tract.

Eosinophil counts per high power field (HPF) were evaluated for esophagus (A), stomach (B), ileum (C), and colon (D). Sensitized animals were challenged by oral gavage with 10 g HEWP in water (Group 1), water alone (Group 2), or left unchallenged (Group 3). A fourth group of age-matched control animals were housed separately and neither sensitized nor challenged (Group 4). In challenged animals groups, GI tissues were harvested, processed, stained with H&E, and number of eosinophils counted. The pathologist remained blinded to the treatments throughout the experiment and analysis periods. Means $\pm$ SEM are shown for each group of six animals in addition to the individual values. Asterisk indicates significant difference of the mean ($p < 0.05$).

Figure 4. Eosinophil infiltration into the esophagus.

Representative sections of esophagus had eosinophilic infiltration demonstrating eosinophil infiltration into the esophagus from sensitized animals that were challenged by gavage with HEWP (A), water alone (B), or left unchallenged (C). The image in panel D is from an animal housed separately and neither sensitized nor challenged. Following challenge, GI tissues were harvested, processed, and stained with H&E. Images were collected by the pathologist who was blind to the treatments throughout the experiment and analysis periods. Arrows indicate the position of eosinophils.

Figure 5. GI tissue inflammation.

Sensitized animals were challenged by gavage with 10 g HEWP in water (Group 1), water alone (Group 2), or left unchallenged (Group 3). A fourth group of animals were housed separately and neither sensitized nor challenged (Group 4). Following challenge, GI tissues were harvested, processed, stained with H&E, and inflammation assessed as described in the Materials and Methods. The pathologist remained blinded to the treatments throughout the experiment and analysis periods. A four step grading system was used to characterize the severity of each symptom; “0” not observed, “1” mild, “2” moderate, and “3” severe. Means $\pm$ SEM are shown for each group of six animals in addition to the individual values. Asterisk indicates significant difference of the mean ($p < 0.05$).

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Figure 7. Increased numbers of eosinophils and lymphocytes after oral exposure to HEWP for 28 days duration following sensitization to HEWP.

Representative sections of proximal and distal esophagus had mild to moderate, patchy infiltration by eosinophils and lymphocytes within the laminae propria subjacent to epithelial hyperplasia or forming clusters within the epithelium (A-I). Arrows=eosinophils; Arrowheads=lymphocytes; Asterisk (*)=focal loss of superficial epithelium and replacement by eosinophils; LP=laminae propria; MM=muscularis mucosa; SG=submucosal glands; SM=submucosa; V=vessels.