Enteric Reovirus Infection Stimulates Peanut-Specific IgG2a Responses in a Mouse Food Allergy Model

Ronald J. Fecek\(^a\), Marisa Marcondes Rezende\(^b\), Ryan Busch\(^a\), Ine Hassing\(^b\), Raymond Pieters\(^b\), and Christopher F. Cuff\(^a,\)*

\(^a\)Department of Microbiology, Immunology, and Cell Biology, Robert C. Byrd Health Sciences Center of West Virginia University, Morgantown, West Virginia USA, 26506
\(^b\)Department of Immunotoxicology, Institute for Risk Assessment Sciences, Utrecht University, Utrecht, The Netherlands

Abstract

IgE-mediated food allergies are an important cause of life-threatening hypersensitivity reactions. Orally administered peanut antigens mixed with the mucosal adjuvant cholera toxin (CT) induce a strong peanut extract (PE)-specific serum IgE response that is correlated with T-helper type 1 (Th1) and T-helper type 2 (Th2)-like T-cell responses. This study was conducted to determine if respiratory enteric orphan virus (reovirus), a non-pathogenic virus that induces robust Th1-mediated mucosal and systemic responses, could modulate induction of PE-specific allergic responses when co-administered with PE. Young mice were orally exposed to PE mixed with CT, reovirus, or both CT and reovirus. As expected, CT promoted PE-specific serum IgE, IgG1, and IgG2a and intestinal IgA production as well as splenic Th1- and Th2-associated cytokine recall responses. Reovirus did not alter PE-specific serum IgE and IgG1 levels, but substantially increased the PE-specific IgG2a response when co-administered with PE. Young mice were orally exposed to PE mixed with CT, reovirus, or both CT and reovirus. As expected, CT promoted PE-specific serum IgE, IgG1, and IgG2a and intestinal IgA production as well as splenic Th1- and Th2-associated cytokine recall responses. Reovirus did not alter PE-specific serum IgE and IgG1 levels, but substantially increased the PE-specific IgG2a response when co-administered with PE. Reovirus did not alter PE-specific serum IgE and IgG1 levels, but substantially increased the PE-specific IgG2a response when co-administered with PE. Additionally, reovirus significantly decreased the percentage of Peyer’s patch CD8\(^+\) T-cells and Foxp3\(^+\) CD4\(^+\) T-regulatory cells when co-administered with PE. These results demonstrate that an acute mucosal reovirus infection and subsequent Th1 immune response is capable of modulating the Th1/Th2 controlled humoral response to PE. The reovirus-mediated increase in the PE-specific IgG2a antibody response may have therapeutic implications as increased levels of non-allergenic PE-specific IgG2a could block PE antigens from binding to IgE-sensitized mast cells.

Keywords

hygiene hypothesis; peanut allergy; reovirus; Th1/Th2 modulation
Introduction

Food allergies pose significant risk of morbidity and mortality, particularly in young children. Among foods that cause allergies, peanuts are a major problem since accidental ingestion is common, allergy to peanuts is severe and sometimes fatal, and peanut allergies resolve in only about 20% of cases (Hourihane et al., 1998; Skolnick et al., 2001). It has been estimated that approximately 1-2% of children develop peanut allergies (Burks, 2008) and over the past several decades, the rate of children that developed peanut allergies had increased in developed countries (Grundy et al., 2002; Sicherer et al., 2003).

The reason why food allergies were increasing in developed countries is not known, but for almost 20 years the hypothesis described by Strachan (Strachan, 1989) and now popularly referred to as the ‘hygiene hypothesis’ has been invoked to explain why populations in developed countries appear to be developing pathologies associated with allergic conditions at increasing rates. Broadly, the hygiene hypothesis suggests that reduced exposure to pathogens or their components early in life results in an increased IgE response to allergens and a subsequent increase in allergic diseases. The mechanism for this increased predilection for mounting IgE responses has been described as an imbalance of Th1/Th2 cells (Romagnani, 1992; Holt, 1994), reduced activation of T-regulatory cells (Tregs) (Wills-Karp et al., 2001), or failures in appropriate activation of innate effector cells and signaling molecules (Prioult and Nagler-Anderson, 2005).

Despite not knowing the precise immune mechanisms that account for increased frequencies of atopic patients, one approach to testing the hygiene hypothesis has been to identify inverse relationships between rates of allergic hypersensitivities and immunity to pathogens in hypersensitive patients. These studies have included analyses of responses to bacterial (Pelosi et al., 2005; Kosunen et al., 2002), parasitic (Yazdanbakhsh et al., 2002; Cooper, 2004), and viral (Matricardi et al., 2002; Linneberg et al., 2003) pathogens. In some studies, seropositivity to Hepatitis A (McIntire et al., 2004) and Epstein Barr virus (Nilsson et al., 2005; Calvani et al., 1997) has been correlated with reduced incidence of atopy. In addition, immunity to herpes virus (Janson et al., 2007) and cytomegalovirus (Nilsson et al., 2005; Janson et al., 2007) have been inversely correlated with atopy. These mucosally transmitted viruses have high frequencies of occurrence in human populations and are frequently transmitted under conditions of hygiene that are less than ideal, suggesting that viruses could be an important role in regulation of hypersensitivity. However, to dissect cellular and molecular mechanisms that are potentially involved in virus-mediated regulation of allergy, an animal model of both allergy and mucosal virus infection is needed.

Previous studies have documented that mice of the C3H strain produce substantial PE-specific serum IgE antibody following oral immunization with PE and the mucosal adjuvant CT (Li et al., 2000; van Wijk et al., 2004). This immune response is characterized by the appearance of PE-specific Th1 and Th2 responses (van Wijk et al., 2004) and activation of innate effectors including dendritic cells (van Wijk et al., 2007a). The present study examines the capacity of a Th1 promoting mucosal virus infection to modulate the allergic response to PE and CT in an animal model of allergy and infection. Reovirus was used as the mucosal viral pathogen because the virus is well characterized, naturally infects humans and mice, and is found ubiquitously in nature. In fact, its presence in water sources is commonly used as a sign of fecal contamination (Abbaszadegan et al., 1993; Fout et al., 2003). Following intestinal infection in mice, reovirus induces stereotypic Th1-driven responses characterized by the development of high titers of virus-specific serum IgG2a antibody (Major and Cuff, 1996), and the induction of interferon-γ (IFN-γ) producing T-cells (London et al., 1987; Fulton et al., 2004). Reovirus activates intestinal dendritic cells in vivo (Errington et al., 2008; Johansson et al., 2007; Fleeton et al., 2004) and under some circumstances reovirus (Rubin et al., 1981) or its hemagglutinin
(Greene and Weiner, 1980) can induce oral tolerance, indicating that the immune response to reovirus in mice has the potential to regulate allergic responses by a variety of mechanisms. Here we found that the robust responses to reovirus modulated the PE-specific humoral immune responses in mice. Although the PE-specific IgE response developed normally in sensitized mice that received reovirus, these mice demonstrated an enhanced PE-specific IgG2a antibody response, suggesting an increased PE-specific Th1 response. These experiments provide evidence for a role of enteric viruses in regulating induction of PE-specific immune responses.

**Methods**

**Virus**

Third passage stocks of reovirus serotype 1, strain Lang were prepared in L929 cells and purified by 1,1,2-trichloro-1,2,2-trifluoroethane (freon) extraction and CsCl gradient centrifugation. (Smith et al., 1969) The concentration of virions in purified preparations was determined by spectrophotometry where 1 optical density U at 260 nm = 2.1 × 10^{12} particles/mL (Smith et al., 1969) and by plaque assays (Major and Cuff, 1996).

**Preparation of peanut extract**

Peanuts from Golden Peanut (Alpharetta, Georgia) were kindly donated by Imko Nut Products, the Nut Company (Doetinchem, the Netherlands). Protein extract was made by blending 100 g of peanuts with 500 mL of 20 mM Tris buffer (pH 7.2) at room temperature for 2 hours in 20 minute intervals. The aqueous fraction was collected by centrifugation (3000 g, at 4°C for 30 min). The aqueous phase was subsequently centrifuged (10 000 g at 4°C for 30 min) to remove residual traces of fat and insoluble particles. Protein concentrations were determined using Bradford analysis with BSA as a standard. Extracts contained typically 32 mg/ml protein and were stored at −20°C. Reducing SDS-PAGE from the extracts showed protein bands between 14 and approximately 100 kDa (not shown).

**Mouse Treatment Protocol**

All experiments were performed under a protocol approved by the WVU Institutional Animal Care and Use Committee. The oral sensitization was performed as previously described (Li et al., 1999; van Wijk et al., 2005) with some modifications. Four week old C3H/HeJ female mice (Jackson Mice, Bar Harbor, ME), were orally gavaged with 0.25 mL of either PBS, PE (6 mg), PE plus CT (1mg/mL, List Biologicals, Campbell, CA), reovirus (10^7 plaque forming units), reovirus plus CT, PE plus reovirus, or PE plus CT and reovirus. The mice were treated on days 1, 2, 3, 8, 15, and 21. All mice were orally dosed with 12 mg of PE alone on day 30. At day 31, all mice were anesthetized and exsanguinated by cardiac puncture, and spleens and small intestines were promptly harvested.

**Splenic cytokine analysis**

Spleen cultures were performed as previously described (van Wijk et al., 2004). Splenocytes were cultured in tissue culture medium (TCM) with or without 200 μg/mL of PE for 96 hours at 37°C in 5% CO₂ atmosphere. Following incubation, the plates were centrifuged for 10 min at 150 × g and supernatants were collected and stored frozen at −70°C for further analysis. Levels of cytokines in the supernatants were determined by sandwich ELISA as described (van Wijk et al., 2004).

**Spleen CD8⁺ effector function**

Single cell suspensions of spleen cells from mice that were infected with reovirus were prepared as previously described (Fecék et al., 2006). Ficoll-Hypaque gradient enriched CD8⁺ effector
cells were treated with GolgiStop™ (BD Pharmingen) and cultured with target L929 cells infected with reovirus at an effector to target ratio of 25:1 in 96 well V-bottom plates. As a positive control, some effector cells were polyclonally activated by incubating with plate bound anti-CD3/anti-CD28 antibodies. Cultures were incubated for 4-6 hours at 37°C at a 5% CO₂ atmosphere. Following incubation, cells were stained for flow cytometry analysis.

**Lamina Propria fragment cultures**

Lamina propria (LP) fragment cultures were established as previously described (Major and Cuff, 1996). Initially, Peyer’s patches (PP) were removed and the small intestines were longitudinally split and cut into approximately 1-cm segments. Luminal contents were removed by washing the intestinal segments in PBS followed by incubation on a rotator at 37°C for 30 minutes in PBS supplemented with 10 mM EDTA and 1 mM dithiothreitol (DTT) to remove the epithelium. The PBS with EDTA and DTT was replaced and the fragment cultures incubated for an additional 30 min on the rotator at 37°C. The LP fragments devoid of epithelium were incubated in TCM for 5 days at 37°C in 5% CO₂. After the 5 day incubation, the supernatants were collected and the total, reovirus-specific, and PE-specific antibody concentrations were measured by ELISA.

**Serum and LP supernatant ELISA**

ELISA was performed as previously described (Major and Cuff, 1996). EIA/RIA flat bottom plates were coated overnight at 4°C with purified reovirus (2.5 × 10¹⁰ particles/mL), 20μg/ml PE, or 1μg/ml goat anti-mouse IgA in 50 μl of 0.1M NaHCO₃ and blocked with 3% BSA in PBS for 1 hour at room temperature (RT). Serially diluted mice sera and LP supernatants were added to the coated wells and incubated overnight at 4°C. Immune reference serum of a known titer was added to additional reovirus coated wells and known concentrations of purified mouse IgA (Southern Biotechnology, Birmingham, AL) were added to anti-IgA coated wells to generate standard curves for determination of antibody titers or quantitation of antibody concentrations as indicated. Biotinylated goat anti-mouse IgG (H + L)-, IgG1-, or IgG2a-specific antibodies (Southern Biotechnology) were added to wells containing serum and biotinylated goat anti-mouse IgA-specific antibody (Southern Biotechnology) was added to wells containing LP supernatant for one hour at RT. Streapavidin peroxidase was added for one hour and reovirus-specific (serum total IgG, IgG1, and IgG2a and LP IgA) antibodies as well as total and PE-specific LP IgA antibodies were detected through the subsequent color development using 2,2’-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) plus 0.03% H₂O₂ and analyzed at 405 nm with an ELISA plate reader. PE-specific serum IgE and PE-specific IgG1 and IgG2a antibodies were determined, respectively, by capture and indirect ELISA as described (van Wijk et al., 2005).

**Peyer’s Patch Lymphocyte Preparation**

Excised PP were mechanically disrupted with 18-gauge syringe needles and enzymatically dissociated with Collagenase Blendzyme Liberase I at 0.28 Wünsch units/mL (Roche, Indianapolis, IN) and DNase 1 grade 1 at 220 U/mL (Roche) for 30 min at 37°C. The disrupted PP were then treated with 0.1M EDTA for 5 min at RT and expressed through a 70 μm cell strainer yielding a single cell suspension that was subsequently stained for flow cytometry analysis.

**Flow cytometric analysis**

Splenic effector and PP cells were stained for expression of CD surface antigens. Spleen CD8⁺ effector cells were stained with anti-mouse CD8α-allophycocyanin (APC) (1:100) (BD Pharmingen) and PP cells were stained with anti-mouse CD8α-FITC (1:100) (BD Pharmingen), CD45R/B220-phycocerythrin (1:300) (BD Pharmingen), or CD4-phycocerythrin (1:200) (BD...
Pharmingen) for 30 min at 4°C in the dark. Cells were twice washed with FACS buffer (1X Dulbecco’s PBS supplemented with 0.5% BSA and 2mM EDTA), and fixed and permeabilized. Splenic effector cells were fixed with Cytofix/Cytoperm™ solution (BD Biosciences) according to the manufacturers’ instructions, intracellularly stained with anti-mouse IFN-γ-phycocerythrin (1:100) (BD Biosciences), and analyzed by flow cytometry. PP cells were fixed for 2 hours at 4°C in the dark with eBioscience Foxp3 fixation/permeabilization solution (eBioscience, San Diego, CA), twice washed with permeabilization buffer (eBioscience), and intracellularly stained with anti-mouse/rat Foxp3-APC (1 μg/10^6 cells) (eBioscience) in permeabilization buffer for 30 min at 4°C. Cells were washed, resuspended in FACS buffer, and analyzed by flow cytometry. All stained cells were analyzed with a FACSCaliber flow cytometer (BD Biosciences). Viable lymphocytes were gated based on forward and side scatter. The number and percentage of individual cell types of the gated population were determined based on expression of CD antigens. Tregs were defined as CD4+/Foxp3 double positive. Data were analyzed with CellQuest Pro, ver. 3.2 (Becton Dickinson).

**Statistical analysis**

Data were analyzed using one way analysis of variance (ANOVA) followed by a Tukey’s or Bonferroni post hoc test. Bonferroni post hoc was used for the PE-specific serum antibody and splenic cytokine analyses. Tukey’s post hoc was used for all other analyses. A p value of <0.05 was considered significant.

**Results**

**Reovirus Induces Robust Th1 responses in the presence of CT and PE**

C3H mice orally infected with reovirus mount a robust anti-reovirus Th1-response. To ensure that mice infected with reovirus in the presence of PE and CT mounted a prototypic systemic and mucosal immune response to reovirus, serum samples and fragment culture supernatants were analyzed for reovirus-specific IgG and IgA, respectively. The anti-reovirus serum IgG titers were very high in serum from infected mice. Although CT significantly increased the IgG response by 3-fold when compared to mice that received reovirus alone, mice that received reovirus with both CT and PE had a statistically significant 3-fold decrease when compared to mice that received reovirus + CT (Fig. 1a). Virus-specific IgG was non-detectable in mice that did not receive reovirus. Intestinal fragment cultures from reovirus-infected mice made substantial reovirus-specific IgA responses, irrespective of whether they received CT and/or PE (Fig. 1b). Mice that did not receive reovirus made no detectable anti-reovirus IgA. While CT did not enhance the anti-reovirus IgA response, mice given PE + reovirus had a decrease in anti-reovirus IgA that was not significant when compared to the IgA response of mice given reovirus alone. However, mice that received PE + CT + reovirus did have a statistically higher anti-reovirus IgA response than mice receiving PE + reovirus. Together these data indicate that reovirus was a potent immunogen in these mice, and neither CT nor PE substantially negatively affected the immunogenicity of reovirus.

To ensure that the prototypic Th-1 dominant anti-reovirus response developed following oral infection with reovirus plus CT and PE, the IgG subclass distribution of reovirus-specific antibody and recall IFN-γ response of CD8+ spleen cells stimulated with reovirus were evaluated. All mice that received reovirus whether mixed with PE, CT, or both, mounted robust virus-specific IgG2a responses, however, a significantly lower titer was observed in mice that received PE + CT + reovirus compared to mice that received CT + reovirus (Fig. 2a), reflecting the levels found when total virus-specific IgG was assessed. Virus-specific IgG1 responses were undetectable (data not shown). Together these data suggest that T-cell help for humoral antibody responses were dominated by IFN-γ producing Th1 cells (Nguyen et al., 1994). That Th1–dominated anti-reovirus responses were found in virus-infected mice was further
supported by the observation that in vitro IFN-γ production was readily detected following in vitro restimulation of CD8+ splenic T-cells from mice infected with reovirus with or without PE and CT (Fig. 2b).

CT promotes mixed PE-specific serum antibody responses and reovirus modulates PE-specific serum IgG2a

To determine the effect of reovirus on the anti-PE response, mice were orally dosed with PE alone, PE + CT, PE + reovirus, or PE + CT + reovirus. As expected, CT promoted production of robust PE-specific IgE, IgG1, and IgG2a serum antibody responses when orally administered with PE (Fig. 3). There was little or no IgE detected in mice receiving PE alone or PE + reovirus. IgG1 and IgG2a were detectable in the PE alone group but were significantly increased when CT was co-administered. Reovirus did not alter the adjuvant effect that CT mediates on PE-specific serum IgE and IgG1 levels. However, when reovirus and PE were co-administered there was a significantly enhanced PE-specific IgG2a response that increased approximately 30-fold when compared to mice given PE alone. Also, mice given reovirus with PE and CT exhibited an 8-fold increase in PE-specific IgG2a when compared to mice administered PE and CT that was trending toward significance.

Reovirus does not affect the CT-mediated PE-specific mucosal IgA response

In addition to promoting systemic antibody responses to PE, CT promotes production of mucosal anti-PE IgA. To determine whether reovirus affects the mucosal anti-PE response, lamina propria fragment cultures of intestinal tissue were established from mice treated with PE mixed with CT, reovirus, or both CT and reovirus (Fig. 4). Anti-PE IgA was readily detected in intestinal cultures from mice receiving PE + CT. There was a slight decrease in the anti-PE IgA when reovirus was co-administered with PE + CT that was not significant. Interestingly, reovirus had no detectable adjuvant properties on the mucosal anti-PE IgA response.

Reovirus does not affect the CT-enhanced splenic recall cytokine responses from mice given PE

Previous studies have indicated that spleen cells from PE-sensitized mice demonstrate a PE-induced Th1/Th2 mixed cytokine response (van Wijk et al., 2004). Therefore the effects of CT and reovirus on the PE-specific recall cytokine response was examined (Fig. 5). Spleen cells from treated mice were stimulated in vitro with or without PE, and the Th1-associated (IFN-γ) and Th2-associated (IL-4, IL-5, and IL-10) cytokine levels were measured in culture supernatants. Spleen cells from mice receiving PE + CT demonstrated significantly increased levels of IL-4, IL-5, and IL-10 compared to the PE alone group. There was an increase in IFN-γ for the PE + CT group albeit not significant. Despite being a potent inducer of Th1 responses, reovirus did not affect the CT-enhanced cytokine response to PE when mice were co-administered PE, CT, and reovirus. Reovirus did not affect the spleen cell cytokine response to PE for the four cytokines tested.

Reovirus modestly affects the PP lymphocyte distribution when co-administered with PE

Despite the development of robust antigen-specific responses to PE and reovirus, the relative percentages of B-cells, T-helper cells, T-cytotoxic cells, and Tregs in the PP were largely unaffected (Table 1). None of the groups had obvious differences in PP size or total cell number. However, there was a slight but statistically significant decrease in the percentages of CD8+ T-cells and CD4+Foxp3+ Tregs when analyzed as individual populations in the PP of the PE + reovirus group. The percentage of CD4+ T-cells also appeared to be trending downward in the PE + reovirus group, but did not reach statistical significance. A commensurate increase in B220+ cells was not observed, suggesting that an increase in macrophages, dendritic cells,
or other undefined cell types probably accounted for this modest decrease in the percentage of CD8+ and Tregs.

**Discussion**

Epidemiological and experimental data support the hygiene hypothesis as an explanation for the observed increase in the prevalence of atopic disorders in developed countries, despite the lack of a well-defined mechanism (Vercelli, 2006). One variant of the hygiene hypothesis suggests that lack of microbial stimulation results in a Th1/Th2 imbalance with a resulting Th2-bias that predisposes individuals to allergic responses. In this study, it was hypothesized that induction of a robust anti-viral Th1-type immune response would inhibit the prototypic Th2-type PE-specific food allergic response. Several experimental efforts biasing the Th1/Th2 balance toward a Th1-type immune response have successfully demonstrated suppression of Th2-type food allergic responses. For example, IL-12, which drives production of IFN-γ that results in cell mediated immunity and class switching to IgG2a, has been shown to inhibit PE-induced anaphylactic reactions, decrease PE-specific IgE levels, and reverse the PE-specific IgG1/IgG2a ratio when orally administered to mice in a model of peanut allergy (Lee et al., 2001). Additionally, in an indirect manner, the toll-like receptor (TLR)-ligands CpG (TLR9) (Adel-Patient et al., 2007) and LPS (TLR4) (Bashir et al., 2004), and the herbal extract food allergy herbal formula-2 (FAHF-2) (Srivastava et al., 2005) induced a Th1-type shift in the immune response resulting in abrogation of PE-specific allergic responses.

The systemic and mucosal responses to virus were highly specific and characteristic of ‘conventional’ Th1-dominant responses (serum IgG2a and IFN-producing cells), even in the presence of CT. Thus it was concluded that virus infection did establish a milieu that promoted formation of antigen-specific Th1-cells following oral exposure. Having established that reovirus induced an immunologic environment that promoted Th1 responses, the next question was what effect this inflammatory setting had on the PE-specific antibody, and particularly the IgE response in the context of CT. Although IgE antibody responses to antigen are typically thought of as driven by Th2 cells, the roles of Th2 and Th1 cells in this model are complex because CT promotes the activation of both PE-specific Th2 and Th1 cells (van Wijk et al., 2004), as well as modifies the activity of regulatory cells (van Wijk et al., 2007b) and APCs (Feng et al., 2008) following exposure to PE. As expected, CT enhanced the PE-specific IgE, IgG1, and IgG2a responses compared to PE and significantly enhanced IL-4, IL-5, and IL-10 cytokine responses by spleen cells stimulated with PE in vitro.

Reovirus did not alter the PE-specific serum IgE or IgG1 responses; however, virus substantially increased the levels of PE-specific IgG2a. This finding would suggest an increase in PE-specific Th1 responses without a concomitant increase in Th2 activity, and is consistent with the hypothesis that reovirus has Th1-promoting activity. However, an increase in PE-specific memory Th1 cells was not observed in the spleens of infected mice as measured by increased IFN-γ production, which argues against the idea that reovirus activates PE-specific Th1-cells. The mechanism for how the virus promoted an enhanced response to an unrelated and physically unlinked antigen has not yet been determined, but virus has been previously shown to enhance humoral immunity to unlinked antigens. Thompson et al., reported mucosal and systemic adjuvant activity by the alphavirus Venezuelan equine encephalitis replicon particles when coinoculated with soluble (ovalbumin) or particulate (influenza virions) antigens (Thompson et al., 2006). One hypothesis that could account for the observations is that virus infection acutely increases local IL-12 production by dendritic cells, macrophages, or other antigen presenting cells either in the intestine or periphery that promotes increased primary responses by PE-specific Th1 cells which results in increased IgG2a production. This hypothesis is consistent with previous findings from our group. Following oral infection with reovirus, increased levels of IL-12 and IFN-γ mRNA were detected in mouse PP and mesenteric
lymph node in the first 96 hours after infection but then begin to diminish (Mathers and Cuff, 2004). The cellular source of this and other proinflammatory cytokines has not been firmly established. Although reovirus did not modify the PE-specific cytokine recall response from spleen cells restimulated with PE in vitro, it is plausible that in vivo, early IL-12 production by innate immune cells and IFN-γ produced by virus-activated T-cells, including CD8+ T-cells, enhanced the PE-specific IgG2a response. An analysis of the kinetics of these cytokines during the sensitization protocol would help determine their possible role in the PE-specific IgG2a enhancement.

Earlier studies demonstrated that reovirus acted as a polyclonal activator of the intestinal IgA responses linked to normal flora or diet in mice if given prior to weaning. This polyclonal response was paralleled by the initiation of germinal centers in the Peyer’s patches in pre-weaned mice (Kramer and Cebra, 1995). The mice used in the present study were young but weaned. Nevertheless, it was thought that perhaps reovirus infection in these young but weaned mice would increase the IgA response to PE antigen, which could prevent the uptake of PE and potentially serve as a barrier to initiation of IgE production and PE-specific allergic responses. Although CT increased the IgA response to PE, reovirus by itself did not enhance the IgA response to PE. It is possible that the admixture of PE and reovirus would be more effective at inducing PE-specific IgA in mice prior to weaning. Alternatively, whether PE-conjugated reovirus could induce strong PE-specific IgA responses in the absence of CT remain to be determined.

The effects of PE on the systemic and mucosal anti-reovirus responses were very limited. Though extremely modest, the virus-specific IgG responses of mice given virus with CT and PE were significantly decreased when compared to mice given virus and CT. Conversely, virus-specific IgA responses were significantly enhanced in mice co-administered reovirus with CT and PE when compared to the PE and reovirus group. Interestingly, these effects by PE are only seen when co-administered with CT. The mechanisms that account for these are not clear and require further investigation.

Reovirus modestly altered the relative distribution of the CD4+Foxp3+ Tregs in the PP of mice treated with PE and reovirus. CD4+CD25+ Tregs have been shown to play an important role in the induction of oral tolerance (van Wijk et al., 2007b). The change in the relative percentage of these cells in the PP of mice given PE and reovirus suggests that reovirus may have an effect on the induction of the oral tolerance response mediated by Tregs, but this remains to be determined.

To the best of our knowledge, this is the first study that examines the effects of an enteric virus on the development of food allergies following oral sensitization. A recent epidemiological study investigating the relation between early-life intestinal viral infections and the development of atopic disorders, pointed to a positive association between infants suffering from recurrent wheeze at age 1 and 2 and increased rotavirus seropositivity in their first year of life (Reimerink et al., 2009). However, the authors also reported an inverse association between norovirus seropositivity and allergic sensitization in the first year of life, highlighting the importance for studying enteric virus infections and their roles in the development of atopic disorders. Overall, our data demonstrates that an acute infection with an enteric virus and the subsequent mucosal and systemic development of a virus-induced Th1 immune response is capable of enhancing the PE-specific IgG2a antibody response. Moreover, the reovirus-mediated increase in PE-specific IgG2a may have therapeutic implications. Strait et al reported that allergen-specific IgG can block IgE-mediated anaphylaxis in vivo through antigen interception and FcγRIIb-mediated inhibition (Strait et al., 2006). Future studies examining the potential of reovirus-induced increased PE-specific IgG2a levels to block or inhibit IgE-mediated allergic responses in a mouse model of peanut allergy are needed. Given that peanut...
allergies can be life-threatening, are rarely outgrown, and avoidance being the only absolute effective treatment, further studies testing mechanisms that have therapeutic prospects need deeper exploration.

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Abbreviations

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<tr>
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<tr>
<td>CT</td>
<td>cholera toxin</td>
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<td>IFN-γ</td>
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Figure 1.
Induction of reovirus-specific systemic and intestinal humoral immune responses. A. Total reovirus-specific serum IgG antibody titers from treated mice were determined as described in materials and methods. B. Reovirus-specific IgA concentrations in lamina propria fragments were determined as described in materials and methods and are presented as picograms of specific antibody per milligram of total IgA. Results shown are combined from two independent experiments. Error bars indicate standard error among 7-10 mice per group. Data were analyzed for statistical significance by ANOVA followed by Tukey’s post hoc test, (**) \( p < 0.01 \).
Figure 2.
Induction of Th-1 dominant reovirus-specific humoral and cell-mediated immune responses.
A. Reovirus-specific serum IgG2a isotype antibody titers from orally treated mice were determined as described in materials and methods. Results shown are combined from two independent experiments. Error bars indicate standard error among 7-10 mice per group. B. Splenic CD8+ T-cells were harvested from reovirus infected mice and restimulated in vitro with reovirus. Activation of CD8+ effector T-cells was assayed by the production of intracellular IFN-γ when challenged with non-infected (open bars) or reovirus-infected (filled bars) L929 mouse fibroblast cell. Bars represent the percentage of the maximum IFN-γ response of CD8+ T-cells following in vitro stimulation. Maximum response of CD8+ T-cells was determined through polyclonal activation with anti-CD3ε/CD28 antibodies. Error bars indicate standard deviation of two replicates. Data were analyzed for statistical significance by ANOVA followed by Tukey’s post hoc test, (*) p<0.05, (**) p<0.01.
Figure 3.
Effects of reovirus on PE-specific serum antibody production. Mice were orally treated as described in the materials and methods. At the end of the treatment protocol, serum from treated mice was collected and the PE-specific serum IgE, IgG1, and IgG2a antibody titers were analyzed by ELISA. Data were analyzed for statistically significant differences by ANOVA followed by Bonferroni post hoc test (**p < 0.01, (***)p < 0.001. Results shown are combined from two independent experiments. Error bars indicate standard error among 7-10 mice per group.
Figure 4. Effects of reovirus on PE-specific IgA production. Lamina propria fragment cultures from treated mice were established *in vitro* as described in the materials and methods. After 5 days, the culture supernatants were collected and the presence of PE-specific IgA was determined by ELISA. Data were analyzed for statistically significant differences by ANOVA followed by Tukey’s post hoc test. Results shown are combined from two independent experiments. Error bars indicate standard error among 7-10 mice per group. PE-specific IgA concentration is shown as picograms per milligram of total IgA.
Figure 5.
Effects of reovirus on IFN-γ, IL-10, IL-4, and IL-5 levels in splenocyte cell culture supernatants. Splenocytes from treated mice were cultured in vitro in the presence (filled bars) or absence (open bars) of 200μg/ml of PE for 96 hours. Following incubation, the cell culture supernatants were harvested and the cytokine levels induced by restimulation were analyzed by ELISA. Cytokine concentration is shown as picograms per milliliter. Data were analyzed for statistically significant differences by ANOVA followed by Bonferroni post hoc test, (*) p<0.05, (**) p<0.01, (***) p<0.001. Results shown are combined from two independent experiments. Error bars indicate standard error among 7-10 mice per group.
Table 1

Lymphocyte distribution in Peyer’s patches

<table>
<thead>
<tr>
<th>Groups</th>
<th>CD4 (Mean Percent, S.D.)</th>
<th>CD8 (Mean Percent, S.D.)</th>
<th>B220 (Mean Percent, S.D.)</th>
<th>CD4/Foxp3 (Mean Percent, S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>21.7 (1.8)</td>
<td>2.7 (0.3)</td>
<td>72.8 (3.0)</td>
<td>1.7 (0.2)</td>
</tr>
<tr>
<td>Peanut</td>
<td>21.8 (3.2)</td>
<td>3.6 (0.8)</td>
<td>70.8 (2.5)</td>
<td>2.6 (0.4)</td>
</tr>
<tr>
<td>Reovirus+CT</td>
<td>23.0 (2.3)</td>
<td>3.6 (1.1)</td>
<td>67.0 (3.9)</td>
<td>2.5 (0.4)</td>
</tr>
<tr>
<td>Peanut+Reovirus</td>
<td>19.1 (1.5)</td>
<td>1.8 (0.2)**</td>
<td>72.0 (2.0)</td>
<td>0.9 (0.1)***</td>
</tr>
<tr>
<td>Peanut+CT</td>
<td>21.7 (2.2)</td>
<td>3.9 (1.0)</td>
<td>69.2 (4.9)</td>
<td>2.4 (0.2)</td>
</tr>
<tr>
<td>Peanut+Reovirus+CT</td>
<td>24.8 (3.9)</td>
<td>3.7 (0.7)</td>
<td>66.4 (4.8)</td>
<td>3.0 (0.5)</td>
</tr>
</tbody>
</table>

a PP cells were stained for expression of CD4, CD8α, CD45R/B220, and Foxp3 and analyzed with flow cytometry.

b Analyzed using ANOVA followed by a Tukey post hoc test

** p<0.01
*** p<0.001