

Pertussis Adjuvant Prolongs Intestinal Hypersensitivity

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Key Words

Intestine · Epithelium · Nerve · Ion transport · Mast cell · Hypersensitivity · Immunoglobulin E

Abstract

Background: Immediate hypersensitivity reactions are a hallmark of allergic disease, and result in the clinical features of food allergy, hayfever, and atopic asthma. The mechanism by which an individual becomes sensitized to an ingested or airborne allergen is not clear, however exposure to bacteria or bacterial products that act as adjuvants may be a contributing factor. The purpose of this study was to examine the role of pertussis toxin (PT) in inducing intestinal hypersensitivity reactions, particularly the ability of the adjuvant to prolong the sensitization. **Methods:** Rats were sensitized to ovalbumin (OA) by injection of OA alone or with 50 ng PT. Secretory responses to OA challenge and nerve stimulation were assessed in jejunal tissues mounted in Ussing chambers. **Results:** Jejunal segments from rats sensitized to OA alone responded to antigen challenge with ion secretion, but sensitization was transient in that specific IgE titers and responses to luminal antigen disappeared by 14 days. In contrast, co-administration of 50 ng PT with OA resulted in long-lasting sensitization. Secretory responses to both luminal and serosal OA challenge were present 8 months after primary immunization. Enhanced secretory responses to nerve stimulation, increased mucosal mast cell numbers, as well as

elevated IgE titers were also induced and may have contributed to the overall responsiveness of the intestine to antigen challenge. **Conclusions:** Our findings indicate nanogram quantities of PT, when administered with a food protein, result in long-term sensitization to the antigen, and altered intestinal neuroimmune function. These data suggest that exposure to bacterial pathogens may prolong the normally transient immune responsiveness to inert food antigens.

Introduction

Allergy is a significant and growing clinical problem. Antigen-triggered immediate hypersensitivity reactions play a role in conditions such as food allergy, hayfever, asthma and atopic dermatitis. The mechanism by which individuals become sensitized to an antigen remains unknown, but specific IgE antibody production is a hallmark of allergic disease. The accepted mechanism of hypersensitivity reactions involves antigen cross-linking of IgE bound to FcεRI on the surface of mast cells, inducing the release of mediators that alter epithelial and smooth muscle physiology [1]. Although IgE antibodies appear to be crucial for intestinal hypersensitivity reactions to food proteins, the barrier function of the epithelium may also play a very important role in regulating luminal antigen access to mucosal immune cells.

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Animal models of intestinal hypersensitivity have been used by our laboratory and others to document the physiological changes that occur after antigen challenge. These studies have shown that *in vivo* antigen challenge is followed by an increase in water secretion driven by active ion transport [2, 3]. Ussing-chambered intestinal segments from sensitized rats respond to antigen with an increase in short-circuit current (Isc) that is indicative of active chloride secretion [4]. Mast cell stabilization, antagonism or inhibition of production of mast cell mediators (histamine, serotonin, or prostaglandins), reduces the ion secretion after antigen challenge [4–6]. Neural blockade also reduces the ion secretory response to antigen challenge, indicating a role for enteric nerves in the hypersensitivity reaction [4, 5].

Much of the emphasis of previous studies using animal models of intestinal hypersensitivity has been on the events after antigen challenge, resulting in limited information on changes induced by the sensitization process. In light of the fact that many allergic conditions are maintained over a lifetime in humans, it is important to utilize a model of hypersensitivity that is also maintained over time. It has been suggested that bacterial or viral factors may play a role in allergic sensitization [7, 8]. Pertussis toxin (PT) [9] and cholera toxin [10] have been demonstrated to have adjuvant effects resulting in intestinal hypersensitivity to a co-administered antigen. PT is the active ingredient of pertussis vaccine and is the material formerly known as histamine-sensitizing factor. In this study, we have demonstrated that co-administration of a very small amount of PT with a food protein antigen, ovalbumin (OA), produces a long-lasting sensitization to OA. Epithelial ion secretion responses to luminal OA challenge were maintained for at least 8 months. In addition, we documented prolonged changes in responsiveness to enteric nerve stimulation, elevated circulating IgE levels, and increased mast cell numbers in the intestinal mucosa. These findings suggest that exposure to very small amounts of bacterial products could not only contribute to inappropriate responses to ingested food proteins, but could lead to a prolonged state of sensitization.

Methods

Sensitization of Animals

Experiments were approved by the Animal Care Committee at McMaster University. Male Sprague-Dawley (SD) rats ($n = 80$, 200–225 g, Charles River Breeding Laboratories, St. Constant, Que., Canada) and Brown-Norway (BN) rats ($n = 20$, 250–300 g, Harlan Sprague-Dawley Inc., Indianapolis, Ind., USA) arrived pathogen-free and were maintained in cages equipped with filter hoods.

Active sensitization: SD rats were injected *i.p.* with 1.0 mg of chicken egg albumin (OA, grade V, Sigma Chemical, St. Louis, Mo., USA) in 1 ml phosphate-buffered saline (PBS), alone (OA group) or co-administered with 50 ng recombinant wild-type pertussis toxin (PT, generously provided by Dr. R. Rappuoli, Sclavo Research Institute, Siena, Italy.) (OA/PT group). Studies with actively sensitized rats were conducted 7, 14, 28, 56, 84 and 226 days sensitization.

Passive sensitization: BN rats were injected *i.p.* with 10 μ g OA plus 50 ng PT, boosted at 28 days with 10 μ g OA, and were bled 4 days later by cardiac puncture. Sera were pooled and aliquots stored at -70°C . The pooled serum used for passive sensitization had an IgE titer of 1:2,048. SD rats were injected *i.p.* with 0.5 ml of serum from actively sensitized BN (high IgE responders) rats, ± 50 ng PT. Studies with passively sensitized rats were conducted 3, 14 and 70 days after sensitization.

Ussing Chamber Experiments

Procedures have been described previously [4] and are outlined here in brief. Rats were anesthetized with urethane, and a 10-cm segment of jejunum was excised beginning 5 cm from the ligament of Treitz. The intestine was placed on a plastic rod, and the external muscle layers with adherent myenteric plexus were stripped off, leaving the submucosal plexus and mucosa intact. The segment was then opened into a flat sheet by cutting along the mesenteric border. Four adjacent pieces of intestine were mounted in Ussing chambers (WPI Instruments, Narco Scientific, Mississauga, Ont., Canada) exposing 0.6 cm^2 of surface area to circulating oxygenated Krebs buffer (in mM) 115 NaCl, 8 KCl, 1.25 CaCl_2 , 1.2 MgCl_2 , 2.0 KH_2PO_4 , 25 NaH_2PO_4 , pH 7.35 ± 0.02), that contained 10 mM glucose on the serosal side, or 10 mM mannitol on the luminal side. An external water jacket maintained the buffers at 37°C . The tissue was clamped at zero volts using a W-P Instruments automatic voltage clamp (Narco Scientific, Downsview, Ont., Canada). The Isc (in μA) was recorded continuously, and the circuit was opened at intervals to measure the potential difference (PD, in mV) and to determine the conductance (G, in mS/cm^2).

Ion Transport Studies

The ion secretory Isc response to OA was calculated as the difference between the baseline Isc and the maximal increase obtained within 5 min after addition of OA (final concentration 100 $\mu\text{g}/\text{ml}$) to either the serosal or luminal side of the tissues. The maximal increase in Isc in response to electrical transmural stimulation (TS) of submucosal plexus neurons (10 Hz, 10 μA , 0.5 ms for a total time of 5 s) was also measured.

Histology

Segments of small intestine were removed for histology from the region just distal to that used for Ussing chamber experiments. Segments were opened along the mesenteric border, stapled flat to cardboard, and fixed in Carnoy's fixative. Tissues were embedded in paraffin and longitudinal sections along the villus/crypt axis were cut at a thickness of 3 μm . Sections were stained with toluidine blue for detection of mucosal mast cells. The numbers of mucosal mast cells were calculated per villus/crypt unit. At least 6–10 well-oriented units were evaluated per section using a computerized videoplan (Kontron, Zeiss, Germany).

IgE Measurement

OA-specific IgE was measured by passive cutaneous anaphylaxis (PCA). SD rats were injected intradermally with 100 μ l of diluted serum from actively immunized rats. Samples were run in duplicate in dilutions from 1:4 to 1:1,024 (to 1:8,192 for BN serum). IgE-positive serum served as a positive control; IgE-negative serum served as a negative control. After 72 h, rats were challenged by i.v. injection of 0.5 ml solution of 1% Evans blue (Sigma) containing 2.5 mg of OA. Blueing of the skin was evaluated 30 min after injection. The highest serum dilution giving a positive reaction was recorded as the PCA titer of that serum and was expressed as the $\log_2$ of the dilution. The mean of duplicate values from different recipient animals were used as the result.

Statistical Analysis

Results were analyzed by ANOVA with posthoc comparisons using Newman-Keuls test or Student's *t* test where appropriate. Results for each group are expressed as means $\pm$ SEM. A *p* value of <0.05 was accepted as significant.

Results

Administration of PT with OVA Induces Intestinal Hypersensitivity to OA That Lasts at Least 8 Months

We have previously shown that co-administration of PT with OA stimulates specific IgE production and intestinal hypersensitivity reactions to this food protein [9], with no hypersensitivity reaction occurring to nonspecific antigen, whether added to the luminal or serosal side of the chambers. In this study, we examined the time frame of the hypersensitivity reaction. OA/PT-sensitized rats maintained a long-lasting intestinal sensitization, as shown by the ion secretory response to OA added to the serosal side of the tissue (fig. 1). Although there was no significant difference in the magnitude of the Isc responses to serosal antigen between OA and OA/PT groups at 7 days (75.8 ± 15.3 and 76.7 ± 8.7 μ A/cm² for OA and OA/PT groups, respectively), PT adjuvant prolonged the intestinal responsiveness. Rats sensitized with PT adjuvant maintained secretory responses to serosal antigen throughout the experimental period, and responses at 8 months were not significantly different from those observed at 7 and 14 days. In contrast, OA rats began to lose responsiveness to serosal antigen challenge at 14 days (approximately 50% of the response observed at 7 days), and it disappeared entirely by 226 days postsensitization.

Responses to luminal antigen were also significantly altered by co-administration of PT (fig. 2). Unlike the responses to serosal antigen, these Isc responses were still increasing beyond day 7 in the OA/PT group. Responses to luminal OA peaked at day 56 in OA/PT rats, in contrast to OA rats that had no detectable responses. The Isc response to luminal OA at 8 months was of a similar magnitude as

that observed at 7 days. In OA rats the responsiveness to luminal OA challenge was short-lived and dropped to negligible levels at 14 days.

Intestinal Hypersensitivity Outlasts Serum IgE

PCA titers of sera from OA rats and OA/PT rats are shown in figure 3. At day 7 postsensitization, both groups showed a vigorous reagenic antibody response to OA. By day 14, sera from OA rats did not invoke a PCA response. In contrast, OA/PT rats maintained elevated IgE titers, which gradually decreased to below the level of detection by 8 months. Heat treatment of the sera (56°C, 1 h) abolished the PCA responses, indicating that the reactive antibodies were of the heat-labile IgE isotype (data not shown).

PT Enhances Epithelial Response to Nerve Stimulation

The impact of sensitization using PT as an adjuvant on enteric nerve activity was examined by measuring the epithelial responsiveness to TS in rats sensitized with OA $\pm$ PT (fig. 4). OA/PT rats demonstrated a significantly increased Isc response to TS at day 14 compared with OA rats. The Isc response to TS continued to be elevated in the OA/PT group at 56 and 84 days postsensitization, and dropped back to control levels by day 226. The pattern of these responses over time mimicked that of the Isc responses to luminal OA, suggesting that altered nerve function may have contributed to these secretory responses.

PT Induces Mast Cell Hyperplasia

The impact of PT adjuvant on mucosal mast cell number was examined. Mucosal mast cells in sections from OA/PT rats were significantly increased at 14 days postsensitization, compared to those in sections from naive rats and OA rats (fig. 5). Mast cell numbers remained elevated at 28 and 56 days, but returned to control levels 84 days postsensitization.

PT Does Not Have Direct Effects on Epithelial Function

Our results suggest that PT enhanced epithelial responsiveness to luminal antigen challenge, perhaps by altering antigen uptake across the epithelium. To determine if PT had direct effects on the intestinal epithelium to enhance responsiveness to luminal antigen, we injected PT in addition to serum in passively sensitized rats. Three days after passive sensitization, rats sensitized with or without PT responded to luminal or serosal OA challenge with equivalent Isc responses (table 1). By 14 days postsensitization, there were no longer any detectable Isc responses in either treatment group, indicating that the IgE had likely been cleared from the intestinal tissue.

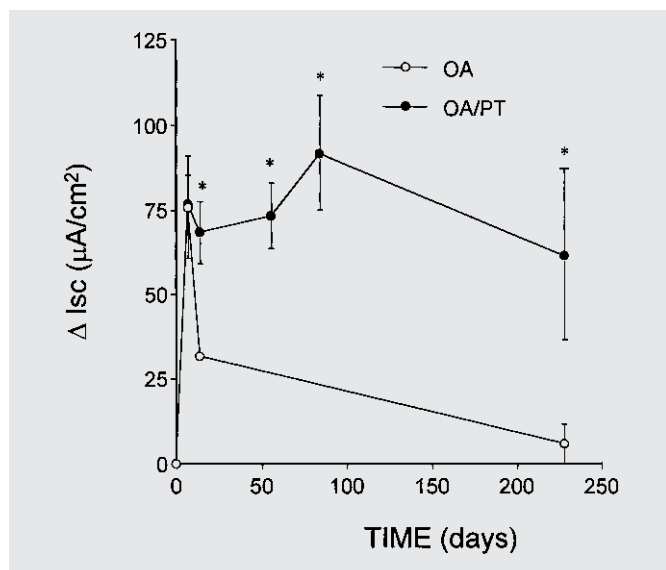


Fig. 1. Effect of PT adjuvant on Isc responses to serosal antigen challenge at various times after sensitization. Rats were sensitized by injection with 1 mg OA $\pm$ 50 ng PT. 7–226 days later, intestinal segments were mounted in Ussing chambers and challenged with 100 μ g/ml OA on the serosal side of the tissues. The peak Isc within 5 min was measured, and expressed as mean $\pm$ SEM. For each point, 4 tissues/rat were evaluated with 4–12 rats/group (except controls day 84 and 226 when 2 rats/group were used). * p < 0.05 compared to OA group at same time point.

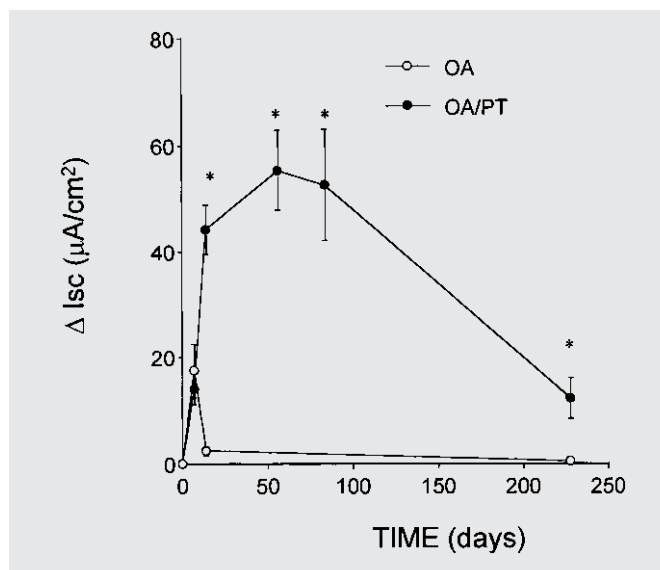


Fig. 2. Effect of PT adjuvant on Isc responses to luminal antigen challenge at various times after sensitization. Rats were sensitized by injection with 1 mg OA $\pm$ 50 ng PT. 7–226 days later, intestinal segments were mounted in Ussing chambers and challenged with 100 μ g/ml OA on the luminal side. The peak Isc was measured within 5 min from challenge, and expressed as mean $\pm$ SEM. * p < 0.05 compared to OA group at same time point.

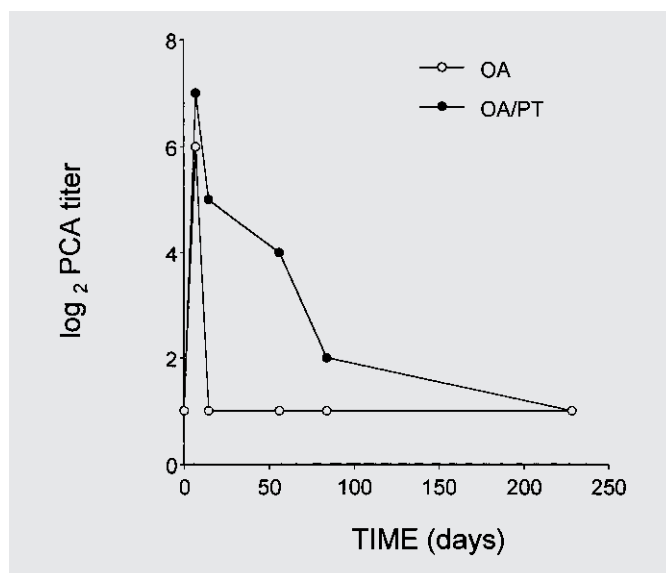


Fig. 3. Effect of PT adjuvant on specific IgE titers at various times after sensitization. Rats were sensitized by injection with 1 mg OA $\pm$ 50 ng PT. 7–226 days later, rats were bled by cardiac puncture. Titers were measured by PCA. The titer was recorded as the highest dilution of serum resulting in blueing of the skin, and expressed as the mean log₂ titer. n = 3–4 rats/group.

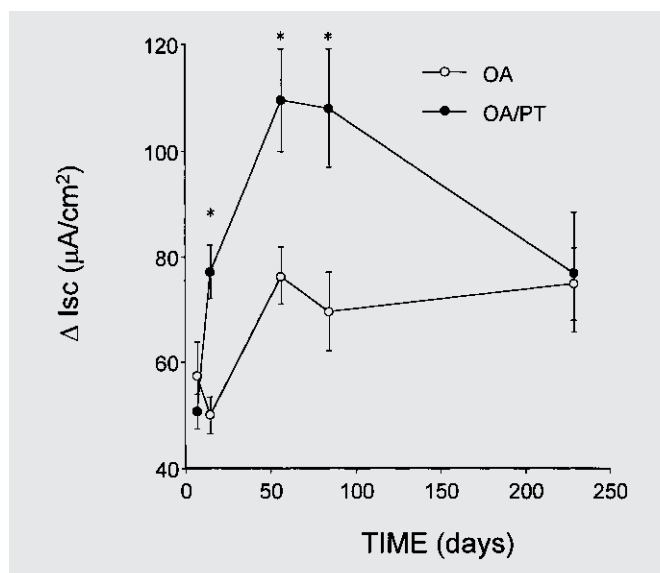


Fig. 4. Effect of PT adjuvant on epithelial Isc responses to TS at various times after sensitization. Rats were sensitized by injection with 1 mg OA $\pm$ 50 ng PT. 7–226 days later, intestinal segments were mounted in Ussing chambers, and the maximal increase in Isc in response to electrical transmural stimulation of enteric nerves in the preparation was measured and expressed as mean $\pm$ SEM. * p < 0.05 compared to OA group at same time point.

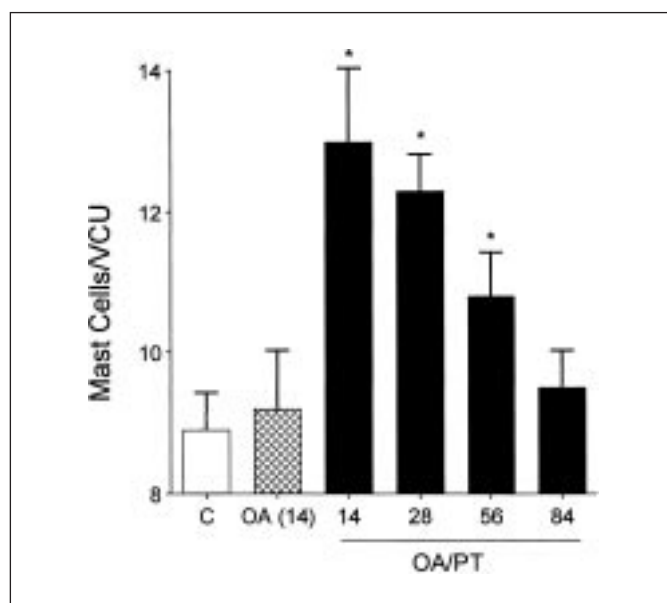


Fig. 5. Effect of PT on mucosal mast cell numbers at various times after sensitization. Rats were sensitized by injection with 1 mg OA $\pm$ 50 ng PT. 14–84 days later, intestinal sections were fixed and stained with toluidine blue. The number of mucosal mast cells were counted in 6–10 well-oriented villus/crypt units (VCU), and expressed as mean number of mast cells/VCU $\pm$ SEM. $n = 5$ –6 rats/group. * $p < 0.05$ compared to naive rats.

Discussion

Diseases involving immediate hypersensitivity reactions such as food allergy, asthma, and rhinitis have a high prevalence in Western society. Both a genetic predisposition and environmental influences are thought to be involved in sensitization of individuals to allergens. One of the environmental influences has been hypothesized to be infection with bacterial organisms, resulting in activation and/or skewing of the immune system in response to a normally inert antigen [7, 8]. In the present study, we examined in rats the effect of co-administration of PT as an adjuvant on the intestinal response to OA. We demonstrated that a single 50-ng dose of PT given together with OA produced long-lasting sensitization. Intestinal segments from these sensitized rats responded to OA challenge 8 months after injection. In contrast, rats given OA alone developed a transient sensitization to OA that subsided within 14 days. In addition, PT adjuvant stimulated production of IgE and increased epithelial secretory responses to nerve stimulation that were observed to persist between 12 weeks and 8 months after injection.

Table 1. Effect of PT on the intestinal Isc response to OA in passively sensitized rats

Time, days	Isc response to luminal OA $\mu\text{A}/\text{cm}^2$		Isc response to serosal OA $\mu\text{A}/\text{cm}^2$	
	serum	serum + PT	serum	serum + PT
3	29.3 $\pm$ 9.2	27.3 $\pm$ 3.3	82.3 $\pm$ 20.7	90.3 $\pm$ 9.2
14	0	0	0	3.8 $\pm$ 3.8

Rats were injected with immune serum $\pm$ 50 ng PT. After 3 or 14 days, jejunal segments were removed and mounted in Ussing chambers where 100 $\mu\text{g}/\text{mL}$ OA was added to the luminal or serosal side of the chamber. The maximum change in Isc (in $\mu\text{A}/\text{cm}^2$) was measured. Values represent mean $\pm$ SEM, $n = 5$ rats/group, 4 tissues/rat.

PT is produced by *Bordetella pertussis*, the pathogen responsible for whooping cough, and is present in some cellular vaccines. PT is a subunit toxin consisting of A and B moieties. The B subunit is involved in binding to the target cell surface, and the enzymatically active A subunit enters the cell and ribosylates a number of G proteins [11]. We have previously shown that an enzymatically inactive mutant does not have any adjuvant activity when co-administered with OA, and provides no greater sensitization than OA administered alone [9]. Therefore, PT must be able to activate one or more target cells in order to generate adjuvant activity. Such target cells include those of the immune system such as T and B lymphocytes that participate in the development of allergic sensitization.

One of the most interesting findings of this study was the longevity of the responsiveness to antigen challenge. Eight months after a single injection of OA with nanogram quantities of PT, intestinal segments still responded to both luminal and serosal antigen challenge with an increase in ion secretion. The responsiveness to serosal antigen challenge was constant over time and had not yet begun to decline at this time point. The response to luminal OA challenge continued to rise to a maximum at 56 days, then subsided somewhat at 8 months. It is well established that IgE cross-linking plays an important role in local hypersensitivity reactions. At the 8-month time point, IgE was no longer detectable in the serum. However, IgE must have still been present in the lamina propria bound to mucosal mast cells in order for the secretory response to occur. In our model, intestinal responsiveness to antigen challenge is clearly the most sensitive marker of sensitization. It has been demonstrated that the half-life of IgE is increased from 12 h in serum to over 7 days in tissue [12]. Binding to Fc receptors

on the surface of mast cells within the lamina propria may have protected IgE from being catabolized. We showed that rats passively sensitized with serum lost sensitization rapidly, and therefore continual production of specific IgE antibodies must have been occurring over the 8-month period to maintain the intestinal hypersensitivity.

IgE cross-linking is not the only important factor in the secretory response to luminal antigen. Antigen presented to the luminal surface of intestinal tissues must cross the epithelial barrier to access and activate subepithelial mast cells. Co-administration of PT with OA caused a 20-fold increase in the Isc response to luminal antigen, compared to a 2.5-fold increase in response to serosal antigen at 14 days postsensitization. This difference in magnitude could be explained by PT enhancing the access of antigen to lamina propria mast cells. We have previously shown that sensitization using pertussis vaccine (containing PT) as an adjuvant increases the amount and rate of antigen transport across the intestinal epithelium [13]. It is clear that PT alone does not directly increase antigen transport across the epithelium, as there was no increase in responsiveness to luminal antigen in passively sensitized rats injected with PT. This suggests that antigen processing and presentation may play an important role in the effects of PT. It has been demonstrated that PT enhances the production of interleukin-4 (IL-4) from helper T cells following primary antigen exposure [14]. IL-4 is an important cytokine involved in B-cell switching for IgE production [15], and is required for IgE production in vivo [16]. We have recently shown that IL-4 increases the transport of antigenic proteins across epithelial cells [17], and we speculate that sensitization using PT adjuvant may enhance the uptake of antigen by stimulating IL-4 production, thus resulting in greater Isc responses to luminal antigens.

The kinetics of the change in responsiveness to luminal antigen paralleled the change in responsiveness to TS. Nerves have been shown to regulate the transepithelial transport of macromolecules: blockade of nerves inhibits the transport of proteins across the intestinal epithelium [18] while stimulation with cholinergic agonists increases transepithelial transport of proteins [19]. It is possible that the altered nerve function reflected by the enhanced TS response increased the uptake of antigen from the lumen.

Sensitization using PT as an adjuvant also induced an increase in numbers of stained mucosal mast cells that remained elevated at least 2 months. Elevated IgE levels have been reported to induce an increase in mast cell FcεRI expression [20], and the combination of elevated mast cell number and elevated FcεRI expression may have contributed to the enhanced sensitization in animals injected with PT

and OA. An increase in mast cell number could also potentially have contributed to the increased Isc response to TS. Previous studies in mast cell-deficient mice have shown that there is bidirectional communication between mast cells and nerves: mast cells contribute to Isc responses evoked by TS, and nerves amplify ion secretion triggered by activation of mast cells [21]. Stead et al. [22] have shown that mast cells and nerves are in intimate contact in the intestinal mucosa of *Nippostrongylus brasiliensis*-infected rats. *N. brasiliensis*-infected rats develop a strong mast cell hyperplasia after infection, and the observation of mast cell-nerve contacts after infection suggests that nerves remodel to increase the total number of mast cell-nerve units.

In summary, co-administration of PT with antigen resulted in prolonged intestinal sensitization characterized by epithelial ion secretion responses to luminal or serosal antigen challenge that were maintained even 8 months after sensitization. Sensitization with PT adjuvant also dramatically increased the magnitude of the ion secretory response to luminal antigen challenge, potentially by increasing the permeability of the intestinal epithelium to luminal antigens. These changes were accompanied in time by an enhanced response to nerve stimulation. These studies suggest that bacterial toxins may contribute to allergic sensitization by prolonging the normally transient responsiveness to co-administered antigens.

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