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Pertussis toxin stimulates hypersensitivity and enhances nerve-mediated antigen uptake in rat intestine

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Kosecka, U., J. S. Marshall, S. E. Crowe, J. Bienenstock, and M. H. Perdue. Pertussis toxin stimulates hypersensitivity and enhances nerve-mediated antigen uptake in rat intestine. *Am. J. Physiol.* 267 (*Gastrointest. Liver Physiol.* 30): G745–G753, 1994.—We previously reported that intestine from rats sensitized to ovalbumin (Ova), using *Bordetella pertussis* vaccine as adjuvant, demonstrated a rapid secretory response [increase in short-circuit current (I_{sc})] to Ova upon secondary challenge. Here, we examined the role of pertussis toxin, the active component of the vaccine, in the response. Sensitization of Sprague-Dawley rats by intraperitoneal injection of recombinant wild-type pertussis toxin (wPT) plus Ova enhanced intestinal responses (at day 14: ~20-fold for luminal antigen, ~2.5-fold for serosal antigen) compared with rats sensitized by injection of Ova alone. In contrast, sensitization with an enzymatically inactive mutant pertussis toxin (mPT, different in two amino acids) produced no significant effect. Ova-specific immunoglobulin (Ig) E and IgG_{2a} antibodies and greater numbers of mucosal mast cells were documented in wPT-sensitized rats. In addition, the I_{sc} response to electrical transmural stimulation of nerves in intestinal preparations was significantly augmented. Neurotoxin inhibited the secretory response to luminal but not serosal antigen. Immunophysiological stimulation by wPT was still evident 8 mo postsensitization. Our studies indicate that pertussis toxin causes long-lasting hypersensitivity to coadministered antigens, involving increased production of reagenic antibodies, hyperplasia of mucosal mast cells, and enhanced neurally mediated uptake of antigen across the intestinal epithelium. These findings suggest a potential role for bacterial products in the development of immunophysiological reactions to ingested antigens.

mast cells; immunoglobulin E

IT IS WELL KNOWN that protein antigens can trigger local immediate hypersensitivity reactions in sensitized hosts. Such allergic reactions have been documented in several organ systems, particularly in the gastrointestinal tract, the airways, and the skin. The generally accepted mechanism is that antigen cross-linking of specific immunoglobulin (Ig) E antibodies bound to the surface of tissue mast cells results in release of potent chemical mediators, either previously synthesized and stored within granules or generated upon activation (33). The mediators act on epithelium and smooth muscle to cause changes in physiology that result in symptoms including diarrhea, cramps, abdominal pain, rhinitis, wheezing, etc.

The factors involved in sensitization in humans are incompletely understood. It has been suggested that hypersensitivity responses may be initiated or enhanced by infections (11, 18). Certainly, infections can result in disruption of the epithelial barrier allowing increased uptake of antigens that may bypass host mucosal immune defense mechanisms (32). In addition, adjuvant

activity has been attributed to bacterial products such as pertussis toxin (23). In fact, pertussis vaccine is commonly used to sensitize experimental animals for IgE production (7, 13).

Several studies in our laboratory have documented the immunophysiological responses to antigen challenge in the gut and airways of rats and mice sensitized to egg albumin [ovalbumin (Ova)] using whole cell *Bordetella pertussis* vaccine and/or alum as adjuvants. We have reported antigen-induced abnormalities in the transport of water and electrolytes in the intestine of sensitized rats studied in vivo (27) and have shown that secretory responses begin within 2–3 min of antigen administration to the luminal surface of tissue preparations of intestine (7) and trachea (34) studied in vitro. In addition, sensitized rats demonstrate increased epithelial permeability both before and after antigen challenge compared with controls, and antigen uptake is sensitive to local neural blockade (8). Studies in mast cell-deficient strains of mice and normal congenic controls confirmed that mast cells contribute to at least 70% of the immunophysiological abnormalities and also indicated the presence of an amplification mechanism involving mucosal nerves (29).

The present study was designed to investigate the role of pertussis toxin, the active ingredient of the vaccine adjuvant, in the antigen-induced hypersensitivity reaction. Wild-type recombinant holotoxin having full enzymatic activity of ADP-ribosyl transferase was compared with a mutant toxin in which substitution of two amino acids in the A subunit renders the holotoxin inactive (31). We examined the ability of both toxins to stimulate reagenic antibody production and antigen-induced physiological abnormalities. We found that a single injection of antigen with nanogram quantities of enzymatically active pertussis toxin resulted in sensitization and immunophysiological changes that lasted for at least 8 mo. In contrast, no significant changes were demonstrated in rats injected with antigen and the mutant toxin.

METHODS

Animals

Male Sprague-Dawley rats (150–200 g, Charles River Breeding Laboratories, St. Constant, Quebec, Canada) arrived pathogen free and were maintained in a separate room in cages equipped in filter hoods. Rats were actively sensitized by an intraperitoneal (ip) injection of 1.0 mg of chicken egg albumin, grade V (Ova, Sigma Chemical, St. Louis, MO) plus different doses of recombinant wild-type pertussis toxin (wPT, 1–200 ng). Unless otherwise indicated, experimental sensitized rats were injected with Ova plus 50 ng wPT. Other groups of rats were injected with Ova (1.0 mg) plus a mutant pertussis toxin (mPT, 1–200 ng). This mutant had two amino acids substi-

tuted in subunit A, and consequently enzymatic activity was not detectable (31). wPT and mPT were kindly provided by Dr. R. Rappuoli (Sclavo Research Institute, Siena, Italy) and were stored at 4°C in ammonium sulfate. All injected components were dissolved in phosphate-buffered saline and mixed together in a final volume of 1 ml. Control rats were injected with saline, only Ova, or with wPT or mPT alone. The experiments were approved by the Animal Care Committee at McMaster University.

Ussing Chamber Experiments

Unless otherwise indicated, studies were conducted 14 days after sensitization. Rats were killed by an overdose of urethane anesthesia, and a blood sample was obtained. A 10-cm segment of intestinal tissue was excised from the jejunum (beginning 5 cm distal to the ligament of Treitz). Some experiments examined responses in the ileum (beginning 5 cm proximal to the cecum) and colon (beginning 1 cm distal to the cecum). The external muscle with adherent myenteric plexus was stripped off, leaving the submucosal plexus and mucosa intact. From each rat, up to four adjacent pieces of intestine were mounted in Ussing chambers (WPI Instruments, Narco Scientific, Mississauga, Ontario, Canada) exposing 0.6 cm² of serosal surface area to Krebs buffer [(in mM) 115 NaCl, 8 KCl, 1.25 CaCl₂, 1.2 MgCl₂, 2.0 KHPO₄, 25 NaHPO₄, pH 7.35 ± 0.02], containing 10 mM glucose on the serosal side osmotically balanced with 10 mM mannitol on the luminal side. A circulating water bath maintained the buffers at 37°C. The current crossing the tissue was measured in the voltage-clamp mode (zero potential difference) and expressed as short-circuit current (I_{sc} , $\mu\text{A}/\text{cm}^2$). At intervals, the circuit was opened to measure the potential difference (PD, mV) and to determine the conductance (G , mS/cm^2) (6, 28). Baseline measurements (I_{sc} , PD, G) were determined at equilibration, ~20 min after mounting.

Immunophysiological Responses

The I_{sc} response to Ova was calculated as the difference between the baseline I_{sc} and the maximal increase obtained within 5 min after Ova addition to either the serosal or luminal side of the tissues. As a control for the specificity of the response, bovine serum albumin (BSA; Boehringer, Mannheim, Germany) was used. The I_{sc} response to electrical transmural stimulation (TS: 10 Hz, 10 mA, 0.5 ms for a total time of 5 s) of submucosal plexus neurons was measured as described (7, 28). Some experiments were conducted in the presence of the neurotoxin tetrodotoxin (TTX, Sigma; 10⁻⁶ M) added to the serosal side of the tissue. After 15 min, the I_{sc} response to TS was abolished. The I_{sc} response to Ova was then compared in tissues treated with TTX or vehicle.

Antibodies

Passive cutaneous anaphylaxis. Sprague-Dawley rats were injected intradermally with 100 μl of diluted serum from actively immunized rats. Samples were run in duplicate in dilutions from 1:4 to 1:1,024. IgE-positive serum served as a positive control; IgE-negative serum served as a negative control. After 72 h, rats were challenged by intravenous injection of 0.5 ml solution of 1% Evans blue (Sigma) containing 2.5 mg of Ova. Blueing of the skin was evaluated 30 min after injection (26). The highest serum dilution giving a positive reaction was recorded as the passive cutaneous anaphylaxis (PCA) titer of that serum. Heat treatment (56°C, 30 min) of sera was used to confirm that positive reactions were due to heat-labile IgE antibodies (2).

Immunoassays. Total and Ova-specific IgE levels were measured in serum by a paper radioimmunosorbent test and a paper radioallergosorbent test, respectively. These assays were conducted as previously described (15). Briefly, following binding of sheep anti-rat immunopurified IgE (ϵ -specific, 20) or Ova to CNBr-activated paper disks (Whatman 1, Whatman International, UK), the coated discs were washed in buffer (0.3% BSA, 0.03% Tween 20, 0.02% NaN₃ in phosphate-buffered saline) and then incubated in tested serum (dilution 1:10) overnight at 4°C. After being washed in buffer, the disks were incubated with ¹²⁵I-labeled sheep anti-rat IgE (20), washed again, and then the radioactivity was counted in a gamma counter. IgE-positive serum served as a positive control, and buffer and IgE-negative rat serum served as a negative control. The radioactivity of the samples was compared with a standard curve obtained from an IgE myeloma (kindly provided by Dr. H. Bazin, Univ. of Ghent, Belgium). The sensitivity of the assay was 6 U/ml.

Ova-specific IgG and IgG_{2a} antibodies in serum were measured by an enzyme-linked immunosorbent assay (ELISA). The ELISA assay was performed as follows: plastic plates (Nunc U96, Nunc, Roskilde, Denmark) were coated with Ova (5 $\mu\text{g}/\text{ml}$) and left overnight at 4°C. After blockade with 1% BSA for 1 h at room temperature, the sera to be assayed were added and incubated for 2 h (room temperature). Anti-IgG antiserum (Cappel, Durham, NC) or anti-IgG_{2a} antiserum (ICN Immunobiologicals, Costa Mesa, CA) was added followed by the developing antibody, alkaline phosphatase-rabbit anti-goat IgG (ZYMED Laboratories, San Francisco, CA). A color reaction was obtained by addition of the substrate, *p*-nitrophenyl phosphate (Sigma) and was read by an automatic ELISA reader (MR 60, Microplate reader, Dynatech Instruments, Torrance, CA). On each plate, samples were compared with standards of IgG-positive serum.

Histology

Segments of jejunum obtained from animals 14 days after sensitization were fixed in Carnoy's fixative. Sections were prepared and stained with hematoxylin and eosin for morphometric measurements and with toluidine blue for detection of mucosal mast cells. The numbers of mucosal mast cells were calculated per villus/crypt unit or villus/crypt area (mm²). At least 6–10 well-oriented units were measured per section using a computerized videoplan (Kontron, Zeiss, Germany).

Statistical Analysis

Differences among and between groups were analyzed using one-way or two-way analysis of variance or Student's *t*-test, as appropriate. Following one-way analysis of variance, post hoc analysis was performed using Student's Newman-Keuls multiple comparisons test. Pearson's correlation coefficient was used to examine the linear relationship between immunophysiological responses and antibody levels. The differences were considered to be statistically significant when $P < 0.05$.

RESULTS

Immunophysiological Responses to Antigen

Baseline electrophysiological parameters were compared in segments of jejunum from rats injected with Ova plus 50 ng wPT or Ova plus 50 ng mPT. Control rats were injected with saline, Ova, wPT, or mPT alone. There was no significant difference in baseline I_{sc} , PD, and G in the various groups of injected rats (data not shown).

To determine an appropriate concentration of Ova to use for the in vitro challenge, a concentration-response curve was performed. In jejunum from rats injected with Ova plus 50 ng wPT, the response was maximal or near maximal at 100 $\mu\text{g}/\text{ml}$ for luminal or serosal Ova, and consequently in subsequent experiments this concentration of Ova was used. After challenge with Ova, the I_{sc} began to increase within 2 min (luminal addition) or within 20 s (serosal addition). Representative tracings are shown in Fig. 1.

The I_{sc} responses to luminal and serosal Ova were compared at day 14 in intestinal preparations from control rats versus those injected with wPT (Table 1). Control rats injected with saline had no jejunal response at all to Ova challenge. Rats injected with Ova had no significant I_{sc} response to luminal Ova; however, serosal challenge did result in an increase in I_{sc} of $31.5 \pm 7.2 \mu\text{A}/\text{cm}^2$. This response was enhanced 2.2-fold (to $68.3 \pm 9.1 \mu\text{A}/\text{cm}^2$, $P < 0.05$) by the injection of rats with wPT in addition to Ova. In jejunum from rats injected with wPT plus Ova, the mean I_{sc} response to luminal Ova challenge was 18.4-fold that in Ova controls (44.2 ± 4.7 vs. $2.4 \pm 1.3 \mu\text{A}/\text{cm}^2$). Challenge with a nonspecific antigen, BSA, to jejunum from rats sensitized with Ova plus wPT evoked no increase in I_{sc} .

Table 1 also shows that jejunum from rats injected with 50 ng mPT plus Ova did not demonstrate a significantly increased I_{sc} response to luminal or serosal Ova compared with jejunum from rats injected with Ova alone.

Sensitization Doses of wPT and mPT

Rats were injected with different doses of wPT (1.0–200 ng) plus 1.0 mg Ova. Figure 2A shows that the magnitude of the I_{sc} response of the jejunum to luminal Ova challenge was increased with increasing doses of wPT. As little as 1.0 ng wPT resulted in a significantly enhanced response compared with Ova alone; the maximum response occurred in rats injected with 50 ng wPT. A similar pattern of enhanced responses was noted to serosal Ova (Fig. 2B). In contrast, mPT did not enhance

Table 1. I_{sc} response to antigen in jejunum

Treatment	n	Challenge	$\Delta I_{\text{sc}}, \mu\text{A}/\text{cm}^2$	
			Luminal	Serosal
Saline	6	Ova	0	0
wPT	6	Ova	0	0
mPT	4	Ova	0	1.9 ± 1.9
Ova	10	Ova	2.4 ± 1.3	$31.5 \pm 7.2^*$
Ova + wPT	6	Ova	$44.2 \pm 5.2^{*\dagger}$	$68.3 \pm 9.1^{*\dagger}$
Ova + wPT	5	BSA	0	0
Ova + mPT	5	Ova	7.8 ± 5.3	$42.9 \pm 21.5^*$

Values represent means $\pm$ SE; n = no. of rats (values from 1–3 tissues were averaged from each rat). Values represent maximum rise in short-circuit current (I_{sc}) occurring during the first 5 min after ovalbumin (Ova) or bovine serum albumin (BSA) challenge (100 $\mu\text{g}/\text{ml}$). $P < 0.05$: *compared with responses in saline injected rats; $\dagger$ compared with responses in rats injected with Ova alone. Animals were injected with 1 ml saline, 50 ng wild-type pertussis toxin (wPT)/mutant PT (mPT), and 1 mg Ova.

immunophysiological responses at any dose up to 200 ng/rat (Fig. 2, C and D).

IgE Levels

PCA titers of sera from Sprague-Dawley rats injected with Ova alone or different doses of wPT or mPT plus Ova are shown in Fig. 3. Sera from rats injected with Ova alone (0 wPT), Ova plus mPT, or Ova plus low doses of wPT had no or very small PCA responses, with the mean for these groups not significantly different from zero. Mean positive PCA responses to Ova were obtained in rats injected with wPT from 10 to 200 ng and were highest in rats treated with 50 ng. Some positive sera were tested again after heat treatment at 56°C for 30 min. Heat treatment virtually abolished positive responses. In two of seven cases, a small degree of responsiveness remained after heat treatment: the titer dropped in one from 1:256 to 1:16 and in the second from 1:16 to 1:4. This finding indicates that the majority of the reaginic response to PCA is mediated by heat-labile IgE antibodies.

At these levels, IgE concentrations were too low to be detected by immunoassays. Immunoassays revealed no significant differences in total and Ova-specific IgE in sera from Sprague-Dawley rats before and after sensitization for all the protocols: Ova plus wPT, Ova plus mPT, or Ova alone. The assay did detect high levels of IgE in pooled sera from sensitized Brown-Norway rats, a high IgE responder strain, where serum with a PCA titer of 1:2,048 had 1,660 U/ml of Ova-specific IgE. Serum with a PCA titer $< 1:256$ had no detectable IgE measurable by the immunoassay.

IgG and IgG_{2a} levels

Since there is evidence that IgG_{2a} can bind to mast cells (2), Ova-specific total IgG and IgG_{2a} antibody levels were measured in sera from rats injected with Ova plus wPT, Ova plus mPT, and Ova alone (Fig. 4). The levels of IgG were significantly increased only by injection of rats with wPT plus Ova; this was almost entirely due to IgG_{2a}. The pattern was similar to that seen for IgE. Rats

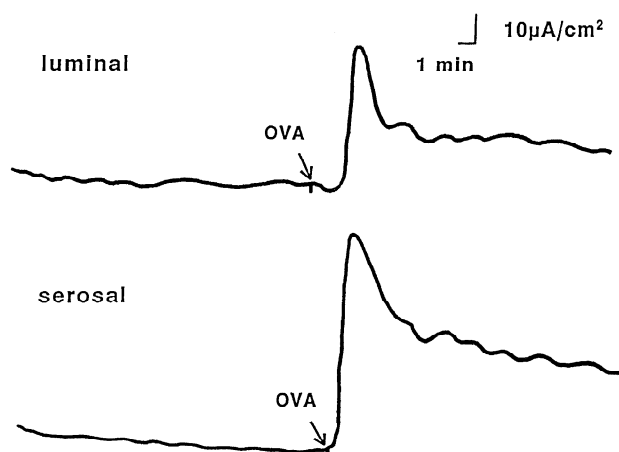


Fig. 1. Representative tracings of short-circuit current (I_{sc}) responses to luminal or serosal ovalbumin (Ova; 100 $\mu\text{g}/\text{ml}$) added to jejunal tissues mounted in Ussing chambers. Tissues were obtained from rats 14 days after injection with Ova + 50 ng wild-type pertussis toxin (wPT).

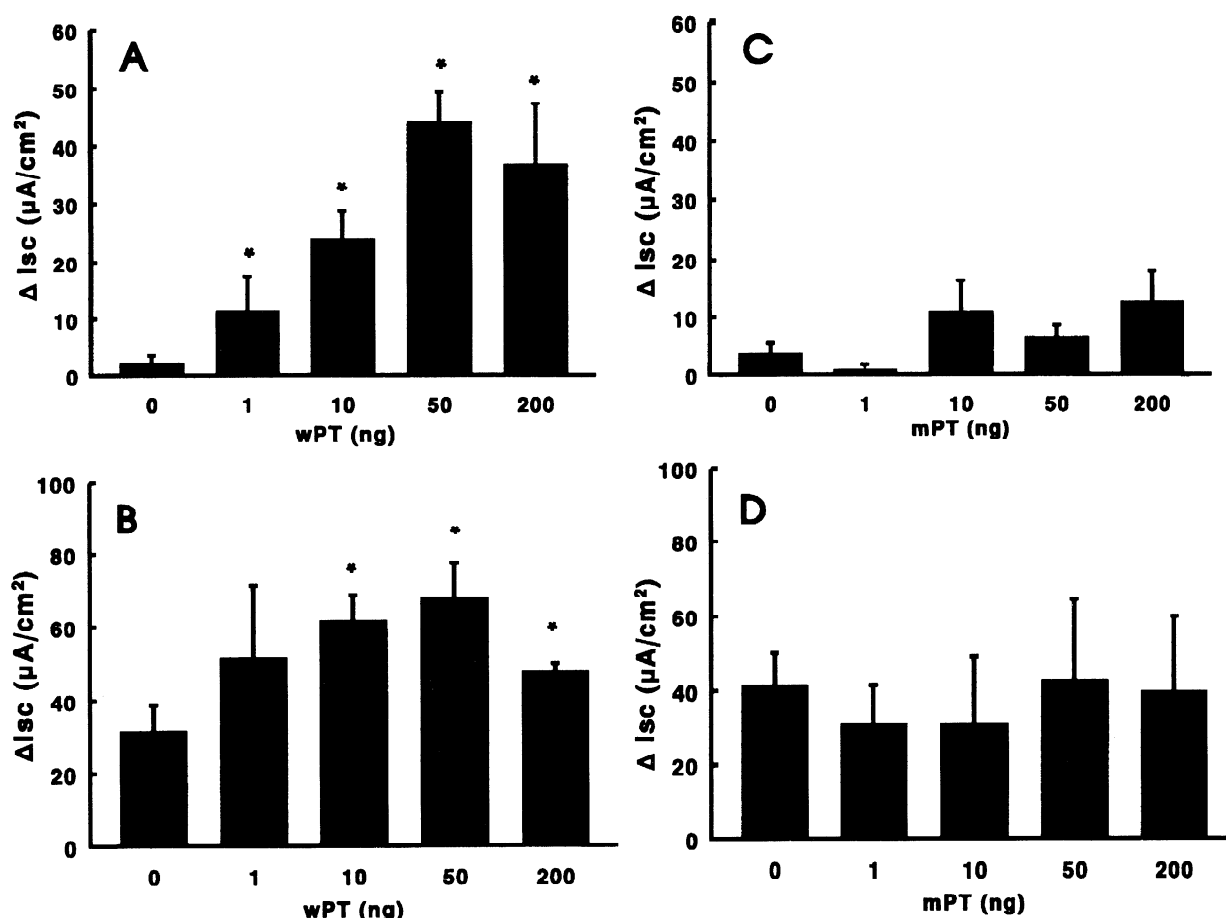


Fig. 2. Responses after sensitization with various doses of wPT (A, B) or mutant pertussis toxin (mPT; C, D). Values represent means $\pm$ SE for increases in I_{sc} in response to luminal (A, C) or serosal (B, D) Ova (100 $\mu g/ml$) added to jejunal tissues from rats 14 days after injection with Ova + wPT (0–200 ng) or Ova + mPT (0–200 ng). $n = 6$ –10 rats/group, 4 tissues/rat; * $P < 0.05$ compared with rats injected with Ova alone. Data represent maximum response within 5 min after Ova challenge.

treated with mPT did not show any elevation of IgG or IgG_{2a} above control levels.

Relation of Circulating Antibody Levels to Immunophysiological Responses

When analyzed by groups, Sprague-Dawley rats sensitized with wPT plus Ova had higher mean values for

both antibody levels and antigen-induced increases in I_{sc} compared with the control groups. However, there was no significant correlation between the magnitude of individual intestinal immunophysiological responses and the level of circulating antibodies [r (36) = 0.34 for luminal antigen addition and 0.20 for serosal antigen addition].

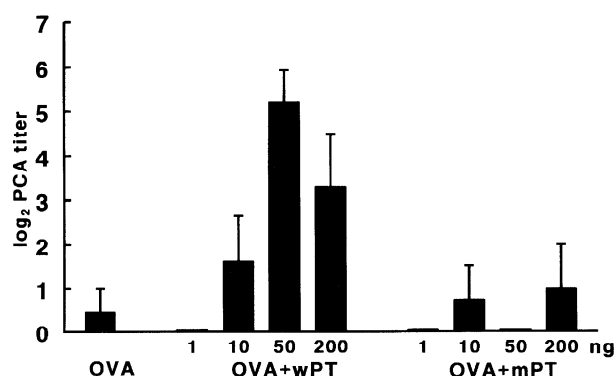


Fig. 3. Ova-specific IgE titers measured by passive cutaneous anaphylaxis. Values represent means $\pm$ SE of titers in serum samples from rats 14 days after injection with Ova only or Ova plus different doses (from 1 to 200 ng) of wPT or mPT; $n = 4$ –6 rats/group.

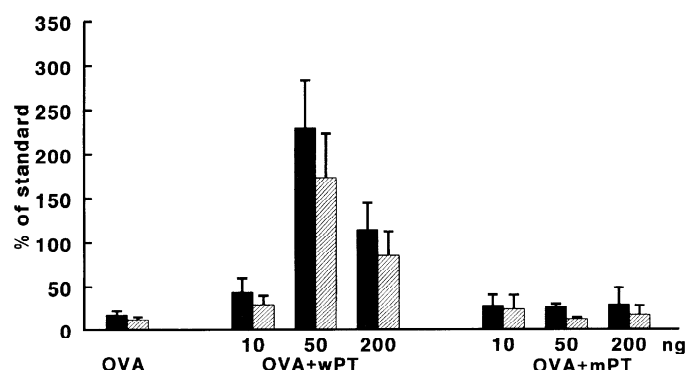


Fig. 4. Ova-specific IgG (solid bar) and IgG_{2a} (hatched bar) in serum samples from rats 14 days after injection with Ova only or Ova plus different doses (from 10 to 200 ng) of wPT or mPT. Data are expressed as % of standard serum; $n = 4$ –6 rats/group.

Table 2. *Morphometric parameters*

Treatment	n	Mast cells/VCU	Villus Area, mm ²	Crypt Area, mm ²	VCU Area, mm ²	Mast cells/mm ²
Saline	5	8.9 ± 0.5	0.40 ± 0.03	0.17 ± 0.02	0.57 ± 0.05	14.6 ± 3.7
Ova	7	9.2 ± 0.8	0.40 ± 0.02	0.18 ± 0.01	0.58 ± 0.03	15.9 ± 1.0
Ova + wPT	6	13.0 ± 1.4*	0.40 ± 0.02	0.16 ± 0.01	0.56 ± 0.03	23.2 ± 2.2*
Ova + mPT	6	8.0 ± 0.5	0.40 ± 0.04	0.17 ± 0.02	0.57 ± 0.06	14.0 ± 2.7

Values represent means ± SE; n = no. of rats [6–10 villus/crypt units (VCU) were evaluated from each rat]. Measurements were made on jejunal sections obtained from rats 14 days after injection with saline, OVA alone, OVA + 50 ng wPT, or OVA + 50 ng mPT. *P < 0.05.

Morphological Studies

Morphological studies were performed to determine if sensitization with wPT altered the number of mast cells in the intestinal mucosa. Granulated mast cell numbers were significantly increased ~40% (P < 0.05) in the lamina propria of jejunum from rats injected with Ova plus wPT compared with control animals injected with saline or Ova alone (Table 2). In contrast, injection of Ova plus mPT did not increase the number of mast cells in the jejunal mucosa. Other morphometric parameters quantitated (villus and crypt areas) were not altered by the sensitization protocol.

Site of Effector Cells

Experiments were conducted to obtain more information regarding the location of the effector cells mediating the responses to luminal antigen. The timing of the initiation of the I_{sc} response and its magnitude were compared for addition of luminal versus serosal antigen. The I_{sc} response began 16.00 ± 0.04 s after addition of Ova to the serosal buffer in the Ussing chamber, whereas the response to luminal Ova began relatively later after a delay of 1.35 ± 0.50 min. In addition, the response to the addition of 100 μ g/ml Ova to the luminal side was ~50% of the response to the addition of the same concentration of Ova to the serosal side (27.0 ± 4.4 vs. 51.2 ± 6.5 μ A/cm², n = 15). After stabilization of the I_{sc} following serosal Ova addition, tissues were challenged with Ova on the luminal side. These tissues demonstrated virtually no capacity to respond (1.2 ± 0.8). These results suggest that subepithelial mast cells medi-

ate the response to antigen whether it is added to the luminal or serosal side of the tissues.

Responses to Nerve Stimulation

I_{sc} responses to electrical transmural stimulation (TS) of nerves were recorded to determine if sensitization with pertussis toxin affected intestinal nerves. Tissues from rats injected with Ova plus wPT had a higher I_{sc} response to TS than tissues from rats injected with saline (Fig. 5). A somewhat greater response was also documented in intestine from rats injected with Ova alone, but this was not significantly different from saline controls. Intestine from rats injected with Ova plus mPT had an I_{sc} response to TS comparable to that in control Ova-injected rats. This finding indicates that wPT, but not mPT, changed the ability of nerves to respond to stimulation. All I_{sc} responses to TS were abolished after 15-min treatment with the neurotoxin TTX.

Neuronal Regulation of Luminal Responses

TTX was used to examine neuronal involvement in the pertussis toxin-induced hypersensitivity reaction. Jejunal tissues from wPT-sensitized rats displayed a 66% lower response to luminal Ova following 15-min pretreatment with the neurotoxin (9.8 ± 1.5 μ A/cm²), compared with tissues treated with vehicle (28.9 ± 4.8 μ A/cm²; P < 0.05) (Fig. 6). However, the serosal response to Ova was not affected by TTX (48.0 ± 4.8 vs.

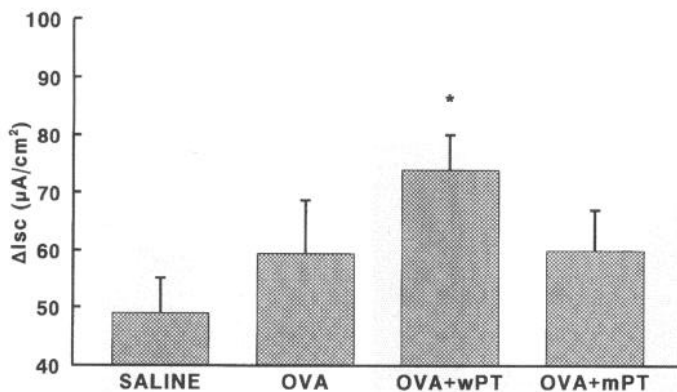


Fig. 5. Values represent means ± SE of increases in I_{sc} in response to electrical transmural stimulation of nerves in jejunal preparations from rats 14 days after injection with saline, Ova only, Ova plus wPT, or Ova plus mPT; n = 6 rats (4 tissues/rat); *P < 0.05 compared with saline control tissues.

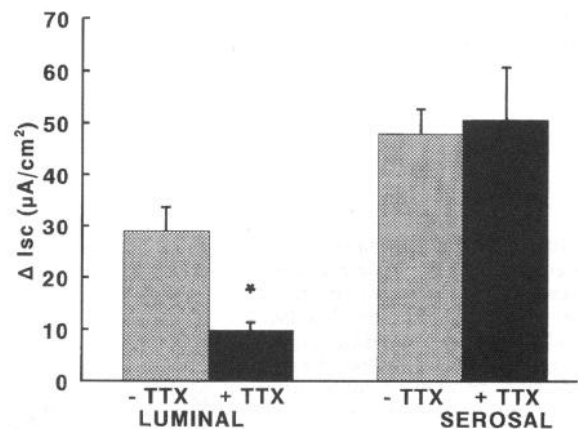


Fig. 6. Values represent means ± SE of increases in I_{sc} in response to Ova (100 μ g/ml) added to the luminal (crosshatched bar) or serosal (solid bar) side of jejunal tissues pretreated with TTX (10^{-6} M) or vehicle. Tissues were obtained from rats 14 days after injection with Ova + 50 ng wPT; n = 9 rats; *P < 0.05 compared with control tissues (not treated with TTX). Data represent maximum response within 5 min after Ova challenge.

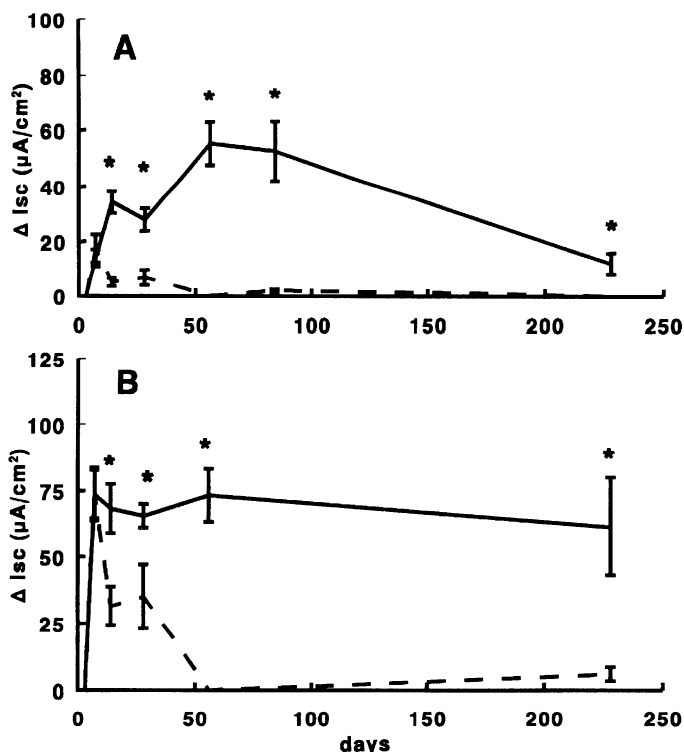


Fig. 7. Time course of immunophysiological responses. Values represent means $\pm$ SE of increases in I_{sc} in response to luminal (A) or serosal (B) Ova added to jejunal tissues from rats at day 3, 7, 14, 28, 56, 84, and 226 after injection with Ova + 50 ng wPT (solid line) or Ova alone (dashed line). $n = 4-12$ rats/group (4 tissues/rat) except for control experiments on day 84 and 226 when $n = 2$ rats; * $P < 0.05$ compared with Ova-injected rats. Data represent the maximum response within 5 min after Ova challenge.

$50.7 \pm 10.1 \mu\text{A}/\text{cm}^2$, for 9 untreated and 9 TTX-treated tissues, respectively). The findings of TTX inhibition of the response to luminal Ova, but not to serosal Ova, were unaffected by reduction of the concentration of antigen added (data not shown).

Time Course of Immunophysiological Responses

To establish how long a single injection of wPT plus Ova predisposes an animal to respond in an enhanced

manner to secondary Ova challenge, rats were killed at different times and increases in jejunal I_{sc} to Ova were examined (Fig. 7, A and B). No response to Ova was recorded in intestine from rats injected with Ova alone or Ova plus wPT at day 3. Jejunum from day 7 rats responded to Ova; however, there was no significant difference in the magnitude of the response in intestine from rats injected with Ova alone versus Ova plus wPT. On day 14, the I_{sc} responses were significantly elevated in Ova plus wPT-injected rats compared with rats injected with Ova alone. Maximum responses to luminal Ova were evident at days 56 and 84. At 8 mo, the Ova plus wPT-injected animals still demonstrated significant responses to luminal and serosal Ova, whereas responses were negligible after ~ 50 days in rats injected with Ova alone.

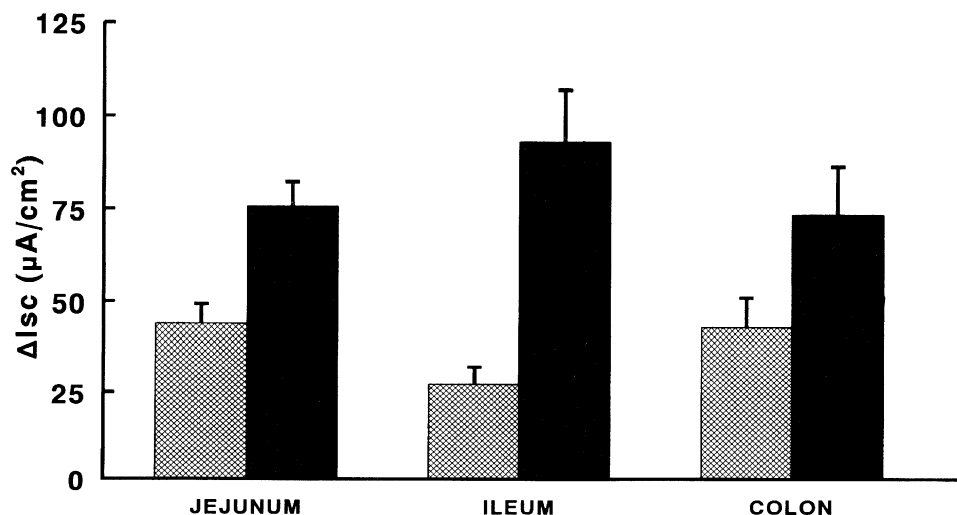
Immunophysiological Responses in Ileum and Colon

Fourteen days after sensitization, ileal and colonic intestinal segments from Ova plus wPT-injected rats responded to Ova in a similar fashion to jejunal segments (Fig. 8). In the ileum, luminal Ova caused a change (Δ) in I_{sc} of $27.4 \pm 5.1 \mu\text{A}/\text{cm}^2$ and serosal Ova caused ΔI_{sc} of $93.0 \pm 14.1 \mu\text{A}/\text{cm}^2$. In the colon, luminal Ova caused ΔI_{sc} of $43.3 \pm 7.9 \mu\text{A}/\text{cm}^2$ and serosal Ova caused ΔI_{sc} of $73.32 \pm 13.1 \mu\text{A}/\text{cm}^2$.

DISCUSSION

We have previously used whole cell pertussis vaccine as an adjuvant in a rodent model of hypersensitivity (7). Our goal in the present study was to examine the role of pertussis toxin, a component of the vaccine, in the sensitization of rats to a bystander antigen and in the immunophysiological response that occurs in response to secondary antigen challenge. This study demonstrated that nanogram quantities of wPT injected with an antigen into rats dramatically enhance the hypersensitivity reaction, particularly stimulating the intestinal secretory response to luminal antigen. We showed that the enzymatic activity of pertussis toxin is essential for its effect. In addition, our results indicate that the

Fig. 8. Immunophysiological responses in different regions of gut. Values represent mean $\pm$ SE increases in I_{sc} in response to luminal (crosshatched bar) or serosal (solid bar) Ova added to jejunum, ileum, and colon from rats 14 days after injection with Ova + 50 ng wPT. $n = 6-12$ rats/group (4 tissues/rat). Data represent maximum response within 5 min after Ova challenge.



wPT-induced immunophysiological response was very long lasting, being evident for at least 8 mo.

Pertussis toxin is a subunit toxin composed of two moieties, designated A and B. Subunit B binds to the *N*-linked oligosaccharides on the cell surface, whereas subunit A enters the membrane and ribosylates several types of G proteins (21). We compared the effects of a recombinant wild-type toxin versus an enzymatically inactive mutant toxin that had two amino acids substituted in subunit A (31). In contrast to the action of wPT, sensitization with mPT as adjuvant did not affect the antigen-induced I_{sc} response in the small intestine. This finding provides evidence for the involvement of G proteins in the mechanism of the gut pathophysiology. G proteins have been identified in a range of immunological and nonimmunological cells, and in vitro studies have documented effects of pertussis toxin on a variety of cell types including T cells (5), mast cells (24), epithelial cells (36), and neuronal cells (14). The action of wPT on some or all of these cell types may enhance hypersensitivity reactions in the gut.

T cells are necessary for antibody production by B cells, and previous studies have shown that IgE antibodies are produced by sensitizing animals using *B. pertussis* vaccine as adjuvant (13). In our model, PCA titers of sera from sensitized rats were significantly higher after injection with wPT in addition to Ova and were consistently reduced or abolished by heat, suggesting that the responsible antibodies were of the heat-labile IgE isotype. The mechanism by which pertussis toxin stimulates IgE antibody production is not clear but may relate to Ig class switching. Recent studies by Hong-Hu and Sewell (12) showed that pertussis toxin increases mRNA expression and secretion of interleukin-4 by spleen and lymph node cells (12); this cytokine is recognized as a switch factor for IgE production (35). In addition, experiments indicate the involvement of cAMP in Ig class switching (19), and pertussis toxin-induced elevation of cAMP by ribosylation of G inhibitory proteins is well recognized (10).

Both the highest mean PCA titer and the largest mean immunophysiological responses to luminal antigen were documented in the group of rats that were injected with Ova plus 50 ng wPT. However, the levels of circulating anti-Ova antibodies were not predictive of the magnitude of the local gastrointestinal response to the antigen in individual rats. A similar result has been reported previously in animals (27) and also in humans when milk-sensitive patients with gastrointestinal symptoms (but undetectable circulating IgE) secreted histamine and demonstrated increased permeability of the small intestine in response to milk challenge (17). Levels of circulating antibodies do not necessarily reflect those bound to mast cells in tissue sites. These findings suggest that the immunophysiological reactions typical of immediate hypersensitivity depend on small but critical levels of reagenic antibodies that may be below the level of detection in the circulation.

In mice, pertussis toxin induced elevated levels of both IgE and IgG₁ (the reagenic IgG subtype in the mouse) antibodies (23). In rats, IgG_{2a} antibodies bind to recep-

tors on mast cells and may participate in anaphylactic reactions (1, 2). In the present experiments, rats that received 50 ng of wPT plus Ova demonstrated elevated levels of Ova-specific IgG_{2a}. Our findings demonstrate that, in contrast to wPT, mPT does not stimulate production of reagenic antibodies of either isotype to a bystander antigen and therefore indicate the requirement for G protein ribosylation. However, our studies were not directed at the mechanism by which wPT stimulates antibody production but rather at the immunophysiological response in the intestine. Further studies are required to determine the relative role of IgE versus IgG_{2a} antibody isotypes in the antigen-induced transport abnormalities.

Mucosal mast cells are important in intestinal anaphylactic responses (27). In mast cell-deficient mice, the response to antigen is diminished by 70% compared with congenic normal mice and is restored after mast cell reconstitution by bone marrow transplantation (29). The number of intestinal mucosal mast cells is increased in certain pathological conditions where anaphylactic reactions to antigens may be involved, such as food allergies, parasitic infection, Crohn's disease, and celiac disease (reviewed in Ref. 6). In our experiments, sensitization with Ova plus wPT resulted in mucosal mast cell hyperplasia. Therefore, the enhancement of intestinal secretory responses could be due, at least in part, to the greater numbers of mast cells participating in the reaction with the release of increased quantities of mediators. In previous studies in which rats were sensitized to Ova with pertussis vaccine as adjuvant, mast cell numbers did not increase significantly (8). The reason for this difference could be the presence of more enzymatically active pertussis toxin injected in the present regime compared with the vaccine. In support of this hypothesis, the antigen-induced I_{sc} responses were generally larger and more consistent following injection of specific amounts of toxin. After wPT injection into rats, mast cell growth may be stimulated by release of T cell or other cytokines (9).

I_{sc} responses to luminal antigens are not normally studied in Ussing chamber experiments since such responses are usually very small (or negligible) or delayed in most models of hypersensitivity, including those induced by parasitic infection. However, luminal responses are important from a physiological viewpoint. After wPT injection, the response to luminal Ova was enhanced ~20-fold, but the response to serosal antigen was enhanced only ~2.5-fold. Although wPT-induced antibody and mast cell effects may explain the enhanced serosal response, obviously additional factors are involved in the greatly augmented luminal response. Our evidence suggests that the I_{sc} change in response to both luminal and serosal Ova was due to activation of subepithelial mast cells for the following reasons: 1) the I_{sc} response to luminal antigen was delayed compared with the response to serosal antigen; 2) the magnitude of the I_{sc} response to luminal antigen was lower than that to serosal antigen; and 3) the response to luminal antigen was abolished after serosal antigen addition. These findings suggest that in this muscle-stripped tissue,

serosal antigen has greater access to subepithelial mast cells and completely activates them, whereas the access of luminal antigen is more limited resulting in only partial activation. The decreased time and magnitude of the I_{sc} response to luminal antigen may be due to the fact that only a portion of added Ova crosses the epithelial barrier in an immunologically intact form and is able to cross-link IgE antibodies on mast cells to activate them. Antigenic uptake across the epithelium may be the crucial factor determining the magnitude of the physiologically relevant hypersensitivity reaction.

We have previously reported neurotoxin inhibition of antigen uptake from the lumen of rats sensitized with pertussis vaccine (8). Inhibition of the secretory response in isolated intestine to luminal Ova was also documented in that model, but the response to serosal antigen was not examined (7). Pertussis toxin has been reported to act on many G proteins coupled to a variety of receptors on nerves (22, 25). In this study, we found that the I_{sc} response to TS in intestine from rats sensitized with wPT was statistically higher than that in the control groups, indicating that wPT affects nerves that regulate ion transport. Furthermore, our studies showed 66% inhibition by TTX of the response to luminal antigen in wPT-sensitized rats, whereas the response to serosal antigen was unaffected. This pattern of inhibition was evident regardless of the concentration of antigen used in the challenge. TTX did not inhibit I_{sc} responses to serosal addition of antigen. Although such inhibition has been reported in some models of hypersensitivity (3, 29, 37), other studies have not found inhibition by TTX (4). Different species, sensitization protocols, or regions of gut having dissimilar innervation may account for the variable effects. Our findings suggest that wPT enhances responses to luminal antigen by acting on nerves that limit antigen uptake. The pathway of increased antigen penetration of the epithelium is not clear from these studies but is the focus of ongoing studies in our laboratory. Neuronal regulation of tight junctions has been suggested in rats, where intravenous stimulation with carbachol increased paracellular movement of horseradish peroxidase (30). Another recent study has described nerve-mediated effects on intact protein transport across gut epithelium (16).

The enhancement of gut hypersensitivity by wPT was very long lasting. In contrast to the rapid decay of the response in rats sensitized without adjuvant, even 8 mo after injection of 50 ng of wPT, there was still significant responsiveness to antigen. No other studies have reported the duration of intestinal hypersensitivity to antigen induced by pertussis toxin or vaccine. However, pertussis toxin was reported to increase sensitivity of mice to histamine, and this phenomenon lasted for at least 84 days (23). Additional studies are required to determine the neuronal and other contributions to the response at the later times.

In conclusion, our experiments indicate that a single injection of wPT with antigen stimulates hypersensitivity to that antigen. Pertussis toxin promotes IgE and IgG_{2a} antibody production, suggesting a role for these antibodies in the induction of intestinal responses lead-

ing to ion secretion. Another factor possibly contributing to the enhanced responsiveness of the gut is the mucosal mast cell hyperplasia. Finally, our results indicate that wPT affects neuronal regulation of epithelial function, resulting in amplification of antigen-mediated secretory responses due to increased antigen uptake. Further dissection of the effects of wPT may prove useful in the elucidation of the precise mechanisms involved in these effects. Although antigen-induced responses in the airways were not investigated in this study, we postulate that similar abnormalities may occur in the respiratory tract since we have previously documented parallel reactions in both systems after injection of whole cell pertussis vaccine (34). Our work in rats also suggests that it may be important to investigate the effects of pertussis toxin on sensitization of humans for allergic tissue responses. The findings that pertussis toxin can cause very long-lasting immunophysiological effects may be of significance in view of the current practice of immunization of children against *B. pertussis* infection.

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