ROLE OF PERTUSSIS TOXIN IN INTESTINAL HYPERSENSITIVITY

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To my dear mom

ABSTRACT

Immediate hypersensitivity (allergy) is a very common disorder, which may develop after exposure of an individual to an antigen. Intestinal hypersensitivity to luminal antigens has been postulated as a possible cause or triggering mechanism in the pathogenesis of ulcerative colitis, Crohn's disease, eosinophilic gastroenteritis, celiac disease and peptic ulcer. The mechanism by which individuals became sensitized is not known but naturally occurring adjuvants (bacteria and their products) may be crucial for the development of hypersensitivity. In my studies, I investigated in the role of pertussis toxin, a product of *Bordetella pertussis*, in intestinal hypersensitivity, since pertussis vaccine containing attenuated bacteria was used previously for the induction of anaphylaxis in experimental animals. Pertussis toxin has the enzymatic activity of ADP-ribosyltransferase, which can block the function of some G proteins, important elements in the transduction of signals into the cell. I sensitized rats or mast cell-deficient mice with ovalbumin (OVA) plus recombinant wild type pertussis toxin (wPT) as adjuvant. The reaction to secondary antigen challenge was evaluated 14 days later by determining changes in short-circuit current (Isc, indication of net ion transport) in small intestinal segments mounted in Ussing chambers. Other parameters measured included evaluation of antibody levels in the circulation and histological enumeration of mast cells in the intestinal

mucosa.

Sensitization of rats with OVA and wPT resulted in enhancement of the jejunal ion secretory response to secondary antigen challenge as compared to rats sensitized with OVA. Injection of doses of wPT as low as 10 ng with OVA caused significantly elevated secondary responses to OVA, while 50 ng wPT resulted in the maximal response. The responses to OVA challenge were enhanced 18.7 fold when OVA was added to the luminal side of the tissues while on serosal side this effect was enhanced 2.2 fold in sensitized rats. The induction of hypersensitivity by wPT adjuvant was dependent on the enzymatic activity of wPT, since the enzymatically inactive mutant (mPT) did not influence responses to secondary antigen challenge. This suggests that ADP-ribosylation of G proteins is involved in the elevation of hypersensitivity by wPT.

Two classes of antigen-specific antibodies, IgE and IgG_{2a}, were measured in the circulation of sensitized rats. The levels of both antibody isotypes were increased at day 14 in wPT but not mPT plus OVA injected rats, which indicates that enzymatically active wPT influences antibody production. The role of particular classes of antibodies was examined by passive sensitization of naive animals with serum from actively sensitized rats. Ion transport responses to secondary antigen challenge of intestine were present in those animals. Blockage of IgE receptors (with myeloma IgE) prior to passive sensitization showed that the response to OVA was totally abolished. This finding shows that in this model of hypersensitivity, IgE antibodies are crucial for the response to

antigen. Experiments on mast cell-deficient mutant mice revealed that mast cells are a necessary element for the responses to antigen. Additionally, the number of stainable mast cells in rat intestinal mucosa was significantly increased by 40% on day 14 in wPT but not mPT plus OVA injected rats. This indicates that wPT-induced increases in mast cell numbers are partially responsible for elevated hypersensitivity responses to antigen. Experiments with the neural toxin, tetrodotoxin (TTX), showed that responses to luminal (but not serosal) OVA were neurally regulated in that these responses to OVA were diminished by 66% after treatment of the tissues with TTX.

Evaluation of the responses to OVA at different days after primary sensitization with OVA plus wPT revealed that the responsiveness to antigen appeared between day 3 and 7 after sensitization, and it was still present on day 228. In rats sensitized with OVA, the responsiveness to OVA was highest on day 7 and than it decreased very rapidly, showing no responses on day 56. Evaluation of IgE levels in rats demonstrated a similar pattern: the IgE levels were detectable for a long time in wPT plus OVA treated rats but only on day 7 in OVA treated rats. This suggest that elevated IgE levels caused by wPT may account for the long lasting hypersensitivity responses.

My data showed that wPT is a very potent adjuvant for hypersensitivity reactions and that the mechanism involves elevation of antigen-specific antibodies, increases in the number of mucosal mast cells and enhanced neurally-mediated antigen uptake.

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ABBREVIATIONS

AA	arachidonic acid
ACh	acetylcholine
ADP	adenosine diphosphate
APCs	antigen presenting cells
BM	bone marrow
cAMP	adenosine 3'5'-cyclic phosphate
cGMP	guanosine 3'5'-cyclic phosphate
CCK	cholecystokinin
cPV	pertussis vaccine
CTMC	connective tissue mast cells
ENS	enteric nervous system
FcεRI	high affinity IgE receptor
FcεRII = CD23	low affinity IgE receptor
G protein	guanyl nucleotide-binding protein
GALT	gut associated lymphoid tissue
GDP	guanosine diphosphate
GM-CSF	granulocyte/ macrophage colony stimulating factor
GTP	guanosine triphosphate
5-HT	serotonin
IgA	immunoglobulin A
IgE	immunoglobulin E
IgG	immunoglobulin G
IgG_{2a}	immunoglobulin G_{2a}
IgM	immunoglobulin A
IELs	intraepithelial leucocytes
IFN	interferon
IL	interleukin
IP3	inositol triphosphate
Isc	ionic shoort-circuit current
LIF	leukemia inhibitory factor
LPS	lipopolisaccharide
LTs	leukotrienes
LTB_4	leukotriene B_4
LTC_4	leukotriene C_4
LTE_4	leukotriene E_4
MC	mast cell
MDCK	Mardin-Darby canine kidney cells
+/+ mouse	mast cell-sufficient mouse
MMC	mucosal mast cell
NAD	nicotinamide-adenine dinucleotide
mPT	recombinant mutant pertussis toxin

PAF	platelet activating factor
PCA	passive cutaneous anaphylaxis
PDE	phosphodiesterase
PGs	prostaglandins
PGD_2	prostaglandin D_2
PGE_2	prostaglandin E_2
PGI_2	prostaglandin I_2
PI	phosphatidyl 4,5-biphosphate
PLA_2	phospholipase A_2
PLC	phospholipase C
PT	pertussis toxin
RMCP I/II	rat mast cell protease I/II
SP	substance P
T84	colon carcinoma epithelial cells
TGF-β	tumor growth factor
TNF-α	tumor necrosis factor
wPT	recombinant wild pertussis toxin
VIP	vasoactive intestinal peptide
W/W^v	mast cell-deficient mouse

CHAPTER I

INTRODUCTION

1.1 Intestinal mucosa

The intestine can be envisaged as a hollow tube consisting of a number of concentric layers (Allen D 1992)(Figure 1). The most external layer is the

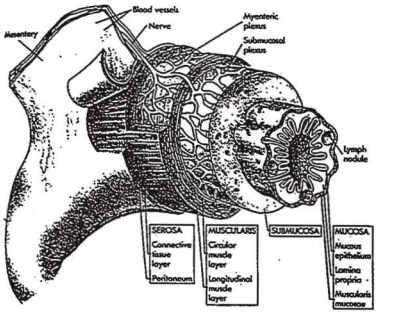

Figure 1. Anatomy of digestive tract shows the concentric layers of the tissue. The most inner is mucosa which is build from epithelial layer, lamina propria and muscularis mucosae. Overlaying the mucosa is submucosa. The muscularis which has circular and longitudinal muscles is covered by serosa (adapted from: Anatomy and Physiology; Allen D, ed.; Mosby Year Book, St. Luis; 1992) .

serosa which is composed of connective tissue. Adjacent to this are two layers of muscle, an outer longitudinal and an inner circular layer, whose primarily function is intestinal motility. The bulk of the intestine is made up of diffuse mucosal and submucosal (lamina propria) tissues that are covered by a continuous one cell thick layer of epithelial cells. The intestine is designed to expose a large surface area to the lumen contents. Expansion of the intestinal surface area is achieved by: the presence of mucosal folds, the presence of villi (finger-like projections) and crypts (depressions) and microvilli (projections coming from the apical membrane of epithelial cells) (Figure 2). These adaptations increase surface area by about 600 fold (Barrett KE 1991).

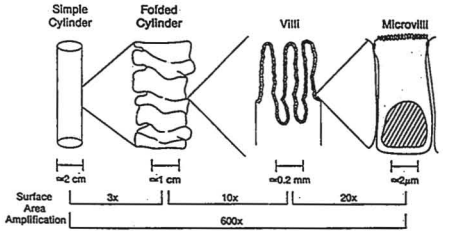

Figure 2. Diagram of expansion of the intestinal surface area by presence of mucosal folds, villi/crypts and epithelial microvilli. Those adaptations increase surface area by about 600 folds (from Barrett KE and Darmsathaphorn K; in: Textbook of Gastroenterology; Yamada T, ed; JB Lippincott Co, Philadelphia; 1991).

1.1.1 Epithelium

The epithelial monolayer consists of a variety of cell types: enterocytes, goblet cells, endocrine cells, Paneth cells, M cells and intraepithelial leucocytes (IELs) (Solcia E 1987, Madara JL 1987). The enterocyte is the predominant cell type and these cells are the regulators of absorption of nutrients and ion exchange between the circulation and the gut lumen (Powell DW 1987). The endocrine cells contain biogenic amines (serotonin) and bioactive peptides (substance P (SP), neuropeptide YY, neurotensin, cholecystokinin (CCK), glucagon-like peptide-1), the release of which may regulate or modulate epithelial function in a paracrine fashion (Roth KA 1992). Goblet cells, interspersed among enterocytes, are filled with secretory granules containing glycoproteins, which can be discharged onto the luminal surface as a mucus (Madara JL 1987). Mucus has a protective function, lubricating the gut and acting as an extrinsic barrier to antigen attachment to the epithelium. The Paneth cells occur at the base of crypts and although they are readily distinguishable morphologically, little is known of their biological function (Satoh Y 1992). The presence of granules with antimicrobial proteins, defensins, suggests a role in host defence (Jones DE 1993). M cells and IELs are related to the immune function of the mucosa (Scott H 1993). M cells are located only on domes of lymphoid follicles interspersed among the absorptive enterocytes and are specialized for antigen delivery to the mucosa (Amerongen HM 1992). IELs are relatively abundant in the epithelium and are most of them located along the epithelial basement

membrane (Halstensen TS 1990). Most IELs posses a Tc receptor and most of them are CD8 (cytotoxic/suppressor) phenotype.

1.1.2 Lamina propria

The lamina propria consists primarily of connective tissue and an array of other cell types: immune cells (mast cells, T cells, B cells/plasma cells, eosinophils, monocytes/macrophages), neurons, fibroblasts, endothelial and muscle cells (Kraehenbuhl JP 1992). The immune and neuronal components of the mucosa are the two major systems which have been shown to influence epithelial ion secretion in normal or pathophysiological conditions (Brown DR 1988, Hinterleitner TA 1991).

1.1.2.1 Immune system

The lumen of the gastrointestinal tract is constantly exposed to many foreign antigens. To deal with this large and diverse array of foreign substances, a local immune system, the gut associated lymphoid tissue (GALT), has developed along mucosal surfaces (Strobel S 1993). GALT consists of both organized lymphoid compartments (Peyer's patches and solitary follicles) and a large cell population distributed diffusely throughout the mucosal lamina propria and epithelium. Peyer's patches are inductive sites, where T cells, B cells and antigen presenting cells (APCs) encounter environmental antigen. These structures which are distributed along the entire intestine are covered by M cells.

In Peyer's patches, antigen presented by APCs (such as macrophages and dendritic cells) activates T cells which help B cells to produce antibodies (Hume DA 1987, Unanue ER 1987). Under normal conditions, most of the immune response (60%) involves IgA production and to much lesser extent IgG, IgM and IgE (James SP 1993).

1.1.2.2 Enteric Nervous System

The enteric nervous system (ENS), which innervates the gastrointestinal tract, is part of the autonomic nervous system (Cooke HJ 1987, 1992). The ENS, although integrated with the central nervous system (receiving sympathetic and parasympathetic inputs) can function independently of the central nervous system. Enteric innervation coordinates epithelial function to maintain normal gut physiology or to respond to physiological or pathological changes. Two major plexi constitute the ENS: the submucosal plexus and the myenteric plexus (Figure 1). Both plexi have projections that ramify throughout the lamina propria to innervate the epithelium, blood vessels, endocrine cells, muscularis mucosa or other submucosal and myenteric ganglia. Anatomical and functional studies have demonstrated that epithelial functions are principally regulated by submucosal neurons, rather than by myenteric neurons (Jodal M 1990).

1.2 Intestinal immediate hypersensitivity

Hypersensitivity describes the exaggerated or inappropriate immune

response to antigen in sensitized individuals (James JM 1992). According to the classification of Coombs and Gell, four types of hypersensitivity can be identified (type I, II, III, IV)(Roitt IM 1989). They differ both in the timing (between exposure to antigen and the occurrence of symptoms) and in the immunological mechanisms involved. Immediate hypersensitivity (allergic reactions) take place within minutes or hours after contact with antigen (allergen). The sensitizing antigen, in the case of intestinal hypersensitivity, is mostly a protein component of the diet (James JM 1992). Studies in Sweden identified food allergy in about 8% of children under age of 6 years. The prevalence of allergy in children to cows milk alone is estimated from 0.3 to 12% (Bahna SL 1978, Dannaeus A 1987, Host A 1990). However, food allergy is less frequent in adults, being estimated at around 0.3 % (Chandra RK 1992). The clinical symptoms, which appear within a few hours after antigen ingestion, can include: diarrhea, vomiting, abdominal cramping, eczema, rhinorrhea, hypotension, urticaria, angioedema and bronchospasm (James JM 1992). Such hypersensitivity can occur in the form of anaphylactic shock, when the response appears very quickly and may have a fatal outcome for the organism (Atkinson TP 1992). Hypersensitivity to food antigen has been suggested as a possible causative mechanism in the pathogenesis of other diseases such as: ulcerative colitis (Fox CC 1993), Crohn's disease (Knutson L 1990), eosinophilic gastroenteritis (Whitington PF 1988) and peptic ulcer (Andre C 1983).

The symptoms of hypersensitivity are not specific, so that differentiation

between hypersensitivity to food and other disorders with similar symptoms, is based on the pathophysiological mechanism (Roitt IM 1989). The classical understanding of immediate hypersensitivity in the intestine includes three main components (Kniker WT 1987)(Figure 3): 1. reaginic antibodies of the IgE class produced in response to primary exposure to antigen bind to mast cells sensitizing them; 2. mast cells activated by secondary contact with the sensitizing antigen release an array of mediators; 3. enterocytes which respond to mast cell mediators stimulate ion secretion into the gut lumen.

Another mechanism of the induction of hypersensitivity may involve complement. Aggregates of IgG antibodies and antigen can activate complement causing production of C3a and C5a, which *in vitro* are able to stimulate mast cell mediator release (Alam R 1992, Mousli M 1992). However, in *in vivo* activation of mast cells by complement has not been shown.

1.3 Components of intestinal immediate hypersensitivity

1.3.1 Immunoglobulin E

An essential element for immediate hypersensitivity reactions is primary contact with antigen. The immune system, which recognizes antigen as allergen, produces sensitizing antibodies of the IgE class, which bind to high affinity IgE receptors ($Fc_{\epsilon}RI$) on mast cells (Sandor M 1993).

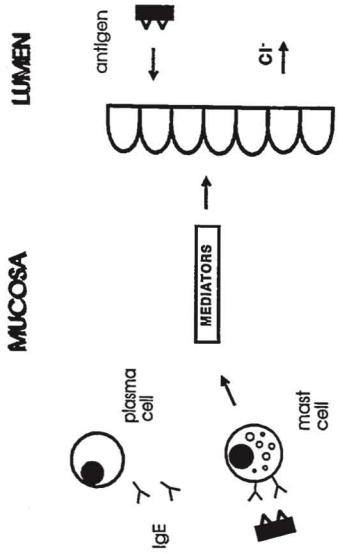

Figure 3. The diagram shows simplified scheme of the mechanism of hypersensitivity. The antigen which enters the luminal space, crosses the epithelial barrier and stimulates plasma cells for production of antigen-specific IgE antibodies. Those antibodies can bind to high affinity IgE receptors on mast cells. When the secondary exposure to the same antigen takes place, antigen cross-bridges mast cell-bound IgE antibodies, causing release of different mediators. Those mediators act on epithelium to stimulate ion (mainly chloride) secretion.

1.3.1.1 Molecular structure

Each immunoglobulin molecule is composed of 2 heavy and 2 light chains. The light chains, designated κ or λ appear the same in all classes of immunoglobulin while the heavy chain is characteristic for the particular classes of immunoglobulin (ε for IgE). The NH_2 terminus, created by both type of chains (F_{ab} portion), is highly variable and determines the specificity of antigen binding. The COOH terminus, composed of heavy chains only (F_c portion), has a constant amino acid sequence and determines the effector function of the immunoglobulin molecules, such as binding to specific F_c receptors (Tharp MD 1990, Geha RS 1992).

1.3.1.2 Physical and biological properties

Rat IgE antibodies (180 kD) are very heat labile and aggregate at 56°C. After 4h at 56°C IgE cannot be precipitated by specific antisera to the rat ε chain. The mean half-life of rat IgE injected into the circulation is about 12 hours, while in the skin the half-life has been determined at 7.4 days. However, the half-life of mast cell-bound IgE in the intestinal mucosa has not been established. The level of IgE in the circulation of non-immunized rat sera is strain-dependent and varies from 10 to 1000 ng/ml (Bazin H 1982, Ovary Z 1986).

1.3.1.3 Regulation of IgE production

The regulation of IgE production is a key question in allergic diseases. IgE,

like other immunoglobulins, are produced by B cells after stimulation by T helper cells (type $CD4^+$)(Keren DF 1992). B cell function is regulated by multiple cytokines and factors. The pattern of cytokines secreted by T cells determines the specific isotype of produced antibodies (Romagnani S 1992, Snapper CM 1993). In the case of IgE production, IL-4 is a crucial cytokine which regulates the level of this immunoglobulin (Roper RL 1990, Savelkoul HFJ 1991, Tonkonogy 1993). IL-4 can exert a direct effect on human B cells, activating transcription through the ε locus (Gauchat JF 1992a). IL-4 also induces expression of the low affinity Fc_ε receptor (Fc_εRII or CD23) which is stimulatory for B cell-$CD4^+$ cell interactions. The stimulatory effect of IL-4 was shown by inhibition of 99% of the primary IgE response *in vivo* after injection of anti-IL-4 antibodies (Finkelman FD 1990). It has been reported that in humans direct T-B cell interactions provide the first signal (priming) for B cells to respond to IL-4. Induction of IgE synthesis by IL-4 is blocked by antibodies to T cell receptor proteins as anti-TcR, anti-CD2, anti-CD4 or anti-LFA-1 antibodies, showing that the T cell receptor and adhesion molecules associated with cell interactions are required for IgE synthesis (Bonnefoy JY 1993, Vercelli D 1989). Of special importance is interaction between CD23 and CD21, which elevates IL-4 induced germline ε transcription (Bonnefoy JY 1993, Flores-Romo L 1993). The requirement of $CD4^+$ cells can be eliminated by direct stimulation of a B cell surface protein, CD40 with gp39 present abudantly on $CD4^+$ and recently described on human mast cells and basophils (Callard RE 1993, Gauchat JF

1993, Marshall LS 1993, Spriggs MK 1992). Three other cytokines, namely IL-5, IL-6 and TNF-α, are stimulatory for IL-4 induced IgE synthesis. IL-5 elevates IgE synthesis only when IL-4 is given in submaximal doses (Pene J 1988, Tonkonogy SL 1989). IL-6 is a non-specific inducer of all antibody subtypes, including IgE (Maggie E 1989). TNF-α is isotype specific and acts at the transcriptional level, inducing transcription of germline ε chain (Gauchat JF 1992b). Such cytokines as IFN-γ, IFN-α, TGF-β and IL-10 block IL-4 induced IgE production. Both IFNs inhibit IgE production via the T cell, since they are effective only in the presence of $CD4^+$ cells (Punnonen J 1993a). IL-10 failed to inhibit IgE synthesis after T cells were activated, indicating that this cytokine effects activation of T cells (Punnonen J 1993b). TGF-β was shown to inhibit IL-4 induced expression of ε germline mRNA expression (Gauchat JF 1992a, Wu CY 1992).

It is important to note that the complexity of the regulation of IgE synthesis is enhanced by the multiple sources of these cytokines. Most of the cytokines are produced by T cells; however, mast cells, macrophages, NK cells can also synthesize significant amount of these peptidergic signals (McGee DW 1993, Nicod LP 1993).

1.3.2. Mast cells

Mast cells (MC) are a type of granulated immune cells which are commonly distributed in tissues. Beneath all surfaces exposed to external antigen, there is an accumulation of MC (Galli SJ 1990). The two largest

mucosal surfaces are the gastrointestinal and respiratory tracts, which possess MC in or directly under the epithelium in the mucosa and also in the connective tissues. Other connective tissues also posses MC and most of these are situated adjacent to blood vessels or nerves (Galli SJ 1990, Stead RH 1990). In the circulation, cells coming from a common myeloid progenitor and having similar characteristics as MC are basophils. Because of their partial similarities (morphology, granule content, activation) and ease of accessibility, basophils constitute a model for mast cell research. Mast cells originate from pluripotential cells in bone marrow. Early bone-marrow derived mast cells possess IgE receptors, although their physiological role is not clear (Rottem M 1991). Regulation of mast cell growth, proliferation, maturation and apoptosis depends on the presence of multiple cytokines (Swieter M 1992) and the *c-kit* ligand, stem cell factor (SCF) (Mekori YA 1993, Wershil BK 1992).

1.3.2.1. Activation

There are two main ways of MC activation: antibody-dependent and antibody-independent. Hypersensitivity reactions involving IgE-dependent MC activation have been known for many years (Dvorak AM 1993, Stanworth DR 1993). The antigen-specific immunoglobulin E, which is produced after exposure to the antigen, binds to MC isotype-specific high-affinity $Fc_\varepsilon RI$ receptor (10^{-10}M) (Raventch JV 1991). Purification of $Fc_\varepsilon RI$ from the rat revealed a 4 subunit structure ($\alpha\beta\gamma\gamma$), of which the α subunit is crucial in binding F_c region of IgE.

After secondary contact with the same antigen, binding of antigen to the F_{ab} portion of the IgE causes cross-bridging of immunoglobulin, MC activation and subsequent release of mediators from granules. There are at least two pathways of second messengers which are activated after cross-linking of IgE (Beaven MA 1993). The first pathway stimulates phospholipase C (PLC) via G protein and with subsequent cleavage of phosphatidylinositol 4,5 biphosphate (PIP_2) and elevation of intracellular calcium. The second pathway involves release of arachidonic acid by stimulation of phospholipase A_2 (PLA_2). Arachidonic acid, is metabolized by the enzymes cyclooxygenase and lipooxygenase to prostaglandins (PGs) and leukotrienes (LTs). The ability of MC to release granule content can be enhanced by the presence of SCF (Bischoff SC 1992) and other cytokines (Coleman JW 1993).

IgG are another class of immunoglobulins which were suggested to activate mast cells. In a few species different subclasses of IgG were found to bind to receptors on mast cells and/or participate in anaphylactic reaction (Bazin H 1982, Daeron M 1992a, 1992b, Halpern GM 1987, Katz HR 1990, Latour S 1992).

The immunoglobulin-independent activation of MC is not clearly defined. It was shown that substances such as neuropeptides (SP, bradykinin, ACTH), pharmacological compounds (mastoparan) can activate MC, presumably via direct activation of a G protein (Mousli M 1990). Recently activation with adenosine via an A_3 receptor was reported for cultured mast cells (rat basophil

leukaemia cells, RBLs)(Ramkumar V 1993). Also such cytokine as IL-8 was shown to release mediators from IL-3 primed basophils, *via* IL-8 receptor (Krieger M 1992). Although many immune cells (neutrophils, mononuclear cells, macrophages) can produce MC-releasing stimuli (Nicod LP 1993), the role of the IgE-independent pathway in physiological or pathological situations is not clear.

1.3.2.2. Mediators

Microscopical examination of MCs reveals multiple granules within the cell which can be released after stimulation (Wershil BK 1991). Released mediators can be classified in three groups (Gordon JR 1990, Katz HR 1991) (Table 1): 1. preformed mediators which reside in the granules, 2. mediators produced upon activation of PLA_2, which results in *de novo* formation of arachidonic acid metabolites from the lipid membrane, 3. cytokines, which can be preformed and/or produced upon activation.

1.3.2.3. Mast cell heterogeneity

Research in the early 1970's has shown that MC in the rat are not a homogenous population but are specific to the type of tissue (Enerback L 1986). Later such heterogeneity became obvious also in other species (Crowle PK 1984, Holgate ST 1991, Lee TDG 1985). The most profound difference in rat MC is between the population residing in the mucosa compared to those in connective tissues (Befus AD 1982, Galli SJ 1990). This division is not only anatomical but

Table 1

Major mast cell mediators in rodents

Mediators		References
1. Preformed granule-derived	proteoglycans	
	heparin	(Tsai M 1991)
	chondroitin sulphate	(Tanish KR 1992)
	amines	
	histamine	(Takei M 1993
	serotonin	(Koike T 1993)
	serine proteases	(Gurish MF 1992, Ghildyal N 1992a,b, Chen Z 1993)
	adenosine	(Marquardt DL 1984)
2. Newly-produced (non-protein)	leukotrienes	
	LTB_4, LTC_4, LTD_4, LTE_4	(Foneth AN 1993, Conti P 1992)
	prostaglandins	
	PGD_2	(Foneth AN 1993, Conti P 1992)
	PGE_2	(Schmaudler-Chock EA 1989)
	PAF	(Hogaboam CM 1993a)
	nitric oxide	(Hogaboam CM 1993b)
3. Cytokines (proteins)	IL-1,4, 6	Burd PR 1989
	IL-3, 5	Seder RA 1991
	GM-CSF	Wodnar-Filipowicz A 1989
	TNF-α	Gordon JR 1991, Ansel JC 1993
	LIF	Marshall JS 1993

includes the requirement for specific fixation, content of mediators, production of cytokines and responsiveness to stimulators. Mucosal mast cells (MMC) in rodents, unlike connective tissue mast cells (CTMC), are unstable in formalin and thus require fixation in Carnoy's solution (Katz HR 1991). The histamine content differs between MMC and CTMC, being ~12-fold higher in CTMC. Although both types of MC possess serine proteases, they differ immunologically; rat mast cell protease I is present in CTMC and rat mast cell protease II in MMC. Mast cells isolated from the peritoneal cavity produce mostly PGD_2 after activation of PLA_2, while MMC release predominantly LTC_4 and LTB_4 (Foneth AN 1993, Katz HR 1992). Progenitors of murine mast cells grown in the presence of IL-3 will differentiate into MMC whereas CTMC tend to develop in the absence of IL-3 (Mekori YA 1993, Tsai M 1993, Wershil KB 1991). Compound 48/80 (Befus AD 1982, Kuno M 1993) and disodium cromoglycate (D'Inca R 1992. Okayama Y 1992) act on CTMC but not on MMC .

1.3.3. Enterocytes

The epithelial lining of the intestine has two major functions: 1. selective transport of nutrients, electrolytes and water and 2. provides a barrier preventing access of the luminal contents (antigens, microbes) into the body.

1.3.3.1. Ion transport regulation

The regulation of directional ion movement across the intestinal tissue

occurs due to the asymmetrical structure of enterocytes, which have a polarized distribution of ion channels, pumps and enzymes on the apical and basolateral surfaces (de Jonge HR 1990)(Figure 4). Additionally, the vectorial movement of ions is possible because of the existence of tight junctions between enterocytes, which act as a barrier to the free movement of ions (Schneeberger EE 1992). In the intestine, the epithelial absorptive and secretory function are spatially distributed.

The villi are covered by predominantly absorptive epithelial cells that possess on their basolateral membranes, sodium-potassium ATPase pumps, and membrane channels for potassium exit (de Jonge HR 1990). This pump exchanges three sodium ions for every two potassium ions which enter the cell, thus creating a low concentration of intracellular sodium. This coupled with the negative intracellular voltage, results in the entry of sodium ions across the apical membrane. As it enters the cell, sodium acts as a cotransporter for different organic molecules and inorganic ions. Those include monocarbohydrates, amino acids, peptides and phosphate and sulphate ions. In the absence of luminal nutrients, sodium/hydrogen exchange which is coupled to chloride/bicarbonate exchange is a principal mechanism for sodium absorption in the jejunum (Rood RP 1988, Hirose R 1988).

Secretion of intestinal fluid occurs mainly in the crypts and results from the active transport of the principal ions, chloride and bicarbonate into the lumen (Hogan DL 1993, Halm DR 1990). The basolateral membrane, in addition to the

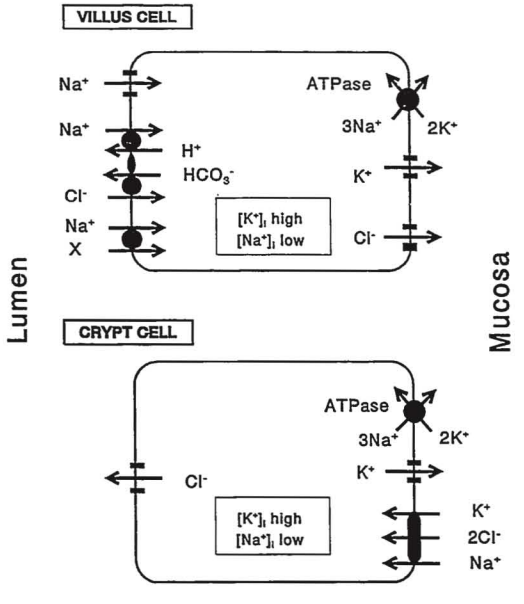

Figure 4. Diagram of organization of ion transport across the villus and crypt cell in intestinal epithelium.

sodium/potassium pump and potassium channels, also contains a cotransporter for the entry of sodium/potassium/chloride (Matthews JB 1992). The presence of this cotransporter on the basolateral membrane and the occurrence of chloride channels on the apical membrane of enterocytes permits chloride to be actively transported across the epithelium from the blood to the intestinal lumen. The active movement of chloride ions is a driving force for net secretion of other ions and water.

1.3.3.2. Extracelluar mediators of ion transport regulation

Many mediators which are stimulatory for ion secretion have been identified in specific cells; yet for some of these the cellular source is more ambiguous. Such mediators include biogenic amines [histamine (Wang YZ 1991), serotonin (5-HT)(Beubler E 1986, Castro GA 1989)], classical neurotransmitters [acetylcholine (ACh)(Wang YZ 1991, Chandan R 1991)], neuropeptides [substance P (Reddix RA 1992, Brown DR 1992) , VIP (Yada T 1989), galanin (Kiyohara T 1993a), neurotensin (Kachur JF 1982)], arachidonic acid metabolites [(PGs, LTs)(Rask-Madsen J 1990, Hyun CS 1993)], cytokines [IL-1, IL-3, (Chang EB 1990), PAF (Hanglow AC 1989)], bradykinins (Lawson LD 1987), adenosine (Barrett KE 1993), endothelin (Kiychara T 1993b) and reactive oxygen metabolites (H_2O_2)(Berschneider HM 1992a,b, Karayalcin SS 1990). The stimulatory action of mediators on enterocytes can be direct or indirect. For example, ACh, histamine, PGE_2, VIP and adenosine are suggested to act at least

in part directly on enterocytes (Yada T 1989). Serotonin was shown to act via direct and indirect (via neuronal) pathways (Beubler E 1990). Many ion secretion mediators act indirectly, at least in part by stimulating release of PGs, and these include histamine (Hardcastle J 1987), serotonin (Beubler E 1989), cytokines (Chang EB 1990), bradykinins (Lawson LD 1987), endothelin (Kiyohara T 1993b) and H_2O_2 (Karayalcin SS 1990). The most important sources of the secretagogues are mast cells and neurons but other cells residing in intestinal mucosa such as lymphocytes, fibroblasts and phagocytes are also possible sources of mediators of ion secretion (Castro GA 1994).

1.3.2.3. Intracellular messengers of ion transport regulation

The mechanism of ion transport depends on intracellular messengers, such as cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP), calcium, PI metabolites, and tyrosine kinase. In villus cells, entry of sodium coupled to chloride but not that coupled with nutrients can be inhibited by an increase in the level of cAMP (Semrad CE 1987). The chloride channels in crypt cells can be opened by cAMP (Anderson MP 1992, Barrett KE 1993). In mammalian intestine, many agents such as cholera toxin (Hyun CS 1982, Rabbani GH 1990), *Escherichia coli* enterotoxin (Forte LR 1992), VIP (Yada T 1989), PGE_1 (Weymer A 1985) and PGE_2 (Musch NW 1987) are able to increase cAMP levels. Cyclic AMP is generated from ATP as a result of stimulation of membrane-bound adenylyl cyclase. The activation of this adenylyl cyclase is

regulated by inhibitory (G_i) or stimulatory (G_s) proteins, whose action is dependent upon the binding and hydrolysis of GTP (Moss J 1990).

Another intracellular mediator, cGMP, causes changes similar to cAMP, inhibiting [Na^+/Cl^-]-linked absorptive processes and increasing electrogenic chloride ion secretion (Forte LR 1992, Binder HJ 1987). The increase of cGMP is associated with pathological conditions, such as the presence of heat-stable *E. coli* enterotoxins. The elevation of cGMP is regulated by stimulation of guanylyl cyclase. Recently, the intracellular natural ligand for activation of guanylyl cyclase, guanylin, was described, but its role in the intestinal physiology reminds to be established (Currie MG 1992).

Calcium is a very potent regulator of ion transport. The first observation of the involvement of calcium in the changes in chloride and sodium transport was that the calcium ionophore A23187 could alter ion transport. In almost all studies of the enterocyte, regardless of the species, an increase in intracellular calcium has one or more of following effects on ion transport: it inhibits Na/Cl absorption, it stimulates anion secretion (Cl^-, HCO_3^-) and modulates apical or basolateral potassium conductance (Rood RP 1988, Binder HJ 1987, de Jonge HR 1989). Some secretagogues like carbachol and serotonin mobilize intracellular calcium, which is a consequence of activation of the PI pathway. Other secretagogues like SP, neurotensin, bombesin increase intracellular calcium level by enhancing of calcium entry into the cell from the extracelluar space (Ziyadeh FN 1988). Intracellular calcium can be also increased by other second

messengers such as cAMP and this results in inhibition of Na-H exchange on the apical membrane of enterocytes (Semrad CE 1987).

Recently, tyrosine kinase activation was shown to have a double effect. First, it was found to inhibit ion secretion induced by carbachol (Vajanaphanich M 1993) and secondly, it enhanced ion secretion evoked by heat stable enterotoxin of *E. coli* (Sears CL 1993).

1.3.2.4. Permeability

The free movement of luminal antigen into the interstitium via the paracellular shunt pathway is largely prevented by the tight junctions that are located in the uppermost 1/3 of the epithelium and join adjacent enterocytes (Schneeberger EE 1992). This barrier is very important in anaphylactic responses to food antigens. Loosening of the tight junctions may allow for excessive antigen entry and thus prolonged and/or exaggerated inflammatory reactions.

The structure of tight junctions includes apical junctions which are associated with actin filaments, intermediate junctions which have circumferential actinomyosin rings and desmosome regions (Madara JL 1990, Marmorstein AD 1992). Although the regulation of the tight junctions seems to be crucial for primary and secondary immune responses to luminal antigen, surprisingly little data is available on the endogenous factors that regulate this gating mechanism. It is believed that apical and intermediate junctions have the ability to contract, regulating molecule permeability through the paracellular

pathway. The work of McRoberts (McRoberts JA 1990) has shown that insulin-stimulated paracellular permeability in T84 cells was related to condensation of the perijunctional actin ring and this effect was dependent on protein synthesis. Similarly, Marmostein (Marmostein AD 1992) has shown condensation of the perijunctional actin ring in Madin-Darby canine kidney (MDCK) cells after stimulation of epithelial permeability with serum proteins. Experiments on T-84 epithelial cell (colon carcinoma cells) monolayers have shown that permeability and the resistance of the monolayer was lowered by IFN-γ, but not by other cytokines (IL-1, IL-2 or TNF-α)(Madara JL 1989, Adams RB 1993). At the cellular level, tight junction conformational changes are influenced by calcium. For example, it has been was shown that tight junctions in MDCK cells are sensitive to changes in external calcium concentration (Gonzalez-Mariscal L 1990).

1.4. Models of intestinal hypersensitivity

1.4.1. Helminth infected rat/mouse

Much of the knowledge on hypersensitivity was accumulated from studies in rat/mouse models of acute inflammation evoked by secondary exposure to antigen from two enteric nematode parasites, namely *Nippostrongylus brasiliensis* or *Trichinalla spiralis* (Castro GA 1989, Perdue MH 1990). The infection of rats causes villus atrophy and crypt hyperplasia, mast cell hyperplasia and elevation of worm antigen-specific and total serum IgE (Harari Y 1987, Perdue MH 1989). Challenge by worm antigen into infected intestine

induces Cl^- secretion, which is due mainly to the release of three types of secretagogues: histamine, serotonin and prostaglandins. The first two are responsible for the early phase (peak in 2 min) of response to antigen, while PGs are responsible for the late phase (peak from 5 to 20 min)(Castro GA 1989). The two amines, histamine and serotonin, are released from MC, since the early phase of the responsiveness to antigen can be restored by passive transfer of immunized serum into recipients with induced mast cell hyperplasia only. PG-induced secretion can be transferred by passive sensitization of naive rats with IgE rich sera. The response to antigen in this model was accompanied by increased epithelial permeability (Bloch KJ 1979, D'Inca R 1992). Research using these models has resulted in elucidation of many of the mechanisms underlying hypersensitivity. However, and perhaps surprisingly, the epithelial responses described above were only observed when antigen was presented to the serosal tissue surface of isolated intestinal tissues; luminal antigen evoked no response from the epithelium (Baird AW 1985). Clearly this is the physiologically relevant exposure to antigen.

1.4.2. Guinea pigs immunized to β-lactoglobulin

A convenient model of food hypersensitivity is the guinea pig sensitized to β-lactoglobulin, a component of cow's milk (Cuthbert AW 1983, Heyman M 1990). Since cow's milk allergy is common in children, this model may be of great socio-medical value. Intestinal responses to serosal β-lactoglobulin addition

could be partially abolished by a histamine blocker and totally by cyclooxygenase inhibitors, suggesting that PGs are key mediators of hypersensitivity in this model (Baird AW 1984). However, the response to luminal antigen was described only in some reports (Baird AW 1984) but not in others (Baird AW 1987), indicating that the luminal response in this model is not always reproducible. Additionally, the responsiveness to antigen was shown to be IgG-dependent in guinea pig, which may be different from that in humans and other rodents (Baird AW 1987).

1.4.3. Rat/mouse immunized with ovalbumin and adjuvants

The third model of hypersensitivity was reported in 1958, when Mota demonstrated allergic enteropathy in rats sensitized to antigen with simultaneous injection of *Bordetella pertussis* vaccine (Mota I 1958). Later, the model of hypersensitivity which was modified for intestinal research was a rodent (rat or mouse) sensitized to ovalbumin (OVA) with two adjuvants: pertussis vaccine and alum. The use of two adjuvants was believed to be crucial in achieving the response to intestinal secondary antigen challenge. The intestine from such animals responded to luminal antigen and this response was, at least partially, IgE mediated (Perdue MH 1984). Changes in ion transport after secondary antigen challenge involved increased Cl^- ion secretion and reduction of Cl^-, Na^+ and K^+ ion absorption (Perdue MH 1986). These changes were accompanied by cAMP accumulation in the intestinal mucosa. Involvement of MC in the response

to antigen challenge was suggested by elevation of histamine content in the circulation, a decrease of histochemically identified MC in the mucosa after antigen challenge and decreased effects of antigen on ion secretion in the presence of MC inhibitors (Perdue MH 1984, Forbes D 1988). Additionally, sensitized animals showed elevated permeability to the inert probe, ^{51}Cr-EDTA, from the intestinal lumen to the circulation (Crowe SE 1993). A similar range of mediators were found to be active in this model as were identified in the helminth/rodent models, that is histamine, serotonin and PGs (Crowe SE 1990).

1.5. Adjuvants

Many substances, naturally occurring or synthetic, can be used to augment immune responses to a particular antigen (Audibert FM 1993). These compounds are termed adjuvants. Many adjuvants are used with the intention of enhancing humoral (mostly IgG or IgA) immune responses to antigens, as in vaccination (Guidice GD 1992). However, some adjuvants can stimulate production of IgE antibodies and result in hypersensitivity. The chemical variety of adjuvants suggests a great diversity in the pathways that stimulate the immune response (Audibert FM 1993). Recently, it was recognized that most adjuvants act via selective activation of one of two types of $CD4^+$ cells, namely Th_1 or Th_2, which regulate the type of immune response by releasing a characteristic spectrum of cytokines. Based on their origin, adjuvants can be divided as follows: vegetal, artificial, cytokine, mineral or bacterial (Audibert FM

1993, Giudice GD 1992).

Some of the oldest and most potent adjuvants are bacteria and their products. In 1937, Freund using a mixture of killed mycobacteria and mineral oil, was the first to employ a bacterial adjuvant (Freund J 1937). This adjuvant is the most potent adjuvant developed but because of its side effects it is only used in experimental conditions. In gram-negative bacteria, the stimulatory effect on the immune response is ascribed to a component of bacterial walls, namely lipopolisaccharide (LPS)(Luderitz O 1982). The main mechanism by which LPS elevates immune response is by stimulation of macrophages to express Ia molecules (important in antigen presentation) and to release IL-1 (Behbehani K 1985, Ziegler HK 1984). Another component of bacteria cell walls which exhibits adjuvant properties is peptidoglycan, P40, from *Corynebacterium granulosum* (Ickovic MR 1984). In addition to the cell wall, other bacterial components such as toxins display adjuvant properties. Cholera toxin, which is a product of *Vibrio cholerae*, enhances local mucosal immunity to orally administered antigens (Holmgren J 1992, Munoz E 1990, Wilson AD 1993). The mechanism of action of cholera toxin includes the switch of B cell antibody production from IgM to IgG and IgA (Lycke N 1990, 1991, 1993) as well as increased ability of peripheral blood lymphocytes to secrete cytokines (Quiding M 1991). Pertussis toxin, which is structurally similar to cholera toxin, was shown to be stimulatory for anaphylaxis (Munoz JJ 1981).

1.6. Pertussis toxin

Pertussis toxin (PT) is one of various toxic components produced by the virulent bacterium, *Bordetella pertussis*, the etiological factor for disease whooping cough. Pertussis toxin is a major contributor to the pathogenesis of the disease and is a protective antigen in pertussis vaccine (Munoz JJ 1989).

1.6.1. Molecular structure

PT belongs to a group of bacterial toxins which display a "dimeric" A-B structure (Tamura M 1982)(Figure 5). The A (active) protomer possesses the enzymatic activity and the B (binding) oligomer allows the toxin molecule to bind to the cell surface. The toxin is a hexamer composed of five dissimilar subunits designated S1 through S5 (Ui M 1990). Moiety A consists of only subunit, S1, while moiety B is composed of five subunits. The molecular ratio of the subunits was calculated as 1(S1):1(S2):1(S3):2(S4):1(S5). The genes coding for the five subunits of pertussis toxin were cloned and sequenced by Locht (Locht C 1986) and Nicosia (Nicosia A 1986). The genes are clustered within 3.2 kilobases in the order S1, S2, S4, S5, S3, forming a single operon. Based on the nucleotide and amino acid sequences, the calculated molecular weight of the toxin is S1=26,220; S2=21,920; S3=21,860; S4=12,060; S5=10,940, which together gives a molecule about 105,06 kD (Nicosia A 1986). Reconstruction of the original toxin from subunits showed that S2 with S4 and S3 with S4 yielded D1 and D2 dimers, respectively. Subunits S2 and S3, which bind to S4, were

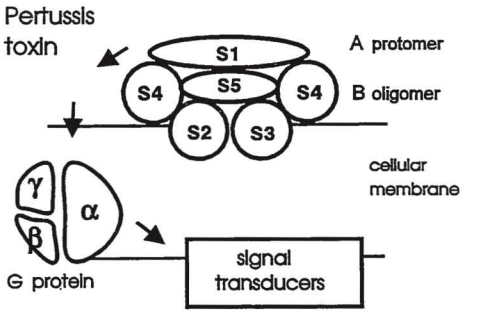

Figure 5. Scheme of pertussis toxin structure and activity. Pertussis toxin display A-B structure, where A protomer consists only of S1 subunit, while B oligomer consists of 5 subunits, S2-S5. The enzymatically active S1 subunit released from the holotoxin ADP-ribosylates α-subunits of G proteins which effect changes in signal transduction into the cell.

shown to be in 70% homologous at the amino acid level. All subunits contain an even number of cysteines which form disulphide bonds within the subunits. Of particular importance is the single disulphide bond within the S1 subunit, the reduction of which activates the enzymatic activity of the toxin (Burns DL 1989, Kaslow HR 1989).

1.6.2. Cellular effects of pertussis toxin action

The oligomer B is involved in the binding of the toxin to the receptor on

the surface of the cells and translocation of the enzymatically active subunit S1 into the membrane (Rappuoli R 1987). The B oligomer binds to glycoproteins such as haptoglobin and fetuin but the identity of the protein to which these carbohydrates are linked is unknown (Kaslow HR 1992, Spangler BD 1993). PT is believed to bind through the D1 and D2 dimers, inserting the S2 and S3 subunits into the membrane. The mechanism of entry of S1 is not clear. Both the A protomer and the B oligomer interact with detergents and phospholipid, so that direct penetration may be a pathway for S1 insertion (Montecucco C 1986, Moss J 1986). The COOH-terminus of the S1 subunit has a hydrophobic region that may promote interactions between the subunit and membrane lipid bilayer. The enzymatic activity of S1 causes, in the presence of nicotinamide-adenine dinucleotide (NAD), transfer of ADP-ribose to a group of guanine-binding (G) proteins which are involved in signal transduction across the cell membranes of all eukaryotic cells (Moss J 1985)(Figure 5). The activity of S1 is composed of two types of enzymatic activity: ADP-ribosyltransferase and NAD glycohydrolase. The first enzyme catalyses the transfer of ADP-ribose from NAD^+ to a cysteine located three amino acids upstream of the carboxyl-terminal of the α subunit. The second hydrolyses NAD^+ to ADP-ribose and nicotinamide and is active in the absence of a suitable acceptor.

1. NAD^+ + protein → ADP-ribose-protein + nicotinamide + H^+
2. NAD^+ + HOH → ADP-ribose + nicotinamide + H^+

1.6.3. Mutants of pertussis toxin

The enzymatic activity of the S1 subunit of PT is crucial for pathogenicity and for the development of side effects in children vaccinated with whole-cell or toxoid pertussis (Monack D 1989). Thus, the approach in the development of a new, safer vaccine was to reduce the enzymatic activity of the toxin (Barbieri JT 1990, Bugnoli M 1989, Pizza M 1989). Using mutagenesis, Rappuoli and coworkers, obtained multiple genetic constructs, with substitution of different amino acids in subunit S1. The mutant (labelled as PTX-9K/129G) with two amino acid substitutions (Arg^9→Lys, Glu^{129}→Gly, had no detectable enzymatic activity, but showed immunogenicity (recognition by protective antibodies and T cells), and it was chosen for further evaluation. Experiments with the mutant toxin allowed the identification of specific physiological or cellular changes that were dependent on enzymatic activity exclusively.

1.6.4. Pertussis toxin substrates - G proteins

1.6.4.1. Structure and activation

As mentioned before, certain G proteins are substrates for PT. Many identified receptors are linked to G proteins, which transduce external signals into effector proteins. G proteins are heterotrimeric proteins made up of α (39-52 kD), β (36kD), and γ (9kD) subunits (Milligan G 1993). Binding of the ligand to the receptor generates changes activating the G protein. This is followed by the exchange of guanosine diphosphate (GDP), bound to the α subunit, for guanosine

triphosphate (GTP) and the subsequent dissociation of the α-GTP complex from the $\beta\gamma$ heterodimer (Figure 6)(Iyengar R 1993). A single receptor can stimulate multiple G molecules, thus amplifying the signal (Simon MI 1991). G protein

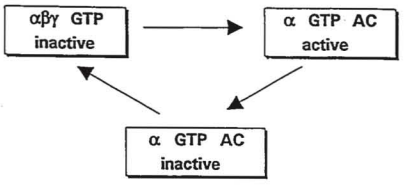

Figure 6. The cycle of adenylyl cyclase activation by α subunit of G protein.

stimulation may effect ion channels and enzymes that generate second messengers. G proteins regulating primary effectors without involvement of a cytosolic second messengers are major signalling mechanisms in the heart (Cohen-Armon M 1991, Dolphin AC 1990, Dunlap K 1987). There, the opening of an inwardly rectifying K^+ channel, via muscarinic receptors requires GTP and a G protein, yet uses no diffusible cytoplasmic second messenger. Low molecular weight second messengers such as cAMP or inositol triphosphate (IP_3) are generated after stimulation of adenylyl cyclase or PLC, respectively. The effects of second messenger actions are intracellular changes including selective protein phosphorylation, gene transcription, cytoskeletal reorganization, secretion and

membrane depolarization (Birnbaumer L 1990).

The heterogeneity of G proteins is ensured by the heterogeneity of each subunit. Currently, at least 16 different genes (20 mRNAs) which encode the α subunit, 4 that encode β subunit and 7 that encode γ subunit are known (Birnbaumer L 1992, Conklin BR 1993). Based on the similarity of the α subunits, heterotrimeric G proteins are grouped into the following broad categories: Gt, Gs, Gi, Go, Gq and G?. The first G protein discovered, transducin (Gt), is coupled to rhodopsin in retinal rods and induces cGMP phosphodiesterase (PDE) activity, which exerted an inhibitory effect on cGMP (Lagnado L 1992). Adenylyl cyclase is regulated by two GTP-binding proteins: Gs which stimulates the activity of adenylyl cyclase and Gi which inhibits the enzyme. Stimulation by the ligand of a particular receptor results in activation of Gs and subsequent adenylyl cyclase activation. These events result in enhancement of cAMP accumulation. Similarly, Gi receives signal information from an inhibitory receptor upon stimulation with an appropriate hormone, and results in inhibition of adenylyl cyclase and inhibits accumulation of basal cAMP or that induced by a stimulatory hormone. Gi was also shown to open K^+ channels (Conklin BR 1993). Go, present in high levels in the brain, closes Ca^{++} channels. Gq protein was shown to stimulate PI and PLC - β subtypes (Hille B 1992). Some G-proteins are not yet characterized but they have been found to regulate PLA_2, followed by production of arachidonic acid (AA) metabolites (Glasser KB 1993).

1.6.4.2. Pertussis toxin-sensitive G-proteins

Of all documented mammalian G proteins, 5 of these were shown to be sensitive to ADP-ribosylation by PT (Gierschik P 1992) (Table 2). PT binds to G proteins in such a way that the protein becomes uncoupled from its receptor (Spiegel AM 1993). The functional consequence of PT activity is that the ADP-ribosylated G protein can no longer interact with its receptor. Effectively, there is lack of inhibition of adenylyl cyclase or lack of stimulation of PLC, D, A_2, PDE or changes in opening and closing of various ion channels. Although the ADP-ribosylation occurs on the α subunit, the presence of $\beta\gamma$ dimer is necessary for full expression of enzymatic activity (Linder ME 1990). When only the α subunit was expressed in E. coli, ADP-ribosylation was only 2% of that in the additional expression of $\beta\gamma$ subunit.

1.6.5. Some physiological effects of pertussis toxin action

The physiological effects of PT are related to the distribution and function of PT-sensitive G proteins (Garcia-Sainz AJ 1985). The diversity of PT effects is best illustrated by research in early 1980s, before PT purification. The product(s) of *Bordetella pertussis*, which later turned out to be PT, were given many designations according to their physiological effects (Munoz JJ 1985), for example, heat-labile adjuvant, histamine sensitizing factor or islet-activating factor. Although many receptors are coupled to PT-sensitive G proteins and a plethora of cellular and physiological effects were documented, few data describe

Table 2

Pertussis toxin-sensitive α subunits of G proteins

α subunit	molecular weight	effector
α_{i1}	40.4	adenylyl cyclase
α_{o1}	40.1	Ca^{++} channels
α_{o2}	40.1	K^+ channels
α_{t1}	40.0	retinal cGMP PDE (rods)
α_{t2}	40.1	retinal cGMP PDE (cones)

From Gierschik P,1992; Curr Top Microbiol Immunol, 175: 69-96.

the relevance of PT in hypersensitivity. I will describe the effects of PT which are related to the cellular components of hypersensitivity but not necessary to hypersensitivity in general sense.

1.6.5.1. Immune effects

In vivo experiments in mice showed that injection of PT with an antigen elevated the sensitivity of mice to anaphylactic shock induced by iv injection of antigen and this elevation appeared with as little as 0.1 ng of PT (Munoz JJ 1981). At least a few studies showed the effect of PT on T cells. Analysis of blood from sensitized mice with PT and antigen revealed a marked leucocytosis and elevated levels of antigen-specific IgE and IgG_1. Haslow (Haslow K 1991) demonstrated that PT can reverse tolerance to an antigen and abolish the capacity of T cells to transfer passively the immune suppression to antigen. Experiments on transgenic mice with S1 subunit of PT expressed in thymocytes, showed that S1 did not influence Tc activation, evaluated by a calcium concentration rise and IL-2 production; however, transgenic lymphocytes did not appear in secondary lymphoid organs, like the thymus or spleen, suggesting that PT-sensitive mechanism regulated thymocyte migration (Chaffin KE 1990). Additionally, PT drove immature lymphocytes through accelerated maturation and changed the ratio of $CD4^+$:$CD8^+$. In the thymus there was a decrease in the number of double positive cells and in the periphery relatively more lymphocytes were $CD4^+$ than $CD8^+$ (Person PL 1992).

PT was shown to be a strong potentiator of inflammation. Co-injection of PT with Freund's adjuvant and antigen, increased the intensity and longevity of the inflammatory response. T cells were shown to be crucial for such stimulatory effects, since in T cell-deficient mice the effect of PT was absent (Munoz JJ 1984).

PT, when injected with antigen, potentiated delayed-type hypersensitivity. Lymphocytes from immunized mice had increased levels of some cytokines. Particularly, there was an elevation of IFN-γ, with smaller effects on IL-3 and IL-2 (Sewell WA 1986). Later studies of Sewell's group (Hong-Hua M 1993) also demonstrated an increase in IL-4 production by spleen cells.

Activation of isolated rat peritoneal mast cells (PMC, connective tissue type) was affected by PT in an inhibitory fashion (Nakamura T 1985). Release of histamine upon stimulation with compound 48/80 was preceded by an increase of intracellular calcium which after PT treatment was markedly decreased. This effect was G protein mediated, since Ca^{++} ionophore, which increases intracellular calcium by influx from the extracelluar space (omitting G protein stimulation), was still effective in histamine release after PT treatment. The inhibitory effect of PT on MC mediator release was also shown in basophils after stimulation with IL-8. This cytokine increased intracellular calcium and released histamine and both effects were markedly inhibited by PT (Krieger M 1992). Similar inhibitory effects of PT on histamine release appear upon stimulation of PMC with complement fragment C3a (Mousli M 1992). However,

IgE-dependent histamine release is unaffected by PT (Krieger M 1992).

1.6.5.2. Neuronal effects

There are many chemical messengers, acting through specific receptors on epithelium, neurons or other cells, which inhibit or stimulate ion transport (Barrett KE 1991). Some of these receptors, such as those for norepinephrine, opioids, neuropeptide Y, somatostatin, dopamine, adenosine and ACh, were identified on neuronal tissues and were shown to PT-sensitive. PT was shown to interfere with norepinephrine receptor-mediated inhibition of Ca^{++} currents in dorsal root ganglia. Also PT blocks the inhibitory action of the enkephalin, analog D-Ala-D-enkephalin (DADLE), and somatostatin on Ca^{++} currents in a neuroblastoma-glioma cell line (NG 108-15)(Dunlap K 1987). Treatment with PT influenced Ca^{++} fluctuations stimulated by the NPY2 receptor in the hippocampal neurons in inhibitory fashion (Bleakman D 1992). Inhibition of D2 receptor activity was reported in caudate neurons in rat brain (Stromberg I 1991) as well as in transfected fibroblasts (Neve KA 1989). In rat superior cervical ganglion, PT reduced the hyperpolarization mediated by adenosine and M_2 receptors (Newberry NR 1989).

There is also evidence for the indirect regulation of secretory neurotransmitter release (such as ACh) by PT. Opiate and α2-adrenergic receptors in guinea pig ileum are coupled to a cAMP system, stimulation of which causes inhibition of ACh release. Electrical field stimulation of ileum

releases ACh which contracts muscles. In tissue from guinea pigs treated with PT, norepinephrine or morphine was not able to inhibit this electrically stimulated ileal contraction (Lujan M 1984).

1.6.5.3. Other effects

PT was shown to be relevant in formation of PGs. Human skin fibroblasts in culture responded to bradykinin by AA release and formation of PGI_2 and PGE_2. Pretreatment of such cultures with PT increased PGs formation by increasing bradykinin stimulated cAMP production (Moss J 1988).

PT, when administrated in vivo, enhanced vascular permeability and sensitivity to histamine. The sensitivity to histamine, evaluated as the number of mice dying after histamine challenge, was elevated with injection of picogram doses of PT per mouse (Munoz JJ 1981). The mechanism of PT-induced histamine sensitivity currently is unknown.

1.7. Purpose of the study

Previous studies have shown that pertussis vaccine acts as a potent adjuvant in the rodent model of immediate hypersensitivity in the intestine. The purpose of my studies was to investigate the role of PT, a component of pertussis vaccine, in hypersensitivity in this model. I measured responses to secondary antigen challenge in intestine from rats sensitized with PT, either recombinant wild type pertussis toxin or a mutant of pertussis toxin as

adjuvants. Subsequently, I conducted experiments to identify the physiological mechanism(s) by which pertussis toxin adjuvant stimulates responses to secondary antigen challenge. Further experiments used mast cell-deficient mice, *W/W^v*, to examine the role of mast cells and reaginic antibodies.

There is at least dual relevance in research into the role of PT in hypersensitivity reactions. First, the use of wPT resulted in a very reproducible model of intestinal hypersensitivity. The special advantage of this model is that sensitivity to antigen is very long lasting, providing the opportunity for long term experiments. Additionally, in this model a luminal challenge with antigen provokes a hypersensitivity response. Secondly, pertussis vaccine, which contains PT is administered into children to induce immunity against whooping cough disease. My experiments provide information regarding possible immunophysiological side effects that may result from injection of PT. These findings should be considered with regard to routine vaccination of children.

CHAPTER II

METHODS

2.1. General experimental plan

The role of pertussis toxin in intestinal hypersensitivity was investigated in rodent models (rat or mouse). Pertussis vaccine (cPV), pertussis toxin (wPT) or a mutant of the toxin (mPT) were injected into animals during the sensitization procedure together with an antigenic protein, ovalbumin (OVA). Experiments were performed 14 days post-sensitization or later. Most experiments involved measurment of the ability of isolated intestinal preparations from actively or passively sensitized animals to respond to secondary antigen challenge. These intestinal responses to antigen were measured in Ussing chambers as increases in short-circuit current (Isc). Additionally, the IgE and IgG antibody levels were measured in the sera from sensitized and control rats and the numbers of mast cells in the intestinal mucosa were counted.

2. 2. Animals

Two strains of rats and one strain of mouse were used: 1. male Sprague-Dawley rats (200-250 g, Charles River Breeding Laboratories, St. Constant, Quebec, Canada); 2. male Brown-Norway rats (250-300 g, Harlan Sprague-

Dawley Inc., Indianapolis, Indiana); 3. male MC-deficient mice (*W/W^v*; WB/ReJ-W/+ × C57BL/6J-*W^v*/+) and congenic normal mice (+/+; WBB6F$_1$-+/+ or WCB6F$_1$-+/+)(4-6 months old, Jackson Laboratories, Bar Harbour, Maine, USA). All animals were housed in separate quarters in standard cages under a controlled temperature (20°C) and photoperiod (12 hours light, 12 hours dark). Water and a standard rodent chow were accessible without limitations.

2.2.1. Rats

Sprague-Dawley are an inbred strain of rats and were used previously as a model for hypersensitivity responses to antigen (Crowe SE 1990). They are low IgE responders (Karlsson T 1979a, 1979b). Brown-Norway rats, also an inbred strain, were used for raising high IgE sera since they demonstrated a high IgE responses after sensitization to antigen (Marshall JS 1993).

2.2.2. Mice

2.2.2.1. Mast cell-deficient *W/W^v* mice

Mast cell-deficient mice (*W/W^v*) have no histochemically identifiable MCs in the intestine (Crowle RK 1984) or in other areas where MCs are commonly found such as spleen, peritoneal cavity, muscularis and mucosal layers of the stomach (Kitamura Y 1989). Some MCs were found in the skin, but the number was very low, 0.3% of that in normal mice. *W/W^v* mice also display other abnormalities such as anemia, lack of skin pigment and sterility.

The MC deficiency is due to a mutation at the W locus on chromosome 5. This locus was shown to be responsible for expression of the *c-kit* proto-oncogene, which regulates production of a transmembrane tyrosine kinase receptor. This tyrosine kinase receptor, by binding stem cell growth factor (SCF), regulates the growth and differentiation of mast cells. In *W/W^v* mice, MCs can be restored by intraperitoneal or intravenous transfer of cultured MCs, or bone marrow cells, containing precursors of MCs (Nakano T 1985). After bone marrow transfer, both mucosal and peritoneal (connective type) mast cells develop. Transfer of bone marrow but not cultured MCs, repairs anemia, and this can serve as a marker of successful reconstitution.

2.2.2.2. *W/W^v* bone marrow reconstitution (BM (+/+) →*W/W^v*)

In our studies, bone marrow (with MCs precursors) from +/+ mice was transferred to *W/W^v* mice. Procedures were performed in a tissue culture hood under sterile conditions. Control +/+ mice were sacrificed and immersed in 5% (v/v) Dettol to prevent contamination during surgery. Hind leg bones were obtained from each mouse and placed in culture media (RPMI 1640)(Moore GE 1967) enriched with 10% fetal calf serum, 9 mM HEPES (without sodium bicarbonate), pH 7.4. Bone marrow was removed by gently flushing bone cavities and subsequently dispersed by pipetting with a syringe using decreasing needle sizes (18-22 guage). Each *W/W^v* mouse received 30 million bone marrow cells injected intravenously in the tail. Mice (BM (+/+) →*W/W^v*) were used for

experiments at least 12 weeks after bone marrow transplantation.

2.3. Sensitization of animals

2.3.1. Rats

2.3.1.1. Active sensitization

Sprague-Dawley rats were sensitized by intraperitoneal (ip) injection of 1.0 mg of chicken egg albumin, grade V (OVA, Sigma Chemical Co., St Louis, MO) mixed with different adjuvants: 1. pertussis vaccine (cPV, 1 ml)(Connaught Laboratories, Willowdale, Ontario, Canada); 2. recombinant wild type pertussis toxin (wPT, 1-200 ng)(PTX, Sclavo Research Institute, Siena Italy); 3. mutant pertussis toxin with two amino acids substitution in S1, which rendered the toxin enzymatically inactive (mPT, 1-200 ng)(PTX-9K/129G, Sclavo Research Institute, Siena, Italy). Unless otherwise indicated, experimental sensitized rats were injected with OVA plus 50 ng wPT. Control rats were either injected with vehicle, injected with OVA (1 mg) only, or injected with 50 ng wPT or 50 ng mPT alone. Animals were used for all experiments 12-14 days after sensitization, with the exception of those rats used in the time course studies.

Sprague-Dawley and Brown-Norway rats were used for raising sera for passive sensitization of other animals. Sera were obtained from Sprague-Dawley rats two weeks after being injected ip with 1 mg OVA plus 50 ng wPT and sacrificed two weeks later. Brown-Norway rats were injected ip with 10 μg of OVA plus 50 ng wPT and boosted twenty eight days later with 10 μg of OVA.

The rats were bled 4 days later. Sera were stored at -70°C. The level of reaginic antibodies in sera was measured by passive cutaneous anaphylaxis (PCA)(see later section).

2.3.1.2. Passive sensitization

Sprague-Dawley rats were passively sensitized ip with 0.3 - 3.0 ml serum from Sprague-Dawley or Brown-Norway rats. The largest volume of serum (3 ml) was split into two doses (2 × 1.5 ml), and given as two injections separated by 8 h. The smaller quantities of serum were injected as a single dose. Measurement of IgE by the PCA technique revealed that the pooled serum obtained from Sprague-Dawley rats had a titre 1:32, while that from Brown-Norway rats had a titre 1:2048. Animals were used for experiments 3 - 70 days after passive sensitization.

2.3.2. Mice

2.3.2.1. Active sensitization

Mast cell-deficient (*W/W*^{v}) and normal mice (+/+) were sensitized by ip injection of OVA (0.1mg) and wPT (5 ng) adjuvant. The injected components were given together in a single inoculation. Animals were used for experiments 2 weeks later.

2.3.2.2. Passive sensitization

W/W^v, +/+ and BM(+/+)→*W/W*^{v} mice were injected with different doses of sera (0.3 -1.5 ml) from actively sensitized Brown-Norway rats. Small volumes of sera (0.3, 0.5 ml) were injected in single doses, while the larger volumes (1.0, 1.5 ml) were split into two equal doses and administered as two injections separated by 8 h on the same day. The concentration of total IgE in the pooled Brown-Norway serum was 3.9 μg/ml and the OVA-specific IgE was 1659 U/ml, as measured by radioimmunoassay. The antigen-specific (anti-OVA) IgE titre measured by passive cutaneous anaphylaxis was 1:2048. Experiments were conducted 3 days later. Three days prior to passive sensitization, some mice were injected ip with 12.5 to 200.0 μl of IR2 rat IgE myeloma (kindly provided by Dr. Bazin, University of Louvain, Belgium). The concentration of total IgE in the stock solution of IgE myeloma was 159.2 μg/ml, thus the maximum dose of injected IgE per mouse was 31.8 μg.

2.4. Measurement of wPT/ mPT concentration

wPT/mPT was obtained in stock solution at concentration of 500μg/ml in 50% ammonium sulphate. Calculation of the amount of injected wPT/mPT was based on evaluation of protein content with the Bio-Rad Protein Microassay. The Bio-Rad Protein Microassay is a dye-binding assay based on differential colour change in response to various concentrations of protein. Standards consisted of several dilutions of bovine serum albumin (BSA) from 0 to 20 μg/ml. The

wPT/mPT sample was diluted so that the concentration would be in the range of the standard curve. Standards and samples were prepared in saline in a total volume of 800μl. To each tube, 200μl of dye reagent (Bio-Rad Laboratories, Richmond, CA) was added. After mixing the tubes and waiting for 15 min, the absorbance was measured against blank at wave length 595 nm by spectrophotometer. The concentration of wPT/mPT was determined from standard curve and proper dilution of the toxin was prepared for injection of animals.

2.5. Ussing chamber experiments

2.5.1. Design of apparatus

The Ussing chamber was introduced by Ussing and Zerahn (Ussing HH 1951) in 1951 in pioneering electrophysiological research to measure ion movements across amphibian skin. The apparatus had the frog skin placed vertically between two adjacent chamber halves and measured potential difference and current across the tissue. The tissue in the chamber was bathed in iso-osmotic physiological buffers on both sides of the tissue, to abolish any osmotic driving forces for the passive movement of ions. Since then, the technique has been modified and used extensively in ion transport research. The Ussing chamber set employed in my studies (WPI Instruments, Narco Scientific, Mississauga, Ontario, Canada) was equipped with two pairs of electrodes. Calomel electrodes (Corning Glass Works, Medfield, MA), connected to agar

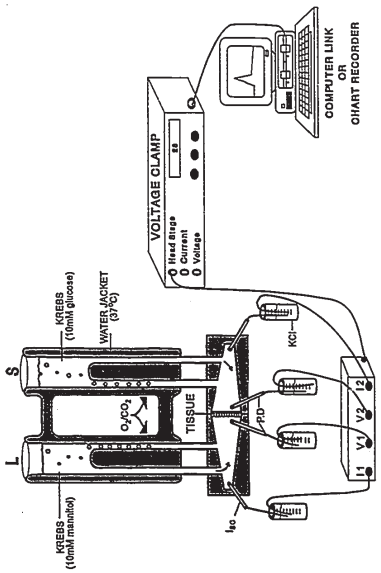

Figure 7. Ussing chamber set.

bridges (polyethylene tubing, PE-320 containing 2M KCl in 1% agar) were placed in close proximity to the tissue (<0.5 cm) (Figure 7). The PD was created by active ion movement across the tissue. In order to measure a current created by active ion movement through the tissue and exclude current created by any preexisting potential difference, the tissue was voltage-clamped at zero voltage, by means of the second pair of silver/silver-chloride electrodes. The injected current is described as short circuit-current (Isc, $\mu A/cm^2$). By applying Ohm's law (PD = IR where R is resistance; G = 1/R) using Isc and PD values (measured at intervals when the circuit is open), tissue ionic conductance (G, mS/cm^2) can be calculated and corrected for tissue surface area.

2.5.2. Basic procedure

Rats were sacrificed by an overdose of urethane anaesthesia and approximately 10 cm segments of intestinal tissue was excised: jejunum (beginning 5 cm distal to the ligament of Treitz); ileum (beginning 5 cm proximal to the cecum); and colon (beginning 1 cm distal to cecum). Mice were sacrificed by cervical dislocation and the mid small intestine was removed. The tissue was immersed in warmed, oxygenated buffer, opened along the mesenteric border and the intestinal contents gently washed out. In rat tissue, the external muscle with adherent myenteric plexuses was stripped off, leaving the submucosal plexus and mucosa intact. Since the mouse tissue is very thin and the stripping procedure damages the tissue, the full thickness intestine was used. Up to four

adjacent pieces of intestine from each animal were mounted in Ussing chambers exposing 0.6 cm^2 of surface area to Krebs buffer (115 mM NaCl, 8 mM KCl, 1.25 mM $CaCl_2$, 1.2 mM $MgCl_2$, 2.0 mM $KHPO_4$, 25 mM $NaHPO_4$, pH 7.35 ± 0.02), containing 10 mM glucose (energy source) on the serosal side osmotically balanced with 10 mM mannitol on the luminal side. A circulating water pump and water jacket maintained the buffers at 37°C.

2.5.3. Electrophysiological measurements

2.5.3.1. Baseline readings

The Isc was measured in the voltage clamp mode at zero PD and continuously recorded on a chart recorder (Houston Instruments, Austin, Texas). At 10 min intervals, the circuit was opened to measure the PD and determine the G. Baseline measurements (Isc, PD, G) were determined at the equilibration, ~20 min after mounting. The Isc 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 and used as an indication of tissue viability.

2.5.3.2. Responses to antigen

Tissues in chambers were challenged with 100μg/ml OVA about 20-30 min after mounting, when the baseline parameters were stable. The Isc response to OVA was calculated as the difference between the baseline Isc and the maximal increase occurring 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. Some experiments were conducted in the presence of the neurotoxin, tetrodotoxin (TTX, 10^{-6} M, Sigma), the $5HT_2$ antagonist, ketanserin (10^{-5} M, Janssen Pharmaceuticals, Belgium), the H_1 antagonist, diphenylhydramine (10^{-5} M, Sigma), or the cyclooxygenase inhibitor, piroxicam (10^{-5} M, Squibb, Princeton, NJ). All inhibitors were added to the serosal side of the tissue and after 15 min, the Isc response to OVA was compared in tissues treated or not treated with inhibitors.

2.5.4. Permeability studies

Intestinal permeability was measured across intestine from unsensitized and sensitized rats by measuring the mucosal to serosal (m→s) movement of two different sized radiolabelled probes: ^{3}H-mannitol (Sigma) and ^{51}Cr-EDTA (Radiopharmacy, McMaster-Chedoke Hospital, Hamilton, Canada). ^{3}H-mannitol has a molecular weight of 180 Da and 6.2Å diameter (Moore R 1990), while ^{51}Cr-EDTA is a larger molecule, with molecular weight of 360 Da with diameter of 10.7Å (Crissinger KD 1990). ^{3}H-mannitol and ^{51}Cr-EDTA were added to the luminal tissue buffer at a final concentration 2.5 μCi/ml (25 μl of 1.0 mCi/ml) and 6 μCi/ml (0.5 ml of 9.0 mM), respectively. Radiolabelled probes were osmotically balanced by adding 0.5 ml of the same concentration of non-radioactive Cr-EDTA to the serosal buffer. Following a 20 min equilibration period, 1 ml samples were

taken from the serosal ("cold") buffer every 25 min and replaced with appropriate amount of buffer. At the beginning, mid-point and end of each experiment 25μl aliquots were sampled from the "hot" luminal buffer for the determination of probe specific activity. Radioactivity of ^{3}H-mannitol and ^{51}Cr-EDTA were counted on β (Beckman LS 5801, Beckman Instruments Inc., Canada) and γ counter (1282 Compugamma, LKB, Wallac, Turku, Finland) respectively and the fluxes were expressed in nM/cm^2/h.

Flux calculations:

net flux = (Δ P cpm × specific activity of P)/(tissue surface area × sample time interval × 60 min × 1000 (unit conversion))

where:

P = probe = ^{51}Cr-EDTA or ^{3}H-mannitol

Δ P cpm (on cold side) = total cpm - previous total cpm + cpm removed with previous sample

total P cpm for cold side = (P cpm (raw counts - background))/(time (min) × chamber volume)

specific activity of the P (μM/cpm)(on hot side) = total P in μM/ total P cpm

total P cpm for hot side = total counts in 10 ml (chamber volume) × aliquot volume (50 μl) × hot probe

hot P = average of three hot counts taken at the beginning, middle and end of experiment

2.6. Assays for immunoglobulins

2.6.1. Radioimmunoassays (RIA)

Total and OVA-specific IgE levels were measured in sera by a paper radioimmunosorbent test (PRIST) and a paper radioallergosorbent test (PRAST), respectively. These assays were conducted as follows: sheep anti-rat immunopurified IgE {ε specific (Marshall JS 1985)) or OVA, to were bound to CNBr-activated filter paper discs (Whatman 1, Whatman International Ltd., England). The coated discs were washed in buffer (0.3% BSA, 0.03% Tween 20, 0.02% NaN_3 in phosphate buffer saline) and then incubated overnight at 4°C in tested serum (dilutions 1:5, 1:10, 1:20). After washing in buffer, the discs were incubated with monoclonal ^{125}I anti-rat IgE (MARE-1, Serotec, Camarillo, CA) or with polyclonal sheep ^{125}I anti-rat IgE (Marshall JS 1985). The discs were washed again and the radioactivity counted in a γ counter. IgE positive serum from Brown-Norway rats served as a positive control and buffer and IgE negative rat serum as negative controls. The radioactivity of the samples was compared with a standard curve obtained from known concentration of highly enriched IgE myeloma (IR 162).

2.6.2. Passive Cutaneous Anaphylaxis (PCA)

The blood, for determination of reaginic antibodies, was obtained from sensitized and control animals by cardiac puncture. Sera were stored at -70°C. Samples of sera were prepared in duplicate in twofold dilutions from 1:4 to

1:8192. One hundred μl aliquots of diluted sera were injected intradermally into the backs of Sprague-Dawley rats. IgE positive serum diluted 1:50 (from sensitized Brown-Norway rats) served as a positive control. IgE negative serum (from unsensitized Brown-Norway and Sprague-Dawley rats) served as negative controls. After 72 h, rats were challenged by iv 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 (Ovary Z 1986). The highest serum dilution giving a positive reaction (a blue spot $\geq$ 0.5 cm in diameter) was recorded as the PCA titre of that serum. Since IgE antibodies are recognized as the only class of antibodies unstable at 56°C, heat treatment (56°C, 30 min) of sera was used to confirm that positive reactions were due to heat-labile IgE antibodies (Rousseaux-Prevost R 1983).

2.6.3. Enzyme-linked immunosorbant assay

OVA-specific IgG and IgG_{2a} antibodies in serum were measured by an enzyme-linked immunosorbant assay (ELISA). The ELISA assay was performed as follows: plastic plates (Nunclon U96, Nunc, Roskilde, Denmark) were coated with OVA (5μg/ml) and left overnight at 4°C. After blocking 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, PA) or anti-IgG_{2a} (antiserum; ICN Immunobiologicals, IL) was added followed by the developing antibody, alkaline phosphatase-rabbit anti-goat IgG (ZYMED Laboratories, Inc, CA). A

colour reaction was obtained by addition of the substrate, p-nitrophynyl phosphate (Sigma), and was read by an automatic ELISA reader (MR 60, Microplate reader, Dynatech Instruments Inc., Torrance, CA). On each plate, samples were compared to standards of IgG positive serum.

2.7. Histology

Segments of jejunum obtained from Sprague-Dawley rats were fixed in formalin or Carnoys fixative (6:3:1 of absolute ethanol: chloroform: glacial acetic acid). Segments placed in Carnoys fixative were transferred to 70% ethanol 24 h later. The tissues were embedded in paraffin wax and the longitudinal sections along villus/crypt axis, at a thickness of 3 μm were cut. Sections were stained with toluidine blue and eosin for detection of mucosal mast cells and cytoplasmic morphology, respectively. The numbers of MMCs were calculated per villus/crypt unit or villus/crypt area (mm^2). At least 6-10 well-orientated units were measured per section using a computerized videoplan (Kontron, Zeiss, Germany).

2.8. Statistical analysis

Differences among and between groups were analyzed using one way analysis of variance or two-sided unpaired 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. Two way analysis of variance were used for some analysis as indicated in the text. 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 if $p < 0.05$.

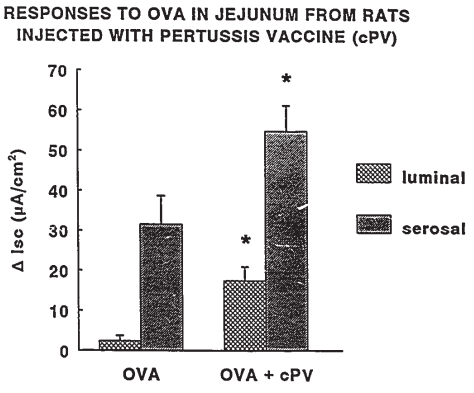

Figure 8. Responses to OVA after sensitization with pertussis vaccine (cPV) adjuvant. Values represent the means ± SEM of increases in short-circuit current (Isc) in response to luminal and serosal OVA (100μg/ml) added to jejunum obtained from rats 14 days after injection with 1 mg OVA + 1 ml cPV or 1 mg OVA only (n=10-11 rats). Unpaired Student's t test, showed that group injected with OVA + cPV had higher responses to both luminal [t(19)=3.7; *p=0.02] and serosal [t(19) = 2.85; *p=0.01] OVA compared to rats injected with OVA alone. Data represent the maximum response within 5 min after OVA challenge.

CHAPTER III

RESULTS

A. RAT MODEL OF HYPERSENSITIVITY

3.1. Establishing the phenomenon

3.1.1. Responses to antigen in rats actively sensitized with pertussis vaccine (cPV) as adjuvant

Previous studies of the rodent model of hypersensitivity have shown that two adjuvants, cellular pertussis vaccine (cPV) and alum injected with antigen (OVA) during sensitization, resulted in subsequent sensitivity of intestinal preparations to secondary challenge with OVA (Crowe SE 1990). To determine if cPV alone could act as an adjuvant for the immunophysiological responses, rats were sensitized with OVA alone (control) or OVA plus cPV. Jejunum from OVA injected rats responded to secondary OVA challenge from the serosal side of the tissue, while the response to luminal OVA was negligible (Figure 8). Injection of rats with OVA plus cPV resulted in presence of a significant response to OVA challenge on the luminal side of the tissue and significantly elevated the serosal response. This demonstrates that cPV alone acts as adjuvant.

3.1.2. Responses to antigen in rats actively sensitized with pertussis toxin (wPT) as adjuvant

Since one of the active ingredients of cPV is pertussis toxin further experiments were conducted to examine the role of pertussis toxin adjuvant in intestinal hypersensitivity.

3.1.2.1. Immunophysiological responses to antigen

After challenge with 100 μg/ml OVA to jejunum from rats injected with OVA plus 50 ng wPT, the Isc began to increase very rapidly; within 2 min after luminal addition and within 20 s after serosal addition. Representative tracings are shown in Figure 9.

The Isc responses to luminal and serosal OVA were compared in intestinal preparations from control rats versus those injected with OVA plus wPT (Table 3). Control rats injected with saline had no jejunal response at all to OVA challenge. Rats injected with OVA had no significant Isc responses to luminal OVA; however, serosal challenge did result in an increase in Isc of 31.5 ± 7.2 $\mu A/cm^2$. This response was enhanced 2.2 fold (to 68.3 ± 9.1 $\mu A/cm^2$, $p<0.05$) by the injection of rats with wPT in addition to OVA. In jejunum from rats injected with wPT plus OVA, the mean increase in Isc after luminal OVA challenge was 18.7 fold that in OVA controls (44.2 ± 4.7 vs. 2.4 ± 1.9 $\mu A/cm^2$). When compared the intestinal responses to OVA in cPV and wPT treated rats, the luminal responses were significantly higher from wPT treated

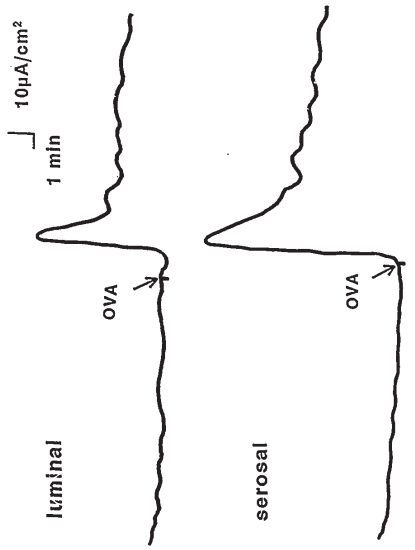

Figure 9. Representative tracings of short circuit current (Isc) responses to luminal or serosal OVA (100 μg/ml) added to jejunal tissues mounted in Ussing chambers. Tissues were obtained from rats 14 days after injection with 1 mg OVA + 50 ng wPT.

Table 3. Short-circuit current (Isc) responses to OVA.

		Challenge	Δ Isc (μA/cm²)	
treatment	n	(*in vitro*)	Luminal	Serosal
saline	6	OVA	0	0
wPT	6	OVA	0	0
OVA	10	OVA	2.4 ± 1.9	31.5 ± 7.2[b]
OVA + wPT	6	OVA	44.2 ± 5.2[a]	68.3 ± 9.1[a]
OVA + wPT	5	BSA	0	0

Values represent maximum rise in Isc occurring during the first 5 min after OVA or BSA challenge (100 μg/ml) in jejunum from rats 14 days after injection with: saline, 1 ml; wPT, 50 ng; OVA, 1mg. Values represent the means ± SEM; n = number of rats (values from 1 to 3 tissues were averaged from each rat). One way analysis of variance revealed differences among groups for the luminal [$F(3,24)=62.01$; $p<0.001$] and serosal [$F(3,24)=21.44$; $p<0.001$] response to OVA: a. luminal responses to OVA in intestine from OVA + wPT injected rats were different compared to all other groups; b. serosal responses to OVA in OVA injected rats were different from OVA + wPT, saline or wPT injected rats. Significance level $p=0.05$.

rats [$t(20)=3.4$; $p=0.003$], while the difference on serosal side did not achieve statistical significance. Challenge with a non-specific antigen, BSA, to jejunum from rats sensitized with OVA plus wPT evoked no increase in Isc, indicating the specificity of the reaction.

3.1.2.2. Baseline jejunal electrophysiological parameters

Baseline electrophysiological parameters were compared in segments of jejunum from rats injected with OVA plus 50 ng wPT. Control rats were injected with saline, OVA, or wPT alone. There was no significant difference in baseline Isc, PD and G in the various groups of injected rats (Table 4), suggesting that the sensitization itself did not change transport parameters of the gut under basal circumstances.

3.1.2.3. Concentration of antigen challenge

To determine an appropriate concentration of OVA to use for the *in vitro* secondary intestinal challenge, a concentration response curve was determined (Figure 10). In intestine from rats injected with OVA plus 50 ng wPT, the Isc response was maximal or near maximal at 100 μg/ml for luminal or serosal OVA, and consequently in all subsequent experiments this concentration of OVA was used.

Table 4. Baseline electrophysiological values in jejunum from control and experimental rats.

Primary		Isc	PD	G
treatment	n	(μA/cm²)	(mV)	(mS/cm²)
saline	6	34.8 ± 3.9	1.3 ± 0.2	28.1 ± 1.1
wPT	12	40.6 ± 4.1	1.4 ± 0.1	30.3 ± 1.4
OVA	13	35.0 ± 4.2	1.2 ± 0.2	29.2 ± 1.3
OVA + wPT	6	32.8 ± 3.7	1.1 ± 0.2	29.8 ± 4.7

Data were obtained at equilibration 20 min after mounting the tissue. Values represent the means ± SEM; n = number of rats (values from 4 tissues were averaged from each rat).

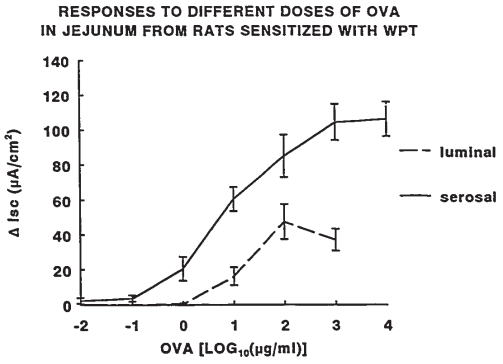

Figure 10. Concentration response curve to luminal and serosal OVA. Values represent means ± SEM increases in short-circuit current (Isc) in response to OVA added to jejunum obtained from rats 14 days after injection with 1 mg OVA + 50 ng wPT. OVA was added in doses from 0.1 to 1000 μg/ml to 6 adjacent fragments of jejunum; n = 5 rats for each data point. Data represent the maximum response within 5 min after OVA challenge.

3.1.2.4. Sensitization doses of wPT

Rats were injected with different doses of wPT (1.0 to 200 ng) plus 1.0 mg OVA. Figure 11A shows that the magnitude of the Isc response of the jejunum to luminal OVA challenge was increased with increasing doses of wPT. As little as 10 ng wPT resulted in a significantly enhanced luminal 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 (Figure 11B).

3.1.2.5. Time course of immunophysiological responses

To established how long a single injection of wPT plus OVA predisposes an animal to respond in an enhanced manner to secondary OVA challenge, rats were sacrificed at different times and increases in jejunal Isc to OVA were examined (Figure 12 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 Isc responses were significantly elevated in OVA plus wPT injected rats compared to rats injected with OVA alone. Maximum responses to luminal OVA were evident at days 56 and 84. At 8 months, the OVA plus wPT injected animals still demonstrated significant responses to luminal and serosal OVA, while in rats injected with OVA alone responses were

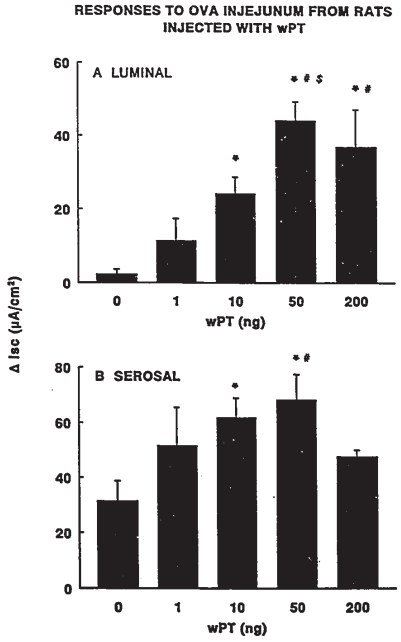

Figure 11. Responses to OVA after sensitization with various doses of wPT. Values represent the means ± SEM increases in short-circuit current (Isc) in response to luminal (A) and serosal (B) OVA (100μg/ml) added to jejunum obtained from rats 14 days after injection with 1 mg OVA + wPT (0 to 200 ng)(n=4-10 rats). Data represent the maximum response within 5 min after OVA challenge. (A). One way analysis of variance on responses to luminal OVA revealed significant effect of wPT treatment [$F(4,30)=14.98$; $p < 0.001$]; responses to luminal OVA: * different from Group 0; # different from Group 1; $ different from Group 10. (B). One way analysis of variance of responses to serosal OVA revealed significant effect of wPT treatment [$F(4,30)=3.73$; $p = 0.014$]; responses to serosal OVA: * different from Group 1, 10, 50, 200; # different from Group 50. Significance level $p<0.05$.

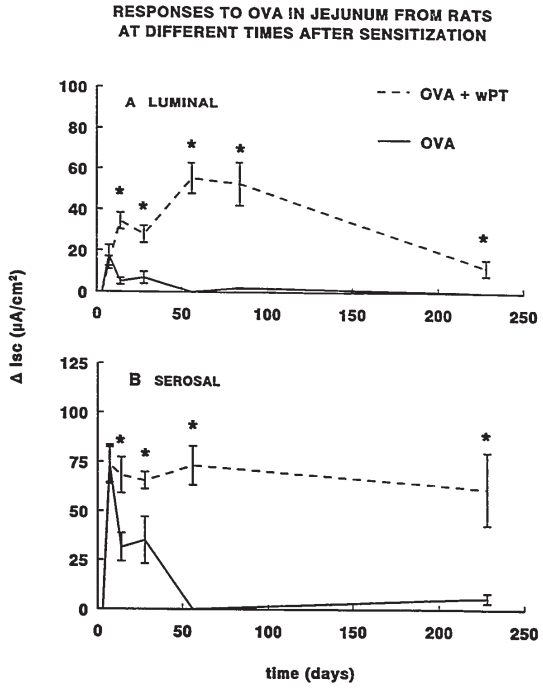

Figure 12. Time course of immunophysiological responses. Values represent the means ± SEM of increases in short-circuit current (Isc) in response to (A) luminal and (B) serosal OVA (100μg/ml) added to jejunum obtained from rats sacrificed at day 3, 7, 14, 28, 56, 84 and 226 after injection with 1 mg OVA + 50 ng wPT or 1 mg OVA alone; n = 4-12 rats (except control experiments on day 84 and 226 when n = 2). Two way analysis of variance of responses to luminal OVA revealed the main effect of injection with wPT [$F(1,49) = 46.6$; $p < 0.001$] and the main effect of time [$F(6,49) = 14.0$; $p < 0.001$]. Similar effects were observed on serosal responses to OVA: wPT effect [$F(1,48) = 57.87$; $p < 0.001$], time effect [$F(6,48) = 7.80$; $p < 0.001$]. There was also synergistic effect between those two factors (wPT and time) as evidence by significant interaction for the luminal [$F(6,49) = 6.13$; $p < 0.001$] and serosal [$F(6,48) = 7.48$; $p < 0.001$] responses to OVA. The differences at specific time points between OVA and OVA+wPT treated rats were evaluated by unpaired Student's t test at significance level *$p < 0.05$. Data represent the maximum response within 5 min after OVA challenge.

negligible after ~50 days.

3.1.2.6. Immunophysiological responses in ileum and colon

To determine if wPT-induced elevated responses to antigen, are present in other segments of the gut, ileal and colonic intestinal segments from sensitized rats with OVA plus wPT were investigated in Ussing chambers. The baseline parameters for ileum and colon are shown in Table 5. Responses to OVA demonstrated in ileum and colon were comparable to those in jejunum. (Figure 13). In the ileum, luminal OVA caused a ΔIsc of 27.4 ± 5.1 $\mu A/cm^2$, serosal OVA 93.0 ± 14.1 $\mu A/cm^2$. In the colon, luminal OVA caused ΔIsc of 43.3 ± 7.9 $\mu A/cm^2$ and serosal OVA, 73.32 ± 13.1 $\mu A/cm^2$.

3.2 Requirement for enzymatic activity of wPT

The enzymatic activity of pertussis toxin was shown to correlate with pathogenicity of *Bordetella pertussis* and the side effects after vaccination with cPV (Monack D 1989). To check if electrophysiological changes after sensitization are dependent on enzymatic activity, the enzymatically inactive mutant was used. Sprague-Dawley rats were sensitized with mutant mPT (1-200ng) plus 1 mg OVA. The baseline parameters (Isc, PD, G) in intestine from OVA plus mPT injected rats, measured after tissue stabilization, did not differ from rats injected with OVA plus wPT (Table 6). The secondary challenge of the jejunum with luminal or serosal OVA did not evoke a significantly higher increase

Table 5. Baseline electrophysiological values in jejunum, ileum and colon from rats sensitized with OVA plus wPT.

Segment	Isc ($\mu A/cm^2$)	PD (mV)	G (mS/cm^2)
jejunum	32.8 ± 3.7	1.3 ± 0.2	35.8 ± 4.7
ileum	12.0 ± 1.2*	0.6 ± 0.1*	19.2 ± 0.9*
colon	60.0 ± 9.9*	2.9 ± 0.4*	20.1 ± 2.7*

Data were obtained at equilibration 20 min after mounting the tissues. Values represent the means ± SEM; 5 - 6 rats (values from 3-4 tissues were averaged from each rat). Analysis of variance revealed differences among groups in Isc, PD and G. Isc and PD in jejunum was different from ileum and colon, while G was comparable in ileum and colon but different from jejunum; (*$p < 0.05$ compared with jejunum).

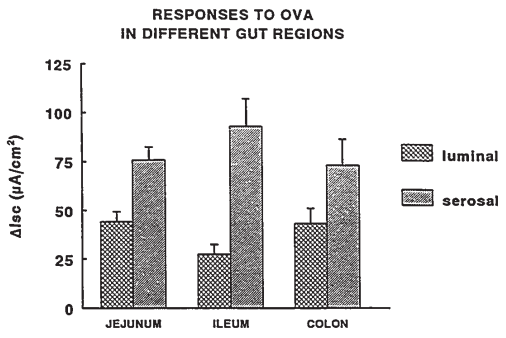

Figure 13. Responses to OVA in different regions of gut. Values represent the means ± SEM of increases in short-circuit current (Isc) in response to luminal and serosal OVA (100μg/ml) added to jejunum, ileum and colon obtained from rats 14 days after injection with 1 mg OVA + 50 ng wPT (n=4-12 rats). Data represent the maximum response within 5 min after OVA challenge.

Table 6. Comparison of baseline parameters (A) and responses to OVA (B) in jejunum from rats injected with mPT or wPT adjuvant.

A)

	OVA	OVA + mPT	OVA + wPT
Isc (μA/cm²)	35.0 ± 4.2	30.9 ± 4.1	32.8 ± 3.7
PD (mV)	1.2 ± 0.2	1.2 ± 0.1	1.3 ± 0.2
G (mS/cm²)	26.8 ± 1.3	28.1 ± 2.7	32.8 ± 4.7

B)

	Δ Isc (μA/cm²)		
	OVA	OVA + mPT	OVA + wPT
luminal	5.7 ± 2.3	7.8 ± 5.3	44.2 ± 5.2*
serosal	31.5 ± 7.2	41.6 ± 8.2	68.3 ± 9.1*

Rats were injected with 1 mg OVA or OVA with 50 ng wPT or mPT. Baseline parameters were obtained at equilibration 20 min after mounting the tissue. The magnitude of the response to OVA was measured within 5 min after OVA challenge. Values represent the means ± SEM; n = 5-6 rats (values from 3-4 tissues were averaged from each rat). One way analysis of variance revealed

differences among groups in response to luminal [$F(2,18) = 23.55$; $p < 0.001$] and serosal [$F(2,18) = 5.44$; $p = 0.014$] OVA. Responses to OVA in intestine from rats which received OVA + mPT did not differ from OVA treated rats. However responses to OVA in OVA + wPT injected rats were significantly higher on luminal and serosal side than OVA + mPT injected rats.

in Isc response than in jejunum from rats injected with OVA alone. This was relevant for all doses of injected mPT (Table 6, Figure 14). The lack of adjuvant effect of the mutant indicates the role of enzymatic activity of the toxin.

3.3. Effects of active sensitization with wPT adjuvant

3.3.1. Permeability

3.3.1.1. Neuronal regulation of luminal responses

The use of tetrodotoxin (TTX), a blocker of fast Na^+ channels and therefore of action potential propagation in neurons (Huot RI 1989), was used to examine possible neuronal involvement in the pertussis toxin-induced hypersensitivity reactions. Jejunal tissue in Ussing chambers displayed a 66% lower response to luminal OVA following pretreatment with the neurotoxin, (TTX, 10^{-6} M)(9.8 ± 1.5 μA/cm²), as compared with tissues without TTX (28.9 ± 4.8 μA/cm²; $p < 0.05$)(Figure 15A). However, the serosal response to OVA was not affected by TTX (48.0 ± 4.8 vs 50.7 ± 10.1 μA/cm², for untreated and TTX-treated tissues respectively).

Lack of sensitivity of responses to serosal OVA could be due to the high challenge concentration of OVA (100μg/ml). To exclude this possibility, experiments were repeated with 10 and 1 μg/ml of OVA (Figure 15B). The results confirmed the previous data: responses to serosal OVA at lower concentrations were insensitive to TTX. The TTX effect could be due to a different population of mast cells that are degranulated after luminal vs. serosal

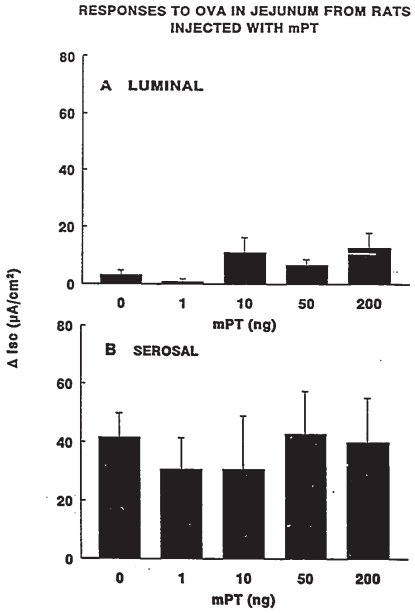

Figure 14. Responses to OVA after sensitization with various doses of mPT. Values represent the means ± SEM increases in short-circuit current (Isc) in response to luminal (A) and serosal (B) OVA (100μg/ml) added to jejunum obtained from rats 14 days after injection with 1 mg OVA + mPT (0 to 200 ng)(n=4-7 rats). Data represent the maximum response within 5 min after OVA challenge.

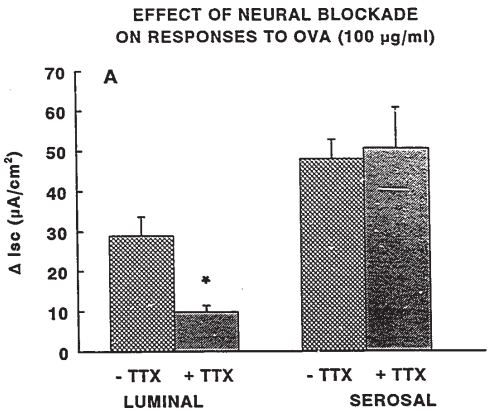

Figure 15. Responses to OVA in the presence of TTX. Values represent the means ± SEM of increases in short-circuit current (Isc) in response to OVA added to jejunum in the absence or presence of TTX (10^{-6}M). Tissue was obtained from rats 14 days after the injection with 1 mg OVA + 50 ng wPT; n=9 rats. An unpaired Student's t test showed that, responses to luminal OVA in TTX treated tissues were significantly lower [t(16)=3.80; *p=0.002] than in untreated tissues. Data represent the maximum response within 5 min after OVA challenge. A. Concentration of (100μg/ml) OVA was challenged to luminal and serosal side of the tissue; B. Concentration of 1, 10, 100 μg/ml of OVA was challenged to serosal side of the tissue.

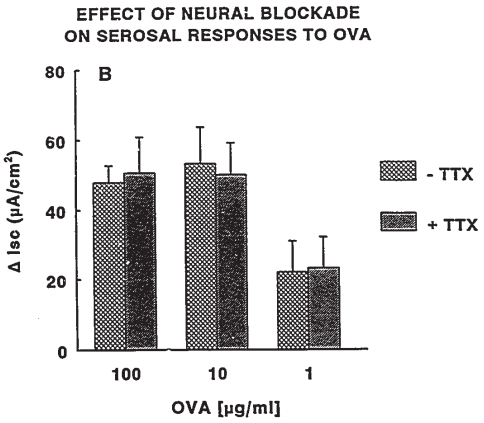

Figure 15B (see text Figure 15)

stimulation with OVA or to regulation of permeability to OVA.

3.3.1.2. Site of effector cells

To determine if the responses to OVA added to either side of the tissue were mediated by the same population of effector MCs, jejunal tissues mounted in Ussing chambers were challenged with OVA first on the serosal side and then on the luminal side. This order of OVA presentation was reversed in parallel experiments using adjacent tissues from the same rat. In the first case, the addition of serosal OVA abolished the subsequent responses to luminal antigen (Table 7). In the second case, the average response to serosal OVA was diminished, but it did not achieved statistical significance. Additionally, the serosal responses to OVA appeared immediately (16.00±0.04 s) after OVA addition to the tissue, while luminal responses appeared after a delay (1.35±0.50 min). This suggests that the same or at least overlapping population of subepithelial MCs responds to antigen addition from either side of the tissue, but that the magnitude of the Isc response to following luminal antigen is reduced due to the fact that less immunologically intact OVA will cross the epithelial tight junction barrier compared with the amount that gains access through the connective tissues on the serosal side after the muscle layers have been stripped off.

Table 7. Luminal versus serosal response to OVA.

ΔIsc (μA/cm²)			
First challenge		Subsequent challenge	
luminal	27.0 ± 4.4	serosal	42.5 ± 4.5
serosal	51.2 ± 6.5	luminal	1.2 ± 0.8*

Rats were sensitized with 1 mg OVA + 50 ng wPT. Fourteen days later jejunal tissues were challenged with OVA (100μg/ml) first on the one side and subsequently on the other side. Separate tissues from the same rats were challenged in the reverse order. Values represent the means ± SEM, n = 15 rats; [t(15)=5.78], *p<0.05 compared to first challenge.

Table 8. Permeability of the jejunum to probe molecules.

Primary treatment	^{51}Cr-EDTA (nM/h/cm²)	^{3}H-mannitol (nM/h/cm²)
saline	10.4 ± 0.4	158.3 ± 12.2
OVA + wPT	11.2 ± 0.5	161.2 ± 10.2

Rats were injected with saline or 1 mg OVA + 50 ng wPT. Fourteen days later two probes, ^{51}Cr-EDTA and ^{3}H-mannitol, were added to luminal side of the isolated jejunum and the fluxes across the tissue were measured. Values represent the means ± SEM; n=5.

3.3.1.3. Permeability to ^{51}Cr-EDTA and ^{3}H-mannitol

The permeability to luminal molecules was determined in the jejunum from sensitized Sprague-Dawley rats with OVA plus wPT and compared with permeability in jejunum from naive rats. The fluxes for ^{51}Cr-EDTA and ^{3}H-mannitol did not reveal statistically significant differences (Table 8) in intestine between treated and control groups of rats.

3.3.2. Responses to nerve stimulation

Isc 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 Isc response to TS than tissues from rats injected with saline (Figure 16). 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 Isc response to TS comparable with that in control OVA injected rats. This finding indicates that wPT, but not mPT changed the ability of nerves to respond to stimulation. All Isc responses to TS were abolished after 15 min treatment with the neurotoxin, TTX.

3.3.3. Mast cell numbers

Morphological studies were performed to determine if sensitization with wPT altered the number of MCs in the intestinal mucosa 14 days post

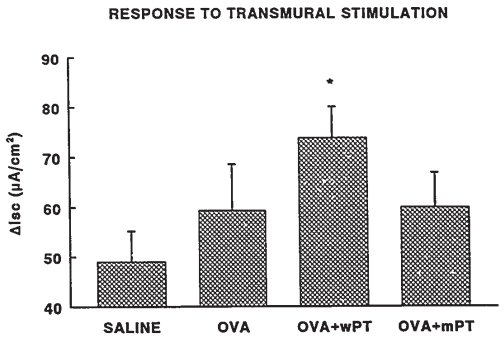

Figure 16. Response to transmural stimulation. Values represent the means ± SEM of increases in short-circuit current (Isc) in responses to transmural stimulation in jejunum obtained from rats 14 days after injection with saline or 1 mg OVA or 1 mg OVA + 50 ng wPT or 1 mg OVA + 50 ng mPT. Data represent the maximum response within 5 min after OVA challenge.

sensitization. Granulated MC numbers per villus/crypt unit were significantly increased ~40% ($p<0.05$) in the lamina propria of jejunum from rats injected with OVA plus wPT compared to control animals injected with OVA alone (Table 9). In contrast, injection of OVA plus mPT did not increase the number of MCs in the jejunal mucosa. The number of MMCs was highest in the jejunal mucosa from rats injected with 50 ng wPT (Table 10). Quantification of MMC along the time course revealed that the number was still elevated on day 28 and back to baseline values on day 84 (Table 11). None of the other morphometric parameters quantitated (villus and crypt areas, length of villus and villus/crypt unit) were altered on day 14 by the sensitization protocol (Table 12).

3.3.4. Antibody profile

3.3.4.1. IgE levels measured by radioimmunoassays

Radioimmunoassays, in which monoclonal antibodies were used as developing antibodies, revealed no significant differences in total and antigen-specific IgE levels in sera taken from Sprague-Dawley rats before and 14 days after sensitization. This result was consistent for all the sensitization protocols, OVA plus wPT, OVA plus mPT or OVA alone. However, the assay did detect high levels of IgE; pooled serum from sensitized Brown-Norway rats with a PCA titre of 1:2048 had 1660 unit/ml of OVA-specific IgE by immunoassay. IgE in serum with PCA titres <1:256 was not detected by the assay. The polyclonal antibodies, used as developing antibodies, gave more sensitivity and could detect

Table 9. Number of mast cells in the mucosa from rats sensitized with wPT or mPT adjuvant.

Primary treatment	n	Mast cells / VCU	Mast cells/ area (mm^2)
OVA	7	9.2 ± 0.8	15.9± 1.0
OVA+ wPT	6	13.0 ± 1.0*	23.2 ± 2.2*
OVA + mPT	6	8.0 ± 0.5	14.0 ± 2.7

Measurements were made on jejunal sections obtained from rats 14 days after injection with OVA alone or OVA + 50 ng wPT or OVA + 50 ng mPT. Values represent the means ± SEM; VCU = villus/crypt unit; n = number of rats (6-10 VCU were evaluated from each rat). One way analysis of variance revealed the differences among groups [$F(2,16)=7.02$; $p=0.006$]. MC numbers in tissues from rats treated with OVA+wPT was higher than in OVA or OVA+mPT treated rats (*$p<0.05$).

Table 10. Number of mast cells in the mucosa from rats sensitized with wPT adjuvant.

wPT dose (ng per rat)	n	Mast cells / VCU
0	7	9.2 ± 0.8
1	4	10.0 ± 0.6
10	5	12.0 ± 0.7
50	6	13.0 ± 1.0*
200	6	12.0 ± 0.7

Measurements were made on jejunal sections obtained from rats 14 days after injection with OVA alone or OVA plus different doses of wPT. Values represent the means ± SEM; VCU = villus/crypt unit; (n = number of rats; 6-10 VCU were evaluated from each rat). One way analysis of variance revealed differences among groups [$F(4,23)=3.86$; $p=0.015$]. OVA + 50 ng wPT injected rats had higher MC numbers compared to OVA alone injected rats at the significance level *$p=0.05$.

Table 11. Number of mast cells in the mucosa from rats sensitized with wPT adjuvant.

Day	n	Mast cells / VCU
14	6	13.0 ± 1.0*
28	5	12.3 ± 0.5*
56	5	10.8 ± 0.6
84	6	9.5 ± 0.5

Measurements were made on jejunal sections obtained from rats at different days days after injection with OVA alone or OVA plus different doses of wPT. Values represent the means ± SEM; VCU = villus/crypt unit; n = number of rats (6-10 VCU were evaluated from each rat). One way analysis of variance revealed differences in MC number [$F(3,18)=4.9$, $p=0.012$]. The MC number on day 14, 28 and 56 differ from day 84 (*$p<0.05$).

Table 12. Morphometric parameters.

	Primary treatment		
	OVA	OVA + wPT	OVA + mPT
n	7	6	6
Villus area (mm^2)	0.042 ± 0.002	0.041 ± 0.002	0.040 ± 0.004
Crypt area (mm^2)	0.018 ± 0.001	0.016 ± 0.001	0.017 ± 0.002
VCU area (mm^2)	0.058 ± 0.003	0.056 ± 0.003	0.057 ± 0.006
Villus length (mm)	0.42 ± 0.01	0.41 ± 0.02	0.43 ± 0.02
Crypt length (mm)	0.16 ± 0.01	0.16 ± 0.01	0.14 ± 0.02
VCU length (mm)	0.57 ± 0.02	0.58 ± 0.03	0.57 ± 0.02

Measurements were made on jejunal sections obtained from rats 14 days after injection with 1 mg OVA alone or OVA + 50 ng wPT or OVA + 50 ng mPT. Values represent the means ± SEM; VCU = villus/crypt unit; n = number of rats (6-10 VCU were evaluated from each rat).

levels of IgE down to 10ng/ml. The RIA with polyclonal antibodies detected IgE levels between 158 and 1773 ng/ml in sera from rats sensitized with OVA plus wPT (Table 13). In contrast, in sera from rats injected with OVA plus mPT, no significant increase in IgE was detected and the values were comparable with control sera (from OVA sensitized rats).

3.3.4.2. IgE measured by passive cutaneous anaphylaxis (PCA)

The IgE antibody levels by PCA were measured in sera from rats sacrified on day 14. PCA titres of sera from Sprague-Dawley rats injected with OVA alone, or different doses of wPT or mPT plus OVA are shown in Figure 17. Sera from rats injected with OVA alone (0 wPT), OVA plus mPT, or OVA plus the low doses of wPT had no or very small PCA responses. The mean positive PCA responses were highest in rats treated with 50 ng wPT. Since IgE antibodies are heat labile, the decrease of PCA titre after heat treatment indicates IgE. Some sera were tested again after heat treatment at 56° for 30 min. Heat treatment virtually abolished positive responses (Table 14). In only two cases out of seven a small degree of responsiveness remained after heat treatment.

The level of IgE was investigated at different times after primary injection (day 28, 56 , 84, 226)(Table 15). In OVA injected rats, the level of IgE decreased very quickly, showing no positive responses after day 28. In OVA plus wPT injected rats, the level of IgE was higher than in controls and it was still detectable on day 84.

Table 13. Concentration of OVA-specific IgE in circulation in different groups of rats.

Primary treatment	IgE concentration (units/ml)	Mean IgE concentration (units/ml)
OVA	25 25 16 0	16.5
OVA + wPT	1733 250 158 199 288	525.6
OVA + mPT	25 50 20 63 0 16	29.0

Rats were sensitized with 1 mg OVA only or 1 mg OVA + 50 ng wPT or with OVA + 50 ng mPT. The sera was obtained 14 days later and assayed by radioimmunoassay, using polyclonal antibodies as developing antibodies. The values represent the concentration of OVA-specific IgE.

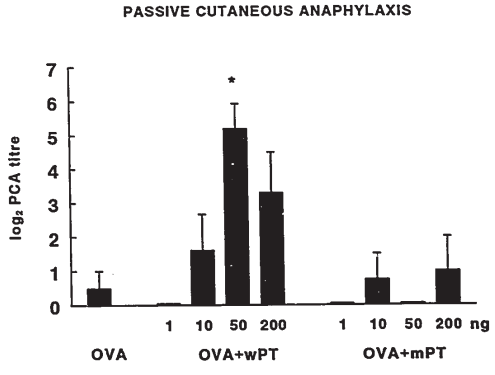

Figure 17. Passive cutaneous anaphylaxis. Values represent IgE titres from sera obtained 14 days after rats were injected with 1 mg OVA only or 1 mg OVA + different doses (from 1 to 200 ng) of wPT or mPT (n=4-6 rats). One way analysis of variance revealed differences in PCA titres among groups [$F(4,20)$; $p=0.001$]. PCA titres in sera from rats injected with 50ng wPT were different from group which received 0, 1, or 10 ng of wPT (*$p<0.05$); however no difference was observed between PCA titre from rats receiving 50 and 200ng of wPT. Treatment with mPT did not elevate the levels of PCA titres compared to controls.

Table 14. Passive cutaneous anaphylaxis (PCA) values from before and after heat treatment of sera.

Before heat treatment	After heat treatment
1:256	1:16
1:16	1:4
1:128	<1:4
1:128	<1:4
1: 64	<1:4
1:16	<1:4
1:16	<1:4

The values represent PCA titres from sera obtained from rats 14 days after sensitization with 1 mg OVA + 50 ng wPT. Each serum was heat treated to 56°C for 0.5 h.

Table 15. IgE levels in circulation at different times after sensitization measured by PCA

Day	Treatment	
	OVA	OVA + wPT
7	64	128
14	<4	32
28	<4	32
56	<4	16
84	<4	4
228	<4	<4

The values represent PCA titres from sera obtained from rats at different days after sensitization with 1 mg OVA alone or 1 mg OVA + 50 ng wPT; n = 3-4.

Since the highest PCA titres were achieved in the rats which showed the highest immunophysiological responses to OVA, the PCA titres in single animals were correlated with Isc responses to OVA. Pearson's correlation between PCA titres and luminal or serosal responses to OVA failed to reach statistical significance [r(36) for luminal = 0.34 and for serosal = 0.20, n=190], suggesting that IgE levels in the circulation are not predictive for the immunophysiological responses to antigen.

3.3.4.3. IgG and IgG_{2a} levels

Since there is evidence that IgG_{2a} can bind to MCs (Daeron M 1992a), 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 (Figure 18). The levels of IgG and IgG_{2a} were highest in rats that received wPT, the maximum increase observed with 50 ng wPT. Rats treated with mPT did not show any elevation of IgG or IgG_{2a} above control levels.

3.4. Passive sensitization

3.4.1. Source of serum

In order to investigate the role of serum factors in intestinal hypersensitivity in this model, the sera from sensitized rats was transferred to naive rats. Three days later, the intestine was removed and challenged with OVA on Ussing chambers. In initial experiments, the sera for passive sensitization

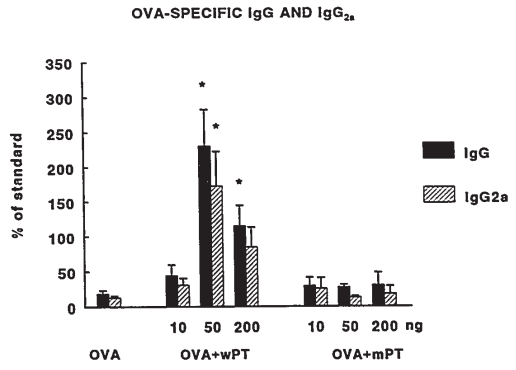

Figure 18. OVA-specific IgG and IgG_{2a} in circulation. The sera samples were obtained from rats 14 days after injection with 1 mg OVA only or 1 mg OVA plus different doses (from 10 to 200 ng) of wPT or mPT. The data are expressed as percent of standard serum (n=3-4 rats). One way analysis of variance revealed differences among groups in IgG [$F(6,46)$; $*p<0.001$] and IgG_{2a} [$F(6,46)$; $*p=0.001$] levels. IgG levels were elevated in sera from rats which received 50 and 200ng wPT, while IgG_{2a} levels were elevated in sera from rats which received 50 ng wPT ($p<0.05$), compared to remaining groups.

were derived from Sprague-Dawley rats after sensitization with OVA plus wPT. The IgE level of this sera, measured by PCA, ranged between 1:32 to 1:256. However, passive transfer of this sera did not induce hypersensitivity to OVA in isolated intestine (data not shown). Brown-Norway rats are known as high IgE responders, thus they were used for raising sera with high IgE content. Rats were injected with OVA plus wPT and boosted with OVA. In pooled serum from these rats, the level of total IgE antibodies was 3.9 μg/ml and OVA-specific IgE levels, measured by PCA, was 1:2048.

3.4.2. Immunophysiological responses to antigen

Naive Sprague-Dawley rats were injected ip with different doses of high IgE content Brown-Norway serum and the intestinal responses to antigen were investigated 3 days later. Baseline parameters were compared in passively and actively sensitized rats; no statistical difference was obtained in Isc, PD or G values (Table 16). The magnitude of intestinal responses to luminal and serosal OVA challenge are shown on Figure 19. The maximum OVA response was achieved with 0.5 ml of Brown-Norway serum. Further increases in the volume of serum did not elevate the response to luminal or serosal OVA.

3.4.3. Time course of responses to antigen

Since the response to OVA could be transferred by passive sensitization, the next experiment was designed to check how long after passive transfer the

Table 16. Baseline electrophysiological values in jejunum from passively or actively sensitized rats.

Sensitization	Isc (μA/cm^2)	PD (mV)	G (mS/cm^2)
passive	39.1 ± 3.7	1.3 ± 0.2	29.9 ± 1.8
active	32.8 ± 3.7	1.1 ± 0.2	29.8 ± 4.7

Data were obtained at equilibration 20 min after mounting the tissues. Values represent the means ± SEM; 5 - 6 rats (values from 2 - 4 tissues were averaged from each rat).

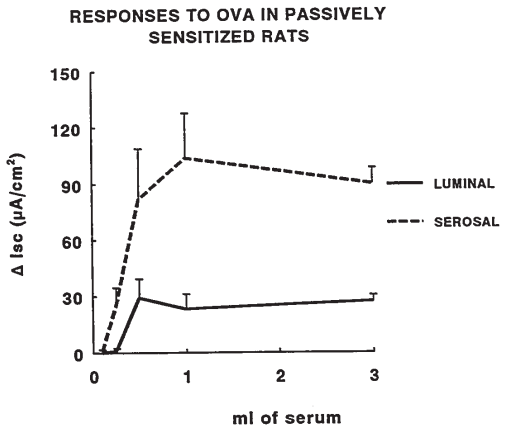

Figure 19. Responses to OVA in jejunum from passively sensitized Sprague-Dawley rats. Values represent the means ± SEM of increases in short-circuit current (ΔIsc) in response to luminal and serosal OVA (100μg/ml) added to jejunum obtained from rats 3 days after injection with 0.3, 0.5, 1 or 3 ml of Brown-Norway sera (n=3-6 rats). Data represent the maximum response within 5 min after OVA challenge.

tissue would respond to OVA. At day 3 after passive transfer, the response to OVA was 29.3±10.0 μA/cm^2 (luminal) and 82.3±14.5 μA/cm^2 (serosal) (Figure 20). At day 14, the luminal response was absent and the serosal response was strongly decreased. On day 70, no response to OVA was noted on either the luminal or serosal side of the tissue.

B. MOUSE MODEL OF HYPERSENSITIVITY

To investigate the role of antibodies and MCs, further experiments were conducted using mast cell-deficient mice (*W/W^v*) and their congenic controls (+/+). This model allowed a comparison to be made for the physiological responses to antigen in the presence or absence of mast cells. Additionally, the smaller body size of the mouse allowed lower doses of sera and antibodies to be used. Both classes of antibodies, IgE and IgG_{2a}, will bind to receptors on mice mast cells (Daeron M 1992).

3.5. Role of mast cells

3.5.1. Responses to antigen in sensitized +/+ and *W/W^v* mice

Groups of +/+ and *W/W^v* mice were actively sensitized with OVA plus wPT and 14 days later the isolated intestine was challenged with OVA in Ussing chambers. The results showed that in the mouse as in the rat model wPT acts as an adjuvant. The average response to OVA added to serosal side of intestine from +/+ mice was 50.5±7.0 μA/cm^2, while no response at all was seen in

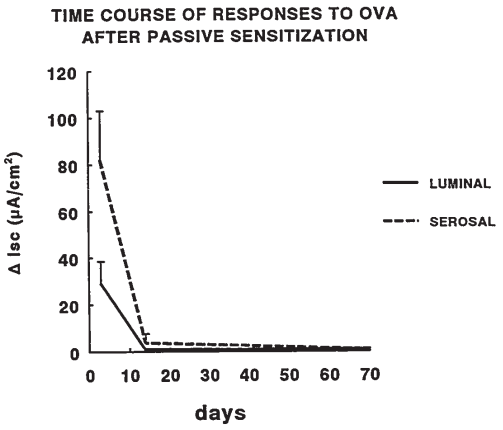

Figure 20. Time course of responses to OVA after passive sensitization. Values represent the means ± SEM of increases in short-circuit current (Isc) in response to luminal and serosal OVA (100μg/ml) added to jejunum obtained from Sprague-Dawley rats sacrificed at day 3, 14, 70 after injection with 0.5 ml of Brown-Norway serum (n=4-6 rats). Data represent the maximum response within 5 min after OVA challenge.

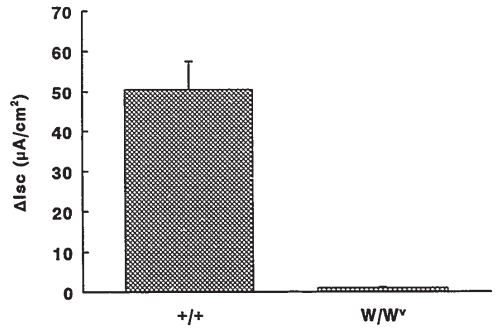

Figure 21. Responses to OVA after active sensitization of +/+ and *W/W*ᵛ mice with wPT adjuvant. Values represent the means ± SEM of increases in short-circuit current (ΔIsc) in response to serosal OVA (100μg/ml) added to mid intestine obtained from mice 14 days after injection with 0.1 mg OVA + 5 ng wPT (n=9-11 mice). An unpaired Student's t test showed that responses to OVA in intestine from *W/W*ᵛ were lower [t(18)=5.118, *p<0.001] than in +/+ mice. Data represent the maximum response within 5 min after OVA challenge.

*W/W*ᵛ mice (Figure 21), demonstrating the role of MCs in this model of hypersensitivity.

3.5.2. Effect of inhibitors on response to antigen

Further indication of mast cell involvement in the response to OVA in the intestine of +/+ mice, injected with OVA plus wPT, came from inhibitor studies. Three inhibitors were used: diphenylhydramine (10^{-6}M, H_1 antagonist), ketanserin (10^{-6}M, $5HT_2$ antagonist) and piroxicam (10^{-6}M, cyclooxygenase inhibitor). Each of these was added to the tissues *in vitro*, 15 min before OVA challenge. The response to OVA was diminished by 62 % in the presence of diphenylhydramine, by 66% in the presence of ketanserin and by 57% by piroxicam (Figure 22). Since the MCs are major source of antigenically released histamine and serotonin in intestinal mucosa, thus the data support the role of MCs.

3.5.3. Responses to antigen in intestine from reconstituted mice

Responses to OVA were compared in +/+, *W/W*ᵛ and BM(+/+)→ *W/W*ᵛ mice. All groups were passively sensitized with 0.5 ml Brown-Norway serum. When *W/W*ᵛ were reconstituted with MCs by bone marrow transfer, the intestine responded to OVA with ion secretion of a similar magnitude to those seen with OVA in +/+ mice (Figure 23).

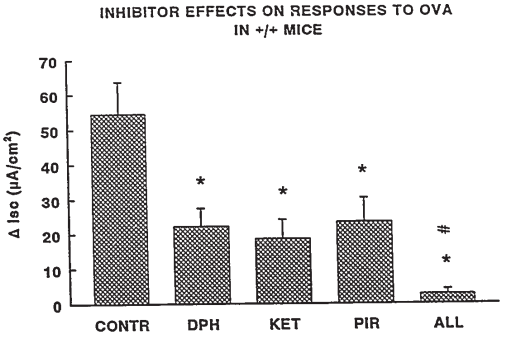

Figure 22. Effect of inhibitors on OVA responses in +/+ mice injected with wPT adjuvant. Mice were sensitized with 0.1 mg OVA + 5 ng wPT 14 days before experiments. Values represent the means ± SEM of increases in short-circuit current (Isc) in response to serosal OVA (100μg/ml) added to mid intestine alone or to mid intestine (jejunum and ileum) pretreated with ketanserin (10^{-6}M), diphenylhydramine (10^{-5}M), piroxicam (10^{-5}M) 15 min before OVA addition (n=6-13 mice). One way analysis of variance revealed differences among groups [F(4,47)=6.84; p<0.001]. Responses to OVA in the tissues treated with inhibitors were different from controls (*); responses to OVA in tissues treated with all inhibitors added together were lower than responses to OVA in tissues treated with single inhibitors and controls (#); significance level p=0.05. Data represent the maximum response within 5 min after OVA challenge.

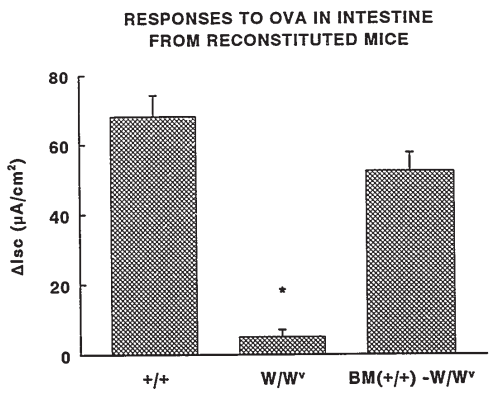

Figure 23. Responses to OVA in reconstituted and passively sensitized mice. *W/W*^{v} mice were reconstituted (BM(+/+)→*W/W*^{v}) with 30 millions bone marrow cells. Values represent the means ± SEM of increases in short-circuit current (Isc) in response to serosal OVA (100μg/ml) added to mid intestine obtained from +/+, *W/W*^{v} and BM(+/+)→*W/W*^{v} mice 3 days after injection with 0.3 ml of Brown-Norway serum (n=3-6 mice). One way analysis of variance revealed a difference in OVA responses [$F(2,20)=11.46$; $p<0.001$] among groups. Responses to OVA in intestine from *W/W*^{v} were lower compared with +/+ or reconstituted mice (*$p<0.05$); there was no difference between responses to OVA in intestine from +/+ and reconstituted mice. Data represent the maximum response within 5 min after OVA challenge.

3.6. Role of antibodies

3.6.1. Passive transfer of responsiveness to OVA to +/+ and *W/W*^{v} mice

To investigate the role of antibodies, +/+ and *W/W*^{v} mice were passively sensitized by ip injection with 0.3 ml high IgE containing serum from Brown-Norway rats. Subsequent challenge of isolated intestine with OVA showed the same pattern as in active sensitization: the presence of a Isc response to OVA in +/+ mice and its lack in *W/W*^{v} (Figure 24).

+/+ mice were injected with different doses (0.1 - 1.5 ml) of serum. The submaximal intestinal responses to OVA was obtained with injection of 0.3 ml of serum and the plateau was achieved with 0.5 ml (Figure 25).

3.6.2. IgE component in response to antigen

To identify the class of antibodies responsible for hypersensitivity, +/+ OVA responses was dependent on the amount of IgE myeloma used; total inhibition was obtained with 200μl of antibody which contained 31.8 μg of IgE (Figure 26, 27). This result indicates that in sensitization by injecting antigen with wPT as adjuvant, IgE antibodies are produced that are a component of the intestinal hypersensitivity.

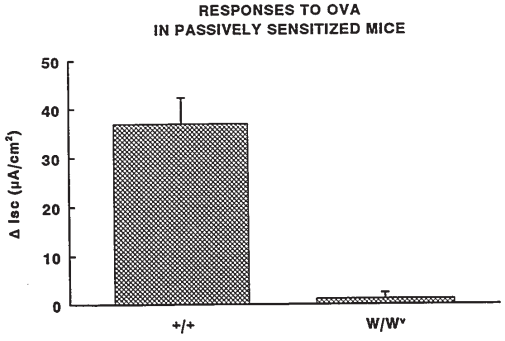

Figure 24. Responses to OVA in intestine from passively sensitized +/+ and *W/W*^{v} mice with Brown-Norway sera. Values represent the means ± SEM of increases in short-circuit current (Isc) in response to serosal OVA (100μg/ml) added to mid intestine obtained from mice 3 days after injection with 0.3 ml of Brown-Norway sera (n=4-6 mice). Response to OVA in *W/W*^{v} mice was lower than in +/+ mice [$t(9)=8.32$; *$p<0.001$]. Data represent the maximum response within 5 min after OVA challenge.

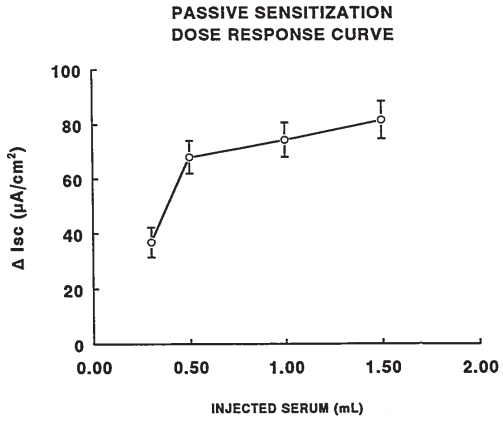

Figure 25. Responses to OVA in +/+ mice passively sensitized with different doses of Brown-Norway sera. Values represent the means ± SEM of increases in short-circuit current (Isc) in response to serosal OVA (100μg/ml) added to mid intestine obtained from mice 3 days after injection with 0.1 - 1.5 ml of Brown-Norway serum (n=4-5 mice). Data represent the maximum response within 5 min after OVA challenge.

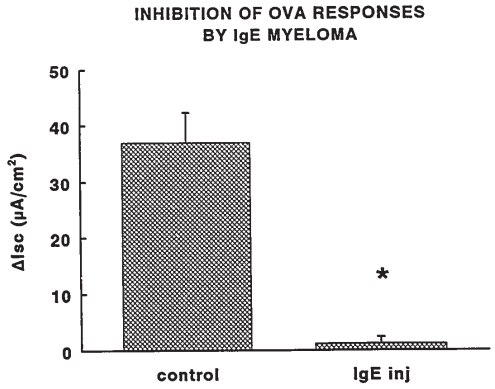

Figure 26. Inhibition of OVA response with IgE myeloma antibodies. Values represent the means ± SEM of increases in short-circuit current (Isc) in response to serosal OVA (100μg/ml) added to mid intestine obtained from mice passively sensitized with 0.3 ml of Brown-Norway serum (control) or injected with 31.8 μg IgE myeloma 3 days prior passive sensitization (n = 4 mice). IgE myeloma injection effected in decrease of the response to OVA [t(6) = 6.26; *p = 0.001] as revealed by an unpaired Student's t test. Data represent the maximum response within 5 min after OVA challenge.

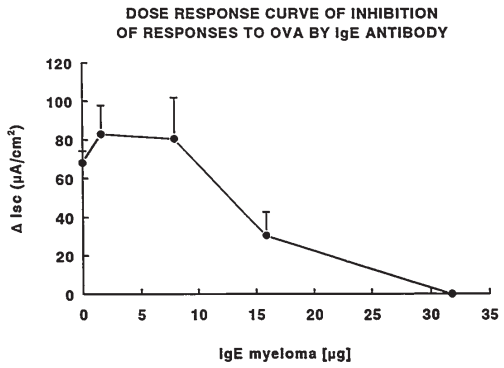

Figure 27. Dose response curve of inhibition of OVA response with IgE myeloma antibodies. Values represent the means ± SEM of increases in short-circuit current (Isc) in response to serosal OVA (100μg/ml) added to mid intestine obtained from mice passively sensitized with 0.3 ml of Brown-Norway sera (control) and injected with 0 - 31.8 μg IgE myeloma 3 days prior passive sensitization (n = 3-6 mice). Data represent the maximum response within 5 min after OVA challenge.

CHAPTER IV

DISCUSSION

4.1. Introduction

Hypersensitivity to food proteins is a very common disorder, particularly among small children (Dannaeus A 1987, Kniker WT 1987). In addition, immune activation via hypersensitivity mechanisms is implicated in such idiopathic disorders as ulcerative colitis (Fox CC 1993), Crohn's disease (Knutson L 1990), eosinophilic gastroenteritis (Whitington PF 1988), celiac disease (Horvath K 1989, Loft DE 1989) and peptic ulcer (Andre C 1983). Thus, the development of animal models of hypersensitivity and elucidation of the mechanism(s) of pathophysiology are very relevant for many disorders. According to the classical scheme of intestinal immediate hypersensitivity, an antigen which gains access to the body can elicit production of antigen-specific IgE antibodies which bind to mast cells. When the same antigen is subsequently encountered, it binds to and cross-links IgE, stimulating degranulation of the mast cells and release of their mediators. Those mediators act on enterocytes (directly or indirectly) resulting in ion secretion and the subsequent movement of water into the gut lumen by creation of an osmotic force. The mechanism of the development of

hypersensitivity is not clear; only some antigens under certain circumstances are recognized by an organism as allergens. Adjuvants are substances which enhance humoral immune responses to co-administered antigen. Thus, adjuvants including those that are components of bacterial cell walls, lipopolysaccharide or muramyl dipeptide, are currently the focus of extensive research to access their usefulness in routine vaccination (Audibert FM 1993). However, some adjuvants have also the ability to increase IgE production. Therefore, the possibility exist that such adjuvants may be a key determinants in allergic sensitization. In experimental animals, a single injection of a protein antigen in the absence of an adjuvant usually does not result in sensitization (Crowe SE 1990). However, when the antigen is given with an adjuvant, a very high percentage of animals became sensitized. There are many naturally occurring adjuvants (such as bacteria and their products) which may have a role in the development of hypersensitivity in humans (Gupta RK 1993).

The whole-cell vaccine used to immunize children against *Bordetella pertussis* infection is an adjuvant which is administered to experimental animals to induce anaphylactic responses (Perdue MH 1984, Forbes D 1988). Also, in the vaccination of children where pertussis vaccine is given with other vaccines, diphtheria and tetanus, pertussis vaccine may act as an adjuvant for the whole composition (Manclarck CR 1984). Early work by Jarrett (Jarrett EEE 1972, 1974, 1976, 1980) showed that injection of bacterial whole-cell vaccine of *Bordetella pertussis* with antigen stimulates antigen-specific IgE antibody

production. A comparison of pertussis vaccine with other adjuvants showed that the vaccine was a better potentiator of IgE production than $Al(OH)_3$ co-precipitated with antigen or Freund's complete adjuvant (Jarret EEE 1980). This was also true for animals sensitized and subsequently boosted with antigen alone.

Previous studies in a rodent model of intestinal hypersensitivity have shown that the adjuvant mixture of whole-cell pertussis vaccine with alum greatly enhances the responsiveness to secondary antigen challenge (Crowe SE 1990). The pathophysiology of intestinal hypersensitivity reactions includes abnormal gut function associated with increased secretion of electrolytes and water (Perdue MH 1984, 1986). Studies on isolated intestine obtained from sensitized animals have shown that antigen challenge increases net negative ion accumulation in the lumen. Specifically, antigen administration stimulated Cl^- secretion and inhibited Na^+ and K^+ absorption. In the circulation, an increase in mucosal mast cell protease (RMCP II) and histamine indicated degranulation of mucosal mast cells after antigen challenge. Moreover sensitized rats displayed increased epithelial permeability as measured by the recovery of ^{51}Cr-EDTA in the blood and urine following injection of this probe into the lumen of isolated gut segment (Crowe SE 1993).

The *Bordetella pertussis* bacterium has many biologically active components such as pertussis toxin, lipopolisaccharide (LPS), adenylyl cyclase, filamentous hemagglutinin, agglutinogens, dermonecrotic toxin and tracheal

cytotoxin (Prasad SM 1993, Weiss AA 1986). All those components, when introduced into a host organism stimulate a humoral immune response directed against those components. Purification of pertussis toxin in 1981 has allowed the adjuvant and other properties of this molecule to be separated from other components of the bacteria (Munoz JJ 1981). At least two components from *Bordetella pertussis*, pertussis toxin and LPS, were shown to have adjuvant activity (Caroff M 1990, Mountzouros KT 1992). The findings of Munoz (Munoz JJ 1981) demonstrated that purified pertussis toxin injected with antigen makes mice more susceptible to systemic anaphylactic shock in a subsequent intravenous challenge with the same antigen.

My studies examined the role of pertussis toxin in intestinal hypersensitivity. Initial experiments were designed to investigate if pertussis vaccine (cPV) used as a single adjuvant added to antigen during sensitization is a potentiator for the intestinal immunophysiological responses to antigen in the rat. Subsequent studies examined if a recombinant of the wild type pertussis toxin (wPT) alone, a component of the vaccine, is stimulatory for these responses.

The results of my studies showed that the adjuvant effect of cPV, stimulated the secretory response to antigen challenge in rat intestine. Injection of rats with wPT plus antigen enhanced hypersensitivity reactions in a similar manner to cPV. The enzymatic activity of wPT was essential for this effect as no enhancement was evident following sensitization with an enzymatically

inactive mutant toxin. Importantly, the enhanced antigen-induced intestinal response lasted for at least 8 months. wPT injection of rats increased the number of mucosal mast cells and the levels of antigen-specific IgE, IgG and IgG_{2a} antibodies in the circulation. Experiments in mast cell-deficient mice showed that the use of wPT as adjuvant resulted in reactions typical of the classical mechanism of immediate hypersensitivity involving mast cells and IgE antibodies. In addition, other changes were evident following wPT sensitization that may have enhanced the intestinal anaphylactic reactions.

4.2. Pertussis toxin enhances intestinal hypersensitivity

The immediate hypersensitivity reaction was compared in intestine from rats which were sensitized with OVA alone or with OVA plus adjuvant. Fourteen days post-sensitization, segments of intestine were mounted on Ussing chambers, challenged with OVA and the changes in Isc evaluated. Intestine from rats injected with OVA alone responded to antigen challenge from the serosal side of the tissue, but not from the luminal side. Addition of cPV adjuvant into the sensitization protocol induced a responsiveness to luminal OVA as shown by an increase in Isc. This shows that OVA injected alone is able to induce minimal hypersensitivity to a luminal antigen and the presence of an adjuvant is necessary for the full development of responses. wPT produced a similar response, indicating that wPT is the active ingredient in pertussis vaccine. The magnitude of the responsiveness to luminal OVA was actually higher in intestine

from rats that received nanogram quantities of wPT compared to those that received cPV. cPV contains bacteria attenuated by heat and formalin treatment and it is not clear how much active pertussis toxin is present per unit volume of vaccine. This may explain the decreased ability of cPV plus OVA treated rats to respond to luminal antigen.

The wPT was very potent in the stimulation of intestinal hypersensitivity. When compared with responses in rats sensitized with OVA alone, as little as 10 ng of wPT injected with OVA into adult rats led to a significant increase in ion secretion from the gut in response to antigen challenge. The maximum response occurred in rats sensitized with 50 ng wPT. At this dose of wPT, intestinal secretion increased 18.7 fold in response to luminal antigen as compared to rats injected with OVA alone. This demonstrates that wPT has potent adjuvant activity in the induction of intestinal hypersensitivity.

4.3. Relevance of enzymatic activity of the toxin

To investigate whether the enzymatic activity of pertussis toxin is required for the enhanced immunophysiological responses, a mutant form of the toxin (mPT) was used. Pertussis toxin is composed of two moieties, designated A and B. Subunit B binds to the N-linked oligosaccharides on the cell surface, while subunit A enters the membrane and ADP-ribosylates several types of G proteins (Milligan G 1989, Moriarty TM 1990). mPT had two amino acid substitutions in subunit A that rendered it enzymatically inactive (Barbieri JT 1990). Sensitization

with mPT as an adjuvant did not result in antigen-induced Isc responses of a greater magnitude than those recorded in control tissues. This provides evidence that pertussis toxin-sensitive enhancement of hypersensitivity is dependent upon the ADP-ribosylation of G proteins. G proteins have been identified in a range of immunological and non-immunological cells and *in vitro* studies have documented pertussis toxin effects on a variety of cell types including T cells (Clark CG 1990, Aussel C 1988, Person PL 1992), mast cells (Krieger M 1992, Mousli M 1992, Nakamura T 1985), epithelial cells (van de Berghe 1991) and neuronal cells (Kanaho Y 1989, Newberry NR 1989). Some effects of pertussis toxin on these cell types may be involved in enhancing hypersensitivity reactions in the gut and these possibilities will be discussed in later paragraphs. Further studies were conducted to examine changes in specific components of the hypersensitivity reaction that may have contributed to the enhanced antigen-induced responses documented in animals sensitized with wPT.

4.4. Role of antibodies

Previous studies have shown that IgE antibodies are produced following sensitization of animals using *Bordetella pertussis* vaccine as an adjuvant (Jarrett EEE 1980). When purified pertussis toxin was used as an adjuvant in the immunization of mice, it induced elevated levels of antigen-specific antibodies of both, IgE and IgG_1 isotypes in the serum (Munoz JJ 1981). In my experiments, three different approaches were used to measure IgE; radioimmunoassay with

monoclonal or polyclonal developing antibodies and passive cutaneous anaphylaxis (PCA). The use of radioimmunoassay with monoclonal antibodies did not reveal any increase in IgE in sera from Sprague-Dawley rats sensitized with OVA plus wPT compared to IgE before sensitization or to controls sensitized with OVA alone. The use of monoclonal antibodies results in high specificity but lower sensitivity, since these antibodies bind to only one epitope on the heavy chains of the IgE molecule. The IgE levels in Sprague-Dawley rats were probably too low to be detected by this assay. Sprague-Dawley rats are recognized as low IgE responders (Karlsson T 1979b). The assay did detect anti-OVA IgE in serum from sensitized Brown-Norway rats, a strain recognized to be a high IgE responder (Diaz-Sanchez D 1991). When polyclonal antibodies were used as developing antibodies in the radioimmunoassay, increased levels of IgE were detected in sera from Sprague-Dawley rats sensitized with OVA plus 50 ng wPT compared to controls. The polyclonal antibodies were raised against the whole IgE molecule, resulting in the presence of anti-IgE antibodies specific for many IgE epitopes providing more opportunities for binding and detection of IgE. Using polyclonal antibodies in the immunoassay, the IgE levels in sera from OVA plus mPT injected rats were not significantly different from sera from rats that received OVA only. The lack of antibody production after injection of mPT is unlikely to be due to lack of antigenicity of this molecule, since the mutant toxin has previously been shown to be as immunogenic as wPT for systemic protective antibody production (IgG) against *B. pertussis* infection (Pizza M

1989). Thus, these findings provide evidence that wPT acts as an adjuvant for IgE production to a bystander antigen. In contrast, the fact that mPT is inactive indicates a role for G protein ADP-ribosylation in IgE immunoglobulin production.

Since the polyclonal antibodies were raised against the whole IgE molecule, their specificity may cross-react with epitopes which are common for all classes of immunoglobulins (eg. light chains). However, the polyclonal anti-IgE antibodies were absorbed against all other classes of rat immunoglobulins and other serum proteins to prevent non-specific binding. To obtain further evidence for IgE antibody stimulation, the results from the radioimmunoassay were verified by the PCA technique. This method provides information about the biological activities of the antibody measured, and RIA only quantifies the antibody protein. In PCA technique, aliquots of diluted serum from sensitized rats were injected intradermally into the dorsal skin of a naive rat. The antibodies in the injected serum bind to mast cells in the skin of the recipient rat and cause degranulation in response to iv antigen. Since Evans blue is injected with antigen, extravasation mediated by histamine increasing endothelial permeability results in an easily observed blue area on the skin.

Two classes of antibodies, IgE and IgG, can bind to mast cells; however, the half-lives of these antibodies on mast cells are considerably different, being short for IgG (hours) and longer for IgE (days)(Ovary Z 1986). In studies on mice, positive responses by PCA, using high doses of injected IgG, resulted in a positive titre, 1.5 h after injection but the reaction was not detectable after 48

h (Inagaki N 1988). IgE binds to mast cells, for at least 12 days as evaluated by PCA. Based on these observations, the development of PCA 72 hours after injection of sera is recognized as sufficient time for the evaluation of mast cell-bound IgE.

In my model, PCA titres were significantly higher after sensitization of rats with OVA plus wPT compared to those injected with OVA only. Additionally, PCA titres were reduced or abolished by heat treatment of the sera. IgE antibodies are recognized as being heat-labile at 56°C. This temperature changes the carboxy-region causing polymerization of molecules (Rousseaux-Prevost R 1983). Such polymers have very low cytotropic effects in PCA. Thus, the decrease of PCA titres after heat treatment suggests that the responses were due to heat-labile antibodies of the IgE isotype. Some remaining PCA responsiveness after heat treatment may have resulted from the presence of IgG antibodies or binding of IgE polymers to FcεRI, since the residual cytotropic activity for polymers has been documented (Rousseaux-Prevost R 1983). Enhancement of PCA titres did not occur in rats sensitized with mPT plus OVA. These data confirm the results obtained by radioimmunoassay using polyclonal antibodies, namely, the elevation of IgE levels in rats that received with wPT as adjuvant and the lack of elevation in those which received mPT. This finding indicates the necessity of the ADP-ribosylation properties of wPT for IgE production.

Both the highest PCA titres and the largest immunophysiological responses

to luminal antigen were documented in Sprague-Dawley rats that were injected with OVA plus 50 ng wPT. However, the amount of circulating anti-OVA IgE was not predictive of the magnitude of the local gastrointestinal response to the antigen in individual rats. The correlation between individuals PCA titres and responses to luminal OVA was 0.34 and for serosal OVA was 0.2. This finding has also been reported previously in rats (Perdue MH 1986) and also in humans in which 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 (Knutson TW 1993). These results suggest that the responses to antigen relate to the presence of antibodies but not necessarily to specific concentration of antibodies in the circulation. Furthermore, levels of circulating antibodies do not indicate the amount of IgE bound to mast cells in tissue sites. Additional support for this hypothesis comes from experiments in which rats were passively sensitized with different doses of serum, showed a threshold dose below which there was no response to secondary antigen challenge and above which increasing doses resulted in Isc responses of increasing magnitude. However, injection of larger doses of serum (above 1 ml) did not further enhance the Isc response. The data suggests that immunophysiological reactions typical of immediate hypersensitivity depend on small but critical levels of reaginic antibodies. However, other factors in addition to antibodies cannot be excluded in determining the magnitude of the immunophysiological response to antigen.

The mechanism by which pertussis toxin stimulates antibody production is not clear but may reflect an effect of pertussis toxin on T cells (Aussel C 1988, Verschueren H 1991, Arora PK 1987). Pertussis toxin (holotoxin but not the B subunit) accelerates the maturation of thymocytes. Analysis of thymic and circulating T cells showed that pertussis toxin decreased the number of immature double positive $CD4^+CD8^+$ cells in the thymus, while in the periphery more single positive $CD4^+$ helper type T cells were evident (Person PL 1992). These cells, as their name indicates, help B cells in the production of immunoglobulins, thus the increased number of $CD4^+$ cells may account for the elevation of the humoral immune response. Production of T cell cytokines, such IL-5 or IL-6 that stimulate immunoglobulin synthesis, may be involved (Leung DY 1993, Tonkonogy SL 1993). Additionally, there is evidence of a specific effect of pertussis toxin on IgE production related to IgE class switching. Experiments performed with cholera toxin and prostaglandins suggest the involvement of cAMP in immunoglobulin class switching (Lycke N 1990, Phipps 1990, Munoz E 1990). Pertussis toxin-induced elevation of cAMP by ADP-ribosylation of G inhibitory proteins is well recognized (Gierschik P 1992). In our model, G proteins were undoubtedly affected by wPT since injection of OVA with the mPT did not stimulate IgE antibody production or intestinal hypersensitivity. Interestingly, in allergic disorders such as atopic dermatitis (where an elevated level of IgE was observed) the adenylyl cyclase activity of mononuclear leucocytes is increased and there is a significant decrease in the Gi protein content (Hanifin JM 1992).

My model of hypersensitivity may represent an analogous situation, where wPT may block some Gi proteins. The regulation of IgE production is determined by the whole network of cytokines and membrane proteins but the balance between two cytokines, namely IFN-γ and IL-4, seems to be crucial. IFN-γ plays the role of negative regulator (Snapper CM 1993) and IL-4 acts as a positive stimulus for IgE production (Wang YZ 1991). Furthermore, studies by Hong-Hu and Sewell (Hong-Hu M 1993) showed that pertussis toxin increased mRNA expression and secretion of IL-4 by spleen and lymph node cells. Pertussis toxin was also shown to stimulate IFN-γ production (Sewell WA 1986), but probably the balance of IL-4 and IFN-γ is more important than the absolute amount of each cytokine released. Thus, wPT could increase the levels of IgE by general elevation of the immune response as well as by specific action on antibody production of the IgE isotype.

Although IgE is thought to be necessary to induce hypersensitivity reactions, some studies (as mentioned before) did not show the presence of circulating IgE in individuals or report a correlation between antigen-specific IgE and sensitivity to antigen. In a few species, IgG antibodies were found to bind to receptors on mast cells and/or participate in anaphylactic reactions (Bazin H 1982, Daeron M 1992a, 1992b, Halpern GM 1987, Katz HR 1990, Latour S 1992). Early studies (Halper J 1976, Bach MK 1970) have suggested that histamine from peritoneal mast cells or rat basophil leukaemia cells (RBLs) can be released by IgG stimulation; however, technical limitations resulted in

unconvincing data. In the studies of Halper et al., the IgG_{2a} antibody preparation used for rat basophil leukaemia cells (RBLs) was contaminated with other classes of antibodies and only partial inhibition of RBL stimulation with non-specific IgG_{2a} could be achived. In the studies of Bach et al., the IgG_{2a} enriched sera was used for RBL activation. Thus, in both studies, the RBL activation could not be accounted for specifically by IgG_{2a}. Later, physiological studies showed the involvement of IgG in antigen-induced ion secretion in guinea pig colon, in which passive sensitization of the tissue with heat-treated serum for three hours (ie. heat-labile IgE was denaturated) was still able to transfer responsiveness to antigen (Baird AW 1987). In mice, IgE, IgG_1 and IgG_{2a} are subclasses which have been shown to bind to mast cell receptors and evoke mediator release (Benhamou M 1990, Katz HR 1990). The reports of the ability of murine IgG_{2b} antibodies to activate MCs are conflicting, with evidence both refuting (Daeron M 1982), and supporting this putative immunological function of IgG_{2b} (Hirayama N 82). Recently, Daeron (Daeron M 1992a, 1992b) and Latour (Latour S 1992) have reported that RBLs transfected with murine recombinant IgG III receptors can release TNF-α and 5-HT from mast cell granules after stimulation of IgG receptors with antigen-rat IgG_{2a} complexes.

In my experiments, rats that received 50 ng of wPT plus OVA demonstrated elevated levels of OVA-specific IgG and IgG_{2a} above control values, whereas the levels of IgG and IgG_{2a} antibodies were not enhanced in rats receiving mPT plus OVA. This shows that wPT induces antigen-specific IgG

production and also that this increase is related to the enzymatic activity of the adjuvant. The regulation of immunoglobulin class switching to IgG, similar to that of IgE, is dependent on cytokines released by different populations of T helper cells. In the mouse, IL-4 stimulates not only IgE but also IgG_1 antibody production; however there is no effect on IgG_2 (Snapper CM 1988, 1993). In humans, the same cytokine induces B lymphocytes to increase the transcription of isotypic chains for IgE and IgG_4 (Kotowicz K 1993; Purkerson J 1992). However the role of IgG_4 in allergic conditions is still controversial (Halpern GM 1987). The regulation of IgG_{2a} subclass in the rat is, as yet not fully understood.

Since at least 2 classes of antibodies in the rat are possibly relevant in hypersensitivity reactions, further experiments tried to assess which class was involved in mediating the secretory responses in my model. First, I examined if the intestinal response to OVA in actively sensitized rats could be passively transferred via serum to naive rats. Passive sensitization with serum from Sprague-Dawley rats that had been injected with OVA plus 50 ng wPT failed to restore responses to OVA. Effective passive sensitization was achieved with serum from Brown-Norway rats, which were sensitized with OVA plus wPT and boosted with OVA only. The difference between the effectiveness of these two preparations of pooled serum coming from different rat strains was likely to have been due to increased concentrations of IgE antibodies as measured by PCA: 1:32 in Sprague-Dawley rats and 1:2048 in Brown-Norway rats. The passive sensitization experiments suggested that a serum factor (presumably antibodies),

was crucial for hypersensitivity.

Subsequently, to investigate the class of antibody responsible for hypersensitivity in my model, normal (+/+) mice were injected with rat IgE myeloma antibody prior to passive sensitization. Because of the limited supply of IgE myeloma antibodies, I used mice in this part of the investigation. Additionally, the cross-reactivity of antibodies has been demonstrated between rat and mouse species: mouse IgE, IgG_1 and IgG_{2a} transferred the cutaneous reaction to the rat (Hirayama N 1982). More importantly, rat IgE (Ortega E 1991) and IgG_{2a} (Daeron M 1992b) were shown to cross-react in binding and activation of mouse mast cells. The purpose of the injection of IgE myeloma in my experiments was to block all available IgE receptors with IgE myeloma antibodies. The results showed that blocking IgE receptors prevented the antigen-specific IgE from binding to mast cells and subsequently no response to OVA was achieved. This indicates that the hypersensitivity reaction in rodents sensitized with wPT and antigen involves IgE antibodies. The possibility exists that the role of other subclasses of IgG, besides IgG_{2a}, may have gone undetected in this experimental design, since information is incomplete regarding the cross-reactivity of all subclasses of IgG. IgG antibodies may also account for hypersensitivity reactions via stimulation of complement, of which products C3a and C5a can release connective tissue mast cells. However, there is no evidence for stimulation of mucosal type of mast cells by complement.

To summarize, my data suggest that wPT induced production of antigen-

specific IgE, IgG and IgG_{2a} is mediated via ADP-ribosylation of G proteins. Also, in my model of hypersensitivity, IgE antibodies were shown to be sufficient for the intestinal immunophysiological responses to antigen.

4.5. Role of mast cells

Mucosal mast cells are key cells in intestinal hypersensitivity. The number of intestinal mucosal mast cells is increased in certain pathological conditions where anaphylactic reactions to antigens may be involved, such as parasitic infection (Stead RH 1991), Crohn's disease (Dvorak AM 1983) and celiac disease (Adenis A 1992, Marsh MN 1985). Previous experiments in Dr. Perdue's laboratory in experimental models of hypersensitivity and inflammation have provided evidence for their role by demonstrating antigen-induced release of histamine and mast cell protease II (Perdue MH 1984). In mast cell-deficient mice (*W/W*^{v}) sensitized with antigen and the two adjuvants, whole-cell pertussis vaccine and alum, the response to antigen was diminished by 70% compared with congenic normal mice (Perdue MH 1991), showing that most of the response to antigen was due to mast cells. In my experiments in rats, sensitization with OVA plus wPT resulted in increased numbers of mast cells in the mucosa compared to the number of mast cells in intestinal mucosa from rats sensitized with OVA alone. Morphological measurements of areas of villi and crypts in the intestinal mucosa did not reveal any differences between rats in different sensitization protocols indicating that the change in number of mast

cells did not result from changes in the anatomy of the small intestine. This suggests that the enhancement of intestinal secretory responses could be due, at least in part, to an increase in the quantity of mediators released from mast cells. In tissues from rats injected with OVA plus mPT, the mast cell number was not increased compared to OVA alone, demonstrating the role of the enzymatic activity of wPT in the mast cell hyperplasia.

The proliferation of mast cells is regulated by cytokines and growth factors produced by immune cells (Galli SJ 1990). IL-3, produced predominantly by activated T cells, can influence mast cell proliferation. Mice injected repeatedly with IL-3 developed mucosal mastocytosis (Tsai M 1993). In pathological conditions such as nematode infection, marked mastocytosis develops, which can be decreased by 50% by injection of anti-IL-3 antibodies (Madden KB 1991). IL-4, produced by T cells and mast cells, acts as a cofactor in stimulating mast cell proliferation. Another T cell product, IL-9, was shown to act synergistically with IL-3 to promote proliferation of cultures of primary mouse bone marrow cells, which differentiate into mucosal mast cells (Renauld JC 1993). As with IL-9, IL-10 (coming mainly from activated T cells and normal B cells) is inactive itself but in combination with IL-3 and IL-4 can stimulate murine mucosal-like mast cell proliferation (de Waal Malefyt R 1992). It is likely that some or all of those regulators of mast cell proliferation are produced after sensitization of animals (Powrie F 1993).

Mast cell-deficient mice (*W/W*^{v}) and their normal controls (+/+) are an

excellent model for investigating the role of mast cells in various responses. The mutation on chromosome 5 at the *c-kit* gene locus results in a lack of mast cells in all organs investigated (except skin where the number of mast cells is 0.3% of those in normal mice) (Kitamura Y 1989, Galli SJ 1993). My experiments in which mice were sensitized with wPT plus OVA, showed the presence of ion secretion in response to OVA in intestine from +/+ mice and the lack of any response in *W/W*^{v} mice. This suggests that the hypersensitivity reaction induced by wPT depends on mast cells. This is in contrast to previous studies (Perdue MH 1991) in which mice were sensitized with cPV and alum plus OVA. In those experiments, intestine from *W/W*^{v} mice demonstrated a secretory response to antigen challenge that was 30% of that in +/+ controls, suggesting that other cells were involved in addition to mast cells. This discrepancy may be due to the different adjuvants used in the sensitization process. Further support for the relevance of mast cells in my study was obtained in *W/W*^{v} mice that had their mast cell populations restored by transfer of bone marrow cells. *W/W*^{v} reconstituted mice that were passively sensitized with serum from actively sensitized animals, demonstrated restoration of secretory response to secondary antigen challenge of the intestine. These results confirm that mast cells are important for hypersensitivity reactions in my model.

Further experiments to elucidate the role of mast cells in hypersensitivity revealed three major components in the response to antigen. Intestinal tissues from mice actively sensitized with wPT as adjuvant were challenged with OVA

after addition of selective inhibitors to the Ussing chamber buffer. Use of a histamine H_1 receptor antagonist, diphenhydramine, decreased the response to OVA by 62%, while the use of the serotonin $5HT_2$ receptor antagonist, ketanserin, decreased the response to OVA by 66%. Addition of the cyclooxygenase inhibitor, piroxicam, decreased the response to OVA by 43% indicating that production of PGs was involved. These results confirmed those of a previous study. Two secretagogues, histamine and serotonin, were shown to be released by mast cells in the rat model of hypersensitivity (Castro GA 1987), and doxantrazole, a stabilizer of mucosal and connective tissue mast cells, significantly decreased the intestinal response to antigen, in a similar manner as inhibition by ketanserin and diphenhydramine (Crowe SE 1990).

At least 3 types of histamine receptors have been identified (Rangachari PK 1992). H_1 is present on the basolateral surface of epithelial cells, while H_2 and H_3 are present on nerves. Studies in rat jejunum have shown that histamine induced a transient increase in Isc which could be inhibited totally by a H_1 but not a H_2 receptor antagonist (Hardcastle J 1987). H_3 receptors have been implicated in the regulation of somatostatin secretion in the stomach (Schubert ML 1993). Moreover, in guinea pig H_3 receptors were identified on presynaptic surfaces on neurons in the submucosal plexuses, where they were suggested to suppress release of ACh, epinephrine and 5-HT (Frieling T 1993). However, the role of H_3 receptors in antigen-induced ion secretion remains to be established. Stimulation of intestine with histamine causes chloride ion secretion

and sodium malabsorption via an increase in cytosolic calcium in enterocytes (Hardcastle J 1987).

Serotonin, has 10 distinct subtypes of receptors (Humphrey PPA 1993) but only two of them have been shown to be involved in the stimulation of ion secretion in the gut. Serotonin receptors, like histamine receptors, are present on enterocytes ($5HT_2$) (Branghek T 1984) as well as on nerves ($5HT_3$)(Beubler E 1990), and activation of both types can result in increased ion secretion. Both serotonin receptors, $5HT_2$ and $5HT_3$, were shown to be involved in secretory responses caused by cholera toxin (Beubler E 1990). The second messenger, in epithelial cells stimulated by serotonin is calcium (Beubler E 1986), elevation of which resulted in Cl^- secretion and inhibition of NaCl absorption (Hardcastle J 1981).

The source of PGs involved in response to antigen is uncertain since many cells in the intestinal mucosa can produce PGs after activation of phospholipase A_2 (PLA_2)(Berschneider HM 1992a, Weinreich D 1992). PGs inhibit neutral NaCl absorption and stimulate electrogenic Cl^- secretion in both the small intestine and colon through the cAMP-mediated protein kinase A system (Smith GG 1987). The most potent PGs in stimulation of ion secretion are PGE_2 and PGI_2, while PGD_2 is of lower potency or may even inhibit secretion (Hinterleitner TA 1991). Although the antigenic stimulation of mucosal mast cells via the antigen/IgE-dependent pathway involves activation of PLA_2, the main products are not PGs but LTB_4 and LTC_4 (Foneth AN 1993). Thus, stimulation of mast cells probably

does not directly account for the PG-induced ion secretion. The major source of PGs in the mucosa is the subepithelial tissue. Recent studies (Berschneider HM 1992b) have shown that fibroblasts modulate secretory responses of crypt cells (T84, colon carcinoma cell line) to bradykinin, serotonin, histamine and hydrogen peroxide. This effect was present when the T84 cells were co-cultured with fibroblasts or juxtaposed for the time of experiment. This stimulatory effect was abolished by indomethacin, implicating PGs. Additionally, fibroblasts treated with inflammatory mediators, such as histamine, serotonin, IL-1 or bradykinin, were shown to release significant amounts of PGE to the media (Berschneider HM 1992b). Studies with pertussis toxin and fibroblasts showed that pertussis toxin acted on fibroblasts such that subsequent stimulation with bradykinin caused enhanced accumulation of PGI_2 and PGE_2 (Moss J 1988). Thus, the fibroblast may also be a component of wPT-enhanced hypersensitivity reaction.

The pathways by which epithelial cells were stimulated after exposure to antigen are not clear. In my experiments, when all three inhibitors were added together, the response to OVA was abolished by 95%, while each of them added separately resulted in less inhibition (ketanserin 66%, diphenhydramine 62%, piroxicam 43%). Two putative secretagogues, histamine and 5-HT, stimulate ion secretion in epithelial cells via intracellular calcium as a second messenger. It is possible that the same pools of calcium were utilized by those two secretagogues. However, since two antagonists were not used in combination, this conclusion cannot be assumed. As mentioned above, a

possible source of PGs are fibroblasts. Two mechanisms of secretory stimulations are possible: histamine and serotonin released from mast cells may act on a third cell producing PGs which stimulates ion secretion from the epithelium; or histamine and serotonin act both directly and indirectly on epithelium. Studies on epithelial cell lines has demonstrated directs effect of histamine and serotonin. The human intestinal cell line (407) responded to direct action of histamine and serotonin by elevation of intracellular calcium (Yada T 1989). In addition, both histamine and serotonin were shown to increase Isc when applied to T84 monolayers (Berschneider HM 1992b). However, studies on responsiveness to histamine in rat small intestine (measured as an increase in Isc) have shown that another cyclooxygenase inhibitor, indomethacin, abolished the response to histamine by 50%, suggesting involvement of the PG pathway in secretion to histamine (Hardcastle J 1987). Similar reports relate to the action of 5-HT. Stimulation of rat jejunum with 5-HT caused fluid secretion into the gut lumen associated with the production of PGs (Beubler E 1986, 1989). 5-HT-induced fluid secretion was partially abolished by indomethacin. Those results fit with my data and indicate that both mast cell secretagogues, histamine and serotonin, stimulate ion secretion in part by direct action on epithelial cells and in part indirectly via activation of other cells, possibly fibroblasts, to release PGs.

4.6. Changes in epithelial barrier function

Responses to antigens present in the gut lumen are especially important from a physiological point of view. The epithelial covering of the gastrointestinal tract represents a "first line" protective barrier preventing easy access of luminal antigens into the underlying mucosa, where they can initiate an immune response and possibly initiate deleterious inflammatory reactions. Therefore, the "breakage" of this barrier is of pathophysiological relevance. My data showed that the response to luminal antigen was strongly enhanced from negligible values of 2.4 ± 1.9 to 44.2 ± 4.7 $\mu A/cm^2$ (18.7 fold increase) following sensitization with wPT. In contrast, the response to serosal antigen was increased from 31.5 ± 7.2 to 68.3 ± 9.1 $\mu A/cm^2$ (2.2 fold increase). Addition of antigen to the serosal surface of muscle-stripped intestine permits easy access to mucosal mast cells in the lamina propria. While this experimental approach provides information on the sensitization of animals, it is not representative of the *in vivo* situation. The enhanced responses to serosal antigen in wPT injected animals probably reflects increased IgE and mast cells numbers. However, the much greater enhancement of the response to luminal antigen indicates that other changes have also taken place following wPT administration. My results suggest that the regulation of antigen uptake across the epithelium is crucial in the determining the magnitude of the hypersensitivity reaction.

The final reacting cells in hypersensitivity reactions are enterocytes, which may be changed after sensitization. However, I believe that such changes were

not likely to be important in the responses to antigen. Although enterocytes possess pertussis-sensitive G proteins ($Gi\alpha_2$, $Gi\alpha_3$)(van den Berghe N 1991), the short life span of enterocytes (3 days from proliferation to desquamation)(Lipkin M 1987) rather excludes them as a component responsible for enhanced hypersensitivity reactions that are maximal 14 days after sensitization and persist for at least 8 month. In support of this idea is the finding that *in vitro* permeability to two molecules, ^{51}Cr-EDTA and ^{3}H-mannitol, in intestine from sensitized vs naive rats did not show any statistical differences.

My data suggest that the luminal responses to antigen are neuronally regulated. In my study, neural blockade with TTX did not inhibit the response to serosal antigen, unlike the situation in parasite-sensitized animals (Castro GA 1987, Russel DA 1989, Wang Y 1991). In *Trichnella spiralis* infected guinea pigs, the intestinal secretory response to serosal addition of parasite antigen, was markedly diminished after treatment with TTX. However, the lack of effect of TTX on the serosal response in my experiments was consistent and independent of the concentration of OVA used for the stimulation. This might be due to differential innervation between guinea pig colon and rat small intestine or due to changes that occur during parasite infection. In contrast to the lack of inhibition the response to serosal antigen, TTX did significantly inhibit the response to luminal antigen in wPT sensitized rats by 66%. The different effects of TTX on responses to luminal vs serosal antigen could be due to the stimulation of different populations of mast cells in the mucosa by antigen added

to opposite sides of the tissue. Recent observation showed that mast cells in the mucosa are in close proximity to nerve fibres (Stead R 1987), suggesting the possibility for bidirectional interactions. A different innervation of mast cell populations could result in different sensitivity to neuroblockade. However, this appears unlikely, as I have shown that addition of OVA to the serosal buffer markedly diminished the subsequent response to luminal OVA in the same tissue preparation. This implies that the same or at least overlapping populations of mast cells are involved in the response to antigen, regardless of the side of challenge.

The enteric nervous system appears to be a target of wPT action that might amplify intestinal hypersensitivity responses. Pertussis toxin has been reported to ADP-ribosylate many G proteins coupled to a variety receptors on nerves (Morris J 1991, Neve KA 1989, Newberry NR 1989). Moreover, neuronal regulation of tight junctions has been suggested in rats, where intravenous stimulation with carbachol increased paracellular movement of horseradish peroxidase (Phillips TE 1987). In addition, it was demonstrated that TTX inhibited macromolecular probe uptake *in vivo* in rats sensitized with antigen and pertussis vaccine (Crowe SE 1993). My data also suggests that the neuronal component of the mucosa in wPT injected rats is somehow changed since the response to transmural stimulation was elevated in this group. Thus, is possible that the nervous system elevates accessibility of macromolecules from the lumen to the gut mucosa in intestine from rats injected with wPT.

Based on the permeability of isolated rat intestine to probe markers, no evidence of changes in molecular transport through the epithelial barrier was obtained. However, experiments with neural blockade strongly suggest that neural regulation of the epithelial barrier is altered after sensitization with OVA plus wPT.

4.7. Longevity of wPT-enhanced secretory responses to antigen

The wPT enhanced intestinal hypersensitivity to luminal and serosal antigen was very long lasting. Eight months after a single injection of 50 ng of wPT with OVA, intestinal tissues still demonstrated significant secretory responses to antigen. Not many studies have reported the longevity of the physiological effects of pertussis toxin or vaccine. The longest observation was reported by Munoz (Munoz JJ 1981), in which pertussis toxin was reported to increase the sensitivity of mice to systemic injection of histamine (resulting in anaphylactic death) for at least 84 days. However, the mechanism of this effect was not examined.

A comparison of actively and passively sensitized rats demonstrated that the prolonged responsiveness to OVA occurred only in intestine from actively sensitized rats. The secretory response to OVA was evaluated in intestine from passively sensitized rats on days 3, 14 and 70 after sensitization. The response to OVA was present on day 3 but even at day 14, there was virtually no response to secondary antigen challenge. This indicates that injection of IgE into

circulation can not substitute the effects induced by active sensitization with wPT.

Some aspects of the long lasting effects of active sensitization may be thought to be accounted for by increased numbers of mast cells since the number in the intestinal mucosa was elevated at day 14 and 28. However, at later days after primary sensitization the numbers were not increased in intestine from wPT sensitized rats above those in control rats. This is in agreement with studies of Enerback (Enerback L 1979) who evaluated the time course of increased numbers of mucosal mast cells after injection of compound 48/80 in intestine of Sprague-Dawley rats, and reported the turn over as being 39.5 days. In my experiments, sensitization with wPT increased the number of mast cells for a similar length of time. However, the response to antigen lasted for much longer. Therefore, the increased numbers alone cannot account for the long lasting effect. The possibility exists that changes in mast cell function were elicited by sensitization with wPT. Some cytokines were shown to influence not only growth but also mast cell function. For example, IL-3 enhances the synthesis of 5-HT in murine bone marrow-derived mast cells (Ziegler I 1993). In addition, purified peritoneal mast cells from mice incubated with IL-3 or IL-4 changed their ability to release 5HT after antigen challenge (Coleman JW 1992, 1993). IL-10 was also shown to up regulate expression of granule serine protease gene in mouse mucosal and serosal mast cells (Ghildyal N 1992a, 1992b). Sensitization with antigen and adjuvant obviously activates T cells and

other immune cells and may stimulate release of cytokines which can effect mast cell growth or function. In addition, the number of IgE receptors may have been influenced directly or indirectly by sensitization with wPT. This possibility remains to be investigated.

The longevity of the sensitization by wPT also depends on the presence of antibodies. The highest PCA titres were achieved at day 7, and after this time there was a consistent decrease. Control rats injected with OVA showed no positive titre on day 14. However, in OVA plus wPT injected rats, a positive titre was still present on day 84, indicating that antigen-specific IgE antibodies were still in the circulation. These kinetics of IgE disappearance are somewhat different from those described by work of Meacock (Meacock SCR 1976), who investigated the levels of IgE 30 days after primary sensitization of Wistar rats with OVA plus *Bordetella pertussis* bacteria. IgE peaked and plateaued in the circulation at 12 days. My results demonstrated that addition of wPT to a sensitization protocol significantly prolongs the presence of reaginic antibodies in the circulation. The fact that no IgE was detected in circulation on day 228 may have been due to a real lack of IgE or the level of IgE being below the sensitivity of the assay. Another issue is the relationship of IgE in the circulation vs tissue sites. On day 228, when there was no IgE in circulation, there was still a significant quantity of antibodies attached to Fcε receptors on mast cells in the intestinal mucosa since cross-linkage of these antibodies by antigen stimulated ion secretion.

To summarize, the prolonged hypersensitivity after single injection of antigen with wPT adjuvant may result from the presence of altered factors (perhaps cytokines) in the environment that stimulated mast cell growth and production of IgE antibody.

4.8. Summary and conclusions

In conclusion, my results indicated that wPT is the active ingredient in the whole-cell pertussis vaccine responsible for stimulating hypersensitivity. A single injection of wPT with antigen dramatically enhanced intestinal hypersensitivity reactions to that antigen. wPT stimulated increases in IgE and IgG_{2a} antibody levels; however, IgE alone appeared to be sufficient to induce induction of intestinal responses leading to ion secretion. Other factors possibly contributing to the enhanced responsiveness were the increased numbers of mucosal mast cells and neuronally-mediated decrease of epithelial barrier function to antigen. Further dissection of the effects of wPT may prove useful in the elucidation of the precise mechanisms involved in IgE/mast cell-mediated changes in the gut. The work on this rodent model also suggests that it may be important to investigate the effects of pertussis toxin on sensitization of humans for allergic responses. The findings that injection of wPT can cause very long lasting effects on immunophysiological responses to co-administred antigen may be of significance in view of the current practice of immunization of children with *Bordetella pertussis* vaccines.

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