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INTESTINAL ANAPHYLAXIS
EFFECT OF IgE-MEDIATED REACTIONS ON GUT FUNCTION

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled, "INTESTINAL ANAPHYLAXIS. EFFECT OF IgE-MEDIATED REACTIONS ON GUT FUNCTION", submitted by Mary H. Perdue in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

The effect of intestinal anaphylaxis on small intestinal function was investigated.

Hooded Lister rats were sensitized to egg albumin by intraperitoneal injection of this protein plus adjuvant and developed high titers of specific IgE antibodies. Response to intraluminal challenge with egg albumin was examined in sensitized rats and sham-treated litter-mate controls fourteen days later. Intraluminal antigen resulted in epithelial cell injury in sensitized rats as evidenced by the appearance of enterocyte constituents in the lumen concomitant with decreased mucosal sucrase activity. Histamine in mucosal homogenates and numbers of specifically stained granulated mast cells in intestinal sections prepared after perfusion with antigen-containing solution also decreased significantly in sensitized rats. These changes did not occur in control rats.

In experimental animals, net absorption of water and electrolytes during in vivo antigen perfusion was reduced compared to perfusion with non-antigen containing solution. These changes were specific for the antigen and persisted after the antigen was removed. No transport abnormalities were observed during antigen perfusion in control rats. The rapid onset of the abnormalities suggested involvement of mediators released from intestinal mucosal mast cells. This hypothesis was verified by inhibition of the response by doxantrazole, an inhibitor of histamine release from isolated gut mast cells.

Unidirectional ion fluxes were measured across sheets of intestine

in Ussing chambers under short-circuited conditions. Addition of egg albumin to the medium bathing intestine from sensitized rats resulted in abnormal movements of chloride ions producing net chloride secretion. Net sodium ion movements were not affected. The chloride secretion was accompanied by an increase in potential difference and short-circuit current. Intestine from controls did not demonstrate these changes. Incubation of jejunal slices with antigen resulted in no changes in intracellular enterocyte levels of cyclic nucleotides in control or experimental rats.

Our studies demonstrated that important changes in intestinal function took place during IgE-mediated reactions. The abnormalities were most likely produced by the interaction of mast cell mediators with enterocytes in the intestinal epithelium. These studies have clinical relevance for individuals who suffer from hypersensitivity to food protein.

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"The doubter is a true man of science; he doubts only himself and his interpretations but he believes in science."

Claude Bernard

An Introduction to the Study of Experimental Medicine

TABLE OF CONTENTS

PAGE

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

GENERAL INTRODUCTION

1.1 Food Allergy

Food allergy is a major health problem. Gastrointestinal symptoms related to ingestion of certain foods in sensitive individuals include nausea, vomiting, diarrhea, abdominal pain and occasionally blood in the stools[1]. True allergy to foods, as differentiated from intolerance to foods, is an immune response based on an antigen and IgE antibody reaction. Acute allergic reactions to foods which are IgE-mediated can affect any organ of the body. However, gastrointestinal food allergy exists when any part of the gastrointestinal tract is the predominant site of the reaction.

Many reactions to food constituents are not true allergic reactions. Some reactions may be toxic or pharmacologic due to natural or added chemical constituents. Bacterial or fungal contamination can produce similar symptomology. Food intolerance caused by intestinal enzyme deficiencies leading to malabsorption of certain food nutrients is often mistaken for hypersensitivity to food antigens. Consequently, a great deal of confusion has been created regarding the clinical entity of food allergy. Certainly, true hypersensitivity reactions to food proteins do occur as indicated by elevation of IgE in intestinal juice of patients with consistent gastrointestinal reactions to certain foods[2].

Age seems to be a factor in food allergy, possibly due to decreased levels of secretory IgA in the young or poorly developed suppressor mechanisms for IgE-producing plasma cells[3]. Cow's milk protein is a fairly frequent allergen in young children. Other proteins introduced at an early age, such as soya bean protein or egg albumin, can produce allergic reactions.

Diagnosis and study of true allergic reactions in the gastrointestinal tract of humans are difficult. Skin testing does not provide reliable results. Elimination diets are often used as a diagnostic tool. However, fluctuations in reactivity, cummulative and additive effects, and lack of correlation with quantity make results difficult to interpret. Reactivity may be influenced by season, infection or emotions[4]. Few well-controlled animal studies have been attempted and no literature exists which defines the pathophysiology of transport abnormalities during IgE-mediated reactions in the small intestine.

1.2 Intestinal Immunology

Despite the fact that approximately 25% by weight of the intestinal tract is immune tissue, possible interactions between the absorptive epithelium and the underlying cells of the gut-associated lymphoid tissue (GALT) are not appreciated. Besides Peyer's Patches, aggregates of lymph follicles found primarily in the ileum, isolated lymphoid follicles are located throughout the length of the small intestine. At intervals overlying this lymph tissue are specialized epithelial cells called microfold or "M" cells. These M cells seem to

be antigen-sampling sites, engulfing foreign macromolecules and transporting them intact to the contiguous lymphoid tissue beneath[5]. All the elements necessary for antigenic stimulation of antibody production are located in this region: macrophages, T lymphocytes and B lymphocytes. To proliferate and become plasma cells committed to production of a specific antibody, the stimulated B cells must travel through the lymph circulation to the thoracic duct and mesenteric lymph node before they finally "home" to the lamina propria and other mucosal sites[6].

Ninety-five percent of plasma cells in the lamina propria are IgA-producers and under normal circumstances only 2% produce IgE. However, atopic individuals have a larger proportion of cells which produce reaginic antibodies. The lamina propria also contains other immune-type cells: mast cells, eosinophils, macrophages and, under certain circumstances, neutrophils. Intraepithelial lymphocytes have also been demonstrated.

1.3 Immediate Hypersensitivity

An immediate hypersensitivity reaction involves antigen-specific IgE antibodies bound to mast cells. Exposure to the antigen results in bridging of the IgE molecules by antigen and, following a series of biochemical reactions, loss of mast cell granules and subsequent mediator release[7]. The mediators are biologically active compounds, the best known being histamine, which act on target organs to produce profound affects. Some mediators have chemotactic properties for

inflammatory cells whose entry into the site may augment the reaction[8]. Increased numbers of mast cells, production of mediators or elevation of IgE antibody levels have been noted in inflammatory bowel disease[9], celiac disease[10], cystic fibrosis[11], irritable bowel syndrome[12], and infant colic[13], as well as in food allergy[2,14]. The significance of these findings is unclear.

1.3.1 IgE

IgE was discovered in 1966 by Ishizaka et al.[15], who isolated it from sera of ragweed-allergic patients. Its unique properties corresponded to the properties of reaginic antibody defined by Prausnitz and Kustner. Subsequently, the discovery of human IgE myeloma proteins confirmed that reaginic antibodies were indeed IgE. IgE is present, although in very low concentrations, in the serum of normal individuals[16].

IgE, like other immunoglobulins, is composed of two light and two heavy chains that are covalently linked by disulphide bonds. Light chains may be k or λ. Heavy chains, termed epsilon chains (ε) are unique to IgE and contain five complete domains, each with an intradomain disulfide bond. Its amino acid sequence is similar to the μ chain of IgM except for the c-terminal 19 amino acids. The ε chain consists of one variable (V) region and four constant (C) domains. The skin-fixing, or mast cell cytotropic, activity resides in the C ε 3 or C ε 4 domains and the well-known heat lability of the reaginic skin fixation involves alteration of these regions. IgE is rich in carbohydrate (12%) and has a molecular weight of 196,000[8].

IgE myeloma proteins have been used to prepare antisera which when labelled with fluorescein or ^{125}I, allow the identification of IgE-producing plasma cells. These cells have been found extensively in the mucosal surfaces of the body: the bronchi and bronchioles of the respiratory tract, the gastrointestinal mucosa, as well as in the tonsils and adenoids, and in the urinary bladder[17]. This distribution parallels that of IgA-producing plasma cells.

The production of IgE-secreting plasma cells is regulated in a complex fashion and controlled by soluble factors produced by different T cell types. There appear to be class-specific helper and suppressor T cells as well as antigen-specific regulators[18]. Genetic and environmental factors are involved. In most individuals IgE production is suppressed. Sublethal whole body irradiation, adult thymectomy, T cell immunosuppression with antithymocyte serum, and radiomimetric drugs all enhance IgE production due to absence of suppressor T cells[19].

Katz has proposed the concept of "allergic breakthrough" in which IgE antibody production is minimal in normal non-allergic individuals and maintained at low levels by IgE "damping mechanisms" such as suppressor T cells or their soluble factors[20]. Under certain circumstances, or in atopic individuals, this damping mechanism may be disturbed. Escape of IgE helper T cells could then stimulate IgE-forming B cells and contact with antigen would result in proliferation of specific IgE-producing cells. In this way, failure of normal suppression can initiate allergic sensitization. In humans, events such as viral

infections and inflammatory states may be sufficiently stressful to pose a disturbing effect on the damping mechanism controlling IgE.

1.3.2 Mast Cells

Theoretically, mast cells originate from embryonic connective tissue and reside in skin, in connective tissue which surrounds blood vessels, in sheaths around peripheral nerves, and in mucosal and submucosal tissues of the gastrointestinal and respiratory system. Mast cells are 10-30 μm in diameter, have unsegmented nuclei and are densely packed with metachromatic granules[7].

Receptors with high affinity for IgE have been demonstrated on mast cells and basophils but not on other cell types. The receptor *in situ* is unclustered, mobile, univalent and its aggregation into dimers and higher oligomers triggers degranulation. The receptor is a glycoprotein composed of two subunits. There are 1-5 x 10^5 receptors per mast cell of which about 10% are occupied by IgE *in vivo*. The extremely high binding constant of IgE to its receptor, Ka 10^9-10^{12} M^{-1} combined with the long residence time of approximately six weeks permits the mast cell to concentrate IgE antibodies on the cell surface. The binding is reversible and temperature dependent[21].

Mast cells within the gastrointestinal tract layers vary in distribution. Usually mast cells occur randomly throughout the lamina propria. In the duodenal mucosa they are especially prominent in the loose connective tissue in and around Brunner's glands. Mast cells in the submucosa, muscular layer and serosa occur both close to blood vessels and at sites distant from them. In the mucosa, the number of

mast cells varies according to the region examined with stomach, duodenum and ileum containing more than other regions[22].

Mast cells are a heterogenous population of cells and differences are noted from species to species and from tissue to tissue within the same species. The content of the granules is also different depending on the source. Mast cells in the rat dermis, serosa and intestinal tract submucosa are 12.6 μm in diameter and the granules 0.2-0.4 μm[23]. In the rat intestine mucosal mast cells are morphologically different. They contain granules with different staining properties and the granules are smaller and more amorphous[24]. Other differences exist for gut mast cells with respect to agents which trigger release of granules and those which inhibit degranulation[25,26]. Another possible type of mast cell is the intraepithelial mast cell or globule leukocyte. These cells may be derived from mucosal mast cells which have migrated into the epithelium[23].

1.3.3 Release of Mediators

Exposing IgE-fixed mast cells to antigens against which the IgE molecule is directed results in the secretion of mast cell granules from which the mediators of anaphylaxis are derived. The bridging of two IgE molecules by antigen initiates a biochemical cascade involving movement of calcium into the cell, activation of a cell membrane-associated serine esterase, microtubule assembly and exocytosis of the granule[27].

Methylation of membrane phospholipids produces a calcium channel,

permitting calcium to enter the cell; calcium is necessary for mediator release[28]. Membrane phospholipid releases free arachadonic acid and stimulates both cycloxygenase and lipoxygenase pathways to form prostaglandins and leucotrienes. Increased fluidity of the membrane allows coupling of the IgE receptor to adenylate cyclase and activated catalytic protein converts cytosol ATP to cyclic AMP. The peak rise in cAMP occurs within 15 seconds - concommitantly with phospholipid methylation - and results in feedback suppression of further phospholipid methylation which inhibits further calcium influx and histamine release[29]. Phosphorylation of perigranular membrane proteins causes an increase in granule membrane permeability to water and calcium that cause granule swelling. Calcium also causes contraction of microfilaments that move the swollen granules to the mast cell surface. Fusogens then cause fusion of mast cell granules to form secretory granules and channels and then fuse with the plasma membrane, which results in secretory expulsion of the granule contents[30].

Approximately 20 mediators of immediate hypersensitivity have been defined. Some are preformed; others are synthesized following membrane activation of mast cells; a third group are generated secondarily from other cells (Table 1)[31]. Preformed mediators such as histamine are released in the tissue fluid immediately. Several of the secondary mediators are formed immediately (prostaglandins) or within minutes (slow-reacting substances - SRS). The granule associated mediators remain in the tissue for hours until dissolution occurs or they are phagocytosed[29].

TABLE 1. Mediators of Immediate Hypersensitivity

Mediators	Known Actions
A. Performed, rapidly eluted	
1. Histamine	Smooth muscle contraction, increased vascular permeability
2. Eosinophil chemotactic factor	Selective attraction of eosinophils
3. Neutrophil chemotactic factor	Selective attraction of neutrophils
4. Serotonin	Increased vascular permeability in rats
5. Superoxide anions	Toxin for cell membranes
B. Preformed, more slowly released	
1. Heparin	Anticoagulation, inhibition of complement activation
2. Chymotrypsin	Proteolysis
3. Trypsin	Proteolysis
4. Arylsulfatase	Inactivation of SRS-A
5. Peroxidase	Protein inactivation
6. Kininogenase	Protein inactivation Kinin generation
C. Newly synthesized by mast cells	
1. SRS-A, slow reacting substance, Leucotrienes C_4, D_4, E_4	Contraction of pulmonary muscle Increased vascular permeability Attraction of neutrophils and eosinophils Stimulation of release of lysosyme from white blood cells
2. Prostaglandins	Bronchoconstriction

TABLE 1 (continued)

3.	HETE	Attraction of eosinophils and neutrophils
4.	HHT	Attraction of eosinophils and neutrophils
5.	Platelet activating factor	Platelet aggregation and degranulation

D. Newly synthesized by other sources

1.	Acetylcholine	Bronchoconstriction, mucous secretion, augments mediator release
2.	Serotonin (5-HT)	Increased vascular permeability and anaphylaxis in rats.

The events initiated by the mediators depend on the tissues into which they are released. Thus histamine in the skin and nose may produce primarily vasopermeability while in the lung it may also induce bronchospasm. Mediator discharge clearly initiates the events associated with immediate hypersensitivity, which are apparent within minutes and may be explosive in nature. Late-phase allergic reactions occur in response to granule-derived mediators as well. Thus two to eight hours after degranulation there is an influx of polymorphonuclear leukocytes followed by mononuclear cells over the subsequent twenty-four hours[31].

1.3.4 Histamine

Histamine is the classical mediator usually associated with immediate hypersensitivity reactions. In studies using isolated mast cells from various species and tissues, measurement of increased histamine in the extracellular medium is used as an indication of degranulation[26,32].

Histamine (β-aminoethylimidazole) is a bioactive amine which causes smooth muscle contractions of human bronchioles and small blood vessels, increased permeability of capillaries, and increased secretion by nasal and bronchial mucous glands. It is also involved in gastric acid secretion.

This amine is formed by the action of histidine decarboxylase on histidine. Histamine is metabolized by two major enzymatic pathways, N-methyl transferase and histaminase or diamine oxidase. Both enzymes are found in the small intestinal mucosa[33].

The gastrointestinal tract is a rich source of histamine. Tissue concentrations are particularly high in the duodenal and gastric mucosa with lower concentrations reported in the small and large intestine. Although located mainly in mast cells there is some evidence that other cells, such as neuroendocrine cells and granulated lymphocytes, may also contain histamine[34]. In human stomach histamine has been localized to conventional mast-type cells by histochemical techniques.

Histamine produces its effects by interacting with two distinct receptors, H_1[35] and H_2[36]. The H_1 effects are those originally attributed to histamine and include edema formation, and contraction of smooth muscle. H_1 antihistamines block the effects mediated by these receptors. Such antihistamines include diphenhydramine, pyrilamine and chlorpheniramine. H_2 receptors are found in the stomach and their interaction with histamine results in increased gastric acid secretion. These effects are blocked by H_2 antihistamines, such as cimetidine. The ability of H_2 receptor antagonists to block all forms of gastric secretion indicates an essential role for histamine in regulation of gastric secretion.

Histamine-induced changes in vascular permeability are "proinflammatory" in that they may allow the ingress of plasma proteins and leukocytes[37]. Anti-inflammatory effects of histamine are mediated through H_2 receptors and include inhibition of mouse T-lymphocyte cytotoxicity, inhibition of neutrophil lysosomal enzyme release, inhibition of antigen-induced release of histamine from human peripheral

leukocytes (but not human mast cells), and suppression of antigen-induced migration-inhibitory factor produced by T cells that possess histamine receptors[38].

Receptors for histamine are presumed to be a component of the cell surface. They have not yet been identified by physical or chemical means and the knowledge of their structural properties is restricted to conclusions drawn from pharmacological studies of structure-activity relationships of histamine agonists and antagonists. How interaction of histamine with its receptors elicits the appropriate cellular response is also uncertain. Histamine seems to have actions that allow increased permeability of plasma membranes to sodium and calcium, which allows them to enter the cell down electrochemical gradients. Free intracellular calcium may also be released from intracellular stores. These effects are critical for changes in muscle tension[39] and secretion[40]. Those effects of histamine mediated through H_2 receptors have been associated with elevated concentrations of cyclic adenosine monophosphate (cAMP)[38].

The effects of histamine on intestinal transport have been studied *in vivo* and *in vitro*. Direct infusion of histamine into the mesenteric arterial system of dogs resulted in copious secretion of fluid into the lumen[41]. Lee and Silverberg suggested that a major part of this effect was the result of a capillary leak causing a filtration process rather than stimulation of an active ion secretion. Fromm and Halpern[42] demonstrated a transient rise in short circuit current in response to histamine using isolated rabbit ileal mucosa. This effect

was blocked by H_1 receptor antagonists but not by H_2 receptor antagonists and was not associated with changes in Na^+ and Cl^-transport. However, Lindaker et al[43], using the same tissue, showed an inhibition of Cl^- flux from mucosa to serosa which resulted in net Cl^- secretion. Alkali secretion was also inhibited. Both electrical and ion responses to histamine were blocked by the H_1 receptor blocker diphenhydramine, but not by the H_2 receptor blocker cimetidine. These studies supported the possibility of a role for endogenous histamine in influencing ion transport and indicate a possible mechanism by which absorption might be impaired in situations in which histamine is liberated locally in the intestine.

1.3.5 Inhibitors of Mast Cell Degranulation

Therapy to control symptoms of immediate hypersensitivity reactions with antagonists of histamine is not entirely effective. This is because histamine is only one of many autocoids released or formed during the reaction which together elicit the symptoms. To suppress the effects of all these agents would require a battery of blocking agents. For this reason treatment of allergic reactions using inhibitors of mast cell degranulation is advantageous - preventing production or release of the autocoids by inhibiting responses of sensitized mast cells to specific antigens.

Sodium cromoglycate (SCG) or cromolyn inhibits antigen-induced secretion of histamine from human pulmonary mast cells and from mast cells at certain other sites[44]. Curiously, SCG does not protect basophils or mast cells isolated from the intestine of parasite-infested

rats[26,45].

SCG is the disodium salt of 1,3-bis (2-carboxy-chromone-5-yloxy)-2-hydroxypropane. In the lung its therapeutic effects are due to direct action on the pulmonary mast cells. Inhibition of antigen-induced release of histamine and SRS-A can be demonstrated with isolated peritoneal mast cells[46]. The inhibitory effect seems to involve preventing the release of granules rather than interfering with IgE or antigen binding to the cell. It may block calcium channels in the membrane, although other explanations have been suggested. There are remarkable species and tissue differences in responsiveness. When used to treat asthma, the drug must be administered before the antigen is encountered to be effective[47].

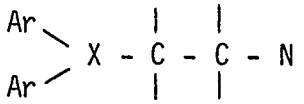
In situations in which the intestine seems to be the target organ for hypersensitivity reactions, response to SCG has been varied and SCG certainly has not been uniformly effective in preventing abnormalities. Recently SCG has been shown to be ineffective in inhibiting release of histamine from isolated intestinal mucosal mast cells[26]. In spite of these findings SCG continues to be used to treat food allergy.

Doxantrazole is a pharmacologically active agent shown to inhibit histamine release from antigen-challenged mucosal mast cells isolated specifically from the intestine of rats[26]. Many other inhibitors, such as SCG, are known to block degranulation of pleural and peritoneal mast cells but not gut mast cells. However, doxantrazole seems to be equally effective on all types of mast cells.

Doxantrazole (3-(5-tetrazolyl)-thioxanthone 10, 10 dioxide) inhibits phosphodiesterases of human lung and guinea pig lung and beef heart[48]. It has been suggested that the antiallergic activity of doxantrazole is due to its ability to elevate intracellular cAMP levels in mast cells by inhibiting phosphodiesterase. Maintaining high cAMP in mast cells prevents degranulation. However, we do not know why other phosphodiesterase inhibitors such as theophylline prevent histamine release from peritoneal mast cells but not from intestinal mast cells[26]. The different actions of agents with different mast cell types has yet to be explained. Since mast cells can now be isolated from the gut, future studies should elucidate some of these perplexing findings.

1.3.6 Antihistamines

Diphenhydramine was first used as an antihistamine in 1946[49]. By the late 1940's numerous other compounds with significant histamine blocking properties had been reported. While these drugs reduced many important responses to histamine, they failed to inhibit others, namely gastric acid secretion. The action of receptor blocking drugs is to occupy receptors on the effector cell without initiating a response. H_1 blocking agents contain a substituted ethylamine moiety:



H_1 receptor blockers such as diphenhydramine inhibit most responses of smooth muscle to histamine[50]. Within the gastrointestinal tract, the guinea pig ileum has been extensively studied *in vitro*.

Antagonism of constrictor action of histamine on respiratory smooth muscle can be shown in vivo and in vitro. However, in man allergic bronchoconstruction is mediated more by leucotrienes than by histamine so treatment with H_1 blockers is not effective[51]. Diphenhydramine, given orally, reaches a maximum concentration in the blood in about two hours. This drug does have some antimuscarinic activity and may induce sedation[52]. However the incidence of gastrointestinal side effects is low. The disadvantage of H_1 blockers is that they are effective only against histamine and not against the effects of other autocoids.

Allergies of the respiratory tract are most amenable to therapy with H_1 blockers. Clinically, gastrointestinal allergies are seldom benefited. However, use of H_1 blockers is useful in ascertaining whether histamine binding to H_1 receptors is a significant cause of the problem (allergic symptoms).

Cimetidine is an H_2 blocker currently used to treat ulcers. The discovery and introduction of drugs which block H_2 receptors was speeded by indications that endogenous histamine has a role in gastric secretion and by the clinical evidence that hypersecretion of acid occurs in peptic ulcers. The discovery of these drugs provided undisputed evidence of the importance of endogenous histamine in the physiological control of gastric secretion.

Burimamide was the first H_2 receptor blocker to be synthesized[36] but was poorly absorbed. Metiamide was effective clinically but caused granulocytopenia. Replacement of the thione (=S) group yielded

cimetidine. Cimetidine is a very polar, hydrophilic molecule. The imidazole ring is believed to be important for receptor recognition, while both the cyanoguanidine and imidazole portions of the molecule contribute to affinity[53].

Cimetidine is a reversible competitive antagonist of the actions of histamine that are exerted on H_2 receptors. It is not known to influence responses to drugs or autocoids acting through other receptors. It is well absorbed orally. Concentrations in blood after oral administration are maximal in 1 to 1.5 hours and a single dose is effective for about 4 hours. Most of the oral dose is excreted unchanged in urine within 24 hours. There are infrequent side effects[50].

1.4 Intestinal Transport of Water and Electrolytes

Absorption can be defined as the lumen to plasma (or mucosal to serosal) movement of fluid or solutes and secretion as the plasma to lumen (or serosal to mucosal) movement of fluids or solutes. Absorption and secretion occur simultaneously in the intestine and their algebraic sum results in luminal disappearance of fluid and solutes, defined as net absorption, or luminal accumulation of fluid and solutes, defined as net secretion.

There is considerable indirect evidence to suggest that in the small intestine absorption occurs in the villus cells and secretion occurs in the crypts, the proliferative gland-like structures at the base of the villi. Under normal circumstances villus absorption exceeds crypt secretion, resulting in net absorption across the entire

intestine.

1.4.1 Absorption

Nearly all absorption in the intestine, except passive non-ionic diffusion, is linked to sodium transport[54]. Sodium ion enters the brush border of the enterocyte passively down its electrochemical gradient. This condition is generated by the extrusion of sodium from the cell by the sodium pump, the Na-K-ATPase on the basolateral membrane. The action of this pump maintains a low sodium concentration inside the cell and a large potential difference (interior negative). Other solutes enter the cells coupled to sodium entry, against their electrochemical gradient. Therefore the uphill transport of glucose, amino acids, and chloride is coupled to the existing sodium gradient. Other solutes utilizing the sodium gradient for absorption are bile salts, water-soluble vitamins, and small peptides. The movement of these solutes out of the enterocyte toward the plasma has not been as extensively studied but is generally believed to be a non-energy requiring step involving diffusion or facilitated diffusion down respective electrical and/or chemical gradients.

1.4.2 Secretion

Secretion in the small intestine implies an abnormal accumulation of water and electrolytes in the lumen. However, secretion is also a normal function of the gut, and fluid movement from the serosa (blood side) to the mucosa (lumen) takes place on a constant basis being balanced by movement of water and ions in the opposite direction. When the contents of the intestine are hypertonic, water is attracted into

the lumen by osmosis. There are reasons to believe that active secretion may be a normal process. The contents of the small intestine must be maintained in a fluid state in order to be propelled. Active secretion might act to dilute and wash away potentially injurious substances from the epithelial surface[55]. Also, fluid might be required to distribute IgA from the crypts where it is secreted to other mucosal surfaces.

Under certain circumstances, fluid movement into the lumen overwhelms the absorptive forces and an abnormal secretory state results. Secretion of this type can be induced by agents which act on the mucosal or on the serosal side of the epithelium or in conditions which result in an increased rate of epithelial cell renewal[56]. Table 2 is a list of the various agents and conditions which result in abnormal net secretion in the small intestine.

1.4.3 Role of Cyclic Nucleotides

Both cyclic adenosine monophosphate (cAMP) and cyclic guanine monophosphate (cGMP) can act as intracellular second messengers in the intestinal tract. In many situations in which net secretion is produced resulting in diarrhea, an agent has been identified which elevates enterocyte cAMP or cGMP. The best example of this phenomenon is cholera. Cholera enteroxtoxin, as well as vasoactive intestinal peptide, prostaglandins and theophylline all result in increased cAMP and all produce similar effects: inhibition of Na^+ and Cl^- absorption and stimulation of Cl^- secretion[55]. These ion movements are accompanied by increases in potential difference and short circuit current.

TABLE 2. Agents and Conditions Producing Net Secretion

Mucosal Stimulation

- cholera toxin
- E. coli toxins
- viruses
- cationic amino acids
- hydroxy fatty acids
- laxatives
- bile salts

Serosal Stimulation

- vasoactive intestinal peptide
- prostaglandins
- serotonin
- acetylcholine
- calcitonin
- A 23187
- substance P
- bombesin
- mast cell mediators

Alteration in Cell Turnover Kinetics

- viral infections
- celiac disease
- cytotoxic drugs
- irradiation

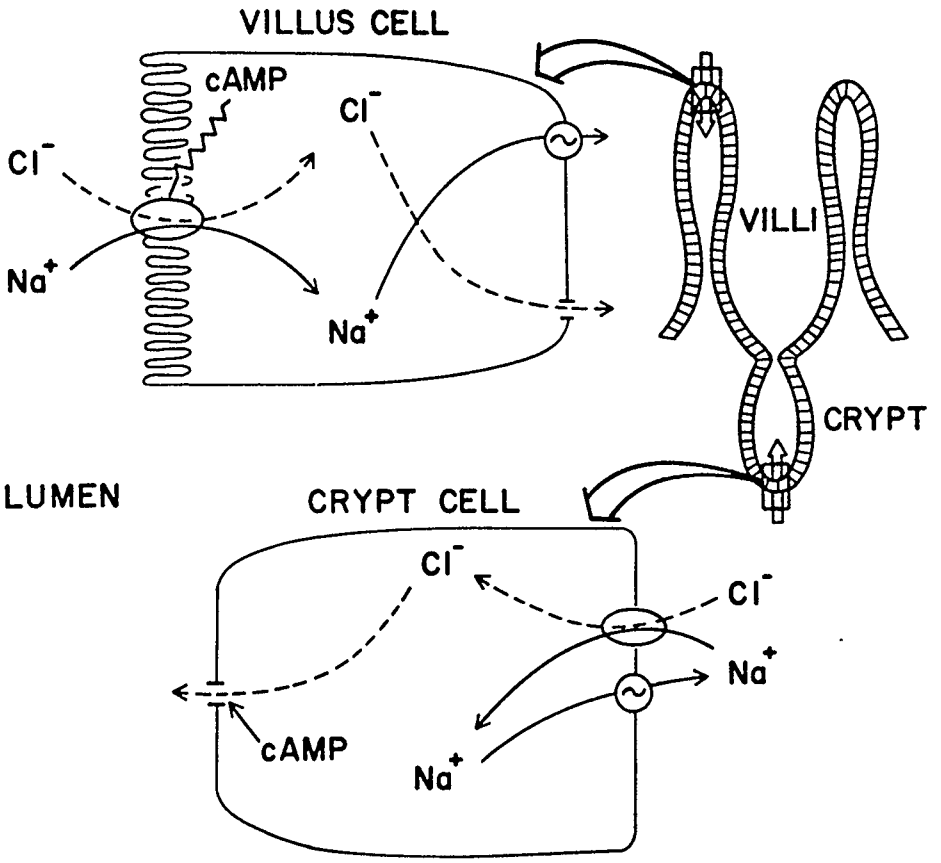
Figure 1 is a diagram proposed by Field to explain the effects of cAMP on ion fluxes. He suggested that cAMP causes inhibition of coupled active NaCl absorption in villus cells, but Cl^- secretion in crypt cells[57].

In the upper (villus) cell of the diagram Na^+ that enters the cell along with Cl^- is actively extruded across the contraluminal membrane by Na-K-ATPase. Cl^- diffuses out across the contraluminal membrane resulting in electrically neutral transcellular absorption of NaCl (this Cl^- flux may be KCl or HCl symports or Cl/HCO_3 or Cl/OH antiports rather than simple diffusion[58]). Thus the potential energy of the Na^+ gradient is used for absorption of Cl^- as well as absorption of glucose and amino acids.

To explain the net secretion of Cl^- that takes place in the presence of cAMP, Field presents the lower (crypt) cell. He suggests that cAMP has a second relevant effect; directly stimulating secretion from crypt cells. In Field's description of crypt secretion, Cl^- entry is coupled to Na^+ entry (serosal NaCl cotransport) with the Na gradient again providing the driving force for Cl^- accumulation. Normally Cl^- diffuses back into the serosal medium. However, the conductive permeability of the luminal membrane to Cl^- is increased by secretory stimuli, and under these circumstances Cl^- moves out into the lumen. Na^+ is attracted by the electrical forces now established and moves into the lumen through the paracellular route.

Additional evidence for Field's model comes from studies in tissue with predominent villus-like epithelium (gallbladder[59] and

Figure 1: Field's model of effects of cAMP on transport of Na and Cl. For explanation see text (From reference 57).


VILLUS CELL
cAMP
Cl⁻
Cl⁻
Na⁺
Na⁺
VILLI
CRYPT
LUMEN
CRYPT CELL
Cl⁻
Cl⁻
Na⁺
cAMP
Na⁺

flounder intestine[60]). In these tissues cAMP inhibits salt absorption but does not stimulate secretion. Furthermore in the distal colon of the rabbit where crypts predominate, cAMP stimulates Cl^- secretion only[61].

The addition of the calcium ionophore A23187 to ileal mucosa produces similar changes in ion flux to those produced with cAMP but these changes are dependent on the presence of extracellular Ca^{++}[62]. In addition cAMP can stimulate release of Ca^{++} from intracellular stores[63].

The molecular events by which the intracellular cAMP and Ca^{++} cause secretion in the intestine are obscure. Cyclic nucelotide phosphorylation of proteins in both brush border and basolateral plasma membranes has been reported at concentrations of cAMP which stimulate secretion[64]. Further work must be done before the details of these mechanisms can be ascertained.

Although cGMP has not been as extensively studied as a second messenger in ion secretion from the small intestine, it has been implicated in several instances. Heat-stable Escherichia coli enterotoxin decreases Na^+ absorption, stimulates Cl^- secretion and increases mucosal cGMP levels[65]. The time course for the increase in cGMP levels is well correlated with the appearance of active Cl^- secretion. Therefore, it appears that cGMP can also induce active Cl^- secretion and probably inhibits Na^+ Cl^- coupled influx. However, epinephrine and insulin stimulate active ion absorption and also increase mucosal cGMP levels[66]. There is no evidence that links cGMP changes to cyto-

solic calcium concentrations. In some tissues (e.g. pancreas) there is a pattern of cGMP response to field stimulation and VIP which is the reverse of the cAMP response[67]. This pattern may suggest a complicated negative feedback mechanism. In only a few reported studies in the intestine were both cAMP and cGMP response patterns measured. As is the case for cAMP, cGMP may stimulate a membrane-bound protein kinase, which in turn results in phosphorylation of endogenous membrane proteins[64].

1.5 Goblet Cell Mucus

1.5.1 Protective Barrier

Mucus is secreted as a viscoelastic gel that adheres to the mucosal surface and acts as a flexible covering. It contrasts with other gastrointestinal secretions which are water soluble. Because of its gelatinous nature and structure of complex glycoprotein molecules, mucus is difficult to study and, therefore, despite the ubiquity of mucus throughout the gastrointestinal tract, its role under normal conditions and in diseased states is poorly defined.

The main function of intestinal mucus is to protect the delicate epithelium from mechanical damage due to the passage of contents. The physical properties of mucus, a non water-soluble viscous gel, allow it to provide a slimy lubricant surface, yet ensures that most of the gel remains stuck to the mucosa. The gel retains water providing an aqueous environment for the absorptive cells. There is circumstantial evidence that gastric mucus protects against ulceration. Gastric

mucus, which contains HCO_3^-, probably acts as a first line of defense against acid. Mucus may, to a certain extent, protect the underlying mucosal cells from proteolysis. However, the luminal surface of mucus is constantly being solubilized and degraded resulting in a dynamic balance between erosion of the mucus and its secretion by goblet cells.

It has been suggested that mucus acts as an antibacterial and antiviral agent. Mucus could provide a barrier between the pathogenic organism and the mucosa or combine directly with the organism or its toxin and inactivate it. Cholera toxin has been shown to be bound to mucus[68]. Mucus does contain secreted IgA which complexes with microbial agents and undegraded macromolecules[69]. Mucus also seems to be important for normal flora in the gut, being a nutrient source for endogenous bacteria[70].

The molecules responsible for the viscous and gel-forming properties of mucus are distinct glycoproteins or mucins, which constitute between 1 and 10% by weight of the gel[71]. Water constitutes 95%, and dialyzable salts, with electrolyte content comparable to plasma, approximately 1%. Small quantities of proteins, nucleic acids and other glycoproteins are also found. Mucus isolated from the intestinal tract is heterogenous and contains material from sloughed epithelial cells, bacteria, digested food, plasma proteins, digestive enzymes, secretory IgA, bile, and other constituents of intestinal juice.

The mucins themselves are high molecular weight molecules from

10^5 to 10^6 daltons, and consist of protein cores with carbohydrate side chains. Mucins are distinct from membrane-bound glycoproteins and serum glycoproteins in that mucins contain no mannose. They do contain N-acetylglucosamine, N-acetylgalactosamine, galactose, glucose and sialic acids[71].

1.5.2 Control of Goblet Cell Secretion

Goblet cells in the intestine respond to certain stimuli with increased secretion of mucus. The factors controlling secretion seem to vary from species to species and from region to region, and are even different depending whether crypt or villus goblet cells are examined. Cholinergic stimulation of increased mucin exocytosis from crypt goblet cells throughout the small and large intestine has been demonstrated by Specian and Neutra[72]. They also examined the effect of other putative secretagogues on secretion in vivo and in organ culture. Adrenergic agents, gastrointestinal regulatory peptides (caerulein, CCK, neurotensin, gastrin, secretin, somatostatin, substance P, and VIP), serotonin, histamine, and dibutyryl cyclic nucleotides were tested over wide concentration ranges. Only histamine at 10^{-4} M had any effect, stimulating mucus release by crypt goblet cells in mucosal explants from the colon but not from the small intestine[73]. Chemical irritants including mustard oil, alcohol, hypertonic saline, bile salts, triglycerides and mechanical trauma (distention) have been shown to elicit local mucus release from goblet cells although the nature of the interaction of chemicals with goblet cell surface membranes is unexplored. Certain bacterial enterotoxins, such as cholera

toxin, which bind to small intestinal brush border membranes and activate adenylate cyclase in intestinal absorptive cells also elicit goblet cell secretion[74]. Increased secretion of mucus into the intestinal lumen has been reported during anaphylactic reactions in rat small intestine.[75]

1.6 Purpose of the Study

The present study was undertaken to examine intestinal responses to antigen during intestinal anaphylaxis.

We established a model in which immunized rats produced antigen-specific IgE antibodies and we challenged them by in vivo intraluminal perfusion with the antigen. Our investigations were designed to define morphological and biochemical abnormalities as well as effects on the transport of water and electrolytes.

The use of antiallergic agents allowed us to examine the role of mast cells and mast cell mediators in producing the abnormalities, while the in vitro flux experiments provided the means to identify the driving forces for the unidirectional ion movements. The measurement of cyclic nucleotides in antigen-challenged intestine yielded information about intracellular events during the reaction.

These studies provided scientific evidence that IgE-mediated hypersensitivity reactions lead to specific pathophysiology in the small intestine.

CHAPTER 2

MATERIALS AND METHODS

2.1 The Model

2.1.1 Sensitization

Four strains of rats were examined with respect to production of IgE antibodies specific for the sensitizing antigen, chicken egg albumin. Three of the strains had been used by other investigators in studies relating to anaphylaxis[75-77], although not specifically involving the intestine as a target organ. These strains were Sprague Dawley, Brown Norwegian and Hooded Lister rats. Because Hooded Long Evan rats were often used at our institution, rats of this strain were also included in the initial study. Sprague Dawley rats were purchased from the University of Calgary, Medical Vivarium; Brown Norwegians from Johns Scientific Breeders; Hooded Long Evans from Charles River Breeders. Hooded Lister rats were not available locally; therefore, breeder rats were purchased from Oxfordshire Breeding Laboratories, Oxfordshire, England. An outbred colony was established at the Medical Vivarium, the University of Calgary. New animals were imported (approximately 12 per year) at intervals from the English breeding company. Breeding pairs were selected from non-related rats and each pair produced about 6 litters during a one-year period. These rats were housed in a separate room.

The rats were sensitized by intraperitoneal (i.p.) injection of

chicken egg albumin (Sigma, Grade V) 10 μg in 1.0 ml of sterile saline plus 1.0 ml of Bordetella pertussis vaccine, 10^{10} organisms (Connaught Laboratories)[78]. Control animals received saline plus vaccine only. In later experiments we used aluminum hydroxide as adjuvant[79]. The egg albumin (EA) was precipitated with aluminum hydroxide as follows: To 1 ml of distilled H_2O containing 1.0 mg/ml of EA was added 1.0 ml of 10% $AlK(SO_4)_2 \cdot 12H_2O$, followed by 2 drops of .02% phenol red. The solution was titrated with 1.0 N NaOH until the colour changed to pink. The aluminum hydroxide formed, which contained the antigen, was centrifuged at 2000 x g for 10 minutes and the supernatant discarded. The precipitate was washed once with sterile saline and resuspended in sterile saline to a total volume of 10 ml. One ml of this stock antigen solution contained 10 μg of protein and 1 mg of aluminum hydroxide. Rats were injected i.p. with 1.0 ml of this solution. Control rats were injected with material prepared in the same manner but without EA.

2.1.2 Passive Cutaneous Anaphylaxis (PCA)

Thirteen days after i.p. injection blood was drawn from rats by cardiac puncture for determination of anti-EA IgE titers. Serum was removed from centrifuged (3000 x g, 15 min) clotted blood and stored at -20°C until at least 8 samples were accumulated. Thawed serum was diluted by 2-fold serial dilutions (1/2, 1/4, ...1/64, 1/128) in saline. Shaven areas of skin on the backs of non-sensitized rats were injected with 0.1 ml of each dilution of serum. Each serum sample was

injected into 2 rats with one area being injected with saline only. Two to three days later (48-72 hours), time for IgE to bind onto skin mast cells, 2.5 mg of EA in 1.0 ml saline and 0.5 ml of 1% Evans blue dye in saline were injected intravenously. Skin reactions were recorded after 60 min. Titers were recorded as the greatest dilution of serum producing a coloured reaction measuring 5 mm or more in diameter[80]. To test for the heat-labile nature of the IgE antibodies, sera were heated to 56° for 3 hours and the PCA repeated.

2.2 *In Vivo* Perfusion

2.2.1 Experimental Protocol

Rats, 14 days after primary immunization and fasted for 24 hours, were anaesthetized with an intramuscular injection of 25% urethane (Sigma) in saline, 5 ml/kg. The rats were maintained at 37° during the surgical procedure by means of a rectal temperature probe and heating pad. A tracheostomy was performed, the abdomen opened by midline incision and a 15 cm segment of proximal jejunum, starting 10 cm distal to the ligament of Treitz, was cannulated at both ends. The segment was washed out with warmed isotonic electrolyte solution at a rate of 0.5 ml/min for 5 min using a Harvard peristaltic pump. The intestine was returned to the abdominal cavity and the incision closed. The intestinal segment was then perfused *in situ* at 0.15 ml/min with the same solution which contained [^{14}C] polyethylene glycol, (PEG) 4000 as the non-absorbable marker. The solution collected for

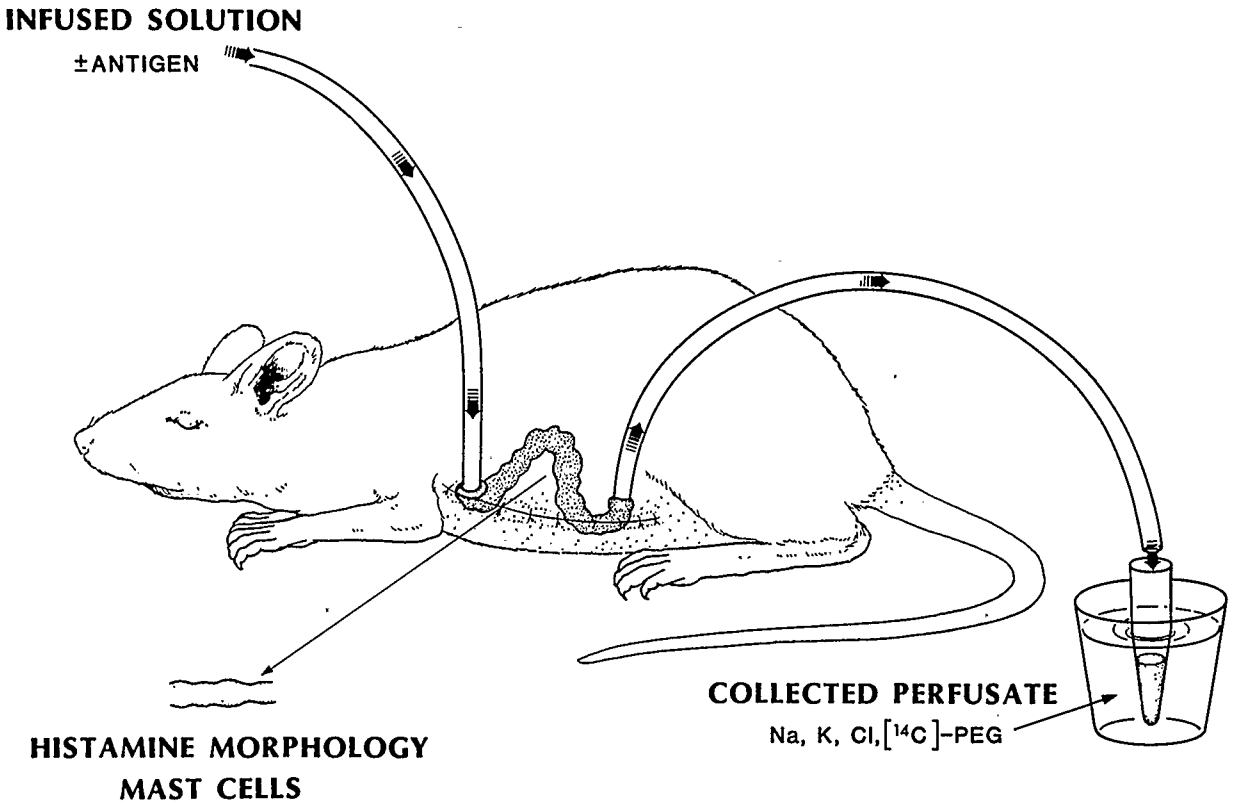
the first 60 min was discarded. Thereafter consecutive 20 min samples were collected from the distal end of the segment by gravity flow into chilled containers (4°C). Samples were stored at -20°C for later determination of Na^+, K^+, Cl^- and [^{14}C] PEG for each 20 min period (Figure 2).

During preliminary experiments certain technical problems were experienced. Occasionally intraluminal pressure increased during perfusion due to partial obstruction. Distension of the intestine had deleterious effects on intestinal absorption and stimulated increased mucus secretion into the lumen. Therefore a pressure-sensing device, consisting of a vertically-mounted 0.2 ml pipet, was incorporated into the perfusion tubing with a "T" connection just prior to the inflow cannula. Increased intraluminal pressure was indicated by fluid rising in the pipet. If this occurred the pump was stopped and measures were taken to unblock the intestinal lumen. The animal was excluded from the study if the manipulation was not successful or if the pressure rose above 2 cm H_2O. Criteria for inclusion of data from a perfused rat were that the three baseline collection periods demonstrated stable absorptive fluxes of H_2O, Na^+, K^+ and Cl^-.

2.2.2 Solutions

Washing and baseline perfusion solutions contained 140 mM Na^+, 10 mM K^+, 120 mM Cl^-, 30 mM HCO_3 and 10 μCi/ml of [^{14}C] polyethylene glycol (PEG) 4000 as the non-absorbable marker. When the antigen, chicken egg albumin (Sigma, grade V) was added after the first 60 min

Figure 2: Experimental procedure for *in vivo* marker perfusion. Rats were perfused for one hour (0-60 min) with basal antigen-free solution. Then the solution was changed to one which included the antigen, egg albumin 10 μg/ml, and the perfusion continued (60-160 min). Absorption of water, sodium, chloride and potassium during one hour of antigen-containing perfusion (100-160 min) was compared with the basal antigen-free perfusion (0-60 min).


INFUSED SOLUTION
±ANTIGEN
COLLECTED PERFUSATE
Na, K, Cl, [14C]-PEG
HISTAMINE MORPHOLOGY
MAST CELLS

of collection, its concentration was 10 μg/ml. Bovine serum albumin (BSA, Sigma, fraction V) when examined was also used at a concentration of 10 μg/ml. In the withdrawal studies the antigen-free electrolyte solution was reintroduced after perfusion with antigen for 100 min. In studies examining the effect of inhibitors, the agent was solubilized at the indicated concentration (several fold above the therapeutic dose for humans) and was present throughout the entire perfusion period. Sodium cromoglycate was also administered intravenously, 20 mg/kg, 20 min before the antigen perfusion was initiated. Sodium cromoglycate (Intal) was purchased from Fisons; doxantrazole was a gift from Burroughs Wellcome; diphenhydramine and cimetidine were purchased from Sigma.

Sodium and potassium concentrations were measured by flame photometer (Radiometer) and chloride by chloride analyzer (Corning, Model 925). Measurement of [^{14}C] PEG was carried out on 200 μl samples of perfusates in Aquasol II (New England Nuclear) in a Beckman scintillation counter, LS 9800.

2.2.3 Calculations

In vivo water absorption was calculated by standard formulae[81] as follows:

$$H_2O = R \left(1 - \frac{[PEG]i}{[PEG]o}\right) \times 1000$$

and solute or electrolyte absorption as follows:

$$S = R \left([S]_1 - [S]_2 \times \frac{[PEG]i}{[PEG]o}\right) \times 1000$$

where R = rate of fluid entering the test segment in ml/20 min

$[PEG]_i$ = concentration of PEG, the non-absorbable marker in DPM/min/ml entering the test segment

$[PEG]_o$ = concentration of PEG in DPM/min/ml leaving the test segment

$[S]_1$ = concentration of solute entering the test segment

$[S]_2$ = concentration of solute leaving the test segment

Results are expressed as μl or μmoles (μEq for univalent ions) absorbed per cm per 20 min period. A negative sign indicates net secretion.

2.2.4 Morphology

Intestinal tissue slices (approximately 1 cm in length) were slit open along the mesenteric border with a pair of fine blunt-tipped scissors and the opened intestine pinned out on a block of paraffin. Tissues were fixed in Carnoy's fixative for 18 hours and stained with periodic acid Schiff's (PAS) stain for morphometric assessment or toluidine blue for identification of intestinal mucosal mast cells[82]. Section numbers were covered to prevent subjective interpretation. Granulated mast cells, defined as cells containing at least five darkly staining cytoplasmic granules, were counted in nine villus-crypt units for each section. Villus height and crypt depth of five representative and well-oriented villi per section were measured by a calibrated micrometer.

2.2.5 Biochemical Determination

Mucosal homogenates were prepared from the perfused segments by scraping the mucosa from the underlying muscularis with a glass slide and homogenizing it with a Weaton glass homogenizer (Sargent-Welch) in

2.5 mM disodium ethylenediaminetetraacetate (EDTA), 10 ml/g. These homogenates were quick frozen in a dry ice and acetone bath and stored at -70°. Thawed samples were sonicated (Fisher, Model 300) for 10 sec on ice before they were analyzed for enzyme activities. Changes in the activity of enterocyte enzymes may indicate damage to the epithelium. Sucrase was measured by the method of Dalqvist[83], alkaline phosphatase by the method of Kelly[84], thymidine kinase by the method of Weiser[85], and Na-K-ATPase by the method described by Kelly[86]. Enzyme activities were expressed as units per mg of homogenate protein, measured by the method of Lowry[87], using bovine serum albumin as standard.

Histamine was measured by the method of Beaven[88]. The assay involves transfer of a [^{14}C] methyl group to histamine from S-adenosylmethionine-[^{14}C]-methyl using a histamine-N-methyltransferase enzyme prepared from guinea pig brains. Histamine was measured in solutions collected from the perfused segments and in mucosal homogenates prepared after EA perfusion. Homogenates were placed in a boiling water bath for 10 minutes to release histamine and the assay was run on 100 µl aliquots of supernatant after centrifugation at 2000 x g for 20 min. Perfusates, which contained no particulate material, were not pre-treated. A standard histamine solution consisting of histamine dihydrochloride (Sigma) diluted in 0.01 N HCl was used to prepare a standard curve. The reaction was stopped by the addition of 0.4 M perchloric acid containing excess methylhistamine (Sigma). The [^{14}C] methylhistamine was extracted 2 times with chloroform and allowed to

dry in a fume hood for 24 hours. Aquasol II (New England Nuclear) was added and the samples counted in a Beckman Scintillation Counter.

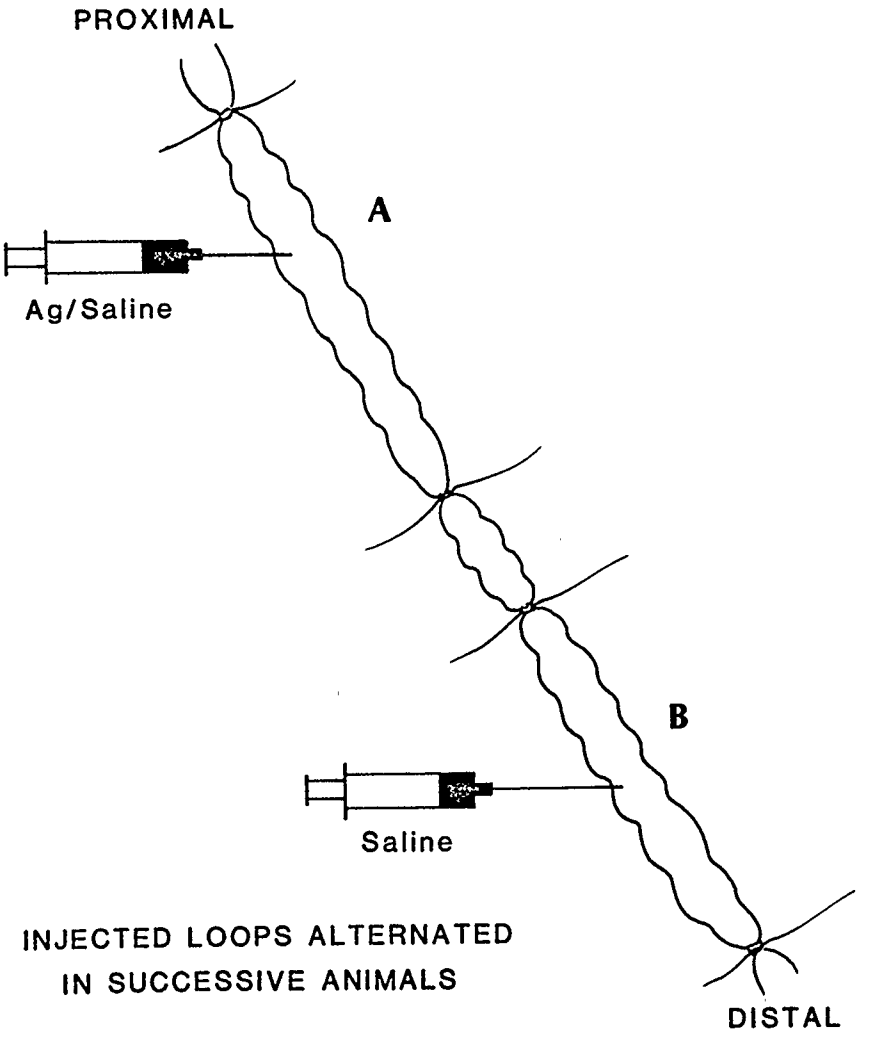
Since preliminary experiments demonstrated that EDTA interfered with the histamine assay separate experiments were run to determine the effect of intestinal anaphylaxis on mucosal histamine. Scraped mucosa for these assays was homogenized in phosphate buffer, pH 7.9.

2.3 *In Vivo* Isolated Loops

2.3.1 Experimental Protocol

In this experiment, rats were sensitized and prepared for surgery as described for the marker perfusion studies, page 32. The animals were kept at 37° by means of a rectal temperature probe and heating pad. A segment of jejunum, 25 cm in length, was exteriorized and perfused with buffered saline pH 7.4, 37° for 15 min at a rate of 0.5 ml/min to clear intraluminal contents. Care was taken not to distend the segment and intraluminal pressure was monitored and kept below 2 cm of water. Following the washing procedure the liquid was drained by gravity flow. Two 10 cm (measured exactly with a length of suture) loops were tied off, separated by 4-5 cm. One loop was injected with 100 μg EA in 0.5 ml saline; the other loop was injected with saline alone. Pilot studies had indicated that volume: loop length was critical in avoiding intestinal distension. In successive animals the EA-injected segment was alternated between the proximal and distal loops (see Figure 3). The intestine was returned to the abdominal cavity and the injections repeated hourly for 4 hours. The volume of

Figure 3: Experimental procedure for injection of *in vivo* isolated loops. Two 10 cm loops of proximal jejunum were tied off. One was injected with 0.5 ml saline; the other was injected with 100 μg EA in 0.5 ml saline. Injections were repeated hourly for 4 hours. The antigen-injected loop was alternated from proximal to distal in successive animals.


PROXIMAL
A
Ag/Saline
B
Saline
INJECTED LOOPS ALTERNATED
IN SUCCESSIVE ANIMALS
DISTAL

fluid of these later injections was 0.1 ml. At the end of the experiment the loops were removed while still intact, rinsed in saline, blotted and length measured. The intestine was slit open and a 1 cm section removed for histological processing (pinned on a paraffin block and fixed in formalin). Each loop was placed in a beaker containing 20 ml of cold saline plus 4 drops of 50 mM PMSF (phenylmethyl sulfonyl fluoride, Sigma), a protease inhibitor. The beaker was swirled to remove secreted mucus and then the mucosa was brushed gently with a sable brush to remove adherent mucus. This material was homogenized using a Weaton glass homogenizer (Sargent-Welch) and frozen. The mucosa was scraped off the muscularis with a glass slide, homogenized (10 ml/g) in phosphate buffer pH 7.9, and aliquoted in plastic vials and frozen at -70°. Results from sensitized rats with titers $\geq$ 64 were compared with results from sham-treated litter-mate controls.

2.3.2 Mucin Radioimmunoassay

Mucin was analyzed by a competitive solid phase radioimmunoassay, developed in the laboratory of Dr. Janet Forstner, Department of Biochemistry, Hospital for Sick Children, Toronto, using a specific rabbit antibody characterized to rat mucin[89]. Microtiter plates were used. The wells were first coated with antigen (mucin, containing 50 ng of protein in 50 μl phosphate buffered saline (PBS)). After 2 hours incubation at 25° the fluid was aspirated and the wells were washed with PBS. Bovine serum albumin (BSA 20 mg/ml in PBS) was added to each well and incubated for 2 hours to saturate the binding sites.

The fluid was aspirated and the wells were washed with PBS. The specific anti-mucin antibody was incubated for 2 hours at 37° with standard purified mucin or unknowns and this mixture was then added to the antigen-coated wells and incubated overnight. After washing the wells, ^{125}I-Protein A (0.01 μCi), which specifically binds IgG antibodies, was added and incubated for 4 hours at 25°. Drained, washed and dried wells were cut from the microtiter plate and counted in a Beckman 8000 Gamma counter. The CPM were plotted versus standard purified mucin (protein) present in the pre-incubation mixtures to give a standard curve on semi-logarithmic paper. The unknown samples were read from the curve. The slope of the diluted unknown samples was ensured to be parallel to the standard curve.

2.3.3 Biochemical Determinations

Protein, histamine and sucrase were measured as described (page 38). DNA was measured by the fluorometric method of Hinegardner[90] using thymus DNA (Sigma) as standard. Albumin was measured using bromocresol green binding[91], and serum electrophoresis was performed by the Biochemistry Department, Alberta Children's Hospital, using the Beckman Paragon System (Beckman) employing agarose gels. Total mucosal homogenate per loop was a quantitative measurement of loop size and was used as a base for the luminal determinations.

2.4 *In Vitro* Ussing Chamber Studies

2.4.1 Tissue Preparation

Rats, either sensitized 14 days previously or sham-treated

litter-mate controls, were killed by decapitation. After opening the abdomen a 15 cm segment of jejunum beginning 10 cm distal to the ligament of Treitz was removed. The excised segment was then immersed in warmed oxygenated Krebs buffer and rapidly opened by cutting along the mesenteric border. The exposed mucosal surface was rinsed free of intestinal contents and the tissue clamped between two leucite half-chambers. Four adjacent pieces of full-thickness jejunum were mounted from each rat. Full-thickness intestine was used because procedures to strip the mucosa were difficult and time-consuming and resulted in reduced viability of the tissue. The elapsed time between excision and the onset of perfusion was approximately six minutes.

Perfusion of each surface of the tissue was accomplished employing a gas-lift circulating system which provided adequate aeration with water-saturated 95% O_2, 5% CO_2 gas mixture. The perfusing solutions circulated through glass reservoirs (MRA Corp) which were enclosed in a second chamber through which water circulated from a constant temperature circulating pump. By this means the solutions were maintained at 37°. The reservoirs were capped with glass condensors to minimize evaporative losses.

The composition of the bathing solution was NaCl 115 mM, KCl 8.0 mM, $CaCl_2$ 1.25 mM, $MgCl_2$ 1.2 mM, KH_2PO_4 2.0 mM, and $NaHCO_3$ 25 mM. Glucose was included at a concentration of 1.0 mM because preliminary experiments with rat tissue indicated improved stability of PD under these conditions. The pH of the buffer was adjusted to 7.40 at 37°.

2.4.2 Electrical Measurements

The tips of KCl-in-agar bridges were placed within 1 mm of each surface of the membrane at the centre and the potential difference (PD) between these bridges was measured by a pair of matched calomel electrodes (Fisher) leading into a potentiometer (Keithley, Model 600B). The calomel electrode and bridge junction potentials measured in the experimental medium prior to mounting the intestinal sheet were usually 0 - 0.2 mv. External current was applied to the system employing Ag-AgCl electrodes connected to a variable electromotive force. The magnitude of the applied current was determined by means of a General Electric microammeter. The area of the exposed tissue in the leucite chambers was 0.4 cm^2.

The determination of the short-circuit current (Isc, the external current required to abolish the transmural PD when both sides of the tissue were bathed with identical solutions) in rat intestine was complicated by the low tissue resistance, so that the resistance of the perfusion solution between the tissue and the bridge tips contributed significantly to the total resistance of the system. To obtain the true transmural PD when an external current was applied to the system, correction for the potential drop between the bridge tips and the tissue surface was made[92]. In order to evaluate the magnitude of this correction, the resistance of the fluid gap between the bridge tips was measured in the absence of the tissue. The PD was recorded when the current imposed was 50 μamps. Since the current and PD are linearly related, the resistance (R) for each chamber could be calculated

(PD = IR). This resistance (R_s) was subtracted from the total resistance (R_t) determined at 10 min intervals during the experiment from measured PD and I with tissue in place and the Isc calculated:

$$Isc = PD/(R_t - R_s)$$

This calculation was programmed into a Hewlett Packard desktop computer which printed PD, Isc and G (conductance, 1/(R x A), where A equals area of membrane exposed) during each 10 min period. In these experiments the tissue was maintained in the short-circuited condition throughout.

2.4.3 Ion Fluxes

Unidirectional Na^+ and Cl^- fluxes from mucosa to serosa (ms) and serosa to mucosa (sm), were determined using ^{22}Na and ^{36}Cl (10 μCi in NaCl, Amersham). In two of four chambers, isotopes were added to the mucosal side; in the other two chambers, they were added to the serosal side. The total volume added to each side of the reservoir was 10 ml. After a period of 20 min, time required to reach a steady state, samples were drawn, 1.0 ml from the unlabelled side and 0.1 ml from the labelled side. After each withdrawal from the unlabelled side 1.0 ml of fresh buffer was added to the reservoir to maintain a constant volume. In most experiments, four samples were withdrawn at 10 min intervals; then 1.0 ml of buffer containing 1.0 mg of antigen, chicken egg albumin (EA, Sigma, Grade V) was added to each side (the volume had previously been adjusted to 9.0 ml) so that the final concentration was 100 μg/ml. Four additional samples were withdrawn at 10 min intervals and were replaced with 1.0 ml buffer containing 100 μg EA.In

some experiments, EA was added at the beginning of the experiment. Four samples were withdrawn at 10 min intervals starting at 20 min. Fluxes during this shorter experiment were compared with those in the longer experiment when EA was added after 60 min. Samples were counted in a Compugamma (LKB-Wallac 1282) gamma counter if only ^{22}Na was present, in a Beckman LS 9800 scintillation counter with 5 ml of Ready Solv fluorescent cocktail (Beckman) if only ^{36}Cl was present, or in both counters if both isotopes were present simultaneously. ^{22}Na emits both β and γ radiation so β counting included both isotopes. The efficiency of counting ^{22}Na was determined in both counters and γ CPM was corrected to obtain equivalent β CPM. Subtraction of this value from β CPM yielded CPM for ^{36}Cl.

2.4.4 Calculations

Unidirectional flux (J) when the isotope was placed on the mucosal side was calculated as follows[93]:

$$J_{ms} = v_s(p_{s2} - p_{s1} \cdot c)/(\Delta t p_m A)$$

where v_s = volume on serosal side = 10 ml

p_s = CPM/ml of serosal fluid

c = correction factor for dilution

A = area of membrane = 0.4 cm^2

Δt = time between samples 1 and 2 in hr

p_m = CPM/µmol in the mucosal fluid (specific activity)

The results are expressed as µmol/cm^2/hr. The conductance of the tissue, 1/(R x A), was calculated from the spontaneous PD and corrected Isc and was expressed as mmhos/cm^2. Net fluxes were calculated as the

difference between Jms and Jsm. A negative sign indicates net movement toward the serosal side. Data from 2 adjacent pieces of tissue were used to obtain net fluxes. However, if the individual conductances differed by more than 25% the pair was excluded.

2.5 Cyclic Nucleotides

2.5.1 Experimental Protocol

Experimental rats (titers $\geq$ 64) were used 14 days after sensitization. The rats were killed by decapitation and 18 cm of upper jejunum removed and placed in warmed oxygenated Krebs buffer plus 1.0 mM glucose, pH 7.40 at 37°. The jejunum was opened, rinsed clean and cut into 18 - 1 cm lengths weighing about 200 mg each. The pieces were pre-incubated in clean buffer for 30 min. Then pieces were incubated in each of the following oxygenated solutions: 1) buffer alone, 2) buffer plus 100 µg/ml EA, or 3) buffer plus 5 mM theophylline. Incubation times and conditions were chosen to correspond to Ussing chamber studies. After incubation pieces were placed in test tubes containing 2 ml of hot (85°) 6% trichloroacetic acid (TCA) in a heating block. After 15 min at 85° the test tubes were allowed to cool and the contents homogenized with a Weaton glass homogenizer (Sargent Welch). Aliquots, 50 µl, were removed for protein estimation. Supernatants after sedimentation of precipitated cellular protein were extracted 3 times with water-saturated ethyl ether and the final liquid evaporated under a stream of air in a 70° heating block. The residue was resuspended to a total volume of 0.5 ml with distilled water. Recovery of cyclic nucleotides was monitored by including [^{3}H] cyclic

AMP in the TCA before adding the tissue.

2.5.2 Assays

Cyclic AMP was measured by a competitive binding assay[94] and cyclic GMP by a radioimmunoassay[95] using kits from Amersham.

2.6 Statistics

All quantitative measurements were compared by analysis of variance, analysis of co-variance, Student's t test or paired t test where applicable.

CHAPTER 3

ESTABLISHING THE MODEL

3.1 Introduction

In order to study the effects of IgE-mediated reactions on intestinal function it was necessary to establish an animal model in which the animals would respond to immunization with antigen by production of antigen-specific IgE antibodies. A number of strains of rats and mice can be stimulated to produce IgE antibodies following intraperitoneal injection of small quantities of protein plus the appropriate adjuvant[75,77,79]. Bordetella pertussis vaccine or aluminum hydroxide, stimulate production of antibodies of the IgE class specifically[96]. Although mice have been used extensively to examine factors controlling IgE production, their small size limits the type of physiological studies which can be performed. Rats are large enough for examination of intestinal absorption by in vivo marker perfusion and the diameter of their small intestine allows sheets of intestine to be mounted in Ussing chambers for in vitro studies. Intestinal responses to intraluminal antigen that have been demonstrated previously by others are increased mucosal permeability and mucus release from goblet cells[75]. Other responses which are consistent with immediate hypersensitivity reactions such as histamine release or mast cell degranulation have not been reported in the intestine.

The present studies were undertaken to find a rat strain and

appropriate conditions to elicit a consistent and reproducible reaginic response and to document the occurrence of intestinal anaphylaxis during antigen challenge by means of a biological response.

3.2 Results

3.2.1 Specific Antigen Titers

3.2.1.1 Genetic Effects

Sprague Dawley Rats. Six litters of rats were immunized. As seen in Table 3, rats of this strain required 1 mg EA/ml as primary antigen challenge before any consistent response was observed. Using this dose the majority of rats (8/18) had a positive titer at a dilution of 8; 4 rats had a titer at 16. At a dose of 10 µg/ml only 2/12 rats had a positive titer.

Brown Norwegian Rats. Two litters of rats were sensitized. Of these, all rats in one litter produced no IgE antibodies (titer = 0). In the second litter 2/6 rats had a titer of 64; 2/6 had a titer of 32; 2 had a negative response.

Hooded Long Evan Rats. Three litters of rats were used. Of these, 12/20 rats had no response. The others had titers of 4 to 64.

Hooded Lister Rats. In the first group of 12 a series of concentrations were used. These rats produced the most consistent response with a primary EA challenge of 10 µg; 5/6 rats injected with 10 µg had a titer $\geq$ 64. Even rats sensitized with only 1.0 µg had a titer of 64. In the next group of 16 rats, all injected with 10 µg, over half had a titer $\geq$ 64. Heat treatment, 56° for 3 hours, of sera with an

TABLE 3. Anti-Egg Albumin Titers in Serum of Sensitized Rats

Rat Strain	Weight (g)	Dose EA (µg)	n	PCA Titer
Sprague Dawley	184 ± 3	1.0	4	0
		10	10	0
			1	2
			1	8
		100	3	0
			2	4
			1	8
		1000	2	0
			2	2
			2	4
			8	8
			4	16
Brown Norway	120 ± 8	10	8	0
			2	32
			2	64
Hooded Long Evan	137 ± 3	10	12	0
			1	4
			1	8
			2	16
			4	64
Hooded Lister	168 ± 4	1.0	2	64
		10	2	0
			1	4
			2	8
			1	16
			1	32
			14	64
		100	1	32
			1	64
		1000	1	32
			1	64

Numbers represent x ± SE, EA was injected i.p., adjuvant was Bordetella pertussis vaccine.
Method for sensitization, page 30.
Method for PCA, page 31.

anti-EA titer of $\geq$ 64 completely abolished the reactivity in the PCA assay. This indicates that the antibodies responsible for the reaction were IgE[123].

3.2.1.2 Weight Effects

Further studies examined the effect of age and weight on eliciting an IgE response. We found that smaller younger rats had a more consistent response to sensitization. Rats with titers $\geq$ 64 weighed significantly less (185 ± 6 g, $\bar{x}$ ± SE) than rats with minimal (4 to 16) or no IgE titers (207 ± 7 g).

Based on these results we decided to use Hooded Lister rats weighing 140 - 180 g that developed titers $\geq$ 64 as our experimental group. Controls would be sham-treated litter-mates having no anti-EA IgE antibodies.

3.2.2 Mast Cells

Numbers of specifically-stained granulated mast cells (method, p. 37) in the mucosa after antigen perfusion (method, p. 32) are given in Table 4. Degranulation after antigen challenge in sensitized rats was suggested by a significant reduction in the number of granulated cells in the mucosal sections from the experimental compared to the control group. Intraluminal exposure to EA for one hour in experimental rats did not lead to any significant change in villus height or crypt depth when compared to controls.

3.2.3 Histamine and Protein

Histamine and protein (method, page 38) concentrations in collected perfusates before and after antigen challenge are shown in Figure

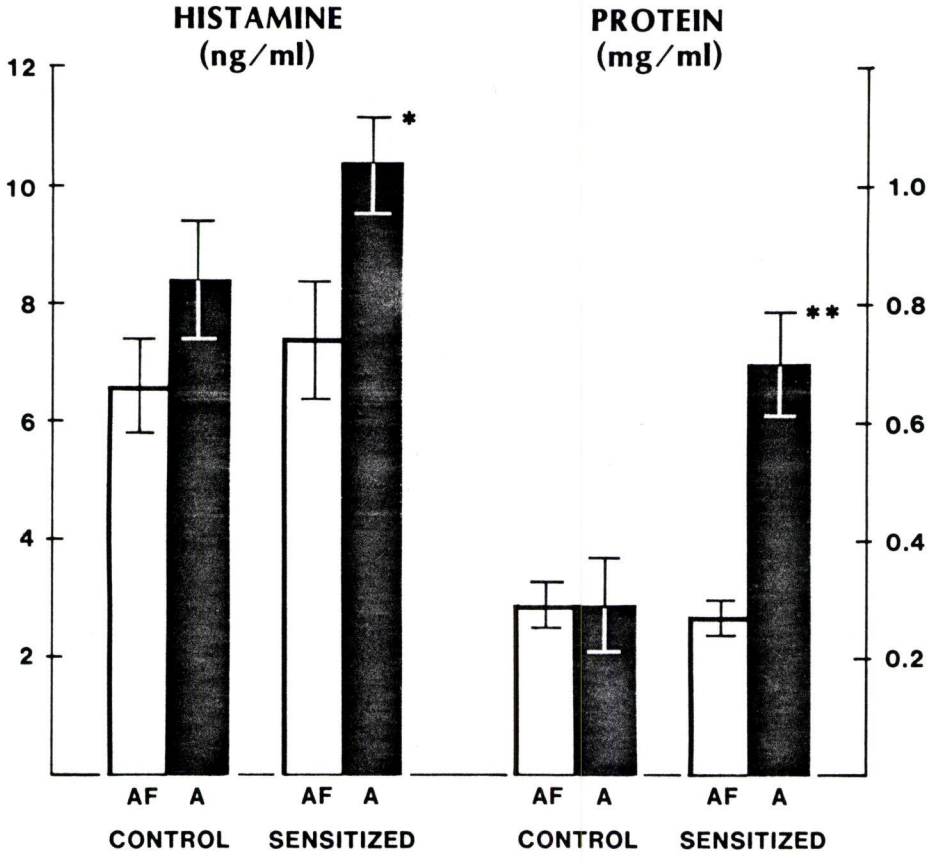
TABLE 4. Morphological Determinations

	Experimental (6)	Control (6)	p
Villus height μm	305 ± 34	321 ± 20	n.s
Crypt depth μm	146 ± 13	139 ± 8	n.s
Mast cells in 9 V-C units	25 ± 2	41 ± 3	<0.001

Rats were challenged by *in vivo* perfusion of a 15 cm segment of jejunum for one hour with EA, 10 μg/ml, in isotonic electrolyte solution. Sections were fixed in Carnoy's fixative and stained with PAS and toluidine blue. Five well-oriented villus-crypt units were measured per section. Mast cells were counted in nine units.

( ), number of rats; ns, not significant; numbers represent $\bar{x}$ ± SE

Figure 4: Histamine and protein in perfusates. Perfusates were collected for one hour before and during antigen perfusion; antigen is EA, μg/ml; bars represent $\bar{x} \pm SE$; AF, antigen-free hour; A, antigen hour; *$p < 0.05$, ** $p < 0.025$. Six rats were studied in each group.


HISTAMINE
(ng/ml)
PROTEIN
(mg/ml)
12
10
8
6
4
2
1.0
0.8
0.6
0.4
0.2
*
**
AF A
CONTROL
AF A
SENSITIZED
AF A
CONTROL
AF A
SENSITIZED

4. In sensitized rats, histamine increased significantly ($p < 0.05$) in the perfusate after antigen exposure. In controls histamine also tended to increase after antigen exposure but this increase was not significant. Protein in collected perfusates increased significantly ($p < 0.025$) after antigen challenge in experimental rats, while controls showed no change.

Figure 5 compares histamine in the mucosal homogenates of both groups after EA perfusion. Histamine, expressed as ng/mg mucosal homogenate protein, was reduced ($p < 0.005$) following antigen challenge in sensitized rats compared to controls. A similar decrease in numbers of granulated mast cells was observed in specifically fixed and stained sections prepared after EA exposure.

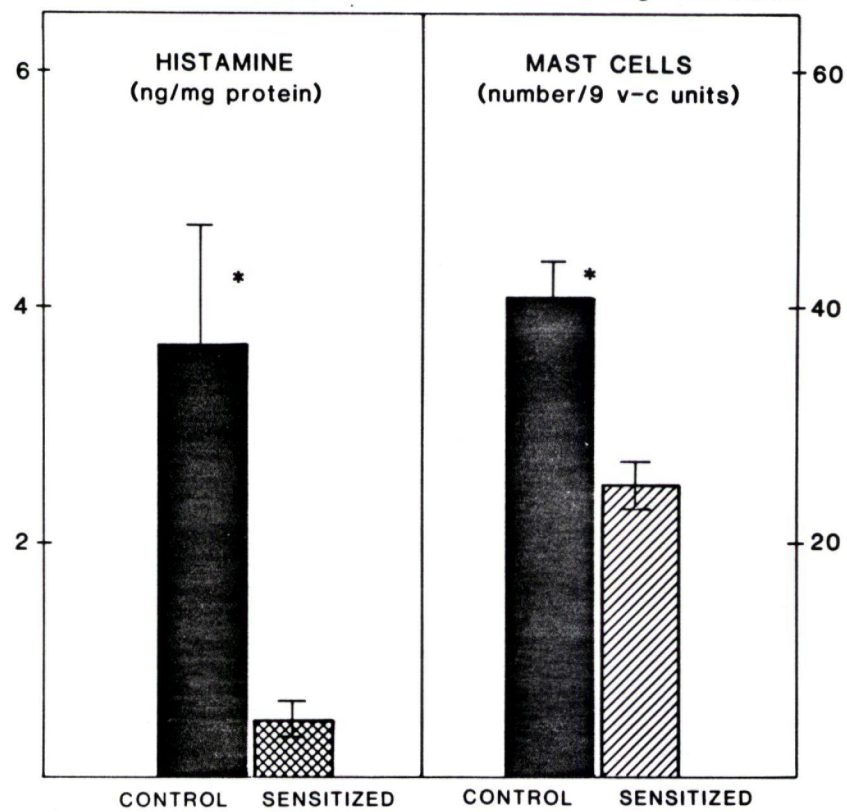
3.3 Discussion

Immediate hypersensitivity reactions are thought to occur because of the actions of mast cell mediators acting on target organs. The mediators are released following antigen-bridging of specific IgE antibodies bound to mast cells. In our model sensitized rats developed significant anti-EA antibodies in their serum as measured by PCA. The PCA reaction was eliminated by heating the serum to 56° for 3 hours indicating that these antibodies were of the IgE class[97].

Rat weight was found to be an important factor in eliciting a selective IgE response, a finding previously reported by Pauwels[98]. The reasons younger animals are more responsive are not clear but may reflect reduced suppressor T cell activity[3]. IgE production is

Figure 5: Histamine and granulated mast cells in intestinal mucosa. Homogenates and sections were prepared from mucosa of rats perfused with EA, 10 μg/ml, for one hour. Sections were fixed in Carnoy's fixative and stained with toluidine blue. Nine villus-crypt units per section were counted. Six rats were included in each group. Bars represent $\bar{x} \pm SE$;
* $p < 0.005$.

HISTAMINE AND GRANULATED MAST CELLS IN INTESTINAL MUCOSA OF CONTROL AND SENSITIZED RATS AFTER Ag PERFUSION



controlled by a complex interaction of helper and suppressor cells with suppression usually predominating[20]. In addition, younger animals have a decreased production of secretory IgA and an increased capacity for the absorption of intact macromolecules[99]. These factors may contribute to an increased likelihood of a reaginic response in the young.

Numbers of granulated mast cells and amounts of histamine were decreased in the intestinal mucosa of sensitized rats compared to controls after antigen perfusion. These results suggest that antigen triggered the release of mast cell mediators, including histamine. Histamine and protein were elevated in perfusates collected from antigen-perfused experimental rats. The increased protein suggests an increase in vascular permeability. These findings are in keeping with a model of anaphylactic (IgE-mediated) reactions in the intestine.

CHAPTER 4

DEFINING THE ABNORMALITIES

4.1 Epithelial Injury

4.1.1 Introduction

Continuous feeding of an antigen to an allergic individual for a prolonged period of time can result in chronic diarrhea and malabsorption. In instances where a jejunal biopsy has been performed a lesion has often been demonstrated which consists of mild to moderate villus blunting and infiltration of the lamina propria with lymphocytes and eosinophils[100,101]. The events involved in producing this epithelial damage are unknown. The studies described in this section were designed to examine whether IgE-mediated hypersensitivity reactions could lead to intestinal epithelial abnormalities.

Sections of jejunum from sensitized rats after intraluminal antigen challenge were examined for evidence of morphologic changes. Damage to the luminal membrane of intestinal epithelial cells was estimated by measuring the activity of brush border enzymes, sensitive markers of luminal membrane disturbances. The contents of the intestinal lumen were examined for evidence of enterocyte constituents. Since release of goblet cell mucus (mucin or glycoprotein) can be stimulated by certain noxious substances in the lumen[74], and since increased mucus secretion has been reported in intestinal anaphylaxis[75], we also measured mucin in the intestinal lumen after antigen challenge.

4.1.2 Results

4.1.2.1 Morphology

Although there were no alterations in villus height or crypt depth (Table 4) following antigen challenge in sensitized rats there was evidence of villus edema, defined as separation of the villus core from the lamina propria. This effect ranged from extremely mild to severe. Figure 6 shows two areas of the same section from an experimental rat after antigen perfusion (method, p. 32). Figure 6A demonstrates no or mild edema while Figure 6B demonstrates moderate edema. Also evident in 6B is some cellular debris over the tips of the villi.The PAS stain which is specific for glycoprotein, shows darkly stained material in goblet cells on the villi and in crypts. There is no evidence of increased goblet cell secretion in sensitized rats compared to controls.

4.1.2.2 Mucosal Enzymes

Values for enzyme activities (method, p. 37) in mucosal homogenates prepared from the perfused segment are shown in Table 5. Sucrase activity was significantly reduced ($p < 0.025$) in homogenates from experimental rats compared to controls. Activities of Na^+-K^+-ATPase, thymidine kinase and alkaline phosphatase were not significantly altered.

4.1.2.3 Intraluminal Contents

Mucin. Mucin measured by radioimmunoassay (method, p. 42) in the luminal fluid of isolated loops expressed per total mucosal loop protein is shown in Figure 7. Antigen challenge in sensitized rats

FIGURE 6: Intestinal morphology in a sensitized rat after 2 hours of antigen perfusion.

A - minimal edema

B - moderate edema

(Edema is defined as separation of the epithelium from the villus core - shown by arrow).

Magnification x 50

Stain PAS

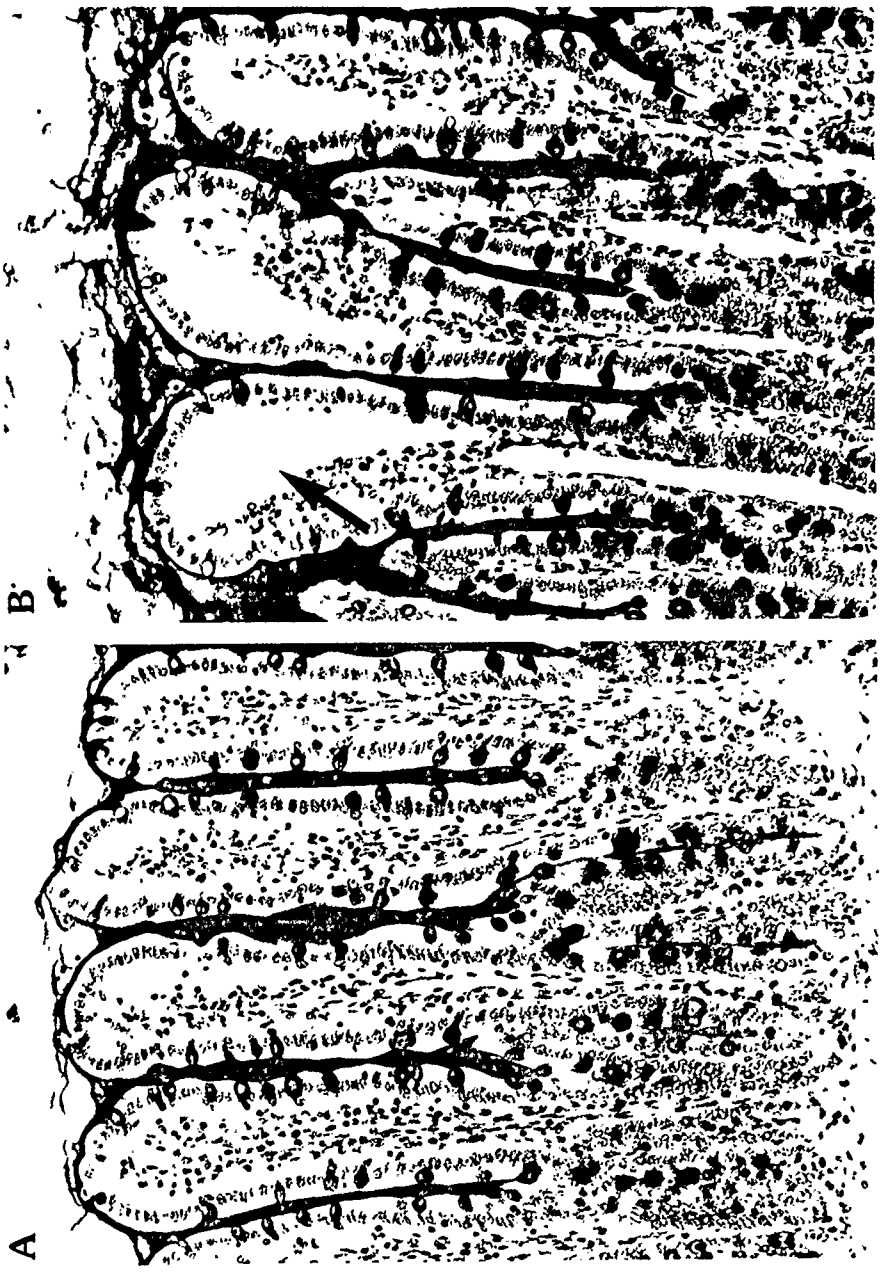
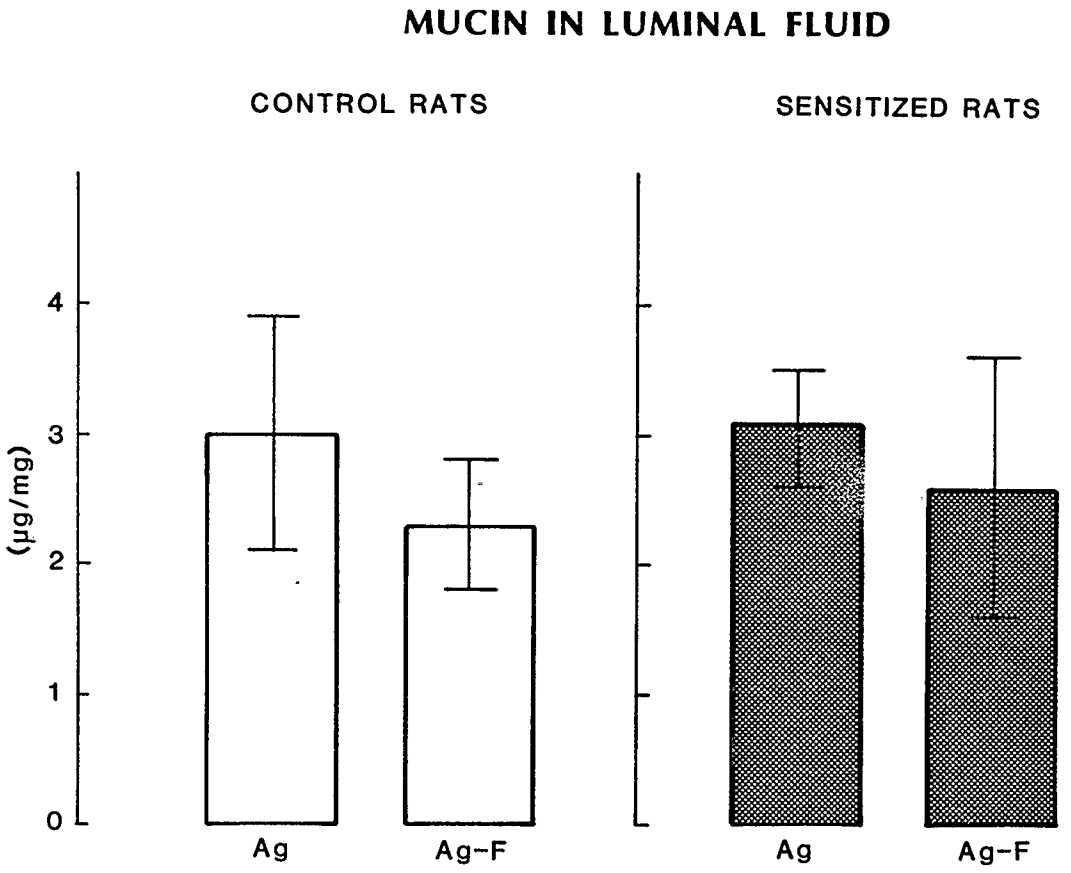

B
A

TABLE 5. Mucosal Enzymes

	Control (6)	Experimental (9)	p
Sucrase	95 ± 3 (91.0 - 99.8)	75 ± 9 (71.4 - 80.7)	<0.05
Alkaline Phosphatase	0.19 (0.16 - 0.23)	0.18 (0.17 - 0.19)	ns
Na-K-ATPase	0.14 (0.12 - 0.15)	0.11 (0.10 - 0.13)	ns
Thymidine Kinase	3.1 (2.9 - 3.4)	4.6 (4.0 - 5.3)	ns

Enzyme data are expressed as the antilog of log mean and range of 1SE and are units per min per mg of protein; ( ), number of animals; ns, not significant

Figure 7: Mucin in luminal fluid during antigen challenge. Mucin was measured by radioimmunoassay in luminal contents from tied-off 10 cm loops of jejunum in sensitized and control rats. Loop A was injected with 100 μg EA in 0.5 ml saline (Ag); B was injected with saline alone (Ag-F). Nine control rats were compared with nine sensitized rats. Bars represent $\bar{x}$ ± SE. Units are μg/mg mucosal protein.


MUCIN IN LUMINAL FLUID
CONTROL RATS
SENSITIZED RATS
(µg/mg)
0
1
2
3
4
Ag
Ag-F
Ag
Ag-F

did not lead to increased secretion of immunoreactive mucin from goblet cells.

DNA. DNA was significantly increased ($p < 0.05$) in the luminal fluid of antigen-challenged segments in sensitized compared to control rats (Figure 8). It was also significantly increased ($p < 0.025$) compared to non-challenged segments in sensitized rats.

Protein. Total protein in the luminal fluid was increased in the antigen-challenged loops in sensitized rats ($p < 0.005$) compared to non-challenged segments (Figure 9). Similarly, protein was greater in challenged intestinal loops from sensitized rats compared to challenged loops from controls ($p < 0.005$). Specific plasma proteins were estimated in samples which had increased total protein. Albumin was negligible. No serum protein bands were evident on electrophoretic gels even when samples were concentrated 2.5 fold.

Sucrase. Sucrase activity is shown in Figure 10. Again the same pattern emerged. Only segments challenged with EA from sensitized rats had increased sucrase activity in the luminal fluid.

4.1.3 Discussion

These studies demonstrated that epithelial damage did occur in antigen-challenged intestine of sensitized rats. Although no structural lesion apart from edema was observed by light microscopy, biochemical determinations provided evidence of injury. Sucrase, a brush border enzyme, was decreased in mucosal homogenates of sensitized rats exposed to egg albumin. The luminal contents from isolated intestinal segments injected with antigen contained increased quantitites of

Figure 8: DNA in luminal fluid during antigen challenge. For legend see Figure 7.

$p < 0.05$ compared to control

$p < 0.025$ compared to Ag-F loop

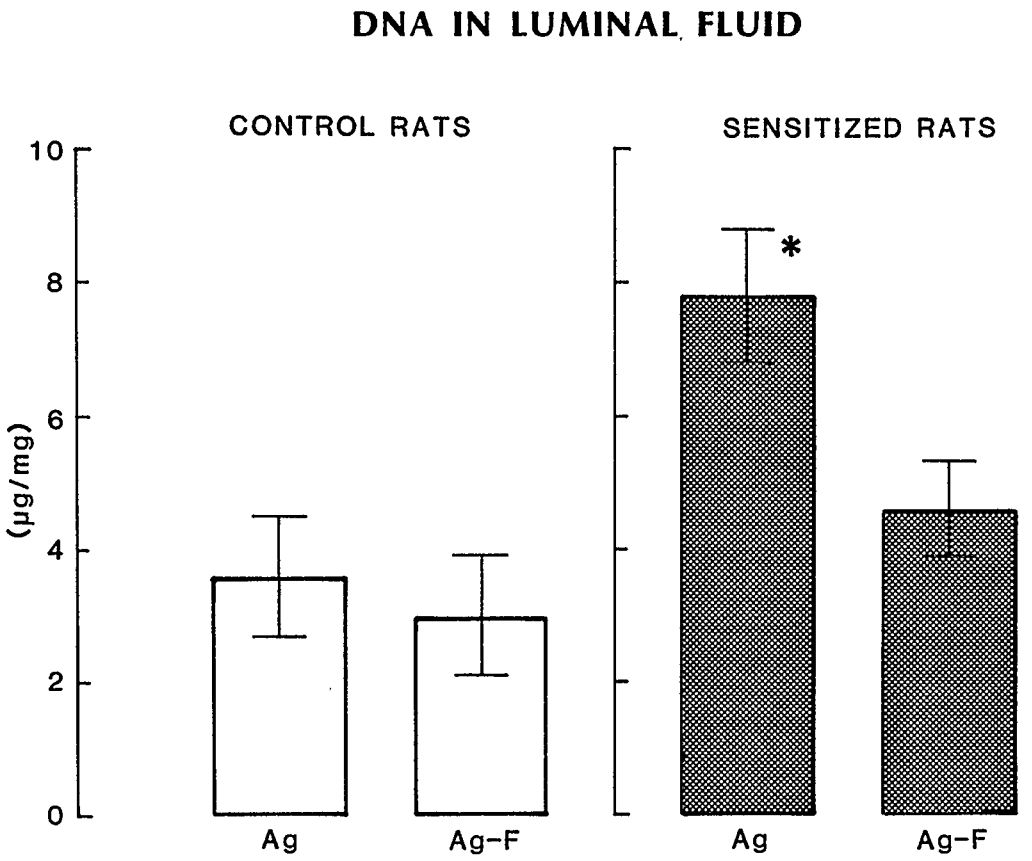

DNA IN LUMINAL FLUID
CONTROL RATS
SENSITIZED RATS
10
8
6
4
2
0
(µg/mg)
*
Ag
Ag-F
Ag
Ag-F

Figure 9: Protein in luminal fluid during antigen challenge. For legend see Figure 7.

$p < 0.005$ compared to control or to Ag-F

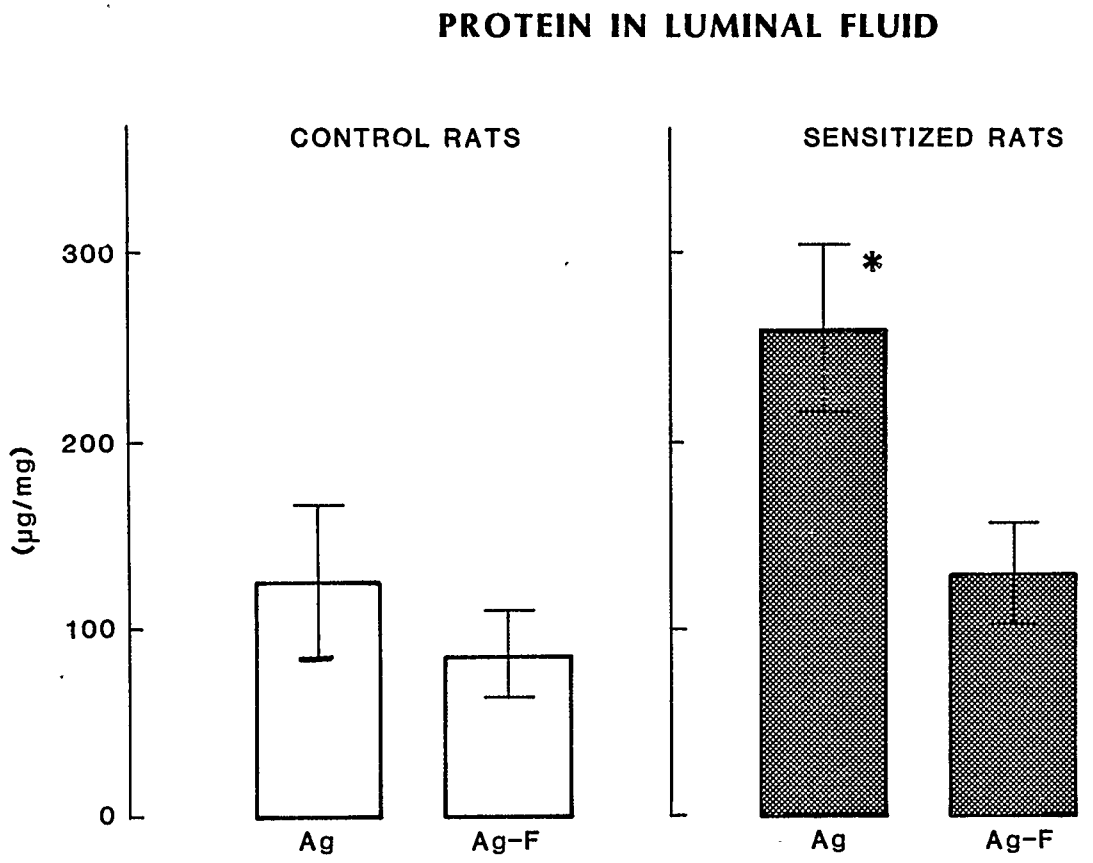
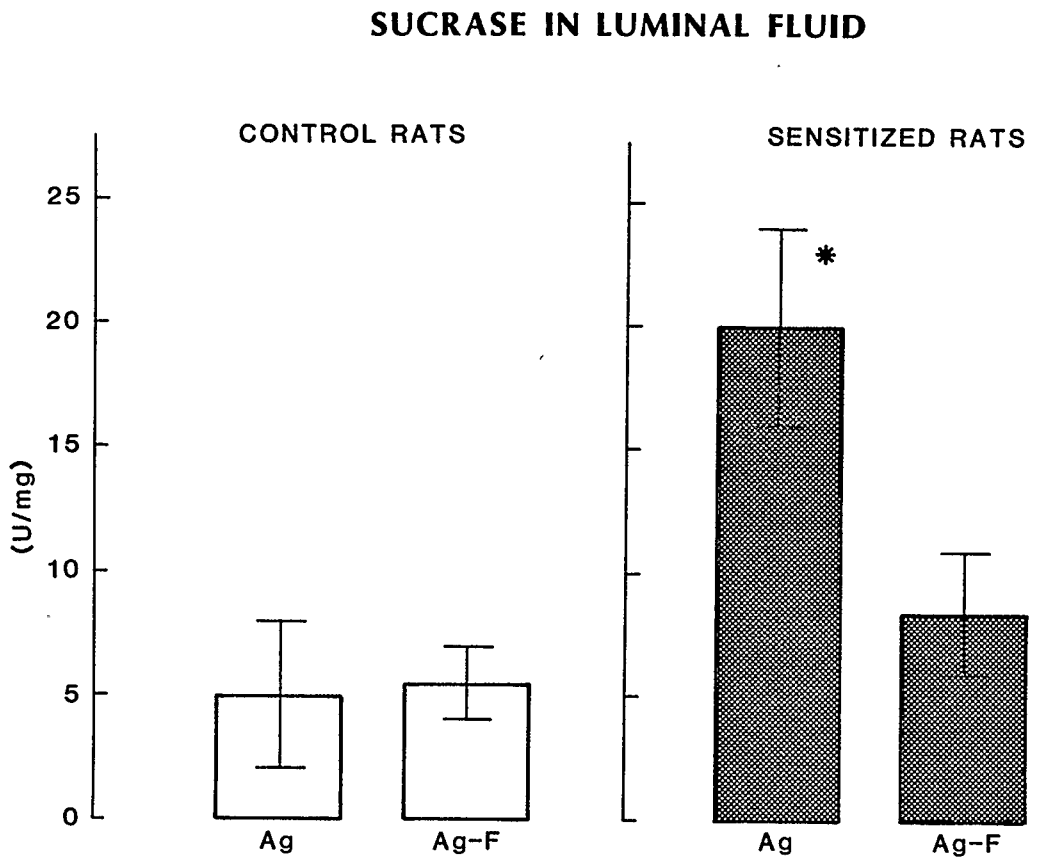

PROTEIN IN LUMINAL FLUID
CONTROL RATS
SENSITIZED RATS
300
200
100
0
(µg/mg)
*
Ag
Ag-F
Ag
Ag-F

Figure 10: Sucrase in luminal fluid during antigen challenge. For legend see Figure 7.

$p < 0.005$ compared to control or to Ag-F


SUCRASE IN LUMINAL FLUID
CONTROL RATS
SENSITIZED RATS
(U/mg)
25
20
15
10
5
0
*
Ag
Ag-F
Ag
Ag-F

sucrase, as well as DNA and protein of non-serum origin.

Using a very specific radioimmunoassay we found no evidence of enhanced mucin secretion from goblet cells during intestinal anaphylaxis. Our results contrast with those from Lake and colleagues[75] who documented increased mucus release during anaphylaxis. They actually measured an increase in the lumen of ^{35}S-labelled glycoprotein after duodenal instillation of antigen in sensitized rats. However, ^{35}S is incorporated non-specifically into all glycoproteins including components of the luminal membrane of enterocytes such as sucrase itself. Their results are therefore compatible with our findings of enterocyte lysis during IgE-mediated reactions in the intestine (Figure 11). Neutra[73] has also shown, using special staining techniques and electron microscopy, that goblet cell mucin secretion is not a feature of intestinal anaphylaxis.

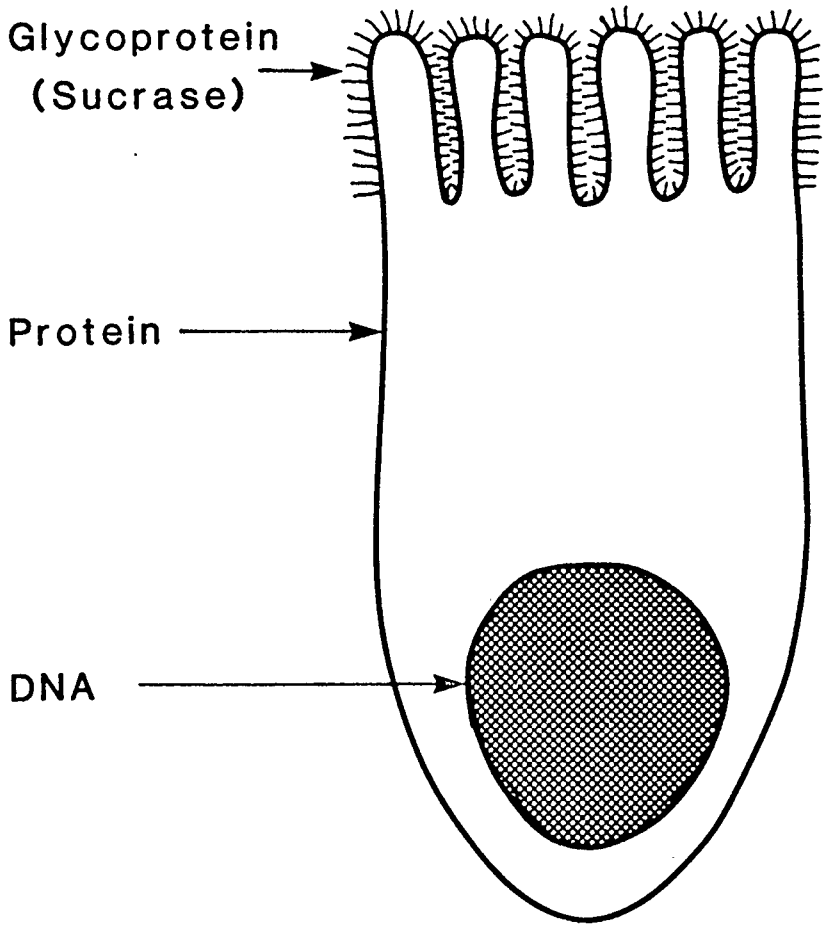
4.2 Transport Abnormalities

4.2.1 Introduction

The clinical symptom of diarrhea after ingestion of antigen in allergic individuals and the experimental finding of epithelial injury during intestinal anaphylaxis suggest that IgE-mediated reactions in the small intestine can cause abnormalities of water and electrolyte transport. The effect of intraluminal antigen challenge on absorption of water, Na^+, K^+ and Cl^- was assessed by <u>in vivo</u> perfusion (method, p. 32).

FIGURE 11: An intestinal epithelial cell demonstrating biochemical components which were increased in the lumen after intestinal anaphylaxis.

VILLUS EPITHELIAL CELL



4.2.2 Results

4.2.2.1 Experimental versus Controls

PEG Recovery. The recovery of the non-absorbable marker, PEG is shown in Table 6. Within each group recovery of [^{14}C] PEG before and after antigen challenge did not differ significantly. In addition, recovery was similar in experimental and control groups. Body weight, and weight and length of the perfused segment of intestine are also shown in Table 6 for control and experimental animals. There were no significant differences in any of these parameters.

Ion Transport. Net transport of H_2O and electrolytes during in vivo perfusion in experimental (titers $\geq$ 64) and sham-treated littermate controls is shown in Figure 12. During the antigen-free period (0-60 min), net absorption of H_2O, Na^+, Cl^- and K^+ did not differ significantly between the two groups. However, 20 min after initiating intraluminal exposure to the antigen, EA 10 μg/ml in the perfused solution, net H_2O and electrolyte absorption began to decrease in the experimental animals; by 40 min net absorption of H_2O, Na^+, K^+ and Cl^- was significantly less ($p < 0.01$) than values obtained during the antigen-free period. With continued exposure to the antigen net fluxes remained depressed in the experimental group. In control rats there were no significant differences in fluxes between the antigen-free and the antigen periods although there was a tendency for fluxes to gradually decrease. Data from these experiments comparing absorption during the antigen-free period (0-60 min) with an antigen-containing period (100-160 min) is shown in Table 7 for comparison

TABLE 6. Weight and PEG Recovery

	Control (6)	Experimental (9)	p
Body weight (g)	171 ± 3	185 ± 6	ns
Perfused intestine			
length (cm)	13.0 ± 0.5	11.2 ± 0.5	ns
weight (g)	22.0 ± 1.6	18.9 ± 1.6	ns
weight/length	1.7 ± 0.1	1.7 ± 0.1	ns
Recovery [^{14}C] PEG			
overall (%)	112.3 ± 2.8	108.0 ± 7.0	ns
antigen-free (%)	113.3 ± 5.5	109.7 + 13.0	ns
antigen (%)	111.4 ± 8.5	106.3 ± 4.4	ns

Each value represents $\bar{x}$ ± SE; ( ), number of rats;

ns, not significant

Figure 12: Jejunal water and electrolyte transport in 6 control (——) and 9 experimental (– – –) rats before and after antigen challenge. Results are expressed as μl or μEq absorbed per cm in 20 min periods. Negative values represent secretion.

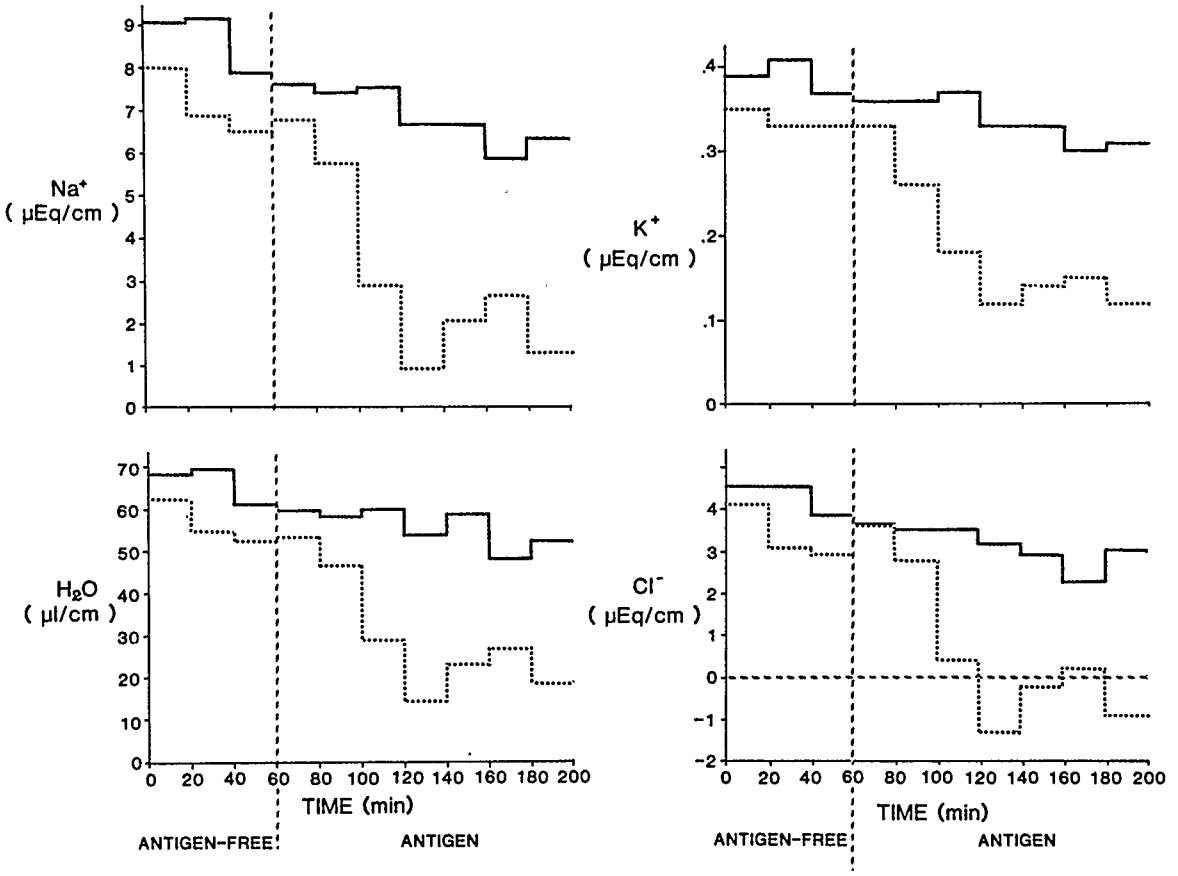

Na+
(μEq/cm)
K+
(μEq/cm)
H2O
(μl/cm)
Cl-
(μEq/cm)
TIME (min)
ANTIGEN-FREE
ANTIGEN

TABLE 7. Net Absorption During Intestinal Anaphylaxis

	Control (6)			Experimental (9)		
	Ag - F	Ag	p	Ag - F	Ag	p
H_2O ($\mu l \cdot cm^{-1} \cdot h^{-1}$)	200 ± 14	168 ± 13	ns	170 ± 29	67 ± 25	<0.005
Na^+ ($\mu Eq \cdot cm^{-1} \cdot h^{-1}$)	26.2 ± 3.3	20.9 ± 2.7	ns	21.5 ± 4.4	6.0 ± 3.7	<0.005
K^+ ($\mu Eq \cdot cm^{-1} \cdot h^{-1}$)	1.2 ± 0.1	1.0 ± 0.1	ns	1.0 ± 0.2	0.4 ± 0.2	<0.005
Cl^- ($\mu Eq \cdot cm^{-1} \cdot h^{-1}$)	13.0 ± 2.5	9.7 ± 1.9	ns	10.2 ± 4.1	-1.0 ± 3.3	<0.01

Each value represents $\bar{x}$ ± SE; ( ), number of rats;

Ag-F is antigen-free period (0-60 min);

Ag is antigen period (100-160 min);

ns, not significant

with later studies.

4.2.2.2 Rats with Minimal IgE Response

Heavier older rats (207 ± 7 g) with titers $\leq$ 16 were also perfused with the antigen-containing solution to see whether serum IgE antibodies correlated with intestinal response. The results of these *in vivo* perfusion studies are shown in Table 8. Regardless of body weight net fluxes of H_2O, Na^+, K^+ and Cl^- in animals who showed no IgE response to immunization were similar to control animals. Fluxes during the antigen period were not significantly different from those found during the antigen-free period. In contrast, rats with even a minimal reagenic response (4 - 16) showed significant reduction of net absorption of H_2O and Na^+ with intraluminal antigen challenge. Values for K^+ and Cl^- decreased but did not reach levels of significance.

4.2.2.3 Specificity for the Antigen

To ensure that the abnormalities of electrolyte transport observed were antigen-specific, we studied the intestinal response following challenge with bovine serum albumin (BSA). Only experimental rats were studied. Rats were sensitized to egg albumin as described and developed specific IgE titers of $\geq$ 64. Net fluxes of H_2O, Na^+, K^+ and Cl^- did not differ between the initial antigen-free period and after intraluminal challenge with BSA, 10 μg/ml (Table 9).

4.2.2.4 Removal of Antigen

In a further series of experiments net fluxes of water and electrolytes were determined before (0-60 min), during (60-160 min) and after (160-260 min) antigen challenge in control and experimental

TABLE 8. Net Absorption By Non- and Poor Responders

	Non-Responders (11)			Poor Responders (7)		
	Ag - F	Ag	p	Ag - F	Ag	p
H_2O ($\mu l \cdot cm^{-1} \cdot h^{-1}$)	146 ± 11	136 ± 12	ns	170 ± 37	95 ± 28	<0.025
Na^+ ($\mu Eq \cdot cm^{-1} \cdot h^{-1}$)	21.1 ± 1.7	20.0 ± 1.7	ns	21.4 ± 7.0	10.6 ± 5.4	<0.05
K^+ ($\mu Eq \cdot cm^{-1} \cdot h^{-1}$)	0.9 ± 0.1	0.9 ± 0.1	ns	1.0 ± 0.2	0.7 ± 0.2	ns
Cl^- ($\mu Eq \cdot cm^{-1} \cdot h^{-1}$)	8.1 ± 1.1	9.1 ± 1.4	ns	11.2 ± 5.9	2.6 ± 4.7	ns

Each value represents $\bar{x}$ ± SE; ( ), number of rats;

Ag-F is antigen-free period (0-60 min);

Ag is antigen period (100-160 min);

ns, not significant

PCA titer of non-responders = 0;

PCA titer of poor responders 4-16

TABLE 9. Net Absorption During BSA Perfusion

	Ag - F	Ag	p
H_2O ($\mu l \cdot cm^{-1} \cdot h^{-1}$)	162 ± 9	156 ± 14	ns
Na^+ ($\mu Eq \cdot cm^{-1} \cdot h^{-1}$)	22.2 ± 2.9	21.2 ± 2.2	ns
K^+ ($\mu Eq \cdot cm^{-1} \cdot h^{-1}$)	1.0 ± 0.1	1.0 ± 0.1	ns
Cl^- ($\mu Eq \cdot cm^{-1} \cdot h^{-1}$)	4.9 ± 2.3	5.4 ± 1.4	ns

Each value represents $\bar{x}$ ± SE; n = 9; ns, not significant;

Ag-F is antigen-free period (0-60 min);

Ag is antigen period containing BSA (100-160 min)

animals (Table 10). As before, intraluminal exposure to the specific antigen, EA, in the experimental group led to a signficiant reduction in net fluxes of H_2O and electrolytes compared to the initial antigen-free period. Removal of the antigen had no effect. Net fluxes of H_2O, Na^+, K^+ and Cl^- after removal of the antigen remained significantly less than values obtained during the initial antigen-free period while not significantly different from the antigen period. In controls net fluxes did not differ significantly between any of the three study periods.

4.2.3 Discussion

The findings indicate that important alterations in H_2O and electrolyte transport occur during reactions to antigen in the small intestine. Intestinal challenge with the specific antigen in sensitized rats with serum IgE titers $\geq$ 64 led to significant inhibition of net absorption of Na^+, K^+, Cl^- and H_2O. These alterations in transport function occurred within minutes of intraluminal exposure and persisted even after antigen withdrawal. The intestinal response was shown to be specific for the sensitizing antigen. Intestinal antigen challenge in sham-treated controls had no effect on transport function. Also, in rats sensitized to egg albumin intraluminal challenge with a different food antigen, BSA, did not alter transport.

Since other investigators have shown changes in mucosal permeability[75,102] during intestinal antigen challenge the PEG recovery findings are important. PEG recovery did not differ between control and sensitized rats and more importantly within the experimental

TABLE 10. Net Absorption Before, During and After Antigen Perfusion

	Control (6)			Experimental (8)		
	Ag - F	Ag	Ag - F	Ag - F	Ag	Ag - F
H_2O ($\mu l \cdot cm^{-1} \cdot h^{-1}$)	158 ± 15	139 ± 19	130 ± 17	165 ± 21	95* ± 18	86* ± 22
Na^+ ($\mu Eq \cdot cm^{-1} \cdot h^{-1}$)	22.9 ± 2.1	19.5 ± 2.6	18.2 ± 2.6	21.1 ± 3.7	11.5* ± 3.1	8.9* ± 2.2
K^+ ($\mu Eq \cdot cm^{-1} \cdot h^{-1}$)	0.9 ± 0.1	0.8 ± 0.1	0.7 ± 0.2	0.8 ± 0.2	0.5* ± 0.1	0.4* ± 0.1
Cl^- ($\mu Eq \cdot cm^{-1} \cdot h^{-1}$)	10.0 ± 1.4	9.7 ± 0.1	8.8 ± 2.2	5.8 ± 3.5	3.2 ± 2.5	1.6* ± 2.8

Each value represents $\bar{x}$ ± SE; ( ), number of animals;

Ag-F is antigen-free period (0-60 min) or post Ag (200-260 min);

Ag is antigen period (100-160 min);

ns, not significant;

* $p < 0.01$ compared to 0-60 min period

group, [^{14}C] PEG recovery was not significantly different before and after antigen challenge. These findings ensure that the observed alterations in transport function are not related to variation in recovery of the non-absorbable marker.

Our data suggest that the changes in gut function occurring after EA exposure are related to IgE-mediated reactions. In heavier rats that did not develop a serum IgE response, intestinal antigen challenge had no effect on H_2O and electrolyte transport. In contrast, in those animals with even a minimal response (titer 4-16), intraluminal challenge did lead to abnormalities of intestinal H_2O and electrolyte absorption. The magnitude of transport abnormalities after antigen challenge in the minimal responders, however, was less than that found in the experimental group with IgE titers $\geq$ 64. In animals with titers of $\geq$ 64 antigen challenge reduced net absorption of H_2O, Na^+, K^+ and Cl^- by 60, 73, 60 and 100% respectively, compared to 44, 48, 30 and 72% in rats with titers of 4-16. These findings suggest that the intestinal response is related to serum levels of specific IgE antibody.

The rapid intestinal response to intraluminal antigen challenge and persistence of transport abnormalities after antigen withdrawal are in keeping with release of a chemical mediator. Anaphylactic reactions are thought to be produced by pharmacologically active mediators released from mast cells[7]. Many of these substances such as histamine, serotonin, vasoactive intestinal peptide (VIP) and prostaglandins have been shown to produce abnormalities of intestinal transport.[103]

CHAPTER 5

EXAMINING THE MECHANISMS

5.1 Inhibitors

5.1.1 Introduction

The results of the in vivo perfusion studies demonstrated dramatic decreases in the net absorption of water and electrolytes in antigen-challenged intestine of sensitized rats. The rapid onset of these changes and their persistence after the antigen was withdrawn was suggestive of an immediate hypersensitivity reaction involving mediators released from mast cells. The studies to be described examined the effect of antiallergic compounds on the transport abnormalities. Sodium cromoglycate and doxantrazole inhibit histamine release from antigen-challenged isolated mast cells under certain conditions[26]. These compounds were used to determine whether mast cell mediators could be implicated in producing the absorptive defect. We also assessed the effect of the histamine receptor blockers, H1-diphenhydramine and H2-cimetidine, as a first step in examining which of various mast cell mediators might be involved.

5.1.2 Results

5.1.2.1 Inhibitors of Mast Cell Degranulation

Sodium Cromoglycate. The effect of sodium cromoglycate (SCG) on the transport abnormalities produced during antigen perfusion in sensitized rats was examined in two ways: inclusion of SCG in perfused

solutions at a concentration of 5 x 10^{-3} M, or intravenous administration of SCG 20 mg/kg 20 min before introducing the antigen-containing solution. The results are shown in Table 11. Sodium cromoglycate did not prevent decreases in absorption following antigen challenge in sensitized rats. Comparison of H_2O and electrolyte transport for 1 hour before (0-60 min) and after (100-160 min) addition of EA shows a significant decrease in absorption in response to the antigen. These changes occurred whether SCG was included in the perfused solutions or intravenously administered. In control non-sensitized rats SCG did not affect normal absorption.

Doxantrazole. The effect of doxantrazole (DOX) on transport abnormalities was determined by including DOX in the perfused solutions at a concentration of 10^{-4} M or 10^{-3} M (see Table 12). DOX at 10^{-4} did not prevent the transport abnormalities produced by antigen in sensitized rats. However, 10^{-3} M DOX included in the perfused solution resulted in stable absorption of H_2O, Na^+, K^+ and Cl^-. Antigen exposure did not alter absorption rates. This is shown in Figure 13 which compares transport abnormalities in experimental rats without DOX to transport when DOX was included in perfusion solutions at 10^{-3} M. DOX had no effect on absorption in control rats as seen in Table 12.

5.1.2.2 Histamine Receptor Antagonists

Diphenhydramine. The effect of diphenhydramine at 5 x 10^{-4} M in perfused solutions is shown in Table 13. Transport abnormalities occurred despite the inclusion of this H1 receptor blocker. Diphen-

TABLE 11. Effect of Sodium Cromoglycate on Net Absorption

	Control (7)			Experimental					
				SCG in Solution (7)			SCG IV (7)		
	Ag - F	Ag	p	Ag - F	Ag	p	Ag - F	Ag	p
H_2O ($\mu l \cdot cm^{-1} \cdot h^{-1}$)	213 ± 10	197 ± 8	ns	181 ± 13	93 ± 20	<0.005	209 ± 16	134 ± 19	<0.005
Na^+ ($\mu Eq \cdot cm^{-1} \cdot h^{-1}$)	28.6 ± 1.2	25.4 ± 2.6	ns	24.0 ± 1.9	12.3 ± 3.2	<0.025	30.3 ± 2.3	17.6 ± 3.0	<0.005
K^+ ($\mu Eq \cdot cm^{-1} \cdot h^{-1}$)	0.99 ± 0.04	0.87 ± 0.10	ns	0.76 ± 0.05	0.45 ± 0.12	ns	1.13 ± 0.07	0.87 ± 0.12	ns
Cl^- ($\mu Eq \cdot cm^{-1} \cdot h^{-1}$)	14.8 ± 1.0	14.1 ± 1.0	ns	12.9 ± 2.5	4.0 ± 2.8	<0.01	13.6 ± 2.8	3.1 ± 2.5	<0.01

Each value represents $\bar{x}$ ± SE; ( ), number of rats;
Ag-F is antigen-free period (0-60 min);
Ag is antigen period (100-160 min);
p, Ag period compared to Ag-F period; ns, not significant;
SCG is sodium cromoglycate either 5 x 10^{-3} M in perfused solution or 20 mg/kg I.V.;
Controls received 20 mg/kg SCG I.V. following the Ag-F period; no Ag was included in solutions.

TABLE 12. Effect of Doxantrazole on Net Absorption

	Control (6)			Experimental 10^{-4} M (5)			Experimental 10^{-3} M (6)		
	Ag - F	Ag	p	Ag - F	Ag	p	Ag - F	Ag	p
H_2O ($\mu l \cdot cm^{-1} \cdot h^{-1}$)	185 ± 15	178 ± 18	ns	174 ± 14	103 ± 15	<0.005	165 ± 18	164 ± 19	ns
Na^+ ($\mu Eq \cdot cm^{-1} \cdot h^{-1}$)	24.3 ± 2.1	21.7 ± 3.3	ns	23.0 ± 1.9	13.7 ± 2.1	<0.025	22.0 ± 1.4	21.2 ± 2.6	ns
K^+ ($\mu Eq \cdot cm^{-1} \cdot h^{-1}$)	1.1 ± 0.1	1.1 ± 0.1	ns	1.2 ± 0.3	1.1 ± 0.2	ns	0.9 ± 0.1	1.1 ± 0.2	ns
Cl^- ($\mu Eq \cdot cm^{-1} \cdot h^{-1}$)	9.5 ± 2.1	9.5 ± 2.0	ns	8.5 ± 2.2	1.5 ± 3.3	<0.001	8.9 ± 1.4	10.2 ± 2.5	ns

Each value represents $\bar{x}$ ± SE; ( ), number of rats;
Ag-F is antigen-free period (0-60 min);
Ag is antigen period (100-160 min);
p, Ag period compared to Ag-F period; ns, not significant;
Doxantrazole was 10^{-4} M or 10^{-3} M in perfused solution;
Controls were perfused with doxantrazole 10^{-3}M added after the Ag-F period,—no Ag was included in solutions.

Figure 13: Jejunal water and electrolyte transport in sensitized rats before and after antigen challenge without (..., n = 9) or with (---, n = 6) doxantrazole 10^{-3} M in perfused solutions. Results have been equalized to initial values of 100 for purposes of comparison. Negative values represent net secretion.

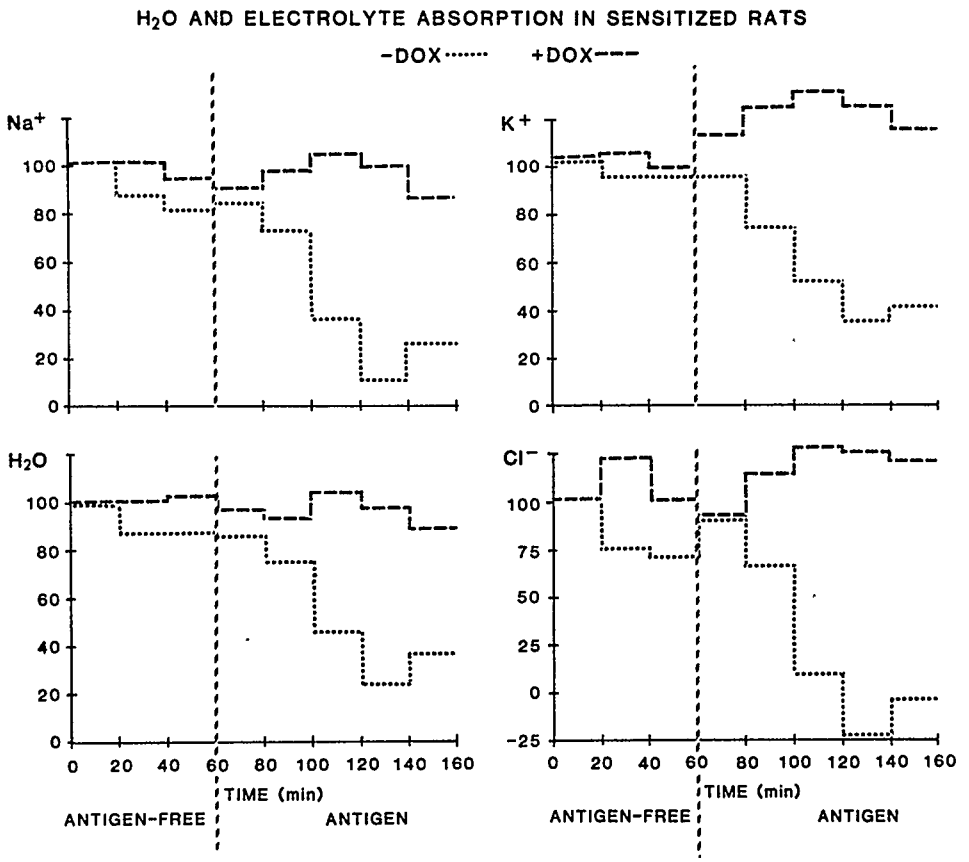

H2O AND ELECTROLYTE ABSORPTION IN SENSITIZED RATS
-DOX
+DOX
Na+
K+
H2O
Cl−
100
80
60
40
20
0
75
50
25
-25
0 20 40 60 80 100 120 140 160
TIME (min)
ANTIGEN-FREE
ANTIGEN

TABLE 13. Effect of Diphenhydramine on Net Absorption

	Control (5)			Experimental (7)		
	Ag - F	Ag	p	Ag - F	Ag	p
H_2O ($\mu l \cdot cm^{-1} \cdot h^{-1}$)	160 ± 15	150 ± 25	ns	191 ± 12	99 ± 13	<0.005
Na^+ ($\mu Eq \cdot cm^{-1} \cdot h^{-1}$)	22.3 ± 2.4	21.9 ± 3.8	ns	24.5 ± 2.3	11.6 ± 1.7	<0.001
K^+ ($\mu Eq \cdot cm^{-1} \cdot h^{-1}$)	0.59 ± 0.12	0.68 ± 0.20	ns	0.85 ± 0.06	0.68 ± 0.06	<0.025
Cl^- ($\mu Eq \cdot cm^{-1} \cdot h^{-1}$)	13.1 ± 1.5	10.5 ± 3.4	ns	10.0 ± 1.3	1.7 ± 1.4	<0.005

Each value represents $\bar{x}$ ± SE; ( ), number of rats;

Ag-F is antigen-free period (0-60 min);

Ag is antigen period (100-160 min);

p, Ag period compared to Ag-F period; ns, not significant;

Diphenhydramine was 5 x 10^{-4} M in perfused solutions;

Controls were perfused with 5 x 10^{-4}M diphenhydramine added after the Ag-F period,-no Ag was included in solutions.

hydramine did not produce any transport changes in control rats.

Cimetidine. Cimetidine was included in perfused solutions at 10^{-4} M. Results are shown in Table 14. The inclusion of Cimetidine, an H2 receptor blocker, did not eliminate the changes in transport produced by antigen in sensitized rats. Cimetidine alone produced no absorptive abnormalities in control rats.

5.1.3 Discussion

We used several antiallergic compounds to identify the role of mast cells and histamine in producing the intestinal abnormalities which we documented in this model. Certainly mast cells were involved. The intestinal tract probably contains a heterogeneous population of mast cells: serosal mast cells, mucosal mast cells and intraepithelial mast-like cells. From the work of Bienenstock and his colleagues we know that not all types of mast cells are protected from antigen-induced degranulation by sodium cromoglycate[23]. In their studies sodium cromoglycate protected only connective tissue peritoneal mast cells and had no effect on isolated mucosal mast cells. However, both mucosal and peritoneal mast cells were protected by doxantrazole. If mast cells *in situ* react to antigen in a similar manner we would have to assume that the transport abnormalities in sensitized rats were due to mediator(s) released from mucosal mast cells since doxantrazole, but not sodium cromoglycate, inhibited those effects in a dose dependent manner.

While we have demonstrated histamine release associated with the transport and epithelial abnormalities, it does not necessarily follow

TABLE 14. Effect of Cimetidine on Net Absorption

	Control (6)			Experimental (6)		
	Ag - F	Ag	p	Ag - F	Ag	p
H_2O ($\mu l \cdot cm^{-1} \cdot h^{-1}$)	191 ± 25	169 ± 25	ns ns	163 ± 10	108 ± 18	<0.01
Na^+ ($\mu Eq \cdot cm^{-1} \cdot h^{-1}$)	29.3 ± 4.1	27.3 ± 4.1	ns	25.6 ± 0.9	16.3 ± 2.8	<0.01
K^+ ($\mu Eq \cdot cm^{-1} \cdot h^{-1}$)	0.90 ±0.09	1.08 ±0.15	ns	0.81 ±0.07	0.56 ± 0.11	<0.05
Cl^- ($\mu Eq \cdot cm^{-1} \cdot h^{-1}$)	13.6 ± 1.7	15.2 ± 2.3	ns	12.1 ± 1.7	8.2 ± 2.3	<0.05

Each value represents $\bar{x}$ ± SE; ( ), number of rats;

Ag-F is antigen-free period (0-60 min);

Ag is antigen period (100-160 min);

p, Ag period compared to Ag-F period; ns, not significant;

Cimetidine was 10^{-4} M in perfused solutions;

Controls were perfused with 10^{-4}M cimetidine added after the Ag-F period, no Ag was included in solutions.

that the abnormalities were caused by histamine. Neither the H1 receptor antagonist, diphenhidramine, nor the H2 receptor antagonist, cimetidine, prevented decreases in net absorption in response to the antigen. H1 receptors have been demonstrated on smooth muscle in the small intestine of guinea pig[104],but have not been demonstrated on absorptive epithelial cells. H2 receptors are present in gastric mucosa[36] but possibly not in the jejunum. The antihistamines are effective only against the actions of histamine and not against the actions of other mast cell autocoids. At least 20 chemical mediators of immediate hypersensitivity have been defined including other agents such as vasoactive intestinal peptide, serotonin, leukotrienes and prostaglandins. These substances appear to have a more important role than histamine in producing intestinal dysfunction during anaphylactic reactions.

5.2 *In Vitro* Studies

5.2.1 Introduction

The *in vitro* studies employing Ussing Chambers were undertaken to provide more information about the nature of the transport abnormalities produced during antigen challenge in sensitized rats. Results from the *in vivo* perfusion studies demonstrated consistent and dramatic decreases in net absorption of H_2O, Na^+ and Cl^- but provided no information about changes in unidirectional fluxes or driving forces. This information was obtained by studying ion movements across sheets of intestinal epithelium under imposed conditions of electrical and

chemical neutrality in the absence and presence of the antigen. Changes in electrical parameters of the tissue from sensitized rats after addition of the antigen also yielded information about ion movements. The short-circuit current, Isc, equals the sum of net ion fluxes across the tissue, taking valence into account. Therefore, effects of antigen on Isc and tissue conductance were also examined.

In vitro net chloride secretion is often associated with activation of adenylate cyclase and increased intracellular cyclic AMP levels. Agents such as VIP and prostaglandins are known to exert their effects in this manner. We measured enterocyte cyclic nucleotide levels to determine whether either cyclic AMP or cyclic GMP was involved as a second messenger.

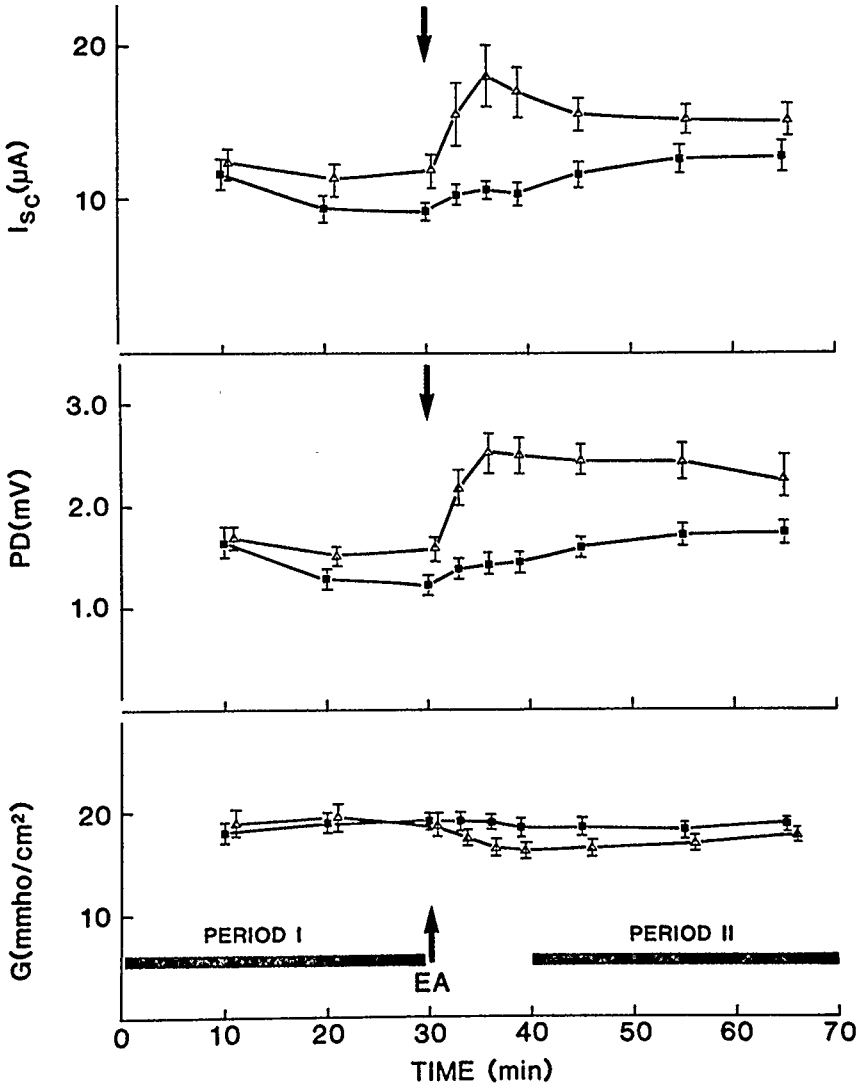
5.2.2. Results

5.2.2.1 Electrical Parameters

The electrical data from Ussing chamber experiments (method page 43) are plotted in Figure 14. Base line measurements were similar in tissue from control and sensitized rats. The addition of EA to the medium bathing tissue from sensitized rats led to dramatic increases in Isc and PD which became significantly different from controls within 3 min, the earliest time point at which measurements were made. The increases peaked 6 min after adding the antigen. The Isc then decreased slightly and levelled off but was still significantly greater than control values. The PD remained high and significantly different from controls throughout the duration of the experiment.

The addition of EA to control tissue did not result in any

Figure 14: Electrical measurements during in vitro Ussing Chamber experiments. Circles (o) represent jejunum from control rats; triangles (Δ) represent jejunum from sensitized rats. The antigen (EA, 100 μg/ml) was added at 30 minutes. The bars at the bottom represent time periods during which 4 samples of buffer were taken for Na^+ and Cl^- flux determinations. Short circuit current (Isc) is expressed as microamps; potential differences (PD) as millivolts; conductance (G) as mmhos per cm^2. $\bar{x} \pm SE$ are shown; n = 18. [For complete method see page 43].


20
10
I_{SC}(μA)
3.0
2.0
1.0
PD(mV)
20
10
G(mmho/cm²)
PERIOD I
PERIOD II
EA
0
10
20
30
40
50
60
70
TIME (min)

immediate changes. In the control tissue the PD and Isc tended to rise slightly during the last 30 min but the mean of all values during the second sampling period (40 to 70 min) was not significantly different from the mean of all values obtained during the first sampling period (0 to 30 min).

A second series of shorter experiments were undertaken to rule out possible effects of tissue deterioration. In these experiments EA was added to medium bathing intestine from sensitized rats 10 min before the first sampling period. The electrical data obtained were similar to those parameters measured in the second period of the longer experiments: the PD increased 165% and the Isc increased 151% compared to the antigen-free period (0 - 30 min) of both control and sensitized rats in the original experiment.

Conductance was stable throughout the short-circuited chamber studies and only minor changes were noted during antigen challenge in tissue from sensitized rats. During the basal period conductance was similar in tissue from control and sensitized rats. After adding antigen conductance initially decreased slightly ($p < 0.05$ at 36 and 39 min) in tissue from sensitized animals but then returned to control values during the second sampling period (40 - 70 min)

5.2.2.2 Ion Fluxes

Sodium and chloride ion movements across short-circuited intestine (method page 46) are shown in Table 15. The fluxes of Na^+ were similar in control and sensitized rats in the basal period. Jms was

TABLE 15. Effect of Antigen of Fluxes of Na^+ and Cl^- In Vitro

		Jms	Jsm	net
Na^+				
Controls (9)	I	8.21 ± 0.45	7.89 ± 0.39	0.32 ± 0.20
	II	8.46 ± 0.44	8.65 ± 0.58	-0.20 ± 0.25
	p	ns	ns	ns
Experimental (6)	I	8.04 ± 0.62	8.20 ± 0.55	-0.16 ± 0.29
	II	7.00 ± 0.46	7.61 ± 0.48	-0.58 ± 0.35
	p	<0.025	ns	ns
Cl^-				
Controls (7)	I	9.10 ± 0.60	8.91 ± 0.71	0.33 ± 0.30
	II	10.05 ± 0.35	9.54 ± 0.61	0.51 ± 0.51
	p	ns	ns	ns
Experimental (8)	I	10.21 ± 0.57	9.06 ± 0.78	1.15 ± 0.33
	II	7.01 ± 0.36	10.33 ± 0.52	-3.08 ± 0.31
	p	<0.001	<0.025	<0.001

Results are expressed as $\mu Eq \cdot h^{-1} \cdot cm^{-2}$; Jms is flux from mucosa to serosa; Jsm is flux from serosa to mucosa; I is basal period, antigen-free, 0-30 min; II is antigen-containing period (EA, 100 µg/ml), 40-70 min; each value is $\bar{x}$ ± SE; p values compare II to I; ( ), number of paired tissues; ns, not significant.

decreased by addition of EA in experimental rats; Jsm was not affected. Net Na^+ transport was not significantly different from zero in either group. In contrast the addition of EA to tissue from sensitized rats resulted in net Cl^- secretion. This secretion was due to a significant decrease in Jms ($p < 0.001$) and an increase in Jsm ($p < 0.025$). No changes were observed in Cl fluxes across control tissue after antigen addition.

Chloride fluxes in experiments in which EA was added before the first sampling period are shown in Table 16. Only sensitized rats were used. There were no differences between Cl^- fluxes in these experiments and those from the previous experiments after EA was added to experimental rat tissue.

5.2.2.3 Cyclic Nucleotides

Results of cyclic nucleotide assays (method, page 48) are shown in Table 17. The addition of theophylline to intestinal tissue slices from sensitized animals produced a rise in cyclic AMP levels of about 2 fold at 15 min. In contrast, the addition of the antigen, EA, had no effect on intracellular levels of this nucleotide at any time. Similarly, no changes in cyclic GMP levels were observed.

5.2.3 Discussion

Full thickness jejunum was used for the in vitro studies to more closely resemble the in vivo situation. The serosa and muscularis of rat intestine adhere very tightly to the mucosa and prolonged manipulations are necessary to strip the mucosa from the underlying tissue.

TABLE 16. Effect of Antigen on Cl^- Fluxes in Sensitized Rats

(12)	Jms	Jsm	net
I	6.46 ± 0.31	9.72 ± 0.33	-3.32 ± 0.20
p*	<0.005	ns	<0.005
p**	ns	ns	ns

Jms is flux from mucosa to serosa; Jsm is flux from serosa to mucosa; I, sampling period is 0-30 min; EA (100 μg/ml) was added 15 min previously; each value is $\bar{x}$ ± SE; ( ), number of paired tissues; * compared to basal period I or antigen period II in controls in Table 15; ** compared to antigen period II in experimental rats in Table 15; ns, not significant.

TABLE 17. Cyclic Nucleotides During Antigen Challenge

		Time (min)				
	Addition	0	1	3	6	15
cAMP	EA	0.88 ±0.17	0.98 ±0.25	0.95 ±0.09	0.87 ±0.17	0.89 ±0.15
	Theo					1.88* ±0.17
cGMP	EA	0.48 ±0.07	0.59 ±0.08	0.54 ±0.02	0.52 ±0.02	0.60 ±0.04
	Theo					0.99* ±0.09

values represent $\bar{x}$ ± SE from 4 sensitized rats and are expressed as pmol/mg of protein; full thickness jejunum was used; EA (egg albumin) was 100 µg/ml, Theo (theophylline) was 5 mM in Krebs buffer; * $p < 0.005$ compared to values at 0 time.

Previous studies have shown that permeability of stripped mucosa is greatly increased.[105] The inclusion of 1 mM glucose was found to improve the viability of the tissue, possibly because rat gut has a high rate of glycolysis.

The flux experiments were begun after an equilibration period of 30 min. at which time the potential difference had stabilized. The addition of 100 μg/ml EA to medium bathing tissues from sensitized rats resulted in a rapid and dramatic increase in both PD and Isc which was significantly different by 3 min, the earliest time measurements were made. These parameters peaked at 6 min and declined somewhat thereafter but were still significantly greater than values in control tissues. These changes signify a change in ion movements. The addition of EA to the medium bathing control tissues resulted in a very gradual increase in the potential difference (PD) and short-circuit current (Isc) but means for all the values during the first antigen-free period preceding the addition were not significantly different those during the post-antigen period. The mean conductances of tissues from both groups were not significantly different during the two experimental periods.

Ion fluxes, both Na^+ and Cl^-, were not significantly different across intestinal sheets from sensitized rats or controls during period I, the antigen-free period. Egg albumin had no effect on ion fluxes across control rat intestine. However, the addition of egg albumin to the medium bathing intestine from sensitized rats resulted in net Cl^- secretion against its electrochemical gradient which was evident

within 10 min. The net Cl^- secretion was related to both a decrease in Jms and an increase in Jsm. Transport of Na^+ did not change significantly, but a trend toward a decrease in Jms was noted. To ensure that these flux changes were not due to deterioration of the tissue Cl^- fluxes were measured in experiments in which the antigen was added to tissues from sensitized rats immediately after equilibration. Results of Cl^- fluxes in these experiments were similar to those in which the antigen had been added after a 30 min equilibration plus a 30 min basal period. Again net Cl^- secretion occurred.

The rapidity of the antigen-induced changes in electrical and transport parameters in sensitized rat intestine was particularly impressive. The antigen was added to both sides of unstripped jejunum and would have had to have passed through several tissue barriers to reach IgE-bound mast cells in the lamina propria, regardless of direction. Presumably mediators released from these mast cells acted on enterocyte receptors to activate an intracellular second messenger which in turn affected Cl^- transport and Isc. In our model neither cyclic AMP nor cyclic GMP appeared to be involved. Intestinal levels of these second messengers did not increase. However, theophylline, a phosphodiesterase inhibitor, did result in an increase in cyclic AMP. This finding ensures that the experimental techniques were appropriate.

Cyclic nucleotides are not the only intracellular messengers whose action results in intestinal net Cl^- secretion. An increase in free intracellular Ca^{++} can cause the same effect without elevating

cyclic nucleotides. Both the Ca ionophore A23187[62] and agents which increase enterocyte Ca^{++} produce net Cl^- secretion.[57] This results from inhibition of Cl^- and Na^+ absorption (Jms) and stimulation of Cl^- secretions (Jsm). Agents which are known to produce secretory effects in this manner are serotonin, carbacol, substance P and neurotensin[106]. Undoubtedly other compounds will be identified in the future which will be shown to produce effects in a similar manner. Sensitized mast cells in the intestine when challenged by antigen may release mediator(s) capable of causing increased entry of Ca^{++} into intestinal enterocytes and net secretion.

CHAPTER 6

CONCLUSIONS AND SPECULATIONS

In this chapter I will summarize the intestinal responses which we have documented during intestinal anaphylaxis and will speculate on the pathophysiology involved in producing these abnormalities. Finally, I will present some suggestions on directions for future experimentation.

6.1 Summary

We demonstrated that IgE-mediated immune reactions occurred in the small intestine of sensitized rats. Histologic sections prepared after antigen challenge consistently showed villus edema but no changes in villus height or crypt depth. However, epithelial damage was suggested by a decrease in sucrase activity in mucosal homogenates and an increase in the quantity of enterocyte constituents in the intestinal lumen. Antigen perfusion of cannulated segments of jejunum in sensitized rats resulted in decreased numbers of granulated mast cells in the lamina propria as well as decreased histamine levels in the mucosa. Forty minutes after beginning intraluminal antigen perfusion in vivo net absorption of water and electrolytes decreased significantly and absorption remained depressed even after the antigen was removed from the perfusate. In some animals a net secretory state was produced. The transport abnormalities could be blocked by the inclusion of doxantrazole, an inhibitor of mast cell degranulation, in the

perfusate. Sodium cromoglycate and histamine receptor antagonists did not prevent the abnormalities.

Results from the in vitro experiments showed that the initial effect of the antigen on intestine from sensitized rats took place almost immediately. The potential difference and short-circuit current increased significantly within three minutes of adding the protein to both sides of full-thickness jejunum. These electrical changes coincided with net movements of chloride toward the mucosal side of the intestine against its electrical gradient. The chloride secretion was not associated with changes in intracellular cyclic nucleotides.

6.2 Speculations

Examination of the events which occur during intestinal anaphylaxis suggests that the pathophysiology is complex, perhaps involving several processes.

In the in vitro experiments changes in the electrical parameters of the intestine were evident within minutes, whereas in the in vivo marker perfusion studies the transport abnormalities did not become apparent until at least 20 minutes after intraluminal instillation of the antigen. The time lag in the in vivo studies probably reflects the time involved for passage of the antigen across the epithelium to the lamina propria where the mast cells reside. Keljo, who studied the transport of horseradish peroxidase (HRP) across piglet intestinal epithelium in vitro, showed a lag phase of approximately 20 minutes between addition of HRP to the mucosa and its appearance on the

serosal side of the tissue[107]. This lag was the same regardless of the presence or absence of a Peyer's patch in the segment studied, although the presence of lymphoid tissue did result in an increased transport rate of HRP following the lag. In our in vivo studies the 15 cm length of jejunum perfused invariably contained several patches, while in the Ussing chamber studies patches were avoided. However, in the chamber studies antigen was added to both sides of the intestine. If passage through or between epithelial cells presents the main barrier for immunologically reactive protein then we would have to assume that the muscularis and serosa are far more permeable to large molecules.

Even after the antigen gains access to the mucosal mast cells a series of events must take place before degranulation occurs. Using rat peritoneal mast cells, Ishizaka showed that membrane methylation reactions and calcium entry into the cell precede mediator release [108]. The time for these events to reach a maximum was shown to be approximately two minutes. However, released histamine could be measured as soon as 30 seconds following bridging of IgE receptors. After mediator release another series of membrane events involving the enterocyte must take place before changes in intestinal electrical parameters could become evident. Consideration of the numerous reactions and interactions involved makes the three minute interval between antigen addition and observed effect seem amazingly short but still within the realm of physiological processes.

Which of the many mast cell mediators was responsible for the

ion transport abnormalities? The effect of exogenous application of many of these agents on ion transport across intestinal tissue in vitro has been examined by others. Those which have been shown to produce net chloride secretion are histamine[43], serotonin, VIP and prostaglandins[103]. Both prostaglandins and VIP result in increased levels of intracellular cyclic AMP in enterocytes. Since we did not demonstrate increases in cyclic AMP we would have to discount the effect of either of these agents as being of prime importance. However our results do not rule out the possibility that Ca^{++}-calmodulin was involved in increasing the turnover of intracellular cyclic nucleotides. The mode of action of histamine has not been defined, but our results demonstrated that antihistamines did not prevent the decreases in absorption which occurred in response to antigen in sensitized rats. Serotonin is contained in rat mast cells, and produces chloride secretion by a mechanism that operates by stimulation of entry of calcium into enterocytes, rather than by increases in cyclic nucleotides[103]. Therefore, serotonin may be involved in producing transport changes in our model.

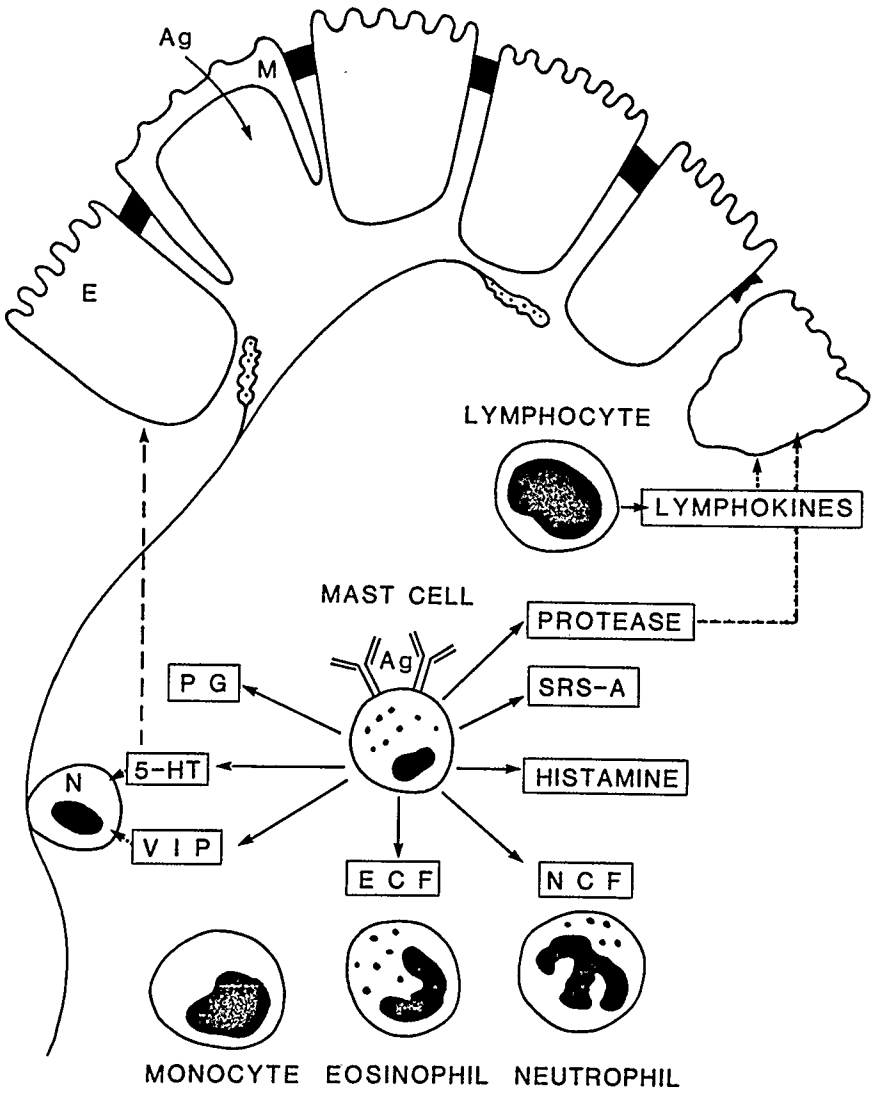
Another possibility should be considered. In a section of intestine prepared before antigen challenge the number of mast cells is relatively small compared to the number of epithelial cells (5-6 compared to > 120 in a villus-crypt unit). Also the mast cells are not located in immediate juxtaposition to the enterocytes. Therefore, an amplification system may exist which magnifies the response. This amplification system could be endocrine, paracrine or neurocrine in

nature. A likely possibility is that a mediator released from mast cells acts on elements of the enteric nervous system which results in an expanded response. Acetylcholine (ACh) may be the final mediator which exerts its effects on the enterocytes (Figure 15). Cholinergic agonists, when administered on the serosal side of intestinal tissue, cause increased calcium entry into enterocytes and electrical and ion flux changes similar to those produced in our system[107]. Intracellular levels of cyclic nucleotides are not effected. Studies by Morris and Turnberg have shown that inhibition of ACh enzymatic degradation by neostigmine or eserine causes secretion[109]. Indeed, Gaginella has demonstrated a lack of receptors for serotonin on mucosal enterocytes [110] but serotonin does stimulate ACh release from guinea pig myenteric plexus[111]. An alternative explanation is that mast cell mediators inhibit release of norepinephrine (NE). Tapper suggests that a balance exists between absorptive tone, due to NE and secretory tone caused by ACh[112]. Prostaglandins inhibit release of NE[113], and secretion could result from an unopposed cholinergic tone.

Donowitz has studied the relation between intracellular free calcium and ion movements. Early studies in rabbit ileum demonstrated that increases in cellular calcium inhibited absorption of sodium and chloride and resulted in chloride secretion[57]. An effect both on the microvillus membrane and on the basolateral membrane seem to be involved[114,115]. Evidence also exists that calcium-calmodulin induces phosphorylation of transport proteins[116]. Calcium may have an effect on the link between the Na/H and the Cl/OH absorptive processes since

Figure 15: Diagram of possible mechanisms responsible for pathophysiology during intestinal anaphylaxis.

E = enterocyte; M = microfold "M" cell; N = neuron of the enteric nervous system; ECF = eosinophil chemotactic factor; NCF = neutrophil chemotactic factor; SRS-A = slow reacting substance: leucotrienes; PG = prostaglandins; S-HT = serotonin; VIP = vasoactive intestinal peptide; Ag = antigen.


Ag
M
E
LYMPHOCYTE
LYMPHOKINES
MAST CELL
PROTEASE
Ag
P G
SRS-A
5-HT
HISTAMINE
N
V I P
E C F
N C F
MONOCYTE
EOSINOPHIL
NEUTROPHIL

free calcium and cellular pH are also linked and pH effects Na and Cl absorption[117].

In our experiments net chloride secretion, involving reduced movement toward the serosa and increased movement toward the mucosa, appears to be the driving force for the other transport abnormalities observed in vivo. Movement of chloride into the lumen would create an electrical gradient attracting cations such as sodium and potassium. These ions may move into the lumen from the intracellular fluid and/or from the bloodstream via the paracellular route. Water would follow passively attracted by the osmotic gradient.

Most experimental situations which result in changes in ion transport similar to those which we have documented do not involve epithelial damage. However, intestinal biopsies in clinical situations of food protein hypersensitivity have demonstrated that a lesion often exists. In one case study a sensitive child developed a lesion consisting of severe villus blunting within 24 hours of challenge with egg albumin[100]. The mechanism responsible for this type of injury is unclear but probably reflects the effect of immune factors on the epithelium. When mast cells degranulate chemotactic chemicals are released which attract eosinophils and neutrophils to the site. Monocytes and basophils are attracted by factors released from lymphocytes. In general, villus atrophy and changes in epithelial structure occur when the intestinal mucosa becomes inflamed, regardless of the initiating stimulus. Processes that lead to tissue injury may be initiated by substances that are produced by immunologically committed lymphocytes

upon exposure to the antigen. One described lymphokine, lymphotoxin, has been shown to cause cellular destruction[118].

The experiments in which we demonstrated enterocyte constituents in the lumen were relatively long involving four injections of antigen in a four-hour period. However, close examination of sections prepared after only one hour of antigen perfusion also revealed cellular material over the tips of the villi. Miller has recently described the release of a protease as an initial event during mast cell degranulation[119]. This protease is extremely specific in its action, attacking only collagen-like material present in the epithelial basement membrane of the intestine[120]. When present in high concentration, this protease can result in detachment of enterocytes[121]. In our isolated loop studies perhaps protease action resulted in a more fragile epithelium. This type of effect could explain the lesion of villus atrophy frequently encountered in individuals who suffer from food protein hypersensitivity.

6.3 Future Research

The experiments which I have described have only begun to explain the mechanisms producing pathophysiology during IgE-mediated reactions in the intestine. The identity of the mediator(s) involved can be further defined by the use of additional inhibitors. Indomethacin and benoxaprophen inhibit metabolism of arachadonic acid and could be used to examine the effect of prostaglandins and leucotrienes. A specific antagonist, cyproheptadine, also exists for

serotonin. Tetrodotoxin, which inhibits fast sodium channels in nerves only and blocks release of neurotransmitters, could be used to examine the role of the enteric nervous system. Nicotinic and muscarinic receptor blockers would yield information on the action of ACh. The role of calcium in producing chloride secretion could be investigated by eliminating calcium from the bathing solution in the Ussing chambers or adding verapamil, a calcium channel blocker.

The epithelial injury should be examined in more detail and related to measurement of protease in the tissue and in serum. The effect of increasing doses of antigen and lengths of time of exposure would provide useful information. As well as the parameters we have already examined the integrity of the epithelium could be determined by examining response to glucose at various times. Damage to villus cells can be detected by an absence of glucose-stimulated sodium transport. The increased mucosal permeability during anaphylaxis previously reported by others might also be caused by protease action. We could measure the appearance in the lumen of intravenously-injected Evans blue dye bound to serum albumin.

Results from Ussing chamber studies could yield information on the route the protein takes to reach the mast cells. Effects of addition of antigen to the mucosal side of tissue with and without Peyer's patch lymphoid tissue could be compared.

These proposed studies would still involve the upper small intestine. Undoubtedly, other regions of the small and large intestine would demonstrate pathophysiology under similar provocation.

Defining the abnormalities produced by immune factors acting on the intestinal epithelium could have enormous clinical relevance. Many entities have been described in which mucosal mast cell numbers are increased. If the pathophysiology of even a small proportion of these can be explained by the events we have described we will have made a substantial contribution to medical science.

CHAPTER 7

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