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Transepithelial Transport of Antigen: Novel Mechanisms in Food Allergy

By

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Abstract

Food allergies are a significant clinical problem, with symptoms including diarrhea, vomiting, or systemic anaphylaxis. To elicit allergic reactions, antigens must first cross the intestinal epithelium. The purpose of my studies was to examine macromolecular transport across intestinal epithelium, and the effect of sensitization and immune activation on transepithelial antigen transport.

Rats were sensitized to a model protein antigen, horseradish peroxidase (HRP) by injection with adjuvants. Intestinal segments were removed and mounted in Ussing chambers for the study of transepithelial movement of HRP. Electron microscopy analysis of HRP transport showed that specifically sensitized rats transported HRP across the epithelium in greater amounts, and more rapidly, than naïve controls or rats sensitized to an irrelevant antigen. After the hypersensitivity response, there was a significant increase in HRP flux across the intestinal epithelium in HRP sensitized, but not control rats. This was accompanied by an opening of the epithelial tight junctions to allow paracellular flow of antigen. Sensitized mast cell deficient rats also had an enhanced initial uptake of antigen, but did not develop a non-specific decrease in epithelial barrier function.

The role of interleukin-4 (IL-4) in the regulation of transepithelial antigen transport was examined. Treatment of human epithelial monolayers with IL-4, or with serum from atopic patients, caused a significant increase in transepithelial transport of HRP. Antibodies against IL-4 abolished the effect of atopic serum on

transepithelial HRP transport. Electron microscopy analysis showed an increase in both transcellular and paracellular HRP transport.

These studies show that transepithelial transport of antigen is profoundly altered by sensitization, and that mast cells and interleukin-4 enhance the delivery of antigen across the intestinal epithelium.

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Epithelial Function in Health and Allergy

Food Allergy

Adverse reactions to food are perceived to be a major health problem in developed countries. It has been reported that up to one third of American households have made changes to their food choices due to the belief that a member of the household has a food allergy (Sloan and Powers, 1986). Abnormal reactions to food can be classified as allergy (having an immunological basis) or intolerance (due to host characteristics or toxic properties of the food). Clinical symptoms can be similar between allergy and intolerance including diarrhea, abdominal pain, vomiting, and skin rashes. Food allergy is diagnosed by demonstrating that withdrawal of the food leads to symptom resolution, and subsequent challenges with the food lead to consistent presentation of symptoms. The current gold standard for food allergy diagnosis is a double-blind placebo-controlled oral food challenge (Sampson, 1997). Using this method of diagnosis, the prevalence of food allergy in European and North American populations has been estimated at 1-2% (Niestijl Jansen *et al.*, 1994, Young *et al.*, 1994). The rising prevalence of allergy to certain foods such as peanut raises considerable concern due to the more severe clinical outcome (high incidence of anaphylaxis) (Sampson, 1997). The prevalence of food allergy is higher in children, and a proportion of individuals outgrow their allergies with age. The mechanism responsible for both the development and loss of sensitization to food proteins remains unclear. Patients with other atopic disorders (atopic dermatitis, asthma, allergic rhinitis) have a higher prevalence of food allergy compared to the general population, indicating that the susceptability to IgE production may be a very important factor. The observation of a familial pattern of food allergy suggests a genetic component, as has been hypothesized for

other atopic disorders. The intestinal epithelium plays a very important role in food allergy, as it is the primary barrier between the allergen and the mucosal immune system, and also is responsible for many of the intestinal symptoms observed after allergen contact. Currently the only means of food allergy management is allergen avoidance, however this remains problematic due to "hidden allergens" present in processed foods. By clarifying the role of the epithelium in antigen transport to the mucosal immune system, and the impact of immune activation on epithelial physiology, therapeutic strategies targetting the epithelium may become feasible.

The Intestinal Epithelium

Throughout the GI tract, a single layer of epithelial cells provides a barrier separating the external environment (luminal contents) from the internal milieu. The intestinal epithelium is composed of a number of cell types derived from a homogeneous stem cell population found in the intestinal crypts. The majority of cells are columnar enterocytes that mature as they travel up the crypt-villus axis, changing from an ion secretory phenotype in the crypt region to an absorptive phenotype on the villus (Goodlad, 1989). Interspersed between the enterocytes are mucin-producing goblet cells and enteroendocrine cells. Epithelial cells migrate up from the crypt and are shed at the villus tip, with an average lifespan of approximately 4-7 days. Paneth cells migrate to the base of the crypts from the stem cell population and secrete defensin-like molecules termed cryptdins that have anti-microbial properties and may function in host defense (Ouellette *et al.*, 1997). Interspersed between epithelial cells are intraepithelial lymphocytes (IEL) that comprise a phenotypically unique subset of lymphocytes (Jarry *et al.*, 1990). Microfold, or M cells are very thin cells that are located in dome regions of

lymphoid patches and are specialized for transport of particulate antigens to the underlying immune cells (Amerongen *et al.*, 1992).

Nutrient Absorption

One of the key functions of the epithelium is to absorb nutrients from the luminal contents. Most nutrient absorption is linked to an inward sodium gradient established by the Na^+/K^+ ATPase (transports 3 Na^+ out and 2 K^+ into the cells) located in the basolateral membrane. After proteins and carbohydrates are digested by pancreatic and brush-border enzymes, amino acids and monosaccharides are transported across the apical membrane by sodium-linked co-transporters, and peptides are taken up by proton-linked transporters. Exit from the epithelium occurs via facilitated diffusion, and nutrients then diffuse into the capillaries located at the core of each villus (Alpers, 1994). Larger proteins can also enter the epithelium in an intact form by endocytosis and undergo digestion within lysosomes. There is evidence that a small quantity of protein can escape lysosomal degradation and exit the cell by exocytosis across the basolateral membrane (Cornell *et al.*, 1971, Heyman *et al.*, 1982). This process may be important in triggering an immune or inflammatory response.

Ion Transport

As stated earlier, nutrient absorption and ion transport are intimately linked processes. The Na^+/K^+ ATPase located at the basolateral membrane establishes an inward gradient for Na^+, an outward gradient for K^+, and a net negative charge inside the cell. Na^+ absorption at the apical membrane occurs through Na^+ channels and co-transporters. In villus cells, Na^+ and solute absorption provide an osmotic force for water absorption. In crypt cells, a $Na^+/K^+/Cl^-$ cotransport in the basolateral membrane creates a high intracellular concentration of Cl^-.

Opening of Cl^- channels in the apical membrane upon stimulation produces an outward flow of Cl^- ions. Na^+ ions and water passively follow the flow of Cl^- through the tight junctions, resulting in a net secretion of ions and water (Chang and Rao, 1994). Secretion of HCO_3^- also occurs through apical membrane channels, and is of significant importance in the duodenum where it contributes to the neutralization of gastric secretions (Flemstrom, 1994). Secretion of ions and water is an important normal physiological process that provides ions for absorptive co-transporters, and water to solubilize luminal contents. Water secretion is also of value in host defense, by washing away mucosal irritants or pathogenic microbes. If secretion exceeds absorption, the net result is water loss, leading to diarrhea and subsequent dehydration.

Barrier Function

The intestinal epithelium carries out the opposing functions of selective absorption and maintenance of a barrier against luminal contents. Goblet cells secrete mucin that forms a gel over the epithelium. The intestinal epithelial glycocalyx (glycoprotein molecules anchored in the brush border membrane) has been shown to trap larger molecules and restrict contact with the epithelial membrane (Frey *et al.*, 1996). This physical barrier may be an important component of host defense against luminal microbes. However, most antigenic proteins are capable of diffusing through the glycocalyx to reach the cell surface. The lipophilic cell membrane is impermeant to charged molecules, and therefore most molecules can only enter the cell if there is a specific transporter present. The route between cells is occluded by a junctional complex consisting of tight junctions, adherens junctions, and desmosomes. Although all of the components of the junctional complex appear to be important for maintaining epithelial continuity, studies utilizing electron-dense tracers have demonstrated that larger

molecules can diffuse through the paracellular space to the level of the tight junction, where they are stopped (Cornell *et al.*, 1971, Cerejido, 1991). Tight junctions are composed of anastamosing grooves and ridges that provide a continuous seal capable of blocking macromolecules as small as 1900 Da (Madara and Trier, 1982).

The Mucosal Immune System

The intestine comprises the largest immune organ in the body, containing approximately 40% of the total number of immune cells. The gut-associated lymphoid tissue, or GALT, is located in three compartments: organized in lymphoid follicles or Peyer's patches, diffusely distributed throughout the lamina propria, and within the epithelial layer. Many immune cells lie in close proximity to the epithelium, and in the past 10 years it has become increasingly clear that immune cells are capable of modulating epithelial function, particularly ion transport functions. Considerably less is known about immunomodulation of epithelial barrier function, particularly in relation to transport of antigenic proteins.

Cells and Mediators

T cells can be subdivided based on expression of the CD4/CD8 surface markers. $CD8^+$ cytotoxic/suppressor cells recognize antigen associated with MHC class I, while $CD4^+$ helper cells recognize antigen associated with MHC class II, and secrete cytokines that coordinate cell-mediated and humoral immunity. T helper cells can be further subdivided into Th1 and Th2 cells based on their cytokine profile: IL-2 and IFN-γ secreting cells are termed Th1, while IL-4, -5, -6, and -10 secreting cells are termed Th2. Th1 and Th2 cells promote cell-mediated and humoral immunity, respectively. These divisions are not absolute, it has been demonstrated that CD8 cells can function as helper cells (Seder *et*

al., 1998), and cytokine profiles falling in between the Th1/Th2 categories have been found (Kelso, 1995). Th0 cells producing both IL-4 and IFN-g have been suggested to be precursor cells that are capable of differentiating into Th1 or Th2 cells (Miner *et al.*, 1998). Thr cells are regulatory T cells that produce TGFβ and IL-10 (Groux *et al.*, 1997). Several cytokines produced by T cells have been demonstrated to modulate epithelial function (discussed in further detail below). Intraepithelial lymphocytes (IELs) are primarily $CD8^+$ and T cell receptor α/β+, but a proportion of IELs are TCR γδ+, and this cell population increases in some gastrointestinal diseases (Jarry *et al.*, 1990). IELs are granular cells, and have been shown to be capable of secreting cytokines that can affect epithelial function (Yamamoto *et al.*, 1993). It remains to be determined if IELs have a unique function in mucosal immunology as suggested by their unusual phenotype.

Stimulation of B lymphocytes within the intestinal mucosa induces secretion of antibodies, primarily of the IgA isotype. Secretory IgA (sIgA) is transported across the intestinal epithelium and can interact with antigens in the lumen to restrict their penetration through the epithelium (Manzanec *et al.*, 1993). Other immunoglobulin isotypes may also be produced, such as IgM. The lamina propria appears to have a higher proportion of IgE producing B-cells compared to other peripheral lymphoid organs (Brown *et al.*, 1975), and there is evidence that IgE secreted into the intestinal lumen is produced by mucosal B cells (Negrao-Correa *et al.*, 1996). IgE can function in a host-protective manner against intestinal parasites (Ahmad *et al.*, 1991), but also plays a pathophysiological role in food allergy (Sampson, 1997).

Macrophages, dendritic cells, and B cells participate in acquired immunity by presenting antigen to T cells within the context of MHC class II. Macrophages and neutrophils can also participate in innate immunity by phagocytosing

microbes and releasing proinflammatory mediators in response to bacterial products. Mast cells and eosinophils are normally found within the intestinal mucosa, and increase in number in response to helminth infection (Befus *et al.*, 1979, Capron *et al.*, 1986). In addition to playing a role in parasite rejection, they have been implicated in the pathophysiology of allergic diseases.

Normal Response to Food Proteins

In normal individuals, the ingestion of food does not lead to activation of the mucosal immune system, despite the ability of small amounts of intact protein to reach the lamina propria. Food-specific antibodies of the IgG, IgM, and IgA isotypes are commonly found even in normal individuals (Johansson *et al.*, 1984). Patients with inflammatory diseases of the intestine have elevated levels of food-specific antibodies (May et al, 1977), likely reflecting the increased exposure of the mucosal immune system to food antigens due to a defect of epithelial barrier function. Despite these elevated levels of food-specific antibodies, no clinical symptoms are observed in such patients after ingestion of the antigenic food. Normally, intake of proteins by the oral route leads to a state of antigen-specific peripheral tolerance, a process termed oral tolerance. Single or multiple feeds of antigen inhibit IgE production, delayed type hypersensitivity, T cell proliferation, contact sensitivity and CD8+ T cell responses (reviewed by Strobel and Mowat, 1998). Transfer of oral tolerance has been shown with CD4+ cells (Hornquist *et al.*, 1996), and CD8+ TCR γδ cells (McMenamin *et al.*, 1995), indicating a role for regulatory cells. There is evidence that antigens presented by epithelial cells preferentially stimulate CD8+ suppressor cells (Mayer and Shlien, 1987). Epithelial cells can also present antigen to CD4+ T cell hybridomas (Hershberg *et al.*, 1997). However, the lack of costimulatory molecules on epithelial cells may result in T cell anergy. There is also some evidence that

mucosal dendritic cells can function as tolerogenic antigen presenting cells (Viney *et al.*, 1998). Aberrations in this normal mechanism of tolerance induction may be an important factor in the development of food allergy.

Intestinal Hypersensitivity

Description of Models

A number of rodent models of food allergy have been developed to study the impact of hypersensitivity reactions on intestinal physiology. These models have in common an immediate hypersensitivity gut reaction in response to antigen challenge, and intestinal manifestations include water and ion secretion and altered intestinal motility (Perdue and McKay, 1993). These alterations in intestinal functions provide a means for studying mechanisms underlying clinical symptoms of food allergy such as diarrhea, cramping, and abdominal pain. There are two main categories of models of intestinal hypersensitivity: 1) animals sensitized by oral or systemic exposure to food antigens, and 2) animals sensitized following parasite infections.

Parasitized rodent models have yielded interesting information on the impact of hypersensitivity reactions on intestinal physiology. Rats previously infected with the nematodes, *Nippostrongylus brasiliensis* or *Trichinella spiralis*, develop an anaphylactic response to worm antigen challenge after they have cleared the primary infection (King *et al.*, 1984, Harari *et al.*, 1987). The parasitized rodent models develop an exaggerated immunological response, characterized by very high IgE levels, eosinophil infiltration and mastocytosis (Befus *et al.*, 1979) that are very useful for studying the impact of mast cell activation on epithelial physiology, but may not be an ideal model of food allergy

given that parasite infection produces a marked inflammation that has a prolonged effect on intestinal function (Barbara *et al.*, 1997).

Guinea pigs that are given cow's milk to drink for approximately 3 weeks become sensitized to β-lactoglobulin (β-LG) and develop anaphylactic responses to β-LG challenge 3 days after milk withdrawal (Cuthbert *et al.*, 1983). Sensitization can be passively transferred to naive animals by serum, and is mediated by antibodies of the IgG1 isotype (Baird *et al.*, 1987). The β-LG model has been useful for determining the epithelial response to intestinal hypersensitivity reactions, and many of the findings have been confirmed in other models of intestinal hypersensitivity.

Another model of intestinal hypersensitivity is the systemically sensitized rat or mouse. Rodents injected with ovalbumin (OVA), using alum ± pertussis vaccine as adjuvants develop intestinal hypersensitivity responses to OVA by 10-14 days post-injection (Perdue *et al.*, 1984, Perdue and Gall, 1986). This model has been used not only to study intestinal hypersensitivity responses, but is also one of the most frequently utilized animal models of asthma. Injection of serum from a sensitized to a naive rodent passively transfers the sensitization, and there is evidence for both IgE and IgG1 involvement in the hypersensitivity response (Kosecka and Perdue, unpublished observations, Oshiba *et al.*, 1996). This model has allowed the use of gene-targetted "knock-out" mice to determine the role of a number of immune mediators in the sensitization process.

Regulation of IgE Production

Immediate hypersensitivity reactions are mediated by multivalent antigen cross-linking IgE antibodies bound to the surface of mast cells via high affinity receptors (FcεRI). Patients with food allergy do not always have elevated levels of IgE, but most will have detectable levels of antigen-specific IgE, and

immediate skin reactivity to intradermal antigen (Crowe and Perdue, 1992). Sensitized or parasitized rodents generate elevated levels of antigen-specific and total IgE antibodies that are detectable 7 days after infection or sensitization. (Kosecka *et al.*, 1994, Negrao-Correa *et al.*, 1996). B cells present antigen to T helper cells through MHC II, and the production of IL-4 from T cells and CD40/CD40 ligand interaction stimulates production of IgE (Romagnani, 1997). IL-4 is necessary for the generation of an IgE response in response to parasite infection or systemic sensitization, as demonstrated with the use of neutralizing anti-IL-4 antibodies (Finkelman *et al.* 1988) and IL-4 knockout mice (Kopf *et al.* 1993). The cytokine IL-13 mimics the function of IL-4 on B cells and monocyte/macrophages, but not T cells (Zurawski *et al.*, 1994). The role of IL-13 in IgE production in rats is as yet unknown. The finding that mast cells, basophils, and eosinophils express CD40 ligand (Ohkawara *et al.*, 1996), and produce IL-4 has led to the suggestion that these cells may also have a role in stimulation of IgE production, and mast cell stimulated IgE production has been demonstrated *in vitro* (Gauchat *et al.*, 1993).

As IL-4 is an important regulatory cytokine in IgE production, it would be expected that it would be elevated in allergic disease. In atopic humans, IL-4 expression is upregulated in peripheral blood mononuclear cells (Tang and Kemp, 1994), and at mucosal sites in allergic patients, including airways (Bradding *et al.*, 1993) and intestine (Hauer *et al.*, 1997). T cells, eosinophils, and mast cells have been shown to produce IL-4 at mucosal sites by *in situ* hybridization and immunohistochemical staining (Bradding *et al.*, 1993, Nonaka *et al.*, 1995). The stimuli for IL-4 production in allergic humans, and in sensitized rodent models, remains to be clarified. Many of the animal models utilize pertussis vaccine or toxin as an adjuvant, and pertussis toxin stimulates IL-4 production from isolated T cells (Mu and Sewell, 1993). However, sensitization

can be achieved in the absence of pertussis toxin, and therefore other adjuvants (i.e. pollutants, or molecules that retain proteins in tissues) may be involved. Other factors, such as the nature of the antigen itself or the type of antigen presenting cell, may also determine the cytokine output of the T helper cell.

Epithelial Response to Antigen Challenge

The impact of the intestinal hypersensitivity reaction on epithelial ion transport has been extensively studied using *in vitro* segments of intestine placed in Ussing chambers. This apparatus voltage clamps the tissue to a potential difference of zero (i.e., electrogenic active transport across the tissue is matched by an equal and opposite short-circuit current (Isc) to maintain the zero potential difference). Thus, Isc is a measure of the net active ion transport across the tissue. The Ussing chamber apparatus also effectively maintains separate luminal and serosal compartments, allowing for flux measurements of permeability markers across the tissue.

Intestinal segments from ovalbumin sensitized rats respond to antigen challenge with a rapid increase in Isc. Delivery of antigen to the serosal side of the tissue produces a large biphasic increase in Isc, with a large peak beginning 10-15 seconds after challenge, followed by a sustained increase in Isc compared to baseline readings (Crowe *et al.*, 1990). This biphasic increase in ion secretion has also been observed in tissues from β-LG sensitized guinea pigs after serosal β-LG challenge (Javed *et al.*, 1992), and *T.spiralis* infected rats after worm antigen challenge (Castro *et al.*, 1987). Studies using chloride channel blockers and chloride-free buffers indicate that the majority of the increase in Isc is due to chloride secretion (Crowe *et al.*, 1990, Cuthbert *et al.*, 1983, Harari *et al.*, 1987). *In vivo* studies using rodents prepared with intestinal loops to measure fluid secretion have shown that challenge with antigen (OVA, β-LG, worm larvae)

leads to prolonged hypersecretion (Perdue *et al.*, 1984, Cuthbert *et al.*, 1983, Castro *et al.*, 1979), indicating that the changes in ion secretion observed *in vitro* result in alterations in fluid handling by the small intestine. This increase in luminal fluid, together with disrupted motility patterns also observed after challenge in sensitized rats (Scott *et al.*, 1988), are likely contributing factors to symptoms of diarrhea after exposure of food-allergic patients to dietary allergens.

The majority of *in vitro* studies determining the effect of antigen challenge on epithelial transport function have used serosal antigen delivery. Although this route of challenge is adequate for determining the effect of mast cell activation on the epithelium, it bypasses the first step of antigen challenge *in vivo,* namely the transport of antigen across the intestinal epithelium. Crowe *et al.* (1990) characterized the ion secretory responses in response to luminal OVA challenge to intestinal segments from OVA sensitized rats. Luminal antigen challenge produces a rapid increase in Isc, that is slightly delayed compared to serosal challenge, ie beginning within 3 min after challenge compared to 15 seconds after serosal challenge (typical Isc tracings are shown in Fig 1.1). The delay is thought to be due to the time required to cross the epithelium, since luminal and serosal antigen challenge appear to degranulate the same population of mast cells (Kosecka and Perdue, unpublished observations). Luminal Isc responses can be classified as biphasic, similar to the serosal response but with a lag period, or monophasic, with a slower sustained increase lacking the rapid initial peak. Clearly, the normal intestinal epithelium does not provide an adequate barrier to prevent the hypersensitivity response to luminal antigen.

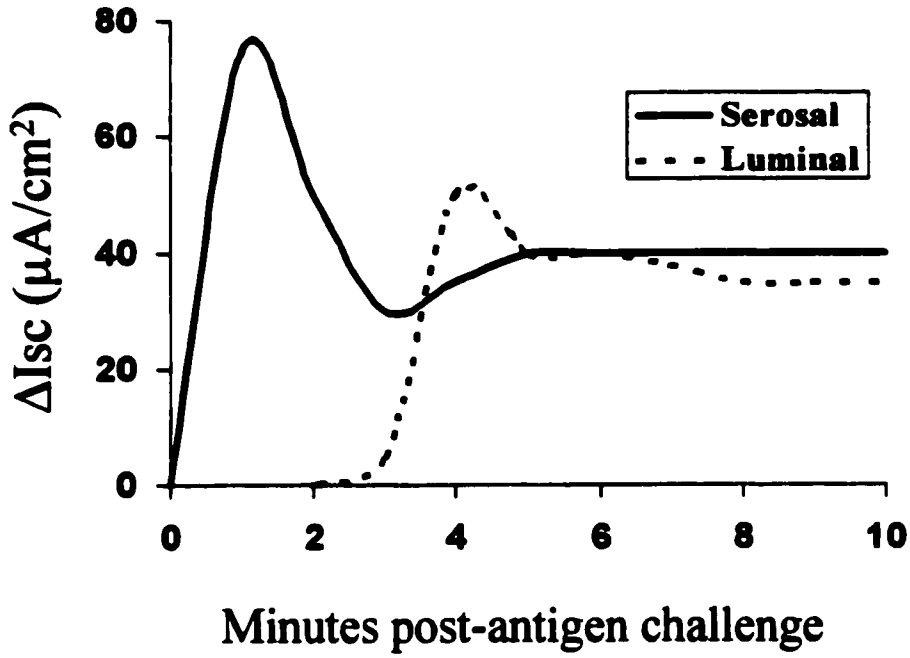


Figure 1.1: Representative short-circuit current (Isc) tracings from Ussing chambered intestinal segments removed from ovalbumin (OVA)-sensitized rats after addition of OVA to the luminal or serosal buffer. The Isc response began approximately 15 s after serosal challenge, and 3 min after luminal challenge.

The impact of the hypersensitivity reaction on epithelial barrier function has been of considerable interest. *In vivo* studies using OVA sensitized rats demonstrated that addition of OVA to the lumen of intestinal loops resulted in a large increase in lumen-to-blood transport of the permeability marker, ^{51}Cr-EDTA (Crowe *et al.*, 1993). Heyman *et al.* (1990) challenged intestinal segments from β-LG sensitized guinea pigs with serosal β-LG and showed an increase in the luminal to serosal transport of intact horseradish peroxidase (HRP). Intravenous challenge of *N. brasiliensis* infected rats with worm antigen also produces an increase in absorption of ^{51}Cr-EDTA and OVA from the lumen (Ramage *et al.*, 1988), and leak of albumin and mast cell protease to the lumen (Scudamore *et al.*, 1995). A number of other studies have also demonstrated an enhanced uptake of "bystander proteins" after systemic antigen challenge in sensitized rodents (Bloch *et al.*, 1979, Bloch *et al.*, 1987, Kleinman *et al.*, 1989). Permeability studies have also been done in allergic patients, showing an enhanced uptake of inert probes after direct luminal challenge with antigen (Knutson *et al.*, 1996). Clearly, antigen challenge in a sensitized host has a profound impact on both ion transport and barrier functions of the intestinal epithelium.

Role of Mast Cells and Nerves

Antigen challenge to the serosal side of intestinal segments produces an almost immediate epithelial ion transport response, while addition of antigen to the luminal side induces a slightly delayed response, indicating that effector cells mediating the hypersensitivity response are located below the level of the epithelium. Mast cells are located in the lamina propria immediately below the level of the epithelium, in an ideal position to modulate epithelial function. High affinity receptors for IgE (FcεRI) are found on the surface of mast cells. Studies

with isolated mast cells have shown that cross-linking of IgE bound to FcεRI by multivalent antigen leads to the release of a number of pre-formed and rapidly synthesized mediators such as amines (histamine, serotonin), lipid metabolites, proteases, nitric oxide, and cytokines (Marshall and Bienenstock, 1994, Saperas, 1996). Other resident immune cells such as eosinophils and macrophages possess low-affinity receptors for IgE (FcεRII), and can be activated by IgE-antigen immune complexes to release mediators similar to those released by mast cells.

Mast cell stabilizers such as doxantrazole (a mast cell stablizer that acts on all mast cells, including mucosal mast cells), but not sodium cromoglycate (a mast cell stabilizer with a greater efficacy on connective tissue mast cells) inhibit the fluid accumulation in intestinal loops of OVA sensitized rats after antigen challenge (Perdue *et al.*, 1985, Mourad *et al.*, 1995). Doxantrazole has also been shown to effectively inhibit the fluid secretion observed in the β-LG model (Theodorou *et al.*, 1994). Although these studies provide strong support for the hypothesis that mast cells mediate the ion secretory response to antigen challenge, more conclusive evidence has come from studies on mast cell deficient mice (Perdue *et al.*, 1991). *W/W^v* mast cell deficient mice and +/+ control littermates were sensitized to OVA, and intestinal segments were challenged on the serosal surface with OVA *in vitro*. *W/W^v* mice had a significantly reduced ion secretion response to OVA that could be restored by reconstitution of bone marrow cells from +/+ donors. Interestingly, *W/W^v* mice maintained a small response to antigen challenge, indicating that another cell type in the sensitized mouse could also respond to specific antigen.

Mast cells release a wide array of mediators in response to IgE cross-linking, and a number have been implicated in the hypersensitivity response through the use of pharmacological inhibitors. Receptor antagonists against the

H_1 histamine receptor and serotonin receptors have been used to effectively inhibit the ion secretory response to antigen challenge in all the main models of intestinal hypersensitivity (Castro *et al.*, 1987, Crowe *et al.*, 1990, Perdue *et al.*, 1991, Javed *et al.*, 1992). Inhibition of cyclooxygenase by indomethacin or piroxicam also inhibits the ion secretion response (Castro *et al.*, 1987, Perdue *et al.*, 1991). The typical biphasic response to antigen challenge appears to be differentially mediated, with histamine and serotonin regulating the initial Isc peak, and prostaglandins playing a larger role in the sustained elevation of Isc after challenge (Castro *et al.*, 1987, Perdue *et al.*, 1991, Mourad *et al.*, 1995). Other mediators such as leukotriene D_4 and platelet activating factor (MacNaughton *et al.*, 1992), interleukin-1 (Theodorou *et al.*, 1994), and nitric oxide (Fargeas *et al.*, 1996) also contribute to the ion and water secretory responses to antigen challenge.

Bidirectional signals between mast cells and nerves appear to be important in amplification of the allergic response. Activation of enteric nerves stimulates a rapid transient epithelial ion secretion (Perdue *et al.*, 1986). Mast cell mediators have been shown to activate enteric nerves (Frieling *et al.*, 1991 and 1993). Nerves contribute to the ion secretion response to antigen challenge, but the exact role of nerves remains somewhat unclear. Castro *et al.* (1987) inhibited the Isc response to serosal worm antigen in intestinal segments from *T. spiralis* infected rats with the neutrotoxin tetrodotoxin (TTX, a blocker of fast sodium channels) and the muscarinic inhibitor atropine. These neuronal inhibitors also blocked the ion secretion response to exogenous histamine and serotonin, but not prostaglandin E_2, suggesting that mast cell mediators produce an ion secretion response by acting directly on the epithelium, and indirectly through enteric nerves. Javed *et al.* (1992) reproduced these results in the β-LG sensitized guinea pig model. Perdue *et al.* (1991) showed that pretreatment of

intestinal segments from OVA sensitized mice with TTX inhibited the response to serosal OVA challenge. This effect was dependent on the presence of mast cells, as only intestine from mast cell replete, and not mast cell deficient mice was sensitive to TTX. The initial peak, but not the sustained elevation of Isc, was sensitive to TTX. The above studies support the hypothesis that nerves can amplify the effect of mast cell activation.

In contrast to the above results, two studies in OVA sensitized rats indicated that the response to serosal antigen was not mediated by nerves. Crowe *et al.* (1990) and Kosecka *et al.* (1994) showed that TTX pretreatment inhibited the response to luminal, but not serosal antigen challenge. These studies suggested that nerves might contribute to the hypersensitivity response by enhancing uptake of antigen across the intestinal epithelium, rather than acting as a functional unit with mast cells. Some of the conflicting evidence on the role of nerves in the response to serosal antigen could potentially be explained by species differences or different modes of sensitization. Although there is conflicting evidence about the exact role of nerves in the hypersensitivity response, there does appear to be clear support for the hypothesis that nerves participate to some degree in the intestinal ion secretion response to antigen. Fig 1.2 summarizes the role of mast cells and nerves in the regulation of epithelial ion secretion during intestinal hypersensitivity.

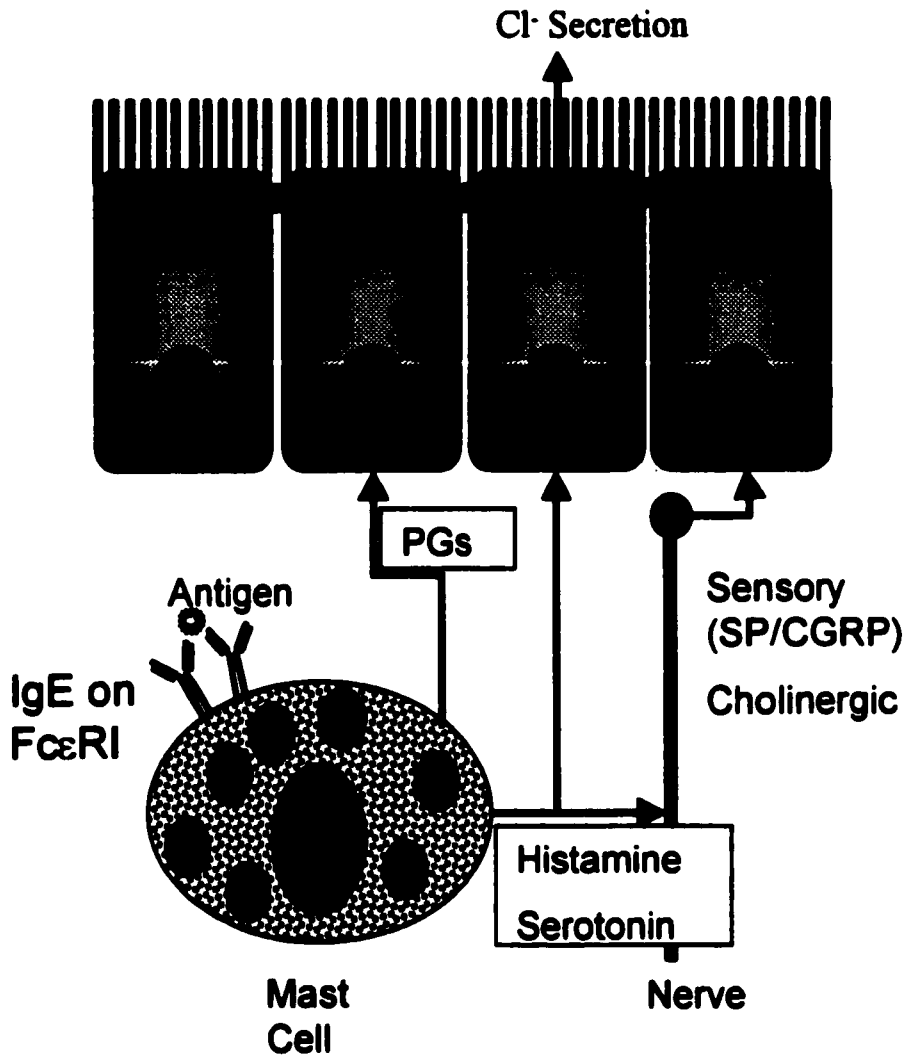


Figure 1.2: Schematic showing mechanisms involved in the intestinal hypersensitivity response. Antigen activates mast cells via IgE bound to FcεRI. Mediators released from the mast cell can activate Cl^- secretion from the epithelium directly (i.e. Prostaglandins, PGs, histamine, and serotonin) or indirectly through nerves (histamine and serotonin). Sensory nerves (containing substance P (SP) or CGRP (calcitonin gene related peptide) are involved in the secretory response in the OVA sensitized rat, while cholinergic nerves are involved in the β-LG sensitized guinea pig and *T. spiralis* infected rat.

The mechanisms responsible for antigen-induced changes in epithelial barrier function remain poorly defined. Crowe *et al.* (1993) used an *in vivo* preparation to examine the lumen-to-blood transport of the inert probe ^{51}Cr-EDTA after antigen challenge in OVA sensitized rats. Treatment of isolated intestinal segments with TTX inhibited the antigen-induced increase in ^{51}Cr-EDTA uptake, indicating a role for nerves in the regulation of epithelial barrier function. Challenge of *N. brasiliensis* infected rats with worm antigen i.v. produces a protein leak into the intestinal lumen that can be mimicked by the infusion of rat mast cell protease II (RMCP II) (Scudamore *et al.*, 1995). Inhibitor studies have not been done, but the capability of RMCP II to alter barrier function, along with the documented rise in RMCP II levels in the OVA sensitized and challenged rat (Crowe *et al.*, 1993), suggest that mast cells may also contribute to the epithelial barrier dysfunction.

Effect of Sensitization on Epithelial Physiology

The first interaction between allergen and host occurs at the epithelial surface, and there is some indication that sensitized individuals may have a reduced epithelial barrier function that could facilitate contact between allergen and mucosal immune cells. Studies on the permeability of intestinal epithelium from OVA sensitized rats (under baseline conditions, i.e. prior to antigen challenge) demonstrated a significant increase in ^{51}Cr-EDTA uptake from the lumen compared to control rats (Crowe *et al.*, 1993). Heyman *et al.* (1990) used the β-LG sensitized guinea pig to show that intestinal segments from sensitized animals had a significantly higher flux of both degraded and intact HRP (a "bystander" protein antigen in this model) across the intestinal epithelium compared to controls. ^{51}Cr-EDTA is commonly accepted as a marker of the paracellular transport pathway between epithelial cells, and the study by Crowe

et al. (1993) suggests that sensitization may induce a "leakiness" in the intestinal epithelium by altering the tight junctions. Heyman *et al.* (1990) suggested that their results supported the hypothesis that sensitization enhanced the endocytic pathway, but an increase in paracellular pathway permeability could also explain the elevated flux of degraded protein broken down by brush border proteases rather than lysosomal enzymes. Two studies in human patients have also suggested that the sensitized state may be accompanied by changes in epithelial barrier function. Majamaa *et al.* (1996) found that HRP transport across intestinal biopsies from children with food-sensitive atopic dermatitis was elevated compared to non-atopic controls. Benard *et al.* (1996) measured intestinal epithelial permeability *in vivo* and demonstrated that patients with bronchial asthma had a signficantly higher clearance of ^{51}Cr-EDTA compared to patients with chronic obstructive lung disease. Changes in barrier function in response to sensitization are of significant interest because the initiating step in any mucosal hypersensitivity reaction is the transport of antigen from the intestinal lumen to subepithelial mast cells. An alteration in epithelial barrier function could explain how antigen added to the luminal side of intestinal segments from sensitized rats initiates a hypersensitivity response (requiring subepithelial mast cells) in such a rapid manner.

Impact of Cytokines on Epithelial Physiology

Sensitization in humans and in animal models is associated with a change in immune phenotype that may be responsible for some of the changes observed in epithelial physiology. A number of studies have examined the cytokine production by ELISPOT or ELISA of culture supernatant from peripheral blood mononuclear cells (PBMC), or allergen-specific T cell clones, derived from food allergic patients. An increase in IL-4 and IL-5 production, and decreased levels of

IFN-γ in PBMCs from patients allergic to egg and cow's milk has been documented (Noma *et al.*, 1996, Hauer *et al.*, 1997). Peanut and egg specific clones produce IL-4 and IL-5, but not IFN-γ in response to allergen stimulation (de Jong *et al.*, 1996, Katsuki *et al.*, 1996). Hauer *et al.* (1997) quantified cytokine producing cells in biopsies from children with cow's milk sensitive enteropathy and found an increase in both IL-4 and IFN-γ producing cells. TNF-α has also been shown to be produced by PBMCs from children with cow's milk allergy after stimulation with milk proteins (Heyman *et al.*, 1996). Of the cytokines that may be overexpressed in food allergic individuals, a number have been documented to have direct effects on the intestinal epithelium. IFN-γ and TNF-α, predominant cytokines in cow's milk sensitive enteropathy, have been shown to decrease transepithelial resistance and increase permeability of isolated epithelial monolayers to tracer molecules (Madara and Stafford,1989, Heyman *et al.*, 1996). IL-4, which appears to be more pronounced in IgE mediated immediate food allergy, also induces a drop in transepithelial resistance across epithelial monolayers, and increases flux of small molecular weight probes, presumably by opening the tight junctions between epithelial cells (Colgan *et al.*, 1994, Sanders *et al.*, 1995, Zund *et al.*, 1996). IL-4 and IFN-γ also reduce the ion secretory response of epithelial monolayers to secretagogues such as carbachol and forskolin (Colgan *et al.*, 1994, Holmgren *et al.*, 1989). There are currently no reports on the direct effects of IL-5 on epithelial function.

Although the majority of studies examining the effect of cytokines on epithelial function have been conducted *in vitro* to eliminate any indirect effects, Ramaswamy *et al.* (1994) showed that IL-4 could induce the transport of IgE across rat intestinal epithelium *in vivo*. This effect was not a non-specific effect on epithelial barrier function, as other isotypes were not transported into the intestinal lumen. The authors proposed that this may be an important host-

defense mechanism against intestinal parasites, but it may also be relevant to food allergy in which antigen-IgE complexes are important triggers of pathophysiology.

Transepithelial Transport of Proteins

The intestinal epithelium provides a barrier to prevent the mass influx of antigenic material from the lumen, but it is well established that immunologically relevant proteins can be absorbed intact. One of the first studies to examine the uptake of antigens from the intestine was published in 1927 by Brunner and Walzer. They passively sensitized 65 human subjects to fish proteins by subcutaneously injecting them with serum from individuals with documented fish allergy. After 1 day, subjects were fed a meal of raw herring, and after a short period of time (minutes to two hours), a wheal and flare reaction was observed at the site of injection in 64 of 65 subjects tested. This study demonstrates that in normal individuals, antigen (intact enough to be able to cross-link IgE bound to mast cells) readily crosses the intestinal epithelium in immunologically significant quantities. These studies have been repeated in rodent models that demonstrate that passive cutaneous anaphylaxis can be achieved by oral delivery of the antigen (Bernstein and Ovary, 1968). Introduction of foodstuffs into different regions of the GI tract demonstrated that the small intestine was most effective in the uptake of antigens (Walzer, 1941). The small intestine contains Peyer's patches, scattered lymphoid aggregates covered with microfold epithelium (M cells) that are very efficient at transcellular uptake of antigens, and form a site of entry for a number of bacterial and viral pathogens. Although some have argued that the Peyer's patch is the only site of absorption of macromolecules under physiological conditions (Mayrhofr, 1994), there is considerable evidence for macromolecular uptake across intestinal enterocytes. Keljo and Hamilton (1983)

compared transport of HRP across intestinal segments of piglet jejunum with or without a Peyer's patch, and although they found that segments with a patch had a higher flux rate compared to non-patch tissue, it was only three-fold higher. Considering the relative surface area of enterocytes compared to M cells, the contribution of enterocytes to total uptake of macromolecules may be considerable.

Macromolecular uptake across the small intestine has been studied using Ussing chamber preparations of intestinal segments without Peyer's patches. Heyman *et al.* (1982) examined the transport of HRP across rabbit jejunum, and found that luminal to serosal transport of HRP was an energy-dependent process, required intact microtubules, and was increased by interfering with lysosomal acidification. These results suggest that HRP was actively transported by an endocytic mechanism across epithelial cells, and that intact protein escaped from lysosomal breakdown. Although the majority of studies have been done using HRP, intestinal absorption of intact bovine serum albumin (BSA) has also been examined, with similar findings (Kimm *et al.*, 1994). BSA transport across rat jejunum was also energy dependent and inhibited by microtubule disrupting agents. Addition of glucose, which upregulates the paracellular transport of small molecules, had no effect on BSA transport, suggesting that transepithelial transport occurred through a transcellular process. Energy-dependent macromolecular uptake has also been demonstrated across monolayers of the intestinal epithelial cell line CaCo-2 (Heyman *et al.*, 1990).

Transcellular Transport of Proteins

The above studies suggest that macromolecular uptake across the intestinal epithelium occurs by endocytic transport, which is supported by electron microscopy studies using HRP as a macromolecular tracer. Cornell *et al.*

(1971) injected HRP into ligated segments of rat small intestine, and monitored the absorption of HRP. HRP was observed absorbed to the apical surface membrane, within intracellular vesicular structures, in the basolateral spaces around epithelial cells, and in the lamina propria. Tight junctions maintained their integrity, and did not appear to allow the passage of HRP. This was confirmed by Walker *et al.* (1972) using everted gut sacs from rat small intestine. Invaginations of the apical membrane bud off to form phagosomes, that fuse with lysosomes to form large phagolysosomes. Although the majority of internalized protein is broken down in the phagolysosome, some is exocytosed across the basolateral membrane (Sanderson and Walker, 1993). Figure 1.3 summarizes the findings from morphological studies examining the transepithelial transport of HRP.

Although it is clear that intact antigens can cross the epithelial barrier by transcytosis both *in vivo* and *in vitro*, it remains uncertain if this transport pathway is involved in the transport of antigen to mast cells in the hypersensitivity response. Intestinal segments respond to luminal antigen challenge with an increase in Isc in a very rapid time frame, within 3 minutes. It is difficult to conceive that endocytosis, transport across the cell, and exocytosis could occur within such a short time. No careful morphological studies have been done to determine the kinetics of fluid phase transcytosis across intestinal epithelium, and many of the studies have examined intestinal epithelium hours after exposure to macromolecular tracers. One study of the kinetics of transcytosis has been done in Madin-Darby Canine Kidney (MDCK) cells, showing that HRP added to the apical side of the cells was first detectable in the basal media 10 min after an initial 5 min pulse period (Bomsel *et al.*, 1989). Even this fairly rapid transcytosis does not explain the response to luminal antigen 3 min after challenge. There is some indication that the transcellular pathway can be regulated. Bijlsma *et al.* (1996) showed that cholinergic stimulation of intestinal

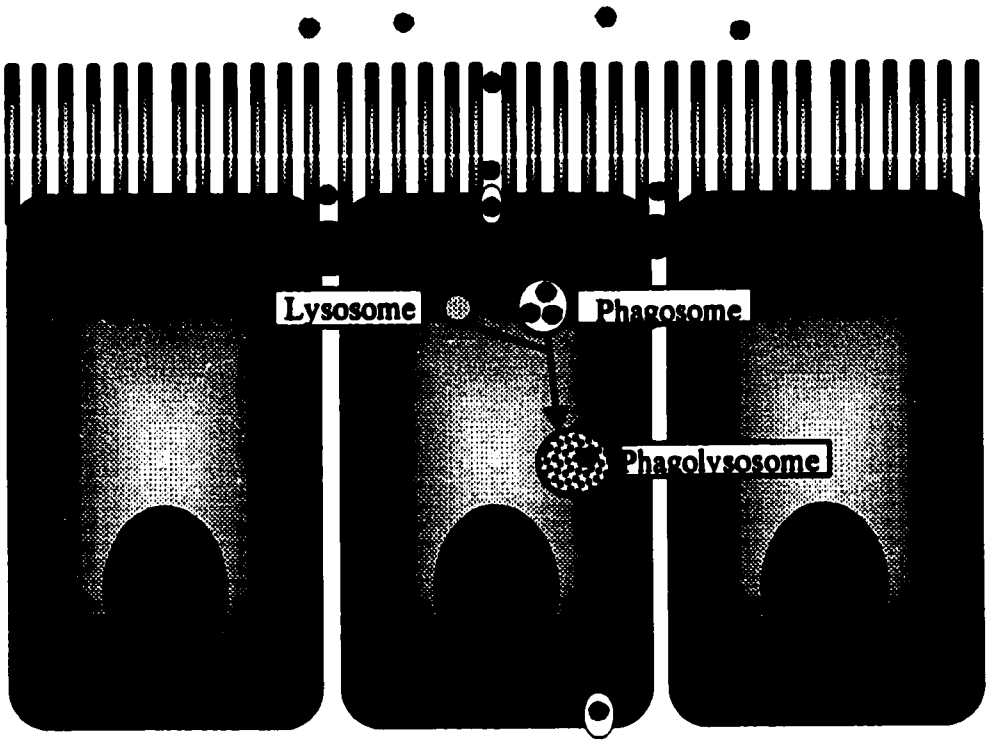


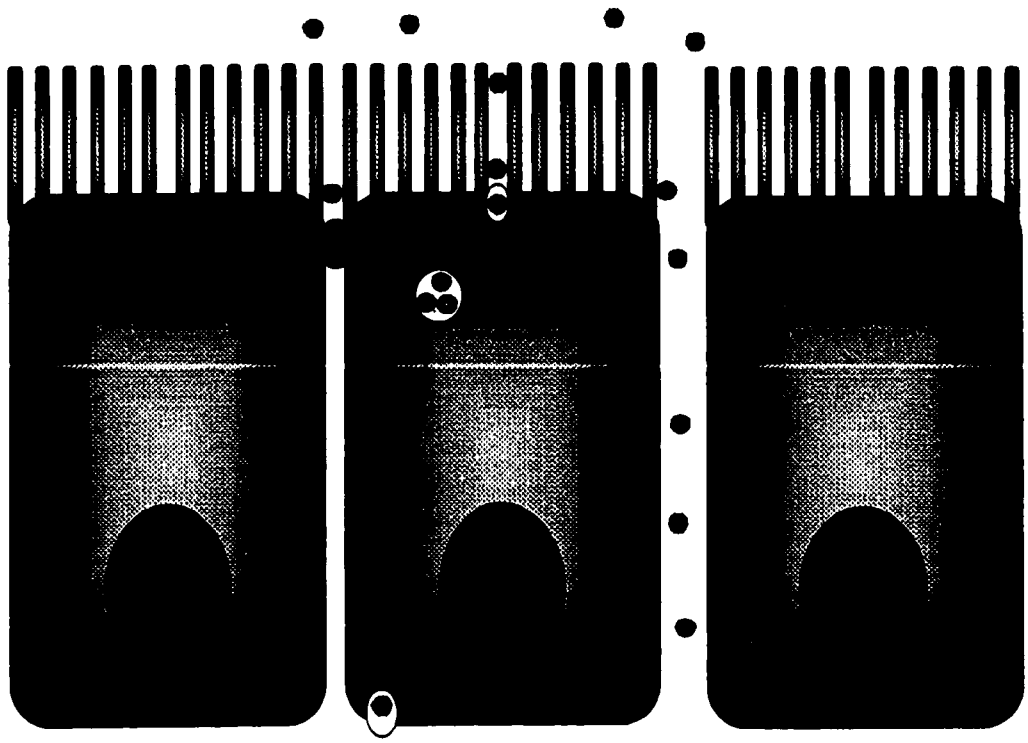
Figure 1.3: Schematic illustrating macromolecular transport across normal intestinal epithelium. Macromolecules are taken up at the apical surface by endocytosis, and fuse with lysosomes to form phagolysosomes. The majority of protein is broken down in lysosomes, but a small fraction escapes degradation and is exocytosed across the basolateral membrane. Tight junctions normally restrict passage of macromolecules.

epithelium *in vitro* resulted in enhanced endocytic uptake of HRP. Studies in other polarized cells (MDCK cells) have demonstrated that elevation of intracellular PKC stimulates the transcytotic pathway (Holm *et al.*, 1995), suggesting that many factors could stimulate the endocytic uptake of antigen across the epithelium.

Regulation of the Paracellular Transport Pathway

Morphological studies using electron dense tracers to examine macromolecular uptake across the intestinal epithelium unequivocally show that tight junctions between epithelial cells prevent diffusion through the paracellular pathway in the normal rat intestine (Cornell *et al.*, 1971, Walker *et al.*, 1972, Gonnella and Neutra, 1984, Mayrhofer *et al.*, 1990). However, in the last decade the concept of physiological regulation of tight junction permeability has emerged. Luminal nutrients, such as glucose, can decrease the electrical resistance across the intestinal tissue by reversably altering the permeability of the tight junction (Madara *et al.* 1992). More relevant to the issue of intestinal permeability in the sensitized individual, neuroimmune factors have also been demonstrated to alter the barrier function of the intestinal epithelium. Phillips *et al.* (1987) showed that stimulation of rats *in vivo* with carbachol (a cholinergic agonist) induced the paracellular movement of HRP across intestinal epithelium. This finding has recently been repeated *in vitro* (Bijlsma *et al.*, 1996). The impact of a number of immune mediators on epithelial permeability has been assessed *in vitro* using colonic epithelial cell lines grown as monolayers. As outlined previously, the cytokines IFN-γ, TNF-α, and IL-4 decrease the electrical resistance of epithelial monolayers, suggesting regulation of the tight junction (Madara and Stafford, 1989, Heyman *et al.*, 1994, Colgan *et al.*, 1994). Other immune mediators such as reactive oxygen metabolites and prostaglandins also

reduce the resistance of intestinal epithelium (Baker *et al.*, 1995, Perdue and McKay, 1994), indicating that they likely act on the epithelial tight junction. A change in epithelial resistance does not necessarily indicate that the epithelium is permeant to macromolecules; for example, glucose can increase the paracellular permeability to small molecules, but not macromolecules such as protein antigens (Madara *et al.*, 1992). Although the paracellular pathway is impermeant to macromolecules in the normal intestine, sensitization may induce changes in immune or neural function that could increase the permeability of the tight junctions. Diffusion of macromolecules through the paracellular pathway could potentially explain the very rapid response to antigen observed after luminal antigen challenge. Figure 1.4 is a schematic summarizing the state of knowledge on the regulation of transcellular and paracellular macromolecular transport pathways in intestinal epithelium.



Transcellular Pathway

Stimulated by:

cholinergic agonists

↑ intracellular PKC

Paracellular Pathway

Stimulated by:

cholinergic agonists

inflammatory cytokines

luminal nutrients

Figure 1.4: Schematic showing regulation of transepithelial transport of macromolecules. In normal intestinal epithelium, macromolecules are taken up by a transcellular transport pathway. To date, only cholinergic agonists have been shown to upregulate this pathway, although it is believed to be stimulated by elevation of PKC, and therefore may be regulated by a large number of factors . The paracellular pathway is normally impermeant to macromolecules, but cholinergic stimulation opens the tight junctions to allow passage of proteins. Indirect evidence suggests that the cytokines IFN-γ, TNF-α, and IL-4 also regulate the paracellular pathway.

Aims of Thesis Research Project

Previous studies have shown that intestine from rats sensitized to a model protein antigen develops a rapid (within 3 min) ion secretory response after luminal antigen challenge. Our current understanding of transepithelial macromolecular transport does not adequately explain how antigen introduced on the luminal side could activate mast cells in the lamina propria within such a short time. My first aim was to determine if sensitization altered the rate or the route of transepithelial antigen transport. I examined this using HRP, a model protein that can be visualized by electron microscopy, as a sensitizing antigen in a rat model of intestinal hypersensitivity. I determined the impact of sensitization on transepithelial antigen transport by quantifying the uptake of HRP prior to the ion secretory response, and comparing HRP transport to that in rats sensitized to an irrelevant antigen and naïve rats. In my first study, quantitative findings were obtained by measuring luminal to serosal flux of HRP, combined with electron microscopy analysis and electrophysiological measurements in rats sensitized and challenged with HRP. I found that sensitization and the intestinal hypersensitivity reaction both have profound impact on transepithelial antigen transport.

Mast cells have been shown to mediate the ion and water secretion changes observed after antigen challenge, and a few mast cell mediators are capable of altering epithelial barrier function. Therefore, in the second study I examined the role of mast cells in the regulation of transepithelial antigen transport by determining the effect of sensitization and antigen challenge in sensitized mast cell deficient (*Ws/Ws*) rats compared to control mast cell replete (+/+) littermates.

Upregulation of IL-4 expression is associated with sensitization in rodent models and human allergic diseases, and IL-4 has been shown to alter the resistance of epithelial monolayers. In my third study, I examined the impact of IL-4 on transepithelial macromolecular transport by culturing human intestinal epithelial cell monolayers in the presence of IL-4 or serum from atopic patients. HRP was again used as a model protein antigen, and transepithelial antigen transport was determined by flux measurements and electron microscopy analysis.

By developing a better understanding of transepithelial antigen transport in allergy, and examining the regulation of macromolecular uptake by immune factors, we may be able to eventually target therapeutics to prevent disrupted epithelial barrier function in allergic conditions. Clarifying the mechanisms of antigen uptake across the epithelium is important not only for food allergy, but may also be important for understanding the involvement of luminal antigens in the pathophysiology of inflammatory bowel disease.

Chapter 2

Rapid Transepithelial Antigen Transport in Rat Jejunum: Impact of Sensitization and the Hypersensitivity Reaction

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Comment: The experiments were conducted and data collected by the author M.C. Berin, with the exception of the electron microscopy analysis which was performed by A.J. Kiliaan, with additional data provided by P-C Yang.

Abstract

Intestine from sensitized rats develops a rapid secretory response to luminal antigen challenge that depends on activation of subepithelial mast cells. The aims of this study were to determine the timing and route of the transepithelial protein antigen transport. Rats were sensitized to horseradish peroxidase (HRP). After 10-14 days, jejunal segments were resected, mounted in Ussing chambers and challenged with HRP on the luminal side. Electron microscopy of tissues fixed at 2 min (before mast cell activation) showed enhanced endocytic uptake of HRP in enterocytes of HRP-sensitized rats compared with ovalbumin-sensitized or saline-injected controls. At this time, HRP was distributed throughout epithelial cells and was already evident in the lamina propria. In contrast, HRP was restricted to the apical region of enterocytes in controls. At 30 min (after mast cell activation), in HRP-sensitized rats only, HRP was also located within tight junctions and the paracellular region between epithelial cells. Tissue conductance was elevated in HRP sensitized rats beginning 30 min after HRP addition, and correlated with the overall flux of HRP across the tissue. Our results show that specific sensitization enhances the initial uptake and transcytosis of antigen across intestinal epithelium. Subsequent to activation of mast cells, antigen transport is further enhanced by penetration through the paracellular pathway.

Introduction

Animal models of intestinal hypersensitivity to food proteins have demonstrated that antigen challenge of the sensitized intestine produces alterations in ion transport, permeability, and motility (1). Antigen absorbed into the mucosa cross-links IgE bound to FcεRI receptors on the surface of mucosal mast cells, resulting in the release of a number of active mediators including histamine, serotonin, and prostaglandins that stimulate net ion secretion from epithelial cells (2). Studies involving the inhibition of mast cell mediators (2,3), and the use of genetically mast cell-deficient mice and congenic controls (4), have clearly testified to the central role of the mast cell in mediating the effects of intestinal anaphylaxis.

In our previous experiments using rats sensitized to ovalbumin (OVA), we have shown that antigen added to the luminal side of isolated segments of jejunum evokes a secretory response, beginning within approximately 3 min (2). This rapid secretion cannot readily be explained by our current understanding of macromolecular transport across the epithelium by either the paracellular or the transcellular pathway. The paracellular pathway between adjacent epithelial cells has been demonstrated to be impermeable under normal circumstances to macromolecules such as protein antigens (5,6). Kinetic studies of the transcytotic pathway in polarized epithelial cells have indicated that transport across the epithelial barrier takes approximately 20 minutes (7). Antigen sampling M cells are not required for this rapid secretory response, as Peyer's patch-free intestinal segments are routinely used in these experiments. Therefore, the route by which luminal antigens gain access to mast cells in the subepithelial compartment of the mucosa in such a short time is unclear.

The present study was designed to examine the effect of 1) specific sensitization and 2) non-specific sensitization and 3) the immediate hypersensitivity reaction on antigen transport across the intestinal epithelium. For these experiments, we sensitized rats to a model protein antigen, horseradish peroxidase (HRP), since the intact protein can be measured by kinetic enzymatic assay (8) and its reaction product visualized by electron microscopy (9). We determined HRP uptake into endosomes and its rate of transport across epithelial cells in rats sensitized to HRP and compared results with those from rats sensitized to an irrelevant antigen, OVA or non-sensitized saline-injected controls. We found that sensitization of rats to HRP profoundly altered the transport kinetics of this protein antigen across the epithelium. HRP was transported via a transcellular pathway in both enterocytes and goblet cells such that it reached effector cells in the lamina propria by 2 min. In addition, our study showed that subsequent to activation of mast cells, a large increase in the flux of HRP across the tissue occurred that correlated with increased conductance, and was associated with penetration of antigen into the paracellular region.

Methods

Animals

Pathogen free male Sprague-Dawley rats (250-275 g, Charles River Breeding Laboratories, St. Constant, Quebec, Canada) were housed in cages equipped with filter hoods. Rats were sensitized to HRP (type II, Sigma Chemical Co, St. Louis, MI) by a subcutaneous injection of 1 mg HRP in 1 mL alum plus an intraperitoneal injection of 1 mL Bordetella pertussis vaccine (Connaught Laboratories, Willowdale, Ontario, Canada) (HRP group). Control rats were either sensitized (using the same protocol) to OVA (grade V, Sigma) as an irrelevant antigen (OVA group), or injected with saline alone (SALINE group). Rats were studied 10 to 14 days after sensitization. All animal experiments were conducted with approval from the McMaster University Animal Care Committee.

Ussing Chamber Experiments

Rats were anesthetized with Urethane (Aldrich Chemical Company, Milwaukee, WI) and a 15 cm segment of jejunum was excised (beginning 5 cm distal to the ligament of Treitz). The external muscle layers were stripped off, leaving the submucosal plexus and mucosa intact. From each rat, 8 pieces of intestine were mounted in Ussing chambers (WPI Instruments, Narco Scientific, Mississauga, Ontario, Canada). Care was taken to avoid tissue containing Peyer's patches. The chamber opening exposed 0.6 cm^2 of serosal surface area to 8 mL of circulating oxygenated Krebs buffer, pH 7.35, 37° C. The serosal buffer contained 10 mM glucose osmotically balanced with 10 mM mannitol in the luminal buffer. The tissue was clamped at zero volts using a W-P Instruments

automatic voltage clamp (Narco Scientific, Downsview, Ontario, Canada). The short-circuit current (Isc, in μA) was recorded continuously, and at regular intervals, the Isc was turned off in order to record potential difference, so that tissue conductance (mS/cm^2) could be determined. Tissues were allowed to equilibrate until Isc stabilized and then HRP was added to the luminal buffer.

HRP Flux

HRP was added to the luminal buffer at a final concentration of 5×10^{-5} M. Duplicate samples of serosal buffer (500 μL) were obtained at 0, 30, 60, and 90 minutes and were replaced with Krebs buffer. HRP activity was determined by assaying enzyme activity using a modified Worthington method (10). Briefly, 150 μL of sample was added to 800 μL of phosphate buffer containing 0.003% H_2O_2 and 80 μg/mL o-dianisidine (Sigma). Enzyme activity was determined from the rate of increase in optical density at 460 nm over a 2 min period. Fluxes were calculated according to standard formulae and were expressed as $pmoles/cm^2/hr$.

Electron Microscopy

To determine the route and timing of transepithelial transport of HRP, tissues from 4 rats in each group were obtained for electron microscopy at 2, 5 and 30 minutes after HRP addition to luminal side of tissue. Jejunal segments were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 2 hours at room temperature, washed and left overnight (at 4° C) in the same buffer, and washed three times for 5 minutes each in 0.05 M Tris buffer (pH 7.6). Tissues were incubated for 30 minutes in 5 mg 3,3'-diaminobenzadine

tetrahydrochlorine (Sigma) in 10 mL 0.05 M Tris buffer and 0.01% H_2O_2 (pH 7.6, 22° C), and were subsequently processed for routine electron microscopy. Tissues were oriented such that villus epithelial cells were cut longitudinally. Photomicrographs were prepared from the mid-villus region. As an indication of the rate of transcytosis, the incidence of HRP was recorded at 2 and 30 min after addition of HRP within specific regions of enterocytes: apical (above the nucleus), mid (beside the nucleus), or basal (below the nucleus). Five enterocytes per rat, 20 per rat group, were used. The percentage of HRP positive enterocytes were calculated for each rat, and the mean and standard error were calculated for each rat group. To determine the amount of HRP initially taken up by enterocytes, HRP-containing endosomes within a 4 x 6 μm window in the apical region of the cell (immediately below the microvilli) were counted and measured in coded photomicrographs (9). Twenty photomicrographs were examined per rat, 80 for each rat group. Analysis was performed by one investigator (A.J.K.), who was unaware of the treatment group. The area of HRP within endosomes was calculated and averaged for each rat before determining group means.

As a further control, intestine from rats sensitized to HRP but not challenged with HRP was fixed for electron microscopy and the epithelium was examined for endogenous peroxidase activity. There was no HRP evident in this group, indicating that endogenous peroxidase was not a factor in these experiments.

Statistics

Statistical significance was tested by ANOVA, with the Neuman-Keuls used for post-hoc analysis. Pearson's correlation coefficient was used to examine the linear relationship between HRP flux and conductance measurements. A value of $P < 0.05$ was considered significant.

Results

Electrophysiological Parameters

Baseline Isc did not differ between the rat groups (19.5 ± 3.3, 26.0 ± 7.0, 15.9 ± 1.7 $\mu A/cm^2$ for SALINE, OVA, and HRP respectively). Intestine from rats sensitized to HRP showed a marked increase in Isc in response to luminal antigen challenge with HRP. A representative Isc tracing is shown in Fig 1. The increase in Isc at 5 minutes after HRP challenge was 32.1 ± 2.5 $\mu A/cm^2$ (mean ± SEM) and the time from antigen challenge to initial rise in Isc was 3.4 ± 0.4 min. The magnitude of this response was similar to that of tissue from OVA sensitized rats challenged with OVA. In contrast, HRP challenge to tissues from either control groups (SALINE or OVA) produced no change in Isc.

Initial baseline conductance was also not different between groups. However, conductance was significantly elevated in HRP sensitized rats at 90 min, both compared to the original baseline, and compared to values in SALINE and OVA rats at 90 min (Table 1). Conductance in the HRP sensitized group was stable for the first 10 min after HRP challenge, then slowly increased such that it was significantly elevated at 30 min, and rapidly increased thereafter (Fig 2). In contrast, SALINE and OVA rats maintained a stable conductance throughout the experimental period.

HRP Flux Measurements

To obtain an overview of transepithelial transport of antigen, luminal to serosal flux of HRP across the tissues was measured over a 90 minute period. The flux of HRP reached steady-state at 30 to 60 minutes in both control groups, but flux across intestine from HRP sensitized rats continued to rise throughout

the 90 minute experimental period (Fig 3). The HRP flux value was not significantly different between groups during the initial 30 minute period, but was significantly increased in the HRP sensitized group during the 30 to 60 minute period compared to control groups (32.9 ± 10.6 vs 10.4 ± 2.5 and 8.6 ± 1.7 pmoles/cm^2/hr, mean ± SEM, for HRP sensitized, OVA and SALINE respectively), and was five-fold higher (64.3 ± 11.0 vs 12.4 ± 2.4 and 11.9 ± 2.6 pmoles/cm^2/hr) at the 60 to 90 minute flux period. The control groups did not differ from each other in each time period. The relationship between conductance and flux was examined by correlation analysis with data from the three treatment groups in the final flux period, resulting in a correlation coefficient of 0.80 ($p < 0.001$).

Electron Microscopy

Two Minute Timepoint: To determine if antigen was reaching cells in the lamina propria prior to mast cell activation and the subsequent ion secretory response, tissues were fixed 2 minutes after HRP challenge (Fig 1). Electron photomicrographs of enterocytes and the underlying lamina propria were used to assess the distribution of HRP (examples shown in Fig 4). In HRP sensitized rats, HRP was found to be distributed throughout enterocytes, such that 65% of enterocytes examined had HRP within endosomes in the apical region, 50% were positive for HRP in the mid-region, and 45% of enterocytes contained endosomes positive for HRP in the basal region of the cell, adjacent to the lamina propria (Fig 5). OVA rats also had HRP distributed throughout enterocytes, but the incidence of HRP-containing endosomes was much lower than that observed in the HRP sensitized group. HRP positive endosomes in the

apical region were found in 45% of enterocytes in OVA rats, 20% contained HRP in the mid-region, and only 15% contained HRP in the basal region. SALINE rats had a more limited distribution of HRP within enterocytes than the HRP or OVA sensitized rats. Enterocytes from SALINE rats did contain HRP-positive endosomes, but these were predominantly in the apical region. Of the enterocytes examined, 30% contained HRP within the apical region, 10% were positive for HRP in the mid-region, but there was zero incidence of HRP within the basal region of enterocytes. In addition to enterocytes, HRP was also observed within goblet cells (Fig 4) and Paneth cells (not shown) of HRP sensitized rats. Intestinal epithelial cells from rats sensitized to HRP but not challenged showed no HRP reaction product in any location.

To determine if sensitization altered the amount of HRP taken up into enterocytes, the diameter of HRP in endosomes within the apical region was measured (Fig 6). The mean area of endosomal HRP was significantly greater in HRP sensitized rats compared to SALINE rats (257 ± 80 vs 72 ± 16 nm^2). OVA sensitized rats did not significantly differ from either group (111 ± 45 nm^2).

At 2 min after HRP challenge, HRP was already present in the lamina propria (Fig 7A) in 40 % of photomicrographs examined from the HRP sensitized group, compared to 10% and 0% of photomicrographs from OVA and SALINE rats respectively (Fig 5). At 5 min after HRP challenge, there was more HRP evident within the lamina propria and mast cells showed early signs of activation (Fig 7B). The source of lamina propria HRP appeared to be transcellular antigen transport, as there was no evidence for paracellular transport of HRP. Tight junctions were negative for HRP in all three groups at the 2 minute post-challenge timepoint.

Thirty Minute Timepoint: In tissues fixed for electron microscopy analysis at 30 minutes post-HRP challenge, mast cells were obviously activated. Transcellular movement of HRP across the epithelium was again observed, with HRP distributed throughout enterocytes in all three treatment groups (Fig 8). HRP sensitized rats had an increased incidence of HRP in the basal region of enterocytes compared to OVA and SALINE rats (50% of enterocytes positive for HRP-containing endosomes compared to 25% and 20% respectively). HRP was observed in the paracellular region and tight junctions between adjacent epithelial cells in 3 of 4 rats sensitized to and challenged with HRP (Fig 9). Epithelium from OVA sensitized or SALINE rats showed no HRP crossing the tight junctions or within the paracellular region.

	Initial G	Final G
SALINE	31.9 ± 1.9	33.5 ± 2.4
OVA	32.7 ± 1.1	34.5 ± 1.9
HRP	29.9 ± 1.8	48.8 ± 3.9*

Table 2.1: Impact of intestinal hypersensitivity on jejunal conductance (G, mS/cm^2). Values represent the means ± SEM for 6 rats per group, 2 to 4 tissues per rat. Readings were taken after equilibration (time = 0 min), and at the end of the experiment (time = 90 min). * $p < 0.05$ compared with horseradish peroxidase (HRP) sensitized rats at t= 0, and compared with saline-injected control and ovalbumin (OVA) sensitized rats at t=90.

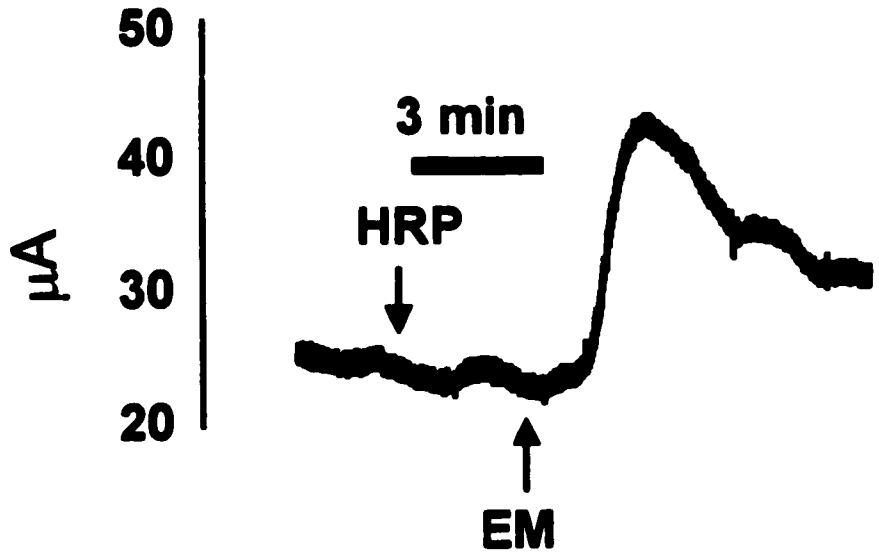

Figure 2.1: A representative tracing of Isc in intestine from a rat sensitized to HRP and challenged with 50 μmol/L HRP on the luminal side of the tissue. In experiments examining the transepithelial transport of antigen before the ion secretory response, tissues were removed from the Ussing chamber and fixed for electron microscopy 2 minutes after HRP addition.

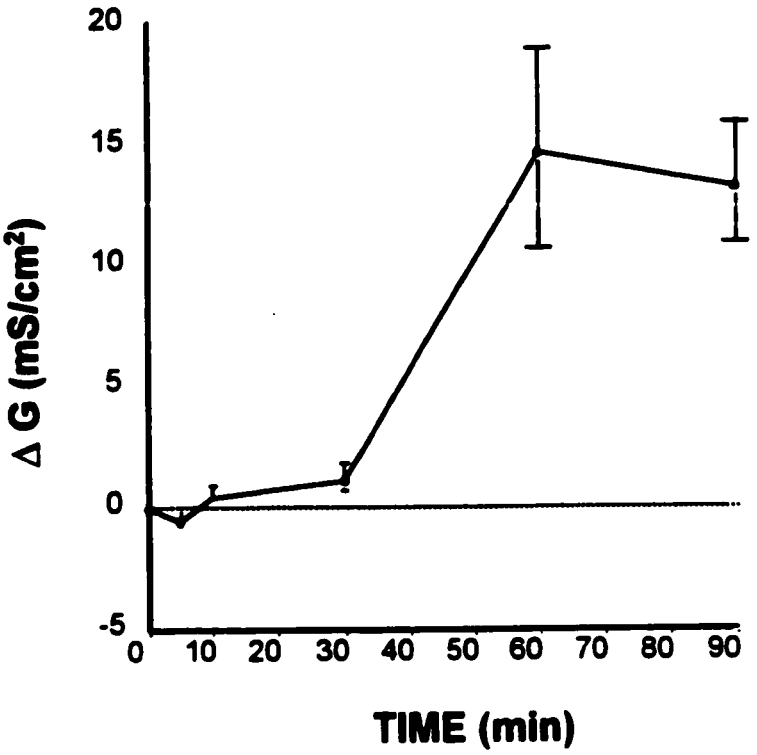

Figure 2.2: Change in conductance in response to HRP challenge in HRP-sensitized rats. Conductance was measured immediately before (zero time) and at intervals after luminal HRP challenge. The change in conductance at each time period was determined, and data are expressed as mean change ± SEM for 8 rats. 2-4 tissues per rat.

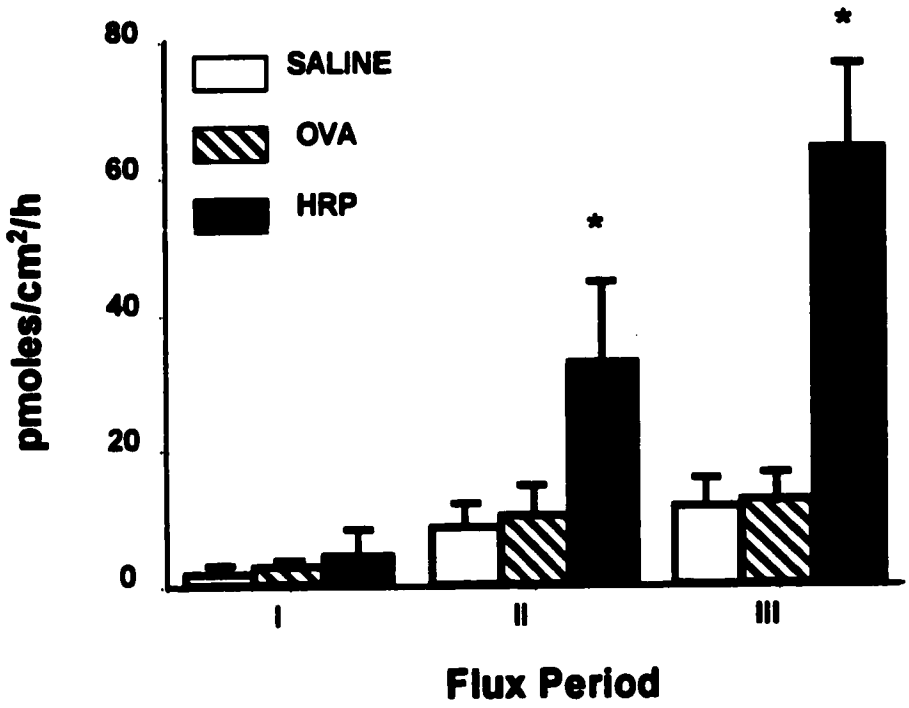

Figure 2.3: Effect of sensitization and antigen challenge on mucosal to serosal flux of HRP. Flux of HRP was measured across intestine obtained from rats injected with saline, rats sensitized to OVA, or rats sensitized to HRP. HRP was measured in serosal buffer by kinetic enzymatic assay at 0-30 (I), 30-60 (II), and 60-90 (III) minutes after addition of 50 μmol/L HRP to the luminal buffer. Data are expressed as mean ± SEM for 8 rats per group, 2-4 tissues per rat. *$p<0.05$ compared with saline controls.

Figure 2.4 (Next Page): Representative photomicrographs prepared from tissues fixed 2 minutes after addition of HRP to the lumen, showing HRP-containing endosomes within epithelium from (A) HRP-sensitized rats and (B) OVA-sensitized rats. The lumen (LU) and microvilli (MV) are indicated on the left side of the photomicrographs. Short arrows indicate the presence of HRP-containing endosomes; long arrows indicate the endosomes shown in the higher magnification insets. Bars = 2 μm (inset bars = 5 μm).

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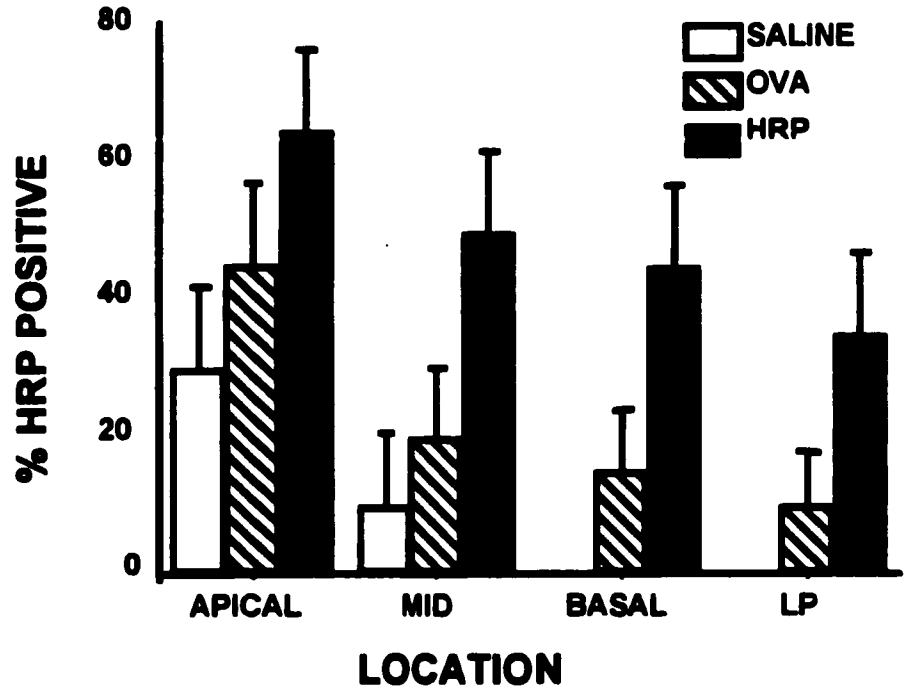

Figure 2.5: Distribution of HRP within enterocytes and in the lamina propria. Tissues from saline, OVA-sensitized, and HRP-sensitized rats were fixed for electron microscopy 2 minutes after luminal HRP addition. Coded photomicrographs (20 for each of 4 rats/group) were examined for incidence of HRP (percentage of sections with HRP-containing endosomes) in the apical, mid, or basal region of enterocytes, or in the lamina propria (LP), and data are expressed as percentage (mean ± SEM) of photomicrographs examined in each group that were positive for HRP.

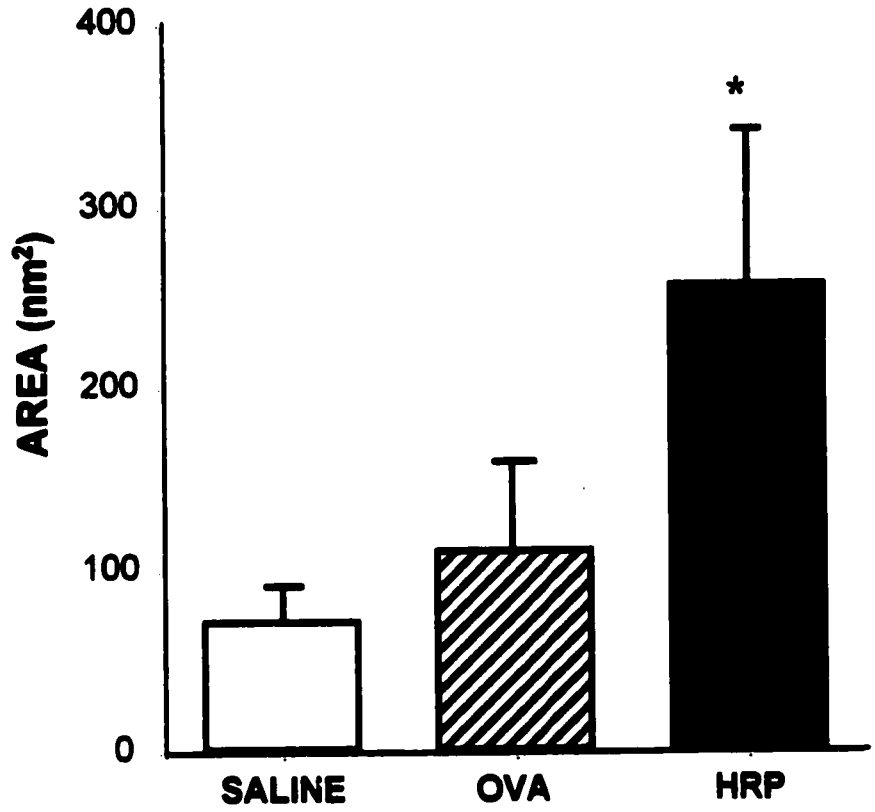

Figure 2.6: Effect of sensitization on uptake of HRP into enterocytes. The area of endosomal HRP within 4 x 6 μm windows was determined in coded electron photomicrographs taken from intestine of saline-injected, OVA-sensitized, or HRP-sensitized rats. Tissue was fixed 2 minutes after luminal addition of HRP, and 20 photomicrographs per rat (4 rats per group) were used for quantification. *Significance at $p < 0.05$ compared with saline-injected control rats.

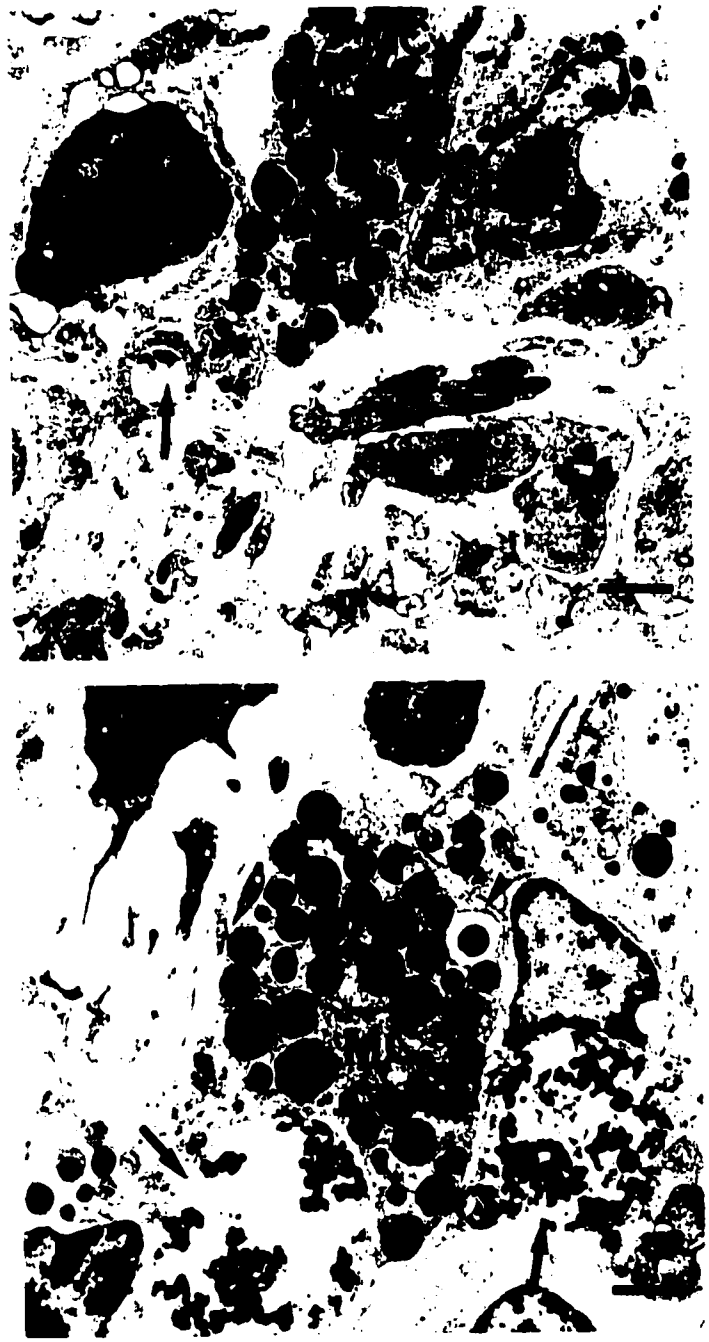

Figure 2.7: Representative photomicrographs showing HRP within the lamina propria in HRP-sensitized rats at (A) 2 minutes and (B) 5 minutes after luminal addition of HRP. Curved arrows indicate the presence of HRP; the straight arrow indicates a clear area surrounding a mast cell (MC) granule, an early sign of activation. Bars = 2 μm.

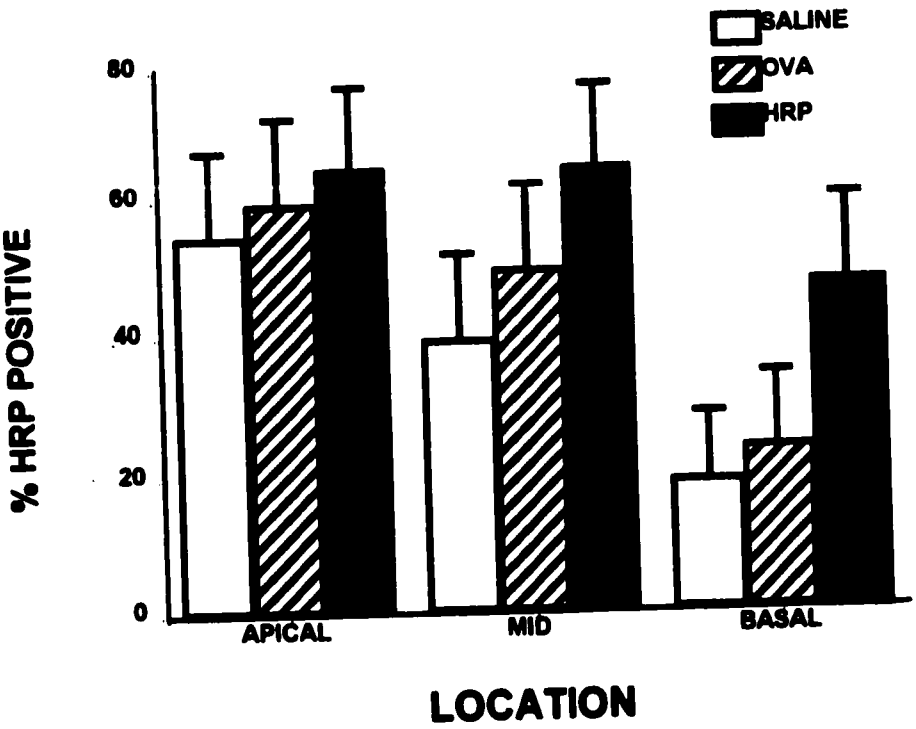

Figure 2.8: Distribution of HRP within enterocytes after effector cell activation. Tissues from saline, OVA-sensitized, and HRP-sensitized rats were fixed for electron microscopy 30 minutes after luminal HRP addition. Coded photomicrographs (20 for each of 4 rats/group) were examined for incidence of HRP (percentage of sections with HRP-containing endosomes) in the apical, mid, or basal region of enterocytes; data are expressed as percentages (mean ± SEM) of photomicraphs examined in each group that were positive for HRP.

Figure 2.9: Representative photomicrograph of an enterocyte from an HRP-sensitized rat 30 minutes after addition of HRP to the luminal buffer. Arrows indicate the paracellular space between enterocytes, which is filled with HRP. Bar = 1 μm.

Discussion

Previous experiments in our laboratory have shown that intestine from rats sensitized to OVA responds to antigen challenge on the luminal side of the tissue with a rapid (beginning in ~ 3 min) increase in Isc that is due to chloride ion secretion (2,11). Mast cells are required for the response (2,4). These findings suggest that antigen is transported across the epithelial barrier to activate subepithelial mast cells within a very short time. However, the recognized kinetics of transepithelial protein movement indicate that approximately 20 min are required for transcytosis (7). The aims of the current study were to determine the route and timing of antigen transport across the intestinal epithelium in sensitized rats. We demonstrated that protein transport across the intestinal epithelium was increased by non-specific sensitization, and dramatically enhanced by specific sensitization such that antigen gained access to effector cells in the lamina propria within 2 min after its addition to the luminal surface. Our finding that transcytosis was maximal for the sensitizing antigen suggests that there may be a component of recognition in the antigen transport process. In addition, morphological documentation of HRP between tight junctions and adjacent epithelial cells together with increased tissue conductance suggest that after activation of effector cells antigen penetrated the epithelium via the paracellular pathway.

In order to examine antigen transport across the epithelium, we used HRP (a protein which can be readily measured by enzymatic assay and visualized by electron microscopy) as a sensitizing antigen. Intestine from HRP sensitized rats responded to HRP challenge with similar ion transport responses as we previously observed with rats sensitized to OVA (2), with respect to the

magnitude of the Isc increase and the timing of the secretory response.

To determine the effect of sensitization on initial antigen uptake (i.e. prior to mast cell activation), tissues were fixed for electron microscopy at 2 min, approximately 1 min before the beginning of the Isc rise induced by luminal HRP antigen challenge. Quantification of the amount of HRP within a fixed apical region of enterocytes revealed that there was significantly greater uptake of HRP into enterocytes of HRP-sensitized rats compared with OVA-sensitized and non-sensitized control rats. Although there was a trend showing increased uptake of HRP in OVA sensitized rats, this did not reach the level of statistical significance. However, when the distribution of HRP within enterocytes was examined, HRP was located in endosomes throughout enterocytes and in the lamina propria in both HRP sensitized and OVA sensitized rats, although the incidence of HRP in all regions was lower in OVA sensitized rats. In contrast, HRP within the epithelium of saline-control rats was confined to the apical region of enterocytes. These findings indicate that the rate of antigen transport across epithelial cells and into the lamina propria was altered by specific sensitization, and that the rapid initial uptake of antigen may have been influenced by specific recognition. The data suggest that non-specific sensitization enhances transepithelial antigen transport, but not to the same extent as specific sensitization. These results are in agreement with previous *in vivo* experiments from our laboratory demonstrating that intestine from OVA sensitized rats had a greater baseline transport of ^{51}Cr-EDTA than control rats (12). Taken together, these studies imply that sensitization alone alters the barrier properties of the intestine.

Studies in control animals have shown that there is significant transcytosis of macromolecules to the basal membrane in polarized epithelial cells, but that the time from apical uptake of protein to exocytosis across the basal membrane

is slow, requiring at least 20 minutes (7). This is consistant with our observations of antigen transport across intestine from saline-control rats. It is not clear how sensitization induces changes in the kinetics of transcytosis, but it is possible that cytokines released from immunocytes could regulate endocytic transport. Atopy is associated with an increase in Th2 cell cytokines, particularly IL-4, IL-5, and IL-10 (13). Although a number of cytokines have been demonstrated to have profound effects on ion secretion and epithelial barrier resistance, the impact of these immunological mediators on the transcytotic pathway has yet to be addressed. Inflammatory cytokines (IL-1, TNF-α) have been shown to upregulate the endocytic rate in hepatic endothelium (14), indicating that immunological regulation of endocytic pathways is possible. In addition, milk protein has been shown to stimulate secretion of cytokines (including TNF-α) from peripheral blood mononuclear cells obtained from infants with cow's milk allergy, and epithelial cells cultured with supernatants from these cells demonstrated increased flux of intact HRP (15).

The specificity of the increased uptake of antigen into the enterocyte raises the intriguing possibility that there is recognition of antigen by the enterocyte, possibly through an antibody-mediated mechanism. If uptake of HRP was occurring through a receptor-mediated transport mechanism, this could explain both the specificity and increased rate of transport observed. Binding to a receptor might reduce lysosomal degradation of the protein and thus explain the increased incidence at 30 min of HRP in endosomes in HRP-sensitized rats compared with the other rat groups.

At the early timepoint (2 min), it is also possible that a small number of HRP molecules, too few to be detected morphologically, could have leaked across the tight junctions. This possibility cannot be ruled out in this study,

although the conductance data suggest that there was no opening of tight junctions until after 5 min at the earliest. Further studies are in progress to accurately determine the contribution of each pathway to transepithelial antigen transport prior to mast cell activation.

Although numerous studies have demonstrated an increased flux of specific antigen (12), bystander antigens (16-19), or small molecular weight probes (12) during intestinal anaphylaxis, the route of the uptake has not been clearly delineated. Therefore, we examined the uptake of HRP across the intestine by electron microscopy 30 minutes after HRP challenge, well after the mast cell-mediated increase in Isc. As indicated above, HRP transport via endosomes was enhanced by specific antigen challenge. In addition, the antigen-induced hypersensitivity response was associated with an increase in tissue conductance and the presence of intact HRP in the paracellular regions between enterocytes. HRP within the lateral spaces could be attributed to either penetration through tight junctions and/or exocytosis of intracellular HRP. High magnification electron microscopy demonstrated HRP within the tight junctional area. Although paracellular HRP was not present in all sections examined from HRP sensitized rats, HRP in the paracellular regions was never documented in any sections from SALINE or OVA rats. In addition, the conductance was elevated in HRP, but not SALINE or OVA rats. Measurements of conductance (thought to reflect the permeability of the tight junction (19)), correlated highly with the flux of HRP acdross tissues from all three treatment groups. This suggests that the elevated flux in specifically sensitized and challenged rats was related to the increased conductance of the tight junctions, and that the pathway of HRP transport included a paracellular component.

Previous studies in our laboratory have demonstrated that receptor

antagonism of mast cell mediators or neural blockade attenuates the ion secretory response (2, 20), indicating that release of mast cell and nerve products occurs following antigen challenge. The mast cell protease RMCP II has been shown to decrease epithelial barrier function (21). In addition, a number of studies have established that neural activation or neurotransmitters can alter epithelial permeability to macromolecules. Stimulation of rat intestine with cholinergic agonists (6,9) increased tight junctional permeability, and stress was also shown to impair barrier function via cholinergic mechanisms (22). In those studies, electron microscopy indicated HRP within the tight junctions and paracellular regions. Neural blockade with tetrodotoxin and atropine inhibited macromolecular transport across the normal small intestine (23). Evidence also suggests that Substance P and neuropeptide Y (NPY) can modify the epithelial barrier (2, 24). In our study, since HRP was observed in the paracellular space only after the ion secretory response, we hypothesize that activation of mucosal mast cells and/or their interaction with nerves mediated the paracellular permeability to antigen.

Mucosal to serosal flux of HRP was significantly elevated in rats specifically sensitized to HRP, compared to OVA-sensitized and saline control rats. This is consistent with previous findings which demonstrated increased serum levels of antigen after anaphylaxis (12). This could be due to enhanced uptake via both the transcellular and paracellular routes. In addition, the flux of HRP continued to rise throughout the entire experimental period in rats sensitized to HRP, whereas in the other rat groups the flux of HRP reached equilibrium after 30 minutes. The escalating rate of antigen transport may indicate that after crossing the epithelial barrier, HRP was activating mast cells and perhaps nerves, thereby continuing to augment the epithelial barrier defect.

In summary, transepithelial antigen transport in sensitized rats appeared to be a highly complex process composed of two phases. The initial phase occurred prior to mast cell activation. This phase involved enhanced endocytic uptake of specific antigen into enterocytes and its rapid transcytosis into the lamina propria. The second phase occurred after mast cell activation and was associated with a markedly increased conductance, the presence of antigen in tight junctions and paracellular regions, and increased overall flux of antigen across the tissue. Further experiments are necessary to fully define the mechanisms responsible for phase I and II of transepithelial antigen transport.

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Chapter 3

The Influence of Mast Cells on Pathways of Transepithelial Antigen Transport in Rat Intestine

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Comment: The experiments were conducted and data collected by the author M.C. Berin. Electron microscopy analysis was performed by M.C. Berin under supervision of A.J. Kiliaan.

Abstract

Luminal antigen challenge of intestinal segments from sensitized rats results in a rapid (~3min) secretory response. We previously showed in horseradish peroxidase (HRP) sensitized rats that the initial phase of transepithelial antigen transport occurred via a transcellular route and was enhanced by sensitization. However, following the hypersensitivity reaction, antigen also crossed between epithelial cells. The aim of this study was to determine the role of mast cells in the altered transepithelial antigen transport. *Ws/Ws* mast cell deficient rats and +/+ littermate controls were sensitized to HRP. After 10-14 days, jejunal segments were resected, mounted in Ussing chambers, and challenged with HRP on the luminal side. Electron microscopy of jejunum fixed at 2 min showed similarly enhanced endocytic transport of HRP in sensitized +/+ and *Ws/Ws* rats compared to naive controls. In sensitized +/+ rats, a secretory response occurred ~ 3 min after challenge and thereafter tissue conductance increased. Naive +/+ and sensitized *Ws/Ws* rats did not demonstrate a secretory response to HRP challenge and conductance remained at baseline levels. The flux of HRP was elevated across tissue from sensitized +/+ rats but not naive controls or sensitized *Ws/Ws* rats. The results indicate that sensitization enhances the initial phase of transepithelial uptake of antigen by transcytosis in a mast cell-independent manner. However, subsequent recruitment of the paracellular pathway for antigen transport in sensitized rats is dependent on the presence of mast cells and occurs after their activation.

Introduction

Local hypersensitivity reactions at mucosal surfaces play an important role in the pathophysiology of allergic diseases, including food allergy, atopic asthma, and rhinitis. Antigen challenge in sensitized individuals leads to mast cell activation by cross-linking IgE bound to FcεRI on the cell surface. Released mast cell chemicals such as histamine, serotonin, proteases, and lipid mediators produce alterations in epithelial and smooth muscle physiology (1) that are responsible for many of the acute symptoms of allergic disease. Such symptoms develop very rapidly (within min) after encounter with antigen (2). However, because the mucosal epithelium is believed to provide a selective barrier restricting the influx of ingested or inhaled antigens, it is unclear how luminal antigens are transported across this barrier to reach effector cells.

Soluble luminal antigens can be taken up across epithelium by two routes: the transcellular pathway or the paracellular pathway. The transcellular pathway involves endocytic uptake of antigen at the apical membrane and its transport in endocytic vesicles through the cell to the basolateral membrane where it is released into the extracellular space (3). The paracellular pathway is the pathway between epithelial cells. However, this route is restricted by intercellular tight junctions at the apical pole of epithelial cells that limit passage of macromolecules (4,5)

We recently reported (6) enhanced transepithelial transport of antigen in a rat model of intestinal hypersensitivity. Electron photomicrographs clearly demonstrated accelerated transport of protein antigen (horseradish peroxidase (HRP)) within endosomes, such that antigen was present in the lamina propria at 2 min (~ 10 times faster than normal) after its addition to the mucosal buffer

bathing tissues in Ussing chambers. By 3 min, a secretory response was evident, indicated by an increase in the short-circuit current (Isc) associated in time with evidence of mast cell activation (clear zones around granules). Subsequently (>30 min post-challenge), a large increase in flux of antigen across the tissue was documented, and a larger conductance value suggested decreased resistance of the paracellular pathway. This was confirmed by electron microscopy that showed antigen in the paracellular regions and tight junctional areas.

A large body of evidence indicates that mast cells regulate epithelial ion transport (7). However, there is little information on whether mast cells are involved in the regulation of epithelial permeability. Support for this concept includes studies showing that antigen challenge of sensitized rats results in increased transport of a range of probes, from small molecular weight markers (8) to proteins (9). In addition, infusion of the mast cell mediator, rat mast cell protease II (RMCP II), caused enhanced leakage of protein into the intestinal lumen (10). However, the exact role of mast cells in the alteration of rate or route of transepithelial antigen transport has not been clearly defined.

Therefore, the aim of the current study was to directly assess the role of mast cells in transepithelial antigen transport across small intestine from sensitized rats, comparing results in mast cell-deficient rats and controls. *Ws/Ws* rats have a genetic mutation at the *c-kit* locus resulting in the lack of mast cells within the intestinal mucosa, whereas the +/+ littermates have been shown to have normal numbers of mast cells (11). As before, we chose HRP as our model protein antigen since it can be measured quantitatively by enzymatic assay and visualised by electron microscopy. Transepithelial transport of HRP across jejunal segments was assessed after its addition to the luminal side of tissues in

Ussing chambers. Our analysis of EM photomicrographs demonstrated similar results in both sensitized +/+ and *Ws/Ws* rats in the initial phase of antigen transport: enhanced uptake and transport of antigen in endocytic vesicles, indicating that mast cells are not involved in this phase. However, a hypersensitivity response occurred only in the +/+ rats, indicating an absolute requirement for mast cells in antigen-stimulated ion secretion. In addition, only +/+ rats showed increased overall flux of antigen, increased conductance, and the presence of HRP in the paracellular spaces. These findings provide evidence that mast cells regulate the permeability of the epithelial paracellular pathway.

Methods

Animals

Ws/Ws and +/+ rats were obtained by breeding male and female *Ws/+* heterozygous rats (from the original colony developed by Y. Kitamura, Osaka, Japan). A spontaneous mutation (*Ws/+*) was first identified in a BN/fMai rat colony, and the heterozygous rats were bred with female rats of the Donryu strain to obtain viable *Ws/Ws* rats (11). *Ws/Ws* rats have a 12-base deletion in the tyrosine kinase domain of the *c-kit* gene (12) that results in a lack of melanocytes, erythrocytes, and mast cells. By 10 weeks of age, no mast cells can be detected in skin (13) or intestine (14) from *Ws/Ws* rats, whereas +/+ rats have normal numbers of mast cells. Rats (> 12 weeks of age), were maintained on a 12 hour light/dark cycle, and were given food and water *ad libitum*. Some experiments were repeated with Sprague-Dawley rats to confirm our previous findings (6). Experiments were approved by the Animal Care Committee at McMaster University.

Rats were sensitized to horseradish peroxidase (HRP) by subcutaneous injection with 1 mg HRP (type II, Sigma-Aldrich Canada Ltd, Oakville, Ontario, Canada) in 1 ml alum (10% $AlK(SO_4)_2 \cdot 12H_2O$), and intraperitoneal injection of 1 ml *Bordetella pertussis* vaccine (Connaught Laboratories, Willowdale, Ontario, Canada) as adjuvants to stimulate IgE production (15). Naive rats, sham sensitized by injection of saline, served as controls. Experiments were conducted 10-14 days after sensitization. Rats were anaesthetised and a blood sample was obtained for measurement of IgE. A laparotomy was performed, and a 15-20 cm segment of jejunum was excised, beginning 5 cm distal to the ligament of Treitz, and immediately placed in warmed oxygenated Krebs buffer.

Ussing Chambers

Intestinal segments were placed on a plastic rod and the external muscle layers were stripped off, leaving the submucosal plexus and mucosa intact. From each rat, 4 - 8 pieces of intestine were mounted in Ussing chambers (WPI Instruments, Narco Scientific, Mississauga, Ontario, Canada). Care was taken to avoid tissue containing Peyer's patches. The chamber opening exposed 0.6 cm^2 of serosal surface area to 8 ml of circulating oxygenated Krebs buffer, pH 7.35, 37 °C. The serosal buffer contained 10 mM glucose osmotically balanced with 10 mM mannitol in the mucosal buffer. The tissue was clamped at zero volts using a W-P Instruments automatic voltage clamp (Narco Scientific, Downsview, Ontario, Canada). The short-circuit current (Isc, in $\mu A/cm^2$) was recorded continuously. At 5 min intervals, the tissue was voltage clamped at 1 mV (for a duration of 1 s), and the Isc deflection was used to determine the conductance (G, mS/cm^2) according to Ohm's law. Tissues were allowed to equilibrate until the Isc stabilized before HRP (5×10^{-5} M) was added to the luminal buffer. The Isc response to HRP was measured as the peak increase in Isc within 15 minutes after addition of HRP to the luminal buffer.

HRP Flux

To determine mucosal to serosal flux of HRP, duplicate samples (500 ml) of serosal buffer were obtained at 0, 30, 60, and 90 minutes after addition of HRP, and were replaced with Krebs buffer. HRP activity was measured by assaying enzyme activity using a modified Worthington method (16). Briefly, 150 µl of sample was added to 800 µl of phosphate buffer containing 0.003% H_2O_2 and 80 µg/ml o-dianisidine (Sigma). HRP concentration was calculated using

enzyme activity (the rate of increase in optical density at 460 nm over a 2 min period). Fluxes were calculated according to standard formulae and were expressed as pmoles/cm^2/hr.

Electron Microscopy

To examine the route and extent of initial antigen uptake across the intestinal epithelium, tissues were removed from Ussing chambers 2 min after HRP challenge (approximately 1 min prior to the Isc response). To examine antigen transport after the hypersensitivity reaction, tissues were removed at 90 min. Tissues were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 2 hours at room temperature. Tissues were incubated overnight at 4 °C in 0.1 M sodium cacodylate buffer, and then washed three times for 5 minutes each in 0.05 M Tris buffer (pH 7.6). Segments were incubated for 30 min in 3,3'-diaminobenzedine tetrahydrochlorine (Sigma) (5 mg in 10 ml 0.05 M Tris buffer and 0.01% H_2O_2 , pH 7.6, 20° C). Samples were then processed for routine electron microscopy and embedded in epon. Tissues were oriented such that villus epithelial cells were cut longitudinally, and photomicrographs were prepared from the mid-villus region. To assess the distribution of HRP across the epithelium, the incidence of HRP within the apical or basal regions of the cell, or in the lamina propria was recorded. The total area of HRP within endosomes was also quantified in windows of fixed area (4 x 6 μm) (see Fig 1 for schematic representation). Ten micrographs per region were used for each rat, 40 for each rat group. For each rat, the percentage of windows positive for HRP in each region were determined, and then mean values were calculated for each rat group. Analysis was performed by one investigator (MCB) who was unaware of the treatment group.

IgE Measurement

IgE was determined in serum by passive cutaneous anaphylaxis as described previously (17). Briefly, Sprague Dawley rats were injected intradermally with 100 µl of diluted serum from *Ws/Ws* and *+/+* rats. Samples were run in duplicate dilutions from 1:8 to 1:512. Sera were also heat treated (56 °C, 60 min) and injected intradermally as above. After 72 h, rats were challenged by intravenous injection of a 0.5 ml solution of 1% Evans blue (Sigma) containing 2.5 mg of HRP. Bluing of the skin was evaluated 30 min after injection. The highest serum dilution giving a positive reaction was recorded as the PCA titre of that serum.

Statistics

Statistical significance between the treatment groups was assessed using ANOVA, with Dunnett T test for post hoc analysis. Analysis of conductance measures was performed using a repeated measures ANOVA. Correlation between HRP flux values and conductance measurements was assessed using Pearson's correlation test. Differences between groups were considered significant at $p < 0.05$.

Results

Luminal antigen challenge increases ion secretion in a mast cell-dependent manner

Mucosal mast cells were confirmed to be present in intestinal tissue sections from +/+ rats, but none were visible in intestinal tissues of *Ws/Ws* rats.

In Ussing chambers, intestinal segments from HRP sensitized +/+ rats responded to luminal HRP challenge with an increase in Isc (13.8 ± 2.0 $\mu A/cm^2$, mean ± SEM) beginning 3.4 ± 0.5 min after challenge. These results were similar to but of lower magnitude compared with those obtained from HRP sensitized Sprague-Dawley rats (mean increase in Isc of 32.1 ± 2.5 $\mu A/cm^2$, 3.4 ± 0.4 min after challenge). Tissues from naive rats and sensitized *Ws/Ws* rats showed no Isc response at all to HRP challenge, and maintained a stable Isc baseline for the duration of the 90 min experimental period. Representative Isc tracings for tissues from sensitized +/+ and *Ws/Ws* rats are shown in Fig 2. To ensure that the sensitization of *Ws/Ws* rats was successful, specific antibody titers were measured by passive cutaneous anaphylaxis (PCA). Sensitized +/+ and *Ws/Ws* rats had similar mean antibody titers of 7.4 ± 0.4 and 8.2 ± 0.2 respectively ($\log_2$ PCA titer). Heat treatment of the serum abolished the PCA response, indicating that the reactive antibodies were of the IgE isotype (18).

Initial transepithelial antigen transport (phase I) occurs by transcytosis and is enhanced by sensitization, but is mast cell independent

As shown above, luminal HRP challenge elicited a very rapid Isc response that was dependent on the presence of mast cells. We have previously shown that sensitization of Sprague-Dawley rats enhances antigen transcytosis across

intestinal epithelium to the extent that HRP reaches the lamina propria within 2 min (6). To confirm this finding and to examine the role of mast cells in enhanced antigen transcytosis, results were compared in tissues from naive and sensitized Sprague-Dawley and +/+ rats, and sensitized *Ws/Ws* rats. In EM photomicrographs of tissues from all sensitized rats, the distribution of HRP-containing endocytic vesicles was similar: HRP-containing vesicles were identified in the apical and basal regions of enterocytes, and in the lamina propria as soon as 2 min after HRP challenge. HRP was also found in goblet cells below the level of the theca. Examples are shown in Fig 3A-C. HRP was not visualized within any tight junctions or paracellular regions in photomicrographs from any of the rat groups at this timepoint. Compared to results from unsensitized naive rats, sensitization of rats increased the incidence of HRP-containing vesicles in both the apical and basal region of enterocytes. Sensitized Sprague-Dawley rats had HRP within endosomes in 65 ± 11 % of apical windows, and 45 ± 11 % of basal windows, compared to 30 ± 11 and 0 % in apical and basal regions of epithelium from naive Sprague-Dawley rats. Naive +/+ rats had endosomal HRP in the apical region of enterocytes in only 18 ± 3 % of photomicrographs examined, compared to 41 ± 9 % and 48 ± 6 % in sensitized +/+ and *Ws/Ws* rats respectively. Basal regions of enterocytes contained endosomal HRP in 13 ± 3 % of photomicrographs from naive +/+ rats, compared to 29 ± 6 % and 36 ± 9 % of photomicrographs from sensitized +/+ and *Ws/Ws* rats respectively (Fig 4). Area of endosomal HRP was also not statistically different between sensitized *Ws/Ws* and +/+ rats in all regions (apical: 180 ± 42 vs 120 ± 53; basal: 125 ± 90 vs 107 ± 58 nm^2 for +/+ and *Ws/Ws* rats respectively). The lack of significant differences between sensitized +/+ and *Ws/Ws* rats indicated that mast cells do not influence the initial uptake and transport of antigen.

Subsequent to the intestinal hypersensitivity reaction (phase II), amplified transepithelial antigen transport requires sensitization, occurs via the paracellular route, and is mast cell dependent

We have previously shown that the mucosal to serosal flux of HRP across jejunal segments (measured over a 90 min period after challenge) was significantly increased in Sprague-Dawley rats sensitized to HRP compared to naive controls or rats sensitized to an irrelevant antigen. To determine the role of mast cells in antigen transport at this stage mucosal to serosal flux of intact HRP and tissue conductance were compared in tissues from naive and HRP-sensitized +/+ and *Ws/Ws* rats. HRP flux was significantly higher across intestine from sensitized +/+ rats compared to unsensitized +/+ rats beginning in the second 30 min flux period (53.9 ± 10.6 vs 25.4 ± 3.6 pmoles/cm^2/h), and was even more pronounced in the third flux period (114.0 ± 19.5 vs 45.2 ± 5.7 pmoles/cm^2/h). Intestine from sensitized *Ws/Ws* rats had a HRP flux comparable to unsensitized controls (21.5 ± 4.2 and 21.7 ± 3.5 pmoles/cm^2/h in the second and third flux periods respectively), and significantly less than sensitized +/+ rats (Fig 5). Conductance measures at 90 min post-challenge correlated closely with the HRP flux (correlation coefficient, r = 0.83). Conductance gradually increased throughout the experimental period in sensitized +/+ rats, but not naive or *Ws/Ws* rats. A statistically significant increase was observed 60 min after HRP challenge, that was maximal at 90 min (conductance 12.0 ± 2.0 mS/cm^2 for sensitized +/+ rats vs -0.3 ± 0.4 for sensitized *Ws/Ws* rats) (Fig 6).

Electron microscopy of tissues fixed 90 min post-challenge demonstrated HRP in paracellular regions of sensitized +/+ rats, but not *Ws/Ws* or naive +/+ rats (Fig 7). This is consistent with previous findings in Sprague-Dawley rats that demonstrated that increased HRP flux and conductance were associated with

the presence of HRP within the tight junctions and paracellular regions between epithelial cells.

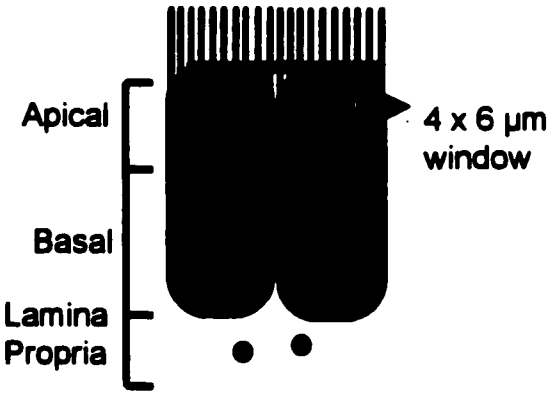

Figure 3.1: Schematic of Endosomal HRP Quantification: Photomicrographs were prepared from mid-villus epithelium. Incidence and area of endosomal HRP were measured in 4 x 6 µm windows (shown as rectangles) placed immediately below the apical membrane, or immediately above or below the basal membrane. 10 photomicrographs (per area) per rat, 40 per rat group, were used for quantification.

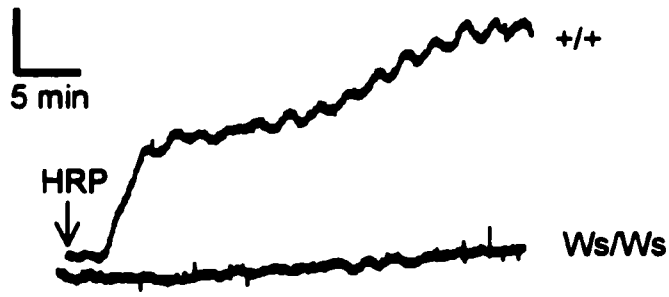

Figure 3.2: Representative short-circuit current (Isc) tracings. Isc responses to HRP (5×10^{-5} M) in jejunum from a sensitized +/+ or Ws/Ws rat.

Fig 3.3 (A-C): Legend on next page

Figure 3.3 (previous and this page): Representative photomicrographs (2 min after HRP challenge). Electron micrographs of tissues 2 min after luminal challenge with HRP. (A) Apical view of enterocyte in tissue from a sensitized +/+ rat. Arrows indicate four endosomes containing HRP. MV = microvilli. Bar = 1 μm. (B) Basolateral view of an enterocyte in tissue from a sensitized Ws/Ws rat, showing a large endosome containing HRP between the nucleus (nu) and lamina propria (lp). Bar = 1 μm. (C) Full thickness view of epithelium in tissue from a sensitized +/+ rat, showing a goblet cell containing HRP in the basal region of the cell, as indicated by the arrows. Bar = 2 μm.

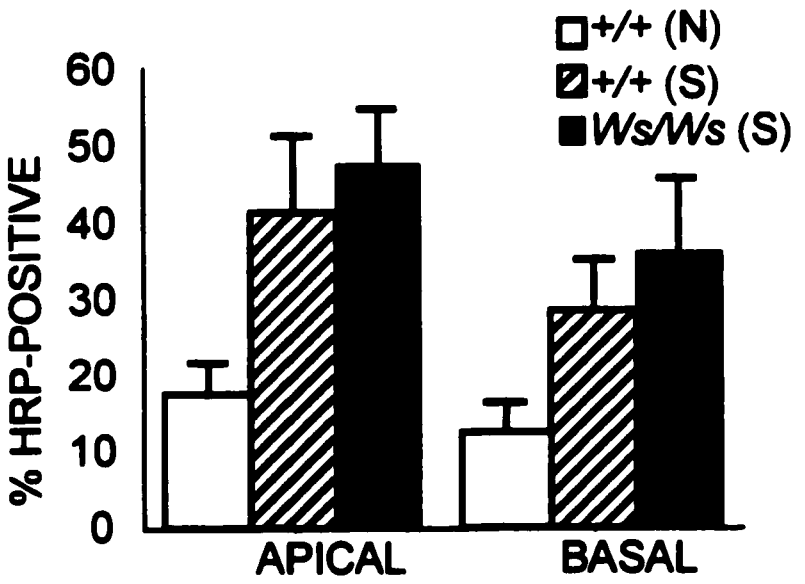

Figure 3.4: Distribution of HRP-positive endosomes 2 min after HRP challenge. Electron photomicrographs were analysed from tissue taken 2 min after luminal challenge with HRP. Incidence of HRP within 4 x 6 µm windows in the apical, or basal region was recorded for 10 micrographs/rat, 40/group. +/+ (N) = naive +/+ rats, +/+ (S) = sensitized +/+ rats, Ws/Ws (S) = sensitized Ws/Ws rats. Data are expressed as the mean ± SEM for 4 rats per group.

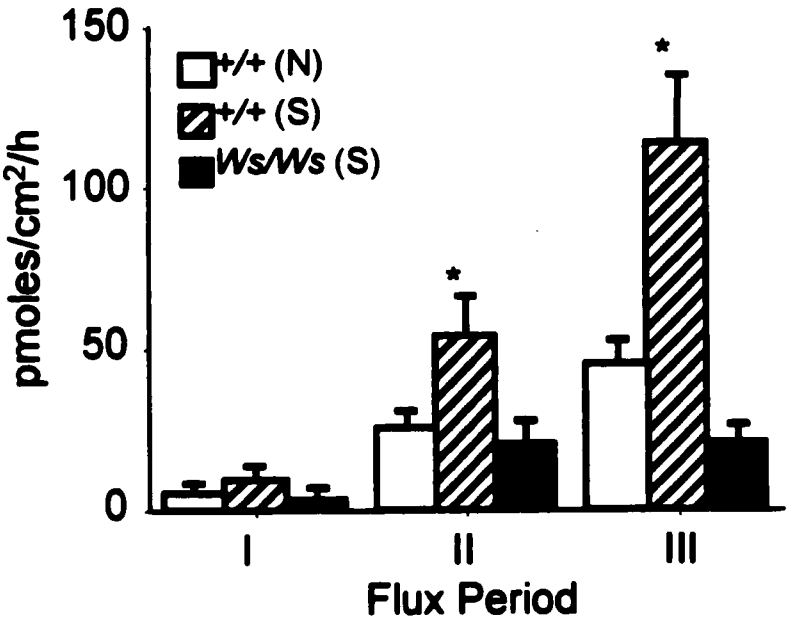

Figure 3. 5: Mucosal to serosal horseradish peroxidase (HRP) flux. HRP flux across jejunum in period I (0-30 min), II (30-60 min) and III (60-90 min) after HRP addition. +/+ (N) = naive +/+ rats, +/+ (S) = sensitized +/+ rats, Ws/Ws (S) = sensitized Ws/Ws rats. Data are expressed as the mean ± SEM, * $p < 0.05$ compared to control rats at the same time point, n = 8 rats/group.

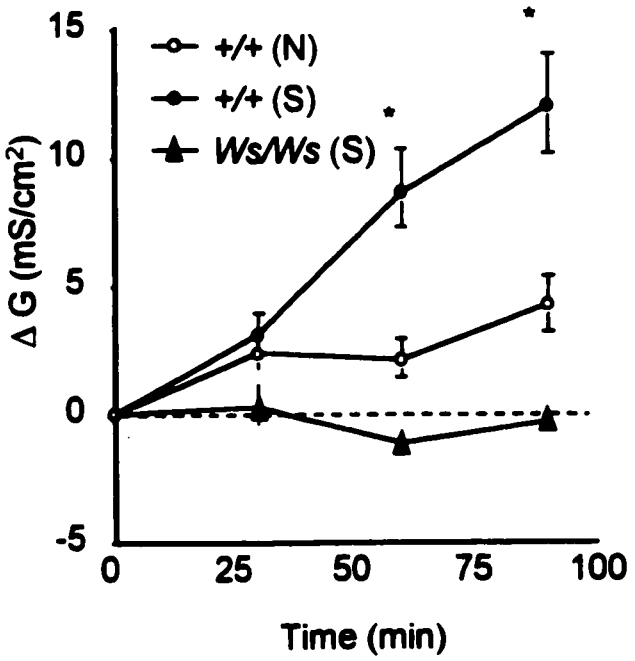

Figure 3.6: Time course of tissue conductance after HRP challenge. Change in tissue conductance (G, in mS/cm2) after luminal HRP challenge (t=0). +/+ (N) = naive +/+ rats, +/+ (S) = sensitized +/+ rats, Ws/Ws (S) = sensitized Ws/Ws rats. Data are expressed as mean ± SEM, * $p < 0.05$ compared to baseline reading, n = 8/group.

Figure 3.7: Representative photomicrograph from an HRP-sensitized +/+ rat, 90 minutes after luminal HRP challenge. Arrows indicate the paracellular space between enterocytes, which is filled with HRP. Bar = 1 μm.

Discussion

In this study, we demonstrated that intestinal transepithelial antigen transport occurred in two phases, an initial phase of antigen uptake and translocation and a second phase that followed after the hypersensitivity reaction. The initial phase was increased by sensitization and was mast cell independent. The second phase was dependent on both sensitization and the presence of mast cells. Initially, HRP was transported by transcytosis in endocytic vesicles. In this phase, uptake of HRP at the apical membrane and its transport through the cell was significantly greater in rats sensitized to HRP. The similar data in +/+ and *Ws/Ws* rats indicated that mast cells did not affect this phase. After the hypersensitivity reaction, both the HRP flux and the tissue conductance values were significantly elevated only in mast cell containing intestine from rats sensitized to HRP, indicating that mast cells were required for this second phase of antigen transport.

Intestine from sensitized *Ws/Ws* mast cell-deficient rats did not respond to luminal antigen challenge with an increase in Isc. This finding is consistent with those from previous studies on the critical role for mast cells in the intestinal hypersensitivity reaction. Doxantrazole, a mast cell stabilizer, blocked the Isc response to antigen challenge (19). Experiments in *W/W^v* mast cell-deficient mice and +/+ littermate controls showed that mast cells were responsible for the majority of the antigen-induced increase in Isc; however, a small component remained in intestine from *W/W^v* mice (20). We obtained similar findings in our current experiments using *Ws/Ws* and +/+ rats, but the entire Isc response to antigen was eliminated in the absence of mast cells, suggesting that this is a better model to study mast cell-mediated changes in physiology. The absent hypersensitivity reaction in *Ws/Ws* rats was not due to an inability of these rats to

mount an IgE antibody response since both *Ws/Ws* and +/+ rats had similar PCA titers that were abolished by heat treatment.

Analysis of electron photomicrographs of tissues fixed 2 min after luminal HRP challenge demonstrated that the route of initial antigen uptake was transcellular. The incidence of HRP-containing endocytic vesicles throughout enterocytes was greater in sensitized versus naive rats. However, the absence of mast cells in *Ws/Ws* rat intestine did not reduce either the amount (measured by area) or incidence of HRP in various regions of the cell. These findings imply that the total amount of HRP transported across the epithelium was enhanced by sensitization but was not influenced by the presence of mast cells.

Bockman and Winborn (21) reported that sensitization of hamsters to ferritin upregulated intestinal absorption of ferritin after luminal exposure. We previously documented that endocytic transport of protein was enhanced only for the antigen to which the rats had been sensitized and not for an irrelevant antigen. Taken together, their results and those from our studies provide support for recognition of antigen by epithelial cells, possibly by surface-bound immunoglobulin in sensitized animals (for further discussion see ref (6) and related editorial, ref (22)). This study was conducted to examine if mast cells, known to bind antigen via IgE and high affinity FcεRI receptors and occasionally described within epithelium (23,24), are involved in the early phase of antigen transport that occurs prior to the hypersensitivity reaction. Our findings of similar amount of HRP uptake and distribution in sensitized *Ws/Ws* and +/+ rats rule out a contribution of mast cells to this initial phase of transepithelial antigen transport.

In mast cell-containing intestine from HRP-sensitized rats, a large increase in flux of intact HRP was observed beginning at 30 min after the hypersensitivity reaction. The increased HRP flux was associated in time and

correlated with increased tissue conductance, a measure of the integrity of the tight junctions. This was supported by electron photomicrographs showing HRP in the paracellular regions. A number of studies, both in sensitized animal models (9,25) and allergic patients (26,27) have shown that intestinal permeability to small molecular weight probes and "bystander" antigens increases following antigen challenge. In contrast to the specificity observed with initial transcytosis of antigen, this phase of antigen transport appears to be a non-specific permeability defect. We have observed that ovalbumin-sensitized Sprague-Dawley rats challenged with luminal ovalbumin also develop an increased luminal to serosal flux of HRP (M.C. Berin, unpublished observations), confirming the lack of specificity of this antigen transport pathway. The increased HRP flux and conductance we observed in HRP sensitized +/+ and Sprague-Dawley rats associated with the presence of HRP in the paracellular regions and within the tight junctions, suggests a recruitment of the paracellular pathway. Heyman et al also showed increased intestinal flux of HRP post-antigen challenge across biopsies from children with cow's milk allergy (28), and intestinal segments from sensitized guinea pigs (29), but apparently in the absence of an alteration in tissue conductance. No electron microscopy analysis was carried out in those studies to examine the route of HRP transport.

As indicated above, HRP challenge to mast cell-containing intestine from sensitized rats resulted in an elevated Isc, followed by increased conductance and flux of HRP. However, transport parameters in intestine from sensitized *WsWs* mast cell-deficient rats were indistinguishable from those in intestine from unsensitized +/+ controls. This result indicates that both mast cells and sensitization are required for the later phase of non-specific antigen transport. The role of mast cells in regulation of epithelial ion secretion has been well

studied, and it has been clearly established that mast cell mediators such as histamine and prostaglandins can act via specific receptors on the intestinal epithelium to initiate chloride ion secretion (7). Although a number of studies have reported that antigen challenge in sensitized animals produces an increase in intestinal epithelial permeability, this study is the first to directly show that the development of the antigen-induced barrier defect is mast cell dependent. It should be noted that c-kit deficiency can have effects on cell populations other than mast cells. Intraepithelial lymphocytes (IEL) also express the c-kit receptor, and interactions with SCF-producing intestinal epithelial cells may be important for their normal development. Although IEL populations have not been examined in *Ws/Ws* rats, *W/W^v* mast cell-deficient mice demonstrate age-dependent changes in IEL subsets. As mice age, there is a decrease in the percentage of TCR$\gamma\delta$ IEL, and an increase in TCR$\alpha\beta$ IEL in *W/W^v* compared to +/+ mice (30). As there is not a dramatic depletion of IEL in *c-kit*-deficient animals, it is unlikely that they are responsible for the recruitment of the paracellular antigen transport pathway we observe in +/+ but not *Ws/Ws* rats. However, it cannot be ruled out that an alteration in IEL function in *Ws/Ws* rats may play a role in transepithelial antigen transport. Reconstitution experiments with mast cells obtained from +/+ bone marrow would confirm that mast cells are responsible for the increased antigen transport observed after the hypersensitivity reaction, however due to the heterogeneous genetic background of the Ws/Ws and +/+ animals (F_2 generation of 2 inbred rat strains) reconstitution is not feasible.

Although the mechanism of mast cell-induced increase in antigen transport has not been explored in the current study, it has been shown that a number of mast cell products such as cytokines, prostaglandins, nitric oxide and proteases, can alter epithelial permeability (31). Alternately, mast cells could

potentially regulate tight junction permeability indirectly through nerves. Nerves have been shown to be activated by mast cells or specific mast cell mediators and may act to amplify the hypersensitivity response (32). Stimulation of intestinal tissue with a cholinergic agonist, carbachol, has been shown to increase paracellular permeability to HRP (5,33) and stress-induced barrier disruption has also been demonstrated to be mediated by cholinergic nerves (34). We have previously shown that pretreatment of intestinal segments with tetrodotoxin prevents the antigen-induced increase in ^{51}Cr-EDTA flux in ovalbumin-sensitized rats (25). Therefore, it is possible that mast cells recruit the paracellular transport pathway for antigen via the activation of enteric nerves.

In conclusion, our study suggests that transepithelial antigen transport occurs in two distinct phases in sensitized rat jejunum. We have provided evidence that in Phase I, antigen is initially taken up rapidly and transported across the epithelium by an endocytic mechanism that is enhanced by sensitization, but is independent of the presence of mast cells. In phase II, antigen transport is sensitization and mast cell dependent, and leads to a large flux of antigen across the epithelial barrier likely through the recruitment of the paracellular pathway. These findings suggest that in an allergic individual, even small amounts of antigen within the lumen can be preferentially transported to the lamina propria where subsequent activation of mast cells further induces a non-specific barrier defect. This sequence of events may be extremely important in initiating and sustaining allergic inflammation not just in the gastrointestinal tract but at all mucosal sites in the body.

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Chapter 4
Role for IL-4 in Macromolecular Transport Across Human Intestinal Epithelium

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Abstract

Increased epithelial permeability is associated with intestinal inflammation, but there is little information on factors that regulate barrier function in the absence of or prior to inflammation. We examined if the cytokine, IL-4, or serum from atopic individuals, could alter epithelial barrier function to antigenic-sized macromolecules. Monolayers of human intestinal T84 epithelial cells were grown ± IL-4 or serum for 1-3 days. T84 resistance and transepithelial transport of horseradish peroxidase (HRP) were subsequently measured. IL-4 and atopic serum significantly decreased T84 monolayer resistance and increased transepithelial HRP transport. Electron microscopy analysis demonstrated that transcellular as well as paracellular pathways were affected. Anti-IL-4 antibodies abolished the increase in HRP transport in response to both IL-4 and serum. We speculate that enhanced production of IL-4 in allergic conditions may be a predisposing factor to inflammation by allowing uptake of luminal antigens that gain access to the mucosal immune system.

Introduction

The intestinal epithelium functions as a selective barrier that facilitates nutrient absorption while limiting uptake of antigenic or noxious material from the lumen. The lamina propria immediately below the epithelial layer is densely populated with immune cells capable of responding to antigens that breach the epithelial barrier. Mediators released from activated immune cells have been shown to alter normal epithelial transport and barrier function, and may possibly amplify the influx of antigenic material from the lumen. It has been well documented that inflammatory disorders of the human intestine are associated with epithelial barrier dysfunction (29,34), but it is not clear if the epithelial perturbation is a consequence of inflammation or a predisposing factor.

Epithelial barrier dysfunction in the absence of overt inflammation has been documented in atopic patients and in animal models of allergy. Majamaa et al (23) demonstrated that small intestinal biopsy specimens from patients with atopic eczema had a greater transepithelial transport of intact proteins than biopsies from non-atopic controls. *In vivo* studies on patients with bronchial asthma have also indicated that intestinal epithelial permeability is elevated compared to controls (1). We recently demonstrated that sensitized rats had enhanced macromolecular uptake across the intestinal epithelium (2). In those studies, basal epithelial permeability was altered in the absence of intestinal inflammation or mast cell activation.

Atopic diseases are characterized by an increase in interleukin-4 (IL-4) producing T-lymphocytes. These can be detected in the systemic circulation (33), and at respiratory (4) and intestinal (12) mucosal surfaces. IL-4 functions as a switch factor for IgE synthesis from B lymphocytes (8) and stimulates the production of other Th2 cytokines (20). IL-4 has also been shown to have direct

effects on human intestinal epithelium, reducing ion secretory responses, decreasing electrical resistance and increasing permeability to small molecular weight probes (6,36).

In this study, we examined the direct effect of IL-4 and serum from atopic humans on the barrier function of a model intestinal epithelium to an antigen sized protein. Horseradish peroxidase (HRP) was chosen as the model protein for several reasons: 1) HRP can be readily measured by kinetic enzymatic assay, 2) HRP can be visualized by electron microscopy, and 3) we have previously used HRP as a sensitizing antigen in a rodent model of food allergy (2). Apical to basal transepithelial transport of intact HRP was used to assess the ability of T84 monolayers to restrict movement of antigenic macromolecules. We found that T84 cells grown in the presence of either IL-4 or atopic serum responded with a large increase in the transepithelial movement of HRP. Monoclonal antibodies against human IL-4 completely prevented this increased HRP uptake. Electron microscopy indicated that multiple macromolecular transport pathways were affected: endocytic uptake of HRP was significantly increased, and tight junctions were perturbed, allowing movement of HRP through the paracellular regions between epithelial cells. These findings suggest that stimulated IL-4 production in atopic disease states may be sufficient to alter epithelial barrier function, thereby allowing an influx of antigenic material from the intestinal lumen.

Methods

Cell Culture

T84 epithelial cells were seeded (10^6 cells) on tissue culture-treated Transwell filter supports (0.4 μm pore size, 1.2 cm^2 surface area; Costar Corporation, Cambridge, MA). Culture media consisted of a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and F-12 nutrient mixture (GIBCO, Grand Island, NY) supplemented with 10% (vol/vol) heat-inactivated fetal calf serum (FCS, Cansera International, Rexdale, ON, Canada), 1.5% Hepes (GIBCO), and 2% penicillin-streptomycin (GIBCO). Cells were grown at 37 °C in 5% CO_2 for 7 days to attain polarized epithelial monolayers with transepithelial resistance (TER) measurements of > 1000 Ω/cm^2. Monolayer resistance was monitored throughout the experimental period using a Millicell-ERS system (Millipore, Bedford, MA). TER is a measure of the ability of T84 cells to restrict passive movement of ions.

Serum Samples

Blood was obtained from atopic patients during a routine visit to the allergy clinic at McMaster Medical Centre. Patients used were age 27 - 62 (n = 4), with active atopic dermatitis, documented to have immediate skin test reactivity to multiple antigens, including ragweed and dust mite. Patients were not currently taking oral corticosteroids. Sera from healthy volunteers with no history of allergy or asthma were obtained for control experiments. All serum samples were snap-frozen in liquid nitrogen and stored at - 70°C prior to use in experiments.

Treatment of Monolayers with IL-4 or Serum

Confluent monolayers were cultured with 10 ng/ml recombinant human IL-4 (R & D Systems, Minneapolis, MN) added to the basal compartment (vol. 1.5 ml) of the Transwell unit. Atopic or control serum was added to the basal compartment at 50 µl/ml (5 %). Cells were treated for 1,2, or 3 days prior to study. In some experiments, media containing IL-4 or serum was preabsorbed with anti-human IL-4 (1 µg/ml, R & D Systems) 2 h prior to addition to the epithelial monolayers.

Ussing Chamber Experiments

T84 monolayers on filter supports were mounted in Ussing chambers and bathed in oxygenated Krebs buffer (pH 7.35, 37 °C). Krebs buffer bathing the basal surface contained 115 mM NaCl, 8 mM KCl, 1.2 mM $MgCl_2$, 1.25 mM $CaCl_2$, 2.0 mM KH_2PO_4, 25 mM $NaHCO_3$, and 10 mM glucose as an energy source. In the apical buffer, 10 mM mannitol was substituted for glucose. After a 15 min equilibration, the potential difference across the monolayers was clamped at 1 mV (differential pulse method, 1 pulse/30 seconds), and the resulting current deflection was measured and TER calculated according to Ohm's law.

Transepithelial Transport of HRP

HRP (MW 44 000, type VI-A, Sigma) was added to the apical side of the monolayers at a concentration of 10^{-5} M and monolayers were incubated overnight (18 h) at 37 °C. Media samples were obtained from the apical and basal compartments and assayed for HRP concentration by kinetic enzymatic assay as previously described (2). Briefly, 150 µl of sample was added to 800 µl of phosphate buffer containing 0.003% H_2O_2 and 80 µg/ml o-dianisidine (Sigma). Enzyme activity was determined from the rate of increase in optical density at

460 nm during a 2 min period. HRP transport was expressed as percent recovery of apical HRP present in the basal compartment.

Electron Microscopy

To examine the route of transepithelial transport of HRP, T84 monolayers to which 10^{-5} M HRP had been added on the luminal side were subjected to transmission electron microscopy. Briefly, 60 min after HRP addition T84 monolayers were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) and then processed for 3,3'-diaminobenzidine tetrahydrochloride (DAB) cytochemistry using 0.5 mg/ml of DAB and 0.01% H_2O_2 in Tris buffer. After washes in Tris buffered saline, samples were dehydrated through a series of graded ethanol washes and embedded in Epon. Ultrathin sections were cut and placed onto mesh copper grids and stained with uranyl acetate and lead salts. Random photomicrographs were prepared by an observer unaware of the treatment group (PCY). For each experimental group, intracellular HRP was quantified by determining the number of HRP-containing endosomes and total endosomal HRP area in windows (25 x 30 μm, 20/group). Tight junctions and paracellular regions were also examined for the presence of HRP.

IL-4 Measurement

IL-4 concentration in serum samples was measured using a commercially available human IL-4 ELISA kit (BioSource International, Camarillo,CA). The limit of detection of the assay was > 15 pg/ml.

Statistics

Data was expressed as mean ± SEM. Due to variability in control monolayer TER between experiments, data were expressed as percent of control. Results were

analyzed using ANOVA and individual comparisons made by Student's *t*-test; p values less than 0.05 were accepted as significant.

Results

Treatment with IL-4 or atopic serum decreases transepithelial resistance of T84 monolayers

TER of control monolayers increased throughout the 3 day experimental period, and ranged from 1200 to 2400 Ω/cm^2. Treatment of monolayers with IL-4 resulted in a significantly lower TER (53.8 ± 3.6 %, 46.8 ± 3.1 %, and 38.7 ± 3.5 % of control for day 1,2, and 3 respectively) (Fig 1). Resistance was also lower after treatment with atopic serum (82.6 ± 2.1 %, 73.7 ± 3.8 %, and 68.1 ± 5.3 % at days 1,2, and 3 respectively). The maximum change in TER occurred at day 3, therefore HRP transport experiments were conducted at day 3.

Treatment with IL-4 or atopic serum increases transepithelial transport of HRP

Apical to basal transepithelial movement of HRP was measured 3 days after culture of the T84 monolayers with IL-4 or atopic serum. In control monolayers, intact HRP was detected at low levels in the basal compartment of the transwell. Treatment with IL-4 increased HRP transport to 657 ± 174 % of matched control monolayers. Atopic serum also increased HRP transport to 675 ± 247 % of time-matched control monolayers (Fig. 2). Serum from non-atopic controls did not significantly alter HRP transport compared to controls (104 ± 7 % of untreated time-matched monolayers).

Treatment with IL-4 or atopic serum affects both transcellular and paracellular pathways

The route of apical to basal transepithelial HRP transport was assessed using transmission electron microscopy. Representative photomicrographs are shown in Fig. 3. Analysis of HRP within paracellular regions demonstrated that

IL-4 and atopic serum induced transport through the tight junctions. While no paracellular regions examined in control monolayers contained HRP, 70 % of IL-4 treated, and 45% of atopic serum treated monolayers were positive for HRP (Table 1). At 60 min after addition of HRP to the luminal side of T84 monolayers, HRP was located within endosomal vesicles in control, IL-4-treated and atopic serum-treated T84 cells. Quantitative analysis of intracellular HRP (by measuring mean area of endosomal HRP) showed that treatment with IL-4 and atopic serum significantly increased the amount of endosomal HRP. The mean area of HRP in epithelial cells increased from 2.54 ± 0.30 μm^2/window in control monolayers to 8.24 ± 1.7 μm^2/window in IL-4 treated monolayers and 6.75 ± 1.3 μm^2/window in atopic serum treated monolayers (Fig 4).

Antibodies against IL-4 inhibit the increase in transepithelial HRP transport induced by IL-4 or atopic serum

IL-4 induced a drop in TER that was inhibited by addition of anti-IL-4 antibodies at a minimum concentration of 1 μg/ml (94 ± 6% of control resistance compared to 47 ± 6% of control with IL-4 alone). Preincubation of the IL-4 or atopic serum with anti-IL-4 antibody abolished the increase in HRP transport, such that HRP transport was not significantly different from control (151 ± 31 and 119 ± 7 % of control for IL-4 and atopic serum groups respectively) (Fig. 5). The concentration of IL-4 in the atopic serum was measured by a sensitive ELISA for human IL-4. IL-4 was below the level of detection in all of the sera used in the experiments.

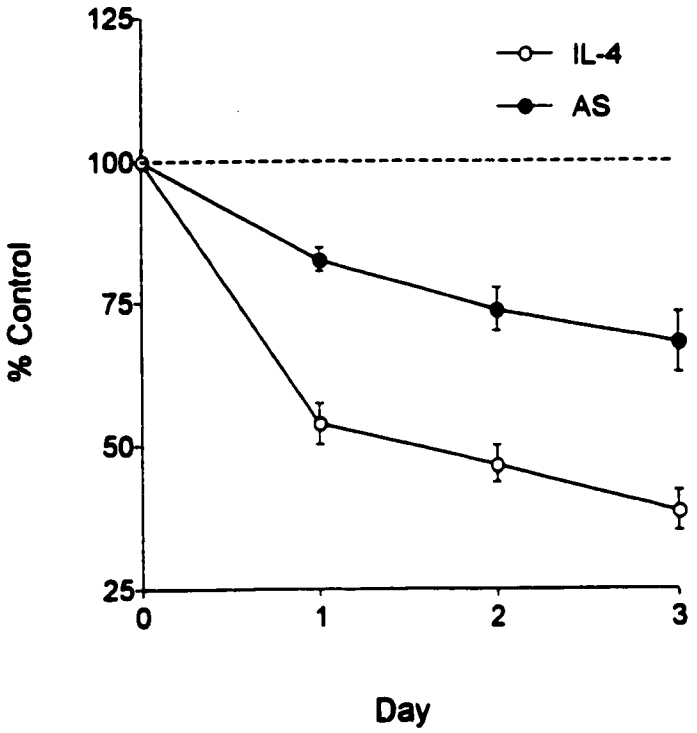

Figure 4.1: T84 monolayer transepithelial resistance (TER, in Ω/cm^2) after 1, 2, or 3 days treatment with 10 ng/ml IL-4 or 5 % atopic serum (AS). TER was significantly reduced after 1 day treatment, and maximal percent change from control was observed after 3 days treatment (values expressed as mean ± SEM, n=4-14).

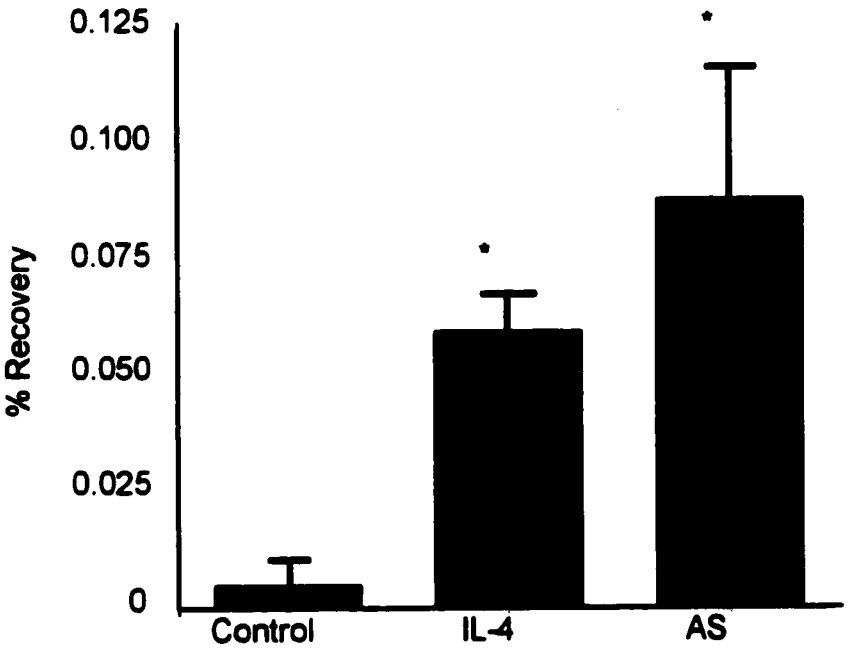

Figure 4.2: Movement of HRP across T84 monolayers after treatment with IL-4 or atopic serum for 3 days. HRP (10^{-5} M) was added to the apical compartment of monolayers. Samples from the apical and basal compartments of Transwells were taken after 18 h of incubation at 37 °C. HRP concentration in the basal compartment was expressed as percent recovery of apical HRP (n = 9-15 monolayers). *p < 0.05 compared to controls.

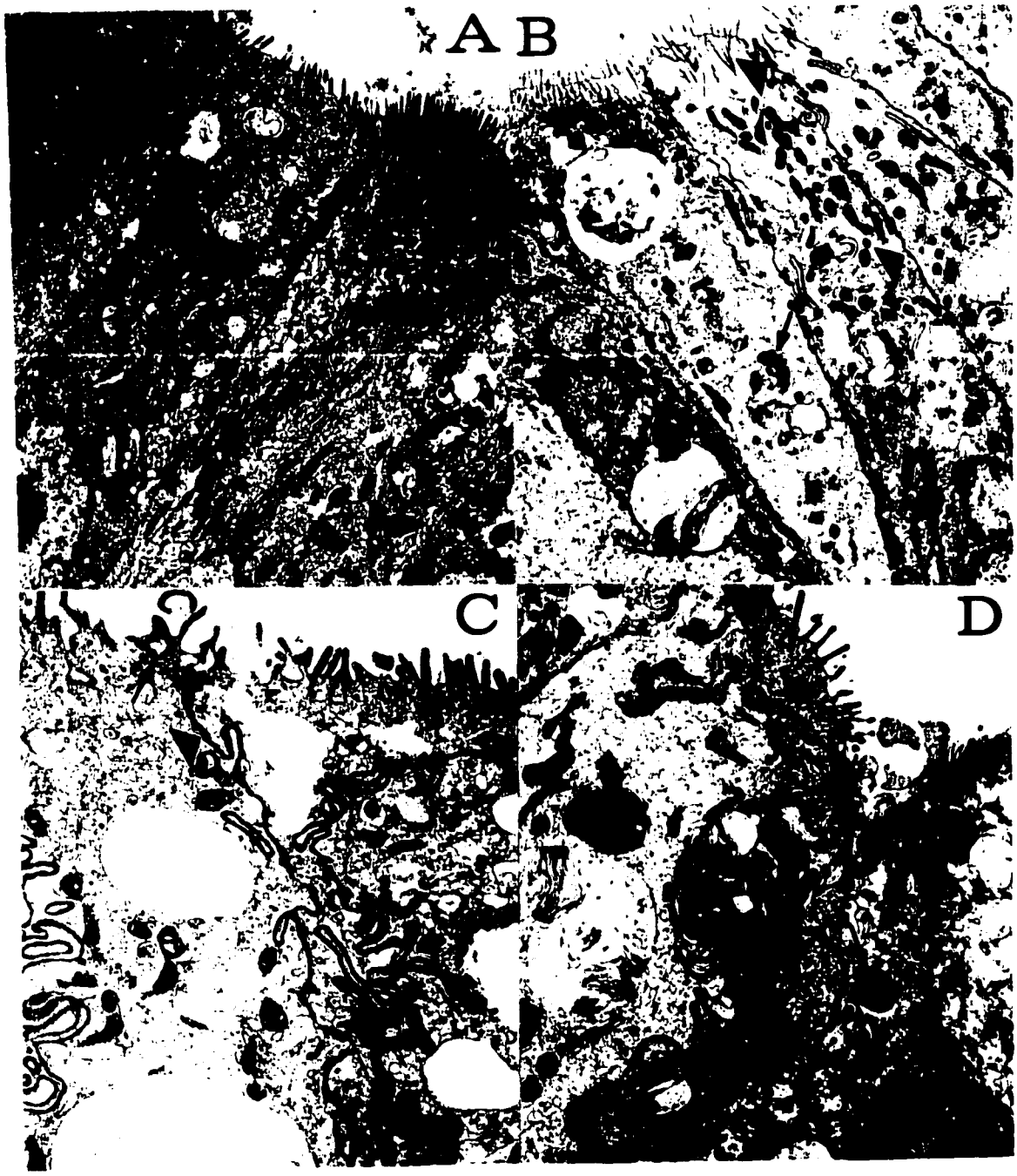

Figure 4.3: Transmission electron photomicrographs of control (A), IL-4 treated (B), and atopic serum treated (C,D), T84 cells exposed to 10^{-5} M HRP for 60 min and stained for peroxidase activity. Arrows indicate HRP reaction product in vesicles, arrowheads mark HRP-containing paracellular regions.

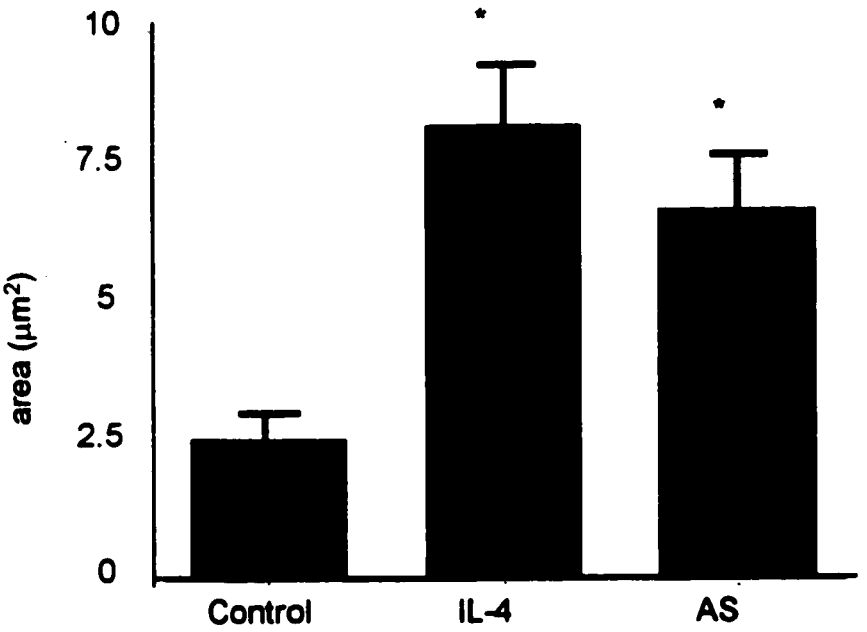

Figure 4.4: Quantification of intracellular HRP. T84 monolayers were treated with IL-4 or atopic serum for 3 days, then exposed to HRP on the apical side for 60 min. Endosomal HRP area was quantified in 25 x 30 μm windows using 20 photomicrographs per treatment group. (n = 2 monolayers/group). *$p < 0.05$ compared to controls.

Group	Total Regions Examined	HRP-Positive Regions	% HRP Positive
Control	65	0	0
IL-4	71	51	70
AS	73	32	45

Table 4.1: Quantification of HRP within paracellular regions of T84 monolayers. T84 monolayers were cultured for 3 days in the presence of IL-4 (10 ng/ml) or atopic serum (AS, 5%), or with media alone (control). Monolayers were fixed for electron microscopy 60 min after addition of HRP to the luminal side of Ussing chambers.

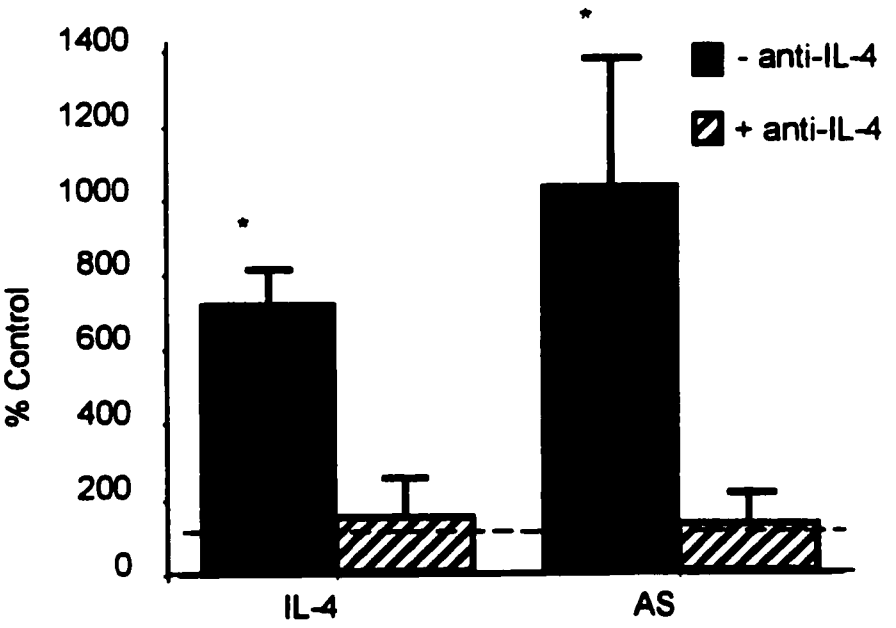

Figure 4.5: Effect of an anti-IL-4 antibody on transepithelial HRP movement in the presence of IL-4 or atopic serum. T84 monolayers were treated with IL-4 or atopic serum, ± 1 μg/ml anti-IL4 for 3 days. HRP was added to the apical compartment of Transwells, and basal recovery of HRP was measured after 18 h. Data are expressed as percent of HRP movement across control monolayers (n = 4-10). *p < 0.05 compared to untreated control.

Discussion

It is well established that gastrointestinal inflammation is associated with reduced barrier function (29,34). The observation of increased intestinal epithelial permeability in a sub-group of healthy relatives of patients with Crohn's disease (25,28) has led to the hypothesis that increased epithelial permeability may be a predisposing factor in the development of inflammatory bowel disease. However, much of the information to date on regulation of the epithelial barrier has focused on inflammatory factors rather than possible predisposing non-inflammatory factors. In this study, the effect of IL-4 and serum from atopic patients on transepithelial transport of a model protein antigen, HRP, was examined. IL-4 significantly reduced epithelial barrier function, as demonstrated by decreased resistance and increased apical to basal movement of HRP. Atopic serum mimicked the effects of IL-4 on HRP transport. Our studies also suggest that the effects of atopic serum were mediated by an IL-4 dependent mechanism, as shown by complete inhibition of enhanced HRP transport with anti-IL-4 antibodies. Analysis of the route of HRP transport by electron microscopy revealed that both transcellular and paracellular transport routes were affected.

IL-4 was initially described as a T helper lymphocyte-derived growth factor for B-lymphocytes (18). It has since been shown to be produced not only by T cells, but also mast cells (5) and eosinophils (27). IL-4 is required for IgE production (9) , and the generation of a Th2 cytokine profile (20), characterized by IL-4, IL-5, and IL-10 production. It is considered an anti-inflammatory cytokine, due to its inhibitory action on inflammatory mediator production from macrophages (10). IL-4 is elevated in allergic disorders (33) and helminth infections (32), and in ileal biopsies from Crohn's disease patients in early stages of relapse (7). IL-4 is produced by immune cells at mucosal sites in close

proximity to intestinal (12) and respiratory (4) epithelium, ideally situated to modify epithelial physiology.

IL-4 clearly has effects on epithelial cells. Intestinal epithelial monolayers cultured with IL-4 have decreased TER, as well as decreased ion secretory response to secretagogues, effects that were inhibited by an antibody against the IL-4 receptor (6,36). We have previously demonstrated enhanced transcellular antigen uptake across the intestinal epithelium in a rat model of food allergy (2). Sensitized rats are characterized by elevated IL-4 production from isolated spleen cells (Berin and Perdue, unpublished observations). The current studies were designed to determine the role of IL-4 in the regulation of macromolecular transport across intestinal epithelium.

We confirmed the findings of Colgan et al (6) by demonstrating that culture of T84 cells with IL-4 caused a significant drop in TER. The time course and magnitude of resistance changes were similar to those reported previously. TER was also monitored in monolayers cultured with atopic serum. The time frame of the drop in resistance was similar to that observed with IL-4, but the magnitude of the resistance drop was smaller. All treatments were added to the basal side of the cells, as there is evidence for basolateral polarization of the IL-4 receptor (6), reflecting the physiological route of cytokine delivery.

Although resistance changes suggested an alteration in tight junctional permeability, it was unclear if these changes would translate into greater permeability of a macromolecule such as HRP. Previous studies have indicated that T84 tight junctions can maintain their barrier function against macromolecules such as HRP (MW 44 000) even at low resistance (13). Apical to basal movement of HRP was assessed at day 3, when relatively large resistance changes compared to control were observed. Culture of T84 cells with IL-4 caused a large 7-fold increase in HRP transport compared to controls.

Culture with atopic serum also altered monolayer barrier function, and a 10-fold increase in intact HRP transport was observed. Although proteins are normally transported across epithelial cells by endocytosis (31), the increased TER together with the large increase in HRP flux suggested that HRP was crossing the epithelial monolayer by the paracellular route. Examination of the monolayers by electron microscopy confirmed this, showing HRP within the tight junctions and in the paracellular regions between epithelial cells in IL-4 and atopic serum-treated monolayers but not controls. The opening of the epithelial tight junctions to macromolecules could potentially have pro-inflammatory effects *in vivo* by allowing an non-specific influx of luminal antigens, including large bacterial products such as lipopolysaccharide, into the lamina propria where they could stimulate resident immune cells.

Analysis of the route of HRP transport by electron microscopy indicated that IL-4 and atopic serum also enhanced transcellular transport pathways, similar to our previous observations in sensitized rats (2). Area of endosomal HRP was increased approximately 3-fold in IL-4 and atopic serum-treated monolayers. In control monolayers, HRP was observed within intracellular membrane-bound vesicles, and was never identified in the paracellular spaces between cells. Transcytosis was clearly occurring even in the control monolayers, as measurable amounts of HRP appeared in the basal compartment after addition to the apical side of the cells. Previous studies using intestinal epithelial monolayers (15) and intact tissues (17,19) have shown that small amounts of intact protein cross epithelial cells, and this process was inhibited by metabolic inhibitors and microtubule disrupting agents (19). Although the majority of intact HRP transported across IL-4 and atopic serum-treated monolayers likely travelled unimpeded between the cells, the relatively large increase in endosomal HRP suggested that the transcellular pathway could also contribute to a defect in

epithelial barrier function.

There is a lack of information on the regulation of the transcellular transport pathway, or if the transcellular and paracellular pathways are controlled by similar mechanisms. Increases in paracellular permeability are associated with cytoskeleton rearrangement (21) that may also impact on the endocytic pathway. A concurrent increase in paracellular and transcellular HRP transport has been reported in rat jejunum after treatment with carbachol (3), but other studies examining the impact of proinflammatory factors on epithelial barrier function have not addressed the transcytotic pathway. However, many of the proinflammatory cytokines that increase epithelial paracellular permeability have also been shown to alter the endocytic pathway in other systems. TNF-α, which has been shown to decrease TER of HT29 intestinal epithelial monolayers (16), enhances endocytosis in isolated hepatocytes (24). IFN-γ perturbs tight junctions of T84 epithelial monolayers (22), and has recently been shown to cause a greater and more rapid uptake of antigen by endocytosis in nasal epithelium (35). Our studies demonstrate that the non-inflammatory immunoregulatory molecule IL-4 can also regulate antigen uptake by the intestinal epithelium. In addition to increasing the amount of antigen reaching lamina propria immune cells by the paracellular route, IL-4 exposure could also potentially regulate mucosal immunity by altering epithelial antigen presentation by increasing the amount of internalized antigen. Antigen presentation by epithelial cells has been shown to occur through either a conventional IFN-γ -dependent MHC II restricted pathway that activates CD4+ cells (14), or an alternate pathway that activates suppressor $CD8^+$ T cells (26). Further studies are necessary to determine if IL-4 alters the nature of antigen presentation by the intestinal epithelium.

Patients with allergic diseases have been shown to have elevated intestinal permeability. This is observed even when the involved site is skin (23)

or lung (1), indicating that a circulating factor may be responsible for the alteration in epithelial barrier function. IL-4 has been measured in serum from atopic patients, and previously reported levels range from 231 to 2900 pg/ml in atopic patients (11). We were unable to detect IL-4 within the serum by ELISA, suggesting that if IL-4 was present it was in very low concentration. However, preabsorption of the serum with anti-IL-4 antibodies completely abolished the effect of atopic serum on HRP transport, indicating that an active factor in the serum was in fact IL-4. It is difficult to reconcile the complete inhibition of the effects of atopic serum by anti-IL-4 antibodies with our inability to detect IL-4 within the serum, as previously published dose-response curves with IL-4 indicate that picogram levels of IL-4 are insufficient to alter TER (6). However, it is possible that other factors present within serum act as either "priming" agents or in a synergistic fashion to enable very small doses of IL-4 to alter epithelial barrier function. Cytokines other than IL-4, such as TNF-α and IL-5, have been shown to elevated in allergic diseases, and peripheral blood mononuclear cells from children with cow's milk allergy induce an increase in HRP flux across a model epithelium after stimulation with milk protein, through a TNF-α-dependent mechanism (16). However, our experiments suggest that if other cytokines are present in serum, they are not sufficient to alter barrier function.

A decrease in epithelial barrier function has been hypothesized to be a predisposing factor in the development of inflammation. IL-4 expression in ileal biopsies from Crohn's disease patients is increased in early stages of relapse (7), suggesting a potential role for IL-4 in the initiation of mucosal inflammation. We have clearly demonstrated that IL-4 compromises epithelial barrier function against a model antigenic protein by increasing both paracellular permeability and transcellular uptake. IL-4 could participate in mucosal inflammation not only by increasing exposure of mucosal immune cells to luminal contents, but also

potentially through increasing antigen presentation by intestinal epithelium.

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Chapter 5: Discussion

Immunomodulation of Transepithelial Antigen Transport

Food allergy is a highly prevalent disorder with symptoms that involve the gastrointestinal tract and may also affect other organ systems. Symptoms can occur very rapidly after allergen ingestion and are thought to be mediated by immediate hypersensitivity reactions involving mast cell activation and release of mediators that act locally, altering epithelial and smooth muscle physiology in the gastrointestinal tract. The first step in an allergic reaction is the passage of antigen across the intestinal epithelial barrier. The overall aim of the studies described in this thesis was to examine the transepithelial transport of intact immunogenic antigens in experimental models of intestinal allergy.

The Model: HRP as a Sensitizing Antigen

In order to study transepithelial antigen transport prior to the hypersensitivity reaction in sensitized rats, we used horseradish peroxidase (HRP), as an antigen. HRP is a protein that can be visualized by cytochemistry and electron microscopy (Cornell *et al.*, 1971, Bijlsma *et al.*, 1996). HRP has a molecular weight of 44 000 daltons, in the same range as ovalbumin (OVA) and other common food allergens (Sampson, 1997). Rats were sensitized according to the protocol used previously in the laboratory, substituting HRP for OVA (Crowe *et al.*, 1990, Crowe *et al.*, 1993). Intestine from rats sensitized to HRP responded to luminal antigen challenge with an increase in Isc that was similar to that observed with OVA sensitized rats, with respect to magnitude and timing. Antigen-specific IgE titres were also similar to those measured in OVA-sensitized rats (Kosecka et al., 1994). Using mast cell deficient *Ws/Ws* rats we showed that the ion secretory response was completely dependent on the presence of mast cells, as has been shown with OVA sensitized rats (Perdue and Gall, 1985). Therefore, we demonstrated that the HRP sensitized rat model was a suitable

model for the study of the impact of sensitization on transepithelial antigen transport.

Sensitization-Induced Alterations in Antigen Transport

The specific aim of my first study was to determine the route and timing of antigen uptake across the intestinal epithelium in the HRP-sensitized rat model of hypersensitivity. Previous studies have shown that Ussing chambered segments of intestine from OVA sensitized rats respond to luminal antigen challenge with an increase in ion secretion within approximately 3 min (Crowe *et al.*, 1990, Kosecka *et al.*, 1994), compared to 15 s after serosal challenge. The increase in ion secretion was shown to be dependent on the presence of mast cells (Perdue *et al.*, 1991), and prior serosal antigen challenge abolished the response to luminal antigen challenge (Kosecka and Perdue, unpublished), suggesting that the same subepithelial population of mast cells mediates the response to serosal and luminal antigen challenge. Macromolecular uptake by the epithelium had been shown to occur in small quantities through the transcellular route in normal tissue (Cornell *et al.*, 1971, Walker *et al.*, 1972, Mayrhofer *et al.*, 1990), a process generally believed to be quite slow (requiring 15-30 minutes) (Bomsel *et al.*, 1989). Protein transport via the paracellular route between tight junctions has never been demonstrated in normal healthy gut. Although there is a large body of evidence suggesting that the hypersensitivity reaction induces a change in epithelial barrier function (Bloch *et al.*, 1979, Ramage *et al.*, 1988, Kleinman *et al.*, 1989, Heyman *et al.*, 1990, Crowe *et al.*, 1993), it was not clear if antigen uptake across the epithelium is altered in the sensitized rat prior to mast cell activation.

We chose to examine antigen uptake across the epithelium visually, using electron microscopy to locate antigen 2 min after challenge (approximately 1 min

prior to the initiation of the epithelial ion secretion response). HRP uptake across the intestinal epithelium was examined in 3 groups of rats: HRP sensitized, OVA sensitized (to examine the effect of sensitization on non-specific uptake of bystander molecules), and naive animals. In all tissues fixed 2 min after HRP addition to the luminal side of the tissue, HRP was located in endosomes within the epithelial cells, but never in the paracellular spaces between epithelial cells. The key observation from my studies was that in tissue from OVA and HRP sensitized rats, HRP was located throughout epithelial cells and in the lamina propria at 2 min. In contrast, tissue from naive rats contained HRP only in the apical region of epithelial cells, indicating that the sensitization process enhanced the rate of antigen transport across the intestinal epithelium. However, the amount of HRP was quantified within epithelial cells (by measuring area of endosomal HRP), was dramatically different in these two groups. HRP sensitized rats had significantly greater epithelial uptake of HRP compared to OVA rats, and OVA rats were not significantly different from naïve controls.

These results suggest that 1) sensitization increases the rate of macromolecular transport across the tissue in a non-specific manner, and more importantly, 2) the specific antigen itself is preferentially taken up by the epithelium. There is some evidence for prior immunization altering the uptake of antigen by the intestinal epithelium. Walker and colleagues demonstrated that multiple parenteral immunizations (but not single immunizations) inhibited the uptake of the sensitizing antigen, but not irrelevant antigen, across the small intestinal epithelium of rats (Walker et al., 1972, Walker et al., 1973). The mechanism of this inhibition was shown to be binding of antigen to IgG1 in the intestinal lumen (Walker *et al.*, 1975). Evidence for enhanced antigen uptake after previous antigen exposure comes from a study by Bockman and Winborn (1965). They sensitized hamsters to ferritin by injection with Freund's adjuvant

and observed that there was increased intraepithelial ferritin in sensitized animals after addition to the lumen of ligated intestinal loops. The outcome of prior antigen exposure on subsequent antigen uptake by the intestinal epithelium may depend on the type of immunoglobulin response generated. Our results strengthened the observation of enhanced antigen uptake in sensitized animals by quantifying the increase in antigen uptake, and comparing it to animals sensitized to an irrelevant antigen. We were also the first to show that antigen could cross the epithelium of sensitized rats by the transcellular route in such a short period of time.

The antigen-specific nature of the enhanced uptake was a novel finding. We ruled out the possibility that mast cell-IgE interactions were involved in the initial phase of antigen uptake by demonstrating that it occurred in mast cell-deficient *Ws/Ws* rats. This observation leads to the hypothesis that recognition of antigen occurs at the epithelial surface, potentially through an immunoglobulin-mediated interaction. This was also a speculation raised by Bockman and Winborn (1965), although they did not examine the specificity of the enhanced uptake by sensitizing hamsters to an irrelevant antigen. Immunoglobulins are present in the lumen of the intestinal tract, predominantly IgA, but also IgG and IgE (Belut *et al.*, 1980, Brown, 1978). Luminal IgE has been shown to be elevated in patients with food allergy (Belut *et al.*, 1980), and in parasitized rats (Negrao-Correa et al., 1996). The IgA receptor (pIgR), transcytosis of IgA, and regulation of this process by cytokines has been fairly well characterized (Manzanec *et al.*, 1993, Casanova, 1992, Denning, 1996), but it is not clear if IgA/pIgR can function to bring antigen back to the lamina propria from the intestinal lumen. Receptors for IgG (the neonatal IgG receptor, FcRn) (Blumberg *et al.*, 1996) and IgE (Kaiserlian *et al.*, 1993) have been demonstrated on the apical membrane of adult human intestinal epithelium, but their function and

possible role in antibody/antigen transcytosis remains to be elucidated. In summary, the components required for antibody-mediated antigen transport across the intestinal epithelium appear to be present, and further studies are needed to investigate the nature of the specificity of antigen uptake across rat intestinal epithelium in sensitized rats.

Recruitment of the Paracellular Antigen Transport Pathway After The Intestinal Hypersensitivity Reaction

Previous studies had shown that the hypersensitivity response is associated with a reduced epithelial barrier function, shown by an increase in flux of tracers ranging in size from small radiolabelled molecules to proteins (Bloch *et al.*, 1979, Ramage *et al.*, 1988, Kleinman *et al.*, 1989, Heyman *et al.*, 1990, Crowe *et al.*, 1993). The route of transepithelial transport was not clear from these studies, but it appeared to be non-selective as most of the studies used a tracer molecule different from the antigen. We found that HRP sensitized rats had a significantly greater flux of HRP over a 90 minute period (encompassing the hypersensitivity reaction) compared to OVA sensitized and naive controls. Challenge of intestine from OVA-sensitized rats with luminal OVA also induced an increase in overall HRP flux, demonstrating the non-specific nature of the increased HRP transport. Conductance of the tissue increased throughout the experimental period, beginning at approximately 30 minutes post-challenge, in HRP sensitized but not OVA sensitized or naive animals, and correlated with the flux measures. Conductance is a measure of the ionic permeability of the tissue, and although it is a measure of tight junction permeability (Madara, 1989), an increase in conductance does not always equate with increased paracellular transport of macromolecules (Madara *et al.*, 1992). Therefore, we examined the transepithelial transport of HRP by electron microscopy at 30 min post-challenge,

at the beginning of conductance changes and well after the ion secretory response. We found HRP in the tight junctions and paracellular regions of HRP sensitized, but not OVA sensitized or naive rats. Taken together, these results provide convincing evidence that specific antigen challenge in sensitized rats produces a non-specific epithelial barrier defect by recruiting the paracellular pathway. The observation that this increase of paracellular permeability occurred only after the hypersensitivity response suggested that it was mediated by activated immune cells. These studies show that there are two phases of transepithelial transport of macromolecules in the sensitized rat. Phase I is transcellular, is enhanced by sensitization, and uptake is maximal for the sensitizing antigen. Phase II involves recruitment of the paracellular pathway, and occurs only after specific antigen challenge in sensitized rats.

Evidence for Mast Cell Regulation of the Paracellular Transport Pathway

Mast cell mediators (histamine, serotonin, and prostaglandins) have been shown to be responsible for the ion secretory response observed after intestinal antigen challenge (Crowe *et al.*, 1990, Perdue *et al.*, 1991), but the role of mast cells in the regulation of epithelial antigen transport has not been addressed. To examine the role of mast cells in the regulation of epithelial antigen transport in sensitized rats, I used mast cell-deficient *Ws/Ws* rats and +/+ mast cell-replete litttermates as controls. *Ws/Ws* rats have a spontaneous mutation in *c-kit* that prevents the development of mast cell precursers into mature mast cells (Tsujimura *et al.*, 1991). Transepithelial antigen transport across tissue from HRP sensitized *Ws/Ws* and +/+ rats was compared to that across intestine from saline-injected +/+ rats. Electron microscopic analysis of endosomal HRP in tissues fixed 2 min after luminal HRP challenge confirmed the finding that sensitization increases HRP uptake, since there was significantly greater

endosomal HRP in sensitized +/+ rats compared to naive +/+ controls, in both apical and basal regions of the enterocyte. In tissue from sensitized *Ws/Ws* rats, HRP uptake was similar to that observed in sensitized +/+ rats, indicating the mast cell-independence of the initial enhanced antigen uptake, and demonstrating that HRP was rapidly reaching the lamina propria in sensitized *Ws/Ws* rats.

Tissue from sensitized +/+ rats responded to antigen challenge in a similar manner as tissue from the HRP-sensitized Sprague-Dawley rats used in our first study. HRP challenge of HRP-sensitized intestine resulted in an increased conductance, HRP flux, and the observation of HRP in the paracellular regions by electron microscopy. This second phase of antigen transport was not observed in the absence of mast cells, as sensitized *Ws/Ws* rats did not show any increase in HRP flux or conductance, and tissue maintained its tight junctional integrity. Tissue from sensitized *Ws/Ws* rats was identical to tissue from naive +/+ rats with respect to flux, conductance, and lack of paracellular HRP transport. These studies confirm that two phases of macromolecular transport occur across sensitized rat intestine. Phase I is transcellular, upregulated by sensitization, and is mast cell-independent. Phase II involves recruitment of the paracellular pathway after antigen challenge of intestine from sensitized rats, and is dependent on the presence of mast cells.

Reconstitution experiments, where mast cells isolated from +/+ rat bone marrow are injected into *Ws/Ws* rats to restore mast cells, were not feasible as the *Ws/Ws* rat colony has a heterogenous genetic background and previous attempts at reconstitution by other investigators had met with limited success. However, the results obtained from the above experiments strongly support a role for mast cells in the regulation of epithelial barrier function. Two other cell populations have been shown to be affected by *c-kit* deficiency, interstitial cells of

Cajal (ICC) (Mikkelsen *et al.*, 1998) and intraepithelial lymphocytes (IEL) (Laky *et al.*, 1997). ICC are not present in the tissue preparation I have used in experiments, as they are stripped off the tissue with the external muscle layers. Although IEL populations have been shown to be altered in the *W/W^v* mast cell-deficient mouse (a decrease in TCR$\gamma\delta$, and increase in TCR$\alpha\beta$), there is no evidence of depletion of any subset of IEL (Laky et al., 1997). Therefore, it is extremely unlikely that a shift in the IEL subsets could be responsible for the complete abolishment of the Phase II response in sensitized Ws/Ws rats.

There is supportive evidence for the concept of mast cell regulation of epithelial barrier function. Scudamore *et al.* (1996) examined the effect of rat mast cell protease (RMCP) II on epithelial integrity. They observed an increased leak of protein and RMCP II into the intestinal lumen after intravenous worm antigen challenge in *N. brasiliensis* infected rats (which elicits an IgE mediated hypersensitivity reaction). Injection of RMCP II directly into the intestinal circulation replicated the effects of worm antigen challenge on luminal protein leak, suggesting that RMCP II could increase epithelial permeability. Recently, RMCP II has also been shown to increase tissue conductance of intestinal segments mounted in Ussing chambers (Vergnolle *et al.*, 1998). RMCP II levels increase in the serum of sensitized rats after luminal antigen challenge (Crowe et al., 1993), and it is possible that local release of RMCP II from mucosal mast cells in the Ussing chambered intestinal segments could mediate a change in epithelial permeability. Histamine, which is released by antigen stimulated mast cells, causes an increased epithelial permeability to ^{51}Cr-EDTA in rabbits (Miller *et al.*, 1991). Mast cells are also capable of producing a number of cytokines after IgE cross-linking (IFN-γ, TNF-α, IL-4) (Burd *et al.*, 1989, Gordon and Galli, 1990) that have been shown to increase epithelial tight junctional permeability *in vitro* (Madara and Stafford, 1989, Heyman *et al.*, 1994, Colgan *et al.*, 1994).

Release of mast cell mediators can activate enteric nerves (Frieling *et al.*, 1993), and mast cells and nerves have been shown to act as a functional unit in the regulation of epithelial ion transport (Perdue *et al.*, 1991). Treatment of intestinal segments with the neurotoxin tetrodotoxin has been shown to reduce macromolecular transport across the normal rat jejunum (Kimm *et al.*, 1994), and inhibit luminal to serosal uptake of ^{51}Cr-EDTA in the sensitized rat after challenge (Crowe *et al.*, 1993). Inhibition of muscarinic receptors with atropine inhibits basal macromolecular uptake, and cholinergic stimulation results in an opening of the epithelial tight junctions (Phillips *et al.*, 1987, Bijlsma *et al.*, 1996). This evidence suggests that nerves could participate in or amplify the change in antigen transport after activation of mast cells.

Role of Interleukin-4 in Epithelial Barrier Defects Following Sensitization

A number of experimental and clinical studies have suggested that epithelial barrier function is altered in sensitized individuals even prior to antigen challenge. Patients with atopic eczema and bronchial asthma have been shown to have elevated intestinal permeability (Majamaa *et al.*, 1996, Benard *et al.*, 1996). Using animal models of intestinal hypersensitivity, Crowe *et al.* (1993) and Heyman *et al.* (1990) demonstrated increased epithelial permeability in the absence of antigen challenge. These studies suggest that sensitization is associated with decreased barrier function that allows an increase in non-specific uptake of luminal contents.

Sensitization in humans and animal models is associated with a shift in immune phenotype to a Th2 cytokine profile, predominantly an increase in IL-4 expressing cells (Ackerman *et al.*, 1994, Bittleman and Casale, 1994, Bradding *et al.*, 1994, Hauer *et al.*, 1997). IL-4 treatment of epithelial monolayers results in a decreased transepithelial resistance (reflecting a change in tight junction

permeability) (Colgan et al., 1994, Sanders et al., 1995). It was not known if IL-4 could contribute to the barrier function perturbations observed in sensitized individuals, and the aim of the third study of my thesis was to examine the effect of IL-4 on transepithelial transport of macromolecules in an *in vitro* model system.

We used T84 cells, a human colonic epithelial cell line that forms monolayers with high electrical resistance, as a model epithelium. We confirmed that IL-4 treatment of epithelial cells caused a reduction in transepithelial resistance. Some studies have shown that macromolecular transport is not elevated even in the condition of a substantially reduced epithelial resistance (Hecht *et al.*, 1992), indicating that a change in the ionic permeability of tight junctions does not always allow the passage of large molecules. To examine the effect of IL-4 on transepithelial antigen transport, we again used HRP as a model protein. We found that overall HRP transport was significantly elevated in IL-4 treated monolayers compared to controls, demonstrating that IL-4 is capable of altering the transepithelial transport of macromolecular proteins. To examine the route of HRP transport, we performed electron microscopy analysis of monolayers exposed to HRP after treatment with or without IL-4. Control monolayers took up HRP by endocytosis, and HRP could be observed within intracellular vesicles. At no time did we observe HRP in the paracellular regions of control monolayers. In monolayers treated with IL-4, HRP was found in a large proportion of paracellular regions, indicating that the change in tight junction permeability (measured by the decrease in transepithelial resistance), was of sufficient magnitude to allow the passage of intact protein. We also observed that IL-4 treatment led to an increase in endocytic uptake of HRP, there was a significant increase in the area of HRP within endocytic vesicles. Therefore, IL-4 could potentially contribute to a decreased intestinal epithelial barrier function by increasing both paracellular and transcellular transport of antigen.

Since passive sensitization of rats with serum can transfer increased antigen uptake (unpublished observations), we postulated that IL-4 may circulate in quantities that can affect protein uptake. We therefore examined the impact of serum from patients with atopic dermatitis, an allergic condition characterized by high IL-4 levels (Tang and Kemp, 1994) on epithelial barrier function. Growth of epithelial monolayers in the presence of atopic serum resulted in decreased transepithelial resistance, increased HRP transport, increased endocytic HRP area, and recruitment of the paracellular transport pathway, in short replicating the effects of recombinant IL-4. Addition of anti-IL-4 antibodies abolished the effects of atopic serum, providing persuasive evidence for a physiological role for IL-4 in the regulation of epithelial barrier in allergic disease.

The finding that IL-4 can directly modulate transepithelial antigen transport suggests that it could potentially play a role in the sensitization-induced changes in epithelial barrier function in the rat model of intestinal hypersensitivity. A feature of the hypersensitivity model is an elevation of total and antigen-specific IgE (Kosecka *et al.*, 1994). IgE production requires the presence of IL-4, and spleen cells isolated from sensitized rats and stimulated *in vitro* produce higher levels of IL-4 compared to unsensitized rats (Berin and Perdue, unpublished observations). It is possible that basal levels of IL-4 production from immune cells within the intestinal mucosa are sufficient to alter the normal transport of macromolecules in sensitized rats. In addition, antigen challenge could potentially induce the release of IL-4 by local immune cells. Antigen processing and presentation to Th2 lymphocytes in the lamina propria would stimulate the release of IL-4 from antigen-specific T cells. IL-4 could also be produced from mast cells stimulated with antigen/IgE via the FceRI receptor, or eosinophils stimulated with antigen/IgE through the FceRII receptor. This antigen-stimulated release of IL-4 could potentially contribute to the non-specific barrier defect

observed in sensitized rats after challenge. Although these effects may not be immediate, the non-specific epithelial barrier defect after antigen challenge in sensitized rats has been shown to be extremely long-lived (Yang and Perdue, unpublished observations), and may be due to cytokine release from antigen-stimulated immune cells. Experiments are currently underway in Dr. Perdue's laboratory using IL-4 knockout mice to determine the *in vivo* role of IL-4 in transepithelial antigen transport in the rodent model of intestinal hypersensitivity.

Regulation of transepithelial antigen transport in the context of specific immunity

It is clear that prior exposure to an antigen can alter the uptake of that antigen from the intestinal lumen. The mechanisms that determine the nature of the immune response to the antigen (i.e. tolerance versus immune activation, Th1 versus Th2 cytokine profile) remain to be elucidated. The type of immunoglobulin response may in part contribute to the effect on transepithelial antigen uptake. For example, IgA and IgG1 in the intestinal lumen have been shown to inhibit antigen uptake across the intestinal epithelium (Manzanec *et al.*, 1993, Walker *et al.*, 1975). IgE is elevated in serum and luminal secretions in allergic rodent models and atopic diseases, but it is not yet clear if this immunoglobulin isotype can contribute to antigen transport across the intestinal epithelium. My experiments have shown selective uptake of antigen occurs in sensitized rodents, and this suggests that in an allergic individual small amounts of antigen could be preferentially taken up across the intestinal epithelium.

Antigen reaching immune cells within the intestinal mucosa could elicit a number of different responses, with different outcomes on epithelial barrier function. I have shown that activation of mast cells causes a non-specific epithelial barrier defect by opening of the tight junctions. Further studies have been done to examine the time course of the changes in barrier function after

antigen challenge (Yang and Perdue, unpublished). Luminal antigen challenge *in vivo* produces an increase in epithelial permeability to ^{51}Cr-EDTA that can be observed 30 min after challenge, and is still present 72 h after challenge. An infiltration of mononuclear cells into the lamina propria is also observed. An increased influx of luminal antigens, or bacterial products, together with an accumulation of newly recruited immune cells that are more reactive than resident immune cells (Mahida *et al.*, 1989) could set the stage for chronic inflammation. These results demonstrate that there are connections between allergy and inflammation in the intestine. Mast cell activation has been documented in inflamed intestine from patients with IBD (Fox *et al.*, 1990, Knutson et al., 1990, Raithal et al., 1995), although it is not clear if this is a result or a contributing factor to inflammation.

Prior exposure to an antigen could also expand the population of antigen-specific T cells, or change their cytokine repertoire. Antigen reaching the lamina propria could then result in local production of cytokines capable of modifying transepithelial antigen transport. Activation of regulatory T cells (hypothesized to mediate oral tolerance) would result in TGF-β production. TGF-β is a cytokine that has direct effects on the epithelium and prevents disruption of epithelial barrier function (McKay and Singh, 1997). Activation of Th1 or Th2 cells by antigen crossing the epithelium could have detrimental effects on epithelial barrier function. Release of IFN-γ from antigen-specific Th1 cells could increase transepithelial antigen transport by loosening epithelial tight junctions (Madara and Stafford, 1989). My studies show that release of IL-4 from antigen-specific Th2 cells could also potentially cause an influx of antigen across the epithelial barrier.

The observation that IL-4 can increase transepithelial transport of luminal antigens provides another link between intestinal hypersensitivity and

inflammation. Increased epithelial permeability has been suggested to be a predisposing factor to the development of IBD (Peeters *et al.*, 1997), and there is evidence that luminal contents (particularly normal flora) play a pathophysiological role in the onset of inflammation (Rutgeerts *et al.*, 1991, D'Haens *et al.*, 1998). Therefore, factors that increase epithelial barrier function prior to development of inflammation may be important in the pathogenesis of IBD. Recent studies have shown that IL-4 expression is upregulated in the ileal mucosa of patients with Crohn's disease in early stages of relapse, but not in patients with chronic inflammation (Desreumaux *et al.*, 1997). Sources of mucosal IL-4 (identified histologically by double staining) include T cells, mast cells, and eosinophils. IgE expression was also elevated in biopsies from patients with early relapse, but not chronic inflammation (Desreumaux *et al.*, 1998). I speculate that specific transcellular uptake of antigen may lead to the activation of antigen-specific Th2 cells, release of IL-4, and the development of a non-specific barrier defect. The influx of luminal contents, including bacterial flora or bacterial products, could then activate resident immune cells (including macrophages), and the resulting release of proinflammatory cytokines would further augment the delivery of antigen across the intestinal epithelium. This is shown schematically in Fig 5.1.

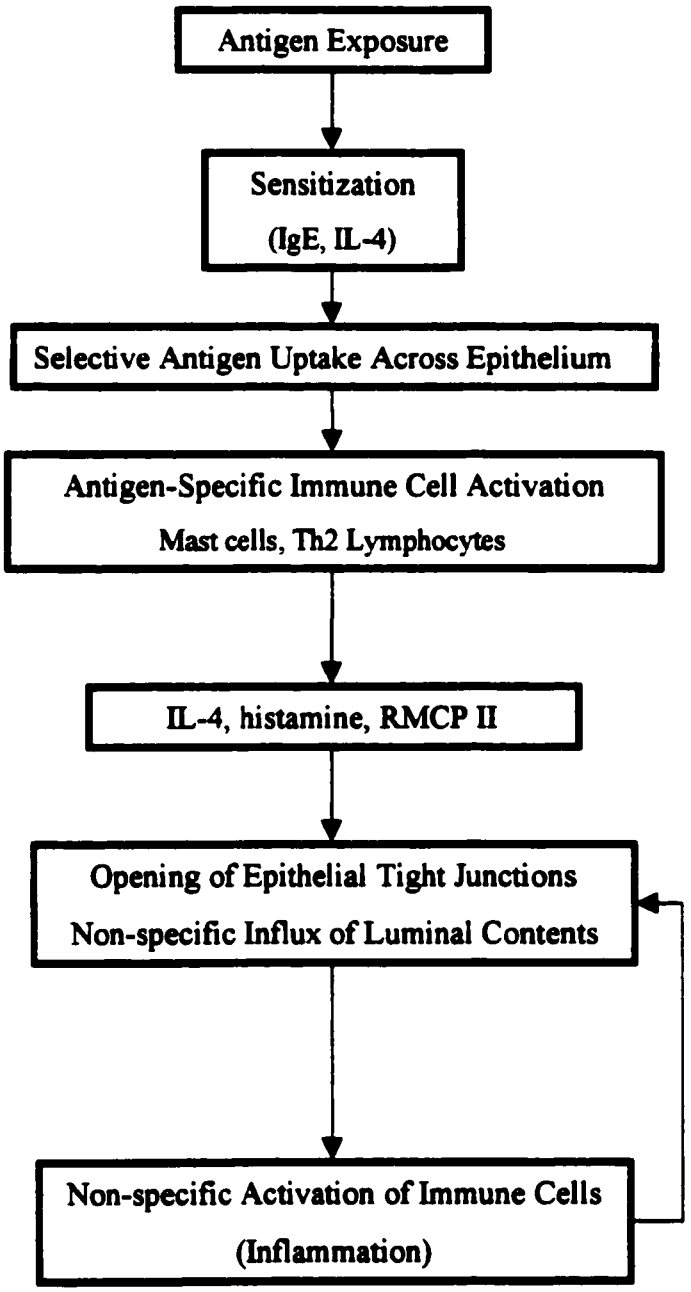

Figure 5.1: Speculations on the Regulation of Transepithelial Antigen Transport in the Context of Specific Immunity

Future directions

My studies on the regulation of transepithelial transport of antigen in allergy have shown that: a) sensitization increases the rate of macromolecular transport across the intestinal epithelium; b) sensitization increases antigen-specific uptake by epithelial cells; c) activated mast cells increase uptake of antigen across the intestinal epithelium by recruiting the non-specific paracellular pathway; and d) IL-4 acts directly on the epithelium to increase transcellular and paracellular transport of macromolecules. These findings have raised a number of important questions, some of which are currently being examined in Dr. Perdue's laboratory.

The most novel finding of my studies was the specificity of antigen uptake by epithelial cells in sensitized rats. I have discussed the potential mechanisms of this specific uptake, and experiments are in progress to determine the mechanism of this enhanced uptake, including identification of the immunoglobulin isotype and receptor involved.

The regulation of epithelial tight junction permeability by mast cells remains to be explored. The role of mast cell mediators, and the potential interaction between mast cells and nerves could be determined by pharmacological inhibitor studies, and may be of benefit in the prevention of intestinal allergic inflammation.

The observation that IL-4 regulates transepithelial transport of macromolecules *in vitro* obviously leads to the examination of the role of IL-4 in transepithelial antigen transport *in vivo*. These studies are currently underway. My studies on the effect of IL-4 on epithelial cells *in vitro* were focused on the delivery of antigen across the epithelium, but one area that remains to be explored is the impact of IL-4 on epithelial antigen presentation. Epithelial cells can present antigen to $CD8^+$ (Mayer and Shlien, 1987) or $CD4^+$ (Hershberg *et al.*,

1997) T cells through CD1d or MHC II respectively. Presentation through the MHC II pathway (activating CD4+ helper cells instead of CD8+ suppressor cells) is dependent on prior exposure of the cells to IFN-γ (Hershberg *et al.*, 1997). Although it has been shown that IL-4 does not have any effect on MHC II expression by epithelial cells (Colgan et al., 1997), it is possible that IL-4 could enhance presentation by increasing the amount of antigen internalized. The impact of enhanced transcellular transport on epithelial antigen presentation both *in vitro* and in the rat model of hypersensitivity is a research area that has yet to be addressed.

Summary Statement

Taken together, the studies that form my thesis provide evidence that transepithelial antigen transport is significantly altered in sensitized animals, and that regulation of both paracellular and transcellular transport pathways can occur. Regulation of epithelial barrier function by immune mediators has been a focus of research interest for some time, but much of the emphasis has been placed on regulation of tight junction permeability. My studies demonstrate that the transcellular transport pathway should also be considered in the context of epithelial barrier function, and that upregulation of this pathway may occur concurrently with regulation of paracellular permeability. Enhanced endocytic uptake of antigen may contribute to mucosal immunity not only by increasing the amount of antigen reaching the lamina propria, but could also affect antigen presentation by epithelial cells. Antigen handling by the intestinal epithelium plays a significant role not only in allergic disease, but may also be an important factor in the pathophysiology of intestinal inflammation.

Appendix:

Expanded Description of Selected Methods

The Ussing Chamber

The Ussing chamber was developed by Ussing and Zerahn (1951) for electrophysiological recordings across frog skin. The basic method involves pinning a flat sheet of tissue between two chamber halves containing iso-osmotic physiological fluid, and measuring potential difference (PD) and current across the tissue with electrodes. Fig A.1 illustrates the Ussing chamber apparatus used in my experiments. In the current studies, rat jejunum (beginning 5 cm from the ligament of Treitz) was placed on a plastic stripping rod, and the external muscle layers removed by dissection leaving the mucosa and submucosal plexus intact. The tissue was then pinned as a flat sheet onto the chamber halves. Krebs buffer containing 10 mM glucose as an energy source was added to the serosal side of the tissue, and Krebs buffer containing 10 mM mannitol as an osmotic balance was added to the luminal side. A circulating water pump and water jacket maintained the buffers at 37 °C, and the buffers were aerated with 95% O_2/ 5% CO_2. PD was measured by two pairs of calomel electrodes connected to agar bridges (polyethylene tubing, PE-320 containing 2M KCl in 1% agar) placed in close proximity to the tissue. The tissue was voltage clamped at zero voltage by a pair of silver/silver-chloride electrodes. The current injected to maintain the PD at zero (short-circuit current, Isc) was equal and opposite to the current generated by the tissue by active transport, and therefore is a measure of net active ion transport. Tissue ionic conductance (G, mS/cm^2), a measure of the permeability of the tissue to ions, is calculated using Ohm's law (V=IR, where V = PD, I = Isc, and R = 1/G). Conductance in my experiments was measured by the differential pulse method, in which the tissue is transiently clamped from 0 mV to 1 mV, and the short-circuit current deflection

is used to calculate G. Isc readings were recorded using a data aquisition program (Acknowledge, Biopac Systems Inc., Goleta, CA).

Electrophysiological Recordings

Intestinal segments from rats (4 - 8 pieces per rat) were mounted in Ussing chambers, and allowed to equilibrate for approximately 15 min, when the Isc stabilized. Baseline Isc and G readings were determined prior to the addition of 5×10^{-5} M HRP to the luminal buffer. Isc responses to HRP were measured as the maximal increase in Isc within 15 minutes after challenge. The tissue was pulsed to determine conductance every 5 min throughout the 90 min experimental period.

HRP Flux

To determine flux of HRP, samples (2 x 250 µl) were obtained from the serosal buffer at 0, 30, 60, and 90 minutes, and the volume was replaced with fresh Krebs buffer. Samples (100 µl) were also taken from the luminal buffer at 0 and 90 minutes to determine a reference activity. Luminal samples were diluted 1:10 000 (5×10^{-9} M) prior to assaying for enzymatic activity. Concentration of HRP was determined by kinetic enzymatic assay. The assay buffer was composed of 0.1 M phosphate buffer containing 0.003% H_2O_2 and 80 µg/ml *o*-dianisidine. 150 µl of sample was added to 800 µl assay buffer, and the absorbance at 460 nm was immediately measured over a 2 minute period. If the increase in absorbance was not linear over this time period, the samples were diluted until they fell within the linear range. Flux of HRP was calculated using luminal samples as standards, and expressed as pmol/cm^2/h. This method was a modified Worthington method (Maehly and Chance, 1954), and is routinely used in macromolecular absorption studies (Heyman *et al.*, 1984, Bijlsma *et al.*, 1996).

Electron Microscopy

To examine HRP absorption across the intestinal epithelium morphologically, tissues were removed from Ussing chambers and fixed for electron microscopy. Tissues were removed at 2, 30, or 90 min after HRP addition, and fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer for 3 hours at room temperature. Tissues were transferred to 0.1 M sodium cacodylate at 4 °C overnight. Tissues were cut into small segments parallel with the villi, and rinsed in Tris buffer. To develop HRP into an electron dense reaction product, tissues were incubated in 0.5 mg/ml 3,3'-diaminobenzidine tetrahydrochlorine in Tris buffer for 30 min. Tissues were postfixed for 1 h in osmium tetroxide, and stained with uranyl acetate for 30 min. After dehydration in ethanol and propylene oxide, tissues were embedded in Epon. Semi-thin sections were cut to obtain a proper orientation of the tissue (sections of mid-villus, and longitudinal sections of epithelial cells), followed by cutting of ultrathin sections with a diamond knife. Sections were placed on copper grids, and stained with lead salts.

Coded tissues were observed by electron microscopy, and random photomicrographs of properly oriented epithelium were taken for quantification of intracellular HRP. Area of HRP within endosomes was measured within a standard 4 x 6 μm window placed immediately below the apical membrane or immediately above the basal membrane. Mean area of HRP was determined for each rat, and data was expressed as mean ± SEM of rats within each group.

Cell Culture

The intestinal epithelial cell line T84 was used as a model epithelium in these studies. T84 cells were originally derived from lung metastases in a patient

with colonic carcinoma. When grown on filter supports, T84 cells form polarized monolayers with high resistance tight junctions, typically > 1000 ohms/cm^2. Other human intestinal epithelial cell lines, such as CaCo-2 and HT-29 cells, also form monolayers, but of a much lower resistance, typically 100-200 ohms/cm^2. The high resistance of T84 cells makes them an ideal cell line for examining changes in barrier function, particularly the regulation of tight junctions. Functionally, T84 cells are crypt-like epithelial cells that secrete chloride ions in response to a number of secretagogues (Dharmsathaphorn *et al.*, 1984).

T84 cells were grown as monolayers in a 1:1 mixture of Dulbecco's modified Eagle's medium and F-12 nutrient mixture, supplemented with 10% heat-inactivated fetal calf serum, 1.5% HEPES buffer, and 2% penicillin-streptomycin. Cells were grown at 37 °C in 5% CO_2, and subcultured every 7-14 days by trypsin treatment (0.1% trypsin and 0.9 mM EDTA in Ca^{2+}- and Mg^{2+}-free phosphate buffered saline). For HRP transport experiments, 10^6 cells were seeded onto tissue culture-treated Transwell filter supports (0.4 μm pore size, 1.2 cm^2 surface area). Epithelial cells form a confluent monolayer covering the entire surface of the filter, forming a barrier between apical and basal compartments (see Fig A.2). Media was changed daily, and monolayers were used in experiments 7-10 days after seeding, when transepithelial resistance measurements were > 1000 ohms/cm^2.

T84 monolayers can be mounted in Ussing chambers (designed for use with epithelial monolayers and modified to preserve the integrity of the cell monolayers by minimizing turbulence from the air-lift system). Epithelial ion transport and barrier function can be assessed by electrophysiology and flux measurements as described above for intestinal tissue. In my studies, HRP flux experiments were attempted using the Ussing chamber apparatus, but the

amount of HRP crossing the monolayers within the experimental period (2 h) was too small for accurate flux measurements. HRP transport experiments were instead done by adding HRP to the media in the apical compartment of the transwell unit, and after an overnight incubation sampling the media in the basal compartment for HRP enzymatic activity (Hecht *et al.*, 1992, Philpott *et al.*, 1997). Rather than calculating a flux rate, HRP transport was expressed as percent of apical HRP appearing in the basal compartment. The assay for HRP activity was performed as described above, and the culture media did not interfere with the enzymatic assay. Preparation of monolayers for electron microscopy was also performed as described above for intestinal tissue, with the exception of a shorter fixation time (2 h) in 2% glutaraldehyde.

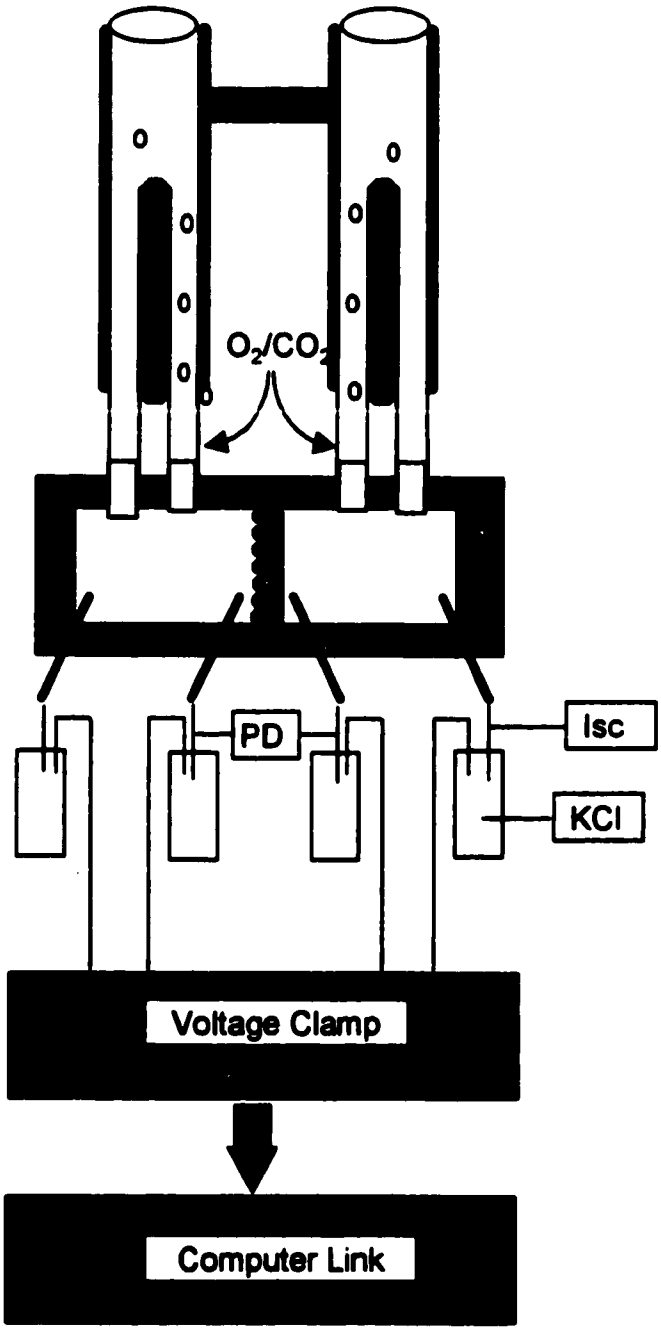

Figure A.1: Ussing chamber apparatus.

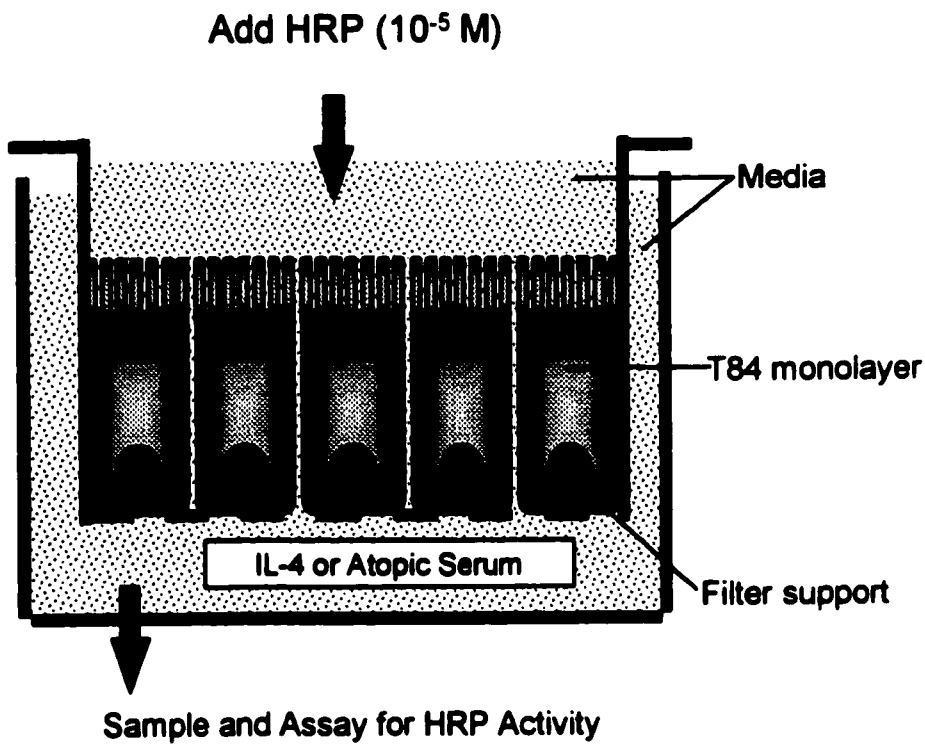

Fig A.2: Diagram of the T84 model epithelium used for examination of the regulation of transepithelial HRP transport by IL-4 (Chapter 4). T84 cells were cultured with IL-4 or serum from atopic patients added to the basal media. Horseradish peroxidase (HRP) was added to the apical media, and after an overnight incubation, the basal media was sampled and assayed for HRP enzymatic activity.

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