MECHANISM OF ENHANCED INTESTINAL TRANSEPITHELIAL ANTIGEN TRANSPORT IN FOOD ALLERGY: ROLE OF IgE, IL-4, AND CD23/FcεRII

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TITLE: Mechanism of enhanced intestinal transepithelial antigen transport in food allergy: role of IgE, IL-4 and CD23/ FcεRII

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ABSTRACT

Enhanced transepithelial antigen transport in intestine of sensitized rats was previously demonstrated using horseradish peroxidase (HRP) as a model antigen. In sensitized rats, transepithelial antigen transport was rapid (< 2 min) and of greater magnitude (three-fold) compared to controls.

In my project, the essential components involved in the enhanced antigen transcytosis were investigated using mice with genetic deletions for IL-4 and CD23/FcεRII. Increased transepithelial antigen transport was demonstrated in actively sensitized IL-$4^{+/+}$ mice, but not IL-$4^{-/-}$ mice, in which the phenomenon paralleled the expression of CD23 protein on enterocytes. Passively sensitized mice (both IL-$4^{+/+}$ and IL-$4^{-/-}$ mice) displayed greater antigen transport after transfer of immune serum unless the serum was first depleted of IgE or IL-4. IL-4 added to cultures of epithelial cells upregulated expression of CD23 mRNA. Finally, this augmented antigen uptake system was inhibited by luminal anti-CD23 and was absent in sensitized $CD23^{-/-}$ mice. RT-PCR showed that cultured cells expressed only the *b* isoform of CD23. Sequencing revealed classical and alternative CD23*b* transcripts lacking exon 5 (*b*Δ5) or 6 (*b*Δ6), in which all forms were translated into functional IgE receptors. Endocytosis of the *b*Δ5 and *b*Δ6, but not the classical CD23*b* protein, was observed after binding with saturating anti-CD23, suggesting continuous endocytosis of the alternative forms that agrees with their intracellular localization at steady state. Classical CD23*b* proteins were expressed on the cell surface and only endocytosed upon antigen-induced IgE cross-linking of the receptor.

Taken together, the results demonstrated that IgE/CD23 mediates enhanced transepithelial transport of antigen in sensitized mouse intestine, and that IL-4 plays a major regulatory role. We identified the expression of classical and alternative CD23*b* transcripts in intestinal epithelial cells, and demonstrated that the translated proteins display distinct endocytic functions. We concluded that antigen binding to epithelial CD23/IgE facilitates its entry into the body resulting in intestinal anaphylaxis.

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PREFACE

The first chapter is an introduction and background related to the project. The second chapter describes co-authored study in which I made a significant contribution. The experiments in this paper were conducted in a rat model where the phenomenon of enhanced transepithelial antigen transport was first reported. I was involved in the discussion forming the hypothesis for the mechanism, and performed the experiments of anti-IgE conjugation to sepharose beads, immunoprecipitation of IgE from immune serum, and isolation of monoclonal anti-CD23 antibodies from hybridoma cells. Subsequently, my major project was conducted in mice as illustrated in the next three chapters (three first-author papers). The mouse model offers the advantage of the availability of gene-deficient mice, mouse epithelial cell lines, species specific reagents, such as antibody. I conducted all of the experiments and performed data analysis for these papers, with the exception of imaging techniques, electron microscopy and confocal microscopy. The discussion synthesizes the information obtained from the four papers, and illustrates a comprehensive view regarding the contribution of the project to its research field. The discussion is included as the sixth chapter and is followed by references for the introduction and discussion.

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CHAPTER 1: Introduction - intestinal permeability in food allergy

1.1 Food allergy

Hypersensitivity reactions, including food allergy, asthma, allergic rhinoconjunctivitis, and atopic dermatitis, are the largest group of immune disorders that affect 20-30% of the population in North America and Europe, and the prevalence of such conditions is increasing in industrialized countries (Sampson, 1996; The International Study of Asthma and Allergies in Childhood (ISAAC) Steering Committee, 2001). Food allergy is one type of atopic disorder that involves 2-5% of adults and an even greater proportion of children (Sampson, Burks, 1996; The International Study of Asthma and Allergies in Childhood (ISAAC) Steering Committee, 2001). Sensitized individuals develop gastrointestinal symptoms, such as nausea, vomiting, cramping and diarrhea, and in some cases respiratory and cutaneous symptoms, after ingesting specific foods (Sampson, Burks, 1996). The onset of hypersensitivity reactions in humans and animal models of food allergy is extremely rapid (within minutes) after exposure to antigens (Berin et al. 1997; Sampson et al. 1992; Amlot et al. 1987). The foods most commonly responsible for inducing intestinal allergy include milk, egg, shellfish, tree nuts, and peanuts (Anderson, 1997; Bock et al. 2001). In severe cases, such as peanut allergy, systemic anaphylaxis can occur with fatal consequences (Sampson et al. 1992; Bock et al. 2001).

Allergic individuals are characterized by the presence of high levels of immunoglobulin E (IgE) to specific food antigens, mostly water-soluble glycoproteins ranging from 10,000-60,000 daltons (D) (Sampson, Burks, 1996). Elevated IgE in the circulation has long been recognized in all atopic diseases. Local synthesis of IgE was also

found to be high in the mucosa of the intestinal tract in food allergic patients (Belut et al. 1980). Allergen-specific IgE binds to the cell surface of mucosal mast cells. After exposure to food, the antigen cross-links IgE bound to mast cells, leading to degranulation and release of mediators. These mast cell mediators, including histamine, prostaglandins, serotonin etc., induce local anaphylactic responses (Anderson, 1997). Several hypotheses of the immunopathogenesis of food allergy have been reported. These mechanisms include a shift in T helper 1/ T helper 2 (Th1/Th2) cells, a malfunction of T regulatory (Tr) cells, a lack of oral tolerance, and an increased antigen load (Sampson, 1999b; Black, 2001b). T lymphocytes are subgrouped according to the cytokines expressed. The Th1 cell type expresses mainly IL-12 and IFNγ, whereas Th2 expresses mainly IL-4, -5,-13. Major roles of IL-4 in atopic diseases include promotion of IgE synthesis from B cells, and direction of naive T lymphocytes to differentiate into Th2 cells; both effects can be antagonized by IFNγ. The shift of Th1 to Th2 cells may account for the development or exacerbation of food allergic symptoms (Wills-Karp et al. 2001; Black, 2001). In addition, Tr cells express immunosuppresive factors, such as TGFβ and IL-10. In atopic disease, Tr cells may fail to downregulate immune responses to inert foodstuffs, and hence, be partially responsible for the lack of oral tolerance (Wills-Karp et al. 2001; Black, 2001). Moreover, elevated influx of intact antigens in the intestinal tract has been documented in food allergic individuals (Jalonen, 1991; Dupont et al. 1989) and sensitized animal models (Berin et al. 1997). The increased antigen load implicates impaired barrier function of the epithelial

layer (Berin et al. 1997). My thesis research was directed at examining this concept in detail.

Treatments for food allergy currently include antihistamine and epinephrine which mainly inhibit the effect of mast cell mediators in the intestine and airway to remedy diarrhea, wheezing, and breathing difficulties, induced by food allergen ingestion (Sicherer, 1999; Anderson, 1997). However, the effectiveness of anti-histamines and epinephrine are greatly reduced in long-term users and severely anaphylactic individuals. Allergen immunotherapy involving intradermal injection of allergen and administration of larger quantities of antigen, has been used to correct the specific immune responses by attempting to shift or reduce the Th2 response. However, immunotherapy has not been shown to be effective for food allergy unlike inhalant and other types of allergens and the safety of this treatment has been questioned (Sampson, 1999a). Other alternative methods include the mutation of IgE-binding epitopes on major peanut proteins, and the use of peanut protein cDNAs encoded into plasmid vectors, opting for desensitization of the patients (Sampson, 1999a). The most recent treatment in progress is probiotic therapy. The use of probiotics has been suggested to relieve the severity of anaphylactic reactions (Kirjavainen et al. 1999; Majamaa, Isolauri, 1997). None of these treatments are very effective therapies for food allergies. To date, the only effective treatment of food allergy is an elimination diet involving avoidance of suspected foods. However, particularly in young children, the diet may become so restricted that nutrition is compromised (Zeiger, 1997; Anderson, 1997). Therefore, it is important to have a clear understanding of the

pathophysiology of food allergic reactions in order to develop novel therapeutic approaches.

1.2 Physiology of intestinal mucosa

The intestinal mucosa is composed of a single cell epithelial layer and the underlying lamina propria. The epithelium separates the external environment from the internal milieu of the body and thus forms an important barrier in preventing noxious substances in the lumen from gaining access to the body. The epithelium contains mainly transporting intestinal epithelial cells (enterocytes), in addition to other cell types, such as intraepithelial lymphocytes (IEL), M cells, Paneth cells, goblet cells, and enteroendocrine cells (Yu, Perdue, 2001; Perdue, McKay, 1994) (Figure 1.1). M cells are flattened epithelial cells covering the Peyer's patches where their main function is thought to be antigen sampling. Paneth cells that lie at the base of the crypt contain abundant lysozymes and secrete defensin that has antimicrobial functions (Ganz, 2000). Goblet cells secrete mucus that plays an important role in mucosal defense by trapping pathogens and antigens (Perdue, McKay, 1998). Enteroendocrine cells, including enterochromaffin cells, G cells, etc., are flask-shaped specialized cells that produce mediators and hormones (Cooke, 1987).

The three main functions of the intestinal epithelial cells are ion and water transport, nutrient absorption, and barrier function. Enterocytes are derived from stem cells found in the crypt. Crypt cells have a secretory phenotype and as they migrate toward the villus tip they mature into an absorptive phenotype (Perdue, McKay, 1993).

Tight junctions between adjacent epithelial cells produce a physical barrier to luminal antigens. This physical barrier is not permeable to molecules larger than 500 daltons or 11.5 Å in Stokes radius, thus excluding macromolecules from passing through the paracellular pathway (Madara, 1989). Some macromolecules are taken up into epithelial cells by endocytosis. Most macromolecules or proteins are degraded either by digestive enzymes in the lumen or by lysosomal enzymes inside the enterocytes. Macromolecules endocytosed into epithelial cells are degraded in an acidic compartment following fusion of endosomes with lysosomes (Walker, 1981). Enterocytes have the capacity to transport a very small proportion of antigenic material from the intestinal lumen to the underlying tissues via transcytosis, a process that takes ~20-30 min (Keljo, Hamilton, 1983). Barrier function also involves mucus secretion from goblet cells, peristalsis, defensin production from Paneth cells, and immunoglobulin (Ig) A secretion which binds to pathogens and prevents them from gaining access to the body (Yu, Perdue, 2001; Ganz, 2000; Perdue, McKay, 1994).

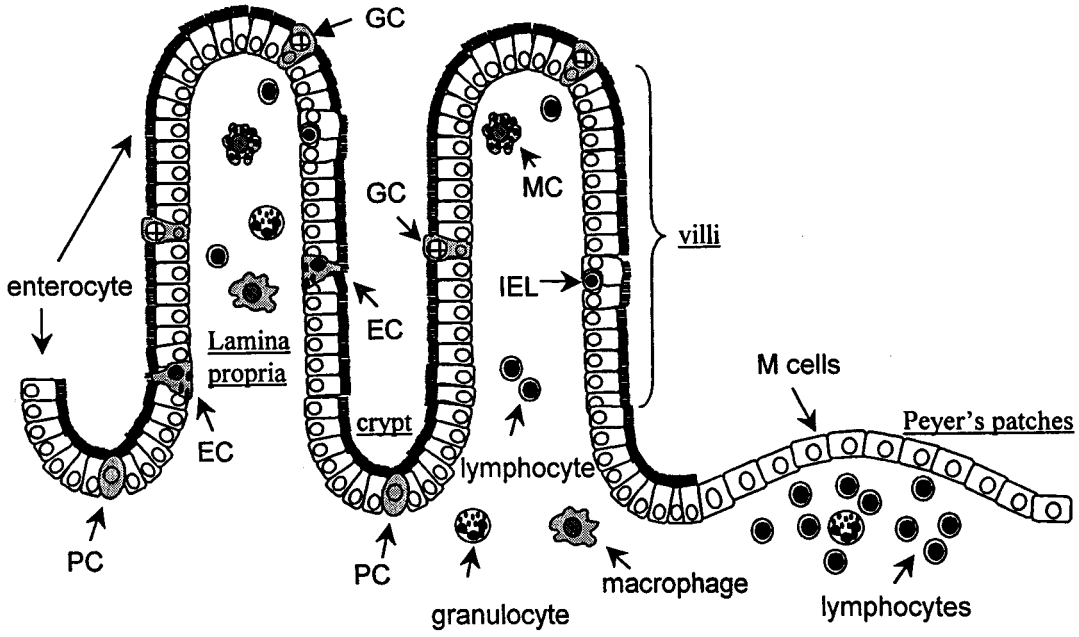

Figure 1.1 Schema of intestinal mucosa. The intestinal epithelium is formed mainly by a single layer of enterocytes, as well as other cell types, e.g. intraepithelial lymphocytes (IEL), M cells, Paneth cells (PC), goblet cells (GC), and enteroendocrine cells (EC). M cells are flattened epithelial cells covering the Peyer's patches where lymphocytes aggregate. Paneth cells lie at the base of the crypt and secrete antimicrobial defensin. Goblet cells which secrete mucus are dispersed among enterocytes. Enteroendocrine cells, e.g. enterochromaffin cells, G cells, which are flask-shaped specialized cells, produce mediators and hormones. In the lamina propria, various immune cells, such as lymphocytes, granulocytes, mast cells (MC), and macrophages, are present.

1.2.1 Endocytosis of macromolecules in enterocytes

Epithelial cells internalize macromolecules either non-selectively through fluid-phase endocytosis or selectively through receptor-mediated endocytosis. The apical membrane region of epithelial cells that is involved in endocytosis is localized at the bases of the microvilli, where deep membrane invaginations form. The fluid phase macromolecules and receptor-ligand complexes are initially accumulated at the clathrin-coated pits on the plasma membrane, where membrane invaginations occur and subsequently bud off to form clathrin-coated vesicles (Mellman, 1996). These vesicles rapidly lose their clathrin coat before fusion with the early endosomes in the peripheral cytoplasm. The early endosomes are regarded as sorting facilities, where contents are sorted for either degradative or recycling pathways (van Ijzendoorn et al. 2000; Mellman, 1996).

The fluid phase macromolecules endocytosed into intestinal epithelial cells are mostly directed to the late endosomal/lysosomal compartments for degradation. Early morphological studies have demonstrated that soluble proteins (e.g. horseradish peroxidase (HRP)) loaded into the lumen of rat ileum were taken up by intestinal epithelial cells into vesicles of various sizes (early endosomes) beneath the apical membrane, and subsequently delivered into large electron-dense vacuoles in the supranuclear regions, which were identified to be lysosomes by acid phosphatase histochemistry (Gonnella, Neutra, 1984). Markers of early endosomes such as transferrin (Tf) and the transferrin receptor (TfR), are used frequently for co-localization studies to verify the location of the

internalized molecules (Odorizzi et al. 1996; Brown et al. 2000). Moreover, studies using filter-grown polarized Madin Darby canine kidney (MDCK) epithelial cells have demonstrated the time course of intracellular trafficking (Parton et al. 1989). At 5-10 min after apical administration of HRP, the marker was restricted to early endosomes close to the apical surface of the cells. After 15 min, HRP was found in the late endosomal compartments, identified by positive staining with mannose-6-phosphate receptor (MPR), located in the supranuclear region of these cells. After longer incubation periods, the internalized marker was presented in MPR-negative, electron-dense lysosomal structures (Parton et al. 1989).

Intracellular trafficking of receptor-mediated endocytosis is well studied in both non-polarized cells, as well as polarized cells, such as MDCK cells (Matter, 2000; Mellman, 1996). Proteins taken up by receptor-mediated endocytosis are delivered either to the lysosomal degradative pathway or the recycling pathway. One example of receptor-mediated endocytosis sorted to the degradative pathway is the low-density lipoprotein (LDL) receptor (Goldstein et al. 1985). Once LDL particles are endocytosed into the early endosomes, they dissociate from their receptor due to the low pH in early endosomes (pH= 5.8). The free receptors are directed to recycling endosomes and returned to the plasma membrane, whereas the LDL ligand is delivered from the early endosomes to the late endosomes/lysosomes where the low pH (pH = 5) in the compartment facilitates the degradation of contents by lysosomal enzymes (Goldstein et al. 1985).

The recycling pathway in non-polarized cells involves the delivery of receptor-ligand complexes from early endosomes to recycling endosomes that are destined to return to the plasma membrane (Mellman, 1996). In polarized cells, such as epithelial cells, the plasma membrane is separated into the apical and the basolateral domains, structurally maintained by the tight junctions as well as constant sorting of specific proteins to the two separate membranes (van Ijzendoorn et al. 2000; Matter, 2000). Endosome membrane molecules in polarized epithelial cells are either directed to the surface of origin, or to the opposite cell surface (transcytosis) (Mellman, 1996). Transcytosis has been suggested to be a special form of recycling (van Ijzendoorn et al. 2000). Macromolecules are either internalized from the apical or basolateral membrane, and delivered to distinct apical early endosomes (AEE) or basolateral early endosomes (BEE) (Odorizzi et al. 1996). The endocytosed contents are either recycled to the surface of origin directly from early endosomes, both AEE (Figure 1.2, step 1a) and BEE (Figure 1.2, step 1b), or the contents enter special recycling endosomes (Figure 1.2, step 2a and 2b) before being sorted either to the original surface or the opposite membrane (Figure 1.2, step 3a,b) (Odorizzi et al. 1996; Brown et al. 2000).

Examples of receptor-mediated transcytosis of macromolecules across intestinal epithelial cells include transport of immunoglobulin (Ig) A and IgG (Hunziker, Kraehenbuhl, 1998; Casanova, 1992). IgA transport by polymeric immunoglobulin receptor (pIgR) has been well-characterized in polarized cells from the basolateral to the apical membrane (Apodaca et al. 1994; Brown et al. 2000). Transfection studies of pIgR in MDCK cells have demonstrated that pIgR is internalized at the basolateral membrane at

steady state without binding to IgA ligand, and targeted to the same surface (recycling) or opposite surface (transcytosis) of the cells (Hirt et al. 1993). The binding of dimeric IgA to pIgR provides a dimerization signal for inducing enhanced transcytosis of the ligand-receptor complex (Singer, Mostov, 2002; Song et al. 2002). In addition, in human infants, IgG in the colostrum is absorbed through the enterocytes by specific receptors, Fc receptor neonatal (FcRn) (Casanova, 1992). Studies in human intestinal epithelial T84 cells have demonstrated that IgG binds to FcRn on the apical surface of enterocytes, and is transported to the basolateral membrane of cells (Praetor et al. 1999; Stefaner et al. 1999; Dickinson et al. 1999). The FcRn binds IgG at the apical surface of epithelial cells at low luminal pH and the complex is endocytosed. After transcytosis to the basolateral surface of cells, the receptor ligand complex dissociates due to the high pH environment in the serosal fluid, and IgG is thus released (Rodewald, 1980; Praetor et al. 1999; Stefaner et al. 1999).

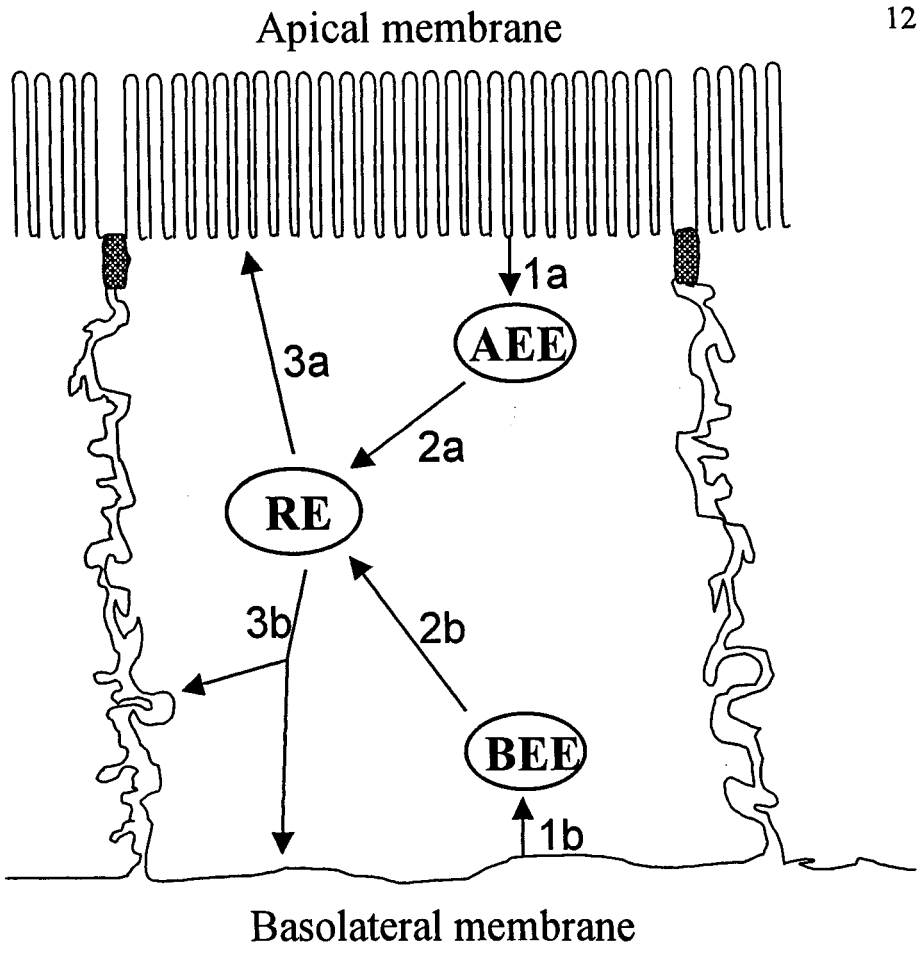

Figure 1.2 Schematic diagram of recycling pathway in polarized cells. Macromolecules are either internalized from the apical or basolateral membrane, and delivered to distinct apical early endosomes (AEE) or basolateral early endosomes (BEE). The endocytosed contents are either recycled to the surface of origin directly from early endosomes, both AEE (step 1a) and BEE (step 1b), or the contents enter special recycling endosomes (RE) (step 2a and 2b) before being sorted either to the original surface or the opposite membrane (step 3a,b).

1.2.2 Measurement of permeability of intestinal mucosa

Non-invasive absorption tests that involve orally administered inert or bioactive probes into the intestinal tract are widely used in human subjects in health and disease for measurement of intestinal permeability. Sugars, including disaccharides (e.g. lactulose, sucrose, sucralose; MW: 320-360) and monosaccharides (e.g. mannitol; MW: 160-180), have been used as inert probes in clinical studies. A mixture of disaccharides (5-10 g) and monosaccharides (0.5-5 g) were ingested after an overnight fast and urinary excretion was measured after 5-6 h (Söderholm et al. 1999a; Bjarnason et al. 1986a). Disaccharides and monosaccharides permeated the intestinal epithelial cells via different pathways. Disaccharides are transported through the paracellular space between adjacent epithelial cells, whereas monosaccharides have been suggested to be passed through the aqueous pores on the brush border membrane of enterocytes (Bjarnason et al. 1995; Bjarnason et al. 1986). Based on site-specific degradation of different disaccharides by luminal enzymes and bacteria, the comparative absorption of each disaccharide indicates permeability changes at different sites of the gastrointestinal tract. For example, sucrose is degraded to glucose and fructose by sucrase in the jejunum, lactose is degraded to glucose and galactose by bacterial fermentation in the colon, and sucralose remains undigested throughout the whole gastrointestinal tract (Meddings, Gibbons, 1998). All of these sugars are not naturally present in the urine and urinary excretion is complete following intravenous instillation. The increased level of urinary sucrose indicates increased permeability of the stomach, since the majority of sucrose is destroyed beyond the proximal small intestine (Meddings, Gibbons, 1998). The ratio of lactulose vs. mannitol

(L/M ratio) is widely used in clinical studies as a measurement of small intestinal permeability (Meddings, Gibbons, 1998; Söderholm et al. 1999). Sucralose, which is a nonabsorbable and nondigestible sweetener produced by chlorination of sucrose molecules, indicates the permeability of the small intestine and colon when it is found in the urine, since the absorption surface of the stomach is small and considered negligible in the test. Comparison of sucralose to lactulose levels or to the L/M ratio, provides information regarding whether the impaired permeability is in the small intestine or colon (Meddings, Gibbons, 1998).

Other inert probes for measuring permeability in humans include ^{51}Cr-EDTA, dextran, and polymeric ethylene glycol (PEG). An oral dose of 100 μCi of ^{51}Cr-EDTA with 200-300 ml of water is administered to subjects after an overnight fast, and urine is collected over the next 24 h. The size of ^{51}Cr-EDTA (MW = 342) permits transport via the paracellular pathway (Crowe et al. 1993; Bjarnason et al. 1986). The urinary excretion over 24 h is representative of the paracellular permeability from the upper to lower gastrointestinal tract, since it is not degraded by bacteria or enzymes in the lumen (Bjarnason et al. 1986). However, radioactivity makes the probe less desirable in human subjects. Another inert probe, fluoresceinated or radiolabeled dextran (MW =40,000) is a large molecule that normally does not pass through the tight junction, and is used for measurement of intestinal permeability to macromolecules in patients with Crohn's disease (Söderholm et al. 1999b). Moreover, early work has reported the use of PEGs for measurement of intestinal permeability. PEGs are mixtures of polymers ranging from MW 100 to 4000, the most widely used mixture is PEG 400 (including polymers of 194-502)

(Bjarnason et al. 1986). Due to the shortcomings of variable urine excretion levels of the polymers and unclear pathways of transport, PEGs are not used in recent studies (Bjarnason et al. 1995).

Proteins used for the measurement of transcellular and paracellular permeability of intestine in animal models include horseradish peroxidase (HRP, MW = 44,000) and ovalbumin (OVA, MW=45,000) (Heyman et al. 1990; Crowe et al. 1993; Berin et al. 1997). Protein probes are suitable model antigens for study of permeability in intestinal allergy, due to their similar sizes to the antigens. The ratio of intact and degraded protein was used as the parameter for intestinal permeability of cow's milk allergic patients (Heyman et al. 1988; Heyman et al. 1994). The kinetic chromatic assay of HRP allows for the measurement of the rate of transport as well as for the visualization of the protein in the transcellular and paracellular pathways (Heyman et al. 1990; Berin et al. 1997).

In vitro studies of intestinal permeability in epithelial cell culture or tissue segments involve the use of all of the aforementioned probes added to either the luminal or serosal surface. Unlike the *in vivo* studies in which the probes were measured in the urine or the blood, the probes were collected on the opposite side of the layers in *in vitro* studies. Therefore, inflamed tissues are not suitable for permeability studies *in vitro* due to the thickness of the smooth muscles in inflammation which may completely block the transport of the probes to the opposite side of the tissue. However, the examination of intestinal permeability in inflamed tissues is feasible *in vivo*, since the probes that are transported across the intestinal epithelial layer are routed to the blood vessels in the mucosa or submucosa, and are eventually collected in the blood or urine for detection.

1.3 Mucosal immune system

It has become increasingly clear in recent years that the intestine is the largest lymphoid organ in the body, containing ~ 40% of immune cells in the body (as reviewed in Strobel, 1993; Perdue, McKay, 1993). The gastrointestinal-associated lymphoid tissue (GALT) includes intraepithelial lymphocytes (IEL), lymphocyte aggregates in Peyer's patches, and lymphocytes diffusely distributed throughout the lamina propria (Strobel, 1993) (Figure 1.1). The GALT not only serves as the front line of immune responses to luminal antigens, but is also involved in regulating intestinal epithelial physiology. IELs are dispersed between intestinal epithelial cells. They are mainly $CD8^+$, with T cell receptors (TcR) of either the αβ- or γδ-type (Jarry et al. 1990). Underlying the intestinal epithelium, the lamina propria contains a wide variety of immune cells, including lymphocytes, mast cells, granulocytes, and macrophages (Berin et al. 1999a; Yu, Perdue, 2001a; Perdue, McKay, 1994a).

1.3.1 Immunologically Mediated Alteration of Intestinal Functions

The involvement of the immune system in modification of intestinal morphology and function was first suggested in studies of parasite infection. Rats infected with *Nippostrongylus brasiliensis* (Nb) and *Trichinella spiralis* (Ts) showed enterocyte detachment, partial or subtotal villous atrophy and crypt hyperplasia. However, the expected enteropathy during infection was ablated in rats that were previously depleted of T cells by thymectomy and irradiation (Manson-Smith et al. 1979; Ferguson, 1976). It was later shown that activated mucosal T cells are associated with crypt cell hyperplasia in an explant human small intestine culture system (McDonald, Spencer, 1987). Recognition

of the involvement of immune cells in the regulation of intestinal function has resulted in the use of a new term, immunophysiology.

1.3.2 Role of specific immune mediators

A wide array of cytokines and immune mediators have been reported to alter epithelial transport (summarized in Table 1.1). The summary is based on studies of both epithelial cell lines and tissue segments.

Table 1.1 Cytokines and mediators that alter intestinal epithelial permeability.

Cell source	Cytokines and mediators
Mast cell	IL-4, RMCP II
Monocyte/ Macrophage	IFNγ, TNFα
T cell	IL-4, IL-13, IFNγ, TNFα
Granulocyte	IFNγ, TNFα

Abbreviations: IL, interleukin; TNFα, tumor necrosis factor α; IFNγ, interferon γ; RMCP II, rat mast cell protease II

1.3.2.1 Intestinal epithelial cell lines

A number of cytokines have shown direct effects on the permeability of intestinal epithelial monolayers. Confluent T84 cells (a human colonic epithelial cell line) exposed to 10 ng/ml IL-4 for 48-72 h exhibit an attenuation in transepithelial barrier function as assessed by decreased resistance and this effect was inhibited by neutralizing anti-IL-4 or anti-IL-4 receptor antibodies (Colgan et al. 1994). Increased transepithelial flux of macromolecules has also been demonstrated in T84 cells after the addition of IL-4 (Berin et al. 1999b). The treatment with IL-4 upregulated the transepithelial transport of HRP through both transcellular and paracellular pathways (Berin et al. 1999b). Furthermore, IL-13, which shares similar biological activity with IL-4, attenuates epithelial resistance and increases the transport of radiolabeled dextran tracer in human epithelial cell lines (Zünd et al. 1996).

It has been shown that HT29 cell lines (human intestinal epithelial cells) incubated with TNFα for 48 h, in the presence of a low concentration of IFNγ, displayed a reduced transepithelial resistance (Mullin, Snock, 1990; Mahraoui et al. 1997; Heyman et al. 1994), and an increased intact HRP flux (Mahraoui et al. 1997; Heyman et al. 1994), potentially via the paracellular pathway. The synergistic effects of TNFα and IFNγ on barrier function of epithelial monolayers was mediated by the upregulation of the TNFα receptor on intestinal epithelial cells by IFNγ (Tsujimoto et al. 1986). TNFα has also been reported to disrupt actin filaments in endothelial cells (Goldblum et al. 1993), which may account for its alteration of tight junction and transepithelial resistance.

Two cytokines, namely, IL-10 and TGFβ, have been implicated in the prevention or correction of impaired epithelial permeability. By treating T84 cells with IL-10 before and after the addition of IFNγ, IL-10 was shown to prevent the IFNγ-induced increased permeability in T84 cells (Madsen et al. 1997). The addition of TGFβ was also shown to correct the impaired barrier function induced IFNγ in T84 cells (Planchon et al. 1994). The role of IL-10 and TGFβ in reversing disrupted barrier function is suggested to be due to its inhibition of immune responses (Planchon et al. 1994; Madsen et al. 1997).

1.3.2.2 Tissue segments

A role for IFNγ in increasing macromolecular transport in the small intestine has been demonstrated in suckling rat models (Sütas et al. 1997). Rat pups that were injected intraperitoneally with IFNγ prior to experiments have shown increased transport of intact HRP across the jejunal segments. Increased HRP flux was found only in segments containing Peyer's patches while no effect was seen in patch-free segments (Sütas et al. 1997). Furthermore, rat mast cell protease (RMCP) II has been suggested to increase macromolecular transport via the paracellular pathways (Berin et al. 1999a; Scudamore et al. 1995a).

1.3.3 Activated epithelium

Intestinal epithelial cells are able to secrete a range of cytokines and mediators (Table 1.2) (as reviewed in Perdue, McKay, 1998; Perdue, McKay, 1994). These epithelial cell-derived mediators may then act on epithelial cells themselves through an autocrine mechanism or act to stimulate subepithelial immune cells, myofibroblasts, or

nerves to secrete factors that further modulate epithelial functions (Perdue, McKay 1993). Receptors on intestinal epithelial cells include IL-2 (Ciacci et al. 1993), IL-4 (Zünd et al. 1996; Colgan et al. 1994), IL-13 (Sanders et al. 1995; Zünd et al. 1996), TNFα (Taylor et al. 1998; Heyman et al. 1994), IFNγ (Taylor et al. 1998; Madara, Stafford, 1989) and TGFβ (Barnard et al. 1989; Kurokawa et al. 1987), and protease activating receptor (PAR)-2 (Vergnolle et al. 1998; Kong et al. 1997), implying an autocrine effect for some cytokines and mediators to alter the epithelial function, an effect that may play a role in the exacerbation or maintenance of the inflammatory response. Protease activating receptor (PAR)-2 is a recently characterized G-protein-coupled receptor expressed on small intestinal epithelial cells. Luminal pancreatic trypsin and PAR-2 activating agonists induce the release of prostaglandin E_2 (PGE_2) leading to either a direct or indirect effect on ion secretion from epithelial cells (Vergnolle et al. 1998; Kong et al. 1997).

Table 1.2 Cytokines and mediators derived from intestinal epithelial cells.

Epithelial cell mediators	
cytokines	IL-1α, IL-1β, IL-1 receptor antagonist, IL-6, IL-8, TNFα, TGFα, TGFβ, MCP-1, IGF -II, IGF-binding protein 1-4, GM-CSF, NGF, eotaxin
lipid metabolites	arachidonic acid, PGE_2, $PGF_{2\alpha}$, LTB_4, PAF
Reactive species	nitric oxide, superoxide, hydrogen peroxide

Abbreviations: TGF, transforming growth factor; MCP, monocyte chemoattractant peptide; IGF, insulin growth factor; GM-CSF, granulocyte/monocyte colony-stimulating factor; NGF, nerve growth factor

1.4 Animal models of intestinal hypersensitivity

Animal models of food allergy include guinea pigs orally fed with cow's milk, and rats or mice intraperitoneally injected with HRP or OVA plus adjuvants. A number of studies utilized the intestinal segment ligation technique and luminal fluid collection for measurement of ion secretion (Crowe et al. 1993; Pène et al. 1988; Perdue et al. 1984). Our group and others employ Ussing chambers to assess the physiology of the intestine (Perdue et al. 1991; Crowe et al. 1990; Berin et al. 1998; Berin et al. 1997). The Ussing chamber apparatus is comprised of two separate chambers facing the luminal and serosal sides of the intestine (Figure 1.2). Oxygenated isotonic buffers are circulated separately in the two chambers, allowing the administration of chemical agents to an isolated single side

of the intestine. Electrodes close to the tissues measure net ion transport indicated by the short-circuit current (Isc). The parameter of the ionic permeability is measured as the conductance (G) of the tissue, which is the inverse of the resistance. The conductance value reflects the permeability of the tight junctions and paracellular spaces of the tissue. Ussing chambers are connected to voltage clamps to inject the current required to offset the potential difference across the tissue, to preamplifiers for the display of electrophysiological parameters, and also to a transmural stimulator that generates small voltages for examining the nerve function or viability of the tissue. A computer program records the electrophysiological parameters over a period of time, thus, allowing researchers to monitor the time course of each response of the tissue upon antigen challenge or electric stimulation.

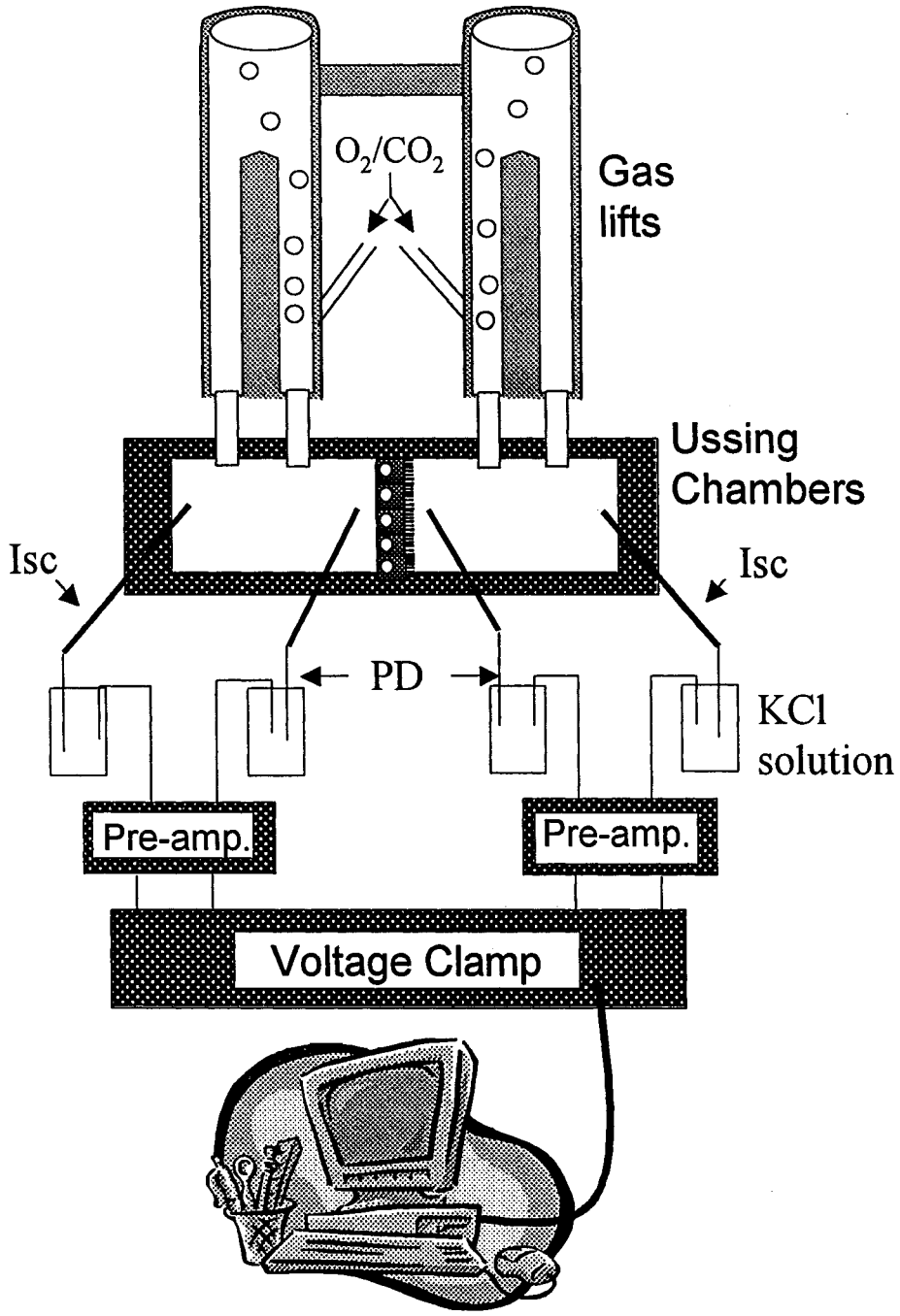

Figure 1.3 Ussing chamber apparatus

Figure 1.3 Ussing chamber apparatus. The basic method involves pinning a flat sheet of tissue between two chamber halves containing iso-osmotic physiological fluid, and measuring the electrophysiological parameters across the tissue with electrodes. The intestinal segments used in studies were cut longitudinally into a flat sheet and pinned onto the chamber halves. The two chamber halves are facing the luminal and serosal sides of the intestine and are connected to gas lift systems where physiological buffers are circulated separately. The buffers are aerated with 95% O_2/ 5% CO_2. A circulating water pump and water jacket maintain the buffers at 37°C. The potential difference (PD) is measured by two pairs of calomel electrodes connected to agar bridges placed in close proximity to the tissue. The tissue is voltage clamped at zero voltage by a pair of silver/silver-chloride electrodes via agar bridges placed at the far ends of chamber halves. The electrical connections from the electrode wells of KCl solutions are run to pre-amplifiers that interface between the chambers and voltage clamp. The current injected to maintain the PD at zero is termed short-circuit current (Isc) which is equal but opposite to the current generated by the tissue by active transport. The Isc represents the net active ion transport. Tissue ionic conductance (G, mS/cm^2) is a measure of the permeability of the tissue to ions, and is the inverse of resistance (R, ohms (Ω)). The G is calculated using Ohm's law ($V = IR$, where V = PD, I = Isc, and R = 1/G). The Isc readings are recorded using a data aquisition program operated on a computer.

1.4.1 Epithelial response to allergen challenge

Early *in vivo* studies in OVA-sensitized rats showed a dramatic reduction of the absorption of water, Na^+, Cl^-, and K^+ in the cannulated small intestinal segments after intraluminal antigen challenge. The response was specific for the sensitizing antigen and was IgE-mediated (Perdue et al. 1984). Reduced net ion absorption results from either a decrease in mucosal-to-serosal flux, or an increase in serosal-to-luminal flux, or a combination of both. Using radiolabeled sodium and chloride ions, it was clearly shown that antigen challenge to sensitized intestine induces decreased mucosal-to-serosal flux along with increased serosal-to-mucosal flux of Cl^-; while fluxes of Na^+ did not change (Perdue, Gall, 1986). *In vitro*, the Isc of sensitized rat intestine increased after antigen challenge (Perdue, Gall, 1986). The change in Isc depends mainly on Cl^- secretion as demonstrated by inhibition with chloride channel blockers and chloride-free buffer (Cuthbert et al. 1983; Crowe et al. 1990; Perdue, Gall, 1986). It has been demonstrated that the epithelial ion transport is not modulated by sensitization *per se* since no significant changes in the baseline Isc and baseline net fluxes of ions are seen in the intestine prior to antigen challenge in the OVA-sensitized rats (Crowe et al. 1990).

The reduction of ion and water absorption and increased chloride secretion after antigen challenge corresponds with reduced mucosal histamine content and numbers of stained mucosal mast cells, suggesting mast cell activation (Perdue et al. 1984). The involvement of mast cells in altered ion transport in intestinal anaphylaxis is further confirmed using mast cell-deficient animals (Perdue et al. 1991) and mast cell stabilizing agents (Crowe et al. 1990; Perdue, Gall, 1986; Perdue, Gall, 1983). In mast cell-deficient

mice (W/W^v), the change in Isc in the jejunum of OVA-sensitized W/W^v rats at antigen challenge was ~70% less than that seen in +/+ rats; while replenishing mast cells by bone marrow precursors from +/+ rats completely restored the Isc response to antigen stimulation (Perdue et al. 1991). Similar inhibition of the change in Isc was found by treating rats with doxantrazole, an agent that inhibits histamine release from mucosal mast cells (Perdue, Gall, 1983). Moreover, histamine-1 antagonists, serotonin antagonists and cyclooxygenase inhibitors also reduce the Isc response (Crowe et al. 1990). These results suggest that active mediators released from mast cells are responsible for antigen-induced epithelial ion secretion.

Antigen challenge to the serosal side of the intestine in Ussing chambers induces an immediate increase (~30 sec) in Isc; while the addition of antigen to the luminal side of the tissue results in a lag phase of ~3 min (Crowe et al. 1990). The luminal Isc response is abolished when antigen is added to the serosal side first, suggesting that the Isc change resulting from both luminal and serosal challenge is via a common mechanism or terminal target, which is likely to be mast cells (Baird et al. 1984). The lag phase seen in luminal challenge has been suggested to reflect the time for antigen to be transported across the epithelial layer to activate mast cells in the lamina propria (Crowe et al. 1990).

The mechanism of this rapid rise of Isc in response to luminal antigen challenge is puzzling since transcellular transport of macromolecules appears to take ~20-30 min to reach the serosal side (Keljo, Hamilton, 1983) while paracellular transport is limited to small molecules (<500 D) in normal physiological conditions (Madara, 1989). In rats sensitized to OVA, recovery of ^{51}Cr-EDTA in blood was 60-80% higher than in control

animals (Crowe et al. 1993). In contrast, increased permeability to the protein antigen was seen only after luminal challenge. After challenge, the intestinal uptake of OVA (MW = 45,000) into blood was elevated 14-fold in OVA-sensitized rats compared to that in controls (Kitagawa et al. 1995). Thus, increased intestinal permeability to luminal antigens was suggested to account for the rapid rise of Isc (~3 min) post challenge in the sensitized animals.

The question of whether this enhanced permeability is via transcellular or paracellular pathways has been addressed in a subsequent study from the Perdue group (Berin et al. 1997). Similar to OVA-sensitized animals, the rapid rise of Isc was also demonstrated in Sprague Dawley rats sensitized to horseradish peroxidase (HRP) (Berin et al. 1997). The use of HRP allows the researcher to monitor and visualize the transepithelial transport of antigen using kinetic enzymatic assays and electron microscopy, respectively. Segments of jejunum from rats sensitized to HRP were mounted in Ussing Chambers and luminally challenged with HRP antigen. Electron micrographs showed that at 2 min post challenge (before the rise of Isc at ~ 3 min), there were increased numbers and a greater area of the HRP-containing endosomes in enterocytes of HRP-sensitized rats compared to controls, suggesting enhanced uptake of the specific antigen (Berin et al. 1997). The rate of transepithelial transport of antigen was also faster in the intestine of sensitized animals compared to controls. In HRP-sensitized rats, HRP-containing endosomes were distributed throughout the enterocytes (from apical to basolateral regions of cells and lamina propria); while in OVA- and saline-treated animals, HRP endosomes were restricted to the apical region (Berin et al. 1997). The

phenomenon of enhanced antigen uptake and transport was shown to be specific for the sensitizing antigen in this period (within 3 min post challenge), and was denoted as phase I. After 30 min post challenge (denoted phase II), HRP was transported not only transcellularly in endosomes, but also through the tight junctions and paracellular spaces in sensitized rats (Berin et al. 1997). In contrast, epithelium from sham-sensitized and OVA-sensitized rats did not show HRP in paracellular regions. Moreover, a several-fold increase of luminal-to-serosal HRP flux was detected in the sensitized intestine, which was associated with increased tissue conductance (Berin et al. 1997).

The mechanism of this enhanced transepithelial transport of macromolecules has been studied using genetically modified animals (Berin et al. 1998). The rapid and increased transcellular transport of HRP within 3 min, phase I, was not abolished in mast cell-deficient rats (WS/WS), whereas the phenomena of increased transepithelial transport and flux of HRP in phase II were inhibited in these rats (Figure 1.4). These results suggest that phase I is mast cell-independent and is modulated by sensitization *per se*, whereas phase II relies on the presence of mast cells (Berin et al. 1998) (Figure 1.5). In phase I, the uptake of antigen is specific to the sensitizing antigen, indicating that an immunoglobulin recognition mechanism may be involved. The precise mechanism is not fully understood. Macromolecular transport in phase II is dependent on the activation of mast cells leading to increased permeability of the tight junctions resulting in the influx of antigen through the paracellular pathways (Berin et al. 1998; Berin et al. 1997). A mast cell mediator, i.e. RMCP II, was suggested to be responsible for alteration of the paracellular permeability (Berin et al. 1999a; Berin et al. 1998a).

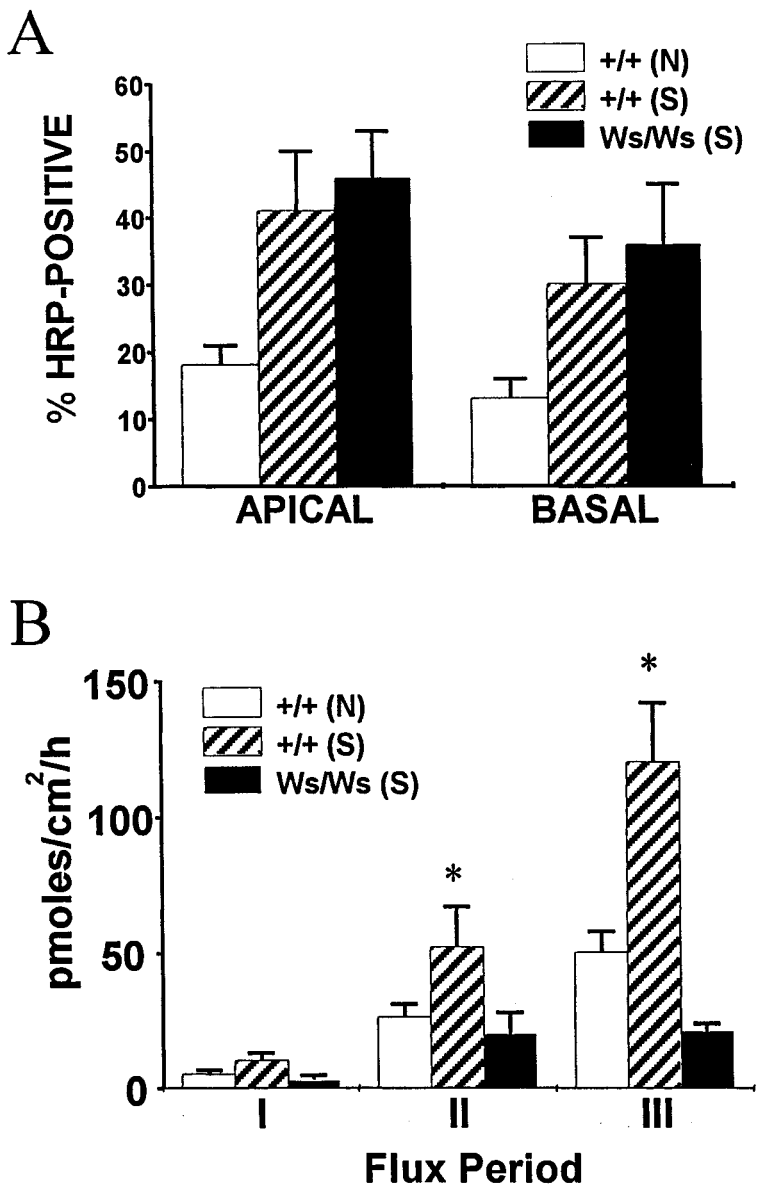

Figure 1.4 The role of mast cells in enhanced transepithelial antigen transport in intestine of sensitized rats

Figure 1.4 The role of mast cells in enhanced transepithelial antigen transport in intestine of sensitized rats. (A) Distribution of HRP-positive endosomes at 2 min post HRP challenge. The incidence of HRP-positive endosomes in apical or basal regions of enterocytes in non-sensitized (+/+ (N)) and sensitized (+/+ (S)) wild type rats and sensitized mast cell-deficient rats (WS/WS (S)) were analysed in electron photomicrographs (10 micrographs per rat for 4 rats). Data are expressed as mean ± SEM. (B) Transmucosal HRP flux in the three groups as above. The HRP flux across jejunum after antigen challenge for the period of 0-30 min (I), 30-60 min (II), and 60-90 min (III). Data are presented as the mean ± SEM, * $p < 0.05$ with control rats at the same time period. n = 8 rats per group.

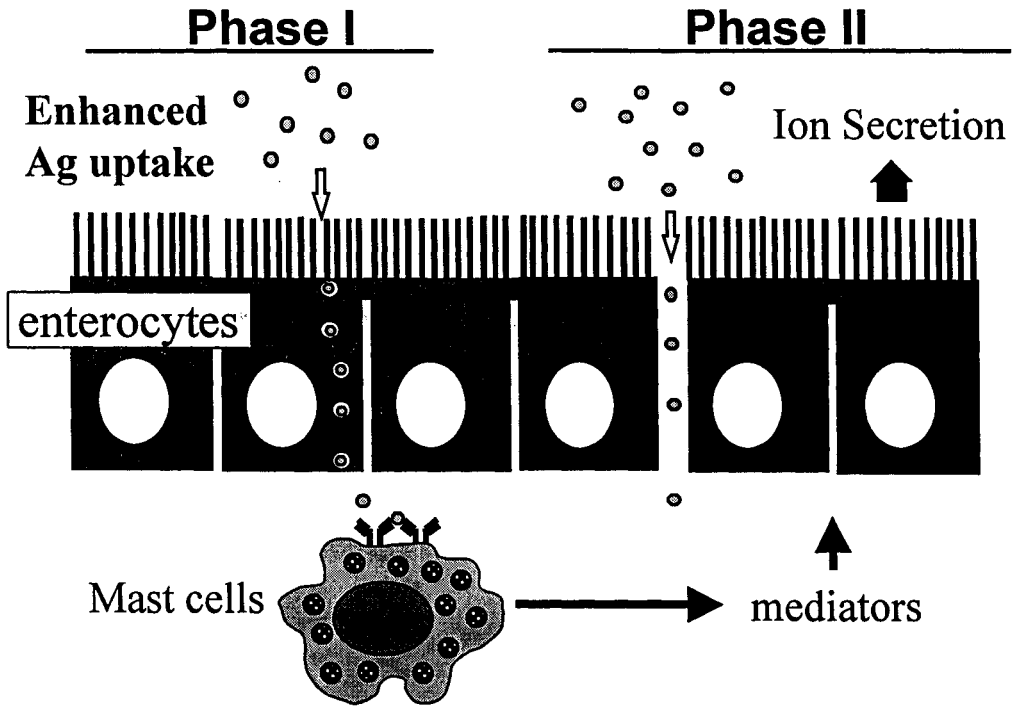

Figure 1.5 Schematic diagram of enhanced transepithelial antigen transport in intestinal allergy. In sensitized intestine, the specific antigens are taken up by the intestinal epithelial cells in a greater amount and are rapidly transported across the cells within 3 min after luminal antigen challenge. When antigen reaches the lamina propria, it activates mast cells by cross-linking IgE receptors on the cell surface. The activation leads to mast cell degranulation and release of mediators that induce ion secretion from the intestinal epithelial cells and increased permeability of the tight junctions, thus allowing more antigen to come through the paracellular pathway. The initial phase of antigen transport before mast cell activation is termed phase I; the period after mast cell activation is termed phase II.

Evidence from guinea pigs orally sensitized to cow's milk further supports the concept of immunomodulation of intestinal permeability to macromolecules in phase II. Antigen challenge in cow's milk sensitized guinea pigs showed that serosal challenge with β-lactoglobulin (βLG) induced an increased influx of intact bystander protein, HRP, while no significant difference was seen in the level of degraded HRP (Heyman et al. 1990). Luminal challenge of specific antigen in OVA-sensitized rats also showed an increased amount of gavaged bystander protein (HSA) in the serum (Bloch, Walker, 1981). These results suggest that antigen challenge in the intestine of sensitized animals may result in enhanced penetration of immunologically intact macromolecules (food antigens, bacterial products, etc.) across the barrier, which may lead to allergic inflammation.

1.5 Immune factors in hypersensitivity: IgE, IL-4 and IgE receptors

1.5.1 Role of IgE and IL-4

Elevated synthesis of IgE is the hallmark of atopic diseases. The presence of high levels of serum IgE has been documented in food allergic patients (Sampson, Burks, 1996) and animal models of hypersensitivity (Berin et al. 1997). Moreover, an increased concentration of IgE in intestinal luminal fluid was also reported in food allergic individuals, concomitant with an elevated serum IgE level (Negrao-Corrêa et al. 1996; Brown, Lee, 1976; Belut et al. 1980). In addition, radiolabeled IgE (but not IgG) translocated from the serum into the intestine and intestinal lumen in rats after infection with *T. spiralis* or after infusion with IL-4 (Negrao-Corrêa et al. 1996; Ramaswamy et al. 1994). IL-4 regulates the synthesis of IgE in B cells by inducing germ line ε transcript and

promotes the differentiation of T lymphocytes to a Th2 cell type (Paul, 1991). Elevated production of IL-4 by mononuclear cells isolated from blood and intestinal mucosa of atopic individuals has been demonstrated (Hauer et al. 1997). IL-4 also plays a role in upregulating the expression of IgE receptors.

1.5.2 Role of IgE receptors

1.5.2.1 The high affinity IgE receptor: FcεRI

Two types of IgE receptors have been identified: FcεRI and FcεRII. In human, the high affinity IgE receptors, FcεRI, are expressed on a variety of cells, e.g. mast cells, basophils, monocytes, epidermal Langerhans' cells and dendritic cells (Kinet, 1999; Metzger, 1992; Maurer et al. 1995; Stingl, Maurer, 1997). In mouse, the expression of FcεRI is more restricted and was only found on mast cells and basophils (Kinet, 1999). There is no evidence of the expression of FcεRI on intestinal epithelial cells. FcεRI was first identified on mast cells and its expression on these cells is strongly correlated with allergy. IL-4 upregulates the expression of FcεRI on mast cells (Toru et al. 1996). In atopic patients, the binding of allergens to IgE causes cross-linking of the FcεRI on the cell surface leading to the activation and degranulation of mast cells, resulting in anaphylaxis (Sampson, Burks, 1996). Moreover, IgE-dependent allergen focusing and presentation via FcεRI has been demonstrated in monocytes, epidermal Langerhans' cells, and peripheral blood and dermal dendritic cells isolated from atopic patients (Maurer et al. 1996; Maurer et al. 1995; Stingl, Maurer, 1997). Allergens are more efficiently taken up, processed, and presented to activate T cells by these antigen-presenting cells via

IgE/FcεRI binding in human (Maurer et al. 1995). The FcεRI-facilitated antigen presentation was not seen in rodents.

1.5.2.1 The low affinity IgE receptor: CD23/FcεRII

The low affinity IgE receptor, CD23/FcεRII, has been identified in human and mice. CD23 is a type-II integral membrane glycoprotein with an extracellular C-terminal sequence homologous to C-type animal lectins (Delespesse et al. 1991; Delespesse et al. 1992). Expression of the murine CD23 has been reported mainly on B cells, T cells, and follicular dendritic cells (Conrad, 1991), whereas human CD23 was found in a wide range of cells, including B cells, monocytes, eosinophils, platelets, epidermal Langerhans cells, dendritic cells, keratinocytes and bronchial and intestinal epithelial cells (Kaiserlian et al. 1993; Campbell et al. 1994; Bécherel et al. 1994; Bonnefoy et al. 1993).

The expression of human CD23 on various cell types is upregulated by IL-4, including B cells (Pène et al. 1988b; Defrance et al. 1987b), T cells (Prinz et al. 1990), monocytes (Vercelli et al. 1988), eosinophils (Arock et al. 1994), keratinocytes (Bécherel et al. 1994b), and epidermal Langerhans' cells (Bieber et al. 1989). Murine IL-4 also increases the expression of CD23 on B cells (Hudak et al. 1987). Other cytokines have been reported to either increase or decrease the expression of CD23, depending on the different cell types. IL-13, which shares similar bioactivity to IL-4, is known to promote the expression of CD23 on human B cells and monocytes (Steinsvik et al. 1999; Defrance et al. 1994; Punnonen et al. 1993; McKenzie et al. 1993). In contrast, mouse IL-13 has no effect on CD23 expression on B cells (Lai, Mosmann, 1999). In addition, human IFNγ

decreases the expression of CD23 on B cells (Rousset et al. 1988; Pène et al. 1988), whereas it increases the expression on T cells (Prinz et al. 1990), monocytes (Kawabe et al. 1988), keratinocytes (Bécherel et al. 1994b), platelets (Pancre et al. 1998), and Langerhans cells (Bieber et al. 1989). The IgE ligand was also found to increase surface expression of CD23 by inhibiting its membrane turnover in mouse B cells (Ruzek, Mathur, 1995; Kisselgof, Oettgen, 1998), human B cells, T cells and monocytes (Kawabe et al. 1988).

Two isoforms of CD23, *a* and *b*, differing in their cytoplasmic amino-terminal tail, were found in humans and mice. In humans, CD23*a* is constitutively expressed on mature B cells (IgM^+ IgD^+) (Delespesse et al. 1991). CD23*b* is expressed on various human cell types after IL-4 induction, including B cells, monocytes, T cells, eosinophils, and keratinocytes (Delespesse et al. 1991; Delespesse et al. 1992). Mice express the homologue of the human CD23a mainly on B cells (Conrad, 1991). In contrast to humans, treatment with IL-4 upregulates CD23 isoform *a* in murine B cells. The existence of an IL-4-inducible *b* like isoform remains controversial in mice (Conrad et al. 1993; Richards, Katz, 1994). Only one group has reported the presence of CD23 *b* isoform in a mouse spleen non-B cell population after IL-4 and LPS stimulation by sequence analysis of plasmids encoding RT-PCR products (Kondo et al. 1994).

CD23 is a multifunctional receptor that plays a role in facilitating antigen focusing and presentation (Van der Heijden et al. 1995; Santamaria et al. 1993; Pirron et al. 1990; Kehry, Yamashita, 1989), regulating IgE synthesis (Yu et al. 1994) and which serves as a growth factor (Luo et al. 1991) and an adhesion molecule (Bonnefoy et al. 1995) in B

cells, and which is also involved in IgE-mediated cytokine production and cytotoxicity in monocytes (Paul-Eugène et al. 1992; Borish et al. 1991), eosinophils (Capron et al. 1984; Arock et al. 1994), platelets (Matsuda et al. 1997), and keratinocytes (Bécherel et al. 1997; Bécherel et al. 1994; Bécherel et al. 1994). The IgE/CD23-mediated antigen focusing and presentation in B cells was demonstrated in both *in vitro* and *in vivo* studies. Evidence in murine cells showed that monomeric IgE bound to CD23, in contrast to IgG1 bound to FcγR, allows B cells to bind specific antigen and to present very small amounts of antigen that are sufficient to activate T cells (Kehry, Yamashita, 1989). The binding of preformed IgE-antigen complexes to CD23, compared to antigen alone, also allows B cells to present low quantities of antigen to activate T cells. The addition of anti-CD23 antibody abolished this effect (Kehry, Yamashita, 1989). Similar findings were documented in human cell *in vitro* studies (Santamaria et al. 1993; Pirron et al. 1990). In mouse *in vivo* studies, passive sensitization of mice with trinitrophenyl (TNP)-specific IgE before bovine serum albumin (BSA)-TNP antigen challenge, led to enhanced B cell antigen capture and presentation in which the anti-BSA IgE production was 10^5-fold stronger than in mice without passive sensitization (Gustavsson et al. 1994; Heyman et al. 1993). The IgE-mediated enhanced antigen uptake in B cells and antibody response were antigen-specific, and were completely inhibited in the presence of anti-CD23 (Gustavsson et al. 1994; Heyman et al. 1993).

Immunohistochemical studies showed the expression of CD23 proteins on intestinal epithelial cells of normal individuals, and enhanced expression was found in patients with inflammatory bowel disease, cow's milk protein intolerance or severe

autoimmune enteropathy (Kaiserlian et al. 1995; Kaiserlian et al. 1993). Immunostaining of CD23 was also demonstrated on bronchial epithelial cells of asthmatic patients, but not in normal subjects (Campbell et al. 1994). Although the presence of CD23 has been identified on various mucosal epithelial cell surfaces, the functions of the epithelial CD23 remains unknown.

1.6 Aim of project

The aim of this project was to investigate the mechanism of enhanced antigen transport across intestinal epithelial cells using sensitized genetic deficient mice. My study focused on the immune factors that are likely to be involved in mediating and regulating the transepithelial antigen transport in phase I (within 3 min post antigen challenge). We hypothesized that IgE and the low affinity IgE receptor, CD23, may mediate the enhanced antigen transport across intestinal epithelial cells, and IL-4 may play a regulatory role in the mechanism.

CHAPTER 2: Enhanced intestinal transepithelial antigen transport in allergic rats is mediated by IgE and CD23 (FcεRII).

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ABSTRACT

We previously reported that active sensitization of rats resulted in the appearance of a unique system for rapid and specific antigen uptake across intestinal epithelial cells. The current studies used rats sensitized to horseradish peroxidase (HRP) to define the essential components of this antigen transport system. Sensitization of rats to HRP stimulated increased HRP uptake into enterocytes (significantly larger area of HRP-containing endosomes) and more rapid transcellular transport compared with rats sensitized to an irrelevant protein or naive control rats. Whole serum but not IgE-depleted serum from sensitized rats was able to transfer the enhanced antigen transport phenomenon. Immunohistochemistry demonstrated that sensitization induced expression of CD23, the low affinity IgE receptor (FcεRII), on epithelial cells. The number of immunogold-labelled CD23 receptors on the enterocyte microvillous membrane was significantly increased in sensitized versus control rats, and was subsequently reduced following antigen challenge when CD23 and HRP were localized within the same endosomes. Finally, pre-treatment of tissues with luminally-added anti-CD23 antibody significantly inhibited both antigen transport and the hypersensitivity reaction. Our results provide evidence that IgE antibodies bound to low affinity receptors on epithelial cells are responsible for the specific and rapid nature of this novel antigen transport system.

INTRODUCTION

Allergic diseases are the most common of all immunologically-mediated conditions, affecting 20-30 percent of the U.S. population, and are increasing in prevalence in most countries of the developed world (1). Food allergy is one type of allergic disorder which affects both adults and children. Following ingestion of a specific food antigen, a sensitized individual may experience local gastrointestinal symptoms such as nausea, vomiting and diarrhea; extra-intestinal symptoms can occur in the skin and airways (2,3). In severe reactions, such as those to peanut antigen, systemic anaphylaxis can occur and be life-threatening. Treatment of food allergies usually consists of avoidance of suspected foods. In some cases, particularly in children, elimination diets can become so restrictive that nutrition may be compromised. Therefore, it is important to have a clear understanding of the mechanisms involved in food allergic/intestinal hypersensitivity reactions in order to develop effective therapeutic strategies.

The accepted sequence of events in allergy is that symptoms are triggered when antigen cross-links IgE antibodies bound to the surface of mast cells. Released bioactive mediators then act on receptors on other cell types to alter function. Studies in humans and animal models of food allergy have shown that intestinal reactions occur very quickly and result in dramatic physiological changes. Within minutes, epithelial secretion of ions, water and mucin begins, vasodilation and vascular permeability increase and contraction of smooth muscle occurs. (4-6). The rapid nature of the response has resulted in allergic reactions being termed 'immediate' hypersensitivity. The exact mechanism responsible for the rapidity of the allergic symptom production has never been fully explained, since the epithelial lining of the gastrointestinal tract should theoretically restrict access of macromolecular antigens to effector cells such as mast cells, located in the subepithelial lamina propria.

Normally, macromolecules penetrate the epithelium in very limited quantities. M cells, specialized cells in the epithelium covering Peyer's patches (located mainly in the distal small and large intestine), transport antigens from the lumen to immune cells in the patch (7). This process is thought to be important in the generation of oral tolerance, a mechanism to actively suppress immune responses (8). However, the number of M cells is relatively small compared to the number of columnar epithelial cells (enterocytes) which line the entire intestinal tract. Enzymes anchored in the enterocyte microvillus membrane degrade most ingested proteins into non-antigenic amino acids and peptides. Enterocytes do take up some intact protein into endosomes which are transported across the cells, but the majority of endocytosed protein is hydrolyzed by lysosomal enzymes following fusion of endosomes with lysosomes (9). Thus, the amount of immunologically intact protein that arrives in the circulation comprises <0.01% of that ingested. Transepithelial protein transport has been shown to be relatively slow, requiring 20-30 min (10).

In contrast to this normal process, our previous results indicated a much faster event in sensitized rats. In early studies of rats sensitized to ovalbumin (OVA), *in vivo* perfusion of the small intestine with antigen-containing buffer resulted in significantly altered transport of ions and water within 20 min, associated with mast cell activation (11). Subsequently, we examined segments of small and large intestine (rodents and humans) in Ussing chambers and demonstrated that challenge with antigen or anti-IgE resulted in active Cl^- ion secretion (the driving force for water secretion leading to diarrhea), indicated on-line by an increase in the short-circuit current (Isc) (12-15). Surprisingly, when antigen was added to the luminal surface of intestinal tissues from sensitized rats, the change in Isc began in only 3 min (16). In addition, intestinal responsiveness in this animal model was retained for at least 8 months (17).

Recently, we conducted studies designed to determine effect of sensitization on the rate and route of transepithelial antigen transport. We sensitized rats to horseradish peroxidase (HRP) and subsequently challenged tissues in Ussing chambers with this protein. HRP was used as a model antigen since it is similar in size to typical food antigens and its reaction product can be visualized in cells and tissues by electron microscopy. We found that endosomal uptake of HRP by enterocytes was enhanced in rats sensitized to this protein but not in those sensitized to OVA (18). Both the amount of HRP antigen endocytosed and its rate of transport across the cells was significantly increased by specific sensitization, such that at 2 min HRP was already present in the lamina propria in tissues from sensitized rats, whereas HRP-containing endosomes remained confined to the apical region of enterocytes in control tissues. The specificity of the antigen uptake phenomenon in sensitized rats suggested that it was mediated by an immunoglobulin recognition mechanism.

The present study was designed to investigate the essential components of this enhanced specific antigen transport system in sensitized rats. We found that rapid transepithelial transport could be passively transferred to naive rats by injection of serum from actively sensitized rats, but that depletion of IgE from the serum eliminated the effect. Expression of the low affinity IgE receptor, FcεRII/CD23, on the enterocyte microvillus membrane was stimulated by sensitization. Immunogold receptor localization showed that the number of receptors on the apical membrane was increased by sensitization and then reduced by antigen challenge. Subsequently, we localized CD23 and HRP antigen within the same endosomes. Finally, pre-treatment of tissues with anti-CD23 monoclonal antibody before HRP challenge concentration-dependently inhibited both transepithelial transport of antigen and the hypersensitivity reaction. Our results provide

evidence that an IgE-CD23 mechanism accounts for enhanced transepithelial antigen transport in sensitized rats.

METHODS

Animals

All studies were approved by the McMaster University Animal Care Committee. Pathogen-free male Sprague-Dawley rats (mean weight 300 g, Charles River, St. Constant, Quebec, Canada) were actively sensitized to HRP (type II) or OVA (type VI) (both from Sigma Chemical Co. St. Louis, MO) by injection of 1 mg protein in alum (sc) plus pertussis vaccine or toxin (ip) adjuvants to induce IgE production, as previously described (16-18). Controls were rats sham-sensitized by injection of saline. Experiments were conducted 14 days after active/sham sensitization.

Rats were passively sensitized to HRP by injection (ip) of 1.5 ml of high IgE-containing serum (anti-HRP IgE titer = 1:1024) generated in HRP-sensitized and boosted Brown-Norway rats (Harlan Sprague-Dawley Inc, Indianapolis, IN), a high responder strain. In pilot studies, we determined that heat-treatment (56?C for 2 h) of this serum abolished its ability to passively transfer enhanced antigen uptake, suggesting the involvement of heat-labile IgE antibodies. Therefore, we specifically depleted IgE by immuno-precipitation using monoclonal mouse anti-rat IgE (MARE-1, Serotec, Raleigh, NC) coupled to Sepharose 4B beads. Serum and beads were incubated overnight at 4°C, and then centrifuged to remove the IgE-bead complexes. Controls were rats sham-sensitized by injection of serum from naive rats. Experiments were conducted 3 days after passive/sham sensitization.

Ussing chambers

A 15 cm segment of jejunum (beginning 10 cm distal to the ligament of Treitz) was removed from anesthetized rats. The external muscle layer was stripped off and mucosal sheets (usually 4/rat) were mounted in Ussing chambers (surface area 0.6 cm^2) (16). Care

was taken to avoid segments with Peyer's patches. The tissues were bathed in 10 ml of oxygenated Krebs buffer (in mM: 115.0 NaCl, 8.0 KCl, 1.25 $CaCl_2$, 1.2 $MgCl_2$, 2.0 KH_2PO_4, and 25.0 $NaHCO_3$, pH 7.35 ± 0.02, 37 ° C). The buffer in the serosal compartment contained 10 mM glucose osmotically balanced by 10 mM mannitol in the mucosal (luminal) compartment of the chamber. Tissues were short-circuited at zero volts by injection of Isc (in mA/cm^2) using an automated voltage clamp (WPI Instruments, Narco Scientific, Mississauga, ON, Canada). At intervals, the Isc was turned off and the spontaneous potential difference was recorded. Tissue conductance (in mS/cm^2) was calculated according to Ohm's Law. After an equilibration period of 20 min, 5 x 10^{-5} M HRP (or in some cases OVA as a non-specific antigen control) was added to the mucosal buffer (luminal side of the tissue).

Epithelial transport of antigen

The increase in Isc, indicating the ion secretory response, began ~ 3 min after the addition of HRP to the mucosal compartment of chambers containing jejunum from HRP-sensitized rats (18). Therefore, to examine epithelial uptake of HRP before the hypersensitivity reaction, tissues were removed from the chambers at 2 min and fixed for electron microscopy. (We previously showed that mast cells were not activated 2 min after addition of antigen to the mucosal buffer of chambers containing tissues from sensitized rats (18)). Tissues were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4), washed, incubated in 3,3'-diaminobenzadine tetrahydrochlorine (Sigma) and H_2O_2,, and subsequently processed for transmission electron microscopy. Ultrathin sections of mid-villus epithelium (cut in the longitudinal plane) were stained with uranyl acetate and lead citrate. Photomicrographs of epithelial cells were taken at a magnification of 8,000. To quantify epithelial HRP uptake, the total area of HRP-containing endosomes in fixed

size windows (206 mm^2) in the apical region of enterocytes was measured using a computerized image processing system. Measurements were made by one investigator (PCY, who was unaware of the rat treatment) on 12-24 coded photomicrographs per group (obtained from 4-6 rats per treatment group).

To examine transport of HRP protein across the tissues, HRP was added to the mucosal buffer and samples of serosal buffer (1 mL) were collected at 30 min intervals for 90 min. The concentration of HRP was determined by a kinetic enzymatic assay (18). Briefly, 150 ml of sample was added to 800 ml of phosphate buffer containing 0.003% H_2O_2 and 80 mg/ml o-dianisidine (Sigma) and the enzyme activity was determined from the rate of increase in optical density at 460 nm. The mucosal-to-serosal flux was calculated using a standard formula and expressed as pmol/cm^2/h.

Epithelial expression of CD23

In rodents, the high affinity receptor for IgE (FcεRI) is restricted to mast cells and basophils due to the necessity of the beta chain for receptor stability and transport to the cell surface, while in humans, expression requires only the alpha and gamma chains and distribution is more extensive (19). However, expression of FcεRI on epithelial cells has not been reported in either humans or rodents. In contrast, the low affinity receptor (FcεRII/CD23) has been identified on epithelial cells in the intestine (20) and airways (21). Therefore, we determined the effect of sensitization of rats on epithelial expression of CD23. The hybridoma cell line, B3B4, producing a well-described anti-CD23 monoclonal antibody (IgG2a anti-mouse CD23, cross-reactive with rat) (22), was grown in DMEM with 5% FCS. The antibody was isolated from culture supernatant by ammonium sulfate precipitation followed by affinity purification in a protein G-Sepharose column (Pharmacia Biotech, Uppsala, Sweden). In preliminary experiments, we confirmed that this antibody

similarly identified CD23 expression on rat as well as mouse splenic B cells. The B3B4 antibody was used for immunohistochemical detection of CD23 on intestinal epithelial cells. For light microscopy, segments of jejunum were immersed in OCT, snap frozen in liquid nitrogen and stored at -70°C. Cryo-sections were dried at room temperature overnight and fixed with acetone for 15 min. Endogenous peroxidase was extinguished by treating sections with 0.01% H_2O_2 for 10 min, and with 1% bovine serum albumin (BSA) for 30 min. CD23 was identified by the biotin-streptavidin method, (B3B4 primary antibody, secondary biotinylated antibody from DACO Corporation, Mississauga, ON, Canada). Controls included sections where the B3B4 primary antibody was omitted or replaced with an irrelevant isotype-matched antibody (Rockland Immunochemicals, Gilbertsville, PA). No immuno-reactive cells were observed in the control sections.

For immuno-electron microscopy, tissues were fixed in 2% paraformaldehyde/0.75% glutaraldehyde and dehydrated with a series of graded ethanols. The specimens were saturated in 50%, 75% and 100% LR White at 4°C and embedded with LR White, and then polymerized in a freezer under UV radiation. Ultrathin sections were treated with 1% BSA mixed with 5% rabbit serum for 30 min, incubated with primary antibody for 1 h at room temperature, then incubated with gold-conjugated rabbit anti-rat IgG antibody for 1 h. Sections were post-fixed in osmium and stained with uranyl acetate and lead citrate. Sections were observed with the electron microscope and coded photomicrographs prepared. Immunogold labels were counted on the microvillus membrane of epithelial cells and expressed per 100 mm. Controls for electron microscopy included sections where the B3B4 primary antibody was omitted or replaced with the isotype-control antibody. No labels were detected in these control sections.

Effect of blocking CD23 on antigen uptake and transport

B3B4 antibody has been shown to block/displace binding of IgE to its receptor (22). Therefore, we examined the effect of excess antibody on antigen uptake. Thirty min before HRP challenge, B3B4 antibody (10-40 mg/ml) or isotype control antibody was added to the mucosal buffer bathing tissues from rats actively sensitized to HRP. To examine the effect of anti-CD23 on transepithelial antigen transport before the hypersensitivity reaction, tissues were removed 2 min after challenge and processed to identify HRP in tissues. The area of HRP-containing endosomes in enterocytes was measured in 12 windows/rat group. To examine the effect of anti-CD23 on the transport of HRP protein across the tissues, samples of serosal buffer were collected at for three 30 min periods. The concentration of HRP was determined and the mucosal-to-serosal flux was calculated.

Effect of blocking CD23 on the hypersensitivity reaction

To examine the effect of anti-CD23 on the hypersensitivity reaction itself, we determined the following responses to luminal HRP challenge of sensitized tissues following pre-incubation with various concentrations (10-40 mg/ml) of B3B4 or isotype control antibody: 1) the increase in Isc (maximum change within 15 min after HRP challenge) indicating ion secretion, and 2) the increase in conductance (change at 30 min after HRP challenge) indicating enhanced permeability mainly of the paracellular pathway.

Statistics

Differences between groups were tested by ANOVA, with posthoc analysis using Newman Keuls test or Student's t test where appropriate. The data were expressed as mean $\pm$ SEM. A value of $p < 0.05$ was considered to be significant.

RESULTS

Sensitization enhances epithelial uptake of specific antigen.

To examine the effect of sensitization on the uptake of specific versus bystander antigen, tissues from control, HRP-sensitized and OVA-sensitized rats were fixed for electron microscopy 2 min after HRP addition to the mucosal compartment of Ussing chambers. In electron photomicrographs from rats actively sensitized to HRP, many large HRP-containing endosomes were present in enterocytes (example shown in Fig. 2.1a). Such endosomes were observed in all regions of the cell and some HRP was also identified in the lamina propria. However, in photomicrographs from control rats (Fig. 2.1c) and those sensitized to OVA, only a few HRP-containing endosomes were identified in the apical region of enterocytes. Image analysis revealed that the total area of epithelial HRP endosomes in fixed size apical windows was increased several fold in HRP sensitized rats compared with naive rats ($p < 0.01$) or rats sensitized to OVA (Fig. 2.1d, upper panel). This result (similar to our previous finding (18)) indicates the specific nature of the enhanced antigen uptake phenomenon and implicates an immunoglobulin recognition mechanism.

IgE is required for the enhanced epithelial uptake of antigen.

Rats passively sensitized to HRP by injection of high IgE-containing serum also demonstrated large HRP-containing endosomes in enterocytes (example shown in Fig. 2.1b). Image analysis confirmed that the area of endosomes was significantly greater ($p < 0.01$) than those in controls (Fig. 2.1d, lower panel), although the magnitude of the increase was less than in actively sensitized rats. Both heat-treatment of the serum to destroy heat-labile IgE, and immuno-precipitation to remove antibodies of the IgE isotype specifically, completely abolished the enhanced HRP uptake restoring the area of

HRP-containing endosomes to the control value. This result indicates that IgE was mediating the enhanced epithelial uptake of HRP antigen in sensitized rats, most likely by binding to an IgE receptor expressed on the microvillus membrane of enterocytes.

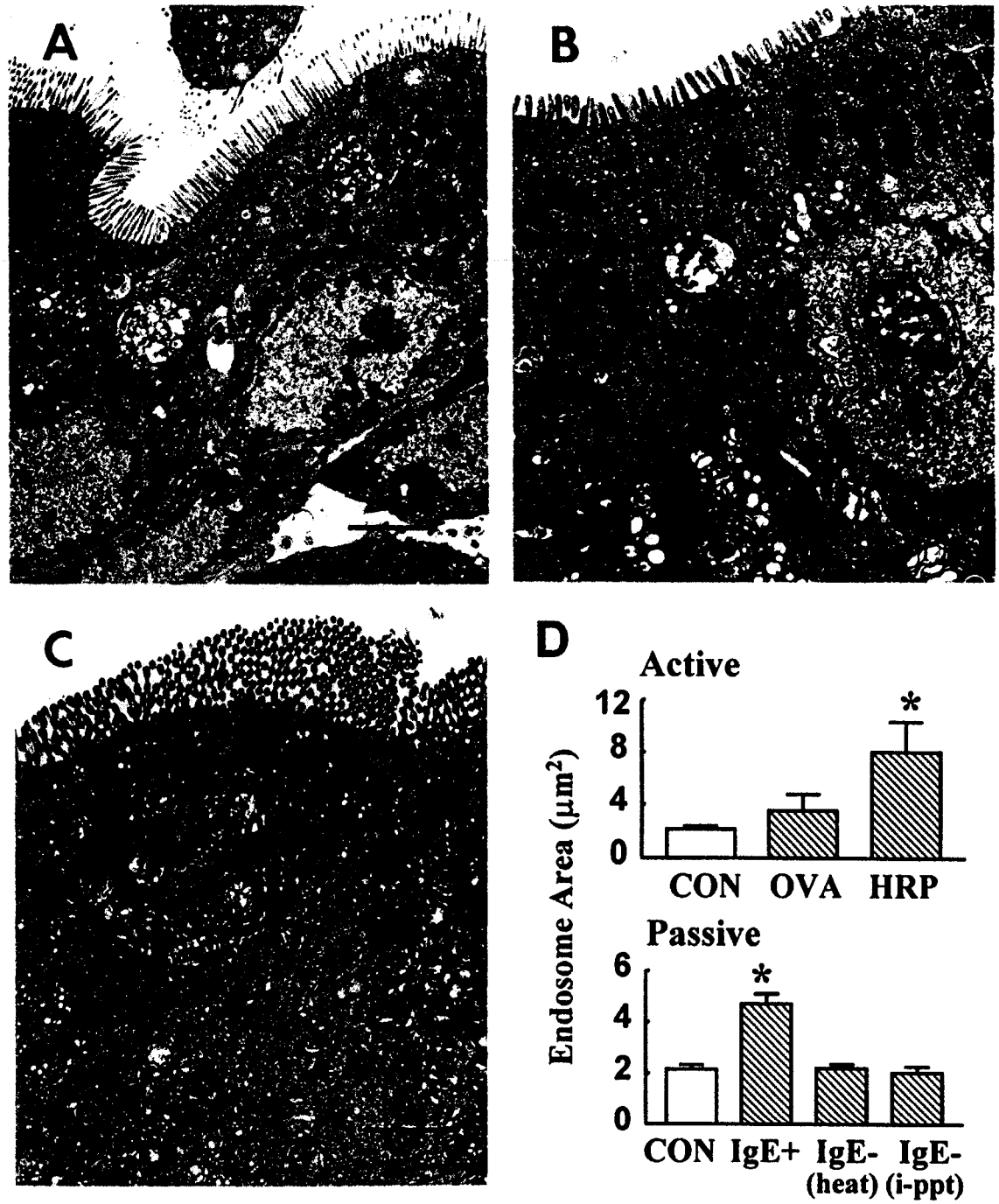
A
B
C
D
Active
12
8
4
0
*
CON
OVA
HRP
Passive
6
4
2
0
*
CON
IgE+
IgE-
(heat)
IgE-
(i-ppt)
Endosome Area (μm²)

Figure 2.1 HRP uptake into enterocyte endosomes. HRP was added to the mucosal buffer of chambers containing jejunal tissues obtained from naive control rats or rats actively sensitized to OVA or HRP. Tissues were fixed for electron microscopy 2 min after HRP challenge and processed to visualize HRP reaction product. Representative photomicrographs are shown for tissues obtained from: **a)** a rat actively sensitized to HRP, **b)** a rat passively sensitized to HRP, or **c)** a naïve control rat. HRP-containing endosomes are indicated by arrowheads (bars indicate 1 μm.). These photomicrographs are representative of those used to obtain the quantitative measurements of endosomal area shown in **d**. *Upper Panel: Active Sensitization.* Rats, actively sensitized to OVA or HRP, were compared with naive controls (CON); jejunal tissues from all rats were challenged with HRP. The total area of HRP endosomes was measured in fixed size windows in the apical region of enterocytes. *Lower Panel: Passive Sensitization.* For passive sensitization, experimental rats were injected ip with serum from actively sensitized rats, either untreated (IgE+), heat-treated (IgE- (heat)), or IgE-depleted (IgE-(i-ppt)). Values represent means ± SEM; * $p < 0.01$ compared with control; n = 12-24 views analyzed for each group (from 4 rats/group).

Sensitization stimulates the expression of CD23 on intestinal epithelial cells.

Immunohistochemical staining to examine expression of FcεRII/CD23 on epithelial cells was performed using anti-CD23 monoclonal antibody. In sections from naive control rats, minimal immunoreactivity was evident on enterocytes, although some cells (appeared to be mononuclear cells) in the lamina propria were positive (Fig. 2.2a). However, in sections from sensitized rats, immunoreactivity was clearly observed on epithelial cells (Fig. 2.2b). The most prominent staining was located on the apical membrane, with lighter staining in the apical portion of the cells.

At the ultrastructural level, gold labelling indicated CD23 immunoreactivity along the microvilli of enterocytes with many labels present in sensitized rats (Fig. 2.2c) and relatively few in control rats. The number of labels per 100 mm of microvillus membrane was significantly greater ($P < 0.01$) in actively sensitized rats compared with controls (Fig 2.3). Specific antigen challenge with HRP, but not OVA, reduced ($p < 0.01$) the presence of CD23 on the surface of enterocyte microvilli; however, the number of labels was still greater ($p < 0.05$) than the control value. Similar results were obtained for passively sensitized rats where compared with controls the number of CD23 labels was increased ($p < 0.01$), and then reduced following HRP challenge (16 ± 1, 132 ± 9 and 52 ± 1 labels per 100 mm microvillus membrane, respectively; $n = 20$ views analyzed in each group (from 4 rats/group) . These results suggest that antigen challenge caused a redistribution of CD23, perhaps by internalization into the epithelial cells.

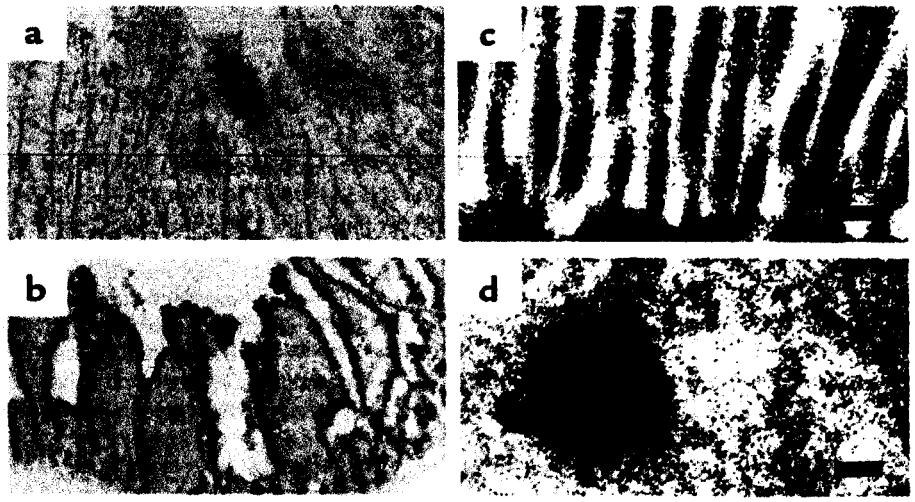
a
c
b
d

Figure 2.2 Light and electron photomicrographs showing expression of CD23 on intestinal epithelial cells. Jejunal tissues for light microscopy were obtained from **a)** a naive control rat or **b)** an actively sensitized rat. Sections were cryofixed and stained with anti-CD23 antibody. Strong immunoreactivity for CD23 is demonstrated on epithelial cells in the section from the sensitized rat but not in the section from the control rat (magnification x 40). **c)** Immuno-electron photomicrograph of enterocyte microvilli from a HRP sensitized rat prior to HRP challenge demonstrating gold-labelled CD23 receptors on the microvillus membrane (bar 200 nm). **d)** After HRP challenge, endosomes containing HRP with several CD23 labels on the endosome membrane are visualized (bar 100 nm). These photomicrographs are representative of those prepared from 4 rats/group.

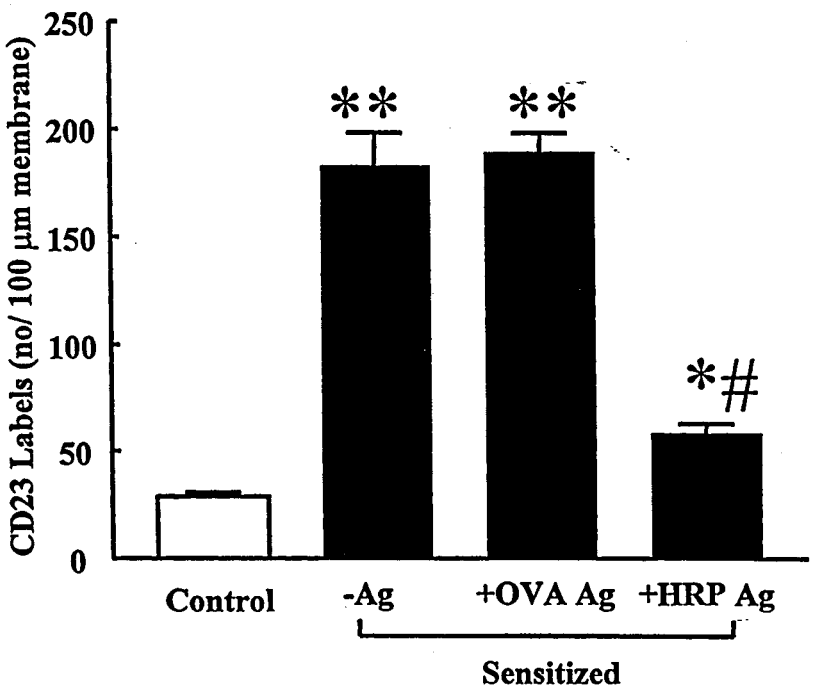
CD23 Labels (no/ 100 µm membrane)
250
200
150
100
50
0
**
**
*#
Control
-Ag
+OVA Ag
+HRP Ag
Sensitized

Figure 2.3 Effect of sensitization and antigen challenge on CD23 expression on the enterocyte microvillus membrane. Rats were actively sensitized to HRP (black bars) and either unchallenged (-Ag), or challenged with OVA (+OVA Ag) or HRP (+HRP Ag); antigens were added to the mucosal buffer 30 min before tissues were removed and processed for immuno-gold labelling using anti-CD23 antibody. The open bar indicates the value for naive control rats. Values represent means $\pm$ SEM; * $p < 0.01$,** $p < 0.01$ compared with control, # $p < 0.01$ compared with -Ag; n = 12 views analyzed for each group (from 4 rats/group).

CD23 and antigen are co-localized in endocytic vesicles following antigen challenge.

To examine if CD23 was internalized into endocytic vesicles following HRP antigen challenge, tissue sections were processed to visualize both HRP and CD23. In HRP-sensitized and challenged rats, numerous vesicles were identified which contained both HRP and CD23 (example shown in Fig. 2.2d). No such vesicles were observed in tissues from control or OVA sensitized rats.

Anti-CD23 antibodies inhibit epithelial antigen uptake and transport.

To confirm the role of the low-affinity IgE receptor, FcεRII/CD23, in the enhanced epithelial uptake and transport of antigen, tissues from HRP sensitized rats were incubated with increasing concentrations of anti-CD23 or control antibody added to the mucosal buffer prior to HRP challenge. Anti-CD23, in a concentration-dependent manner, reduced the total area of HRP containing endosomes within enterocytes (Table 2.1). At the highest concentration of antibody used (40 mg/mL), the inhibition of antigen uptake was 83%, restoring the value for the area of HRP-containing endosomes in sensitized rats to that in unsensitized rats (Fig. 2.1d). In contrast, the isotype control antibody had no significant effect on endosomal area (6.0 ± 0.5 versus 5.7 ± 0.6 mm^2 at 40 mg/mL, n = 12). In addition, the transmucosal flux of HRP protein was inhibited by anti-CD23 as indicated by the significantly reduced flux values (Table 2.1). For example, with 40 mg/mL in period III, the flux value was inhibited 71% from 44.3 ± 1.6 to 12.8 ± 0.9 $pmol/cm^2/h$ (n = 5 rats in each group). This value in sensitized rats was then similar to that in unsensitized rats (11.9 ± 1.6 $pmol/cm^2/h$). This data provides evidence that the mechanism responsible for enhanced sampling and transport of antigen by epithelial cells in sensitized rats involves CD23.

TABLE 2.1 Effect of anti-CD23 on epithelial uptake and transport of HRP antigen

Anti-CD23 (μg/ml)	HRP Endosome Area (μm2)	HRP Flux (pmol/cm^2/h)		
		I	II	III
0	6.0 ± 0.5	9.7 ± 2.1	21.9 ±1.5	44.3 ±1.6
10	4.3 ± 0.2*	7.1 ± 3.1	19.6 ± 1.2	37.6 ± 2.0
20	2.1 ± 0.2**	5.4 ±0.9*	10.7 ± 1.0**	21.0 ± 1.7**
40	1.0 ± 0.1**	4.7 ±1.1**	8.3 ±1.0*	12.8±0.9**

Thirty min prior to luminal challenge with HRP, various concentrations of anti-CD23 were added to the mucosal buffer of Ussing chambers containing jejunal tissues from rats actively sensitized to HRP. To determine HRP uptake into endosomes, tissues were removed 2 min after HRP challenge, immediately fixed for electron microscopy and processed to visualize HRP reaction product. HRP-containing endosomes were measured within 206 μm^2 windows in the apical region of enterocytes. For HRP fluxes, buffers were sampled at 30 min intervals; the periods shown are period I (0-30 min), period II (30-60 min) and period III (60-90 min). Values represent means ± SEM; * $p < 0.05$, ** $p < 0.01$ compared with no anti-CD23; for endosome area n = 12 views analyzed for each group (from 4 rats/group), for HRP flux n = 5 rats/group.

Anti-CD23 antibodies inhibit the intestinal hypersensitivity reaction.

Anti-CD23 antibody added to the luminal surface of epithelial cells before antigen challenge also inhibited functional changes characteristic of the intestinal hypersensitivity reaction (Fig. 2.4). The Isc response to HRP challenge of tissues from sensitized rats was reduced, and the magnitude of the inhibition depended on the concentration of antibody used (Fig. 2.4a). In fact, at the highest concentration (40 mg/mL), there was no secretory response at all to luminal antigen. Fig 2.4b shows that the rise in conductance was similarly inhibited by the anti-CD23 antibody. In contrast, the isotype control antibody had no significant effect on either of the two measures of antigen-induced intestinal pathophysiology. These results suggest that CD23 on epithelial cells plays an important functional role in the intestinal hypersensitivity reaction to luminal antigen.

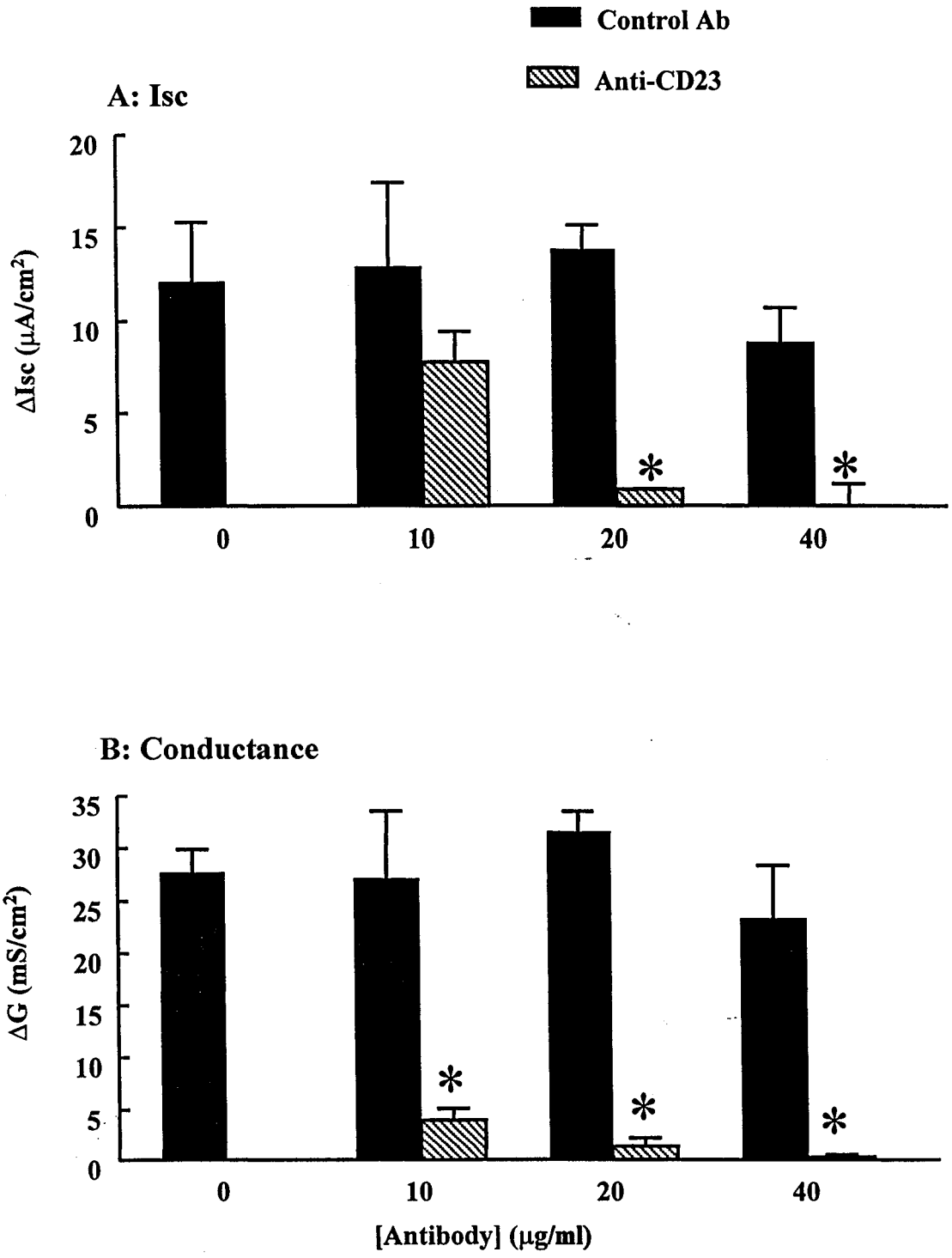
Control Ab
Anti-CD23
A: Isc
ΔIsc (μA/cm²)
20
15
10
5
0
0
10
20
40
*
*
B: Conductance
ΔG (mS/cm²)
35
30
25
20
15
10
5
0
0
10
20
40
*
*
*
[Antibody] (μg/ml)

Figure 2.4 Effect of anti-CD23 on Isc and conductance values following antigen challenge. Thirty min prior to luminal challenge with HRP, various concentrations of anti-CD23 (hatched bars) or isotype control antibody (black bars) were added to the mucosal buffer of Ussing chambers containing jejunal tissues from rats actively sensitized to HRP. **A)** The increase in Isc (Δ Isc) is the difference between the peak value (within 15 min) post challenge and the baseline value. This antigen-induced secretory response was significantly reduced by anti-CD23 in a dose-dependent manner. **B)** The increase in conductance (Δ G) is the difference between the value at 30 min post challenge and the baseline value. This antigen-induced permeability increase was significantly reduced by anti-CD23 in a dose-dependent manner. Values represent means ± SEM; * $p < 0.01$ compared with control values; n = 5 rats/group.

DISCUSSION

Our studies in a rat model of food allergy provide evidence that enhanced specific uptake of antigen by intestinal epithelial cells is mediated by IgE antibodies binding to the low affinity receptor, CD23/FcεRII. Although CD23 expression has been noted previously on epithelial cells, this study is the first to define a role for CD23 in facilitated antigen transport by enterocytes. In addition, we showed that anti-CD23 interferes both with antigen uptake and the subsequent allergic reaction in intestinal tissues. This information may be important in understanding and treating food allergic conditions.

One of the main roles of the intestinal epithelium is to act as a barrier to limit the influx into the body of antigens and other potentially noxious material in the gut lumen. Under normal conditions, delivery of luminal antigens to the gut mucosal immune system results in oral tolerance (8). However, in food allergic humans and animals, antigen transported into the mucosa results in a local hypersensitivity reaction. Studies from our group and others have documented that luminal antigen challenge stimulates secretion of ions and water and increases motility associated with the development of diarrhea (reviewed in ref 5). These changes occur faster than would be expected based on the normal physiology of endosomal protein transport across epithelial cells to reach and activate lamina propria mast cells, shown to be the key effector cells in the intestinal hypersensitivity reaction (13,23). In fact, Ussing chamber studies of jejunal tissues from sensitized rats demonstrated that the Isc response to luminal antigen begins in only ~ 3 min (16,18). In addition, we found that *in vivo* intestinal transport of antigen from lumen to blood was increased in sensitized rats when compared with naive animals (24), although the mechanism was not elucidated.

Recently, we designed a series of studies to investigate if sensitization alters transepithelial antigen transport in a qualitative or quantitative manner. Using rats

sensitized to HRP, we visualized HRP within intestinal cells/tissues by electron microscopy and determined the route and rate of antigen transport (18). Compared with rats sensitized to an irrelevant protein (OVA), rats sensitized to HRP preferentially endocytosed this antigen and rapidly delivered significantly greater amounts of this protein across the epithelium. This finding suggested a mechanism for recognition of antigen at the level of the epithelium. However, because mast cells bear IgE receptors and reside in close proximity to epithelial cells in intestinal tissues, it was possible that mast cells were responsible for the identification of specific antigen. Therefore, we conducted similar studies in mast cell-deficient *Ws/Ws* rats (23). We confirmed that these animals had no mast cells in the intestinal tract, but produced normal levels of IgE antibodies. Jejunal tissues from sensitized *Ws/Ws* rats also demonstrated enhanced epithelial uptake of HRP, but did not display any hypersensitivity reaction based on the complete lack of Isc or conductance response following luminal antigen challenge. In addition, after antigen challenge, tissues from mast cell-replete rats demonstrated HRP in the paracellular spaces between epithelial cells and the overall flux of HRP across tissues was significantly increased; however, such findings were never observed in tissues from *Ws/Ws* rats.

Taken together, our previous studies suggested two phases of enhanced antigen transport: an early phase (*phase I*) before the hypersensitivity reaction, and a later phase (*phase II*) subsequent to activation of mast cells (25). In *phase I*, antigen is transported via the transcellular route in endosomes which rapidly traverse epithelial cells; this phase is induced by sensitization and is specific for the sensitizing antigen, but is mast cell-independent. In contrast, *phase II* begins after mast cell activation and involves recruitment of the paracellular route which amplifies the barrier defect, resulting in non-specific uptake of antigens and other luminal molecules. Here, we designed experiments to investigate the mechanism responsible for the first phase of specific antigen uptake across

the intestinal epithelium. In addition, because the first phase may be the most crucial in delivering antigen into the body, we determined the effect of inhibiting *phase I* antigen transport on the hypersensitivity reaction.

IgE is present in human intestinal secretions of individuals with food allergies (26). Impressively, in nematode parasitized rats, the concentration of IgE was shown to be greater in intestinal fluid than in serum or mesenteric lymph (27). In addition, a luminally-directed transepithelial transport pathway for labeled myeloma IgE (but not IgG_1) was induced in rats by nematode infection or iv infusion of IL-4 (28). These results suggest a receptor-mediated mechanism for transepithelial transport of IgE into the lumen. In this study, we documented enhanced epithelial uptake from the lumen of a model antigen, HRP, in rats sensitized to the specific antigen compared with those sensitized to an irrelevant protein, OVA, or naive rats. This finding also implicates an immunoglobulin recognition system at the level of the epithelium. In addition, passively sensitized rats demonstrated enhanced uptake of HRP antigen into enterocyte endosomes with an absolute requirement for IgE. Based on this background, we postulated that a functional epithelial receptor for IgE may be present on the apical membrane of gut epithelial cells in sensitized rats.

Intestinal hypersensitivity reactions are mediated by antigen cross-linking of IgE antibodies bound to its high affinity receptor (FcεRI) on mast cells. However, this receptor has not been demonstrated on epithelial cells. The low-affinity IgE receptor (FcεRII, also known as CD23) has been identified on the apical membrane of enterocytes in biopsies from humans, with increased expression in individuals with food allergy and inflammatory bowel disease (20). CD23 has also been demonstrated on airway epithelial cells in asthmatics (21). Therefore, we determined the effect of sensitization of rats on epithelial expression of CD23. The B3B4 anti-CD23 antibody we used in the expression studies has

been well-characterized as identifying CD23 on mouse B cells and blocking binding or displacing IgE from its receptor (22). There is a high degree of homology between mouse and rat (and even human) CD23 (29). We confirmed that the B3B4 antibody similarly identifies CD23 on splenic B cells from rats and mice. Immunohistochemistry of light microscopic sections showed minimal CD23 expression on epithelial cells in control animals, although there was some positive staining in the lamina propria. In sensitized rats, CD23 immunoreactivity on enterocytes was dramatically enhanced; the expression appeared to be mainly on and immediately below the apical membrane. A clearer picture of expression of the IgE low affinity receptor emerged in the electron microscope where immunogold labelling showed a small number of CD23 receptors on the microvillus membrane of enterocytes in control rats relative to the number in sensitized rats. In addition, the number of such labels was decreased in sensitized rats following antigen challenge. Receptor internalization was confirmed by the finding of numerous endosomes containing both CD23 and HRP antigen. The increased expression of CD23 induced by sensitization is most likely due to activation of the IL-4 enhancer element on the CD23 gene (30). IL-4 is produced in excess by intestinal mucosal T lymphocytes following sensitization (31), and is also present in serum of atopic individuals (32). Therefore, IL-4 (and/or possibly other unidentified factors) in the serum used to passively sensitize rats may have stimulated expression of epithelial CD23. Studies in progress in IL-4 deficient mice confirm the requirement for IL-4 for both elevated CD23 expression and enhanced epithelial antigen uptake. CD23 has previously been described as mediating endocytosis of antigen in human B cells (33) and therefore, it is not unreasonable that it might play a similar role in enterocytes.

Our results suggest that the initial phase of enhanced transepithelial antigen transport in sensitized rats is mediated by HRP binding to anti-HRP IgE attached to low

affinity receptors on the apical membrane of enterocytes. In confirmation of the role of CD23 in transepithelial antigen transport, anti-CD23 monoclonal antibody inhibited enterocyte uptake and transport of HRP antigen in a concentration-dependent manner. Taken together, these findings and those from our previous studies (18,23) suggest that binding to IgE/CD23 protects antigen from degradation during transepithelial transport resulting in large quantities of immunogenic protein gaining access to the lamina propria in a short period of time. This antigen then is available to activate mast cells and initiate the hypersensitivity reaction. In confirmation of this hypothesis, luminal treatment of tissues with anti-CD23 inhibited the intestinal responses characteristic of gut hypersensitivity. The increase in Isc after luminal HRP challenge of tissues from sensitized rats was reduced indicating inhibition of the secretory response. In addition, the elevated conductance was decreased by anti-CD23, indicating inhibition of non-specific epithelial permeability. Thus inhibition of *phase I* of transepithelial antigen transport interfered with the intestinal anaphylactic reaction, including the secretory response and the increase in conductance/permeability.

Although several aspects of the specific antigen transport system remain to be defined, our results provide convincing evidence that enhanced uptake of antigen by enterocytes in sensitized rats is mediated by IgE and CD23. The specificity of the uptake can be explained by the involvement of IgE antibodies. The increased quantity of antigen delivered across the epithelium into the body can be explained by a receptor-mediated process which protects antigen from degradation. Our study also demonstrates that this system for uptake of specific antigen is important in the allergy since blocking the binding of IgE to its receptor inhibits both transepithelial antigen transport and the hypersensitivity reaction. Preliminary experiments in sensitized mice suggest the existence of a similar

mechanism for antigen uptake. If this process also occurs in humans, it may be a novel target for treatment of food allergic conditions.

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CHAPTER 3: Enhanced transepithelial antigen transport in intestine of allergic mice is mediated by IgE/CD23 and regulated by IL-4

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Abstract

Background & Aims: We previously described a system for enhanced transepithelial transport of antigen where both the amount of specific antigen and its rate of transport were dramatically increased in intestine of sensitized rats compared to controls. This study investigated the essential components mediating antigen uptake in mice genetically deficient for IL-4 or CD23. *Methods:* Mice were actively or passively sensitized to horseradish peroxidase (HRP). Jejunal segments from control or sensitized mice were mounted in Ussing Chambers and challenged with HRP from the luminal side. Tissues were processed for electron microscopy and photomicrographs were analyzed for antigen uptake (location and area of HRP-containing endosomes). Immunohistochemistry and RT-PCR were used to detect epithelial CD23 expression. *Results:* Actively sensitized IL-$4^{+/+}$, but not IL-$4^{-/-}$ mice, displayed increased transepithelial antigen transport and CD23 expression on enterocytes. Passively sensitized IL-$4^{+/+}$ and IL-$4^{-/-}$ mice displayed elevated antigen transport after transfer of immune serum but not if the serum was depleted of IgE or IL-4. IL-4 added to cultured IEC-4 cells upregulated expression of CD23 mRNA. The augmented antigen uptake was inhibited by anti-CD23 and was absent in sensitized $CD23^{-/-}$ mice. *Conclusions:* Our studies indicate that IL-4 regulates IgE/CD23-mediated enhanced transepithelial antigen transport in sensitized mouse intestine.

Introduction

Allergic diseases affect 20-30% of the population in the United States, and their prevalence is increasing in developed countries.[1] Food allergy is one type of allergic disorder that involves 2-5% of adults and an even greater proportion of children.[2] Sensitized individuals develop gastrointestinal symptoms, such as nausea, vomiting, and diarrhea, and in some cases respiratory and cutaneous symptoms, after ingesting specific foods. In severe cases, such as peanut allergy, systemic anaphylaxis can occur with fatal consequences. The onset of hypersensitivity reactions in humans and animal models of food allergy is extremely rapid (within minutes) after the exposure to antigens.[3-5] The rapid nature of the reactions has resulted in the use of the term "immediate" hypersensitivity. However, the mechanism accounting for the rapid response has never been completely explained.

It is generally accepted that intestinal anaphylactic reactions are caused by biological mediators released from mucosal mast cells after antigen cross-linking of IgE bound to the cell surface.[5] However, macromolecular food antigens must first cross the intestinal epithelial barrier before gaining access to mast cells in the subepithelial compartment. This barrier is composed of a single layer of cells, mainly enterocytes, linked by tight junctions which restrict molecules larger than 500 daltons from passing through the paracellular pathways.[6] Enterocytes themselves have the capacity to transport a small proportion of antigenic material from the intestinal lumen to the underlying tissues via transcytosis, a process which takes ~20-30 min.[7] However, most protein antigens are digested either in the lumen or by lysosomal enzymes inside the cell. Therefore, most food proteins lose

their antigenic properties before or during transepithelial transport. In contrast, we recently reported our findings of greatly enhanced transepithelial transport of intact antigen in less than 2 min in an experimental rat model of food allergy.[8-11]

We previously examined the route and timing of intestinal transepithelial antigen transport in sensitized rats using horseradish peroxidase (HRP) as a model antigen.[8] HRP was used since the intact molecule can be accurately measured and its reaction product can be visualized in tissues. We showed that both the quantity of antigen taken up by epithelial cells and its rate of transport into the lamina propria were several fold increased in sensitized rats compared with naive control rats. Electron microscopy indicated that the route of transepithelial antigen transport during the initial phase (phase I) was intracellular via endosomes. Once the antigen was released into the lamina propria and mast cells were activated, a second phase (phase II) of antigen transport occurred involving recruitment of the paracellular pathway. Phase II augmented permeability, allowing for even greater antigen uptake. Phase I was shown to be mast cell-independent, occurring before the antigen-induced ion secretion caused by release of mast cell mediators, but specific for the antigen to which the rats were sensitized.[8,9] Phase II required mast cells since it did not occur in mast cell-deficient rats.[9,10]

Our finding that enhanced antigen transport across enterocytes in sensitized rats was antigen specific suggested an immunoglobulin recognition mechanism at the level of the epithelium. We identified that sensitization induced the expression of the low affinity IgE receptor, CD23/FcεRII, on epithelial cells in the rat jejunum.[11] In addition, following challenge with HRP antigen to the luminal surface of intestinal tissues, the receptor was

internalized and was localized on the membrane of enterocyte endosomes containing HRP. Importantly, anti-CD23 antibody inhibited phase I antigen uptake leading to the reduction of the hypersensitivity reaction and phase II antigen transport in rat intestine.[11]

The aim of the present study was to identify whether the phase I enhanced transepithelial antigen transport is present in another species and to investigate the essential components involved in the mechanism of this augmented antigen uptake system utilizing genetic deficient mice. IL-4 is a major isotype switching factor for IgE synthesis in B lymphocytes[12], and increased IL-4 production in allergic patients has been reported.[13,14] IL-4 also plays a role in upregulating the expression of IgE receptors, including CD23, on B cells.[15-18] Therefore, we designed experiments to examine the role of IL-4 in epithelial CD23 expression and the consequence of genetic deletions for IL-4 and CD23 on phase I transepithelial antigen transport. Our studies indicate that 1) the phenomenon of enhanced intestinal transepithelial antigen transport is reproducible in sensitized mice, 2) IgE/CD23 mediates the augmented antigen transport in enterocytes, and 3) IL-4 plays an major regulatory role in the mechanism through both stimulation of IgE synthesis and upregulation of CD23 expression on intestinal epithelial cells.

Methods

Animals

Adult male IL-$4^{+/+}$ and IL-$4^{-/-}$ mice, and $CD23^{+/+}$ and $CD23^{-/-}$ mice were used in this study. IL-$4^{-/-}$ mice (BALB/c background) were from a colony maintained in the Central Animal Facility at McMaster University (initially obtained from H. Bluethmann, Pharmaceutical Research Gene Technology, F. Hoffmann-La Roche, Switzerland). IL-$4^{+/+}$ (BALB/c) mice were purchased from Harlan Breeding Laboratories, Indianapolis, IN. IL-$4^{+/+}$ and IL-$4^{-/-}$ mice were actively sensitized by ip. injection with 100 μg of horseradish peroxidase (HRP) (type II; Sigma Chemical Company, St. Louis, MO) or ovalbumin (OVA) (Grade V; Sigma) in 0.2 ml of alum, and 0.1 ml of pertussis vaccine (Connaught Laboratories, Willowdale, Ontario, Canada) as adjuvants.[4] Experiments were conducted on day 14.

$CD23^{-/-}$ mice (C57BL/6 background) were initially obtained from M. Lamers, Max-Planck-Institut fur Immunbiologie, Freiburg, Germany.[19] $CD23^{+/+}$ (C57BL/6) mice were purchased from Harlan breeding Laboratories, Indianapolis, IN. C57BL/6 mice required boosting to induce active sensitization. $CD23^{+/+}$ and $CD23^{-/-}$ mice were injected with HRP as above except that 50 ng of pertussis toxin[20] was substituted for vaccine and mice were boosted with HRP on days 7 and 14. Experiments were conducted on day 21.

Mice (IL-$4^{+/+}$ and IL-$4^{-/-}$) were passively sensitized by ip. injection of 0.5 ml of high-IgE containing serum (titer 1:1024) obtained from actively sensitized Brown Norway rats, a high responder strain. The serum was either untreated or depleted of IgE by passing the serum through a column containing sepharose 4B beads (Pharmingen, Missisauga, ON,

Canada) conjugated to anti-IgE (MARE-1, anti-rat IgE heavy chain; Serotec, Oxford, England). In some experiments, the serum was incubated with a neutralizing anti-IL-4 antibody (polyclonal rabbit anti-rat; Biosource, Camarillo, CA) for 1 h at room temperature. Experiments were conducted 3 days after passive sensitization. In all studies, non-sensitized naive mice served as controls. The experiments described were conducted with approval from the McMaster University Animal Care Committee.

Ussing Chambers

Mice were killed by cervical dislocation and a laparotomy was performed. Four pieces of mid-jejunum were mounted in Ussing Chambers (WPI, Narco Scientific, Missisauga, ON, Canada). Care was taken to avoid Peyer's patches. An area of 0.6 cm^2 of intestine was exposed to 8 ml of circulating oxygenated Krebs buffer (pH 7.33-7.37) at 37°C. The serosal buffer contained 10 mM of glucose osmotically balanced by 10 mM of mannitol in the luminal buffer. The tissue was clamped at 0 V using a W-P Instruments automatic voltage clamp (Narco Scientific). The short-circuit current (Isc, $\mu A/cm^2$) was recorded on line. At 5 min intervals, the tissue was pulsed with 1 mV for a duration of 1 s. The change of Isc caused by the pulse was used to determine the tissue conductance (mS/cm^2) according to Ohm's law. Preliminary experiments indicated that baseline Isc and conductance values of tissues from each group of mice were similar. Therefore, consistent baseline electrophysiological values and the Isc responses to electrical transmural

stimulation (10 Hz, 10 mA, 0.5 ms for a total time of 5 sec) were used to confirm tissue viability. HRP antigen was added to the luminal buffer at 5×10^{-5} M.

Epithelial Uptake and Transport of Antigen

Tissues were removed from the Ussing chamber 2 min after HRP antigen addition to the luminal buffer to examine HRP transport across the epithelium.[8-11] Jejunal segments were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4), washed, incubated in 3,3'-diaminobensidine tetrahydrochlorine (Sigma) and H_2O_2 (pH 7.6), and subsequently processed for transmission electron microscopy. Ultrathin sections of mid-villus epithelium (cut in the longitudinal plane) were stained and photomicrographs were taken at a magnification of 3,000. The total area of HRP-containing endosomes in enterocytes (120 μm^2 apical window) was quantified in photomicrographs using computerized image analysis.[8-11] In addition, the distribution of HRP-containing endosomes within enterocytes was determined and expressed as the percentage of cells containing endosomes in the apical, mid, or basal regions of enterocytes and also in the lamina propria. This analysis was performed for 100 photomicrographs containig well-oriented enterocytes (tissues from 4 mice per group) by one investigator (P.C.Y) who was unaware of the origin of the tissues. In some experiments, the effect of adding the well-characterized anti-CD23 antibody (B3B4, rat IgG2a anti-mouse CD23) [21-23] to the mucosal buffer 30 min prior to antigen challenge was examined. B3B4 was isolated from the supernatant of cultured hybridoma cells [21] by ammonium sulphate precipitation followed by affinity purification in a protein G column (Mab Trap G II, Pharmingen). The

control for the anti-CD23 was an isotype-matched irrelevant antibody purchased from Rockland Immunochemicals, Gilbertsville, PA.

Epithelial CD23 Protein Expression

A 1.5 cm segment of jejunum was snap frozen in OCT, cut into 5 μm sections and processed for immunohistochemistry.[11] Endogenous peroxidase was quenched by treating sections with 0.01% H_2O_2. CD23 expression was identified by the biotin-streptavidin method using B3B4 as the primary antibody at a dilution of 1:1000. The secondary antibody, biotinylated mouse anti-rat IgG (1:300) and streptavidin-HRP (1:600) were purchased from DAKO, Carpinteria, CA. Spleen tissues served as positive controls. Negative controls included sections where the primary antibody was omitted or replaced with the isotype control antibody.

Epithelial CD23 mRNA Expression

Mouse small intestinal epithelial cells of the IEC-4 cell line [24] were cultured in DMEM (Gibco BRL, Gaitherburg, MD) supplemented with 5% FCS, 0.01M HEPES, 20 mM L-glutamine, 0.1 U/ml penicillin G, and 5 μg/ml streptomycin. Confluent cells were treated with 10 ng/ml of recombinant mouse IL-4 (R&D Systems, Minneapolis, MN) for various periods of time. The RNA was extracted from cells using an RNeasy® Mini kit (Qiagen, Missisauga, ON, Canada) and 2 μg of RNA was reverse-transcribed by Oligo $d(T)_{16}$ using Perkin-Elmer RNA PCR core kit (Perkin-Elmer, Missisauga, ON, Canada).

The resulting cDNA (20 μl) was then subjected to PCR by addition of 80 μl of a master mix containing 1.5 mM (for CD23 primers) or 2 mM (for G3PDH primers) of $MgCl_2$ solution, 1x PCR buffer (50mM KCl, 10 mM Tris-HCl, pH 8.3), 2.5 U AmpliTaq DNA polymerase, 0.5 μM upstream primer and 0.5 μM of downstream primer. The upstream primer of CD23: 5'-AGAAAGCGTTGCTGCTGTG-3' (exon 3) and downstream primer of CD23: 5'-GCCAATCCAGGAATCCTTC-3' (exon 11) were used to amplify a 695 bp DNA product. The DNA thermal cycler (Teche PHC-3; Mandel Scientific Company Ltd. Guelph, ON, Canada) was programmed to perform a protocol as follows: 94°C for 3 min for 1 cycle; 94°C for 1.5 min, 60°C for 2 min, and 72°C for 3 min for 30 cycles; and 72°C for 7 min for final extension. To control for sample-to-sample variation, primers for glyceraldehyde-3-phosphate dehydrogenase (G3PDH) (upstream primer: 5'-CCATGGAGAAGGCTGGGG-3', downstream primer: 5'-CAAAGTTGTCATGGATGACC-3') were used to amplify a 193 bp product. The PCR protocol for G3PDH was as follows: 94°C for 3 min for 1 cycle; 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min for 25 cycles; and 72°C for 7 min for 1 cycle. Negative controls were performed with samples lacking RNA or samples with RNA that were not reverse transcribed. The positive control was RNA from spleen cells of actively sensitized mice (presence of CD23 previously demonstrated by FACS). PCR products were then electrophoresed in 1.5 % agarose gel in the presence of 0.5 μg/ml ethidium bromide. The amplified DNA bands were visualized with a UV transilluminator and photographs were

taken. The intensity of the DNA bands was analyzed using a densitometer with software from KODAK Digital Science 1D, GIBCO, Rockville, MD.

IgE Assay

The level of HRP-specific IgE in mouse serum was determined by passive cutaneous anaphylaxis (PCA). Sprague-Dawley rats were injected intradermally with 0.1 ml of serum in duplicate dilutions from 1:1 to 1:1024. After 72 h, rats were challenged by iv. injection with 0.5 ml of HRP (5 mg/ml) in 1% Evan blue. Positive reactions were evaluated as blue spots after 30 min. The PCA titer was recorded as the highest serum dilution showing a positive result. Heat treatment (56°C for 3 h) abolished the reaction indicating that the immunoglobulin was heat-labile IgE.

IL-4 Assay

Mouse spleens were meshed through a metal screen to isolate single cells. Erythrocytes in the cell mixture were lyzed with ammonium chloride potassium buffer and the remaining cells were washed and incubated in 24 well plates with Concanavalin A (10 μg/ml; Sigma) for 48 h. The supernatant was collected and tested for IL-4 by ELISA (Biosource). The limit of detection was 5 pg/ml.

Statistics

Data were analyzed by analysis of variance (ANOVA). When appropriate, statistical significance was tested by two-way Student's t tests for comparison between groups. Values are presented as mean ± SEM. A value of $p < 0.05$ was considered significant.

Results

Sensitization enhances epithelial uptake of specific antigen

Preliminary experiments indicated that luminal antigen challenge of tissues from sensitized mice caused a rise in Isc, indicating the secretory response, which began after 2 min. Therefore, to examine antigen uptake in enterocytes before the hypersensitivity reaction, tissues from HRP-sensitized IL-$4^{+/+}$ mice were removed at 2 min after HRP challenge and immediately fixed for electron microscopy. The number and size of HRP-containing endosomes in enterocytes were greater in tissues from HRP sensitized mice (Fig. 3.1C) compared with tissues from naive control mice (Fig. 3.1A) and mice sensitized to OVA (Fig. 3.1B). When quantified by image analysis, the area of endosomes in enterocytes of HRP-sensitized mice was 8-fold greater ($p<0.05$) than values in naive controls and 6-fold greater than values in OVA-sensitized mice (Fig. 3.1D). These findings suggest recognition of antigen at the level of the epithelium, potentially by cell bound immunoglobulin.

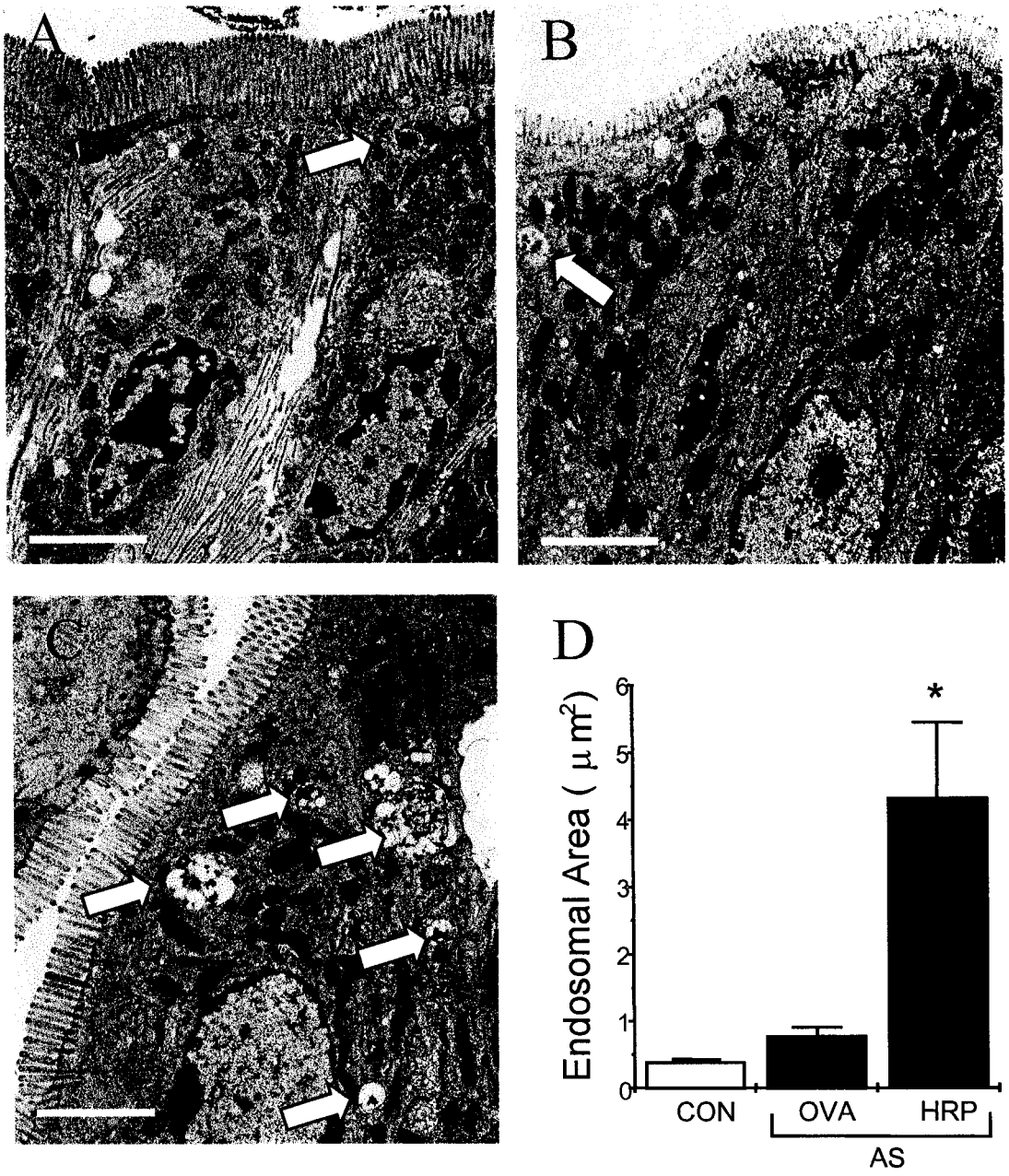
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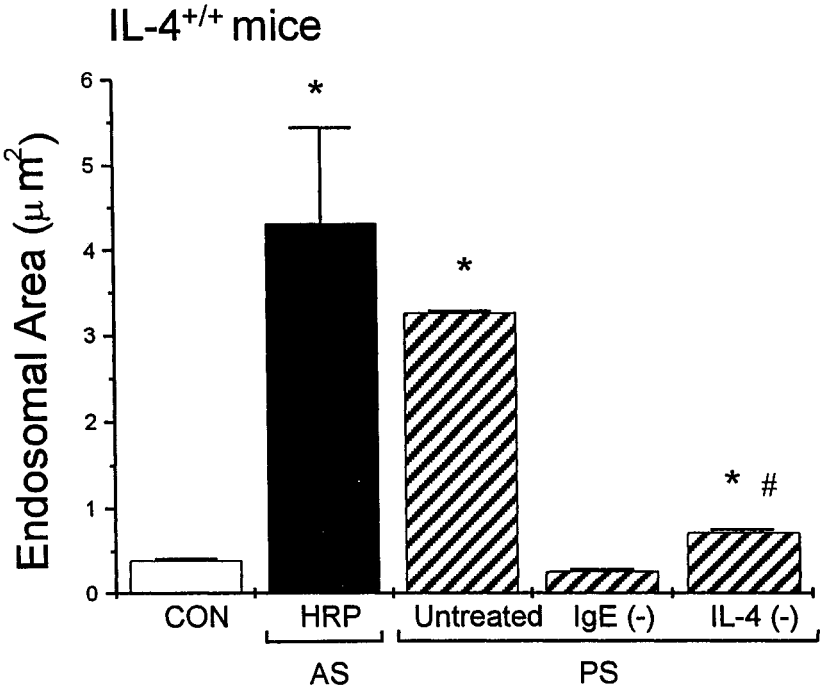
Figure 3.1 HRP uptake into enterocyte endosomes in control and sensitized mice. HRP (5×10^{-5} M) was added to the mucosal (luminal) buffer of Ussing chambers containing jejunal tissues obtained from different groups of mice. Tissues were fixed for electron microscopy 2 min after HRP challenge and processed to visualize HRP uptake into endosomes. Representative photomicrographs are shown for tissues obtained from (A) naive control IL-$4^{+/+}$ mice, (B) OVA sensitized IL-$4^{+/+}$ mice, (C) HRP sensitized IL-$4^{+/+}$ mice. HRP endosomes are indicated by arrowheads (bars indicate 2 μm). Magnification was 3000x. These photomicrographs are representative of those used for quantitative measurements of endosomal area in (D). The total area of HRP endosomes was measured in electron photomicrographs by computerized image analysis within a fixed size window of 120 μm^2 in the apical region (above the nucleus) of mid-villus enterocytes from naive control mice (CON), mice actively sensitized (AS) to HRP or OVA. Values represent the means ± SEM; * $p < 0.05$ compared with controls; n = 12 photomicrographs from 4 mice per group.

IgE and IL-4 are required for enhanced epithelial uptake of antigen

To investigate if circulating factors mediate the enhanced antigen uptake in sensitized mice, HRP endosomes were evaluated in enterocytes of tissues from passively sensitized IL-$4^{+/+}$ mice after luminal HRP challenge. Values for tissues from mice sensitized with whole serum were compared with those in which the serum was specifically depleted of IgE or IL-4. At 2 min post-challenge, enterocytes from mice passively sensitized with whole serum demonstrated significantly increased ($p<0.05$) antigen uptake (greater total area of HRP endosomes) compared with values for naïve control mice (Fig. 3.2A). In fact, these values were comparable to those in actively sensitized mice. However, depletion of IgE from the serum abolished the enhanced uptake. This finding indicates that IgE is necessary for the uptake of the specific antigen into epithelial cells. Moreover, significant reduction of antigen uptake was demonstrated in mice sensitized with IL-4-neutralized serum, in spite of the presence of IgE, compared to values in mice injected with whole serum. This finding indicates that IL-4 is also involved in the enhanced transepithelial antigen uptake, apparently by a mechanism independent of its role in promoting IgE synthesis.

A


IL-4+/+ mice
Endosomal Area (μm2)
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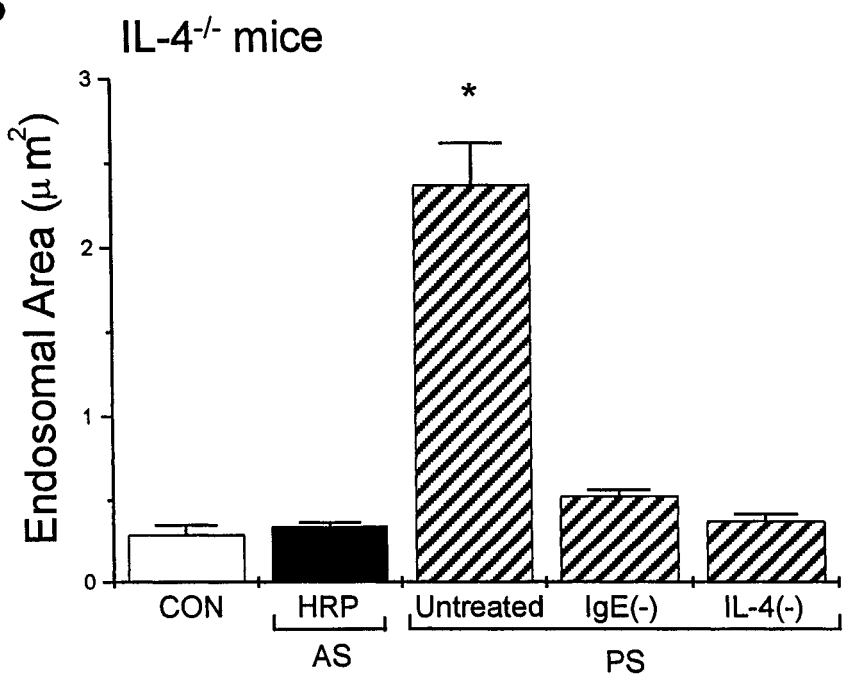
IL-4-/- mice
Endosomal Area (μm2)
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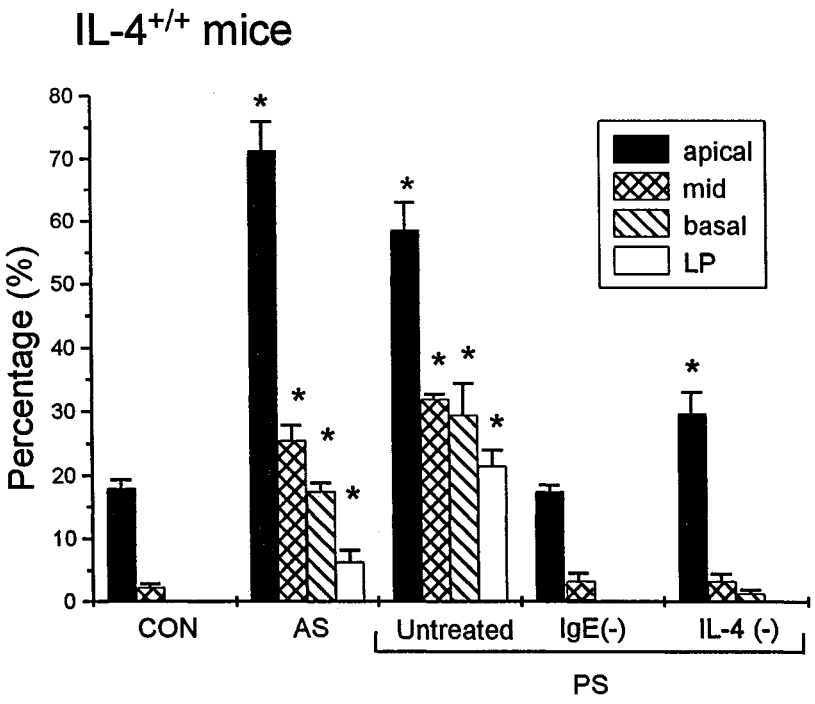
Figure 3.2 HRP uptake in enterocytes of actively and passively sensitized mice. Intestinal tissues from each group of mice were challenged with HRP (5×10^{-5} M) from the luminal side and then fixed at 2 min post-challenge and processed for electron microscopy. The total area of HRP endosomes in enterocytes was measured in a fixed sized apical window in electron photomicrographs and quantified in tissues obtained from (A) IL-$4^{+/+}$ mice, either naive controls (CON), actively sensitized (AS), or passively sensitized (PS) using untreated, IgE-depleted (IgE (-)) or IL-4-neutralized serum (IL-4 (-)), and (B) IL-$4^{-/-}$ mice, groups as above. Values represent the means ± SEM; * $p < 0.05$ compared with controls; # $p < 0.05$ compared with passively sensitized with untreated serum; n = 12 photomicrographs from 4 mice per group.

Sensitization also increases the rate of transepithelial antigen transport

In naïve control mice, ~20 % of enterocytes showed HRP-endosomes in the apical region of cells and ~2 % in the mid region, whereas no HRP was visualized in the distal regions or lamina propria at 2 min after HRP luminal challenge (Fig. 3.3A). The percentage of enterocytes containing HRP-endosomes in all cell regions was significantly greater in actively sensitized mice, with HRP uptake in the apical region in > 70% of enterocytes, ~ 25 % in the mid region, and ~ 20 % in the basal region and ~5 % in the lamina propria. Similarly, values were also significantly higher in passively sensitized mice compared with controls (Fig. 3.3A). These results indicate that in both actively and passively sensitized mice, antigen was transported more rapidly across intestinal epithelial cells than in controls. Similar to results for antigen uptake, mice that were injected with IgE-depleted serum showed a slower rate of antigen transport, with the majority of HRP-endosomes confined to the apical region. In addition, mice injected with IL-4-neutralized serum demonstrated a reduced rate of transport, almost to the control values. These data provide evidence that IgE and IL-4 were involved in increasing the speed of transport of antigen across intestinal epithelial cells in sensitized mice.

A


IL-4+/+ mice
Percentage (%)
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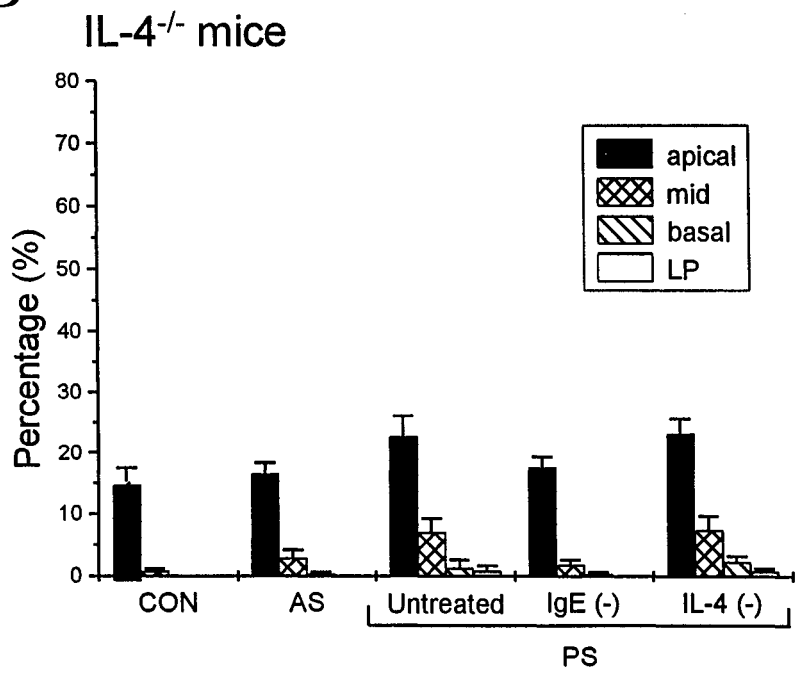
IL-4-/- mice
Percentage (%)
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Untreated
IgE (-)
IL-4 (-)
PS

Figure 3.3 Rate of antigen transport across intestinal epithelial cells. Intestinal tissues from each group of mice were challenged with HRP from the luminal side and then fixed at 2 min post challenge and processed for electron microscopy. The percentage of enterocytes containing HRP-endosomes in the apical, mid, basal regions of cells and in the lamina propria was quantified in tissues obtained from (A) IL-$4^{+/+}$ mice, either naive controls (CON), actively sensitized (AS), or passively sensitized (PS) using untreated, IgE-depleted (IgE (-)) or IL-4-neutralized serum (IL-4 (-)), and (B) IL-$4^{-/-}$ mice, groups as above. Values represent means ± SEM; n = 4 tissues examined from 4 mice per group; * $p < 0.05$ compared with controls.

Transepithelial transport of antigen is not enhanced in sensitized IL-4$^{-/-}$ mice

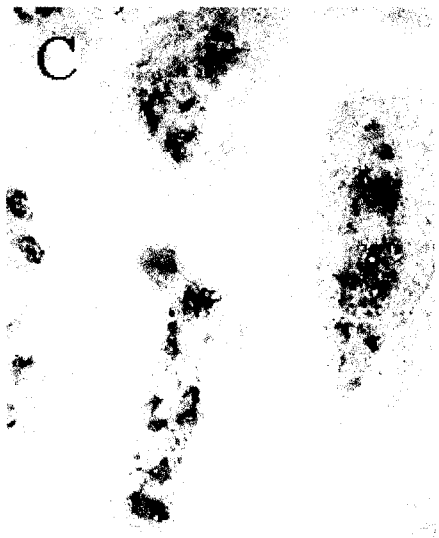
To further investigate the role of IL-4 in enhanced transepithelial antigen transport, HRP uptake into enterocyte endosomes was examined in IL-4$^{-/-}$ mice actively sensitized to HRP. The absence of IL-4 production in IL-4$^{-/-}$ mice was confirmed by ELISA in culture media from spleen cells (undetectable for both naive and sensitized IL-4$^{-/-}$ mice vs. 39.3 ± 1.5 pg/ml for sensitized IL-4$^{+/+}$ mice). In contrast to the 8-fold increase in the area of HRP-containing endosomes in enterocytes of HRP actively sensitized IL-4$^{+/+}$ mice (Fig. 3.2A), no enhanced HRP uptake was found in enterocytes of actively sensitized IL-4$^{-/-}$ mice compared with naive control IL-4$^{-/-}$ mice (Fig. 3.2B). The area of endosomes in both sensitized and non-sensitized IL-4$^{-/-}$ mice was similar. In addition, actively sensitized IL-4$^{-/-}$ mice did not demonstrate an enhanced rate of transport compared with control values (Fig. 3.3B). The absence of increased transepithelial antigen transport may have been due to the lack of IgE synthesis in these mice. In fact, HRP-specific IgE was not detectable by PCA in serum from actively sensitized IL-4$^{-/-}$ mice. Therefore, additional experiments were conducted in passively sensitized IL-4$^{-/-}$ mice.

The area of HRP-endosomes in enterocytes of passively sensitized IL-4$^{-/-}$ mice was significantly enhanced compared with the value in control IL-4$^{-/-}$ mice (Fig 3.2B). Depletion of IgE from the injected serum abolished the enhanced antigen uptake in enterocytes. In addition, the area of HRP-endosomes in enterocytes of IL-4$^{-/-}$ mice injected with IL-4-neutralized serum was not different from the value in control mice (Fig. 3.2B).

Since IL-4 is known to upregulate the expression of the low affinity IgE receptor, CD23, on immune cells,[15-17] we examined the role of CD23 in enhanced antigen uptake.

Expression of the IgE low affinity receptor, CD23/FcεRII, on intestinal epithelial cells is stimulated by sensitization

The IgE-mediated uptake of specific antigen in enterocytes of sensitized mice suggests the involvement of an immunoglobulin receptor on intestinal epithelial cells. CD23/FcεRII, the low affinity IgE receptor has been shown on intestinal and airway epithelial cells of humans,[25-27] and on intestinal epithelial cells in sensitized rats.[11] In this study, immunohistochemistry using anti-CD23 antibody [21-23] showed a low level of expression of CD23 on the apical membrane of enterocytes in non-sensitized control mice (Fig. 3.4A). The expression of CD23 was upregulated by sensitization, shown by increased staining on the apical membrane as well as in intracytoplasmic regions of enterocytes in actively sensitized IL-$4^{+/+}$ mice (Fig. 3.4B). No staining of CD23 was seen on the apical membrane of epithelial cells in both control and actively sensitized IL-$4^{-/-}$ mice (Fig. 3.4C and 3.4D) (semi-quantitative analysis shown in Table 3.1). No staining was seen on the surface of intestinal epithelial cells in the negative controls including sections where the primary antibody was omitted or replaced with the isotype-matched irrelevant antibody.


C


D

Figure 3.4 Expression of CD23 protein on intestinal epithelial cells. Jejunal tissues were cryofixed, sectioned, and stained with anti-CD23 using a biotin-streptavidin method. Photomicrographs shown are tissues obtained from (A) a control IL-$4^{+/+}$ mouse, (B) a HRP-sensitized IL-$4^{+/+}$ mouse, (C) a control IL-$4^{-/-}$ mice, and (D) a HRP-sensitized IL-$4^{-/-}$ mice. These photographs are representative of those prepared from 4 mice per group. Magnification was 200x. CD23 staining is evident on the apical membrane of enterocytes in sensitized IL-$4^{+/+}$ mice, but not in naive controls. No staining of CD23 was seen on enterocytes of either control or sensitized IL-$4^{-/-}$ mice. Spleen tissues from actively sensitized mice were used as a positive control; whereas negative controls included sections where the primary antibody was omitted or replaced with an isotype-matched irrelevant antibody (not shown).

Table 3.1 Expression of CD23 protein on apical membrane of intestinal epithelial cells

Mice	IL-$4^{+/+}$	IL-$4^{-/-}$
Naïve control	+	-
Active sensitization	+++	-/+

Jejunal tissues from naïve or actively sensitized IL-$4^{+/+}$ and IL-$4^{-/-}$ mice were snap frozen, sectioned, and stained with anti-CD23 (B3B4) as primary antibody for immunohistochemistry. n = 4 mice per group. Spleen tissues were used as positive control. Negative controls included sections where the primary antibody was omitted or replaced with an irrelevant isotype control antibody. Semi-quantitative analysis of relative staining for CD23 on epithelial cells was performed by 2 separate investigators on coded sections.

IL-4 stimulates expression of CD23 mRNA in cultured mouse intestinal epithelial cells

RT-PCR was performed to investigate if IL-4 induces or upregulates CD23 transcript expression in mouse intestinal epithelial cells. Treatment of a mouse small intestinal epithelial cell line, IEC-4 cells (24), with 10 ng/ml of IL-4 induced increased expression of CD23 mRNA, with peak expression at 8-24 h (Fig. 3.5).

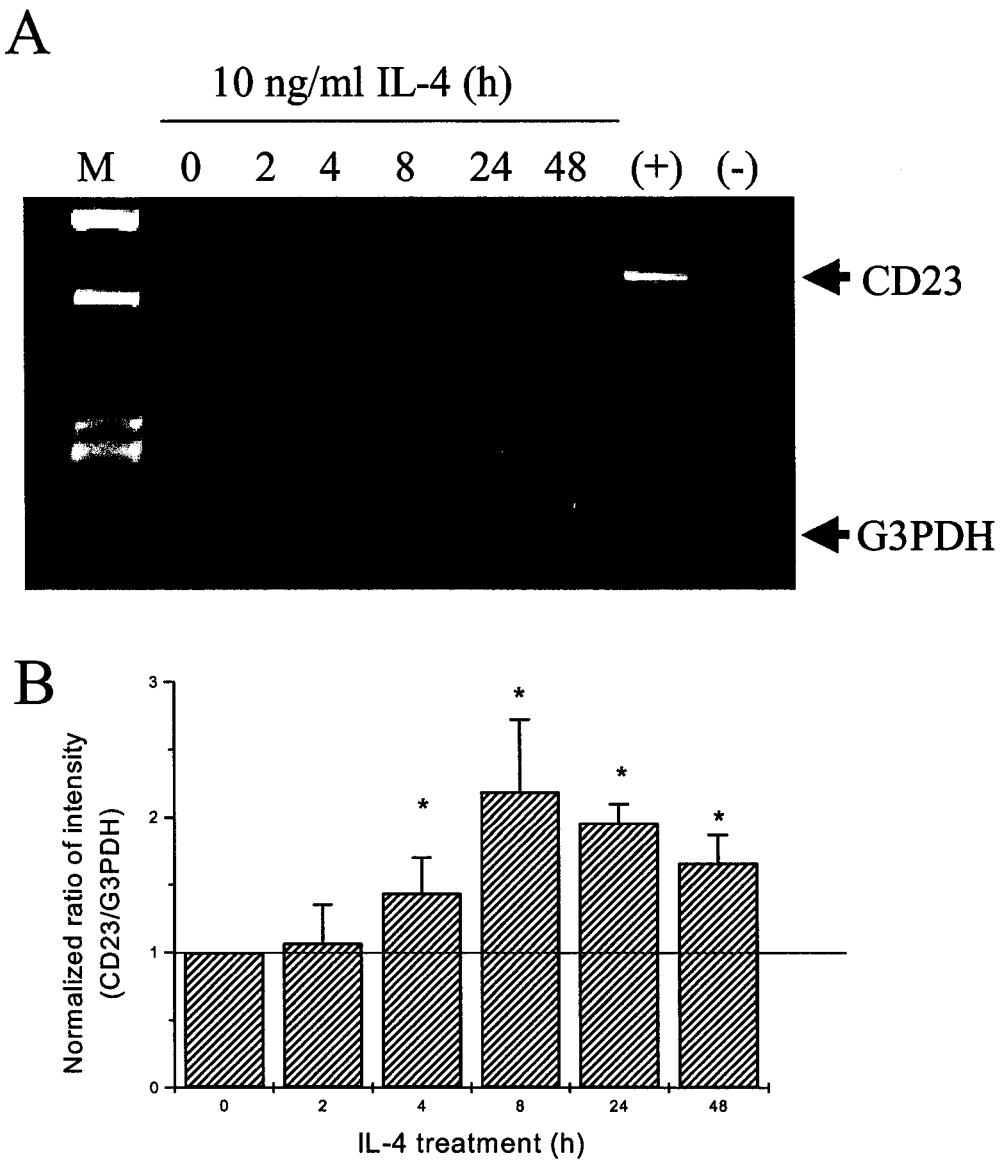


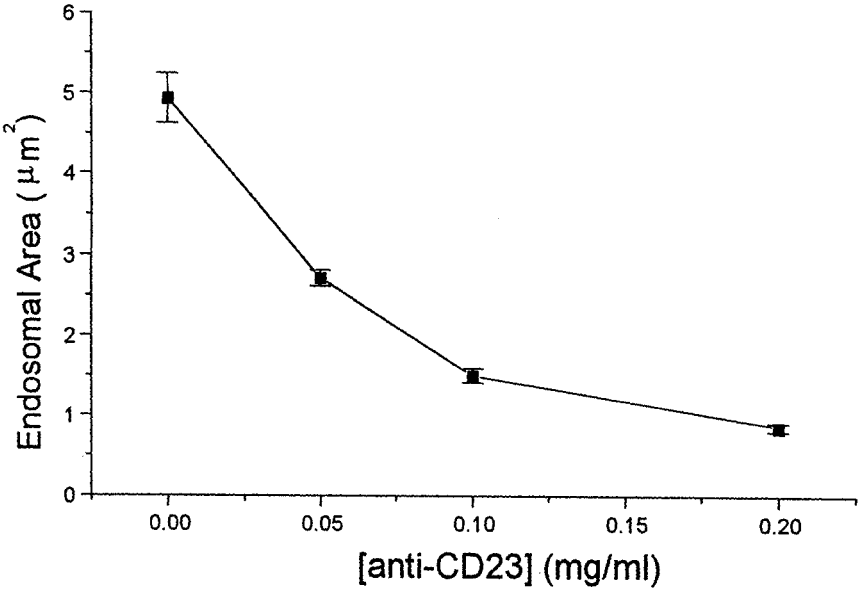
Figure 3.5 Effect of IL-4 on expression of CD23 mRNA by cultured mouse intestinal epithelial cells.

Figure 3.5 Effect of IL-4 on expression of CD23 mRNA by cultured mouse intestinal epithelial cells. (A) IEC-4 cells were treated with 10 ng/ml of recombinant mouse IL-4 for 0, 2, 4, 8, 24, and 48 h. The mRNA was subjected to RT-PCR using primers for CD23 and G3PDH. PCR products were electrophoresed in a 1.5 % agarose gel. Molecular weight markers (Ready load ϕ X174 RF DNA/*Hae* III Fragments; GIBCO, BRL) were added in the first lane. The intensity of bands was then analyzed using densitometry. (B) Normalized ratio of intensity of CD23/G3PDH at different time points after IL-4 treatment. Values represent means $\pm$ SEM; n = 3 individual experiments; * $p < 0.05$ compared with controls (0 h).

Enhanced transepithelial transport in sensitized mice is inhibited by anti-CD23 antibody.

To investigate the role of epithelial CD23 in the enhanced transepithelial transport of antigen in intestine of sensitized mice, we utilized the anti-CD23 antibody added to the luminal side of the intestine in Ussing chambers 30 min prior to antigen challenge. There was a dose-dependent inhibition of antigen uptake in enterocytes by the antibody. The enhanced antigen uptake in enterocytes of sensitized mice was significantly reduced at 0.05 mg/ml of antibody (2.7 ± 0.1 μm^2) compared with values of untreated tissues (4.9 ± 0.3 μm^2) (Fig. 3.6A). The rate of transepithelial transport of antigen was also diminished significantly at 0.05 mg/ml of antibody with ~50% of enterocytes containing endosomes in apical area, ~10% in the mid region, ~3% in the basal region, and ~1% in lamina propria compared to control values (~75%, ~20%, ~12%, and 6%) (Fig. 3.6B). Isotype control antibody up to 0.2 mg/ml did not reduce the enhanced transepithelial antigen transport (data not shown).

A


Endosomal Area (μm 2)
0
1
2
3
4
5
6
0.00
0.05
0.10
0.15
0.20
[anti-CD23] (mg/ml)

B

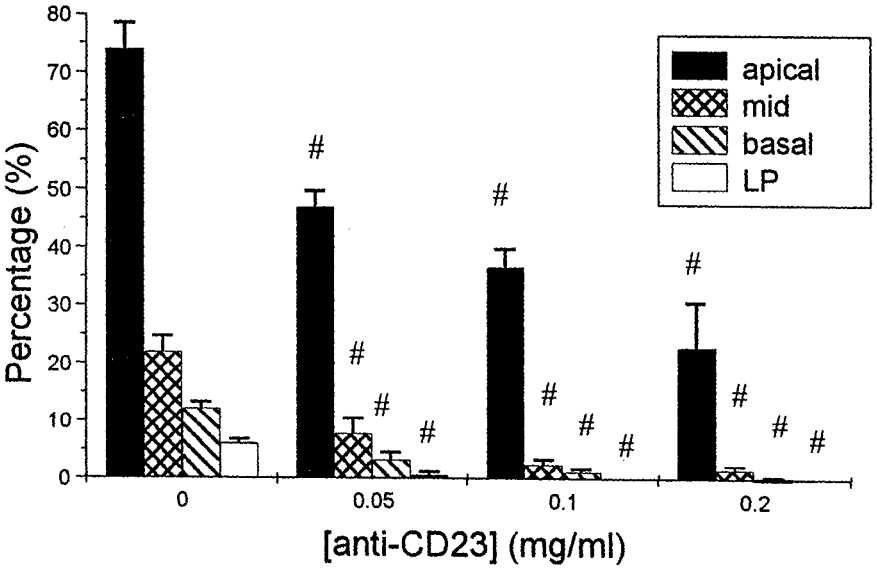
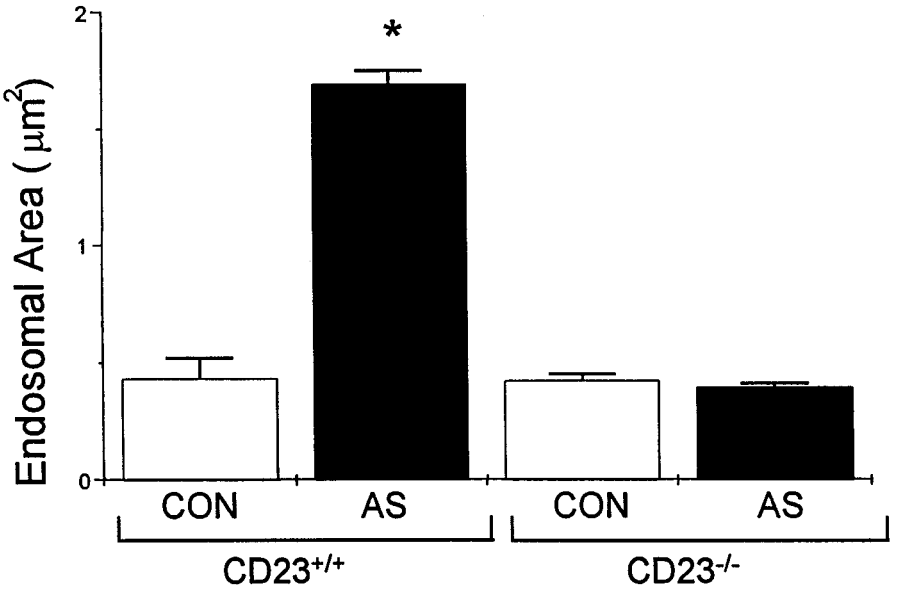

Percentage (%)
0
10
20
30
40
50
60
70
80
apical
mid
basal
LP
#
0
0.05
0.1
0.2
[anti-CD23] (mg/ml)

Figure 3.6 Effect of anti-CD23 antibody on transepithelial antigen transport. Various concentrations of anti-CD23 were added to the luminal side of Ussing chambers containing tissues from sensitized $CD23^{+/+}$ mice 30 min prior to HRP challenge. Intestinal tissues from each group of mice were challenged with HRP (5×10^{-5} M) from the luminal side and then fixed at 2 min post challenge and processed for electron microscopy. (A) Area of HRP-endosomes in enterocytes of tissues pretreated with anti-CD23. n = 12 photomicrographs from 4 mice per group. (B) Percentage of enterocytes containing HRP-endosomes in apical, mid, basal regions and lamina propria in tissues pretreated with anti-CD23. n = 4 tissues examined from 4 mice per group. Values represent means ± SEM; # $p < 0.05$ compared with control tissues (0 mg/ml of anti-CD23).

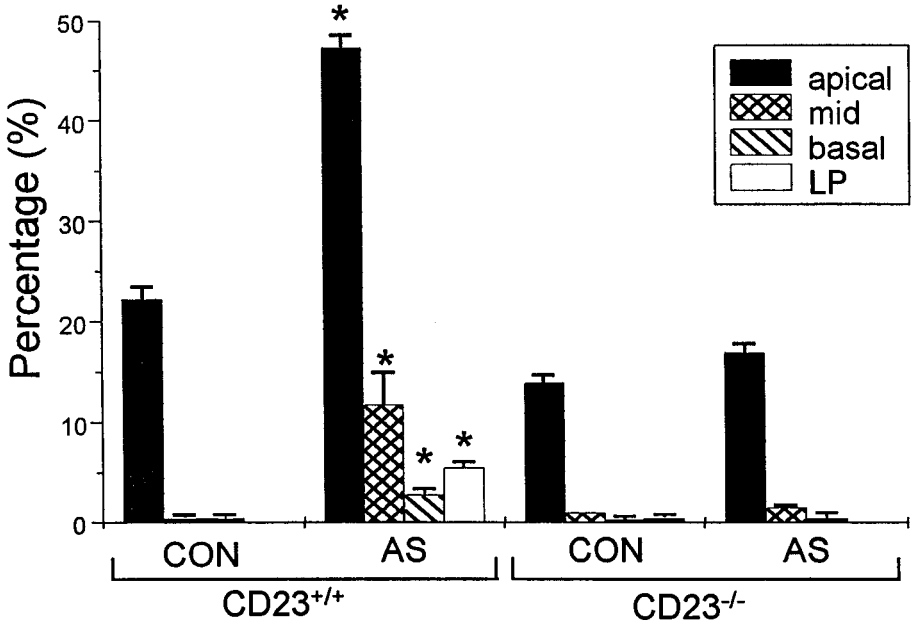
Transepithelial transport of antigen is not enhanced in CD23$^{-/-}$ mice.

Genetically targeted mice that lack CD23 were used to investigate the role of CD23 in enhanced antigen uptake. The titer of IgE in serum measured by passive cutaneous anaphylaxis was 1:256 (median value) for actively sensitized CD23$^{-/-}$ versus 1:128 (median value) for actively sensitized CD23$^{+/+}$ mice. A significantly ($p<0.05$) enhanced area of HRP endosomes in enterocytes was documented in actively sensitized CD23$^{+/+}$ mice compared to naive controls (4-fold increase) (Fig. 3.7A). The rate of antigen transport across enterocytes was also greater in actively sensitized mice (Fig. 3.7B). However, in actively sensitized CD23$^{-/-}$ mice, the area of HRP-endosomes and rate of antigen transport across enterocytes were comparable to values of naive CD23$^{-/-}$ mice (Fig. 3.7A and 3.7B).

A


Endosomal Area (μm²)
2
1
0
*
CON
AS
CON
AS
CD23+/+
CD23-/-

B


Percentage (%)
50
40
30
20
10
0
*
*
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*
apical
mid
basal
LP
CON
AS
CON
AS
CD23+/+
CD23-/-

Figure 3.7 Transepithelial antigen transport in $CD23^{+/+}$ and $CD23^{-/-}$ mice. Intestinal tissues from actively sensitized (AS) and naive control mice (CON) were challenged with HRP (5×10^{-5} M) from the luminal side and then fixed at 2 min post challenge and processed for electron microscopy. (A) Area of HRP-containing endosomes in enterocytes. n = 12 photomicrographs from 4 mice per group. (B) Percentage of enterocytes containing HRP-endosomes in apical, mid, basal regions and lamina. n = 4 tissues examined from 4 mice per group. Values represent means ± SEM; * $p < 0.05$ compared with controls.

Discussion

Our previous studies documented enhanced uptake and rapid transepithelial transport of specific antigen in jejunum of actively sensitized rats.[8,9,11] Here, we showed that both phenomena were reproducible in another species. The use of genetically targeted mice allowed us to investigate the essential components involved in phase I antigen transport. In the current study, we provide evidence that IgE/CD23 mediates the enhanced transepithelial antigen transport in sensitized mouse intestine. We also show that IL-4 is a major factor regulating the mechanism by both stimulation of IgE synthesis and CD23 expression on intestinal epithelial cells.

We previously showed in sensitized rats that the quantity of antigen taken up by intestinal epithelial cells and the rate of antigen transport into the lamina propria were increased several fold above values in naïve control rats, so that at 2 min after luminal challenge, the antigen was already present in the lamina propria.[8] Similarly, in HRP-sensitized mice, we found an eight-fold increase in HRP antigen uptake by enterocytes compared to naïve mice or mice sensitized to an irrelevant antigen, OVA. Moreover, a greater percentage of enterocytes in HRP-sensitized mice contained HRP endosomes in the mid and basal regions of the cells and also in the lamina propria compared to control and OVA-sensitized mice where endosomes were restricted to the apical area. These results indicate that both the quantity of antigen taken up and the rate of transepithelial antigen transport were enhanced in mice sensitized to that protein, suggesting a recognition phenomenon at the level of epithelium.

Passive sensitization of mice confirmed that factors in the serum contribute to the effect of increased uptake and rapid transport of specific antigen. In contrast to whole serum, IgE-depleted serum completely abolished the increased transepithelial antigen transport, indicating that IgE antibodies mediate the enhanced luminal-to-serosal antigen transport in sensitized mouse intestine. IgE has been reported in the intestinal fluid in food allergic patients.[28] In rats post-parasite infection, when IgE production was stimulated, the concentration of IgE in the intestinal fluid was greater than in the serum or mesenteric lymph.[29] In addition, radiolabeled IgE (but not IgG) translocated from the serum into the intestine and intestinal lumen after infusion of rats with IL-4.[30] Those findings suggested a receptor-mediated mechanism for IgE transport into the lumen. In our study, antigen uptake across the epithelium in the opposite direction, i.e. from lumen into the mucosa, required the presence of IgE. Therefore, our result also implies the involvement of an IgE receptor on the surface of epithelial cells in sensitized animals.

IL-4 regulates the synthesis of IgE in B cells by inducing germ line ε transcript.[12] Elevated production of IL-4 by mononuclear cells isolated from blood and intestinal mucosa of atopic individuals has been demonstrated.[13,14] To investigate if IL-4 is involved in the phenomenon of enhanced transport of antigen across enterocytes in sensitized animals, we used genetically targeted IL-$4^{-/-}$ mice and IL-4-neutralized serum for passive sensitization. The absence of the enhanced uptake of HRP into enterocyte endosomes in actively sensitized IL-$4^{-/-}$ mice suggests that IL-4 is involved in increased transepithelial antigen uptake in sensitized animals. However, as shown previously [31] and confirmed by us, IL-$4^{-/-}$ mice do not produce IgE, which may be one factor accounting for the lack of

effect. Importantly, IL-$4^{+/+}$ mice passively sensitized with IL-4 neutralized serum also showed diminished enterocyte uptake of HRP antigen compared with mice sensitized with untreated serum, although the value was greater than that in naïve controls. In contrast, IL-4 neutralization completely abolished enhanced uptake of antigen in enterocytes of IL-$4^{-/-}$ mice. Since IgE remained in the IL-4-neutralized serum, it is clear that IL-4 is involved in the enhanced antigen uptake by inducing changes in addition to stimulation of IgE production. Moreover, we showed that passive sensitization using IL-4-neutralized serum abolished the rapid transport of antigen across enterocytes in IL-$4^{+/+}$ mice in comparison to those injected with untreated serum, indicating that IL-4 was crucial not only for enhancement of antigen uptake, but also for rapid transepithelial antigen transport.

One of the important functions of IL-4 is upregulation of the expression of IgE receptors on immune cells.[15-18] The high affinity IgE receptor, FcεRI, is mainly expressed on mast cells and basophils.[32] In atopic diseases, the binding of allergens to IgE causes cross-linking of FcεRI on the mast cell surface which leads to cell activation and degranulation.[32] However, this receptor has not been reported on enterocytes. The low affinity IgE receptor, CD23, has been identified on epithelial cells. Bronchial epithelial cells from brushings of the airway of asthmatic patients, but not control subjects, showed positive staining for CD23.[27] Human cultured keratinocytes expressed CD23 after IL-4 treatment.[33] Importantly, expression of CD23 was demonstrated on apical membrane of enterocytes in biopsies from normal individuals and appeared to be greater in individuals with food allergy and other enteropathies.[25] In our recent study,[11] we showed that sensitization of rats induced the expression of CD23 on the apical membrane of

enterocytes, as detected by both immunohistochemistry and immunogold labeling. In addition, the number of CD23 labels on the cell surface was decreased after antigen challenge, and internalization of CD23 was demonstrated by the presence of CD23 labels on the membrane of endosomes containing antigen.[11]

In this study of sensitized mice, we identified the expression of CD23 protein on the apical membrane of intestinal epithelial cells by immunohistochemistry, with upregulated expression in actively sensitized IL-$4^{+/+}$ mice compared to naive controls. In contrast, low to negligible expression of CD23 was seen on the surface of enterocytes in actively sensitized IL-$4^{-/-}$ animals. The absence of CD23 expression on the surface of enterocytes paralleled the lack of enhanced transepithelial antigen uptake. The increased expression of epithelial CD23 induced by sensitization in IL-$4^{+/+}$ mice, but not IL-$4^{-/-}$ mice, is likely due to activation of the IL-4 enhancer element on the CD23 gene.[34] IL-4 is produced in excess in lymphocytes isolated from intestinal mucosa in allergic individuals,[14] leading to a high local concentration of IL-4 in the lamina propria that may activate the CD23 gene in intestinal epithelial cells. We also demonstrated that IL-4 upregulates CD23 mRNA expression in a mouse intestinal epithelial cell line, IEC-4, after 8-24 h. Due to the difficulties of isolating a pure population of epithelial cells from mouse intestine for RT-PCR studies, we did not measure the level of CD23 mRNA in enterocytes of sensitized and control mice. Taken together, our results in IL-$4^{+/+}$ and IL-$4^{-/-}$ mice and cultured cells suggest that IL-4 affects transepithelial antigen transport by stimulating both IgE production and CD23 expression on enterocytes.

The role of CD23 in facilitating enhanced antigen transport by intestinal epithelial cells in sensitized mice was substantiated by dose-dependent antibody inhibition. The addition of the well-characterized anti-CD23 antibody, B3B4, [21-23] to the luminal surface of intestine 30 min prior to antigen challenge, reduced the enhanced antigen uptake in enterocytes and significantly decreased the rate of transport across the epithelium. In contrast, the isotype control antibody did not inhibit enhanced antigen transport. CD23 has been previously reported to mediate antigen uptake and focusing in B cells and this effect was inhibited by pretreatment with B3B4.[23] B3B4 has been shown to compete with IgE for binding to CD23 with a higher affinity,[21] therefore, we suggest that this antibody displaces IgE from its receptor on the epithelial cells that leads to reduced endocytosis of antigen.

Studies in sensitized $CD23^{-/-}$ mice provided further evidence of the involvement of CD23 in enhanced antigen uptake and transport. No enhanced uptake and transport of antigen was seen in enterocytes of sensitized $CD23^{-/-}$ mice compared to sensitized $CD23^{+/+}$ mice or naive controls. $CD23^{-/-}$ mice have been reported to display normal lymphocytic differentiation and were able to mount normal antibody responses, including IgE responses, upon immunization and parasitic infection.[19,35-37] In agreement with others,[19,37] sensitized $CD23^{-/-}$ mice exhibited higher or comparable specific IgE levels compared to sensitized $CD23^{+/+}$. In spite of the presence of a high titer of HRP-specific IgE in the serum of actively sensitized $CD23^{-/-}$ mice, there was no enhanced uptake of HRP in intestinal epithelial cells upon luminal antigen challenge. Together with the anti-CD23

inhibition, this result strongly supports the role of epithelial CD23 in binding IgE and mediating enhanced antigen uptake and rapid antigen transport in sensitized intestine.

Normally, proteins internalized in endosomes are transported to the supranuclear region of enterocytes where the vesicles fuse with lysosomes. Lysosomal enzymes degrade these macromolecules in an acidic compartment.[38] However, transepithelial transport of IgA and IgG has been demonstrated under physiological conditions. Polymeric immunoglobulin receptor (receptor for IgA) and FcRn (receptor for IgG), have been identified on the basal and apical surface of intestinal epithelial cells.[39,40] It has been suggested that receptor-mediated transport of protein within endosomes across intestinal epithelial cells may bypass degradative lysosomal pathways.[39,40] Whether CD23 enhances transepithelial antigen transport of intact antigens and IgE by a similar mechanism in hypersensitivity requires further investigation.

In summary, our study has documented enhanced transport of specific antigen across intestinal epithelial cells following luminal challenge in both actively and passively sensitized mice. Our results provide evidence that IgE/CD23 mediates the enhanced transepithelial antigen transport in sensitized intestine since both IgE depletion and anti-CD23 antibody reduced epithelial uptake of antigen. Moreover, we showed that IL-4 plays a major role in the mechanism of phase I enhanced transepithelial antigen transport by regulating both IgE synthesis and expression of CD23 on intestinal epithelial cells. Finally, we suggest that antigen binding to IgE/CD23 may bypass the intracellular degradative pathway for proteins resulting in large quantities of antigen penetrating the epithelial barrier. An understanding of the mechanism involved in enhanced transepithelial

antigen transport may allow the development of novel therapeutic interventions to reduce adverse anaphylactic reactions in food allergy.

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CHAPTER 4: Murine intestinal epithelial CD23/FcεRII is required for enhanced transepithelial antigen transport: evidence for novel alternative splice forms

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Abstract

We previously demonstrated enhanced transepithelial antigen transport in the intestine of allergic rodents, associated with elevated expression of the low affinity IgE receptor CD23/FcεRII protein expression on enterocytes. Here, we examined the role of CD23 in the transport phenomenon using $CD23^{-/-}$ mice, and characterized the isoform of intestinal epithelial CD23. $CD23^{-/-}$ mice and wild type controls were sensitized to horseradish peroxidase (HRP). Jejunal segments were challenged with HRP in Ussing Chambers, and the area and location of HRP containing endosomes in enterocytes were measured in electron photomicrographs. The absence of CD23 abolished enhanced uptake and rapid transport of antigen in enterocytes, leading to reduced transmucosal antigen flux. RT-PCR showed that cultured epithelial cells expressed only the *b* isoform of CD23, which was upregulated by IL-4. Sequencing revealed classical CD23*b* and alternative CD23*b* transcripts lacking exon 5 or 6, all of which were translated into functional IgE receptors in transfected cells. IL-4 upregulated the transcript lacking exon 5; the protein encoded by this form was endocytosed upon ligand binding. Our findings suggest that unique splice forms of CD23*b* display distinct endocytic properties, which may be important in mediating enhanced transepithelial antigen transport. We conclude that antigen binding to epithelial CD23/IgE facilitates its entry into the body and results in intestinal anaphylaxis.

Introduction

Allergic conditions, including food allergy, asthma, allergic rhinoconjunctivitis and atopic dermatitis, are the largest group of immune disorders affecting 20-30% of the population in North America and Europe, and there is evidence that such conditions are increasing in prevalence (1,2). Food allergy is a disorder that leads to gastrointestinal symptoms, and in some cases, extraintestinal symptoms in the skin and airways, in sensitized individuals after food antigen ingestion. In severe cases, such as peanut allergy, systemic anaphylaxis can occur with fatal consequences (3). To date, the only effective treatment of food allergy is an elimination diet involving avoidance of suspected foods. However, particularly in young children, the diet may become so restricted that nutrition is compromised. Therefore, it is important to have a clear understanding of the pathophysiology of food allergic reactions in order to develop novel therapeutic approaches.

Intestinal anaphylactic symptoms develop extremely rapidly (4,5). Such reactions are known to be mediated by mast cell activation induced by antigen cross-linking of IgE bound to the cell surface via high affinity receptors (6). However, mast cells in the intestinal mucosa are located in the lamina propria beneath the epithelial lining of the gut, which in theory, should prevent access of antigenic molecules to these effector cells (7). Epithelial cells (enterocytes) are held together at their apical poles by tight junctions that restrict molecules larger than 500 daltons from passing through the paracellular pathways (8). In addition, although a small quantity of macromolecules is taken up into enterocytes by endocytosis, most proteins are degraded during transcytosis, thus reducing their

antigenic properties (9). However, enhanced epithelial permeability and antigen uptake have been reported in food allergic patients and sensitized animals (10-15). Therefore, several years ago, we began studies to examine the pathway and mechanism by which macromolecular antigens enter the body.

Our previous experiments in allergic rodents suggested that a unique mechanism was responsible for enhanced transport of intact antigen across the epithelial barrier. Rats were sensitized to horseradish peroxidase (HRP), and subsequently jejunal segments were challenged with antigen on the luminal surface. Enhanced antigen uptake (several-fold control values) into enterocyte endosomes and rapid transport across the cell were documented (12,14). As early as 2 min, HRP antigen was already present in the lamina propria (12,14), an extremely rapid rate of transcytosis compared with normal values of 20~30 min (16). We termed this phase I of enhanced transepithelial antigen transport. Further studies revealed that phase I antigen transport was specific for the sensitizing antigen and IgE-dependent, but mast cell-independent since similar findings were obtained in mast cell-deficient rats (12-14). Immunohistochemical staining demonstrated expression of the low affinity IgE receptor (FcεRII/CD23, originally described in B cells (17)) on jejunal enterocytes (14,15). Subsequently, we found that gut epithelial CD23 expression was associated with rapid antigen uptake into enterocytes in sensitized wild type IL-$4^{+/+}$ mice, but neither CD23 expression nor enhanced antigen uptake was demonstrated in sensitized IL-$4^{-/-}$ mice (15). This finding implies that IL-4, a Th2 cytokine elevated in allergic conditions, regulates the expression of CD23 in intestinal epithelial cells. Finally, in

confirmation of the role of CD23 in enhanced antigen uptake into enterocytes in phase I, results were negative in sensitized $CD23^{-/-}$ mice (15).

A second phase of antigen penetration through the epithelium was evident > 30 min after challenge in sensitized rats (12). This phase (termed phase II) involved antigen transport via the paracellular pathway as well as the transcellular pathway. HRP was visualized in the paracellular spaces and tight junctions, and a significant increase in the overall flux of antigen across the mucosa was documented. Mast cells were shown to be activated at this time as indicated by electron microscopy. Phase II antigen transport did not occur in sensitized mast cell-deficient rats, implying that the augmented epithelial permeability in this phase was mast cell-dependent (13).

The aim of the current study was to continue our examination of the role of epithelial CD23 in augmented intestinal antigen transport (phase I and phase II) using CD23-deficient mice, and to characterize the isoform of CD23 expressed on mouse intestinal epithelial cells. In humans, *a* and *b* isoforms of CD23 have been described on a wide range of cells (18-23). However, in mice, expression of the *a* isoform of CD23 has been reported on B cells (17,24,25), but the existence of the *b* isoform remains controversial. Here, we demonstrated both phase I and II of enhanced intestinal antigen transport, including paracellular transport and transmucosal flux, in sensitized $CD23^{+/+}$ mice, but not in sensitized $CD23^{-/-}$ mice or non-sensitized controls. Only the *b* isoform of CD23 was identified in mouse epithelial cells. In addition to the classical CD23*b* transcript, we also identified novel alternative forms lacking exon 5 or 6. All three forms of CD23*b* were translated into functional IgE receptors. IL-4 upregulated the alternative

CD23*b* transcript lacking exon 5, and the protein encoded by this form was endocytosed upon anti-CD23 and IgE binding. Taken together, our results suggest that unique splice forms of CD23*b* may display distinct endocytic properties, important in mediating enhanced transepithelial antigen transport in the intestine of sensitized mice.

Methods

Animals. $CD23^{-/-}$ mice (C57BL/6 background) were bred in the Central Animal Facility at McMaster University (original breeders obtained from M. Lamers, Max-Planck-Institut fur Immunbiologie, Freiburg, Germany) (26). $CD23^{+/+}$ (C57BL/6) mice and BALB/c mice were purchased from Harlan breeding Laboratories, Indianapolis, IN. Mice were used at 7-14 weeks of age. Mice were sensitized by i.p. injection with 100 μg of HRP (Type II; Sigma) in 0.2 ml of alum, and with pertussis toxin as adjuvants (15). On day 7 and 14, mice were boosted with HRP. Experiments were performed on day 21. Naive mice served as controls. All animal experiments were conducted with the approval from the McMaster University Animal Care Committee.

Ussing Chambers. Mice were killed by cervical dislocation and a laparotomy was performed. From each mouse, 4 pieces of jejunum were mounted in Ussing Chambers (WPI instruments, Narco Scientific Corp., Mississauga, ON, Canada). Care was taken to avoid tissues with Peyer's patches. An area of 0.6 cm^2 of intestine was exposed to 8 ml of circulating oxygenated Krebs buffer (pH 7.33-7.37) at 37°C. The serosal buffer contained 10 mM of glucose as an energy source osmotically balanced with 10 mM of mannitol in the luminal buffer. The tissue was clamped at 0 V using a W-P Instrument automatic voltage clamp (Narco Scientific). HRP (5×10^{-5} M) was added into the luminal buffer and 500 μl of serosal buffer samples were collected at 30 min intervals for 120 min for

measurement of HRP flux. The concentration of HRP was measured by a kinetic enzymatic assay and the flux calculated by a standard formula (12,14).

Antigen Uptake by Enterocytes. Tissues were removed from the Ussing chambers at 2 min (phase I) and 60 min (phase II) after HRP challenge and processed for electron microscopy to determine the route and rate of HRP uptake across the epithelium (12,14,15). Jejunal segments were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4), washed, incubated in 3,3'-diaminobensidine tetrahydrochlorine (Sigma) and 0.01% H_2O_2 (pH 7.6), and subsequently processed for transmission electron microscopy. Ultrathin sections of midvillus epithelium (cut in the longitudinal plane) were stained and photomicrographs were taken. The total area of HRP containing endosomes in the apical region of enterocytes in a 120 μm^2 window was quantified in photomicrographs using computerized image analysis. In addition, the distribution of HRP containing endosomes within enterocytes was determined and expressed as the percentage of cells containing endosomes in the apical, mid, basal regions and also in the lamina propria (12,14,15). This analysis was performed for 100 well-oriented enterocytes in tissues from 4 mice per group by one investigator (P.C.Y.) who was unaware of the origin of the tissues.

Passive cutaneous anaphylaxis. The level of HRP-specific IgE in mouse serum was determined by passive cutaneous anaphylaxis. Sprague-Dawley rats were injected intradermally with 0.1 ml of mouse serum in duplicate dilutions from 1:1 to 1:1024. After

72 h, rats were challenged by i.v. injection with 0.5 ml of HRP (5 mg/ml) in 1% Evan blue. Positive reactions were evaluated as blue spots present after 30 min. The titer was the highest serum dilution showing a positive result. Heat treatment (56°C for 3 h) abolished the reaction indicating that the immunoglobulin was heat-labile IgE.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR). RT-PCR was performed on RNA extracted from mouse jejunal segments, enterocytes isolated from mouse jejunum, or cultured mouse intestinal epithelial cells of the IEC-4 cell line. Segments of jejunum from control or sensitized BALB/c mice were washed in phosphate-buffered saline (PBS) and cut into 3 mm cubes for RNA extraction using a RNeasy® Mini kit (Qiagen, Mississauga, ON, Canada). Enterocytes were isolated from the mouse small intestine by previously reported methods with modifications (27,28). The jejunal segment was slit open, washed and incubated in RPMI 1640 media (Invitrogen Corporation, Carlsbad, CA) containing 1 mM dithiothreitol (DTT; Sigma) for 15 min at room temperature to remove mucus. Peyer's patches were removed. The tissues were then incubated with pre-warmed isolation solution (0.05% trypsin, 0.53 mM EDTA in PBS) (Invitrogen) for 20 min at 37°C and gently shaken every 5 min. The isolated cells were collected and washed in RPMI (as above without DTT), following with filtration through nylon mesh (Nytex, Tetko, Elmsford, NY). Epithelial cells were purified by density gradient centrifugation on a Percoll gradient (Amersham Pharmacia Biotech, Quebec, Canada). Intestinal epithelial cells were collected, washed and resuspended in RPMI. The

viability of enterocytes (trypan blue negative) was > 95%. The estimated purity of epithelial cells was determined to be ~90% by flow cytometry using cytokeratin as the epithelial cell marker (27,28). RNA was extracted from the isolated enterocytes using the RNeasy® Mini kit (Qiagen) (see below).

IEC-4 cells were cultured in DMEM media (Invitrogen) supplemented with 5% FCS, 0.01M HEPES, 20 mM L-glutamine, 0.1 U/ml Penicillin G sodium, and 5 μg/ml streptomycin sulfate (29). 10^6 cells/ml were seeded in a 60 mm diameter cell culture plate (Corning, Corning, NY) for 3 days. Confluent cells were either untreated or treated with 10 ng/ml of recombinant mouse IL-4 (R&D Systems, Minneapolis, MN) for 24h. The RNA was extracted from cells using RNeasy® Mini kit (Qiagen) according to the manufacturer's instructions. RNA (2 μg) was reverse-transcribed with Oligo $d(T)_{16}$ using Perkin-Elmer RNA PCR core kit (Perkin-Elmer, Mississauga, ON, Canada). The resulting cDNA (in 20 μl) was then subjected to PCR by addition of 80 μl of a master mix containing 2 mM of $MgCl_2$ solution, 1x PCR buffer, 2.5 U AmpliTaq DNA polymerase, 0.5 μM upstream primer and 0.5 μM of downstream primer.

To determine the isoform of CD23 expressed by intestinal epithelial cells, two sets of primers were used (30). As upstream primers, the *a* isoform specific oligo-A (5'-CCTCATCACTGAAAGGATCCAAACAAG-3') and the *b* isoform specific oligo-B (5'-GAAAGCCAATTTGAACGGGAACTTGG-3') were used. As a common downstream primer, oligo-E (5'-GGAGCCCTTGCCAAAATAGTAGCAC-3') was used. The DNA thermal cycler (Teche PHC-3; Mandel Scientific Company Ltd. Guelph, ON, Canada) was

programmed to perform a protocol as follows: 94°C for 3 min for 1 cycle; 94°C for 1.5 min (denaturation), 60°C for 2 min (annealing), and 72°C for 3 min (extension) for 35 cycles; and 72°C for 7 min for final extension. To amplify full-length coding sequence of CD23b, we designed a new primer set including oligo-B' (5'-ATGAATTCTCAAAACCAGGGA-3') and oligo-F' (5'-TCAGGGTTCACTTTTTGGG-3'). The DNA thermal cycler was programmed as follows: 94°C for 5 min for 1 cycle; 94°C for 30 sec, 58°C for 30 sec and 72°C for 1 min for 35 cycles; and 72°C for 5 min. Negative controls were performed with samples lacking cDNA or samples with mRNA that were not reverse transcribed. RT-PCR products were then electrophoresed in a 0.8 % agarose gel in the presence of 0.5 μg/ml ethidium bromide, visualized with a UV transilluminator and photographs were taken. Molecular weight markers, Ready load ϕ X174 RF DNA/*Hae* III Fragments (Invitrogen) was used. The intensity of the DNA bands was analyzed using a densitometer with software from KODAK Digital Science 1D (GIBCO, Rockville, MD).

DNA Sequence analysis. PCR products were extracted from the electrophoresed gel, cloned into pCR®3.1 plasmids and amplified by transforming TOP 10F' competent cells using a eukaryotic bidirectional TA cloning ® Kit (Invitrogen). Transformed competent cells were plated on an LB agar plate containing 50 μg/ml of ampicillin and incubated overnight. Individual colonies were grown in ampicillin-containing LB broth overnight and plasmidic DNA was purified using the Qiaprep® Miniprep kit (Qiagen). Clones

containing CD23 cDNAs were sent for nucleotide sequencing (Eurogentec, Seraing, Belgium). Clones with cDNAs in the correct orientation were selected using appropriate restriction sites and used for transient transfection (see below).

Transfection, immunofluorescence and endocytosis. HeLa cells were cultured in DMEM media supplemented with 10% FCS, 20 mM L-glutamine, and 5 μg/ml streptomycin sulfate to subconfluency on coverslips. HeLa cells were transfected with CD23 encoding plasmids using a calcium phosphate transfection kit (Invitrogen) and were processed for immunofluorescence studies the following day as previously described (31,32).

Briefly, transfected HeLa cells were washed with PBS and fixed with 4% paraformaldehyde and 0.03 M sucrose at 4°C for 30 min and quenched with 50 mM NH_4CL in PBS at room temperature for 10 min. Cells were incubated with a primary antibody B3B4 (20 ug/ml) (rat IgG2a anti-mouse CD23 (33)) in a permeabilizing buffer (PBS containing 0.1% bovine serum albumin (BSA) and 0.05% saponin (Sigma)) at room temperature for 30 min, washed twice with the permeabilizing buffer, and then incubated with goat anti-rat IgG secondary antibody conjugated with Alexa Fluor™ 488 or 594 (1:100) (Molecular probes, Eugene, OR) in permeabilizing buffer, and washed twice. Cells were mounted on microscope slides in 100 mg/ml Mowiol (Calbiochem, La Jolla, CA), 25% glycerol, 100 mM Tris-HCl, pH 8.5. Negative controls included staining where the primary antibody was omitted as well as mock transfected HeLa cells.

For IgE binding studies, HeLa cells transiently transfected with CD23 isoforms were incubated with monoclonal mouse anti-DNP IgE (5 μg/ml) (Sigma) in an IgE-binding solution (DMEM media containing 0.4 mM $Ca(NO_3)_2 \cdot 4H_2O$, and 0.1% BSA (34,35)) at 4°C for 1 h, washed twice in cold IgE binding solution and then fixed with 4% paraformaldehyde as described above. To reveal membrane bound IgE, cells were incubated with 10 μg/ml monoclonal rat IgG1 anti-mouse IgE antibodies (Southern Biotechnology, Birmingham, AL) in PBS containing 0.1% BSA (50 μl) at room temperature for 30 min, washed, and stained with secondary goat anti-rat IgG antibodies (1:100) conjugated with Alexa Fluor™ 488 or 594 (Molecular Probes).

Endocytosis of membrane-bound anti-CD23 and transferrin was examined in subconfluent HeLa cells grown on coverslips one day after transfection. The cells were first incubated for 20 min at 37°C in DMEM to eliminate receptor-bound transferrin, washed in cold PBS and then incubated for one hour at 4°C in the presence of the anti-CD23 antibody (50 μg/ml in DMEM, 1mg/ml BSA (DMEM-BSA)). Cells were washed two times in DMEM-BSA and then incubated in DMEM-BSA containing 100 nM Alexa Fluor™ 594-conjugated transferrin (Molecular probes). After incubation at 37 °C for 30 min, the cells were rapidly cooled to 4 °C using cold DMEM-BSA, washed twice in cold PBS and then fixed for one hour at 4°C. The anti-CD23 antibody was revealed using the Alexa Fluor™ 488-labelled goat anti-rat IgG secondary antibody as described above.

The samples were examined under an epifluorescence microscope (Axioplan II, Zeiss) attached to a cooled CCD-camera (Spot-2, Diagnostic Instruments) or under a

confocal microscope (LSM 510, Zeiss). Alexa Fluor™ 488 and 594 corresponding staining were observed using the classical FITC and rhodamine/TexasRed filters respectively.

Statistics. Data are presented as mean ± SEM. Statistical significance was tested by ANOVA, with post-hoc analysis using Newman Keuls tests or Student's t tests when appropriate. A value of $p < 0.05$ was considered significant.

Results

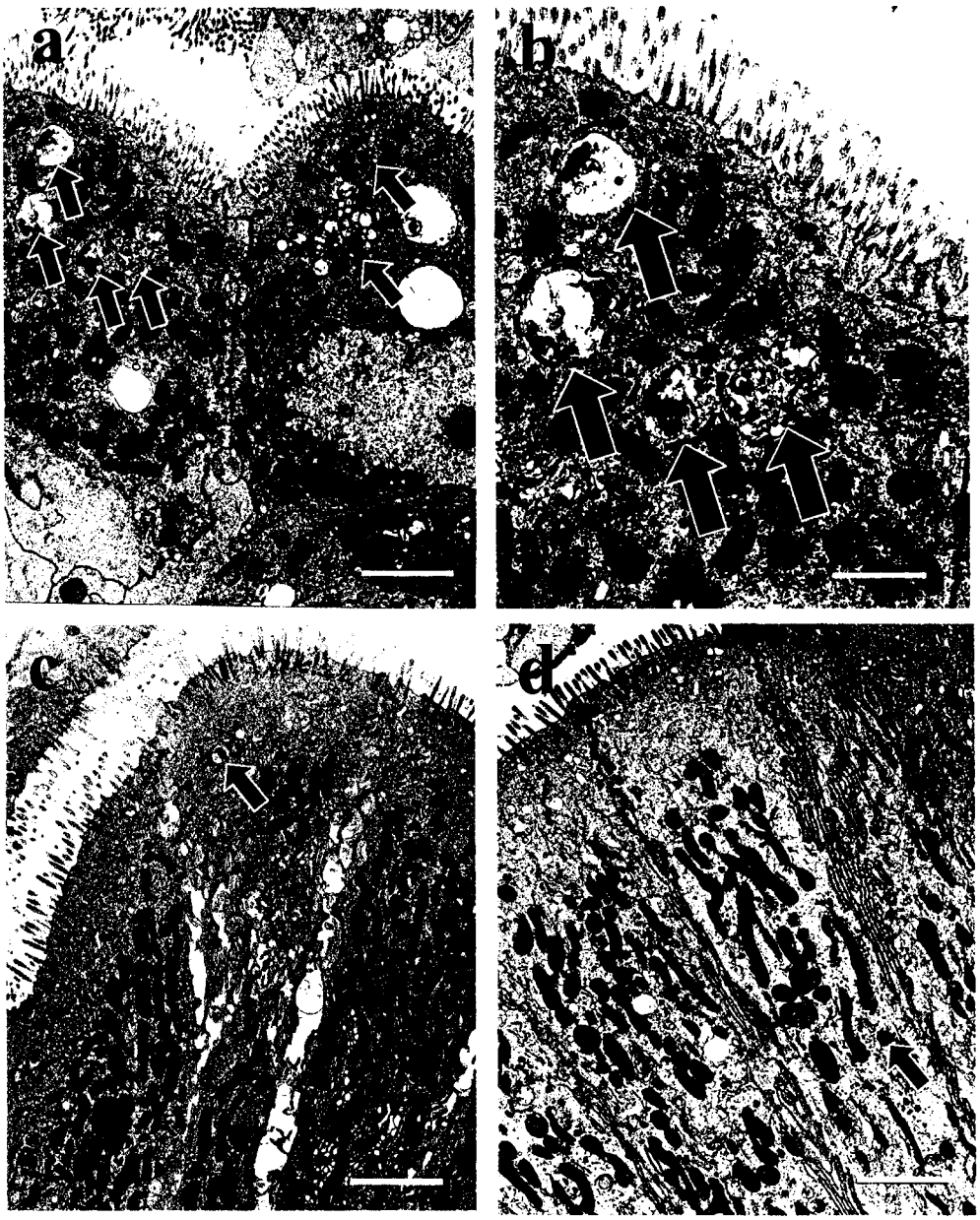
Sensitized CD23$^{+/+}$ mice demonstrated both phase I and phase II of transepithelial antigen transport.

The titer of anti-HRP IgE in serum in sensitized wild type CD23$^{+/+}$ mice was 1:128 (median value), whereas the value was undetectable for non-sensitized CD23$^{+/+}$ controls.

At 2 min after luminal HRP antigen challenge (phase I), electron photomicrographs of jejunal tissues revealed an increased size and number of HRP containing endosomes within enterocytes in sensitized mice (Fig.4.1a and 4.1b) compared to non-sensitized mice (Fig. 4.1c). By image analysis, the total area of HRP containing endosomes was found to be 3.5-fold greater ($p<0.05$) (Fig. 4.2a), indicating enhanced antigen uptake in sensitized mice. The rate of antigen transcytosis was also elevated in sensitized animals, since at 2 min HRP containing endosomes were distributed throughout the cells and HRP was observed in the lamina propria. In sensitized mice, the values for the percentage of enterocytes containing endosomes in various epithelial cell regions were: apical 50 %, mid 12 %, basal 3%, and lamina propria 6%; whereas in control animals the endosomes were restricted to the apical region (apical 22%, mid 1%, basal 1%, and lamina propria 0%) (Fig. 4.2b). No HRP was demonstrated in the paracellular regions.

At 60 min after HRP challenge (phase II), antigen uptake by enterocytes in sensitized mice was further enhanced. A 5-fold increase ($p<0.01$) in the area of HRP containing endosomes was demonstrated compared to controls (Fig. 4.2a), and the values for the percentage of cells containing endosomes in cell regions were: apical 61%, mid 21%, basal 15%, and lamina propria 20% versus apical 28%, mid 1%, basal 1%, and

lamina propria 0% in controls (Fig. 4.2b). HRP was also present in the paracellular spaces at 60 min post challenge in sensitized mice (Fig. 4.1e). Furthermore, a significant increase in HRP flux across the tissues (2.3-fold) ($p<0.05$) at 60-120 min post antigen challenge was demonstrated in sensitized $CD23^{+/+}$ mice compared to controls (Fig. 4.2c).


a
b
c
d

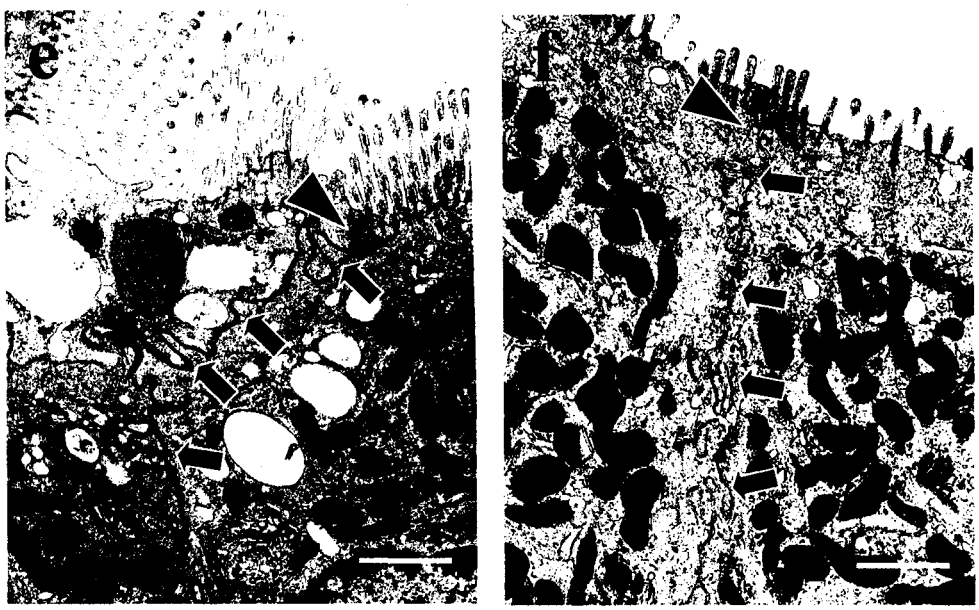

e
f

Figure 4.1 Electron photomicrographs showing HRP uptake into enterocyte endosomes and paracellular spaces in the intestinal epithelium. Jejunal tissues mounted in Ussing Chambers were challenged with HRP added to the luminal buffer. Tissues were fixed at 2 or 60 min post challenge, and processed for electron microscopy to visualize HRP containing endosomes or paracellular spaces. Representative photomicrographs show HRP containing endosomes (arrows) in tissues obtained from (a and b) HRP sensitized $CD23^{+/+}$ mice, (c) control $CD23^{+/+}$ mice, (d) HRP sensitized $CD23^{-/-}$ mice. Panel (b) is an enlargement of panel (a), showing HRP containing endosomes (circled with dotted lines). Bars indicate 2 μm, except in panel (b) (bar indicates 1 μm). Panels (e) and (f) show the paracellular spaces (arrows) between enterocytes, which are filled with HRP in tissues of (e) HRP sensitized $CD23^{+/+}$ mice, but not in (f) HRP sensitized $CD23^{-/-}$ mice. Tight junctions are indicated by arrowheads. Bars indicate 1 μm. These photomicrographs are representative of those used for quantitative measurements of endosomal area (see Figure 4.2a).

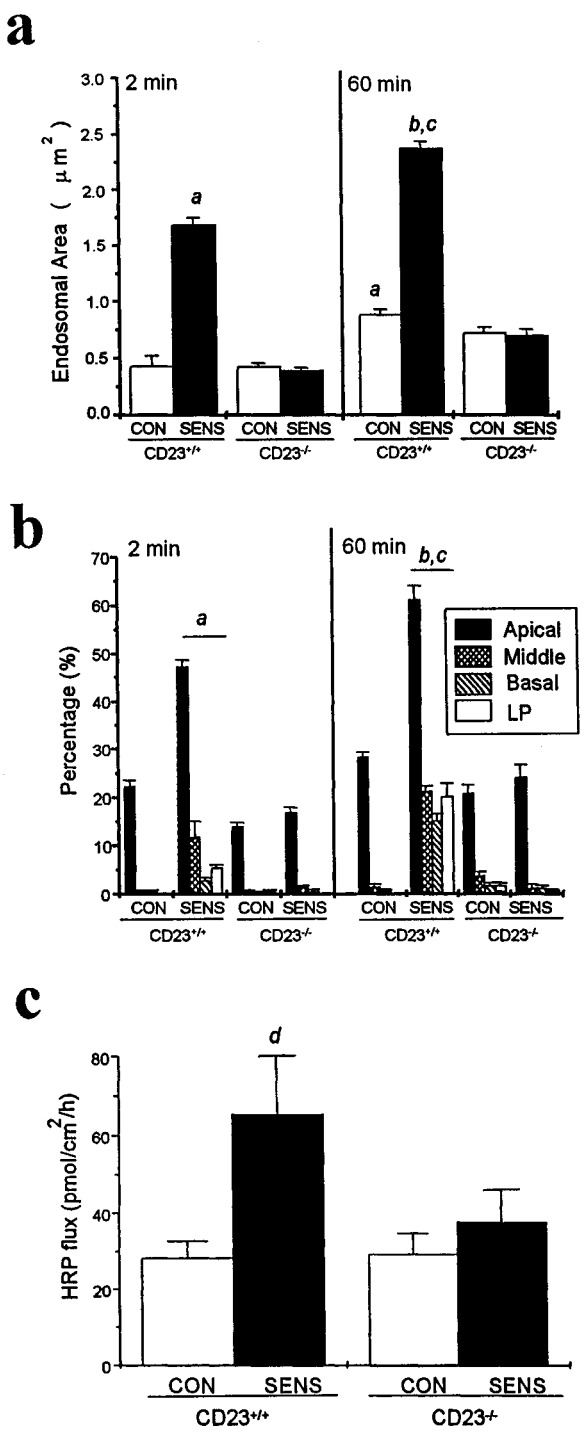

a
2 min
60 min
Endosomal Area (μm²)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
b,c
a
CON SENS CON SENS CON SENS CON SENS
CD23+/+ CD23-/- CD23+/+ CD23-/-
b
2 min
60 min
b,c
a
Percentage (%)
70
60
50
40
30
20
10
0
Apical
Middle
Basal
LP
CON SENS CON SENS CON SENS CON SENS
CD23+/+ CD23-/- CD23+/+ CD23-/-
c
d
HRP flux (pmol/cm²/h)
80
60
40
20
0
CON SENS CON SENS
CD23+/+ CD23-/-

Figure 4.2 HRP transport across the intestinal epithelium of control and sensitized $CD23^{+/+}$ and $CD23^{-/-}$ mice. Intestinal tissues from control (CON) and sensitized (SENS) mice were challenged with HRP from the luminal side and then fixed at 2 or 60 min post-challenge and processed for electron microscopy. (a) The total area of HRP containing endosomes in enterocytes measured in a fixed sized (120 μm^2) apical window in electron photomicrographs (n = 12 photomicrographs from 4 mice per group). (b) The percentage of enterocytes containing HRP containing endosomes in the apical, mid, basal regions of cells and in the lamina propria (LP) (n = 12 tissues examined from 4 mice per group). (c) HRP flux across intestine from control and sensitized $CD23^{-/-}$ and $CD23^{+/+}$ mice (n= 12 tissues from 4 mice per group). Values represent means ± SEM; $p < 0.05$, ***a*** compared with control $CD23^{+/+}$ mice at 2 min, ***b*** compared with control $CD23^{+/+}$ mice at 60 min, ***c*** compared with sensitized $CD23^{+/+}$ mice at 2 min, ***d*** compared with control $CD23^{+/+}$ HRP flux.

Neither phase I nor phase II of enhanced transepithelial antigen transport was demonstrated in sensitized CD23$^{-/-}$ mice.

The titer of anti-HRP IgE in serum was 1:256 (median value) for sensitized CD23$^{-/-}$ mice, whereas no IgE was detected in non-sensitized CD23$^{-/-}$ mice.

At 2 min post challenge, HRP containing endosomes in enterocytes of sensitized CD23$^{-/-}$ mice (Fig. 4.1d) appeared similar to those in control CD23$^{+/+}$ (Fig. 4.1c) and CD23$^{-/-}$ mice (data not shown). HRP uptake in enterocytes based on the total area of HRP containing endosomes was not statistically different in sensitized versus non-sensitized CD23$^{-/-}$ mice (Fig. 4.2a). Rapid transcytosis of antigen across enterocytes was not seen in sensitized CD23$^{-/-}$ mice. Values for the percentage of enterocytes containing HRP containing endosomes in apical, basal, mid regions and lamina propria were 24%, 1%, 1%, and 0%, respectively, in sensitized mice versus 14%, 1%, 0%, and 1%, respectively, in non-sensitized CD23$^{-/-}$ mice (Fig. 4.2b).

At 60 min post antigen challenge, the area of HRP containing endosomes within enterocytes was not significantly different in sensitized CD23$^{-/-}$ mice compared to non-sensitized CD23$^{-/-}$ mice (Fig. 4.2a). Moreover, the endosomes remained restricted to the apical region of cells in both sensitized and non-sensitized CD23$^{-/-}$ mice (Fig. 4.2b). Values for the percentage of enterocytes containing endosomes within various cell regions in sensitized mice were: apical 24%, mid 1%, basal 1%, and lamina propria 1%, values not statistically different from those in non-sensitized mice (apical 21%, mid 4%, basal 2%, and lamina propria 2%). In addition, HRP was not observed in the paracellular spaces between enterocytes in sensitized CD23 $^{-/-}$ mice (Fig. 4.1f) or in control mice (data not

shown). Finally, the transmucosal flux of HRP was similar for sensitized and control $CD23^{-/-}$ mice (Fig. 4.2c).

The b isoform of CD23 was the only transcript expressed in mouse intestinal epithelial cells.

CD23 is expressed as two major isoforms, *a* and *b*, that show differences in their expression pattern and cellular functions (18-20). To better understand the role of CD23 in enhanced antigen transport, we characterized the isoform(s) expressed by mouse intestinal cells. RT-PCR was performed on mRNA using two different pairs of primers that were designed to amplify specifically isoform *a* or *b* of murine CD23 (30). RNA prepared from both full thickness jejunum and isolated enterocytes expressed CD23 isoform *b* (data not shown). However, even isolated enterocytes are not a pure preparation of epithelial cells (~90%), and may contain immune cells present either within the epithelium (e.g., intraepithelial lymphocytes) or in the lamina propria (e.g., T cells or B cells). Therefore, we concentrated our efforts on a well-characterized mouse epithelial cell line, IEC-4 (29). Again, a band of the expected size was obtained only with the *b* isoform specific primers (Fig. 4.3). In contrast, mRNA isolated from mouse spleen cells yielded a positive result only for isoform *a*, as would be expected from published studies (24,25). These results suggested that the *b* isoform was the only subtype expressed constitutively by intestinal epithelial cells. IL-4 treatment of cultured epithelial cells upregulated (~2 fold) the expression of CD23*b* mRNA (Fig. 4.3).

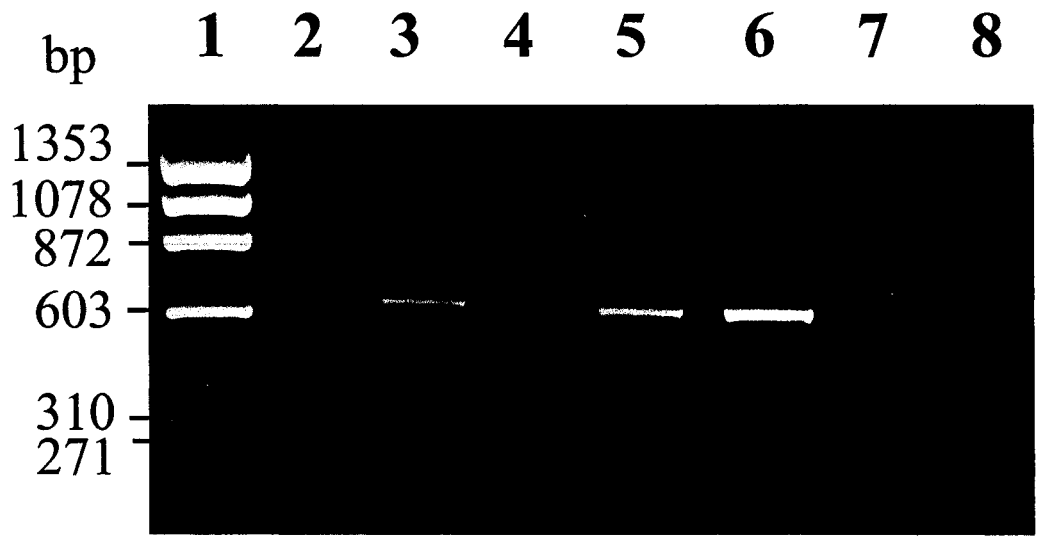


Figure 4.3 Characterization of the isoforms of CD23 transcripts in cultured epithelial IEC-4 cells. Lane 1, molecular weight markers (Ready load Φ X174 RF DNA/*Hae* III Fragments). Lanes 2 and 3, cDNA derived from untreated cells where RNA was subjected to primer sets for CD23*a* and CD23*b* transcripts, respectively. Lanes 4 and 5, cDNA derived from IL-4-treated IEC-4 cells where RNA was subjected to primer sets for CD23*a* and CD23*b* transcripts, respectively. Lanes 6 and 7, cDNA derived mouse spleen cells where RNA was subjected to primer sets for CD23*a* and CD23*b* transcripts, respectively. Lane 8, negative control where no template was added. This figure is representative of 3 individual experiments.

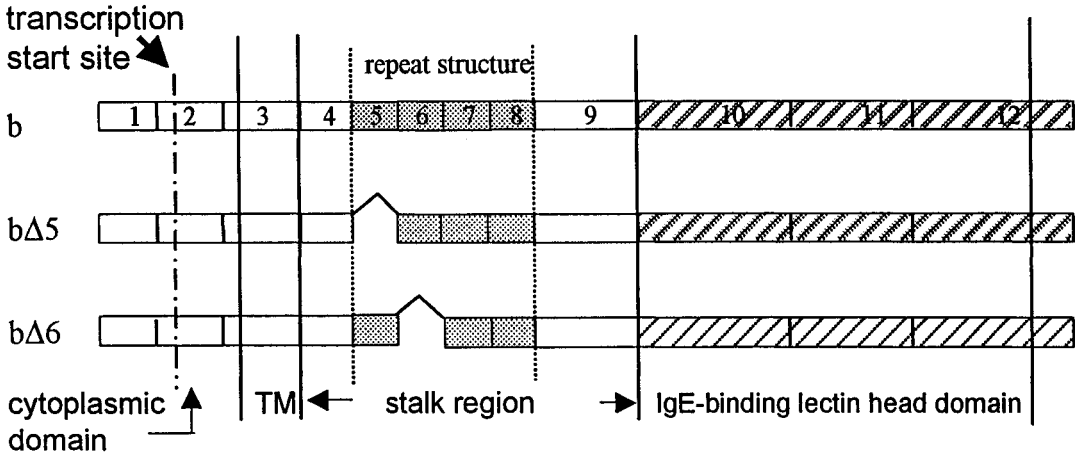
Intestinal epithelial cells expressed classical CD23b and novel alternative splice forms lacking exon 5 and 6.

To further confirm that the amplification products corresponded to CD23*b*, they were purified, subcloned into a PCR3.1 vector and individual clones were sequenced. The majority of the clones displayed the exact sequence of CD23*b* (clone pERB452, GenBank entry: X64223), denoted here as the classical CD23*b* transcript (30). In addition, several clones demonstrated internal deletions, between base 229 to 291 or base 291 to 354 (base numbers according to the sequence of pERB452). The missing regions corresponded to the entire sequences of exon 5 or exon 6, respectively, which are part of the extra-cellular stalk region (Fig. 4.4a). To determine if these deletions corresponded to functional splice events, mRNA from IEC-4 cells was subjected to RT-PCR using a new set of primers which amplified the full-length coding region from ATG to the stop codon. The PCR products were processed as described above and a total of 34 clones were analyzed from 4 individual experiments. The sequencing results confirmed the presence of full-length alternative splice forms lacking only exon 5 or exon 6, designated *bΔ5* (GenBank accession number: AY069980) or *bΔ6* (GenBank Accession number: AY069981), respectively. From the 34 analyzed clones, 23 contained classical *b* transcripts (68 %), whereas 8 clones corresponded to *bΔ5* (24 %) and 3 to *bΔ6* (9 %) (Fig. 4.4b).

To further characterize these new splice forms, we examined if their expression could be modified by IL-4, known to modulate classical CD23*b* in B and non-B cells (16,17). RT-PCR products amplified from IL-4-treated cells were analyzed as described

above. From 25 clones, 15 contained classical CD23*b* transcripts (60%) and 10 contained the alternative *bΔ5* form (40%). No *bΔ6* transcript was found, suggesting that IL-4 may downregulate this form while *bΔ5* was upregulated (from 25% to 40%) under the same conditions (Fig. 4.4b). Moreover, although we experienced technical difficulties in amplifying a thick RT-PCR band from RNA obtained from isolated enterocytes of sensitized mice, we did isolate two positive clones and sequencing revealed that both clones contained the exon 5-lacking isoform of CD23*b*.

a



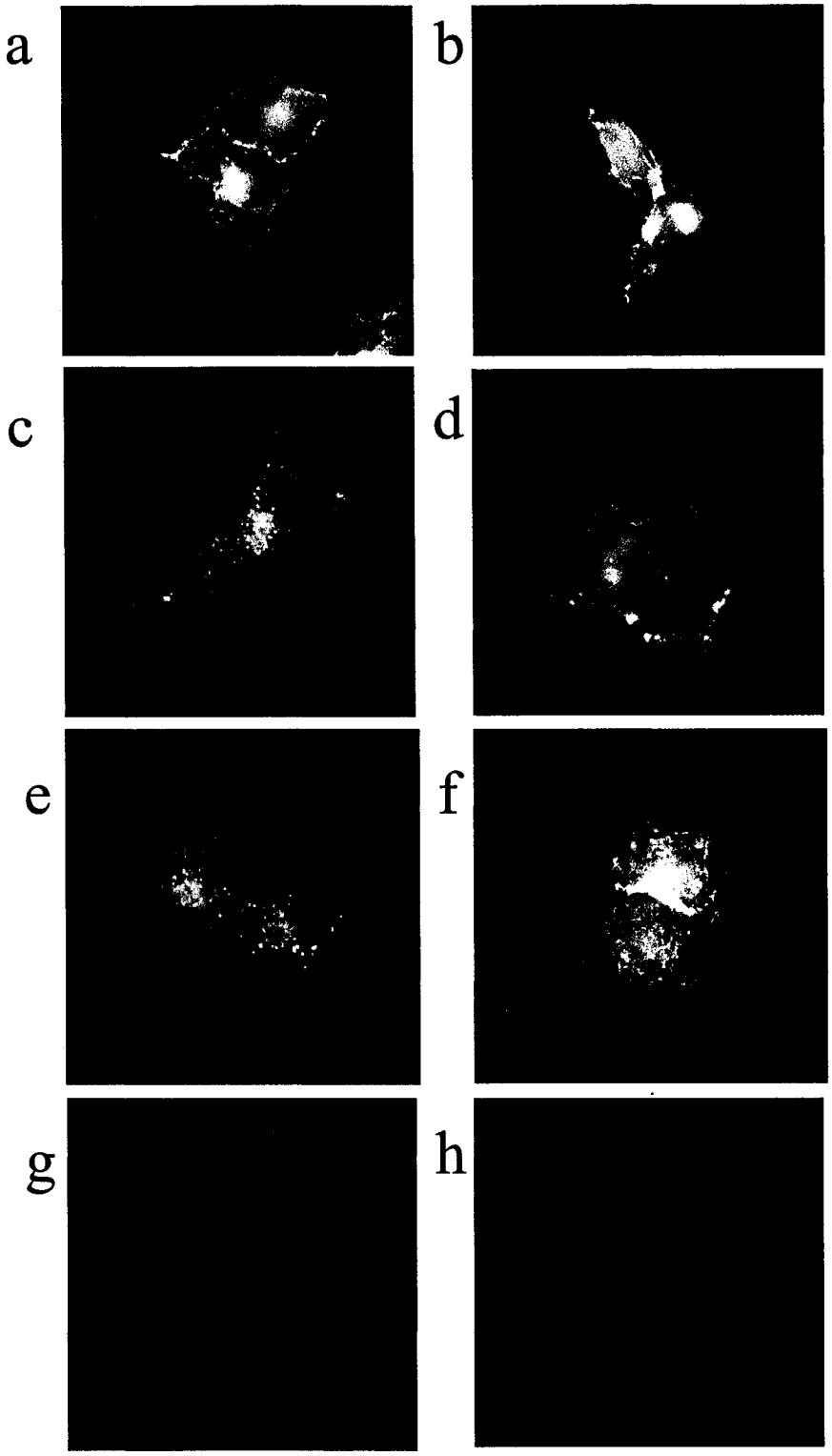
b

CD23b transcripts	b	bΔ5	bΔ6	total clones
Control cells	23 (67.7 %)	8 (23.5 %)	3 (8.8 %)	34
IL-4-treated cells	15 (60.0 %)	10 (40.0 %)	0	25

Figure 4.4 Classical and alternative CD23b transcripts expressed in IEC-4 cells. (a) Schematic sequences of the classical *b*, and alternative *Δ5*, and *bΔ6* CD23 transcripts. (TM: transmembrane region). (b) Number of clones expressing classical *b*, *bΔ5*, and *bΔ6* CD23 transcripts obtained from control and IL-4-treated IEC-4 cells.

Classical and alternative transcripts of CD23b are all capable of binding IgE, but show different endocytic properties.

To investigate whether the novel alternative transcripts of CD23*b* encode for functional proteins, plasmids containing the cDNA of the alternative and classical forms were used to transiently transfect HeLa cells. Immunostaining (using permeabilizing buffer) revealed that the different transcripts were translated into proteins and correctly folded since they were recognized by a well-characterized anti-CD23 antibody (33) (Fig. 4.5a,c,e). The protein encoded by the classical CD23*b* transcript was localized mainly on the cell surface at steady state, whereas the proteins encoded by both the *bΔ5* and *bΔ6* forms were found on intracellular vesicular structures as well as on the cell surface (Fig. 4.5a,c,e). These findings were confirmed by confocal microscopy (data not shown). No staining was seen in mock-transfected cells and neighboring nontransfected cells (Fig. 4.5g). To confirm that the expressed CD23 proteins were functional, CD23- and mock-transfected cells were incubated with monoclonal mouse IgE at 4°C, and stained with a secondary anti-IgE antibody. Bright membrane staining was observed in cells transfected with the classical *b* isoform (Fig. 4.5b), as well as in cells transfected with the alternative *bΔ5* and *bΔ6* forms (Fig. 4.5d and 4.5f). IgE binding was specific since it was not observed in mock-transfected cells (Fig. 4.5h). Taken together, these results indicate that all of the CD23b transcripts expressed in intestinal epithelial cells are translated into functional IgE receptors.


a
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h

Figure 4.5 Expression of and IgE binding to proteins encoded by different CD23*b* transcripts in transfected HeLa cells. HeLa cells were transfected with plasmids encoding classical CD23 *b* (panels a,b), *bΔ5* (panels c,d) or *bΔ6* (panels e,f) transcripts or with empty vector (panels g,h). Protein expression was identified using a monoclonal anti-CD23 antibody (panels a,c,e,g). Monoclonal mouse IgE was used for the examination of IgE binding (panels b,d,f,h).

We next examined if the *bΔ5* protein, that is upregulated by IL-4, demonstrated functional differences compared with the classical CD23*b* protein. Hela cells were transiently transfected with the classical *b* or the *bΔ5* transcript; the proteins expressed on these cells were tested for their ability to mediate the internalization of bound anti-CD23 antibodies (Fig. 4.6). Confocal microscopy showed that after 30 min incubation at 37°C, membrane bound anti-CD23 was found in intracellular vesicles in *bΔ5* expressing cells (Fig. 4.6b). These vesicles were identified as early endosomes by colocalization with internalized transferrin (Fig. 4.6d and 4.6f). This was not the case in classical *b* expressing cells where the bound anti-CD23 antibodies remained on the plasma membrane surface while transferrin was internalized (Fig. 4.6a,c,e). The same results were obtained for IgE uptake (data not shown). These results provide evidence that the protein encoded by *bΔ5*, but not the classical *b* transcript, is endocytosed upon ligand binding, suggesting different functions for *bΔ5* and classical *b* in epithelial cells.

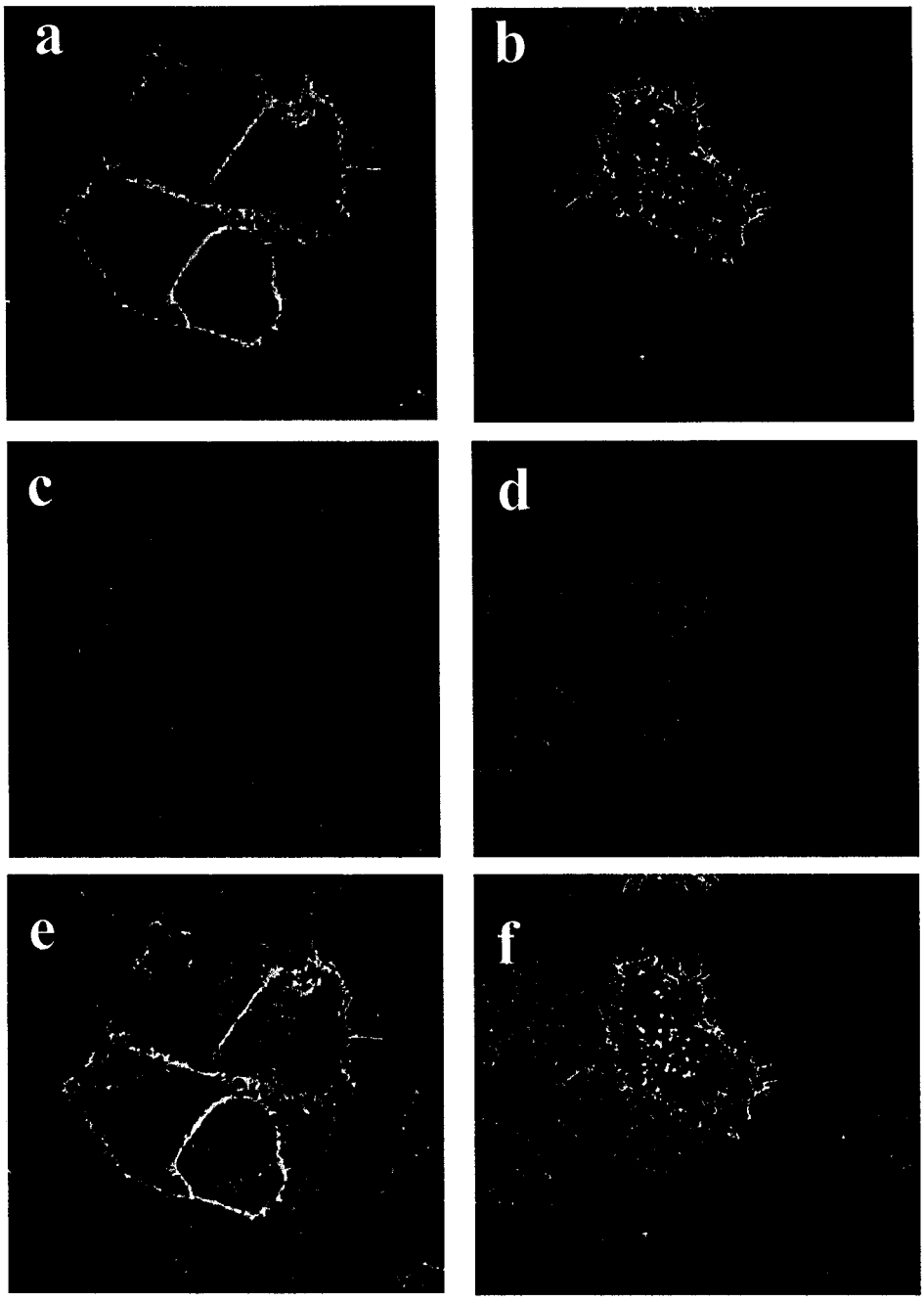
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Figure 4.6 Endocytic properties of proteins encoded by classical *b* and *bΔ5* CD23 transcripts. HeLa cells transfected with plasmids encoding classical *b* (panels a,c,e) and *bΔ5* (panels b,d,f) CD23 transcripts were incubated with anti-CD23 antibody at 4°C for 1 h, washed, and then incubated in the presence of an early endosomal marker, transferrin, at 37°C for 30 min to allow for internalization. Fluorescence images were obtained by confocal microscopy. CD23 is indicated by the green staining (panels a,b); transferrin by the red staining (panels c,d); colocalization of CD23 and transferrin is indicated by the yellow color (panels e,f).

Discussion

We previously reported increased transepithelial antigen transport (~20 fold normal) in the intestine of sensitized rodents, resulting in rapid production of allergic symptoms (12-15). In this study, we confirmed that CD23 is critical for both phase I and phase II of enhanced transepithelial antigen transport since neither occurred in $CD23^{-/-}$ mice. In addition, we characterized the murine epithelial CD23 isoform as type *b* and showed that IL-4 upregulates a unique splice variant that binds IgE and undergoes endocytosis.

The high serum titer of HRP-specific IgE verified that both $CD23^{+/+}$ and $CD23^{-/-}$ mice were similarly sensitized. $CD23^{-/-}$ mice have been reported to display normal differentiation and phenotypes of lymphocyte populations, and to mount normal antibody responses to immunization and parasite infection (36,37). HRP uptake in jejunal enterocytes was significantly enhanced in sensitized $CD23^{+/+}$ mice, but not $CD23^{-/-}$ mice, compared to non-sensitized controls at 2 min post challenge. In addition to the enhanced uptake, the rate of antigen transport across enterocytes in sensitized $CD23^{+/+}$ mice was extremely rapid, with HRP already present in the lamina propria at 2 min post challenge. In contrast, HRP containing endosomes were limited to the apical region of enterocytes in sensitized $CD23^{-/-}$ mice at the 2 min time point. A further increase in antigen uptake was seen in sensitized $CD23^{+/+}$ mice at 60 min post challenge, and HRP containing endosomes were widely distributed within the enterocytes, as well as in the lamina propria. In contrast, in non-sensitized $CD23^{+/+}$, and non-sensitized and sensitized $CD23^{-/-}$ mice, HRP was only found in endosomes in the apical region of the cells. Several studies have shown

that soluble proteins internalized from the apical side of polarized epithelial cells are transported to the late endosomes/lysosomes found in the supranuclear region (9,38,39). Our results showing that HRP in basal enterocyte endosomes at 60 min in $CD23^{+/+}$ mice suggest that binding of antigen to IgE/CD23 protects it from intracellular degradation in late endocytic compartments.

At 60 min post challenge, HRP was also found in the paracellular spaces between intestinal epithelial cells in sensitized $CD23^{+/+}$ mice, but not in sensitized $CD23^{-/-}$ mice. In addition, there was a significant increase in the overall transmucosal HRP flux in sensitized $CD23^{+/+}$ mice compared with the other groups. This enhanced overall flux probably involves HRP transported via both the paracellular and transcellular pathway. Taken together, these results suggest that the lack of CD23 abolished the enhanced antigen uptake and rapid transcellular transport in phase I leading to the absence of enhanced antigen flux in phase II (previously reported to be mediated by antigen-induced mast cell activation (13)).

Expression of murine CD23 has been reported in B cells, T cells, and follicular dendritic cells (17), and we recently demonstrated CD23 protein expression by immunostaining in intestinal epithelial cells in sensitized rats and mice (14,15). In B cells, CD23 facilitates antigen uptake and focusing (40,41). Results presented here and in our previous studies (14,15) show that CD23 plays a similar role in facilitating antigen entry into and transport across enterocytes. We previously showed in sensitized rats by immunogold labeling of enterocyte CD23 that ligand binding induced internalization of both CD23 and antigen within the same endosomal compartment (14). Moreover,

transcytosis of IgA and IgG across intestinal epithelial cells is mediated by specific Fc receptors, i.e. pIgR and FcRn, and binding of immunoglobulin to its receptor circumvents degradation during intracellular endosomal transport (42,43). Therefore, it is likely that CD23 is responsible for transepithelial transport of intact antigens by internalization of IgE/antigen complexes at the apical surface and intracellular transport of these complexes across the cell.

Two isoforms of CD23, *a* and *b*, have been reported in humans. The *a* isoform is constitutively expressed in B cells, whereas IL-4 induces the expression of isoform *b* in B cells and non-B cells including monocytes, eosinophils, and keratinocytes (18,19). The amino acid sequences of the CD23 proteins encoded by the different isoforms *a* and *b* differ only in their 6/7 N-terminal residues, a region that corresponds to the cytoplasmic domain (20), suggesting that this region regulates divergent intracellular trafficking and/or signaling pathways. In mice, B cells express CD23*a* (17), but the existence of an IL-4-inducible *b* like isoform remains controversial (24,25). Only one group has reported a murine *b* isoform (30). In our studies, we found that spleen cells isolated from sensitized mice expressed only the CD23*a* transcript; whereas intestinal epithelial cells expressed exclusively isoform *b*. The CD23*b* transcript was constitutively expressed in IEC-4 cells and its expression was upregulated by IL-4. Moreover, we also detected the presence of CD23*b* transcripts in both murine intestine and isolated enterocytes, indicating that isoform *b* exists not only in cultured mouse intestinal epithelial cells but also *in vivo*.

In addition to the classical transcript of CD23*b*, we identified two novel alternative transcripts lacking the entire sequence of exon 5 (*bΔ5)* or exon 6 (*bΔ6*). In human and

mouse B cells, there have been reports of alternative splice forms of CD23 transcripts, mainly lacking exon 3 encoding the transmembrane region of the protein (44,45), but to our knowledge no reports of the novel transcripts we identified. The repeated heptad amino acid sequences derived from exon 5 to 8 in the mouse CD23 transcript make up the hydrophobic core of the stalk region of the protein and are important for regulating the affinity of IgE binding (46,47). The existence of a number of alternative transcripts of CD23 may imply functional discrepancies for the different forms of the protein. We demonstrated that IL-4 treatment promoted the expression of *bΔ5* transcript, suggesting that sensitization involving increased IL-4 production, alters the expression of CD23, not only quantitatively but also qualitatively. Furthermore, we detected the presence of *bΔ5* transcript in enterocytes isolated from sensitized mice.

Immunofluorescence studies using transfected cells demonstrated that the classical and novel CD23*b* transcripts were translated into proteins. The localization of classical CD23*b* proteins was mainly on the cell surface, whereas the *bΔ5* and *bΔ6* proteins were found in intracellular vesicular structures and on the cell surface. The intracellular location of the alternative CD23*b* proteins at steady state may represent either retention of the newly synthesized proteins in intracellular compartments or increased turnover of membrane proteins due to constitutive endocytosis. We identified that both the classical and alternative CD23*b* proteins expressed on the cell surface are functional IgE receptors.

We demonstrated that the proteins encoded by the classical CD23 *b* and *bΔ5* transcripts display different endocytic properties in transfected cells. The *bΔ5*, but not the

classical *b* form, translated into protein that was endocytosed upon ligand binding. The anti-CD23 antibody, previously shown to attach to the IgE binding site on the lectin domain of the CD23 receptor (33), induced internalization by the *bΔ5* protein, as did IgE. These data are in agreement with the results obtained in human B cells showing that the classical *b* isoform does not mediate the internalization of membrane bound anti-CD23 antibody (20). Therefore, the *bΔ5* form of the CD23 receptor may be responsible for enhanced transepithelial transport of IgE or IgE/allergen complexes following sensitization.

In summary, our study demonstrated a functional role for CD23, most likely the protein encoded by a novel splice form of CD23*b*, in facilitating the IgE-mediated enhanced antigen transport across mouse intestinal epithelium. Our findings suggest that antigen binding to IgE/CD23 bypasses the lysosomal degradative pathway resulting in large quantities of antigen penetrating the epithelial barrier. This antigen is then available to activate mast cells resulting in further increased transmucosal antigen flux and the symptoms of intestinal anaphylaxis.

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CHAPTER 5: Endocytic properties of classical and alternative splice forms of intestinal epithelial CD23/FcεRII isoform b

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Abstract

We previously demonstrated CD23 as the receptor mediating enhanced transepithelial antigen transport via IgE binding in rodent models of intestinal hypersensitivity (food allergy). The aim of the current study was to correlate the expression of different transcripts of CD23 with their endocytic properties. RT-PCR and plasmid cloning revealed the presence of the classical form of CD23*b* transcripts, and also novel alternative forms lacking exon 5 (*b*Δ5) or exon 6 (*b*Δ6), in cultured mouse intestinal epithelial cells. Transfection studies showed that classical CD23*b* proteins were expressed mainly on the cell surface, whereas *b*Δ5 and *b*Δ6 proteins were expressed in intracellular locations as well as on the cell surface, suggesting continuous cycling of the alternative forms between the cell membrane and intracellular compartments. All three forms of CD23*b* proteins were capable of IgE binding, and displayed unique endocytic properties. Endocytosis of the alternative *b*Δ5 and *b*Δ6 proteins, but not the classical CD23*b* protein, was observed after binding with saturated concentrations of anti-CD23 or monomeric IgE, suggesting constitutive endocytosis of the two alternative forms that agrees with their intracellular localization at steady state. Classical CD23*b* proteins were only endocytosed upon IgE cross-linking of the receptor induced by antigen binding. Endocytosis of *b*Δ5 proteins, but not the *b*Δ6 protein, was also observed following antigen-induced IgE cross-linking. These results suggest that the different forms of CD23*b* proteins may play distinct roles in conditions such as intestinal hypersensitivity.

Introduction

Food allergy is a disorder that leads to gastrointestinal symptoms, such as nausea, vomiting, abdominal pain, and diarrhea, in sensitized individuals rapidly after food antigen ingestion. The anaphylactic reactions are induced by mediators released from submucosal mast cells that are activated by undigested food antigens (1). Increased permeability of the intestinal epithelial barrier and enhanced transepithelial transport of specific antigen have been documented in patients with food allergy (2), and in animal models of hypersensitivity (3-6). Recently, the novel mechanism accounting for the enhanced antigen transport was identified to be mediated by IgE binding to a low affinity IgE receptor, CD23/FcεRII, expressed on the intestinal epithelial cells (5,6). However, the molecular and functional characteristics of the epithelial CD23 is not fully understood.

We previously demonstrated that following sensitization, increased expression of intestinal epithelial CD23 paralleled the phenomenon of enhanced transepithelial antigen transport (5,6). Moreover, mice passively sensitized by injecting immune serum showed increased antigen uptake in intestinal epithelial cells, but not if the serum was first depleted of IgE (6). The enhanced transport phenomenon was totally abolished in the intestine of active sensitized CD23-deficient mice (6,7). Furthermore, electromicrographic studies revealed that after luminal antigen challenge to sensitized intestine, the numbers of immunogold-labeled CD23 decreased along the apical surface of epithelial cells, followed with co-localization of CD23 and antigen in the same endosomal compartments inside the cells (5). Taken together, these results indicated a novel transepithelial transport system

involving antigen binding to IgE that is bound to CD23 at the apical membrane of enterocytes, and this binding induces endocytosis of CD23/IgE/allergen complexes.

Two isoforms of CD23, *a* and *b*, were identified in human and mouse. In human, CD23 isoform *a* is constitutively expressed in B cells, isoform *b* is expressed upon IL-4 treatment in B cells, monocytes, eosinophils, and keratinocytes (8). In mice, the homologue of the isoform *a* is also constitutively expressed in B cells (8,9) but in contrast to human, it is upregulated by IL-4 treatment (9). Moreover, differential endocytic properties between CD23 isoform *a* and *b* have been demonstrated in human. Transfection studies showed that CD23*a* is constitutively endocytosed through clathrin-coated pits, whereas CD23*b* is involved in phagocytosis of IgE opsonized particles, suggesting different functions of the two isoforms (10). Using RT-PCR, we previously demonstrated that mouse intestinal epithelial cells constitutively express isoform *b* (7). In addition, sequencing analysis demonstrated the presence of classical CD23*b* transcripts and also two unique alternative forms lacking exon 5 (*b*Δ5) or 6 (*b*Δ6). Treatment of IL-4 to cultured cells upregulated the expression of the classical *b* and *b*Δ5 CD23 transcript, with a proportional increase of the latter, while decreased the expression of *b*Δ6 transcript (6,7).

The aim of the current study was to elucidate the endocytic properties of the classical and alternative forms of CD23*b* proteins. Plasmids encoding the cDNAs of various forms of CD23*b* were transfected into HeLa cells. The endocytic manner of the CD23 isoforms was observed following the uptake of anti-CD23 antibody and of monoclonal murine IgE cross-linked or not by the antigen. The diversified patterns of

endocytosis in classical CD23*b*, *b*Δ5 and *b*Δ6 proteins may suggest distinct functions on intestinal epithelial cells.

Methods and Materials

Cell lines. Mouse small intestinal epithelial cells of the IEC-4 cell line (6,11) were cultured in DMEM (Invitrogen Corporation, Carlsbad, CA) supplemented with 5% FCS, 0.01M HEPES, 20 mM L-glutamine, 0.1 U/ml Penicillin G sodium, and 5 μg/ml streptomycin sulfate. 10^6 cells/ml were seeded in 60 mm diameter cell culture plates (Corning, Corning, NY) for 3 days until confluent. HeLa cells were cultured in DMEM media supplemented with 10% FCS, 20 mM L-glutamine, and 5 μg/ml streptomycin sulfate to subconfluency on coverslips for transfection studies.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR). RT-PCR was performed on RNA extracted from cultured mouse intestinal epithelial cells of the IEC-4 cell line. Confluent IEC-4 cells were processed for RNA extraction using RNeasy Mini kit (Qiagen, Missisauga, ON, Canada) according to manufacturer's instruction. RNA (3 μg) was reverse-transcribed with Oligo $d(T)_{12-18}$ (Invitrogen). cDNA (150 ng) was subjected to PCR by mixing with 0.4 mM of each dNTP (Pharmacia), 2 mM $MgCl_2$ (Perkin-Elmer, Roche Molecular Systems Inc., Branchburg, NJ), 1x PCR buffer (Perkin-Elmer), 0.8 μM of both upstream and downstream primers (see below), and 1 unit of AmpliTaq DNA polymerase (Applied Biosystems, Roche Molecular Systems Inc.).

Two sets of primers were used for PCR. The first set of primers, including upstream primer Oligo-B' (5'-ATGAATTCTCAAAACCAGGGA-3') and downstream

primer Oligo-F' (5'-TCAGGGTTCACTTTTTGGG-3'), was used to amplify the full-length coding region from ATG to the stop codon of CD23. A DNA thermal cycler (Biometra thermal cycler) was programmed to perform a protocol as follows: 94°C for 5 min for 1 cycle; 94°C for 30 sec (denaturation), 58°C for 30 sec (annealing) and 72°C for 1 min (extension) for 35 cycles; and 72°C for 5 min for final extension. The second set of primers, including upstream primer Oligo-B (5'-GAAAGCCAATTTGAACGGGAACTTGG-3') and downstream primer Oligo-E (5'-GGAGCCCTTGCCAAAATAGTAGCAC-3'), was used to amplify a shorter region of CD23 cDNA from base 1 to 642 (base number according to sequence of clone pERB452, GenBank entry: X64223). Negative controls were performed with samples lacking cDNA templates. RT-PCR products were then electrophoresed in either 0.8 % or 1 % agarose gel in the presence of 0.5 μg/ml ethidium bromide and visualized with a UV transilluminator. Molecular weight markers, Ready load ϕ X174 RF DNA/*Hae* III Fragments (Invitrogen) were used. RT-PCR bands encoding the full length of CD23 cDNAs were cut from the gel and kept frozen at –20°C overnight in a Spin-X centrifuge tube filter (0.4 μm cellulose acetate; Corning Costar Corp., Cambridge, MA). The cDNA was extracted using the classical phenol-chloroform methods, and cloned into plasmids (see below).

Plasmids and DNA Sequence analysis. PCR products were extracted from the electrophoresed gel, cloned into pCR3.1 plasmids and amplified by transforming TOP

10F' competent cells using a eukaryotic bidirectional TA cloning Kit (Invitrogen). Transformed competent cells were plated on a Luria-Bertani (LB) agar plate containing 50 μg/ml of ampicillin and incubated overnight. Individual colonies were grown in ampicillin-containing LB broth overnight and plasmidic DNA was purified using the Qiaprep Miniprep kit (Qiagen). Clones containing CD23 cDNAs were sent for nucleotide sequencing (Eurogentec, Seraing, Belgium). Clones with cDNAs in the correct orientation were selected using appropriate restriction sites and used for transient transfection (see below). Plasmids containing CD23 isoform *a* cDNA were obtained from Dr. D.H. Conrad, and were used for transient transfection as positive controls for experiments.

Transfection, immunofluorescence and endocytosis. HeLa cells were transfected with CD23 encoding plasmids using a calcium phosphate transfection kit (Invitrogen) and were processed for immunofluorescence studies the following day as previously described (12,13). Briefly, transfected HeLa cells were washed with phosphate-buffered solution (PBS) and fixed with 4% paraformaldehyde and 0.03 M sucrose at 4°C for 30 min and quenched with 50 mM NH_4Cl in PBS at room temperature for 10 min. Cells were incubated with monoclonal anti-CD23 antibody (B3B4, rat IgG2a anti-mouse CD23 (14)) (20 ug/ml) in a permeabilizing buffer (phosphate-buffered solution containing 0.1% bovine serum albumin (BSA) and 0.05% saponin (Sigma)) at room temperature for 30 min, washed twice with the permeabilizing buffer, and then incubated with goat anti-rat IgG antibody conjugated with Alexa Fluor 488 (1:100) (Molecular probes, Eugene, OR) in

permeabilizing buffer, and washed twice. Cells were mounted on microscope slides in 100 mg/ml Mowiol (Calbiochem, La Jolla, CA), 25% glycerol, 100 mM Tris-HCl, pH 8.5. Negative controls included staining where the primary antibody was omitted as well as mock transfected HeLa cells.

For IgE binding studies, HeLa cells transiently transfected with CD23 isoforms were incubated with monoclonal mouse anti-DNP IgE (5 μg/ml) (Sigma) in an IgE-binding solution (DMEM media containing 0.4 mM $Ca(NO_3)_2 \cdot 4H_2O$, and 0.1% BSA (15,16) at 4°C for 1 h, washed twice in cold IgE binding solution and then fixed with 4% paraformaldehyde as described above. To reveal membrane bound IgE, cells were incubated with 10 μg/ml monoclonal rat IgG1 anti-mouse IgE antibodies (Southern Biotechnology, Birmingham, AL) in PBS containing 0.1% BSA (50 μl) at room temperature for 30 min, washed, and stained with goat anti-rat IgG antibodies (1:100) conjugated with Alexa Fluor 488 (Molecular Probes).

Endocytosis of CD23 and transferrin was performed on subconfluent HeLa cells grown on coverslips one day after transfection. The cells were first incubated for 20 min at 37°C in DMEM to eliminate receptor-bound transferrin, and washed in cold PBS. For anti-CD23 antibody uptake, cells were incubated with saturated amount B3B4 (50 μg/ml) in DMEM containing 1 mg/ml BSA (DMEM-BSA) for one hour at 4°C. Cells were washed two times in DMEM-BSA and then incubated in DMEM-BSA containing 100 nM Alexa Fluor 594-conjugated transferrin (Molecular probes). After incubation at 37°C for 15 or 30 min, cells were rapidly cooled to 4 °C using cold DMEM-BSA, washed twice in

cold PBS and then fixed for one hour at 4°C. The anti-CD23 antibody was revealed using the Alexa Fluor 488-labeled goat anti-rat IgG secondary antibody (1:100) (Molecular probes). For IgE uptake, cells were incubated with the monoclonal anti-DNP mouse IgE (5 μg/ml) (Sigma) in an IgE-binding solution at 4°C for 1 h, twice in the cold solution, and then incubated in the solution containing 100 nM Alexa Fluor 594-conjugated transferrin (Molecular probes) at 37°C for different times before fixing. The location of IgE was then stained with rat IgG1 anti-mouse IgE (Southern Biotechnology) and goat anti-rat IgG antibodies conjugated with conjugated with Alexa Fluor 488 (Molecular Probes) in the permeabilizing buffer.

For studies of endocytosis of CD23 induced by antigen cross-linking of bound IgE, transfected cells (eliminated with endogenous transferrin) were incubated with monoclonal anti-dinitrophenyl (DNP) IgE (Sigma) in IgE-binding solution at 4°C for 1 h, washed twice in the cold solution, and then incubated in IgE-binding solution containing 0.01 μg/ml of DNP-BSA (dinitrophenylated bovine serum albumin; Molecular Probes), and 100 nM Alexa Fluor 594-conjugated transferrin (Molecular probes) at 37°C for different times. After incubation, the cells were rapidly cooled to 4 °C using cold IgE-binding solution, washed twice, and fixed for one hour at 4°C. Fixed cells were stained with 10 μg/ml monoclonal rat IgG1 anti-mouse IgE antibodies (Southern Biotechnology) in the permeabilizing buffer at room temperature for 30 min, washed, and stained with goat anti-rat IgG antibodies (1:100) conjugated with Alexa Fluor 488 (Molecular Probes) in permeabilizing buffer. Prior to the experiments, the preservative (sodium azide) in

monoclonal anti-DNP IgE was eliminated using a Centricon centrifugal filter device YM-10 (Millipore Corp., Bedford, MA) following the manufacturer's instruction.

The samples were examined under an epifluorescence microscope (Axioplan II, Zeiss) attached to a cooled CCD-camera (Spot-2, Diagnostic Instruments) or under a confocal microscope (LSM 510, Zeiss). Alexa Fluor 488 and 594 corresponding staining were observed using the classical FITC and rhodamine/TexasRed filters respectively.

Results

1) Expression of classical and alternative forms of CD23*b* transcripts in mouse intestinal epithelial cells

We previously identified the isoform of CD23 expressed in cultured mouse intestinal epithelial cells to be subtype *b* using RT-PCR. The PCR band corresponding to full-length CD23 were extracted and cloned into the PCR3.1 vector for further sequence analysis. The majority of the clones displayed the exact sequence of CD23*b* (clone pERB452, GenBank entry: X64223), denoted here as the classical CD23*b* transcript (17). In addition, several clones demonstrated internal deletions, between base 229 to 291 or base 291 to 354 (base numbers according to the sequence of pERB452). The missing regions corresponded to the entire sequences of exon 5 or exon 6, respectively, which encode for part of the extracellular stalk region of the receptor (Fig. 5.1a). The alternative CD23b transcripts lacking exon 5 is denoted CD23*b*Δ5 (GenBank accession number: AY069980), and the one lacking exon 6 is denoted CD23*b*Δ6 (GenBank Accession number: AY069981). Using a second set of primers that amplify a shorter region of CD23 cDNA between base 1-642, the resulting RT-PCR products showed the expected two bands of 642 and 579 bp on the agarose gel (Fig. 5.1b).

```
CD23b    1-atgaattctcaaaaccagggatactgggaacctcctagaaagcgttgctgctgtgcaaga
bΔ5        atgaattctcaaaaccagggatactgggaacctcctagaaagcgttgctgctgtgcaaga
bΔ6        atgaattctcaaaaccagggatactgggaacctcctagaaagcgttgctgctgtgcaaga

CD23b   61-cgtgggacacagctcatgttggtggggctgctgagcacagcaatgtgggctggcctgctg
bΔ5        cgtgggacacagctcatgttggtggggctgctgagcacagcaatgtgggctggcctgctg
bΔ6        cgtgggacacagctcatgttggtggggctgctgagcacagcaatgtgggctggcctgctg

CD23b  121-gccctgcttcttctgtggcactgggaaacggagaagaatctaaaacagctgggagacact
bΔ5        gccctgcttcttctgtggcactgggaaacggagaagaatctaaaacagctgggagacact
bΔ6        gccctgcttcttctgtggcactgggaaacggagaagaatctaaaacagctgggagacact

CD23b  181-gcaattcagaatgtctctcatgttaccaaggacttacaaaaattccagagtaatcaattg
bΔ5        gcaattcagaatg.............................................
bΔ6        gcaattcagaatgtctctcatgttaccaaggacttacaaaaattccagagtaatcaattg

CD23b  241-gcccagaagtcccaggttgttcagatgtcacaaaacttgcaagaactccaagctgaacag
bΔ5        ..............ttgttcagatgtcacaaaacttgcaagaactccaagctgaacag
bΔ6        gcccagaagtcccagg.........................................

CD23b  301-aagcaaatgaaagctcaggactctcggctctcccagaacctgaccggactccaggaggat
bΔ5        aagcaaatgaaagctcaggactctcggctctcccagaacctgaccggactccaggaggat
bΔ6        ................actctcggctctcccagaacctgaccggactccaggaggat

CD23b  361-ctaaggaacgcccaatcccagaactcaaaactctcccagaacctgaacagactccaagac
       421-gatctagtcaacatcaaatccctgggcttgaatgagaagcgcacagcctccgattctcta
       481-gagaaactccaggaagaggtggcaaagctgtggatagagatactgatttcaaagggaact
       541-gcatgcaacatatgtcccaagaactggctccatttccaacagaagtgctactattttggc
       601-aagggctccaagcagtggatccaggccaggttcgcctgcagtgacctgcaagggcgacta
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       721-tcctggattggcctccaggatctcaatatggagggagagtttgtatggtcggacgggagc
       781-cctgtgggttatagcaactggaatccaggggagcccaataacgggggccagggtgaggac
       841-tgtgtgatgatgcggggatccggccagtggaacgacgccttctgccgcagctacttggat
       901-gcatgggtgtgtgagcagctggcaacatgtgagatatctgccccttagcctctgtgact
       961-ccaacaaggcccaccccaaaaagtgaaccctgacaaacttctgctcacactcttctgga
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Figure 5.1 (A) The presence of classcical and alternative CD23*b* transcripts in mouse intestinal epithelial cells.

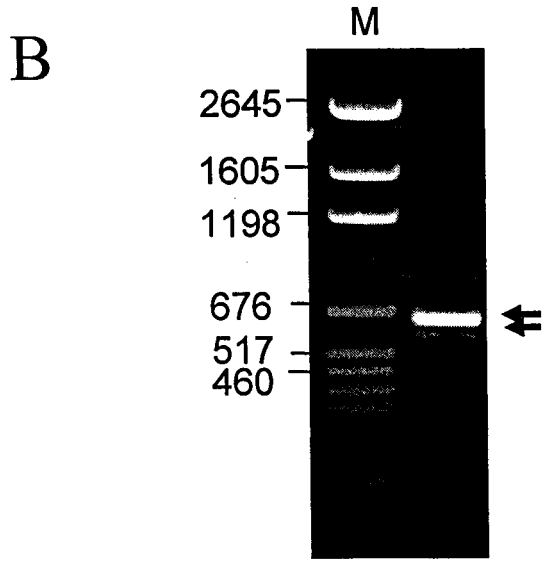


Figure 5.1 The presence of classical and alternative CD23*b* transcripts in mouse intestinal epithelial cells. (A) Nucleotide sequences of the classical and alternative CD23*b* transcripts lacking exon 5 (bΔ5) or exon 6 (bΔ6). RNA extracted from IEC-4 cells were reverse-transcribed and subjected to PCR using Oligo-B' and Oligo-F' primers to obtain the full-length encoding region of CD23 cDNA. The RT-PCR product was electrophoresed and cut from the 1% agarose gel, and cloned into pCR3.1 plasmids. The nucleotide sequences were analyzed by Sanger-based dideoxy sequencing strategy. The nucleotide sequences in bΔ5 and transcripts after base 321 were the same as the classical form, and therefore, were not repeated in the figure. (B) Electrophoresed gel showing two bands of RT-PCR products encoding for a shorter region of the CD23*b* transcripts. RNA was reverse-transcribed and subjected to PCR using Oligo-B and Oligo-E primers to obtain a shorter region of CD23*b* cDNA. Two RT-PCR bands (642 and 571 bp) were observed on the 0.8% agarose gel.

2) Classical and alternative forms of CD23*b* were translated into functional IgE receptors on cell surface of transfected HeLa cells.

To confirm that these classical and alternative transcripts of CD23*b* were translated into functional proteins, plasmids encoding for the various CD23*b* cDNAs were transfected into HeLa cells. Plasmids containing CD23*a,* the isoform mainly expressed in murine B cells, were also used in transfection for staining as a positive control.

First, cells were fixed and stained with anti-CD23 antibody in a permeabilizing buffer to examine their subcellular localization. The monoclonal anti-CD23 antibody (B3B4) was originally raised against the C-terminal IgE-binding lectin head domain of CD23 expressed on B cells (14), therefore, staining of CD23*a* proteins in transfected cells served as the positive controls here. We showed that classical CD23*b* proteins were localized mainly on the cell surface at steady state, whereas both *b*Δ5 and *b*Δ6 were found in vesicular structures as well as on the cell surface (Fig. 5.2). The intracellular localization of alternative CD23*b* isoforms was confirmed using confocal microscopy (data not shown). No staining was seen in mock-transfected cells and negative controls (Fig. 5.2).

Following incubation of monoclonal IgE with transfected cells, the presence of IgE on the surface of all three forms of cells was revealed using anti-IgE antibody. Immunofluorescence staining demonstrated that all forms of CD23*b* proteins bound IgE (Fig. 5.3). IgE was also bound to the positive control cells transfected with CD23*a*-encoding plasmids (Fig. 5.3). No staining was seen in mock-transfected cells and negative controls (Fig. 5.3). The same results were obtained in MDCK cells (data not shown),

indicating that murine CD23 isoforms expressed in non-murine cell lines are functional murine IgE receptors.

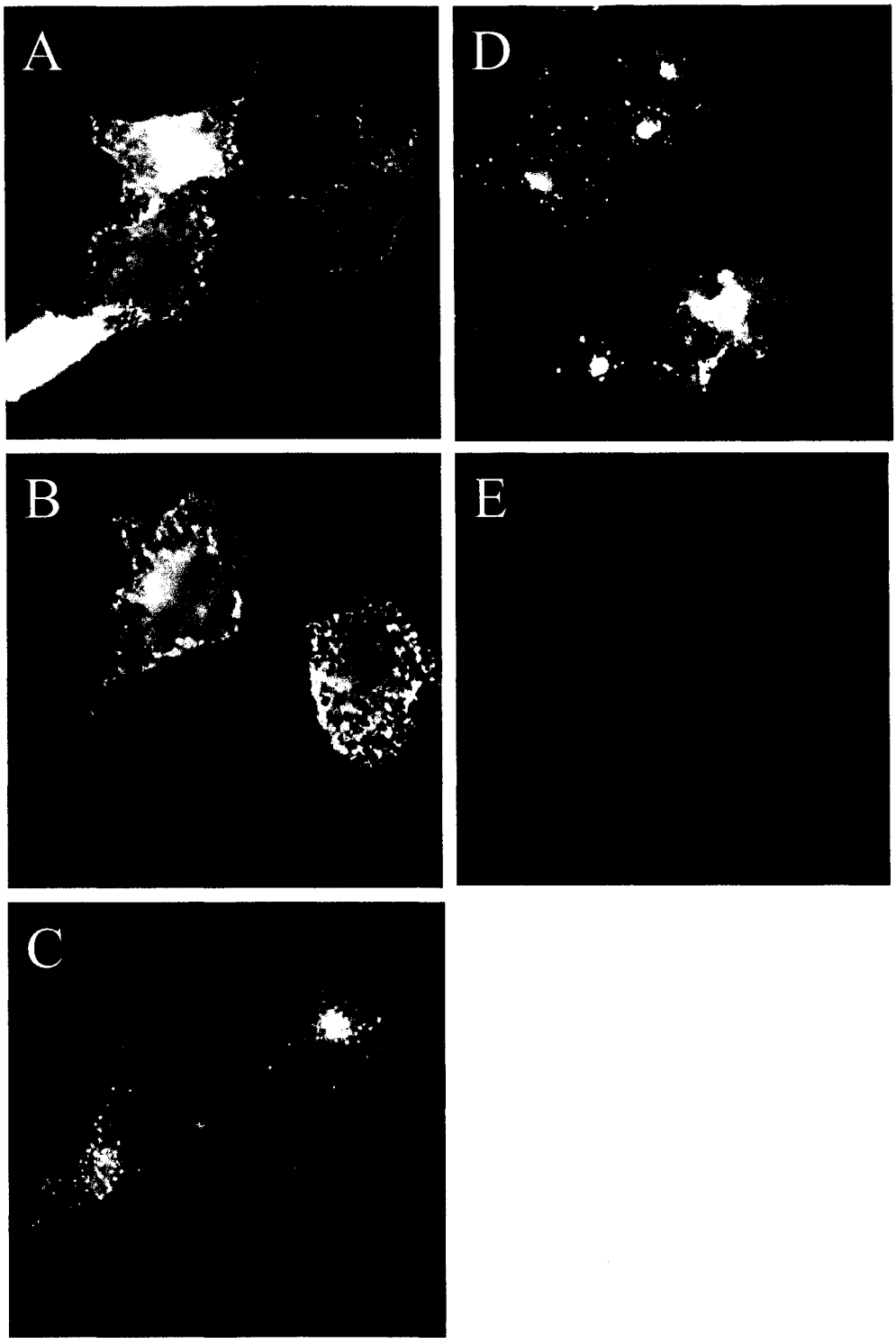

A
D
B
E
C

Figure 5.2 Expression of different forms of CD23 proteins in transfected cells. HeLa cells were transiently transfected with plasmids encoding for various forms of CD23, i.e. CD23*a*, CD23*b*, *b*Δ5, and *b*Δ6, and processed for immunofluorescence studies. Transfected cells were stained with anti-CD23 antibody in a permeabilizing buffer. Cells transfected with CD23*a*-encoding plasmids were used as positive controls, whereas mock-transfected cells were used as negative controls. These immunofluorescence images represent staining of CD23 on cells transfected with CD23a-encoding plasmids (A), classical CD23b-encoding plasmids (B), *b*Δ5-encoding plasmids (C), *b*Δ6-encoding plasmids (D), and mock plasmids (E). Classical CD23*b* proteins were expressed mainly on the cell surface, whereas alternative CD23*b*Δ5 and *b*Δ6 proteins were localized in intracellular compartments as well as on the cell surface.

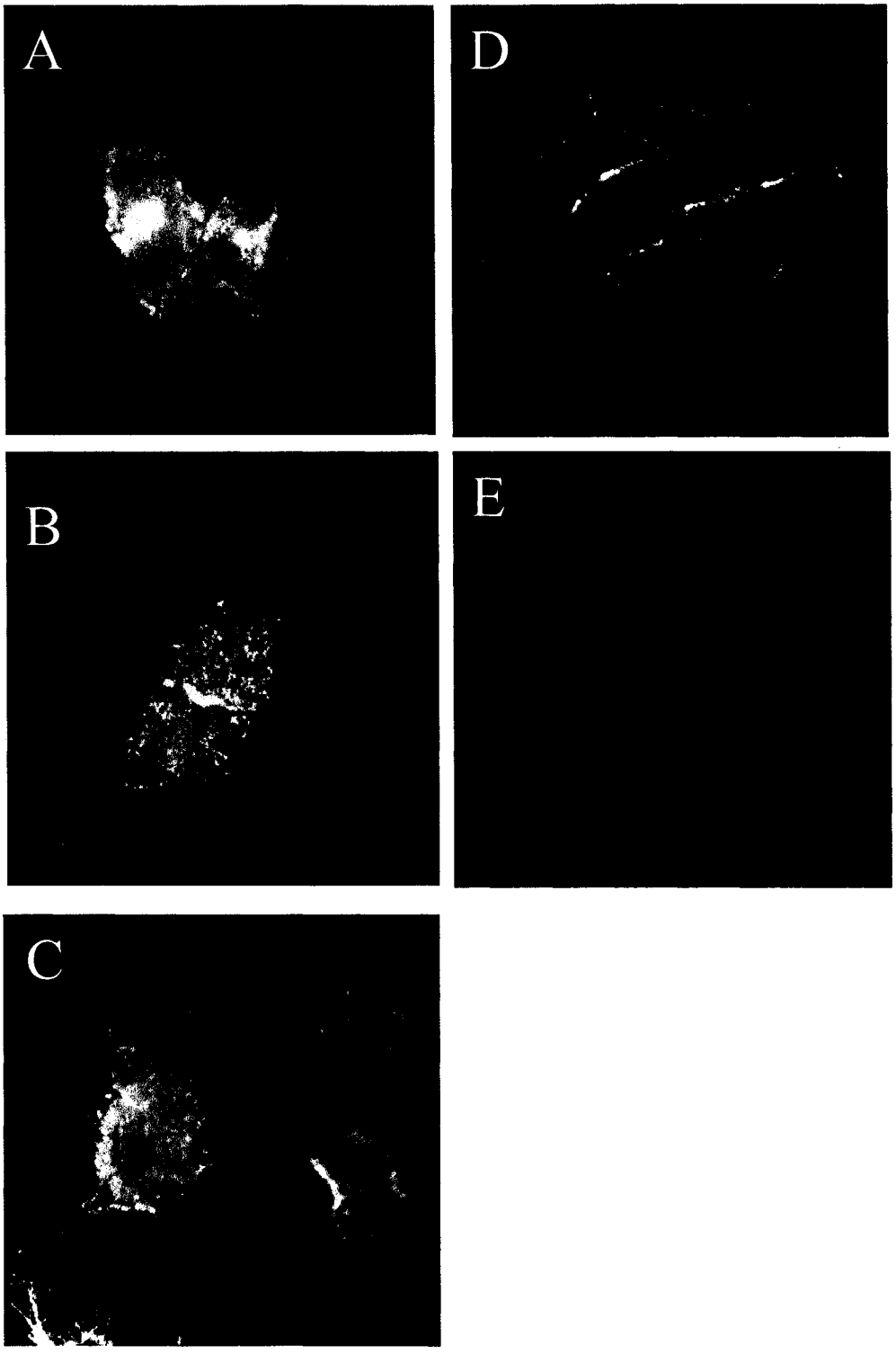
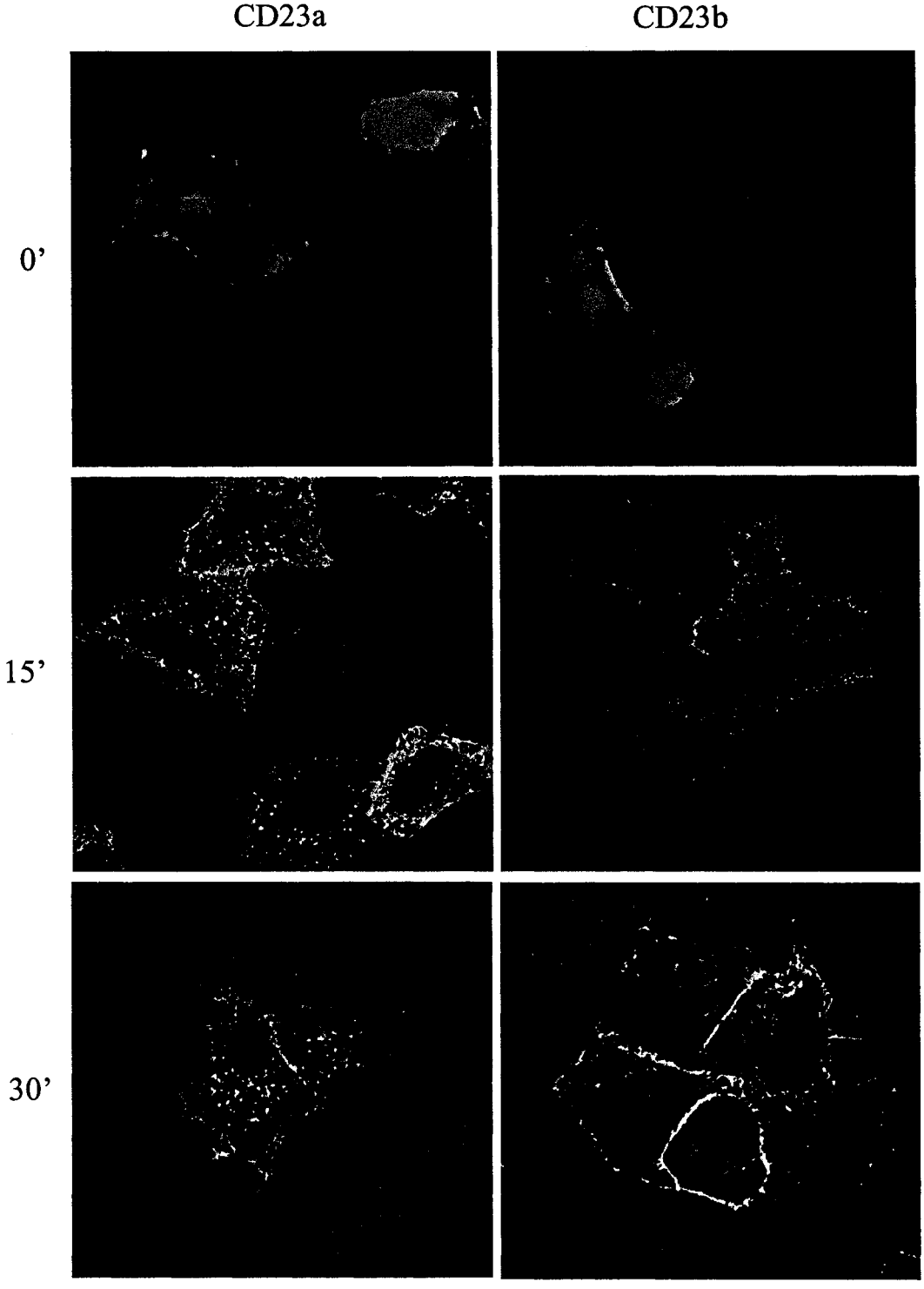

A
D
B
E
C

Figure 5.3 IgE binding of different forms of CD23 proteins on transfected cells. HeLa cells were transiently transfected with plasmids encoding classical and alternative transcripts of CD23, including CD23*a*, CD23*b*, *b*Δ5, and *b*Δ6. Monoclonal IgE was incubated with transfected cells at 4°C for 1 h for surface binding, fixed, and subsequently, stained with anti-IgE antibody. CD23*a*-transfected cells were used as positive controls, whereas mock-transfected cells were used as negative controls. These immunofluorescence images represent staining of IgE on cells transfected with CD23*a*-encoding plasmids (A), classical CD23*b*-encoding plasmids (B), *b*Δ5-encoding plasmids (C), *b*Δ6-encoding plasmids (D), and mock plasmids (E). Immunostaining showed that all forms of CD23 bound IgE on the cell surface.

3) Endocytosis of cell surface CD23*b*Δ5 and *b*Δ6, but not classical CD23*b* proteins, upon binding with saturated concentrations of anti-CD23 or monomeric IgE.

To examine the endocytic properties of the different isoforms, transiently transfected Hela cells were incubated with saturated amount of monoclonal anti-CD23 antibody. This protocol was chosen to be able to compare the obtained results with the only one published study that used a similar approach to compare the endocytic properties of human a and b isoform (10). Immunofluorescence studies revealed that CD23*a* was internalized after both 15 and 30 min of incubation at 37°C. The internalized receptor co-localized with transferrin, indicating that CD23*a* was endocytosed into early endosomes (Fig. 5.4). In agreement with the results found in humans (10), no internalization of the classical CD23*b* was seen up to 30 min incubation (Fig. 5.4). In contrast to the classical CD23*b*, both alternative forms, *b*Δ5 and *b*Δ6 showed internalization of receptor at 15 min and 30 min incubation (Fig. 5.4). Co-localization studies showed that CD23*b*Δ5 and *b*Δ6 were internalized into early endosomal compartments containing transferrin (Fig. 5.4). Similar findings were obtained when monomeric IgE was used to bind CD23 (not shown).


CD23a
CD23b
0'
15'
30'

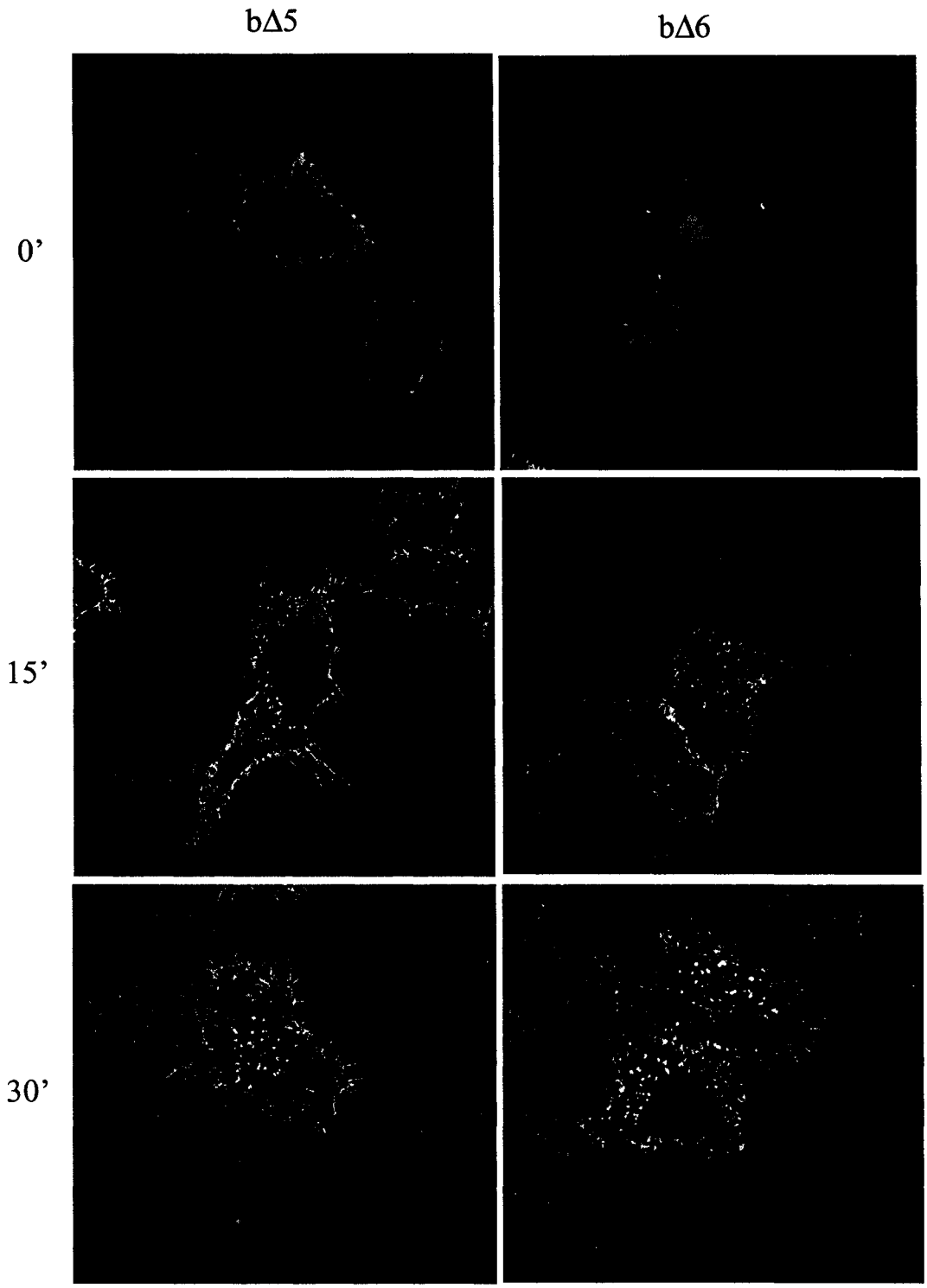
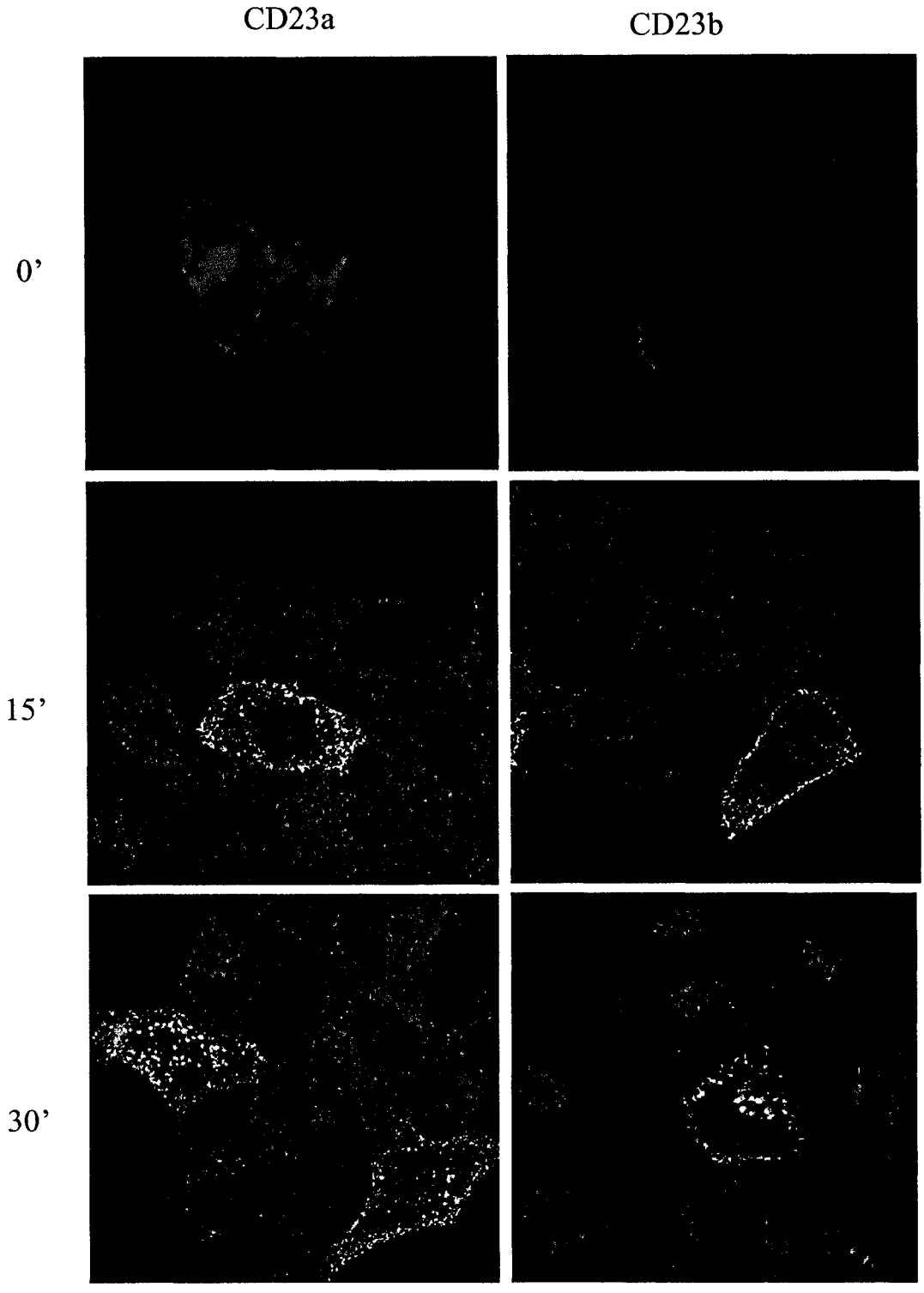

bΔ5
bΔ6
0'
15'
30'

Figure 5.4 Endocytosis of various forms of CD23 proteins upon binding with a saturating concentration of anti-CD23. Saturated amount of anti-CD23 antibody was incubated with cells transiently transfected with plasmids encoding cDNAs of CD23*a*, CD23*b*, *b*Δ5, and *b*Δ6 at 4°C for 1 h for surface binding, washed, and then incubated with fluorescence-conjugated transferrin (Tf) at 37°C for 0, 15, and 30 min for internalization. Transferrin (Tf) was used as an early endosomal marker. Binding of anti-CD23 on the cell surface (0 min incubation at 37°C) was shown using an epifluorescence microscope. The results of internalization after incubation at 37°C for 15 and 30 min were investigated by confocal microscopy using vertical optical sections. CD23 is indicated by the green staining; transferrin by the red staining; colocalization of CD23 and transferrin is indicated by the yellow color.

4) Antigen-induced IgE cross-linking results in endocytosis of classical CD23*b* and *b*Δ5, but not *b*Δ6 proteins.

To examine the effect of cross-linking of the receptor, anti-DNP IgE was bound to cell surface CD23, and subsequently subjected to cross-linking of DNP-BSA antigen. Following this treatment, CD23*a* was internalized at 15 and 30 min incubation. The internalized CD23*a* co-localized with transferrin in early endosomes (Fig. 5.5). The classical CD23*b* and *b*Δ5 showed internalization into early endosomes only at 30 min incubation. In contrast, CD23*b*Δ6 was not internalized and remained on the cell surface even after 30 min incubation (Fig. 5.5).


CD23a
CD23b
0'
15'
30'

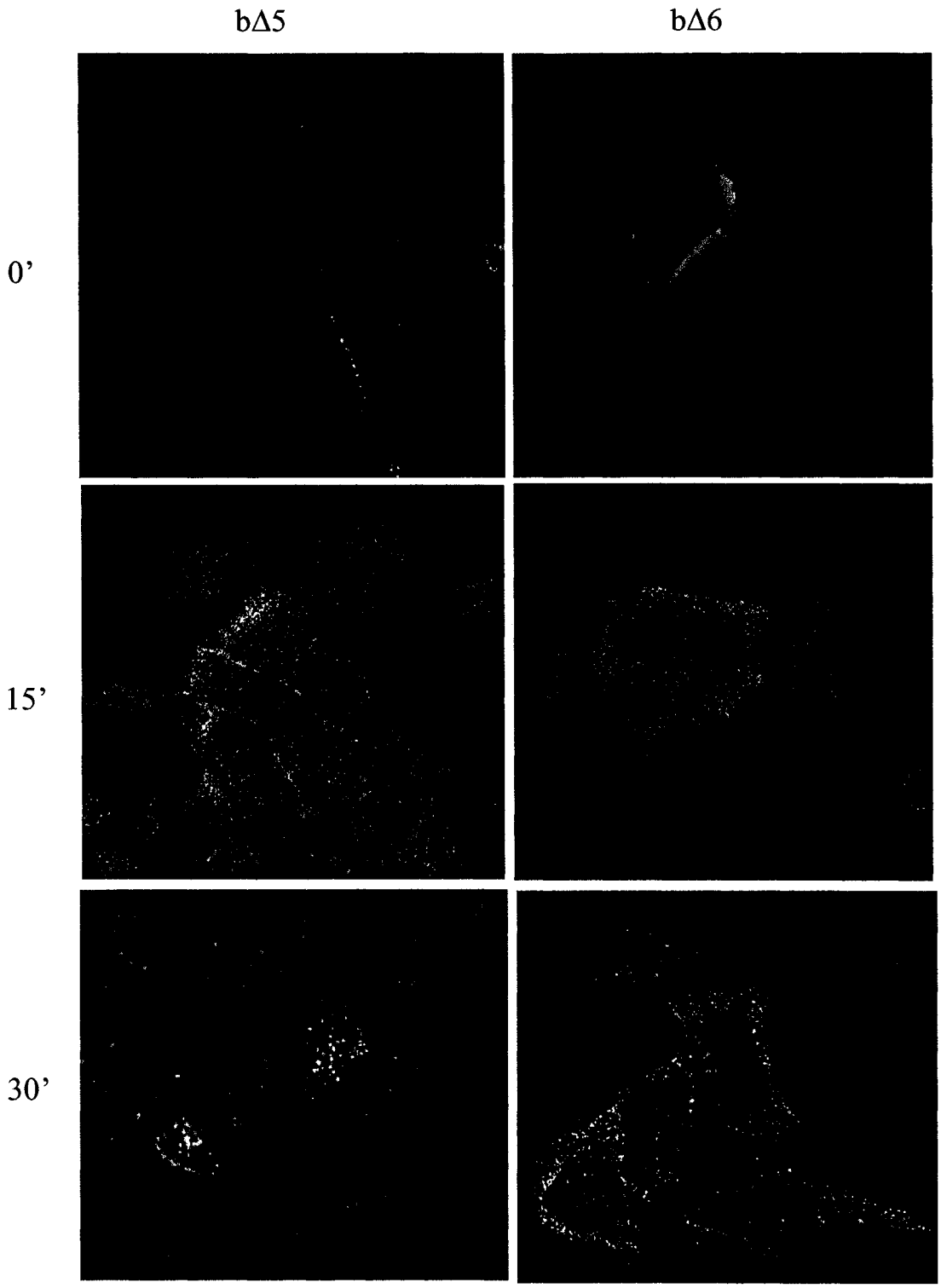

bΔ5
bΔ6
0'
15'
30'

Figure 5.5 Endocytosis of various forms of CD23 proteins after IgE/antigen cross-linking. Cells were transfected with plasmids encoding cDNAs of CD23*a*, CD23*b* or *b*Δ5, and *b*Δ6. Cells were incubated with monoclonal anti-DNP IgE at 4°C for 1 h for surface binding and then with DNP-BSA and transferrin (Tf) at 37°C for 0, 15, and 30 min. Transferrin was used as an early endosomal marker. Binding of IgE on the cell surface (0 min incubation at 37°C) was shown using an epifluorescence microscope. The results of internalization after incubation at 37°C for 15 and 30 min were investigated by confocal microscopy using vertical optical sections. IgE is indicated by the green staining; transferrin by the red staining; colocalization of IgE and transferrin is indicated by the yellow color.

Discussion

Epithelial CD23 plays a crucial role in mediating enhanced transepithelial transport of specific antigen via IgE binding in the sensitized intestine. We previously identified the expression of CD23 isoform *b* transcript in mouse intestinal epithelial cells, including the classical form, and two novel alternative splice forms. Here, transfection studies have demonstrated that all three forms of CD23*b* in intestinal epithelial cells are translated into functional IgE receptors, with different locations of expression and distinct manners of endocytosis following anti-CD23 binding and antigen-induced IgE cross linking.

By gel electrophoresis and sequence analysis, we identified the expression of the classical CD23*b* transcripts as well as two alternative forms lacking the entire sequence of exon 5 or 6 on cultured intestinal epithelial cells. The repeated heptad amino acid sequence derived from exon 5 to 8 in mouse CD23 forms the hydrophobic core of the stalk region and constitutes part of the leucine zipper domain that is important for the stabilization of CD23 trimers (18,19). Studies using CD23 mutant constructs have shown that exons 6 to 8 are required for oligomerization of the receptor and therefore contribute to receptor avidity for IgE, by inducing oligomerization of the CD23 molecules (19). Moreover, the two N-linked glycosylation sites on the CD23 stalk region resides to amino acid residue 65 (corresponds to the end of exon 4) and residue 114 (exon 7) (20). The N-glycosylation sites on the stalk region are required for cell surface expression of CD23 (21) and also inhibit the cleavage of membranous CD23 into soluble forms of CD23 (sCD23) (22). The initial cleavage site on mouse CD23 to release the 38kD form has been identified to be between N93 and L94 (nucleotide base 277 ~ 282, exon 6) (23).

Therefore, the lack of exon 5 or 6 may perturb the conformation of the neighboring regions on the coiled-coil stalk leading to changes of biogenesis, oligomerization and processing of CD23.

Our immunofluorescence studies have demonstrated that classical CD23*b*, and alternative CD23 *b*Δ5 and *b*Δ6 transcripts, were translated into functional IgE receptors. The localization of classical CD23b proteins was mainly on the plasma membrane, whereas *b*Δ5 and *b*Δ6 proteins were found on the cell surface as well as in intracellular compartments. The alternative CD23*b*Δ5 and CD23*b*Δ6 proteins were visualized as vesicular structures. This suggests that at steady state, either the newly synthesized protein products of *b*Δ5 and *b*Δ6 were partially retained in intracellular compartments, or the mature protein was cycling between the plasma membrane and intracellular compartments.

We previously showed by RT-PCR that treatment of IL-4 augmented the quantity of CD23*b* mRNA 2 to 3-fold in IEC-4 cells (6,7), and *in vivo* studies demonstrated that the lack of IL-4 in gene-deficient mice abolished the enhanced expression of CD23 proteins on enterocytes following sensitization (6). These studies indicated that IL-4 upregulates the expression of both the transcripts and proteins of CD23*b* in intestinal epithelial cells (6,7). Moreover, by analysing the sequence of a number of cloned plasmids in our previous study, we found that IL-4 treatment did not induce changes in the percentage of clones containing the classical CD23*b* transcript, but promoted the percentage containing *b*Δ5 transcript, and decreased that of *b*Δ6. These results suggest

that altered expression of different forms of CD23*b* may take place following sensitization where IL-4 production increases (7). Overall, IL-4 increased the expression of both classical CD23*b* and *b*Δ5 transcripts quantitatively, with an increased ratio of the alternative form.

Other alternative CD23 transcripts and proteins have been identified in human and mouse cells. An alternative CD23 transcript lacking exon 3 was found in human B cells, T cells, and eosinophil cell lines (24,25). In mouse B and T cells, alternative splice forms of CD23 transcripts lacking exon 3-encoded sequences were also reported (26). Exon 3 corresponds to the transmembrane region of the CD23 molecule and the lack of this region leads to a change in the amino acid at position 48 from histidine to aspartic acid that renders the truncated form of CD23 hydrophilic (24,26). Studies in transfected cells showed that the protein products encoded by the CD23 transcript lacking exon 3 were retained in the cytoplasm, and it was suggested that these alternative forms may play a regulatory role in the expression of the classical full length CD23 (24). The presence of a number of alternative transcripts of CD23 in mouse may implicate functional discrepancies among these isoforms and that differential expression is tightly regulated.

One of the multiple functions of CD23 is its involvement in IgE-mediated antigen focusing and presentation. *In vitro* studies of murine B cells have suggested a role for cell surface CD23 in focusing IgE immune complexes and facilitating antigen uptake and presentation (27-29). Monomeric IgE pre-bound to cell surface CD23, but not IgG1 pre-bound to FcγR, facilitated B cells to uptake a low amount (100-fold less than control) of specific antigen that was further processed for antigen presentation leading to induction of

T cell activation (27). The addition of anti-CD23 antibody, B3B4, abolished this effect. Similar findings were reported in human B cells (28). Moreover, the binding of preformed IgE-antigen complexes to CD23 also allowed human and mouse B cells to present low levels of antigen to activate T cells, compared to binding to antigen alone (29). Similar findings were demonstrated in *in vivo* mouse studies (30). Passive sensitization of mice with trinitrophenyl (TNP)-specific IgE before bovine serum albumin (BSA)-TNP antigen challenge, caused enhanced B cell antigen capture and presentation in which the anti-BSA IgE production was 10^5-fold stronger than in mice without passive sensitization (30,31). The IgE-mediated enhancement of antibody response was completely inhibited in the presence of anti-CD23 (30-33). The facilitating role of CD23 in antigen focusing implies endocytosis of the molecules into B cells. It has been demonstrated that the proteins encoded by the two isoforms of CD23 transcripts, *a* and *b*, display differential endocytic properties (10). Transfection studies have demonstrated that only CD23*a* protein is constitutively internalized, whereas CD23*b* mediates phagocytosis of IgE-opsonized sheep red blood cells. The results suggest that IgE cross-linking is necessary for the *b* isoform to be internalized (10).

Luminal proteins endocytosed into enterocytes are normally transported in apical endosomes in the supranuclear region of cells, then the macromolecules are degraded by lysosomal enzymes in acidic compartments (34,35). Transferrin is a widely used marker of the clathrin-dependent endocytosis and of the early endosomes (36,37). Physiologically, iron-loaded transferrin binds to its receptor (TfR) on the cell surface. TfRs are constantly internalized with or without binding to transferrin. Iron dissociates from receptor bound

internalized with or without binding to transferrin. Iron dissociates from receptor bound transferrin in early endosomes due to their relatively acidic pH and transferrin and its receptor are recycled back to the plasma membrane through early/recycling endosomes. Our studies showed that the alternative CD23 *b*Δ5 and *b*Δ6 proteins were internalized into early endosomes following binding with saturating amounts of anti-CD23 and monomeric IgE; in contrast, no endocytosis was seen for the classical CD23*b* protein. Endocytosis of the classical CD23*b* proteins was only seen after IgE cross-linking induced by antigen binding. This is in agreement with previous human studies showing that CD23*b* was internalized only after binding with complexes of IgE conjugated to antigen, but not upon binding with anti-CD23 (10). Endocytosis of the *b*Δ5, but not of *b*Δ6, proteins was also observed after antigen-induced IgE cross-linking.

These diversified patterns of endocytosis may represent distinct functions of the classical and alternative CD23*b* proteins in hypersensitivity. In our previous studies in sensitized mouse intestine, we demonstrated that the rapid enhanced transepithelial transport of antigen was mediated by CD23 and required the stimulation of specific antigen binding to IgE, possibly by inducing a cross-linking signal of the receptor (6,7). Previous electromicrographic studies using immunogold labeling of CD23 molecules have shown endocytosis of CD23 into enterocytes following luminal antigen challenge in sensitized rat intestine (5). Decreased numbers of immunogold-labeled CD23 on the apical region of enterocytes paralleled the phenomenon of co-localization of CD23-labels and antigen in the same intracellular endosomal compartments (5). In previous rat and mouse studies, the addition of monoclonal anti-CD23 antibodies (B3B4) to the luminal surface of

the intestinal segments in Ussing chambers significantly reduced the phenomenon of enhanced antigen uptake, most likely by their competition to IgE binding on the CD23 molecules (5,6). Taken together, the prerequisites of the form of CD23*b* protein to mediate the enhanced transepithelial antigen uptake are 1) ready-loaded with IgE ligand, 2) expressed on the apical membrane of epithelial cells, 3) able to be endocytosed after antigen binding. In this study, we showed that classical CD23*b* protein was targeted to the apical surface of transfected polarized MDCK cells for expression (data not shown), and was internalized only upon antigen-induced IgE cross-linking. This result suggests that the classical CD23*b* proteins may be the form mediating the enhanced transepithelial transport of specific antigen in intestinal hypersensitivity. Moreover, the alternative forms of CD23*b* proteins may be involved in other functions. In allergic patients, an increased concentration of IgE in intestinal luminal fluid was found to be associated with high serum IgE levels, suggesting serosal-to-luminal transepithelial transport of IgE (38,39). Augmented transport of radiolabeled IgE, but not IgG, into the intestinal lumen was documented after parasitic infection or intravenous infusion of IL-4 in rats (40). Intact IgE (MW 190,000) in the intestinal lumen was detected by non-denaturing electrophoretic analysis, indicating the IgE molecules were not degraded during transepithelial transport (40), suggesting the involvement of a receptor, likely to be CD23. Based on the endocytic manner of the various CD23*b* proteins in this study, the IL-4-inducible form *b*Δ5 that is continously internalized is most likely to account for the transport of monomeric IgE in hypersensitivity. Although *b*Δ5 proteins were also endocytosed after binding with IgE/Ag, its ability to internalize without the IgE ligand would make a less efficient system for the

specific and rapid enhanced transepithelial antigen transport reported in sensitized intestine. In contrast, classical CD23*b* proteins remained on the cell surface after binding with monomeric IgE, therefore, ensure a high level of CD23*b* expression ready loaded with IgE on the membrane that are sufficient for endocytosis upon luminal antigen challenge and for mediating the increased transepithelial antigen transport. Futhermore, other studies documented the existence of an additional alternative CD23 transcript lacking exon 3 that encodes for proteins that are retained in the cytoplasmic compartments, and suggested that they play a role in the regulation of the expression of full-length classical CD23. We previously showed that the expression of *b*Δ6 transcripts in IEC-4 cells was downregulated after stimulation of IL-4 (a cytokine that is elevated in allergy), and here, we demonstrated that their encoded proteins were constitutively internalized in steady state and upon monomeric anti-CD23 or IgE binding, but not after antigen-induced IgE cross-linking (scenario of antigen challenge in hypersensitivity). Therefore, the presence of *b*Δ6 transcripts may be regulatory in intestinal hypersensitivity, and may affect the expression of the classical or the other splice form. It is noteworthy that the differential endocytic properties of CD23 *b*Δ5 and *b*Δ6 proteins confirm that exons that were supposed to be part of the repeat structure responsible for oligomerization, may also account for distinct functions of the proteins.

The caveat of our speculation for the function of the classical CD23*b* proteins lies at the rate of transport. Our previous models of intestinal hypersensitivity in mouse and rat jejunal tissues showed an extremely rapid rate of antigen uptake (<3 min) mediated by IgE

bound to CD23 in enterocytes in the Ussing Chamber (5,6). The endocytosis study here demonstrated that the classical CD23*b* endocytosed 30 min after antigen-induced cross-linking of IgE, yet very limited internalization was seen at the 15 min time point. There are several possible explanations for the descrepancy. The epithelial HeLa cells used for our transfection and endocytosis studies are well-characterized and relatively easy to transfect compared to intestinal epithelial cell lines, e.g. Caco-2 cells and T84 cells. The lack of endogenous CD23 expression (shown by negative immunohistochemical staining) in HeLa cells further provides the advantage of ruling out non-specific binding. However, unlike T84 and Caco-2 cells, HeLa cells do not polarize *in vitro*. The lack of an unidentified transport machinery that is responsible for the rapid apical to basolateral transport in polarized cells cannot be excluded. Furthermore, differential rates for receptor-mediated transport have been documented in other immunoglobulin receptors, such as IgA transport via polyermeric immunoglobulin receptor (pIgR) in intestinal epithelial cells (41-43). It was shown that pIgR itself is constitutively transcytosed across the cells; however, binding of dimeric IgA increased its rate of transcytosis *in vitro* and *in vivo* (42-45). The binding of dimeric IgA to the pIgR induced dimerization of the receptor and this signal is crucial and sufficient to stimulate IgA transcytosis via pIgR (42,43). Therefore, there may be unidentified signals needed for the rapid endocytosis of CD23*b* proteins which may be provided by allergen binding to receptor bound IgE.

In summary, we correlated the molecular characteristics of different forms of intestinal epithelial CD23*b* proteins with their endocytic properties. All forms of CD23*b* transcripts, including the classical and two unique alternative forms, were translated into

functional IgE receptors, displaying differential patterns and rates of endocytosis following binding with anti-CD23, or binding of IgE with or without cross-linking by antigen. These results suggest that classical CD23*b* proteins may be involved in augmented uptake of specific antigen via IgE binding, whereas the alternative *b*Δ5 proteins may mediate transepithelial IgE transport, and *b*Δ6 proteins may play regulatory roles of CD23*b* expression, in conditions of intestinal hypersensitivity.

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CHAPTER 6: Discussion - immune factors in enhanced transepithelial antigen transport

Food allergic patients develop anaphylactic symptoms in the gastrointestinal tract, airways and skin, rapidly after ingestion of specific foods. Previous studies in sensitized rat models have demonstrated the phenomenon of enhanced and rapid transcellular transport of antigen across the intestinal epithelial barrier (< 3 minutes) that leads to the mast cell-induced anaphylactic responses and increased paracellular transport of antigen. The aim of my study was to explore the mechanism of enhanced transepithelial antigen transport in intestine of sensitized mice and to define the role of specific immune factors that are involved in this antigen transport system.

6.1 Mouse models of food allergy

The aim of the first study was to produce a mouse model of hypersensitivity that displayed the phenomenon of enhanced transepithelial antigen transport in the intestine and to investigate the mechanism by utilizing gene deficient mice. Previous studies (Berin et al. 1997) in actively sensitized rats have shown that the enhanced transepithelial antigen transport in phase I, occurred via the transcellular route, is extremely rapid (<3 min post challenge) and is mast cell-independent; while in phase II, antigen transport via the paracellular route is delayed (>30 min post challenge) and is mast cell-dependent. The phase I transport is specific for the sensitizing antigen, suggesting an immunoglobulin recognition mechanism. Passive sensitization of rats with immune serum reproduced the enhanced antigen transport phenomenon, but not if serum was first heat-treated or IgE-depleted (Yang et al. 2000), suggesting that IgE is one of the immune factors that is involved in the mechanism.

Due to the availability of various gene deficient mice, we decided to utilize mouse models to further investigate the antigen transport mechanism. The phenomenon of enhanced transepithelial antigen transport was reproduced in the intestine of sensitized wild type mice, including two mouse strains, BALB/c and C57BL/6. We demonstrated an 8-fold and 3-fold increase of antigen uptake in enterocytes of sensitized BALB/c mice (controls for the IL-$4^{-/-}$ mice) and C57BL/6 mice (controls for the $CD23^{-/-}$ mice), respectively, compared to their non-sensitized counterparts. We found that BALB/c mice were easier to actively sensitize compared to C57BL/6 mice. One injection of HRP plus adjuvant i.p. to BALB/c mice was sufficient to induce the production of specific IgE as well as the intestinal symptoms of hypersensitivity, whereas two boosts were required to sensitize the C57BL/6 mice. The BALB/c strain was shown to generate a Th2 type response with production of IL-4 (Kopf et al. 1996; Chatelain et al. 1992), whereas C57BL/6 mice were more prone to Th1 type response involving production of IFNγ (Heinzel et al. 1989), after parasitic infection. These two strains of mice are frequently used in the study of differential development of $CD4^+$ T helper cell subsets (Th1 and Th2) in relation to disease progression (Reiner, Locksley, 1995; Noben-Trauth et al. 1996; Heinzel et al. 1989). However, we were able to overcome such strain differences by modifying sensitization procedures.

6.2 Role of IgE, IL-4, and CD23 in enhanced transepithelial antigen transport

To investigate the role of specific immune factors in the mechanism of enhanced antigen transport in sensitized mice, adoptive transfer of immune serum into naive mice

was performed. Passive sensitization of mice with immune serum reproduced the phenomenon of enhanced antigen uptake in enterocytes, and similar to rats, injection of IgE-depleted serum failed to induce the phenomenon. We also observed that neutralization of IL-4 in the serum significantly reduced the enhanced antigen uptake, suggesting that IL-4 is involved in the mechanism. Antigen challenge to the intestine of sensitized IL-$4^{-/-}$ mice failed to induce enhanced antigen uptake compared to controls. The abolishment of enhanced antigen uptake in sensitized IL-$4^{-/-}$ mice was not simply due to the lack of IgE synthesis, since passive sensitization of IL-$4^{-/-}$ mice reproduced the phenomenon. However, in spite of the presence of IgE in the IL-4-neutralized serum, mice injected with the IL-4-neutralized serum did not display enhanced antigen uptake into enterocytes. Therefore, these results suggest that IL-4 plays a role in the enhanced transepithelial antigen transport by a mechanism that is separate from its stimulation of IgE synthesis.

In my study, immunohistochemical staining for the low affinity IgE receptor, CD23, was observed in the apical area of intestinal epithelial cells following sensitization of wild type mice, but not of IL-$4^{-/-}$ mice. RT-PCR studies confirmed the upregulation of CD23 mRNA in mouse intestinal epithelial IEC-4 cells after treatment with IL-4. Studies in $CD23^{-/-}$ mice (C57BL/6 background) demonstrated that the deficiency of CD23 completely abolished phase I enhanced antigen transport leading to the lack of phase II paracellular transport and transmucosal antigen flux. Addition of anti-CD23 antibodies, but not isotype control antibodies, to the luminal side of the intestine prior to antigen challenge significantly inhibited the phase I enhanced antigen uptake in enterocytes of

sensitized mice in this study. Taken together with previous findings in rats, our results suggest that CD23 mediates increased transepithelial transport of specific antigen via IgE binding and IL-4 plays a regulatory role in the mechanism by both stimulating IgE synthesis and upregulating CD23 expression in intestinal epithelial cells.

One typical concern of using gene deficient mice is the possibility of other physiological abnormalities. The gene deficient mice used in this project were homozygous animals deficient of a single gene. They are denoted as -/-, in comparison to their wild type counterpart termed +/+. IL-$4^{-/-}$ mice have been reported to have a decreased serum level of IL-13 after infection (Noben-Trauth et al. 1996; Bancroft et al. 1998). However, murine IL-13 does not increase CD23 expression on B cells (Lai, Mosmann, 1999) and is unlikely to affect expression of CD23 in intestinal epithelial cells. $CD23^{-/-}$ mice were reported to display normal differentiation and phenotypes of lymphocyte populations, and to mount normal antibody responses to immunization and parasite infection (Fujiwara et al. 1994; Yu et al. 1994). Our studies confirmed those of others that $CD23^{-/-}$ mice are able to produce a normal or a slightly increased level of IgE upon sensitization compared to wild type mice (Fujiwara et al. 1994; Yu et al. 1994). The lack of enhanced transepithelial antigen transport in sensitized $CD23^{-/-}$ mice, albeit in the presence of high serum IgE levels, indicates a critical role of the CD23 receptor *per se*, and corroborates the anti-CD23-induced inhibition of enhanced transepithelial antigen transport.

The results obtained from gene knock out mice implicate crucial roles for IL-4 and CD23 in the unique mechanism of this IgE-dependent phase I enhanced transepithelial

antigen transport. Increased local production of IL-4 in the intestinal mucosa has been reported in food allergic patients (Hauer et al. 1997), probably due to augmented IgE synthesis from lamina propria B cells. In addition, increased expression of CD23 on the apical surface of intestinal epithelial cells has also been reported in food allergic patients (Kaiserlian et al. 1995; Kaiserlian et al. 1993), but no function for the receptor was identified. Our postulated mechanism for the events is depicted in Figure 6.1. After luminal antigen challenge, antigen attaches to specific IgE bound to CD23 on enterocytes. The IgE/CD23-binding mediates endocytosis and transcytosis of specific antigen across intestinal epithelial cells of sensitized mice. This receptor-mediated binding increases the rate of transepithelial antigen transport and protects the antigen from degradation resulting in a large quantity of immunogenic material entering the body.

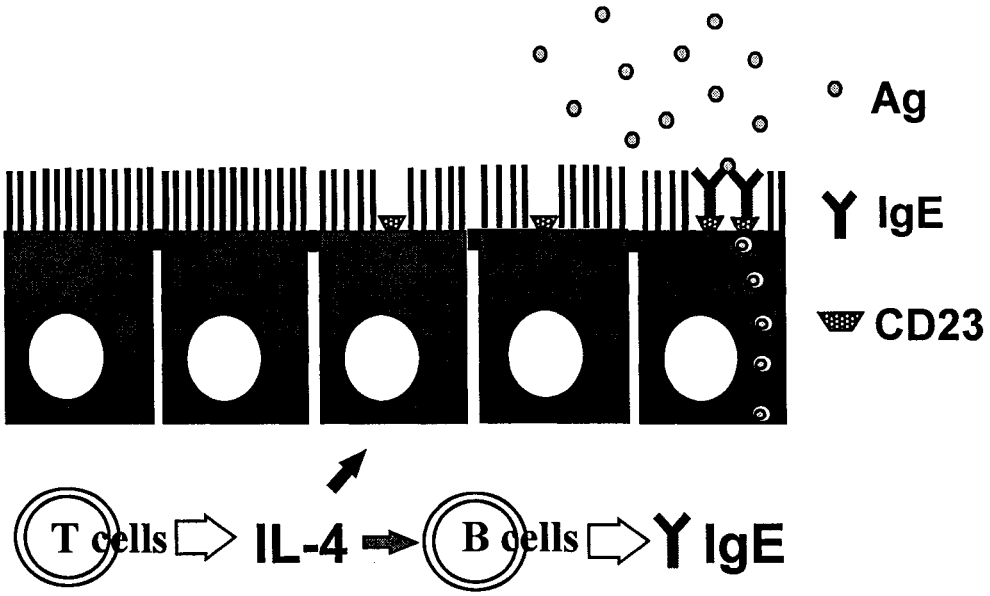

Figure 6.1 Schematic diagram of enhanced transepithelial antigen transport mediated by IgE/CD23 and regulated by IL-4. In sensitized intestine, increased production of IL-4 from Th2 cells in the mucosa leads to 1) isotype switching and increased synthesis of IgE in B cells, and 2) elevated production of CD23 on the intestinal epithelial cells. Following luminal antigen challenge, the specific antigen binds to IgE that is in turn bound to CD23 on the surface of enterocytes. The binding of antigen to IgE/CD23 induces the enhanced transepithelial antigen transport.

6.3 Molecular characteristics of CD23 on mouse intestinal epithelial cells

Next, we investigated the molecular characteristics of the CD23 receptor. Two isoforms of CD23 have been reported to be expressed in human cells. CD23 isoform *a* is constitutively expressed in B cells; whereas IL-4 induces the expression of isoform *b* in B cells as well as in other cell types (Delespesse et al. 1991). In mouse cells, the expression of the homologue of CD23 isoform *a* is expressed constitutively in B cells and is upregulated by IL-4 (Conrad, 1991; Delespesse et al. 1991). Until my studies, the existence of murine CD23 isoform *b* was controversial (Conrad et al. 1993; Richards, Katz, 1994). Only one group reported the expression of the murine homologue of CD23 isoform *b* in a B cell-depleted population of spleen cells after stimulation with IL-4 and LPS (Kondo et al. 1994). RT-PCR studies utilizing primers specific to either isoform *a* or *b* demonstrated that spleen cells isolated from sensitized mice expressed only CD23*a*; whereas mouse intestinal epithelial cells express exclusively isoform *b*. Moreover, the expression of CD23*b* transcript was further upregulated 2-3-fold upon stimulation with IL-4.

The amino acid sequences of the two isoforms of CD23, *a* and *b*, differ only in their N-terminal intracytoplasmic tail of 6/7 residues in both mouse and human (Yokota et al. 1992). The discrepancy of the sequences in the intracytoplasmic ends suggests differentiated internalization or signaling pathways. To examine this issue, I performed cell transfection studies with mouse CD23*a* and CD23*b*. These studies demonstrated functional differences between the two isoforms. CD23*a* was endocytosed following ligand binding with anti-CD23, whereas CD23*b* was internalized only after antigen-

induced cross-linking of bound IgE. Previous studies (Yokota et al. 1992) using the wild type and mutated forms of human CD23*a* and CD23*b* showed similar results, in which CD23*a* was endocytosed following binding with anti-CD23, whereas CD23*b* was internalized only after binding with complexes of IgE conjugated with antigen. Mutation studies suggested that the functional difference results from the lack of an aromatic amino acid in the cytoplasmic tail of the CD23*b* molecule (Yokota et al. 1992).

Plasmidic cloning of cDNAs and sequence analysis further confirmed the presence of CD23 isoform *b* in the intestinal epithelial cells. The key observation in this study was the identification of two alternative splice forms of CD23*b* (*b*Δ5 and *b*Δ6) in addition to the expression of the classical CD23*b* transcript in a mouse intestinal epithelial cell line. IL-4 treatment upregulated the expression of the classical CD23*b* and *b*Δ5 transcripts, with a proportional increase in the latter. Interestingly, IL-4 downregulated the expression of *b*Δ6. These results indicate both quantitative and qualitative modification in the expression of CD23*b* deletion forms in conditions such as hypersensitivity where IL-4 production increases.

The sequence of the two alternative splice forms of CD23*b* transcripts does not lead to a change in the open reading frame. By transfection studies, we showed that all three of the classical and alternative CD23b transcripts were translated into proteins correctly folded as demonstrated by anti-CD23 binding. The expression of the classical CD23*b* protein was mainly on the cell surface, whereas the two alternative forms were identified in intracellular locations as well as on the cell surface. The intracellular

localization of the alternative CD23*b* proteins suggests retention of proteins in the cytoplasmic compartments or increased turnover of membranous proteins. All three forms of CD23*b* proteins were shown to be functional receptors based on IgE binding to the cell surface. Studies from other laboratories have identified different CD23 transcripts lacking exon 3 in human and mouse B cells and T cells (Nunez, Lynch, 1993; Yoshikawa et al. 1999). The presence of various forms of CD23 in intestinal epithelial cells and other cell types may suggest tight regulation of the expression of different forms or functional redundancies of the proteins.

6.4 Endocytic properties of classical and alternative splice forms of CD23*b*

Previous studies in sensitized rats clearly showed that antigen challenge resulted in endocytosis of CD23 in enterocytes. Immunohistochemical staining of CD23 and immunogold-labeled CD23 on the apical surface of intestinal epithelial cells was demonstrated following sensitization (Yang et al. 2000). After luminal antigen challenge, the number of immunogold-labeled CD23 molecules decreased on the cell surface associated with co-localization of immunogold-labeled CD23 and antigen in the same endosomal compartment in enterocytes (Yang et al. 2000).

Distinct endocytic properties of the classical and alternative splice forms of CD23*b* were documented in my study. In the transfected HeLa cells, the classical CD23*b* proteins were endocytosed after antigen-induced cross-linking of bound IgE, but not after anti-CD23 or monomeric IgE binding. These findings agree with others (Yokota et al. 1992) showing CD23*b* internalization only after binding with complexes of IgE conjugated with

antigen, but not after binding with anti-CD23. The *b*Δ5 proteins were endocytosed upon anti-CD23 or monomeric IgE binding, as well as after antigen-induced IgE cross-linking. Although the sequence structure of *b*Δ6 is similar to that of *b*Δ5, *b*Δ6 proteins were endocytosed upon anti-CD23 or monomeric IgE binding, but not after antigen-induced IgE cross-linking. However, the mock-transfected cells showed no staining of IgE on the cell surface and absence of IgE staining in intracellular locations after IgE/antigen binding. Taken together, these results indicate that both IgE and antigen are endocytosed with CD23.

The observation of various CD23*b* transcripts in mouse epithelial cells that display structural and functional discrepancies suggests that their differential expression regulated by cytokines, e.g. IL-4, may account for different functions of the proteins under different conditions. In our sensitized mouse studies, we demonstrated that the CD23-mediated transcytosis of antigen requires the stimulation of specific antigen binding to IgE, possibly by inducing a cross linking signal. Therefore, both the classical CD23*b* and *b*Δ5 proteins may be able to serve this purpose. In addition, in human studies, increased concentrations of IgE in intestinal luminal fluid were found to be associated with high serum IgE (Negrao-Corrêa et al. 1996; Belut et al. 1980). Also, augmented transport of radiolabeled IgE into the intestinal lumen was documented in rats after parasitic infection or intravenous infusion of IL-4. These studies suggest the involvement of a receptor, likely to be CD23, in the serosal-to-luminal transport of IgE (Ramaswamy et al. 1994). Based on the endocytic properties of different forms of CD23*b* proteins, the IL-4-inducible form

bΔ5 is most likely to account for the transport of monomeric IgE. Other studies have documented the existence of an additional alternative CD23 transcript lacking exon 3 in B cells, T cells and eosinophils (Nunez, Lynch, 1993; Nunez et al. 1995; Yoshikawa et al. 1999). The exon 3-lacking transcripts encoded for proteins that were retained in the cytoplasmic compartments, which led to the suggestion that they play a role in the regulation of the expression of the full-length classical CD23 (Yoshikawa et al. 1999).

Endocytosis of CD23 was also reported in murine and human B cells and has been implicated in facilitating antigen focusing and presentation. The presentation of extremely low concentrations of antigen to T cells is augmented by IgE-dependent antigen focusing via CD23 expressed on activated B cells (Van der Heijden et al. 1995; Santamaria et al. 1993; Pirron et al. 1990; Kehry, Yamashita, 1989). Studies using murine B cells demonstrated that monomeric IgE bound to CD23 (but not IgG1 bound to FcγR) allowed B cells to bind specific antigen at a very low concentration that was sufficient for antigen presentation and activation of T cells. The addition of anti-CD23 abolished this effect (Kehry, Yamashita, 1989). Moreover, the binding of preformed IgE-antigen complexes to CD23, compared to antigen alone, also allowed Epstein Barr virus-transformed human B cells (constitutively expressing high level of CD23) to present low levels of antigen to activate T cells (Santamaria et al. 1993).

It was reported in human transformed B cells that CD23 is non-covalently associated with the major histocompatability complex (MHC) class II, human leukocyte antigen HLA-DR on the cell surface (Flores-Romo et al. 1990; Bonnefoy et al. 1988). Binding of antigen-IgE complex to CD23 on the B cell surface led to endocytosis of CD23

as well as the spatially associated HLA-DR. The CD23 label was observed in cytoplasmic organelles that resembled the class II vesicle (compartments for peptide loading onto MHC), and subsequently, the HLA-DR-CD23 complex was found to return to the cell surface on a time scale (3-6 h) consistent with the recycling of HLA-DR for antigen presentation (Karagiannis et al. 2001). Studies using murine B lymphoma cell lines showed that antigen/IgE complexes induced endocytosis of cell surface CD23, and the internalized IgE ligands were routed into high density lysosomal vesicles containing acid hydrolases where they were degraded (Chen, 1992; Chen, 1991). These results indicate that antigen/IgE immune complexes were sorted into lysosomal compartments (Chen, 1992; Chen, 1991), and CD23 was recycled to the cell membrane along with the HLA-DR molecules (Karagiannis et al. 2001). It was suggested that antigen peptide loading may be facilitated by the proximity of the HLA-DR molecules and antigen, and the recycling of CD23 with HLA-DR to the cell surface may enhance B cell-T cell interaction by adhesion function of the CD23 molecules (Karagiannis et al. 2001).

In polarized epithelial cells, transcytosis is recognized as a form of cycling of cell surface molecules from one side of the membrane towards the opposite side. Although normally proteins and macromolecules endocytosed non-specifically from the apical membrane of enterocytes are sorted into late endosomal/lysosomal compartments for degradation (Abrahamson, Rodewald, 1981), transcytosis of intact immunoglobulins across epithelial cells by selective receptors has been documented, for example, for IgA and IgG (Hunziker, Kraehenbuhl, 1998; Casanova, 1992). Transepithelial transport of IgA and IgG is mediated by pIgR and FcRn expressed on the adult and infant intestinal

epithelial cell surface, respectively. The transcytotic route for immunoglobulins has been mainly characterized by morphological and biochemical findings reported in early papers. More recent studies using specific endosomal markers have helped to resolve conflicting results related to membrane receptor trafficking in polarized cells.

The route of IgG transepithelial transport was documented in early studies by injecting IgG conjugated to a tracer into lumen of the ligated small intestinal segments in neonatal rats, and the uptake of IgG-tracer was analysed in electron photomicrographs. The apical uptake of IgG into epithelial cells was selective and was localized in endocytic coated pits at the base of the microvilli where membrane invaginations occur (Berryman, Rodewald, 1995; Rodewald, 1973; Rodewald, 1970). One group studied the transport route of a non-selective fluid phase marker (HRP) in comparison to the receptor-mediated endocytosis marker (IgG) across enterocytes of newborn rats (Abrahamson, Rodewald, 1981). Both markers were injected into the lumen of cannulated segments of intestine *in situ* for various periods of time and their cellular distribution was determined by electron microscopy. HRP was endocytosed and confined to the apical region of cells in late endosomal/lysosomal structures as identified by acid phosphatase and aryl sulfatase activities, both of which were lysosomal enzymes. In contrast, IgG was initially present in early endosomes in the apical area and subsequently in coated vesicles at the basolateral cell region or bordering the basolateral membrane. IgG was also detected free within paracellular spaces, suggesting that it is released from cells (Abrahamson, Rodewald, 1981). Information regarding the endosomal trafficking during apical-to-basal transcytosis is still limited and the exact transport route is not well understood.

In contrast, the pathway of basal-to-apical transcytosis of another immunoglobulin is well-characterized. Studies in pIgR-transfected MDCK cells have shown that pIgR expressed on the basolateral membrane was internalized without the binding of a ligand, however, the amount of transcytosis was greatly increased by the binding of dimeric IgA that induced a dimerization signal to the receptor (Singer, Mostov, 2002; Song et al. 2002). Futher studies tracing the route and time course of IgA transcytosis have demonstrated that IgA is endocytosed into basolateral early endosomes (BEE) at 5 min pulse (presence of ligand), and sorted into tubulovesicular endosomes in the apical region of the cells at 25 min chase (absence of ligand), before fusion with the apical plasma membrane for exocytosis (Apodaca et al. 1994). The tubulovesicular endosomes were inaccessible to fluid phase markers that were destined to late endosomes/lysosomes, suggesting that these vesicles are recycling endosomes (Apodaca et al. 1994). The recycling endosomes were further differentiated into the common recycling endosomes (CE) and apical recycling endosomes (ARE) (Brown et al. 2000) (Figure 6.2). No intermediate step of the fusion with high-density lysosomes was involved in IgA transcytosis (Brown et al. 2000; Apodaca et al. 1994).

Therefore, in contrast to B cell CD23 that facilitates antigen focusing by sorting endocytosed antigen into lysosomes for antigen processing and into class II vesicles for antigen presentation, intestinal epithelial CD23 may sort the endocytosed antigens into recycling endosomes for transcytosis. The transepithelial antigen transport mediated by IgE/CD23 is extremely rapid (< 3 min post challenge) compared to normal transcytosis which takes ~20-30 min, suggesting a unique pathway of transport that involves

protection from lysosomal degradation and rapid endosomal trafficking by altered interactions with the cytoskeleton. It is likely that CD23-mediated endocytosis of IgE/antigen bypasses the lysosomal pathways to deliver antigen to the serosal membrane, leading to the phenomenon of enhanced transepithelial antigen transport in sensitized mouse intestine. The intracellular compartments through which endocytosed epithelial CD23 passes are unknown and require further investigation.

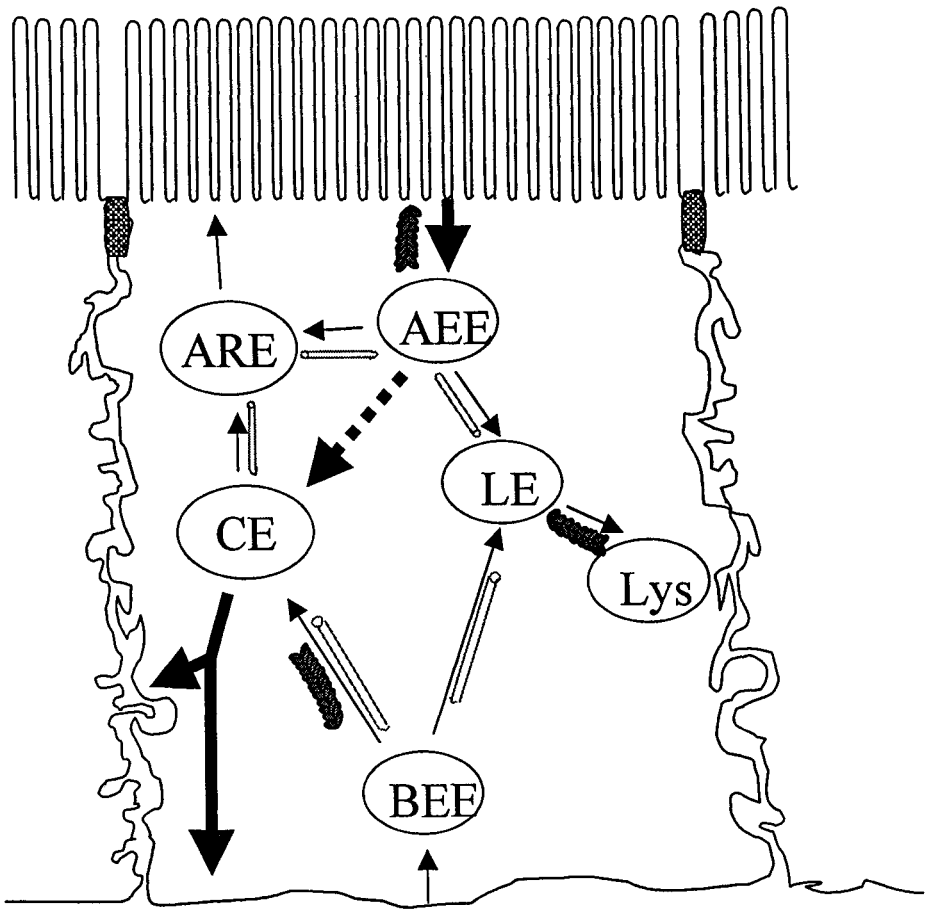

Figure 6.2 Lysosomal degradation, transcytosis, and recycling pathways in polarized epithelial cells

Figure 6.2 Transcytosis and recycling pathways in polarized epithelial cells. Extracellular molecules are either taken up as fluid volume or via the specific receptors on the plasma membrane. These molecules are endocytosed into early endosomes in the apical or basal regions of cells, denoted as apical early endosomes (AEE) and basal early endosomes (BEE), respectively. Actin microfilaments are required for the endocytosis to AEE, but not BEE. Fluid phase molecules are transported from these early endosomes into late endosomes and lysosomes for degradation. The transport between early endosomes to late endosomes requires microtubules; whereas the transport between late endosomes and lysosomes involve actin microfilaments. Receptor-mediated endocytosis may involve the previously mentioned lysosomal degradative pathway, or the recycling or transcytosis pathways. The apical recycling pathway includes the cargo transport from AEE to the apical recycling endosomes (ARE) and then returned to the apical plasma membrane. The step between AEE to ARE involves microtubules, whereas the exocytosis step is microtubule-independent. The basolateral recycling pathway involves the cargo transport from BEE into common recycling endosomes (CE), in which this step is dependent on actin and microtubules. Cargo in the CE is then recycled back to the basolateral membrane. Transcytosis in polarized cells is suggested to be a form of recycling. The route of basolateral-to-apical transcytosis, e.g. IgA transport, is more well-characterized than the apical-to-basolateral transcytosis, e.g. IgG transport. For an example of IgA transport, IgA was endocytosed into BEE via binding of polymeric Ig receptor (pIgA) on the basolateral surface of epithelial cells, then transported to CE, and ARE, and subsequently delivered to the apical membrane. The basolateral-to-apical transport

requires microtubules in the post-endocytic trafficking. The apical-to-basolateral transcytosis of IgG and its receptor, FcRn, may include the initial step of AEE, and the transcytosis pathway was reported to be microtubule-independent. Our results of IgE/CD23-mediated enhanced transepithelial antigen transport implicate apical-to-basolateral transcytosis. It is postulated that molecules transported from the apical to basolateral sides of the membrane involved the transport from AEE to the CE (broken arrow) followed by basolateral exocytosis. The hypothesis is indicated by the bold arrow lines.

6.5 Possible evolutionary explanation for food allergy: relationship to expulsion of enteric parasites

One theory may explain the increasing incidence of food allergy in the developed world. The hygiene hypothesis suggests that increased hygiene of individuals in industrialized countries limits development of the immune system that evolved to combat microbial and parasitic infections. Thus, the immune system overreacts in response to inert food and environmental antigens (Wills-Karp et al. 2001). Allergic reactions, characterized by increased serum IgE and mast cells mediator-induced anaphylaxis responses, and eosinophils recruitment, are features similar to responses against parasitic helminth infection (Metcalfe et al. 1997). Animal parasites include protozoa (e.g. *Leishmania major*), helminths (e.g. *Trichinella spiralis*, *Nippostrongylus brasiliensis*, *Schistosoma mansoni* and toxoplasma), and ectoparasites (e.g. ticks and mites). In general, pathogenic protozoa have evolved to survive within host cells, therefore, the immune response against these organisms is mainly through cell-mediated cytotoxicity and phagocytosis, including cytototic T cells (CTL) and macrophages. In contrast, helminths survive in extracellular tissues, and defense against helminth infection is mediated mainly by a special type of antibody-dependent cellular cytotoxicity, in which IgE antibodies coat the surface of helminth and recruit eosinophils via binding to Fcε receptors. These eosinophils are subsequently activated to secrete granule enzymes that destroy the parasites. Moreover, numbers of mast cells increase greatly in the lamina propria of helminth-infected intestine (Metcalfe et al. 1997). Challenge of post-infected intestine with soluble parasite antigen caused mast cell degranulation and mediator release to

induce an immediate ion secretory reaction, resembling an anaphylactic response, in order to flush out the luminal parasites.

The increased CD23 expression on intestinal epithelial cells of sensitized animals may have originally evolved to serve as a first line warning system of enteric helminth infection. Following an initial infestation, anti-helminth IgE antibody production and CD23 expression on epithelial cells is stimulated. Upon subsequent enteric infection, the helminth antigens in the intestinal lumen may bind to antigen-specific IgE bound to CD23 on the intestinal epithelial cells which mediates the rapid enhanced transport of intact antigen across the epithelial barrier. Therefore, the subepithelial mast cells are quickly activated to release mediators, such as histamine, 5-HT and prostaglandins, cytokines and chemokines. Some mediators induce ion and water secretion to flush out the parasites, while several proinflammatory cytokines, e.g. TNFα, recruit inflammatory cells (neutrophils and macrophages) into the lamina propria or lumen. However, in food allergic patients, the CD23-mediated enhanced antigen transport induces adverse anaphylactic responses against ingested food proteins.

6.6 CD23 as a potential therapeutic target

To date, the only effective treatment for food allergy is the avoidance of suspected allergic foods in the diet. However in some infants and children, the diet becomes so restrictive that nutrition is compromised. Therefore, a clear understanding of the mechanisms responsible for the pathophysiology of food allergies is needed for the development of novel therapeutic agents. In this study, we demonstrated that luminal

administration of anti-CD23 antibodies prior to antigen challenge significantly reduces the amount of antigen uptake in enterocytes in phase I in sensitized mice. Previous rat studies showed that anti-CD23 not only inhibited the phase I enhanced endosomal antigen transport, but also reduced phase II ion secretion and transmucosal antigen flux (Yang et al. 2000). These results suggest CD23 as a potential target for the development of novel therapeutic interventions to limit antigen influx into the body in diseases where enhanced permeability of the intestine is a prominent feature, such as food allergy.

6.7 Future directions

The phenomenon of enhanced transport of intact antigen across the intestinal epithelial barrier suggests that the transepithelial antigen transport mediated by IgE/CD23 may bypass the lysosomal degradative pathway. The IgE/CD23-mediated antigen transport across the intestinal epithelial cells is extremely rapid compared to the rate of normal protein transcytosis (Keljo, Hamilton, 1983). This rapid transepithelial transport of antigen may be due to one or both of two factors: 1) reduced degradation of endosomal antigen by lysosomal enzymes, or 2) increased transcytosis of endosomes by their altered interactions with the cytoskeleton. In keeping with the endosomal trafficking pathway of IgA and IgG transcytosis in polarized cells, IgE was endocytosed into apical early endosomes (AEE) identified by the presence of transferrin, and we postulated that it is to be sorted directly into recycling endosomes without fusion with lysosomes (Apodaca et al. 1994; Abrahamson, Rodewald, 1981). The endocytic trafficking in polarized cells requires the cytoskeletal structures, such as actin and microtubules, to translocate the cargo from

early endosomes near the margin of the cell membrane to late endosome/lysosomes in the supranuclear region, or to recycling endosomes at the opposite pole of the cells (Apodaca, 2001). Actin microfilaments play a critical role in endocytosis of fluid phase markers and receptor-bound ligands in the apical, but not basolateral, membrane of polarized epithelial cells, such as MDCK cells (Gottlieb et al. 1993) and Caco-2 cells (a human intestinal epithelial cell line) (Jackman et al. 1994). The delivery of cargo between late endosomes and lysosomes also requires actin as demonstrated in a human carcinoma cell line, HEP-2 epithelial cells (van Deurs et al. 1995). The involvement of microtubules has been reported in several steps of the endosomal sorting and trafficking pathway in polarized cells. In MDCK cells, microtubule-depolymerizing agents blocked the transport of cargo between early endosomes and late endosomes (Bomsel et al. 1990), and thus may impair the degradation pathway. Studies using pIgA-transfected MDCK cells have shown that microtubules are required for the sorting of IgA from basolateral early endosomes (BEE) into recycling endosomes (CE and ARE), but are not required for basolateral endocytosis into early endosomes and apical exocytosis from recycling endosomes (Hunziker et al. 1990; Apodaca et al. 1994) (Figure 6.2). Studies of the pathway of transferrin (Tf) recycling indicated that the transport between BEE and CE is impaired by microtubule-depolymerizing agents and actin disrupting agents (Maples et al. 1997; Apodaca et al. 1994). Order-of-addition experiments indicate that the actin-dependent step precedes the microtubule-dependent step in postendocytic trafficking of pIgR-IgA complexes, and blockage of both actin and microtubules inhibit the transport (van Deurs et al. 1995). In contrast, apical-to-basolateral transcytosis of IgG is not dependent on microtubules

(Apodaca et al. 1994), reflecting the spatial organization of polarized cells that results in distinct transcytotic routes depending on sides of entry. It is likely that apical-to-basolateral transport of immunoglobulins, such as IgG and IgE, involves the translocation of cargo from AEE directly to CE for transcytosis (Figure 6.2, broken arrow). Future colocalization studies using markers for each endosomal compartments or pH-dependent markers will give us further insight into the pathway of this rapid IgE/CD23-mediated transcytosis. Inhibition studies using actin-disrupting agents and microtubule-depolymerizing agents in cell culture systems will address the possible involvement of individual cytoskeleton proteins in the process of intracellular trafficking. The CD23/IgE model would be a unique tool to characterize the apical to basolateral transcytosis pathway.

Our results in sensitized mice implicate apical-to-basal transport of IgE, whereas basal-to-apical IgE transport has also been documented in other studies. Increased IgE concentrations in the intestinal lumen have been demonstrated in food allergic patients and animal models (Berin et al. 1997; Sampson, Burks, 1996). In rats, specific translocation of serum IgE into the intestinal wall and intestinal lumen after parasitic infection or i.v. IL-4 perfusion suggests the involvement of an IgE receptor in the basal-to-apical transport of IgE (Ramaswamy et al. 1994). The mechanism and regulation of the bidirectional transcytosis of IgE and the recycling of the potential receptor, CD23, has yet to be addressed. We hypothesized that the IL-4-inducible *b*Δ5 proteins may be involved in the transport of monomeric IgE. The sorting and endocytic pathway of bΔ5 proteins will be

examined and the use of microtubule depolymerizing agents (Apodaca et al. 1994) may potentially differentiate the apical-to-basal or basal-to-apical translocation of the proteins.

CD23 is also involved in induction of cytokine and mediator release from epidermal keratinocytes and monocytes. In isolated human keratinocytes, cross-linking of CD23 with IgE/anti-IgE immune complexes induced the production of several pro-inflammatory cytokines, such as IL-6 and TNFα, in a dose-dependent manner (Bécherel et al. 1997; Bécherel et al. 1994; Bécherel et al. 1994). The IgE-dependent stimulation of CD23 leads to *de novo* synthesis and release of IL-1β, IL-6, TNFγ and thromboxane B_2 in circulating blood monocytes of asthmatic patients (Paul-Eugène et al. 1992; Borish et al. 1991). Production of several cytokines and chemokines (including IL-1β, TNFα, IL-8, MIP-1α, and MCP-1α) induced by cross-linking of CD23 was also reported in human alveolar macrophages (Gosset et al. 1999). All the cell types mentioned above that release cytokines and chemokines in response to CD23 cross-linking stimulation were either cells isolated from allergic patients or cells pretreated with IL-4, and therefore, were activated cells. It was shown that CD23 expression can be upregulated in the murine intestinal epithelial cells after sensitization or IL-4 stimulation, in enterocytes of food allergic patients (Kaiserlian et al. 1995; Kaiserlian et al. 1993), in bronchial epithelial cells of asthmatic patients (Campbell et al. 1994) and in nasopharyngeal epithelial cell lines and keratinocytes after IL-4 stimulation (Bécherel et al. 1994b; Rousselet et al. 1990b). Since the intestinal epithelium can also be activated to secrete a wide range of cytokines and chemokines, including IL-1, -6, -8, TNFα, TGFβ, MCP-1 and eotaxin (Yu, Perdue,

2001), it is reasonable to postulate the involvement of CD23 in the mechanism. Moreover, increased CD23 expression on enterocytes has also been reported in other diseases that showed increased permeability of the intestine, such as inflammatory bowel disease (IBD) (Kaiserlian et al. 1995; Kaiserlian et al. 1993). Stimulation of CD23 may activate the intestinal epithelium in IBD patients to induce cytokine and mediator release leading to increased permeability of the intestine (TNFα) or induction of inflammatory responses (IL-1, IL-6, IL-8). Therefore, it appears that CD23 may play a very important role in food allergy, parasitic infection and intestinal inflammation.

6.8 Summary statement

Taken together, the studies of my project identified the crucial immune factors that are involved in the mechanism of enhanced transepithelial antigen transport in sensitized mice, namely IgE, CD23, and IL-4. Our studies are the first to define the mechanism of this unique antigen transport system, which provides an explanation of the rapid nature of anaphylactic responses in food allergy. The data indicating the molecular characteristics and functional differences of the various forms of CD23 in intestinal epithelial cells has furthered the understanding of the role of specific isoforms of epithelial CD23 in hypersensitivity.

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