

THE UNIVERSITY OF CALGARY

**The Role of Cytoskeletal Proteins in *Giardia lamblia*-  
Induced Epithelial Injury**

by

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## **Abstract**

*Giardia* is the most common intestinal parasite worldwide, causing malabsorptive diarrhea. Malabsorptive diarrhea is seen in several infectious and non-infectious diseases such as yersiniosis, rotaviral infections, Crohn's disease and Celiac's disease. The mechanisms of epithelial injury in giardiasis remain unknown. This study examines the effects of *G. lamblia* on electrical resistance and the cytoskeletal proteins: filamentous and globular actin, villin, ezrin and  $\alpha$ -actinin of human intestinal and colonic epithelial monolayers. *G. lamblia* significantly decreased electrical resistance of cell monolayers. Exposure to *Giardia* lysates or trophozoite spent medium induced localized condensation of F-actin in the terminal web region while G-actin remained unchanged, and a reorganization of the cytoskeletal proteins  $\alpha$ -actinin, ezrin and villin. Rearrangement of F-actin, villin and ezrin were not affected by verapamil or cytochalasin D. *G. lamblia* reduces electrical resistance of human intestinal epithelial monolayers, at least in part via unidentified trophozoite products as sonicated trophozoites produced similar results. The epithelial injury is associated with F-actin and  $\alpha$ -actinin rearrangements in the terminal web, reorganization of villin and concurrent disorganization of ezrin by mechanisms independent of extracellular  $\text{Ca}^{2+}$  or actin polymerization.

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## **Dedication**

To: Troy *kekasihku*,  
my family, especially Diane  
and beloved Frisky

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## **List of Abbreviations**

Caco2	Colonic Adenocarcinoma Cells
CD	Crohn's Disease
DMEM	Dulbecco's Modified Eagles Medium
EGF	Epidermal Growth Factor
ERM	Ezrin, radixin, moesin
F-actin	Filamentous Actin
G-actin	Globular Actin
HLA	Human Leukocyte Antigen
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
NO	Nitric Oxide
PBS	Phosphate Buffered Saline
SCBN	Small Intestinal Cells of B.N.
TNF	Tumour Necrosis Factor

# 1. INTRODUCTION

## 1.1. The Small Intestine

### 1.1.1. Small Intestinal Architecture

The small intestine is unique in its capacity to absorb nutrients, electrolytes and water and is remarkably well adapted for its primary role in absorption. The absorptive epithelium of the human intestine receives a luminal load averaging 9L per day, and as much as 8.8 L are absorbed, resulting in less than 200g/day of stool output (67). The efficient ability of the small intestine to absorb 98% of the fluid load is partly due to the unique architecture of the small intestine. The structural specialization of the human small intestine begins with folds of submucosa termed *plica circularis* or *valvuli conaventes* which are approximately 1cm in height and 5cm in length (241). The *plica circularis* amplifies the intestinal area by up to 3-fold. In addition to the *plica circularis*, numerous mucosal villi extend into the lumen like “fingers” throughout the small intestine, further increasing the absorptive area by some 7- to 14- fold (402). The shape of the villus varies between species, and in humans are leaf or finger shape, ranging between 0.5 to 0.8mm in height (372). Finally, the specialization of the small intestine architecture in absorption is amplified by closely packed microvilli lining the apical surface of absorptive cells, which overlay the villus and crypt of the intestine. Each epithelial cell has as many as 3,000 to 6,000 microvilli visible only under electron microscopy (339). The microvilli are responsible for amplification of surface area by 14- to 40- fold (48, 410). Cumulatively, the specialized architectural structures within the small intestine, increase the absorptive surface area of the 3m long gut by 600 fold or to greater than 200m<sup>2</sup> (164).



### **1.1.2. Absorption and Secretion of Water in the Small Intestine**

Water transport in the small intestine is closely coupled with solute and electrolyte movement. In the epithelium water transport occurs through the paracellular pathway (116) and not via transcellular water flow or by glucose transporters (390). Most water absorption depends on the active  $\text{Na}^+/\text{K}^+$ -ATPase pump located at the basolateral membrane which exchanges 3  $\text{Na}^+$  out of the cell for 2  $\text{K}^+$  into the paracellular spaces thus creating an electrogenic potential difference which is favourable for more  $\text{Na}^+$ , and also powers transport of all other electrolytes coupled to sodium entry. An environment of high osmotic pressure from the active absorption of electrolytes and solutes, induces water from the lumen to flow to the paracellular region therefore reducing osmotic pressure by raising the hydrostatic or fluid pressure. The increased hydrostatic pressure drives water through the basement membrane into the interstitial compartment and finally to the capillaries.

## **1.2. The Mucosal Cell Population**

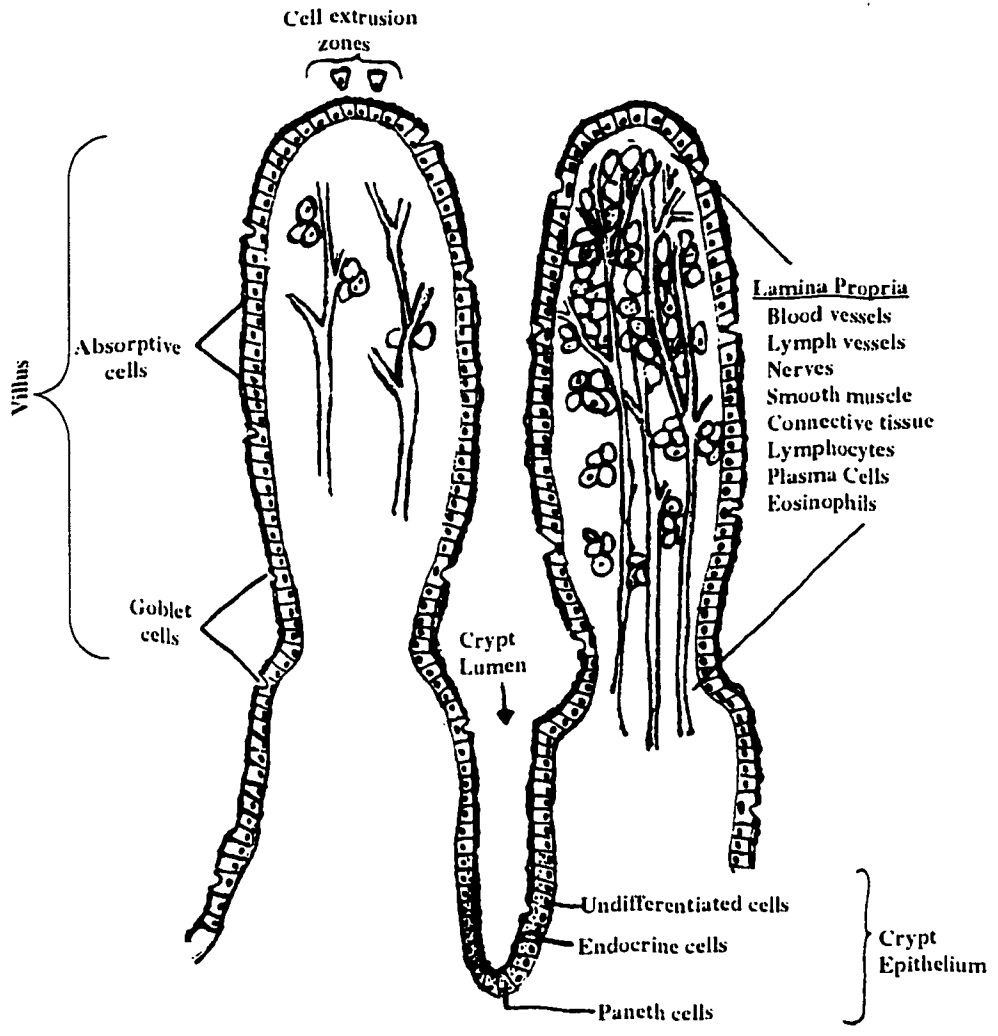
Apart from the capacity of the small intestine to absorb water and nutrients, the small intestine is also a highly differentiated structure whose constituent cells exhibit complex, morphological specializations that facilitate their diverse functions. The mucosa of the small intestine is made of the epithelium, the lamina propria and the muscularis mucosa. This chapter will focus on the intestinal epithelium and the predominant five cell populations that constitute an intact epithelium.

### **1.2.1. The Intestinal Epithelium**

The epithelium acts as an efficient physical barrier that allows exchanges between the lumen and interstitial compartment comprised of blood and lymph vessels, while

protecting the host from environmental pathogens (219). The epithelial cells in the digestive tract have three main functions: i) to digest food and absorb nutrients, ii) to protect against pathogens and iii) to maintain continuous homeostatic cell renewal and cell death. The five cell populations in the intestine responsible for achieving and maintaining intestinal function include goblet cells, entero-endocrine cells, Paneth cells, M cells and absorptive cells. All five populations are derived from undifferentiated or stem cells located in the shallow invaginations between villi known as the crypts of Lieberkühn (Fig. 1). Each crypt in the small intestine is monoclonal and derived from a single stem cell (307). There are 4-16 anchored stem cells in the base of crypts and these serve as precursors for other intestinal epithelial cells including undifferentiated crypt cells (78). Crypt cells are vital in the secretion of  $\text{Cl}^-$  and water into the lumen. The morphology of crypt cells including short microvilli, a less developed glycocalyx where oligosaccharidases and peptidases are normally found, as well as a disordered terminal web, sparse endoplasmic reticulum and abundant unattached ribosomes and polysomes, all indirectly indicating the secretory role of crypt cells (373). Furthermore, prominent within the cytoplasm of crypt cells are secretory granules as well as granules rich in glycoprotein (373, 374). As the crypt cells mature and differentiate, they migrate from the crypt to villus where their function changes to absorption (116). Per day, 1,200-1,400 epithelial cells migrate into each villus of the mouse intestine, matched by a similar rate of apoptosis or programmed cell death, at the tip of the villus to maintain homeostasis within the tissue (151). Cell turnover from the time of cell formation to apoptosis and sloughing off at villus tip takes 3-5 days (151). Spontaneous apoptosis also occurs in the

**Figure 1:** Schematic diagram of two sectioned villi and a crypt, illustrating the histologic organization of the mucosa of the small intestine. Adapted from *Madara and Trier, Physiology of the Gastrointestinal Tract, 1994*.



proliferative region of each crypt allowing an escape route for cells with genetic alterations (264).

#### **A. Goblet Cells**

Goblet cells are polarized mucus secreting cells present throughout the epithelium, and increases in numbers in the proximal jejunum to distal ileum (268). Goblet cells are responsible for producing mucus which serve to cross-link mucin glycoproteins in the stabilization of mucin gels (29). Mucin in conjunction with the negatively charged glycoproteins, acts as a barrier in protecting the epithelium from noxious intraluminal substances and binding organisms, as well as provides a protective coat during mucosal restitution (397). Mucus release is achieved by exocytosis where mucus granules fuse together and are released at the apical membrane via microtubular functions (181, 289). The goblet cell is aptly named, as the cell has an appearance of a wine goblet due to the distension of the apical two-thirds of the cell by clear mucin. As goblet cells are involved in the secretion of mucus, they have sparse microvilli and a poorly developed terminal web and glycocalyx (236).

#### **B. Entero-endocrine Cells**

The entero-endocrine cells are narrow, columnar cells with a slender apex found throughout the small intestine. Entero-endocrine cells are classified by shape and content of their secretory granules which regulate secretory or excretory functions and include such substances as serotonin, secretin, substance P and many others that are able to exert paracrine or endocrine effects (346). These granules are found to be subnuclear, membrane bound secretory granules and are able to contain more than one peptide or amine product. The granules are released upon stimulation of cells by external or internal

factors via exocytosis. As the entero-endocrine cells are also involved in secretion of granules, cells have sparse microvilli and by electron microscopy their cytoplasm appear non-electron dense (236).

### **C. Paneth Cells**

Paneth cells are found throughout the small intestine but are increased in numbers in the duodenum and ileum. The cells resemble truncated pyramids, being widest at the basal area where nuclei are located, and have rudimentary microvilli and undeveloped terminal web concurrent with their secretory nature (236). Prominent eosinophilic granules are also seen in the apical compartment of the cell (236). The renewal rate of paneth cells can be as extensive as 18-22 days in mice (79).

Paneth cells secrete several bactericidal substances, including lactoferrin which chelates iron required for bacterial growth, peroxidase which produces free radicals able to attack microorganisms (219), lysozyme able to digest the lipopolysaccharide coat of Gram-negative enterobacteria (255), and cryptidin which are defensins capable of forming pores in bacterial and parasite membrane leading to lysis (334). In addition, paneth cells have been demonstrated to contain immunoglobulins and cytoplasmic trypsin-like material as well as  $\alpha_1$ -anti trypsin mRNA (34, 218, 319).

### **D. M Cells**

Mucosal lymphoid follicles are found through out the intestine. When found in isolated follicles or aggregates they are termed Peyer's patches. The Peyer's patch appears as a rounded mound or 'dome' that is devoid of villi. The overlying epithelium consist of specialized absorptive cells. These specialized cells are known as M cells, or follicle-associated epithelial cells as they are only found associated with lymphoid

follicles (294). By electron microscopy, M cells have shorter but more abundant microvilli (294). The terminal web of M cells is incomplete and allows organelles such as ribosomes and mitochondria to approach the apical membrane (241). As the lumen is continuously exposed to foreign materials which have the potential to be dangerous, M cells serve to sample antigens from the lumen. Several pathogens, including *Shigella*, *Yersinia enterocolitica* and *Salmonella* utilize M cells as portals of entry into the host circulating system. A 1 $\mu$ m to 5 $\mu$ m bridge of cytoplasm serves as the only barrier between the antigen and microflora in the intestinal luminal contents and the underlying immunocompetent cells (241). Lymphocytes, macrophages and plasma cells are located in the intercellular spaces between the M cells and neighbouring cells. With the capacity to transport via transcytosis, M cells pass macro-molecular antigens and microorganisms to underlying macrophages which stimulate the adjacent lymphocytes and plasma cells. Microorganisms such as reovirus, poliovirus type 1 and cholera vibrios have been observed to be transported in vesicular structures by M cells (241). Less clear is the ability of M cells to process and present antigens to immunocompetent cells. Currently, M cells are known to contain acidic endosomal and acid-phosphatase containing prelysosomal and lysosomal compartments, express class II major histocompatibility complex and are able to secrete IL-1 (6, 296).

### **1.3. Absorptive Enterocyte**

As the absorptive enterocyte comprises the topic of this research, it will be covered as a chapter by itself. The absorptive enterocytes of the gut are highly polarized columnar cells capable of vectorial transport of nutrients and electrolytes. The apical pole of absorptive cells are characterized by closely packed microvilli, approximately

0.5-1.5  $\mu\text{m}$  in height (236). Due to the special nature of absorptive enterocytes in vectorial transport of nutrients, the apical plasma membrane is very distinct from the basolateral membrane.

### **1.3.1. Enterocyte Membranes**

#### **A. Apical Microvillus Membrane**

The apical membrane of the enterocyte has a width that is greater than most eukaryotic plasma membrane, in addition, there is a high protein to lipid ratio (176). The apical membrane also has a high cholesterol to phospholipid ratio and an increased content of glycosphingolipids (386). Together the higher protein to lipid ratio, in addition to the different lipids present, contributes to a more rigid apical membrane. This has functional implications as a more rigid, or less fluid membrane implies a lower passive membrane permeability and an increased mechanical stability (179).

Overlying the apical membrane is the carbohydrate rich glycocalyx. The glycocalyx is formed in part by an array of digestive enzymes such as disaccharidases and peptidases (Table 1) which play a crucial role in terminal digestion of carbohydrates and peptides prior to absorption. The digestive enzymes are predominantly glycoproteins that are anchored to the lipid bilayer of the apical membrane by a hydrophilic N-terminal sequence at the cytoplasmic surface of the membrane or by a C-terminal amino acid attached to a phosphatidylinositol glycan (232). Concurrent with the digestion of nutrients, the apical membrane also contain transport proteins required for the transport of glucose (via SGLT-1), fructose (via GLUT 5), amino acids, bile acids, di- and tri-



**Table 1:** Brush border enzymes of the apical microvillus membrane.

<b>Function</b>	<b>Protein</b>
<b>Glycosidase</b>	Maltase-glucoamylase Sucrase-isomaltase Lactase-phlorizin hydrolase Trehalase
<b>Peptidase</b>	Aminopeptidase A, N, W Carboxypeptidase P Dipeptidyl aminopeptidase IV Peptidyl dipeptidase Pteroyl polyglutamate hydrolase Enteropeptidase Enteropeptidase-24.11 Enteropeptidase-2 $\gamma$ -Glutamyl transferase
<b>Phosphatase</b>	Alkaline phosphatase Phosphodiesterase-I

*Adapted from Holmes and Lobley, 1989*

peptides, the regulatory proteins guanylate cyclase as well as receptor proteins that selectively bind calcium and cobalamine complexed to intrinsic factor vital for the absorption of vitamin B<sub>12</sub> (86, 241). The asymmetric distribution of ion motive pumps, channels and transporters on the apical membrane surface allows for the vectorial transport from the lumen to the interstitial compartment.

## **B. Basement and Basolateral Membranes**

The basolateral membrane overlays a basement membrane that is a continuous sheet of fine fibrillar material with fenestrations to allow the migration of chylomicrons and lymphocytes into the intercellular spaces (377). The major component of the basement membrane extracellular matrix includes type IV collagen, laminin, entactin, heparan sulfate proteoglycan and interstitial matrix components (221). It has been reported that direct cell contacts with the extracellular matrix via integrin-dependent signals are vital in providing survival signals to the enterocyte, thus delaying apoptosis (127, 311).

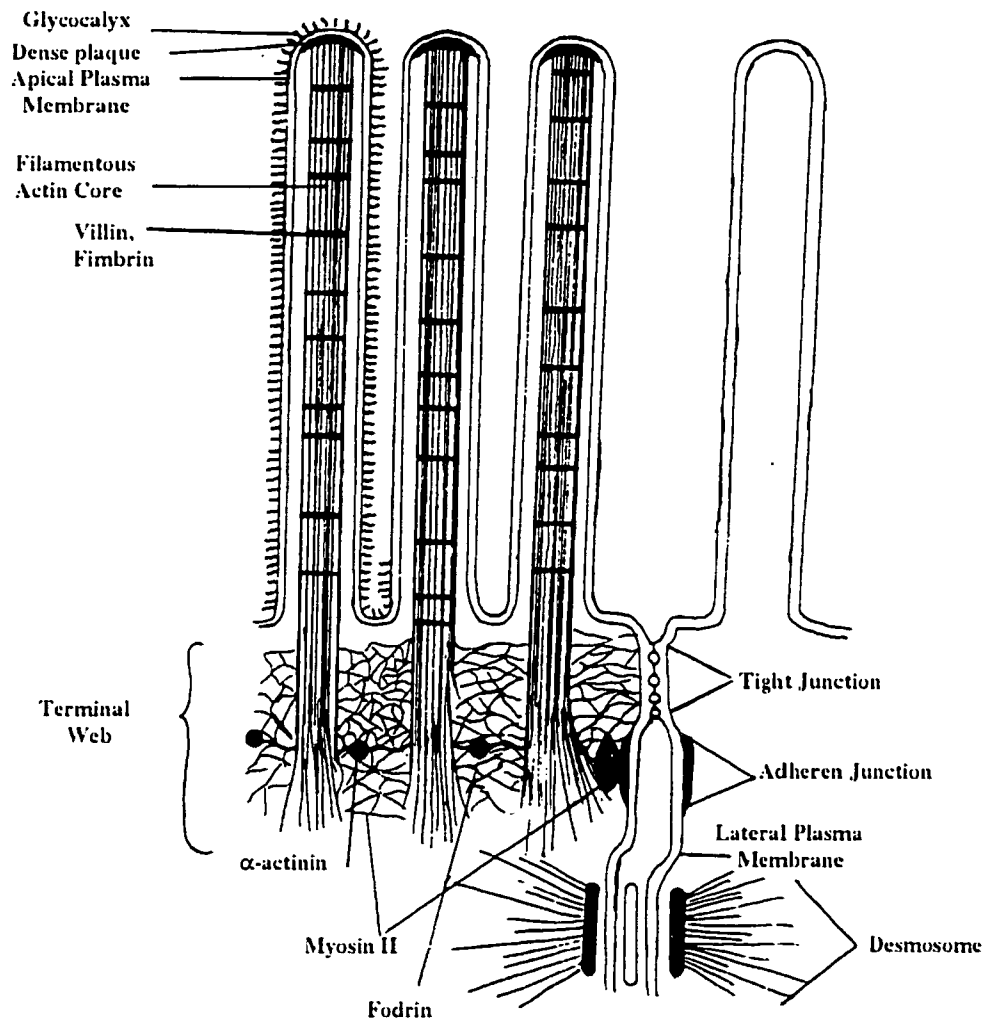
The basolateral membrane contains several pumps and transporter or carriers including the transferrin receptor for transport of iron, basolateral amino acid and di- and tri-peptide carriers, glucose and fructose carriers, a Ca<sup>2+</sup>-ATPase pump for active transport of calcium during low Ca<sup>2+</sup> conditions as well as a Na<sup>+</sup>, K<sup>+</sup>-ATPase pump which is an energy requiring pump that transports Na<sup>+</sup> into the intercellular space and maintain electrochemical gradients favouring Na<sup>+</sup> absorption and Na<sup>+</sup> coupled transport of nutrients by the resident transporters of the microvillus membrane. Also located at the basolateral membranes are adenylate cyclase and glycosyltransferases (212).

### 1.3.2. The Enterocyte Cytoskeleton

The microvillus membrane is supported by an underlying cytoskeleton, that not only serves as a supporting structure to maintain the architecture of the entire apical pole to the enterocyte, but also plays a role in the uptake of some nutrients into the cell and in the control of paracellular permeability (180). The cytoskeleton is primarily composed of axial bundles of 20-30 actin filaments and associated proteins and extend down into a transverse fibrillar mesh work known as the terminal web (14, 272) (Fig. 2). The microvillar core bundle and the terminal web regions consist of different actin associated proteins which are unique to each region.

Most microfilament associated proteins are known to be substrates of serine/threonine and tyrosine protein kinases whose activities *in vitro* are effected by phosphorylation, and include Ras and Rho proteins which belong to the G-protein family distributed apically in the enterocyte membrane (43). Rho GTPases have been known to regulate the organization of the cytoskeleton (150, 151, 287). Rho has been shown to regulate filamentous actin organization at the apical pole of polarized intestinal epithelial cells thus influencing permeability of the associated tight junction (287). Exactly how rho functions in regulating cell cytoskeleton assembly and function is beyond the scope of this introduction, suffice to say that rho acts as a molecular switch to control signal transduction pathways that link membrane receptors to the cytoskeleton (149). In the microvilli of the enterocyte, F-actin core bundles are hexagonally arranged by bridging filaments known as actin bundling proteins, and include villin, fimbrin, a 110-kD protein calmodulin complex (30, 99), and a family of ERM (ezrin-radixin- moesin) proteins (14, 328, 329). The actin core bundles extend into the terminal web, where other proteins

**Figure 2:** Schematic diagram of the structural features of the apical plasma membrane, the apical cytoskeletal proteins of the absorptive enterocyte, and the tight junctional complex of intestinal absorptive cells. Adapted from *Hardin and Gall, Annals of the New York Academy of Sciences, 1992*.



such as myosin, and a nonerythroid isoform of spectrin known as fodrin form a dense meshwork (14, 99, 180). Also located within the terminal web region is a set of filaments that extend along the cytoplasmic aspect of the zonula adherens, below the zonula occludens, to form a contractile circumferential filament band (56) that is composed of actin filaments and contains  $\alpha$ -actinin, tropomyosin and myosin (46, 130, 172, 173). The circumferential filament band is known as the perijunctional actomyosin/actin-myosin ring and is important in regulating tight junction permeability and paracellular flow across both native and cultured intestinal epithelia (239, 240). Within the terminal web, microtubules may extend from below to further associate with the cytoskeletal proteins. This chapter will focus on the major cytoskeletal proteins in this study.

#### **A. Actin**

Actin based cytoskeleton appears to be ubiquitous among eukaryotes and its appearance is said to be a key step in the earliest history of eukaryotic lineage (366). In eukaryotes, actin is found in two forms: monomeric actin, also known as globular actin (G-actin), and filamentous actin (F-actin) which are polymerized forms of G-actin. Actin monomers polymerize in a head to tail fashion to form long helical filaments whose two ends are structurally and dynamically distinct (366). Core actin filaments point unidirectionally away from the membrane allowing them to pull the plasma membrane inward when needed (276). It is known that changes in actin concentration within an enterocyte corresponds to changes in microvillus length (275, 353). Mooseker *et. al.* (1982) demonstrated that addition of excess actin monomers to isolated membrated brush border causes an acute increase in microvillus length (275).

G-actin forms a pool that is distributed diffusely throughout the cell cytoplasm whereas F-actin is found at distinct sites such as i) the terminal web and the microvilli core, ii) in association with cadherins at the zonula adherens forming the actin-myosin ring, iii) along the lateral cell surface including tight junctions and iv) in large bundles at the base of the cytoplasm in association with integrins at focal adhesion sites (400). F-actin cores have binding 'hot spots' where certain bundling proteins are more likely to bind than others (262). Villin and other bridging filaments attach laterally in a regular array, spread every 33nm down the length of the core (44). Many proteins are found to be associated with the microvillus actin core, including an 80kD epidermal growth factor (EGF) receptor protein tyrosine kinase substrate termed p81 (141, 385). EGF addition to rabbit jejunum is able to increase microvilli height within five minutes indicating the rapidity of cytoskeleton in responding to cellular signals (80, 156). In addition, focal adhesions where actin attaches to the substrates through the plasma membrane are known to be enriched in tyrosin-phosphorylated proteins such as vinculin, talin, paxillin, tyrosine kinase pp<sup>60v-src</sup> and pp125<sup>FAK</sup> (331). These proteins serve as sites for actin regulation and function, which are extremely rapid.

F-actin plays a critical role in the enterocyte cytoskeleton. Abnormal intracellular expression of actin has been implicated in cell transformation, metastatic potential, motility, fibrotic diseases, familial amyloid polyneuropathy and scar formation (191). In fact, the internalization of many bacteria, including *Salmonella typhimurium*, *Proteus mirabilis* and *Escherichia coli* depend on F-actin (117). F-actin is a common target for many pathogens and intestinal pathogens, such as shiga toxin-producing *E. coli* (STEC), *Shigella*, *Listeria*, *Yersinia*, and *Clostridium difficile* which cause diseases

associated with the rearrangement of F-actin in intestinal cell cultures (28, 161, 162, 188).

### **B. Villin**

Villin is a 95kD polypeptide with a 'core' domain made up of six repeats and a unique thermostable COOH-terminal headpiece required for F-actin bundling activity (126, 274). Villin saturates actin filaments at 2 or 3 molecules of villin to one molecule of actin, and decorates the full length of actin filaments, even in the rootlet, to cross link actin (47, 256). In the enterocyte, actin is bundled into hexagonal arrays by bundling proteins such as villin, fimbrin and a recently discovered 100kD protein known as epsin (22). Of these, villin has been implicated to have a critical regulatory role in the organization of epithelial brush border microfilaments. During embryonic development, villin is the first cytoskeletal protein to be apically localized (84). Similarly, in the course of enterocyte migration and differentiation from the crypt towards villus, the amount of villin increases dramatically and is concurrent with the appearance of brush border microvilli (35). Villin is also important during the restitution process where it is found at the leading edges of migrating cells *in vivo* and *in vitro* (239). There is also a temporal decrease in cortical F-actin and an increase in cytoplasmic villin which parallel the phenotypic changes in cell shape throughout the restitution process (5).

The importance of villin to the cytoskeleton architecture was further demonstrated in an elegant experiment by Franck and his colleagues (1990) using cultured cells that do not normally express villin but do contain actin. When villin was microinjected into these cells, there was a rapid and long lasting change in cell morphology, including reorganization of myosin, tropomyosin,  $\alpha$ -actinin, and fimbrin normally associated with



stress fibers, as well as the reorganization of actin into cortical structures such as microspikes and large surface microvilli (122). In separate experiments, Friederich *et al.* (1989) demonstrated that microinjection of villin into fibroblast also led to the formation of microvillar-like structures (125). Additionally, the use of villin anti-sense mRNA in Caco2 cells blocks the formation of microvilli, and the localization of enzymes sucrase-isomaltase to the apical plasma membrane (84). Evidence also exists for the redundancy of villin as a bridging protein. The targeted disruption of the villin gene in mice indicated no impairment to the morphogenesis of microvilli (304). This was expected as the developing intestinal cells *in vivo* were likely able to respond to, and compensate for the loss of villin by other actin bundling proteins such as fimbrin or epsin (304). In addition, another actin bundling protein, advillin, which is also a member of the gelsolin family that villin belongs to, has been implicated to play a compensatory role which may explain the near normal phenotype observed in villin deficient mice (252).

Villin has dual properties in response to  $\text{Ca}^{2+}$  binding and has been described as a schizophrenic protein in regulating the integrity of the cytoskeleton (256). At intracellular  $\text{Ca}^{2+}$  concentrations less than 10nM villin cross-links actin filaments into bundles, however at concentrations greater than 1 $\mu\text{M}$  villin binds to and caps the barbed fast assembly end of the filament (101, 256). By capping the barbed ends of actin, villin fragments actin, and also plays a role in regulating actin turnover.

A pool of villin is associated with the plasma membrane, capable of tyrosine phosphorylation and association with phospholipase C- $\gamma$ 1, which is involved in early aspects of cell signalling via the signal transduction pathway. In addition, villin in response to stimuli has been shown to associate with polyphosphoinositides, especially

phosphoinositol 1,4,5-bisphosphate (PIP<sub>2</sub>) (211). *In vitro* studies have demonstrated that the major effect of PIP<sub>2</sub> on villin is to inhibit its ability to sever actin filaments (192). Unequivocally, the differential activation of severing and nucleating activities in response to changes in calcium and polyphosphoinositides which are often the immediate consequence of cell stimulation, place villin directly in the pathway between receptor activation and cytoskeletal remodelling, and as indicated by Ca<sup>2+</sup>-dependence, villin-mediated actin cytoskeletal disruptions during pathophysiological states.

### **C. Ezrin**

Ezrin is a cytoskeletal protein which belongs to the ERM (ezrin-radixin-moesin) family and crosslinks actin core bundles to the overlying plasma membrane via specific groups of integral membrane proteins such as CD44, CD43 and ICAM2 (215). These integral membrane proteins are involved in cell-cell adhesion. For example, CD44 is a transmembrane receptor for hyaluronate which is involved in the homing and binding of proinflammatory cells (160). ERM proteins are found at the cytoplasmic surface at crucial locations between the plasma membrane and the underlying cytoskeleton (328, 329). At these locations, ERM proteins are able to participate in reorganization of the cortical actin cytoskeleton, signal transduction and growth control (254). Only about half of the ERM proteins are located just beneath the plasma membrane where they function as membrane-actin cross-linkers; the other half assumes a soluble form in the cytoplasm and do not tightly associate with actin filaments (171, 329).

Ezrin was first identified as a constituent of microvilli and in intestinal epithelial cells only ezrin and moesin are expressed (42, 375). The suppression of ERM proteins by anti-sense oligonucleotides causes a complete loss of microvillar structure from the

cell surface indicating that these proteins play a key role in microvillar formation in general (362). Ezrin which is initially found in an inactive folded state is activated by tyrosine phosphorylation, serine/threonine phosphorylation or phosphoinositides (244). Researchers have yet to determine the exact mechanism of ezrin activation as ezrin is a phosphoprotein with multiple phosphorylation sites (45). *In vivo* and *in vitro*, ezrin has been shown to be phosphorylated by various tyrosine kinases, even by the EGF receptor (45). To complicate matters further, the GTPase protein, rho, has also been implicated in the activation of ezrin for actin core binding, and may be involved upstream of the regulator from the ezrin-CD44 association (171). As rho reportedly regulates phosphatidylinositol turnover, control of actin based cellular events by phosphoinositides may in fact involve ezrin (97). There is also a good correlation between phosphorylation of tyrosine or serine/threonine and the formation of microvilli and membrane ruffles that contain abundant ezrin (369).

Interestingly, the correlation between epithelial physiology and ERM proteins was further strengthened by an experiment conducted by Kondo *et. al.* (1997) who showed via immunofluorescence microscopy and biochemical analysis, that in the early phase of Fas ligand-induced apoptosis in cells expressing the Fas ligand, ERM proteins, including ezrin, translocate from the plasma membrane of microvilli to the cytoplasm concomitant with dephosphorylation of ERM proteins. Interleukin 1 $\beta$ -converting enzyme (ICE) protease inhibitors were able to suppress the dephosphorylation as well as the cytoplasmic translocation of ERM proteins (215). The disappearance of microvilli has long been recognized as one of the common early events of apoptosis. In this study the researchers were able to indicate that during apoptosis, microvillar disappearance is

directly due to the cytoplasmic translocation of ERM proteins, particularly ezrin in intestinal epithelial which reflects changes that occur in the enterocyte cytoskeleton due to physiological processes. Recently, ezrin was found to be redistributed to the cell membrane and phosphorylated in epithelial cells infected with enteropathogenic *Escherichia coli* (106). The authors speculated that the phosphorylation of the redistributed ezrin is involved in transducing signals in the host cell (106).

#### **D. $\alpha$ -Actinin**

$\alpha$ -Actinin is ubiquitous in cells with F-actin, which  $\alpha$ -actinin binds, cross-links and stabilizes (57). When bound with phosphatidylinositol 4,5-bisphosphate,  $\alpha$ -actinin is activated and responds via an actin-gelating activity (129). The cytoskeletal protein  $\alpha$ -actinin, is found closely associated with the zonula adherens, which is the junctional complex at the lateral margins of the cells (100). Specifically,  $\alpha$ -actinin is found associated with the actin-myosin perijunctional ring where it may participate in anchoring the F-actin-containing ring to the membrane at this site (100). By cross linking the actin filaments in arrays of opposing polarities,  $\alpha$ -actinin plays a pivotal role in the contractility of the actin-myosin ring (99). At the plasma membrane,  $\alpha$ -actinin interacts with diacylglycerol or palmitic acid (154).  $\alpha$ -Actinin can also be found in microvillar core rootlets within the terminal web (99). However, in endothelial cells,  $\alpha$ -actinin also mediates linkages between the plasma membrane and cytoskeleton via association with ICAM-1, L-selectin,  $\beta_1$ - and  $\beta_2$ - integrins in the intracellular focal contact-associated protein talin, vinculin and zyxin (163).

Due to its close proximity to the plasma membrane,  $\alpha$ -actinin can be utilized as a candidate protein in defining cytoskeletal responses underneath the plasma membrane.

Experiments conducted utilizing attaching and effacing *E. coli* (EPEC) which causes lesions characterized by the destruction of the microvillus membrane and intimate contact between bacteria and host plasma membrane, resulted in an aggregation of host  $\alpha$ -actinin into the cytoplasm of epithelial cells (189). In EPEC infected cells, the rearrangement of  $\alpha$ -actinin occurred in conjunction with rearrangement of cytoskeletal F-actin and is likely indicative of the pathogenic effects of the bacteria (189).

### **1.3.3. Enterocyte Tight Junctions**

The enterocyte tight junction is responsible for maintaining the barrier function and polarity of the epithelium. Tight junctions are located at the apex of epithelial cells where they join their neighbours and measure approximately 1-2  $\mu\text{m}$  in depth (243). Tight junctions are relative, not absolute seals to passive permeation through the paracellular space, thus representing the rate limiting barrier to passive permeation. The tight junctions are actually a series of barriers of 'kisses' where neighbouring cells join and lie adjacent to the perijunctional actin-myosin ring. Solute permeation is restricted based on size and charge. As a consequence all epithelia with tight junctions have a measurable resistance and this resistance across the tight junction provides the easiest way to assess junctional ion permeability. The value of the transepithelial resistance depends on the true area of the epithelium, as such to correct for macroscopic or microscopic foldings which increase the area of the epithelium, the transelectrical resistance is expressed relative to the capacitance (81, 227). Monolayers of epithelial cells develop transepithelial electrical resistance as their tight junctions become assembled and sealed.

In freeze-fracture replicas, tight junctions appear as a network of anastomosing strands parallel to the free surface of the epithelium, a pattern suggesting that each strand constitutes a barrier to the passage of ions and molecules through the paracellular pathway (174). There is evidence that actin is involved in the functional regulation of the tight junction. Drugs such as phalloidin and cytochalasins B and D which disrupt actin organization within the cell have been shown to cause concomitant perturbations of paracellular resistance and in junction freeze-fracture fibril organization (41, 237). In addition, the apical membrane  $\text{Na}^+$ -coupled transporter of glucose and amino acids into the enterocyte, triggers via an unknown mechanism, a contraction of the actin-myosin cytoskeleton associated with junctional complexes which then causes an increase in tight junction permeability in the presence of glucose (239). It is likely that tight junction association to the cytoskeleton of enterocytes occur within the terminal web and at sites where cells adjoin, known as kiss sites, with microvillus actin rootlets. In fact an intact apical submembranous cytoskeleton is a prerequisite for proper functioning of the tight junction. More specifically, microfilaments, rather than microtubules are involved since colchicine does not affect assembly of tight junctions *in vitro* (41). The microfilaments are thought to attach to the tight junction from the terminal web below it and as microfilaments contract or relax, junctional permeability is altered (238).

There are several proteins associated with tight junctions. These include zonula occludens-1 (ZO-1), cingulin, and BG91 (9). Of these, ZO-1 is the best described. ZO-1 is the first protein shown to be a unique component of tight junctions and is distributed exclusively along the margins of cell-cell contact in a continuous network (10). During replating of *in vitro* cultures, junction reassembly involves an upregulation in ZO-1

mRNA and protein levels (10). *In situ* hybridization of ZO-1 performed in mouse small intestine indicate that ZO-1 mRNA expressions occur predominantly over the crypt zone in readiness for ZO-1 assembly into tight junctions as cells mature and migrate up the villus (242).

In addition to creating barriers to the diffusion of membrane proteins and lipids, tight junctions also serve as an indicator of membrane integrity. Altered assembly and functional properties of tight junctions have been documented in a wide range of pathologic states including cancer, Crohn's disease and intestinal food anaphylaxis (9). Many researchers have also utilized the transepithelial electrical resistance created by tight junctions as reliable markers of epithelial permeability during disease states (117, 118, 161, 162, 301, 302, 310).

#### **1.3.4. Epithelial Cell Cultures**

##### **A. Human Colonic Adenocarcinoma (Caco2)**

The colonic adenocarcinoma cell line Caco2 is a cell line that is able to spontaneously differentiate in culture, and provides a reliable tool with which to study brush border assembly and intestinal cell functions (121, 300, 305, 412). As an *in vitro* model, Caco2 cells are useful as they form polarized monolayers and express well developed microvilli (10, 143, 184). Although colonic, Caco2 also express several markers characteristic of normal small intestinal cells, such as microvillar hydrolases and polarity (305). Currently, Caco2 cells are used widely in research including investigations in drug absorption, transport properties, interleukin production, microbial pathogenesis and many others.

## **B. Small Intestinal Cells (SCBN)**

This cell line is the first non-tumorigenic human small intestinal epithelial cell line to be isolated and grown in tight monolayers (295). SCBN was originally obtained from a duodenal biopsy of a male patient (B.N.) with diarrhea of unknown aetiology. SCBN has been shown to form polarized monolayers, to express junctional complexes and disaccharidase activities as well as to have well developed microvilli (295). In addition, SCBN express cytokeratins, mucin antigen, mRNA for EGF, interleukin-6 and vascular cell adhesion molecule-1 (295). Recently, SCBN has been shown to express a functional interleukin-2 receptor where IL-2 was able to modulate ion transport and cellular proliferation (290). Without doubt, SCBN is a novel, non-transformed epithelial cell line that has great potential within the research arena.

### **1.4. Intestinal Disease States – Diarrhea**

It has often been noted that the gut is capable of colonization by a variety of pathogenic organisms, but is limited in its means of expressing an injured state. Diarrhea is a common manifestation in intestinal injury. In some instances, such as the response to noxious and injurious agents, diarrhea is appropriate as it may act to flush out pathogens and toxins. *Vibrio cholera* produces an enterotoxin capable of binding to enterocyte  $G_{M1}$  gangliosides located on the apical membrane (224). Once bound, the enterotoxin enters the host system to ADP-ribosylate the  $G_s$  subunit of the G-protein, which normally regulates host cell adenylate cyclase in a hormone dependent manner. GDP-ribosylation renders adenylate cyclase inactive thus increasing cyclic AMP such that  $Cl^-$  is actively secreted into the lumen and  $Na^+$  passively follows (224, 278). Physiologically, this



simulates a high  $\text{Na}^+\text{Cl}^-$  secretory condition within the lumen culminating in the secretion of water and the formation of secretory diarrhea.

Malabsorptive diarrhea, on the other hand, are consequences of any disorder that interrupts one or more of the states of digestion and absorption which include hydrolysis, membrane transports, cellular processing and substrate transport into the bloodstream or lymph system (38). The lack of nutrient or electrolyte absorption can be due to i) mucosal morphology abnormalities, ii) abnormalities in intestinal contractility and transit or iii) a lack of intestinal digestive enzymes or nutrient transporters due to genetic abnormalities in expression, increase in immature enterocyte numbers from abnormal epithelial cell turnover, or in response to disease states. In addition to decreased water absorption, the surplus of nutrients or electrolytes in the lumen can create an osmotic imbalance such that via osmosis, water will flow from the basolateral regions into the lumen of the intestine, enhancing the diarrhea associated with malabsorption. Furthermore, undigested nutrients can be fermented by luminal flora which also act to increase intraluminal osmotic loads and by its acidity decrease transit times of bowel contents, further exacerbating malabsorptive diarrhea (38). The focus of this chapter is to present differing conditions of both non-infectious, and infectious malabsorptive diarrhea, involving some of the morphological and physiological conditions described above.

#### **1.4.1 Noninfectious Malabsorptive Diarrhea**

##### **A. Crohn's Disease**

Crohn's disease (CD) is the major form of chronic inflammatory bowel disease in developed countries. Crohn's disease occur in young adults with an estimated prevalence

of more than one per thousand inhabitants (59). To date, researchers remain uncertain as to how CD develops. However with an incidence in the United States increasing from 1 per 100,000 to as high as 10 per 100,000 over the past 30 years, CD remains a cause of concern for industrialized countries where it occurs in vastly higher numbers than in developing countries (214). Despite this, CD is not considered communicable but it is recognized to occur in rates that are higher in Caucasians than people of African descent, Hispanics, Asians and American Indians (214, 258). In either case, both sexes are affected equally with the majority diagnosed before age 30 (214). There is a familial tendency in CD with a concordance of about 50% in genetically identical twins and to a lesser degree with non identical twins and more remote relationships (376). So far the loci that appear to be associated with the condition is an area on chromosome 16 and on chromosome 3, 7 and 12 (185). The genes in these areas were found to include genes controlling growth factors and responses to growth factors as well as those regulating the structure of mucin glycoprotein adhesion molecules and cytokine receptors. Other factors associated with an increased incidence of developing CD include nutritional or dietary factors such as breastfeeding, sugar and food additive intake as well as environmental factors such as cigarette smoking, oral contraceptives, hygiene, environment, climate, pollution and stress (119, 226, 349).

The symptoms associated with CD includes abdominal distention, flatulence, cramps and bouts of malabsorptive diarrhea associated with abdominal pain, fever and weight loss (65, 214). Also associated with CD is an increase in intestinal permeability (279, 312, 364, 380). CD can occur anywhere in the gastrointestinal tract and has a predilection for the terminal ileum and ascending colon, but affected patients often

demonstrate well demarcated segments of diseased bowel separated by healthy (skip) zones (202, 356). In the diseased regions, a combination of longitudinal and transverse fissures/ulcers with intervening mucosal edema produces the characteristic cobblestone appearance which aid in the diagnosis of CD (33). Other complications of CD include recurrent, symptomatic bowel obstruction, toxic megacolon, sepsis associated with persistent abscesses or fistulas and medication failures (214). Unfortunately more than half of all CD patients require at least one surgical procedure involving the removal of intestinal sections riddled with lesions or fistulas (214).

How CD is initiated or persists remain unknown. However, researchers have suggested a role for pathogenic organisms. It was found that isolated bacterial cell wall products injected into the wall of the intestine, thus bypassing the barrier function of the epithelium, was able to cause a chronic relapsing inflammatory condition which superficially resembles CD (407). In addition, 'knockout' mice that lack the genes for interleukin-10 are also prone to developing chronic inflammation of the bowel, but what is most interesting is that this inflammatory condition can be prevented if the animals were reared in a bacterial free environment (220, 326, 355). This lead researchers to conclude that there may be a single exogenous agent, such as *Mycobacterium paratuberculosis* or a combination of antigens in the gut, perhaps bacteria derived, which could initiate an inflammatory response, enhancing mucosal permeability and permitting greater antigen ingression and the further stimulation of the inflammatory process (175, 320).

That the inflammatory host response plays a critical role in the disease progress is clearly illustrated by research that shows i) an increase in T cells (321), ii) increase in

plasma cells expressing IgG and IgM in the lamina propria (37), iii) a denser population of macrophages (352) and iv) an increase in the number of acute inflammatory cells, predominantly polymorphonuclear leukocytes (330). In parallel there is also an increase in the number of proinflammatory molecules present within the cells, in interstitial fluid and in the lumen of adjacent gut (175). Abnormal numbers of activated B lymphocytes have also been detected in the peripheral blood of CD patients (406). Activated inflammatory cells within the mucosa can further initiate or perpetuate inflammation by direct cell-mediated processes, including cytotoxicity for other cell types via the release of immune mediators such as IL-1, IL-2, IL-6, IL-8, TNF- $\alpha$ , activated oxygen radicals, and reactive nitrogen metabolites which have been shown to induce oxidation and inhibition of essential epithelial cell functions as well as disrupting actin cytoskeleton and tight junctions (36, 40, 128, 147, 259, 263, 327). Undoubtedly, the immune function of the patient plays a large part in prolonging as well as perpetuating the disease state. However apart from the immune mediators involved in directly initiating diarrhea, malabsorptive diarrhea associated with Crohn's Disease is largely due to immune mediated villus atrophy and crypt epithelial cell destruction (356) as well as a diffuse shortening of enterocyte microvilli with the latter seen in models of Crohn's disease (103). It is well established that Crohn's patients can suffer from a reduction in brush border enzyme activities (15, 148). The loss of physiologic function of the intestine due to these morphological abnormalities and loss of enzyme activities, dramatically reduces the potential for nutrient and water absorption.

To date, corticosteroids remain the mainstay treatment of active CD, effectively dampening the host immune response, and results in a rapid initial reduction of symptoms

in 70% of patients. However, focus is now being turned to cytokine therapy including the use of anti-cytokines such as anti-TNF- $\alpha$  or by using modified cytokines such as recombinant IL-10 which act to inhibit pro-inflammatory cytokines (356, 358, 387).

## **B. Celiac Disease**

Celiac disease also called gluten enteropathy is an intolerance in genetically susceptible individuals to certain storage proteins found in cereals including wheat, barley and rye (21, 134). A change in diet with the complete avoidance of gluten intake can result in a rapid response within one to two weeks, with disaccharidase deficiencies resolving upon epithelial regeneration. In a multicenter study involving 36 centers from 22 European countries, celiac disease was demonstrated in an average of about 1 in 100 live births with a range of one in 250, to one in 4,000 in the participating countries (144). In Asia and Africa, celiac disease is rare, but in all populations celiac disease in females outnumber males two to one (21).

The storage proteins in gluten associated cereals are found in the alcohol soluble fraction as gliadins (wheat), secalin (rye) and horden (barley) (134, 249). Collectively they are known as prolamines, and are thought to be the most responsible for triggering or exacerbating celiac disease. Gluten is found in bread, cereals, pasta, biscuits and even as a binder in some medications (177). Oats which have 5-10% of prolamines, versus 50% with wheat, are considered to be either less toxic or not toxic at all (340). More distantly related grains such as maize, rice and millet show no evidence of toxicity (134).

The intolerance in celiac disease is genetically predisposed with the prevalence of celiac among first degree relatives being approximately 10%, with as many as 75% of monozygotic twins found to be concordant with the disease (16, 306). Researchers have

since found a strong association of celiac disease with HLA class II molecules, in particular HLA-DQ2 and -DQ8 where more than 90% of celiac patients have the -DQ2 heterodimer encoded by A1\*0501, B1\*0201 (347). Yet, celiac disease is not limited to HLA susceptibility. In a study involving the predisposition of siblings to celiac it was found that the theoretical relative risk calculated from the known carrier frequency for HLA-DQA1\*0501, B1\*0201 is approximately 1 in 20, however, the actual risk of celiac in siblings is as high as 1 in 4, thus indicating that celiac disease is a polygenic disorder requiring both HLA and non-HLA susceptibility genes (183). This observation is supported by the fact that 20% of the general population who do not suffer from celiac disease also carry the risk alleles encoding the HLA-DQ, further indicating that other genes outside the HLA region are involved in susceptibility of celiac disease (249).

In celiac disease the mucosal folds are greatly diminished and thickened in the small intestine (371). Other small intestinal abnormalities seen in celiac disease include i) partial to total villus atrophy, ii) elongated crypts, iii) increased mitotic index in the crypts, iv) increased intraepithelial lymphocytes, v) infiltration of plasma cells, lymphocytes, mast cells, eosinophils and basophils in the lamina propria, vi) loss of nuclear polarity with pseudostratification of epithelial cells, vii) loss of brush border and viii) abnormalities in epithelial cell shape, which becomes flattened and cuboidal (39, 371). Undoubtedly, these abnormalities are associated with immune mediated intestinal injury. It is thought that the prolamines in gluten-containing cereals bind to extracellular transglutaminase which catalyzes the transfer of an acyl group from gliadin, the glycine donor, to a nonspecific lysine acceptor, resulting in protein crosslinking. Here, antiendomysial antibody is directed to the transglutaminase and the T-cell epitopes of

gliadin (95). In this way the transglutaminase acts as an autoantigen. Antigen presenting cells with the specific HLA-DQ2 class II molecules on their surface present the autoantigen to T cells thereby activating an auto-reactive T-cell population (196, 233, 248). Activation of the immune response leads to upregulation of IL-2 receptor expression and the production of proinflammatory cytokines IFN- $\gamma$ , TNF- $\alpha$  and IL-6 which may further stimulate the release of injurious inflammatory mediators (216, 217, 357). In other studies, gliadin was found to have an early and immediate effect on cultured mucosal biopsies. This included an upregulation of ICAM-1 and HLA-DR expression on enterocytes and adjacent macrophages (247). The direct toxicity of gliadins is said to aid in precipitating the chain of events that activate T cells in the lamina propria which orchestrate the damage leading to enteropathy. Whether it is by a direct effect or immune mediated, the damage to the intestine of celiac patients includes the loss of normal intestinal mucosal architecture. In celiac patients the architecture of the small intestine is closer to that found in the colon, where villi is not as tall and crypts are much deeper. In addition the increased crypt mitotic activity indicates premature epithelial cell turnover, where the majority of cells are immature and lack disaccharidase enzyme expression. More importantly, there is a dramatic loss of absorptive surface area due to the loss of enterocyte brush border (89). The intestinal permeability in celiac untreated patients is typically higher, and is found to correlate with histological changes caused by celiac disease (393, 394). Cumulatively, these physiological abnormalities result in the perpetuation of malabsorptive diarrhea.

## 1.4.2. Infectious Malabsorptive Diarrhea

### A. *Yersinia enterocolitica*

*Yersinia enterocolitica* is recognized as a major cause of bacterial enteritis in the pediatric population and has been recovered from humans, animals, especially pigs and environmental sources such as infected water and in food including tofu, cow, and goat milk (251, 332, 337). The infection cycle involves the fecal-oral route from animal to man, person-to-person or from contaminated food or water (291). Once ingested, *Yersinia enterocolitica* colonizes and enters epithelial cells, reaches the lamina propria and muscularis mucosa, proliferates in reticuloendothelial cells, particularly in peyer's patches and causes ulceration (155). Invasion by *Yersinia enterocolitica* is also dependent on F-actin, where the use of cytochalasin D will block the entry of *Yersinia enterocolitica* into the cell (409).

The replication of *Yersinia enterocolitica* in host lymphoid tissue is dependent on the presence of a 70kb virulence plasmid that encodes a set of secreted proteins termed Yop's or *Yersinia* outer proteins which are responsible for mediating resistance to the host defense mechanism (309, 354). Yop E is capable of disrupting the actin microfilament structure which induces cell rounding and detachment from the extracellular matrix (324). Yop H on the other hand dephosphorylate host cell proteins thus subverting normal signal transduction processes of the eukaryotic cell (11, 31). Together, Yop E and H are largely responsible for the inhibition of phagocytosis and suppression of the oxidative burst of phagocytes (325). *Yersinia enterocolitica* has been found to produce an enterotoxin, but this is not essential for disease production as strains without the enterotoxin is still capable of causing intestinal damage (332).



*Yersinia enterocolitica* is characterized by weight loss and malabsorptive diarrhea with marked histologic and biochemical changes in intestinal mucosa which include i) extensive mucosal abscess formation and occasional ulcer formation in the absorptive epithelium, ii) crypt hyperplasia, iii) villus atrophy (291, 292), iv) loss of enterocyte brush border (55) and v) decrease disaccharidase enzyme activity (55, 292). The severe and diffuse loss of small intestinal brush border was found throughout the small intestine. Brush border height and surface area, determined by electron microscopy, were markedly decreased in infected animals even in areas where there were no surrounding abscesses (55). In addition, the increase in crypt proliferation indicated that there was no delay in cell maturation determined by  $\text{Na}^+\text{K}^+\text{ATPase}$  and thymidine kinase activity which are markers of mature enterocytes (291, 292). Furthermore, decrease in disaccharidase activity was out of proportion to the degree of mucosal injury, indicating that the loss of villi alone cannot account for the degree of disaccharidase impairment (55, 291, 292). Cumulatively, this suggests that the malabsorptive diarrhea associated with *Yersinia enterocolitica* cannot be accounted for by morphological alteration to the mucosa or to increased epithelial cell turnover with an increase in immature enterocyte numbers. Instead the pivotal cause of malabsorptive diarrhea in *Yersinia enterocolitica* infection appears to be due to a diffuse loss of brush border, directly decreasing available absorptive surface area resulting in maldigestion. The mechanism of disease injury is suggested to be due to the direct effect of the organism, to the production of cytotoxins or proteases, or as a result of the inflammatory response from proteolytic inflammatory agents (291). It is attractive to further speculate that the damage and decrease in microvilli likely involves enterocyte regulation of its architectural cytoskeletal proteins,

especially in light of the fact that *Yersinia enterocolitica* is known to affect cellular actin (324).

## **B. Rotavirus**

Rotavirus belongs to the *Reoviridae* family and is the most common pathogen responsible for hospitalization due to diarrhea (297). In the United States alone this amounts to more than 26,000 people a year (297). Viral gastroenteritis is the leading cause of infant mortality in developing countries, where globally, rotavirus is the most common cause of malabsorptive diarrhea in children under two (91). Like most infectious agents of the gastrointestinal tract, rotavirus is spread via the fecal-oral route where virus particles shed in large quantities in stool for up to eight days and is able to survive for weeks on environmental surfaces including those found in hospitals and institutions (91).

Rotaviral enteritis is characterized by fever and vomiting with a duration of one to three days, which usually precedes the onset of malabsorptive diarrhea capable of lasting four to seven days (178). In children, diarrhea of this duration is capable of causing severe dehydration, as such treatment usually involves the use of oral rehydration therapy.

Rotaviruses have limited tissue tropism. The virus infects mostly mature enterocytes located towards the apical area lining the villi of the small intestine (145). The infection is associated both *in vivo* and *in vitro* with a series of subcellular pathological alterations. *In vitro*, cytopathic process by rotaviruses include features coincidental with apoptosis, such as plasma membrane blebbing, peripheral condensation of chromatin and nuclear fragmentation (359). Physiologically, cytopathic processes in

rotaviral infection causes a flattening of epithelial cells, villus atrophy, crypt hyperplasia, microvilli aberrations and an increase in inflammatory cells in the lamina propria (145, 199, 271). The host inflammatory response is likely to perpetuate diarrhea as rotaviral infection is able to induce increased secretion of IL-8 and RANTES (regulated upon activation normal T cell expressed and secreted *in vitro*) (64). Incidentally, IL-8 and RANTES are the most potent chemoattractant for intraepithelial lymphocytes able to release more inflammatory mediators as well as toxic metabolites. The initial serum antibody response to rotaviral infection is the IgM class, but the local IgA immune response remains the critical factor in generating protective immunity after natural infection (91, 112).

The malabsorptive diarrhea associated with rotaviral infection was initially thought to be due solely to an increased number of immature enterocytes (92, 145, 271). Rotaviral infection in piglets, suggests that malabsorptive diarrhea occurs due to cells that fail to differentiate and mature as they migrated at an accelerated rate up from the crypts to replace shed cells (92). Concordant with these findings, it has also been shown that activities of sucrase-isomaltase, lactase and maltase-glucoamylase are frequently decreased during and after infection (83, 92). Interestingly, the low disaccharidase enzyme activity occurs without significant enterocyte destruction at the apical portion of villi (20). In light of this, efforts have been made to identify the mechanisms involved in malabsorptive diarrhea during rotavirus infection. In a study conducted on Caco2 cells, researchers showed that rotavirus infection specifically and selectively decreased the activity and expression of apical sucrase-isomaltase without altering the activity and apical expression of other brush border hydrolases including  $\gamma$ -Glutamyl-transpeptidase,

alkaline phosphatase, amino phosphatase and dipeptidyl peptidase IV (198). The selectivity of rotavirus in decreasing sucrase-isomaltase activity stems from the fact that sucrase-isomaltase, unlike the other brush border hydrolases, is directly targeted from the transgolgi network to the apical membrane thus circumventing delivery to the basolateral membrane and subsequent delivery to the apical membrane via transcytosis (257). The authors suggest that rotaviral infection perturbs the transport of sucrase-isomaltase from the transgolgi network to the brush border membrane. Concurrent with sucrase-isomaltase decrease in activity and expression, rotavirus was able to disrupt apical F-actin and villin, where villin staining in infected cells had irregular subcortical staining (198). Past research conducted in cultured cells have also indicated rotaviral-induced F-actin and villin rearrangement, where rearrangement was due to an indirect change in the organization of the cytoskeleton via biochemical events involving the increase of intracellular calcium which is known to modulate microvilli alterations (266, 274, 370).

Taken together, the research on rotaviral infections indicate that malabsorptive diarrhea is multifactorial, involving an increase in immature enterocyte number, host immune response, a decrease in absorptive surface area via microvilli disruption and the selective decrease in sucrase-isomaltase expression and activity, with the latter factors likely due to cytoskeletal rearrangement in the apical portion of the enterocyte. As rotaviral infection is also able to cause cytopathic responses in enterocytes emulating apoptosis, it is tempting to speculate that the cytoskeletal rearrangement associated with infection is indirectly due to increased intracellular calcium release initiated by rotaviral induced cell apoptosis.

### C. *Cryptosporidium*

*Cryptosporidium parvum* is an apicomplexan parasite first reported in humans in 1976 (87). The parasite is able to cause self limiting diarrhea in immunocompetent persons, but severe life threatening disease in immunocompromised individuals (69, 210). The prevalence of cryptosporidiosis is highest among young children and Acquired Immune Deficiency Syndrome (AIDS) patients (87, 231, 285). Infection in Europe and North America is between 1% and 3% but is much higher in underdeveloped countries, ranging from 5% in Asia to 10% in Africa (88). Transmission occurs by the fecal-oral route with the ingestion of the thick walled oocysts which are highly resistant to the environment (136). Oocyst can be found in as many as 87% of raw water samples and 27% of drinking water samples, as oocyst are resistant to chlorination (222, 223). Consequently, outbreaks involving swimming pool as well as chlorinated drinking water, such as the outbreak of cryptosporidiosis in Milwaukee (1993) where 400,000 people were infected, can occur (253, 260, 350).

Once ingested, oocyst are able to excyst and release sporozoites which penetrate enterocytes to develop into trophozoites beneath the host cell membrane while remaining extracytoplasmic. In this location the parasite can derive nutrients while minimizing immunologic detection (88). The sporozoites are also able to travel up the biliary tract of AIDS patients to infect epithelial cells lining the gall bladder and bile ducts to cause injury (231). *Cryptosporidium* has also been detected in the pharynx, esophagus, stomach, duodenum, jejunum, ileum, appendix, colon, rectum, and respiratory tract of humans (90). As such it is not difficult to understand the potential for resulting in life threatening disease in AIDS patients or other immunocompromised patients, not solely

from malabsorptive diarrhea, but also due to other complications. Apart from diarrhea, clinical symptoms of cryptosporidiosis include anorexia, vomiting, fever, abdominal pain, loss of weight and mild to moderate degree of dehydration with the severity of symptoms dependent on intensity of oocyst shedding (90, 315). Malabsorption of fat and carbohydrates is commonly seen in cryptosporidiosis (378).

T cells have been shown to have a protective role in the clearance of the parasite (76). Athymic mice develop a persistent cryptosporidial infection; specifically, selective depletion of CD4<sup>+</sup> lymphocytes in mice causes chronic infection whereas mice depleted of CD8<sup>+</sup> lymphocytes or B lymphocytes were not susceptible to infection (261, 360, 381). Additionally, elevated anti-*cryptosporidium* IgM, IgG and IgA antibodies have been demonstrated in the sera of infected patients (60).

The pathogenesis of malabsorptive diarrhea in cryptosporidiosis is postulated to be due to several factors including villus atrophy and crypt hyperplasia as well as a defect in microvilli and to the release of metabolites of inflammatory nature, including TNF- $\alpha$  and prostaglandin E<sub>2</sub> which may promote intestinal secretion associated with malabsorption (12, 13, 137, 146). In Caco2 monolayers, *Cryptosporidium* was able to induce injury to the brush border as well as increase transmonolayer permeability with a significant fall in electrical resistance 24h and 48h post infection (146). Incidentally, *Cryptosporidium* in human biliary epithelial cells were found to induce apoptosis (77). Whether this indicates a potential mechanism for epithelial injury affecting permeability as well as defects in the microvilli which plays a pivotal role in malabsorption of fats and carbohydrates, has yet to be determined.

## 1.5. *Giardia lamblia*

*Giardia* was first identified in 1681 by Anton van Leewenhoek in his own diarrheal stool using one of his home made lenses (98). Currently we classify *Giardia* as belonging to the phylum zoomastigophora and it is thought to be the most primitive eukaryotic organism by molecular classification with the small rRNA subunit (345). The quadrinucleated cyst which is the infective stage, can be oval or round and is approximately 10  $\mu\text{m}$  (293). Upon ingestion of cysts, excystation is triggered in the duodenum by exposure to acidic gastric pH and pancreatic enzymes trypsin and chymotrypsin (123). Each binucleated trophozoite can divide by binary fusion, causes mucosal damage and, in the ileum, encysts via mechanism initiated by limited cholesterol availability in the latter regions of the intestine (403). The pear shape trophozoite is dorsally convex with a ventral sucking disk that is aided by the beating of its four pairs of flagella in creating a negative pressure to attach to mucosal surfaces (107, 403). The motile trophozoites are capable of erratic tumbling and colonize the whole length of the small intestine to be encysted at the latter regions and excreted in the feces of asymptomatic and symptomatic *Giardia*-infected individuals.

### 1.5.1. Epidemiology

Giardiasis is the most common protozoal infection of the human intestine worldwide with a prevalence of the parasite infecting the upper intestine of some 200 million people worldwide (1, 398). The parasite is most commonly found in infants and young children, and in developing countries where prevalence is as high as 20% - 30%, nearly 100% of children acquire *G. lamblia* infections during the first two years of life (108, 165). Reinfection with different strains of *G. lamblia* can occur, compounding the

high infection rates with cycles of infection, clearance and reinfection with a corresponding nutrition insufficiency which acts as an additional risk factor in susceptibility to *G. lamblia* infection. In developed countries, infections are sporadic in campers and hikers who ingest contaminated water. Direct transmission is most common in infants and children in day care centers, schools and other institutions, as well as in public swimming pools or other circumstances where there is a breakdown in personal hygiene procedures (1, 293, 308, 333, 365, 403). The current prevalence in industrialized countries is 2% - 5% but a study in the United States indicates that this number is increasing (206).

In all cases, reservoirs of *Giardia* include infected humans, contaminated surface water supplies and many wild and domestic animals are able to carry *Giardia sp.* indistinguishable morphologically, phenotypically and genotypically (51, 368, 404, 405). The prevalence of *Giardia*, apart from the many reservoirs and the ease of transmission, is also due to the environmentally resistant cysts which remain viable for at least two to three months in cold water and are relatively resistant to killing by iodine, ultraviolet light, and concentrations of chlorine used in water processing plants (165, 197).

### **1.5.2. Clinical Manifestations**

The majority of individuals who acquire *Giardia lamblia* are asymptomatic but capable of shedding cysts (379). In most symptomatic patients, symptoms begin within 3 – 20 (mean of 7) days and is self limiting within a two week period (107). Immunodeficiency syndromes such as with AIDS, hypogammaglobulinemia or agammaglobulinemia, and nutritional deficiencies are major contributors to the development of chronic infection (7, 107, 230, 344, 399). In acute and chronic giardiasis,



symptoms include malabsorptive diarrhea with foul smelling, greasy stool, weight loss, flatulence and abdominal cramps, malaise, nausea and anorexia (107, 108, 170, 403). *Giardia lamblia* is also known to increase gastrointestinal transit and smooth muscle contractility, which is a likely cause of cramping and decreased food intake (93). Uncommonly, extraintestinal manifestations such as reactive arthritis and biliary tract disease may occur (82, 135, 336).

Malabsorption due to *Giardia* infection has been widely reported as early as 1926 and can lead to malabsorptive diarrhea (8, 53, 58, 66, 102, 132, 133, 267, 269, 277, 361, 363, 391). In chronic giardiasis, steatorrhea, vitamin A and B<sub>12</sub> as well as protein and carbohydrate malabsorption may also occur (7, 19, 61, 85, 245, 348). Even when infection is asymptomatic, malabsorption of fats, carbohydrate sugars and vitamins may occur with reduced intestinal disaccharidase activities persisting even after parasite eradication (170).

### 1.5.3. Immunology

Host factors, especially immunologic factors, are important in determining the severity of the response to parasites (344, 399, 411). The regulation of *Giardia lamblia* infection by the host immune response has been suggested based on these observations: i) development of severe symptoms in hypogammaglobulinemia patients (389), ii) acquisition of partial resistance to reinfection by experimental animals or spontaneous clearance of infection (190, 203, 282, 317, 396), iii) inability of experimental hosts immunosuppressed by prior treatment with corticosteroids, irradiation or antilymphocytic serum to eradicate the infection (3, 4, 25, 193), and finally iv) by passive transfer of immunity via immune cells (392).

Partial resistance to reinfection in giardiasis is primarily due to anti-*Giardia* antibodies including IgG, IgA and IgM in human sera, with the latter two also actively secreted (142, 167, 187, 194, 284). The theoretical protective effects of anti-*Giardia* antibodies is in the potential inhibition of trophozoite attachment or in aiding the opsonization of trophozoites by complement (313, 322). This suggestion is supported by research indicating that serum titers of IgG and IgA were higher in asymptomatic carriers indicating an increased ability to mount a specific response (299). Furthermore, it has been shown that patients who suffer from chronic giardiasis fail to develop significant levels of antibodies to surface associated trophozoite antigens (388). Research conducted using *G. muris* infections in rats and mice found IgA and IgG were able to coat the trophozoite flagella and adhesive disc, thus reducing parasite motility and adhesion to the epithelium (109, 205, 229). The coating of *G. muris* by the immunoglobulins also aided opsonization by phagocytes and macrophages of the Peyer's patches (23, 166, 313, 367). The immunoglobulins in these rodents, arise from plasma cells that originate from Peyer's patches B lymphocytes (62). In experimental giardiasis, B cell-deficient transgenic mice were unable to produce anti-*Giardia* IgA and could not resolve the infection (351). Other experiments using *G. muris* infected rodents further indicated a vital role for T-lymphocytes in the clearance of the parasite (166, 168, 204). Heyworth *et. al.* (1987) determined the population of T-lymphocytes most critical for clearance of the parasite is the CD4<sup>+</sup> lymphocytes, as selective depletion of this population resulted in a chronic infection with excretion of large numbers of cysts (168). Corresponding research has also established that nude mice, in which *Giardia* infection is chronic, have a more profound deficiency of CD4<sup>+</sup> lymphocytes than CD8<sup>+</sup> lymphocytes (63, 235,

318). Other research has also supported these findings, postulating that the activation of CD4<sup>+</sup> lymphocytes may be responsible for the induction of local antibody dependent effector responses since there is a corresponding increase in induction of CD4<sup>+</sup> cells during the decline phase or latter phase of giardiasis (209, 392). Our current knowledge of a *Giardia*-induced immunological response has lead to the production of a vaccine (Drs. M.E. Olson, D.W. Morck, H. Ceri, University of Calgary, AB, Canada).

Despite the ability of M cells to ingest trophozoites, *Giardia* is too large a parasite to be ingested by macrophages and killed intracellularly via toxic reactive oxygen metabolites. The host immune system compensates by producing an easily diffusible chemical such as nitric oxide (NO) (1). Apart from producing NO, activated macrophages also produce TNF- $\alpha$ , IL-1 and reactive oxygen metabolites which are potent chemoattractors capable of also causing mucosal damage (270, 283). NO has been shown to be responsible for killing *Giardia* trophozoites (115).

The clearance and reinfection of *Giardia* is further complicated by the ability of the trophozoite to express variant specific surface proteins (VSP) (2, 131, 282). VSP's can spontaneously change, are resistant to trypsin and chymotrypsin, and are also shed into the environment (280). Antigenic variation in *Giardia* is known to occur during human infections, where the loss of VSP's occur at the time of humoral responses, hence enabling the trophozoite to escape host immune response (281, 282).

#### **1.5.4. Pathology**

Mucosal biopsies and histological study of the small intestinal mucosa has revealed a complete spectrum of mucosal changes, from mild partial villus atrophy to sub-total villus atrophy which occurs in about 10% of patients with giardiasis (107).

Crypt hyperplasia has been observed concurrently with the reduction of villus height (52, 54, 66, 102, 132, 317). In addition, *Giardia* affects mucosal morphology differently in various intestinal regions as well at different time points of infection. For example, during the acute phase of infection, it has been shown that elongation of the villi in the distal small intestine is seen simultaneously with villus atrophy in the upper gut (52, 54, 113). Conversely, jejunal villus hypertrophy maybe accompanied by villus atrophy in the ileum later in the course of murine giardiasis (133). Apart from demonstrating the amazing, compensatory abilities of the gut for alterations in other regions, these observations also suggest that villus atrophy and the subsequent loss of mature absorptive epithelial surface, may contribute to malabsorptive diarrhea in giardiasis. Yet, clinical manifestations, especially malabsorptive diarrhea have been seen in giardiasis in the absence of villus atrophy (54, 113, 182). Researchers postulated that *Giardia* might induce an increase in epithelial cell turnover, resulting in an immature epithelium incapable of expressing intestinal enzymes. This could result in the malabsorptive diarrhea similar to mechanisms of malabsorption seen in viral gastroenteritis (338). Undoubtedly, *Giardia* is capable of causing damage to epithelial cells. *In vitro* studies have indicated that *Giardia* causes: i) large lipid droplets in the cytoplasm of MDCK cells (70), ii) a reduction in alkaline phosphatase and disaccharidase activities of cultured epithelial cells (111), and iii) changes in cytoplasmic granulation, vacuolation and pyknotic nuclei in epithelial (HeLa) and fibroblastic (Vero) cells (201, 314). In addition, Radulescu *et. al.* (1980) was able to illustrate cellular injury using only cell free filtrates of *Giardia* trophozoites (314). However, using *in vivo* models of giardiasis, increased numbers of immature enterocytes were found only in the duodenum, but not in the

jejunum or ileum, where there was a marked decrease in disaccharidase deficiencies. This indicates that delayed maturation of enterocytes may contribute to diarrhea but is unlikely to be the sole basis for malabsorptive diarrhea in giardiasis (54).

It is widely accepted that *Giardia* is able to impair both mucosal and luminal intestinal enzyme activities, including mucosal maltase, sucrase, lactase, saccharase, trehalase and alkaline phosphatase (8, 52, 54, 66, 102, 111, 133, 195, 341). Brush border and luminal enzyme deficiencies were shown to occur *in vivo* and *in vitro*, and can be induced by live parasites as well as soluble trophozoite extracts (24, 111, 207, 269, 335). Luminal enzymes such as trypsin and chymotrypsin, lipase and amylase are also impaired in giardiasis and are partly responsible for fat malabsorption (68, 73, 207, 288, 335).

The controversy over whether *Giardia* is capable of causing secretory diarrhea continues today. Research on *G. muris* infected mice have reported  $\text{Cl}^-$  secretion and lack of  $\text{Na}^+$  absorption in association with increased  $\text{Ca}^{2+}$  uptake and elevated calmodulin activity (138, 139). However, *Giardia* infected mice also experience a significant reduction in food intake during the acute stage of infection (52, 54). Starvation is able to sensitize the intestine to cholinergic agents or other secretagogues capable of increasing  $\text{Cl}^-$  and fluid secretion (408). Additionally, malnutrition alters the host parasite relationship to the detriment of the host (114). As such, in pair fed studies using *G. lamblia* infected gerbils, no secretory functions were found to be significantly effected (50, 54, 343).

Initially, the abnormal microvilli ultrastructure seen in *Giardia* infected animals, were thought to be due to trophozoite adherence. The ventral adhesive disk in contact with the microvilli was postulated to have caused a focal shortening and bending of

microvilli. Once the trophozoite releases its hold on the microvilli, 'footprints' of the area of attachment were visible. *Giardia* trophozoites directly adhering were thought to cause a depletion of microvilli with a concurrent reduction in mucosal disaccharidase enzyme activity and loss of absorptive surface area (71, 72, 265). However, it is not likely that these limited changes can account for the extensive, persistent and diffuse nature of enzyme deficiencies seen in this disease. In addition, the enterocyte is capable of rapid increase in microvilli height during EGF treatment indicating that the limited focal distortions created by the suction disk of trophozoites can be easily and rapidly recovered *in vivo* (80, 156). Buret *et. al.* (1990;1991;1992) have since shown by comparing transmission electron micrographs of the small gut of *Giardia*-infected rodents at the same magnification, that *Giardia* causes a diffuse shortening of brush border of enterocytes along the entire small intestine, at sites of trophozoite attachment as well as in other sites. Most importantly, the reduction in microvilli height can be observed in the absence of any change in the height of mucosal villi. Moreover, persistent parasitic colonization of rodent duodenum correlates with persistent reduction of the brush border surface area and disaccharidase deficiencies, and as trophozoite colonization declines in the jejunum, recovery of the disaccharidase activities is paralleled by a simultaneous recovery of brush border alteration. This indicates that the pivotal limiting factor for small intestinal malabsorption and maldigestion is due to the diffuse loss of microvillus surface area.

#### **1.5.5. Pathogenesis**

There are many factors indicated in the pathogenesis of *Giardia* induced epithelial injury and associated diarrhea. The next section will cover some of the factors considered to be involved in the disease process.

#### **A. Colonization Factors**

*Giardia* has a surface mannose-binding lectin which may potentially contribute to epithelial damage (186, 208, 225). Experiments have indicated that dietary lectins are able to cause direct damage to microvillus membrane similar to those in giardiasis (107). Another colonization factor initially postulated in past research is that large trophozoite numbers bound to the intestinal absorptive epithelium created a mechanical barrier to the diffusion and digestion of nutrients (19, 265, 277, 391). Currently, malabsorption by mechanical obstruction is no longer considered pertinent as studies utilizing soluble trophozoite extracts were able to impair intestinal enzyme activities in rodents, as well as damage epithelial cells *in vitro* (24, 201, 207, 269, 314, 335).

#### **B. Host Response**

Histological analysis of biopsies conducted in symptomatic patients indicate infiltration of the lamina propria by plasma cells, lymphocytes and polymorphonuclear lymphocytes, indicating the importance of host factors (403). The activation of the host immune response has the potential to affect mucosal architecture and morphology. The release of cytokines and other inflammatory metabolites can certainly cause similar physiological changes within the small intestine in giardiasis reflective of the morphological alterations seen in other inflammatory small intestinal diseases. Currently we know that IL-2 and IFN- $\gamma$  by themselves are unable to induce change in mucosal architecture but it is not known what role, if any, these cytokines as well as others

released during acute infection may have in the pathogenesis of diarrhea during giardiasis (27, 213).

### **C. Bile Salts**

*Giardia* trophozoites are able to take up bile salts, which play an important role in the *Giardia* life cycle (110, 152, 153). The uptake of bile salts by trophozoites may reduce intraluminal bile salt concentrations, therefore decreasing pancreatic lipase and micellar solubilization of fat, thus contributing to fat malabsorption. An early study conducted in humans found an association between *Giardia* induced steatorrhea and free bile salts in their intestinal lumens (363). The investigation suggested that *G. lamblia* could deconjugate bile salts. However, unequivocal evidence has now illustrated in humans as well as *in vitro* that *Giardia* is unable to deconjugate bile salts (152, 342).

### **D. *Giardia* Secretory/Excretory Products**

Lysosome-like vacuoles line the plasma membrane of trophozoites. These vacuoles release hydrolases, including a thiol dependent proteinase similar to a cytotoxin found in *Entamoeba histolytica* (124, 228, 234, 298). Other secretory/excretory trophozoite products include two cysteine proteases found in trophozoite homogenates (401). A separate research group found that *Giardia* trophozoites produced a cysteine-rich protein which carries multiple repeats of a sarafotoxin-like motif at a telomeric position (74, 75, 384). In an *in vivo* experiment conducted utilizing oral administration of an avian strain of live *Giardia* trophozoites in adult mice, there was weight loss in the absence of an established infection in the mice (382, 383). The researchers concluded that this is consistent with either a toxin secreted/excreted by the parasite or a toxic response caused by the degradation of the parasite. Their former conclusion is justified



by experiments conducted *in vivo* as well as *in vitro* which utilized soluble trophozoite extracts or lysed trophozoites, and successfully induced damage in the absence of whole trophozoites (24, 70, 71, 201, 269, 314). Despite evidence for a secretory/excretory product, experiments indicate that *Giardia lamblia* does not produce an enterotoxin which resembles the cholera toxin or *E. coli* heat-labile classes of toxins (343). However, *Giardia* secretory/excretory products may act by promoting clinical manifestations via damage to the protective mucus layer of the intestine, by interfering with host enteric defense mechanisms (50) or by disrupting host regulation of normal enterocyte kinetics, cytoskeletal architecture or cell cycling.

#### **1.5.6. Summary**

In summary, the mucosal and epithelial injury witnessed in giardiasis and a number of other disorders cause malabsorptive diarrhea. The pathogenesis or mechanistic of malabsorptive diarrhea is multifactorial, involving mucosal architectural abnormalities, parasite colonization factors, the host immune response, and potential secretory/excretory products. Of these, research has indicated that the pivotal cause of malabsorptive diarrhea is a diffuse loss of brush border which effectively decreases the available absorptive surface area, as well as removes the sites required to insert both disaccharidases utilized in carbohydrate reduction, and sodium-nutrient co-transporters involved in sodium and carbohydrate uptake from the lumen.

### 1.6.0. Objectives of This Study

Using an *in vitro* model system, the overall objective of this study is to determine the mechanistics of *Giardia*-induced epithelial injury. As diffuse loss of enterocyte brush border plays a vital role in causing malabsorptive diarrhea, the role of enterocyte cytoskeletal proteins involved in *Giardia*-induced epithelial injury will be investigated.

The specific aims of this study are:

1. To determine the transepithelial electrical resistance of cell monolayers grown on Transwell™ filters, pre- and post- incubation with *Giardia lamblia* trophozoites
2. To investigate the effect of *Giardia lamblia* on G-actin, F-actin, villin, ezrin and  $\alpha$ -actinin
3. To investigate the effect of actin polymerization inhibitors and calcium channel blockers on *Giardia lamblia*-induced epithelial injury
4. To determine if *Giardia lamblia* produces a secretory/excretory product responsible for epithelial injury

## **2. MATERIALS AND METHODS**

### **2.1. Tissue Culture**

#### **2.1.1. Small Intestinal Cells**

##### **A. Small Intestinal Cells of B.N**

The small intestinal cell line used in this study was previously isolated from an individual with diarrhea of unknown aetiology (initials B.N) in New Castle, Australia (295). The cell line was called SCBN, and cultures were set up at the University of Calgary. Cells were used between passages 15 and 30.

##### **B. Growth and Maintenance**

SCBN cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma, St. Louis), supplemented with 5% (or 10%) fetal calf serum, 100 µg/ml streptomycin, 100 U/ml penicillin, 0.08 mg/ml tylosin and 200 mM L-glutamine (all from Sigma, St. Louis). With the above supplements the medium is known as complete DMEM. Cells were incubated at 37°C and 5% CO<sub>2</sub> in 96% humidity (Nuair<sup>TM</sup> US Autoflow, Plymouth, MN) and grown in tissue culture treated vessels appropriate for each experiment. The media was replenished every 2-3 days. Cell monolayers were confluent by 3 days at which time the cells can be passaged, frozen down or utilized in experiments.

##### **C. SCBN Passaging**

Cells were passaged from monolayers grown on 25 cm<sup>2</sup> tissue culture treated flask (Falcon) that had reached confluency. The monolayer was initially rinsed with sterile PBS depleted of calcium and magnesium (Sigma) before addition of 1x-Trypsin-Ethylenediamine Tetraacetic Acid (EDTA) (0.5 g porcine trypsin and 0.2 g of EDTA • 4

Na per litre) in cation free PBS (Sigma). Monolayers were incubated with trypsin for approximately 30 minutes or until the monolayer had detached from its surface. Trypsinization was terminated utilizing 5% DMEM and the cells were diluted to a maximum passage ratio of 1 to 10 or a seeding cell count of  $5 \times 10^5$  cells /ml. Cell numbers were obtained using a hemacytometer.

#### **D. Cell Freezing and Storage**

SCBN cells were maintained and passaged as described above in 75 cm<sup>2</sup> flasks (Falcon 3111). After cell detachment, individual cells were spun down at 30 x g (4°C) (I.E.C Centra-7R, Refrigerated Centrifuge). Supernatant was aspirated off before cells were resuspended in 1.5 ml serum free DMEM supplemented with 10% tissue culture grade dimethyl sulfoxied (DMSO) (Sigma). Cell suspensions were aliquoted into sterile cryovials before step wise freezing, -20°C (2 h), -85°C (overnight), and finally transferred into liquid nitrogen.

Recovery of frozen cells involved the removal of the cryovial from liquid nitrogen and the immediate immersion of the vial into sterile 37°C water, while agitating the vial. Once thawed the cells were added drop by drop into a 25 cm<sup>2</sup> flask which already contained 10% DMEM pre-warmed to 37°C. The cells were incubated as described previously.

#### **2.1.2. Colonic Cells**

##### **A. Colonic Adenocarcinoma Cells**

Colonic adenocarcinoma cells (Caco-2) were obtained from the American Type Culture Collection (ATCC HTB-37). Passage number indicated was 30, and all experiments were performed from passage 30 to 50.

## **B. Growth and Maintenance**

Caco-2 cells were grown and maintained using procedures described for SCBN. Caco-2 cells were fed every 2-3 days and achieved confluency approximately 5 days after initial seeding. At this time Caco-2 cells could be passaged, frozen down or were ready to be utilized in experiments at a separate time.

## **C. Caco-2 Passaging**

Caco-2 monolayers were passaged from large flask in the manner similar to SCBN described above. Caco-2 cells were diluted to a maximum passage ratio of 1 to 5 or a seeding cell count of  $1.6 \times 10^6$  cells /ml.

## **D. Cell Freezing and Storage**

Caco-2 cells were passaged from 75 cm<sup>2</sup> flasks according to procedures described above with the exception of a shorter trypsinization time of 20 minutes. Recovery of Caco-2 cells from frozen storage involved procedures previously described.

## **2.2. Parasites**

### **2.2.1. Growth and Maintenance**

Two strains of *Giardia* were used in all experiments. The *Giardia lamblia* strain NF obtained during an epidemic outbreak of human giardiasis in Newfoundland (Canada) was kindly provided by Dr. M. Olson (University of Calgary, Calgary (AB)). The *Giardia lamblia* strain S2, was originally recovered from a sheep (51).

Trophozoites were grown axenically at 37°C in Diamonds TYI-S-33 (94) supplemented with 2.5 mg/ml Pipracil<sup>®</sup> (Cyanamid Canada Inc. PQ), 100 µg/ml streptomycin, and 100 U/ml penicillin in glass tubes. To maintain the lines trophozoites

were passaged every 2 to 3 days. Confluent trophozoite tubes were placed on ice or 'cold shocked' for 20 minutes before transfer of 5000 trophozoites to 7.5 ml new media.

### **2.2.2. Harvesting of Trophozoites**

Trophozoites were harvested at log phase by cold shock on ice (20 min) and centrifugation (10 min, 500 x g, 4°C) (Beckman Centrifuge). The pellet was washed twice in 4°C sterile PBS then resuspended in 5% DMEM, enumerated with a hemocytometer, and diluted to a concentration of  $1 \times 10^6$  trophozoites/ml. Experiments with lysed trophozoites required sonication (550 Versonic Dismembrator, Fischer Scientific) on ice for 3 bursts, maximum setting, at 1 minute each. Trophozoite lysis was confirmed with a light microscope.

### **2.2.3. Growth of Trophozoites in Reduced Medium**

As TYI-S-33 is toxic to epithelial cells (personal communication, Dr. Ann McDonnell, Queensland Medical Research Institute) trophozoites were grown in serum free DMEM reduced with 5mM L-Cysteine (Sigma), supplemented with 10% CLEX (Dextran Products), 100 µg/ml streptomycin, and 100 U/ml penicillin and allowed to reduce for at least 2 h before exposure to trophozoites.

TYI-S-33 was decanted from glass tubes containing trophozoites grown to log phase and replaced with equal amounts of the reduced medium. Trophozoite tubes were left to incubate at 37°C on its sides to encourage adherence to the glass sides. After 15-18h the trophozoite spent medium is collected and swimming or dead trophozoites removed via centrifugation (10 min, 500 x g, 4°C). To verify sterility, spent medium as well as the reduced medium was plated on blood agar plates and incubated overnight at 37°C.

## **2.3. Effects of *Giardia lamblia* on Epithelium and the Cytoskeleton**

### **2.3.1 Transwell™ Experiments**

#### **A. Caco-2 and SCBN Transwell™ Cultures**

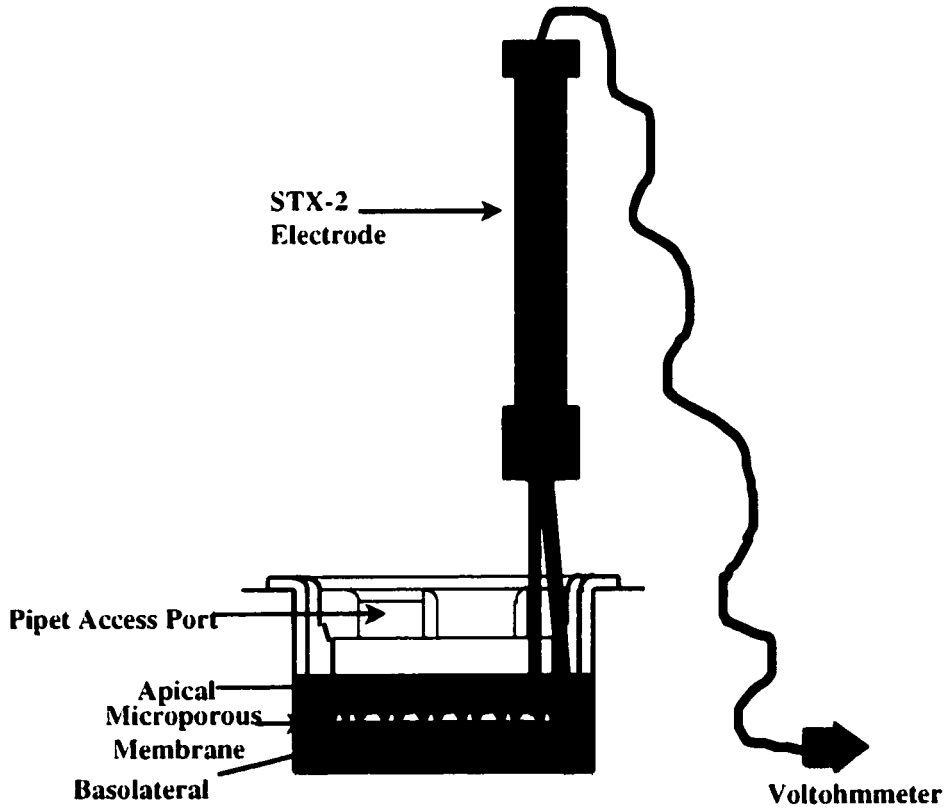
Cells were grown on Transwell™ filter units (Costar, Cambridge MA) which contained a 0.33 cm<sup>2</sup> semi-permeable filter membrane (3.0µm pore size) (Fig. 3). The filter units were incubated in 24-well cluster plates (Costar). Trypsinized SCBN (200µl; 1.5x10<sup>5</sup> cells/ml) or Caco2 (200µl; 3x10<sup>5</sup> cells/ml) cell suspensions in culture medium containing 10% fetal calf serum were added apically to the filter units. Each unit was placed in 500µl fresh 10% DMEM (basolateral) and incubated at 37°C, 5% CO<sub>2</sub> in 96% humidity (Nuaire™).

#### **B. Confluence Analysis via Electrical Resistance**

Baseline electrical resistance for SCBN and Caco2 monolayers was determined at intervals over 13 or 20 days respectively to determine optimal time of confluency. Transepithelial electrical resistance was measured using an STX-2 Electrode (World Precision Instruments, FL) and an Epithelial Voltohmmeter (World Precision Instruments, FL). Resistance for each well was measured 3 times at each entry port for the STX-2 Electrode. Transepithelial resistance was expressed as net  $\Omega\text{cm}^2$  after subtraction of baseline resistance for Transwell™ membranes (33.6  $\Omega\text{cm}^2$ ). Experiments were carried out when confluent cell monolayers reached >100  $\Omega\text{cm}^2$  resistance.

**Figure 3:** Diagrammatic representation of the Transwell™ filter system and the STX-2 Electrode used to measure transepithelial electrical resistance. Resistance was obtained at each of three portals of entry towards the basolateral region and represented as an average.





### **C. Co-incubation with *Giardia lamblia* and Electrical Resistance**

Monolayers that reached transepithelial resistance reflecting confluency were used in all experiments. Trophozoites were harvested as previously described and diluted to a final concentration of  $1 \times 10^6$  trophozoites/ml DMEM and 200 $\mu$ l of whole trophozoites or trophozoite lysates which were diluted after enumeration to  $1 \times 10^6$  trophozoites/ml, were added to the apical side of confluent monolayers grown on Transwell™ filters. Controls received 200 $\mu$ l DMEM vehicle. Preparations were incubated for 24 h at 37°C, 5% CO<sub>2</sub>.

Prior to measuring transepithelial resistance of confluent experimental monolayers, adhering trophozoites were removed with five washes of cold (4°C), sterile PBS. Monolayers exposed to vehicle alone or trophozoite lysates were also washed. This procedure ensured that adhering trophozoites would not affect electrical resistance measurements. Monolayer resistance for each well was measured from 3 STX-2 Electrode entry ports.

#### **2.3.2 Cytoskeletal Proteins Involved in *G. lamblia* Induced Injury**

To circumvent the difficulties associated with the autofluorescence of Transwell™ filter membranes (not shown), monolayers were grown on 96 well tissue culture treated microtiter plates (Becton Dickinson Labware, NJ), or on Lab-tek tissue culture treated chamber slides with removable chambers (Nalge Nunc International) for epifluorescence analysis. Experiments were performed 4 days after seeding for SCBN and 9 days for Caco2 time at which each cell line exhibited high electrical resistance when grown on Transwell™ filters.

Similar cell suspensions of whole trophozoites, trophozoite lysates or trophozoite spent medium were added to 96 well tissue culture treated microtiter plates (200 $\mu$ l) and left to incubate for 24 h at 37°C, 5% CO<sub>2</sub>. Difquick staining (Baxter Health Care Corporation, Miami FL) for visualization under brightfield was conducted on monolayers incubated with trophozoite spent medium. Monolayer fixation for fluorescent microscopy was performed after trophozoites were removed by cold shocking, or in experiments using lysed products or trophozoite spent medium, immediately after removal of the suspension.

Monolayers were fixed with 2% Paraformaldehyde in PBS for 60 min at 21°C, and washed three times in sterile PBS. Cells were permeabilized (0.5% Triton X, 10 min 21°C) and washed in sterile PBS before fluorescent staining.

#### **A. Filamentous Actin (F-actin)**

F-actin staining was performed using methods described for G-actin as below. Fluorescent stain specific for F-actin, BODIPY R6G Phalloidin (1:40 in PBS) (Molecular Probes, OR) was added apically to monolayers and left to incubate in the dark for 75 min at 25°C, and washed with PBS before visualization.

#### **B. Globular Actin (G-actin)**

The fluorescent stain for G-actin, Oregon Green DNase I (0.3 $\mu$ M in PBS) (Molecular Probes, OR) was added apically to the monolayers and allowed to incubate for 75 min at 25°C in the dark. Excess stain was washed off using sterile PBS before visualization on a Zeiss Axiovert 25 (Zeiss Canada) inverted microscope equipped with a FITC filter. Photomicrographs were obtained on Kodak Elite III Ektachrome 400 film (Eastman Kodak Co., NY).

### **C. Villin**

As the visualization of villin involves indirect immunofluorescence whole fetal bovine serum (FBS) was used to block nonspecific binding (20 min, 37°C). Before the addition of the antibodies, the monolayers were rinsed three times in sterile PBS. Monoclonal mouse anti-human villin antibody (1:50 in DMEM) (Chemicon International, CA) was allowed to incubate for 1 h at 37°C. Excess antibody was rinsed off three times with sterile PBS before addition of Cy-3 conjugated anti-mouse IgG polyclonal antibody (1:200 in DMEM) (Sigma, St. Louis) for 1 h at 37°C. Excess antibody was rinsed off three times in PBS with aspiration of the final rinse. Negative controls for the monoclonal antibodies in question as well as for the polyclonal antibody-Cy-3 conjugated was also conducted to negate the occurrence of nonspecific staining.

The plastic divisions of the chamber slides were removed and the slides were mounted with cover slips using aqueous mounting media (Aqua Poly/Mount, Polysciences Inc., Warrington, PA), and allowed to set overnight in the dark before examination under fluorescence.

### **D. Ezrin**

Indirect immunofluorescence and wash procedures for ezrin was conducted using similar methods as for villin. Non-specific antibody binding was blocked using FBS before anti-human ezrin antibody (0.5 µg/ml in DMEM) (Transduction Laboratories, KY) was added apically and allowed to incubate for 1 h at 37°C. Cy-3 conjugated anti-mouse IgG polyclonal antibody (1:200 in DMEM) (Sigma, St. Louis) (1 h at 37°C) was used to visualize the protein.

## **E. $\alpha$ -Actinin**

Monolayers were exposed to whole fetal calf serum to block nonspecific binding, and incubated with  $\alpha$ -actinin monoclonal antibody (1:50 in DMEM) (Chemicon International, CA) for 1 h at 37°C. Excess antibody was rinsed off with sterile PBS before addition of Alexa™ 350 conjugate Anti-Mouse IgG (Molecular Probes, OR) polyclonal antibody (1:100 in PBS) for 1 h at 37°C. In separate experiments, monolayers were double-stained with BODIPY R6G for F-actin and for  $\alpha$ -actinin using the successive steps for individual staining procedures as described above. Excess antibody was rinsed off three times in PBS before visualization.

## **2.4. Molecular Mechanism of Cytoskeletal Injury**

### **2.4.1. Role of Extracellular Calcium**

Experimental monolayers were pre-treated with the  $\text{Ca}^{2+}$  channel blocker Verapamil hydrochloride (Fluka Biochemicals, Switz.) at a concentration of 100 $\mu\text{M}$  in 5% DMEM as previously indicated (159). Control monolayers were incubated with the vehicle alone. After incubation live or sonicated trophozoites, or trophozoite spent medium, or the vehicle were added and co-incubated with the monolayers for 24 hours. Monolayers were stained for F-actin, human villin and human ezrin.

### **2.4.2. Role of Actin Polymerization**

Monolayers to be used in experiments were pre-treated with the actin polymerization inhibitor Cytochalasin D (Fluka Biochemicals, Switz.) at a concentration of 1 $\mu\text{M}$  in tissue culture media for 10 min (at 37°C). Control monolayers were pre-treated with the vehicle. After incubation with the inhibitor live or sonicated trophozoites, or trophozoite spent medium, or the vehicle were added and left for 24

hours. Visualization of F-actin, human villin and human ezrin were performed on these monolayers.

## **2.5. Scanning Electron Microscopy**

SCBN cells ( $1 \times 10^5$  cells/ml; 1 ml) were grown on sterile 18 mm round, glass coverslips in 10% DMEM. After 5 days, concentrated *Giardia lamblia* ( $9 \times 10^6$  trophozoites) in 5% DMEM harvested as per methods described previously were added and allowed to co-incubate for 24 h at 37°C in 5% CO<sub>2</sub>. Control monolayers were incubated in 5% DMEM alone. After incubation, samples were fixed in 2% (weight/volume) gluteraldehyde in PBS (pH 7.3), rinsed in PBS and postfixed with 2% osmium tetroxide at 25°C for 2 h. Samples were rinsed in PBS and dehydrated via stepwise dehydration in alcohol: once in 70% ethanol, once in 90% ethanol and three times in 100% ethanol for 15 min each time. Dehydration was completed in 100% freon, and samples were left to air-dry, sputter coated with gold-palladium (3 min.), and mounted on aluminum stubs. Samples were examined with a Hitachi 450 Scanning Electron Microscope at an acceleration voltage of 20 kv.

## **2.6. Confocal Laser Microscopy**

All micrographs for confocal laser microscopy were obtained at 1 µm increments.

### **A. F-actin**

Monolayers stained for F-actin were analyzed by confocal laser microscopy (Imaging Facility, University of Calgary Medical School) using a Zeiss Axioplan 2 confocal microscope (Excitation = 529nm; Emission = 547nm).

### **B. Villin and Ezrin**

Monolayers stained for villin or ezrin were analyzed using a Biorad View Scan DVC-50 confocal laser microscope ( Excitation = 552nm; Emission = 565nm) with a Diagnostic Spot II digital camera courtesy of Dr. D Muench (Biological Sciences, University of Calgary).

### **C. $\alpha$ -Actinin**

Monolayers stained for  $\alpha$ -actinin were analyzed with UV filters on Zeiss Axiovert 100M Inverted LSM 510 confocal microscope (Excitation = 347nm; Emission = 441nm) courtesy of Dr. X. Sun (Cross Cancer Institute, University of Alberta).

## **2.7. Statistical Analysis**

Results were expressed as mean  $\pm$  Standard error (SE) and compared by one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls test for multiple comparison where appropriate. Significance levels were established at  $P \leq 0.01$ .

## 3. RESULTS

### 3.1. Tissue Culture

SCBN and Caco-2 cells formed confluent monolayers (Fig. 4). SCBN reached confluency after 3 days whereas Caco-2 required 5 days after initial seeding. The typical 'cobblestone' pattern was clearly seen when monolayers achieved confluency.

### 3.2. Effects of *Giardia lamblia* on the Epithelium

#### 3.2.1. Electrical Resistance

##### A. Confluency via Transepithelial Resistance

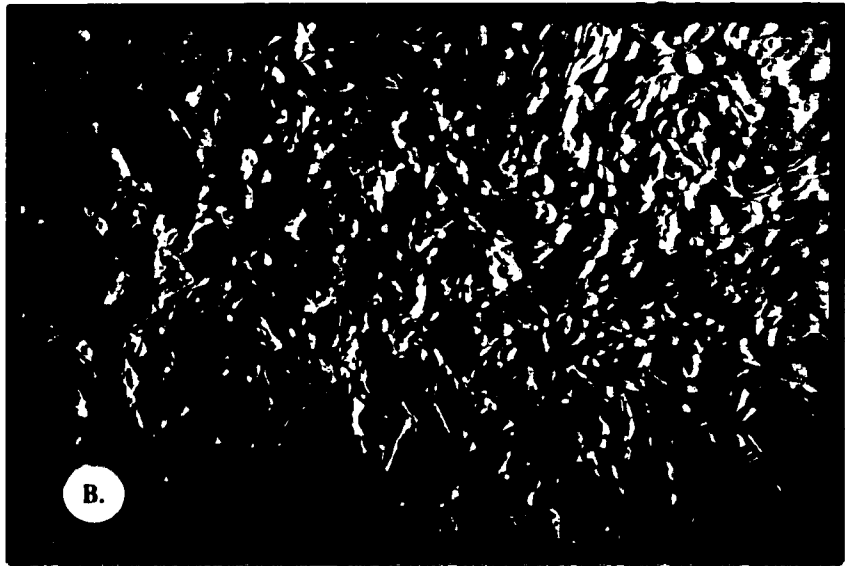
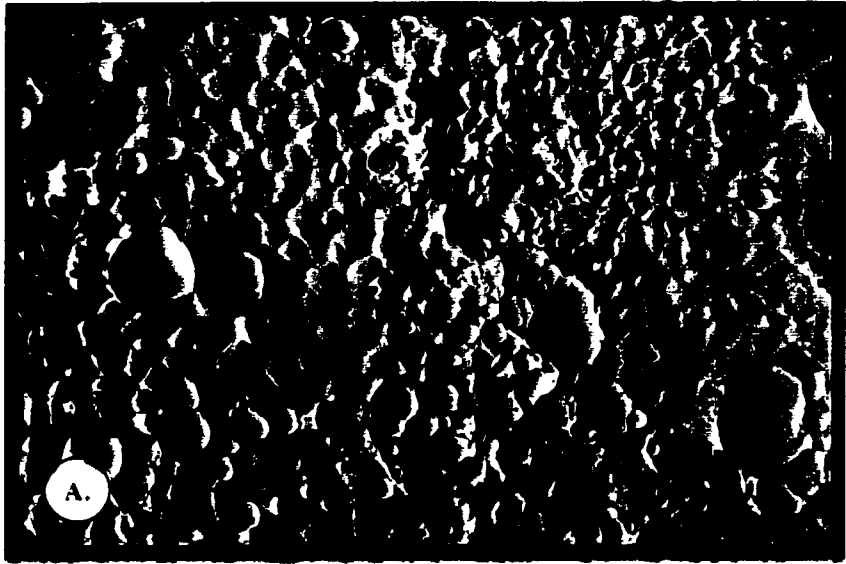
In order to determine the optimal period for experimentation of confluent monolayers, electrical resistance of SCBN and Caco2 was measured over several days. Confluent SCBN monolayers reached highest electrical resistance ( $106\text{-}123\ \Omega\text{cm}^2$ ) 3 to 4 days post seeding (Fig. 5). Highest resistance in Caco2 ( $119\text{-}135\ \Omega\text{cm}^2$ ) was measured 6 to 9 days after seeding (Fig. 6). Unlike SCBN, Caco2 maintained high electrical resistance ( $>77\ \Omega\text{cm}^2$ ) for up to 20 days post seeding. All subsequent experiments used monolayers at the time of peak electrical resistance.

##### B. Transepithelial Resistance after Co-incubation with *G. lamblia*

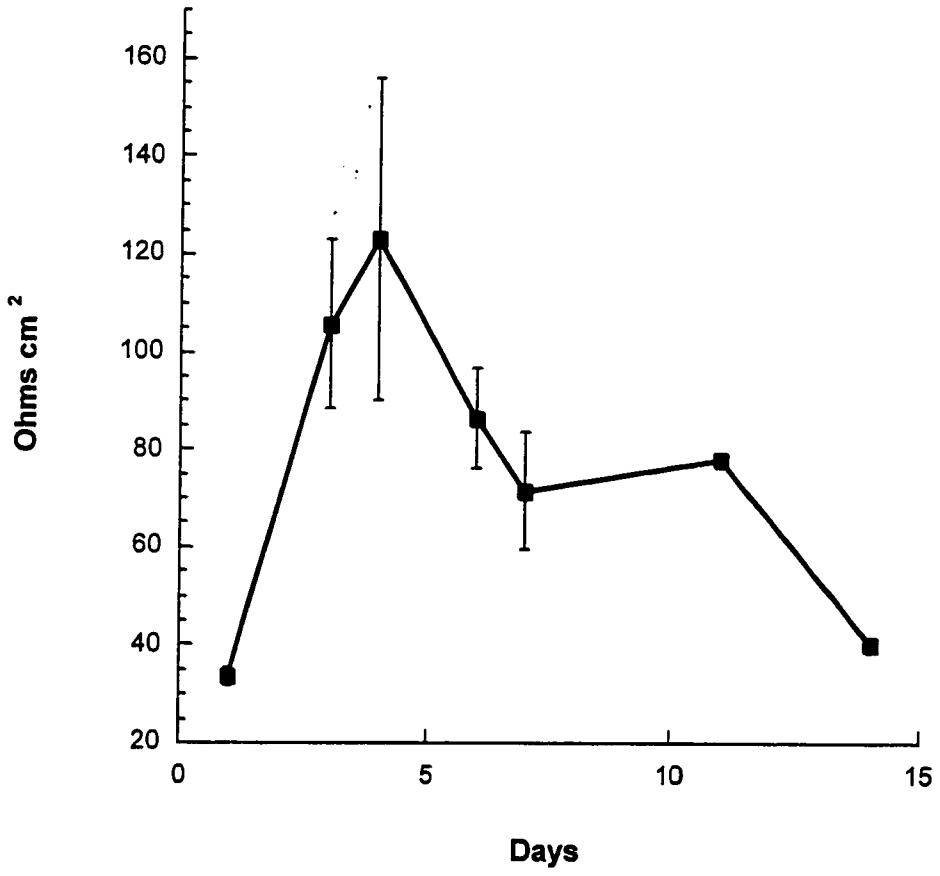
Transwell™ filters with confluent monolayers at high transepithelial resistance ( $\geq 100\ \Omega\text{cm}^2$ ) were co-incubated for 24 h with *G. lamblia* trophozoites. Exposure to whole *G. lamblia* trophozoites (S2 or NF) significantly reduced transepithelial electrical resistance of washed SCBN and Caco2 monolayers (Fig. 7 and 8). In separate experiments, as the washing procedures significantly reduced baseline electrical resistance, polarized Caco2 monolayers were incubated for 24 h and 48 h with sonicated



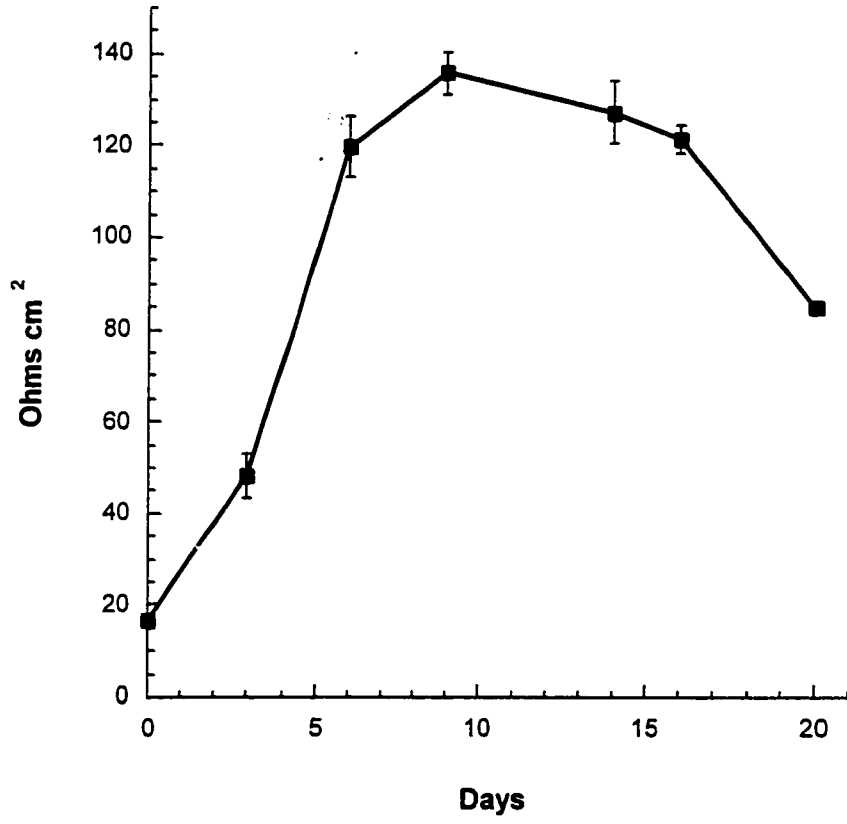
**Figure 4:** Micrographs of SCBN (A) and Caco2 (B) enterocytes grown to confluency on flasks. The typical 'cobblestone' morphology is clearly seen in both cell lines. Original magnification, 200X.



**Figure 5:** Electrical resistance of SCBN as a measure of confluency. Highest electrical resistance was observed between days 3 and 4. Values are mean  $\pm$  SEM from at least four monolayers.

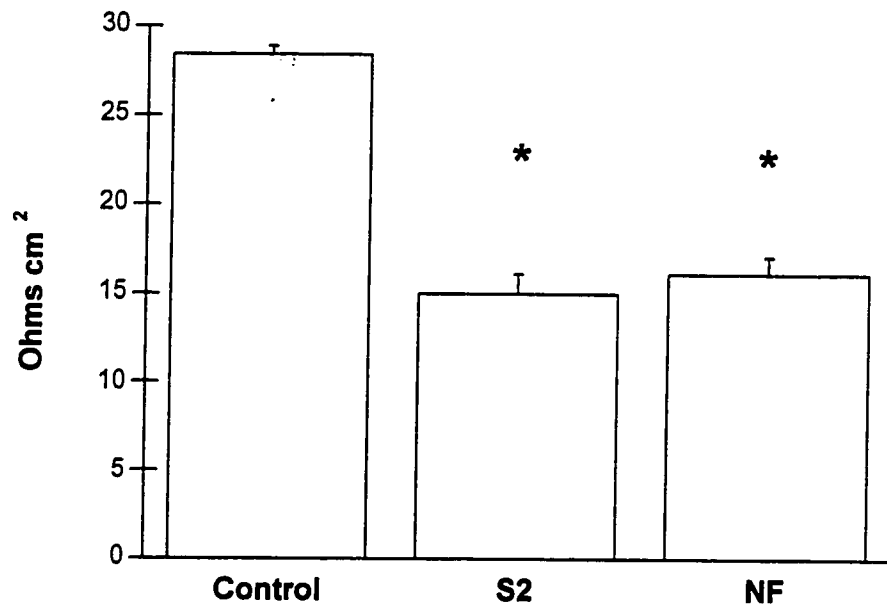


**Figure 6:** Electrical resistance of Caco2 as a measure of confluency. Highest electrical resistance was observed between days 6 and 9. Values are mean  $\pm$  SEM from at least three monolayers.



**Figure 7:** Electrical resistance of SCBN monolayers, exposed for 24 h to vehicle (control) or to whole *G. lamblia* S2 or NF trophozoites, after wash with cold PBS to remove adhering trophozoites. Values are mean  $\pm$  SEM from at least three monolayers.

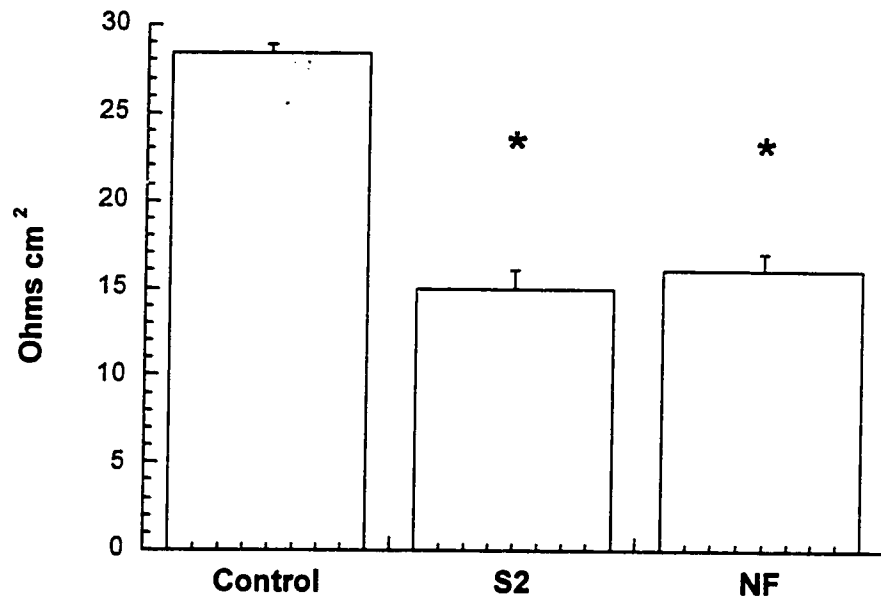
\* $P < 0.001$  vs. control.





**Figure 8:** Electrical resistance of Caco2 monolayers, exposed for 24 h to vehicle (control) or to whole *G. lamblia* S2 or NF trophozoites, after wash with cold PBS to remove adhering trophozoites. Values are mean  $\pm$  SEM from at least three monolayers.

\* $P < 0.001$  vs. control.



samples of *G. lamblia* strain S2 and NF without the washing steps. Again, exposure to parasite lysates significantly decreased the transepithelial electrical resistance of Caco2 monolayers (Fig. 9). The loss of electrical resistance was similar in either cell line, whether exposed to the *G. lamblia* strain S2 or NF.

### **3.2.2 The Role of Cytoskeletal Proteins**

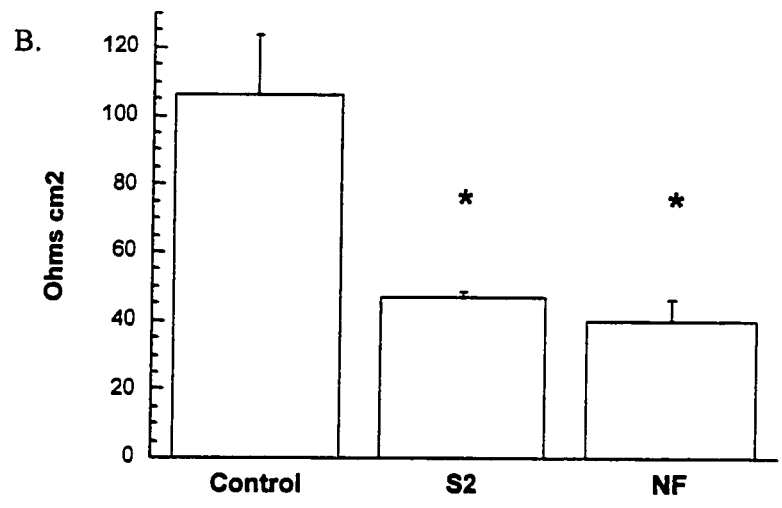
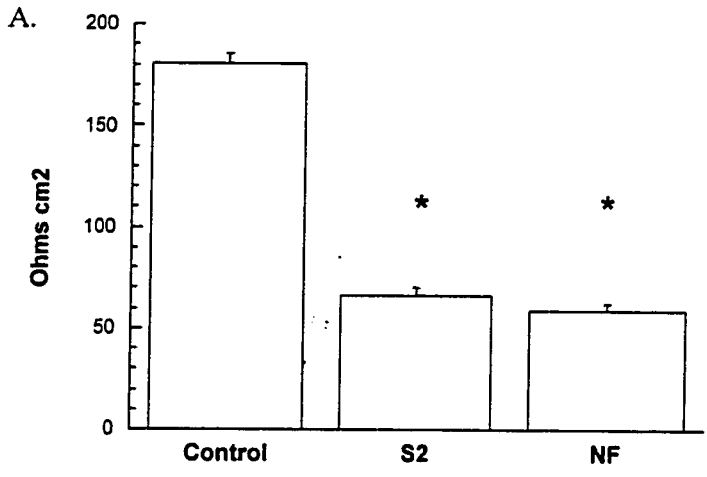
#### **A. Filamentous and Globular Actin**

In order to assess the effects of *G. lamblia* on the epithelial cytoskeletal proteins F- and G-actin, confluent SCBN and Caco2 monolayers were exposed to whole or sonicated *G. lamblia* and stained for G-actin. Exposure of monolayers to *Giardia* induced flocculation of F-actin in SCBN or Caco2 (Fig. 10 and 11). A similar pattern of F-actin reorganization was seen in SCBN or Caco2 monolayers exposed to sonicated *G. lamblia* regardless of the strain used (Fig. 12). Staining patterns for G-actin were not different between control or experimental monolayers exposed to *G. lamblia* in either SCBN or Caco2 cells (Fig. 13).

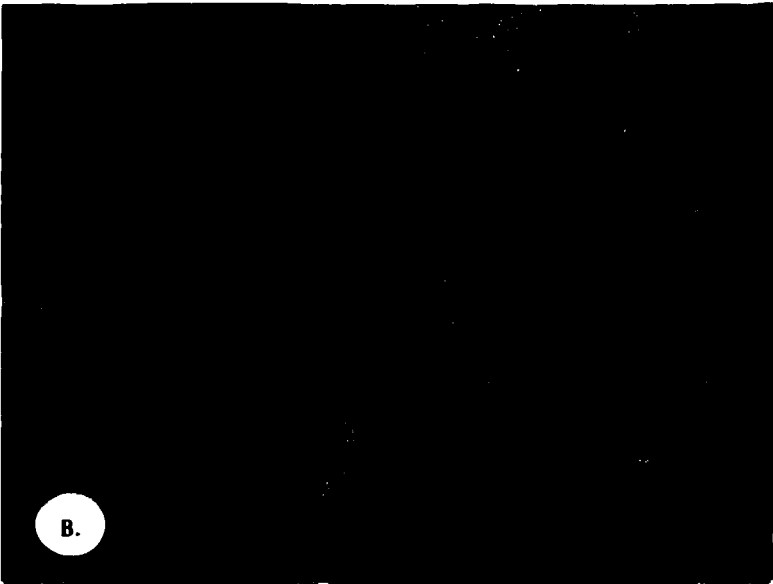
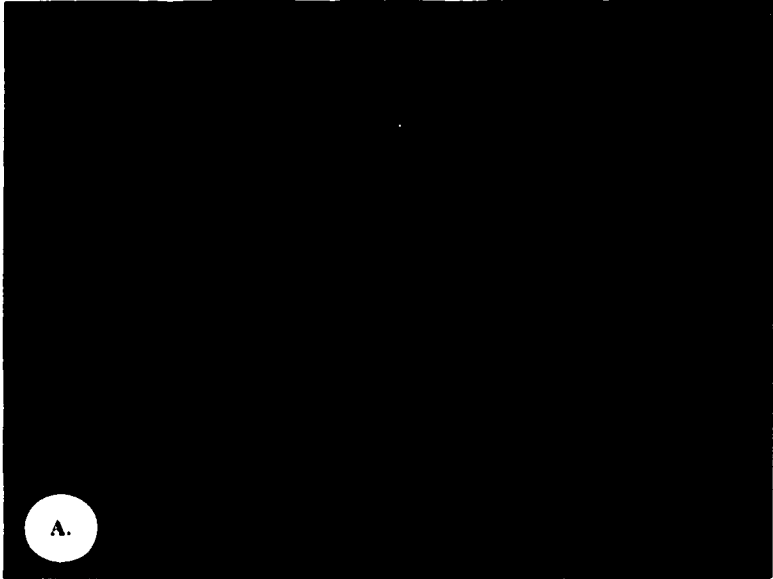
#### **B. Villin and Ezrin**

Whole *G. lamblia* trophozoites, lysates as well as spent medium affected the arrangement of the cytoskeletal proteins ezrin and villin. Control monolayers stained for ezrin revealed a perijunctional localization of ezrin with a clear central area with no staining (Fig. 14). In monolayers exposed to trophozoite lysates or products there is a disruption of ezrin staining towards a more dispersed pattern. In addition to the abrogation of the central clearing distinct in control monolayers, exposed monolayers

**Figure 9:** Electrical resistance of Caco2 monolayers exposed for 24 h (A) or 48 h (B) to the vehicle (control) or to sonicated *G. lamblia* trophozoites. Values are mean of at least four monolayers  $\pm$  SEM. \* $P$ <0.002 vs. control.

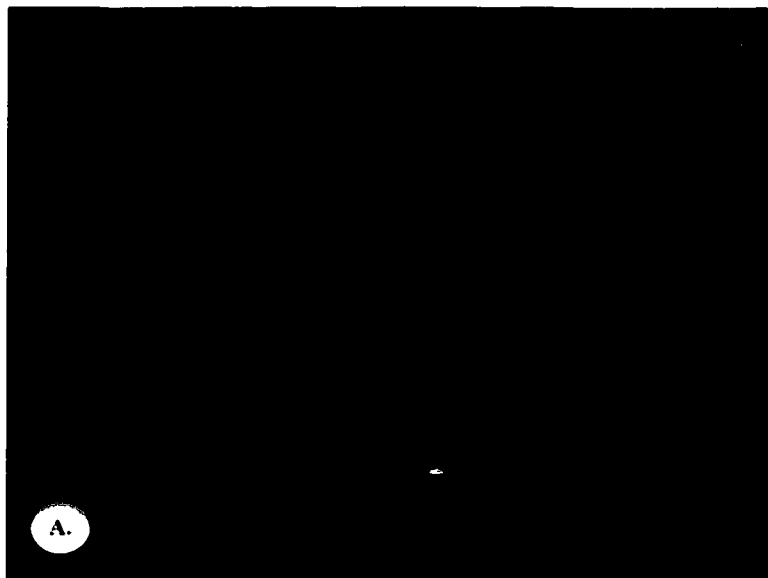


**Figure 10:** Representative epifluorescence micrographs of SCBN monolayers stained for F-actin with rhodamin-phalloidin. In control cells (A), F-actin appears as diffuse filaments located throughout each cell, as well as in areas representing the perijunctional actin ring. The cellular distribution of F-actin appears flocculated in monolayers exposed for 24 h to whole trophozoites (B). Original magnification, 400X.

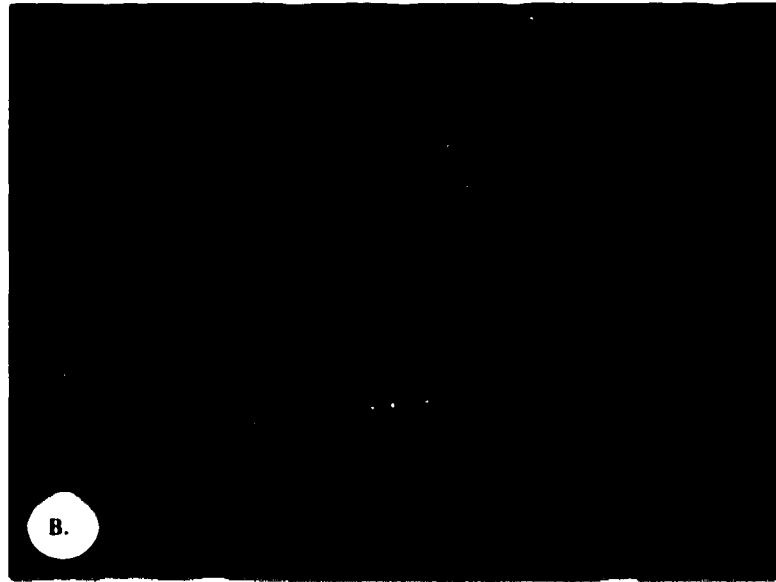
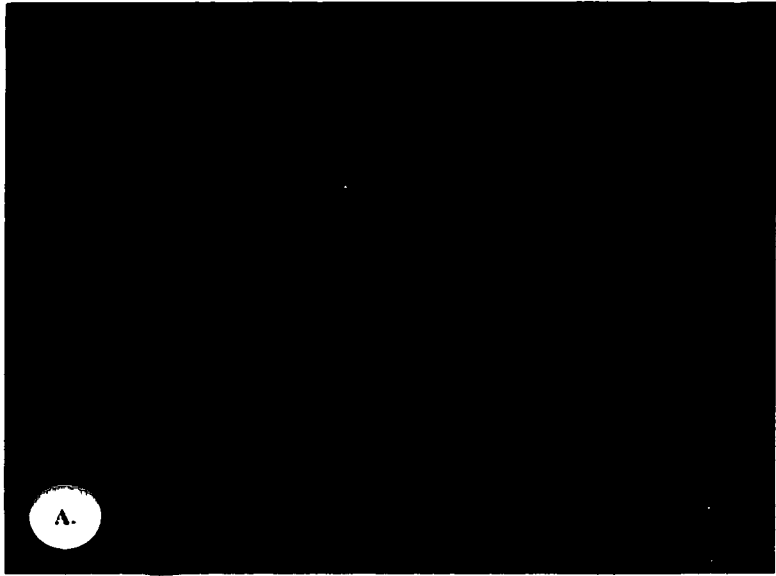


**Figure 11:** Representative epifluorescence micrographs of Caco2 monolayers stained for F-actin with rhodamin-phalloidin. Control cells (A) have diffuse F-actin staining in the central region becoming more concentrated at the perijunctional actin ring. Monolayers exposed to whole trophozoites exhibit F-actin flocculation (B). Original magnification, 400X.





**Figure 12:** Representative epifluorescence micrographs of Caco2 (A) or SCBN monolayer (B) stained for F-actin with rhodamin-phalloidin after exposure to lysed *G. lamblia* trophozoites for 24 h. Flocculation of F-actin occurs in both colonic as well as small intestinal cells *in vitro*. Original magnification, 400X.



**Figure 13:** Representative epifluorescence micrographs of SCBN monolayers immunostained for G-actin. Staining patterns were similar for sham treated control monolayers (A), or monolayers exposed to whole *G. lamblia* trophozoites (B). In all cases, G-actin appeared as a pervasive green fluorescence throughout the cytoplasm of epithelial cells. Similar results were obtained with Caco2 cells (not shown). Original magnification, 400X.



also had dense staining of the membrane areas (Fig. 14). Reorganization of ezrin was *G. lamblia* strain independent and occurred in monolayers exposed to live trophozoites as well as in monolayers co-incubated with trophozoite products (Fig. 14).

Visualization of villin revealed a disruption from an organized perijunctional location to one that is disrupted and reorganized towards the nucleus (Fig.15). In monolayers exposed to *Giardia* and stained for villin there were areas of cell-cell junction disruption, loss of contact with neighbouring cell as well as loss of cell integrity (Fig.15). The staining patterns of villin in experimental monolayers were consistent irrespective of the *G. lamblia* strain used, or whether exposed to live trophozoites or trophozoite products (Fig. 15).

### **3.3. Difquick Staining**

Difquick staining of SCBN monolayers exposed to trophozoite spent medium for 24 h showed a loss of cell-cell contact (Fig. 16).

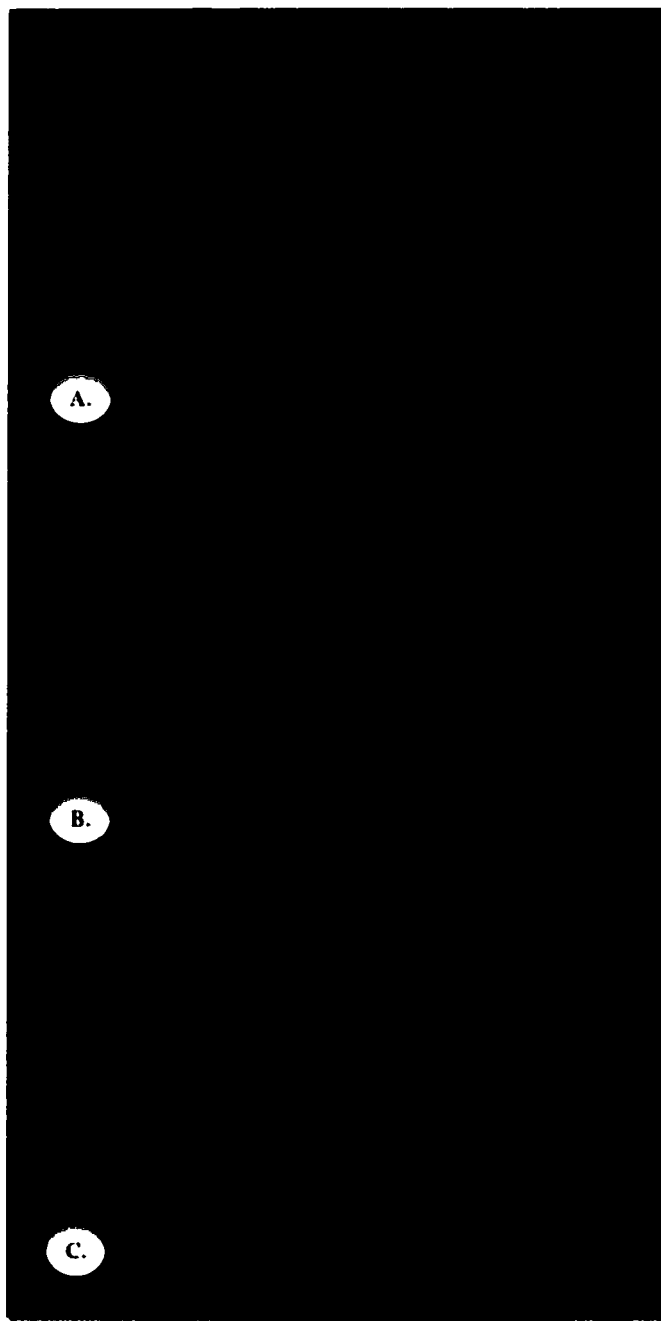
### **3.4. Molecular Mechanisms of *G. lamblia* Induced Injury**

Confluent monolayers pre-treated with either verapamil or cytochalasin D and exposed to *G. lamblia* for 24 h also to exhibited localized F-actin flocculation (Fig. 17) as well as reorganization of the cytoskeletal proteins villin and ezrin (Fig. 18). Similar cytoskeletal reorganization patterns were observed whether SCBN or Caco2 monolayers were exposed to whole *G. lamblia* trophozoites or to sonicated trophozoites alone. Exposure of monolayers to drugs alone had no effect (Fig. 17 and 18).

### **3.5. Scanning Electron Microscopy**

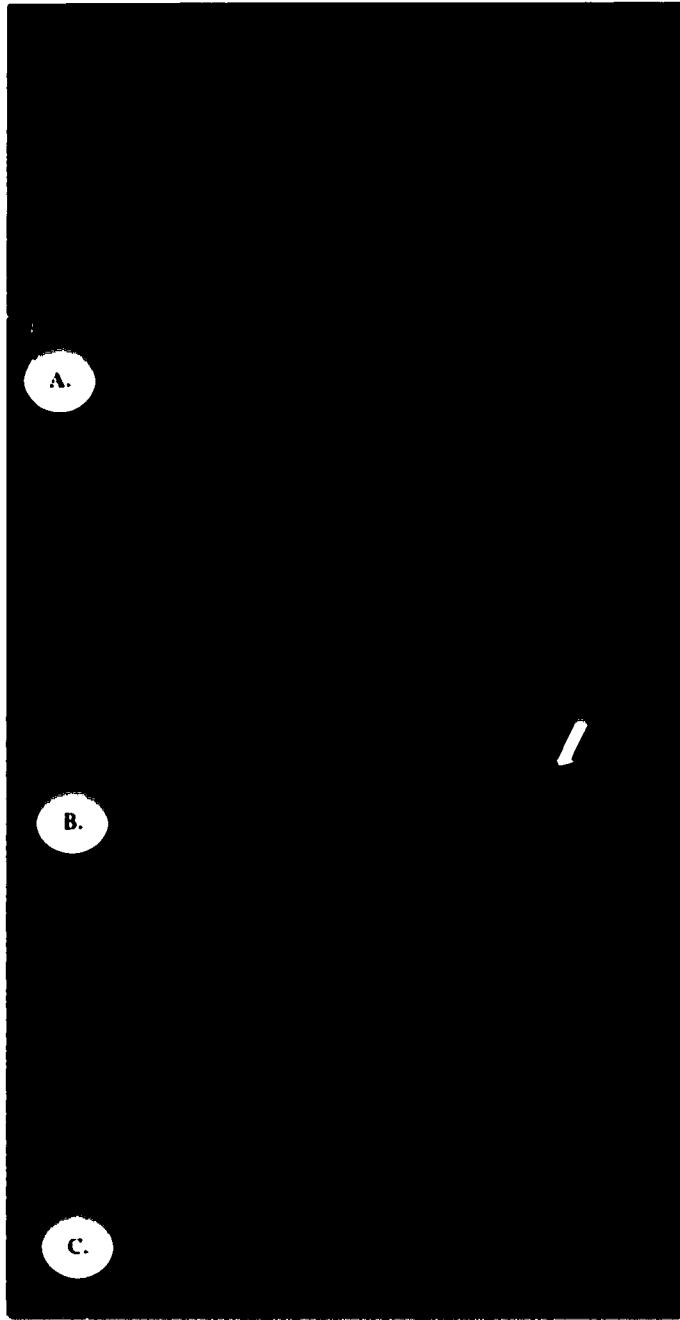
Ultrastructural analysis by scanning electron microscopy revealed a difference between control epithelial monolayers and monolayers co-incubated with *Giardia*

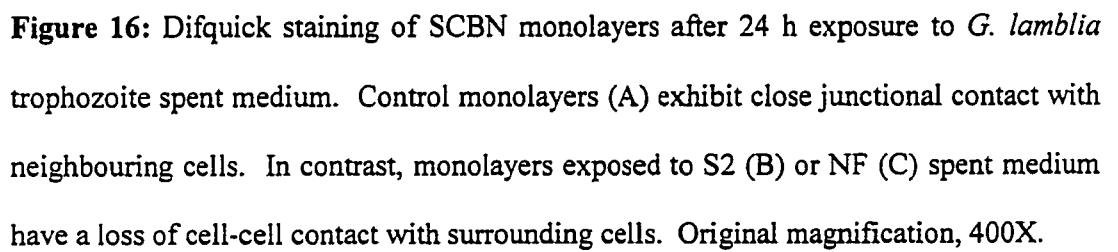
**Figure 14:** Confocal laser micrographs of SCBN monolayers immuno-stained for the cytoskeletal protein ezrin. Control monolayers (A) exhibited perijunctional localization of ezrin with a clear central area. In monolayers exposed to trophozoite lysates (B) or spent medium (C), ezrin is dispersed within the central region and highly concentrated at membrane areas. Original magnification, 600X.



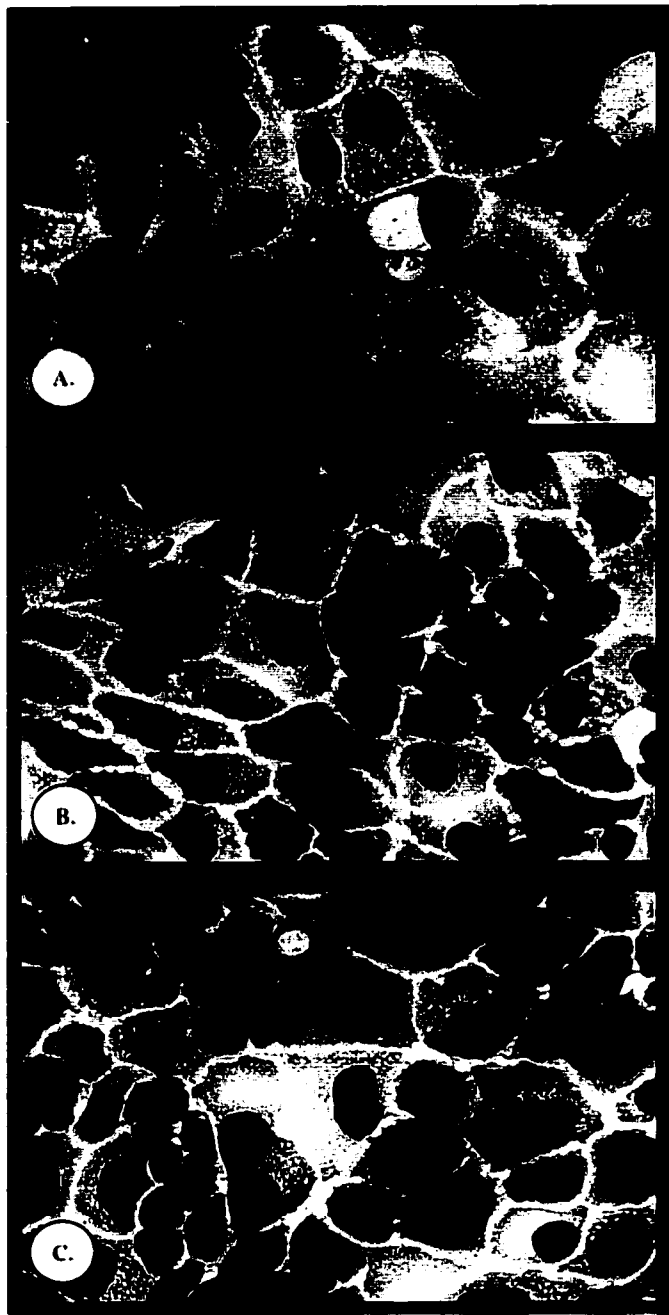


**Figure 15:** Confocal laser micrographs of SCBN monolayers immuno-stained for the cytoskeletal protein villin. In control monolayers (A), villin is organized in a perijunctional location. Upon exposure for 24 h to lysed *G. lamblia* (B) or spent medium (C), villin is disrupted and reorganized towards the nucleus. Exposed monolayers stained for villin exhibit areas of cell-cell junction disruption, and loss of contact with neighbouring cells (arrow). Original magnification, 600X.

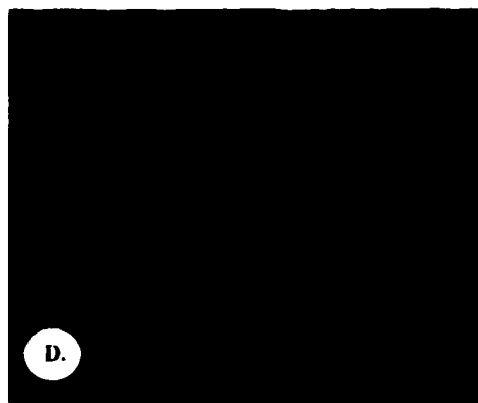
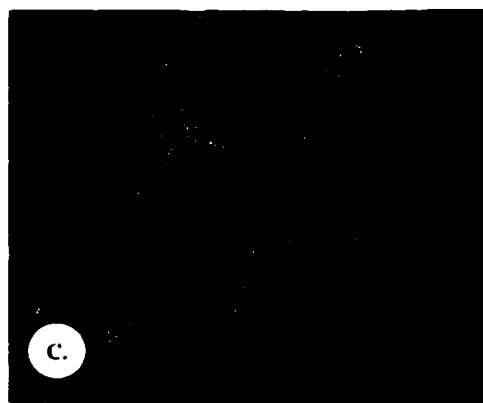
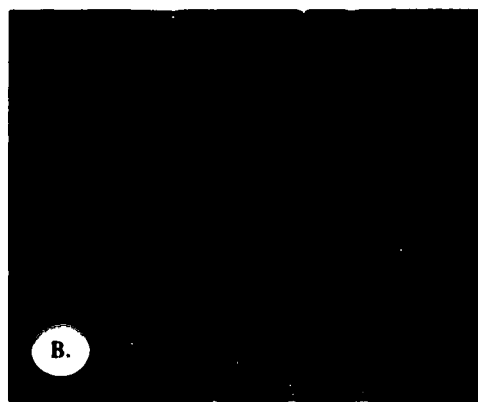
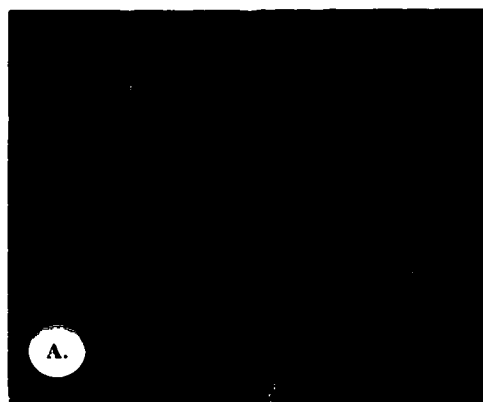




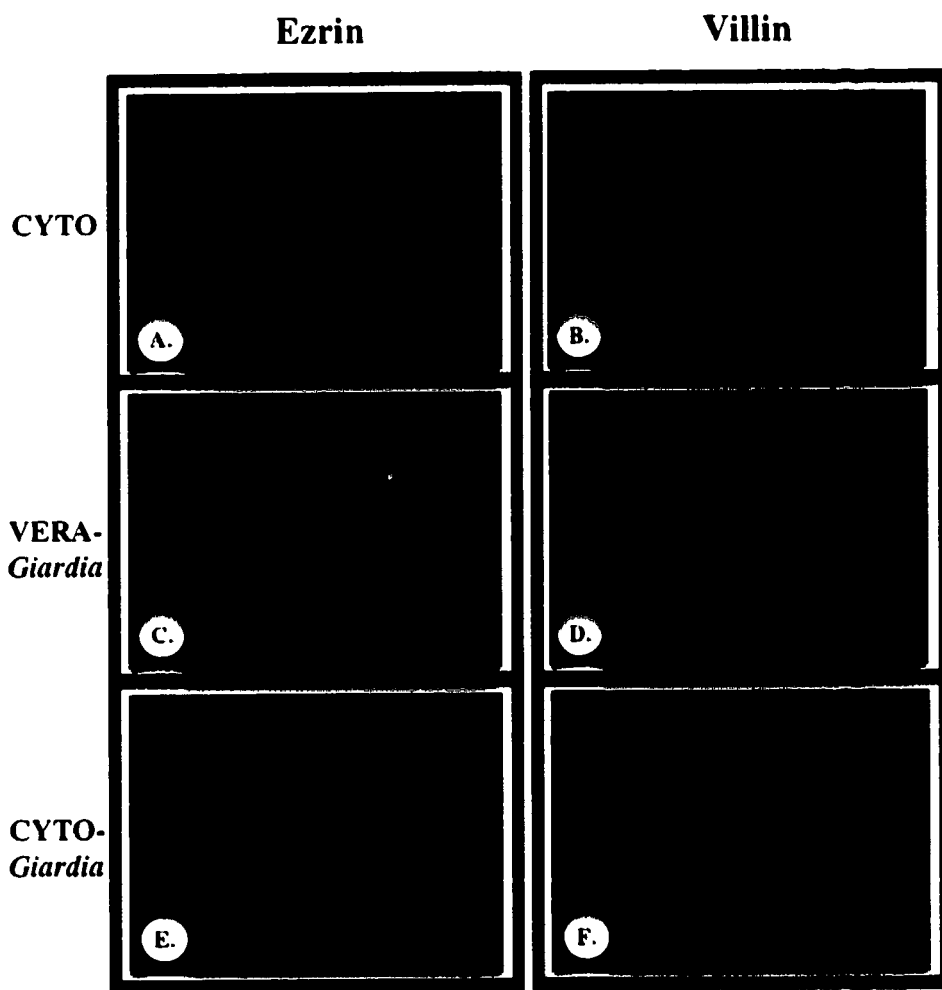
**Figure 16:** Difquick staining of SCBN monolayers after 24 h exposure to *G. lamblia* trophozoite spent medium. Control monolayers (A) exhibit close junctional contact with neighbouring cells. In contrast, monolayers exposed to S2 (B) or NF (C) spent medium have a loss of cell-cell contact with surrounding cells. Original magnification, 400X.



**Figure 17:** Representative confocal laser micrographs of SCBN monolayers pre-treated with verapamil (A and B) or cytochalasin D (C and D) and stained for F-actin with rhodamin-phalloidin. After 24 h exposure to lysed trophozoites (B and D), despite pre-treatment with inhibitors, F-actin flocculation is still observed. Inhibitors alone did not affect F-actin staining (A and C). Original magnification, 400X.



**Figure 18:** Representative epifluorescence micrographs of SCBN monolayers stained for villin and ezrin pre-treated with 100  $\mu$ M verapamil or 1  $\mu$ M cytochalasin D and exposed for 24 h to sonicated *G. lamblia*. Exposure to verapamil (not shown) or cytochalasin D (CYTO) alone did not affect villin or ezrin. Addition of verapamil (C and D) or cytochalasin D (E and F) does not inhibit rearrangement of villin or ezrin in experimental monolayers.



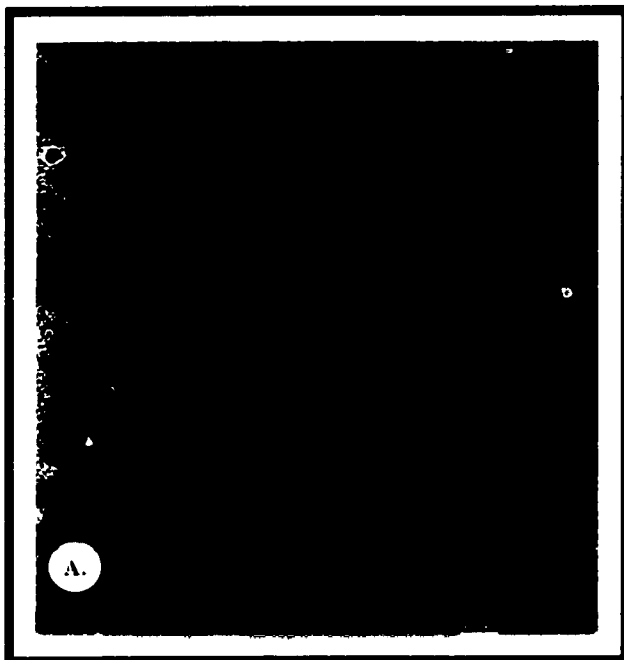


*lamblia* (Fig. 19). Trophozoites were successful in attaching to the epithelial cells *in vitro* and were abundant on the epithelial surface but no “footprints” or disc impressions were seen in monolayers exposed to epithelial cells as previously found (70-72). In exposed monolayers there was aberrant cell architecture or ‘blebbing’ on the surface, which was not apparent in control monolayers (Fig. 19). In addition, the microvilli of exposed monolayers appeared blunted and were less distinct at the same magnification, in contrast to control epithelial cells which had clearly distinguishable microvilli (Fig 19). Trophozoites revealed the typical asymmetrical spiral ridge of their suction disc as well as the phlange and four pairs of flagella used in motility (Fig. 20).

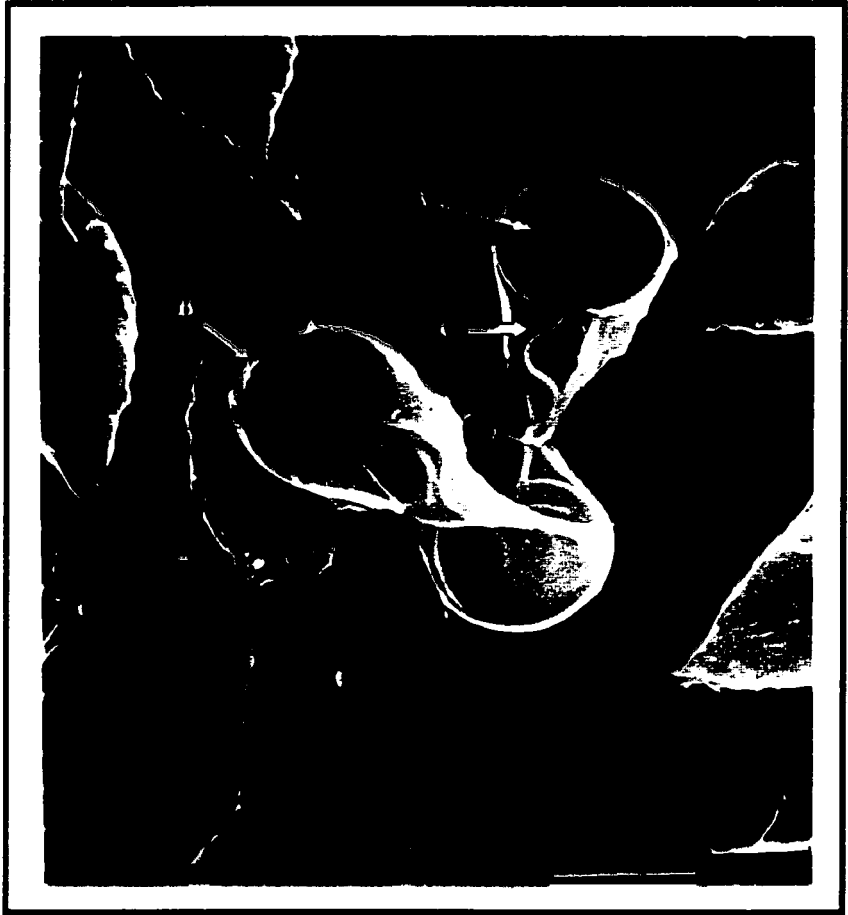
### **3.6. Confocal Laser Microscopy**

To help localize the intracellular site of F-actin rearrangement, monolayers were observed with confocal laser microscopy (Fig. 21). Despite varying cell heights within a monolayer, flocculated F-actin was consistently seen within the apical third quarter of enterocytes (Fig. 21). Confocal microscopy also revealed that staining of rearranged F-actin overlapped staining of terminal web-associated  $\alpha$ -actinin over a  $2\mu\text{m}$  cell region immediately beneath the epithelial brush border (Fig. 22). Staining for either flocculated F-actin or  $\alpha$ -actinin were not observed above or below this area of the cell (Fig. 22). Confocal microscopy also indicated that trophozoite lysates displaced peripheral  $\alpha$ -actinin (Fig. 23).

**Figure 19:** Scanning electron micrographs of SCBN control (A), and monolayers exposed to *G. lamblia* (B). Monolayers with *Giardia* exhibit membrane blebbing (arrows) and less distinct microvilli. Bar = 5  $\mu\text{m}$ .



**Figure 20:** Scanning electron micrograph of SCBN monolayer exposed to *Giardia lamblia* strain S2 at a high magnification. The typical ventral adhesive disk (A), the phlange (B) and flagella (C) of a trophozoite are clearly visible. Bar = 5  $\mu\text{m}$ .



**Figure 21:** Confocal laser micrographs of a SCBN monolayer exposed to lysed *G. lamblia* trophozoites and stained for F-actin. Highest density of flocculated F-actin within the enterocyte (arrow) is seen at a height of 6  $\mu\text{m}$  and 7  $\mu\text{m}$ . Total height of the cell was 9  $\mu\text{m}$ . Flocculated F-actin was not seen below 5  $\mu\text{m}$  or above 8  $\mu\text{m}$  in cell shown by arrow. Original magnification, 400X.

$h = 5 \mu\text{m}$

$f$

$h = 6 \mu\text{m}$

$f$

$h = 7 \mu\text{m}$

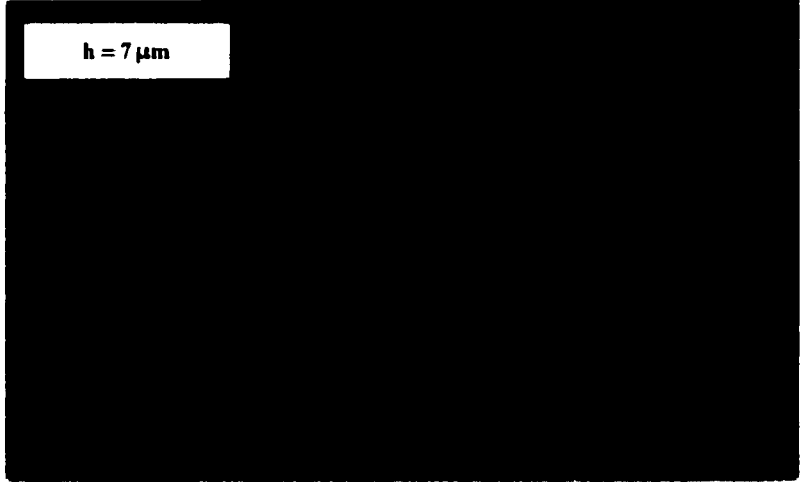
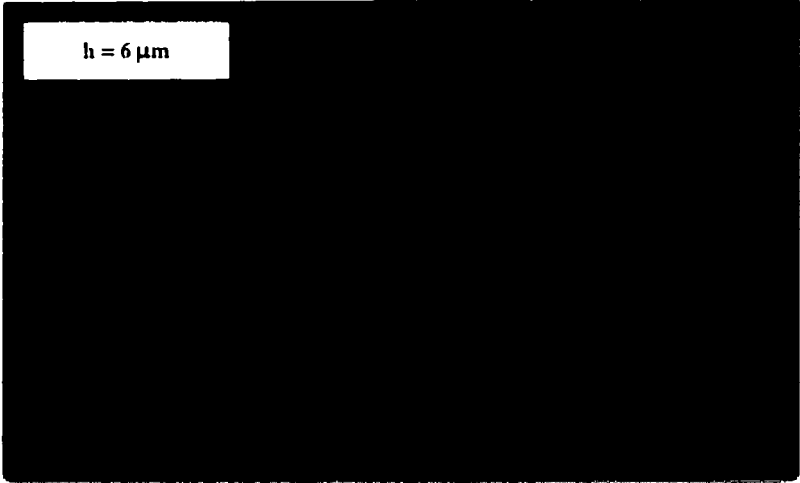
$f$

$h = 8 \mu\text{m}$

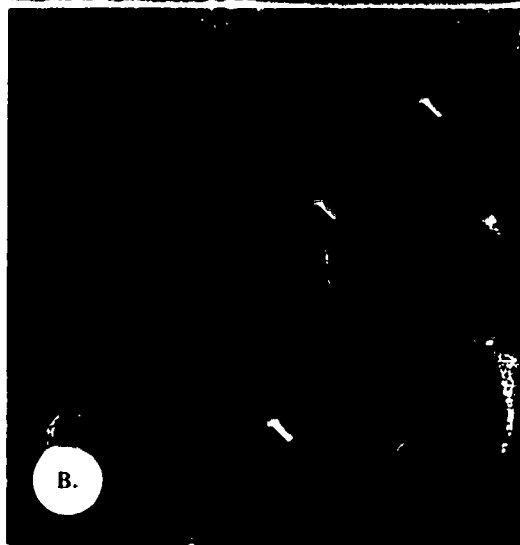
$f$

**Figure 22:** Confocal laser micrographs of a SCBN monolayer exposed to lysed *G. lamblia* trophozoites and strained for both F-actin and  $\alpha$ -actinin. In these cells (total height = 8  $\mu\text{m}$ ) terminal web  $\alpha$ -actinin and flocculated F-actin colocalize within a 2  $\mu\text{m}$  region. Original magnification, 400X.





**Figure 23:** Confocal laser micrographs of SCBN monolayers exposed to vehicle (control) or lysed *G. lamblia* trophozoites and stained for  $\alpha$ -actinin. In control monolayers (A)  $\alpha$ -actinin staining outlines the cell periphery. Monolayers exposed to *G. lamblia*-sonicates (B) exhibit focal loss of peripheral  $\alpha$ -actinin (arrows). Original magnification, 400X.



#### 4. DISCUSSION

*G. lamblia* injures human intestinal epithelial cells *in vivo* and *in vitro*. Using the well studied colonic carcinoma Caco2 line as well as a novel human non-transformed epithelial duodenal cell line, this study demonstrates that *Giardia* reduces epithelial resistance and that the pathophysiology of this injury implicates the cytoskeleton of enterocytes. Results indicate that the loss of transepithelial electrical resistance induced by *Giardia* is associated with a rearrangement of F-actin in the epithelial terminal web and loss of peripheral  $\alpha$ -actinin. In conjunction, there is a disarrangement of the regulatory cytoskeletal proteins, ezrin and villin. These abnormalities could be observed when intestinal cells were exposed to the live parasites or to dead trophozoite products, as well as to trophozoite spent medium. The findings also suggest that the rearrangement of F-actin, ezrin and villin occurred via mechanisms independent of extracellular  $\text{Ca}^{2+}$  or actin polymerization.

This study reports for the first time the effects of an enteric pathogen on transepithelial electrical resistance, cellular actin,  $\alpha$ -actinin, ezrin and villin of a non-tumorigenic human small intestinal cell monolayer. This cell line, called SCBN, was the first non-tumorigenic human small intestinal epithelial cell line to be grown to confluency *in vitro* (295). Originally isolated from a duodenal biopsy of a patient (B.N.) with diarrhea of unknown etiology, SCBN has been shown to form polarized monolayers, to express junctional complexes and disaccharidase activities as well as have well developed microvilli (295). In addition, SCBN also contains cytokeratins, mucin antigen, and messenger RNA for epidermal growth factor, IL-6 and vascular cell adhesion molecule-1 (295). This study compared the pathophysiological effects of *G. lamblia* on

SCBN, with its effects on the better characterized and more widely used colonic adenocarcinoma cell line, Caco2, (10, 143, 184). Results indicate that both in SCBN and in Caco2, *Giardia* induces a loss of transepithelial electrical resistance, in association with F-actin flocculation in the terminal web, loss of peripheral  $\alpha$ -actinin and a rearrangement of the cytoskeletal proteins ezrin and villin. The similarities in these findings further underscore that SCBN represents a valuable tool for the characterization of cellular mechanisms implicated in intestinal pathophysiology.

Previous studies have demonstrated that a number of intracellular bacterial or viral pathogens induce actin polymerization during cell invasion (169). Recently, epithelial injury caused by enteropathogenic *Escherichia coli* (EPEC) or *Clostridium difficile* Toxin A and B was shown to involve intracellular flocculation of F-actin via mechanisms independent of actin polymerization (161, 301, 316). This injury occurred independently from cellular invasion. Similarly, *Giardia* causes intestinal disease without invading the epithelium. This protozoan induces diffuse epithelial brush border shortening, an injury also observed in bacterial enteritis, chronic anaphylaxis, Crohn's, or Celiac's disease (52, 89, 103, 303). In giardiasis, this generalized shortening of microvilli is observed along the entire epithelial lining, at sites of trophozoite attachment as well as in other areas (52-54). Such ultrastructural alterations are at least in part responsible for disaccharidase impairment and malabsorption of electrolytes, water, and nutrients (12, 52-54). Conversely, in response to luminal Epidermal Growth Factor, increased absorption is associated with diffuse lengthening of epithelial microvilli (156) via mechanisms that involve F-actin polymerization (80). Together, these observations

clearly indicate that epithelial microvilli constitute a kinetic interface, and that they may alter their length in response to various luminal stimuli.

This present study characterizes permeability and cytoskeletal changes associated with *Giardia*-induced epithelial alterations. Findings from previous experiments have shown that measurements of whole gut tissue permeability to [<sup>51</sup>Cr]EDTA were unable to detect alterations in intestinal permeability during giardiasis (157). However, the effect of *Giardia in vivo*, utilizing non-digestible disaccharides such as sucrose and mannitol, on intestinal permeability has not been determined. *In vitro* experiments were warranted in order to assess the effects of *Giardia* on the permeability of the epithelial lining alone.

Observations from the present study unequivocally demonstrate that *Giardia lamblia* induces a significant loss of electrical resistance in pure epithelial monolayers, that this epithelial injury is associated with rearrangement of F-actin, ezrin, villin and  $\alpha$ -actinin, and that flocculated F-actin co-localizes with terminal web  $\alpha$ -actinin, while G-actin remain unaffected. Consistent with the F-actin changes seen in enterocytes exposed to EHEC, EPEC, *Salmonella*, or *Clostridium difficile* Toxin A and B, the *Giardia*-induced F-actin, ezrin and villin reorganization appears to be independent of extracellular  $\text{Ca}^{2+}$ . Previous observations have shown that the pathology due to giardiasis in mice requires the involvement of  $\text{Ca}^{2+}$  from intracellular sources (140), and the apical membrane alterations induced by *Salmonella* and EPEC in epithelial cells are also associated with a marked increase in intracellular calcium (32).

In addition, pretreatment of enterocytes with cytochalasin failed to inhibit F-actin reorganization in cells exposed to *Giardia*. Hence, this observation contrasts with the F-actin dependent membrane lesions seen in enterocytes exposed to *Salmonella* and EHEC,

where actin polymerization inhibitors block bacterial entry (32, 49). Additionally, the pretreatment of enterocytes with cytochalasin D also failed to inhibit the relocalization of ezrin or villin. Taken together, these observations suggest that the epithelial injury associated with exposure to *Giardia* requires intracellular but not extracellular calcium, and that the cytoskeletal rearrangement seen in this process is independent of actin-polymerization.

In keeping with the diffuse nature of microvillus shortening seen in giardiasis *in vivo* (52-54), the cytoskeletal protein rearrangements reported herein could be observed in the absence of live trophozoites, when enterocytes were exposed to sonicated parasite products or even to trophozoite spent medium alone. Intriguingly, experiments using MDCK cells co-cultured with *Giardia lamblia* have suggested that monolayers exposed to trophozoites may exhibit either no change or an increase in electrical resistance (70, 72). Whether this discrepancy is due to the fact that, in these studies, monolayer confluency prior to exposure to trophozoites was confirmed solely by visualization, or, whether these observations reflect the existence of strain-dependent virulence factors, has yet to be clarified. Regardless, the findings presented here show that *Giardia lamblia* can increase epithelial permeability, which is associated with a rearrangement of F-actin in the terminal web, and that this disruption of epithelial integrity may result from exposure to parasite products alone. Moreover, in separate experiments, exposure of SCBN or Caco2 monolayers to live or sonicated *Giardia* trophozoites for only 2 h was sufficient to induce F-actin re-arrangement (not shown), further highlighting the rapidity of the epithelial response to these parasite products.

Purified Toxin A or B from *Clostridium difficile* increases epithelial permeability by condensing F-actin within the perijunctional ring (161, 162, 316). Recent reports have described that increased epithelial permeability during infection with a variety of bacterial species may be associated with formation of ruffles on the apical membrane, and that this alteration is accompanied by profound rearrangement of cytoskeletal proteins, including F-actin and  $\alpha$ -actinin (32). Although *Giardia* is known to contain a variety of proteinases (158, 298), the possible pathogenic implications of excretory-secretory products released by the parasite remain subject of controversy (24, 26, 74, 108, 382, 384). *Clostridium difficile* Toxin A and B are known to target the GTP-binding rho protein in host cells (96, 200, 286). The loss of epithelial integrity in cells exposed to *Clostridium difficile* toxin is postulated to be due to an apoptotic response via rho regulatory G proteins (120, 246). It has been suggested that rho and other GTPases may regulate nuclear as well as cytoplasmic effects based on the observation that chronic activation of rho by deregulated exchange factors induces cell morphological changes (395). Within the enterocyte, GTP-binding proteins are responsible for stimulating the protein kinase cascade which is involved in the second messenger system and signal transduction cascade utilizing  $\text{Ca}^{2+}$ . It has been shown that epithelial colonization with EPEC induces signal transduction responses, which involve elevated intracellular  $\text{Ca}^{2+}$ , second messenger molecules and inositol 1,4,5-bisphosphate (18, 104), as well as phosphorylation of tyrosine and threonine-serine residues on cellular proteins (17, 250, 323). Moreover, it has been suggested that other cytoskeletal proteins, including villin and fimbrin, when activated within the cell, may act as actin-severing components (273). In the context of the study reported here, rearrangement of villin, via elevated



intracellular  $\text{Ca}^{2+}$  may in part contribute to the loss of F-actin scaffolding, which in turn could lead to the disruption of microvillus support thereby causing microvillus shortening.

It is intriguing to note that ezrin, which can serve as a tyrosine kinase phosphorylation site, is also rearranged in response to *Giardia* (375). Ezrin is readily phosphorylated by the Epidermal Growth Factor receptor and mediates the regulatory functions of the cell in response to growth factors by its interaction with actin (45). Furthermore, the association of ezrin as a linker of F-actin core bundles to the overlying plasma membrane implicates phosphatidylinositol turnover and rho-dependent signalling pathways (171). Potentially, F-actin rearrangement could also involve ezrin disassociation from the overlying membrane via rho, intracellular  $\text{Ca}^{2+}$  increase, and the signal transduction pathway. Kondo *et. al.* (1997) leads us one step further in understanding the response of F-actin to ezrin activation upon exposure of either the ezrin filament- or membrane- binding domains (215). Using mouse fibroblasts capable of expressing recombinant human Fas antigen receptor, Kondo *et. al.* showed that addition of the Fas antigen induced apoptosis within 1 h. Induction of apoptosis was associated with dephosphorylation of ezrin via rho pathways involving elevated levels of  $\text{Ca}^{2+}$ , which induced the translocation of ezrin from the plasma membrane to the cytoplasm and resulted in F-actin staining patterns similar to those seen in this study (215). The authors concluded that the cytoplasmic translocation of ERM proteins is responsible for the microvillar breakdown at an early phase of apoptosis (215). In addition, during EPEC infections, ezrin was found to be redistributed toward enterocyte membranes where it is likely to play a role in transducing signals to the cell (106). In both situations, ezrin is

relocated within the cell. *Giardia* are known to have cysteine proteases, this is pertinent as apoptotic cell death is usually accompanied by the activation of interleukin-1 $\beta$  converting enzyme (ICE) and other members of the cysteine protease family (105, 401). One of the characteristics of apoptosis is membrane blebbing. In light of the blebbing seen in monolayers observed under SEM, it is attractive to suggest that *Giardia* causes a diffuse shortening of brush border microvilli via trophozoite secretory/excretory products capable of inducing apoptosis, where upon induction, rho is deactivated and concurrently stimulates release of intracellular Ca<sup>2+</sup> while affecting ezrin relocalization. The elevated Ca<sup>2+</sup> could indirectly affect F-actin by rearranging villin which can also sever and cap F-actin filaments. Once the F-actin enterocytic scaffolding is lost, other cytoskeletal proteins, including  $\alpha$ -actinin respond by rearranging. Whether binding of *Giardia* excretory-secretory products to host enterocyte rho is implicated in the pathogenesis of giardiasis, and/or whether *Giardia* is capable of inducing apoptosis resulting in the loss of microvilli and increased epithelial permeability reported here has yet to be uncovered.

In conclusion, the mechanistics or molecular mechanisms of *Giardia*-induced epithelial injury remain unknown. This study characterizes permeability and cytoskeletal changes in a human non-transformed small intestinal epithelial cell line and Caco2 monolayers exposed to *Giardia lamblia*. The findings demonstrate that loss of electrical resistance in epithelial monolayers exposed to this Protozoan is associated with flocculation of F-actin in the terminal web and loss of peripheral  $\alpha$ -actinin. *Giardia lamblia* also causes a rearrangement of  $\alpha$ -actinin and of the important regulatory cytoskeletal proteins ezrin and villin, which likely may act directly on F-actin to cause disarrangement. In this system, cytoskeletal protein rearrangement is independent of

actin polymerization and of extracellular  $\text{Ca}^{2+}$ , and the cytoskeletal changes can be induced by exposure to parasite products alone, further supporting a role for a *Giardia* trophozoite secretory/excretory products in pathogenesis. Additional research using this model will not only help unravel the pathogenic basis of giardiasis, but will also improve our understanding of the mechanisms responsible for brush border injury and malabsorption in other small intestinal malabsorptive disorders, such as bacterial enteritis, Crohn's disease, and Celiac's disease. Finally, the *in vitro* model used in this study, including the Transwell™ filter system and fluorescent staining of important cytoskeletal proteins, can be utilized as a novel model of small intestinal epithelial injury to study the effects of various pathogenic organisms.

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