In vitro evaluation of the biologic changes induced by a beta-hemolysin from *Serpulina hyodysenteriae*

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INTRODUCTION

The development of an in vitro system that utilizes a cell line similar to that which is affected by disease would enable us to examine potential mechanisms of pathogenicity like those associated with gastrointestinal diseases. A model, utilizing intestinal epithelial cell cultures, was established to facilitate the study of the biologic effects of a beta-hemolysin, on epithelial permeability. The Caco-2 human colon carcinoma cell line, which forms polarized monolayers, well defined brush borders and tight junctions (**1**), was used to examine changes in intestinal permeability induced by beta-hemolysin (**1**, **2**). Following addition of the beta-hemolysin to cultures of Caco-2 cells, relative transepithelial permeability was examined by the measurement of electrical resistance across these monolayers.

Serpulina hyodysenteriae, originally named *Treponema hyodysenteriae* (**3**), is the primary agent of swine dysentery which induces a severe mucohemorrhagic diarrheal disease in growing pigs. Beta-hemolysin, a toxin isolated from the organism, is thought to be a primary factor in the induction of lesions and has proven to be cytotoxic for several types of eukaryotic cells (**4**). The exact biologic characteristics of beta-hemolysin are still unknown. The hemolysin is an oxygen stable, heat labile, small polypeptide with a reported molecular weight ranging from 19 kDa to 23 kDa (**5**). Epithelial cell damage has been demonstrated, following exposure to beta-hemolysin preparations, in ligated ileal and colonic loops of germ free pigs (**6**). Similar results were observed in ligated rat ileal loops injected with the hemolysin (**7**) and in pig colonic loops injected with viable cultures

of *S. hyodysenteriae* (**8**, **9**). These studies demonstrated that beta-hemolysin, synthesized by *S. hyodysenteriae,* is a contributing factor to the virulence of swine dysentery.

The systemic effects of swine dysentery are fluid and electrolyte loss caused by colonic malabsorption, ultimately leading to dehydration and death of untreated swine (**10**, **11**). Net solute and water transport are abolished in the colon as a result of decreased lumen-to-blood fluxes of sodium and chloride ions (**12**). A "leakiness" of the intestinal mucosa caused by disruptions in the tight junctions, which are important in regulating transepithelial water and solute flow, could be a factor in the malabsorption.

Cell plasma membranes maintain a specific cytosolic ion composition which usually differs greatly from that of the surrounding extracellular fluid. A significant fraction of available energy in every cell is required to maintain the concentration gradients of ions across the plasma and intracellular membranes. Changes in ion levels may play an important role in regulating intracellular pathways.

Calcium ions are important as intracellular messengers in many signal transduction pathways (**13**, **14**). Elevations of intracellular calcium are part of the regulation of diverse cellular processes. Eukaryotic cells have an internal calcium ion concentration that is usually maintained at approximately 100 nM which is far below the extracellular levels of calcium (**15**). Calcium ATPase (i.e., the calcium pump) plays an important role in transporting calcium out of the cell to maintain low cytosolic concentrations. Calcium binding proteins, located in vesicles of the sarcoplasmic reticulum (SR), serve as reservoirs for intracellular calcium and thereby reduce the concentration of free calcium ions in the cytosol. This decreases the energy required to sequester calcium ions in the cytosol (**13**,

16).

Calcium influx is thought to be initiated by membrane depolarization which opens voltage gated channels or by the binding of ligands to receptor operated ion or calcium channels (**14**). Following membrane depolarization, a cascade of intracellular events are initiated in which calcium may be released from intracellular stores. The presence of cytosolic calcium may then activate enzymes that are part of essential metabolic pathways, such as smooth muscle contraction, glycogen breakdown and exocytosis (**14**). Any damage to cell membranes caused by infectious agents may induce changes in the intracellular calcium ion concentration, thereby, disrupting calcium activated functions as well as disrupting the integrity of the epithelium. The present studies were performed in order to examine changes in epithelial monolayer permeability and in calcium flux in cells incubated in the presence of the beta-hemolysin of *S. hyodysenteriae*.

LITERATURE REVIEW

Basic Histology of the Digestive Tract

Tissues are collections of cells that frequently have similar morphological characteristics and similar functions. Despite the complexity of the mammalian body, it is composed of only four different basic types of tissue; epithelial, connective, muscular and nervous (**17**). These tissues associate with one another in variable proportions to form different organs and systems in the body.

The stomach and intestine in mammals are lined with epithelial cells which differ in morphology according to their location and carry out the digestive, secretory and absorptive functions of the gastrointestinal tract (**18**, **19**). These cells form a barrier between the lumen and other body tissues. A process of selectivity prevents the absorption of some harmful or toxic substances. The enterocytes, epithelial cells, are joined together at their luminal poles by dense areas called tight junctions. The cell membranes and the tight junctions existing between the cells form a barrier where specific transport processes or size specifications determine which luminal contents are allowed to cross through cells or pass between cells before eventually entering the blood or more specifically the portal system (**19**).

Epithelial tissue

Epithelial tissues are composed of closely aggregated polyhedral cells with very little intercellular space (**20**). Adhesion between these cells is strong. Thus, cellular sheets (i.e., monolayers) are formed that cover the surface of the body and line cavities of

organs.

Epithelial cells take on specific roles according to their location and function, for example; covering and lining (skin); absorption (intestine); secretion (epithelial cells of glands); sensation (neuroepithelium); and contractility (myoepithelial cells). An important feature of epithelia is their polarity, where the apical membrane of this polarized cell monolayer is in contact with the luminal space of the structure within which it is situated, while the basolateral (or serosal) membrane is in contact with the tissue fluid which provides nutrients for the cell (**17**).

Epithelial cells function in fluid homeostasis and the active and regulated transport of solutes and electrolytes; but, in general, they function as barriers to the unrestricted diffusion of solute molecules across the monolayer. Tight junctions situated between the cells aid in maintaining this barrier function.

Epithelial cells are extremely cohesive (intercellular adhesion) and relatively strong mechanical forces are required to separate them (**17**). This is due in part to the binding action of the glycoproteins, which are integral membrane proteins of the plasma membrane, and because of a small amount of intercellular proteoglycan.

All epithelial cells come in contact with an extracellular ground substance at their basal surfaces called the basal lamina. This membrane provides a selective barrier, limiting or regulating exchanges of macromolecules between the connective tissue and the epithelial tissue. The basement membrane is the combination of the basal lamina and the reticular lamina.

As mentioned previously, epithelial cells adapt specifically to perform their various functions. In the intestine, the absorptive epithelial cells have numerous projections or microvilli arising from the surface to increase the area for absorption. These microvilli are arranged in an orderly array of many hundreds to form a brush border.

Tight junctions

Intestinal epithelial cells are attached to one another at the uppermost part of their lateral cell borders by structures referred to as tight junctions (zonula occludens) (**20**). The tight junctions are made up of ridges, half of which extends from one cell and half from the other, that connect the cells so strongly at the junctions that they almost obliterate the space between the cells. These adjacent plasma membranes are fused, resulting in the typical pentalaminar appearance of the tight junctions. One to several fusion sites may be observed along the lateral borders of two adjacent epithelial cells depending upon the section of tissue (**20**). The intercellular junctions serve not only for adhesion, but as seals to prevent the flow of material through the intercellular space and to provide a mechanism for intercellular communication (gap junctions) (**17**). Another function of the tight junctions is maintenance of cell polarity, where the ridges prevent the lateral movement of proteins in the cell membrane, thereby ensuring that proteins remain a part of the apical or basal cell membranes (**20**) such as sucrase-isomaltase, lactase and aminopeptidase N which are brush border associated hydrolyses located on the apical cell membrane of small intestinal cells (**21**).

Tight junctions are formed partly by intramembranous particles (IMPs). The nature of the IMPs is uncertain; however, some studies have concluded that IMPs were

proteinaceous while others suggest they are composed of lipid micelles (**19**). Electrophysiological and freeze fracture studies of a variety of epithelia indicate a general correlation between the electrical resistance across the epithelial cells and the number of rows or strands of IMPs making up the tight junctions (**19**). Movement of substances across the epithelia occurs via either a transcellular route or a paracellular pathway. Tight junctions are important as a mechanism in the regulation of paracellular transport of water and solutes (**19**). The tight junctions form a barrier to the movement of ions and other solutes from one side of the epithelium to the other. The extent and selectivity of this barrier varies from tissue to tissue, where some epithelia are more "leaky" than others. As mentioned previously, the number of tight junctions correlates with the relative "leakiness" of the epithelium (i.e., epithelia with few fusion sites are more permeable to water and solutes than epithelia with numerous fusion sites).

The number of junctional strands which make up part of the structure of tight junctions, averages 4.4 but vary not only from cell to cell but also along the length of one cell (**17**). The greater the number of junctional strands, the tighter the epithelium. In the guinea pig distal colon, the belt formed by the zonulae occludens between superficial columnar epithelial cells is substantially thinner (285 nm) than that found in the proximal colon (358 nm); however, the number of junctional strands is greater, suggesting an electrically tighter epithelium in the distal colon (**17**).

Physiological studies indicate that the paracellular path provides higher resistance in the ileum than in the jejunum and between villus cells as compared with crypt cells (**20**). The tight junctions have fixed negative charges which would allow cations to be

more permeable than anions. The tight junction permeability for small ions greatly exceeds that of the cell membrane (**20**); therefore, small ions are transported passively across the intestinal mucosa through the paracellular path.

Solutes taken up across the apical brush border of the epithelium move into the lateral intercellular space via the basolateral cell membrane. This causes a rise in osmotic pressure in the intracellular space, thereby establishing an osmotic gradient that is separated from the intestinal lumen by the tight junction (**19**). Active sodium pumps, requiring energy, located along the lateral and basal membranes pump sodium in exchange for potassium into the intercellular spaces, which also causes a rise in osmotic pressure. This causes water flow across the tight junctions accompanied by solutes from the luminal fluid to move through the basement membrane where they are then taken up by the capillaries (**19**). They are allowed passage through the channel pores in the tight junctions depending on the size of their hydrated radius. The movement of water and electrolytes generally favors the direction from the lumen to the tissue because of the regulatory function of the tight junctions (**19**).

Absorption

The intestinal mucosa and digestive tract secretory organs provide the enzymes, electrolytes and water necessary for digestion and absorption to take place in the lumen. These processes require energy and are under the control of the neuroendocrine system. The absorptive cells are capable of maintaining stable non equilibrium ion gradients where the intracellular ion content differs from that of the extracellular pool. The cationic

selectivity which maintains the intracellular ion concentrations requires a significant proportion of the cells available energy (**22**).

Various active processes are involved in absorption of food from the digestive tract; however, a large portion of nutrient movement occurs by passive diffusion as molecules move down electrical and chemical concentration gradients. The absorption of solutes across the intestinal wall takes place by several mechanisms and are listed in Table 1 (**22**).

The large absorptive capacity of the small intestine is made possible by the presence of villi, which are covered by mature absorptive cells or enterocytes. The absorptive area is made even larger by the presence of brush border microvilli located on the apical plasma membrane of enterocytes (**19**). The motor function of the gut, which is also controlled by the neuroendocrine system (**22**), ensures a continuous changing contact between the intestinal lining and the luminal contents thus facilitating absorption. Many of the important enzymes responsible for terminal digestion are situated on the luminal plasma membrane of mature enterocytes.

Absorption in the small intestine

The proximal end of the small intestine is the main site of absorption where most of the vitamins, minerals and digestive end products are absorbed (**23**). Water and bile acids are mostly absorbed by enterocytes in the distal small intestine or ileum. The passive absorption of electrolytes across the intestinal wall depends on electrical as well as concentration gradients. Electrolytes can cross the epithelium either by transcellular or paracellular pathways (**22**).

Table 1. Mechanisms of absorption

Mechanism	**Description**
Diffusion (Passive)	Diffusion of molecules occurs through the membrane by the partition into the lipid if they are fat soluble.
Solvent Drag (Passive)	Low molecular weight solutes accompany the osmotic movement of water which occurs through either the paracellular or transcellular pathway.
Carrier Mediated or Facilitated Diffusion (Passive)	The solute and sodium combine with a membrane bound carrier to form a soluble complex which diffuses through the membrane.
Electro-chemical Gradient (Passive)	Movement of ions occurs due to the differences in concentrations and charges between the ions in the extracellular fluid and the cytosol.
Active Transport	It involves the use of ATP to transport sodium out of the cell on the basolateral membrane.

Sodium is actively absorbed by intestinal epithelial cells due to the activity of a sodium/potassium ATPase on the basolateral membrane or by a sodium/hydrogen exchange mechanism on the apical epithelial membrane (**24**). The small and large intestine absorb sodium into enterocytes down an electrochemical gradient. Sodium is then pumped out by active transport into the intercellular space across the basolateral membrane of the enterocytes which contains an active sodium/potassium exchange pump, which is thought to exchange 50% more sodium than potassium. Another transport process is the coupling of sodium entry with hexose sugars, such as glucose, or amino acids and small peptides from the lumen. The epithelial cells also exhibit neutral exchange of sodium in exchange for hydrogen ions.

In the jejunal epithelium, where tight junctions are leaky, intracellular potassium absorption accompanies the osmotic influx of water by solvent drag. In the ileum, where the junctions between the cells are tighter than those in the jejunum, solvent drag is a less efficient mechanism for electrolyte absorption.

Absorption in the colon

The stagnant conditions which predominate in the colon are ideal for bacterial fermentation of food residues (**25**). Carbohydrates and other substrates which were not digested and absorbed in the small intestine are fermented in the colon to volatile fatty acids, such as acetate, which are absorbed and metabolized as a source of energy by the colonic epithelium (**12**). In this regard, the main function of the colonic epithelium is the absorption of water and electrolytes that were not absorbed or were secreted by the small intestine.

The absorption of water and electrolytes in the colon occurs through various mechanisms. Acetate, which is formed and absorbed by the pig colon as well as other volatile fatty acids, facilitates the absorption of sodium and water (**22**). Volatile fatty acids which are absorbed in the unionized form, dissociate once inside the cell, making hydrogen ions available for exchange with the sodium ion through a sodium/hydrogen exchanger (**22**). Sodium is actively secreted by colonic epithelial cells into the intercellular space due to the activity of a sodium/potassium ATPase pump on the basolateral membrane or by a sodium/hydrogen exchange mechanism. Osmotic gradients are produced by the active transfer of solute from the lumen into the cells. Both sodium and chloride ions are absorbed through the brush border into columnar epithelial cells

lining the intestinal tract. This absorption is followed by movement of these solutes through the basolateral membranes into the intercellular fluid between these cells. This creates a hypertonic fluid and establishes an osmotic gradient. Water then flows from the intestinal lumen into the intercellular space through the tight junctions. Water, therefore, moves passively between the cells through the pores of the tight junctions and thereby distends the intercellular space. Elevated hydrostatic pressure aids in the movement of water and other components across the basement membrane into the intercellular fluid and finally into the capillaries. The intercellular space becomes less tense, and the cell walls of adjacent cells return again to close proximity (**24**).

The intercellular junctions in the colon are relatively tight when compared to those in the small intestine. As a result, the colon has low osmotic permeability and is capable of transporting sodium into the cell against an electrochemical gradient (**22**). The tightness of the colonic epithelium plays an important role in preventing back-diffusion of materials already transported across the epithelium because of their relative impermeability to ions, water and larger molecules.

Potassium movement into the colonic lumen probably occurs due to passive diffusion down electrical and concentration gradient, even though some secretion occurs as a result of the release of mucous from goblet cells. These movements as well as water absorption account for the low sodium and high potassium concentrations in the normal stool (**25**). Nevertheless, loss of ileal or colonic fluids in chronic diarrhea can lead to severe hyperkalemia (**24**).

Besides the efficient absorption of nutrients from the intestinal lumen, the reabsorption of all digestive secretions, composed mainly of water and electrolytes, is critical and an equally important function of the colonic epithelium. The movement of water in the intestine is a passive process and occurs secondary to osmotic pressure gradients.

Diarrhea

Diarrhea is a condition resulting from an excessive elimination of semifluid feces (**24**). It may be the result of an infection in the gastrointestinal tract (gastroenteritis) or of an excessive parasympathetic stimulation of the small and/or large intestine (neurogenic diarrhea) commonly induced by stress. The irritability of inflamed mucosa in the presence of infection may promote peristalsis, which results in rapid voiding of intestinal contents from the body, in an attempt to rid the body of the infection. If this protective mechanism is prolonged malabsorption will ensue (Table 2). The end result of malabsorption is dehydration and, in severe cases, circulatory collapse which can lead to death. Although diarrhea is a common sign associated with diseases of the large intestine, the pathophysiologic mechanisms causing it vary depending on the organism.

Pathogenesis of organisms which cause secretory diarrhea

Vibrio cholerae produces a toxin which activates adenylate cyclase, thereby causing an increase in intracellular cyclic AMP (cAMP). The intracellular accumulation of cAMP increases chloride ion secretion from crypt cells and inhibits the function of the villus mucosal carrier for neutral sodium chloride co-transport resulting in a net decrease in

Table 2. Causes of impaired water absorption

Cause	Description and Examples
Indigestible Solutes	Ingestion of solutes which can not normally be absorbed . Therefore, water remains in the lumen. (Osmotic laxatives, magnesium sulfate and indigestible polysaccharides from fruits and vegetables such as pectin)
Maldigestion	Impaired digestion of normal food constituents. (Lactose in lactase deficiency and pancreatic insufficiency)
Malabsorption	Impaired absorption of normal food material. (Due to loss of absorptive cells or impairment of transport processes or rapid transit of food through the intestine; diarrhea)

sodium/chloride absorption. Sodium moves from the intercellular space past the cationic selective tight junctions into the lumen. This causes isotonic secretions into the lumen, which overwhelms the absorptive capacity of the intestine, leading to diarrhea (**24**). Enterocytes take in 1 sodium ion, 1 potassium ion and 2 chloride ions through a co-transporter (**24**).

Escherichia coli produces a similar toxin which is heat labile and highly antigenic and also activates cAMP. *Escherichia coli* also produces another enterotoxin, that is heat stable which activates the cyclic GMP (cGMP) system and causes electrolyte secretion (**12**). Enterotoxigenic *Vibrio cholerae* and *E. coli* have similar effects on the intestine causing an accumulation of isotonic water and electrolytes in the lumen due to active secretion. No permeability changes or changes in tissue architecture have been associated with the activity of cholera toxin or the heat labile enterotoxin of *E. coli* (**26**).

Salmonellosis, a diarrheal disease where the bacteria invade the ileal and colonic mucosa, causes chloride secretion and a reduction in sodium absorption. *Salmonella* spp. have been shown to increase cAMP levels in epithelial cells of ileal loops of rabbits. Prostaglandin mediated activation of adenylate cyclase was postulated as the means in which cAMP levels are elevated (**12**). *Shigella* spp. also produce diarrhea, but only after the invasion of the colonic mucosa by the bacteria. An enterotoxin from *Shigella* has been isolated but its exact involvement as a pathogenic factor is not fully understood (**26**); however, an increase in cAMP does occur. In contrast to chloride toxin, this increase does not happen until after the secretion of fluids and electrolytes has taken place (**26**).

Pathogenesis of swine dysentery

Swine dysentery, a transmissible mucohemorrhagic diarrheal disease of the large intestine in growing pigs, was estimated to cost the United States from 30 to 50 million dollars annually in 1980 (**27**) and 100 million dollars annually in 1986 (**28**). The disease is caused by *S. hyodysenteriae*, formally known as *Treponema hyodysenteriae*, an anaerobic, motile, gram negative, oxygen tolerant spirochete which produces beta-hemolysis on blood agar (**29**). Swine dysentery, which lasts for several days, sometimes causes reduced growth rate in weaned pigs. Clinically, the disease induces diarrhea containing mucus which may be accompanied by blood (**30**).

Serpulina hyodysenteriae requires the combined action of one or more of the indigenous colonic anaerobic bacteria in addition to the presence of the spirochete, so that colonization of the colon results in the onset of disease (**29**, **31**). Abnormal levels of *Campylobacter* spp., a gram negative bacillus, *Bacteroides* spp., *Fusobacterium* spp., and

two protozoa, *Balantidium* and *Trichomonas* (**27**), have been detected and appear to contribute to the severity of swine dysentery.

Serpulina hyodysenteriae produces two toxins, lipopolysaccharide (LPS) and hemolysin, that are thought to play a role in lesion production (**29**). Lipopolysaccharide is a molecule which is situated on the outer membrane of gram-negative bacteria and induces many biologic effects upon the immune system in mammals (**32**). The LPS may contribute to the pathogenicity of swine dysentery (**32**). Results from studies performed on mice using LPS preparations suggest that the endotoxin elicits an inflammatory response by stimulating interleukin-1 and tumor necrosis factor (**5**).

Hemolysin is an oxygen stabile, heat labile, small polypeptide with a reported molecular mass ranging from 19 kDa to 23 kDa (**4**). The exact biologic characteristics of beta-hemolysin are still unknown but it has been shown to be cytotoxic for several types of eukaryotic cells (**6**, **7**). Methods have been developed to extract a more purified hemolysin from cell cultures that may be administered to pigs or placed into ligated colonic loops to determine its ability to induce tissue damage or fluid accumulation (**4**). Epithelial cell damage has been demonstrated in ligated ileal and colonic loops of germ free pigs when exposed to beta-hemolysin (**6**). Similar results were observed in both ligated rat ileal loops injected with hemolysin (**7**) and in pig colonic loops injected with cultures of *S. hyodysenteriae* (**8**). Together, these studies indicate that beta-hemolysin produced by *S. hyodysenteriae* is a contributing factor in the virulence of swine dysentery. Negative mutants of *S. hyodysenteriae* do not induce disease in swine (L. A. Joens, Department of Animal Science, University of Arizona, 1994).

Lesions, in pigs infected with *S. hyodysenteriae*, develop and are confined to the cecum and large bowel (**3**) while the small intestine is not affected (**27**). Most of the damage occurs in the upper one third of the mucosa (**12**). Inflammatory mediators released by mucosal mast cells were thought to play a role in lesion production, but studies performed using mast cell deficient mice also developed lesions (**33**). Production of excess mucous as well as crypt elongation, surface necrosis and pseudomembrane formation (**34**) are some of the morphological changes induced by *S. hyodysenteriae*. As the disease progresses, the epithelial cells lose their attachment to the underlying structures and are sloughed into the lumen. The epithelial necrosis which occurs in the colon accounts for the severe diarrhea in the affected animals (**12**).

One or more of the different factors which cause the onset of secretory diarrhea were thought to mediate the diarrhea associated with swine dysentery. But, studies with nonabsorbable markers to monitor the effect of this disease on colonic fluid and electrolyte transport in the intestine of infected pigs demonstrated that water and ion absorption was completely abolished (**12**). Increased mucosal permeability was suspected, because of the lesions in the intestine of infected pigs, but results from studies using ligated colonic loops in pigs infected with *S. hyodysenteriae* failed to demonstrate that possibility (**10**). Prostaglandins were suspected to play a role in the induction of diarrhea because of the inflammation occurring in the intestine, but cAMP and cGMP levels were measured in the intestinal mucosa of pigs with swine dysentery and found to be within normal levels (**12**). This suggests that a mechanism different than that associated with disease caused by *E. coli* or *Salmonella* spp. mediates the disease caused by *S. hyodysenteriae*.

Small intestinal functions, such as the glucose-stimulated fluid absorptive mechanism, in pigs infected with *S. hyodysenteriae* was examined and found to be unaffected (**29**). Therefore, it was concluded, that the systemic effects of swine dysentery are the result of fluid and electrolyte loss caused by colonic malabsorption. It was speculated that affected pigs failed to absorb their own endogenous secretions, due to the damage of the absorptive enterocytes and possibly increased tissue hydrostatic pressure, which ultimately result in dehydration and possibly death (**10**, **11**).

A decrease in serum sodium, chloride and bicarbonate levels and loss of extracellular sodium and water have been demonstrated in pigs with swine dysentery (**26**). The low concentration of sodium results in an increase of potassium in the extracellular fluid leading to hyperkalemia, whereas the decrease in bicarbonate leads to acidemia. Both factors, metabolic acidosis and hyperkalemia, are thought to be significant as a cause of death in swine with severe swine dysentery (**29**).

As mentioned previously, the biologic role of beta-hemolysin in the onset or progression of swine dysentery is unclear. A gene believed to encode for a beta-hemolysin from *S. hyodysenteriae* has been cloned, sequenced and expressed (**35**). Mutations of the *tly* gene, which encodes the hemolysin, caused a reduction in hemolytic activity in the mutated strains. All pathogenic strains of *S. hyodysenteriae* were found to contain the *tly* gene. Non-pathogenic strains of *S. innocens*, in contrast, did not contain this gene (**35**). The mutants had reduced hemolytic activity which suggests there may be more than one hemolysin. It seems that damage produced by the direct exposure of enterocytes to the hemolysin was a direct effect and did not depend on mechanisms such

as protein synthesis because of the relatively fast cytotoxic effects (**6**). Damage does not seem to occur due to lysis of the cell membrane either, where disruption of the internal organelles occur first, followed by the swelling and shedding of the epithelial cells (**6**).

Hypothesis

It is proposed that the beta-hemolysin produced by *S. hyodysenteriae* disrupts the integrity of the epithelial monolayer which would contribute to the onset of disease and to the severity of the diarrhea observed in affected animals. Therefore, in this study, an in vitro model was developed utilizing an intestinal cell line to simulate the effects that occur in vivo when the epithelium is exposed to the beta-hemolysin produced by *S. hyodysenteriae*.

The effect of the beta-hemolysin preparations on altering the transepithelial resistance across an epithelial monolayer, was measured and monitored over a 24 hour period. The changes in electrical resistance across the epithelial monolayers would reflect changes in the integrity of the tight junctions or depolarization of the cellular membrane. These changes would then alter paracellular transport processes suggesting that the hemolysin plays a role in disrupting epithelial integrity.

Exposure of cells to the beta-hemolysin may not only affect membrane integrity but may also affect intracellular events. Calcium has been proven to be an ion which mediates essential signal transduction pathways in the cell. Changes in intracellular calcium ion concentrations are therefore strictly regulated and sudden changes can dramatically affect cellular functions. Hemolysin has been shown to have cytotoxic effects

on various eukaryotic cells (**4**), but its effect on intracellular concentrations has not been well documented. An increase in intracellular calcium beyond the tolerable concentration for the cell would effect many of the metabolic pathways regulated by calcium and levels of byproducts from these pathways to potentially damaging or cytotoxic ranges. These changes could in turn affect membrane integrity due to intracellular changes in cytosolic calcium concentrations.

MATERIALS AND METHODS

Cell Lines

Caco-2 cells

The human adenocarcinoma cell line (HTB 73) was obtained from American Type Cell Culture (ATCC) and used between passage 18 and 50. Cells were grown in Eagles minimal essential medium (MEM) with non-essential amino acids and Earl's salts and supplemented with 20% fetal calf serum (FCS) with penicillin (100 μg/ml) and streptomycin (100 μg/ml). The cells were grown in tissue culture flasks at 37°C in a 5% CO_2 atmosphere until confluent. The media in the flasks was changed twice each week and passed to a new flask once a week. Media was removed from the flasks and discarded. The cells in the flask were then washed with 5 mls of phosphate buffered saline (PBS) which was removed and discarded. One half ml of trypsin solution (Sigma) containing ethylenediamine tetraacetic acid (EDTA) in 4.5 mls PBS was placed in the flask and incubated 5 to 10 minutes at 37°C and 5% CO_2. Ten drops of the cell suspension were placed in a new flask with fresh media. The flask was then incubated at 37°C in a 5% CO_2 atmosphere.

IPEC-1 cells

This continuous epithelial cell line was derived from the small intestine of a 12 hour old unsuckled mixed breed piglet. This cell line was obtained from H. M. Berschneider, College of Veterinary Medicine and Core Center in Diarrheal Diseases, North Carolina State University, Raleigh, NC, and used between passage 70 and 82. Cells

were grown in DMEM/F12 with insulin (5 *f*g/l), transferrin (5 *f*g/l), selenium (5 ng/1, premix, Collaborative Research Inc.), epidermal growth factor (5 *f*g/l, Collaborative Research Inc.) and 5% fetal calf serum. Penicillin (100μg/ml) and streptomycin (100 μg/ml) were added to the media. These cells, an adherent cell line, were grown in tissue culture flasks at 37°C with 5% CO_2. Cells were subcultured every 7 to 14 days and treated with trypsin as described above.

Human and pig peripheral blood lymphocytes

Whole human blood was drawn from two healthy volunteers, a 27 year old AB^+ male and a 24 year old A^+ female. Whole porcine blood was collected from two pigs by venipuncture. Nine mls of diluted whole blood (1:3 in PBS) was layered onto 3 mls of Histopaque 1077 (Sigma) in a 15 ml centrifuge tube. The tubes were then centrifuged for 45 minutes at 350 x g at room temperature. The buffy coat was then carefully removed from the interface and placed in a 50 ml centrifuge tube. The cells were washed with PBS by centrifugation for 10 minutes at 200 x g. The supernatant fluid was discarded and the cell pellet was resuspended in 45 mls of MEM and centrifuged again for 10 minutes at 200 x g. The pellet was resuspended in 10 mls of MEM and divided into two equal portions in separate tubes and spun at 200 x g for 10 minutes. The pellet in each tube was resuspended in 2 mls MEM or Jockliks modified MEM, respectively. Cells were then counted and stored at 37°C in a dry block tube incubator. Later the cells were used for flow cytometric analysis.

Hemolysin

The beta-hemolysin derived from *Serpulina hyodysenteriae* strain B204 (serotype 2) was kindly provided by D. L. Hutto, Veterinary Medical Research Institute, Iowa State University, Ames, IA. Log phase cultures of *S. hyodysenteriae* were suspended in a PBS extraction buffer containing 0.05% RNA core, 1μM glucose and 1 μM $MgSO_4$. Crude hemolysin was concentrated by ultra filtration, then purified by DEAE column chromatography and HPLC size exclusion chromatography. A fraction, with a molecular mass of 19-22 kDa, was shown to contain all the hemolytic activity. A hemolytic unit (HU) was defined as the amount of hemolysin required to cause 50% hemoglobin release in a 1% suspension of sheep red blood cells in 2 mls PBS (**6**).

Electrical resistance assay

The Caco-2 cell culture was performed as described by Finlay and Falkow (**36**). Briefly, transwell filter units (0.33 cm^2) were placed in 24 well microtiter plates (Costar). Tissue culture treated transwell filter units (Model No 3415; Costar, Cambridge, MA), were placed in 24 well plates (Costar), with 3.0 μm pores in a porous membrane were used. Caco-2 cells were trypsinized and a cell suspension, containing 1.5 x 10^5 cells per 150 μl in MEM supplemented with 20% fetal calf serum and antibiotics, was added to the upper compartment (apical side of the cells) in the transwell filter units. Six hundred microliters of the same media without any cells were added to the lower compartment (basal side) of the transwell units. The plates containing the inoculated transwell inserts were incubated at 37°C in 5% CO_2 and both the apical and basolateral media were

changed every other day for the next 10-15 days, or until cell confluency was reached (Cell confluency was measured using the Millicel-ERS after the tenth day of incubation, to determine if the transwells had sufficient cells to form a complete monolayer with an electrical resistance of 200-300 Ωcm^2). Prior to the addition of hemolysin, both apical and basolateral media were changed and replaced with MEM containing no fetal calf serum. Electrical resistance measurements were used to monitor the loss of epithelial integrity across the Caco-2 monolayers. The electrical resistance of Caco-2 monolayers was measured using a Millicell-ERS (Electrical Resistance System), (Millipore, Bedford, MA) (**36**). The resistance measurements were taken at half hour intervals. Between each measurement, culture plates were incubated for 15 minutes at 37°C in 5% CO_2. The plates were then placed at ambient room temperature for 15 minutes prior to measuring the electrical resistance because temperature changes affected the resistance and caused voltage drift. The hemolysin preparation was added apically at different concentrations and time points. The resistance measurements were taken at half hour intervals for 6 hours, then at 12 and 24 hours following the addition of hemolysin. A well was set up with no cells present (blank). Each reading was preceded by a blank measurement to ensure that no voltage drift occurred. The electrical resistance of untreated wells (controls) were also measured regularly. Transepithelial resistance measured for a well containing a monolayer was multiplied by the area of the filter (0.33 cm^2) after the blank was subtracted (~150 Ω). The area times resistance (Ωcm^2) was the result. All the samples were run in triplicate, averaged, then all the readings were normalized by dividing each reading (T_n) by the initial time measurement (T_0).

Flow Cytometric Analysis

Caco-2 and IPEC-1 cells were cultured as described above. A single cell suspension was obtained after treatment with trypsin. The cells were harvested by centrifugation at 200 x g for 10 minutes. Caco-2 cells were resuspended in MEM containing 20% FCS, and IPEC-1 cells were suspended in DMEM/F12 with 10% FCS in a 15 ml polypropelene tube filled to the brim and sealed. The tubes were then placed on a rotating tube rack in a 37°C incubator for 4 hours, to allow the cells to recover from the effect of trypsin. Human and pig lymphocytes were also used in the assay and were collected as previously described.

Indo-1 AM (I-1223)

Indo-1 (Molecular Probes, Eugene, OR) is a highly fluorescent calcium chromophore that exhibits large changes in fluorescence emission wavelengths when bound to calcium or manganese and excited by ultraviolet light (**15**). The absorption maximum of Indo-1 is between 330 nm and 350 nm.

Cells were incubated in 3-5 μM of Indo-1 for 30 minutes at 37°C. After loading the cells with Indo-1 they were diluted 1/10 in the media prior to flow cytometric analysis at a concentration of 1.5 x 10^5 cells/ml. The cytometer used was an EPIC 752 (Coulter Cytometry, Hialeah, FL) with 100 mW laser power at the Cell and Hybridoma Facility at Iowa State University. A UV laser blocker filter (351 nm-365 nm) was used. A 440 nm dichroic long pass filter (DCLP), a 515 nm long pass filter (LP) and a 395 band pass filter (BP) were also used. Each sample was analyzed for 600 seconds on the flow cytometer. After establishing a baseline reading for the initial 100 seconds, the hemolysin was added

at various concentrations (1, 5, 25 and 50 HU/ml) and the effect was monitored for 500 seconds. The ratio of [bound Indo-1/free Indo-1] was measured by the computer software connected to the cytometer. The results were recorded as a ratio of the reading at the various time points [$MPCt_{(n)}$] divided by the baseline reading [$MPCt_{(0)}$].

4-Bromo A-23187

This calcium ionophore (Molecular Probes, Eugene, OR) was used to bring about an increase in intracellular calcium (positive control). This ionophore has 200 times more affinity for manganese than calcium, and therefore, is also used as a positive effector for manganese flux studies (**37**). After 100 seconds of a baseline reading on the flow cytometer, the ionophore was added and the effect was monitored for 500 seconds.

Divalent ion flux

Each cell type was suspended in culture media and centrifuged at 200 x g for 10 minutes. The cells were counted and placed in a 37°C dry block tube incubator where they were ready to be loaded with Indo AM. Manganese flux was monitored by resuspending loaded cells taken from a Jockliks (modified MEM/calcium-free media) cell suspension and diluting them into a one molar solution of manganese Jockliks solution. Cell autoflourescence and manganese quenching of Indo-1 were monitored under a fluorescence microscope. Cells were also analyzed in Jockliks (modified MEM/calcium-free media) which contains no FCS supplemented with EDTA to monitor calcium flux in calcium-free medium. Cell suspensions loaded with Indo-1 were treated with hemolysin (1, 5, 25 and 50 HU/ml) after a 100 second base-line reading was established. Each sample was analyzed for 600 seconds on the flow cytometer. The results were recorded as

a ratio of the reading at the various time points [$MPCt_{(n)}$] divided by the baseline reading [$MPCt_{(0)}$].

Membrane integrity assay

Cell membrane integrity was monitored using fluorescein diacetate (FDA). For cell loading, 4 uls of a 1-10 mM stock solution of FDA in DMSO was used. Cells were incubated and allowed to load for 20 minutes at 37°C. Loaded cells were then removed and diluted to a final concentration of 1.5×10^5 cells/ml. Cell suspensions loaded with FDA were treated with hemolysin (1, 5, 25 and 50 HU/ml) after a 100 second base-line reading was established. Each sample was analyzed for 600 seconds on the flow cytometer. The results were recorded as a ratio of the reading at the various time points [$MPCt_{(n)}$] divided by the baseline reading [$MPCt_{(0)}$]. Fluorescein diacetate release from cells due to treatment with hemolysin was monitored by flow cytometric analysis.

Each treatment was represented by two tubes containing 1.5×10^5 cells/ml loaded with either Indo-1 or FDA depending on the assay. The measurements at each time point were averaged for each one of the treatments. The average measurements for each time point (T_n) were then divided by the initial reading taken at the beginning of the experiments before treatments were added (T_0). Normalized readings were obtained this way for each treatment.

RESULTS

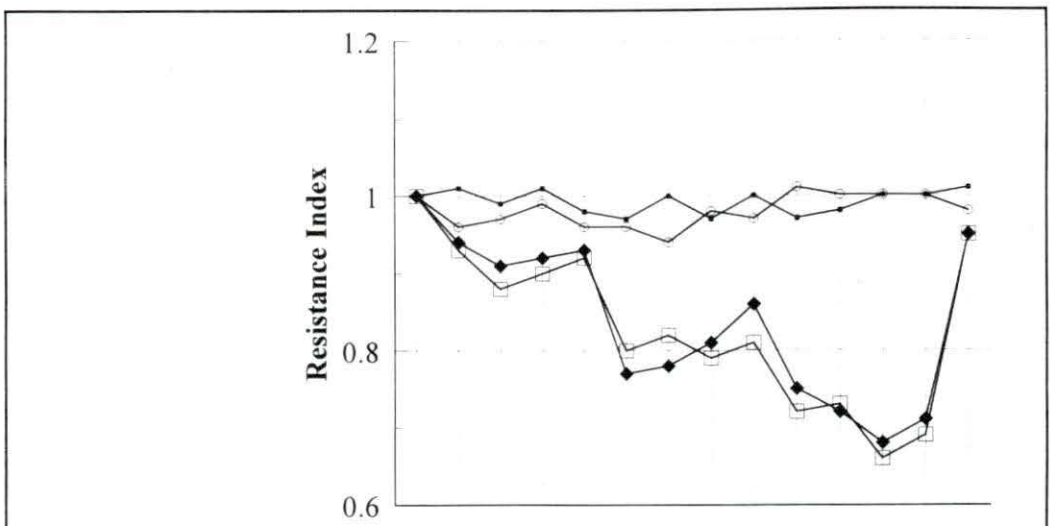
Changes in Electrical Resistance of Intact Monolayers Exposed to Beta-Hemolysin

A model utilizing Caco-2 cells which form epithelial monolayers was established to examine the changes in electrical resistance as a result of exposure to a beta-hemolysin isolated from *S. hyodysenteriae*. The ability of the hemolysin to alter the integrity of an epithelial monolayer or confluency in a dose-dependent manner, was reflected by changes in transepithelial resistance. Confluent monolayers of Caco-2 cells grown on treated support filters were divided into groups of three and subjected to various concentrations, 25, 50 and 100 hemolytic units (HU), of hemolysin added to their apical side at 30 or 60 minute intervals.

The data depicted in Figures 1 and 2 illustrate the effects of a beta-hemolysin from *S. hyodysenteriae* on the transepithelial resistance of a Caco-2 cell monolayer. During preliminary experiments it was noted that a single addition of the hemolysin (50, 100 or 200 HU/ml) had no detectable affect on the epithelial resistance of the monolayer (data not shown). Since it is more likely that the organism would continuously produce hemolysin during the course of the infection, hemolysin was added to each culture well at 30 or 60 minute intervals for a period of 6 hours after the initiation of the experiment.

The half hourly additions (50 HU/ml) and the hourly additions (100 HU/ml) of the beta-hemolysin caused a significant effect in reducing monolayer resistance (Figure 1). However, all of the monolayers that were treated with hemolysin were able to reestablish

their transepithelial electrical resistance, if it was affected, and continued to grow after cessation of hemolysin addition. The continuous growth of the monolayers was reflected as an increase in electrical resistance and was very pronounced at the 24 hour time point (Figures 1 and 2).

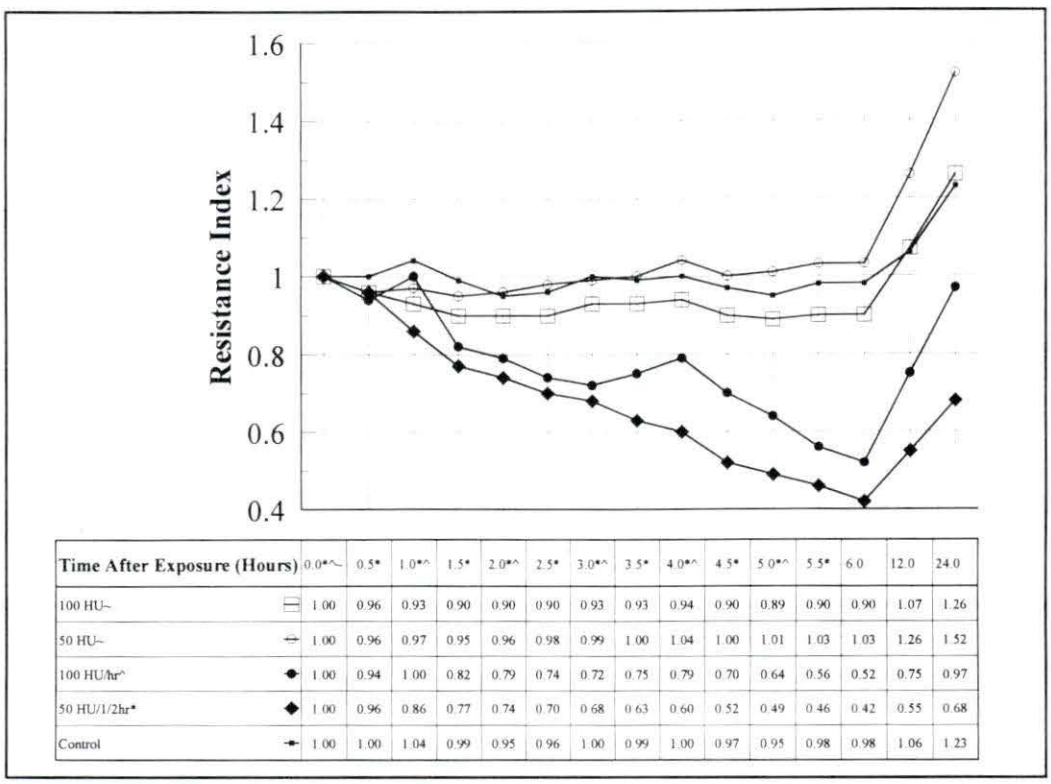


Time After Exposure (Hours)	0.0^	0.5	1.0^	1.5	2.0^	2.5	3.0^	3.5	4.0^	4.5	5.0^	5.5	6.0	24.0
100 HU / hr ^	1.00	0.93	0.88	0.90	0.92	0.80	0.82	0.79	0.81	0.72	0.73	0.66	0.69	0.95
50 HU / hr ^	1.00	0.94	0.91	0.92	0.93	0.77	0.78	0.81	0.86	0.75	0.72	0.68	0.71	0.95
25 HU / hr ^	1.00	0.96	0.97	0.99	0.96	0.96	0.94	0.98	0.97	1.01	1.00	1.00	1.00	0.98
Control	1.00	1.01	0.99	1.01	0.98	0.97	1.00	0.97	1.00	0.97	0.98	1.00	1.00	1.01

Figure 1. The effect of a beta-hemolysin from *S. hyodysenteriae* on the transepithelial resistance of Caco-2 monolayers. Hemolysin was added at either 50 or 100 HU/ml to the apical side of Caco-2 cells supported on a transwell membrane. The hemolysin was added either once at the beginning of the experiment (~), every 0.5 hour for the first 5.5 hours (*), or every hour for the first 5 hours (^). Resistance measurements were averaged and normalized (T_n/T_0) for the three samples of each treatment group and reported as a resistance index.

Hemolysin concentrations that exceeded 25 Hu/ml (Figure 1) and were added at 30 minute intervals (Figure 2) had more of an effect on reducing the transepithelial resistance of the monolayers. Electrical resistance measurements taken at 12 and 24 hours revealed an increase and reestablishment of resistance.

After the addition of at least 150 HU, in the 50 and 100 HU/ml groups, there was a decrease in the electrical resistance. In contrast, there was no decrease detectable at the 5.0 hour time point in the cells receiving 25 HU/ml every 30 minutes (Figure 2). No decrease in electrical resistance was observed when hemolysin concentrations of 50 HU/ml were added every hour up to 5 hours to the lower compartment (i.e., basolateral side of the Caco-2 cells) (data not shown).



Time After Exposure (Hours)	0.0*^~	0.5*	1.0*^	1.5*	2.0*^	2.5*	3.0*^	3.5*	4.0*^	4.5*	5.0*^	5.5*	6.0	12.0	24.0
100 HU~	1.00	0.96	0.93	0.90	0.90	0.90	0.93	0.93	0.94	0.90	0.89	0.90	0.90	1.07	1.26
50 HU~	1.00	0.96	0.97	0.95	0.96	0.98	0.99	1.00	1.04	1.00	1.01	1.03	1.03	1.26	1.52
100 HU/hr^	1.00	0.94	1.00	0.82	0.79	0.74	0.72	0.75	0.79	0.70	0.64	0.56	0.52	0.75	0.97
50 HU/1/2hr*	1.00	0.96	0.86	0.77	0.74	0.70	0.68	0.63	0.60	0.52	0.49	0.46	0.42	0.55	0.68
Control	1.00	1.00	1.04	0.99	0.95	0.96	1.00	0.99	1.00	0.97	0.95	0.98	0.98	1.06	1.23

Figure 2. The effect of a beta-hemolysin from *S. hyodysenteriae* on the transepithelial resistance of Caco-2 monolayers. Hourly additions (^) of 25, 50 or 100 HU/ml were made to the apical side of Caco-2 cells supported on a transwell membrane. Resistance measurements were averaged and normalized (T_n/T_0) for the three samples of each treatment group and reported as a resistance index.

Induction of Calcium and Manganese Flux in Cells Treated with Beta-Hemolysin

The changes induced by beta-hemolysin on single cells was measured by flow cytometry. The effect of beta-hemolysin on calcium and manganese flux in Caco-2 cells, IPEC-1 cells, human or pig lymphocytes was monitored in MEM (to monitor calcium flux), Jockliks modified MEM with EDTA (to monitor direction of calcium flux) or Jockliks containing manganese (to determine the effects on other divalent ions). The beta-hemolysin (1, 5, 25 or 50 HU/ml) was added 100 seconds after the initiation of the assay and calcium flux in both calcium free and calcium containing media was monitored. The calcium ionophore A-23187 was used as a positive control.

The data in Figures 3, 4, 5 and 6 illustrate the effects of a beta-hemolysin from *S. hyodysenteriae* on calcium flux in Caco-2 cells, IPEC-1 cells, pig lymphocytes and human lymphocytes, respectively. Changes in calcium flux in Caco-2 cells were detectable following the additions of 25 and 50 HU/ml of beta-hemolysin with the latter amount having a more pronounced effect (Figure 3). The addition of 1or 5 HU/ml produced no detectable changes in the calcium flux. Figure 4 illustrates that all hemolysin concentrations used (1, 5, 25 and 50 HU/ml) produced a change in calcium flux in the IPEC-1 cells. A dose response was obvious where the higher doses had more of an effect than the lower doses used.

The concentrations of hemolysin used in the experiment conducted using pig lymphocytes illustrated by Figure 5, all produced changes in intracellular calcium levels. The two lower doses (1 and 5 HU/ml) and the two higher doses (25 and 50 HU/ml), can

be divided into two groups according to the magnitude of their responses.

Figure 6 illustrates the pattern of the response in the human lymphocytes, which resemble the responses which occurred with the pig lymphocytes, these responses, however, were more dramatic than those observed for porcine lymphocytes.

An increase in intracellular calcium levels in the cells was observed after hemolysin was added to the various cell types. The same test was performed on all cell types in Jockliks (modified MEM/calcium-free media with EDTA) to determine whether the calcium was released from intracellular stores or entered from the outside. Figures 7, 8, 9 and 10 illustrate these effects.

Figure 7 indicates that no apparent changes in calcium flux occur in Caco-2 cells treated with beta-hemolysin from *S. hyodysenteriae* suspended in media containing no calcium. Figure 8 indicates that no intracellular changes in calcium levels can be detected when IPEC-1 cells were suspended in calcium free medium and subjected to the four hemolysin doses (1, 5, 25 and 50 HU/ml).

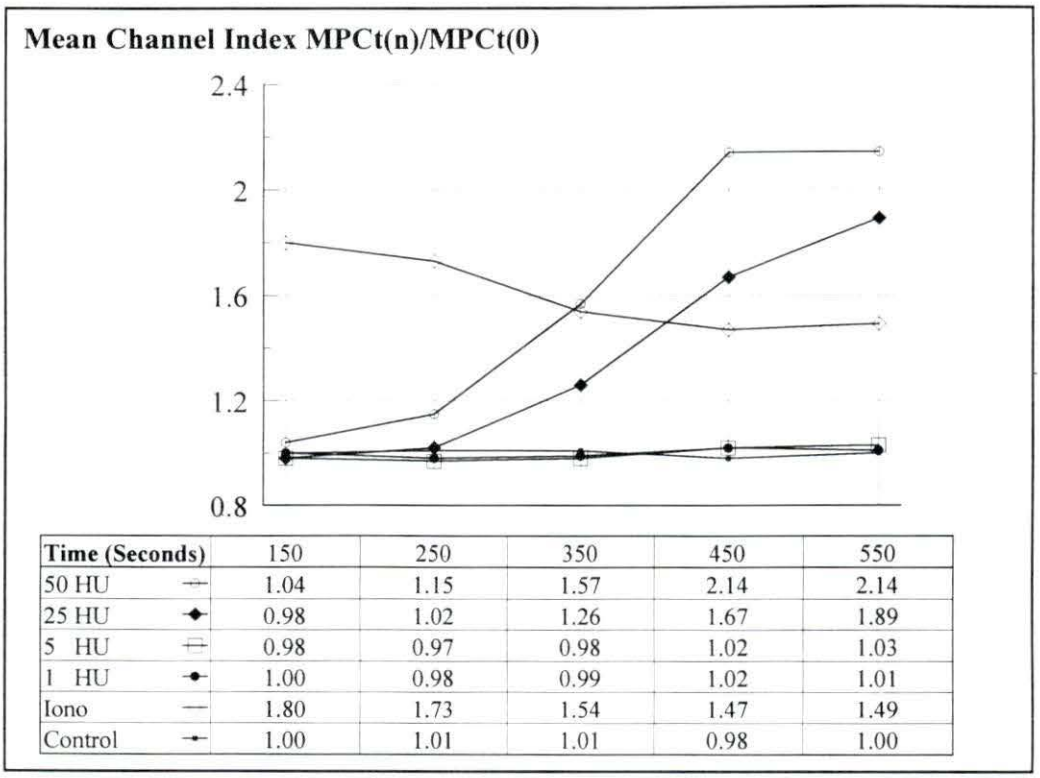
Treatment with the beta-hemolysin in calcium free media (Figures 9 and 10) resulted in a reduction of cellular volume. Due to the reduction in cell size, the [dye/cell size] ratio was altered to produce an effect resembling the occurrence of calcium flux (see data in figure). Otherwise there was no actual change in the concentration of intracellular calcium in porcine or human lymphocytes treated with the beta-hemolysin.

To determine if the beta-hemolysin had a calcium specific effect on ion flux, the assay was conducted using Caco-2 cells, IPEC-1 cells, pig and human lymphocytes in Jockliks (modified MEM/calcium-free media) containing manganese (Figures 11, 12, 13

and 14), respectively. The ionophore, A-23187, was used as a positive effector to produce an influx of manganese. The assay was performed on all previously mentioned cell types, but only the highest dose (50 HU/ml) of hemolysin was used.

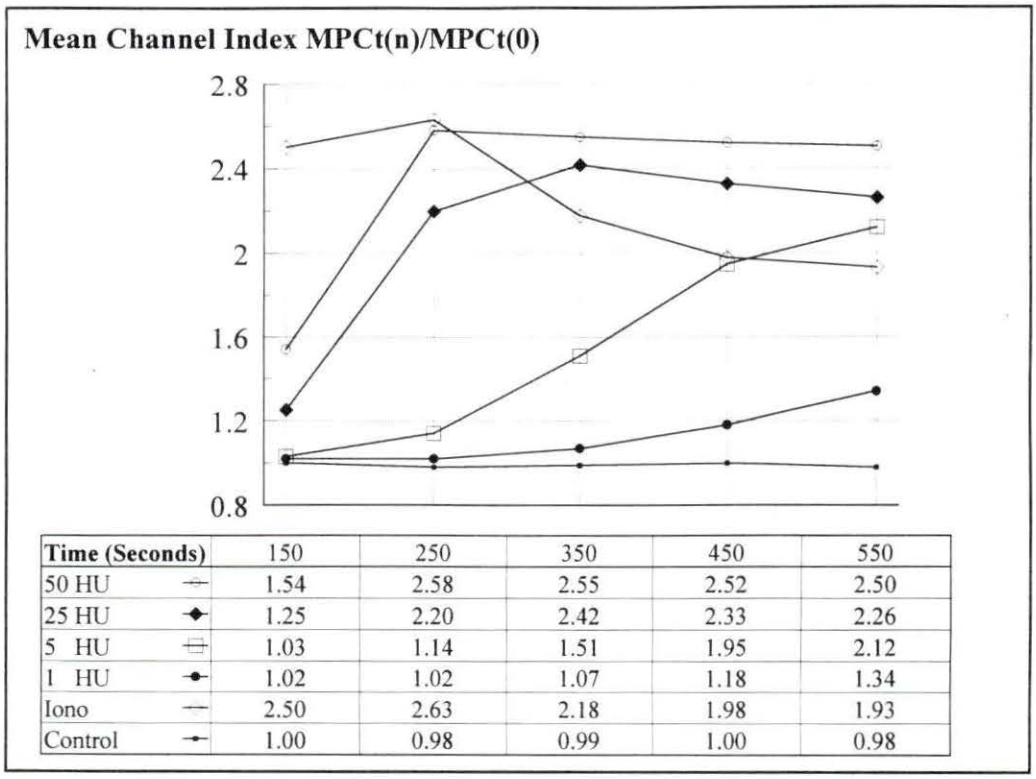
No change occurred in manganese levels following the addition of the beta-hemolysin. The response or apparent shift in intracellular manganese concentrations that occurred after 350 seconds or at 250 seconds in the case of the pig lymphocytes (Figure 13) was the result of cellular shrinkage. This shrinkage occurred in all cells suspended in calcium free medium containing manganese following the addition of beta-hemolysin, as apposed to the cells suspended in Jockliks (modified MEM/calcium-free media) where the cell shrinkage occurred in only the pig and human lymphocytes (Figures 9 and 10).

In order to determine whether cytoplasmic contents would leak from cells treated with the beta-hemolysin, cells were loaded with FDA and then treated with 50 HU/ml of the beta-hemolysin. Cells treated with the beta-hemolysin failed to leak or release FDA indicating that the cells remained intact (data not shown).



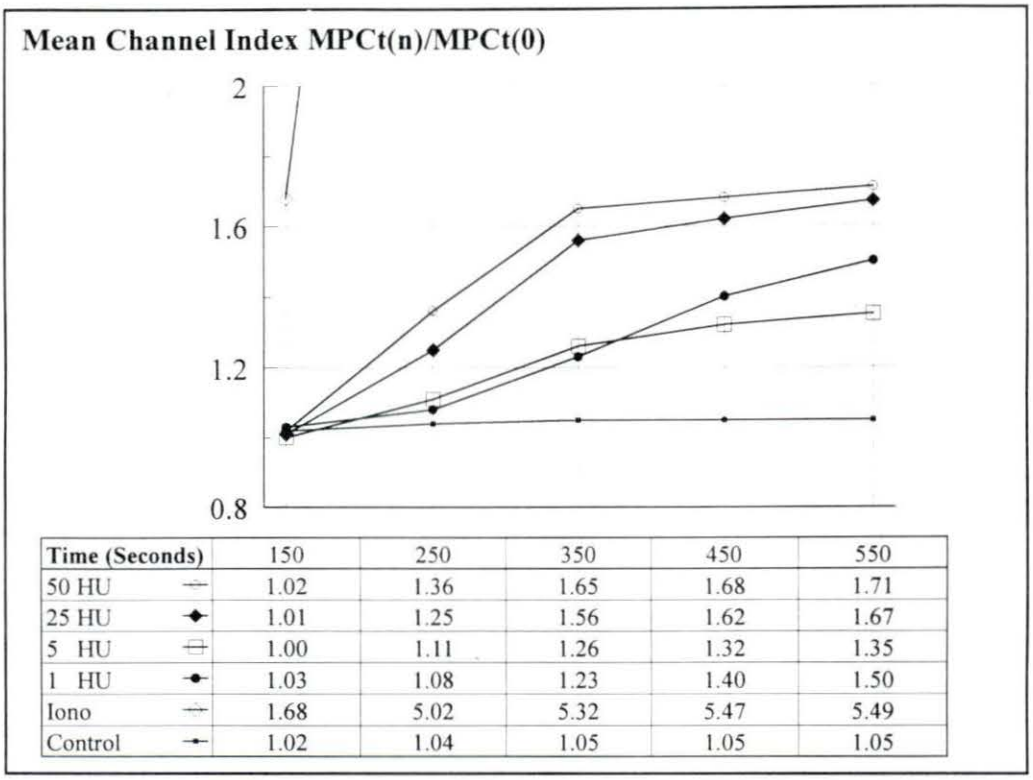
Time (Seconds)	150	250	350	450	550
50 HU	1.04	1.15	1.57	2.14	2.14
25 HU	0.98	1.02	1.26	1.67	1.89
5 HU	0.98	0.97	0.98	1.02	1.03
1 HU	1.00	0.98	0.99	1.02	1.01
Iono	1.80	1.73	1.54	1.47	1.49
Control	1.00	1.01	1.01	0.98	1.00

Figure 3. The effect of a beta-hemolysin from *S. hyodysenteriae* on calcium flux in Caco-2 cells. Aliquotes of Caco-2 cells (1 x 10^5 cells/ml) loaded with Indo-1 were analyzed on the flow cytometer. The beta-hemolysin (1, 5, 25 or 50 HU/ml) was added 100 seconds after the initiation of the assay. Each treatment was performed in duplicate and the mean channel index was determined. The mean channel index was calculated by dividing the ratio of the mean peak channel at time n [$MPC_{t(n)}$] by the ratio of the mean peak channel at time 0 [$MPC_{t(0)}$]. The calcium ionophore (Iono), A-23187, was used as a positive control.



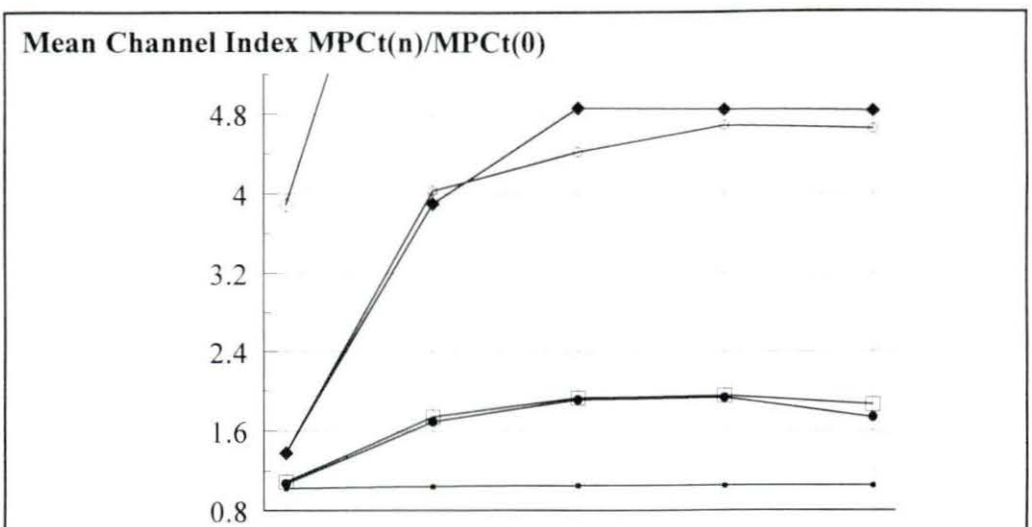
Time (Seconds)	150	250	350	450	550
50 HU	1.54	2.58	2.55	2.52	2.50
25 HU	1.25	2.20	2.42	2.33	2.26
5 HU	1.03	1.14	1.51	1.95	2.12
1 HU	1.02	1.02	1.07	1.18	1.34
Iono	2.50	2.63	2.18	1.98	1.93
Control	1.00	0.98	0.99	1.00	0.98

Figure 4. The effect of a beta-hemolysin from *S. hyodysenteriae* on calcium flux in IPEC-1 cells. Aliquotes of IPEC-1 cells (1 x 10^5 cells/ml) loaded with Indo-1 were analyzed on the flow cytometer. The beta-hemolysin (1, 5, 25 or 50 HU/ml) was added 100 seconds after the initiation of the assay. Each treatment was performed in duplicate and the mean channel index was determined. The mean channel index was calculated by dividing the ratio of the mean peak channel at time n [$MPC_{t(n)}$] by the ratio of the mean peak channel at time 0 [$MPC_{t(0)}$]. The calcium ionophore (Iono), A-23187, was used as a positive control.



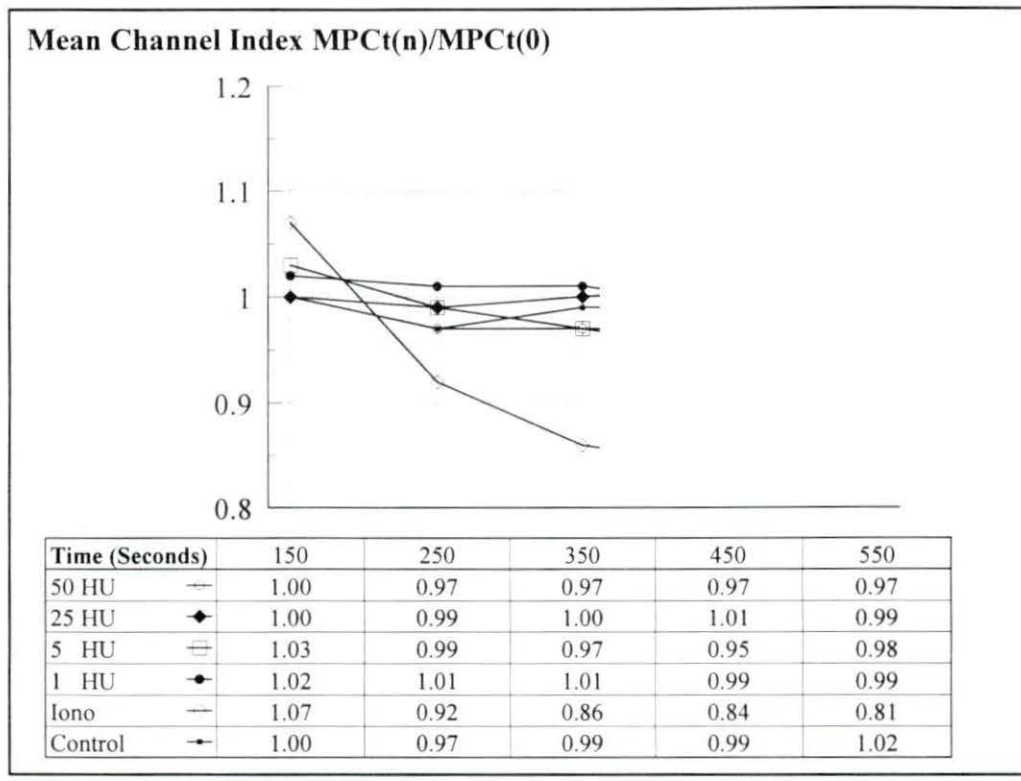
Time (Seconds)	150	250	350	450	550
50 HU	1.02	1.36	1.65	1.68	1.71
25 HU	1.01	1.25	1.56	1.62	1.67
5 HU	1.00	1.11	1.26	1.32	1.35
1 HU	1.03	1.08	1.23	1.40	1.50
Iono	1.68	5.02	5.32	5.47	5.49
Control	1.02	1.04	1.05	1.05	1.05

Figure 5. The effect of a beta-hemolysin from *S. hyodysenteriae* on calcium flux in pig lymphocytes. Aliquotes of pig lymphocytes (1 x 10^5 cells/ml) loaded with Indo-1 were analyzed on the flow cytometer. The beta-hemolysin (1, 5, 25 or 50 HU/ml) was added 100 seconds after the initiation of the assay. Each treatment was performed in duplicate and the mean channel index was determined. The mean channel index was calculated by dividing the ratio of the mean peak channel at time n [$MPC_{t(n)}$] by the ratio of the mean peak channel at time 0 [$MPC_{t(0)}$]. The calcium ionophore (Iono), A-23187 was used as a positive control.



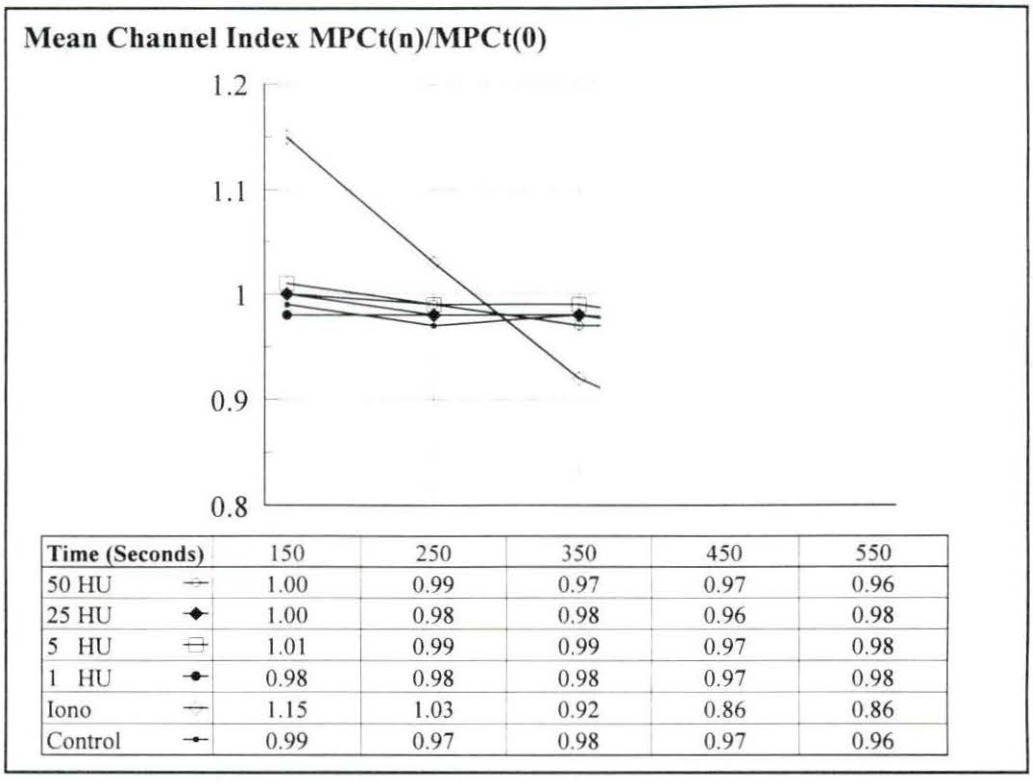
Time (Seconds)	150	250	350	450	550
50 HU	1.38	4.03	4.42	4.68	4.64
25 HU	1.38	3.90	4.86	4.84	4.82
5 HU	1.09	1.74	1.93	1.95	1.86
1 HU	1.07	1.69	1.91	1.93	1.73
Iono	3.89	8.48	8.69	8.83	9.03
Control	1.02	1.04	1.05	1.05	1.05

Figure 6. The effect of a beta-hemolysin from *S. hyodysenteriae* on calcium flux in human lymphocytes. Aliquotes of human lymphocytes (1×10^5 cells/ml) loaded with Indo-1 were analyzed on the flow cytometer. The beta-hemolysin (1, 5, 25 or 50 HU/ml) was added 100 seconds after the initiation of the assay. Each treatment was performed in duplicate and the mean channel index was determined. The mean channel index was calculated by dividing the ratio of the mean peak channel at time n [$MPC_{t(n)}$] by the ratio of the mean peak channel at time 0 [$MPC_{t(0)}$]. The calcium ionophore (Iono), A-23187, was used as a positive control.



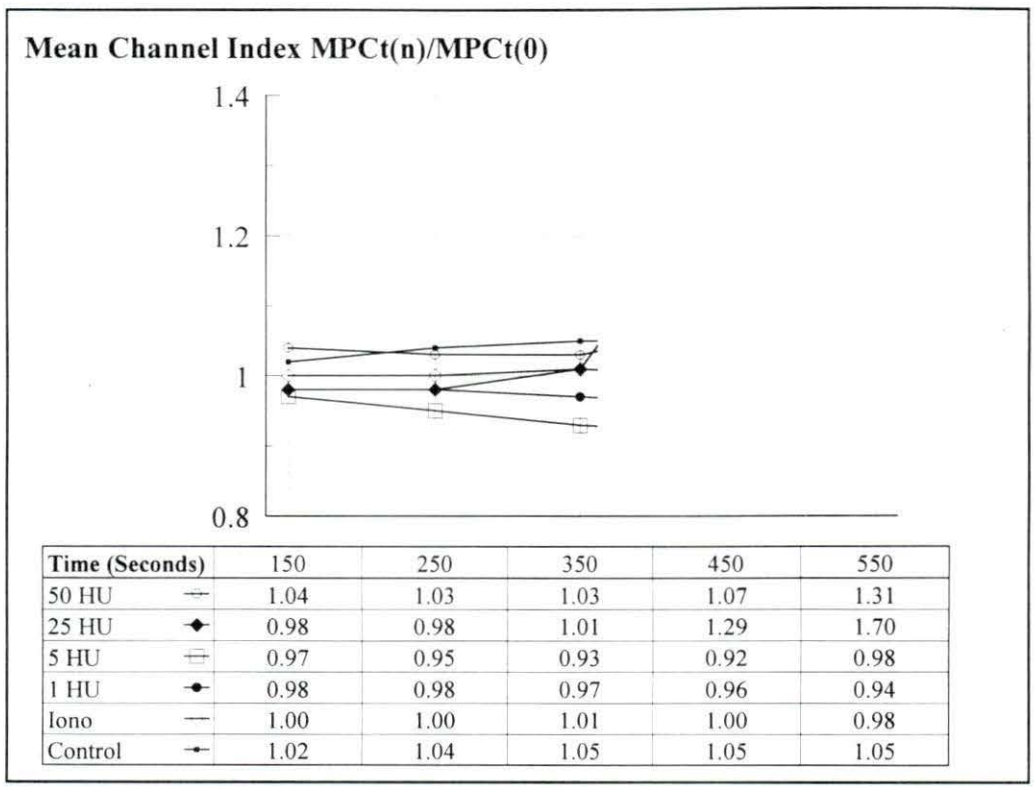
Time (Seconds)	150	250	350	450	550
50 HU	1.00	0.97	0.97	0.97	0.97
25 HU	1.00	0.99	1.00	1.01	0.99
5 HU	1.03	0.99	0.97	0.95	0.98
1 HU	1.02	1.01	1.01	0.99	0.99
Iono	1.07	0.92	0.86	0.84	0.81
Control	1.00	0.97	0.99	0.99	1.02

Figure 7. The effect of a beta-hemolysin from *S. hyodysenteriae* on calcium flux in Caco-2 cells in calcium free medium (Jockliks modified MEM with EDTA). Aliquotes of Caco-2 cells (1 x 10^5 cells/ml) loaded with Indo-1 were analyzed on the flow cytometer. The beta-hemolysin (1, 5, 25 or 50 HU/ml) was added 100 seconds after the initiation of the assay. Each treatment was performed in duplicate and the mean channel index was determined. The mean channel index was calculated by dividing the ratio of the mean peak channel at time n [$MPC_{t(n)}$] by the ratio of the mean peak channel at time 0 [$MPC_{t(0)}$]. The calcium ionophore (Iono), A-23187, was used as a positive control.



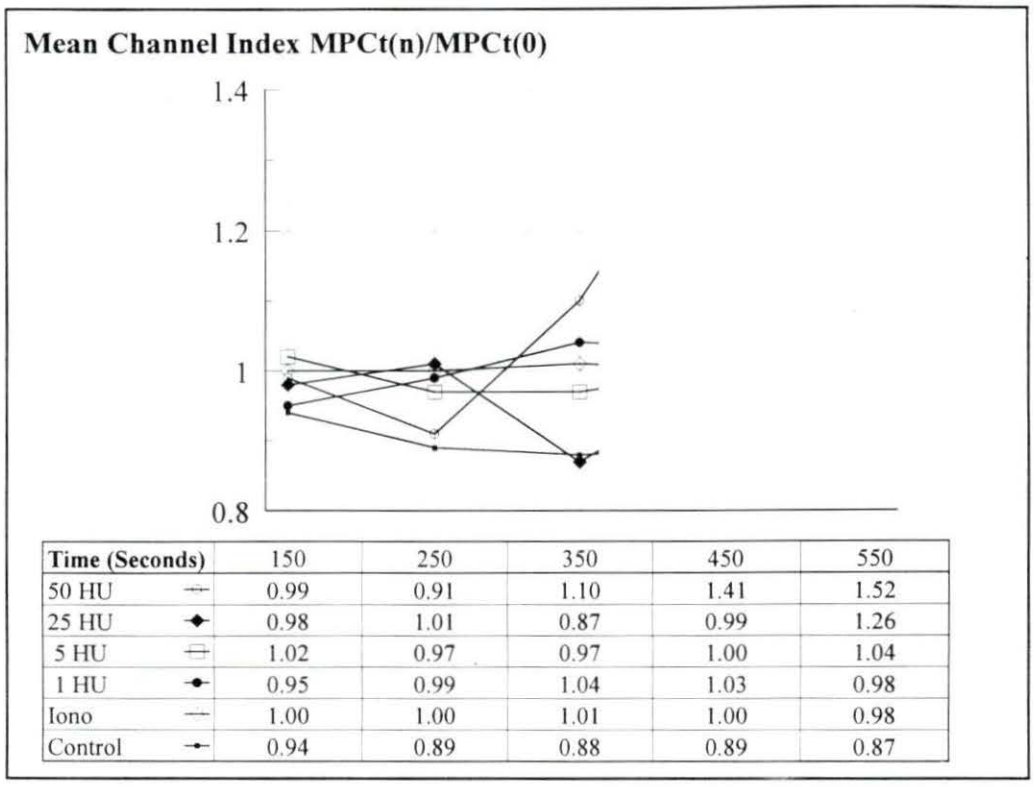
Time (Seconds)	150	250	350	450	550
50 HU	1.00	0.99	0.97	0.97	0.96
25 HU	1.00	0.98	0.98	0.96	0.98
5 HU	1.01	0.99	0.99	0.97	0.98
1 HU	0.98	0.98	0.98	0.97	0.98
Iono	1.15	1.03	0.92	0.86	0.86
Control	0.99	0.97	0.98	0.97	0.96

Figure 8. The effect of a beta-hemolysin from *S. hyodysenteriae* on calcium flux in IPEC-1 cells in calcium free medium (Jockliks modified MEM with EDTA). Aliquotes of IPEC-1 cells (1 x 10^5 cells/ml) loaded with Indo-1 were analyzed on the flow cytometer. The beta-hemolysin (1, 5, 25 or 50 HU/ml) was added 100 seconds after the initiation of the assay. Each treatment was performed in duplicate and the mean channel index was determined. The mean channel index was calculated by dividing the ratio of the mean peak channel at time n [$MPC_{t(n)}$] by the ratio of the mean peak channel at time 0 [$MPC_{t(0)}$]. The calcium ionophore (Iono), A-23187, was used as a positive control.



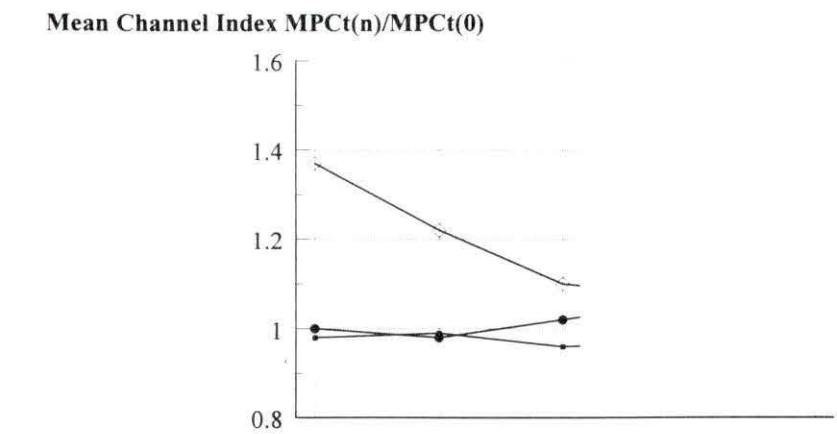
Time (Seconds)	150	250	350	450	550
50 HU	1.04	1.03	1.03	1.07	1.31
25 HU	0.98	0.98	1.01	1.29	1.70
5 HU	0.97	0.95	0.93	0.92	0.98
1 HU	0.98	0.98	0.97	0.96	0.94
Iono	1.00	1.00	1.01	1.00	0.98
Control	1.02	1.04	1.05	1.05	1.05

Figure 9. The effect of a beta-hemolysin from *S. hyodysenteriae* on calcium flux in pig lymphocytes in calcium free medium (Jockliks modified MEM with EDTA). Aliquotes of pig lymphocytes (1 x 10^5 cells/ml) loaded with Indo-1 were analyzed on the flow cytometer. The beta-hemolysin (1, 5, 25 or 50 HU/ml) was added 100 seconds after the initiation of the assay. Each treatment was performed in duplicate and the mean channel index was determined. The mean channel index was calculated by dividing the ratio of the mean peak channel at time n [$MPC_{t(n)}$] by the ratio of the mean peak channel at time 0 [$MPC_{t(0)}$]. The calcium ionophore (Iono), A-23187, was used as a positive control.



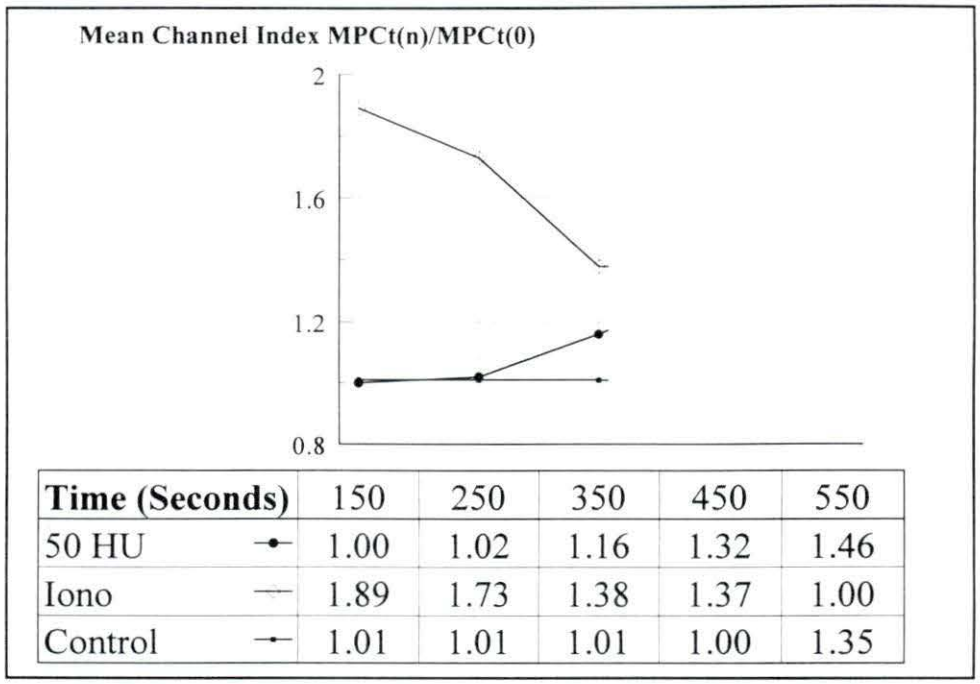
Time (Seconds)	150	250	350	450	550
50 HU	0.99	0.91	1.10	1.41	1.52
25 HU	0.98	1.01	0.87	0.99	1.26
5 HU	1.02	0.97	0.97	1.00	1.04
1 HU	0.95	0.99	1.04	1.03	0.98
Iono	1.00	1.00	1.01	1.00	0.98
Control	0.94	0.89	0.88	0.89	0.87

Figure 10. The effect of a beta-hemolysin from *S. hyodysenteriae* on calcium flux in human lymphocytes in calcium free medium (Jockliks modified MEM with EDTA). Aliquotes of human lymphocytes (1 x 10^5 cells/ml) loaded with Indo-1 were analyzed on the flow cytometer. The beta-hemolysin (1, 5, 25 or 50 HU/ml) was added 100 seconds after the initiation of the assay. Each treatment was performed in duplicate and the mean channel index was determined. The mean channel index was calculated by dividing the ratio of the mean peak channel at time n [$MPC_{t(n)}$] by the ratio of the mean peak channel at time 0 [$MPC_{t(0)}$]. The calcium ionophore (Iono), A-23187, was used as a positive control.



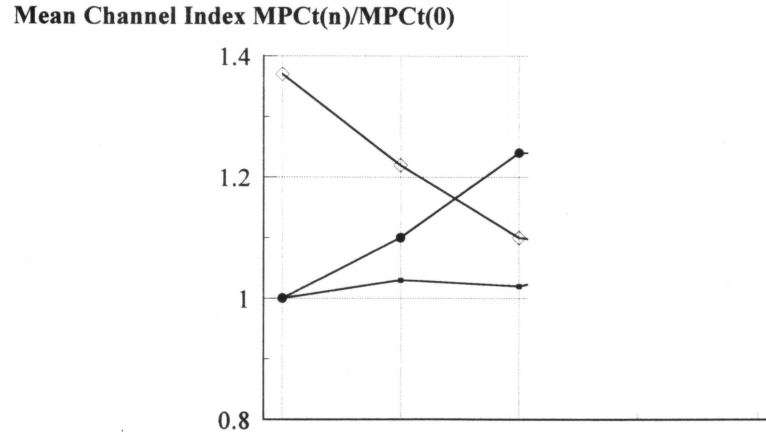
Time (Seconds)	150	250	350	450	550
50 HU	1.00	0.98	1.02	1.06	1.79
Iono	1.37	1.22	1.10	1.07	0.92
Control	0.98	0.99	0.96	0.97	1.60

Figure 11. The effect of a beta-hemolysin from *S. hyodysenteriae* on manganese flux in Caco-2 cells in calcium free medium (Jockliks modified MEM) containing manganese. Aliquotes of Caco-2 cells (1 x 10^5 cells/ml) loaded with Indo-1 were analyzed on the flow cytometer. The beta-hemolysin (50 HU/ml) was added 100 seconds after the initiation of the assay. Each treatment was performed in duplicate and the mean channel index was determined. The mean channel index was calculated by dividing the ratio of the mean peak channel at time n [$MPC_{t(n)}$] by the ratio of the mean peak channel at time 0 [$MPC_{t(0)}$]. The calcium ionophore (Iono), A-23187, was used as a positive control.



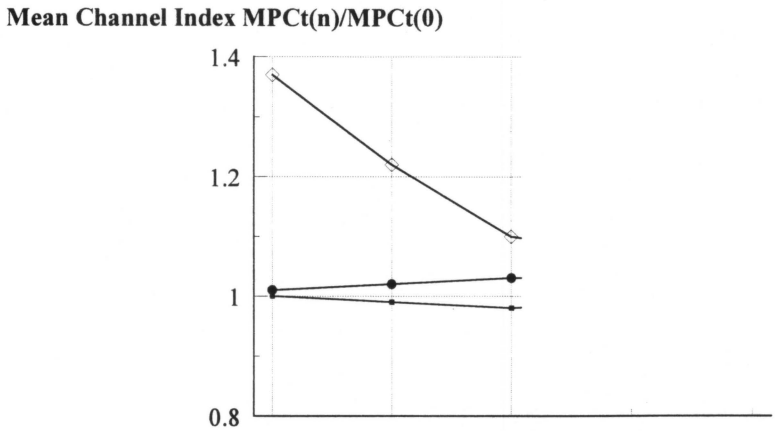
Time (Seconds)	150	250	350	450	550
50 HU	1.00	1.02	1.16	1.32	1.46
Iono	1.89	1.73	1.38	1.37	1.00
Control	1.01	1.01	1.01	1.00	1.35

Figure 12. The effect of a beta-hemolysin from *S. hyodysenteriae* on manganese flux in IPEC-1 cells in calcium free medium (Jockliks modified MEM) containing manganese. Aliquotes of IPEC-1 cells (1 x 10^5 cells/ml) loaded with Indo-1 were analyzed on the flow cytometer. The beta-hemolysin (50 HU/ml) was added 100 seconds after the initiation of the assay. Each treatment was performed in duplicate and the mean channel index was determined. The mean channel index was calculated by dividing the ratio of the mean peak channel at time n [$MPC_{t(n)}$] by the ratio of the mean peak channel at time 0 [$MPC_{t(0)}$]. The calcium ionophore (Iono), A-23187 was used as a positive control.



Time (Seconds)	150	250	350	450	550
50 HU	1.00	1.10	1.24	1.24	1.25
Iono	1.37	1.22	1.10	1.07	1.60
Control	1.00	1.03	1.02	1.06	1.06

Figure 13. The effect of a beta-hemolysin from *S. hyodysenteriae* on manganese flux in pig lymphocytes in calcium free medium (Jockliks modified MEM) containing manganese. Aliquotes of pig lymphocytes (1 x 10^5 cells/ml) loaded with Indo-1 were analyzed on the flow cytometer. The beta-hemolysin (50 HU/ml) was added 100 seconds after the initiation of the assay. Each treatment was performed in duplicate and the mean channel index was determined. The mean channel index was calculated by dividing the ratio of the mean peak channel at time n [$MPC_{t(n)}$] by the ratio of the mean peak channel at time 0 [$MPC_{t(0)}$]. The calcium ionophore (Iono), A-23187 was used as a positive control.



Time (Seconds)	150	250	350	450	550
50 HU	1.01	1.02	1.03	1.03	1.04
Iono	1.37	1.22	1.10	1.07	1.60
Control	1.00	0.99	0.98	0.99	0.98

Figure 14. The effect of a beta-hemolysin from *S. hyodysenteriae* on manganese flux in human lymphocytes in calcium free medium (Jockliks modified MEM) containing manganese. Aliquotes of human lymphocytes (1 x 10^5 cells/ml) loaded with Indo-1 were analyzed on the flow cytometer. The beta-hemolysin (50 HU/ml) was added 100 seconds after the initiation of the assay. Each treatment was performed in duplicate and the mean channel index was determined. The mean channel index was calculated by dividing the ratio of the mean peak channel at time n [$MPC_{t(n)}$] by the ratio of the mean peak channel at time 0 [$MPC_{t(0)}$]. The calcium ionophore (Iono), A-23187 was used as a positive control.

DISCUSSION

Serpulina hyodysenteriae, the causative agent of swine dysentery, produces a beta-hemolysin that is thought to play a role in lesion production (**29**). This toxin may also be a contributing factor in the morphological changes and systemic effects which are associated with this colonic disease. The systemic effects are mainly due to the fluid and electrolyte imbalance, which result because of the failure of the transepithelial transport system, which moves the sodium and chloride ions from the lumen to the blood. This deficiency results in malabsorption which often leads to dehydration, the ultimate cause of death in these animals.

Variations in membrane integrity may be measured which reflect changes in transepithelial electrical resistance. A reduction in measured transepithelial electrical resistance across the Caco-2 monolayers confirms the positive role beta-hemolysin plays in altering monolayer integrity. A dose dependant reduction in the resistance was evident throughout the first 6 hours of the experiments where the higher doses had a greater effect and continued to decrease as long as the toxin was added.

Hemolysin addition once at the beginning of the assay (as much as 200 HU/ml) had no measurable effect on the reduction of transepithelial resistance of the Caco-2 monolayers. Hemolysin additions were, therefore, applied to the apical side of the transwells every 30 minutes for 5 hours in an attempt to reproduce or simulate what occurs in the pig intestine during an ongoing infection. This resulted in a decrease of the electrical resistance across the monolayers that was not observed following a single

addition of hemolysin.

The beta-hemolysin did not have a permanent effect on the viability of Caco-2 cells with the concentrations used. The monolayers were able to reestablish their electrical resistance within 12 hours after the final addition of hemolysin (Figure 1 and 2). Some of the monolayers, such as the ones contained in the control wells, continued to grow, causing an increase in their electrical resistance over 24 hours. We concluded that the amount of beta-hemolysin added to the cells cultured on the transwell membranes was insufficient, and therefore did not cause permanent disruption of the monolayer or death of cells. Higher concentrations or additions of the hemolysin over longer time periods may have had an irreversible effect. The ability of the cells to reestablish the electrical resistance may be due to the high tolerance and resilience of this cell line (Caco-2 human adenocarcinoma cell line).

The reduction in transepithelial resistance does not seem to be due to the accumulation of hemolysin in the transwells. In wells where 25 HU/ml were added every 60 minutes for 5 hours, a total of 150 HUs were added, and still no effect was detected. Whereas the same total amount (150 HUs) produced a detectable decline by the third application in wells receiving 50 HU/ml every 60 minutes.

The mechanism through which the hemolysin induces its effects is not clear and may be one of many. It has been suggested that the beta-hemolysin is a pore-forming molecule. However, that would be hard to confirm just by measuring electrical resistance changes in epithelial monolayers. Monitoring changes in ion flux due to hemolysin exposure in single cells, would be a more appropriate way to determine its role as a pore-

forming molecule as will be discussed later.

Since the number of tight junctions correlates with the transepithelial resistance of the tissue (**19**), the hemolysin of *S. hyodysenteriae* may be able to interfere with the functions or structures of the tight junctions, thereby causing "leakiness" in the epithelium. The formation of pores or openings across the monolayers, where the cells are connected by tight junctions, could produce such a decrease in resistance by allowing more of the electrical current to pass.

The beta-hemolysin has been shown to be cytotoxic to various cell types (**4**). If it causes death of epithelial cells, the integrity of epithelial monolayers would be disrupted and would be reflected as a decrease in the electrical resistance across the monolayer. The percentage of dead cells necessary to cause such a result is unclear, but the addition of the beta-hemolysin does not affect all the cells since the monolayers or cell populations continue to grow and divide, thereby reestablishing the electrical resistance of the monolayer within 12 hours of the last addition of hemolysin. If cells are dying because of their exposure to the hemolysin, applying a mitotic blocker to the monolayers to prevent their recovery of resistance would confirm cell death and reestablishment of electrical resistance as a result of cell division. On the other hand, if the hemolysin only depolarizes the cell membrane, the Caco-2 cells would be able to reestablish the electrical resistance even in the presence of the mitotic blocker.

Changes in intracellular calcium levels mediate cellular signal transduction pathways and regulate diverse cellular processes. Calcium flux also plays an important role in maintaining membrane integrity. Unregulated changes could interrupt normal

cellular functions regulated by calcium. It could be that the hemolysin effects the processes that regulate calcium levels inside the cell such as calcium channels that regulate calcium entry or pathways that result in calcium release from internal stores. Preliminary results indicate that the addition of hemolysin to swine peripheral blood lymphocytes results in tyrosine phosphorylation of several proteins. This indicates the initiation of at least one signal transduction pathway (data not shown).

Flow cytometry was utilized to test the hypothesis that beta hemolysin exerts its actions on host tissues by causing disruptions in the plasma membrane which lead to changes in ion flux. The hemolysin caused changes in calcium levels in both human (Caco-2 and lymphocytes) and porcine (IPEC-1 and lymphocytes) cell types and thereby does not seem to be species specific. The lymphocytes, in general, seemed to be more sensitive to the effects of the beta-hemolysin than the epithelial cells.

The same experiments were done in calcium free medium to determine if the intracellular levels of calcium increased solely as a result of the influx of extracellular calcium, or because of release from bound calcium intracellular stores. Because no significant changes in calcium flux occurred in this calcium free medium, we were able to deduce that the hemolysin caused changes in the cell membrane which allowed the ions in the extracellular media to flow into the cells.

To determine if these changes were targeted specifically toward calcium, the test was done in media where changes in other ions were also monitored. Manganese was chosen because of low intracellular concentrations and also because it is easily detected by the same fluorescent indicator (Indo-1) which was used to detect the calcium. No

manganese flux was detected in cells treated with the hemolysin. However, a reduction in cell size occurred almost immediately after treatment with hemolysin, but only when the cells were in calcium free medium.

Cells treated with hemolysin while suspended in calcium free media showed an almost immediate decrease in size as soon as the hemolysin was added while no change in calcium levels were detectable. This reduction in size may be the result of ions moving out of the cell where the hemolysin is acting on specific calcium channels where ion exchange occurs. These channels are triggered and remain open so that afflux of ions such as sodium, potassium or even bicarbonate occurs and results in cell shrinkage because of the unavailability of the calcium in the extracellular media and imbalance of ion movement that is occurring.

Fluorescein diacetate (FDA) was used to detect cell death by monitoring the "leakiness" of the cells to FDA after being subjected to the hemolysin. Since a manganese flux was not detectable and fluorescein diacetate did not leak from the cells following treatment with beta-hemolysin, it is possible that calcium specific channels are affected. These studies indicate that the hemolysin may have a somewhat specific effect on calcium ions, calcium channels, or calcium homeostasis but to make sure, using calcium blockers and the effect of hemolysin on other ions needs to be studied.

We may conclude that the hemolysin causes biologic changes in the various cell membranes which were reflected as changes in electrical resistance. The changes in calcium flux in the individual cells, as a result of hemolysin addition, may be the reason for the change in the electrical resistance. If hemolysin is acting on calcium channels and

cell death is occurring due to the addition of hemolysin in the electrical resistance assay, then it may be that hemolysin is triggering calcium specific channels. As a result, an increase in intracellular calcium levels and the byproducts of the pathways, which calcium regulates, to amounts which exceed the tolerable levels that the cells can withstand occurs, thereby causing cell damage and even death.

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