Abstract

COLE, JEFFREY THOMAS. Cyclooxygenase inhibition and glutamine addition to *Cryptosporidium parvum* infected calf ileal tissue. (Under the direction of Robert Argenzio).

The research contained herein examines the relationship between *Cryptosporidium parvum* infection, glutamine administration and cyclooxygenase (COX) inhibition in calf ileal tissue. Ileal mucosa from healthy and *C. parvum* infected calves was mounted in Ussing chambers and treated with COX inhibitors or glutamine. Radiolabeled isotopes were used to determine the unidirectional flux of ^{22}Na and ^{36}Cl, to calculate net fluxes in response to both treatment and infection. Electrical data was collected, and, in conjunction with the isotope data, aided in the determination of electroneutral versus electrogenic transport.

Treatment of infected tissue with indomethacin (10^{-6} M), a non-selective COX inhibitor, increased electroneutral Na^+ uptake ($P< 0.05$, ANOVA), whereas selective inhibition of COX-1 (SC-560, 10^{-6} M) or COX-2 (NS-398, 10^{-6} M) had no effect. Treatment with SC-560 and NS-398 (10^{-6} M) yielded similar results to that of the indomethacin treatment, indicating that blockade of both enzymes is required to restore electroneutral Na^+ absorption. Exogenous glutamine increased Na^+ uptake ($P< 0.02$, control tissue; $P= 0.17$, infected tissue; ANOVA), also increasing short circuit current in the infected tissue (I_{sc}) ($P< 0.05$, ANOVA) suggesting electrogenic Na^+-glutamine absorption in the infected tissue. The absence of an electrical response following glutamine administration in healthy tissue suggests stimulation of an electroneutral transport mechanism. Immunohistochemistry revealed the presence of both COX isoforms in healthy

and infected tissue. Further, NHE-3 was found in the control, but not in the infected tissue, while NHE-2 was found in neither healthy nor infected tissue. For both COX-2 and NHE-3, Western blot analysis confirmed the immunohistochemical findings. This study indicates that the blockade of both COX isoforms is necessary to release the prostaglandin-mediated inhibition of electroneutral Na^+ uptake in *C. parvum* infected calf ileal tissue. The absence of both NHE-2 and NHE-3 in infected tissue, despite the complete restoration of electroneutral sodium transport following prostaglandin inhibition, suggests the presence of an unidentified sodium transporter in infected calf ileum. Further, glutamine increases Na^+ uptake by an electrogenic mechanism in infected tissue and an electroneutral mechanism in healthy tissue.

CYCLOOXYGENASE INHIBITION AND GLUTAMINE ADDITION TO *CRYPTOSPORIDIUM PARVUM* INFECTED CALF ILEAL TISSUE

by

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Approved by

Robert Argenzio Chair of Advisory Committee	Anthony Blikslager Co-Chair of Advisory Committee
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Dedication

This dissertation is dedicated to the Cole family.
May I make the family as proud of me as I am of them.

Biography

Born November 22, 1974, Jeffrey Thomas Cole is the son of George W. and Brenda M. Cole. After being born and raised in Orlando, Florida, Jeffrey followed his father and uncle in attending North Carolina State University. In 1996, Jeffrey received a Bachelor of Science in Animal Science, with a minor in Nutrition, graduating Summa cum laude. Afterwards, he attended the University of Illinois, graduating in 1998 with a Master of Science in Animal Science. His studies focused primarily on canine nutrition. Jeffrey then returned to North Carolina State University, studying gastrointestinal physiology under the supervision of Drs. Robert Argenzio and Anthony Blikslager, in completing the requirements for a doctoral degree. In 2002, Jeffrey graduated with a Doctor of Philosophy degree in Physiology, again completing the requirements for a graduate minor in Nutrition.

Acknowledgements

As my primary advisor, **Robert Argenzio** has been one of the finest mentors a student could ask for. In addition to his expertise in gastrointestinal physiology, the knowledge he has shared with me about the myriad of things a doctoral candidate should be conversant in has been an invaluable addition to my education. I consider it the highest privilege to have been his final graduate student.

My co-advisor, **Anthony Blikslager**, is perhaps the perfect complement to Dr. Argenzio as an advisor. He has been unshakably encouraging in my graduate work, as well as greatly improving my technical writing and presentational skills.

Having **Robert Harrell** as my nutrition minor advisor has greatly benefited me as a researcher, by encouraging the integration of my knowledge of gastrointestinal physiology with nutrition.

Samuel Jones was the final member of my graduate committee. He graciously took the time to read a manuscript prior to submission, and his contributions to the paper made a much more effective manuscript.

I would especially like to express my appreciation to both Martha Armstrong and **Marjory Gray**. Their laboratory expertise was critical to the completion of my degree. However, more important than their skill in various laboratory techniques was the working environment fostered by these two individuals. In future years I am certain to remember fondly my time spent in the laboratory with both women.

Karen Young provided a thorough instruction is one of the techniques used in my studies. Her patience in teaching me both the technique, as well as providing a thorough grounding in the theory behind it, is much to be admired.

A former graduate student of Dr. Argenzio, **Jody Gookin** was instrumental to my joining his laboratory. She demonstrated several laboratory techniques, and served as a guide to completing the many requirements necessary to completing a doctoral degree.

Before her retirement, **Elaine Hunt** shared some of her expertise in daily calf care and treatment, which was an integral part of my studies.

In addition to the individuals mentioned above, I would be remiss in not mentioning my many friends who provided a much needed source of entertainment and encouragement. In particular I would like to thank **Lezah Pagels, Greg** and **Constance Lyda**, and **Paul Arnold**.

Finally, and most importantly, I need to mention my family, whose support and encouragement enabled the completion of this degree.

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Introduction: Cryptosporidiosis is a widespread enteric disease, with a significant economic impact in both the livestock and human health industry. *Cryptosporidium parvum* infection results in a severe, watery diarrhea via two mechanisms. First, villous atrophy results in the loss of the full array of nutrient and electrolyte transporters; in particular, the loss of sodium/hydrogen exchangers impairs fluid absorption, resulting in malabsorptive diarrhea. Second, the inflammatory response to the infection, including cyclooxygenase-elaborated prostaglandins, results in the secretion of electrolytes, draws fluid into the lumen of the gastrointestinal tract (GI tract). This does have a protective role, as the quantity of fluid secreted is thought to be an attempt to flush the pathogen from the hosts GI tract. However, the severity of the malabsorptive diarrhea coupled with the secretory diarrhea can cause severe, lethal dehydration in the host.

The range of deleterious effects of cryptosporidiosis has led to the development of several disparate treatments. The primary treatment is the administration of oral rehydration solutions, intended to rehydrate the host. Typically, these solutions are glucose-based, although recent evidence suggests that glutamine would be more efficacious than glucose. Additional treatments include the administration of non-selective cyclooxygenase inhibitors, to minimize the inflammatory response, which would lessen the severity of the secretory diarrhea. Additionally, prostaglandins are known to have inhibitory effects on transporters heavily involved in sodium, and thus fluid absorption, such as the sodium/hydrogen exchangers (NHE). However, to date, little research has been

conducted to examine the cumulative effects of COX inhibition and glutamine administration in *C. parvum* infected calves; nor has the effect of infection on the primary vectorial sodium transporter (NHE-3) been elucidated.

A long-term goal of this laboratory is the determination of mechanisms by which intestinal absorptive processes adapt to pathogenic injury, focusing primarily on the upregulation of crypt-based transporters following villous atrophy. Several researchers, both in this laboratory and others have demonstrated a number of absorptive responses in injured tissues, mediated by glutamine, alanine, and alanylglutamine, suggesting either crypt based transport, or upregulation of transport activity in villi remnants. Typically, H^+-peptide and Na^+-glucose transporters are expressed in villous cells, and absent in the proliferating enterocytes populating the intestinal crypts. Additionally, transport of the amino acids leucine and lysine appear to be limited to the upper third of the villi (Mordrille et al., 1997). This information supports the paradigm that intestinal transport is limited to the mature enterocytes residing on the terminal villus, while secretory function is limited to the crypt cells. Due to the extent of the villous damage seen during injury, in conjunction with stimulatory responses to certain amino acids, induction of a crypt-based transporter is a likely explanation. Further, several researchers have demonstrated the presence of transporters in crypt epithelium, and we intend to further elucidate the extent of this adaptive response, and possible mechanisms regulating this response (Blikslager et al. 2001; Binder et al., 2000).

These findings have led to the development of theories that challenge current paradigms of intestinal transport. Formerly, absorptive mechanisms were thought to be limited to the terminal villi, with secretory responses found predominately in the crypts of intestinal mucosa (Figure 1). However, we propose an alteration to this model; following injury, and subsequent villous atrophy, we believe the intestine adapts by the production and membrane insertion of some transporters into crypt apical membranes (Figure 2). Further, these results should have applicability to any number of intestinal injuries, such as short bowel syndrome following intestinal resection, or other infectious diseases, such as rotavirus.

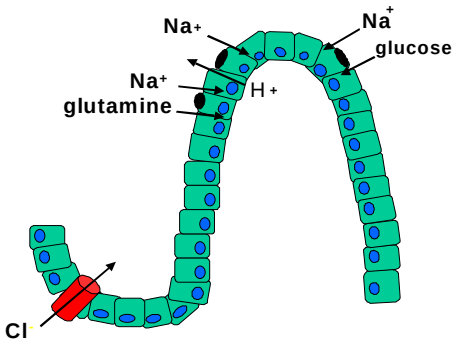

Figure 1. Graphical representation of current paradigms in intestinal nutrient transport

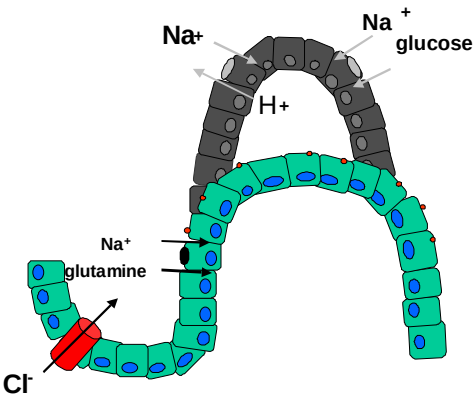

Figure 2. Graphical representation of proposed paradigm for intestinal nutrient transport in injured ileal tissue

In this laboratory, we use a *Cryptosporidium parvum*-infected calf model to examine the effects of injury on ileal tissue. This model is ideal for studying this zoonotic pathogen, since the calf is the natural reservoir for C. parvum and exhibits the same severe, watery diarrhea seen in human hosts. The widespread

nature of this pathogen is perhaps responsible for the number of large outbreaks of cryptosporidiosis in humans; in recent years, six outbreaks of over 18,000 individuals have been reported (Smith and Rose, 1990).

Cryptosporidiosis: The genus *Cryptosporidium*, one of approximately 20 members of the Apicomplexan phylum of protozoans, was first discovered in 1907; *C. parvum* was identified in 1911. Together, the members of the genus *Cryptosporidium* infect over 170 species of animals; specifically, *C. parvum* can be infective in 79 species of mammals (Atwill et al., 1999; O'Donogue, 1995). Many members of this genus can be distinguished from one another by gross morphological examination; others, such as *C. parvum* and *C. wrairi*, are virtually identical genetically and phenotypically and can only be differentiated by sequence analysis of the Cryptosporodium Outer Wall Protein (COWP) gene (Patel et al., 1999). A wide variety of diagnostics based on morphological features have been developed to identify the various species, including the size and shape of the oocysts or sporocysts, the presence or absence of a micropyle cap and Stieda body and the activities of various isoenzymes, such as glucose phosphate isomerase and others (El-Ghaysh and Barret, 1999; Morgan et al., 1999). Additionally, determination of the 18S RNA sequence can be used; however several species, such as *C. parvum* and *C. wrairi*, demonstrate identical sequences (Patel et al., 1999). Practically, however, it is usually sufficient to identify the pathogen as a representative of the Cryptosporidium genus; the source (i.e. host species, location of infection) will suffice to assign the species being dealt with.

Following its discovery, almost a half-century lapsed before the association between *Cryptosporidium* and disease in diarrheic turkeys was discovered (de Graaf et al., 1999). It was another 20 years before two species known as *C. parvum and C. muris* were identified in cattle (Scott et al., 1995; Uga et al., 2000). To date these are the only two species that are known to colonize mammals (Upton and Current, 1985). *Cryptosporidium parvum* is typically located in the intestine, although it can also be found elsewhere in immunocompromised individuals, while *C. muris* is invariably found in the glands of the stomach (Laurent et al., 1999; Anderson, 1998). Despite inhabiting the same host, these two pathogens can be readily identified morphologically, *C. parvum being* 4-6 μm in diameter, while *C. muris* is much larger. Additionally, *C. parvum* has a single, thin wall about 0.4 μm thick, and contains 4 sporozoites (Upton and Current, 1985).

In addition to the various species of *Cryptosporidium*, there are strains within species that vary in some key aspects- virulence, pathogenesis, antigenic variances and drug sensitivity (Morgan et al., 1999). Unfortunately, these differences are also responsible, in part, for the difficulty in developing an effective vaccine or microbiocidal treatment. Awad-El-Kariem et al (1998) analyzed DNA from a number of *C. parvum* oocysts isolated from outbreaks of cryptosporidiosis in both cattle and humans. They determined that there are two general strains within the *C. parvum* species- a “human” strain and an “animal” strain. The animal strain can be zoonotic, but the human strain does not appear to be anthroponotic.

Other researchers refer to a “porcine” strain and a “bovine” strain (Morgan et al., 1999b). One indicator for the relative virulence of the particular strain being studied, is its ability to generate an infectious response in mice and rats, which are highly resistant to *C. parvum.* Morgan et al. (1999b) reported that the porcine strain was incapable of infecting nude mice, although the bovine type did result in infection. A study of the 18S rDNA gene sequence indicates that there is definite variation between the bovine, porcine, and human strains of *C. parvum* (Morgan et al., 1999b).

INCIDENCE: Cryptosporidiosis is a worldwide concern, especially in developing countries, in which 6.1% of human diarrheic samples contain *C. parvum* oocysts (de Graaf et al., 1999b). Quilez et al (1996) reported that over 50% of Latin Americans have anti-*C. parvum* serum IgG, despite having no history of exposure. Between 15 and 44% of the population in industrialized

Table 1. Global incidence rates of cryptosporidiosis on livestock operations.

Incidence (%)	Detection Method	Location	Farm Type	Source
92	sucrose flotation	United Kingdom	Mixed[+]	Scott et al., 1995
93	sucrose flotation	Japan	Dairy	Uga et al., 2000
95	microscopy	France	Beef	Naciri et al., 1999
45*	microscopy	California	Mixed	Atwill et al., 1999
48	microscopy	California	Dairy	Harp et al., 1996
92	microscopy	California	Dairy	Atwill et al., 1998
59	microscopy	California	Dairy	Harp and Goff, 1998
56	microscopy	Idaho	Dairy	Anderson and Hall, 1982
75	microscopy	Maryland	Dairy	Leek and Fayer, 1984
49	antibody test	Central US	Dairy	Garber et al., 1994
56	antibody test	Northern US	Dairy	Garber et al., 1994
56	antibody test	Southeast US	Dairy	Garber et al., 1994
74	antibody test	Western US	Dairy	Garber et al., 1994

* This percentage is the number of herds with at least one animal shedding oocysts

[+] Mixed farms contain both beef and dairy livestock

countries has anti-*C. parvum* serum, and 2.1% of the diarrheic samples in these countries contains *C. parvum* oocysts (de Graaf et al., 1999b).

Calves are the primary reservoir of *C. parvum*; indeed, the most probable cause of diarrhea in calves less than 4 weeks of age is *C. parvum* (O'Handley et al., 1999). It would appear that dairy cattle are more susceptible to infection than beef cattle, although this may simply be a management difference (Atwill et al., 1999). There do appear to be some regional differences; generally speaking, the United States appears to have lower incidence rates than other regions of the world (Table 1). However, there are several breed differences that must be noted when considering susceptibility to the infection. For example, Belgian Blue-White, French Limousin, and Charolais all have much higher mortality rates than do other breeds (de Graaf et al., 1999b).

In calves, infection is most commonly seen at approximately 2 weeks of age, and, in non-experimental settings, cryptosporidial diarrhea is usually limited to animals between 1 and 4 weeks of age (O'Handley et al., 1999; Atwill et al., 1999). Typically the animals begin shedding oocysts as early as 5 d after infection, and continue asymptomatic shedding until 53 d of age (Faubert and Litvinsky, 2000). A wide array of factors can impact the spread and infective risk of *C. parvum* contamination, most of which are sanitary or calf rearing concerns. Unfortunately, *C. parvum* is resistant to most commerically available disinfectants, making eradication difficult (Harp and Goff, 1998). The most effective means of eliminating the organism is by extremes of temperature. Fayer et al (1998) reported that placing *C. parvum* in a 73^{O}C environment for 5

seconds, a –70 ^{O}C environment for 1 h, or a –20 ^{O}C environment for 24 h are all equally effective in killing the pathogen. Faubert and Litvinsky (2000) discovered that on farms where calves frequently display cryptosporidial diarrhea, over 60% of samples collected from manure specimens, floors, walls, water, and surrounding soil were positive for oocysts. A study conducted by Scott et al (1995), determined that 60% of fecal smears contained oocysts, detecting between 35 and 7000 oocysts per g of feces.

In contrast to humans and calves, which can both become readily infected, piglets have a low prevalence of natural infection, apparently due to high lactogenic immunity (Quilez et al., 1996). However, if the infective dose is high enough, piglets do demonstrate cryptosporidial diarrhea.

TRANSMISSION: *C. parvum* is typically transmitted by a fecal/oral route, and, in primates, the minimum infective dose is as few as 10 oocytes (Scott et al., 1995). The infectious dose for 50% of the human population is 132 oocytes (88% of population can be infected by as few as 300 oocytes) (Pell, 1997). In humans, the primary route of infection is by water-borne oocysts, initially caused by fecal contamination of the drinking supply, or inadvertent consumption of other sources of water, such as swimming pool water. For example, while not definitively confirmed, the outbreak of cryptosporidiosis in Milwaukee, which infected over 400,000 individuals, is thought to be the result of run-off from dairy operations in the vicinity of the water treatment plants, following higher than normal amounts of rainfall (MacKenzie et al., 1999). Dairy operations have a high risk of contaminating surrounding areas, especially during periods of

excessive rainfall, for a number of reasons. Oocysts shed into the environment can remain infective for up to 6 months (Naciri et al., 1999; Harp et al., 1996). Not only can the organism remain viable for long periods, but cryptosporidial calves can shed between 10^6 and 10^7 oocysts per g feces (Mohammed et al., 1999; Harp and Goff, 1995). During the course of a typical outbreak of cryptosporidial diarrhea, a single calf can shed a total of 6×10^{11} oocysts (Uga et al., 2000). In addition to oocysts shed by diarrheic calves, assymptomatic adults can shed between 750,000 and 720,000,000 oocysts per day with about 50% being viable (Naciri et al., 1999).

LIFE CYCLE: Typically the protozoan is ingested in the oocyst form. Once ingested, the organism undergoes excystation, caused by the reducing conditions in the gastrointestinal tract (GIT), the presence of pancreatic enzymes, or bile salts (Laurent et al., 1999; de Graaf et al., 1999b). However, this is not strictly required for infectivity, since the respiratory tract and conjuctiva can also be infected, although this usually occurs only in immunocompromised individuals. In mice, the entire *C. parvum* life cycle is completed in 72 h (Riggs and Perryman, 1987). The organism has a merogonic life cycle (two generations of meronts), a gametogenic cycle (including macrogametes, microgametes and zygotes) and sporogeny, with the type 1 meronts, and the thin walled oocysts being the two infective stages (de Graaf et al., 1999b). Thin walled oocysts can be formed during intracellular reproduction, release, and re-infect the same host. Thick walled oocysts are produced by the same process, but are not auto-

infective, and are excreted into the environment. However, once re-ingested, these oocysts can be infective (Gookin et al., 2002).

INFECTION AND INVASION: Since *C. parvum* typically only enters the epithelial cells lining the intestine, it is considered a minimally invasive pathogen. However, infection can spread to the biliary tract, pancreatic duct, stomach, esophagus and respiratory tract in immunocompromised hosts (Laurent et al., 1999). Typically, healthy individuals experience mild, self-limiting diarrhea; however, immunosuppressed individuals experience much more severe symptoms, such as bile duct obstruction, jaundice, severe abdominal cramps, nausea, vomitting, fever, pancreatitis, cholera-like diarrhea, and severe dehydration, all of which can ultimately lead to death (Laurent et al., 1999). In developing countries, 24% of diarrheic samples obtained from AIDS patients contain the *C. parvum* pathogen (de Graaf et al., 1999b). Goodgame et al (1995) reported a lower rate, indicating that between 15 and 20% of the stools obtained from diarrheic AIDS patients contains *C. parvum*, a value that is perhaps reduced by including the data from industrialized countries

Most Apicomplexan zoites have similar machinery for entering host cells, *C. parvum* included, with the first step to entering the host being the attachment to the epithelial cells. A number of potentially interactive proteins have been isolated on both the pathogen and host epithelium, which may mediate *C. parvum* attachment. Joe et al (1998) speculated that a surface lectin on the sporozoite mediates the attachment. Chen and LaRusso (2000) implicated a Gal/GalNAc specific lectin as the primary initiator of attachment, inducing other

mechanisms to begin the procedure for anchoring and entering the cell. Interestingly, both the *C. parvum* surface protein (47 kDa) partly responsible for binding to the host, and the host cell protein (57 kDa) that binds to the *C. parvum* antigen are manganese sensitive (Nesterenko et al., 1999). Another potential attachment mechanism is the Cryptosporidium sporozoite ligand (CSL; 1300 kDa), which can attach to the host epithelial cells. Langer and Riggs (1999) demonstrated that when isolated and administered to a potential host, in this case Caco-2 cells, CSL bound to the epithelial layer and prevented the attachment of *C. parvum*. Additionally, the host proteins that appear to recognize *C. parvum* are expressed at a higher concentration in the ileum, when compared to the duodenum or jejunum, which may explain in part why *C. parvum* colonizes this region of the intestine at a much higher concentration (Nesterenko et al., 1999). Another explanation for this preferential colonization of the ileum is the activation by the reducing conditions and digestive enzymes in the proximal intestine. It is highly likely that normal motility of the host intestine deposits the oocysts in the distal small bowel during the process of excystation.

The pathogen has three types of secretory organelles adapted for providing entry into the epithelial cells- micronemes, rhoptries, and dense granules (Petry and Harris, 1999). Micronemes, specialized secretory organelles that secrete adhesins that interact with cellular receptors, release their contents during the preliminary attachment phase of zoite and host interaction, when the parasite is demonstrating a gliding motility along the host cell (Chen et al., 1998; Meissner et al., 2002). During invasion, rhoptries, of which Cryptosporidium have

only one per organism, release their contents. The contents of rhoptries are key to the formation of parasitopherous vacuoles (Blackman and Bannister, 2001). Dense granule contents are released after the parasite/host interaction is already established, and expand the membrane enclosing the parasitopherous vacuoles (Blackman and Bannister, 2001; Petry and Harris, 1999). Once attached, the pathogen can enter the cell and form a protective, parasitopherous vacuole within 20 minutes; the peak rate of entry occurs approximately 2 h after consumption or administration of an infectious dose. The vacuole membrane consists of an inner, parasite-derived layer and an outer layer derived from the host apical epithelial membrane, providing a protected environment for the *C. parvum* to reproduce (Chen et al., 1998).

Following infection, there are changes in the host cell in addition to formation of the parasitopherous vacuole. Kapel et al (1997) first reported a rearrangement of the host cytoskeleton following infection, resulting in the formation of a dense band of modified host proteins (known as the electron dense band) underlying the vacuole. Later work specified more exactly what alterations in the cytoskeleton were occurring following infection. At every focus of invasion by *C. parvum*, Elliot and Clark (2000) reported a plaque-like, host-derived, accumulation of filamentous actin (f-actin). This formation serves as an anchor for the pathogen, and is most likely induced by the *C. parvum* organism, as a component of the host/parasite interface (Elliot and Clark, 2000). In fact, Chen and LaRusso (2000) speculate that this rearrangement of the host cytoskeleton is required for infection to occur.

CLINICAL MANIFESTATION: Most hosts that develop *C. parvum* infection generally display similar symptoms, including secretion of excess fluid into the GIT, failure to resorb this fluid in the colon, villous atrophy, malabsorption due to decreased surface area and maldigestion, from altered digestive enzyme secretion and concentration (Anderson 1998). Obviously, the time course of symptom expression varies amongst host species, infecting strain, and number of the organisms consumed. In humans, the typical incubation period is 2-14 d, and can affect individuals of all ages (Laurent et al., 1999). In contrast, calves under 30 d of age are primarily affected by *C. parvum* infection (Mohammed et al., 1999). Typically, animals begin shedding oocysts about 4 days post infection, with maximal excretion occurring at day 7. Although the clinical manifestation of disease may soon disappear, oocysts will continue to be shed for an additional 4 weeks (Toyoguchi et al., 2001).

TREATMENT: To date, researchers have tested 79 antibiotics and anticoccidials, most of which have proven to be completely ineffective (Peeters et al., 1993). In cattle, most treatments are limited to the administration of oral rehydration solutions and anti-inflammatory compounds, such as flunixin meglumine (Banamine®). Currently, there is no effective antibiotic to this pathogen, which, due to its naturally self-limiting nature, is perhaps not vitally important. With a death loss from all causes, including *C. parvum* infection, of less than 2%, it would not be economically viable to administer a more aggressive treatment regime. However, a greater concern is with dual infections, which may synergize and result in high mortality. Although, should a treatment

be discovered for cattle, it would greatly facilitate the development of similar treatments in humans, in which this pathogen can be lethal, if the host has an inadequate immune system. The complexity of the life cycle makes it difficult to determine the most effective target for treatment, although the extracellular stages would obviously be the most susceptible to treatment. One difficulty with treating this stage, however, is the short length of time between excystation and infection, which would make it difficult to appropriately time an effective treatment (de Graaf et al., 1999b). The oocyst, due to its inert nature, both physically and chemically, is perhaps the least promising avenue of research (de Graaf et al., 1999b). However, it should be feasible to develop an inhibitor of excystation, or perhaps an antibiotic specific to the host ligands, which would competitively exclude *C. parvum*. Another potential candidate for treatment are the zoite stages, which do have 3 surface antigens that have already been identified; typically, these stages are readily phagocytized by M cells, which is in fact one of their primary means of entering the host epithelial layer (de Graaf et al., 1999b).

There is some research indicating that it may be possible to develop an effective treatment. Fayer and Ellis (1993) reported that paromycin, a broad-spectrum antibiotic, greatly reduced both the severity and duration of diarrhea, although the mechanism of action is unknown. Other potential candidates include sulfaquinoxaline, which decreased oocyst shedding in mice and rabbits; arprinocid, which decreased the infection rate in rats and hamsters (although these species have been proven difficult to deliberately infect as adults), and halofuginone lactate, which decreased both oocyst shedding and clinical signs in

calves (Peeters et al., 1993). Another possibility is the compound difluoromethylornithine (DFMO) which has shown some ability to limit cryptosporidiosis in AIDS patients (Holland et al., 1992). However, due to its mode of action (inhibiting ornithine decarboxylase activity and limiting polyamine synthesis), this drug causes identical damage to the host as does the organism it is trying to eliminate. For example, DFMO has been shown to cause villous atrophy in several species, interfere with enterocyte maturation and proliferation, and decrease nutrient absorption. These effects are all due to the key role of polyamines in gene transcription, mRNA synthesis and nucleotide production (Holland et al., 1992).

Administering pooled samples of high anti- *C. parvum* titer colostrum is of dubious efficacy in preventing or limiting infection. Harp and Goff (1998) reported that colostrum was ineffective in preventing or limiting infection; although Perryman et al. (1999) demonstrated that it was completely protective against diarrhea, and also greatly reduced oocyst shedding following experimental infection. Joe et al (1998) reported that antibodies present in hyper-immune bovine colostrum inhibited the attachment of the pathogen by 30%. Incidentally, in the same study, the researchers reported that porcine gastric mucin and bovine submaxillary mucin also inhibited attachment, by 97 and 75%, respectively (Joe et al., 1998).

Arginine administration has been shown to be efficacious in decreasing oocyst shedding following infection (Waters et al., 1997). Arginine is converted to nitric oxide, by nitric oxide synthase, which then reacts with reactive oxygen

metabolites to form peroxynitrites, which are cytotoxic to the pathogen and can thus limit infection. This response is eliminated by the administration of L-NAME, a nitric oxide synthase inhibitor (Waters et al., 1997). This work agrees with that presented by Leitch and He (1994), perhaps the first researchers to study the affects of arginine in limiting *C. parvum* infection. Leitch and He (1994) determined that feeding arginine prior to infection delayed the onset and reduced the extent of oocyst shedding, and that feeding arginine after infection also decreased oocyst shedding, although the more efficacious treatment was delivery before infection. These results were also determined to be the effect of the conversion of arginine to nitric oxide. Should arginine prove efficacious in treating cryptosporidiosis, this may have a beneficial side effect. One potential clinical treatment is the administration of glutamine based oral rehydration solutions. However, at higher doses, glutamine can be toxic, due to excessive ammonia production. It is highly likely that concurrent administration of arginine would allow higher levels of glutamine to be fed, by disposal of the NH3 via the urea cycle.

Leitch and He (1999) followed up their earlier work by determining the specific enzymes involved in the production of nitric oxide. The researchers discovered that inducible nitric oxide synthase (iNOS) typically localize to the terminal villi, with little found in the crypts. Additionally, infected animals typically have higher levels of epithelial cell iNOS, due to upregulation by hormones such as IL-1, IL-2, TNF-α and IFN-γ, all of which are increased in response to *C. parvum* infection (Leitch and He, 1999). The researchers were able to

thoroughly demonstrate the role of nitric oxide in combating *C. parvum* infection. Using two different NOS inhibiters, L-NAME (a general NOS blocker) and L-NIL (a specific iNOS blocker), Leitch and He (1999) were able to heighten the severity of the infection. Additionally, treating the animals with anti-oxidants such as ascorbic acid or superoxide dismutase (a superoxide scavenger) eliminated the beneficial effects of nitric oxide by inhibiting their reaction with reactive oxygen metabolites (Leitch and He, 1999).

Another orally administered compound that can potentially delay or prevent cryptosporidiosis is putrescine (Waters et al., 1997). This polyamine is produced from ornithine by ornithine decarboxylase, and is involved in cellular proliferation, differentiation and maturation (Waters et al., 1997). Mice fed this polyamine prior to and during infection had delayed onset and reduced oocyst shedding in the preliminary stages of infection. However, after 10 d, there was no difference between the rats consuming putrescine and those that were not (Waters et al., 1997). The mechanism by which this compound acts to delay infection is unknown at this time, although the researchers speculate that it may be due to either alterations in the rate of maturation of the host epithelial layer, or interference in the *C. parvum* life cycle. The efficacy of feeding putrescine must be studied further, to determine its potential as either a prophylactic or treatment for infection.

One possible treatment currently used is the administration of cyclooxygenase inhibitors. Prostaglandin levels increase following *C. parvum* infection as a part of the inflammatory response (Argenzio et al., 1996; Laurent et

al., 1998). In fact, mucosal inflammation can result in a three-fold increase in the tissue levels of PGE_2 (Cosme et al., 2000). However, prostaglandins have deleterious effects on electroneutral NaCl transport, and accompanying fluid uptake, which is of vital importance due to the severity of the diarrhea induced by infection. Laurent et al (1998) speculated that the increase in PGE_2 may stimulate intestinal secretion of both chloride and mucin, which could be protective against further infection, and elimination of the current pathogen population. This topic will be discussed below; briefly, prostaglandin production can be deleterious due to inhibition of immune surveillance, or heightened apoptotic mechanisms.

DAMAGE: Infection with *C. parvum* consistently results in a number of deleterious effects readily observed in the intestine. Perhaps the most obvious, and important, is moderate to severe villous atrophy and accompanying crypt hyperplasia. (Laurent et al., 1999; de Graaf et al., 1999a). Elliot and Clark (2000) reported the loss of microvilli in regions infected by *C. parvum*. This alteration in epithelial architecture begins within 12 h of infection and is completed by 36-48 h following infection, with 85% of the infected, exfoliated enterocytes, sloughing off the villi tip (Moore et al., 1999).

In infected ileal tissue, the villi contract to approximately one-third the height of normal, healthy tissue (Argenzio et al., 1990). Furthermore, the reduction in height and accompanying crypt hyperplasia alters the villi height:crypt depth ratio, from 5:1 in healthy tissue to 1:1 in infected tissue, a change accompanied by alterations in surface area (Argenzio et al., 1990). As

might be expected, the extent of the damage is correlated to the infectious dose. Another physical effect of the pathogen is the generation of apical membrane defects large enough for the passage of 1000 kDa compounds (Griffiths et al., 1994). These defects can allow the entry of other injurious compounds or pathogens, exacerbating the effects of infection. In Caco-2 cells, the researchers reported a severe decrease in paracellular resistance following infection, the magnitude of which was highly correlated to the infectious dose (Griffiths et al., 1994). However, due to the inability of Caco-2 cells to form tight junctions in vitro, this has limited applicability to an in vivo situation. Indeed, in the studies performed by this author, *C. parvum* infection and the subsequent damage increased tissue resistance, most likely by a combination of villous atrophy and collapse of the para-cellular spaces.

One additional effect of *C. parvum* infection is the impairment of Vitamin A absorption, most likely due to the villous atrophy characteristic of infection. Since retinol and carotenoids are absorbed via passive diffusion, any reduction in the surface area would have a limiting effect on the amount being absorbed. Further, the villus would be populated by immature cells lacking the intracellular machinery necessary for esterification and chylomicron synthesis, thus interfering with net movement of Vitamin A into the host body. Due to the complexity of the intracellular conversion of retinol to retinyl esters, involving acyl-CoA:retinol acyltransferase, cellular retinol-binding protein type II, and lecithin-retinol acyltransferase in particular, the most likely limiting step in Vitamin A metabolism in damaged tissue is retinyl ester formation (Combs, 1998).

Accompanying the morphological damage caused by *C. parvum* infection is an inhibition of intestinal glucose and glutamine coupled sodium absorption (Argenzio et al., 1996; Argenzio et al, 1990). These effects are eliminated in vitro, via inhibition of prostaglandin synthesis, although in vivo inhibition worsens intestinal damage (Argenzio et al., 1996). Several researchers have shown that within four days of infection, prostaglandins, in particular, PGE_2, $PGF_{2\alpha}$ and PGI_2, are increased by up to 50-fold during infection, and act via an enteric neuronal mechanism (PGI_2) or an endocrine mechanism (PGE_2) (Argenzio and Rhoads, 1997; Argenzio et al., 1996; Laurent et al., 1998). Prostaglandin E_2 most likely acts via a cAMP-dependent pathway to regulate electroneutral NaCl uptake; PGI_2 acts via VIPergic and cholinergic neuronal pathways (Argenzio et al., 1996).

One tissue response that has yet to be fully elaborated is the elevation of the anti-inflammatory cytokine, TGF-β, following *C. parvum* infection (Robinson et al., 2000). This cytokine aids in tissue repair following damage, specifically restitution and the deposition of the extracellular matrix; however, TGF-β was isolated only from the jejunum, while *C. parvum* infection is typically heaviest in the ileum (Robinson et al., 2000). Additionally, the researchers could not determine any variance in the symptoms (severity of the diarrhea and oocyst shedding), despite differences in the levels of TGF-β. However, it may be possible that TGF-β does have a protective effect, which is further explanation for the preferential colonization of the ileum, as opposed to the jejunum, despite the preponderance of attachment sites in the ileum. This topic will be explored in greater detail below.

Additional damage is caused by increased epithelial apoptosis in the first 24 h following infection (McCole et al., 2000). In studies using a human intestinal cell line, researchers found that 93% of the cells undergoing apoptosis were infected with *C. parvum* (McCole et al., 2000). Although infection caused 12% apoptosis, the pathogen appears to attenuate apoptosis by increasing NF-KB, which prevents the programmed cell death (McCole et al., 2000; Chen et al., 1999). This apoptotic response to infection is apparently initiated by the translocation of Fas-L to the membrane, the first step in a cascade of events that leads to caspase dependent apoptosis (Chen et al., 1999).

IMMUNE RESPONSE: Currently no vaccine for *C. parvum* infection exists; however, the search for both treatments and vaccines has garnered extensive knowledge of the immune response to *C. parvum* infection. In immunocompetent hosts, infection with *C. parvum* confers immunity to a second infection (Toyoguchi et al., 2001; Wyatt et al., 2000; Harp et al., 1990). It is believed that this resistance to a second infection is conferred by CD^+ T-cells (You and Mead, 1998). During a secondary infection, the hosts excrete anti- *C. parvum* IgA, IgM and IgG produced locally in the intestine (Wyatt et al., 2000). The anti-*C. parvum* titers are to a specific 23 kDa sporozoite surface protein (P23) and may implicate that protein as a potential antigen for antibiotic or vaccine development (Wyatt et al., 2000). The native microflora of the gut may also play a preventative role in developing resistance; mature flora in rats appears to be protective against infection (Harp et al., 1990).

Resistance to, and recovery from infection, is dependent on the presence of IFN-γ, IL-12, and both α/β and γ/δ T lymphocytes (both $CD4^+$ and $CD8^+$) and the infiltration of polymorphonuclear (PMN) cells (Adjei et al., 2000; Abrahamsen et al., 1997; de Graaf et al., 1999b; Wyatt et al., 1999; You and Mead, 1998; Wyatt et al., 1997). Perhaps the first line of defense against *C. parvum* infection is the increase in mononuclear cells in the lamina propria within 36 h, and PMN within 48 h of infection (Moore et al., 1999). Argenzio et al (1990) reported that the number of neutrophils infiltrating the tissue is directly correlated to the numbers of oocysts. Wyatt et al. (1997) and McDonald et al (1996) reported that *C. parvum* infection resulted in elevated levels of $CD8^+$, activation of $CD4^+$, increased IFN-γ, and down regulation of tumor necrosis factor-α.

Aguirre et al (1994) reported that a MHC-II deficiency resulted in a more severe and prolonged infection, due to decreased $CD4^+$ levels. $CD4^+$ are helper cells that interact with antigens via α/β and γ/δ heterodimer T cell receptors, with the γ/δ heterodimer typically found in the lamina propria and intra-epithelial lymphocytes (Eichelberger et al., 2000). When researchers attempted to deliberately infect mice deficient in either α/β or γ/δ heterodimer receptors, they discovered that both receptors were not strictly required for resistance to infection, a result attributed to compensation by other immune mechanisms (Eichelberger et al., 2000). Following infection with *C. parvum*, γ/δ T cell levels increased, upregulating the release of chemoattractants to draw immune cells, such as macrophages, to the infected tissue (Adjei et al., 2000; Eichelberger et al., 2000). Perryman et al (1994) confirmed the role of $CD4^+$ in infection

clearance by administering spleen cells, bone marrow cells and thymocytes, all of which have high levels of $CD4^+$, all of which reduced infection. However, when these same cells were depleted of $CD4^+$ and administered, there was no improvement in the ability to clear or reduce the infection.

Aguirre et al (1998) reported that IFN-γ inhibition in infected mice prolonged infection, and increased oocyst shedding, although the animals were eventually able to resolve the infection. Other researchers, using both IFN-γ knockout mice or inhibitors of IFN-γ, found that a lack of IFN-γ increases the susceptibility to infection, with a 20-fold increase in parasite burden and increased mortality rates (Griffiths et al., 1998; McDonald and Bancroft, 1994) This agrees with evidence presented by Clinton et al (2000), which indicates that IFN-γ and IL-12 are both critical to controlling murine cryptosporidiosis. The exact mechanism by which IFN-γ acts has yet to be elucidated, although it is known to have a wide array of potentially beneficial actions. These can include activating macrophages, and increasing the Major Histocompatibility Complex (MHC) class II expression, NOS activity in macrophages, natural killer (NK) cell activation, neutrophil adhesion, and increased B cell activity (Clinton et al., 2000; Aguirre et al., 1998; Urban et al., 1996). The only two sources of IFN-γ are T-lymphocytes and NK cells, indicating some type of feedback mechanism linking IFN-γ and NK cells (Hayward et al., 2000).

It has also been demonstrated that the administration of IL-12 has a protective effect against *C. parvum* infection, if given at the appropriate time (Urban et al., 1996). Injection of IL-12 simultaneous with administration of an

infectious dose completely prevented the development of cryptosporidiosis, by inducing a IFN-γ dependent immune response; immediately following administration, IFN-γ mRNA increased 20-fold (Urban et al., 1996). By inhibiting IFN-γ production, the researchers were able to clearly demonstrate that IL-12 acts indirectly via IFN-γ to prevent infection.

It is currently believed that soluble antigens released from the organism stimulate a mucosal immune response, including the release of IgA antibodies (Adjei et al., 1999). Twelve days after deliberate infection, anti-*C. parvum* IgA levels increased, and remained elevated until day 24 (Toyoguchi et al., 2001). Tarazona et al (1998) reported that IgA depletion in mice leads to more severe diarrhea and increased susceptibility to infection. This IgA response may be induced by any of the contents of rhoptries, micronemes, or dense granules, all of which secrete their contents during the infectious process (Toyoguchi et al., 2001).

The typical host responds to infection by a multifactorial mechanism. Following infection, the mucosa becomes inflamed, and neutrophils and macrophages are attracted to the lamina propria by the C-X-C chemokines, in particular Il-8 and GROα that are produced following *C. parvum* infection (Laurent et al., 1999). Like many other responses, the magnitude of chemokine release is proportional to the inoculum size (Seydel et al., 1998; Laurent et al, 1997). Adjei et al. (2000) speculate that γ/δ T-cells activate the macrophages attracted to the site of infection. The basolateral secretion of IL-8 is limited to

those epithelial cells infected by *C. parvum*, and is not induced by either cell lysis or the simple presence of cellular contents of infected cells (Laurent et al., 1997).

Another potential mechanism for the clearance of *C. parvum* infection is CD40L, a TNF-like factor transiently expressed on T-cells after activation. Cosyns et al (1998) reported that CD40L (the natural ligand to CD40) and CD40 are required to clear the pathogen, using at least one of two potential mechanisms. First, the binding of CD40L to CD40 presented by macrophages could contribute to T-cell activation. Second, binding could be a means of signal delivery between T-cells and antigen bound $CD4^+$ cells, allowing them to more effectively assist B-cells. Later work, by Lukin et al (2000), appears to support both theories. CD40 transduces signals promoting isotope switching in B-lymphocytes, and increases the expression of adhesion and accessory molecules on macrophages (Lukin et al, 2000). However, further work is needed to better elucidate the mechanisms of action and specific roles in the clearance of *C. parvum.*

VACCINE: To date, no effective vaccine has been developed for *C. parvum* infection, although there is some promising research that indicates that it may be possible. Lyophilized *C. parvum* oocysts decrease the duration of the diarrhea and oocyst shedding Harp and Goff (1995). Unfortunately, the mechanism by which this was accomplished could not be elucidated. However, a later study by the same researchers, using a variety of potential immunogenic treatments, including the same lyophilized *C. parvum* organisms were ineffective in affecting the disease (Harp et al., 1996). This latter trial was a clinical trial, and

the researchers speculated that, due to the ubiquitous nature of this pathogen, it is entirely likely that the calves were infected before they could be vaccinated (Harp et al., 1996).

Another interesting finding which provides support for the possibility of discovering a *C. parvum* vaccine, is the fact that intestinal scrapings from adult rats transfers resistance to infection in infant rats (Atkill and Harp, 2000). Irradiation, sufficient to kill any intestinal bacteria in the scrapings, did not interfere with this ability to confer resistance, although boiling the tissue removed any protective effect (Atkill and Harp, 2000). The scrapings of adult cattle, but not previously infected calves, had the same protective effect in rats (Atkill and Harp, 2000). It remains to be seen what, if any, tissue component can be isolated that confers this protection on young tissue.

Cryptosporidium parvum, a widespread enteric pathogen, causes severe, watery diarrhea in both humans and calves. This pathogen acts in a myriad of ways to infect the intestine and cause damage. As of yet, no effective vaccine or antibiotics exists, which has led, in part, to the search for the most efficacious treatment regime.

Glutamine

Many intestinal injuries result in the need for the administration of oral rehydration solutions; in fact, a common treatment for cryptosporidiosis in calves is the provision of a glucose-based electrolyte solution. However, the loss of glucose transporters, and the absence of a sodium-absorptive response following glucose administration, suggests that an alternative base for the solution may be more efficacious. For a number of reasons, such as the stimulatory effect on sodium absorption in injured tissue and the proliferative responses by injured epithelium, it has been postulated that a glutamine-based solution may be ideal for rehydration following damage.

Two mechanisms account for glutamine-mediated stimulation of sodium, and thus fluid, absorption- one electrogenic and one electroneutral. The first is a relatively simple mechanism; glutamine stimulates the activity of a Na-glutamine tranporter (ASC) that is electrogenic in nature. The second mechanism is subsequent to glutamine uptake via ASC. Once absorbed, the five-carbon amino acid is metabolized to CO_2 and NH_3. Then, having altered intra-cellular levels of these two compounds, particularly CO_2, the enterocyte uses intracellular H_2O to convert CO_2 to HCO_3^- and H^+ via a carbonic

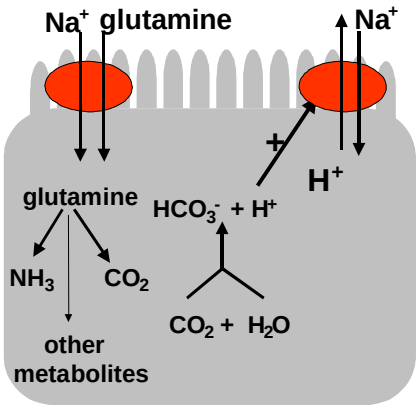

Figure 3. Graphical representation of the mechanism by which glutamine stimulates electroneutral sodium transport.

anhydrase mediated reaction. This alteration in intracellular H^+ levels stimulates NHE activity, thus accounting for electroneutral sodium absorptive responses (Figure 3).

Glutamine Background: Glutamine is the most abundant amino acid in blood and muscle tissue, as well as in the total free amino acid pool (Ersin et al., 2000; Wernerman and Hammarqvist, 1999). Combined with glutamate, GLN comprises 30% of the amino acids in meat, and by itself represents approximately 50% of the free amino acid pool in the body (Rhoads et al., 1990). Amongst its various functions, many of which will be discussed below, are promoting intestinal growth, improving gut healing following injury, decreasing bacteremia, increasing T-cell levels, increasing DNA synthesis, ureagenesis and villous atrophy (Ersin et al., 2000; Moinard et al., 1999). Due to their roles in ureagenesis, GLN and alanine represent 70% of all amino acids transported from the periphery to the viscera. Perhaps the most important function of GLN, however, is its role as the preferred fuel for any rapidly dividing cell, such as lymphocytes, macrophages, and enterocytes (Coeffier et al., 2001).

Although not defined as an “essential” amino acid, in that the body can typically synthesize sufficient amounts to meet its minimum requirements, certain tissues do appear to have a “requirement” for glutamine. One explanation is the low levels of glutamine synthetase activity in the enterocytes, which typically extract 20-30% of the circulating glutamine (Yang et al, 2000). In the enterocyte, glutamine plays a major role in stimulating intestinal Na^+ absorption, acts as a preferential energy substrate, and is involved in the metabolic processes of

intestinal repair (Abely et al., 2000). Plauth et al (1999) indicated that the absence of glutamine did not result in protein catabolism, as measured by leucine release, the typical response to amino acid deficiency. However, the researchers reported functional impairment and decreased intestinal viability following the removal of glutamine from the diet. Additionally, enterocyte glutamine synthetase appears to play no role in the provision of endogenous glutamine to the epithelial cells (Plauth et al., 1999). Due to the fact that glucose consumption increased by up to a factor of 7.2, there was no alteration in energy usage by the cell, so glutamine was providing a substantial portion of the cells energy requirement. Additionally, glutamine appeared to be heavily involved in the transfer of amino groups during nucleotide biosynthesis and other anabolic pathways, ureagenesis, and the production of arginine and citrulline (Plauth et al., 1999).

Glutamine Absorption: Glutamine is transported by several different systems, including ASC, y^+L and b^{o+}. Of special interest here is the role of ASC, which has been shown to be upregulated in the crypts of C. parvum infected calf tissue (Blikslager et al., 2001). One means of monitoring glutamine uptake is the administration of competitors for ASC and b^o, such as Ala, Ser, and Thr (Pawlik et al., 2000). ASC is apparently regulated by protein kinase C (PKC); following induction by phorbol esters, PKC has an inhibitory effect on glutamine uptake, which is post-translational in origin (Pawlik et al., 2000).

The sodium-independent b^{o+} transporter is specific for neutral and cationic amino acids, including GLN (Rajan et al., 2000). It has two subunits, only one of

which (rBAT) has been identified and characterized. The y^+L transporter is a sodium-independent transporter for cationic amino acids; however, it is sodium-dependent for neutral amino acids such as GLN (Rajan et al., 2000). Like b^{o+}, y^+L has two subunits, a heavy chain cell surface antigen (4F2) and a chain identified as LAT1, which does not appear in the small intestine (Rajan et al., 2000). However, a similar chain linked to 4F2 has been identified in the small intestine, and tentatively named LAT2. For both b^{o+} and y^+L, maximal transport requires the presence of both subunits.

Glutamine can be absorbed as either a single amino acid or as a di- or tri-peptide. One indicator of transport is the alteration in short circuit current (I_{sc}) which is indicative of electrogenic, Na-dependent transport, although I_{sc} can also be due to the movement of chloride ions. For example, Winckler et al (1999) demonstrated that the dipeptide Gly-GLN results in a large increase in I_{sc}, and, furthermore, that this uptake was by a transporter distinct from that used by either glucose or glutamine alone. It appears that, although approximately 20% of the dipeptide was hydrolyzed and absorbed separately, the dipeptide transporter has a single binding site for one of the two amino acids and transports it based on that linkage (Winckler et al., 1999).

Glutamine and Sodium Absorption: Glutamine has long been implicated as one of the primary substrates that is co-transported with sodium into enterocytes. However, it was not until recently that it was seen that GLN administration could stimulate the absorption of sodium. This inductive effect could play a key role in the treatment of diarrheal diseases. Due to the fluid loss

accompanying diarrhea, a primary concern to the health of the animal is maintaining adequate hydration. The most common treatment for diarrheal illness, in both humans and animals, is the administration of rehydration solutions, which are usually glucose based. However, should glutamine prove more efficacioius than glucose in stimulating sodium uptake, a main driving force for water absorption, it would have important clinical ramifications.

Rhoads et al (1991) conducted a study to clarify the role of glutamine in sodium absorption, using a rotavirus infected piglet model. Treatment with GLN increased electroneutral sodium uptake, in contrast to the electrogenic sodium uptake generated by glucose administration or alanine (ALA). Furthermore, GLN and ALA were equally proficient in stimulating sodium transport, albeit by separate mechanisms.

Using a similar model, cryptosporidial piglets, Argenzio et al (1994) demonstrated that the administration of glutamine to healthy ileal tissue generated an increase in the electrogenic absorption of sodium. However, when this same treatment was applied to infected tissue, the researchers noted an increase in both electrogenic and electroneutral sodium absorption, with the electrically silent movement responsible for the greater part of the sodium uptake. However, this latter mechanism is inhibited by the presence of prostaglandins, which will typically be elevated during the course of infection; the response of infected tissue to glutamine administration was greatly amplified when prostaglandin production was inhibited (Argenzio et al., 1994).

Glutamine Metabolism: One of the keys to understanding the role of glutamine in the intestine, especially when considering potential beneficial effects following injury, is to clearly understand the metabolism of glutamine in the enterocyte. Perhaps the most lucid study conducted on this topic was by Abely et al (2000) who used rabbit ileal tissue to determine the fate of GLN in both undamaged tissue, and tissue damaged by cholera toxin. In healthy tissue, 48% of the glutamine is metabolized to other compounds, such as glutamate, ornithine, polyamines and alanine, for use within the cell. Additionally, intact glutamine (the remaining 52% of absorbed glutamine) and any metabolites can be completely transported across the tissue and delivered across the basolateral membrane. Within the enterocyte, 34% of the glutamine is completely oxidized to CO_2 during the course of energy production. In cholera toxin-infected tissue, glutamine generated a similar response, with a slight increase (51%) in the amount being metabolized within the enterocyte. Furthermore, Abely et al (2000) determined that 95% of the energy produced during the oxidation of glutamine was still available to the enterocyte, and was not released across the basolateral membrane. Of key interest to the studies conducted by the author, glutamine induced a 3.5-fold increase in electrogenic transport, compared to electroneutral mechanisms.

Yang et al (2000) also studied the fate of GLN being metabolized by the enterocyte for energy production. Once absorbed and de-aminated, GLN is metabolized to α-ketoglutarate and enters the TCA cycle, yielding 30 mol ATP/mol GLN (Yang et al., 2000; Wernerman and Hammarqvist, 1999). During

the course of this study, the researchers determined that glutamine addition increased the absorptive surface of the bowel. One finding indicating the importance of dietary glutamine is the enterocyte response following addition of glutamine to either side of the polarized cells. Glutamine added to the mucosal side only caused a significant decrease in the enterocyte ATP levels; however, addition to both serosal and mucosal sides of the cells maintained the ATP level within the cell.

Kong et al (2000a) reported that 46% of the CO_2 released from the jejunum is provided by glutamine metabolism. Following starvation, the activity of glutaminase decreased in the jejunum, but not the ileum. The glutaminase enzyme converts glutamine to glutamate, ammonia and water. Of the two glutaminase isoforms, a hepatic type and a kidney type, the kidney type is found in the intestine, where it regulates both enterocyte metabolism and nucleotide synthesis (Kong et al., 2000a). Glutamine synthetase levels increased during starvation, indicating alterations in de novo synthesis to meet the enterocyte requirement, although villi atrophy and decreased mucosal protein and DNA content would lower requirements.

Total parenteral nutrition (TPN), while not exactly starvation, does mimic many of the effects of a lack of digesta, such as decreased villous height, mucosal weight, protein and DNA content. Addition of a glutamine supplement to the TPN solution increases glutaminase activity in the intestine, while minimizing villous atrophy (Kong et al., 2000b). This suggests that the enterocyte is able to

meet at least some of its needs by uptake of glutamine across the basolateral membrane.

Plauth et al (1999) studied the effect of either luminal or vascular GLN administration on utilization of glutamine by the enterocyte and determined that both routes of delivery were equally efficacious. Vascular glucose administration greatly decreased GLN utilization, leading researchers to conclude that up to 35% of the glutamine that can potentially be absorbed is used for fuel (Plauth et al., 1999). One key result is the observation that the production of glutamine metabolites (particularly alanine, citrulline, ammonia) is not altered by the route of delivery, indicating a single production pool. Additionally, when the same amount of GLN was added simultaneously to each side of the cell, metabolite output doubled (Plauth et al., 1999). However, the interaction between the two sites of administration is unknown. The vascular administration of glutamine may have resulted in greater metabolism of the apically delivered glutamine by sparing its use for energy production.

Another means of determining the role of glutamine is the incubation of cells in glutamine-free media. LeBacquer et al (2001) reported a strong correlation between free glutamine concentrations and protein synthesis, although other evidence indicates otherwise. In Caco-2 cells, there is a high rate of de novo glutamine synthesis, as even when incubated without glutamine in the media, the cells maintained their intracellular glutamine concentration and the normal rate of protein synthesis. However, one interesting observation made by LeBacquer et al (2001) was the relative levels of glutamine synthetase and

glutaminase. In these cells, glutamine synthetase activity was 152-fold less than that of glutaminase, suggesting that in vivo, the tissue would be unable to meet the demand for glutamine without exogenous amino acid being present.

Intestinal Proliferation: Glutamine has been shown to play an important role in epithelial proliferation after injury. The effect may be the result of glutamine providing fuel to the enterocytes, or its role in DNA, RNA, or protein synthesis. Another possibility is by serving as a precursor to polyamines, which have also been shown to induce proliferation.

Perhaps one of the most well-studied cell lines in intestinal physiology, Caco-2 cells have been used in a variety of studies to determine the effect of glutamine on cellular proliferation. Murnin et al. (2000) used this cell line in several studies, one of which was an attempt to determine the effect of both L- and D-glutamine on cellular parameters. The addition of either stereoisomer accelerated cellular proliferation. Unfortunately, since the authors were more interested in simply determining if D-glutamine would be as effective as the L-isomer, there was no exploration of potential mechanisms by which this might occur. Possible mechanisms include simple energy provision to the cell, or conversion of glutamine to polyamines necessary for nucleotide production.

Wiren et al. (1998) also conducted a study to elucidate the effects of glutamine on the Caco-2 line, as well as the HT-29 cell line, both of which are derived from colon cancer cells. The main avenue of study was the effect of glutamine on growth response in the cells, in comparison to other energy substrates, to determine if the response was due to the function of glutamine as

an energy source. As might be expected, the lack of all energy sources resulted in inhibition of cellular multiplication; additionally, treatment of cells with glutamine but without glucose, also resulted in inhibition. However, when the cells were provided with adequate glucose, there was a significant increase in cellular proliferation following glutamine addition (Wiren et al., 1998). The authors reported that at no time was any cellular proliferation observed in the absence of glutamine, indicating a functional requirement for this amino acid. While the results of this study make it clear that glutamine is required for stimulation of cellular proliferation, there are several flaws in the design that lessen the impact of the results. For example, Wiren et al. (1998) reported that glutamine failed to replace glucose as an energy source, since it did not stimulate cell proliferation in its absence. However, the levels added to the cells were most likely inadequate to function both as an energy source and as a stimulant to cellular proliferation. Another valuable piece of information that was lacking was the effect of the glucose free solution on the cells. For instance, the authors reported only that there was inhibition of cellular proliferation in the absence of glucose. However, one might expect that in the total absence of an energy source, there would be some cell death occurring, a fact that was not discussed.

Another means of investigating the effects of glutamine on intestinal morphology is to treat patients with glucocorticoids, which induce a catabolic state (up to 25% whole body proteolysis during a fast) that mimics pathological stress. Bouteloup-Demange et al. (2000) used such a model to investigate the effects of enteral glutamine administration on intestinal protein synthesis, with the

expectation that it would increase intestinal protein synthesis. Following cortisol injection, glutamine addition had no significant effect on protein synthesis; however, there was a strong trend (P=0.058) towards significance. In evaluating these results, several factors must be considered. Bouteloup-Demange et al. (2000) used two different tracers to monitor protein synthesis- phenylalanine and leucine. While the difference between the glutamine and glutamine-free feeding regime only approached significance, there was a significant effect seen between the tracers, indicating some variability in the system, with leucine resulting in higher synthesis rate calculations. Comparison of this study to other similar studies conducted in healthy patients not receiving cortisol injections revealed a protein synthesis rate two to three times higher than in human patients not treated with cortisol. This result makes it more imperative that the researchers should have included control groups. Since cortisol is a proteolytic stimulant, the addition of glutamine should not have increased protein synthesis above that seen in non-treated humans. However, the increase in protein synthesis seen here does have potential as a stimulant to intestinal growth, which obviously would not occur without protein being manufactured.

Zhou et al (2001) used another common means of studying potentially intestinotrophic mechanisms- intestinal resection. Despite 85% resection, the administration of glutamine and somatotropin resulted in mucosal hyperplasia and improved absorption. Most importantly, despite the severity of the resection, the treatments actually resulted in increased body weight. Glutamine alone was insufficient to induce these beneficial responses; but glutamine and somatotropin

together improved intestinal characteristics beyond levels achieved by somatotropin alone, suggesting a synergistic mechanism (Zhou et al., 2001).

Various growth factors (ie. EGF and TGF-α) have been shown to possess potent mitogenic effects, as well as stimulating cellular migration. Ko et al. (1993) conducted an in vitro study to determine the essentiality of glutamine for the stimulatory effects of EGF on intestinal cells. The first component of the study determined that EGF requires glutamine for its mitogenic response, due to the complete lack of stimulation of DNA synthesis (as shown by measurements of tritiated thymidine uptake) when EGF was added alone. However, the addition of glutamine completely restored the stimulatory effects of EGF on DNA synthesis. A glutamine treatment, with no EGF, insured that the response was not due solely to glutamine; it was evident that both are required to stimulate DNA synthesis, as well as RNA and protein synthesis. One important observation was made about the expression of early growth response genes. EGF alone resulted in the upregulation of these genes (zif268, jun-B, cmyc); this effect was also observed when both EGF and glutamine were added, but the duration of the response was much shorter. In fact, EGF alone prolonged the elevation in expression for 2 hours longer than did both compounds together. This is actually a key result, as the early response gene mRNA typically has a very short half-life (15-30 minutes). It would appear, based on the results of Lau and Nathans (1987), that if protein synthesis is inhibited or interfered with, the mRNA will persist for a much longer period. From these results, it can be theorized that the importance of glutamine on the EGF response is primarily

involved in protein synthesis, and not due to its role in energy metabolism. Importantly, the maximal responses to glutamine all occurred in the physiologically normal range for plasma glutamine levels (.42-.68 mmol/L) (Ko et al., 1993).

Following the study by Ko et al. (1993) it was speculated that perhaps TGF-α would generate a similar response. Both TGF-α and EGF have a 35% homology, as well as activating the same receptor, but TGF-α is produced locally in the small intestine (Blikslager et al., 1999). To determine the effect of concurrent administration of glutamine and TGF-α, Blikslager et al. (1999) used weaned pigs to monitor the course of intestinal recovery following 2 hours of ischemic damage, and the effect of glutamine on intestinal morphology in Thiry-Vella loops. Glutamine treatment maintained normal villi morphology for 10 days, while the glucose treatment resulted in villous and crypt atrophy. The addition of both glutamine and TGF-α to ischemic injured tissue resulted in a significantly higher villous surface area when compared to glutamine, glucose or glucose plus TGF-α, strongly indicative of an increased rate of intestinal repair.

Although the intestinotrophic effects of GLN have been well-documented, little has been done to determine potential mechanisms by which this response occurs. Rhoads et al (2000) compared the mechanisms by which glutamine and EGF stimulate intestinal proliferation and determined that mitochondrial glutamine is required for stimulation of proliferation. EGF and glutamine, both of which increase thymidine incorporation, act via a cAMP inhibitable, extracellular signal related kinase (ERK) pathway. However, despite these similarities, these

two compounds do not use identical intracellular signaling pathways to generate their response. The main difference being that EGF uses a Raf-dependent mechanism, whereas glutamine is Raf-independent.

Antibacterial Function: Other researchers have pursued an avenue of research indicating that glutamine has an anti-microbial effect in tissue. For instance, Foitzik et al (1999) demonstrated that in animals with pancreatitis, glutamine addition stabilized gut permeability and decreased the translocation of the enteric bacteria into the pancreas. However, the researchers were unable to develop a putative mechanism by which this occurs. The most likely explanation is that glutamine stimulates epithelial cell differentiation and proliferation, and may act by restoring villi morphology.

One interesting fact, that appears to belie the anti-microbial aspect of glutamine administration, is its effect on cytokine release. In patients with inflammatory bowel disease (IBD), glutamine reduced IL-8 levels by up to 60%, with a lesser reduction of IL-6 (Coeffier et al., 2000). Due to the lack of response in mRNA, this effect appears to be post-transcriptional in nature. However, Coeffier et al (2000) were unable to determine either a mechanism by which glutamine reduces cytokine levels, or a plausible reason for this reduction.

The role of glutamine in the immune system is further implicated by the finding that hypoglutaminaemia contributes to the impairment of immune function (Moinard et al, 1999). Researchers have been able to demonstrate that in vitro glutamine administration increases the phagocytic capability of macrophages, and theorized that glutamine improves phagocytosis and increases the

respiratory burst by meeting the increased demand for energy during both activities. This would appear to be a likely explanation, due simply to the fact that glutamine is the preferred energy substrate for macrophages. Interestingly, both arginine and ornithine-2-oxoglutarate, a precursor to glutamine and arginine, increased TNF-α release from the macrophages. This could potentially be a concern to treatment of cryptosporidiosis with glutamine based oral rehydration solutions, since TNF-α is heavily involved in macrophage activation and pro-inflammatory cytokine production, with the attendant side effects, which will be discussed elsewhere (O'Shea et al., 2002; Moinard et al., 1999).

Glutamine and Apoptosis: Intestinal growth is a balance between cellular proliferation, cellular migration, apoptosis, and epithelial sloughing. Therefore the beneficial effect of glutamine on growth obviously involves at least some of these characteristics. Based on evidence indicating that glutamine starvation resulted in enterocyte apoptosis, Papconstantinou et al. (2000) used rat intestinal epithelial cells to further elucidate the potential mechanisms by which this would occur, specifically, the role of caspases. There are four families of caspases, of which the researchers selected one from each to monitor during the course of glutamine denial. The researchers observed that starving the cells of glutamine increased the activity of caspase 2 and 3, while not affecting caspase 1 or 8, a finding confirmed by adding caspase inhibitors, which abolished the apoptotic effect of glutamine starvation. The authors speculated that since glutamine is a primary metabolic fuel for the enterocytes, glutamine deprivation could result in damage to the mitochondrial membrane. This may

allow the release of cytochrome c from the mitochondria to the cytoplasm, where it has been seen to play a role in caspase-induced apoptosis (Papaconstantinou et al., 2000; Soeda et al., 2001). Limitation of apoptosis, by providing adequate glutamine could conceivably induce a mild growth response. This could also play a role in enteric infections, such as *Cryptosporidium parvum*, which has been shown to induce apoptosis (McCole et al., 2000). Due to the accompanying diarrhea and nutrient malabsorption, it is possible that the enterocytes may be glutamine deficient, allowing for an easier upregulation of apoptosis by the pathogen.

Electroneutral Transporters

There are a number of electroneutral transport mechanisms, such as sodium/hydrogen exchangers, sodium/bicarbonate co-transporters, and chloride/bicarbonate exchangers. Of particular interest here are the sodium/hydrogen exchangers, which appear to be stimulated in part by glutamine addition, as described above, due to their potential role in sodium and fluid absorption following intestinal damage. These exchangers can, variously, function as vectorial sodium transporters, or as environmental regulators, adjusting intracellular pH and volume. The isoforms located predominately in the intestine, NHE-3 and NHE-2 will be the focus of the following study.

Physical Characteristics: The sodium/hydrogen exchanger family (NHE) is one of the primary means of moving sodium across membranes, both cellular and mitochondrial. Six isoforms of this glycophosphoprotein have been isolated thus far, and share between 45 and 70% amino acid homology, in addition to many of the same physical characteristics (Biemesdorf et al., 1998; Orlowski and Grinstein, 1997). However, the exchanger of concern here, NHE-3, is the most differentiated, least related, isoform identified as yet (Wakabayashi et al., 1995).

The transporters have two structural domains, and the hydrophobic N-terminus has 10-12 membrane spanning α helices, while the hydrophilic C-terminus has no α helices (Biemesdorf et al., 1998). Sodium transport is mediated by the N-terminal domain, with the C-terminal end interacting with cytosolic proteins. The membrane spanning regions M3-M12 have great identity among the isoforms, with M6 and M7 being the most highly conserved (95%)

(Orlowski and Grinstein, 1997; Levine et al., 1995). Consequently, most researchers believe the actual sodium/hydrogen exchange occurs in region M6 and M7. Researchers have been able to determine that amino acid residues 636-656 are high affinity calmodulin binding sites, and residues 579-684 are vital for protein kinase A inhibition of NHE-3 (Biemesdorf et al., 1998).

The exchangers appear to exist in the plasma membrane as homodimers, never as heterodimers (Farfournoux et al., 1994). Evidence indicates that within the homodimer pair, the subunits may function separately; it is unknown if the pair can operate independently. NHE-3 is an 85 kDa protein, and functions independently of NHE-2 (ie. an alteration in one does not stimulate a change in the other)(McSwine et al., 1998; Wormmeester et al., 1998).

One means of differentiating between the exchangers is by their susceptibility to various inhibitors, such as amiloride (and its analogues) or cimetidine (Orlowski and Kandasamy, 1996). Generally speaking, the sensitivity is usually NHE-1 > NHE-2 >> NHE-3 > NHE-4 > NHE-5 and -6, although with recent advancements in the understanding of their structure and control, isoform specific inhibitors are being developed (Orlowski and Grinstein, 1997). Differences in drug sensitivity appears to be primarily linked to the M9 transmembrane domain, although the other domains play a role (Orlowski and Kandasamy, 1996).

Function: One of the primary functions of electroneutral exhangers is to regulate the internal environment, such as cell volume or pH. These effects are in addition to their role in nutrient, electrolyte and fluid absorption. Amongst the

NHE isoforms, the different transporters have different roles. NHE-1, which is ubiquitous on basolateral membranes, plays a major role in intracellular environmental regulation. NHE-2 and NHE-3, of which NHE-3 is the only one found in the calf, are both responsible for vectorial Na transport. In fact, in the rat, up to 98% of Na absorption is contributed by NHE-3 activity (Kiela et al., 2000).

The six isoforms vary in their expression between both tissues and membranes, and amongst species. For instance, in dogs, all basal ileal Na absorption is due to NHE-3, while in rabbits, sodium absorption is split evenly between NHE-2 and NHE-3 (Janecki et al., 1998). Further, NHE-3 activity changes as the animal ages; for instance, as the intestine matures the glucocorticoid surge appears to induce NHE-3 expression (Kiela et al., 2000).

A chemical gradient causes the flux of sodium and hydrogen across NHE a process that does not require ATP to function (Orlowski and Grinstein, 1997). However, ATP is required for maximal transport activity, perhaps by influencing the affinity of the exchanger for intracellular hydrogen.

One interesting fact about NHE was determined by Repishti et al (2001). Following inhibition of NHE-3 in duodenal tissue, the researchers determined that the tissue responded by significantly increasing net duodenal HCO_3 secretion. This is perhaps due to the increase in extracellular/luminal Na, which would electrochemically attract Cl from the cell via CFTR channels. However, Cl/HCO_3 exchangers would be activated by increases in luminal Cl concentrations, thus secreting HCO3- and returning Cl to the enterocyte. However, this is only

speculation; no definitive explanation has yet been offered. Repishti et al (2001) suggested that if the gradient of either Na and HCO_3^- could be increased by enough in the “wrong” direction, the NHE isoforms would reverse their direction of transport. Essentially, the transporters would then excrete Na and absorb H, instead of the opposite, “normal” activity.

Due to their roles in regulating transcellular movement of fluid and nutrients, all the NHE isoforms play a substantial role in determining transepithelial resistance (TER). Turner et al (2000) demonstrated that NHE inhibition increases TER, a response that was not due to cellular swelling and subsequent collapse of lateral intracellular spaces.

Membrane Recycling: Much evidence indicates that NHE-3 is transported to and from the membrane in response to changing intracellular conditions. Janecki et al (2000) reported the existence of three intracellular locations for NHE-3- the plasma membrane, juxtanuclear accumulation vesicles, and a population of particles spread throughout the cytoplasm. Kurashima et al (1997) demonstrated that approximately 90% of the transporters are found in intracellular membrane vesicles in the juxtanuclear accumulation complex (JAC), and cycle between these endosomes and the plasma membrane. In contrast, Janecki e tal (2000) determined that only 75% of the NHE-3 resides in the JAC, and Cavet et al (2001) reported that 85% was intracellular. Kurashima et al (1998) further determined that the half-life of NHE-3 in the membrane is 15 minutes, indicating a rapid response to environmental changes. Further, Cavet et al (2001) reported the half-life of NHE-3 to be 14 h, allowing for frequent

movement of the exchanger in and out of the membrane. It appears that phosphatidyl inositol-3 kinase (PI3-K) acts as a chaperone, transferring the exchanger to the membrane during exocytosis, a process blocked by the PI3-K inhibitor wortmannin (Kurashima et al., 1998).

Endocytosis appears to be mediated by clathrin coated vesicles (Hu et al., 2001; Chow et al., 1999). These vesicles, which require intact dynamin to form, deliver the exchanger to the intracellular pool; as discussed below, a number of factors stimulate this process, thus inhibiting NHE-3 activity.

Regulation: A number of both intra- and extra-cellular components have regulatory effects on the activity of NHE-3. Although there are six isoforms, they can be regulated individually, due to differences in their cytoplasmic tail (Aharonovitz et al., 2001). Janecki et al (2000) found that one activator was fibroblast growth factor (FGF), which stimulated exocytosis from the JAC to the plasma membrane. The researchers hypothesized that this response was PI3-K mediated, although no evidence was supplied to support this theory.

Similar to FGF, epidermal growth factor (EGF) has also been implicated in the stimulation of NHE-3 activity. By acting on ileal basolateral membrane receptors to increase the amount and activity of PI3-K, EGF apparently mediates the exocytosis of NHE-3 into the membrane (Khurana et al., 1996). Monitoring the movement of radiolabeled Na and Cl revealed that EGF induces an increase in mucosal to serosal flow of both isotopes, inducing a net increase in absorption. These results may help explain those discussed above, that glutamine and EGF together stimulate an increase in electroneutral sodium flux. Within the

enterocyte, glutamine is metabolized to CO_2 and H_2O, increasing HCO_3^- and H^+ by simple Henderson-Hasselbach mechanics, which drives a decrease in intracellular pH. This environmental alteration results in the insertion of the exchanger into the membrane. In concert with the effect of glutamine, EGF will stimulate the activity of PI3-K, to increase the movement of NHE-3 from the JAC to the plasma membrane.

RhoA also upregulates NHE-3 activity. This intracellular mediator is required for optional functioning of the NHE-3, by activating p160 Rho-associated kinase I (ROK), which in turn phosphorylates myosin light chain proteins (Szaszi et al., 2000). Although this is a demonstrable effect, the downstream mechanism has yet to be elucidated. Possibly the end result is phosphorylation of the protein cofilin, which induces actin depolymerization. Without this inhibitory effect, actin assembly greatly increases, which may alter NHE-3 activity, most likely in a mechanical fashion.

Another possible hormonal stimulator of NHE-3 activity is aldosterone, which increases in response to systemic sodium depletion (Ikuma et al., 1999). Sodium depletion has been shown to have a significant effect on NHE-3, increasing its activity, protein and mRNA levels in the proximal colon, all of which combine to produce a 2-fold increase in NHE-3 activity. The NHE-2 isoform appears to be more sensitive to sodium depletion, demonstrating a 7-fold increase in activity. However, despite the definite response of NHE-3 activity to sodium depletion, Ikuma et al (1999) did not present any data to substantiate the role of aldosterone in this response.

In contrast to the stimulatory effect of sodium depletion, chloride depletion plays an inhibitory role in the regulation of NHE-3 (and NHE-2). Optimal NHE activity is halide dependent; further, it is the intracellular chloride concentration that is critical (Aharonovitz et al., 2001). This reduction in activity does not appear to be due to internalization of the exchanger, but instead may be in some way linked to the cell volume; as the cell decreases in size, NHE-3 activity decreases, without altering the amount of protein in the plasma membrane. This effect of chloride is linked to the cytoplasmic tail, since tail truncation removed the inhibitory effect caused by chloride depletion (Aharonovitz et al., 2001). Alterations in the cytoskeletal structure, due to the decreased cellular size may also play a role in the reduction in NHE-3 activity. As discussed elsewhere, the exchanger is intimately related to the actin components of the cytoskeleton; should morphological changes in the cellular framework be mechanically transmitted to the exchanger, it would likely alter its activity.

While the above are all basolateral effectors of NHE-3 upregulation, there are some dietary components that seem to induce exchanger activity. Short chain fatty acids (SCFA) induce a time dependent induction of NHE-3, reaching a maximal stimulation within 12 h (Musch et al., 2000). The SCFA response is seen regardless of the specific compound administered. For instance, butyrate, acetate and propionate all have an equal effect on the NHE-3 activity, and induce NHE-3 activity, mRNA synthesis and protein production. In fact, the effect appears to be metabolically independent, since isobutyrate, a poorly metabolizable SCFA, induces the same response. Given the sensitivity of NHE

isoforms to intracellular pH, it is highly likely that the effect was generated by cellular acidification induced by the absorption of SCFA, although other mechanisms can not be ruled out at this time.

Perhaps the most widely acknowledged inhibitor of NHE activity is cAMP, which can be induced by any number of compounds, including prostaglandins, which will be discussed below. Bookstein et al (1999) demonstrated a decrease in the V_{max} of NHE-3 induced by cAMP, which had, in turn, been stimulated by the addition of phorbol esters. Interestingly, phorbol esters also result in an inhibitory effect on glutamine uptake, as discussed above (Pawlick et al., 2000). A reduced V_{max} indicates a decreased number of transporters in the membrane, so this effect of cAMP may be an effect on PI3-K, although Bookstein et al (1999) did not elaborate on this. Zhao et al (1999) speculated that the cAMP effect is mediated by PKA phosphorylation of NHE-3. Most likely, cAMP induces several responses, all of which act to inhibit NHE activity.

Some inhibitory mediators, such as squalamine, require the cytoplasmic tail to operate. Squalamine is a broad-spectrum antibiotic that is bactericidal, fungicidal and osmotically lyses protozoa, such as *C. parvum*. Incubating tissue with squalamine decreases NHE-3 V_{max} by 47% without affecting either NHE-1 or NHE-2 (Akhter et al.,1999). The effect appears to be reversible, although the inhibition takes at least an hour to develop, since the squalamine apparently acts intracellularly, in contrast to other NHE inhibitors.

Interferon-γ, which is elevated in response to infection, particularly *C. parvum*, also has an inhibitory effect. Treatment of both rat intestine and human

intestinal cells with IFN-γ decreased NHE-3 activity, protein expression and mRNA expression (Rocha et al., 2001). The response took 2 h to begin showing an effect, and by 24 h had reduced the activity of the exchanger to 34% of control. The effect was time and dose dependent. More importantly, this effect was seen both *in vivo* (rat) and *in vitro* (human), and appeared specific to IFN-γ, since sucrase, alkaline phosphatase and villin levels were unaffected (Rocha et al., 2001). The researchers speculated that, based on the protein and mRNA expression data, the effect of IFN-γ was transcriptional in nature.

Another inhibitor of NHE-3 activity is parathyroid hormone (PTH), which acts much more rapidly than does IFN-γ, inhibiting NHE-3 activity within 5 minutes of administration. Collazo et al (2000) and Azarani et al (1996) found that administering PTH increased PKA levels via adenylate cyclase activity, which in turn phosphorylates NHE-3. Furthermore, the administration of PTH appears to stimulate the translocation of PKC from the cytosol to the membrane, where the active NHE-3 is located (Azarani et al., 1996). This has two effects. First, it immediately inhibits the activity of NHE-3; second, it results in the rapid internalization of the now inactive exchanger. Importantly, Collazo et al (2000) reported no changes in the total cellular concentration of NHE-3, indicating no alteration in transcription or production, simply an alteration in the activity and membrane localization of the protein. The phosphorylative inhibiton was reversible, as the inhibited NHE-3 later regained their functionality and returned to the membrane (Collazo et al., 2000).

The role of protein kinase C (PKC) in the inhibition of NHE-3 has not been fully explored, since most research has focused on the activity of PKA. However, PKC does play a role in the inhibition of NHE-3, by phosphorylating the protein, and then causing a redistribution of the exchanger into the subapical cytoplasmic compartments (later renamed JAC- juxtanucleaur accumulation complex)(Janecki et al.,1998). Internalization appears to account for 50% of the inhibitor effect, with maximum PKC induced inhibition occurring within 10 minutes of treatment.

One component of phosphorylative NHE-3 inhibition is the cofactor, sodium-hydrogen exchanger-regulatory factor (NHE-RF). Murthy et al (1998) speculated that NHE-RF mediates PKA inhibition of NHE-3, although this theory was based solely on the fact that NHE-RF links NHE-3 to the actin cytoskeleton. Further refinement to the role of NHE-RF was provided by Lamprecht et al (1998), who investigated the interaction between NHE-3, NHE-RF and E3KARP (NHE-3 Kinase A Regulatory Protein). These three proteins interact at the plasma membrane, and that NHE-RF and E3KARP do indeed link NHE-3 to the actin cytoskeleton. However, neither NHE-RF or E3KARP is phosphorylated by cAMP or protein kinases, nor do either have a cytoplasmic tail capable of acting as an A Kinase Anchoring Protein (AKAP) (Lamprecht et al., 1998). However, the NHE-3/NHE-RF/E3KARP complex does bind to ezrin, which links it to the actin cytoskeleton, and ezrin does have an AKAP, so it is possible that, in an indirect manner, NHE-RF does mediate PKA inhibition of NHE-3. In fact, this theory was confirmed by Zizak et al (1999), who demonstrated that the presence

of either NHE-RF or E3KARP is required for the inhibition of NHE-3 by either cAMP or PKA. The phosphorylation of NHE-RF is not required for this mediation; possibly, the role of NHE-RF is the induction of a conformational change in the NHE-3, making it more susceptible to phosphorylation.

As can be seen, a number of regulatory components either directly or indirectly involve the cytoskeletal framework of the cell. Kurashima et al (1999) attempted to clarify the involvement of the actin cytoskeleton in the regulation of NHE-3 by treating cells with cytochalasins, which are depolymerizing agents. Kurashima et al (1999) reported that depolymerizing the actin filaments resulted in a greatly inhibited activity of NHE-3. However, the inhibition was not due to endocytosis, since the number of transporters in the membrane was not altered. Therefore, it would appear that the relationship between NHE-3 and actin depends on conformational regulation of the exchanger. Additionally, Kurashima et al (1999) speculated that somehow the cytoskeleton regulates the micro-environment surrounding the transport site (or, alternately, the “sensors” which regulate its function), or somehow sequester an autoinhibitory domain, which would maintain the protein in its maximally active state.

Yet another means of inactivation of NHE-3 is by activation of the enteric system. Khurana et al (1997) added carbachol, a muscarinic-cholinergic agonist, and found that it inhibits NHE-3 activity. Further investigation elucidated a partial mechanism for this response. Carbachol activates a basolateral membrane cholinergic receptor, which upregulates PIP_2 specific PLC activity on the brush border by a translocation of PLCγ. Khurana et al (1997) speculate that the

inhibitory effect of PLC may be due to its association with villin, which interferes with actin binding, and may in fact promote actin cleavage. As described above, NHE-3 is intimately associated with actin, and the disruption of the cytoskeletal framework may interfere with the activity of the exchanger.

Rajendran et al. (1995) reported the putative existence of a novel NHE, unrelated to the six isoforms described above. Tentatively named Cl-NHE, (chloride dependent sodium/hydrogen exchanger), this transporter was reported to exist on the apical membrane of crypt cells. Using apical membrane vesicles, Rajendran et al (1995) demonstrated sodium uptake in crypts that appeared to be chloride dependent, H^+ gradient dependent, and amiloride sensitive. However, since amiloride inhibits all the known NHE isoforms, Na channels, and sodium/bicarbonate co-transporters, this in itself demonstrates little. The researchers compared cells isolated from the crypts, to those obtained from the villi tips, and determined that transporters populating the terminal villi were Cl-independent, whereas cells of crypt origin were dependent on the presence of Cl.

Rajendran et al (2001) attempted to clarify the exact nature of the novel Cl-NHE, one its primary characteristics being a relative resistance to amiloride existence, when compared to other NHE isoforms. The Cl dependence was attributed to several possible factors. The first, and simplest, is that an existing NHE isoform had become linked to a Cl transport protein, such as a Cl/anion exchanger (Cl/HCO3), CFTR (Cystic Fibrosis Transmembrane Region) or ORCC (Outward Rectifying Chloride Channel). A second explanation is the existence of

a completely novel transport protein, with both Cl transport and NHE activity. Rajendran et al (2001) reported that Cl-NHE requires the presence at least 5 mM Cl, but not a gradient to stimulates its activity. Since the Cl could be added to either side of the apical membrane vesicles, the researchers speculated that the Cl dependent component was a chloride channel, and not a Cl/anion exchanger.

Geibel et al (2001) further investigated sodium and fluid uptake in colonocytes, and determined that ethyl isopropyl amiloride (EIPA) inhibited both Na and fluid absorption. Further, active sodium absorption in the colonocyte is Cl-dependent. Importantly, for the results obtained by the author, removal of HCO_3 from the bathing solutions reduced fluid absorption, leading Geibel et al (2001) to speculate that the transport mechanism is HCO_3 dependent. During the course of the study, the researchers estimated that basal fluid secretion is 25-30% of the absorptive process.

Prostaglandins

A major consideration of any investigation of intestinal adaptation to injury, particularly infectious injury such as *C. parvum*, is the role of prostaglandins produced as a part of the inflammatory response. Prostaglandin elaboration by one of two cyclooxygenase isoforms has several beneficial, cytoprotective effects; however, accompanying these beneficial effects are numerous deleterious responses. Some of these effects have been discussed above, such as inhibition of electroneutral sodium transport activity; others will be discussed below. Of primary concern here is their cAMP-mediated inhibition of NHE-3

activity; this, coupled with the fact that non-selective COX inhibition restores sodium transport, has led to the investigation of potentially using selective inhibitors to restore transport activity, while maintaining the cytoprotective effects of prostaglandin production.

Production: Prostaglandins are ultimately derived from membrane phospholipids (Figure 4). Liberation of polyunsaturated fatty acids (PUFA) by phospholipase A_2 is the initial step; followed by conversion to arachidonic acid. The availability of these precursors is the rate-limiting step in the production of prostaglandins, a key fact since very little is stored by the cell. Cyclooxygenases, formerly named prostaglandin synthetase, then convert arachidonic acid to PGH_2. Prostaglandin H_2 is the precursor to all other prostaglandins, such as PGE_2, and PGI_2.

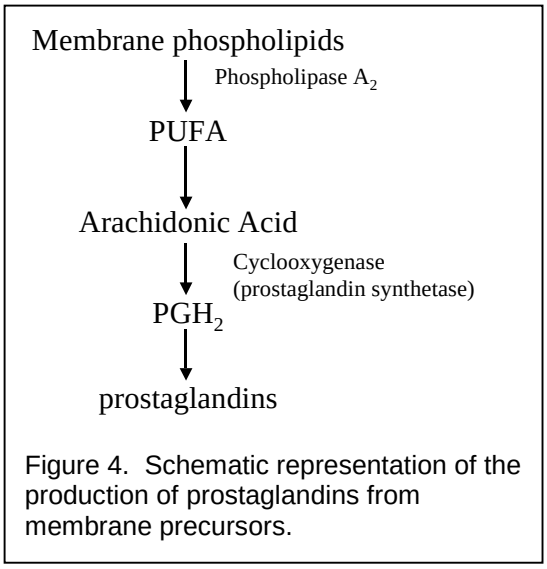

Figure 4. Schematic representation of the production of prostaglandins from membrane precursors.

History: Prostaglandins were first found to have cytoprotective abilities in the mid-1970s. This ability has been attributed to stimulation of both HCO3 and mucous secretion, alterations in blood flow, and enhancement of the ability of epithelium to resist and rapidly repair damage (Wallace and Tigley, 1995). The initial research into the association between prostaglandins and the gastrointestinal tract focused on gastric ulceration. These studies determined that prostaglandins help modulate the inflammatory response of mast cells,

possibly by decreasing TNF-α release (Wallace and Tigley, 1995). A further role of prostaglandins involved the limiting effect they have on neutrophils, which as a major part of any inflammatory response, have a number of potentially deleterious effects, such as recruitment of other immune cells or release reactive oxygen metabolites. (Wallace and Tigley, 1995). Furthermore, prostaglandins decrease the threshold for afferent nerve fibers, and thus exert a hyperalgesic effect during inflammation can decrease intestinal motility following abdominal surgery (Josephs et al., 1999)

Cyclooxygenase (COX): Two enzyme isoforms have been isolated that produce prostaglandins, in the intestine these would be PGE_2, PGI_2 and $PGF_{2\alpha}$, from arachidonic acid- COX-1 and COX-2 (Glinghammar and Rafter, 2001; Gerstle et al., 1994). The former being thought of as constitutive, the latter considered inducible. Early paradigms indicated that COX-1 elaborated prostaglandins that would be primarily responsible for mucosal integrity, while COX-2 was utilized during inflammatory responses (Wallace and Tigley, 1995). However, recent research has rendered this clear delineation between the two isoforms less tenable, with COX-2 appearing to be constitutive in at least some tissues.

However, some data indicates that in those tissues where COX-2 may still be considered inducible, a number of factors can induce an upregulation in the production of this enzyme. For example, “fecal water”, probably due to its component bile acids can induce COX-2 (Glinghammar and Rafter, 2001). This could be a factor in the cryptosporidial model of intestine injury, with its

accompanying villous atrophy, epithelial damage and severe watery diarrhea. Although slight, the possibility exists that, in the distal ileum, refluxed fluid could enter via gaps in the epithelial layer and stimulate the induction of COX-2.

A number of compounds, such as cytokines, growth factors and tumor promoters, have been identified that regulate COX-2 activity (Sheng et al., 2000). Weaver et al (2001) attempted to determine the mechanism by which TNF-α stimulates the expression of COX-2 in colonic epithelium. The effect of TNF-α on COX-2 expression is negatively regulated by PI3-K, as inhibition of PI3-K enhanced the stimulatory effect of TNF-α on COX-2 mRNA and protein levels. A similar inhibitory effect caused by PI3-K was seen when the cytokine IL-1α was added. The effects of TNF-α and IL-1α were due to PI3-K independent upregulation of NF-κB. However, further research indicated that in addition to upregulating NF-κB, TNF-α also stimulated PI3-K activity. This seeming paradox suggests the presence of an autoregulatory negative feedback mechanism.

However, it is highly likely that all these stimulants act via the same intracellular mechanism. In at least one intestinal epithelial cell line (RIE-1), the upregulation of COX-2 is a Ras-dependent mechanism, possibly involving v-src. Using TGF-β as a COX-2 stimulant, Sheng et al (2000) discovered that Ras acts via two mechanisms to upregulate COX-2 activity. First, it stimulates transcription. Second, it prolongs the half-life of COX-2 mRNA.

Additional studies of the regulatory mechanisms governing COX-2 activity focused on the role of the nuclear transcription factor NF-κB. This role was investigated after it was discovered that the promoter for COX-2 has at least two

binding sites for NF-κB (Abate et al., 1998). Inhibition of NF-κB prevented the lipopolysaccharide-dependent (LPS) induction of COX-2 in macrophages, suggesting a key role for this transcription factor in COX-2 regulation. However, LPS does not act only via induction of transcription; LPS appears to stimulate catalytic activity and translation, in addition to transcription (Abate et al.l, 1998). Most likely, NF-κB stimulates COX-2 production by attaching to the promoter region of the appropriate gene, although other possible mechanisms can not be eliminated.

Several other intracellular mechanisms have been implicated in the regulation of COX-2 transcription. Inhibition of both p38 MAPK and protein kinase C (PKC), separately, resulted in total inhibition of COX-2 promoter activity, suggesting a key role for these intracellular signaling mechanisms in the production of COX-2 (Glinghammar and Rafter, 2001). Further, these stimulatory components can be induced by a variety of compounds commonly found in the luminal contents, such as deoxycholic acid, chenodeoxycholic acid, cholic acid and butyric acid, as well as fecal water, which would contain these compounds (Glinghammar and Rafter, 2001).

Localization: Singer et al (1998) attempted to determine the location of both COX-1 and COX-2 in healthy and disease injured human intestinal tissue. The researchers determined that in humans, the COX-2 enzyme is, in fact, inducible. In ileal tissue resected from both healthy patients, and patients with Crohn's disease, COX-1 was found in the cytoplasm of crypt enterocytes and in mononuclear cells in the lamina propria. In contrast, COX-2 could not be

identified in healthy tissue, and in injured tissue was found in the epithelial cells on the terminal villous, but not the crypts.

Pathogenic invasion is a highly likely candidate for induction of COX-2 in intestinal epithelium. For example, *Entamoeba histolytica* infection caused COX-2 expression in tissue previously devoid of this isoform, although the correlation between amebic infection and COX-2 expression was not high. For example, many pathogens were identified in regions completely devoid of COX-2, while other regions every pathogen was accompanied by a large expression of the enzyme in the adjacent epithelium (Stenson et al., 2001). However, this may simply be a temporal effect, that is, the pathogens found without an accompanying COX-2 response in the immediate vicinity may simply have attached immediately prior to tissue collection, with inadequate time to generate a typical response. Alternatively, COX-2 elaboration following infection could be mediated in a paracellular fashion, by interceullar messengers released by infected enterocytes, thus mitigating the need for a response in each infected cell. COX-2, in this case, played a major role in neutrophil infiltration, as inhibition greatly reduced the amount of myeloperoxidase activity, an indicator of neutrophil activity. This may be because COX-2 mediates the increase in IL-8 frequently associated with amebic infections (Stenson et al, 2001).

Electrolyte Movement: Argenzio et al (1993:440) investigated the role of prostaglandins in colostrum deprived neonatal pigs infected with *C. parvum*. Cryptosporidial infection suppressed NaCl absorption, while stimulating Cl secretion, perhaps due to increased levels of PGE_2 (Argenzio et al., 1993).

When prostaglandin production was inhibited, NaCl uptake was restored to levels seen in uninfected, control tissue, however, exogenous PGE_2 administered to indomethacin (a non-selective COX inhibitor) treated tissue, reversed this effect (Argenzio et al., 1993).

Further research into the mechanisms of altered intestinal electrolyte transport involving prostaglandins was conducted by Kandil et al (1994), who examined the effect of tumor necrosis factor α (TNF-α). Among its other effects, this cytokine, which is frequently elevated during inflammatory responses, induces prostaglandin secretion. In cryptosporidial piglets, TNF-α levels increased, and induced a Cl secretory response that was indomethacin inhibitable. Kandil et al (1994) speculated that mononuclear cells responding to the infection release TNF-α, which in turn stimulate fibroblasts to begin producing prostaglandins, inducing the secretory response seen in this study.

Prostaglandin E_2 acts via a cAMP mediated mechanism to induce recovery of barrier function in the intestine following injury Blikslager et al (1999). However, the exact mechanism by which PGE_2 acts is unknown. Possibly, the induction of cAMP increases trans-epithelial resistance (TER) via alterations in the tight junctions. However, one tissue response to PGE_2 is an increase in Cl secretion, inhibition of which abolished reparative effects of PGE_2. Another effect of PGE_2 is the inhibition of Na absorption, such as that discussed previously, which could also play a role in the increased resistance. Both alterations in electrolyte flux could cause the paracellular space to collapse, via osmotic events. The delay between the inhibition of ion movement, and the alteration in

resistance could be due to the osmotic collapse of the paracellular space. However, another possibility is the reassembly of tight junctions. Based on previous results of other researchers, Blikslager et al (1999) speculated that prostaglandins restore tissue resistance via a combination of both osmotic collapse of paracellular spaces, and stimulation of tight junction formation.

Blikslager et al (1997) also conducted a study to determine how PGE_2 and PGI_2 interact to regulate barrier function in injured ileal tissue. The researchers confirmed via histological examination that the initial increase in resistance following prostaglandin treatment was due to alterations in the paracellular spaces, rather than villi contractile or restitutive mechanisms. However, their key finding was that PGE_2 and PGI_2 act synergistically in promoting intestinal repair. Prostaglandin E_2 acts in an endocrine manner, stimulating cAMP to act intracellularly. However, PGI_2 acts neuronally, using cholinergic nerves to elevate intracellular Ca concentrations. Both Ca and cAMP can alter cytoskeletal characteristics, thus modifying tight junction morphology and increasing resistance.

Blikslager et al (2000) studied the effects of genistein, an isoflavinoid with tyrosine kinase receptor inhibitory properties, that can also induce Cl secretion, on intestinal repair, since this is apparently an important component in tissue recovery. Using an ischemic injury model, Blikslager et al (2000) confirmed that tissue treated with PGE_2 responded by demonstrating an elevated TER; further, genistein alone was insufficient to increase TER, but it did act synergistically with PGE_2. Usage of the Na-K-2Cl inhibitor, bumetanide, revealed that both the PGE_2

response, and the synergistic response, were strongly associated with Cl secretion. The induction of Cl secretion resulted in the closure of intra-epithelial spaces, by inducing an osmotic gradient that drew fluid out of the intra-epithelial spaces, thus collapsing the area between cells. Another possibility is that genistein inhibits tyrosine kinase receptors, which have been shown to regulate tight junctions, and thus induced reformation of the linkages that had been opened or damaged during the injury. This could explain why PGE_2 and genistein act synergistically, as both use differing mechanisms to induce repair.

MacNaughton and Cushing (2000) further explored the role of the two COX isoforms in combating intestinal injury. In uninjured mouse colon COX-2 is present in the cells subjacent to the epithelium, indicating that in this tissue and species, COX-2 is constitutively expressed. MacNaughton and Cushing (2000) found that both enzymes contribute to the prostaglandin induced secretory response; however, the bulk of the response seemed to be attributable to the COX-2 isoform. The two isoforms responded to secretagogue administration by producing PGE_2, and not PGD_2; rather, inhibition of the enzymes did not alter PGD_2 levels.

In addition to COX elaborated PGE_2, $PGF_{2\alpha}$ plays a role in regulating glucose absorption in the intestine. By induction of prostaglandins, specifically $PGF_{2\alpha}$, with shear stress, Han et al (1999) observed increased mucosal blood flow, an effect that was indomethacin sensitive. This increase in blood flow stimulated a three-fold increase in glucose uptake, which would not be strictly dependent on the prostaglandin response, as $PGF_{2\alpha}$ treatment without increased

blood flow did not alter glucose absorption. Possibly the increased blood flow altered the electrochemical gradient driving glucose uptake, perhaps by alterations in counter-current exchange.

Protective Effects: Prostaglandins play a major role in cytoprotection in a number of injury models. Inhibition with indomethacin greatly heightens the extent of injury in an ethanol-induced gastric lesion study (Sikiric et al., 1999). However, despite a thorough examination of the effects of a variety of injury models in gastric mucosa, Sikiric et al (1999) did not attempt to postulate any mechanism by which prostaglandins could ameliorate the injury.

One compound frequently found to be upregulated in response to injury are trefoil factor peptides. Their beneficial effects have long been speculated on, however, not until recently has possible mechanisms by which this family acts been investigated. Recent research has discovered that at least one of the trefoil families act via upregulation of the COX-2, but not COX-1, enzyme, with subsequent production of PGE_2 and PGI_2. This upregulation may occur via a nitric oxide (NO) dependent mechanism, as NOS2 produced NO activates COX-2, and trefoil factors stimulates NOS2 activity (Tan et al., 2000). In mediating the cytoprotective effects of trefoil factors, both PGE_2 and PGI_2, which act independently, reduced damage caused via reactive oxygen metabolites (Tan et al., 2000). However, it should be noted that this is not the only mechanism by which trefoil factors act to prevent injury. Since the researchers were injuring the cells with reactive oxygen species, and not adding or stimulating the enzymes responsible for their production, the beneficial effects of both trefoil factors and

prostaglandins must be, in this case, after the potentially injurious compounds are produced. Possible cytoprotective mechanisms may include alteration of mucin production, which would potentially segregate the reactive oxygen metabolites, upregulation of other antioxidant mechanisms to remove the metabolites, or possibly sacrificing the prostaglandins themselves to the metabolites, thus eliminating the more injurious metabolites (Tan et al., 2000).

Additional roles for prostaglandins have been postulated in inflammatory bowel disease models. In particular, PGD_2 production by COX-2 has an anti-inflammatory effect following colitis induction in rats (Ajuebor et al., 2000). The primary role of COX-2 elaborated PGD_2 was reduction of neutrophil infiltration following inflammation. However, the increase in PGD_2 was transient, with production mirroring increases in COX-2 activity, although prostaglandin levels declined despite continued elevation of COX-2 (Ajuebor et al., 2000). This could be a key mechanism to limiting damage, since neutrophils have been implicated in exacerbating tissue injury by causing oxidative damage.

An additional protective effect may be derived from COX-mediated mucin production in the intestine. Administration of indomethacin, compared to exogenous PGE_2, revealed that COX elaborated PGE_2 is critical to the production and maintenance of the gel layer. For example, inhibition of prostaglandin production resulted in premature secretion and delayed basal formation of mucins; further, the gel layer was unstable, presumably due to compositional variances (Akiba et al., 2000). COX-2 plays a central role in the regulation of mucus secretion; a number of mediators, such as bradykinin,

capsaicin, and acid stimulation of vanilloid receptors on afferent nerves all converge on COX-2, which in turn acts via PGE_2 (Akiba et al., 2001).

COX-2 mediated prostaglandins have also been implicated in the prevention of damage following hemorrhagic shock in both the liver and bowel. Following hemorrhagic injury, Tsukada et al (2000) noted an increase in COX-2 mRNA. Several possible mechanisms may explain this interaction between injury and COX-2. The vasodilatory prostaglandins elaborated following injury maximizes perfusion of the ischemic tissue (Tsukada et al., 2000). Another potential mechanism is by inhibiting the release of proinflammatory cytokines.

Additional Prostaglandin Effects: One potential effect of COX-2 products is the inhibition of intestinal motility following abdominal surgery. It is not uncommon for patients to experience inhibited bowel motility following any abdominal surgery; the effect being self-resolving within 3-4 days following the operation (Josephs et al., 1999). These researchers found that inhibition of COX-2 resulted in a significantly increased rate of passage during post-operative ileus; this effect was attributed to the decrease in PGI_2. Addition of exogenous PGI_2 has been shown to delay transit and inhibit intestinal contractions. This hormone directly interacts with both smooth muscle cells and visceral afferent sensory nerves, to play a major role in post-operative ileus (Josephs et al., 1999).

Prostaglandins, particularly PGE_2 and $PGF_{2\alpha}$, have been implicated in the maturation of the epithelial barrier in neonatal animals. Small bowel tissue in rats 22 days of age had higher levels of PGE_2 when compared to rats 10 d of age

(Gerstle et al., 1994). The correlation between PGE_2 levels and increased mucosal barrier function were demonstrated by indomethacin treatment. This could have significant clinical implications, as a common treatment for enteric infection by such pathogens as C. parvum is the administration of non-selective COX inhibitors, such as flunixin meglumine. In dairy calves in particular, infection occurs at a very young age, and administration of these compounds could interfere with proper barrier development and could cause health concerns later in life.

Effects of Cyclooxygenase: In addition to the production of prostaglandins, the COX enzymes have a number of other effects. Hori et al (2001) investigated the relationship between COX-2 and iNOS activity. The researchers determined that LPS induces COX-2 mRNA and protein synthesis, with a subsequent increase in prostaglandin levels. Hori et al (2001) reported that the expression of COX-2 is necessary for the induction of iNOS by LPS, and that the prostaglandins produced by COX-2, but not COX-1 are the mediators of this effect. Further, the prostaglandins bind to a macrophage EP_3 receptor, stimulating cAMP production, which induces PKA activation to induce iNOS expression, probably by activation of NFκ-B.

COX-2 activity has also been implicated in cellular proliferation in both normal epithelial cells as well as malignant cells (Erickson et al., 1999). Initial research suggested that this effect was due to PGE_2 elaboration, although further work, namely that of Erickson et al (1999) revealed that COX-2 stimulates proliferation in a prostaglandin-independent manner. The decrease in cellular

proliferation following COX-2 inhibition was strongly associated with increases in apoptosis; exogenous PGE_2 did not reverse this effect (Erickson et al., 1999). These results were observed in both normal enterocytes and malignant cells.

Prostaglandins and Damage: Despite their cytoprotective effects, prostaglandins can cause tissue damage as well. A variety of tumors can produce prostaglandins, with a subsequent result of angiogenesis, inhibition of immune surveillance and apoptosis and an increase in the invasiveness of malignant cells (Glinghammar and Rafter, 2001).

Perhaps the more serious effect of prostaglandins is the severe secretory diarrhea accompanying enteric infection, by such pathogens as *Vibrio cholerae*. Following infection with *V. cholerae*, COX-2 mRNA and activity were upregulated, with associated production of PGE_2 (Beubler et al., 2001). This increase in prostaglandins causes an elevation in cAMP levels, which would most likely alter CFTR activity, increasing chloride secretion and causing secretory diarrhea.

COX Inhibitors: A variety of compounds have been developed to inhibit COX-2, while leaving COX-1 activity intact. Most inhibitors rely on the different morphology of the COX-2 isoform, that is, a wider active space with a large side pocket. The inhibitors meloxicam, with a methyl group on the thiazoyl ring, and celecoxib ($NH2SO_2^-$) and rofecoxib ($CH3SO_2^-$) all act via this side pocket. However, the inhibitor NS-398 binds the central pocket of COX-2, while not interfering with the side pocket (Tavares, 2000). This may be why NS-398 shows

a loss of specificity at higher doses, such as occurred in the studies by this author.

Summary

The preceding thorough review of the gastrointestinal physiological implications of *Cryptosporidium parvum*, glutamine, electroneutral sodium tranporters, and prostaglandins, has hopefully demonstrated that, while apparently disparate, all interact to regulate sodium tranport in ileal tissue. What follows in a partial investigation of this interaction, using a *C. parvum* infected calf model.

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Cyclooxygenase Blockade and Exogenous Glutamine Enhance Sodium Absorption in Infected Bovine Ileum

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We have previously shown that prostanoids inhibit electroneutral Na^+ absorption in *Cryptosporidium parvum*-infected porcine ileum, whereas GLN stimulates electroneutral Na^+ absorption. We postulated that GLN would stimulate Na^+ absorption via a COX-dependent pathway. We tested this hypothesis in *C. parvum*-infected calves, which are the natural hosts of cryptosporidiosis. Tissues from healthy and infected calves were studied in Ussing chambers, and analyzed via immunohistochemistry and Western blots. Treatment of infected tissue with selective COX inhibitors revealed that COX-1 and COX-2 must be blocked to restore electroneutral Na^+ absorption, although the transporter involved did not appear to be the expected NHE-3 isoform. Glutamine addition also stimulated Na^+ absorption in calf tissue, but although this transport was electroneutral in healthy tissue, Na^+ absorption was electrogenic in infected tissue, and was additive to Na^+ transport uncovered by COX inhibition. Blockade of both COX isoforms is necessary to release the prostaglandin-mediated inhibition of electroneutral Na^+ uptake in *C. parvum* infected calf ileal tissue, whereas GLN increases Na^+ uptake by an electrogenic mechanism in this same tissue.

Introduction

Cryptosporidium parvum is the most common enteric pathogen of young calves and is an important cause of diarrhea in animals and people worldwide (14,30). In the United States alone, a number of large outbreaks of water-borne diarrhea have been reported in recent years, at least six of which involved over 18,000 individuals (2, 24, 29). The ability of this protozoan to cause death in immuno-compromised humans, as well as the economic impact on the livestock industry has resulted in a National Institutes of Health panel ranking this organism as one of the three most important enteropathogens (21, 25). Unfortunately, there is no vaccine or antimicrobial agent which is presently effective; therapy involves oral or intravenous rehydration.

Cryptosporidium parvum infection has been shown to increase tissue prostaglandin concentrations in both pigs and humans, in some cases by up to 50% of baseline levels (6, 22). Prostaglandins, an integral part of the inflammatory response to infection, have been shown to inhibit Na absorption, and their blockade with the non-specific cyclooxygenase (COX) inhibitor indomethacin, restored Na absorption to normal (4, 6). Recent studies have indicated the presence of two COX isoforms- one constitutive (COX-1) and one inducible (COX-2) (13, 31, 33). In humans COX-2 levels increase following infection with *C. parvum*, while COX-1 levels remain unaffected (Laurent et al. 1998). Although attention has increasingly focused on the possible efficacy of inhibiting COX-2, while preserving COX-1 activity for normal homeostatic

mechanisms, recent studies suggest that there may be substantial overlap in the roles of COX-1 and COX-2 (32).

When considering administration of oral rehydration solutions (ORS), one of the main goals is to maximize fluid absorption, to replenish fluids lost with the profuse, watery diarrhea characteristic of cryptosporidiosis. Most solutions are glucose based. However because the enterocytes that transport glucose reside on the villous tips of the small intestine and are damaged by organisms such as *C. parvum*, other potential substrates are being investigated. For example, glutamine has well-documented intestinotrophic effects, as well as improved fluid absorption when compared to glucose based solutions in porcine cryptosporidiosis (5, 18). Previous research has also indicated that glutamine stimulates an electroneutral Na transporter in piglets (5). This transport activity is typically attributed to one of a class of six neutral Na/H exchangers (NHE)(12). Conversely, prostaglandins inhibit NHE activity by stimulating increased intracellular cAMP levels.

The present study examines the hypothesis that inhibition of a single COX isoform, most likely COX-2, would restore electroneutral Na transport following infection with *C. parvum*, and allow the oral administration of glutamine to be maximally effective in increasing Na uptake.

Materials and Methods

Animals and Infection

The University Animal Care and Use Committee approved all procedures. Experimental animals were 1-day-old male, Holstein calves obtained from a local farm and housed in isolation facilities at the North Carolina State University College of Veterinary Medicine. Immediately following arrival, the calves were evaluated for serum colostral antibody status and fed an antibiotic-free synthetic diet (Purina Kid Milk Replacer, Purina Mills Inc, St. Louis, MO) twice per day at a daily volume of 10% body weight.

Pleasant Hill Farm (Troy, Idaho) provided purified *Cryptosporidium parvum* oocysts. Calves designated for the infected group received an oral inoculum of 10^8 oocysts on day 7 of life. Previous research in this laboratory has indicated that the period of maximal diarrhea and intestinal damage is on day 4 post infection (unpublished observations); therefore, both control and infected calves were euthanatized 4 days after infection (day 11 of life) by a lethal overdose of intravenous Na-pentobarbital. Following this, sections of ileum beginning 10 cm proximal to the ileo-cecal valve were taken for in vitro studies.

Ileal sections were opened along the antimesenteric border and bathed in an oxygenated Ringer's solution. Blunt dissection removed the outer muscular layers, in preparation for mounting the mucosa in Ussing chambers. Additional tissue samples were formalin fixed for light microscopy and immunohistochemistry (IHC), or frozen in liquid nitrogen for Western blot analysis.

Tissue Morphology

The formalin-fixed tissues were embedded in paraffin, cut in slices 5 μm thick and stained with hematoxylin and eosin for analysis by light microscopy. Tissue samples from all calves were examined to determine the presence or absence of *C. parvum* organisms. Six well-oriented villi on histologic sections from each animal were measured to determine mean villous height and diameter, as well as crypt depth. These data were converted to measurements describing surface area, as described previously (3). Briefly, the calculations were based on the equation for the surface area of a cylinder. However, the formulae for the two ends of the cylinder were removed, and replaced with the formulation for the surface area of a hemi-sphere, which simulates the cap of the villi. The calculation also includes a correction factor for the characteristic villous folds and variability in the frontal sections of the tissue slices.

Immunohistochemistry

For immunohistochemistry (IHC), tissues were fixed in 10% neutral buffered formalin for 24-h, transferred to a 70% ethanol solution, and embedded in paraffin. Five-μm sections were mounted on slides, deparaffinized and rehydrated. Slides were subsequently incubated in 3% H_2O_2, after which endogenous avidin and biotin were inhibited. After further washing in PBS, slides were incubated for 1 hour at room temperature with a 1:50 dilution of either rabbit anti-NHE-3 or NHE-2 polyclonal antibody, or rabbit anti-COX-1 or COX-2 polyclonal antibody (Chemicon; Temecula, CA). This step was not performed on negative control slides. Following this, slides were incubated with goat anti rabbit

secondary antibody (Zymed; San Francisco, CA) and then labeled with aminoethyl carbazole (Zymed; San Francisco, CA).

Western Blot Analysis

Tissues were stored at –20^{o}C prior to preparation for SDS-PAGE, at which time they were thawed at 4^{o}C. One-gram tissue portions were added to 3 ml of chilled RIPA buffer (0.15M NaCl, 50 mM Tris [pH 7.2], 1% deoxycholic acid, 1% Triton X-100, 0.1% SDS), including protease inhibitors. The sample was homogenized on ice and centrifuged (2500 g, 10 minutes, 4^{o}C). The supernatant was transferred to microcentrifuge tubes, and centrifuged again (10000g, 10 minutes, 4^{o}C). Protein analysis was performed on aliquots (10 μl)(Dc protein assay, Bio-Rad, Hercules, CA). Tissue extracts were mixed with an equal volume of 2X-SDS-PAGE sample buffer. Following boiling for 4 minutes, protein was separated on a 10% polyacrylamide gel, with electrophoresis carried out by standard protocols. Proteins were then transferred to a nitrocellulose membrane (Hybond ECL, Amersham Life Science, Birminham, UK) using an electroblotting mini-apparatus. Prestained molecular weight markers provided an estimate of transfer efficiency. The membrane was blocked overnight with Tris-buffered saline plus 5% dry powdered milk. The membrane was exposed for 2 hours to rabbit polyclonal antibody (anti-NHE-3 or anti-COX-2), (Chemicon, Temecula, CA). After rinsing three times with Tris buffered saline plus 0.05% Tween-20 (TBST), the membranes were exposed to horseradish peroxidase conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 60 minutes. The membranes were washed again three times with TBST, and a final wash

with TBS, and developed for visualization of protein by addition of enhanced chemiluminescence reagent.

Ussing Chambers

In vitro studies in this laboratory using Ussing chamber methodology has been described in detail (4). Briefly, ileal tissue is stripped of the muscularis and mounted in Ussing chambers. Both surfaces were bathed with 10 ml of Ringer solution, oxygenated with 95%O_2/5%CO_2 and maintained in water-jacketed reservoirs at 37^{o} C. Serosal glucose (10 mM) was osmotically balanced with mucosal mannitol (10 mM). In these experiments, the tissues were stripped in either normal Ringer, or Ringer containing the appropriate concentration of COX inhibitor. Additionally, the Ringer solution in the reservoirs contained the appropriate treatments prior to mounting the tissues.

Varying dosages and specificity of cyclooxygenase inhibitors were compared to a normal Ringer solution, which established the baseline. The response of various species and tissues to the non-selective COX inhibitor indomethacin (10^{-6} M) has been thoroughly studied in this laboratory (7, 8, 19). Additional treatments were a selective COX-1 inhibitor, SC-560 (10^{-5},10^{-6} M), a selective COX-2 inhibitor, NS-398 (10^{-5},10^{-6} M) or both (10^{-5},10^{-6} M) (Cayman Chemical;Ann Arbor, MI). Tissue was incubated with the treatments for approximately 30 minutes prior to the initial reading. Following this pre-sampling equilibration period, a 30 min period of sampling evaluated tissue response to COX blockade. After this time period 30 mM glutamine was added to the mucosal side of the tissue, balanced with 30 mM mannitol on the serosal side,

and allowed to equilibrate for 15 minutes. Data were then collected for an additional 30 min.

The spontaneous potential difference (PD) was measured with Ringer-agar bridges connected to matched calomel electrodes and the PD short circuited through Ag-AgCl electrodes, using an automatic voltage clamp corrected for fluid resistance, for measurement of short-circuit current (I_{sc}). After equilibrating the tissue for 30 min, the isotopes ^{22}Na and ^{36}Cl were added to the mucosal or serosal reservoirs bathing paired tissues. If the conductance (G) of the tissue pairs differed by more than 25%, the tissues were discarded from analysis. The isotopes were then equilibrated for 20 minutes, at which time standards and zero time samples were taken from the reservoirs. Samples were then removed after 30 min from the side opposite to that of isotope addition. Following the addition of glutamine and an additional equilibration period, a second 30 min flux period was conducted.

Isotope Quantification

Samples were counted for ^{22}Na in a crystal scintillation counter and for ^{36}Cl in a liquid scintillation counter. The contribution of ^{22}Na counts to ^{36}Cl counts was determined and compensated for. Unidirectional Na and Cl fluxes from mucosa to serosa (J_{ms}) and from serosa to mucosa (J_{sm}) were calculated. From these values, a net ion flux was calculated (J_{net}). Conductance was calculated from the potential difference (PD) and short circuit current (Isc). When PD was between –1 and 1 mV, tissues were clamped at $\pm$ 100μA for 5 sec, and the PD recorded to assure accurate measurement.

Statistical Analysis

Data were analyzed using a paired t-test for paired treatments or a 1-way ANOVA for multiple comparisons followed by a Tukey's test to determine differences among treatments (Sigma Stat, Jandel Scientific, San Rafael, CA). Significance was declared at $P < 0.05$.

RESULTS

Histology and Molecular Biology

Villous atrophy and crypt hyperplasia are two well-substantiated characteristics of intestinal tissue during cryptosporidial infection (15, 23, 26). Ileal mucosa from infected calves examined at the peak of diarrhea demonstrated shortened villi and elongated crypts (Figure 1; $P < 0.05$). However, surface area was not affected, due to the significant increase ($P < 0.05$) in the width of villi following infection (data not shown).

Previous research has indicated that in the intestinal tissue of most species, COX-1 is constitutively expressed in healthy tissue, perhaps acting in a "housekeeping" role, while COX-2 is induced in response to inflammatory stimuli (13, 17, 31, 33). However, IHC conducted in the present study revealed the constitutive presence of both isoforms. In healthy tissue, the enzymes were located along the length of the villi, with the heaviest concentration in the tip (Figure 2A,B). Interestingly, the COX-1 isoform was also concentrated in the goblet cells, while COX-2 was absent from this cell population. In the infected animals, the characteristic villous blunting was noted with COX-1 and COX-2 concentrated in the tips of villi, particularly COX-2 (Figure 2C,D). Neither COX isoform was observed in the crypt enterocytes in either the healthy or infected animals. Western blot analysis confirmed the presence of COX-2 in both control and infected tissue (Figure 3).

Intestinal absorption of NaCl is normally driven by a neutral Na-H exchanger (NHE). Of the NHE family of transporters, only NHE-2 and NHE-3

have been associated with vectorial transport. Immunohistochemistry revealed the presence of NHE-3 in healthy ileal tissue, and was limited to the upper portion of the villi (Figure 4A). The location of NHE-3 would appear to indicate that the exchanger is expressed only in mature enterocytes, since the protein was localized to the tips of the villi. While NHE-3 was identified in the apical membrane, most was found inside the cell. As Kurashima et al. (1998) reported, approximately 90% of NHE-3 protein will be found in juxtanuclear accumulation complexes (20). Infected tissue did not appear to contain NHE-3 (Figure 4B). This lack of NHE-3 in infected tissue was supported by Western blots (Figure 5). Tissues were also examined for the presence of NHE-2 proteins. Neither control nor infected animals demonstrated the presence of NHE-2 (data not shown).

Sodium and Chloride Transport Studies

In both piglets and calves, infection with *C. parvum* reduces villus absorption of Na and Cl (5, 8). In this study, infection completely abolished net Na absorption (Figure 6A), whereas net Cl absorption was not significantly different from control (control 1.414± 0.70; infected 0.53±1.12 $\mu Eq/cm^{2}\cdot h$; $P > 0.05$). The infection also increased the I_{sc} (Figure 7A; $P < 0.05$) and decreased the tissue conductance (Figure 7A; $P < 0.05$). Administration of indomethacin, NS-398 or SC-560 restored Na absorption in infected tissue to levels no different from control tissues (Figure 6A; $P > 0.05$). These increases in net Na absorption were not associated with significant changes I_{sc} (Figure 7A), indicating the COX inhibitor-induced increase in Na absorption was electroneutral. Administration of COX inhibitors did not alter net Na or Cl fluxes or I_{sc} in control tissue.

Preliminary data (not shown) indicated that administration of SC-560 or NS-398 at 10^{-6} M, did not improve sodium absorption in infected animals, whereas indomethacin at 10^{-6} M resulted in a significant increase ($P < 0.05$) in sodium flux when compared to either of these selective COX inhibitors. However, in the current study, simultaneous addition of both selective inhibitors at either 10^{-6} or 10^{-5} M was equally as effective in restoring sodium absorption as indomethacin, suggesting inhibition of both COX-1 and COX-2 is required to restore Na absorption.

Islam et al. (1997) demonstrated that glutamine increases electrolyte absorption; but the mechanism by which it does so appears to vary from species to species (18). In piglets, glutamine appears to stimulate an electroneutral sodium transport mechanism, whereas in calves, electrogenic transport was noted (5, 8). In the healthy tissue examined in the present study, glutamine administration (30 mM) increased sodium absorption when compared to normal Ringer solution alone ($P < 0.05$, Figure 6B). The stimulatory effect of Gln was limited to increased sodium absorption, with no concurrent alteration of net Cl absorption (data not shown) or I_{sc} (Figure 7B), indicating an electroneutral mechanism of action. Treatment of the healthy tissue with COX inhibitors did not increase sodium absorption compared to Gln alone and even decreased Na absorption in the presence of SC-560 (10^{-5} M) or SC-560 and NS-398 (10^{-6} M; Figure 6A,B).

In infected tissue, glutamine administration (30 mM) increased sodium absorption when compared to normal Ringer solution alone ($P < 0.05$, Figure 6

A,B). In contrast to the control tissue, the addition of COX inhibitors further increased Na absorption compared to Gln alone (Figure 6 A,B). In further contrast to control tissue and regardless of inhibitor treatment, the addition of Gln to infected tissue induced an increase in I_{sc} ($P< 0.05$; Figure 7 A,B) equal to the increase in net Na transport, indicating electrogenic Na transport activity. This was confirmed by the lack of response in Cl movement (data not shown).

Discussion

The most significant findings of this study were that despite the villous atrophy and loss of electroneutral transporters (NHE-3), sodium absorption was fully expressed in infected ileum after treatment with COX inhibitors. Furthermore, glutamine stimulated sodium absorption equally in control and infected ileum, but by different mechanisms. Thus, these results suggest an adaptive response in infected tissue, with an upregulation of novel sodium transport mechanisms.

The significant increase in electrically neutral Na uptake in infected tissue following inhibition of prostaglandins by COX blockers, but before the addition of glutamine, occurred despite the absence of IHC or Western blot evidence of NHE-2 and NHE-3, the neutral Na-H exchangers typically found on the apical membrane in ileal tissue (10, 16). Following *C. parvum* infection, the intestine displays severe villous atrophy as a result of both pathogen damage and as a result of villous contraction, a protective mechanism, to reduce the surface area of damaged mucosa. Following clearance of the organism and recovery, the intestine restores the villi to the normal height and repopulates them with enterocytes. The enterocytes populating the villi as recovery begins are most likely not fully mature, differentiated cells, but instead are immature cells whose initial primary role is the restoration of a physical barrier. Thus, cells on the villi may not have acquired transporters such as NHE-3 associated with mature epithelium. Therefore, an alternate sodium transporter in either immature villous or crypt epithelium may be involved; recently, Rajendran et al (2001) and Binder

et al (2000) described a novel Na-H exchanger in crypt epithelium, which has been associated with Na and H_2O absorption (9, 27). Further study will be necessary to determine if such a mechanism is present in calf ileum and is upregulated in the infection.

Previous studies of both pig and calf models of cryptosporidiosis have shown that elevated tissue levels of prostaglandins inhibit electroneutral Na absorption in this infection (4, 8). Because of the potential deleterious effects on the mucosal barrier caused by inhibition of COX-1 and the housekeeping prostaglandins, one of the primary objectives of this study was to determine if selective inhibition of one of the COX isoforms was sufficient to restore neutral Na absorption. However, the lack of response seen when either COX-1 or COX-2 were inhibited alone at an inhibitor concentration of 10^{-6} M indicates that this is probably not sufficient for effectively treating diarrhea. Instead, inhibition of both COX isforms using SC-560 and NS-398 together at 10^{-6} M was required to restore Na absorption. The results of this study, as well as preliminary work conducted in this laboratory, appears to indicate a loss of specificity of the COX inhibitors NS-398 and SC-560 as the concentration increases above 10^{-6} M. Addition at 10^{-5} of either inhibitor resulted in flux data identical to that obtained when the non-specific COX inhibitor indomethacin was added, suggesting a loss of specificity at this higher dose.

In healthy tissue, glutamine administration induces sodium uptake, a response that has been well-documented (1). Glutamine added to control calf tissue induced a significant increase in net Na absorption. The absence of an

associated I_{sc} response indicates the stimulation of an electroneutral transporter, which could be NHE-3, since IHC and Western blot data indicate its presence on the tips of villi. Following glutamine absorption, most will be metabolized, with a subsequent increase in intracellular CO_2 levels (1), increasing the intracellular concentration of H and HCO_3 ions. This mechanism has previously been postulated to account for the stimulation of Na-H exchange by Gln in healthy and *Cryptosporidium*-infected piglet tissue (5, 28). Alternatively, the stimulation of sodium absorption may be the result of alterations in intracellular pCO_2 levels caused by Gln oxidation. CO_2 possibly plays a role in the trafficking of NHE-3 to the membrane from intracellular vesicles, allowing increased sodium uptake, similar to that seen here (11).

Because Gln stimulates neutral Na-H exchange, which was inhibited by prostaglandins in the infected piglet model (5), we reasoned that Gln would be most effective in stimulating Na absorption when combined with prostaglandin inhibition. However, while there was an additive response to GLN and indomethacin in infected tissue (Figure 4), the mechanism by which GLN stimulated Na absorption appeared to be electrogenic. Thus GLN stimulates Na absorption by distinctly different mechanisms in healthy versus infected tissue in calves. The fact that this electrogenic GLN transport is prostaglandin independent may be an important adaptive response, since the transporter will still be operational during the inflammatory response following infection, when tissue prostaglandin levels are increased. Blikslager et al. (2001) demonstrated the upregulation of the amino acid transport system ASC in the crypts of

Cryptosporidium-infected bovine ileum (8). The glutamine stimulation of the electrogenic transporter in the present study resulted in a similar increase in J^{Na}_{net} and I_{sc} as that reported by Blikslager et al. (2001).

Based on the results described above, it is apparent that simply inhibiting one of the two COX isoforms is insufficient to treat cryptosporidiosis in calves. However, blockade of both COX enzymes resulted in the restoration of an electroneutral sodium transporter, which would be expected to increase fluid absorption, an important part of any treatment for this and many other diarrheal diseases. This response, coupled with the additive glutamine-induced stimulation of electrogenic sodium transport activity, has great potential as a treatment for not only *C. parvum*-induced diarrhea, but possibly other diarrheal diseases characterized by villous atrophy and inflammation.

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Figure 1. Villous morphology in control and infected tissue collected 4 days after infection. Infection caused a significant reduction ($P < 0.05$) in the villous height, attributable to villous atrophy characteristic of *C. parvum*-induced damage. Crypt depth significantly increased ($P < 0.05$) following infection, indicative of crypt hyperplasia in response to injury.

Figure 2. Tissue was sampled from both control and infected calves, and analyzed immunohistochemically for the presence of cyclooxygenase isoforms. Panels A (COX-1) and B (COX-2) represent tissue collected from control animals, with both isoforms populating the epithelial cells the length of the villus, with less enzyme expressed in the crypt cells. Panels C (COX-1) and D (COX-2) represent tissue collected from infected animals. Both isoforms are present at higher concentrations in the villous tips, with little found in the crypts. Control tissue was imaged at a 10X magnification; insets viewed at 40X. Infected tissue was imaged at a 20X magnification.

Figure 3. Tissue was sampled from both control and infected calves, and analyzed by Western blot for the presence of cyclooxygenase (COX)-2. A) Each lane (control and infected) is representative of n=3 for each treatment group. The appearance of similar levels of COX-2 in control and ischemic tissues is suggestive of constitutive expression of this protein in calf ileum. B) Intensity of immunoreactive bands was quantified using scanning densitometry software (Scanalytics). Values are density $\pm$ SEM (n=3).

Figure 4. Tissue was sampled from both control and infected calves, and analyzed immunohistochemically for the presence of Na+/H+ exchanger 3 (NHE-3). Panel A represents tissue collected from control animals, with NHE-3 appearing along the villi. The exchanger concentrates in the terminal half of the the villous, although it can also be found in the less mature enterocytes populating the proximal villous. Panel B represents tissue collected from infected animals. There is a complete absence of exchanger in this tissue. Control tissue was imaged at 10X magnification; insets viewed at 40X. Infected tissue was imaged at a 20X magnification.

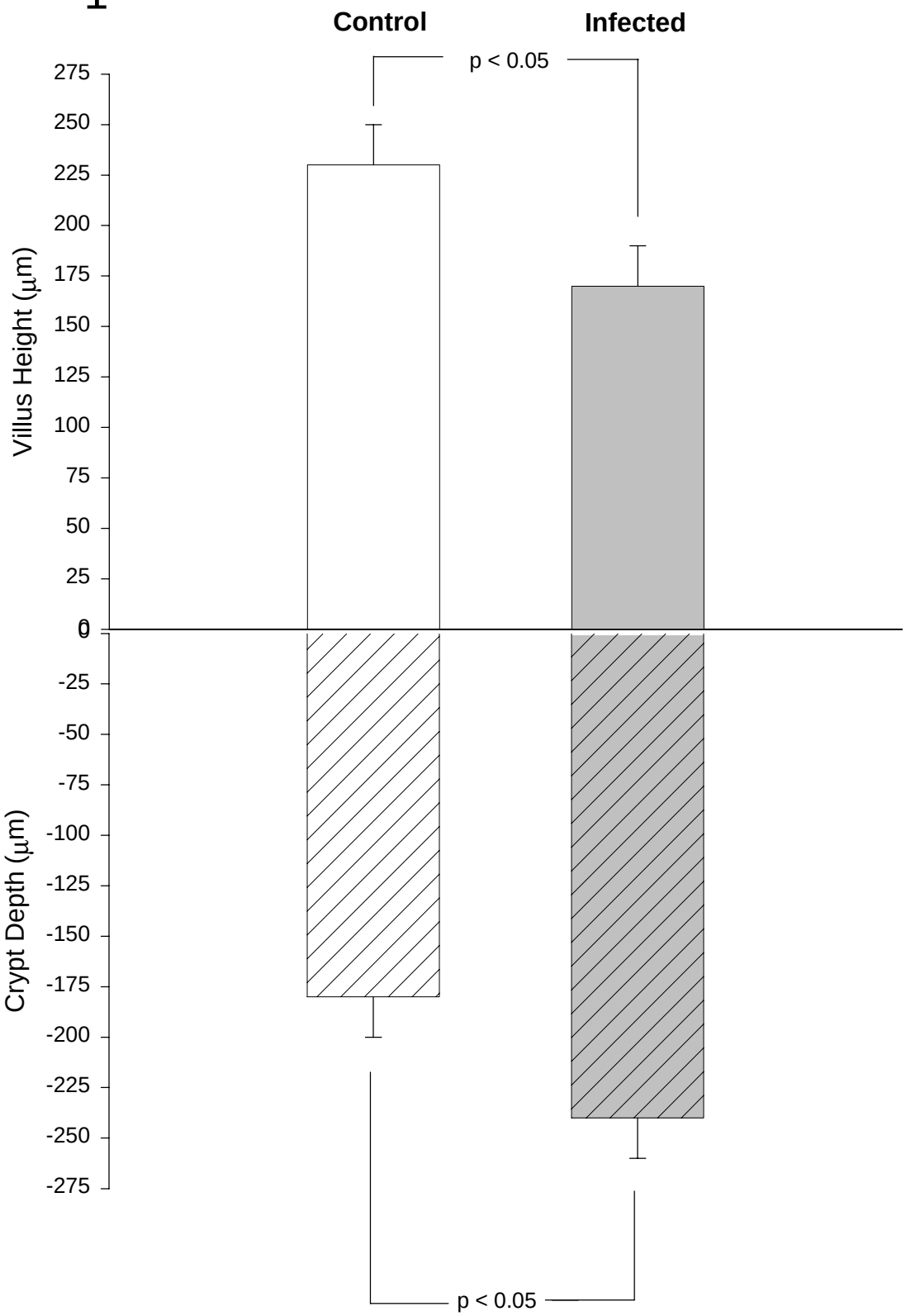
Figure 5. Tissue was sampled from both control and infected calves, and analyzed by Western blot for the presence of Na+/H+ exchanger 3 (NHE-3). A) Each lane (control and infected) is representative of an n=3 for each treatment group. While NHE-3 is present in healthy tissue, there is a near-complete absence of this exchanger in infected tissue. B) Intensity of immunoreactive bands was quantified using scanning densitometry software (Scanalytics). Values are density ± SEM (n=3). Means with a differing superscript are significantly different ($P < 0.05$).

Figure 6. Sodium flux data were measured in both control and infected tissue that was collected 4 days after infection, and treated with cyclooxygenase inhibitors and glutamine. (A). Inhibition of cyclooxygenase activity restored sodium absorption in infected tissue to levels seen in healthy tissue. (B) Glutamine administration significantly ($P < 0.05$) increased sodium absorption in healthy and infected tissue. This effect was prostaglandin independent. For

each panel, values are mean ± SEM (control n=8 animals; infected n=6 animals), and means with differing superscripts differ ($P < 0.05$). NR= normal Ringer solution. IR= Ringer solution containing indomethacin. NS-398= selective COX-2 inhibitor. SC-560= selective COX-1 inhibitor.

Figure 7. Electrical data were measured in both control and infected tissue that was collected 4 days after infection, and treated with cyclooxygenase inhibitors and glutamine. (A) Inhibition of cyclooxygenase activity did not alter the I_{sc} response, although conductance (G) did decrease when compared to the healthy tissue. (B) Glutamine administration significantly ($P< 0.05$) increased I_{sc} in infected tissue when compared to the healthy tissue, although this response was independent of cyclooxygenase inhibition. Conductance was not altered by glutamine treatments. For each panel, values are mean ± SEM, and means with differing superscripts differ ($P < 0.05$).

1


Control
Infected
p < 0.05
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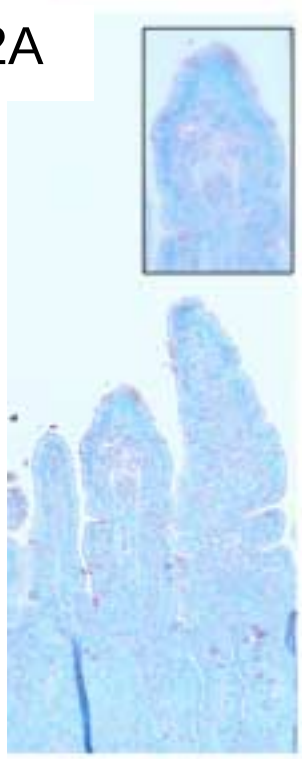
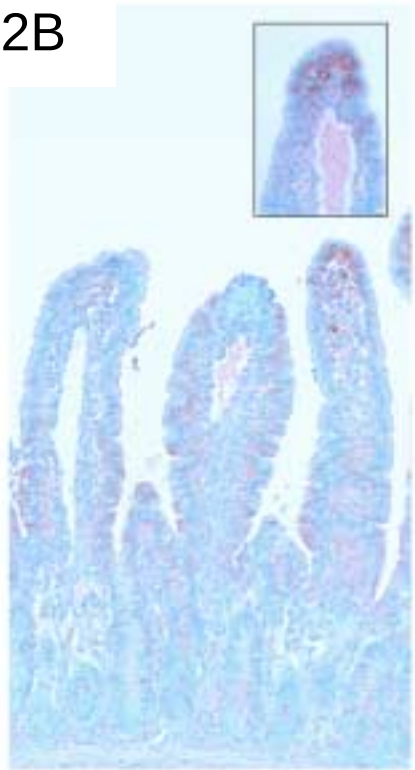
275
250
225
200
175
150
125
100
75
50
25
0
-25
-50
-75
-100
-125
-150
-175
-200
-225
-250
-275
Crypt Depth (µm)
p < 0.05

2A

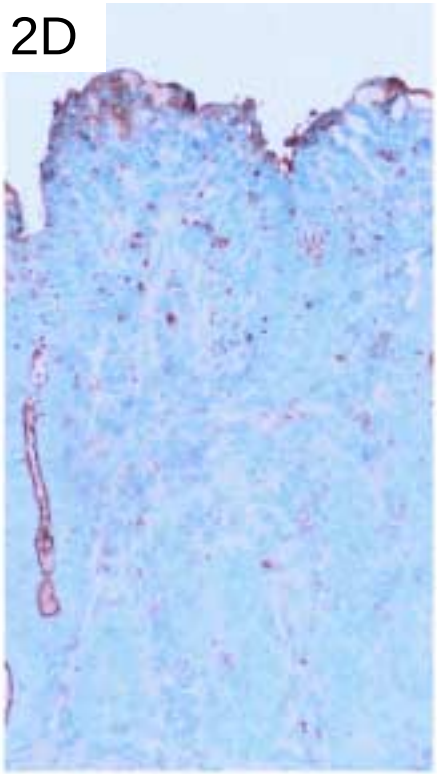


2C

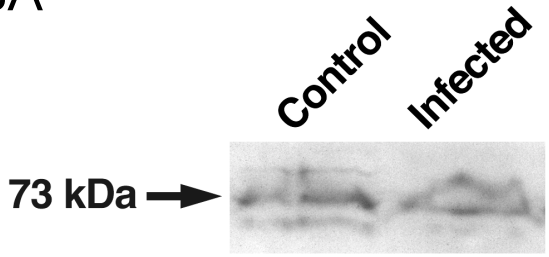
2B



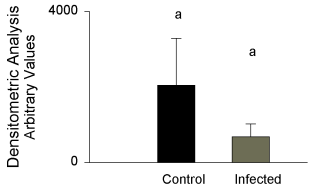
2D



3A

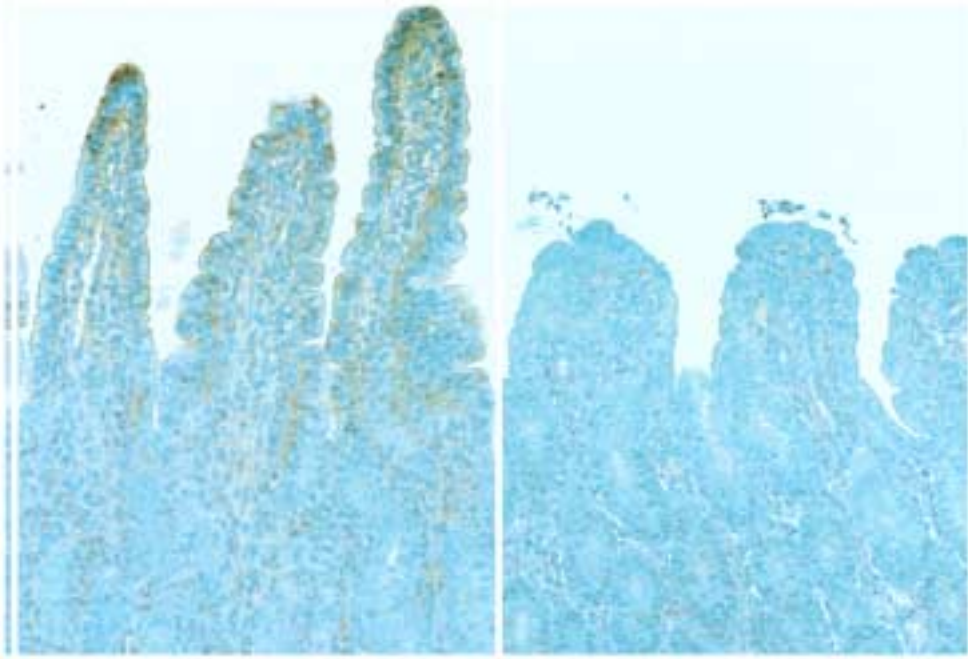

Control
Infected
73 kDa

3B

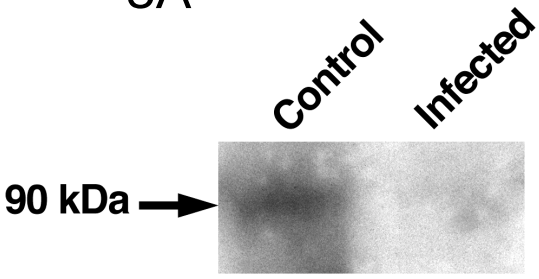

Densitometric Analysis
Arbitrary Values
4000
0
a
a
Control
Infected

4A

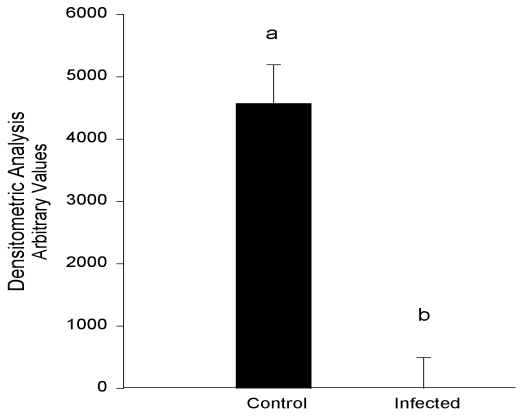
4B



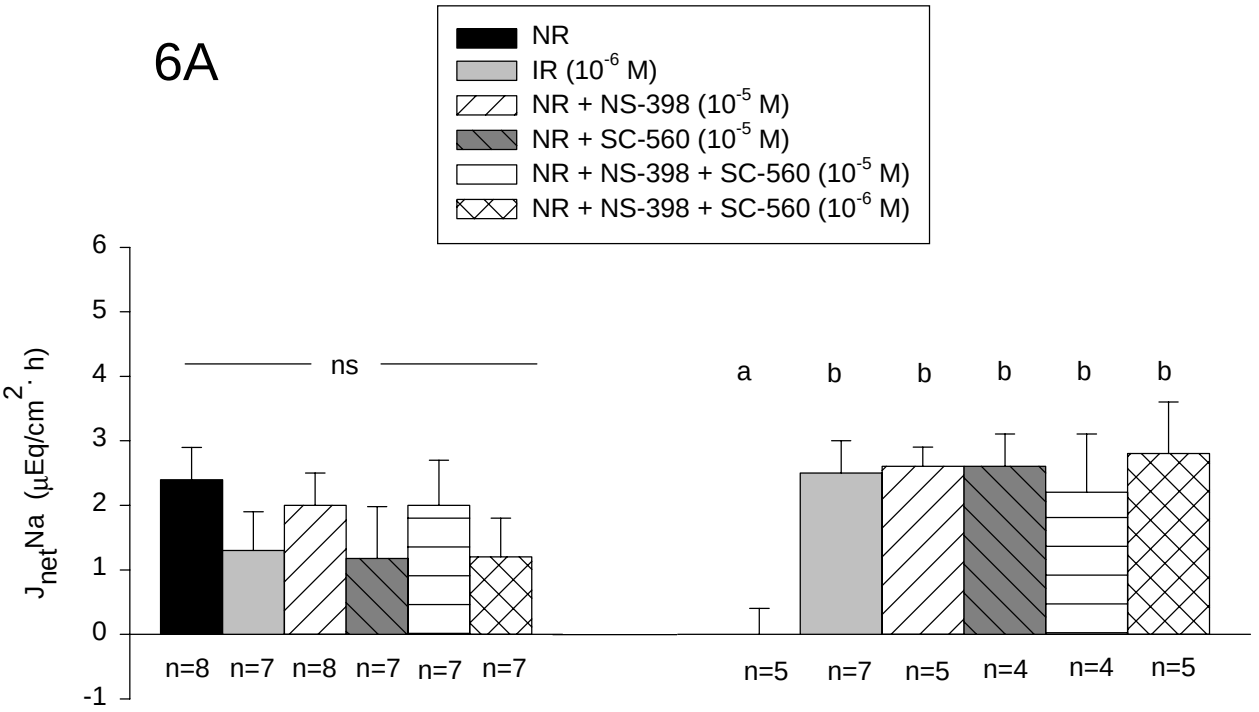
5A


Control
Infected
90 kDa

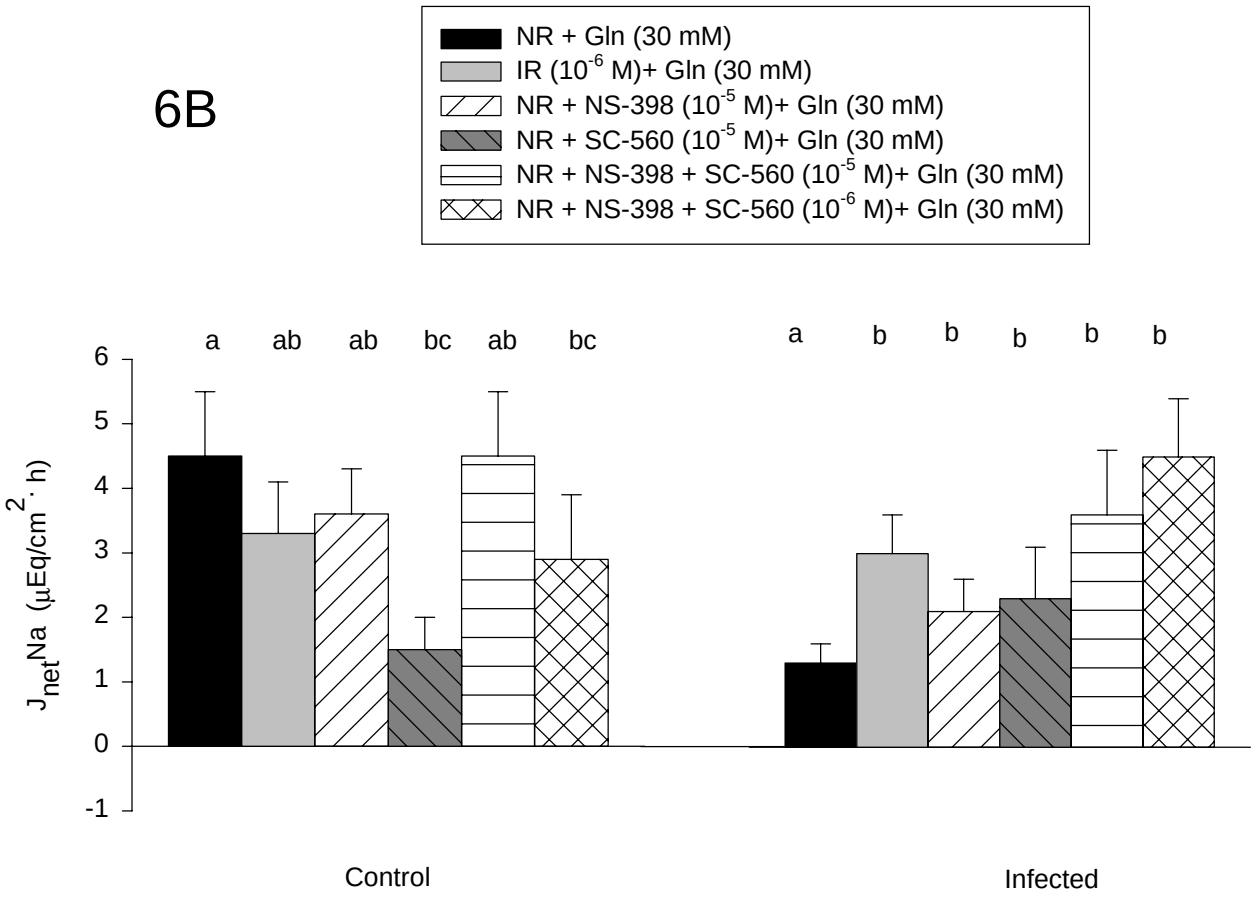
5B

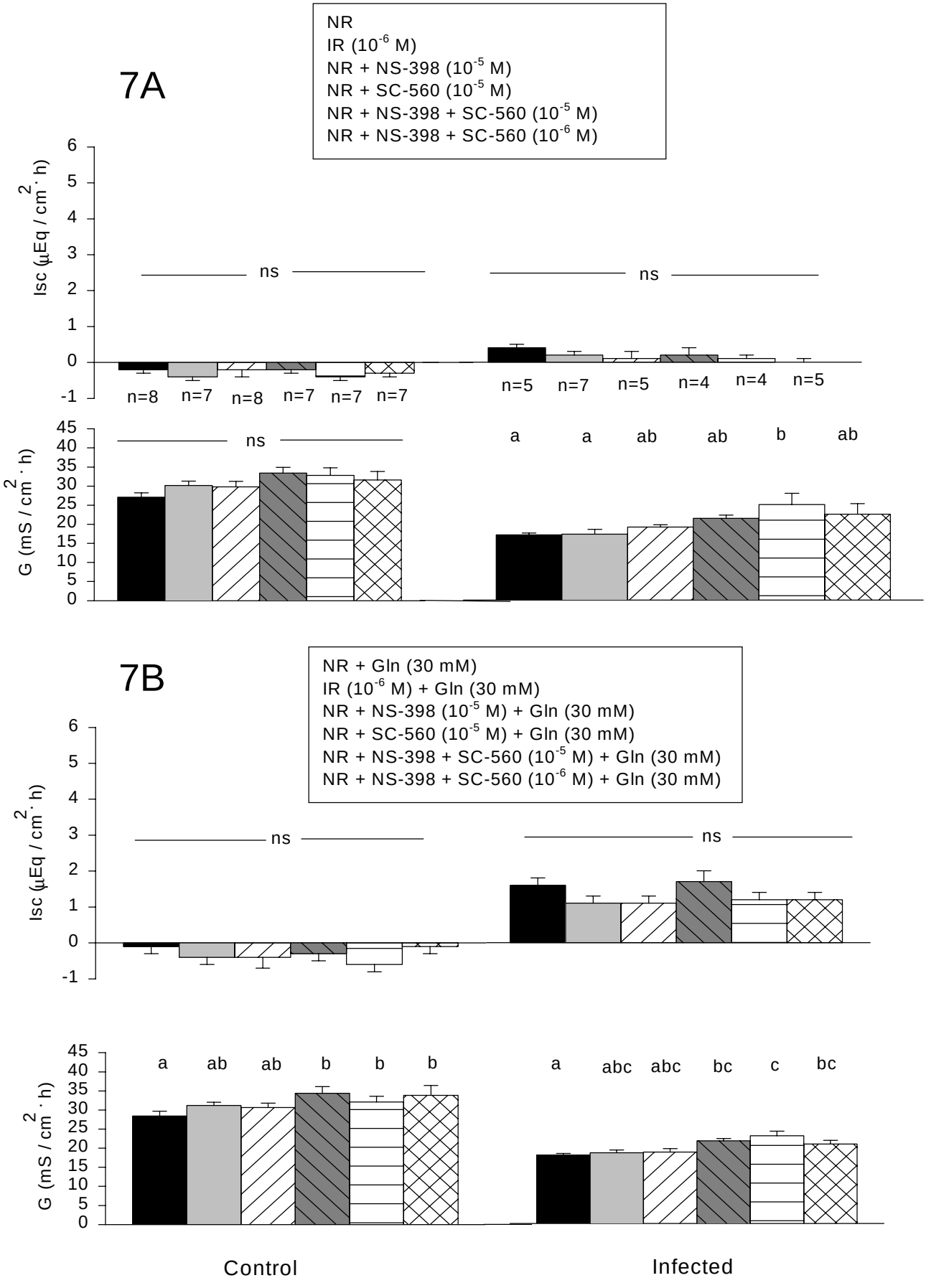

Densitometric Analysis
Arbitrary Values
6000
5000
4000
3000
2000
1000
0
a
b
Control
Infected

6A


NR
IR (10^{-6} M)
NR + NS-398 (10^{-5} M)
NR + SC-560 (10^{-5} M)
NR + NS-398 + SC-560 (10^{-5} M)
NR + NS-398 + SC-560 (10^{-6} M)
$J_{net}Na$ ($\mu Eq/cm^2 \cdot h$)
6
5
4
3
2
1
0
-1
ns
a
b
b
b
b
b
n=8
n=7
n=8
n=7
n=7
n=7
n=5
n=7
n=5
n=4
n=4
n=5

6B


NR + Gln (30 mM)
IR (10^{-6} M)+ Gln (30 mM)
NR + NS-398 (10^{-5} M)+ Gln (30 mM)
NR + SC-560 (10^{-5} M)+ Gln (30 mM)
NR + NS-398 + SC-560 (10^{-5} M)+ Gln (30 mM)
NR + NS-398 + SC-560 (10^{-6} M)+ Gln (30 mM)
$J_{net}Na$ ($\mu Eq/cm^2 \cdot h$)
6
5
4
3
2
1
0
-1
a
ab
ab
bc
ab
bc
a
b
b
b
b
b
Control
Infected


7A
NR
IR (10-6 M)
NR + NS-398 (10-5 M)
NR + SC-560 (10-5 M)
NR + NS-398 + SC-560 (10-5 M)
NR + NS-398 + SC-560 (10-6 M)
Isc (µEq / cm2 · h)
ns
ns
n=8
n=7
n=8
n=7
n=7
n=7
n=5
n=7
n=5
n=4
n=4
n=5
G (mS / cm2 · h)
ns
a
a
ab
ab
b
ab
7B
NR + Gln (30 mM)
IR (10-6 M) + Gln (30 mM)
NR + NS-398 (10-5 M) + Gln (30 mM)
NR + SC-560 (10-5 M) + Gln (30 mM)
NR + NS-398 + SC-560 (10-5 M) + Gln (30 mM)
NR + NS-398 + SC-560 (10-6 M) + Gln (30 mM)
Isc (µEq / cm2 · h)
ns
ns
G (mS / cm2 · h)
a
ab
ab
b
b
b
a
abc
abc
bc
c
bc
Control
Infected

Preliminary NRSA

A long-term goal of this laboratory is the determination of mechanisms by which intestinal absorptive processes adapt to pathogenic injury, focusing primarily on the upregulation of crypt-based transporters following villous atrophy. Several researchers have demonstrated the presence of transporters in crypt epithelium, and we intend to further elucidate the extent of this adaptive response and possible mechanisms regulating this response (Blikslager et al. 2001; Binder et al., 2000).

In this laboratory, we use a *Cryptosporidium parvum*-infected calf model to examine the effects of injury on ileal tissue. This model is ideal for studying this zoonotic pathogen, since the calf is the natural reservoir for *C. parvum* and exhibits the same severe, watery diarrhea seen in human hosts. The widespread nature of this pathogen is perhaps responsible for the number of large outbreaks of cryptosporidiosis in humans; in recent years, six outbreaks of over 18,000 individuals have been reported (Smith and Rose, 1990).

In preliminary studies, using a *C. parvum*-infected calf tissue model, we identified three key findings suggesting an adaptive response in the ileal crypt epithelium. First, ASC protein (a glutamine transporter) is substantially upregulated in the apical membranes of crypt enterocytes. Second, administering a low dose of glutamine (10 mM) stimulated electrogenic absorption of sodium. Third, a higher dose of glutamine (30 mM) stimulates electroneutral absorption of sodium. These last two findings occurred despite the severe villous atrophy and loss of mature enterocytes populating the terminal villi

following infection, suggesting a crypt cell location for the transporters. However, despite the evidence accumulated thus far, a definitive study assigning transport activity following injury to crypt enterocytes has not yet been conducted.

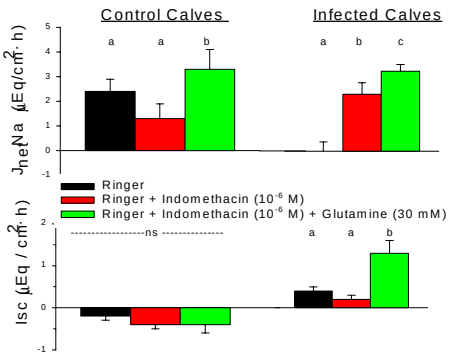
Our central hypothesis is that damaged ileal tissue compensates for the decreased absorptive ability of the terminal villi, by upregulating the production of nutrient transporters in the crypts. Specifically, we postulate that *C. parvum* infected calf ileum has Na^+-linked transporters expressed in crypt epithelium.

Specific Aim 1: Test the hypothesis that intestinal injury results in the adaptive upregulation of crypt based nutrient transporters resulting in near complete restoration of sodium absorption when compared to uninjured controls.
Specific Aim 2: Test the hypothesis that this adaptive upregulation is the result of de novo protein synthesis and insertion into the apical membrane.

Background and Significance: The World Health Organization estimates that, each year, over 5 billion diarrheal episodes affect children and infants worldwide, with a mortality rate of approximately 10 million deaths per year (Levy and Deckelbaum R, 1991; Snyder and Merson, 1982). Although there is a wide range of causative agents of diarrheal disease, one primary culprit is *Cryptosporidium parvum*. Smith and Rose (1990) reported that 20% of diarrheal episodes in developed countries can be attributed to *C. parvum* infection, and that in recent years, 6 outbreaks exceeding 18,000 cases of cryptosporidiosis have been reported in the United States alone. Ciarlet and Estes (2001) report

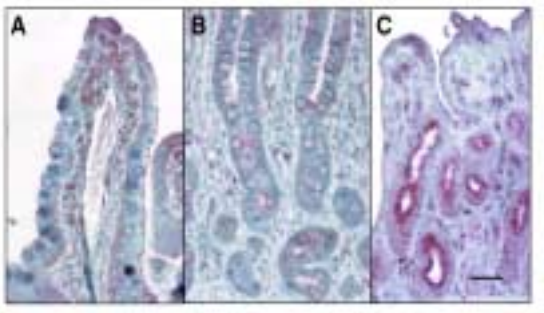
that the cost of diarrhea related hospitalizations for infants and young children in the United States exceeds $1 billion annually. The calf is an excellent model for cryptosporidial diarrhea, since it exhibits the same severe, watery diarrhea characteristic of infection in humans. In addition to serving as a model for investigating the pathogenesis of this disease in humans, *C. parvum* infection in calves has a major economic impact on livestock operations throughout the world. Recent studies have shown that prostaglandin production in this infection inhibits neutral sodium absorption and treatment of infected tissue with indomethacin restores sodium absorption to normal. (Figure 1).

Figure 1. Isotopic flux and electrical response of control and infected tissue following prostaglandin inhibition and glutamine addition.



Intestinal injury and severe diarrhea usually results in severe villous atrophy, and loss of absorptive capability. Additionally, patients maintained on total parenteral nutrition have extreme difficulty being returned to an enteral diet, simply from the loss of the villous architecture necessary for nutrient absorption (Ghatei et al., 2001). However, recent studies have challenged the paradigm that a loss

Figure 2. Immunohistochemical localization of ASC transporter on A) Healthy villous tip, B) Healthy villous crypt, C) Infected villous crypt.

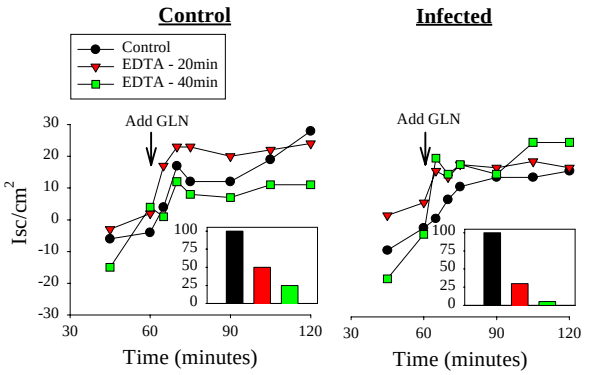


of villi, with a mature enterocyte population, invariably results in malabsorption and subsequent malnutrition. Glutamine has been shown to increase electrogenic sodium transport in *C. parvum* infected calves, whereas in non-infected calf ileum, electroneutral sodium uptake is also stimulated by glutamine addition (Figure 1; Ref 1,2). In the calf, infected tissue responds to injury by the production of ASC transporter in the crypt epithelium (Figure 2). Despite the loss of normal villous architecture, Western blot analysis demonstrated an increase in the amount of ASC protein in infected ileal tissue (Figure 3). This key finding may explain the presence of electrogenic sodium transport in glutamine stimulated infected tissue despite severe villous atrophy. Other compounds, such as alanylglutamine and alanine have also been shown to increase electrogenic Na^+ uptake; alanine may use similar transporters, including ASC, while alanylglutamine likely uses a dipeptide transporter (PEPT1)(Blikslager et al., 2001; Rhoads et al., 1991). Since these compounds are absorbed despite the severe villous atrophy following infection, there is a high probability that crypt cell transport is responsible. Furthermore, data generated using tissue treated with EDTA, which removes the villi and leaves the crypts intact, indicates that the infected intestine retains absorptive capability even in the absence of mature enterocytes, whereas control intestine loses this capacity (Figure 4).

Figure 3. Western blot analysis for the Na^+-glutamine transporter ASC in both control and infected calf tissue.

The finding that glutamine stimulates sodium transport in infected tissue, possibly via crypt epithelial transporters, has tremendous potential to lead to the development of more efficacious nutritional therapies to combat both fluid loss during severe diarrheal episodes and malnutrition resulting from short bowel syndrome. The upregulation of one transporter in crypt cells indicates a high likelihood of other transporters being similarly upregulated. The goal of this study is to determine whether amino acid transporters are upregulated in the crypts as part of an adaptive mechanism to injury.

Figure 4. Tissue response to 10 mM glutamine following removal of the villi via EDTA incubation for 20 and 40 minutes. Inset boxes represent the percent of the villi remaining following incubation.



Experimental Design and Methods

Specific Aim 1: Test the hypothesis that intestinal injury results in adaptive upregulation of crypt based transporters.

Rationale: Previous research indicates that treating injured ileal tissue with absorptive substrates, such as glutamine and alanylglutamine, stimulates sodium-dependent transport. Due to the extent of the villous atrophy caused by infectious diarrhea, and immunhistochemical data localizing various transporters,

we believe that the intestine adapts to injury by upregulation of crypt based transporters. However, it has not been determined whether this response is limited to neutral amino acids, or involves other substrates, such as glucose, cationic amino acids, or dipeptides. Therefore, representatives from several classes of transporters (ASC, SGLT, PEPT1, b^{o+}) have been chosen for study. ASC is a sodium-dependent, neutral amino acid transporter that transports glutamine, among other amino acids. SGLT is a Na-glucose transporter. Preliminary evidence using injured intestinal tissue indicate that there is no stimulation of sodium absorption following the administration of glucose. PEPT1 is a dipeptide transporter, that transports a range of amino acids, including alanylglutamine. b^{o+} is a Na-independent transporter, that transports bipolar, cationic amino acids, such as lysine (Munck et al., 2000). It has not been determined if lysine treatment has an effect on nutrient uptake, although it would not be expected to stimulate sodium absorption. The Na-independent transporter has been chosen in an attempt to determine if the adaptive response is limited solely to Na-dependent transporters, or whether the response is more generalized in nature.

Methods: Sixteen single source newborn Holstein calves will be purchased, fed milk replacer, and tested for adequate colostrum absorption. Inadequate colostral immunoglobulin absorption or previous exposure to *Cryptosporidium parvum* will result in elimination from the study. Eight calves will be sham-infected control animals. A second group of 8 calves will be infected at 7 days of

age by oral administration of 10^8 *C. parvum* oocysts, and housed in strict P2 isolation. At 11 days of age, which previous studies have shown is the time of maximal damage, both groups of calves will be euthanatized via intravenous barbiturate overdose, and the ileum removed. Tissue will immediately be removed and preserved for immunohistochemical localization of ASC, SGLT, PEPT1, and b^{o+}.

In addition to preservation of ileal tissue for IHC analysis, both villi and crypt epithelial cells will be isolated, with the procedure described by Weiser (1973). Briefly, the ileum will be removed, rinsed thoroughly, and incubated at 37^{o}C with a sodium citrate containing solution, followed by an EDTA-based solution. After several cycles of centrifugation, the cells will be isolated. The epithelial cells slough in a temporal gradient, proceeding down the villous, with the crypt cells being removed last. The cells will be separated into two groups-villous tip cells and crypt cells. To ensure proper differentiation, alkaline phosphatase, which is located in the mature villous epithelium, and thymidine kinase, located in the crypts, will be quantified. Sequential cell fractions will also be treated with propidium iodide to ensure that the cells are intact, with functional membranes, to ensure that treatments are absorbed via transporters, and not simply by diffusion across injured membranes.

Cellular fractions will then be incubated with Ringer solution treated with radioisotopically-labeled glucose, glutamine, alanylglutamine, or lysine for 5

minutes. At this time, the cells will be rinsed with ice-cold phosphate buffered saline, which will halt the uptake of labeled treatment, and prevent the metabolism and secretion of by-products. The cells will then be counted on a liquid scintillation counter for the amount of isotope found within the cell, thus determining the extent of transport.

Expected Results: We expect that, in healthy tissues, the transporters will be located in the apical membrane on the terminal villous cell fraction. In infected tissue, we predict that the apical membranes of crypt epithelium will contain both ASC and PEPT1, which will likely not be seen in this location in healthy animals; a large decrease in the amount of SGLT and b^{o+} limited to villi remnants is expected. We predict a decrease in SGLT because of the lack of glucose-stimulated sodium absorption in *C. parvum* infected tissue, where glutamine readily stimulated sodium uptake.

The critical aspect of this study will be the immunohistochemical localization of the transporters to the apical membrane in the crypt cells of infected intestinal tissue. Ample evidence exists that glutamine stimulates sodium uptake in infected tissue, by an electrogenic transport mechanism; however, it has yet to be confirmed that the crypt cells are responsible for this increase. It is expected that the crypt isolates from the healthy tissue will absorb minimal label, while the cells from the infected tissue will absorb an amount of labeled amino acids that approaches the levels seen in villous enterocytes isolated from healthy animals. Demonstration of this fact will open a new arena for the development of more

efficacious nourishment and rehydration regimes for patients suffering from severe diarrheal illnesses.

Pitfalls: One concern is that cells separated from the basement membrane will lose their polarity, and there will be no means of differentiating between apical and basolateral uptake of the amino acid. However, IHC analysis of the tissues will determine if there is an apical transporter for the amino acids being absorbed. Should immunohistochemical analysis prove insufficient to determine the location of the transporters responsible for the uptake of amino acids (ie. if the transporter proteins are revealed on both apical and basolateral membranes), the isolation of enterocytes will obviously be ineffective, as there will be no way to differentiate between apical and basolateral absorption. Therefore, if the preliminary IHC data indicate the localization of protein to both membranes, apical membrane vesicles will be used to determine amino acid absorption.

A second potential pitfall in this study is the number of potential transporters that could be upregulated. With approximately 20 amino acids, 400 dipeptides and 8000 tripeptides, there is a very large number of potential transporters that will not be tested for. However, by analyzing amino acids that have already been demonstrated to generate a response in infected tissue, this study will indicate whether transporters are upregulated in the crypts. A range of transporters has been chosen for analysis to determine if adaptive upregulation extends to more than one type of transporter.

<u>Specific Aim 2:</u> Test the hypothesis that the adaptive response in *C. parvum*-infected intestine is due to de novo protein synthesis of transporters and and membrane insertion into crypt epithelium.

Rationale: The first study should demonstrate that crypt cells respond to infection by increased Na-dependent amino acid transport. However, despite preliminary evidence indicating that sodium absorption is stimulated by amino acid addition to both *C. parvum* and rotavirus infected animals, regardless of the extensive villous atrophy, the mechanism by which this is accomplished is unknown. Several possibilities exist, including de novo synthesis of new transport protein and insertion into the membrane, alterations in intracellular trafficking from a pre-existing pool of transport protein, protein synthesis from a pre-existing mRNA pool, or an alteration in the electrochemical gradient driving the absorptive activity. This study will help determine the basic mechanism by which the intestine responds to injury.

Methods: A group of ten calves will be obtained, housed, and fed as described above. Five of the animals will be sham infected controls; five will be inoculated with 10^8 *C. parvum* oocysts at seven days of age. All animals will be euthanatized at eleven days of age, the ileum removed, and both villi and crypt cells isolated, as described above.

Cellular fractions will be analyzed by Northern and Western blot, as well as a detergent extraction assay Western analysis for the presence and localization of ASC, PEPT1, b^{0+}, and SGLT to determine any alterations due to infection. It is expected that all transporters will be expressed in villous cells from healthy ileal tissue, but not crypt cells. ASC and PEPT1 will be present in crypt cells from infected tissue.

Expected Results: The results of the Western and Northern analysis will aid in determining the mechanisms of adaptation to injury, whether it is an alteration in transcription, resulting in de novo production of transporters accompanied by trafficking of the newly formed protein to the membrane or another possible mechanism (Table 1). Should the injury not result in de novo transporter synthesis, the results can most likely be attributed to an alteration in protein trafficking from an existing intracellular reservoir. However, another possibility exists. The upregulation of crypt absorption could be due to an alteration of the electrochemical gradient driving the movement of electrolytes into the cell, which would occur without the production or membrane insertion of new transporter.

Table 1. Explanation of the increase in nutrient uptake by injured intestinal crypt cells, with possible results of the Western and Northern analysis. Arrows indicate expected difference from the crypt enterocytes in uninjured control tissue.

	Protein Levels	mRNA Levels
1. Increased synthesis	↑	↑
2. Increased trafficking	↔	↔
3. Increased translation	↑	↓
4. Increased electrochemical gradient	↔	↔

Pitfalls: The most likely alternative hypothesis that would explain the presence of Na-dependent amino acid transport, aside from an alteration of de novo synthesis is a change in membrane insertion. It could be that the cells are manufacturing transporters as part of their normal proliferative function, without inserting them into the membrane. This could lead to the formation of an intracellular pool, from which the transporters insert into the membrane when the enterocyte has reached the villi and matured. In the control tissue, there may be no stimuli to induce membrane insertion in the crypt cells, since the transporters populating the terminal villous would be sufficient to meet the animals needs. Villous atrophy during infection could result in inadequate nutrient uptake, and provide the stimuli needed to induce membrane insertion. If this is true, and the infection simply results in the insertion into the membrane, then there will be no difference in the Western or Northern analyses when the healthy and infected cells are compared. Therefore, in the event of a lack of upregulated transport mRNA and protein (Table 1), a detergent extraction assay Western analysis will be conducted. This technique will allow the protein content of the membrane, cytoplasm and cytoskeleton to be differentiated, and after comparison of the infected to the control tissue, yield evidence as to the intracellular location of the protein during injury.

Similarly, the tissue could already contain a pre-existing pool of ASC mRNA as a part of their normal proliferative and developmental response. In healthy tissue, the enterocyte may not manufacture the protein until it has fully differentiated and

migrated onto the terminal villi. However, either the injury itself, or the addition of glutamine, may cause a translational response, and result in the production of ASC protein, with no alteration in mRNA levels.

The least likely hypothesis is that infection and treatment may be altering the electrochemical gradient, resulting in an increased J_{max}. This would not change the amount of mRNA or protein present (Table 1), but simply alter the rate at which the already present transporters function. However, if this were true, then the injured tissue would not demonstrate specificity in its treatment response. Glucose, which also utilizes a Na-dependent transporter, should stimulate a response similar to that of glutamine, which evidence clearly indicates it does not. To determine this, cells in both control and infected tissue will be manipulated to artificially change the gradient, using prostaglandin inhibition of NHE to increase the Na gradient. This should non-specifically increase the nutrient uptake in the crypts of both infected and control tissue.

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Preliminary Data

Background: A study to characterize the unidentified electroneutral sodium transporter reported in the above study is currently underway. The following experiment has been designed with two objectives. First, to localize the transporter in *C. parvum* infected ileum to either the crypts or villous remnants. Second, to functionally identify the transporter uncovered by prostaglandin inhibition.

Materials and Methods: Calves will be obtained, housed, and infected as described above. Briefly, calves to be infected will receive 10^8 *C. parvum* organisms at 7 days of age. Both control and infected calves were euthanatized at 11 days of age, and the ileum removed and mounted in Ussing chambers. Electrical and isotopic flux (^{22}Na and ^{36}Cl) data will be collected, and well as tissue samples for morphology measurements. Treatments are outlined in table 1. With the exception of the normal Ringer solution as a control, all tissues received indomethacin, since non-selective cyclooxygenase inhibition is required for the uncovering of the electroneutral

Treatment	Expected Results	
	Control	Infected
1. Ringer	baseline	↓
2. Ringer + indomethacin (10^{-6} M)	↔	↑
3. 2 + EDTA (1.5 mM)	↓	↑
4. 2 + MIA (10^{-4} M)	↓	↓
5. 3 + MIA	↓	↓
6. Chloride-free Ringer + indomethacin (10^{-6} M)	↔,↓	↔,↓
7. 2 + S-3226	↓	↑
8. 2 + HOE-694	↔	↑
9. HCO_3^--free Ringer + indomethacin (10^{-6} M)	↔,↓	↔,↓

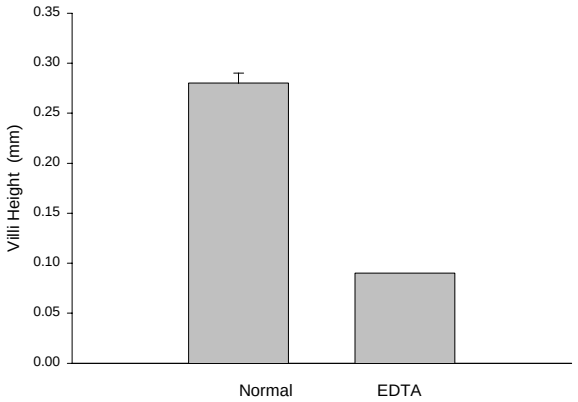
Table 1. Explanation of the treatments and expected results in both uninjured and *C. parvum* infected ileal tissue following treatment addition. Arrows indicate expected alterations in sodium flux following treatment administration to the tissues mounted in the Ussing chambers.

transporter being studied. Ethylenediaminetetraacetic acid (EDTA) treatments, which remove the villi and leave the crypts intact, will localize the transporter to the crypts of the infected tissue. Methyl isobutyl amiloride (MIA), a non-selective inhibitor of sodium channels, sodium-hydrogen exchangers (NHE), and sodium-bicarbonate cotransporters (NBC), will demonstrate that the unidentified protein being dealt with is either an NHE or an NBC. Sodium channels, which are electrogenic, are obviously not responsible for the transport uncovered by prostaglandin inhibition. The chloride-free Ringer solution will, at least partially, determine if the novel transporter is actually the Cl-NHE first reported by Binder et al (2000), which was discussed previously. Since Cl-NHE is thought to be completely dependent on the presence of chloride, the removal of chloride from the solution (replaced by isethionate) should completely inhibit the activity of the transporter under study. Treatment with S-3226, a selective NHE-3 inhibitor, and HOE-694, a selective NHE-2 inhibitor, will confirm the results of the preceding study, that neither NHE-3 nor NHE-2 is present or active in the infected tissue. The final treatment, HCO_3^--free Ringer solution will investigate the possibility that the transporter under study is actually an NBC. This would be a completely novel finding, as no NBC isoform has yet been identified in the apical membranes of intestinal mucosa.

Preliminary Results

Tissue Morphology: Tissue collected from healthy calves was examined to confirm the absence of infection, and that the EDTA treatment was effectively removing the villi. Results are presented in Figure 1. Briefly, EDTA treatment reduced the villous height ($P < 0.01$). In combination with the increased perfusion, EDTA completely removed the villi, while leaving the crypts intact.

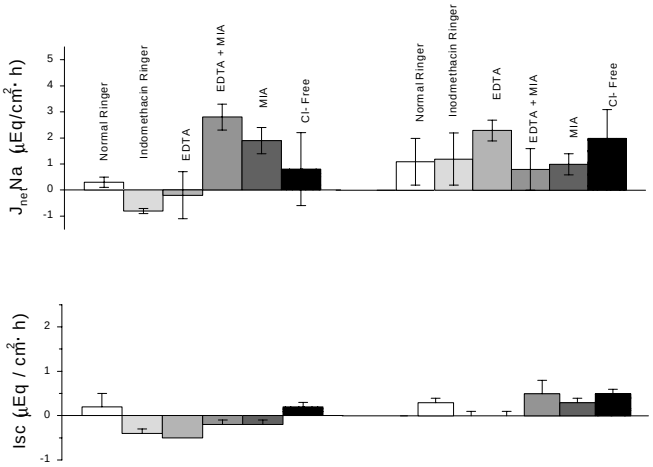
Figure 1. Villi height in healthy calves and healthy calves treated with EDTA and increased perfusion rate.



Electrical and Isotopic Flux Data: Electrical and sodium flux data is presented in Figure 2. The control data will not be commented upon here, as an insufficient number of calves have been examined to make such discussion meaningful. However, some conclusions may be drawn from the data obtained from the infected calves. Based on the preponderance of data obtained in previous studies, such as the one discussed above, one can safely assume that as the numbers increase, the sodium flux in the normal Ringer solution will be closer to 0, instead of the 1 $\mu Eq/cm^{2}\cdot h$ seen here. Further, the indomethacin treatment can be expected to restore sodium transport to approximately 2 $\mu Eq/cm^{2}\cdot h$. The EDTA (with indomethacin) treatment had no effect on sodium uptake, nor did it induce an electrical response. This suggests that the sodium transport is

occurring in the crypt enterocytes, as the treatment and the infection have completely removed the villous tips. Both MIA-containing treatments reduce the indomethacin uncovered sodium transport, without affecting the electrical data, indicating an inhibition of either an NHE or an NBC isoform.

Figure 2. Sodium flux and electrical data collected from both control and infected calves.



The lack of effect seen with the chloride-free Ringer solution suggests that the transporter being characterized here is not the Cl-NHE recently described by Binder et al. (2000).

Preliminary Discussion: While this trial is currently underway, a number of conclusions can be drawn from the data obtained thus far. The EDTA and increased perfusion of the Ussing chambers effectively removes the villi in the control tissue. This is borne out by the complete abolition of sodium transport in these tissues, indicating the loss of the transporters populating the villi. The absence of an EDTA effect in the infected tissue suggests that the sodium transport occuring in these tissues is in fact based in the crypt enterocytes. In infected tissues, MIA does lessen the amount of sodium being absorbed when compared to the EDTA treatment. The lack of an electrical response, combined

with the known subjects of MIA inhibition, NHE and NBC protein, suggests that unidentified crypt based transporter is an isoform for one of these two proteins. The chloride-free Ringer solution had no apparent effect in either tissue, indicating that the transporter is independent of chloride; this would appear to eliminate the possibility of the transporter under study being the Cl-NHE postulated by Binder et al. (2000), although it would be premature at this point to definitively rule it out.