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EFFECT OF TRICHURIS SUIS EXCRETORY/SECRETORY PRODUCTS ON INTESTINAL EPITHELIAL CELLS AND CAMPYLOBACTER JEJUNI INVASION UNDER IN VITRO CONDITIONS

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EFFECT OF *TRICHURIS SUIS* EXCRETORY-SECRETORY PRODUCTS ON INTESTINAL EPITHELIAL CELLS AND *CAMPYLOBACTER JEJUNI* INVASION UNDER *IN VITRO* CONDITIONS

By

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ABSTRACT

EFFECT OF *TRICHURIS SUIS* EXCRETORY-SECRETORY PRODUCTS ON INTESTINAL EPITHELIAL CELLS AND *CAMPYLOBACTER JEJUNI* INVASION UNDER *IN VITRO* CONDITIONS

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Campylobacter jejuni is the leading cause of human diarrheal disease in the United States and throughout the world. *Trichuris suis* is a nematode intestinal pathogen in swine and is essentially identical to *T. trichuria* which parasitizes humans. *T. suis*-infected domestic pigs are susceptible to secondary invasion by resident opportunistic pathogens, such as *C. jejuni*. Thus we use this compound infection model system to gain a better understanding of the pathogenic mechanisms of campylobacteriosis and trichuriasis. The goal of this research was to investigate the contribution of *T. suis* excretory-secretory products to these disease processes using *in vitro* model systems complementary to the animal model.

Dedicated to:

Geraldine L. Abner

1921 - 1999

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PREFACE

This thesis includes two chapters that are in manuscript format. The second chapter entitled "Response of intestinal epithelial cells to *Trichuris suis* excretory-secretory products and the influence on *Campylobacter jejuni* invasion" will be submitted to the Journal of Parasitology with the following co-authors: Dr. Dolores Hill (provided ESP and intellectual input), Dr. Paul Bartlett (assisted with statistical analysis), Dr. Jerrold Turner (electrophysiology experiments were performed in his lab) Dr. Joe Urban (intellectual input, contributed reagents), and Dr. Linda Mansfield. Chapter 3 entitled "Detection of antibacterial activity in excretory-secretory products of adult *Trichuris suis*" will also be submitted to the Journal of Parasitology with the following co-authors: Dr. Dolores Hill (provided ESP), Geetha Parthasarathy (contributed to MIC experiments), and Dr. Linda Mansfield.

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LIST OF ABBREVIATIONS

GI	gastrointestinal
GBS	Guillain-Barre Syndrome
CDT	cytolethal distending toxin
OMP	outer membrane protein
ESP	excretory-secretory products
cRPMI	concentrated RPMI-1640
BSA	bovine serum albumin
HBSS	Hank's balanced salt solution
IEC	intestinal epithelial cell
TER	transepithelial electrical resistance
LGC	lymphoglandular complex
PEN	penicillin
STREP	streptomycin
CAP	chloramphenicol
AMB	amphotericin B
MHB	Mueller-Hinton broth
CFU	colony forming unit
MIC	minimum inhibitory concentration
MBC	minimum bactericidal concentration
AU	activity units
HPLC	high performance liquid chromatography
TFA	trifluoroacetic acid
ACN	acetonitrile
MS	mass spectrometry

CHAPTER 1

LITERATURE REVIEW

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CAMPYLOBACTER JEJUNI

Basic microbiology

Campylobacter jejuni is a Gram-negative curved to spiral rod that exhibits a characteristic corkscrew darting motility mediated by polar flagella. These organisms require enriched media and microaerophilic conditions (3-5% O_2, 3-10% CO_2) for optimal growth (On 1996). They are thermophilic with an optimal growth temperature of 42°C, but growth also occurs at 37°C. These organisms are relatively fragile and sensitive to environmental stresses such as 21% oxygen, drying, heating, disinfectants, and acidic conditions (Doyle and Roman, 1982; Gill and Harris, 1982). Although they are non-sporeforming, rods undergo a degenerate conversion to coccoid forms during stressful conditions (Rollins and Colwell, 1986), which is considered a viable but nonculturable state (Stern et al., 1994). The genome of *C. jejuni* NCTC 11168 has been sequenced and is 1,641,480 bp in length (Karlyshev et al., 1998). The number of cloned genes from *C. jejuni* continues to expand though still lags behind that of other enteric pathogens.

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Campylobacter jejuni is a major public health concern because it is the leading cause of bacterial diarrheal disease reported in the United States and the United Kingdom, more so than *Salmonella spp.* and *Shigella spp.* combined (Parkhill et al., 2000; Fields and Swerdlow, 1999). An estimated 2.1 to 2.4 million cases occur each year in the U.S. (Altekruse et al., 1999). Immunocompromised individuals are particularly susceptible (Moe, 1991; Peterson, 1994a). Consumption of foods of animal origin is usually responsible for transmission of *C. jejuni* to humans (Fang et al., 1991; Butzler and Oosterom, 1991). Most cases of human campylobacteriosis are sporadic, peaking during the summer months in association with improper handling of poultry during food preparation (Adak et al., 1995). Outbreaks have been most often attributed to the consumption of raw milk (Evans et al., 1996; Harris et al., 1987; Kornblatt et al., 1985; Korlath et al., 1985).

The predominant ecological niche for *C. jejuni* is the intestinal tract of a wide variety of domestic and wild vertebrates, particularly birds (Endtz et al., 1991; On 1996). *C. jejuni* efficiently colonizes the intestinal tracts of chickens, which leads to spread to the rest of the flock and carcass contamination during slaughter and

processing (Achen et al., 1998). Practical methods have been aimed at competitively excluding *C. jejuni* to reduce colonization and shedding. Probiotics containing commensal organisms have been tested with success in chickens (Morishita et al., 1997). However, antibiotic resistant *C. jejuni* are a growing concern, which stems in large part from overuse of antibiotics in food animals as growth promotants (Kelley et al., 1998; Koenraad et al., 1995).

Diseases

Campylobacter jejuni can cause a spectrum of diseases in humans, initiated by as few as 800 organisms (Black et al., 1988). Commonly reported symptoms include fever, abdominal cramping, and diarrhea, which commence within 2-3 days following inadvertent ingestion of *C. jejuni* (Wallis, 1994). *C. jejuni*-induced diarrhea may mimic that of *Vibrio cholerae* with copious amounts of water excreted in response to toxin production, and/or by *Shigella* with mucus and blood present in the stool due to invasion of cells (Black et al., 1988; Wallis, 1994). Histological sections of colonic biopsies reveal varying degrees of colitis with crypt abscesses, depletion of goblet cells, and inflammatory infiltrates of the lamina propria composed of polymorphonuclear leukocytes, lymphocytes, and plasma cells

(Lambert et al., 1979). Infection with *C. jejuni* is usually confined to the lower gastrointestinal tract (GI) and is self-limiting within a period of 5-8 days (Black et al., 1988). However, *C. jejuni* infection can result in severe enteric disease and lead to peritonitis, ileitis, and intestinal obstruction (Perkins and Newstead, 1994). Systemic disease is rare, but is a major concern in immunocompromised individuals. Some people have macrophages incapable of killing *C. jejuni*, which may be a risk factor for developing systemic disease due to translocation of *C. jejuni* by these host cells (Wassenaar et al., 1997). Chronic sequelae of *C. jejuni* infection include Guillain-Barre syndrome (GBS), a demylenating neuropathy (Hahn, 1998; Nachamkin et al., 1998; Allos et al., 1998), and Reiter's syndrome, a reactive arthropathy (Peterson, 1994b; Ponka et al., 1981). Both are thought to have an autoimmune etiology. GBS appears to result from cross reactivity of anti-*C. jejuni* antibodies with gangliosides on the surface of host neurons (Yuki and Miyatake, 1998).

Pathogenesis

The molecular mechanisms of *C. jejuni* virulence are incompletely characterized and controversies abound (Mansfield and Abner, 2000; Ketley, 1997). Colonization of

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mucus and adhesion to enterocytes disrupts the normal absorptive capacity of the intestine by damaging epithelial cell function. This can occur directly, by cell invasion or by the production of toxins, or indirectly, following the initiation of an inflammatory response. Infection resulting in disease and pathology depends on multiple bacterial determinants and is influenced by the host's immune status.

Model systems. A variety of animal and tissue culture models have been applied to the study of *C. jejuni* pathogenesis. Chickens are an attractive model because they are the primary source of human infections. However, their response to oral feeding of *C. jejuni* is essentially limited to watery diarrhea (Sanyal, et al., 1984). A great deal of effort has been directed toward developing a mouse model of *C. jejuni* pathogenesis to aid in understanding the contribution of host factors to pathogenesis. Colonization can be achieved in mice, but development of clinical signs and histopathological lesions typical of human campylobacteriosis are difficult to induce unless *C. jejuni* is delivered by non-natural routes to immunodeficient or gnotobiotic mice (Hodgson et al., 1998; Stanfield et al. 1987; Yrios and Balish, 1986). The removable intestinal tie adult rabbit diarrhea procedure has been used to investigate

C. jejuni pathogenesis, particularly to evaluate toxin-producing isolates (Caldwell et al., 1983; Pang et al., 1987). Monkeys have been experimentally infected with *C. jejuni* (Russell et al., 1993; Fitzgeorge et al., 1981), and there are several reports of naturally acquired infections in other laboratory animals (George and Lerche, 1990; Bryant et al., 1983).

Pigs are a promising model and have been used by several investigators (Boosinger and Powe, 1988; Vitovec et al., 1989; Babakhani et al., 1993; Mansfield and Urban, 1996). Although immunocompetent pigs with a full complement of enteric bacteria and immune system components are refractory to infection with *C. jejuni*, gnotobiotic or colostrum-deprived piglets inoculated orally with pathogenic strains of *C. jejuni* develop disease (Boosinger and Powe, 1988; Vitovec et al., 1989; Babakhani et al., 1993). Alternatively, young immune competent pigs exposed to the helminth *Trichuris suis* developed clinical signs and pathology due to naturally acquired *C. jejuni* that closely mimics human disease (Mansfield and Urban, 1996). These pigs develop a self-limiting colitis with watery, bloody diarrhea that correlates with the presence of third stage *T. suis* larvae in the proximal colon and resolves at 50-60 days

of infection following worm expulsion. Colostrum-deprived newborn piglets have also been used to harvest primary colonic epithelial cells to develop *in vitro* assays for the study of *C. jejuni* cellular invasion (Babakhani and Joens, 1993).

Due to their relative ease of maintenance, continuous cell lines derived from various sites of the intestinal epithelium from multiple species and humans have been used more extensively. These cell culture model systems are well-suited to study the interplay between *C. jejuni* and the host cell, facilitating dissection of pathogenic mechanisms at the cellular and molecular levels.

Intestinal colonization. Exposure of pathogens to an *in vivo* environment is important for the expression of virulence genes (Slauch et al., 1994). Adaptation to the changing intestinal environment, such as temperature, pH, and nutrient availability, is required for successful enteric pathogenesis. Heat shock proteins are among the virulence factors needed for *C. jejuni* to cope with the stressful conditions encountered in the intestinal environment, based on the observation that dnaJ mutants were unable to colonize newly hatched chickens (Konkel et al., 1998). Additionally, gastric acid in the stomach provides a

barrier against infection, because ingestion of *C. jejuni* with sodium bicarbonate increases the rate of illness in humans (Black et al., 1988). Once the acid barrier is breached, *C. jejuni* colonizes the distal small intestine and colon. Chemotaxis and motility are critical for *C. jejuni* to establish and maintain a niche within intestinal mucus (Ketley, 1997; Hugdahl et al., 1988). There are multiple reports from *in vivo* model systems that *C. jejuni* is able to colonize mucus, preferentially dwelling within crypts in distal segments of the intestine (Beery et al., 1988; Terzolo et al., 1987; Lee et al., 1986). Colonization of mucus places *C. jejuni* in close proximity to enterocytes, such that toxins and/or adhesins may reach their cellular targets. Spiral cellular morphology and polar flagella provide *C. jejuni* with a distinctive motility well-suited to movement in a viscous environment, which is likely to provide an ecological advantage in intestinal mucus (Ferrero and Lee, 1988; Szymanski et al., 1995). Motility and high-viscosity growth medium mimicking intestinal mucus enhanced binding and invasion of *C. jejuni* in Caco-2 cells (Szymanski et al., 1995). Mucin also enhanced total cell-associated and internalized *C. jejuni* in HEp-2 cells (DeMelo and Pechere, 1988). *C. jejuni* containing mutations in

flagellin genes were unable to colonize the intestinal tract of chickens, indicating that flagella enhance colonization (Nachamkin et al., 1993; Wassenaar et al., 1993).

Toxins. Numerous reports exist on toxins from *C. jejuni* assessing detection among isolates from various human and animal sources in different geographic locations. Most of these studies aim to correlate toxin production with clinical presentation. Animal isolates are less toxigenic than human isolates (Akhtar and Huq, 1989), and among animal isolates *Campylobacter* strains from pigs are less enterotoxigenic than those from chickens (Lindblom et al., 1990). Distinct cholera-like, cytotonic enterotoxin and cytotoxin activities have been described from *C. jejuni*, sometimes being produced concurrently (Johnson and Lior, 1984; Klipstein et al., 1985; Johnson and Lior, 1986; Taylor et al., 1987; Daikoku et al., 1989; Bok et al., 1991; Florin and Antillon, 1992). In general, noninflammatory, acute secretory (watery) diarrhea is associated with the cytotonic enterotoxin in children in developing countries, whereas inflammatory diarrhea and *Campylobacter* invasion are associated with cytotoxin production in sporadic cases in developed countries (Blaser et al., 1979; Klipstein et al., 1985; Walker et al., 1986; Guerrant et al., 1987).

The original description of *C. jejuni* enterotoxin indicated structural and functional similarity to cholera toxin based on elongation of CHO cells, heat lability, inactivation by cholera antitoxin, increased intracellular cyclic AMP levels, and fluid secretion in rat ileal loops (Ruiz-Palacios et al., 1983). Kanwar and colleagues (1994; 1995) have made substantial contributions toward understanding the mechanisms underlying observed increases in intracellular cAMP and fluid accumulation in ligated ileal loops infected with *C. jejuni* (McCardell et al., 1984). The pathophysiology of secretory diarrhea from enterotoxigenic *C. jejuni* infection involves impaired Na^+, K^+-ATPase activity associated with increased Na^+ and Cl^- secretion (Kanwar et al., 1994). Alteration of ion transport is a calcium-dependent process involving protein kinase C activation (Kanwar et al., 1995).

Despite a considerable amount of supporting evidence, several investigators have reported failures to repeat experiments aimed at detecting enterotoxin activity from various sources of *C. jejuni* isolates, casting doubt on the existence of a cholera-like enterotoxin in *C. jejuni*. Until the putative enterotoxin is cloned and sequenced, controversy will remain regarding its existence and

pathobiological significance in *C. jejuni*-mediated enteritis (Wadstrom et al., 1983).

Assays for *C. jejuni* cytotoxins are usually based on morphological alterations of a wide variety of cultured cells. Indicators of cytotoxin activity include cell rounding, elongation, distention, loss of adherence, and cell death. *In vitro* cytopathic effects from cell-free filtrates of polymxyin B-treated and sonicated *C. jejuni* and stool filtrates from infected individuals and animals have been described repeatedly (Johnson and Lior, 1984; Goossens et al., 1985; Pang et al., 1987; Johnson and Lior, 1988; Akhtar and Huq, 1989; Cover et al., 1990; Florin and Antillon, 1992).

The best characterized cytotoxin from *C. jejuni* is the cytolethal distending toxin (CDT) originally described by Johnson and Lior (1988) as causing progressive cell distention and eventual cytotoxicity. Nucleic acid sequence similarity with the *E. coli* equivalent provided primers for PCR amplification, leading to the cloning of three adjacent *C. jejuni* genes (*cdtA*, *cdtB*, and *cdtC*) encoding proteins with predicted sizes of 30, 29, and 21 kDa (Pickett et al., 1996). All three genes were required for toxic activity of CDT in a HeLa cell assay (Pickett et al., 1996). It has

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recently been shown that *C. jejuni* CDT causes HeLa and Caco-2 cells to become arrested in the G_2 cell cycle stage (Whitehouse et al., 1998). In HeLa cells, this block was associated with failure to dephosphorylate CDC2 which leads to accumulation of the inactive form of this kinase necessary for entry into the M phase (Whitehouse et al., 1998).

Adhesion. Several candidate adhesins of *C. jejuni* have emerged over the years. Treatment of bacterial and host cells with proteases and fixatives have been used to document reductions in binding, implicating protein involvement in the adhesin-receptor interaction (McSweegan and Walker, 1986; Maruyama and Katsube, 1994; Moser et al., 1992; Moser and Schroder, 1995). Multiple carbohydrates have been considered to be involved in *C. jejuni* binding to host cells (McSweegan and Walker, 1986; Moser et al., 1992; Russell and Blake, 1994; Szymanski and Armstrong, 1996). Fucose, galactose, mannose, maltose, and glucose may mediate adhesin-receptor interaction, as these sugars have been reported by different investigators to inhibit adhesion of intact or fractionated *C. jejuni* outer membranes to host cell membranes (Moser et al., 1992; Russell and Blake, 1994). *C. jejuni* has an affinity for multiple intact lipid

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structures, particularly unsaturated fatty acids, suggesting that lipids in host cell membranes are involved in mediating *C. jejuni* adhesion (Szymanski and Armstrong, 1996). Despite considerable effort, no single molecular structure has been unequivocally determined to be the primary adhesin.

Flagella have been considered to be involved in the adhesive process (McSweegan and Walker, 1986; Szymanski et al., 1995), although studies with motility defective mutants indicate that flagella have an ancillary role in adhesion (Grant et al., 1993; Yao, et al., 1994; Russell and Blake, 1994). Early work suggested that the oligosaccharides of LPS were involved in *C. jejuni* adhesion, based on reduced cell binding following periodate oxidation of LPS (McSweegan and Walker, 1986). In contrast, Moser and Schroder (1995) reported that the binding of *C. jejuni* outer membrane preparations to INT 407 cells was not significantly altered following oxidation of LPS by sodium metaperiodate nor by pretreatment with LPS-specific monoclonal antibody. Abundant evidence implicates *C. jejuni* LPS in post-infection pathology although it may not have a primary function in adhesion.

Peritrichous pilus-like appendages have been identified in *C. jejuni* (Doig et al., 1996). These are

induced when the bacterium is grown in the presence of bile salts and confer a highly aggregative phenotype. A site-specific insertional mutation within a gene, termed *pspA* which encodes a predicted protein resembling protease IV of *E. coli*, results in the loss of pilus synthesis (Doig et al., 1996). Although the non-piliated mutant showed no reduction in adherence to or invasion of INT 407 cells *in vitro*, disease symptoms in a ferret animal model were significantly reduced (Doig et al., 1996).

Multiple surface and periplasmic *C. jejuni* proteins have been identified and characterized. Although other functions may be inferred based on amino acid sequence analysis, surface-exposed proteins are candidates for adhesins by virtue of their location. The major outer membrane protein (OMP) is arranged hexagonally as a trimer of 42 kDa subunits and is antigenically related to the *E. coli* OmpC porin (Kervella et al., 1992; Amako et al., 1996; Bolla et al., 1995; Zhuang et al., 1997). Inducible OMPs of 55, 35 and 20 kDa, not detected when cultivated in artificial media, were expressed when *C. jejuni* was maintained in implants in chicken peritoneal cavities for one week (Chart et al., 1996). Additionally, a highly conserved, immunogenic 18 kDa OMP with significant

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similarity to other peptidoglycan-associated lipoproteins of gram-negative bacteria has been recently identified in *C. jejuni* (Burnens et al., 1995; Konkel et al., 1996). Based on the homologous proteins, Omp18 is predicted to form a bridge between the outer membrane and peptidoglycan to stabilize the cell wall.

A 28 kDa surface-exposed protein, cell binding factor 1 (CBF1 or PEB1), possibly involved in binding to host cells and facilitating amino acid transport, has been described in *C. jejuni* (Pei and Blaser, 1993; Kervella et al., 1993). CBF1 is highly conserved among *C. jejuni* strains, but is absent in some nonadherent *C. jejuni* strains (Pei and Blaser, 1993; Kervella et al., 1993). CBF1 plays a role in adhesion and invasion of epithelial cells in culture as well as intestinal colonization in a mouse model (Pei, et al., 1998). P29 (HisJ) is a 29 kDa periplasmic protein from *C. jejuni* that functions as a histidine binding protein and has homology to CBF1 (Garvis et al., 1996). However, there was no evidence that P29 promoted binding to eukaryotic cells (Garvis et al., 1996). PEB4 (CBF2) is also a 29 kDa periplasmic protein which may function as an extracellular chaperone (Kervella et al., 1993; Burucoa et al., 1995). Additional external proteins recently identified include a

36 kDa lipoprotein containing the signature sequence for siderophore-binding proteins, which may function in iron acquisition (Park and Richardson, 1995), and CjaC having significant homology to periplasmic solute-binding proteins of the ABC transport system (Pawelec et al., 1998).

Extracellular matrix components, including fibronectin, laminin, and collagens, are potential anchor molecules for *C. jejuni* adhesion (Kuusela et al., 1989; Moser and Schroder 1995; Garvis et al., 1997). In particular, *C. jejuni* binding to fibronectin of the extracellular matrix is mediated by a specific interaction with a 37 kDa outer membrane protein termed CadF (Konkel et al., 1997; Konkel et al., 1999a), which is likely to facilitate invasion of host cells. Flagellin and the major outer membrane protein of *C. jejuni* have also been reported to bind to fibronectin (Moser, et al., 1997).

Invasion. Based on experimentally-infected infant monkeys, cell invasion by *C. jejuni* was concluded to be the primary mechanism of colon damage and diarrheal disease (Russell et al., 1993). *C. jejuni* has been detected in the colonic mucosa and submucosa of experimentally infected infant monkeys and newborn piglets (Russell et al., 1993; Babakhani et al., 1993), and has been recovered from

mesenteric lymph nodes through 23 days of infection in a gnotobiotic mouse model (Fauchere et al., 1985). In these models, *C. jejuni* has been detected inside intestinal epithelial cells, indicating that transcellular migration accounts for at least some of the invasion into underlying tissues (Russell et al., 1993; Babakhani et al., 1993). Monocytes which ingest *C. jejuni* convert them to their coccoid forms, although the bacterium may escape prior to degeneration (Wallis, 1994). Monocytes may play a role in the translocation of the organism to the blood stream, but the role of phagocytosis as a defense mechanism against *C. jejuni* seems to be secondary, with the humoral response having the primary role against infection (Wallis, 1994). Secretory IgA in mucus prevented adhesion of *C. jejuni* to INT407 cells and caused the organisms to aggregate (McSweegan et al., 1987). Additionally, IgA plays a large role in protection against colonization and bacteremia following challenge infections with *C. jejuni* in experimentally infected rabbits (Burr et al., 1988).

Under *in vitro* conditions, attachment of *C. jejuni* to cells is independent of bacterial protein synthesis, although internalization is contingent upon newly synthesized bacterial proteins (Konkel and Cieplak, 1992;

Konkel et al., 1992a; Oelschlaeger et al. 1993; Konkel et al., 1999b). *C. jejuni* synthesizes at least 14 new bacterial proteins within 60 minutes following culture with INT 407 cells, suggesting active engagement in a directed response to facilitate its internalization (Konkel and Cieplak, 1992; Konkel et al., 1993). The precise invasion mechanism of *C. jejuni* has not been elucidated and is confounded by conflicting reports of experiments using various inhibitors of cellular processes. Although de novo protein synthesis by the host cell is not required, internalization does involve active invagination of the target cell membrane (Konkel et al., 1992a). Pretreatment of Caco-2 cell monolayers with filipin III, which disrupts caveolae (plasma membrane invaginations) by chelating cholesterol, significantly reduces the ability of *C. jejuni* to enter these cells (Wooldridge et al., 1996). In one study, internalization of *C. jejuni* by INT 407 cells was inhibited by cytochalasin, which disrupts microfilament formation (Konkel et al., 1992b). However, microfilament depolymerization had no significant effect on entry in another study, although entry was blocked by microtubule depolymerization and inhibitors of coated-pit formation (Oelschlaeger et al., 1993). A separate study argued that

coated-pit formation is not likely to be important since there was no inhibition of *C. jejuni* invasion in Caco-2 cells with monodansylcadaverine or g-strophanthin treatment (Russell and Blake, 1994). In contrast, Konkel and colleagues (1992b) reported that dansylcadaverine, an inhibitor of receptor cycling, did reduce internalization of *C. jejuni* by INT 407 cells.

C. jejuni is found in membrane-bound vacuoles once internalized by INT 407 and HeLa cells (Konkel et al., 1992b; Russell and Blake, 1994). *C. jejuni* has also been detected within enterocytes *in vivo* (Russell et al., 1993; Babakhani et al., 1993). Treatment of INT 407 cells with ammonium chloride and methylamine, two chemicals that inhibit endosomal acidification, did not affect *C. jejuni* internalization, nor did they have a significant impact on their intracellular survival (Konkel et al., 1992b; Oelschlaeger et al. 1993). In the absence of antibiotic in the media, infection of INT 407 cells led to deterioration of monolayers, indicating that *C. jejuni* is able to elicit a cytotoxic effect (Konkel et al., 1992b). Similar observations have been made *in vivo* in that surface epithelial cells are damaged and exfoliated into the intestinal lumen in infected monkey and piglet models

(Russell et al., 1993; Babakhani et al., 1993). However, in these cases the host response contributes to the destruction of mucosal cells.

Translocation of *C. jejuni* has been studied *in vitro* using artificial epithelial barriers of cells grown on microporous membrane filters (Everest et al., 1992; Konkel et al., 1992a; Grant et al., 1993). Clinical isolates of *Campylobacter spp.* vary in their invasion phenotypes and intracellular or paracellular trafficking abilities (Harvey et al., 1999). *C. jejuni* is capable of translocating across polarized Caco-2 cell monolayers not only through but also between cells within 60 minutes following addition to the apical surface (Konkel et al., 1992a). Other investigators have also suggested that *C. jejuni* is capable of penetrating cell monolayers via a paracellular route (Everest et al., 1992), although tight junction integrity is not significantly disrupted in this process (Konkel et al., 1992a; Ketley, 1997). *C. jejuni* translocates across Caco-2 monolayers without decreasing transepithelial resistance nor does penetration of labeled markers increase. However, after incubation for 24 hrs with C. jejuni, the monolayer is damaged. These findings suggest that translocation is through invaded cells instead of a paracellular pathway

(Bras and Ketley, 1999). Translocation is reduced when bacterial protein synthesis is inhibited by chloramphenicol treatment (Konkel et al., 1992a), and intact, functional flagella are necessary for this process (Grant et al., 1993).

TRICHURIS SUIS

Basic parasitology

Trichuris suis, swine whipworm, is a nematode which is found in the cecum and colon of pigs. Adults are approximately two inches long and are shaped like a whip, having a thick posterior end tapering to a long filamentous anterior end. Unfertilized eggs deposited in the feces are barrel- or lemon-shaped with conspicuous bipolar plugs. Embryogenesis is optimal at a temperature of 34°C. Infective first-stage larvae (L1) develop inside the egg within 19 days (Beer, 1973a). As early as 9 hours after ingestion by the pig, infective eggs hatch in the distal small intestine and the large intestine as the L1 larvae pierce through the dissolved polar plugs (Beer, 1973a; Beer, 1973b). Subsequent larval development involves four molts to the L5 or adult stage and can be divided into histotrophic and luminal phases (Beer, 1973a; Beer, 1973b). L1 larvae burrow to the base of intestinal crypts, where they penetrate epithelial and goblet cells (Wakelin, 1969; Beer, 1973a; Panesar, 1981). While embedded in a tunnel of fused host epithelial cells, larvae gradually migrate as they are carried passively to the mucosal surface during the

course of the histotrophic phase of development (Beer, 1973a). By about 20 days post-infection, the posterior tip of the worm body protrudes into the gut lumen, while the anterior region remains in a shallow depression on the mucosal surface covered by a raised syncytial sheath of epithelial cells (Beer, 1973a). Fully formed eggs are present in the female uterus around day 41.

Epidemiology

Trichuris suis is found throughout the world, and swine trichuriasis is a disease of economic importance to the swine industry (Beer, 1973a; Bliss, 1991). Epidemiological surveys have been conducted in multiple countries to determine the prevalence and intensity of *T. suis* infection and transmission patterns in traditional and intensively managed herds. A survey conducted in the U.S. (southern Georgia) between 1977 and 1981 concluded that *T. suis* was rare and not established in swine herds (Marti and Hale, 1986). However, a more recent study in the U.S. found *T. suis* highly prevalent at 45% in pigs at slaughter (Bliss, 1991). Studies conducted in the Nordic countries, particularly Denmark, indicate that *T. suis* occurs only sporadically in traditionally managed swine herds (Roepstorff, et al., 1998; Roepstorff and Jorsal, 1989).

Levels of parasitism by *T. suis* are influenced by a number of management practices, especially the housing system (Pattison et al., 1980).

Even low to moderate levels of infection can cause serious economic losses from pronounced weight loss. Scour problems can occur in young pigs within two-three weeks after ingestion of infective eggs, even though a mature worm may not be present for six weeks post-infection. Diagnosis is difficult because the pathology is often caused by larval worms which are too small to be seen at necropsy and eggs have not yet been produced. However, an antigen with potential application in clinical serologic assays has been identified (Hill et al., 1997). *T. suis* eggs are very hardy and can remain viable for even ten years under adverse conditions, which can lead to recurring, chronic scour problems on a farm.

Diseases

Manifestations of swine trichuriasis include anemia, anorexia, dysentery, growth retardation, dehydration, emaciation, and death (Beer, 1973a; Batte et al., 1977). Clinical signs begin approximately 14 days post-infection, which corresponds to the time that larvae emerge from the surface epithelial cells to initiate the luminal phase

(Beer, 1973a). Involvement of pathogenic microflora in trichuriasis has been clearly established in conventionally reared pigs infected with *T. suis* (Rutter and Beer, 1975; Mansfield and Urban, 1996). Tissue damage is essentially limited to the epithelium immediately surrounding the worms, predisposing this area to secondary infections by invasive gut microflora (Beer, 1973a). Bacteria have been found in close proximity to larval *T. suis* in the damaged mucosa of infected pigs (Beer, 1973a; Mansfield and Urban, 1996). Inflammatory cells and bacteria accumulate at the site of worm attachment (Mansfield and Urban, 1996). However, invasion of opportunistic bacteria exclusively in the proximal colon where worms reside is insufficient to explain the full clinical scenario. The observation that lymphoglandular complexes (antigen processing structures) in the distal colon of *T. suis* infected pigs were enlarged, inflamed, and filled with opportunistic bacterial pathogens, particularly *C. jejuni*, led to the hypothesis that *T. suis* induces suppression of mucosal immunity to resident bacteria (Mansfield and Urban, 1996). Elevation of the status of gut microflora, particularly *C. jejuni*, from opportunist to pathogen during *T. suis* infection likely involves multiple immunological and non-immunological mechanisms.

Pathogenesis

This discussion will focus on the contribution of excretory/secretory products of *Trichuris spp.* to the pathogenesis of trichuriasis. Nematodes, like all metazoan organisms, excrete indigestible material and waste products (for review, see Thompson and Geary, 2000). In most cases, they have an intestine and exhibit defecation, although elimination of soluble waste products of intermediary metabolism and catabolism is mediated by diffusion mechanisms. Nematodes also secrete bioactive molecules that affect their environment in a way that enhances their fitness. Among the effects of molecules secreted by nematodes which could contribute to the disease process are those that mediate immune evasion, extracorporal digestion, host tissue degradation, and modulation of host gut function (Thompson and Geary, 2000; Gamble and Mansfield, 1996; Foster and Lee, 1996). Because several cell types and tissues may serve excretory and secretory functions, it has been difficult to clearly distinguish products of excretion from products of secretion. Hence, the two are often combined in the term excretory/secretory products (ESP).

Protocols used to identify ESP from nematodes usually involve the incubation of intact worms in defined media for

some period, and measuring the appearance in the media of specific types of molecules. Among the ESP from adult *T. suis* maintained in tissue culture media are a zinc metalloprotease (Hill et al., 1993), a thiol protease (Hill and Sakanari, 1997), a phenol oxidase (Fetterer and Hill, 1993; Fetterer and Hill, 1994), and a glycoprotein (Hill et al., 1997). The remaining constituents of *T. suis* ESP, as well as their activity and fate *in vivo* are unknown. In two closely related worms, *T. trichuria* and *T. muris*, at least two serine peptidases have been identified in their ESP (Drake et al., 1994a).

Infective *Trichuris* larvae establish an intracellular niche soon after hatching. Invasion of undifferentiated intestinal epithelial cells is accomplished through the piercing action of a stylet. Secreted molecules such as proteases are likely involved in this process (Drake et al., 1994a). A host syncytial tunnel is formed to carry the developing larvae to the mucosal surface. Initiation and maintenance of this tunnel is likely mediated by ESP. Nutrient acquisition is accomplished by extracorporal digestion. ESP becomes concentrated in the microniche immediately surrounding the whipworms.

INTESTINAL EPITHELIAL BARRIER

The GI tract is a complex ecosystem in which the interface between host and external environment is the intestinal epithelial barrier. The intestinal epithelium is responsible for keeping the intestinal microflora and noxious agents in the lumen at bay. This formidable task is accomplished by a layer of intestinal epithelial cells (IECs) overlaid by a blanket of mucus. Enhanced salt and water secretion, expression of antimicrobial proteins and peptides, and production of intestinal mucins are among the innate defense mechanisms of IECs (Hecht, 1999). Additionally, IECs are capable of responding to antigenic stimulation and are involved in initiating inflammatory and immune responses (Eckmann et al., 1993; Eckmann et al., 2000).

The epithelial barrier function of the large intestine resides in the trans- and paracellular pathways of the surface epithelium and crypts (Gitter et al., 2000). Barrier maintenance depends on the integrity of cell membranes and intercellular tight junctions. The apical and basolateral membranes of individual IECs greatly restrict passive permeation by ions and other hydrophilic solutes, such that permeability across the transcellular pathway is

extremely limited (Madara et al., 1992). The only modulations of the transcellular pathway that would significantly increase the permeability of the epithelium are those that would result in cell death. In the absence of overt cell damage such as ulcers or erosions, abnormal permeability can still occur because the major permeability route across the epithelium is located within the paracellular pathway (Madara, 1998). Neighboring IECs are adjoined by a series of intercellular adhesion complexes that impart cohesiveness to assure continuity of the epithelial barrier (Turner and Madara, 1995). These tight junctions circumferentially wrap the apical pole of each cell, severely restricting the passive movement of small solutes and thus are the rate-limiting barriers for permeability through the paracellular pathway (Turner and Madara, 1995). Tight junctions are formed by many specific proteins and are connected with the cytoskeleton (Gasbarrini and Montalto, 1999). Intestinal tight junctions are highly dynamic areas and their permeability can change in response to both external and intracellular stimuli such as bacterial toxins, cytokines, hormones, and drugs (Gasbarrini and Montalto, 1999; Madara et al., 1990).

Goblet cells interspersed among the epithelial cells throughout the intestine are primarily responsible for the production and maintenance of the protective mucus blanket by synthesizing and secreting high-molecular-weight glycoproteins known as mucins (Specian and Oliver, 1991). The mucins of this "unstirred layer" have a variety of postulated biological functions, including physicochemical protection from toxins and mutagens, adhesion modulation, signal transduction, and regulation of cell growth (Winterford et al., 1999). Lipidic constituents, particularly phospholipid surfactants, impart hydrophobic biophysical characteristics to the colonic mucus for protection against noxious agents in the lumen (Lichtenberger, 1995). Surface mucus plays a role in maintaining the intestinal surface acid microclimate by retaining H+ ions at the intestinal surface (Said et al., 1987).

The intestinal mucus layer is essentially a biofilm that interfaces tissue and digesta, serving as a niche for colonization by bacteria (Costerton et al., 1983; Hecht, 1999). The carbohydrate structures on mucin macromolecules are extraordinarily diverse, providing a vast array of potential binding sites for microorganisms (Hecht, 1999).

Therefore, mucins may protect the colon by offering competing binding sites and preventing attachment of enteric pathogens to the underlying epithelium (Belley et al., 1996). Alternatively, attachment to mucins may provide a temporary foothold for motile microbes to track to the epithelial surface (Hecht, 1999).

The mucus layer and intestinal epithelium turn over rapidly and continuously throughout life (Falk et al., 1998). Proteolytic activity in human and pig feces solubilizes mucus, leading to degradation of the mucus layer (Hutton et al., 1990). Proliferation of IECs occurs in flask-shaped mucosal invaginations known as crypts (Falk et al., 1998). The upward migration of cells from colonic crypts ends with their incorporation into a homolog of the villus - a hexagonal cuff of cells that rings the orifice of each crypt (Falk et al., 1998). As the immature epithelial cells originating from intestinal crypts migrate up the crypt-villus axis, they undergo differentiation from cuboidal to polarized columnar cells with tight junctions, apical microvilli, and a brush border. Once the cells arrive at the surface, they are removed by exfoliation or apoptosis (Falk et al., 1998).

Enteric bacterial pathogens have distinctive mechanisms by which they cause disease. *Shigella* and *Salmonella* are model invasive pathogens (Bloom and Boedeker, 1996). Invasive bacteria can penetrate the intestinal epithelium via transcellular or paracellular pathways by various mechanisms. Hotspots for transcellular invasion are M cells, which are specialized epithelial cells within the follicle-associated epithelium, which act as antigen processing structures. For example, *Shigella flexneri*, which causes bacillary dysentery, penetrates the barrier via M cells in the colonic and rectal mucosa causing a massive inflammatory reaction (Sansonetti and Phalipon, 1999). Macrophages are invaded and undergo apoptosis, which leads to the release of IL-1, a proinflammatory cytokine. Further inflammation and mucosal damage ensues, allowing penetration by additional organisms. These organisms multiply intracellularly and spread cell to cell by actin-dependent mechanisms (Sansonetti, et al., 1999). Salmonella is also invasive, but becomes disseminated systemically because these organisms can survive within macrophages (Bloom and Boedeker, 1996).

A representative noninvasive pathogen is *Vibrio cholerae* (Bloom and Boedeker, 1996). Fimbrial adhesins

mediate attachment and colonization of the apical IEC surface where cholera toxin is secreted. This potent enterotoxin induces a voluminous diarrhea via adenylate cyclase-dependent chloride secretion. Toxin A of *Clostridium difficile* causes severe inflammatory enterocolitis in man and animals that appears to be mediated in part by acute inflammatory cells that migrate into the toxin A-exposed mucosa (Hecht et al., 1988). The mechanism involves selectively increasing tight junction permeability through alterations in the actin cytoskeleton without causing overt cellular damage (Hecht, et al., 1988). Another pathogen with toxin-mediated damage is *Bacteroides fragilis*. This bacterium has a metalloprotease toxin which diminishes monolayer resistance by altering apical F actin and inducing chloride secretion (Chambers et al., 1997).

The common response to infection by enteric pathogens is infiltration of the affected tissue by inflammatory cells. Bacterial invasion of mucosal surfaces results in a rapid influx of polymorphonuclear leukocytes (Eckmann et al., 1993). IECs participate in recruitment of migratory inflammatory/immune cells through elaboration of cytokines, particularly IL-8, upon stimulation by bacteria or other antigens (Eckmann et al., 1993). Among the array of

proinflammatory cytokines produced by IECs are interleukin-8, tumour necrosis factor-alpha, monocyte chemotactic protein-1, granulocyte-macrophage colony-stimulating factor, extractable nuclear antigen-78 and others (Hecht and Savkovic, 1997). High density cDNA array technology was used to characterize the mRNA expression profile of approximately 4,300 genes in human intestinal epithelial cells after infection with the prototypic invasive bacteria, Salmonella (Eckmann et al., 2000). Epithelial infection with Salmonella significantly up-regulated mRNA expression of a relatively small fraction of all genes tested. Of these, several cytokines (granulocyte colony-stimulating factor, inhibin A, Epstein-Barr virus-induced gene 3, interleukin-8, macrophage inflammatory protein-2alpha), kinases (TKT, Eck, HEK), transcription factors (interferon regulatory factor-1), and HLA class I were the most prominent (Eckmann et al., 2000). Cells of the immune system (neutrophils and lymphocytes) are strategically interspersed among the IECs. PMN transmigration occurs via a transjunctional route; this crossing produces a substantial but reversible defect in the tight junction barrier (Madara et al., 1990).

ANTIBACTERIAL AGENTS

Countless numbers of compounds with antibacterial activity have been identified. Entire journals are devoted to the discovery of antimicrobial agents from the natural products of essentially all living organisms. These antimicrobial agents function as growth inhibitors of competing, predatory, or pathogenic microorganisms.

It is common knowledge that vertebrates have an immunological arsenal of extracellular (humoral) and cellular mechanisms to defend against microbial invaders. Antibodies are generally thought of as the humoral branch of immunity, although antibacterial peptides are also produced by a variety of cell types. Best characterized are the defensins, a family of cationic peptides divided into alpha- and beta-defensin subfamilies. The alpha-defensins are produced by neutrophils and intestinal Paneth cells, whereas beta-defensins are mainly produced by epithelial cells (van Wetering et al., 1999; Ouellette, 1999). Granulysin is a broad-spectrum antimicrobial protein produced by human cytolytic T lymphocytes and natural killer cells (Krensky, 2000). Granulysin is functionally related to the defensins and magainins, but along with NK lysin and amoebapores made by *Entamoeba histolytica* it is structurally similar to

saposins, small lipid-associated proteins present in the central nervous system (Krensky, 2000). Much less is known about the primitive immune systems that exist in lower eukaryotic organisms. The remainder of this review will focus primarily on extracellular (secreted) antibiotic peptides produced by lower eukaryotes and the mechanisms by which they affect Gram negative bacteria.

A wealth of information is available on pore-forming polypeptides from the parasitic protozoan *Entamoeba histolytica* (Leippe, 1999). These amoebapores are peptides with antibacterial and cytolytic properties composed of alpha helices and disulfide folds that form pores in membranes, similar to polypeptides in mammalian cytotoxic lymphocytes and lysosomes. Exposed hydrophobic grooves are believed to be critical for ion channel formation in target membranes (Bruhn and Leippe, 1999). They are the effector molecules of primitive phagocytes (Leippe, 1999).

Antibacterial peptides are distributed throughout the animal kingdom and are a key component of innate immunity (Gunn et al., 1998). Recently, there has been a burst of information regarding primitive antibacterial immune proteins from the *Drosophila melanogaster* and *Caenorhabditis elegans* models, respectively representing insects and

nematodes. *Drosophila* responds to infectious agents by activating immunity genes encoding antimicrobial peptides that are secreted into the hemolymph (Meister et al., 1997; Engstrom, 1999).

The *C. elegans* genome contains several putative genes predicted to have antibacterial activity based on similarities to the amoebapores of *Entamoeba histolytica*, granulysin of cytotoxic T lymphocytes and a putative amoebapore-related protein of the liver fluke *Fasciola hepatica* (Banyai and Patthy, 1998). The common feature among these gene products is a saposin-like domain and secretory signal peptide (Banyai and Patthy, 1998).

Perhaps best characterized are the defensins, for which homologs exist in multiple species. Defensins are categorized as alpha defensins (cryptdins; Paneth cells in crypt) and beta defensins (epithelial cells, granulocytes, macrophages) (Ouellette, 1999). They are produced mainly by insects and function to permeabilize microbial cytoplasmic membranes. They tend to be cysteine-rich and form intramolecular disulfide bonds forming hairpin beta-like sheets or alpha-helical-beta sheet mixed structures. Alternatively, they are amphipathic alpha-helices or proline

and/or glycine-rich (Bulet et al., 1999) that tend to be found in arthropods, particularly mosquitoes, and molluscs.

Cationic antimicrobial peptides have amphipathic N-terminal, hydrophobic C-terminal, and a helix-hinge-helix structure (eg, cecropins). There are different levels of anionic content in the target phospholipid bilayers (eg, lipid A of LPS in the outer leaflet of the outer membrane of Gram negative bacteria) into which the cationic antimicrobial peptides insert. Bacterial evasion of the innate immune system is mediated by modification of LPS (Guo, et al., 1998).

PERSPECTIVE

The prevailing hypothesis to explain the dysentery-like syndrome in *T. suis*-infected pigs prior to 1996 was that bacteria invaded underlying tissues as a result of mucosal damage from migrating larval whipworms, particularly when their posterior ends protruded into the lumen of the host gut at about 21 days post-infection (Beer and Rutter, 1972; Rutter and Beer, 1975). At that point, it appeared that the pathology was limited to the proximal colon where the whipworms resided. However, Mansfield and Urban (1996) made the observation that enlarged and inflamed LGCs in the distal colon were targets for invasive bacteria, particularly *C. jejuni*. With this finding, two additional hypotheses were proposed to explain the distal bacteria-laden lesions far-removed from whipworms confined to the proximal colon: (1) biologically active soluble factors released by the whipworms are disseminated downstream, and (2) polarized TH2 cytokine responses to the whipworm infection downregulate TH1 cytokines necessary for resistance to invasive bacterial pathogens (Manfield and Urban, 1996; Urban et al., 1996; Mansfield et al., 2000).

The central focus of this thesis was the release of soluble ESP from *T. suis* and their conceivable influence on

the disease process. Among the hypothesized biological activities of ESP that contribute to the pathology of trichuriasis are degradation of host connective tissue, initiation and maintenance of syncytial tunnel formation, and immune dysregulation (Drake et al., 1994a; Drake et al., 1994b; Hill et al., 1993; Mansfield and Urban, 1996). In the context of data from a dual infection pig model of *T. suis*-dependent *C. jejuni* colitis, *in vitro* experiments were conducted to investigate the effects of ESP from adult *T. suis* on IECs and the interactions of *C. jejuni* with IECs.

Chapter 2 highlights the results of experiments designed to test the effects of *T. suis* ESP on the viability of IECs and the integrity of monolayers simulating the intestinal epithelial barrier. The logical premise underlying these experiments was that IECs are the foremost barrier to invasive bacteria. For bacterial pathogens to reach underlying tissue, the IEC barrier must be overcome either through overt gaps in the epithelium created by cell damage, breaches in paracellular (tight junction) permeability, or enhanced invasion via the transcellular pathway. Novel observations were made regarding the direct cytopathic effects of ESP on IECs grown on solid and semi-permeable substrates. Although increased invasion by *C.*

jejuni could not be demonstrated, an equally interesting and intriguing observation was made in that ESP had a direct growth inhibitory effect on *C. jejuni*.

The discovery of the ESP antibacterial activity led to the refocusing of my project and gave rise to Chapter 3, which describes the preliminary characterization of "trichuricin." This phase of the project was temporarily hampered by unanticipated chloramphenicol contamination at trace levels in the ESP. Rigorous controls were employed to prove that there was antibacterial activity native to *T. suis* ESP.

Chapter 4 summarizes the results and proposes additional hypotheses and experiments for future directions. A model was proposed to extrapolate my *in vitro* results to the *in vivo* scenario. Essentially, it is predicted that ESP contributes to IEC damage in the proximal colon, particularly in the microniche occupied by the anterior end of the whipworms. Despite the potent ESP antibacterial activity, invasion would still occur in the proximal colon if *C. jejuni* is protected from the deleterious ESP effects by being embedded in crypt mucus and if trichuricin is differentially released from the posterior end of the worm into the lumen of the host gut. It will be necessary to use

more sophisticated model systems to uncover the mechanisms by which *C. jejuni* invades distal LGCs in *T. suis*-infected pigs.

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CHAPTER 2

RESPONSE OF INTESTINAL EPITHELIAL CELLS TO *TRICHURIS SUIS* EXCRETORY-SECRETORY PRODUCTS AND THE INFLUENCE ON *CAMPYLOBACTER JEJUNI* INVASION

ABSTRACT

We have previously developed a swine animal model in which natural host resistance to *Campylobacter jejuni* is altered by experimental infection with low numbers of the nematode *Trichuris suis*. Pigs naturally colonized with *C. jejuni* experience colitis due to the invasion of the bacterium approximately 21 days following exposure to *T. suis*. To better understand the mechanism of *T. suis*-dependent *C. jejuni* colitis, we evaluated the effects of *T. suis* excretory-secretory products (ESP) on intestinal epithelial cells (IECs) and the influence of ESP on *C. jejuni* invasion in IECs under *in vitro* conditions. Viability assays, based on the MTT method, revealed a dose-dependent cytotoxic response in ESP-treated IECs. IPEC-1 and INT407 were more sensitive to *T. suis* ESP than Caco-2 cells. Transepithelial electrical resistance (TER) dropped significantly in IPEC-1 cells treated on apical and basolateral surfaces, but not those treated only on apical surfaces. Using the gentamicin killing assay, reduced numbers of intracellular *C. jejuni* were recovered from IECs treated with ESP. This observation can be explained by a direct antibacterial activity in ESP, active at concentrations as low as 10 µg protein/ml, which may function to assist worms in defense against pathogens. In

addition to mechanical damage from worms, these results suggest that soluble products released by *T. suis* contribute to IEC damage at the site of worm attachment.

INTRODUCTION

Campylobacter jejuni is the most commonly isolated human enterobacterial pathogen in the United States, causing an estimated four million cases of enteritis per year (Altekruse et al., 1998). Diarrhea is the most common presenting symptom and is usually self-limiting within 5-7 days, although more severe consequences occur in immunocompromised hosts (Altekruse et al., 1998). *Campylobacter* infection is also the number one antecedent infection associated with development of Guillain-Barré syndrome, an autoimmune neuropathy (Buzby et al., 1997; Nachamkin et al., 1998). The primary source of human infection is improperly prepared poultry, but swine also harbor *C. jejuni* (Borch et al., 1996). The observation that opportunistic *C. jejuni* becomes invasive in conventionally-reared pigs infected with *T. suis*, swine whipworm (Mansfield and Urban, 1996), led us to pursue studies to determine the mechanisms of pathogenesis in this dual infection model. Our central hypothesis is that whipworm infection fosters an environment conducive to *Campylobacter* invasion.

The colonic intestinal epithelium is a primary target for tissue damage by whipworms. Ingested *Trichuris* eggs containing infective L1 larvae hatch in the distal small

intestine and the proximal large intestine (Rutter and Beer, 1975; Panesar, 1981). L1 larvae enter crypts in the cecum and colon, migrate to the base of the lumen, and penetrate the epithelial layer from within the crypt (Wakelin, 1969; Panesar, 1981). *T. suis* larvae, while embedded in a tunnel of fused host epithelial cells, undergo four molts in the process of migration to the mucosal surface (Beer, 1973). Invasion and maintenance of this syncytial niche as the larvae develop and migrate to the mucosal surface may be aided by proteases and pore-forming proteins, based on observations in a *T. muris* infection model (Drake et al., 1994a; Drake et al., 1994b). As the worm matures, the posterior region emerges from the tunnel to lie free in the lumen while the filamentous anterior portion remains buried within a shallow epithelial tunnel. ESP from adult whipworms maintained in tissue culture media contain a zinc metalloprotease (Hill et al., 1993), a thiol protease (Hill and Sakanari, 1997), a phenol oxidase (Fetterer and Hill, 1994), and a glycoprotein (Hill et al., 1997). The remaining constituents of ESP, as well as their activity and fate *in vivo* are unknown.

The interaction between *T. suis* and the normal colonic flora in pigs was first noted by Rutter and Beer (1975). They hypothesized that migrating *T. suis* larvae damage the

epithelium allowing for penetration by microorganisms. Mansfield and Urban (1996) extended these findings when they recovered *C. jejuni* from inflamed lymphoglandular complexes (LGCs) in the distal colon of *T. suis*-infected pigs. The fact that *C. jejuni* invasion occurred at a site far-removed from worms in the proximal colon led to the hypothesis that resistance to *C. jejuni* is diminished by soluble substances released by the worms. The present study tested the hypothesis that *T. suis* ESP affects IECs causing enhanced *C. jejuni* invasion similar to what we observe in our dual infection pig model. Toward this aim, cell lines of pig and human origin were used to investigate the effect of *T. suis* ESP on IECs under *in vitro* conditions. In addition, invasion of *C. jejuni* in IECs was measured in the presence and absence of ESP to determine if ESP rendered IECs more permissive for *C. jejuni* internalization.

MATERIALS AND METHODS

Preparation of *Trichuris suis* ESP. The *T. suis* ESP used in these experiments was prepared from adult whipworms pulled free from the colonic mucosa of experimentally infected pigs as described previously (Hill et al., 1993). After washing in sterile saline pre-warmed to 37°C, worms were washed in sterile Hanks balanced salt solution (HBSS) to remove fine debris not visible under the microscope. This was followed by incubation in a 5x-concentrated antibiotic cocktail in RPMI-1640 for a 16-24hr period. The original 5x cocktail contained 500 U/ml penicillin (PEN), 500 µg/ml streptomycin (STREP), 1.25 µg/ml amphotericin B (AMB), and 350 µg/ml chloramphenicol (CAP). A second incubation in a 1x antibiotic cocktail without chloramphenicol was performed for an additional 16-24hr period. Worms were then washed repeatedly in sterile HBSS, at least 3 changes for a minimum of 2hr each, to remove residual antibiotics. Finally, worms were incubated for 10 days in RPMI-1640 containing 1% glucose (4 worms/ml) at 37°C with humidified 5% CO_2 for collection of whipworm conditioned media containing ESP. To confirm sterility, aliquots of ESP were plated on blood agar plates and incubated aerobically and anaerobically for at least 48

hrs. Batches with contamination were discarded. ESP was collected daily, pooled, and concentrated at 4°C by ultrafiltration using an Amicon stirred cell (Millipore, Bedford, Massachusetts) with a 10,000 MW cutoff to 1/20 of the original volume. Using the Bradford assay (Bio-Rad, Hercules, California), the total protein content of 20x-concentrated ESP was determined, which ranged from 3-4 mg protein/ml. Concentrated ESP was sterile filtered (0.22 μm; Millipore, Bedford, Massachusetts) and stored at -80°C. As a control for the volume reduction step, RPMI-1640 media containing 1% glucose without worms was concentrated under the same conditions. Bovine serum albumin (BSA) added to concentrated RPMI (cRPMI) was also used as a control for protein content.

Maintenance of tissue culture cells. Caco-2 cells are a transformed cell line, originally derived from a human colonic adenocarcinoma (Peterson and Mooseker, 1992). These cells were selected because they spontaneously differentiate into polarized cells on solid substrates. Depending on the degree of confluency, cell populations vary in their state of differentiation. Before confluency cultures contain isolated colonies of undifferentiated cells. As the colonies merge, centrally located cells

begin to assume a columnar shape while peripheral cells remain flat and continue to actively spread. At this stage, there is a mixed population of cells in various stages of differentiation. After confluency, cells become fully differentiated with apical and basal poles, microvilli, and tight junctions. The Caco-2 cell clone used was derived from BBe cells by low-density plating and isolation of individual clones which were screened electrophysiologically for high TER, as well as short-circuit current (a measure of chloride secretion) response to cAMP agonists, such as foskolin (Turner, personal communication).

INT407 cells, derived from embryonic human small intestine, were purchased from ATCC as a model representing undifferentiated cells of intestinal crypts. INT407 cells form homogeneous monolayers of flattened cells. An IEC cell line from the small intestine of a neonatal piglet, IPEC-1 cells (Gonzalez-Vallina et al., 1996), were provided by Dr. Helen Berschneider (North Carolina State University). IPEC-1 cells can be induced to differentiate when grown on collagen-coated transwell filters and fed a serum-free medium with hydrocortisone (1 μmol/L) or dexamethasone (10^{-8} M) (Berschneider, personal communication).

Caco-2 and INT407 cells were grown in MEM containing 15% fetal bovine serum. IPEC-1 cells were maintained in DMEM/F12 supplemented with 5% fetal bovine serum, epidermal growth factor (5µg/ml), insulin (5µg/ml), transferrin (5µg/ml), and selenium (5ng/ml). For routine passaging, cells were washed 1x in versene (PBS + EDTA), followed by incubation in trypsin/EDTA with monitoring, and then quenched in 5 volumes of complete media. For freezer stocks, cells were trypsinized, pelleted (1000 rpm, 5 min, 4°C), resuspended in their respective media containing 5% DMSO at 1-2 x 10^6 cells/ml, and stored in liquid nitrogen. Cells were used between passages 32-46 (IPEC-1), 281-298 (INT407), 59-69 (Caco-2). All tissue culture reagents were purchased from Gibco (Rockville, Maryland) unless otherwise stated.

Viability assays. The response of IECs to ESP was evaluated by testing for growth stimulatory or cell-damaging effects under *in vitro* conditions. Preliminary experiments using trypan blue to selectively stain non-viable cells indicated IEC damage in response to ESP treatment. The MTT method, based on spectrophotometric quantitation of a colored reagent in viable cells, was used for more detailed study to generate data amenable to

statistical analysis. Serial two-fold dilutions of ESP at a starting concentration of 1.5 mg protein/ml were added to confluent cells grown in 96 well plates (n=3) which had been seeded at a density of ~10-20,000 cells/well. IPEC-1 and INT407 cells were used at 2-3 days post-seeding, whereas Caco-2 cells were used 9-10 days after seeding to ensure that they were differentiated. Matched dilutions of cRPMI were used as a negative control to detect any background effect due to osmotic disturbances associated with the volume reduction step in the ESP preparation protocol. To avoid unknown protein interactions between ESP and fetal bovine serum, serum-free medium (QBSF 56, Sigma, St. Louis, Missouri) was the diluent for INT407 and Caco-2 cells, although complete medium had to be used for IPEC-1 cells because serum-free media was insufficient for their growth needs. Fresh ESP and cRPMI were added to cells daily. MTT experiments approximated long-term ESP exposure with endpoint measurements taken at 72 hrs post-exposure. At 72 hrs post-exposure, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) was added to cells at a final concentration of 0.5 mg/ml for 3hrs. The MTT assay is based on endocytosis of a water soluble tetrazolium salt exclusively by viable cells which is converted to an insoluble purple formazan by cleavage of

the tetrazolium ring by dehydrogenases (Liu et al., 1997; Mosmann, 1983). Cells laden with these deposits were homogenized in acidic isopropanol to solubilize the colored product, then measured for absorbance at 562nm in an automated platereader (Bio-Tek Instruments, Inc., Winooski, Vermont). In separate experiments, photographs were taken of cells treated with concentrated ESP (1.5 mg protein/ml), cRPMI at an equivalent dilution, and cRPMI containing BSA (1.5 mg/ml) for 72hrs to examine morphological changes in response to ESP exposure. Photos were taken using the 40x objective on an inverted Nikon TMS microsope with a Nikon FDX camera.

Electrophysiology. IPEC-1 cells were used for electrophysiology experiments because of their sensitivity to ESP in the viability assays. For the growth of IPEC-1 cells as polarized monolayers for these experiments, 0.33 cm^2 transwell permeable supports (Corning-Costar, Acton, Massachusetts) were coated with rat tail collagen (Madara et al., 1988). IPEC-1 cells from a nearly confluent flask were trypsinized to a single cell suspension and plated onto inserts at a final surface area dilution of 1:8. After four days, they were switched to media containing dexamethasone (10^{-8} M) without fetal bovine serum. Medium in these cells was replaced three times per week. To allow

the IPEC-1 cells to differentiate as absorptive enterocytes, monolayers were used at least 15 days after plating.

Electrophysiological measurements of ESP- and cRPMI-treated IPEC-1 cells were made with agar bridges and Ag-AgCl calomel electrodes, as described previously (Turner et al., 1997). Potential differences were measured in HBSS with 15 mM HEPES, pH 7.4, and 25 mM D-glucose before and during application of a 50μA current. TER was calculated using Ohm's law. A drop in TER corresponds to an increase in tight junction permeability or overt cell damage. Cells were stained for actin using a fluorescent anti-actin antibody and photographed using confocal laser microscopy.

Growth of *Campylobacter jejuni*. We tested various media and conditions to optimize the growth of ATCC strain 33292 of *Campylobacter jejuni*, originally isolated from a human with enteritis. To test for virulence and to generate low passage isolates, a 3 day old colostrum-deprived piglet was orally inoculated with ~$5x10^9$ colony forming units (cfu) of *C. jejuni* 33292. Piglets developed clinical signs of diarrheal disease within 2 days post-inoculation and *C. jejuni* were reisolated from feces and amplified to generate low passage bacterial stocks. Freezer stocks were maintained in sheep blood at -80°C. For

in vitro experiments, *C. jejuni* (passage 4-5) was grown overnight (18-24hrs) on Brucella agar supplemented with 5% sheep blood. Bacteria were harvested from plates with a sterile swab into MEM or DMEM/F12 tissue culture medium (for invasion and translocation experiments) or Brucella broth (for antibacterial assays). Absorbance was adjusted to 0.1 OD_{560}, which corresponds to ~$5x10^8$ cfu/ml based on growth curves performed in our laboratory.

Invasion assays. IECs were tested for susceptibility to *C. jejuni* invasion using a standard gentamicin killing assay (Konkel et al., 1992). *C. jejuni* (passage 5-6) was added to IECs in 96 well plates, which had been treated with ESP in a dose-response design (ESP concentrations of 0 - 1 mg protein/ml for 24 hrs). For these experiments, IPEC-1 and INT407 cells were used at 3-4 days post-seeding, and Caco-2 cells were used at 9-10 days to ensure they had reached the differentiated phenotype. Approximately $5x10^7$ cfu of *C. jejuni* was added to each well of cells at roughly 50,000 cells/well to achieve a multiplicity of infection (MOI) of ~1000. Prior to addition of *C. jejuni*, cells were washed in serum-free medium to remove residual ESP. Cells were incubated with *C. jejuni* for six hours to allow internalization followed by aspiration of *C. jejuni*-laden media and treatment with media containing gentamicin (0.1

mg/ml) for one hour to kill extracellular *C. jejuni*. Media was aspirated and cells were washed with media to remove residual gentamicin. Finally, 50µl of 0.5% sodium deoxycholate (Sigma, St. Louis, Missouri) was added to lyse cells and release the intracellular *C. jejuni*. The cell lysate was homogenized in 0.2 ml PBS, serially diluted, and plated to quantitate internalized *C. jejuni*.

Translocation of *C. jejuni* across artificial epithelial barriers. IPEC-1 cells were grown on 0.33 cm^2 transwell filters (3.0 µM pore size) for at least 15 days to achieve a fully differentiated phenotype. ESP and cRPMI were added apically and/or basolaterally at a subtoxic concentration (10 µg protein/ml) for 24hrs. ESP was removed and cells were washed by dipping the transwells into sterile beakers of HBSS. *C. jejuni* was added as described previously at a MOI of ~1000. Aliquots of basal media were diluted and plated at 1 and 4 hrs post-infection to quantitate the numbers of organisms that crossed the artificial epithelial barrier.

Antibacterial assays. After making the observation that reduced numbers of *C. jejuni* were recovered from IECs treated with *T. suis* ESP, a simple agar diffusion method (NCCLS, 1997) was used to test the hypothesis that ESP

directly inhibited the growth of *C. jejuni*. Antimicrobial susceptibility disks were saturated with ESP at a range of concentrations (0 - 4 mg protein/ml) and applied to plates inoculated to confluency with *C. jejuni*. For controls, cRPMI and cRPMI + BSA at matching concentrations were used. After 48hrs of growth, diameters of growth inhibition were measured in millimeters.

Statistical analyses. For the viability assays, multiple analysis of covariance was used to predict the dependent variable, DELTA, defined as the difference between treatment with ESP and cRPMI, on the basis of the categorical variable CELL (IPEC-1, INT407, Caco-2) and the continuous variable DOSAGE. Reported P values are for the type III sum of squares. Specific contrasts were requested between IPEC-1 versus INT407, IPEC-1 vs. Caco-2, and between INT407 and Caco-2.

RESULTS

Viability assays. ESP-treatment consistently reduced cell viability in a dose-dependent fashion, although the dose response was not the same for the three cell types tested (Figure 2.1). IPEC-1 and INT407 cells had a similar response pattern to ESP and were significantly more susceptible to damage by ESP than were Caco-2 cells ($p<0.05$). Reduced cell viability in both INT407 and IPEC-1 cells was evident at doses as low as 50 μg ESP protein/ml final concentration, whereas Caco-2 cells had minimal reductions in cell viability that were limited to the two highest concentrations tested. The effects of ESP were significantly different from those of the cRPMI effect in INT407 and IPEC-1, but not in Caco-2 cells ($p<0.01$).

Morphological changes consistent with cytotoxicity occurred in ESP-treated IPEC-1 and INT407 cells (Figure 2.2). IPEC-1 cell monolayers were destroyed in focal areas; remaining cells were swollen and distorted, often having fibrous extensions. INT407 cells showed diffuse damage and membrane blebbing resulting in a "solubilized" appearance. Caco-2 cells showed no overt damage in response to ESP treatment; instead they appeared to be more irregularly shaped compared to the polygonally shaped control cells. Cells treated with cRPMI and BSA as

controls for volume reduction and protein content, respectively, were no different from untreated controls in all three cell types examined (Figure 2.2).

Electrophysiology. Experiments to determine the TER of ESP-treated IPEC-1 cells revealed differential susceptibility of cells based on which cell surfaces were treated. IPEC-1 cells treated on both apical and basolateral surfaces at 1 mg ESP protein/ml for 72 hrs lost all TER compared to cells treated with ESP only apically and to cells treated with cRPMI (Figure 2.3). Cells were stained for actin using a fluorescent anti-actin antibody and examined using confocal laser microscopy (Figure 2.4). Monolayers of IPEC-1 cells treated apically and basally with ESP had dramatic cell loss with lamellopodia extension, actin microspikes, and prominent stress fibers. All other monolayers were composed of confluent monolayers of tall cells with apical perijunctional actin rings and occasionally evident faint basal stress fibers. A subsequent experiment revealed dose- and time-dependent effects of concomitant apical and basolateral ESP treatment on IPEC-1 cells (Figure 2.5). The data suggests that the effect is transient at lower doses, given the cell recovery at 72 hrs.

Invasion and translocation assays. Instead of the hypothesized enhanced invasion, there was a dose-dependent decrease in the number of internalized *C. jejuni* recovered from IECs treated with ESP (Figure 2.6). This outcome was consistently observed despite a wash step to remove residual ESP prior to addition of *C. jejuni*. The greatest decreases in *C. jejuni* were observed in ESP-treated IPEC-1 cells. Monolayers of all three cell types were intact upon visual inspection after ESP treatment. There was no effect of ESP on *C. jejuni* invasion in cells treated with ESP at concentrations below 0.01 mg protein/ml (data not shown). The "0" dose data illustrate the order of *C. jejuni* invasion efficiency in the IECs tested: INT407 > IPEC-1 > Caco-2 at 0.12, 0.08, and 0.03 *C. jejuni*/cell, respectively.

Measurements of translocated *C. jejuni* in the basal media of IPEC-1 cells grown on transwells and exposed to ESP revealed no difference from cRPMI controls (data not shown).

Antibacterial assays. The hypothesis that ESP directly inhibits the growth of *C. jejuni* contributing to the diminished *in vitro* invasion of *C. jejuni* was tested using an agar diffusion assay. Zones of *C. jejuni* growth inhibition were directly proportional to the concentration

of ESP applied, whereas cRPMI containing BSA and cRPMI alone did not influence *C. jejuni* growth (Figure 2.7).

DISCUSSION

Whipworms are in intimate contact with the intestinal epithelium throughout their lifecycle. It has been hypothesized that ESP is necessary for *Trichuris spp.* to initiate invasion and maintain their syncytial tunnel habitat within IECs (Drake et al., 1998). During the adult *T. suis* stage, the anterior end is embedded superficially in the host epithelium and products are secreted into this syncytial tunnel, whereas the posterior end excretes products into the host gut lumen. Known *in vivo* functions of ESP from nematodes include: immune evasion, extracorporal digestion, host tissue degradation, and modulation of host gut function (Thompson and Geary, 2000). Concentrations of ESP were selected to span a range predicted to be physiologically relevant in the colon of a pig with a moderate whipworm infection, in which it is not unusual to find greater than a thousand whipworms. Whipworms are concentrated in the cecum and proximal colon with numbers declining distally. Conservatively extrapolating from *in vitro* measurements, we estimated ESP concentrations in the proximal colon exceed 1 mg protein. Each adult worm is capable of producing 10-100 μg protein/ml/day under *in vitro* conditions (Hill et al., 1993). Therefore, if each of 100 worms produces 10 μg

protein in a single day, that would yield 1 mg ESP protein in the proximal colon. Regardless of the relative contribution of excreted vs. secreted products, concentrations of ESP exceed 1 mg protein in the proximal colon. Whipworm products orally secreted into the localized microniche likely accumulate to higher concentrations, as adults live for approximately 20 days.

We examined the effect of ESP on epithelial cells simulating different conditions *in vitro*. INT407 (and IPEC-1 when grown on solid substrates) represent cells in the base of intestinal crypts having a flattened to cuboidal morphology. Caco-2 cells (and IPEC-1 when grown on semi-permeable supports) resemble polarized enterocytes on the mucosal surface with microvilli and tight junctions, although the Caco-2 cell clone used did retain a secretory phenotype typical of undifferentiated cells (Turner, personal communication). Transwell permeable supports are designed to produce a cell culture environment that closely resembles the *in vivo* state by allowing polarized cells grown on filters to feed basolaterally and thereby carry out metabolic activities in a more natural fashion. Among their many applications is assessment of artificial epithelial barrier function. With this aim, monolayer

integrity of IPEC-1 cells treated with *T. suis* ESP was measured using standard electrophysiology methods.

ESP from adult *T. suis* caused dose-dependent cytotoxic effects in IECs under *in vitro* conditions, particularly those cells representing undifferentiated cells in the base of crypts. This suggests that ESP damages IECs in the proximal colon, contributing to mechanical damage by adult whipworms (Figure 2.7). Pore-forming proteins from *T. muris* and *T. trichiura* are thought to facilitate invasion and syncytial tunnel formation and maintenance in the host cecal epithelium (Drake et al., 1994b). In fact, they alluded to unpublished results that TT50 lysed caecal epithelial cells *in vitro*.

The substrate on which the cells were grown *in vitro* influenced the response to ESP treatment. IPEC-1 cells grown on solid substrates representing undifferentiated cells, displayed a dramatic response to ESP at 1 mg/ml. However, IPEC-1 cells grown on transwells representing differentiated cells and treated apically with 1 mg/ml ESP were not affected. The complete loss of TER in IPEC-1 cells treated apically and basolaterally with ESP, compared to those treated only apically, suggests that basolateral surfaces of differentiated cells are particularly sensitive to ESP cytotoxin(s). Basolateral surfaces of

differentiated IECs may be exposed to ESP where the epithelium is denuded in the proximal colon or in the microniche occupied by the adult worm. Although impressive, the morphological changes in IPEC-1 cells treated apically and basolaterally with ESP may, in part, represent an attempt at regeneration of the monolayer to compensate for the cell loss.

Because of the location of adult worms, we can surmise that the concentration of ESP diminishes distally, away from the site of worm attachment. We considered it possible that ESP could be disseminated and retain activity over a considerable distance in the pig colon. Biologically active factors in ESP could be passively carried distally while embedded in the unstirred layer at the interface between fecal material and epithelium. We hypothesized that ESP has more subtle effects on downstream IECs, to explain the *in vivo* observation that *C. jejuni* invasion occurs in LGCs in the distal colon. Experiments were conducted to test for ESP-induced mechanisms consistent with enhanced invasion by paracellular and intracellular pathways. It was hypothesized that ESP at lower, non-cytotoxic concentrations would alter IECs, either through decreased tight junction permeability or modification of IEC surface receptors, such that *C. jejuni*

would penetrate the IEC barrier more efficiently. However, the inability to demonstrate increased translocated or intracellular *C. jejuni* in ESP-treated IECs suggests that this hypothesis is inadequate to explain the enhanced *C. jejuni* invasion distal to the site of worm attachment *in vivo*.

The decreased recovery of *C. jejuni* from ESP-treated cells led to the discovery that ESP has a direct growth inhibitory effect on *C. jejuni*, for which precedent exists in another parasitic nematode, *Ascaris suum* (Wardlaw et al., 1994; Kato, 1995; Kato and Komatsu, 1996). The next chhapter describes characterization of the antibacterial activity in more detail.

Collectively, these data suggest it is unlikely that *C. jejuni* penetrates the damaged IEC barrier in the proximal colon immediately surrounding sites where worms are located and where ESP would be at its highest concentration. However, *in vivo* there are other factors that might protect *C. jejuni* such as mucus, association with goblet cells, or association with crypt epithelial cells which underlie adult worms in superficial musocal sites. It is possible that mucus could provide protection from the harmful effects of ESP because *C. jejuni* is known to have a predilection for mucus in the base of intestinal

crypts (Beery et al., 1988). Mucus is chemotactic for *C. jejuni* (Hugdahl et al., 1988) and we have found *C. jejuni* associated with goblet cells in the pig colon (Mansfield, unpublished observations). Perhaps mucus also acts as a substrate to enhance *C. jejuni* proliferation. Intestinal mucus is a diffusion barrier against drug absorption (Larhed et al., 1998), and microcolonies of intestinal bacteria embedded in a glycocalyx matrix are protected against hostile environmental factors (Costerton et al., 1983; Costerton, 1984). *T. suis* infection is known to cause excessive production of mucus (Rutter and Beer, 1975).

T. suis ESP (particularly the zinc metalloprotease) degrades fibronectin which is a component of the extracellular matrix in the basement membrane underlying IECs (Hill et al., 1993). Interestingly, *C. jejuni* has an adhesin, CadF, which binds to fibronectin (Konkel et al., 1997). Fibronectin is differentially expressed in higher amounts in the base of crypts (Beaulieu 1992). Degradation of fibronectin by *T. suis* ESP would contribute to reduced resistance in the epithelial barrier and possible permit *C. jejuni* to penetrate.

Rutter and Beer (1975) hypothesized that migrating larval whipworms damage the epithelium, such that

opportunist pathogens can reach underlying tissues. This damage could be due to any combination of the following: physical damage caused by the invasion and movement of larval and adult worms, soluble ESP released by the worms, and immunopathology as a result of the host response to the worms. We tested the direct effect of ESP on IECs under *in vitro* conditions, in the absence of whipworms and an intact immune system. The experiments described in this paper provide support for a mechanism by which *T. suis* disrupts the integrity of the IEC barrier, which would lead to lowered natural resistance to opportunistic pathogens which can overcome the deleterious ESP effects

These data can be used to argue that *C. jejuni* invasion of LGCs in *T. suis*-infected pigs is due to mechanisms other than ESP-induced alterations in IECs. Host response to worm infection, including both mechanical and ESP-mediated cell damage, may initiate a cascade of events culminating in lowered resistance to *C. jejuni* in the distal colon. There is mounting evidence that complex inflammatory and immune cytokine dysregulation contribute to the enhanced *C. jejuni* invasion downstream from adult worms (Urban et al., 1996; Mansfield et al., 2000). Currently, the favored model is that Th2 cytokines induced by the whipworm infection result in down-regulation of Th1

cytokines necessary for defense against intracellular pathogens such as *C. jejuni* (Urban et al., 1996).

Tissue culture models, particularly the IPEC-1 cells, have been beneficial as a means to screen for factors which contribute to the overall pathological process in the animal model of *T. suis*-dependent *Campylobacter* susceptibility, although they clearly do not reflect the complex *in vivo* anatomy and physiology of the human or pig colon. Additional experiments are needed to explain the differential response of IPEC-1 cells treated apically vs. apically and basolaterally. Future efforts will focus on isolation and characterization of the factor(s) in ESP responsible for decreased viability in eukaryotic and prokaryotic cells.

ACKNOWLEDGMENTS

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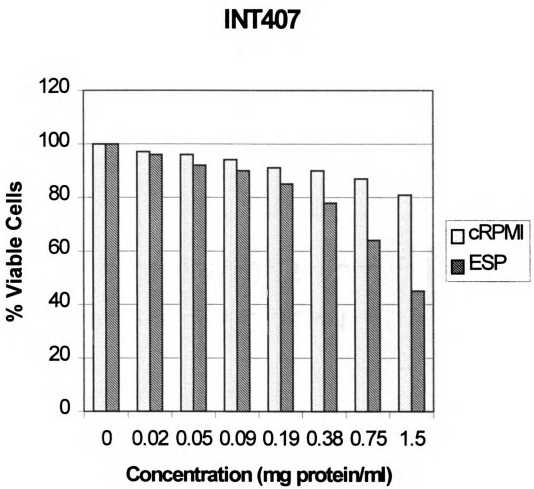
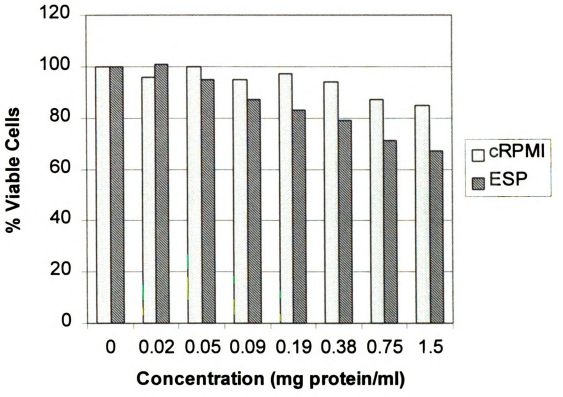
Figure 2.1 - Effect of *T. suis* ESP on viability of IECs in culture. Serial two-fold dilutions of ESP and control cRPMI were applied to INT407, IPEC-1, and Caco-2 cells in 96 well plates (n=3). Viability was measured by the spectrophotometic MTT assay after 72 hrs exposure. INT407 and IPEC-1 cells were more susceptible to ESP than Caco-2 cells ($p<0.05$). The ESP effect was different from the background cRPMI effect in INT407 and IPEC-1 cells, but not Caco-2 cells ($p<0.01$).


INT407
120
100
80
60
40
20
0
% Viable Cells
0 0.02 0.05 0.09 0.19 0.38 0.75 1.5
Concentration (mg protein/ml)
cRPMI
ESP


IPEC-1
120
100
80
60
40
20
0
% Viable Cells
0
0.02
0.05
0.09
0.19
0.38
0.75
1.5
Concentration (mg protein/ml)
cRPMI
ESP

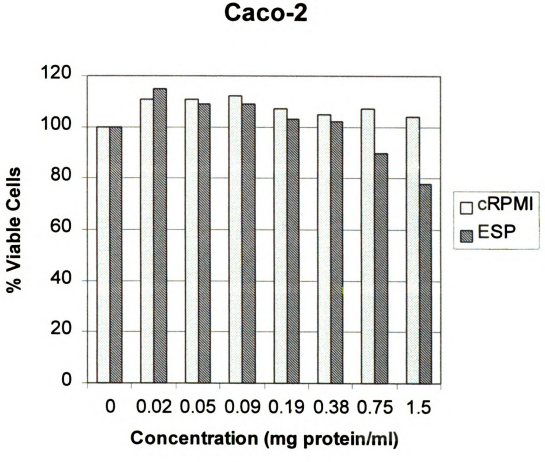

Caco-2
120
100
80
60
40
20
0
% Viable Cells
0
0.02
0.05
0.09
0.19
0.38
0.75
1.5
Concentration (mg protein/ml)
cRPMI
ESP

Figure 2.2 - *T. suis* ESP damages IECs in culture. IPEC-1 (A-D), INT407 (E-H), and Caco-2 (I-L) cells were untreated (A,E,I); treated with ESP at 1.5 mg protein/ml (B,F,J); treated with cRPMI at an equivalent dilution (C,G,K); or treated with cRPMI containing BSA at 1.5 mg/ml (D,H,L) for 72hrs. Cytopathic effects are most apparent in ESP-treated IPEC-1 (B) and INT407 (F) cells. Bar = 25 μm.

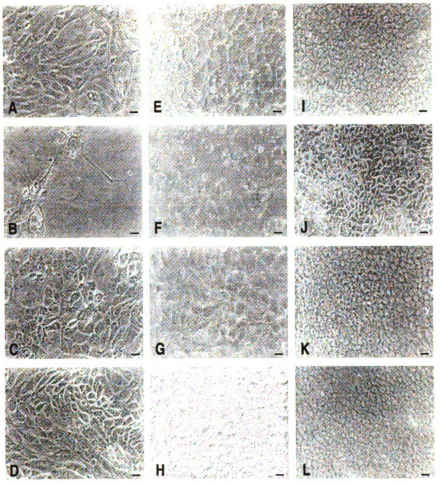


Figure 2.3 - Effect of *T. suis* ESP on TER of differentiated IPEC-1 cells. Cells were treated either on the apical surface alone or on both apical and basal surfaces with ESP at 1 mg protein/ml or cRPMI at an equivalent dilution for 72hrs (n=4). Monolayer integrity was maintained in all treatment groups except ESP-treated apcially and basally.

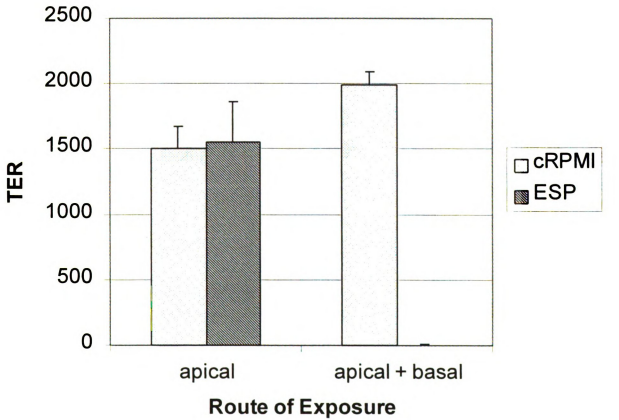


Figure 2.4 - Actin staining of differentiated IPEC-1 cells treated with ESP. Cells were stained for actin using a fluorescent antibody and examined using confocal laser microscopy. ESP apical only (A), ESP apical and basal (B), and cRPMI apical and basal (C). Cells treated with ESP apically and basally responded with dramatic cell loss and residual cells were characterized by lamellopodia, actin microspikes, and prominent stress fibers. Control cells maintained confluent monolayers of columnar cells with apical perijunctional actin rings.

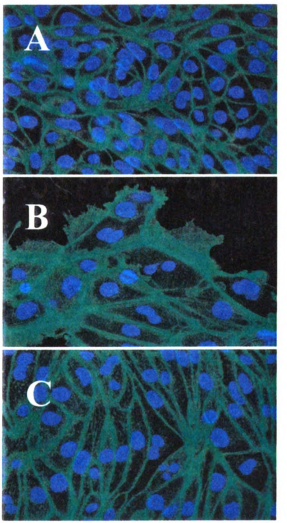


Figure 2.5 - Effect of *T. suis* ESP on TER of differentiated IPEC-1 cells treated apically and basolaterally. Cells (n=3) were treated apically and basolaterally with ESP at 1, 0.1, and 0.01 mg protein/ml or cRPMI at an equivalent dilution for 24 (A) and 72hrs (B). There were dose- and time-dependent effects on monolayer integrity.

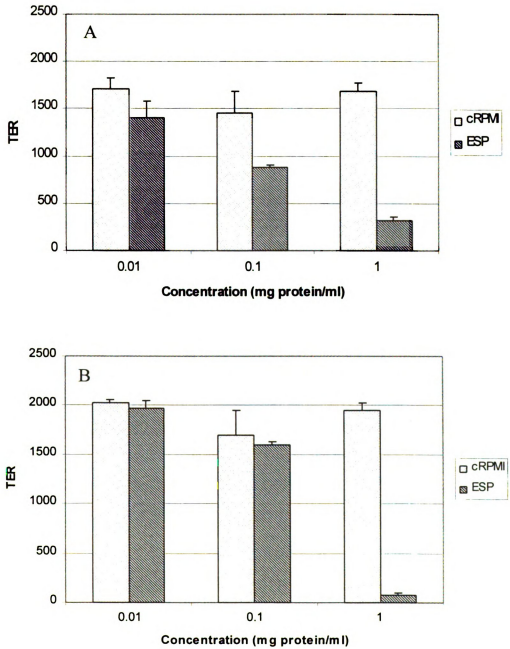


Figure 2.6 - Effect of ESP on *C. jejuni* invasion in IECs. Cells were treated with ESP over a range of concentrations for 24 hrs to simulate subtoxic levels of exposure prior to addition of *C. jejuni* at an MOI of 1000 for 6 hrs to measure internalization. Values represent the mean of six samples ± standard error of the mean.

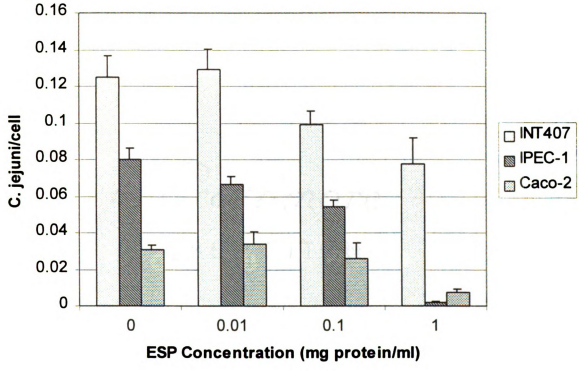


Figure 2.7 - *T. suis* ESP inhibits the growth of *C. jejuni*. Disks were saturated with ESP at 4, 3, 2, and 1 mg protein/ml corresponding to 80, 60, 40, and 20µg total ESP protein/disk (top row), cRPMI containing BSA at equivalent concentrations (middle row), and cRPMI without dilution (bottom disk). Growth inhibition is proportional to the ESP concentration. There was no growth inhibition with cRPMI or cRPMI containing BSA at equivalent concentrations.

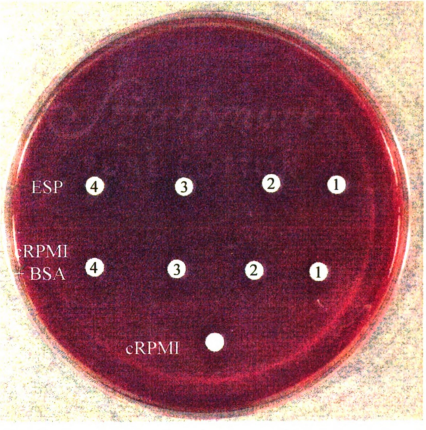
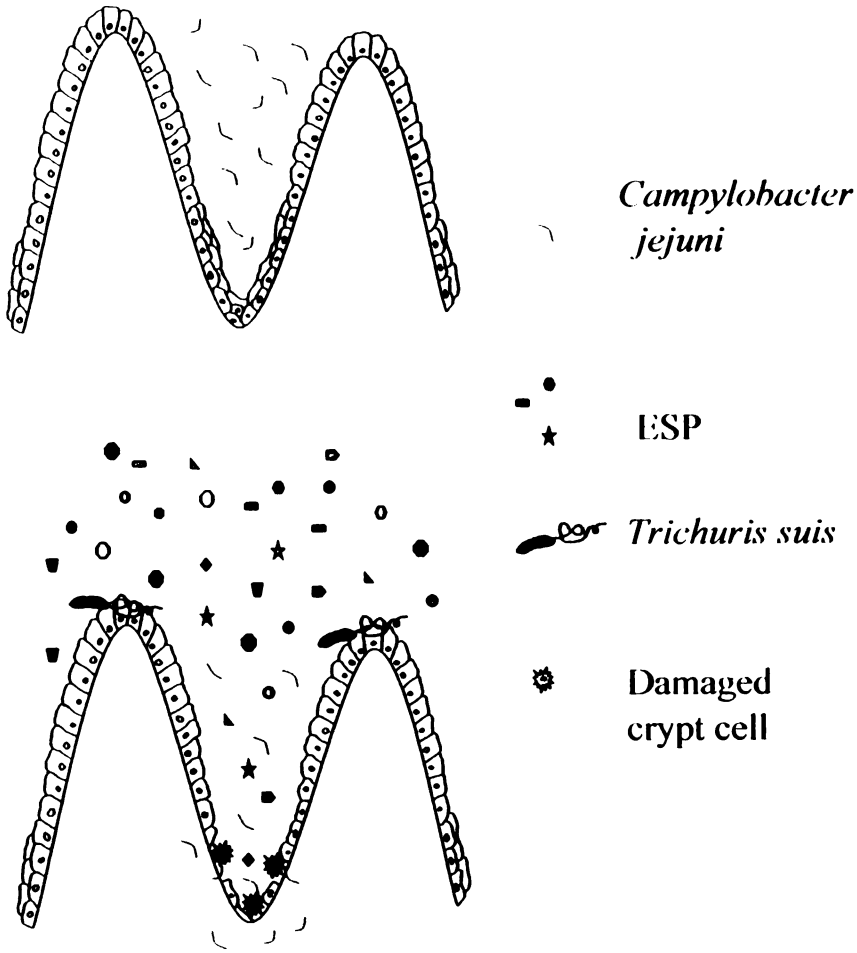


Figure 2.8 - Predictive model of *in vivo* scenario in the proximal colon of pigs infected with *T. suis*. ESP damages IECs, particularly undifferentiated cells in the base of crypts. Opportunistic bacterial pathogens that can overcome the deleterious antibacterial action of ESP (eg, those embedded in mucus within crypts) would have a selective advantage for penetration into the underlying lamina propria.



CHAPTER 3

DETECTION OF ANTIBACTERIAL ACTIVITY IN EXCRETORY-SECRETORY PRODUCTS OF ADULT *TRICHURIS SUIS*

ABSTRACT

Antibacterial activity was detected in excretory-secretory products (ESP) of adult *Trichuris suis* cultured *in vitro* in serum free media. Gram-negative bacteria (*Campylobacter jejuni*, *C. coli*, and *Esherichia coli*) and Gram-positive bacteria (*Staphylococcus aureus*) were sensitive to ESP. Susceptibility was dependent on the dose of ESP but not the concentration of the inoculum. Preliminary assessment of the mode of action suggests a bacteriocidal mechanism. This antibacterial activity was heat stable and resistant to digestion with pronase E and trypsin. Based on ultrafiltration experiments, the activity is less than 10,000 MW. We propose that this excreted/secreted antibacterial activity from *T. suis* be named trichuricin, which is likely a component of a humoral defense system for this helminth.

INTRODUCTION

Compounds with antibacterial activity have been identified from a wide array of invertebrates, including parasitic nematodes (Wardlaw et al., 1994; Kato, 1995). These factors constitute a primitive humoral defense system. It is not surprising that metazoan parasites inhabiting the gastrointestinal tract (GI) produce antibacterial substances, since they are in a microbe-rich environment containing potential pathogens. This is the first report of antibacterial activity from *Trichuris suis*, a parasitic nematode found in the large intestine of pigs. However, antibacterial activity in the body fluid of *Ascaris suum*, a similar nematode parasitizing the pig small intestine, was reported by independent investigators (Wardlaw et al., 1994; Kato, 1995). The original report on *A. suum* described a potent bactericidal activity from the body fluid that was heat stable and less than 14,000 MW in size (Wardlaw et al., 1994). Subsequently, three humoral defense activities (antibacterial, bacteriolytic, and agglutinating) were detected in the body fluid of *A. suum* (Kato, 1995).

The *A. suum* antibacterial factor (referred to as ASABF) has been well-characterized (Kato and Komatsu, 1996). ASABF is a heat-stable and trypsin-sensitive

peptide of 71 amino acids (Kato and Komatsu, 1996). ASABF has structural and functional similarities to the defensins of insects/arthropods. Both are cysteine-rich, cationic peptides that are more effective against Gram-positive bacteria than Gram-negative bacteria (Kato and Komatsu, 1996). ASABF has significant sequence identity with the proteins deduced from a cDNA sequence (yk150c7) and from a putative gene (T22H6.5) of *Caenorhabditis elegans*, a free-living nematode (Kato and Komatsu, 1996).

A gene family of saposin-like proteins has been identified in *C. elegans*, with one of them (T07C4.4) having antibacterial activity when expressed as a recombinant in *E. coli* (Banyai and Patthy, 1998). The putative products of these *C. elegans* genes are similar to the amoebapores of *Entamoeba histolytica* and a putative amoebapore-related protein of the liver fluke *Fasciola hepatica* in that they consist of a single saposin-like domain and a secretory signal peptide (Banyai and Patthy, 1998). Amoebapores of *E. histolytica*, an invasive protozoan pathogen, are pore-forming peptides with antibacterial and cytolytic activities, which function by formation of ion channels in target cell membranes (Andra et al., 1996; Leippe et al., 1994).

Antibacterial activity in invertebrates is quite common. Antibacterial peptides have been described from the silkworm, *Bombyx mori* (Chowdhury, et al., 1995; Hara and Yamakawa, 1995; Kim et al., 1998). A proline-rich antibacterial peptide from the earthworm, *Lumbricus rubellus*, has been reported (Cho et al., 1998). Antibacterial agents have also been detected in two other annelid species, *Nereis diversicolor* (Salzet-Raveillon et al., 1993) and *Eisenia foetida* (Kauschke and Mohrig, 1987).

Most of the antimicrobial agents identified from invertebrates are peptides that exhibit structural similarities. Insect defensins are cationic, cysteine-rich peptides forming intramolecular disulfide bridges that appear in the hemolymph after bacterial challenge or injury (Cociancich et al., 1993). They have potent antibacterial activity against Gram-positive bacteria mediated by disruption of the permeability barrier of the cytoplasmic membrane (Cociancich et al., 1993). The myticins found in the haemocytes of the mussel, *Mytilus galloprovincialis*, are cysteine-rich and exemplify antibacterial peptides originating as precursors with signal sequences that require proteolytic events to activate the mature peptide (Mitta et al., 1999).

The initial observation of antibacterial activity in *T. suis* ESP was against *C. jejuni* in a tissue culture invasion assay (Abner et al., in preparation). In the present study, we describe the preliminary characterization of an antibacterial activity in the ESP collected from *T. suis* adults cultured *in vitro*.

MATERIALS AND METHODS

Experimental infection of pigs and recovery of adult *T. suis*. Weaned pigs were maintained in confinement housing and provided pig chow and water ad libitum. They were experimentally infected with approximately 2500 viable *T. suis* eggs by oral gavage. Pigs were killed 45-50 days after infection with a captive bolt gun. The necropsy procedure involved opening the abdomen and removing the entire GI tract. The GI tract was slit open longitudinally, contents emptied into a bucket, and adult worms plucked from the colon with forceps. Intestinal contents were washed through a 2mm sieve with tap water and additional worms were recovered from the sieve. Worms were picked individually using forceps into successive petri dishes of sterile saline pre-warmed to 37°C. When all damaged or immature worms were eliminated and worms appeared clean visually, they were examined under a dissecting scope and any remaining debris adhering to worms was removed.

Preparation of *T. suis* ESP. The *T. suis* ESP used in the original experiments was prepared from adult whipworms pulled free from the colonic mucosa as described previously (Hill et al., 1993). After washing in sterile saline, worms were washed in sterile Hanks balanced salt solution

(HBSS) to remove fine debris not visible under the microscope. This was followed by incubation in a 5x-concentrated antibiotic cocktail in RPMI-1640 for a 16-24hr period. The original 5x cocktail contained 500 U/ml penicillin (PEN), 500 µg/ml streptomycin (STREP), 1.25 µg/ml amphotericin B (AMB), and 350 µg/ml chloramphenicol (CAP). A second incubation in a 1x antibiotic cocktail without CAP was performed for an additional 16-24hr period. Worms were then washed repeatedly in sterile HBSS, at least 3 changes for a minimum of 2hr each, to remove residual antibiotics. Finally, worms were incubated for 10 days in RPMI-1640 containing 1% glucose (4 worms/ml) at 37°C with humidified 5% CO_2 for collection of whipworm conditioned media containing ESP. To confirm sterility, aliquots of ESP were plated on blood agar plates and incubated aerobically and anaerobically at 37°C for at least 48 hrs. Contaminated batches were discarded. ESP was collected daily, pooled, and concentrated at 4°C by ultrafiltration using an Amicon stirred cell (Millipore, Bedford, Massachusetts) with a 10,000 MW cutoff to 1/20 of the original volume. The total protein content of 20x-concentrated ESP was determined using the Bradford assay, which ranged from 3-4 mg protein/ml. Concentrated ESP was sterile filtered (0.22 µm;

Millipore, Bedford, Massachusetts) and stored at -80°C. As a control for the volume reduction step, RPMI-1640 media containing 1% glucose without worms was concentrated under the same conditions. Bovine serum albumin (BSA) added to concentrated RPMI (cRPMI) was also used as a control for protein content.

Bacterial strains and media. The antibacterial activity in *T. suis* ESP was originally observed using *C. jejuni* (ATCC 33292), and this was the strain on which most experiments were performed for this preliminary characterization. Several *C. jejuni* and *C. coli* isolates were used for MIC determinations (Table 3.1). Additional quality control organisms routinely used for antimicrobial susceptibility assays were tested, including *Enterococcus faecalis* (ATCC 29212), *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), and *Streptococcus pneumoniae* (ATCC 49619). *C. jejuni* and *C. coli* were grown on Mueller-Hinton agar supplemented with 5% sheep blood or in Mueller-Hinton broth (MHB) in humidified 5% CO_2 to achieve a microaerophilic atmosphere. *S. pneumoniae* was grown aerobically on blood agar plates; all other strains were grown on tryptic soy agar plates under aerobic conditions. All organisms were incubated at 37°C.

Antibacterial activity assays. Agar diffusion and broth microdilution methods were used to characterize the antibacterial activity of *T. suis* ESP (NCCLS, 1997). For the agar diffusion assay, susceptibility discs were saturated with 20 µl ESP samples at various concentrations and applied to plates inoculated confluently with the test organism. Plates were observed for zones of growth inhibition surrounding each disc after incubation for 24hr for all organisms except *Campylobacter spp.*, for which results were recorded after 48hr. For the susceptible control organisms listed previously, the effect of the inoculum concentration was assessed. For this experiment, suspensions of the organisms at 10^8 cfu/ml were serially diluted to achieve 10^7, 10^6, and 10^5 cfu/ml suspensions for inoculation of agar plates.

A broth microdilution method in 96 well plates was used to obtain qualitative and quantitative measures of antibacterial activity. Qualitatively, 50µl samples of ESP were added to 50µl suspensions of *C. jejuni* (containing ~$5x10^5$ cfu, as determined by standard serial dilution and plating) in 96 well plates. Plates were evaluated for growth as indicated by the presence of either turbidity or pellets of bacterial cells in the bottoms of U-shaped wells

after 48hr incubation. To quantify activity and determine the minimum inhibitory concentration (MIC) of ESP against test organisms, serial twofold dilutions of 20x-concentrated ESP were prepared in 50µl volumes of MHB (n=2). Each well was then inoculated with 50µl of *C. jejuni* suspended in MHB at a starting inoculum of ~$5x10^5$ cfu, prepared from an overnight (20-24hr) plate culture of actively motile, early log phase organisms. The MIC was assessed as the highest dilution of ESP that resulted in no visible turbidity after 48 hr of incubation. The antibacterial titer was defined as the reciprocal of the MIC and was expressed in activity units (AU) per ml for selected experiments. For each well showing diminished or no turbidity, a 50µl aliquot was subcultured onto a Mueller-Hinton blood plate and incubated for 48 hr. The minimum bactericidal concentration (MBC) corresponded to the highest dilution that showed no growth upon subculturing.

Stability. Unfractionated ESP was subjected to various treatments and then bioassayed for antibacterial activity using the broth microdilution assay to evaluate the stability of the antibacterial activity. Physical treatments included boiling for 15 minutes and freezing-thawing (3 cycles at -70°C). Digestions with trypsin and

pronase E (Sigma, St. Louis, Missouri) were performed with 1mg/ml final enzyme concentration on 100 AU/ml ESP at 37°C for 6hr. In this experiment, BSA (1 mg/ml) was digested as a control and run on a standard 15% SDS-PAGE gel to confirm that the enzymes were functional under these conditions. Prior to bioassay, enzymes were inactivated by boiling for 15 minutes. Control experiments were carried out without ESP to confirm that the heat-inactivated trypsin and pronase E were not inhibitory to growth of the test bacteria.

Ultrafiltration. Ultrafiltration methods were used to nominally size fractionate ESP for an approximate determination of the molecular weight of the antibacterial agent(s). Amicon stirred cells under pressurized nitrogen gas were used to separate ESP into fractions above (retentate) and below (filtrate) 30,000 MW and above and below 10,000 MW with YM30 and YM10 membranes (Millipore, Bedford, Massachusetts), respectively. Prior to bioassay, the concentrated retentate fraction was returned to its original volume in RPMI-1640 media to match the volume of the filtrate so that comparisons of relative activity could be made without distortions due to volume differences. Antibacterial activity was assayed in the retentate and

filtrate fractions using the agar diffusion method as described previously.

High performance liquid chromatography. A Model 173 high performance liquid chromatography (HPLC) system (Perkin Elmer Applied Biosystems, Inc., Foster City, California) at the Michigan State University Macromolecular Structure Facility was used to fractionate *T. suis* ESP for preliminary isolation of the antibacterial activity (Hearn, 1984). Samples (150 µl) of ESP were mixed with an equal volume of 0.1% trifluoroacetic acid (TFA) and centrifuged at full speed (14,000 x g) at room temperature for 15 minutes. Two hundred microliters of the clarified supernatant containing either ~100µg or ~10µg total ESP protein was injected onto a C18 reverse phase column (0.8 mm in diameter and 250 mm long) packed with Vydak 300Å resin (LC Packings, San Francisco, California). Compounds were eluted by a continuous linear gradient of 5% acetonitrile (ACN) in 0.1% TFA to 80% ACN in 0.1% TFA over a three-hour period at a flow rate of 0.04 ml/min. Absorbance of the eluate was monitored at 214 nm, and fractions were collected manually as peaks were detected. Eluted fractions were dried in a Savant Speedvac concentrator, reconstituted in 50 µl sterile ultrapure

water, and filter sterilized (Spin X columns, Costar) prior to bioassay for antibacterial activity by the broth microdilution assay.

To address the concern that one or more of the antibiotics used during the whipworm culture procedure could persist into the final ESP preparation, control HPLC experiments with antibiotic standards were performed for comparison to ESP chromatograms. Initially, 1x concentrations of PEN (100 U/ml), STREP (100 µg/ml), AMB (0.25 µg/ml), and CAP (70 µg/ml) were performed for screening purposes. A subsequent experiment with CAP at 2 µg/ml was then carried out.

Mass spectrometry. Fast atom bombardment-mass spectrometry (MS) was used as an assay for residual antibiotics in ESP potentially carried over in solution or adherent to worms during preparation (Burlingame et al., 1998). Mass spectra were obtained using a JEOL HX-110 double-focusing mass spectrometer (JEOL USA, Peabody, MA) operating in the positive ion mode. Ions were produced by bombardment of samples in a glycerol matrix with a beam of Xe atoms (6 keV) or Cs^+ ions (12 keV). The accelerating voltage was 10 kV and the resolution was set at 1000. The instrument was scanned in 30 seconds from *m/z* 50 to 1000.

Consideration of CAP contamination. After detection of residual CAP in the original ESP, multiple measures were taken to exclude the possibility that the putative antibacterial agent(s) was an artifact of CAP contamination. Using the worst-case scenario, we modeled the predicted maximum amount of CAP absorbed and how much would be released into the medium if the worms dissolved (i.e., released 100%). However, a more realistic scenario is that very little of the drug is released back into the medium due to the equilibrium (plateau) established during uptake. For these theoretical calculations, we used the log PC value (*n*-octanol/water partition coefficient), which is a measure of lipophilicity, and anthelminthic drug absorption kinetic data in model nematodes, *Ascaris suum* and *Haemonchus contortus* (Ho et al., 1990; Ho et al., 1992; Ho et al., 1994). It was assumed that the aggregate worm volume of these eccentrically shaped nematodes occupies 4% of the incubation well volume (4 worms/ml at ~10µl/worm).

PEN and STREP were also included in this assessment, although AMB was excluded because it is an antifungal agent, which does not inhibit the growth of *C. jejuni* or the other test bacteria used in these studies. The size of the drug was also taken into consideration. Additionally,

an experiment to assess the heat stability of the antibiotics was performed.

The definitive test was to prepare ESP in the complete absence of CAP for subsequent control experiments that would parallel selected experiments performed with the original ESP. For these experiments, a local farm with naturally *T. suis*-infected pigs was identified as a source of whipworms to prepare CAP-free ESP. The same protocol was employed except for omission of CAP in the 5x antibiotic cocktail treatment. Also, the 10K filtrate was saved for experimentation.

RESULTS

Antibacterial activity. The original ESP from *T. suis* had a dose-dependent growth inhibitory effect on *C. jejuni* in the agar diffusion assay (Figure 3.1). ESP concentrations of 80, 60, 40, and 20 µg total protein/disk had growth inhibition zones of 27, 25, 18, and 13 mm, respectively. Control cRPMI and cRPMI containing BSA at concentrations equivalent to ESP did not inhibit the growth of *C. jejuni*. The MICs of ESP for available *C. jejuni* and *C. coli* isolates were determined (Table 3.1). *C. jejuni* strains were consistently 2- to 4-fold more sensitive to ESP than *C. coli* isolates (Table 3.1). The MBC of ESP on *C. jejuni* 33292 was equivalent to the MIC at 1:128.

To confirm that the antibacterial activity was not limited to *Campylobacter spp.*, additional organisms were tested by the agar diffusion method (Table 3.2). Two of the five quality control test organisms, one Gram-negative and one Gram-positive, were sensitive to ESP. *Escherichia coli* had a zone of growth inhibition 9 mm in diameter, whereas *Staphylococcus aureus* had a zone of 11 mm. Both organisms were sensitive only to the highest concentration of ESP tested (20µg total protein/disk). In a subsequent experiment, inocula of the susceptible quality control organisms were diluted to determine if sensitivity to ESP

increased with decreasing numbers of organisms present (Table 3.3). The zones of growth inhibition were the same for all 3 inoculum sizes.

Stability and ultrafiltration. The antibacterial activity of the original *T. suis* ESP was unaltered by heat treatment and repeated freeze-thawing (Table 3.4). Furthermore, digestion with trypsin and pronase E did not abolish antibacterial activity (Table 3.4). Fractions above and below 30,000 MW contained antibacterial activity (Table 3.4). However, activity was lost in the 30K retentate after repeated dialysis. Sub-fractionation of the 30K filtrate on a 10,000 MW membrane also indicated activity in the retentate and filtrate.

HPLC and MS. Fractionation of the original ESP on a C18 reverse phase HPLC column demonstrated antibacterial activity in several fractions, with retention times ranging from 20 to 100+ minutes (Figure 3.2A). Control HPLC experiments to test for residual antibiotics revealed that CAP co-eluted with the 20 minute ESP fraction (Figure 3.2B). The other two antibiotics (PEN and STREP) and the antifungal agent (AMB) had no peaks matching those present in ESP (data not shown). By comparison to a CAP standard, MS confirmed that the 20 minute ESP fraction contained CAP or a CAP-like molecule (Figure 3.3). Comparison of peak

heights in the ESP and CAP standard for HPLC an MS experiments indicates that ESP contained less than 2 µg/ml residual CAP or a CAP-like molecule.

Consideration of CAP contamination. The logPC of PEN indicates that it is too hydrophobic to be released back into the media following absorption by the worms (Table 3.5). In addition, PEN is inactivated by heat treatment (Table 3.6), which suggests that it could not be responsible for the heat-resistant activity in ESP. Although boiling did not inactivate STREP (Table 3.6), the log PC indicates that this drug is too hydrophilic to be absorbed by the nematode in any significant quantity (Table 3.5). The larger size of STREP should also contribute to its relative exclusion from absorption across the cuticle (Table 3.5).

However, the logPC of CAP predicts that it would be absorbed and then released by the worms, and it is not heat-inactivated (Tables 3.5 and 3.6). Using the worst case-scenario, we calculated the maximum amount of CAP that would be present at every step in the ESP production protocol (Figure 3.4). We estimated that there would be approximately 1-2 µg CAP/ml in the final ESP preparation after 3 HBSS washes, which is consistent with the HPLC and MS data. Also, we determined experimentally that 2 µg/ml is

the MIC of CAP for *C. jejuni*. The observation that antibacterial activity can be detected in ESP diluted 1:100 indicates that the trace levels of CAP present in the final ESP are insufficient to account for the observed antibacterial activity. A 1:100 dilution of ESP would reduce the concentration of residual CAP to approximately 0.01 - 0.02 μg/ml, which is ~100x less than its MIC.

Although the protein yield of the CAP-free ESP was low, attributable to the limited nematode recovery from naturally infected pigs, antibacterial activity was clearly demonstrated. Despite the discrepancy in protein concentration, the antibacterial titers of the >10,000 MW fraction of original ESP and CAP-free ESP were identical at 2560 AU/ml (Table 3.7). Of particular interest, a high level of antibacterial activity (10,240 AU/ml) was present in the 10K filtrate of the CAP-free ESP (Table 3.7). Comparison of the HPLC profiles from the 10K retentate and filtrate of the CAP-free ESP illustrates that the majority of chemical species is in the filtrate, of which at least three have antibacterial activity (Figure 3.5).

The antibacterial activity in CAP-free ESP had characteristics identical to the original ESP in that it was also resistant to boiling, freezing-thawing, and inactivation by trypsin and pronase E (Table 3.8).

Additionally, HPLC chromatographs of the 10K retentates from original and CAP-free ESP were similar, with the exception of the missing 20 minute CAP peak (Figure 3.6).

DISCUSSION

In this study, we report a potent antibacterial activity in ESP prepared from adult *T. suis*, a nematode which parasitizes the large intestine of swine. ESP from an axenic culture of *T. suis* cannot be prepared without initially high concentrations of antibiotics to prevent the overgrowth of fecal organisms associated externally and internally with the worms. Therefore, it was imperative to exclude the possibility that ESP was contaminated with antibiotics to validate the antibacterial activity we have detected. To this end, experiments employing HPLC, MS, physicochemical properties of the antibiotics in question, and preparation of CAP-free ESP were conducted. As a result of these precautionary measures we can report with confidence that the antibacterial activity detected in ESP is native to *T. suis*.

After detecting residual CAP by HPLC and MS, we performed theoretical calculations to predict the extent to which PEN, STREP, and CAP would be carried over into the final ESP. Uptake of anthelminthic drugs by GI nematodes occurs primarily by absorption across the cuticle (Ho et al., 1992). The cuticle is secreted by a single layer of cells that form the hypodermis, and is composed of a dense collagen and collagen-like protein matrix containing

negatively charged aqueous-filled pores (Ho et al., 1990; Fetterer and Rhoads, 1993). The rate-determining barrier for drug absorption is at the interface of the hydrophilic porous cuticle matrix and the underlying lipophilic hypodermis (Ho et al., 1994). Absorption of anthelminthic compounds across the cuticle generally increases with lipophilicity of the agent and is also influenced by the size of the drug. Molecules with M_r >350 Da are absorbed less efficiently than smaller molecules (Ho et al., 1994).

It is highly unlikely that residual PEN could account for the observed antibacterial activity, due to its logPC and since ESP retains activity after boiling. STREP is not a factor either due to its logPC and size. The theoretical argument that residual CAP did not contribute significantly to the antibacterial activity we described in the original *T. suis* ESP was supported experimentally by showing that the activity persisted in CAP-free ESP.

It was considered highly unlikely that antibiotics could persist into the final ESP preparation, because worms were washed extensively after antibiotic treatments. Nevertheless, low levels of residual CAP were detected by HPLC and confirmed by MS. This observation underscores the importance of being cautious about routinely including

antibiotics in worm incubations. We would recommend that CAP be avoided if possible.

This antibiotic activity was demonstrated against both Gram-negative and Gram-positive bacteria. Sensitivity to ESP was proportional to the concentration of ESP, but was not influenced by the number of organisms in the inoculum. Because the MIC and MBC were equal, we conclude that the ESP antibacterial activity, at least against *C. jejuni*, is bacteriocidal.

The presence of antibacterial activity in ESP subjected to harsh conditions of boiling, freezing-thawing, and exposure to trypsin and pronase E, supports that the activity resides in a very stable molecule(s). Although resistant to proteases, particularly the highly nonspecific pronase E, this does not eliminate the possibility that trichuricin is a short peptide. Furthermore, data from ultrafiltration experiments are consistent with at least one antibacterial agent being <10,000 MW in size, particularly based on the presence of high activity in the 10K filtrate of CAP-free ESP.

Genes encoding antibacterial peptides have been identified in a variety of invertebrate species (Furukawa et al., 1999; Park et al., 1997). Alternatively, antibacterial activity is found in peptide fragments

derived from larger proteins that have been degraded (Bellamy et al., 1992; Strub et al., 1995). It has been documented that *T. suis* ESP proteins are degraded by a constituent zinc metalloprotease, which also undergoes autodegradation (Hill et al., 1993). It is possible that trichuricin is a degradation product of a larger *T. suis* protein. The mechanism of many antibacterial peptides is pore-formation in bacterial cell membranes (Lockey and Ourth, 1996; Maget-Dana and Peypoux, 1994; Leippe et al., 1991; Leippe et al., 1994). Therefore, a likely candidate would be the presumed *T. suis* counterpart of a 50kD pore-forming protein from *T. trichuria* (TT50) (Drake et al., 1994; Drake et al., 1998). TT50 was shown to produce ion channels in lipid bilayers. It contains multiple cysteine residues forming disulphide bonds, which are a common feature of antibacterial peptides.

This report represents the first description of antibacterial activity from *T. suis* and only the second from parasitic nematodes as a whole. Although the first report is from the body fluid of *Ascaris suum*, we cannot rule out the possibility that it too was due to an ESP component that accumulated in the body fluid and the author did not report preparation of ESP. Future efforts will focus on further characterization of the HPLC-purified

fractions of CAP-free ESP containing antibacterial activity.

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Table 3.1 - MICs of *T. suis* ESP on *Campylobacter spp.*

C. jejuni		*C. coli*	
Strain	**MIC**	**Strain**	**MIC**
33292[a]	1:128	1679368	1:32
33560	1:128	17010887	1:64
43430	1:256	18493	1:64
43470	1:128	1935	1:64
19084571	1:64	1777708	1:64
15046764	1:512	17140	1:128
19094451	1:256	43473	1:64
43433	1:64	43474	1:64
33291	1:128	43479	1:32
43429	1:128	43482	1:64
49349	1:256	43134	1:64

Suspensions of *C. jejuni* (~$5x10^5$ cfu) were added to serial twofold dilutions of 20x-concentrated ESP. The MIC was the highest dilution with no growth after 48 hrs incubation.
[a]*C.jejuni* strain 33292 was used in subsequent experiments as the test organism for characterization of the antibacterial activity in *T. suis* ESP.

Table 3.2 - Preliminary inhibitory spectrum of *T. suis* ESP.

Test organism	Susceptibility[a]	
	20µg[b]	2µg[b]
Escherichia coli (gram neg)	+	–
Pseudomonas aeruginosa (gram neg)	–	–
Staphylococcus aureus (gram pos)	+	–
Streptococcus pneumoniae (gram pos)	–	–
Enterococcus faecalis (gram pos)	–	–

Agar plates were inoculated from a suspension of test organisms containing 10^8 cfu/ml.
[a]Susceptibility is referred to by classifying organisms as resistant (–) or sensitive (+) to ESP as determined by the agar diffusion assay.
[b]Susceptibility discs were saturated with 20µl of ESP at concentrations of 1 mg protein/ml (20µg total protein/disk) and 0.1 mg protein/ml (2µg total protein/disk).

Table 3.3 - Effect of inoculum size on ESP sensitivity.

Test organism	Inoculum		
	1:10[a]	1:100	1:1000
Escherichia coli	9[b]	9	9
Staphylococcus aureus	11	11	11

[a]A suspension of organisms at 10^8 cfu/ml was serially diluted to achieve 10^7, 10^6, and 10^5 cfu/ml suspensions for inoculation of agar plates.
[b]Diameters of growth inhibition zones were measured in mm.

Table 3.4 - Physical and chemical properties of ESP antibacterial activity.

Treatment	Activity[a]
Boiling	+
Freezing-thawing	+
Trypsin	+
Pronase E	+
30K retentate	+
30K filtrate	+
10K retentate	+
10K filtrate	+
30K retentate dialyzed[b]	–

[a]Antibacterial activity was measured qualitatively by the broth microdilution assay using *C. jejuni* as the test organism. Activity is classified as no growth (+) or growth (–).
[b]The 30K retentate was dialyzed and concentrated 5x in 100x volumes of RPMI-1640 on a YM30 membrane.

Table 3.5 - Physicochemical properties of antibiotics.

Antibiotic	MW	logPC
Streptomycin sulfate	728.70	-9.53 (-)
Chloramphenicol	323.13	0.92 (8.3x)
Penicillin G	334.40	1.85 (71x)

Molecular weight (MW) and *n*-octanol/water partition coefficient (logPC) were compiled from a drug database at Upjohn. Concentrating factors are in parentheses.

Table 3.6 - Effect of boiling on antibiotics.

Antibiotic	Activity[a]
Streptomycin	+
Chloramphenicol	+
Penicillin	–

Antibiotics (1x concentration) were boiled for 10 minutes.
[a]Antibacterial activity was measured qualitatively by the broth microdilution assay using *C. jejuni* as the test organism.

Table 3.7 - Comparison of antibacterial titers in original and CAP-free ESP.

	[protein][a]	10K retentate[b]	10K filtrate[b]
ESP	3	2560	320[c]
CAP-free ESP	0.1	2560	10,240

[a]Concentration of protein in mg/ml.
[b]Activity units/ml were calculated from the reciprocal of the MIC.
[c]Secondary filtrate because original was not available.

Table 3.8 - Physical and chemical properties of CAP-free ESP antibacterial activity.

Treatment	Activity[a]
Boiling	+
Freezing-thawing	+
Trypsin	+
Pronase E	+

[a]Antibacterial activity was measured qualitatively by the broth microdilution assay using *C. jejuni* as the test organism. Activity is classified as no growth (+) or growth (−).

Figure 3.1 - Agar diffusion assay. Disks were saturated with ESP at 4, 3, 2, and 1 mg protein/ml corresponding to 80, 60, 40, and 20µg total ESP protein/disk (top row), cRPMI containing BSA at equivalent concentrations (middle row), and cRPMI without dilution (bottom disk). Growth inhibition is proportional to the ESP concentration. There was no growth inhibition with cRPMI or cRPMI containing BSA at equivalent concentrations.

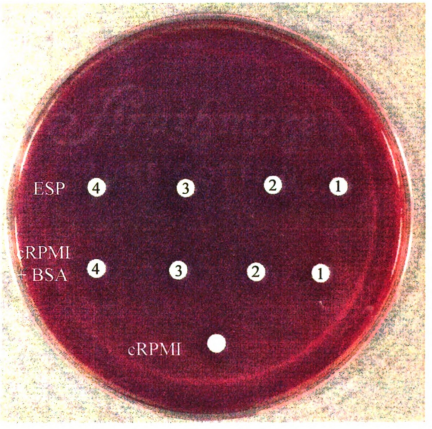


Figure 3.2 - HPLC of original *T. suis* ESP (A) and CAP standard (B). Samples (ESP - 100µg in 0.02ml; CAP - 0.2µg in 0.02ml) were loaded onto a reverse phase (C18) column and eluted with a 5-80% ACN gradient in 0.1% TFA. Asterisks indicate antibacterial activity by the qualitative broth microdilution assay.

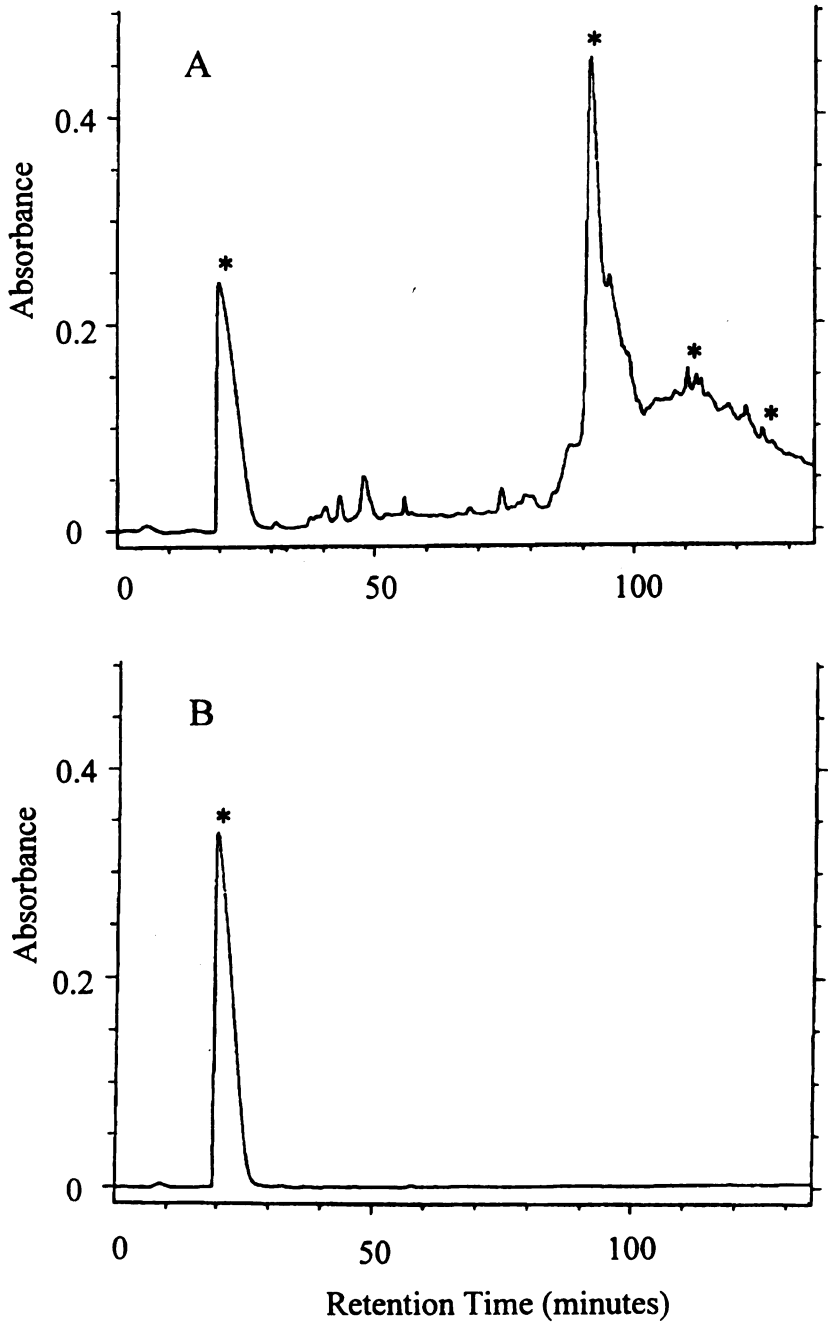


Figure 3.3 - MS of ESP 20 minute fraction (A) and CAP standard (2 µg/ml) (B). Mass spectra were obtained by bombarding samples in a glycerol matrix with a beam of Xe atoms. Boxes indicate characteristic patterns for CAP.

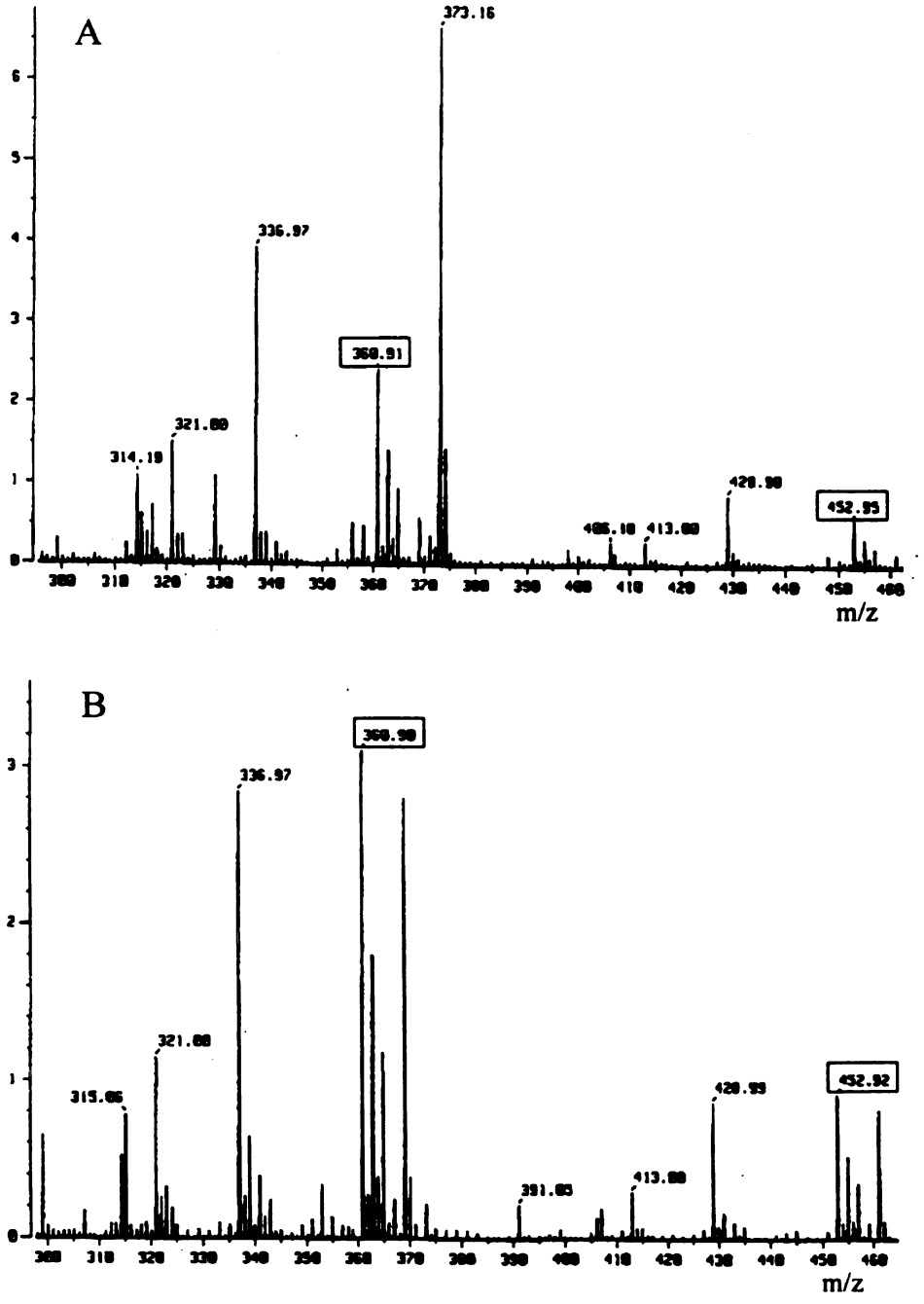


Figure 3.4 - Theoretical calculations estimating residual CAP in the original ESP. LogPC values were used to predict the maximum amount of CAP absorbed by the aggregate worm volume (4% of the total system) and then released back into the medium using the worst-case scenario (i.e., that 100% is released).

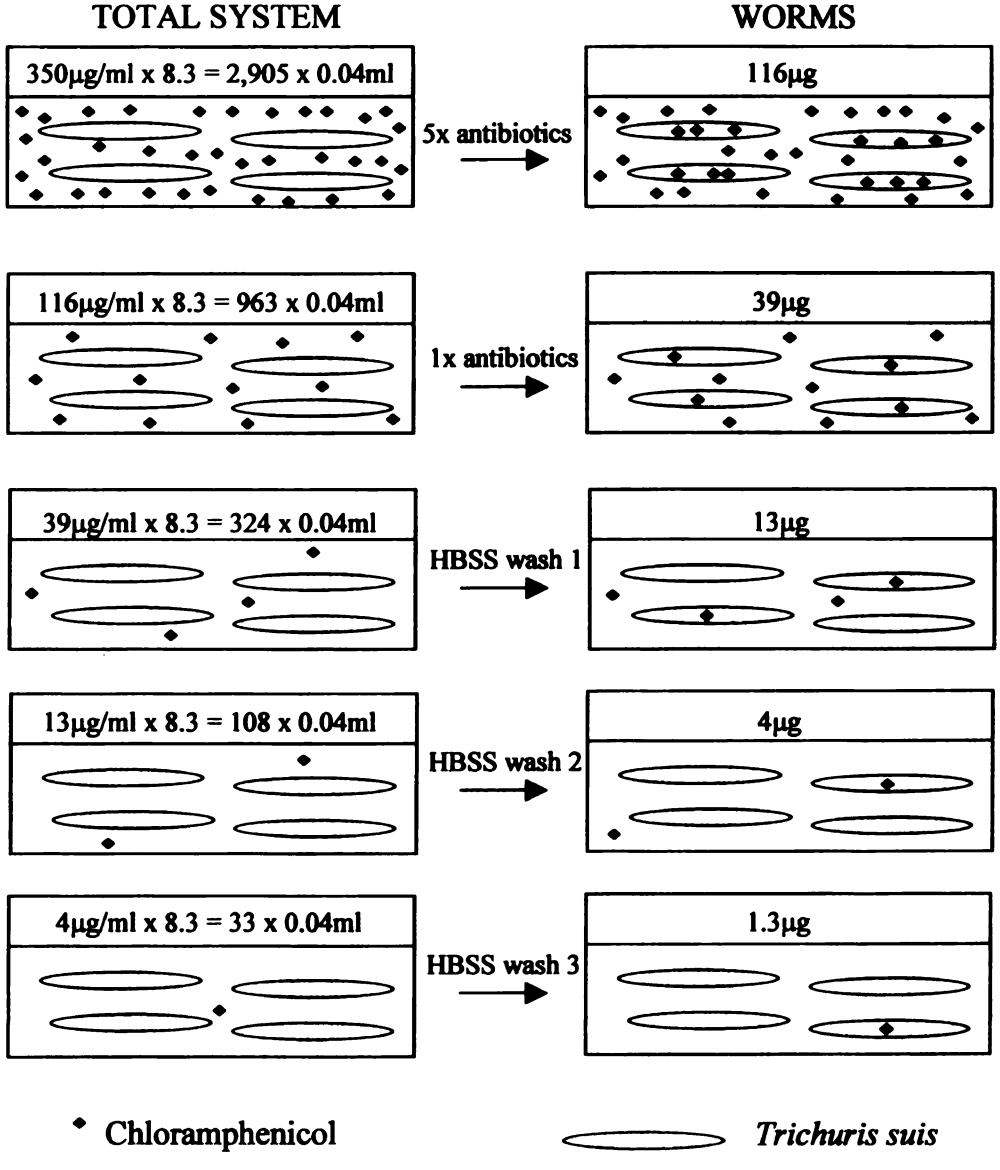


Figure 3.5 - HPLC of CAP-free ESP 10K retentate (A) and CAP-free ESP 10K filtrate (B). Samples (10µg in 0.02ml) were loaded onto a reverse phase (C18) column and eluted with a 5-80% ACN gradient in 0.1% TFA. Asterisks indicate antibacterial activity by the qualitative broth microdilution assay.

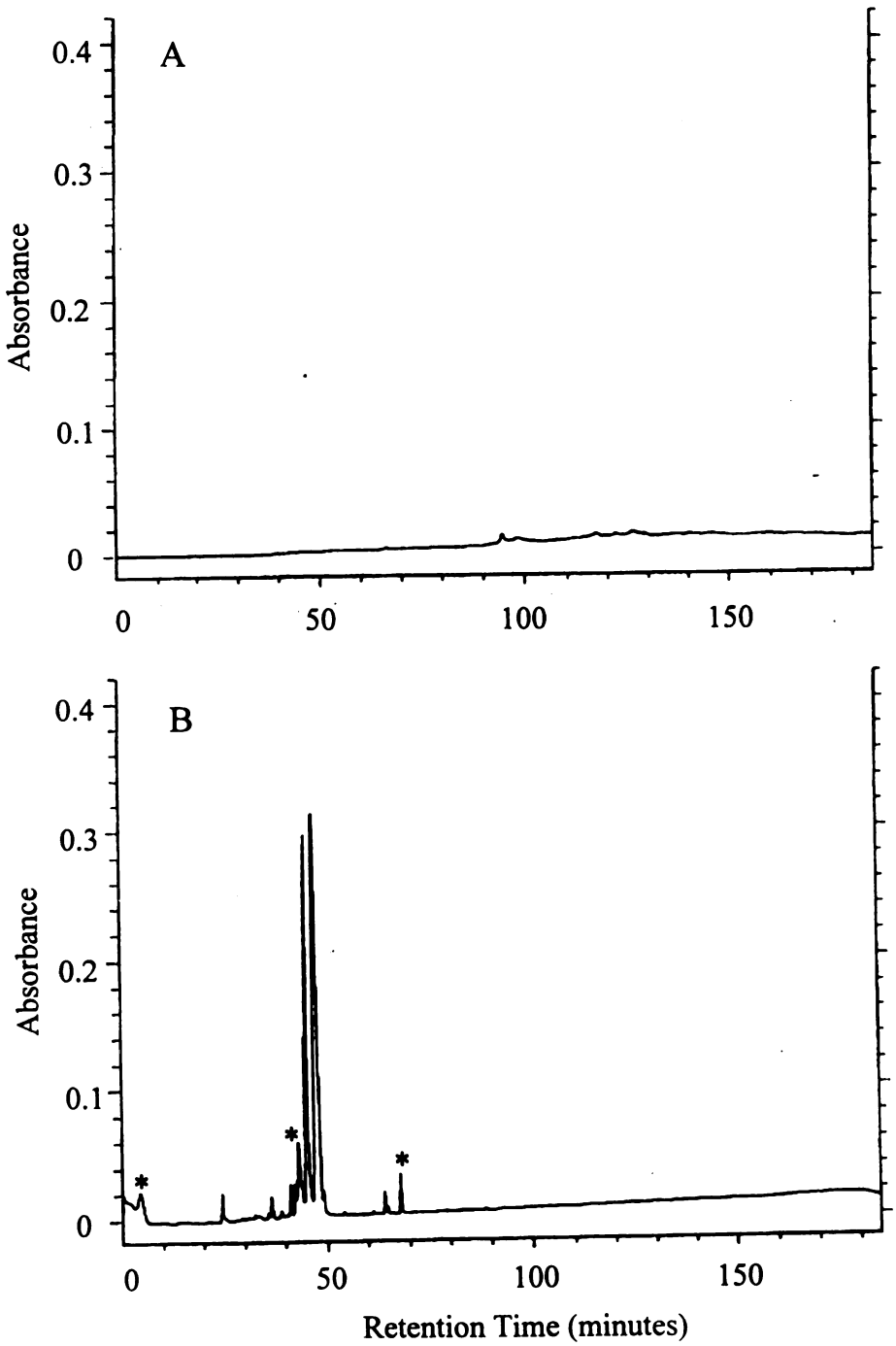
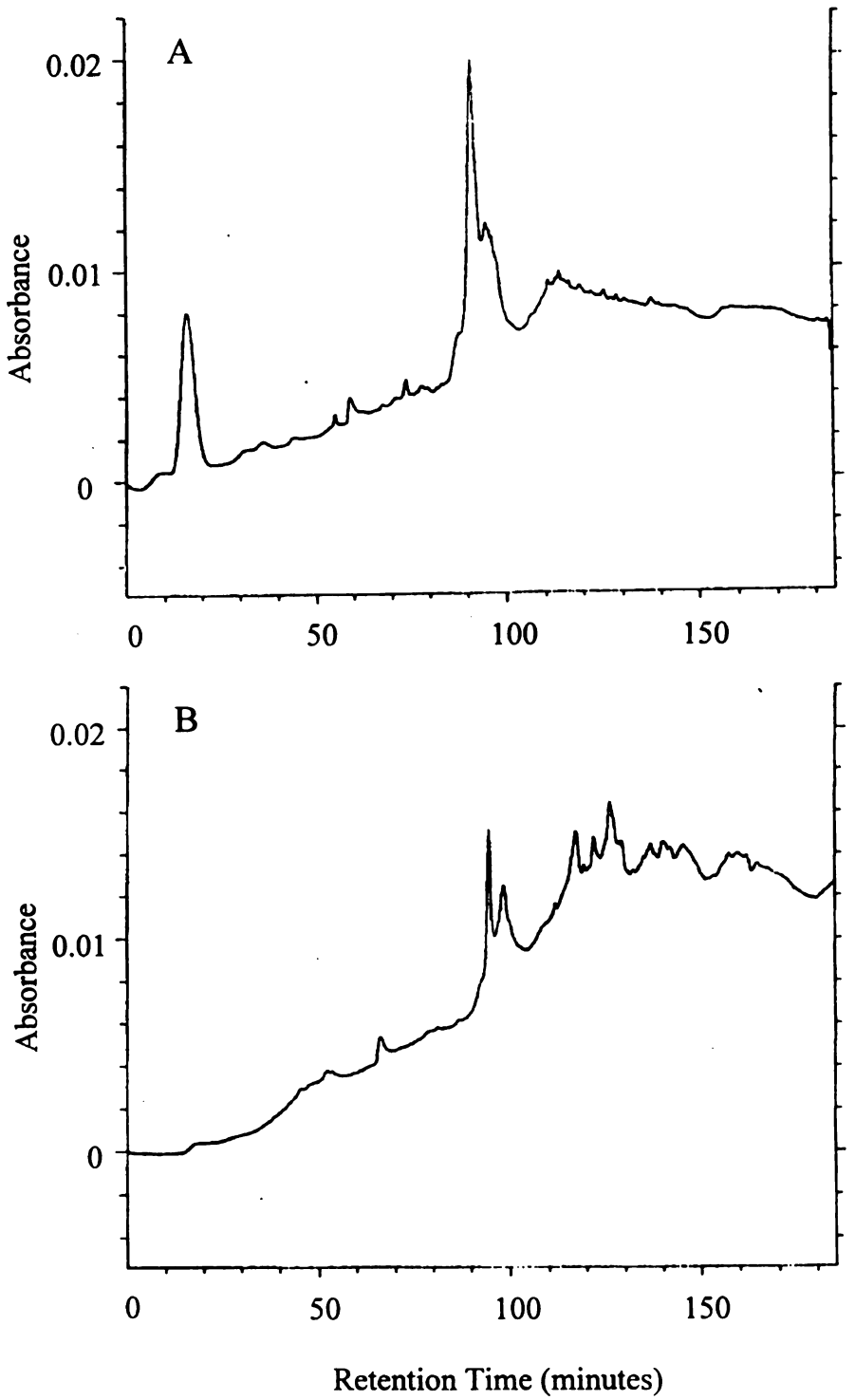


Figure 3.6 - HPLC of original ESP 10K retentate (A) and CAP-free ESP 10K retentate (B). Samples (10µg in 0.02ml) were loaded onto a reverse phase (C18) column and eluted with a 5-80% ACN gradient in 0.1% TFA.



CHAPTER 4

SUMMARY

The long-term objective of our lab is to understand the mechanisms of diminished host resistance to indigenous *Campylobacter jejuni* in the large intestine subsequent to infection of swine with *Trichuris suis*. This model system has relevance to human and veterinary medicine. *Campylobacter jejuni* is the leading cause of colitis in humans in the United States. The pig model is currently the best system to mimic the pathology seen in humans with *C. jejuni* enteritis. Our model is unique in that it is dependent on a nematode infection to set the stage for bacterial invasion. This dual infection model is particularly relevant to third world countries where compound infections involving nematodes and at least one other pathogen cause considerable morbidity and mortality.

From the veterinary perspective, mucohemorrhagic enteritis is a devastating manifestation of swine trichuriasis. Domestic pigs infected with even moderate numbers of whipworms are susceptible to secondary infections with invasive bacterial pathogens. This condition leads to intestinal scours, decreased weight gain, and wasting of the animals, which can amount to a considerable economic burden.

The first published observation of *T. suis* infection having an influence on the status of intestinal bacteria in

domestic pigs dates back only to 1975 (Rutter and Beer). Signs of colitis due to invasion by the bacteria appear at the time the larval worms end their histotropic phase and L3/L4 larvae emerge into the lumen of the host gut at about 21 days post-infection. The bacterial enteritis resolves with expulsion of worms at approximately 45 days post-infection, although chronicity is established if pigs are continually exposed to *T. suis*. Clearly, infection with the whipworms contributes to the disease process. Rutter and Beer (1975) hypothesized that mechanical damage by larval worms in the proximal colon allows penetration of invasive bacteria. However, this does not explain the secondary bacterial lesions due to *C. jejuni* in the distal colon that were later described by Mansfield and Urban (1996). To account for the observation of *C. jejuni* invasion in the LGCs in the distal colon, we established two additional hypotheses. First, soluble *T. suis* ESP influences IECs to facilitate invasion by opportunistic bacteria. Second, dysregulation of cytokine networks due to the host response to the whipworm infection downregulates cytokines necessary for protection against invasive bacteria. These hypotheses are not mutually exclusive, and it is likely that multiple models will converge to explain the observed phenomena.

The goal of my thesis research was to develop *in vitro* model systems to supplement the dual infection pig model in order to test the hypothesis that soluble *T. suis* ESP alters the permeability of IECs and leads to enhanced *C. jejuni* invasion. Confluent monolayers of epithelial cell lines of intestinal derivation serve as useful models for studies of intestinal epithelial structure and function (Madara et al., 1988). At the time this project was initiated, no cell lines for *in vitro* challenge studies with *C. jejuni* had been established in our lab. *In vitro* models were selected based on availability, best representation of crypt and surface enterocyte phenotypes, and relevance to the pig model. In the absence of a cell line from swine large intestine, Caco-2 cells were originally chosen because they were derived from a human colon carcinoma and they undergo spontaneous differentiation such that populations of cells in various stages of differentiation could be obtained. INT407 cells, originating from embryonic human small intestine, were selected to provide a model of basal crypt (undifferentiated) cells. We then obtained IPEC-1 cells, which were derived from embryonic pig small intestine and the most appropriate complement to the animal model. The differentiation phenotype of IPEC-1 cells depends on

culture conditions. IPEC-1 cells are undifferentiated when grown on a solid substrate but can be induced to differentiate when maintained on semi-permeable supports.

In the absence of a fresh pig isolate of *C. jejuni*, strain 33292 originally isolated from a human with enteritis was obtained from the culture collection at a local hospital. This strain was passaged through a piglet to restore virulence and prepare freezer stocks from low passage organisms.

We hypothesized that soluble ESP released from the whipworms contributes to the diminished host resistance to *C. jejuni*. A reductionist approach was taken in which a single host cell type, the IEC, was taken into consideration since IECs represent the foremost barrier to bacterial penetration. *In vitro* experiments were designed to determine if ESP induced alterations in cultured cell models of the IEC barrier that would parallel modulation of intestinal epithelial permeability *in vivo*. IEC responses such as cytotoxicity, enhanced proliferation, or increased tight junction permeability would represent ESP-induced breaches in intestinal integrity. Extrapolated to the *in vivo* state, these sites would be less resistant to *C. jejuni* invasion or translocation.

Assays to assess the viability of IECs after exposure to ESP consistently demonstrated dose- and time-dependent cytotoxic responses in IECs, whereas no enhancement in proliferation of IECs could be established. The *in vitro* cytotoxicity data can be bridged to the proximal colon of *T. suis*-infected pigs where histologic data demonstrates there is damage to the epithelium. The injured host tissue has been attributed to mechanical damage by the worms and the host's reaction to the nematode (Rutter and Beer, 1975; Mansfield and Urban, 1996). Our results indicate that damage to the epithelium can also occur directly from exposure to the ESP. Although our experiments were limited to IECs, other host cell types might be sensitive as well.

Although it is difficult to predict the concentration, a considerable amount of ESP is likely released into the microniches occupied by the anterior end of adult whipworms embedded in syncytial tunnels of host epithelium. ESP secreted orally into this localized microniche accumulates and causes damage because the adults live for approximately 20 days. Conservatively extrapolating from *in vitro* measurements, we estimated ESP concentrations in the proximal colon exceed 1 mg protein with a worm burden of 100 worms. Each adult worm is capable of producing 10-100 µg protein/ml/day under *in vitro* conditions. Therefore, if

each of 100 worms produces 10 μg protein in a single day, that would yield 1 mg ESP protein in the proximal colon. The caveat is that the relative contribution whipworm products secreted orally into the microniche vs. those excreted anally into the luminal milieau is not known. Combined, concentrations of ESP exceed 1 mg protein in the proximal colon.

A plan for subsequent experimentation will now be proposed. A reasonable next step would be to size fractionate ESP to partially isolate the cytotoxin, using IPEC-1 cells in the bioassay to screen for cytotoxic fractions. Following partial purification, polyclonal rabbit antiserum should be prepared for immunodetection purposes. A critical experiment would be to determine more precisely the concentration of the cytotoxin(s) in the proximal colon using immunohistochemistry on available archived tissues. Once the technique was developed using proximal colon, it could be applied to colon sections taken at downstream intervals (eg, every 6 inches) to determine the distal dissemination pattern of the cytotoxin(s). Another critical experiment would be to localize the cytotoxin(s) in histologic sections of the worm using immunohistochemistry to determine if it is secreted from the stichocytes or excreted anally.

Determining the responses of other cell types (neutrophils, macrophages, lymphocytes) to ESP would also be informative. In addition to cytotoxicity, it would be interesting to examine the influence of ESP on immune cell antigen responsiveness by doing lymphoblastogenesis or mitogenesis experiments. The addition of mitogens (LPS or SEB) or ESP to lymphocytes could be done to examine the effect of ESP on proliferation or activation. This would answer questions regarding the potential immunosuppressive properties of ESP. An additional layer of complexity could be added to examine host cell interactions in the presence of ESP using co-cultures. IECs on transwell filters could be cocultured with neutrophils in the basal media to see if the neutrophils are chemoattracted to the IECS on which ESP was applied apically and if they transmigrate through the monolayer of IECs. Commercially available cell-staining kits would be useful to determine if this cytotoxic response involves an apoptotic mechanism. If this ESP component is essential to the worm's survival (eg, initiation or maintenance of the syncytial tunnel) and is immunogenic, it might make a good vaccine candidate for prevention of swine trichuriasis.

Turning to the experiments aimed at assessing tight junction permeability, it is unlikely that ESP affects

tight junctions of IECs when only apical surfaces are exposed. Repeated attempts to document increased tight junction permeability in a well-established Caco-2 model system failed to demonstrate treatment-induced effects when ESP was applied to the apical surfaces of these differentiated cells. Essentially the same outcome was observed when these experiments were repeated using differentiated IPEC-1 cells. However, it was discovered that IPEC-1 cells did respond with a dramatic drop in transepithelial electrical resistance, corresponding to an increase in artificial epithelial barrier permeability, when ESP was applied to both apical and basolateral surfaces. Confocal laser microscopy of actin-stained cells revealed overt damage to cells treated apically and basolaterally, whereas monolayers of control cells were intact. A repeat experiment confirmed this observation and demonstrated dose- and time-dependent effects, which suggested that the increased barrier permeability following apical and basolateral ESP treatment is transient. These findings warrant further investigation and may have relevance to the *in vivo* scenario in that the basolateral surfaces of IECs would be exposed to ESP by the following mechanism. Where the integrity of the epithelial barrier is disrupted in the proximal colon (due to a combination of

mechanical damage from the worms, host response, and ESP cytotoxicity), ESP secreted into the microniche would diffuse into the underlying lamina propria. This would provide a route of ESP exposure for the basolateral surfaces of IECs in various stages of differentiation.

To follow-up on these observations, a plan for additional experimentation has been devised. Again, we would aim to reduce the complexity of the system by localizing the ESP activity to a particular size fraction. It would be interesting to trace the fate of ESP cytotoxin(s) upon interaction with IECs. ESP could be applied apically to IECs grown in transwells and the relative amounts present in the apical media, endocytosed intracellularly, and trancytosed to the basolateral medium could be monitored by western blotting. Additional biological responses should be measured to begin to dissect the mechanism of action leading to cell damage. For example, increased levels of intracellular calcium often signal rearrangements in cytoskeletal actin, which may be associated with cytopathic effects and tight junction alterations.

Another aim was to determine if IECs exposed to subtoxic concentrations of ESP were more permissive for *C. jejuni* invasion. We hypothesized that ESP would facilitate

entry of *C. jejuni* into IECs, perhaps through modification of surface receptors necessary for internalization. Enhanced invasion could not be demonstrated using the gentamicin killing assay. However, during the course of these experiments we did discover that *C. jejuni* recovery was extremely depressed in ESP-treated IECs. It was then determined that ESP directly inhibited the growth of *C. jejuni* and other test organisms. This is the first report of antibacterial activity from *T. suis* as well as the first report of antibacterial activity in the ESP of any nematode. Although the antibacterial activity reported in *Ascaris suum* was from the body fluid, it is possible that it is ESP-derived. We have named this antibacterial agent trichuricin. Trichuricin is a relatively stable molecule less than 10,000 MW, unlikely to be a protein but possibly is a peptide.

Additional experiments will focus on further characterization of the antibacterial activity in fractionated ESP. Using polyclonal antiserum against ESP fractions prepared by immunizing rabbits, it would be interesting to assess the distal dissemination of antibacterial agent in tissue sections and fecal material using immunohistochemistry and western blotting, respectively. The antibodies could also be used to

evaluate trichuricin production within the adults and at different stages in whipworm development. Although subadult stages are hard to recover, it would be feasible to do these experiments with larval whipworms fixed *in situ* while still embedded in the host tissue. This would also give a better estimate of the amount of ESP secreted directly into the syncytial tunnel microniche immediately surrounding the anterior end of the worm. One could predict that larvae don't produce trichuricin because they are less exposed to bacteria while embedded fully within host epithelium in the base of crypts. Antibacterial activity in other invertebrates is upregulated in response to stimulation by bacteria. It would be interesting to determine if trichuricin is induced upon exposure to *C. jejuni* or other potentially pathogenic bacteria that would be encountered in the pig colon.

Initially it seemed that this data was inconsistent with our central hypothesis. How could we postulate that *C. jejuni* invasion is dependent on *T. suis* if there is unequivocal evidence that *T. suis* ESP inhibits the growth of *C. jejuni*? This apparent discrepancy can be reconciled when events in the proximal and distal colon are distinguished. Extrapolation of my *in vitro* experiments to the *in vivo* model is essentially limited to the proximal

colon where worms reside and where ESP would exist at its highest concentrations. Intuitively, this is where IECs would be most affected and the cytotoxic response would be the most dramatic. We consider it unlikely that trichuricin would persist into the distal colon to exert its effect on *C. jejuni* associated with LGCs.

In the hypothetical absence of ESP antibacterial activity in the proximal colon, this would constitute an easy portal of access for bacteria into the underlying tissues. Despite the antibacterial activity, presumed to present *in vivo*, histologic data indicate that bacteria are present in close proximity to the worms and in the immediate underlying lamina propria. Although it is not possible to distinguish viable from non-viable bacteria by looking at H&E stained tissue sections from our earlier studies in pigs, this does suggest that not all bacteria are translocation-inhibited in the presence of *T. suis* ESP. This hypothesis is consistent with our preliminary antimicrobial susceptibility testing in that there was differential susceptibility to ESP among the bacterial organisms tested.

In this model, *C. jejuni* could still penetrate the epithelial barrier in the proximal colon if one or more of the following conditions existed. *C. jejuni* is embedded in

mucus in the base of crypts, which could provide protection from the deleterious ESP effects. Protection afforded by the mucus matrix would allow *C. jejuni* to penetrate IECs in the base of crypts. Mucin has been shown to be chemotactic for *C. jejuni* (Hugdahl et al., 1988), which has a predilection for mucus-filled colonic crypts (Beery et al., 1988). This relates to our interpretation of the electrophysiology data and exposure of IECs from basolateral surfaces. The mucosal surface is often abraded and the barrier is no longer intact where adult worms are present. ESP could diffuse passively into the lamina propria and be disseminated along the basolateral surface of cells in various stages of differentiation causing cytotoxic damage and disturbances in tight junction permeability - when basal surfaces are exposed.

Proteases in *T. suis* ESP may participate in this process. The zinc metalloprotease present in *T. suis* ESP is capable of degrading connective tissue components of basement membranes that form the interface between epithelial cells and lamina propria (Hill et al., 1993). Live *T. muris* degrades basement membrane proteins, which suggests involvement in the invasive process, maintenance of the syncytial niche, and breakdown of epithelial continuity (Drake et al., 1994).

Perhaps the only event needed for invasion by certain bacteria is exposure of components of the extracellular matrix. *C. jejuni* binds to fibronectin, a component of the extracellular matrix, via a 37 kDa outer membrane protein termed CadF (Konkel et al., 1997). Furthermore, fibronectin is differentially expressed in the base of intestinal crypts (Beaulieu 1992). Slight damage to IECs and/or degradation of extracellular matrix components due to ESP would likely cause cell rounding and unmasking of underlying fibronectin. *C. jejuni* could then bind fibronectin and penetrate a discontinuous epithelial barrier into the underlying lamina propria. Our results also show that *C. jejuni* invades undifferentiated cells to a higher degree than differentiated cells. Collectively, these data suggest that conditions are favorable for *C. jejuni* invasion in the base of intestinal crypts.

There could be a differential release of cytotoxic and antibacterial agents from the worms, although it is possible that these activities are mediated by the same agent. IEC cytotoxic factors could be secreted from the mouth of the worm into the epithelial tunnel and antibacterial agents excreted from the anus of the worm into the lumen of the host gut. If so, *C. jejuni* embedded in the crypt mucus would be spared.

It is clear that not all bacteria are equally susceptible to ESP. There could be a differential effect of ESP on intestinal microflora in the proximal colon. It is possible that other bacteria are more susceptible to ESP than *C. jejuni* and then *C. jejuni* would have a growth advantage after trichuricin is exhausted on other bacteria. It is known that *C. jejuni* is invasive at doses of 800 organisms and thus a slight growth advantage may promote pathogenicity. Another likely scenario is that the bacteria that are not sensitive to ESP do penetrate the host epithelium at the proximal colon and contribute to the initiation of a cascade of immunological events that culminate in decreased resistance to invasive bacteria in the distal colon. We return to the key question: How does an event in the proximal colon (whipworm infection) influence an event in the distal colon (*C. jejuni* infiltration and inflammation of LGCs)?

We have obtained interesting answers to discrete questions using a reductionist approach that can be coupled to the animal model and provide the foundation for future studies. The *in vitro* cell models are more representative of the proximal colon and should be considered insufficient for dissecting events in the distal colon because the LGC is essentially an immunological organ composed of multiple

cell types. Because the phenomenon under study (*T. suis*-dependent *C. jejuni* colitis) is so complex and likely involves multiple mechanisms, *in vitro* models composed of single cells will have limited value in addressing many key questions. Reconstitution of more than one cell type *in vitro* or explant cultures may be useful, although there is no substitute for an animal with an intact immune system. The mouse model currently under development will be a useful addition since using pigs is cost-prohibitive and pigs are not inbred.

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