This is to certify that the
dissertation entitled

ROLE OF TH-2 CYTOKINES IN *CAMPYLOBACTER JEJUNI AND TRICHURIS SUIS* MEDIATED PATHOGENESIS IN SWINE

presented by

GEETHA PARTHASARATHY

has been accepted towards fulfillment
of the requirements for the

Ph.D degree in Microbiology and Molecular Genetics

Linda S. Mansfield
Major Professor's Signature

8/27/2004
Date

MSU is an Affirmative Action/Equal Opportunity Institution

ROLE OF TH-2 CYTOKINES IN *CAMPYLOBACTER JEJUNI* AND *TRICHURIS SUIS* MEDIATED PATHOGENESIS IN SWINE

By

Geetha Parthasarathy

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Microbiology and Molecular Genetics

2004

ABSTRACT

ROLE OF TH-2 CYTOKINES IN *CAMPYLOBACTER JEJUNI* AND TRICHURIS SUIS MEDIATED PATHOGENESIS IN SWINE

By

Geetha Parthasarathy

Campylobacter jejuni, an enteric bacterium, is one of the leading causes of bacterial enteritis in the world and *Trichuris suis* is a whipworm helminth of the swine intestinal tract. *C. jejuni* and *T. suis* acted synergistically in the colon of neo-natal germ-free piglets to cause disease more severe than the additive effects of either of the single infection. We hypothesized that cytokine dysregulation could be a contributing factor to this phenomenon, and that Th-2 cytokines might play a role in this polymicrobial disease. Thus the goal of this research was to evaluate the role of Th-2 cytokines such as IL-4, IL-6 and IL-10 in immunity to *C. jejuni* and *T. suis* under *in vitro* and *in vivo* conditions, and further evaluate the role(s) of individual cytokines in *C. jejuni* and *T. suis* mediated pathogenesis in swine.

To my parents, Padma and Parthasarathy, my sister Radha, and my brother-in-law Ravi,

you are my real support and my strength through this unforgettable journey.

ACKNOWLEDGEMENTS

The past six years have been a growing-up experience in many ways, and through it all I was fortunate enough to meet some incredible people who will be milestones in my life's journey.

I would like to thank Dr. Linda Mansfield and Dr. John Linz for their support, encouragement and valuable advice through out these past years. Many a times have I stumbled, and you have been there to lend me a helping hand. I would like to thank my committee members Dr. Richard Schwartz, Dr. Katheryn Meek and Dr. Martha Mulks for their guidance and support through these years.

I would also like to thank the Dept. of Microbiology, Large Animal Clinical Sciences, and the Graduate School for providing me with various fellowships and assistantships over the years. Thanks to Dr. John Baker for believing in me and helping me out at times of financial need.

I had the good fortune to work with many incredible people around me; Lakeisha Cunningham, Dr. Kathryn Jones, Hany Elsheika, Dennis Shubitowski, and Sheila Abner– thanks for listening to my problems; Dave Wilson –thanks for the intellectual and philosophical conversations; Dr. Julia Bell- you are truly a God send to our lab.

My terrific friends; Shikha Gupta, Gauri Jawdekar, Aarthi Rishi, Harini Krishnamurthy and Shibani Mukherjee-you have all touched my life one way or another. The past six years would not be possible but for you all.

Thanks to Pawin Padungtod, Bo Norby and Seth Walk for help with statistics; Dr. Joe Leykam and staff of the Macromolecular Structure Facility for the sometime free-of charge primers; Dr. Terese Best and staff at the Genomic Sequencing Facility for the

sometime free-of-charge sequencing reactions; Russ Somers and Moses Fetters for helping with my computer problems; Dr. Shirley Owens, Dr. Alicia Pastor and Dr. Ewa Danielawicz for facilitating my microscopy experiments; Angie Zell, Jennifer Sysak, Angela Corley, Tonie Brovont, Susan Dies and Margaret Nicholas, for all the administrative support; the custodial staff at the National Food Safety and Toxicology Center for all the night chats.

Last but not the least, thanks to my family and God for being at my side always.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

LITERATURE REVIEW

I - *CAMPYLOBACTER*

A) Microbiology

i) General Characteristics

Campylobacters are microaerophilic, gram-negative, non-spore forming spiral bacteria with a unipolar or bipolar flagellum. Bacterial cells range from 0.5- 5.0 μm in length and 0.2- 0.8 μm in width (122). There are 16 distinct species of Campylobacters known. They include *C. jejuni*, *C. coli*, *C. concisus*, *C. curvus*, *C. fetus*, *C. gracilis*, *C. helveticus*, *C. hominis*, *C. hyointestinalis*, *C. lanienae*, *C. lari*, *C. mucosalis*, *C. rectus*, *C. showae*, *C. sputorum* and *C. upsaliensis* (54, 175). Most species of *Campylobacter* are motile exhibiting corkscrew-like motion and darting motility. However, *C. gracilis* is non-motile (169). The spiral structure is maintained through the exponential phase of growth, and most cells become coccoid after 24 hr of growth in liquid culture. These coccoid structures are deemed viable but non-culturable. However, some researchers consider the coccoid form degenerate. Campylobacters are non-saccharolytic, i.e, they cannot utilize carbohydrates as a source of energy. Instead, amino acids and tricarboxylic acid cycle intermediates are used. On biochemical testing, most species are negative for hippurate hydrolysis except *C. jejuni* subsp. *jejuni* and *C. jejuni* subsp. *doylei.* Also, most can reduce nitrate and are oxidase positive (130, 131). *Campylobacter* genomes are relatively small compared to many bacteria. The genome sizes of *C. jejuni* and *C. coli* are around 1.6 – 1.7 Mb, while those of *C. fetus* subsp *fetus* and *C. fetus* subsp *venerealis* are 1.1 and 1.3 Mb respectively (52, 155). The chromosome is circular, and the GC content of *Campylobacter* DNA varies between 28 to 39% (51). Of all the *Campylobacter* species, only the *C. jejuni* genome has been completely sequenced. There are 1654

predicted coding sequences in *C. jejuni* genome, and ~ 94.3% of the genome codes for proteins, making it the most densely packed bacterial genome so far (135).

ii) Isolation of *Campylobacter*

Campylobacters are normally isolated from fecal samples, environment, food samples, meat and tissue sections. Cary–Blair medium is commonly used as transport medium for most species of *Campylobacter*. Once in the laboratory, they are routinely cultured in enrichment broth cultures such as Preston broth, Campythio and *Campylobacter* enrichment broth and others. Most strains grow optimally between 30 – 37° C, although isolates from some animals grow optimally at 42 °C. Typically, a gas mixture of 5 – 10 % O_2, 5 – 10% CO_2, and 85% N_2 is used. Some species like *C. sputorum*, *C. concisus*, *C. mucosalis*, *C. curvus*, *C. rectus* and *C. hyointestinalis* might additionally require hydrogen (3%) for optimal growth (122). Most strains of *C. jejuni* and *C. coli* are inherently resistant to vancomycin, trimethoprim, penicillins, rifampin and cephalosporins. Hence, some media like cefoperazone deoxycholate agar (CCDA), charcoal-based selective medium (CSM), blood-containing Campy-CVA medium and Skirrow medium might incorporate one or more of these antibiotics for selective growth of these *Campylobacters* (75, 81, 122). For isolation from fecal samples, cefoperazone-containing media have been suggested. However, this selective medium does not allow growth of all species of *Campylobacter*. Hence, a newer method, dubbed "the Cape Town protocol" has been developed, wherein stool samples are filtered through a membrane onto antibiotic free blood plates and subsequently incubated in an H_2-containing atmosphere (122).

iii - a) Identification

Campylobacters can be directly identified in stool samples by microscopic examination and by using the Gram stain. Colony morphology (yellowish-gray, glistening, round colonies) is also used to identify these organisms in a mixed culture. Biochemical assays based on *Campylobacter*-specific traits like oxidase activity are routinely used when pure cultures are available. PCR-based assays are more commonly used in research laboratories for identification of this bacterium. Some of the genes used for analysis include the *fla* gene, 23S rRNA gene and 16S rRNA gene. Both the biochemical assays and PCR assays can distinguish *Campylobacter* at the genus and species levels (24, 74, 121). Immunological assays for identification of *C. jejuni* and *C. coli* are also commercially available (71).

iii – b) Subtyping

Campylobacters exhibit a high degree of genetic strain-to-strain variation even within individual species. Subtyping of the species to identify individual strains is critical, particularly during endemic outbreaks or even during epidemics, for epidemiological investigations. A variety of assays are available to determine individual type strains. Pulsed-field gel electrophoresis (PFGE) is commonly used to identify different isolates of *C. jejuni* and *C. coli*. The assay uses specific restriction enzymes for specific species with subsequent analysis of the pattern profile for each strain (25). PFGE has also proved useful in typing strains of other species such as *C. lari*, *C. fetus*, *C. upsaliensis* and others (22, 125). *fla* typing is yet another common genotypic method employed to distinguish strains. After amplification of the *flaA* gene, the PCR product is subjected to restriction enzyme digestion with subsequent analysis of the fragment

profiles. This assay is different from PFGE in that restriction analysis is performed using a specific gene product rather than the whole genomic DNA. Also, *fla* typing is typically used for *C. jejuni* and *C. coli* strains and not for other species. Yet another genotypic method used to type strains is random amplification of polymorphic DNA (RAPD). This method uses multiple random primers to generate fragments from the entire genome, which are then subjected to agarose gel electrophoresis to visualize patterns. It is useful in typing strains of *C. jejuni*, *C. coli* and *C. lari* (109). Apart from these techniques, two recently introduced genotypic methods include amplified fragment length polymorphism (AFLP) and multilocus sequence typing (MLST) (157, 161). In addition to these genotypic methods, commonly used phenotypic methods include serotyping and phage typing. For most epidemiological studies, a combination of methods is normally used to allow for reliable identification of species and strains.

Genetic variations in *Campylobacter*

Subtyping of *Campylobacter* has revealed tremendous genetic diversity among *Campylobacter* species. In one study, 16S ribotyping showed 77 ribotypes among 261 *C. jejuni* isolates (45). Employment of PFGE in another study revealed 25 different banding patterns among 80 isolates (132), while the use of restriction fragment length polymorphism (RFLP) in another study showed 89 different polymorphism patterns in 120 *C. jejuni* isolates (96). Multiple mechanisms exist for generation of such genetic diversity among bacterial populations, and include horizontal gene transfer within or between bacteria (recombinations), point mutations, deletions, rearrangements, duplications and inversions. *Campylobacter* strains have been shown to employ almost all these mechanisms to generate diversity at multiple loci, although the role of

recombination is thought to be 50 times greater than that of mutation (157). Horizontal gene transfer between heterologous and homologous strains of *C. jejuni* was demonstrated both *in vitro* in liquid cultures and *in vivo* in chickens through the acquisition of antibiotic resistance markers (21, 180). In the *in vivo* study, PFGE analysis of the recombined strains also showed duplications of distinct regions as determined by gain of a large DNA fragment, in addition to the markers (21). A single deletion frame shift mutation in the polymeric T tract of the *flhA* gene, which controls the expression of *flaA* and *flaB*, was shown to confer functional diversity in a *C. coli* strain. The resulting mutants were non-motile, and motility was restored by reversible insertion of the thymine residue (134). Sequence analysis of the lipooligosaccharide (LOS) loci from 11 *C. jejuni* strains showed that gene rearrangements, deletions and single mutations played a role in generation of genetic diversity among these strains (56). In accordance with these results, whole genome comparisons using microarrays have shown that the most divergent genes are the loci that code for lipooligosaccharide and flagellar synthesis, restriction-modification systems, integrases, iron-acquisition, sialylation, sugar transport and metabolism (34, 101, 145). Thus, Campylobacters as a group have been shown to be one of the most divergent bacteria to date. This ability to generate genetic diversity confers this organism a distinct advantage in survival in the host and environment as well as in pathogenicity.

B) Epidemiology

Of the 16 different species of bacteria that comprise the genus *Campylobacter* (family *Campylobacteraceae*), two species, *C. jejuni* subsp. *jejuni* and *C. coli*, are most

commonly associated with gastrointestinal infections in humans (84). Of the two, *C. jejuni* infections are more prevalent (80 – 90% compared to 5 – 10 % by *C. coli*) and are considered one of the leading causes of bacterial enteritis in the world, along with *Salmonella* and *Shigella* (63, 84). It is estimated that around 2.4 million people are affected every year in the United States alone (48). The incidence of *Campylobacter* infections in the developed world is approximately 25-50/100,000 cases of diarrhea reported for all persons and 300/100,000 cases for children between 1 and 4 years of age. *Campylobacter* infections also show a sex-specific incidence pattern, with males being affected more frequently than females. There is also a marked seasonal variation in *Campylobacter* incidence, with infections peaking in the summer months. However, while this is true for most of the developed world, there are marked differences in incident rates and prevalence of *Campylobacter* infections in the developing world. The incidence rate among children less than 4 years of age is much higher, ~ 40,000/100,000 cases reported. There is also no well-documented seasonal variation in infection rates in the developing world. Additionally, the average number of infections /person/ lifetime in the developed countries is 0–1, while that in the developing countries is > 5.

Campylobacters also cause a different spectrum of gastrointestinal infections in these two worlds. In the developed world, inflammatory diarrhea is more commonly associated with *C. jejuni* infections, with symptoms including fever, vomiting, bile-filled discharge, headaches, abdominal cramps and bloody diarrhea. On the other hand, infections in the developing world are much milder, with profuse watery or secretory diarrhea. The differences have been attributed to constant re-infection among people of the developing world, which produces partial immunity (48, 127).

Campylobacters are essentially a food-borne pathogen and are acquired through contaminated raw milk, undercooked poultry, and other foods. Occasionally, campylobacters may be spread through the community water supply (48). The infective dose of *C. jejuni* is as low as 400-500 organisms, as determined from experimental human infections (20). However, this dose might be hard to estimate in the developing world, given the large rate of asymptomatic infections (127). *C. jejuni* infections usually resolve within 1-3 weeks. However, other rare complications can accompany enteritis, including appendicitis, neonatal meningitis, hepatitis, pancreatitis, peritonitis, myocarditis, toxic megacolon, and others (162). Some late-onset syndromes include reactive arthritis and a neuropathological disorder termed Guillain-Barré syndrome. Mortality due to *Campylobacter* infection is estimated to be between 50 and 150 per year in the United States (48). *C. jejuni* is also an opportunistic pathogen and is often isolated from patients with Acquired Immunodeficiency Syndrome (AIDS), hypogammaglobulinemia, and other immunocompromising conditions (82, 163). *Campylobacter* infections are usually treated effectively with antibiotic therapy. The antibiotics of choice include erythromycin and fluoroquinolones like ciprofloxacin. Tetracyclines are normally contraindicated due to the rapid emergence of resistance to this antibiotic (103).

In summary, *Campylobacter* infections are prevalent throughout the world. The geographical area, immune status of the host, seasonal variations, and gender of the individual can affect the rate and incidence of infections. Also, some strains are more pathogenic than others, and the factors that affect pathogenesis will be discussed below. While infections are contained effectively in most cases, the incidence is on the rise.

Also, other species of *Campylobacter*, such as *C. upsaliensis*, are poised to be new human pathogens. With better public health care and public education, *Campylobacter* infections can be mitigated significantly throughout the world.

C) Pathogenesis

C. jejuni is the best studied of all species of campylobacters, though its pathogenic mechanisms are not well understood compared to those of other enteric pathogens. The strain-to-strain variation, the source of isolates, and the variation in eukaryotic cell lines used for analysis make it difficult to interpret results universally. However, some of the pathogenic mechanisms and host factors that contribute to pathogenicity have been documented.

Virulence factors

i) *Campylobacter* toxins

Multiple toxins have been described for *C. jejuni*, including cytolethal distending toxin, cytotoxin, hemolysin, hepatotoxin, and porins.

a) Cytolethal distending toxin (CDT)

This is the most studied toxin of *Campylobacter*. The toxin causes mammalian cells to be arrested at the G_2 phase of the cell cycle, thereby causing the cells to become distended in appearance (179). The holotoxin is composed of three subunits, CdtA, CdtB and CdtC (97). The enzyme activity is associated with CdtB, and was shown to be a nuclease causing double-stranded breaks in DNA when microinjected (62). While CdtB is thought to be essential for the cytotoxic activity, the roles of CdtA and CdtC are controversial. One study with purified subunits showed that all three subunits are

required for cytotoxic activity on Henle-407 intestinal epithelial cells, while another study showed that CdtB and CdtC are sufficient to induce similar activity on HeLa cells (97, 100). All three proteins have leader sequences, suggesting that they are secreted from the cell; however, this remains unproved, since most of the activity remains in bacterial membrane fractions (144). A recent study showed that purified CdtA and CdtC can bind eukaryotic cells but that CdtB does not, suggesting that CDT might be an A: B toxin like cholera toxin. The A subunit of cholera toxin binds eukaryotic cells while the B subunit is translocated into the cells. In *Campylobacter*, the A subunit would include both CdtA and CdtC (100). The *cdt* genes are present in almost all strains of *C. jejuni* and *C. coli*, as well as in the few isolates of *C. fetus*, *C. upsaliensis* and *C. hyointestinalis* strains tested so far (40, 41). However, the amount of cytolethal activity differs between isolates; the reason for these differences remains unclear. Recent studies showed that CDT might play a role in the induction of IL-8 from INT407 cells. Membrane fractions of mutant *C. jejuni* strain 81176 defective in any of the three membrane-associated subunits failed to induce IL-8 (66).

The role of CDT in *C. jejuni* pathogenesis is not clear. A few hypotheses have been proposed based on what is known in other systems. In *Actinobacillus actinomycetecomitans,* an immunosuppressive activity of CDT has been identified by its ability to kill T-cells, and the CDT from *Shigella dysenteriae* has been shown to magnify diarrheal symptoms in suckling mice in a dose-dependent manner (129, 159). In a very recent study, *C. jejuni* strain 81176 CdtB mutant was shown to be defective in colonization of C57BL/129 mice 4 months post inoculation, while the wild type strain was not. Also, in the same study, mice deficient for NF-κB did not develop

gastroenteritis when infected with the CdtB mutant suggesting a potential role for this toxin in pathogenesis (47).

b) Cytotoxin

A 70 KDa cytotoxin has been described that causes HeLa cells and Chinese hamster ovary cells to round up and eventually die. The toxin is heat-and trypsin-sensitive and hence is thought to be a protein. It is also thought to be associated with the bacterial cell membrane (58). Yet another cytotoxic activity has been described recently, which is heat-stable, but sensitive to proteases. This toxin is fairly pH stable and retains its activity after storage at –70°C and –20°C for over 2 years. However, these cytotoxic activities have not been further characterized (99).

c) Hepatotoxin

A hepatotoxic acitivity has been identified in a few strains of *C. jejuni*, which causes mouse liver lesions and hepatitis. The factor also causes massive infiltration of mononuclear cells to the site of lesions. However, only two studies have been published on this activity, and it awaits further characterization (86, 87)

d) Hemolysins

Hemolytic activity has been observed with a few strains of *C. jejuni* and *C. coli*. Two different types of hemolytic activity have been described, one secreted by bacterial cells and one based on physical contact with erythrocytes (144). This activity is not generally seen in *Campylobacter* strains, and one of the reasons suggested is the incubation temperature used for routine growth. In one study, the strains demonstrated hemolytic activity at 42°C but not at 37° (3). Another study found blood agar plates to be an unsuitable medium for detection of hemolysins and proposed the use of blood agarose

plates instead (170). In a recent study, a strain carrying a mutation in phospholipase A gene (*pldA*), which encodes a protein found in the outer membrane of *C. coli* was shown to be deficient in hemolysis; PldA was postulated to play a role in cell-mediated hemolysis of erythrocytes (57).

e) Porins

Only one study has been conducted to date on a transient vacuole-forming cytotoxic activity of porins from *C. jejuni*, which causes rounding and eventual death of HEp-2 cells. The activity is heat labile and trypsin resistant and was found to be associated with lipopolysacharides (8).

ii) Motility

Motility has long been considered a virulence factor in *Campylobacter* pathogenesis, with motile strains being considered more efficient in colonization and more invasive than non-motile strains. In one study, non-motile strains were cleared from the intestinal tract of suckling mice, while the parental wild type colonized efficiently (120). Motility is conferred by a single flagellum or bipolar flagella that enable the bacteria to swim through the mucus layer; motility is also thought to enable the bacteria to seek receptor sites for effective adherence. The flagella are composed of two subunits, FlaA and FlaB, and of the two, FlaA is thought to be essential for motility (59, 178). Motility is also required for invasion, as non-flagellated, non-motile strains are invasion deficient (184). Other factors that affect motility include pH and viscosity. While *C. jejuni* exhibits normal motility at neutral pH and at a pH of 8.5, it is paralyzed at a pH of 5.0 (168). Also, as the viscosity of the environment increases, the motility increases as well.

iii) Adherence and adhesins

Adherence is an important virulence factor in *Campylobacter* pathogenesis. Adherence is required for successful colonization of the host gut, and prevents bacterial cells from being washed away by intestinal fluids and peristalsis. Also, prior to *C. jejuni* invasion, the bacteria bind transiently to the host cells.

Many factors affect adherence. The fibronectin binding protein, CadF, and cell binding factor-1 (CBF1 or PEB1) are two well-known adhesins. Other putative adhesins include the flagella, pili, outer membrane proteins, and lipopolysaccharides (LPS).

a) CadF and PEB1

CadF is a 37-KDa outer membrane protein present in all strains of *C. jejuni* and *C. coli*; it binds the extracellular matrix fibronectin present on host cells (90). Targeted deletion mutants of CadF showed a 100% reduction in colonization of newly hatched Leghorn chickens (185). PEB1 is a 28-KDa surface-exposed protein that is required for adherence and colonization (83). *C. jejuni* with mutation in the *peb1* locus showed a 50 to 100-fold reduction in adherence of *Campylobacter* to HeLa cells and significant inhibition in intestinal colonization of BALB/c mice (142). In addition, PEB1 is thought to be required for amino acid transport in *C. jejuni* (141) and to be an antigenic determinant for production of serum antibodies (143).

b) Flagella and Pili

Apart from their role in motility, flagella also are considered putative adhesins. Non-flagellated mutants have been shown to adhere less efficiently than the corresponding flagellated wild type strains (72). Also, purified flagellar proteins have

been shown to bind INT407 cells (117). In another study, monoclonal antibodies raised against the flagella inhibited adherence and colonization of suckling mice (172).

The role of pili in intestinal colonization or virulence is not clear. In one study, pilus-like structures were reported to be induced in the presence of bile salts, and the corresponding pilus-deficient mutants caused significantly decreased disease symptoms in a ferret animal model (33). However, it was later shown that this pilus-like structure was in fact an artifact (55).

c) OMPs

Multiple outer membrane proteins are present in *Campylobacter*, however, only a few have been shown to play a role in adherence. In addition to PEB1 and CadF, two other OMPs of 26 and 30-KDa that bind HeLa cells are also thought to play a role in attachment of *Campylobacter* to host cells.

d) Lipopolysaccharides (LPS)

LPS are considered major antigenic determinants of most Gram- negative bacteria. Aside from their role in the induction of serum antibodies, LPS/LOS of campylobacters are also thought to play a role in adherence. In the only published study of LPS, purified *C. jejuni* LPS was shown bind INT407 cells as well as intestinal mucus (117).

LPS/LOS biosynthesis

LPS is a major outer surface component of Gram-negative bacteria, and is made of three regions, the Lipid A molecule, the core region (inner and outer) and the O-chains. The Lipid A molecule comprises the endotoxin part of the LPS and is attached to the inner core through ketodeoxyoctulosonic acid or Kdo, which is specific for the inner

core. The outer core region is attached to the O-chains, which are made of 10 to 30 sugar subunits comprised of one to five sugars. Lipooligosaccharides (LOS) unlike the LPS, lack the O-chains (49). LPS biosynthesis is a complex process. The different regions of the LPS are assembled in the inner membrane and then transported (or flipped) to the outer membrane. The Lipid A moiety is synthesized initially, from N- acetylglucosamine (GlcNAc) precursor. Kdo is added directly to the Lipid A molecule, followed by the sugars that comprise the inner core. The outer core is attached next, subsequently followed by the O-chains, and the entire molecule is transported to the outer membrane of the bacterium. In *Campylobacter*, the outer core is thought to be assembled on a lipid carrier, and then transferred to the inner core. A number of genes have been implicated in LPS biosynthesis of *Campylobacter*. These include the *waaC* gene product, a heptosyltransferase, that functions in the attachment of a heptose to Kdo; the *wla* gene cluster, predominantly consisting of glycosyl and galactosyltransferases, and the *galE* gene product, a UDP-glucose-4-epimerse, that function in the synthesis of the core molecules (88, 95, 102, 181). *Campylobacter* strains exhibit variability in the expression of the O-chains. Some are LOS producers, while others produce LPS (49). However, the attachment of the O-chains to the core region is controversial. Some *C. jejuni* strains have been found to produce polysaccharides that are not attached to typical lipid A- core molecules (6, 146). In these cases, it is thought that the O-chains may be attached to a lipid molecule rather than to the core structure. However, this remains unresolved.

The LPS/LOS of *Campylobacter* also has been implicated in the pathogenesis of Guillain Barré Syndrome (GBS). The core structures of Campylobacters contain sialic acid residues, and in multiple serotypes these structures mimic human gangliosides,

particularly GM_1, GM_2, GD_3, GD_{1a} and GQ_{1b} (60). Gangliosides are glycosphingolipid molecules found in nerve ganglia, and ganglioside mimicry in *Campylobacter* core structure results in auto-immune antibodies directed against the gangliosides present in peripheral nerves, resulting in neurological disease. In a recent study, the *cgtA* gene, that encodes a putative N-acetylgalactosaminyl transferase involved in core formation, was shown to undergo slip-strand mutagenesis, resulting in a GM_2 to GM_3 mimicry (61). The *cgtA* gene contains a variable, homopolymeric G tract, and was shown to undergo slip-strand mismatch recombination in this region, resulting in a differential number of G nucleotides in the coding region. This was subsequently shown to transform the ganglioside structure from GM_2 to GM_3. Slip-strand recombination in the *wlaN* gene, also involved in core structure synthesis, has been shown to occur as well. Recombination in this gene affected the LOS core structure, and converted the surface coat from GM_2 to a GM_1 ganglioside (61, 105).

e) Host factors required for adherence

The knowledge of host factors that mediate adherence of *C. jejuni* is limited. One of the well-known mediators mentioned previously is the extracellular matrix protein fibronectin. Intestinal mucus also enhances adherence (30). In one study, the sugars mannose, maltose, fucose, glucose, N-acetylglucosamine and galactose did not significantly inhibit binding of campylobacters to Chinese Hamster ovary cells (CHO), while lipids partly inhibited binding (167). However, in another study, purified LPS was shown to be fucose-sensitive in binding to INT407 cells (117). Besides these putative adhesins, host α-integrin receptors α4β1 and α5β1 also have been implicated (92).

iv) Invasion

Invasion of host cells is considered as one of the most important virulent factors for *C. jejuni* pathogenesis. Invasion has been associated with many models of *C. jejuni* enteritis, including infant chickens, gnotobiotic piglets, chicken embryos, infant mice, and infant monkeys; and most importantly, in patients with *C. jejuni* colitis (72). Clinical strains have been shown to be more invasive in HeLa and Hep-2 cells than environmental and non-clinical isolates (91, 126). Also, isolates from patients with inflammatory diarrhea are more invasive than the ones from non-inflammatory diarrhea. Invasion by *C. jejuni* occurs at low to moderate levels, between 0.01 to 5% of the inoculum (72), and internalization occurs preferentially at the basolateral surface of epithelial cells, as was recently demonstrated with T84 cells (119). Invasion requires *de novo* synthesis of ~ 14 bacterial proteins upon contact with host cells, but not synthesis of host proteins (72). One of these proteins, a campylobacter invasion antigen B (Cia B) is translocated into INT407 cells during invasion. CiaB is a 73 KDa protein with sequence similarity to other secreted proteins such as YopB of *Yersinia*, SipB of *Salmonella,* and IpaB of *Shigella.* Like these type III secreted proteins, CiaB does not have a signal sequence at its amino terminus. Mutation in CiaB results in a 100-fold reduction in invasion and blocks secretion of at least eight proteins, suggesting that CiaB is not only secreted itself, but is also required for the secretion process. However, efforts to identify a Type III secretion system in *C. jejuni* have not been successful so far. Analysis of the complete *C. jejuni* chromosomal sequence (by homology) did not reveal any typical Type III secretion systems other than the flagellar apparatus (135). In addition, Type III secretion genes in other pathogenic organisms are usually organized as operons (93). Physical map

constructs of the coding regions adjacent to the *CiaB* gene have been shown to be monocistronic. Also, the G + C content of the *CiaB* gene is not dissimilar to the *C. jejuni* chromosome, suggesting that the gene is not on a pathogenicity island like other type III secretion genes identified in *Salmonella* and *Shigella* (93).

a) Factors affecting internalization

The factors that affect adherence also affect invasion. Mutations in *cadF* and *peb1* also result in reduced invasion of host cells. Motility is essential, since paralyzed but flagellated mutants (*pflA*) exhibit impaired invasion of INT407 cells (184). However, flagella have been shown to be required for invasion as well. Non-flagellated mutants of *C. jejuni* CF84-340 show a 3-fold decrease in invasion compared to the parental wild type (72). A study by Wassennaar *et al.*, showed that FlaA is required for invasion but not FlaB (177). Since Fla A is required for motility, and motility in turn is essential for invasion, the FlaA effect might be through motility. A recent study by Konkel et al (94) shows that flagella act as the export apparatus for the Cia proteins. They concluded that at least one of the flagellar filament proteins (Fla A or B) is required for Cia protein secretion. Taken together, these data suggest that motility is required for bacteria to contact host cells, while flagellar proteins are involved in adhesion (along with other factors) and in invasion through the secretion of the Cia proteins. In a recent study, campylobacter glycosylated surface proteins have shown to be an additional factor required for adherence and invasion. Inhibition of glycosylation by mutation in the *pglH* gene resulted in significant inhibition of adherence to and invasion of Caco2 cells, as well as chickens *in vivo* (80).

b) Other factors

1) Growth factors

Temperature seems critical for optimal invasion. *C. jejuni* adherence (and therefore invasion) is optimal at 37° C for human epithelial cells and suboptimal at 30 and 42° C. However, optimal temperatures might vary for cell lines of non-human origin. Also, mid-log phase bacteria invade more efficiently than early stationary phase bacteria (89). Growth of bacteria in the presence of mucin or bile salts (deoxycholate) also enhances invasion (30). In addition, iron depletion increases the invasiveness of *C. jejuni* and *C. coli in vitro* (158).

2) The Vir plasmid

A virulence plasmid identified in *C. jejuni* 81176 is thought to play a role in virulence (7). This plasmid of approximately 35 Kbp contains 4 open reading frames that are thought to encode components of a type IV secretion system. Mutation in 2 of these genes, *comB3* and *virB11,* causes three-fold and 11-fold reduction in *C. jejuni* invasion of INT407 cells, respectively, as well as a 3-fold and 6-fold reduction in adherence, respectively. A limited role in clinical significance was revealed by the identification of *virB11* in 6 of 58 clinical isolates.

3) Host factors

The host factors that are required for invasion are beginning to be explored in detail. It is known that invasion is cell-line dependent. Human cell lines are much more susceptible to invasion by *C. jejuni* or *C. coli* than are non-human cell lines. Also, different *C. jejuni* strains do not have similar invasion potentials for multiple cell lines. In

addition, immature semiconfluent cell layers are more susceptible to invasion than are confluent cell layers (72).

C. jejuni invasion is an energy-dependent process. Chemicals such as iodoacetate or dinitrophenol, which inhibit glycolysis and the Kreb's cycle respectively, also inhibit invasion (29). The optimal multiplicity of infection (MOI) of *C. jejuni* for infection of eukaryotic cells has been deduced, at least in one study. In this study, the number of bacteria that invaded INT407 cells increased with increasing MOI, and reached maximal numbers at around 200. However, the maximal invasion efficiency (percentage of inoculum that was internalized) was achieved at an MOI of 0.02, suggesting that *C. jejuni* can be efficient as a solitary invader (73). Since there is considerable laboratory variation in assays and cell lines, whether these results are universally applicable remains to be seen.

C. jejuni invasion is thought to be host-cell-cycle dependent, as peaks of invasion occur at 2- 3 hr and after 7 hr of initial contact with the host cells. *C. jejuni* has been localized near the junctional space of the host cells and inside vacuoles when internalized or intracellular (73). The average number of internalized bacteria within each cell is thought to be 2, however, as mentioned before, this might not be universally true. Bacteria within the host cells are well separated in space, suggesting that that the invasion event for each bacterium is independent of the other.

The host receptors that are required for invasion are not well understood. Inhibition of coated pits and caveolae also inhibit invasion, suggesting these host membrane regions are involved- but no specific receptors have been found (128, 182). Host microtubules (MT), microfilaments (MF), or both are needed for the invasion

process, and the requirement for specific cyto-skeletal structure is strain dependent. *C. jejuni* strain 81176 requires microtubules for internalization. Dyenin, which is associated with MT, might be required for motor activity to transport endosomes containing *C. jejuni* to perinuclear regions or across the cell (92). Other required host factors include signal transduction proteins such as tyrosine kinases, phosphatidylionositol-3-kinase (PI3-kinase) and calcium (19, 84).

Summary of invasion process

C. jejuni strains adhere to host cells through adhesins like CadF and PEB1. Strains that are adherent are not necessarily invasive, suggesting that adherence and invasion are independent processes. Host membrane caveolae and coated pits are involved in invasion, although their roles are unclear. Binding of *C. jejuni* to the host cell presumably causes aggregation of receptors, and *C. jejuni* secretes proteins, including CiaB, into the host cell with the help of the flagellar apparatus, triggering signal transduction events wherein tyrosine kinases phosphorylate host proteins. One of the proteins that are phosphorylated is PI3-kinase; this phosphorylation causes the release of calcium ions, leading to the recruitment of cytoskeletal structures such as microtubules, microfilaments or both, resulting in *C. jejuni* uptake into endosomes. For processes involving microtubules, a dyenin motor is thought to power the endosomal trafficking through the cell.

c) Intracellular survival

C. jejuni has been shown to survive inside HEp-2 cells and INT-407 cells for up to 9 hr and 48 hr, respectively (29, 89). Biopsy specimens from infected macaques and chickens have also been shown to contain *Campylobacter* (151). *C. jejuni* has been

shown to survive for up to 7 days in both human and mouse mononuclear phagocytes (85). Intracellular survival enables *Campylobacter* to replicate and to subsequently disseminate to other tissues. Two genes have been implicated in intracellular survival: the *sodB* gene and the *katA* gene. SodB, superoxide dismutase, catalyzes the conversion of superoxide to hydrogen peroxide and O_2, while KatA, catalase, breaks down hydrogen peroxide to water and O_2. These two genes have been shown to protect *C. jejuni* against the oxidative radicals inside the host cell (92).

A role of iron in intracellular survival of *C. jejuni* has been suggested. *Campylobacter* possesses an enterochelin uptake system, can bind exogeneous siderophores, and can synthesize bacterial ferritin. A ferritin-deficient mutant was shown to be more sensitive to hydrogen peroxide than the parental wild type and had retarded growth under iron deficient conditions, suggesting that ferritin plays a role in iron storage and in the oxidative stress response (176). Recently, a novel iron binding protein, Dps, that provides protection against hydrogen peroxide stress, has been identified. Dps is constitutively expressed and binds free ferrous iron to prevent generation of oxygen radicals. Mutation in this gene caused sensitivity to H_2O_2 *in vitro* (76). In another study, a novel 27-KDa oxidation sensitive protein was shown to degrade gradually in the presence of hydrogen peroxide, with a concomitant decrease in viability (183). This protein shows sequence similarity to non-heme iron proteins rubredoxin oxidoreductase and ruberythrin. Also, in a recent study, after incubation with eukaryotic cells, a *de novo* synthesized 80 KDa protein in *C. jejuni* was identified. This gene is analogous to the *fhuA* gene of *E. coli* and is thought to be involved in iron acquisition - since its expression is repressed in the

presence of iron (53). However, a role for these iron regulation systems in intracellular survival has not been demonstrated.

d) Translocation

C. jejuni exhibits multiple "invasion types" for translocation. Some strains translocate through transcellular pathways, i.e., through the host cell cytoplasm, presumably in endosomal vacuoles. Other strains translocate paracellularly, i.e., between the cell junctions, without disrupting the transepithelial resistance. Some strains exhibit both types of translocation processes (92). A role for translocation as a virulence factor and in pathogenesis has been suggested but has not been studied extensively. In one study 86% of clinical isolates from patients with colitis translocated across Caco-2 cell monolayers. On the other hand, only 48% of the clinical isolates from patients with non-inflammatory diarrhea exhibited a similar phenotype (39). Bacterial translocation through the host cell is also thought to be essential for dissemination to other tissues. Translocation through epithelial cells to underlying cell types like macrophages might spread the organism to extraintestinal locations such as the liver or gall bladder, causing hepatitis or cholecystitis respectively.

D) Immune responses

The role of the immune response in *C. jejuni* infections is known to some extent. Two studies have been published within the last year on innate cytokine responses to *C. jejuni* infection. However, most of the studies published so far have been limited to identification of serum antibodies. Three main classes of antibodies are usually induced in response to *Campylobacter* infection, including IgA, IgG, and IgM. The major surface

antigens that have been implicated in the induction of these serum antibodies include flagella, LPS, and PEB1. Antibodies provide partial immunity to *C. jejuni* infection, since application of anti-*C. jejuni* maternal antibodies been shown to delay colonization of newly hatched chickens (154).

C. jejuni has been shown to induce IL-8 secretion from human intestinal INT407 cells, and live bacteria are required for this process (65). *C. jejuni* has also been shown to induce IL-1α, IL-1β, TNF-α, IL-6, and IL-8 secretion from THP-1, a human monocytic cell line (79). Recently, INT407 cells were also shown to be immunopositive for intracellular IL-4, IL-10, IFN-γ and TNF-α in response to *C. jejuni* infection (2). In a mouse model, a pathogenic strain of *C. jejuni* has been shown to induce TNF-α but not IL-1β production in mouse peritoneal cells (133). On the other hand, patients with *C. jejuni* enteritis were shown to produce significant IL-1β in their stools along with significant luminal nitric oxide (38). Thus, specific cytokine responses induced by *C. jejuni* seems to be host species dependent.

Studies on the role of cell-mediated immunity in *C. jejuni* infections are limited. Lymphocytes are recruited along with polymorphonuclear leukocytes during infection, presumably due to the production of chemokines. Recently, clinical isolates of *C. jejuni* were shown to increase the production of γδT cells *in vitro* (174). However, *C. jejuni* has also been shown to induce apoptosis of chicken lymphocytes and of human Jurkat cell (T-cell) line *in vitro*. In addition, it has also been shown to cause apoptosis of a mouse macrophage cell line J774A.1, and a human monocyte cell line THP-1. In all these cases of apoptosis induction, the CiaB protein has been implicated, as CiaB mutants fail to

induce apoptosis of these cells. It has been postulated that apoptosis of immune cells is a potential immune evasion mechanism for *C. jejuni* (92).

II – *TRICHURIS SUIS*

The genus *Trichuris* (family *Trichuridae*) consists of eight species of whipworms whose hosts are distributed widely among the mammals. *T. trichuira* is a parasite of primates; *T. vulpis* is found in canids (dogs); *T. muris,* in rats; *T. ovis,* in sheep; *T. discolor,* in cattle; *T. felis,* in cats; and, *T. suis*, in swine.

A) Morphology

Trichuris suis is a whipworm helminth with a slender anterior end and a thicker posterior end. The parasites are dioecious, that is, the sexes are separate. Males are smaller than the females and have a distinct curved spicule on the posterior end. The adult worms are ~5 centimeters long. They are also cylindrical and bilaterally symmetrical. The outermost layer is called the cuticle and covers the entire body of the worm. Beneath the cuticle is the hypodermis or subcuticle, and in the anterior end, the hypodermis is comprised of columnar elongations called the bacillary band, which is purported to have glandular activity. The bacillary band protrudes into the cuticle to form pores. On either side of the bacillary bands are vesicular swellings of the cuticle called the cephalic glands. Beneath the hypodermis is the muscle layer that runs longitudinally. Underlying the muscle layer in the anterior section is a large cytoplasmic vacuolar body called the stichosome that surrounds the narrow esophagus. *Trichuris* spp have a complete alimentary canal with a mouth and an anus. The male reproductory organs are comprised of the testis, vas deferens, ejaculatory duct, spicule, and spicule muscle; and the females possess vagina, uterus, oviduct and ovary. The worms also possess a rudimentary nervous system with dorsal and ventral nerve cords that form part of the hypodermis. There is no circulatory or respiratory system. Circulation occurs through

movement of fluids within the pseudocoel, while respiration occurs through the cuticle. The life span of *T. suis* worms is around 4-5 months (77, 137).

B) Life cycle of *T. suis*

The life cycle of *T. suis* starts from eggs and culminates in adults. Sexual reproduction results with the females laying ~ 3000-5000 eggs per day. The eggs are unfertilized and unsegmented and contain two distinct nuclei. They are barrel shaped with two polar plugs and are covered by three thick outer layers and an inner thin vitelline membrane. The eggs exit the host in the feces and develop into an infective larval stage within the egg, in the soil. The two nuclei fuse and multiply resulting in an embryo that subsequently forms the infective larva. The process takes about three to fifteen weeks depending on temperature. The lower the temperature, the longer the development time. The optimal temperature for the development of the embryo is around 34° C, beyond which the eggs begin to degenerate. The infective larva within the egg is characterized by a poorly formed esophagus, an oral spear, and a rudimentary intestinal tract made of granulated material. Infective larvae have been shown to infect pigs even after storage at 5° C for over a year. In nature, the infective larvae can survive for 2–11 years and still retain infectivity. The infective larvae enter the host through the feeding habits of the pig, and proceed to the next stage in life cycle, the infective larva within the host (L1). This transition has been seen to occur only in the pig intestine and not in the open environment. Once inside the host, the infective larvae within the eggs hatch to release the L1 larvae, which may take between 9 hours to 3 days. Hatching takes place predominantly in the cecum and the proximal colon. The L1 larvae have a stylet at the

anterior end, esophagus, a cell body, digestive tract, and rectum at the posterior end. By means of the oral stylet, the L1 larva burrows into the crypt cells of the mucosa and penetrates epithelial cells and goblet cells. By day 7, the larve are embedded deep in the tissues, just under the mucosal epithelium. This marks the beginning of the histotrophic phase in the development of the larvae. This stage can be recovered from the pig intestine for around 10 days; on day 10 the L1 larva undergoes a second molt to form the L2 larva. This stage lasts for 3-6 days, during which body organs are formed, including rudimentary reproductive organs. The old cuticle is shed to expose the new cuticle underneath. Histotrophic migration out of the mucosa and into the lumen begins by day 13. By day 16, the L2 larva undergoes another molt to form the L3 stage. The length of the larvae is considerably longer, with further differentiation of the body organs. This stage lasts up to 4 days, at the end of which the cloacae can be seen in the males. Since the body length is considerable, the larvae are no longer contained beneath the epithelium; the entire posterior end protrudes into the lumen of the large intestine. By day 20, the larvae undergo one more molt to form the L4 stage. The reproductive organs are formed and differentiate into vagina, uterus, oviduct and ovary in the females and the corresponding organs in the males. By day 28, most of the body of the larva is in the lumen of the intestine with the anterior end inside a shallow tunnel covered by a thin layer of mucosal epithelial cells. On day 35, the larvae molt for the final time to form the adult stage. Complete reproductory organs are seen. The prepatency period of the females starts around day 41, and eggs are seen shortly after. The proportion of females in the swine gut is around 0.55-0.66 in natural infections (14, 16, 137). Recently, a phenol oxidase activity has been identified in female worms (42, 43). The activity causes

browning of the female worms when exposed to air. This activity was subsequently localized to the outer shell of the eggs and is activated when the eggs pass out of the host digestive tract into an aerobic environment. Since the eggs remain in pastures for a long time before being ingested, it is thought that this activity might help protect the eggs from the harsh environment (42, 43).

C) Excretory Secretory Products (ESP) of *T. suis*

Adult *T. suis* worms excrete/secrete a variety of products into their environment that are purported to play a vital role during their life cycle. Some of these products have been identified and characterized, and include the zinc metalloprotease, a *Trichuris suis* specific antigen, a thiol protease, a serine protease inhibitor, and a chymotrypsin/elastase inhibitor. A short description of these proteins and peptides follows. Since only the adult worms can be cultured *in vitro*, the types of molecules in the excretions/secretions from other stages of *T. suis* are not known.

i) Zinc metalloprotease

This 45 KDa protein degrades fibrinogen and elastin, two molecules that comprise the extracellular matrix, and is thought to play a role in the burrowing activities of the larva. The protease activity has a pH optimum of 7. 0, which is the same as the pH of the tissue spaces in the intestines. The protease is localized to the stichocytes in the anterior end and is thought to be secreted (67).

ii) *T. suis* specific antigen

This is a 20 KDa glycoprotein that is specific to *T. suis* infection in pigs and does not crossreact with sera from pigs infected with *Toxoplasma gondii*, *Ascaris suum*, or

Trichinella spiralis (68). However, it does crossreact with sera from dogs infected with another *Trichuris* species, *T. vulpis*. The glycoprotein reacts with sera obtained from pigs infected with *T. suis* for 28 days and not with sera obtained early in infection. This suggests that the glycoprotein is secreted/excreted from a larval stage of *T. suis*, most likely L4 (68).

iii) Thiol protease

A thiol protease activity has also been found in the culture fluids of *T. suis* adults (69). This activity has a pH range of 5.5 to 8.0 and was isolated from the gut extracts of adult worms suggesting a role in feeding, nutrient absorption, and digestion. This activity has not been further characterized (69).

iv) Serine protease inhibitor

This peptide of 6.6 KDa selectively inhibits serine proteases such as trypsin and chymotrypsin, but has no effect on thrombin or elastases. Since neutrophils and macrophages have been shown to release molecules with trypsin, and chymotrypsin-like activities, it has been suggested that this inhibitor might have a role in immunomodulation (148).

v) Chymotrypsin/elastase inhibitor

This activity is very similar to the serine protease inhibitor activity described above. It is a peptide of 6.4 KDa that selectively inhibits proteases such as elastase and chymotrypsin, but has no effect on thrombin or trypsin. This inhibitor shows a 48% sequence similarity to the trypsin/chymotrypsin inhibitor activity, and is also purported to play a similar role in immunomodulation (149).

vi) An antibacterial activity

An antibacterial activity of molecular weight < 10,000 has been identified (1). Four different bacterial species were sensitive to this activity, including *C. jejuni*, *C. coli*, *Escherichia coli* and *Staphylococcus aureus*. *C. jejuni* isolates are more susceptible to this activity than *C. coli* isolates. The activity is heat stable and resistant to trypsin. Also, preliminary data suggests that this activity might be bacteriocidal. This activity has not been further characterized (1).

In addition to the proteins and peptides from the ESP of *T. suis*, pore-forming proteins of 47 KDa and 43 KDa in the ESP of *T. trichiura* and *T. muris,* respectively, have also been identified. These proteins might have a significant role in the invasion of mammalian intestinal cells, as they induce ion-conducting pores in lipid bilayers *in vitro* (35).

D) Pathology

T. suis causes a clinical syndrome in pigs called trichuriasis, characterized by anemia, anorexia, pronounced weight loss, watery and a bloody discharge that changes to bloody mucoid diarrhea before death (13). Other clinical signs include cecitis, colitis, inflammation, and hemorrhagic areas in the colon. Disruption of the mucosal epithelium attributable to the parasite is seen, as are nodules in the cecum and colon. Other pathophysiological signs include reduced albumin levels and erythrocyte and eosinophil losses in the periphery. The symptoms begin 13-14 days post infection, coinciding with the emergence of histotrophic larvae. *Trichuris* spp. infections are widespread through the world. In the US and UK alone, is estimated that around 60–85% of the pigs in swine

farms may be infected with *T. suis* (13, 15). Economic losses to the swine industry are considerable. The increase in feed-to-gain ratio due to weight loss is estimated to result in losses of ~ $115 million annually (165). *T. suis* can infect humans as well. Experimental infections in man with a single dose of 1000 infective larvae produced patent infection (Beer RJ), and in another study, the eggs obtained subsequently from a human infection were shown to be infective (15). Apart from potential cross infection with *T. suis*, an estimated 1 billion people through the world are infected with the human *Trichuris* species *T. trichiura*, which also causes dysentery (15), (136, 137).

Multiple factors contribute to the development of patent *Trichuris* infections. Dose of infective larvae and parasite strains can be essential factors. In experimental infections, clinical manifestations in swine have been seen with various doses ranging from 25,000–40,000 infective larvae. Some infections do not produce clinical disease even with 15,000 larvae, while others with moderate doses (2500-3000 (113)) have resulted in disease. Strain differences might be an important factor, as might be the method of inoculation (single vs trickle) (137). Host age also plays a role in the establishment of infection. Weaned pigs are more susceptible to infection than sows (138). In an experimental infection, sows (4 years of age) had less worm fecundity and lower fecal egg excretion than weaned pigs (5 weeks of age). The worms were seen only in the cecum and proximal colon of sows, while they were evenly distributed throughout the colon in weaned pigs. The authors suggested that resistance to infection in sows might be due to acquired immunity and nutritional status. In a follow-up study, nutritional status indeed seemed important, as pigs fed a low protein diet had more *T. suis* worms than pigs fed normal protein diet (140). However, a similar result was not seen in

another study; one of the reasons could be host and /or parasite strain difference(s) (78). Another factor that affects pathophysiology is iron deficiency. In an experimental infection, 10-week-old pigs fed a low iron diet developed more severe infections than pigs fed a normal diet (139). The iron-deficient pigs also exhibited higher total fecal egg count and worm fecundity compared to pigs fed a regular diet.

One additional important factor that contributes to pathology is secondary bacterial infection. In his pioneering study on the life cycle of *T. suis*, Beer suggested that the burrowing activities of *T. suis* into the mucosa might enable secondary bacterial infections to occur, which in turn might enhance pathology (16, 152). In a study conducted in 1996, 8-week-old weaned pigs were given a single dose of 2500 embryonated eggs with or without antibiotic treatments. Only the pigs that did not receive antibiotics developed pathology similar to clinical trichuriasis. Liquid brown diarrhea developed ~ 14 days post infection, as seen in natural infections (113). Since pathology was seen with a moderate dose mimicking natural infective doses, it is likely that a bacterial component is involved in natural disease processes as well. In support of this view, many researchers have observed presence of spiral bacteria from pigs with colitis (17, 171). In addition, *C. jejuni* was isolated from the distal colon of pigs exhibiting pathology in the 1996 study described above. To confirm this hypothesis, a follow-up study was conducted, in which 3-day-old germ-free gnotobiotic piglets were infected either with 10^6 colony forming units of *C. jejuni*, or 3000 *T. suis* infective larvae, or with both. Only piglets that received both the pathogens developed clinical pathology with bloody diarrhea (111). Recently, a human clinical case in Canada provided further support to this dual pathogen hypothesis. A patient with *C. jejuni* infection developed

severe colitis with toxic megacolon and acute renal failure. In addition to *C. jejuni*, *T. suis* ova also were recovered from the patient's feces (160).

Studies on *T. suis* are limited; only a few studies have been conducted to date that explore the various factors affecting pathology and infection. However, with increasing awareness of the possible health hazards posed by *T. suis*, more studies should be forthcoming.

E) Treatment

A variety of anti-helminthic treatments have been studied for efficacy against *T. suis* infections. In earlier studies, two of the benzimidazole class of compounds, Flubendazole and Fenbendazole, had varying effects against *T. suis* infections. Flubendazole administered in the feed of pigs at 1.5 mg/kg for 5 days was 100% effective as measured by fecal egg counts (23). Fenbendazole given at 3 mg/kg for 11 days in the feed was 66% in one study (115) and 100% effective in another study (given the same dose for 3 days in the feed). Drug efficacy in these studies was determined by fecal egg counts and worm counts at necropsy (27). The ivermectin class of antihelminthics show varying degrees of effectiveness. In one study, ivermectin given orally at 300ug/kg was 91.2% effective against *T. suis*, while it was only 78.1% effective when given subcutaneously, as determined by necropsy worm counts (156). In another study, ivermectin was poorly effective, with only 53.9% of the *T. suis* being cleared after 7 days of ivermectin therapy (300 μg/kg, oral) (114). Doramectin, another anti-helminthic belonging to the same class as ivermectin, has also been tested. It too has varying effects. However, the lowest percentage efficacy obtained with doramectin was still higher than

that obtained with other anti-helminthics. In four studies, doramectin given at 300 μg/kg intramuscularly for 14–21days was found to be ~100% effective, while in another study it was found to be 87% effective against adult worms, when administered at the same dose for the same duration with a similar mode of injection (106, 118, 147, 153, 164). Thus, a number of treatment options are available for eradication of *T. suis* infections. However, the choice might depend on the geographical area, spectrum of activity, and the cost effectiveness of the drug.

F) Immune responses

Immune responses to nematode infections have been studied extensively over the last two decades. However, *T. suis* has not received much attention compared to its human and rodent counterparts *T. trichiura* and *T. muris*. Only two studies exist to date on immune responses to *T. suis*. In one study, pigs orally infected with moderate doses of infective larva (2500 eggs) showed a significant increase in IL-10 in the mesenteric lymph nodes compared to IL-12, which was undetectable (112). In another study, pigs given trickle infection (250 eggs) twice weekly for 4 weeks and then challenged with 4000 infective larvae displayed resistance to infection as determined by fecal egg counts, suggesting the presence of acquired immunity to *T. suis* (136). The paucity of information is largely due to the lack of reagents available to test multiple aspects of the pig immune response. Also, the expense of conducting swine experiments has proved prohibitive for most laboratories. Therefore, it is not surprising that most studies on *Trichuris* spp. have been limited to *T. muris* and *T. trichiura.* A review of immune responses to both these gastrointestinal helminthes will be presented here.

i) *T. trichiura*

Studies on populations highly endemic for *T. trichiura* show that antibody responses play a strong role in infections with this parasite. In one of the earlier studies, IgG and IgE antibody classes were elevated in the serum of patients with this parasite (104). Also, IgG1 and IgG2 subclasses predominated compared to other IgG subtypes. In another study, IgG4 and IgA were found to be additionally important, and there was also an age-dependent persistence of these antibody levels. Children had a preponderance of infection, and antibody levels correlated with the infection state. IgG4 and IgE levels persisted into adulthood, and then declined, albeit gradually (123). IgA is thought to be associated with acquired immunity, since secretory IgA in the saliva increases with age; and the higher the IgA levels, the lower the infection intensity (124). With regard to the cytokine response, the role of TNF-α was demonstrated in two studies, and IL-10 in one study, as indicated by their upregulation in serum of patients with *T. trichiura* (107, 123). The roles of other cytokines such as IL-4 in this intestinal disease are not known. However, the presence of IgE, IgG1, and IgG4 suggests that Th-2 responses occur as well.

ii) *T. muris*

Earlier studies indicated that host genetics might play a role in immunity to *T. muris* infections. Certain strains of mice were susceptible to *T. muris* infections, while others were resistant. The susceptible phenotype was characterized by a Th-1 profile with the production of IL-12, IL-18, IFN- γ, and IgG2a (12). The production of these cytokines was dependent on Toll Like Receptor 4 (TLR4) and MyD88 (an adaptor

molecule), as mice deficient in these molecules are highly resistant to *T. muris* infection and develop a strong Th-2 profile (64).

The resistance phenotype on the other hand is characterized by a polarized Th-2 response with the production of IL-3, IL-4, IL-5, IL-9, IL-10, and IL-13 cytokines and IgG1 and IgA antibodies (36, 37, 150). IgA seems more essential than IgG1for resistance because transfer of IgA to susceptible mice provides protection against subsequent infection, while IgG1 does not (150). Eosinophils and mast cells were shown not to be critical in resistance as well. Ablation of IL-5, which recruits eosinophils, and inhibition of c-kit, a stem cell factor receptor involved in mast cell activation, still resulted in the resistant phenotype (18). IL-4 activation requires the costimulatory molecule B7, since suppressing B7 suppresses IL-4 production (173). Also, in the absence of IL-4, IL-13 is critical. IL-4 knockout mice can still expel worms successfully, however, neutralization of IL-13 results in susceptibility (9). A role for TNF-α also has been suggested. Disruption of the TNF-α receptor results in generation of a Th-1 response and failure to expel *T. muris* worms. TNF-α was also shown to be required for the IL-13 mediated expulsion of worms in IL-4 knockout mice (4). Another important factor that affects the resistance phenotype is the transcription factor NF-κB1. Mice with a disruption of this gene are completely susceptible to infection and develop polarized Th-1 responses (5). The role of chemokines in resistance has been elucidated recently. Mice lacking CCL2, a CC chemokine ligand, do not mount resistance to *T. muris* (31). However, the role of other chemokines is not known. In addition to the role of immune factors, non-immune factors play a role. Low-level doses (< 40 eggs) induce a polarized Th-1 response, while higher doses (>200 eggs) induce a Th-2 profile in mice normally resistant to infection

(10, 11). When the doses are given as trickle infections, however, the doses eventually reach a high enough threshold to induce Th-2 responses and resistance.

Thus, Th-2 cytokines play an important role in resistance to *Trichuris* infections. Apart from *Trichuris*, Th-2 cytokines are required for resistance against a variety of other nematode infections. *Ascaris lumbricoides*, a human parasite, induces higher levels of IL-4 and IL-5 in humans harboring this helminth (26). Its swine counterpart, *A. suum*, also induces a distinct Th-2 profile including IL-2, IL-4, IL-5, and IL-10 in mice (108, 116). Th-2 responses, particularly, IL-4 and 1L-13, are required for successful elimination of *Trichinella spiralis*, *Heligosomoides polygyrus,* and *Nippostongylus brasiliensis* infections in mice (110). In addition, MCP-1 and MIP-2 chemokines play a role in the elimination of *T. spiralis* infection (50).

The role of individual Th-2 cytokines in the elimination of nematode infections has been established to an extent. IL-4 has been shown to increase epithelial cell permeability, and this in turn is thought to loosen the worms from the mucosa. IL-4 also stimulates peristalsis in the gut for rapid worm expulsion. Furthermore, IL-4 induces IgE and IgG1 class switching, and along with IL-13 and IL-9, increases mucosal mast cell production. Finally, it also increases the fluid accumulation in the intestinal lumen. IL-5 plays a role in eosinophilia, while IL-13 is thought to play a supportive role for IL-4 (110).

In recent years, a novel use for helminths is being investigated due to their ability to induce a strong Th-2 response. They have been shown to be effective in suppressing pathology associated with inflammatory bowel disease and Crohn's disease, which have a Th-1 etiology in humans (46, 166). Whether this immunosuppressive therapy finds a

wider role or achieves commercial success like that of probiotics remains to be seen. Also, since adverse effects of *T. suis* in both pigs and humans have been shown, this avenue needs to be traveled with caution.

III – RATIONALE FOR THIS STUDY

C. jejuni and *T. suis* are pathogenic organisms that can infect humans and swine. However, the immune responses to these organisms are poorly characterized. Th-2 immune responses like IL-4 and IL-10 have been shown to play a role in a number of helminth infections and IL-6 in a few bacterial infections (4, 9, 28). At the beginning of this study, very little was known about the Th-2 cytokine responses to *C. jejuni,* or to *T. suis* worms, or their ESP. While this is still true of *T. suis*, some advances have been made in recent years with regard to *C. jejuni* infections. However, cytokine responses are host species dependent, pathogen dependent, strain dependent and cell type dependent. In addition, while numerous studies have been conducted using human cell lines *in vitro* with multiple pathogens, studies on swine cells are few. Immune response studies on enteric pathogens using defined cell lines from the animal host, like swine intestinal cells, are even more rare. Hence, an attempt has been made in this study to elucidate the Th-2 cytokine responses from primary intestinal swine epithelial cells in response to both *T. suis* ESP and *C. jejuni*.

While the microbial factors for *C. jejuni* pathogenesis have been elucidated in some detail, the host factors that contribute to its pathogenesis are not very well understood. We have observed that *Trichuris* can dramatically affect the outcome of *C. jejuni* infections (111). However, other indirect or polymicrobial factors that can affect the host responses, and subsequently contribute to *C. jejuni* pathogenesis are not known. In addition, almost nothing is known about *T. suis*-mediated pathogenesis in swine. What is the immune response to *T. suis* infection? What is the consequence of this immune response? How does *T. suis* contribute to pathology? To provide some answers to these

questions, an existing swine model was used to study *C. jejuni*-and *T. suis*-mediated pathogenesis. In this model, pathology is seen in swine only when both organisms are present, and not during either of the single infections. Preliminary studies indicated that the disease might be due to *C. jejuni* and that *T. suis* contributes to this process. Therefore, this model provides an avenue to study the host factors that might be modulated during dual infection and that may contribute to *C. jejuni* (and *T. suis*) mediated pathology. Also, the cytokine immune responses to either infection can be measured using this model, providing some insights to both *C. jejuni* and *T. suis* infections *in vivo*. Thus, this dissertation should make a substantial contribution to understanding the mechanisms underlying these hitherto unknown phenomena.

Hypothesis

The primary hypothesis is that infection with *T. suis* induces a predominantly Th-2 type of cytokine response from the host, and infection with *C. jejuni* induces a predominantly Th-1 cytokine response, accompanied by a Th-2 component. Therefore, in dual infections, synergy in Th-2 components occurs, resulting in upregulation of certain cytokines like IL-4, IL-6 and IL-10 in the host, with one or more of these cytokines playing a role in generating severe disease and pathology. An additional hypothesis is that upregulation of Th-2 cytokines causes a down-regulation in Th-1 cytokine responses needed for resistance to *C. jejuni*.

Short-term goals- The short-term goals are to address the following questions.

1) Do *T. suis* excretory secretory products induce IL-4, IL-6 and IL-10 cytokine responses from the swine intestinal cells *in vitro*?

2) Does *C. jejuni* induce IL-4, IL-6 and IL-10 cytokine responses from the swine intestinal cells *in vitro*?

3) Can synergy occur *in vivo* in any of these cytokines (IL-4, IL-6 and IL-10) as determined from the *in vitro* epithelial cell culture system?

4) What is the Th-2 immune response to *T. suis* and *C. jejuni* infections *in vivo*?

5) What is (are) the role (s) of Th-2 cytokines upregulated during the *in vivo* infection in *C. jejuni* mediated invasion of swine epithelial cells?

In summary, the goal of this study is to determine the role(s) of IL-4, IL-6 and IL-10 in the colonic disease mediated by *C. jejuni* and *T. suis* in swine.

Chapter 2 summarizes studies on *in vitro* Th-2 cytokine responses from intestinal swine epithelial cells in response to two different strains of *C. jejuni* and *T. suis* ESP. Th-2 cytokines including IL-4 and IL-10, have been shown to be essential for resistance against helminth infections, while IL-6 is stimulated as part of the host defense against bacterial infections (28, 32, 44). Additionally, IL-6 has been shown to downregulate anti-leishmanial activity in human macrophages, while IL-4 and IL-10 inhibit Th-1 cytokine responses. However, far from being beneficial, an improper cytokine response can enhance severity of disease as evidenced by IL-6 mediated malignancies and IL-4 mediated pathologies (70, 98). Since our swine dual infection model has both a helminth component and a bacterial component, we decided to analyze the production of IL-4, IL-6, and IL-10 cytokines in response to these pathogens. These cytokine responses were measured in both differentiated and undifferentiated epithelial cells after challenge with *C. jejuni* and/ or *T. suis* ESP. The potential roles of these responses in the dual infection are discussed.

Chapter 3 summarizes studies on *in vivo* Th-2 cytokine responses (IL-4, IL-6, and IL-10) from 4-5 week-old pigs infected with *C. jejuni*, *T. suis* eggs, or both. Inoculations of pigs were carried out either concurrently (short-term experiments) or with inoculation of *T. suis* 21 days prior to infection with *C. jejuni*, followed by *C. jejuni* infection (long-term experiments). The potential roles of the cytokines induced at significant levels during dual infection are discussed.

Chapter 4 summarizes studies on the specific roles of IL-4 and IL-10 in *C. jejuni* invasion of IPEC-1 cells. Invasive strains of *C. jejuni* are considered more virulent than non-invasive strains. While the *C. jejuni*-secreted factors that contribute to invasion are well documented, the host soluble factors that contribute to its invasion are not well understood. In this chapter, evidence of a hitherto unknown role for IL-4 in *C. jejuni* pathogenesis is presented, and its role during dual infection of swine with *C. jejuni* and *T. suis* is discussed.

Chapter 5 provides a summary of all the prior chapters and discusses the possible mechanism of pathogenesis in natural infections with *C. jejuni* and *T. suis* in swine in view of these results.

REFERENCES

1. **Abner, S. R., G. Parthasarathy, D. E. Hill, and L. S. Mansfield.** 2001. *Trichuris suis*: detection of antibacterial activity in excretory-secretory products from adults. Exp Parasitol **99:**26-36.

2. **Al-Salloom, F. S., A. Al Mahmeed, A. Ismaeel, G. A. Botta, and M. Bakhiet.** 2003. Campylobacter-stimulated INT407 cells produce dissociated cytokine profiles. J Infect **47:**217-24.

3. **Arimi, S. M., R. W. Park, and C. R. Fricker.** 1990. Study of haemolytic activity of some *Campylobacter* spp. on blood agar plates. J Appl Bacteriol **69:**384-9.

4. **Artis, D., N. E. Humphreys, A. J. Bancroft, N. J. Rothwell, C. S. Potten, and R. K. Grencis.** 1999. Tumor necrosis factor alpha is a critical component of interleukin 13-mediated protective T helper cell type 2 responses during helminth infection. J Exp Med **190:**953-62.

5. **Artis, D., S. Shapira, N. Mason, K. M. Speirs, M. Goldschmidt, J. Caamano, H. C. Liou, C. A. Hunter, and P. Scott.** 2002. Differential requirement for NF-kappa B family members in control of helminth infection and intestinal inflammation. J Immunol **169:**4481-7.

6. **Aspinall, G. O., C. M. Lynch, H. Pang, R. T. Shaver, and A. P. Moran.** 1995. Chemical structures of the core region of *Campylobacter jejuni* O:3 lipopolysaccharide and an associated polysaccharide. Eur J Biochem **231:**570-8.

7. **Bacon, D. J., R. A. Alm, D. H. Burr, L. Hu, D. J. Kopecko, C. P. Ewing, T. J. Trust, and P. Guerry.** 2000. Involvement of a plasmid in virulence of *Campylobacter jejuni* 81-176. Infect Immun **68:**4384-90.

8. **Bacon, D. J., W. M. Johnson, and F. G. Rodgers.** 1999. Identification and characterisation of a cytotoxic porin-lipopolysaccharide complex from *Campylobacter jejuni*. J Med Microbiol **48:**139-48.

9. **Bancroft, A. J., D. Artis, D. D. Donaldson, J. P. Sypek, and R. K. Grencis.** 2000. Gastrointestinal nematode expulsion in IL-4 knockout mice is IL-13 dependent. Eur J Immunol **30:**2083-91.

10. **Bancroft, A. J., K. J. Else, and R. K. Grencis.** 1994. Low-level infection with *Trichuris muris* significantly affects the polarization of the CD4 response. Eur J Immunol **24:**3113-8.

11. **Bancroft, A. J., K. J. Else, N. E. Humphreys, and R. K. Grencis.** 2001. The effect of challenge and trickle *Trichuris muris* infections on the polarisation of the immune response. Int J Parasitol **31:**1627-37.

12. **Bancroft, A. J., K. J. Else, J. P. Sypek, and R. K. Grencis.** 1997. Interleukin-12 promotes a chronic intestinal nematode infection. Eur J Immunol **27:**866-70.

13. **Batte, E. G., R. D. McLamb, K. E. Muse, S. D. Tally, and T. J. Vestal.** 1977. Pathophysiology of swine trichuriasis. Am J Vet Res **38:**1075-9.

14. **Beer, R. J.** 1973. Morphological descriptions of the egg and larval stages of *Trichuris suis* Schrank, 1788. Parasitology **67:**263-78.

15. **Beer, R. J.** 1976. The relationship between *Trichuris trichiura* (Linnaeus 1758) of man and *Trichuris suis* (Schrank 1788) of the pig. Res Vet Sci **20:**47-54.

16. **Beer, R. J.** 1973. Studies on the biology of the life-cycle of *Trichuris suis* Schrank, 1788. Parasitology **67:**253-62.

17. **Beer, R. J., and J. M. Rutter.** 1972. Spirochaetal invasion of the colonic mucosa in a syndrome resembling swine dysentery following experimental *Trichuris suis* infection in weaned pigs. Res Vet Sci **13:**593-5.

18. **Betts, C. J., and K. J. Else.** 1999. Mast cells, eosinophils and antibody-mediated cellular cytotoxicity are not critical in resistance to *Trichuris muris*. Parasite Immunol **21:**45-52.

19. **Biswas, D., H. Niwa, and K. Itoh.** 2004. Infection with *Campylobacter jejuni* induces tyrosine-phosphorylated proteins into INT-407 cells. Microbiol Immunol **48:**221-8.

20. **Black, R. E., M. M. Levine, M. L. Clements, T. P. Hughes, and M. J. Blaser.** 1988. Experimental *Campylobacter jejuni* infection in humans. J Infect Dis **157:**472-9.

21. **Boer, P., J. A. Wagenaar, R. P. Achterberg, J. P. Putten, L. M. Schouls, and B. Duim.** 2002. Generation of *Campylobacter jejuni* genetic diversity *in vivo*. Mol Microbiol **44:**351-9.

22. **Bourke, B., P. Sherman, H. Louie, E. Hani, P. Islur, and V. L. Chan.** 1995. Physical and genetic map of the genome of *Campylobacter upsaliensis*. Microbiology **141 (Pt 10):**2417-24.

23. **Bradley, R. E., J. Guerrero, H. N. Becker, B. F. Michael, and K. Newcomb.** 1983. Flubendazole: dose range and efficacy studies against common internal parasites of swine. Am J Vet Res **44:**1329-33.

24. **Cardarelli-Leite, P., K. Blom, C. M. Patton, M. A. Nicholson, A. G. Steigerwalt, S. B. Hunter, D. J. Brenner, T. J. Barrett, and B. Swaminathan.** 1996. Rapid identification of *Campylobacter* species by restriction fragment length polymorphism analysis of a PCR-amplified fragment of the gene coding for 16S rRNA. J Clin Microbiol **34:**62-7.

25. **Chang, N., and D. E. Taylor.** 1990. Use of pulsed-field agarose gel electrophoresis to size genomes of *Campylobacter* species and to construct a SalI map of *Campylobacter jejuni* UA580. J Bacteriol **172:**5211-7.

26. **Cooper, P. J., M. E. Chico, C. Sandoval, I. Espinel, A. Guevara, M. W. Kennedy, J. F. Urban Jr, G. E. Griffin, and T. B. Nutman.** 2000. Human infection with *Ascaris lumbricoides* is associated with a polarized cytokine response. J Infect Dis **182:**1207-13.

27. **Corwin, R. M., S. E. Pratt, and R. K. Muser.** 1984. Evaluation of fenbendazole as an extended anthelmintic treatment regimen for swine. J Am Vet Med Assoc **185:**58-9.

28. **de Man, P., C. van Kooten, L. Aarden, I. Engberg, H. Linder, and C. Svanborg Eden.** 1989. Interleukin-6 induced at mucosal surfaces by gram-negative bacterial infection. Infect Immun **57:**3383-8.

29. **De Melo, M. A., G. Gabbiani, and J. C. Pechere.** 1989. Cellular events and intracellular survival of *Campylobacter jejuni* during infection of HEp-2 cells. Infect Immun **57:**2214-22.

30. **de Melo, M. A., and J. C. Pechere.** 1988. Effect of mucin on *Campylobacter jejuni* association and invasion on HEp-2 cells. Microb Pathog **5:**71-6.

31. **deSchoolmeester, M. L., M. C. Little, B. J. Rollins, and K. J. Else.** 2003. Absence of CC chemokine ligand 2 results in an altered Th1/Th2 cytokine balance and failure to expel *Trichuris muris* infection. J Immunol **170:**4693-700.

32. **Dimock, K. A., D. G. Addiss, M. L. Eberhard, and P. J. Lammie.** 1994. Differential proliferative and interleukin-10 responses to fractionated filarial antigens: preferential recognition by patients with chronic lymphatic dysfunction. J Infect Dis **170:**403-12.

33. **Dolg, P., R. Yao, D. H. Burr, P. Guerry, and T. J. Trust.** 1996. An environmentally regulated pilus-like appendage involved in *Campylobacter* pathogenesis. Mol Microbiol **20:**885-94.

34. **Dorrell, N., J. A. Mangan, K. G. Laing, J. Hinds, D. Linton, H. Al-Ghusein, B. G. Barrell, J. Parkhill, N. G. Stoker, A. V. Karlyshev, P. D. Butcher, and B. W. Wren.** 2001. Whole genome comparison of *Campylobacter jejuni* human

isolates using a low-cost microarray reveals extensive genetic diversity. Genome Res **11:**1706-15.

35. **Drake, L., Y. Korchev, L. Bashford, M. Djamgoz, D. Wakelin, F. Ashall, and D. Bundy.** 1994. The major secreted product of the whipworm, *Trichuris*, is a pore-forming protein. Proc R Soc Lond B Biol Sci **257:**255-61.

36. **Else, K. J., G. M. Entwistle, and R. K. Grencis.** 1993. Correlations between worm burden and markers of Th1 and Th2 cell subset induction in an inbred strain of mouse infected with *Trichuris muris*. Parasite Immunol **15:**595-600.

37. **Else, K. J., D. Wakelin, D. L. Wassom, and K. M. Hauda.** 1990. The influence of genes mapping within the major histocompatibility complex on resistance to *Trichuris muris* infections in mice. Parasitology **101 Pt 1:**61-7.

38. **Enocksson, A., J. Lundberg, E. Weitzberg, A. Norrby-Teglund, and B. Svenungsson.** 2004. Rectal nitric oxide gas and stool cytokine levels during the course of infectious gastroenteritis. Clin Diagn Lab Immunol **11:**250-4.

39. **Everest, P. H., H. Goossens, J. P. Butzler, D. Lloyd, S. Knutton, J. M. Ketley, and P. H. Williams.** 1992. Differentiated Caco-2 cells as a model for enteric invasion by *Campylobacter jejuni* and *C. coli*. J Med Microbiol **37:**319-25.

40. **Eyigor, A., K. A. Dawson, B. E. Langlois, and C. L. Pickett.** 1999. Cytolethal distending toxin genes in *Campylobacter jejuni* and *Campylobacter coli* isolates: detection and analysis by PCR. J Clin Microbiol **37:**1646-50.

41. **Eyigor, A., K. A. Dawson, B. E. Langlois, and C. L. Pickett.** 1999. Detection of cytolethal distending toxin activity and cdt genes in *Campylobacter* spp. isolated from chicken carcasses. Appl Environ Microbiol **65:**1501-5.

42. **Fetterer, R. H., and D. E. Hill.** 1994. Localization of phenol oxidase in female *Trichuris suis*. J Parasitol **80:**952-9.

43. **Fetterer, R. H., and D. E. Hill.** 1993. The occurrence of phenol oxidase activity in female *Trichuris suis*. J Parasitol **79:**155-9.

44. **Finkelman, F. D., E. J. Pearce, J. F. Urban, Jr., and A. Sher.** 1991. Regulation and biological function of helminth-induced cytokine responses. Immunol Today **12:**A62-6.

45. **Fitzgerald, C., R. J. Owen, and J. Stanley.** 1996. Comprehensive ribotyping scheme for heat-stable serotypes of *Campylobacter jejuni*. J Clin Microbiol **34:**265-9.

46. **Fox, J. G., P. Beck, C. A. Dangler, M. T. Whary, T. C. Wang, H. N. Shi, and C. Nagler-Anderson.** 2000. Concurrent enteric helminth infection modulates inflammation and gastric immune responses and reduces helicobacter-induced gastric atrophy. Nat Med **6:**536-42.

47. **Fox, J. G., A. B. Rogers, M. T. Whary, Z. Ge, N. S. Taylor, S. Xu, B. H. Horwitz, and S. E. Erdman.** 2004. Gastroenteritis in NF-kappaB-deficient mice is produced with wild-type *Camplyobacter jejuni* but not with *C. jejuni* lacking cytolethal distending toxin despite persistent colonization with both strains. Infect Immun **72:**1116-25.

48. **Friedman, C. R., Neimann, J., Wegener, H.C., and R.V. Tauxe.** 2000. Epidemiology of *Campylobacter jejuni* infections in the United States and other industrialized nations, p. 121-138. *In* I. Nachamkin, and Blaser, M.J (ed.), Campylobacter, Second ed. American Society for Microbiology, Washington, DC.

49. **Fry, B., Oldfield, NJ, Korolik, V, Coloe, PJ and JM Ketley.** 2000. Genetics of *Campylobacter* lipopolysaccharide biosyntheis, p. 381-404. *In* I. Nachamkin, and Blaser, M.J (ed.), Campylobacter, Second ed. ASM Press, Washington DC.

50. **Frydas, S., T. Rallis, I. Theodorides, M. N. Patsikas, C. Trakatellis, M. Di Gioacchino, and M. Felaco.** 2000. *Trichinella spiralis* infection is mediated by MCP-1 and MIP-2, while echinococcus granulosus is strongly mediated by MCP-1, but not MIP-2. Int J Immunopathol Pharmacol **13:**21-26.

51. **Fuglsang, A.** 2003. The genome of *Campylobacter jejuni*: codon and amino acid usage. Apmis **111:**605-18.

52. **Fujita, M., T. Morooka, S. Fujimoto, T. Moriya, and K. Amako.** 1995. Southern blotting analyses of strains of *Campylobacter fetus* using the conserved region of sapA. Arch Microbiol **164:**444-7.

53. **Galindo, M. A., W. A. Day, B. H. Raphael, and L. A. Joens.** 2001. Cloning and characterization of a *Campylobacter jejuni* iron-uptake operon. Curr Microbiol **42:**139-43.

54. **Garrity, G. M., Bell, J.A., and T.G. Lilburn.** 2003. Taxonomic outline of the procaryotes, Second ed. Springer-Verlag, New York.

55. **Gaynor, E. C., N. Ghori, and S. Falkow.** 2001. Bile-induced 'pili' in *Campylobacter jejuni* are bacteria-independent artifacts of the culture medium. Mol Microbiol **39:**1546-9.

56. **Gilbert, M., M. F. Karwaski, S. Bernatchez, N. M. Young, E. Taboada, J. Michniewicz, A. M. Cunningham, and W. W. Wakarchuk.** 2002. The genetic

bases for the variation in the lipo-oligosaccharide of the mucosal pathogen, *Campylobacter jejuni*. Biosynthesis of sialylated ganglioside mimics in the core oligosaccharide. J Biol Chem **277:**327-37.

57. **Grant, K. A., I. U. Belandia, N. Dekker, P. T. Richardson, and S. F. Park.** 1997. Molecular characterization of *pldA*, the structural gene for a phospholipase A from *Campylobacter coli*, and its contribution to cell-associated hemolysis. Infect Immun **65:**1172-80.

58. **Guerrant, R. L., C. A. Wanke, R. A. Pennie, L. J. Barrett, A. A. Lima, and A. D. O'Brien.** 1987. Production of a unique cytotoxin by *Campylobacter jejuni*. Infect Immun **55:**2526-30.

59. **Guerry, P., R. A. Alm, M. E. Power, S. M. Logan, and T. J. Trust.** 1991. Role of two flagellin genes in Campylobacter motility. J Bacteriol **173:**4757-64.

60. **Guerry, P., C. P. Ewing, T. E. Hickey, M. M. Prendergast, and A. P. Moran.** 2000. Sialylation of lipooligosaccharide cores affects immunogenicity and serum resistance of *Campylobacter jejuni*. Infect Immun **68:**6656-62.

61. **Guerry, P., C. M. Szymanski, M. M. Prendergast, T. E. Hickey, C. P. Ewing, D. L. Pattarini, and A. P. Moran.** 2002. Phase variation of *Campylobacter jejuni* 81-176 lipooligosaccharide affects ganglioside mimicry and invasiveness in vitro. Infect Immun **70:**787-93.

62. **Hassane, D. C., R. B. Lee, and C. L. Pickett.** 2003. *Campylobacter jejuni* cytolethal distending toxin promotes DNA repair responses in normal human cells. Infect Immun **71:**541-5.

63. **Healing, T. D.** 1992. The surveillance of communicable disease in the European Community. Commun Dis Rep CDR Rev **2:**R73-7.

64. **Helmby, H., and R. K. Grencis.** 2003. Essential role for TLR4 and MyD88 in the development of chronic intestinal nematode infection. Eur J Immunol **33:**2974-9.

65. **Hickey, T. E., S. Baqar, A. L. Bourgeois, C. P. Ewing, and P. Guerry.** 1999. *Campylobacter jejuni*-stimulated secretion of interleukin-8 by INT407 cells. Infect Immun **67:**88-93.

66. **Hickey, T. E., A. L. McVeigh, D. A. Scott, R. E. Michielutti, A. Bixby, S. A. Carroll, A. L. Bourgeois, and P. Guerry.** 2000. *Campylobacter jejuni* cytolethal distending toxin mediates release of interleukin-8 from intestinal epithelial cells. Infect Immun **68:**6535-41.

67. **Hill, D. E., H. R. Gamble, M. L. Rhoads, R. H. Fetterer, and J. F. Urban, Jr.** 1993. *Trichuris suis*: a zinc metalloprotease from culture fluids of adult parasites. Exp Parasitol **77:**170-8.

68. **Hill, D. E., R. D. Romanowski, and J. F. Urban, Jr.** 1997. A *Trichuris* specific diagnostic antigen from culture fluids of *Trichuris suis* adult worms. Vet Parasitol **68:**91-102.

69. **Hill, D. E., and J. A. Sakanari.** 1997. *Trichuris suis*: thiol protease activity from adult worms. Exp Parasitol **85:**55-62.

70. **Hirano, T., and T. Kishimoto.** 1989. Interleukin-6: possible implications in human diseases. Ric Clin Lab **19:**1-10.

71. **Hodinka, R. L., and P. H. Gilligan.** 1988. Evaluation of the campyslide agglutination test for confirmatory identification of selected *Campylobacter* species. J Clin Microbiol **26:**47-9.

72. **Hu, L., and Kopecko, D.J.** 2000. Interactions of *Campylobacter* with eukaryotic cells: Gut luminal colonization and mucosal invasion, p. 191-216. *In* I. Nachamkin, and Blaser, M.J (ed.), Campylobacter, Second ed. American Society for Microbiology, Washington, DC.

73. **Hu, L., and D. J. Kopecko.** 1999. *Campylobacter jejuni* 81-176 associates with microtubules and dynein during invasion of human intestinal cells. Infect Immun **67:**4171-82.

74. **Hurtado, A., and R. J. Owen.** 1997. A molecular scheme based on 23S rRNA gene polymorphisms for rapid identification of *Campylobacter* and *Arcobacter* species. J Clin Microbiol **35:**2401-4.

75. **Hutchinson, D. N., and F. J. Bolton.** 1984. Improved blood free selective medium for the isolation of *Campylobacter jejuni* from faecal specimens. J Clin Pathol **37:**956-7.

76. **Ishikawa, T., Y. Mizunoe, S. Kawabata, A. Takade, M. Harada, S. N. Wai, and S. Yoshida.** 2003. The iron-binding protein Dps confers hydrogen peroxide stress resistance to *Campylobacter jejuni*. J Bacteriol **185:**1010-7.

77. **Jenkins, T.** 1970. A morphological and histochemical study of *Trichuris suis* (Schrank, 1788) with special reference to the host-parasite relationship. Parasitology **61:**357-74.

78. **Johansen, M. V., H. O. Bogh, H. Giver, L. Eriksen, P. Nansen, L. Stephenson, and K. E. Knudsen.** 1997. *Schistosoma japonicum* and *Trichuris*

suis infections in pigs fed diets with high and low protein. Parasitology **115 (Pt 3):**257-64.

79. **Jones, M. A., S. Totemeyer, D. J. Maskell, C. E. Bryant, and P. A. Barrow.** 2003. Induction of proinflammatory responses in the human monocytic cell line THP-1 by Campylobacter jejuni. Infect Immun **71:**2626-33.

80. **Karlyshev, A. V., P. Everest, D. Linton, S. Cawthraw, D. G. Newell, and B. W. Wren.** 2004. The *Campylobacter jejuni* general glycosylation system is important for attachment to human epithelial cells and in the colonization of chicks. Microbiology **150:**1957-64.

81. **Karmali, M. A., A. E. Simor, M. Roscoe, P. C. Fleming, S. S. Smith, and J. Lane.** 1986. Evaluation of a blood-free, charcoal-based, selective medium for the isolation of *Campylobacter* organisms from feces. J Clin Microbiol **23:**456-9.

82. **Kerstens, P. J., H. P. Endtz, J. F. Meis, W. J. Oyen, R. J. Koopman, P. J. van den Broek, and J. W. van der Meer.** 1992. Erysipelas-like skin lesions associated with *Campylobacter jejuni* septicemia in patients with hypogammaglobulinemia. Eur J Clin Microbiol Infect Dis **11:**842-7.

83. **Kervella, M., J. M. Pages, Z. Pei, G. Grollier, M. J. Blaser, and J. L. Fauchere.** 1993. Isolation and characterization of two *Campylobacter* glycine-extracted proteins that bind to HeLa cell membranes. Infect Immun **61:**3440-8.

84. **Ketley, J. M.** 1997. Pathogenesis of enteric infection by *Campylobacter.* Microbiology **143 (Pt 1):**5-21.

85. **Kiehlbauch, J. A., R. A. Albach, L. L. Baum, and K. P. Chang.** 1985. Phagocytosis of *Campylobacter jejuni* and its intracellular survival in mononuclear phagocytes. Infect Immun **48:**446-51.

86. **Kita, E., F. Nishikawa, N. Kamikaidou, A. Nakano, N. Katsui, and S. Kashiba.** 1992. Mononuclear cell response in the liver of mice infected with hepatotoxigenic *Campylobacter jejuni.* J Med Microbiol **37:**326-31.

87. **Kita, E., D. Oku, A. Hamuro, F. Nishikawa, M. Emoto, Y. Yagyu, N. Katsui, and S. Kashiba.** 1990. Hepatotoxic activity of *Campylobacter jejuni.* J Med Microbiol **33:**171-82.

88. **Klena, J. D., S. A. Gray, and M. E. Konkel.** 1998. Cloning, sequencing, and characterization of the lipopolysaccharide biosynthetic enzyme heptosyltransferase I gene (waaC) from *Campylobacter jejuni* and *Campylobacter coli.* Gene **222:**177-85.

89. **Konkel, M. E., M. D. Corwin, L. A. Joens, and W. Cieplak.** 1992. Factors that influence the interaction of *Campylobacter jejuni* with cultured mammalian cells. J Med Microbiol **37:**30-7.

90. **Konkel, M. E., S. G. Garvis, S. L. Tipton, D. E. Anderson, Jr., and W. Cieplak, Jr.** 1997. Identification and molecular cloning of a gene encoding a fibronectin-binding protein (CadF) from *Campylobacter jejuni*. Mol Microbiol **24:**953-63.

91. **Konkel, M. E., and L. A. Joens.** 1989. Adhesion to and invasion of HEp-2 cells by *Campylobacter* spp. Infect Immun **57:**2984-90.

92. **Konkel, M. E., Joens, L.A., and Mixter, P.F.** 2000. Molecular characterization of *Campylobacter jejuni* virulence determinants, p. 217-240. *In* I. Nachamkin, and Blaser, M.J (ed.), Campylobacter, Second ed. American Society for Microbiology, Washington, DC.

93. **Konkel, M. E., B. J. Kim, V. Rivera-Amill, and S. G. Garvis.** 1999. Bacterial secreted proteins are required for the internaliztion of *Campylobacter jejuni* into cultured mammalian cells. Mol Microbiol **32:**691-701.

94. **Konkel, M. E., J. D. Klena, V. Rivera-Amill, M. R. Monteville, D. Biswas, B. Raphael, and J. Mickelson.** 2004. Secretion of virulence proteins from *Campylobacter jejuni* is dependent on a functional flagellar export apparatus. J Bacteriol **186:**3296-303.

95. **Korolik, V., B. N. Fry, M. R. Alderton, B. A. van der Zeijst, and P. J. Coloe.** 1997. Expression of *Campylobacter hyoilei* lipo-oligosaccharide (LOS) antigens in *Escherichia coli*. Microbiology **143 (Pt 11):**3481-9.

96. **Korolik, V., L. Moorthy, and P. J. Coloe.** 1995. Differentiation of *Campylobacter jejuni* and *Campylobacter coli* strains by using restriction endonuclease DNA profiles and DNA fragment polymorphisms. J Clin Microbiol **33:**1136-40.

97. **Lara-Tejero, M., and J. E. Galan.** 2001. CdtA, CdtB, and CdtC form a tripartite complex that is required for cytolethal distending toxin activity. Infect Immun **69:**4358-65.

98. **Lawrence, C. E., J. C. Paterson, L. M. Higgins, T. T. MacDonald, M. W. Kennedy, and P. Garside.** 1998. IL-4-regulated enteropathy in an intestinal nematode infection. Eur J Immunol **28:**2672-84.

99. **Lee, A., S. C. Smith, and P. J. Coloe.** 2000. Detection of a novel campylobacter cytotoxin. J Appl Microbiol **89:**719-25.

100. **Lee, R. B., D. C. Hassane, D. L. Cottle, and C. L. Pickett.** 2003. Interactions of *Campylobacter jejuni* cytolethal distending toxin subunits CdtA and CdtC with HeLa cells. Infect Immun **71**:4883-90.

101. **Leonard, E. E., 2nd, T. Takata, M. J. Blaser, S. Falkow, L. S. Tompkins, and E. C. Gaynor.** 2003. Use of an open-reading frame-specific *Campylobacter jejuni* DNA microarray as a new genotyping tool for studying epidemiologically related isolates. J Infect Dis **187**:691-4.

102. **Lesse, A. J., A. A. Campagnari, W. E. Bittner, and M. A. Apicella.** 1990. Increased resolution of lipopolysaccharides and lipooligosaccharides utilizing tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis. J Immunol Methods **126**:109-17.

103. **Li, C. C., C. H. Chiu, J. L. Wu, Y. C. Huang, and T. Y. Lin.** 1998. Antimicrobial susceptibilities of *Campylobacter jejuni* and *coli* by using E-test in Taiwan. Scand J Infect Dis **30**:39-42.

104. **Lillywhite, J. E., D. A. Bundy, J. M. Didier, E. S. Cooper, and A. E. Bianco.** 1991. Humoral immune responses in human infection with the whipworm *Trichuris trichiura*. Parasite Immunol **13**:491-507.

105. **Linton, D., M. Gilbert, P. G. Hitchen, A. Dell, H. R. Morris, W. W. Wakarchuk, N. A. Gregson, and B. W. Wren.** 2000. Phase variation of a beta-1,3 galactosyltransferase involved in generation of the ganglioside GM1-like lipo-oligosaccharide of *Campylobacter jejuni*. Mol Microbiol **37**:501-14.

106. **Logan, N. B., A. J. Weatherley, and R. M. Jones.** 1996. Activity of doramectin against nematode and arthropod parasites of swine. Vet Parasitol **66**:87-94.

107. **MacDonald, T. T., J. Spencer, S. H. Murch, M. Y. Choy, S. Venugopal, D. A. Bundy, and E. S. Cooper.** 1994. Immunoepidemiology of intestinal helminthic infections. 3. Mucosal macrophages and cytokine production in the colon of children with *Trichuris trichiura* dysentery. Trans R Soc Trop Med Hyg **88**:265-8.

108. **Macedo, M. S., E. Faquim-Mauro, A. P. Ferreira, and I. A. Abrahamsohn.** 1998. Immunomodulation induced by *Ascaris suum* extract in mice: effect of anti-interleukin-4 and anti-interleukin-10 antibodies. Scand J Immunol **47**:10-8.

109. **Madden, R. H., L. Moran, and P. Scates.** 1996. Sub-typing of animal and human *Campylobacter* spp. using RAPD. Lett Appl Microbiol **23**:167-70.

110. **Mansfield, L. S., Gause, W.C.,Finkelman, F.D., and J.F. Urban, Jr.** 2000. Gastrointestinal nematodes and the immune system, p. 555-567. *In* M. W.

Cunningham, and Fujinami, R.S. (ed.), Effects of microbes on the immune system. Lippincott, Williams and Wilkins, Philadelphia.

111. **Mansfield, L. S., D. T. Gauthier, S. R. Abner, K. M. Jones, S. R. Wilder, and J. F. Urban.** 2003. Enhancement of disease and pathology by synergy of *Trichuris suis* and *Campylobacter jejuni* in the colon of immunologically naive swine. Am J Trop Med Hyg **68:**70-80.

112. **Mansfield, L. S., J. F. Urban, R. R. Holley-Shanks, M. P. Murtaugh, D. S. Zarlenga, D. Foss, A. Canals, W. Gause, and J. K. Lunney.** 1998. Construction of internal cDNA competitors for measuring IL-10 and IL-12 cytokine gene expression in swine. Vet Immunol Immunopathol **65:**63-74.

113. **Mansfield, L. S., and J. F. Urban, Jr.** 1996. The pathogenesis of necrotic proliferative colitis in swine is linked to whipworm induced suppression of mucosal immunity to resident bacteria. Vet Immunol Immunopathol **50:**1-17.

114. **Marchiondo, A. A., and J. Szanto.** 1987. Efficacy of dichlorvos, fenbendazole, and ivermectin in swine with induced intestinal nematode infections. Am J Vet Res **48:**1233-5.

115. **Marti, O. G., T. B. Stewart, and O. M. Hale.** 1978. Comparative efficacy of fenbendazole, dichlorvos, and levamisole HCI against gastrointestinal nematodes of pigs. J Parasitol **64:**1028-31.

116. **Matsumoto, N., S. Katoh, H. Mukae, T. Matsuo, K. Takatsu, and S. Matsukura.** 2003. Critical role of IL-5 in antigen-induced pulmonary eosinophilia, but not in lymphocyte activation. Int Arch Allergy Immunol **130:**209-15.

117. **McSweegan, E., and R. I. Walker.** 1986. Identification and characterization of two *Campylobacter jejuni* adhesins for cellular and mucous substrates. Infect Immun **53:**141-8.

118. **Mehlhorn, H., H. L. Jones, A. J. Weatherley, and B. Schumacher.** 1993. Doramectin, a new avermectin highly efficacious against gastrointestinal nematodes and lungworms of cattle and pigs: two studies carried out under field conditions in Germany. Parasitol Res **79:**603-7.

119. **Monteville, M. R., and M. E. Konkel.** 2002. Fibronectin-facilitated invasion of T84 eukaryotic cells by *Campylobacter jejuni* occurs preferentially at the basolateral cell surface. Infect Immun **70:**6665-71.

120. **Morooka, T., A. Umeda, and K. Amako.** 1985. Motility as an intestinal colonization factor for *Campylobacter jejuni*. J Gen Microbiol **131 (Pt 8):**1973-80.

121. **Nachamkin, I., K. Bohachick, and C. M. Patton.** 1993. Flagellin gene typing of *Campylobacter jejuni* by restriction fragment length polymorphism analysis. J Clin Microbiol **31:**1531-6.

122. **Nachamkin, I., Engberg, J., and Aarestrup, FM.** 2000. Diagnosis and antimicrobial susceptibility of *Campylobacter* species, p. 45-66. *In* I. a. B. Nachamkin, MJ (ed.), Campylobacter, Second ed. American Society for Microbiology, Washington, DC.

123. **Needham, C. S., D. A. Bundy, J. E. Lillywhite, J. M. Didier, I. Simmons, and A. E. Bianco.** 1992. The relationship between *Trichuris trichiura* transmission intensity and the age-profiles of parasite-specific antibody isotypes in two endemic communities. Parasitology **105 (Pt 2):**273-83.

124. **Needham, C. S., J. E. Lillywhite, J. M. Didier, A. T. Bianco, and D. A. Bundy.** 1994. Comparison of age-dependent antigen recognition in two communities with high and low *Trichuris trichiura* transmission. Acta Trop **58:**87-98.

125. **Newell, D. G., Frost, JA., Duim, B., Wagenaar, J.A., Madden, R.H., van der Plas, J., and On, S.L.W.** 2000. New Developments in the subtyping of Campylobacter species, p. 27-44. *In* I. Nachamkin, and Blaser, M.J (ed.), Campylobacter, Second ed. American Society for Microbiology, Washington, DC.

126. **Newell, D. G., H. McBride, F. Saunders, Y. Dehele, and A. D. Pearson.** 1985. The virulence of clinical and environmental isolates of *Campylobacter jejuni*. J Hyg (Lond) **94:**45-54.

127. **Oberhelman, R. A., and Taylor, D.N.** 2000. Campylobacter infections in the developing countries, p. 139-154. *In* I. Nachamkin, and Blaser, M.J (ed.), Campylobacter, Second ed. American Society for Microbiology, Washington, DC.

128. **Oelschlaeger, T. A., P. Guerry, and D. J. Kopecko.** 1993. Unusual microtubule-dependent endocytosis mechanisms triggered by *Campylobacter jejuni* and *Citrobacter freundii*. Proc Natl Acad Sci U S A **90:**6884-8.

129. **Okuda, J., M. Fukumoto, Y. Takeda, and M. Nishibuchi.** 1997. Examination of diarrheagenicity of cytolethal distending toxin: suckling mouse response to the products of the cdtABC genes of *Shigella dysenteriae*. Infect Immun **65:**428-33.

130. **On, S. L.** 1996. Identification methods for campylobacters, helicobacters, and related organisms. Clin Microbiol Rev **9:**405-22.

131. **On, S. L. W. a. B. H.** 1995. Classification and identification of campylobacters, helicobacters and allied taxa by numerical analysis of phenotypic characters. Syst. Appl. Microbiol. **18:**374-390.

132. **Owen, R. J., K. Sutherland, C. Fitzgerald, J. Gibson, P. Borman, and J. Stanley.** 1995. Molecular subtyping scheme for serotypes HS1 and HS4 of *Campylobacter jejuni*. J Clin Microbiol **33:**872-7.

133. **Pancorbo, P. L., M. A. de Pablo, E. Ortega, A. M. Gallego, C. Alvarez, and G. Alvarez de Cienfuegos.** 1999. Evaluation of cytokine production and phagocytic activity in mice infected with *Campylobacter jejuni*. Curr Microbiol **39:**129-33.

134. **Park, S. F., D. Purdy, and S. Leach.** 2000. Localized reversible frameshift mutation in the *flhA* gene confers phase variability to flagellin gene expression in *Campylobacter coli*. J Bacteriol **182:**207-10.

135. **Parkhill, J., B. W. Wren, K. Mungall, J. M. Ketley, C. Churcher, D. Basham, T. Chillingworth, R. M. Davies, T. Feltwell, S. Holroyd, K. Jagels, A. V. Karlyshev, S. Moule, M. J. Pallen, C. W. Penn, M. A. Quail, M. A. Rajandream, K. M. Rutherford, A. H. van Vliet, S. Whitehead, and B. G. Barrell.** 2000. The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. Nature **403:**665-8.

136. **Pedersen, S., and I. Saeed.** 2001. Acquired immunity to *Trichuris suis* infection in pigs. Parasitology **123:**95-101.

137. **Pedersen, S., and I. Saeed.** 2000. Experimental infection of pigs with three dose levels of *Trichuris suis*. Parasite **7:**275-81.

138. **Pedersen, S., and I. Saeed.** 2002. Host age influence on the intensity of experimental *Trichuris suis* infection in pigs. Parasite **9:**75-9.

139. **Pedersen, S., I. Saeed, H. Friis, and K. F. Michaelsen.** 2001. Effect of iron deficiency on *Trichuris suis* and *Ascaris suum* infections in pigs. Parasitology **122:**589-98.

140. **Pedersen, S., I. Saeed, K. F. Michaelsen, H. Friis, and K. D. Murrell.** 2002. Impact of protein energy malnutrition on *Trichuris suis* infection in pigs concomitantly infected with *Ascaris suum*. Parasitology **124:**561-8.

141. **Pei, Z., and M. J. Blaser.** 1993. PEB1, the major cell-binding factor of *Campylobacter jejuni*, is a homolog of the binding component in gram-negative nutrient transport systems. J Biol Chem **268:**18717-25.

142. **Pei, Z., C. Burucoa, B. Grignon, S. Baqar, X. Z. Huang, D. J. Kopecko, A. L. Bourgeois, J. L. Fauchere, and M. J. Blaser.** 1998. Mutation in the *pebIA* locus of *Campylobacter jejuni* reduces interactions with epithelial cells and intestinal colonization of mice. Infect Immun **66:**938-43.

143. **Pei, Z. H., R. T. Ellison, 3rd, and M. J. Blaser.** 1991. Identification, purification, and characterization of major antigenic proteins of *Campylobacter jejuni*. J Biol Chem **266:**16363-9.

144. **Pickett, C. L.** 2000. Campylobacter toxins and their role in pathogenesis, p. 179-190. *In* I. Nachamkin, and Blaser, M.J (ed.), Campylobacter, Second ed. American Society for Microbiology, Washington, DC.

145. **Poly, F., D. Threadgill, and A. Stintzi.** 2004. Identification of *Campylobacter jejuni* ATCC 43431-specific genes by whole microbial genome comparisons. J Bacteriol **186:**4781-95.

146. **Prendergast, M. M., A. J. Lastovica, and A. P. Moran.** 1998. Lipopolysaccharides from *Campylobacter jejuni* O:41 strains associated with Guillain-Barre syndrome exhibit mimicry of GM1 ganglioside. Infect Immun **66:**3649-55.

147. **Reina, D., L. Anderson, M. Habela, A. J. Weatherley, and I. Navarrete.** 2000. Efficacy of doramectin against naturally acquired nematode infection in Iberian swine. Vet Parasitol **89:**139-47.

148. **Rhoads, M. L., R. H. Fetterer, and D. E. Hill.** 2000. *Trichuris suis*: A secretory serine protease inhibitor. Exp Parasitol **94:**1-7.

149. **Rhoads, M. L., R. H. Fetterer, D. E. Hill, and J. F. Urban, Jr.** 2000. *Trichuris suis*: a secretory chymotrypsin/elastase inhibitor with potential as an immunomodulator. Exp Parasitol **95:**36-44.

150. **Roach, T. I., K. J. Else, D. Wakelin, D. J. McLaren, and R. K. Grencis.** 1991. *Trichuris muris*: antigen recognition and transfer of immunity in mice by IgA monoclonal antibodies. Parasite Immunol **13:**1-12.

151. **Russell, R. G., M. O'Donnoghue, D. C. Blake, Jr., J. Zulty, and L. J. DeTolla.** 1993. Early colonic damage and invasion of *Campylobacter jejuni* in experimentally challenged infant *Macaca mulatta*. J Infect Dis **168:**210-5.

152. **Rutter, J. M., and R. J. Beer.** 1975. Synergism between *Trichuris suis* and the microbial flora of the large intestine causing dysentery in pigs. Infect Immun **11:**395-404.

153. **Saeki, H., T. Fujii, S. Fukumoto, K. Kagota, A. Taneichi, S. Takeda, and M. Tsukaguchi.** 1997. Efficacy of doramectin against intestinal nematodes and sarcoptic manage mites in naturally infected swine. J Vet Med Sci **59:**129-32.

154. **Sahin, O., N. Luo, S. Huang, and Q. Zhang.** 2003. Effect of Campylobacter-specific maternal antibodies on *Campylobacter jejuni* colonization in young chickens. Appl Environ Microbiol **69:**5372-9.

155. **Salama, S. M., M. M. Garcia, and D. E. Taylor.** 1992. Differentiation of the subspecies of *Campylobacter fetus* by genomic sizing. Int J Syst Bacteriol **42:**446-50.

156. **Schillhorn van Veen, T. W., and C. D. Gibson.** 1983. Anthelmintic activity of ivermectin in pigs naturally infected with *Ascaris* and *Trichuris*. Am J Vet Res **44:**1732-3.

157. **Schouls, L. M., S. Reulen, B. Duim, J. A. Wagenaar, R. J. Willems, K. E. Dingle, F. M. Colles, and J. D. Van Embden.** 2003. Comparative genotyping of *Campylobacter jejuni* by amplified fragment length polymorphism, multilocus sequence typing, and short repeat sequencing: strain diversity, host range, and recombination. J Clin Microbiol **41:**15-26.

158. **Schwartz, D., R. Perry, D. M. Dombroski, J. M. Merrick, and J. Goldhar.** 1996. Invasive ability of *C. jejuni/coli* isolates from children with diarrhea and the effect of iron-regulated proteins. Zentralbl Bakteriol **283:**485-91.

159. **Shenker, B. J., T. McKay, S. Datar, M. Miller, R. Chowhan, and D. Demuth.** 1999. *Actinobacillus actinomycetemcomitans* immunosuppressive protein is a member of the family of cytolethal distending toxins capable of causing a G2 arrest in human T cells. J Immunol **162:**4773-80.

160. **Shin, J. L., G. W. Gardiner, W. Deitel, and G. Kandel.** 2004. Does whipworm increase the pathogenicity of *Campylobacter jejuni*? A clinical correlate of an experimental observation. Can J Gastroenterol **18:**175-7.

161. **Siemer, B. L., C. S. Harrington, E. M. Nielsen, B. Borck, N. L. Nielsen, J. Engberg, and S. L. On.** 2004. Genetic relatedness among *Campylobacter jejuni* serotyped isolates of diverse origin as determined by numerical analysis of amplified fragment length polymorphism (AFLP) profiles. J Appl Microbiol **96:**795-802.

162. **Skirrow, M., and Blaser, M.J.** 2000. Clinical aspects of *Campylobacter* infection, p. 69-88. *In* I. Nachamkin, and Blaser, M.J (ed.), Campylobacter, Second ed. American Society for Microbiology, Washington, DC.

163. **Sorvillo, F. J., L. E. Lieb, and S. H. Waterman.** 1991. Incidence of campylobacteriosis among patients with AIDS in Los Angeles County. J Acquir Immune Defic Syndr **4:**598-602.

164. **Stewart, T. B., M. C. Fox, and S. E. Wiles.** 1996. Doramectin efficacy against gastrointestinal nematodes in pigs. Vet Parasitol **66:**101-8.

165. **Stewart, T. B., and O. M. Hale.** 1988. Losses to internal parasites in swine production. J Anim Sci **66:**1548-54.

166. **Summers, R. W., D. E. Elliott, K. Qadir, J. F. Urban, Jr., R. Thompson, and J. V. Weinstock.** 2003. *Trichuris suis* seems to be safe and possibly effective in the treatment of inflammatory bowel disease. Am J Gastroenterol **98:**2034-41.

167. **Szymanski, C. M., and G. D. Armstrong.** 1996. Interactions between *Campylobacter jejuni* and lipids. Infect Immun **64:**3467-74.

168. **Szymanski, C. M., M. King, M. Haardt, and G. D. Armstrong.** 1995. *Campylobacter jejuni* motility and invasion of Caco-2 cells. Infect Immun **63:**4295-300.

169. **Tanner, A. C., J. L. Dzink, J. L. Ebersole, and S. S. Socransky.** 1987. *Wolinella recta, campylobacter concisus, bacteroides gracilis,* and *Eikenella corrodens* from periodontal lesions. J Periodontal Res **22:**327-30.

170. **Tay, S. T., S. Devi, S. D. Puthucheary, and I. M. Kautner.** 1995. Detection of haemolytic activity of campylobacters by agarose haemolysis and microplate assay. J Med Microbiol **42:**175-80.

171. **Taylor, D. J., and W. F. Blakemore.** 1971. Spirochaetal invasion of the colonic epithelium in swine dysentery. Res Vet Sci **12:**177-9.

172. **Ueki, Y., A. Umeda, S. Fujimoto, M. Mitsuyama, and K. Amako.** 1987. Protection against *Campylobacter jejuni* infection in suckling mice by anti-flagellar antibody. Microbiol Immunol **31:**1161-71.

173. **Urban, J., H. Fang, Q. Liu, M. J. Ekkens, S. J. Chen, D. Nguyen, V. Mitro, D. D. Donaldson, C. Byrd, R. Peach, S. C. Morris, F. D. Finkelman, L. Schopf, and W. C. Gause.** 2000. IL-13-mediated worm expulsion is B7 independent and IFN-gamma sensitive. J Immunol **164:**4250-6.

174. **Van Rhijn, I., L. H. Van den Berg, C. W. Ang, J. Admiraal, and T. Logtenberg.** 2003. Expansion of human gammadelta T cells after in vitro stimulation with *Campylobacter jejuni.* Int Immunol **15:**373-82.

175. **Vandamme, P.** 2000. Taxonomy of the family Campylobacteraceae, p. 3-26. *In* I. a. B. Nachamkin, MJ (ed.), Campylobacter, Second ed. American Society for Microbiology, Washington, DC.

176. **Wai, S. N., K. Nakayama, K. Umene, T. Moriya, and K. Amako.** 1996. Construction of a ferritin-deficient mutant of *Campylobacter jejuni*: contribution of ferritin to iron storage and protection against oxidative stress. Mol Microbiol **20:**1127-34.

177. **Wassenaar, T. M., N. M. Bleumink-Pluym, and B. A. van der Zeijst.** 1991. Inactivation of *Campylobacter jejuni* flagellin genes by homologous recombination demonstrates that flaA but not flaB is required for invasion. Embo J **10:**2055-61.

178. **Wassenaar, T. M., B. A. van der Zeijst, R. Ayling, and D. G. Newell.** 1993. Colonization of chicks by motility mutants of *Campylobacter jejuni* demonstrates the importance of flagellin A expression. J Gen Microbiol **139 (Pt 6):**1171-5.

179. **Whitehouse, C. A., P. B. Balbo, E. C. Pesci, D. L. Cottle, P. M. Mirabito, and C. L. Pickett.** 1998. *Campylobacter jejuni* cytolethal distending toxin causes a G2-phase cell cycle block. Infect Immun **66:**1934-40.

180. **Wilson, D. L., J. A. Bell, V. B. Young, S. R. Wilder, L. S. Mansfield, and J. E. Linz.** 2003. Variation of the natural transformation frequency of *Campylobacter jejuni* in liquid shake culture. Microbiology **149:**3603-15.

181. **Wood, A. C., N. J. Oldfield, C. A. O'Dwyer, and J. M. Ketley.** 1999. Cloning, mutation and distribution of a putative lipopolysaccharide biosynthesis locus in *Campylobacter jejuni*. Microbiology **145 (Pt 2):**379-88.

182. **Wooldridge, K. G., and J. M. Ketley.** 1997. Campylobacter-host cell interactions. Trends Microbiol **5:**96-102.

183. **Yamasaki, M., S. Igimi, Y. Katayama, S. Yamamoto, and F. Amano.** 2004. Identification of an oxidative stress-sensitive protein from *Campylobacter jejuni*, homologous to rubredoxin oxidoreductase/rubrerythrin. FEMS Microbiol Lett **235:**57-63.

184. **Yao, R., D. H. Burr, P. Doig, T. J. Trust, H. Niu, and P. Guerry.** 1994. Isolation of motile and non-motile insertional mutants of *Campylobacter jejuni*: the role of motility in adherence and invasion of eukaryotic cells. Mol Microbiol **14:**883-93.

185. **Ziprin, R. L., C. R. Young, L. H. Stanker, M. E. Hume, and M. E. Konkel.** 1999. The absence of cecal colonization of chicks by a mutant of *Campylobacter jejuni* not expressing bacterial fibronectin-binding protein. Avian Dis **43:**586-9.

CHAPTER 2

INTERLEUKIN 4 (IL-4), IL-6 AND IL-10 CYTOKINE SECRETION FROM INTESTINAL EPITHELIAL CELLS (IPEC-1) IN RESPONSE TO *CAMPYLOBACTER JEJUNI* AND *TRICHURIS SUIS* EXCRETORY SECRETORY PRODUCTS

ABSTRACT

Swine with dual infections of *Campylobacter jejuni* and *Trichuris suis* developed disease more severe than the additive effect of either disease alone. In this polymicrobial infection, resistance to either organism was altered by the presence of the other. Since Th-2 cytokines play a vital role in resistance to helminth infections, we hypothesized that during dual infection, immune responses are biased to a predominantly Th-2 type and synergy in cytokine production stimulated by these two pathogens results in Th-2-mediated disease. In order to examine the role of *C. jejuni* and *T. suis* in Th-2 immune modulation, Intestinal Pig Epithelial Cells-1 (IPEC-1) were exposed to *C. jejuni* (strains ATCC 33292 and 81176) and *T. suis* ESP, and Th-2 cytokine responses (IL-4, IL-6 and IL-10) were measured. IL-6 was the predominant cytokine produced, followed by IL-10. There was no secretion of IL-4 from IPEC-1 cells. IL-6 was secreted within 24 hr in response to *C. jejuni*, while IL-10 induction took longer (> 24 hr). In addition, IL-6 was secreted from both differentiated and undifferentiated IPEC-1 cells, while IL-10 was secreted predominantly from differentiated cells, compared to negative controls. In all cases, secretion of these cytokines occurred on the cell surface exposed to the bacteria. *T. suis* ESP on the other hand, induced IL-6 and IL-10 within a 24 hr time frame, and both the differentiated and undifferentiated cells secreted these cytokines in response to a low level ESP (0.3 mg/ml) challenge. These data show that *C. jejuni* and *T. suis* ESP induce Th-2 cytokines in swine epithelial cells and suggests that Th-2-mediated synergy in cytokines might occur during concurrent infections, with IL-6 in particular. In addition, preformed IL-6 was demonstrated in IPEC-1 cells for the first time to date, and

this result suggests that swine intestinal cells play a role in immune response during enteric infections.

INTRODUCTION

Campylobacter jejuni (*C. jejuni*) is one of the leading causes of bacterial enteritis in humans, particularly children (7, 15, 26). It is a microaerophlic, gram-negative, spiral or comma-shaped bacterium and is mainly acquired through contaminated food. *Trichuris* spp. are helminth parasites that live in the cecum and proximal colon of many mammals, including humans and swine, and are found commonly throughout the world (4, 5). In an animal model of polymicrobial infection, *C. jejuni* and *Trichuris suis* acted synergistically in the colon of neonatal, gnotobiotic, and naïve conventionally-reared pigs to produce disease and pathological lesions (30, 31). Germ-free piglets infected individually with low doses of either *C. jejuni* (10^6 cfu) or *T. suis* (3000 embryonated eggs) displayed few clinical signs of disease. In contrast, severe colonic pathology was observed in pigs with dual infections; pathology was marked by focal lesions, infiltration of inflammatory cells, excessive mucus secretion, bloody diarrhea and bacterial invasion into epithelial cells and macrophages (30). Lymphoid tissues, especially lymphoglandular complexes (LGCs), appeared to be one important route for *C. jejuni* invasion. LGCs are secondary lymphoid organs present in the distal colon of swine, humans, rats, cattle, and other animals (25, 29, 34, 38) . They are structurally similar to the Peyer's patches in the small intestine and in the sensitized host are composed of a follicle-associated epithelium (FAE) overlying B-cell dependent germinal centers. IgA is generated here after *C. jejuni* challenge and may also serve as sites where the bacterium is harbored or disseminated (32).

This study was designed to understand the role of immunity and immuno-modulation in this dual infection model. Since it is the specific immune reaction against pathogens that keeps dangerous organisms at bay and determines the outcome of the disease state, we hypothesized that cytokine dysregulation may be an important contributor to this polymicrobial synergism. To begin to address this question, we undertook to define the host Th-2 immune responses to these two pathogens. Th-2 cytokines, including IL-4 and IL-10, have been shown to be essential for resistance against helminth infections, while IL-6 is stimulated as part of the host defense against bacterial infections (11-13, 18). Additionally, IL-6 has been shown to downregulate anti-leishmanial activity in human macrophages, while IL-4 and IL-10 inhibit Th-1 cytokine responses (17, 22). However, far from being beneficial, an improper cytokine response can enhance severity of disease, as evidenced by IL-6 mediated malignancies and IL-4 mediated pathologies (14, 21, 28). Since our swine dual infection model has both a helminth component and a bacterial component, we decided to analyze IL-4, IL-6 and IL-10 cytokine levels, and in this study, we focused on *C. jejuni* and *T. suis* mediated *in vitro* Th-2 responses. We posed the following hypotheses: 1) resistance to *C. jejuni* infection requires a predominant Th-1 immune response accompanied by a moderate Th-2 component, while resistance to *T. suis* requires a predominant Th-2 response, and 2) the immune response is shifted towards a predominantly Th-2 type during polymicrobial infections with *T. suis*, which interferes with the Th-1 immunity directed against the bacteria. Since bacterial invasion of intestinal epithelial cells appears to be the first interactive event in *C. jejuni* and *T. suis* infection of swine, and since epithelial cells have been shown to be a significant source of the enteric immune response, IPEC-1 cells were

chosen for the current study (19, 27, 30, 32, 36). IPEC-1 is a non-immortalized, undifferentiated small intestinal cell line from neonatal swine that differentiates when grown on porous substrates for 10-14 days (6, 16). Because IPEC-1 is a non-immortalized cell line, it is a better model of the normal host intestine than a transformed cell line. However, very little is known about the cytokine responses of IPEC-1 cells. Also, ESP from other helminths has been shown to be immunogenic. Hence, in the current study, IPEC-1 monolayers were challenged with pathogenic *C. jejuni* strains and *T. suis* ESP, and the IL-4, IL-6, and IL-10 cytokine responses from these cells were measured. Secreted cytokines were quantified rather than intra-cellular cytokine levels, as autocrine and paracrine effects of cytokines are more likely to interfere with immunity. The data obtained suggests that intestinal epithelial cells (IPEC-1) could be a major source of enteric IL-6 and some IL-10 and hence may play an important role in immunomodulation during enteric *C. jejuni* and *T. suis* infections.

MATERIALS AND METHODS

Bacterial strains and culture

C. jejuni strains ATCC 33292 and 81-176 were used for the challenge studies. Both were initially isolated from humans with enteritis. *C. jejuni* 81-176 was kindly donated by Dr. Carol Pickett, University of Kentucky. Bacterial colonies were grown on Mueller Hinton Agar supplemented with 5% sheep's blood (Cleveland Scientific, Bath, OH) for a period of 48-72 hr. An isolated colony was spread on a fresh plate and further grown as a lawn for 20 hr. Bacterial growth was resuspended in RPMI 1640 (Gibco BRL, Rockville, MD) and the optical density (OD_{560}) adjusted to 0.1 to achieve 5 x 10^8 CFU/ml. Dilutions of the bacteria were made in RPMI 1640 as required for the experimental design.

Cell culture

Both differentiated and undifferentiated IPEC-1 cells were used to determine the IL-4, IL-6 and IL-10 expression in response to *C. jejuni in vitro*. All media and supplements for cell culture were obtained from Gibco BRL, Rockville, MD, unless otherwise stated. transwell and 24-well culture plates were obtained from Corning Costar, Corning, NY.

Differentiated cells

Differentiated cells were used to represent the intestinal villus tip cells and the FAE of the LGCs. The culture protocol was kindly provided by Dr. Jerrold Turner of University of Chicago (personal communication). Briefly, IPEC-1 cells were cultured for routine passage in DMEM/F-12 medium supplemented with 5% Fetal Bovine Serum

(FBS) and 1% Insulin-Transferrin-Selenium in cell culture flasks. After 5–6 days of growth, the cells were washed in versene and trypisinized. Then, they were cultured at a density of $3x10^5$/ well on transwell inserts (6.5 mm diameter, 3 μm pore size) that had been previously coated with fibronectin (20 μg/ml; Sigma, St. Louis, MO). Cells were allowed to differentiate for 10–14 days in RPMI 1640 medium containing phenol red supplemented with 5% fetal bovine serum (FBS). Transepithelial electrical resistance (TEER) across the monolayer was measured using an electrode (EVOMX, World Precision Instruments, Sarasota, FL) to determine confluency and tight junction formation.

Undifferentiated cells

IPEC-1 cells in an undifferentiated state were used to mimic the epithelial cells in the basilar crypt of the intestines. These cells, unlike the differentiated cells, do not have tight junctions or distinct apical or baso-lateral surfaces. Since certain cytokines can be induced only from differentiated cells and not from undifferentiated cells (33), we tested for cytokine secretion from 2-day old undifferentiated IPEC-1 cells. They were cultured similarly to the differentiated cells on fibronectin-coated (20 μg/ml) 24-well plates, at 3x 10^5 cells per well and were grown to confluency (2-3 days).

Scanning Electron microscopy (SEM)

Electron microscopy was done on 12-day IPEC-1 cells grown on transwells, to verify their state of differentiation. SEM was carried out at Center for Microscopy, Michigan State University. After 12 days of growth, media was removed and the membranes were excised and fixed in 4% glutaraldehyde in 0.1M sodium phosphate

buffer. The specimens were subsequently fixed in 1% osmium tetroxide and dehydrated in a graduated ethanol series. The dehydrated membranes were dried in a critical point dryer (Balzers, Lichenstein), mounted on aluminum stubs, and sputter coated with 7 nm of gold (Emscope SC500 sputter coater, UK). Visualization of the cells was carried out with a scanning electron microscope (JEOL 6400V, Japan).

Establishing the optimal Multiplicity of Infection (MOI) for *C. jejuni*

Initially *C. jejuni* strain 33292 was used to determine the optimal bacterial inoculum required to infect the IPEC-1 cells, based on observed increases in IL-6 levels in the medium. The bacterial strain was grown as described previously and adjusted to 0.1 OD_{560}, yielding approximately 5 x 10^8 CFU/ml. This stock solution was further diluted to yield the various MOI inocula (4:1, 40:1, 100:1 and 200:1). These dilution stocks were then used to infect differentiated IPEC-1 cells apically for 8, 12 and 24 hr. *E. coli* LPS O26:B6 (200 μg/ml) was used as a positive control to induce IL-6 expression; the optimal experimental dose was obtained from a dose response experiment. RPMI 1640 without phenol red was inoculated onto IPEC-1 as the negative control. All inocula were in 0.4 ml final volume; three replicates were used for each treatment group. After incubation, supernatants were collected, centrifuged, and analyzed for IL-6 protein by enzyme-linked immunosorbant assay (ELISA).

Infection assays with *C. jejuni*

a) Measurement of secreted IL-6

Infection of IPEC-1 with *C. jejuni* strains 33292 and 81176 was carried out for 8 and 24 hr time intervals with the optimum bacterial dose (40:1 MOI) obtained from previous results. To determine the direction of IL-6 secretion, differentiated IPEC-1 on transwells were challenged with *C. jejuni* strains apically or baso-laterally in separate experiments. *E. coli* LPS O26:B6 served as the positive control and the RPMI 1640 medium without phenol red and FBS served as the negative control. Supernatants were collected as described previously and stored at -80° C until analysis by ELISA. Similarly, undifferentiated IPEC-1 cells growing on 24-well plates were infected with the optimal dose of *C. jejuni* strains for 0, 8, and 24 hr. Since these cells do not have distinct apical or basolateral surfaces, treatments were simply exposed to cells rather than to a specific surface as in differentiated cells. Supernatants were removed from wells and analyzed for IL-6. All treatments used for infection of both differentiated and undifferentiated cells were performed in triplicate for each experiment.

b) Measurement of secreted IL-10

To determine the effect of *C. jejuni* on IL-10 expression from IPEC-1, 10-14 day-old monolayers were challenged with *C. jejuni* strains 33292 and 81176 for 0 hr, 48 hr, and 72 hrs. The time points were chosen based on preliminary experiments, which showed delayed secretion of IL-10 (> 24 hr, data not shown) from *C. jejuni* infected IPEC-1 cells. As with IL-6, the specific direction of secretion (if any) was determined by infecting the monolayers apically or baso-laterally. Concanavalin A (Con A; Sigma, St. Louis, MO) at 100 μg/ml served as a positive control for IL-10 induction. The treatments were suspended in RPMI 1640 media without phenol red and FBS, and the suspension medium served as the negative control. All treatments were performed in triplicate.

Supernatants were collected after the indicated intervals, processed as described previously, and stored at -80° C until analysis. The same treatment design was used for the undifferentiated IPEC-1 cells. Supernatants were removed from wells as indicated, stored, and analyzed later for cytokine levels.

c) Measurement of secreted IL-4

Al-Salloom et al., (2) have recently demonstrated that epithelial cells are immunopositive for IL-4 in response to *C. jejuni* infection. Hence, IL-4 was measured to rule out the possibility that this Th-2 inducing cytokine was produced in our system. Differentiated IPEC-1 cells were exposed apically to the optimal *C. jejuni* inoculum (40:1) for 8 hr, 24 hr, 48 hr, and 72 hr. RPMI 1640 medium served as the negative control. Undifferentiated cells grown on 24 well plates were challenged with the same treatments as the differentiated cells and supernatants processed as described previously. In preliminary trials, differentiated IPEC-1 cells were tested for IL-4 expression using ConA (100μg/ml), phytohemagglutinin (PHA) (20 μg/ml, 50 μg/ml and 100 μg/ml), and pokeweed mitogen (50% v/v) for 8 hr and 24 hrs and were shown not to induce detectable levels of IL-4. Therefore, no positive cell treatment controls were available.

Extraction of ESP from *T. suis* adult worms

ESP was extracted according to the protocol described previously (20). Two to four week old piglets were inoculated with 2500-5000 embryonated *T. suis* eggs. After approximately 45 days, the pigs were humanely euthanized and their colons removed at necropsy. The adult worms were extracted from the cecum and the proximal colon and washed once in 0.85% saline solution, prewarmed to 37°C. The worms were then washed

four times in warm Hanks Balanced Salt Solution (HBSS), for 15 minutes each to remove any fecal contamination and debris. They were then transferred to sterile culture dishes containing RPMI 1640 with 5X antibiotics (penicillin (500 U/ml), streptomycin (0.5 mg/ml), and amphotericin B (1.25 μg/ml), Sigma Chemical Co. St.Louis, MO) at a density of 4 worms/ml, and incubated overnight at 37°C, 5% CO_2. The following morning they were transferred to RPMI 1640 with 1X concentration of the same antibiotics and again incubated overnight. Worms were washed free of the antibiotics in sterile HBSS before being transferred to RPMI 1640 with 1% glucose. The culture medium was collected every two days for 10 days. These supernatants were frozen at -80°C until required, pooled, and concentrated using an Amicon stirred cell with a 10,000 molecular weight cut-off. Different batches of ESP were standardized using Bradford assay (8) and SDS PAGE gel of the total protein.

ESP challenge assays

Exposure of differentiated IPEC-1 cells to *T. suis* ESP was carried out for 12 hr and 24 hr at a concentration of 0.3 mg/ml. Higher doses of ESP have been shown to be cytotoxic to IPEC-1 (1); hence exposures were carried out at this low level dose. *E. coli* LPS 026:B6 (200 μg/ml) served as a positive control for IL-6 secretion, while Concanavalin A (ConA) (100 μg/ml) served as a positive control for IL-10 secretion. The optimal dose of LPS was obtained from preliminary experiments (data not shown). As mentioned before, none of the materials tested, ConA (100 μg/ml), phytohemoagglutinin (PHA) (20 μg/ml, 50 μg/ml and 100 μg/ml) or pokeweed mitogen (50% v /v) induced for IL-4 secretion. Hence no positive control for IL-4 was available. RPMI 1640 medium

without phenol red and FBS and Bovine Serum Albumin (BSA, 0.3 mg/ml) served as negative controls. Supernatants were collected after 12 and 24 hr, and processed as before. All treatments were placed on the apical surface of the differentiated IPEC-1 cells, and experiments were performed in triplicate for each group.

Similar treatments were carried out for the undifferentiated IPEC-1 cells. Supernatants were removed from wells 12 hr and 24 hr after treatment exposure, processed as before, and analyzed later for IL-4, IL-6 and IL-10 cytokine secretions. All treatments were in triplicate. *E. coli* LPS, ConA, and PHA were purchased from Sigma Chemical Co., St. Louis, MO. Pokeweed mitogen was obtained from Gibco-BRL, Rockville, MD.

IL-6 ELISA

Indirect ELISA was performed to measure secreted IL-6. The swine IL-6 cytokine and primary anti-swine IL-6 antibody (rabbit polyclonal, IgG isotype) were purchased from Biosource International, Camarillo, CA. The secondary biotinylated antibody (anti-rabbit IgG (whole molecule)), Bovine Serum Albumin (BSA), extravidin peroxidase, and tetramethyl benzidine (TMB) were purchased from Sigma Chemical Co., St. Louis, MO. Cell culture supernatants removed from both the upper and lower chambers of the transwell inserts (or wells in case of 24-well plates) were coated on 96-well plates and stored overnight at 4°C. Each plate also contained IL-6 protein standard controls (in duplicate), at 100, 50, 25, 12.5, 6.25, 3.125, 1.56, and 0 ng/ml, in RPMI 1640 without phenol red and FBS. Following the overnight incubation, supernatants were discarded and the plates were blocked with 3% BSA solution (in Phosphate Buffered Saline (PBS,

0.01 M) containing 0.1% Tween 20) and incubated for 90 minutes at 37°C. Primary rabbit anti-swine IL-6 antibody (1/100 titer) was used to capture the IL-6 protein (37°C, 90 minutes) and a goat anti-rabbit antibody conjugated with biotin (1/5000) was used as a secondary antibody (37°C, 90 minutes). The optimal titers for the primary and secondary antibodies were determined from preliminary experiments (data not shown). The color reaction was produced using extravidin peroxidase (1 μg/ml, 45 minutes at room temperature) and a ready-to-use peroxide-containing TMB substrate (30 minutes, room temperature). The color reaction was stopped with 6N H_2SO_4. Plates were washed after each step with PBS containing 0.1% Tween-20. A microplate reader (EL800 Universal Microplate Reader, Bio-Tek instruments, Winooski, Vermont) was used to measure the absorbance at 450 nm, and the concentration of IL-6 protein in each sample was quantified using KCjunior® software (Bio-Tek instruments, Winooski, Vermont).

IL-10 and IL-4 ELISA

IL-10 and IL-4 were measured using sandwich ELISA kits obtained from Biosource International (Camarillo, CA), and the assays were performed according to the manufacturer's protocol. Each assay contained the IL-4 and IL-10 standard protein controls, and IL-10 and IL-4 protein were quantified (at 450 nm) as described previously for IL-6.

Measurement of IL-6 mRNA at 0 hr by Reverse Transcriptase- Polymerase chain Reaction (RT-PCR)

IPEC-1 cells growing on transwells for 12 days were treated with LPS (200 μg/ml), *C. jejuni* strains 33292 and 81176, and RPMI 1640 media. Treatments were removed almost immediately, and RNA was extracted using Trizol ® reagent using the manufacturer's instructions (Invitrogen, Frederick, MD). cDNA was constructed from 2 μg of total RNA for each sample, with the First Strand Pro-Star RT-PCR kit (Stratagene, La Jolla, CA), according to the manufacturer's protocol. PCR for IL-6 mRNA was carried out using primers 5' GGAACGCCTGGAAGAAGATG 3', and 5' ATCCACTCGTTCTGTGACTG 3', and for the constitutively expressed beta-2 (β_2) microglobulin gene using primers 5' CTGCTCTCACTGTCTGG 3' and 5'ATCGAGAGTCACGTGCT 3' (39). The assay conditions for the PCR were; 1μl of cDNA, 75ng/μl of each primer, 3 mM $MgCl_2$, 10 mM of each deoxynucleoside triphosphate (dNTP), and 0.625 U of AmpliTaq Gold (Perkin Elmer) in 25 μl reactions. The PCR was run at the following thermocycler conditions: 94°C, 10 minutes; 94°C, 1 minute for denaturing; 55°C, 1 minute for annealing; 72°C, 1 minutes for extension for 40 cycles. The PCR cycles were optimized prior to experimentation (data not shown). The PCR products were run on a 1.8 % agarose gel, stained with ethidium bromide and bands were visualized using the Alpha Imager (Alpha Innotech Corporation, San Leandro, CA).

Effect of cycloheximide (CHX) on IL-6 secretion from IPEC-1 at 0 hr

The effect of inhibition of protein synthesis on IL-6 secretion was determined by treating 12-day old IPEC-1 cells on transwells with CHX. IPEC-1 cells were infected with *C. jejuni* strain 81176 at an MOI of 40:1 with or without different concentrations of

cycloheximide (100 μg/ml, 500 μg/ml and 1000 μg/ml). All treatments were suspended in RPMI 1640 media. To determine the background levels of IL-6 in response to the inhibitor, RPMI medium containing the different concentrations of CHX was included in the assay. Each treatment group was in triplicate, and all treatments were removed from the cells within 30 seconds of addition. The supernatants thus removed were processed as before and stored at -80° C until analysis by ELISA. Additional controls for CHX inhibition were not added, as no swine reagents were available for detection of any constitutively expressed proteins. Effect of CHX on viability of *C. jejuni* also was determined by plating *C. jejuni* 33292 containing 100 μg/ml of CHX on Bolton agar plates after limiting dilutions. In this viability experiment, bacteria were incubated with CHX for ~1 minute (0 hr) and two and a half hours.

Immunoblot Analysis for performed IL-6 protein

Whole cell extracts were made from 5-day old IPEC-1 cells growing in tissue culture flasks. Briefly, cell pellets were washed with Dulbeccos' PBS (without calcium or magnesium) (Cambrex Biosciences, Walkersville, MD), suspended in Jackson's extraction buffer (50 mM NaF, 20 mM HEPES pH 7.8, 450 mM NaCl, 25% glycerol, 0.2 mM EDTA) (pellet:buffer::1:1) and freeze thawed three times in liquid nitrogen-37°C water bath. The suspension was centrifuged at 17,949 x g for 10 minutes. The supernatant or the whole cell extract was separated from the pellet after centrifugation, and total protein was quantified using the Bradford assay. Different concentrations (100 μg/ml and 250 μg/ml) of the total protein were analyzed for preformed IL-6 after electrophoresis on

a 15% SDS polyacrylamide gel and transfer to PVDF membrane (Millipore, Bedford, MA). 80 ng of swine IL-6 protein (Biosource International, Camarillo, CA) was included in the assay as the positive control. A rabbit polyclonal antibody raised against IL-6 was used as the primary antibody (1:1000 dilution). An anti-rabbit antibody (Amersham Biosciences, Piscataway, NJ) labeled with horseradish peroxidase (HRP) was used as the secondary antibody (1:5000 dilution). The membrane was then incubated with a chemiluminescent substrate (ECL, Amersham Biosciences, Piscataway NJ) according to the manufacturer's recommendations, and visualized.

Statistics

A mixed model ANOVA using pair-wise comparisons with Tukey adjustments was used to determine the statistical significance of the cytokine concentrations obtained. The p values were computed using the SAS 8.02 (SAS Institute Inc., Cary, NC) software. P values ≤ 0.05 were considered significant.

RESULTS

Scanning electron microscopy (SEM)

An SEM image of 12 day-old IPEC-1 cells grown on transwells is shown in Figure 2. 1. The TEER values for these cells were in the range of ~600- 800 Ω. cm^2. Cells were confluent, and exhibited distinct microvilli on their apical surfaces, typical of differentiated cells. Such cells were used in subsequent infection assays.

Sensitivity of IL-6, IL-10 and IL-4 ELISAs

The swine IL-6 ELISA was developed in the laboratory, and Figure 2. 2 shows the sensitivity of the assay (~ 1.56 ng/ml) using the swine polyclonal anti-IL-6 antibody (1: 100 titer). The regression curve obtained using KCjunior® software (Bio-Tek instruments, Winooski, Vermont) showed a significant dose response curve (R = 0. 99) at 450 nm with the concentration range and antibody titer used. A similar result was seen for the IL-10 (R= 0.99) and IL-4 (R= 1.0) standard curves as well (Figure 2. 3 and Figure 2. 4). Based on this standard curve, the assays detect ≥ 1 pg/ml of the IL-10 protein, and ≥ 2 pg/ml of the IL-4 protein.

Multiplicity of Infection assays

All MOI of *C. jejuni* 33292 (4:1, 40:1, 100:1 and 200:1) elicited a significantly higher IL-6 response compared to both the positive and negative controls (Figure 2. 5). However, statistical analysis showed that there was no significant increase in IL-6 expression between 40:1 and 100:1 MOI. Therefore a 40:1 MOI was used for all subsequent *in vitro* infections. Also, there was significantly more IL-6 in the apical

chamber at 8 hr and 12 hr after infection in comparison to the lower chamber for most treatment groups. However, by 24 hr after infection, there was no significant difference in IL-6 levels between the apical and basolateral chambers.

Infection assays with *C. jejuni*

a) IL-6 ELISA

Figure 2. 6 shows the effect of apical and baso-lateral infection of IPEC-1 cells with *C. jejuni*. At 8 hr, after apical infection, IL-6 levels were significantly higher in the apical chamber compared to the basolateral chamber in response to strain 33292 ($p < 0.01$), while a similar result was obtained at 24 hr with strain 81176 ($p < 0.01$) (Figure 2. 6A). Similarly, basolateral infections elicited a significant secretion towards the basolateral side with both strains at 8 hr ($p < 0.01$) (Figure 2. 6B). There were no statistically significant differences in IL-6 levels induced by either *C. jejuni* strain given either as apical or basolateral challenges at any time point ($p > 0.05$).

The results of *C. jejuni* challenge of undifferentiated IPEC-1 cells are shown in Figure 2. 7. 0 hr (treatments removed as soon as possible; 30 seconds) was included at this time to determine the earliest time point for IL-6 expression. Like the differentiated cells, the undifferentiated cells also were capable of secreting IL-6 in response to *C. jejuni* infection. Significant IL-6 secretion was seen within minutes to seconds of infection (0 hr) with both strains of *C. jejuni* ($p < 0.05$) and remained elevated at 8 hr and 24 hr ($p < 0.05$) compared to the LPS and RPMI controls. As in bacterial challenge to differentiated cells, there were no statistically significant differences in the IL-6 levels from undifferentiated cells when challenged with *C. jejuni* strains 81176 and 33292.

b) IL-10 ELISA

The effect of *C. jejuni* on apical and baso-lateral secretion of IL-10 is seen in Figure 2. 8. IL-10 secretion in response to apical *C. jejuni* infection was not seen until 24 hr (data not shown) but increased to significant levels ($p < 0.01$) by 72 hr (Figure 2. 8A; later time points were not tested) after challenge with both strains of bacteria. Apical infection produced an increase in IL-10 in comparison to the medium only control, in both the upper and lower chambers by 72 hr (Figure 2. 8A). However, baso-lateral infection elicited IL-10 secretion that was greater on the baso-lateral side than the apical side, and that response was seen only with the *C. jejuni* strain 33292 (72 hr, $p < 0.05$), Figure 2. 8B. The other strain, 81176, did not elicit a significant increase in IL-10 over the negative medium only control when infected baso-laterally at any time.

The secretion of IL-10 from undifferentiated IPEC-1 cells is shown in Figure 2. 9. Unlike the differentiated cells, undifferentiated cells did not secrete significantly increased amounts of IL-10 in comparison to the medium only controls at any time point, and this result was obtained with both the *C. jejuni* strains tested.

c) IL-4 ELISA

There was no IL-4 secreted with any treatment from either differentiated or from undifferentiated IPEC-1 cells at all the time points tested. (Data not shown).

ESP challenge assays

IL-6 ELISA

IL-6 expression levels from differentiated and undifferentiated IPEC-1 cells in response to *T. suis* ESP are shown in Figure 2. 10 and Figure 2. 11 respectively. IL-6 was produced from both differentiated (Figure 2. 10) and undifferentiated IPEC-1 cells (Figure 2. 11), although at different levels, with the differentiated cells producing more IL-6. IL-6 levels from differentiated cells were significantly higher in the apical chamber than in the baso-lateral chamber at 12 hr ($p < 0.001$) and fell to comparable levels in both chambers by 24 hr ($p > 0.05$, Figure 2. 10) in response to ESP. ESP-induced IL-6 levels were also significantly higher than those induced by LPS, BSA and RPMI at both times ($p < 0.001$). The latter two treatments did not elicit detectable amounts of IL-6 from the differentiated IPEC-1 cells.

Similarly, IL-6 levels from undifferentiated IPEC-1 cells in response to ESP was significantly higher compared to the BSA and RPMI induced levels at 12 hr ($p < 0.01$), Figure 2. 11. However, unlike the results seen with differentiated cells, both BSA and RPMI induced detectable amounts of IL-6. The high average level of IL-6 seen in response to BSA at 24 hr from undifferentiated cells was due to a single high value.

IL-10 ELISA

The IL-10 responses from differentiated and undifferentiated IPEC-1 cells due to *T. suis* ESP treatment are shown in Figure 2. 12 and Figure 2. 13 respectively. Similar to IL-6, both the differentiated and undifferentiated cells produced IL-10 in response to ESP by 24 hrs. IL-10 levels from the differentiated cells were significantly higher in response to both ESP and ConA compared to BSA and RPMI induced levels ($p < 0.001$, Figure 2. 12). However, there were no significant differences in IL-10 levels between the two

chambers. The undifferentiated IPEC-1 cells also produced significant levels of IL-10 in response to ConA and ESP but not in response to BSA and RPMI at both 12 hr and 24 hr ($p < 0.001$, Figure 2. 13).

IL-4 ELISA

Unlike IL-6 and IL-10, there was no IL-4 produced either from differentiated or from undifferentiated IPEC-1 cells in response to *T. suis* ESP, at both time points tested (data not shown). IL-4 production, if any, was below the detection limit of the assay.

RT-PCR for IL-6 mRNA at 0 hr

Since IL-6 secretion was seen within minutes of infection (if not seconds) in *C. jejuni* infected cells, (Figure 2. 7), RT-PCR for IL-6 mRNA was conducted to determine if the secretion was due to transcription. IL-6 mRNA was seen in samples treated with RPMI, LPS, *C. jejuni* strains 33292 and 81176, and there were no differences in mRNA bands irrespective of treatment groups (Figure 2.14). RT-PCR for β_2 microglobulin mRNA also showed similar band intensities for all treatment groups, indicating that total mRNA concentrations were similar.

Effect of CHX on IL-6 secretion at 0 hr

Experiments were conducted to determine if the IL-6 secretion into the culture supernatants at 0 hr was due to rapid translation of mRNA. Cycloheximide, an inhibitor of translation, was added in increasing concentrations to determine if there is a decrease in secretion, and if so, whether it is dose dependent. As seen in Figure 2. 15, there was a

decrease in secretion of IL-6 into the apical chamber with the addition of CHX (100, 500 and 1000 μg/ml) with *C. jejuni*. However, there was no dose dependent effect, that is, no significant suppression in IL-6 secretion in response to *C. jejuni* in cells treated with increasing concentrations of CHX. Cycloheximide alone did not elicit IL-6 secretion as RPMI containing the various concentrations of CHX showed no significant increase in IL-6. The decrease in IL-6 secretion in response to CHX treatment was determined not to be a cytotoxic effect of CHX on *C. jejuni*, as validated by viability assays (Figure 2.16). No significant effect of CHX on *C. jejuni* viability were seen either at 0 hr or 2.5hr. Also, inhibition of protein synthesis did not completely abolish secretion as significant amounts of IL-6 were seen in the culture supernatants even after addition of 1000 μg/ml of CHX with *C. jejuni*.

Immunoblot analysis

The results of immunoblot analysis for preformed IL-6 protein in IPEC-1 cells not exposed to any treatment, is seen in Figure 2. 17. Preformed IL-6 protein was seen in whole cell extracts of IPEC-1 cells at the two different total protein concentrations loaded (100 and 250 μg/ml, lanes 3 and 4 respectively, Figure 2. 17). A faint band associated with the cell pellet was also seen; however the band intensity was not as significant as the ones from whole cell extracts (Figure 2. 17, lane 6). The lysis buffer, included as a negative control for proteins, did not show any band as expected (lane 2).

DISCUSSION

Cytokines play a vital role in host defense mechanisms against a variety of infections. The type and amount of cytokines produced by the host varies in response to different infections and also varies within the host according to the tissue types infected (35, 37, 41). Variations can also be host-specific, wherein different mammalian species respond differently to infection with the same organism (10). Hence, it is vital to determine the pattern of secretion of cytokines from a particular tissue in a host-parasite combination, before conclusions about immunoregulation can be drawn. In this study, we examined the ability of IPEC-1 cells to secrete Th-2 cytokines IL-4, IL-6 and IL-10 in response to *C. jejuni* infection and *T. suis* ESP. We hypothesized that infection with *C. jejuni* would drive a predominantly Th-1 cytokine response from the host. However, the *C. jejuni* specific immune response should still have some Th-2 component because IgA has been documented following the infection (32). This Th-2 response may be synergistic with a similar response induced by *T. suis*, resulting in Th-2-mediated pathology. We decided to examine the IL-4, IL-6 and IL-10 responses from swine intestinal epithelial cells, because host pathology is largely confined to the intestinal tract and because epithelial cells are the first major barriers encountered by these enteric pathogens.

Since IPEC-1 is a primary undifferentiated cell-line, we verified its state of differentiation prior to experimentation. The SEM photographs indeed verified their differentiated state when cultured according to our protocol (Figure 2. 1). Also, the sensitivity of ELISA assays were validated by the use of regression curves, and we found that IL-10 ELISA was much more sensitive in our estimate and could detect ~ 1 pg/ml IL-10 compared to the 3 pg/ml sensitivity declared by the manufacturer (Figure 2. 3). IL-

6 and IL-4 ELISAs showed similar regression curves, suggesting that these assays are very sensitive to low concentrations of IL-6 and IL-4 respectively (Figure 2. 2 and Figure 2. 4). Hence, the cytokine concentrations obtained from experimental results were used without bias.

Both *C. jejuni* and *T. suis* ESP elicited Th2 cytokine production from IPEC-1 cells. IL-6 and IL-10 were both secreted by *C. jejuni* and *T. suis* ESP, though at different levels and at different times after infection. IL-6 was the predominant cytokine produced in terms of quantity, followed by IL-10. Although IL-4 is typically induced in T-cells by helminth infections (23), and has not been shown to be secreted by epithelial cells, we decided to measure IL-4 to exclude the possibility that these cells had this potential. However, not surprisingly, secretion levels of IL-4, if any, were below the detection limits of our assay.

IL-6 was secreted in response to both *C. jejuni* and ESP, and IL-6 secretion levels induced by the two agents, were not dissimilar. Also, IL-6 was secreted within a similar time frame (i.e., < 24 hr) with respect to these treatments. While IL-6 levels secreted by ESP were much more consistent, the levels secreted by *C. jejuni* varied between isolates (Figure 2. 5, Figure 2. 6 and Figure 2. 7). However, there were no significant differences in the IL-6 levels secreted by the two different strains of *C. jejuni*. Recently, Jones et al. (24) described infection of a THP-1 monocytic cell line with *C. jejuni* and found IL-6 induction at picogram/ml levels by 24 hr. In our study we found that IPEC-1 cells were an even greater source of IL-6, expressing this cytokine at nanogram levels within a few minutes. In addition, the MOI of *C. jejuni* used in this study is likely more physiologically relevant than the MOI of 100:1or more normally used in other studies (2,

24). However, since other cell lines studied so far have been of human origin, and IPEC-1 is of swine origin, the optimal dose of *C. jejuni* infections might vary between species.

Since *C. jejuni* is invasive, and since epithelial cells are likely to be exposed to this bacterium on both the apical and/or baso-lateral surfaces, infection with *C. jejuni* was carried out on both epithelial surfaces. We found that exposure of either the apical or the baso-lateral surface to *C. jejuni* resulted in IL-6 secretion towards that particular surface, similar to results obtained in another study. Kishikawa et al (27) found that application of transmural pressure on either apical or baso-lateral side of epithelial cells resulted in IL-6 secretion towards that particular surface. This is an important determination for any system, especially if autocrine and paracrine activities of cytokines in immunomodulation are to be analyzed. Here, the ability of the IPEC-1 cells to secrete IL-6 both apically and baso-laterally suggests that while the apical IL-6 might recruit other mediators, the basolaterally secreted IL-6 might have paracrine effects on the underlying tissues. Some of the IL-6 seen in the baso-lateral chamber during apical infection (Figure 2. 6A) could have been due to gravity, as cells seeded on the lower side of transwell inserts did not show a significant level of IL-6 on the baso-lateral side when the infection was apical (data not shown).

IL-10 was also secreted in IPEC-1cells in response to both *C. jejuni* and *T. suis* ESP, and the levels were similar to each other, similar to their IL-6 comparison. While IL-10 secretion by ESP was relatively fast (< 24 hr), IL-10 secretion by *C. jejuni* was much slower, (> 24 hr) similar to that seen by Colgan et al., (9) who found that IL-10 levels peaked at 72 hr post CD1d (a surface receptor) ligation. This suggests that the mechanism of IL-10 induction by *C. jejuni* and *T. suis* ESP might follow different

pathways, and also suggests that while the primary immune response to ESP includes both IL-6 and IL-10 (as evidenced by the time frame), for *C. jejuni* it is predominantly IL-6, with a later secretion of IL-10. However, while *C. jejuni* 33292 induced IL-10 secretion both apically and baso-laterally, the response to *C. jejuni* 81176 was predominantly apical with no baso-lateral secretion, reinforcing the idea that variations exist in host cell responses to different *Campylobacter* strains. The presence of this predominantly apical IL-10 could lead to autocrine effects since epithelial cells have been shown to possess IL-10 receptors (3, 9).

Since some cytokines are produced from cells in a differentiated state and not in an undifferentiated state, we also tested to see if crypt-like cells could produce these cytokines in response to *C. jejuni* and *T. suis* ESP challenge. We found that IL-6 was secreted by undifferentiated IPEC-1 cells in response to *C. jejuni*, but not IL-10 or IL-4. This is contrary to the results reported by Al Salloom et al., (2) who found IL-4 and IL-10 intracellular cytokine production from 3–5 day old INT407 cells, a human intestinal epithelial cell line, by 24 hr in response to *C. jejuni*. However, this contrast might be due to the fact that they measured intracellular cytokines rather than secreted cytokines as in our study. Both IL-6 and IL-10 were induced from undifferentiated IPEC-1 cells in response to *T. suis* ESP, but not IL-4. In fact, we found no evidence of IL-4 secretion in our system, at any of the times tested, with neither *C. jejuni* or *T. suis*, nor with either states of cell differentiation.

Another interesting finding in this study was the short time span for IL-6 secretion by the epithelial cells. IL-6 was seen in the culture supernatants within minutes, if not seconds of *C. jejuni* exposure, but not in the medium only controls (Figure 2. 7). While

the processing time between removal of supernatant and storage (10–15 min) could account for some of the protein secretion, it is still relatively rapid compared to that reported in other cell lines. We tested the possibility that this rapid secretion could be due to transcription of IL-6 mRNA. However, as seen in Figure 2. 14, mRNA was seen in RPMI medium treatments as well, and there were no difference in band intensities with any treatments, suggesting no new transcription. This is in accordance with other results, where IL-6mRNA transcription has been shown to occur only after 30 minutes or more (40). Next, we tested the possibility that translation of this preexisting IL-6 mRNA could account for this rapid secretion. However, while translation accounted for some of the secretion, it could not account for the entire amount seen, as significant levels were still secreted even after inhibition of translation with CHX (Figure 2. 15). And this inhibition of translation was not due to a cytotoxic effect of CHX on *C. jejuni* (Figure 2. 16). However, these levels could be accounted for by the presence of preformed IL-6, as evidenced by Western blot analysis (Figure 2. 17). Significant quantities of IL-6 were seen within IPEC-1 cells, indicating that the rapid IL-6 secretion in response to *C. jejuni* (and *T. suis* ESP) was due to a combination of translation and release of stored IL-6. This appears to be the first demonstration of stored IL-6 cytokine in intestinal epithelial cells. Since these intestinal cells are the first barriers encountered by intestinal pathogens, the production and storage of this immune mediator might provide the host with an immediate defense against enteric infections.

The role of epithelial cells as an important source and initiator of enteric immune responses is a recent concept. Since epithelial cells cover a large surface area, secretion of even picogram amounts of cytokines could make a significant contribution to the overall

immune response. IPEC-1 is a non-immortalized cell line, hence the pattern of secretion should be much more reflective of the physiology in the host than that represented by an immortalized cell line. While IL-10 has been documented to act as an anti-inflammatory cytokine, whether IL-6 behaves in a proinflammatory or anti-inflammatory manner in terms of nitric oxide modulation remains to be tested in our system. Also, as IPEC-1 cells did not secrete IL-4, the question of whether *C. jejuni* or *T. suis* ESP elicits IL-4 from IL-4 competent cells like macrophages or T-cells still remains to be investigated. However, the production of IL-6 and IL-10 by IPEC-1 in response to *C. jejuni* and *T. suis* ESP challenge supports the hypothesis that the concurrent presence of *C. jejuni* and *T. suis* in swine may lead to a Th-2-like environment with subsequent Th-2-mediated pathology. This is also strengthened by the fact that concurrent studies conducted in the laboratory show no induction of IL-1β or IFNγ secretion from (undifferentiated) IPEC-1 cells in response to *C. jejuni* infection by 24 hr (Cunningham L. D and L.S. Mansfield, unpublished data). Because IL-6 was secreted within a similar time frame with respect to these two pathogens, there might be synergy in IL-6 levels *in vivo*. Furthermore, the production of these cytokines and storage of preformed IL-6 within these cells suggest that epithelial cells play a vital role in the induction of inflammation during pathogenic infections in swine.

ACKNOWLEDGEMENTS

This work was supported by the NIH-grant, 61-0954, and the Center for Emerging Diseases grant 61-6387. We thank Dr. Julia Bell for critical review of this article.

REFERENCES

1. **Abner, S. R., D. E. Hill, J. R. Turner, E. D. Black, P. Bartlett, J. F. Urban, and L. S. Mansfield.** 2002. Response of intestinal epithelial cells to *Trichuris suis* excretory-secretory products and the influence on *Campylobacter jejuni* invasion under in vitro conditions. J Parasitol **88:**738-45.

2. **Al-Salloom, F. S., A. Al Mahmeed, A. Ismaeel, G. A. Botta, and M. Bakhiet.** 2003. Campylobacter-stimulated INT407 cells produce dissociated cytokine profiles. J Infect **47:**217-24.

3. **Autschbach, F., J. Braunstein, B. Helmke, I. Zuna, G. Schurmann, Z. I. Niemir, R. Wallich, H. F. Otto, and S. C. Meuer.** 1998. In situ expression of interleukin-10 in noninflamed human gut and in inflammatory bowel disease. Am J Pathol **153:**121-30.

4. **Beer, R. J.** 1973. Studies on the biology of the life-cycle of *Trichuris suis* Schrank, 1788. Parasitology **67:**253-62.

5. **Beer, R. J., B. F. Sansom, and P. J. Taylor.** 1974. Erythrocyte losses from pigs with experimental *Trichuris suis* infections measured with a whole-body counter. J Comp Pathol **84:**331-46.

6. **Berschneider, H. M.** 1989. Development of normal cultured small intestinal epithelial cell lines which transport Na and Cl. Gastroenterology **96:**A41.

7. **Blaser, M. J., D. N. Taylor, and R. A. Feldman.** 1983. Epidemiology of *Campylobacter jejuni* infections. Epidemiol Rev **5:**157-76.

8. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:**248-54.

9. **Colgan, S. P., R. M. Hershberg, Ç. T. Furuta, and R. S. Blumberg.** 1999. Ligation of intestinal epithelial CD1d induces bioactive IL-10: critical role of the cytoplasmic tail in autocrine signaling. Proc Natl Acad Sci U S A **96:**13938-43.

10. **Crabtree, J. E., M. Court, M. A. Aboshkiwa, A. H. Jeremy, M. F. Dixon, and P. A. Robinson.** 2004. Gastric mucosal cytokine and epithelial cell responses to *Helicobacter pylori* infection in Mongolian gerbils. J Pathol **202:**197-207.

11. **de Man, P., C. van Kooten, L. Aarden, I. Engberg, H. Linder, and C. Svanborg Eden.** 1989. Interleukin-6 induced at mucosal surfaces by gram-negative bacterial infection. Infect Immun **57:**3383-8.

12. **Dimock, K. A., D. G. Addiss, M. L. Eberhard, and P. J. Lammie.** 1994. Differential proliferative and interleukin-10 responses to fractionated filarial antigens: preferential recognition by patients with chronic lymphatic dysfunction. J Infect Dis **170:**403-12.

13. **Finkelman, F. D., E. J. Pearce, J. F. Urban, Jr., and A. Sher.** 1991. Regulation and biological function of helminth-induced cytokine responses. Immunol Today **12:**A62-6.

14. **Fireman, P.** 2003. Understanding asthma pathophysiology. Allergy Asthma Proc **24:**79-83.

15. **Friedman, C., Neimann,J, Wegener,HC and Tauxe,RV.** 2000. Epidemiology of *Campylobacter jejuni* infections in the United States and other Industrialized Nations, p. 121-138. *In* N. I. a. B. M (ed.), Campylobacter. ASM.

16. **Gonzalez-Vallina, R., H. Wang, R. Zhan, H. M. Berschneider, R. M. Lee, N. O. Davidson, and D. D. Black.** 1996. Lipoprotein and apolipoprotein secretion by a newborn piglet intestinal cell line (IPEC-1). Am J Physiol **271:**G249-59.

17. **Hatzigeorgiou, D. E., S. He, J. Sobel, K. H. Grabstein, A. Hafner, and J. L. Ho.** 1993. IL-6 down-modulates the cytokine-enhanced antileishmanial activity in human macrophages. J Immunol **151:**3682-92.

18. **Hedges, S., P. Anderson, G. Lidin-Janson, P. de Man, and C. Svanborg.** 1991. Interleukin-6 response to deliberate colonization of the human urinary tract with gram-negative bacteria. Infect Immun **59:**421-7.

19. **Hickey, T. E., S. Baqar, A. L. Bourgeois, C. P. Ewing, and P. Guerry.** 1999. *Campylobacter jejuni*-stimulated secretion of interleukin-8 by INT407 cells. Infect Immun **67:**88-93.

20. **Hill, D. E., H. R. Gamble, M. L. Rhoads, R. H. Fetterer, and J. F. Urban, Jr.** 1993. *Trichuris suis*: a zinc metalloprotease from culture fluids of adult parasites. Exp Parasitol **77:**170-8.

21. **Hirano, T., and T. Kishimoto.** 1989. Interleukin-6: possible implications in human diseases. Ric Clin Lab **19:**1-10.

22. **Janeway, C. T, Walport, M and Capra JD.** 1999. T-cell mediated immunity, p. 7:1- 7:41. *In* M. a. L. Robertson, E (ed.), Immunobiology-The Immune system in health and disease, Second ed. Current Biology Ltd and Garland Publishing Co

23. **Janeway, C. T., P; Walport,M and Capra JD.** 1999. The Humoral Immune Response, p. 307-362. *In* P. a. L. Austin, E (ed.), Immuno Biology-The Immune

System in Health and Disease, Four ed. Current Biology Publications and Garland Publishing.

24. **Jones, M. A., S. Totemeyer, D. J. Maskell, C. E. Bryant, and P. A. Barrow.** 2003. Induction of proinflammatory responses in the human monocytic cell line THP-1 by *Campylobacter jejuni*. Infect Immun **71:**2626-33.

25. **Kealy, W. F.** 1976. Colonic lymphoid-glandular complex (microbursa): nature and morphology. J Clin Pathol **29:**241-4.

26. **Ketley, J. M.** 1997. Pathogenesis of enteric infection by *Campylobacter*. Microbiology **143 (Pt 1):**5-21.

27. **Kishikawa, H., S. Miura, H. Yoshida, M. Hirokawa, H. Nakamizo, H. Higuchi, M. Adachi, R. C. Nakatsumi, H. Suzuki, H. Saito, and H. Ishii.** 2002. Transmural pressure induces IL-6 secretion by intestinal epithelial cells. Clin Exp Immunol **129:**86-91.

28. **Lawrence, C. E., J. C. Paterson, L. M. Higgins, T. T. MacDonald, M. W. Kennedy, and P. Garside.** 1998. IL-4-regulated enteropathy in an intestinal nematode infection. Eur J Immunol **28:**2672-84.

29. **Liebler, E. M., J. F. Pohlenz, and N. F. Cheville.** 1988. Gut-associated lymphoid tissue in the large intestine of calves. II. Electron microscopy. Vet Pathol **25:**509-15.

30. **Mansfield, L. S., D. T. Gauthier, S. R. Abner, K. M. Jones, S. R. Wilder, and J. F. Urban.** 2003. Enhancement of disease and pathology by synergy of *Trichuris suis* and *Campylobacter jejuni* in the colon of immunologically naive swine. Am J Trop Med Hyg **68:**70-80.

31. **Mansfield, L. S., and J. F. Urban, Jr.** 1996. The pathogenesis of necrotic proliferative colitis in swine is linked to whipworm induced suppression of mucosal immunity to resident bacteria. Vet Immunol Immunopathol **50:**1-17.

32. **Mansfield, L. S. a. D. G.** 2004. Lymphoglandular complexes are important colonic sites for IgA induction against *Campylobacter jejuni* in a swine disease model. Comparative medicine.

33. **Marcelo, P., and F. Lefevre.** 2002. Induction of the IFN-gamma gene and protein is linked to the establishment of cell polarity in a porcine epithelial cell line. Exp Cell Res **280:**33-44.

34. **Morfitt, D. C., and J. F. Pohlenz.** 1989. Porcine colonic lymphoglandular complex: distribution, structure, and epithelium. Am J Anat **184:**41-51.

35. **Mukherjee, P., A. K. Ghosh, and A. C. Ghose.** 2003. Infection pattern and immune response in the spleen and liver of BALB/c mice intracardially infected with *Leishmania donovani* amastigotes. Immunol Lett **86:**131-8.

36. **Napolitano, L. M., M. M. Buzdon, H. J. Shi, and B. L. Bass.** 1997. Intestinal epithelial cell regulation of macrophage and lymphocyte interleukin 10 expression. Arch Surg **132:**1271-6.

37. **Nedrebo, T., R. K. Reed, R. Jonsson, A. Berg, and H. Wiig.** 2004. Differential cytokine response in interstitial fluid in skin and serum during experimental inflammation in rats. J Physiol.

38. **O'Leary, A. D., and E. C. Sweeney.** 1986. Lymphoglandular complexes of the colon: structure and distribution. Histopathology **10:**267-83.

39. **Reddy, N. R., B. N. Wilkie, and B. A. Mallard.** 1996. Construction of an internal control to quantitate multiple porcine cytokine mRNAs by RT-PCR. Biotechniques **21:**868-70, 872, 875.

40. **Zhang, J., K. Xu, B. Ambati, and F. S. Yu.** 2003. Toll-like receptor 5-mediated corneal epithelial inflammatory responses to *Pseudomonas aeruginosa* flagellin. Invest Ophthalmol Vis Sci **44:**4247-54.

41. **Zhang, J. Z., M. Sinha, B. A. Luxon, and X. J. Yu.** 2004. Survival strategy of obligately intracellular *Ehrlichia chaffeensis*: novel modulation of immune response and host cell cycles. Infect Immun **72:**498-507.

Figure 2. 1. Scanning electron micrograph of polarized IPEC-1 cells (12 days) on transwell membranes. Top panel is overview of cells; bar represents 20 μm. The apical finger-like microvilli are seen in the bottom panel; bar represents 2 μm. Micrographs are representative of three samples each.

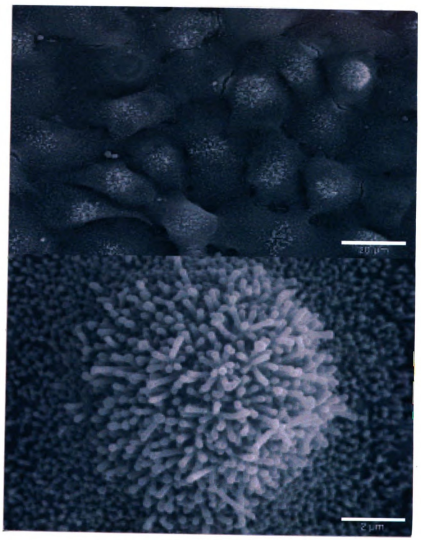


Figure 2. 2. Standard curve for swine IL-6 used in the ELISA assays. Curve is representative of multiple experiments (~10).

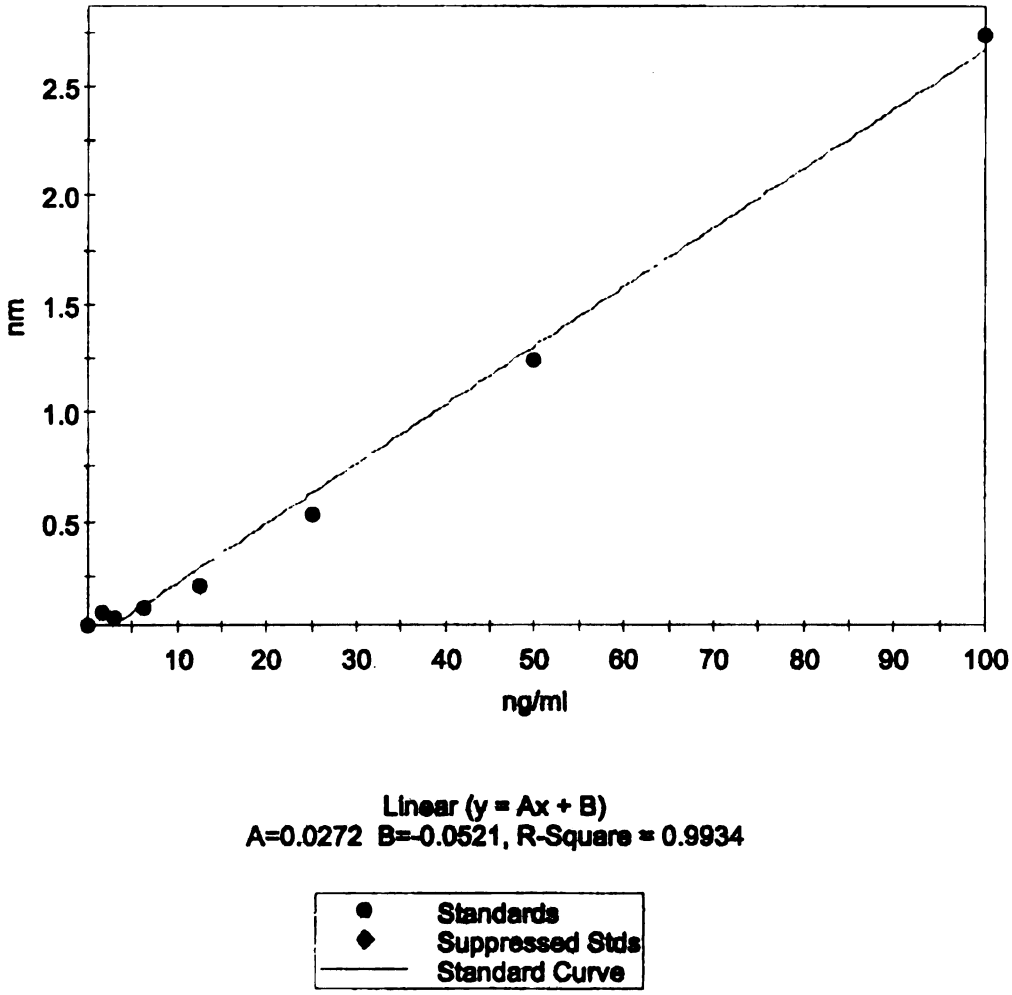
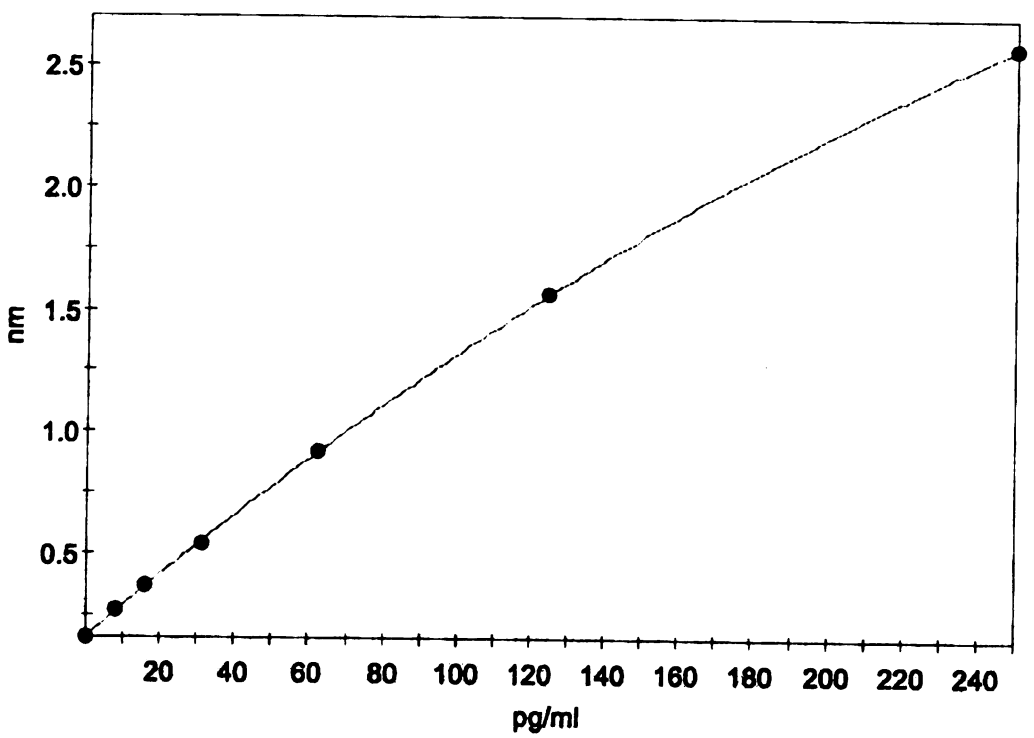


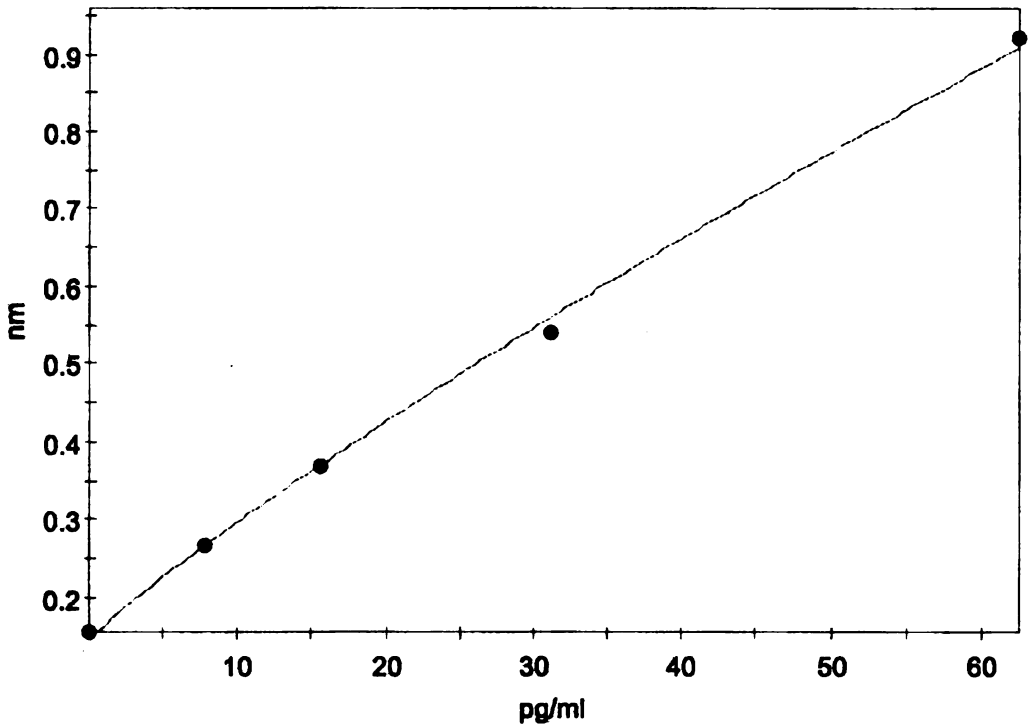
Figure 2. 3. Standard IL-10 curve used in ELISA assays. Figure 2. 3A is the entire standard range, and Figure 2. 3B is the magnification of the range from 0 to 60 pg/ml in Figure 2. 3A. Curve is representative of multiple experiments (~10).

Figure 2. 3A



4 Parameter (y = (A - D) / (1 + (x/C)^B) + D)
A=0.1611 B=1.0217 C=580.5951 D=8.3169, R-Square = 0.9999

Figure 2. 3B



4 Parameter (y = (A - D) / (1 + (x/C)^B) + D)
A=0.1437 B=0.8811 C=12098.715 D=80.0527, R-Square = 0.9981

Figure 2. 4. Standard curve for swine IL-4 used in ELISA. The curve is representative of multiple experiments (3-4).

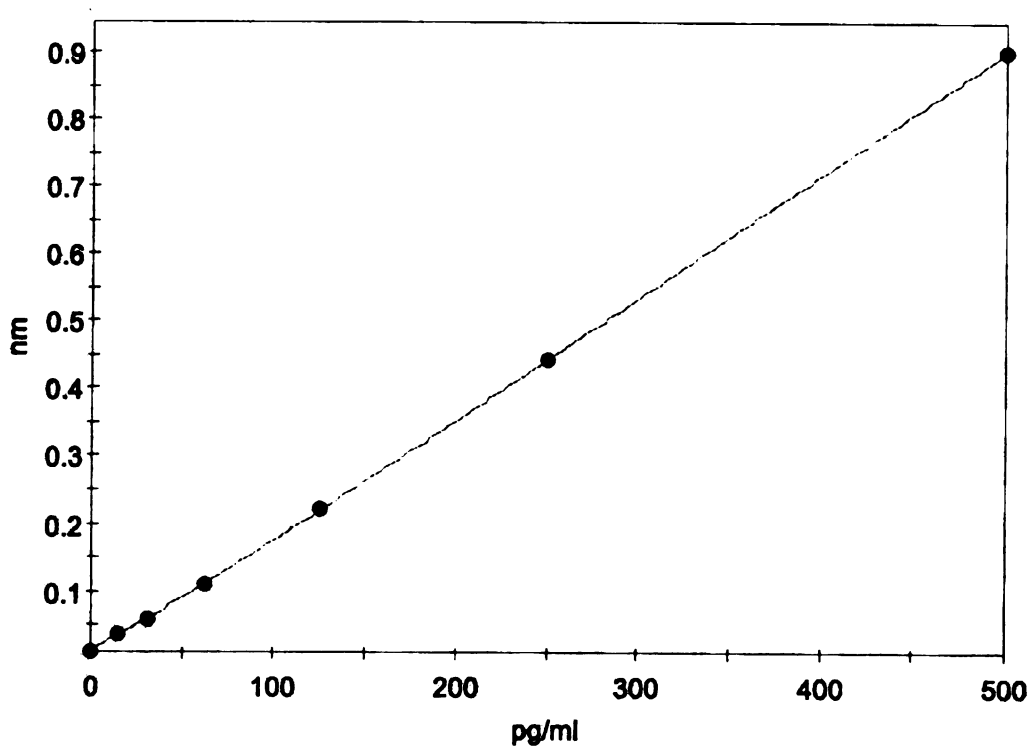


Figure 2. 5. Effect of different MOI on IL-6 expression from IPEC-1 cells. 12-14-day-old IPEC-1 cells were infected apically with different MOI (4:1, 40:1, 100: 1 and 200:1) of *C. jejuni* 33292 for 8, 12 and 24 hr, and supernatants from both chambers were analyzed for IL-6 by indirect ELISA. *E. coli* O26:B6 LPS (200 μg/ml) was used as a positive control and RPMI medium as the negative control. All treatments were performed in triplicate, and are representative of two identical experiments.

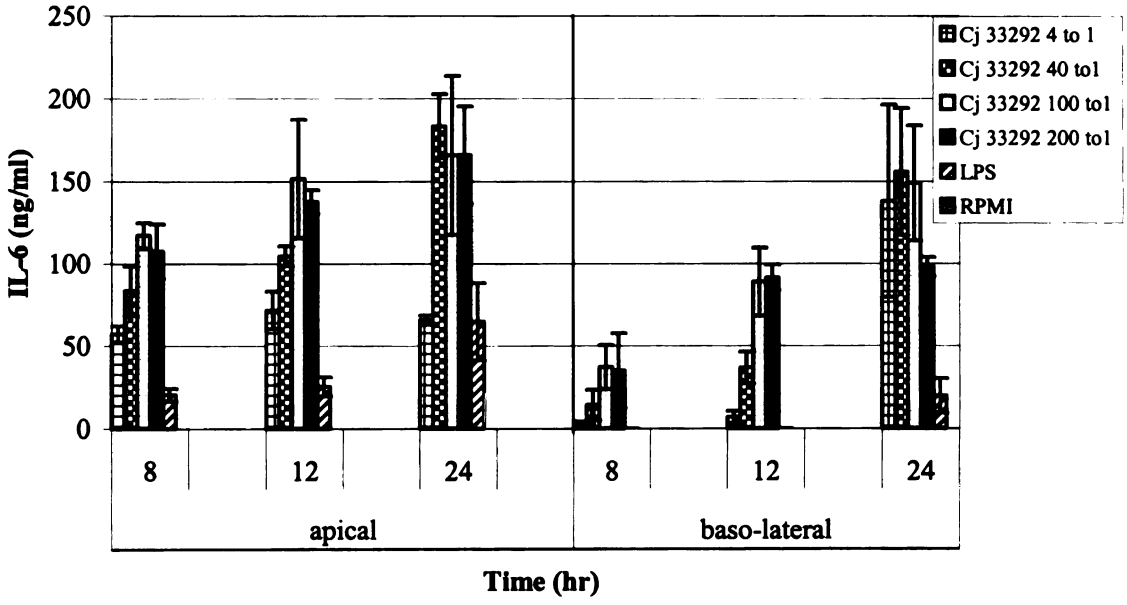


Figure 2. 6. The secretion of IL-6 due to apical (6A) and baso-lateral (6B) *C. jejuni* (Cj) infections is shown. Differentiated IPEC-1 cells were infected with the optimum MOI of *C. jejuni* (40:1) obtained from the experiment shown in Figure 2. 5. The results shown are representative of three identical experiments for Figure 2. 6A, and two identical experiments for Figure 2. 6B, with three replicates in each group. ND- not determined.

Figure 2. 6A

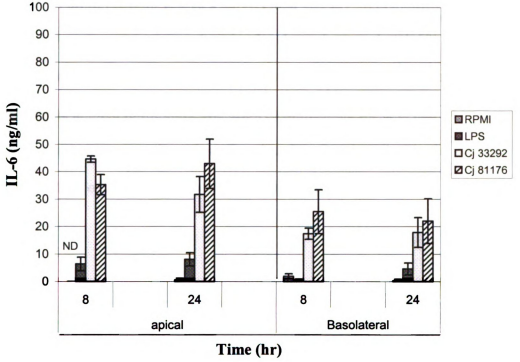


Figure 2. 6B

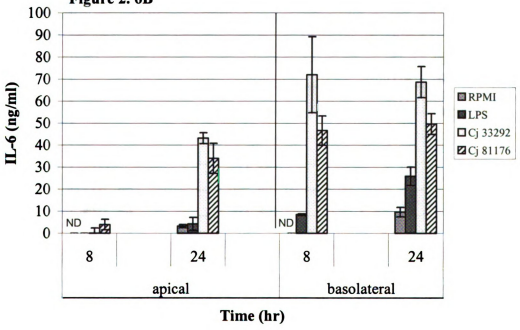


Figure 2. 7. IL-6 secretion from 2-day old undifferentiated IPEC-1 cells. 3 x 10^5 cells were seeded on fibronectin-coated 24-well plates and grown to confluency for 2 days in RPMI 1640 medium containing phenol red and supplemented with 5% FBS. The treatments were suspended at optimal concentrations in RPMI 1640 medium without any supplements, applied to the cells, and allowed to incubate for 0, 8, and 24 hr. Supernatants were removed, processed, and analyzed as described in the materials and methods. Mean of 2 identical experiments is shown. The values shown are mean +/- SEM.

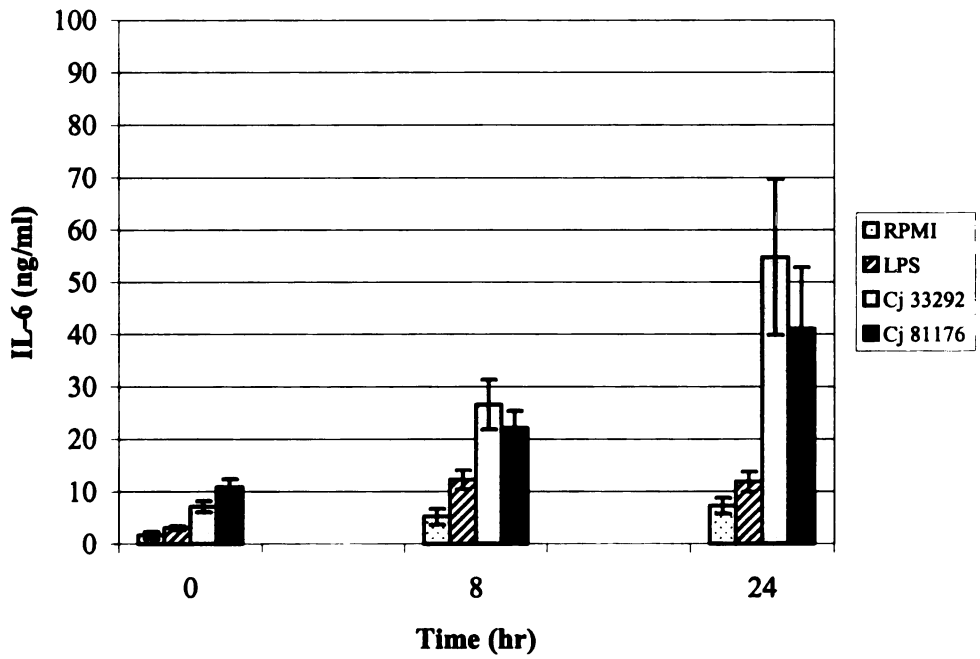


Figure 2. 8 shows IL-10 expression from IPEC-1 cells in response to *C. jejuni* (Cj) infection apically (A) and baso-laterally (B), respectively. Con A (100 μg/ml) served as the positive control and RPMI 1640 medium served as the negative control. The values are mean of two (apical infection) and three (basolateral infection) identical experiments respectively, and mean +/- SEM is shown. ND- not determined.

Figure 2. 8A

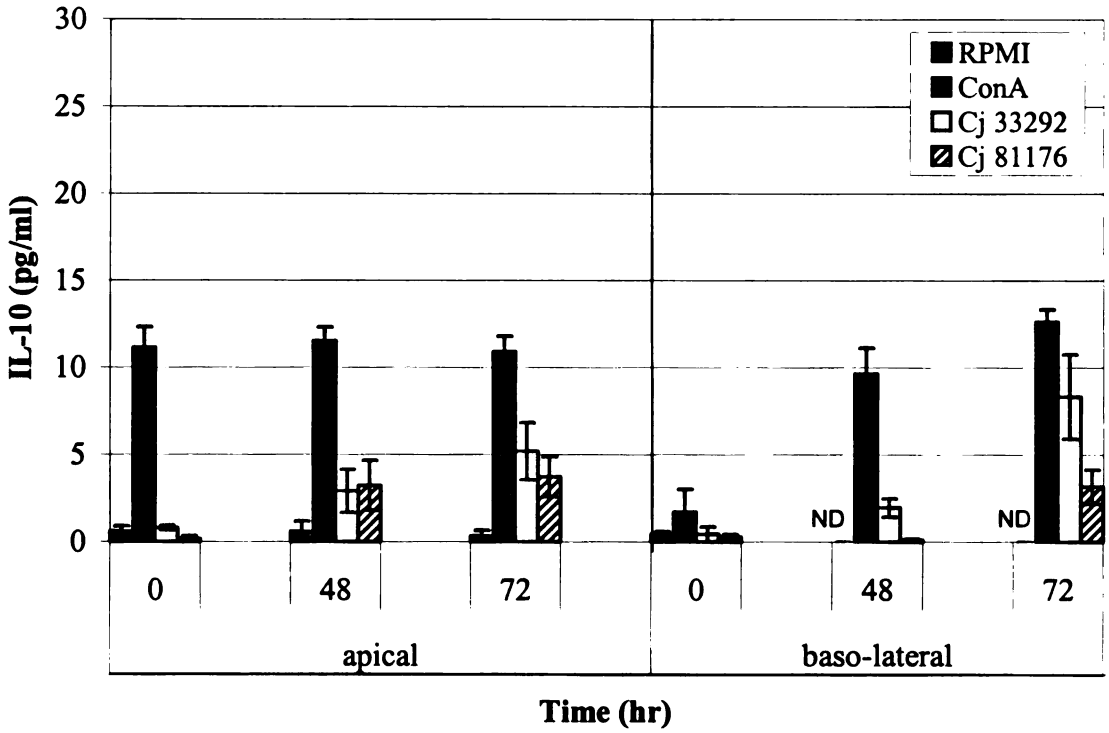


Figure 2. 8B

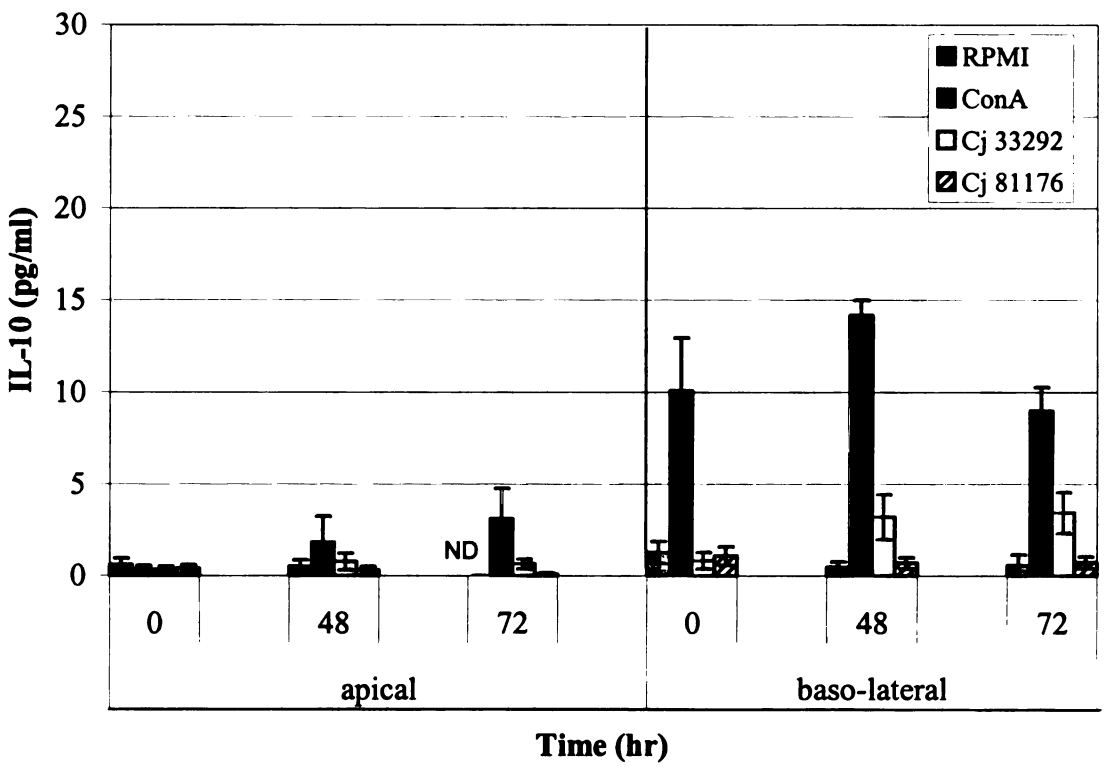


Figure 2. 9. IL-10 expression from undifferentiated IPEC-1 cells in response to *C. jejuni* (Cj) infection. 2 day-old IPEC-1 cells were challenged with optimal dose of *C. jejuni* for 0, 48 and 72 hr, supernatants removed, and analyzed for IL-10 by a sandwich ELISA. Results shown are the mean of three identical experiments. Values are mean +/- SEM.

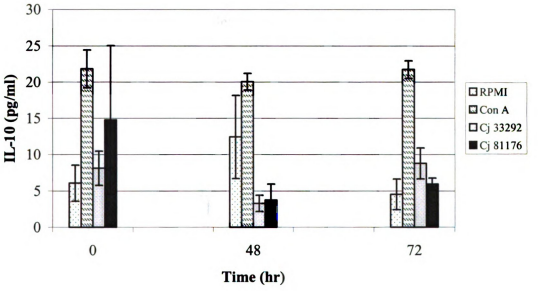


Figure 2. 10. IL-6 secretion from differentiated IPEC-1 cells in response to Excretory Secretory Products (ESP) of *T. suis*. 10-14 day old IPEC-1 cells on porous supports were exposed apically to 0.3 mg/ml of the ESP, *E. coli* LPS (200 μg/ml), BSA (0.3 mg/ml) and RPMI medium. LPS served as the positive control, while BSA and RPMI medium served as negative controls for IL-6 secretion. Supernatants were extracted as described in the materials and methods and analyzed for IL-6 cytokine expression by indirect ELISA. All treatments were in triplicate. Values shown are mean +/- SEM, and are representative of two identical experiments. ND- not determined.

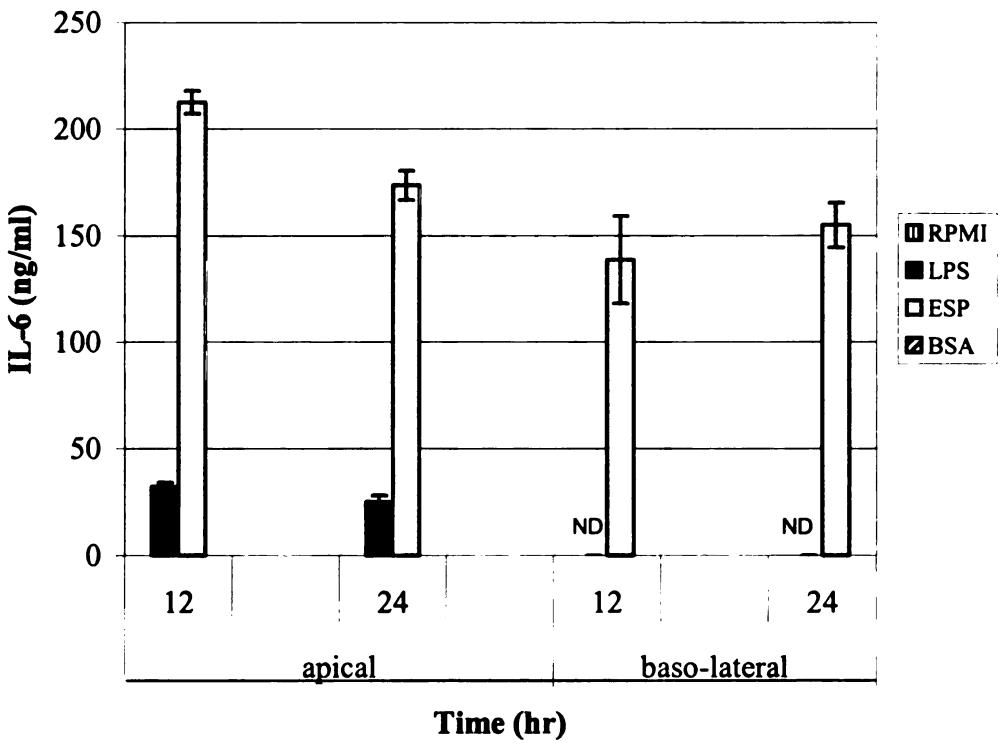


Figure 2. 11. Two-to-three day-old IPEC-1 cells grown to confluency were challenged with 0.3 mg/ml of *T. suis* ESP, 200 μg/ml of LPS, BSA (0.3 mg/ml) and RPMI 1640 medium for 12 and 24 hr. The supernatants were collected as described and analyzed by an indirect ELISA for IL-6 secretion. Values shown are mean +/- SEM, and representative of two identical experiments.

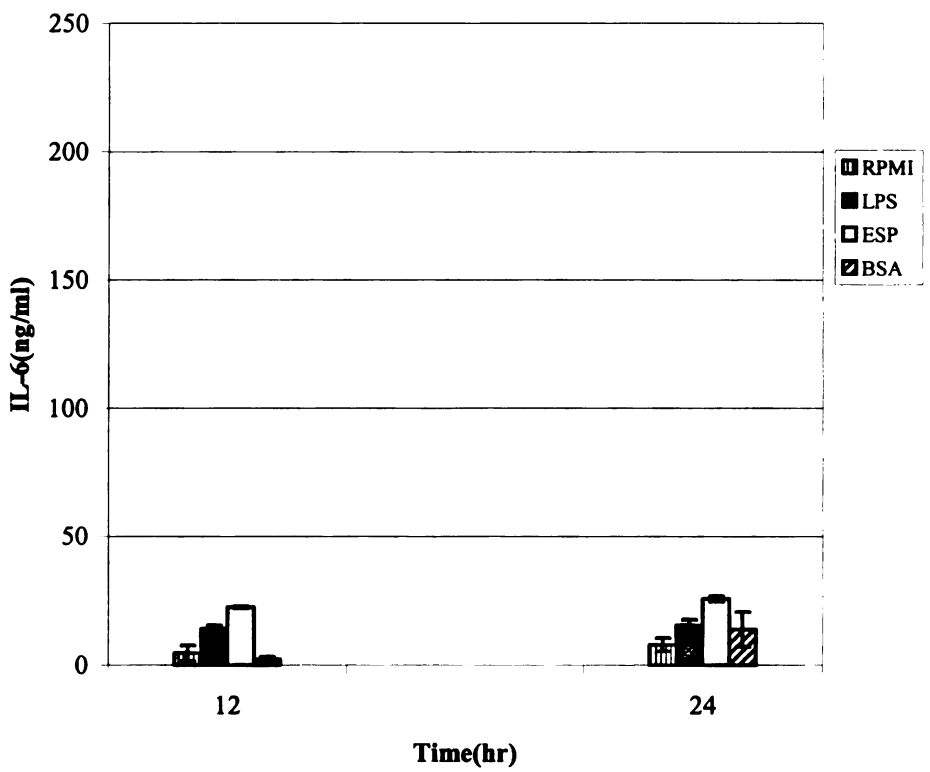


Figure 2. 12. *T. suis* ESP (0.3mg/ml) induced IL-10 expression from differentiated IPEC-1 cells. Confluent IPEC-1 cells on Transwell membranes were exposed apically to *T. suis* ESP (0.3 mg/ml), RPMI, Concanavalin A (ConA), and BSA for 12 and 24 hr. ConA (100 μg/ml) served as the positive control for IL-10 expression while BSA (0.3 mg/ml) and RPMI medium served as the negative controls. A sandwich ELISA was used to analyze the supernatants for IL-10 secretion. Values shown are mean +/- SEM, and representative of two identical experiments.

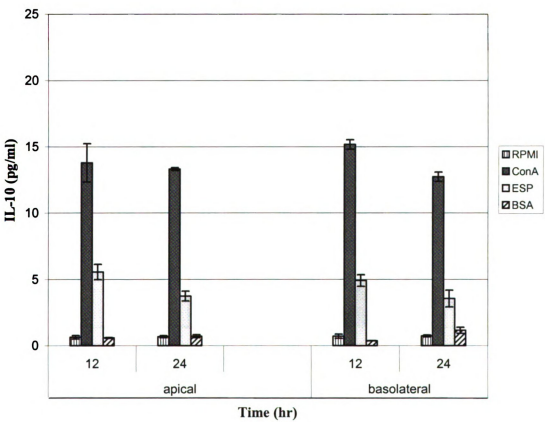


Figure 2. 13. The effect of *T. suis* ESP on IL-10 expression from undifferentiated (crypt-like) IPEC-1 cells is shown. As before, two-to-three day-old IPEC-1 cells were challenged with ConA (100 μg/ml), ESP (0.3 mg/ml), BSA (0.3 mg/ml) and RPMI 1640 medium for 12 and 24 hr, supernatants removed after the indicated time intervals and later analyzed for IL-10 secretion. All treatments were in triplicate, and are representative of two identical experiments. ND- not determined.

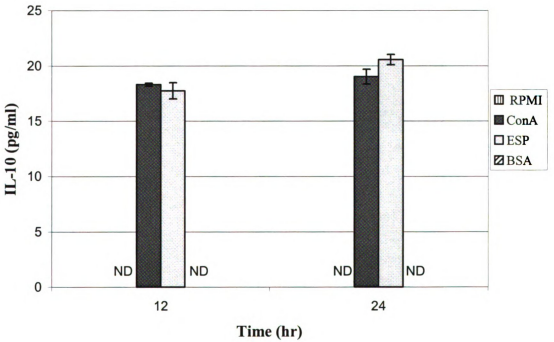


Figure 2. 14. RT-PCR for IL-6 mRNA at 0 hr. RNA was extracted from 12 day-old IPEC-1 cells on Transwells, at 0 hr. cDNA was made from 2 μg of total RNA for each sample. PCR was performed for IL-6 cytokine and the constitutively expressed β2 microglobulin. **Top gel** is RT-PCR for IL-6 mRNA, **bottom gel** for β2 microglobulin mRNA. Lane 1- 100 base pair ladder; lanes 2-3- RPMI treated IPEC-1; Lanes 4-5-LPS treated cells; Lanes 6-7- *C. jejuni* 33292 treated cells; lanes 8-9- *C. jejuni* 81176 treated cells. Lane 10- no RNA control. All treatments were in duplicate. Bands are representative of two identical and independent experiments.

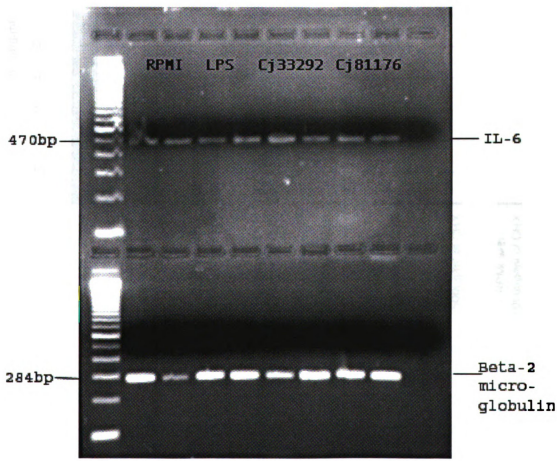


Figure 2. 15. Effect of cycloheximide on IL-6 secretion from IPEC-1 cells, in response to *C. jejuni* 33292 and RPMI 1640 medium. The bacteria were treated with or without different concentrations of cycloheximide (100, 500 and 1000 μg/ml) at 0 hr. Supernatants were removed immediately, processed as described previously, and analyzed for IL-6 by ELISA. RPMI medium was treated similarly and used as control. Values shown are mean +/- SEM, and are representative of two identical experiments.

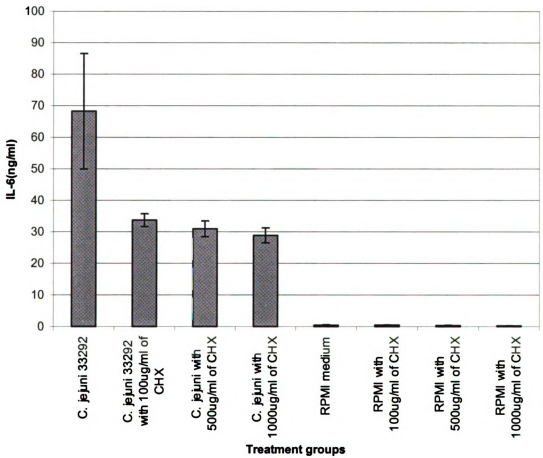


Figure 2. 16. Effect of CHX on viability of *C. jejuni*. Strain 33292 was incubated with 100 μg/ml of CHX for ~ a minute (0 hr) or 2.5 hrs. Bacterial cells were diluted by limiting dilutions, plated and enumerated on Bolton agar plates for 48 hr at 37 C and 5% CO_2. Results are data from a single experiment.

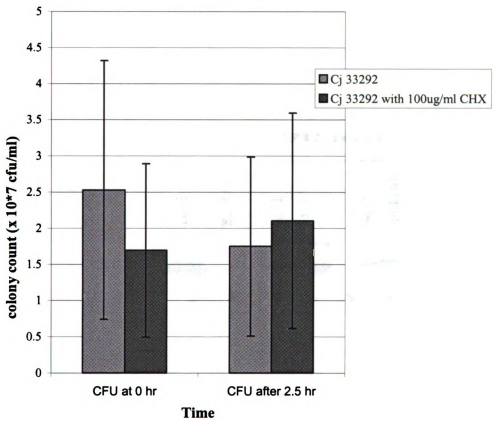
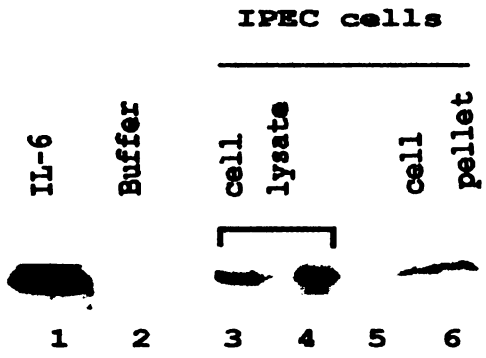


Figure 2. 17. Western blots showing preformed IL-6 protein in IPEC-1 cells prior to any treatment. IL-6 standard was used at a concentration of 80 ng. Lane-1, IL-6 standard; Lane 2, lysis buffer; lane 3, IPEC-1 whole cell lysate (100 μg of total protein); lane 4, IPEC-1 whole cell lysate at (250 μg of total protein); lane 6- cell pellet alone (10 μl, concentration not determined). Blots are representative of two identical experiments.



CHAPTER 3

IL-4, IL-6 AND IL-10 CYTOKINE RESPONSES IN SWINE INFECTED WITH *CAMPYLOBACTER JEJUNI* AND *TRICHURIS SUIS*

ABSTRACT

Campylobacter jejuni is one of the leading causes of bacterial enteritis in the world. It is a gram-negative, spiral or comma-shaped bacterium generally acquired through contaminated food. *Trichuris* sp. is a helminth that dwells in the cecum and proximal colon of swine and humans and is commonly found in all parts of the world. Germ-free piglets infected with either *C. jejuni* or *T. suis* display few clinical signs of disease. In contrast, dual infection results in bloody diarrhea and severe pathology with multifocal lesions, infiltration of inflammatory cells, excessive mucus secretion, and bacterial invasion into epithelial cells and macrophages. In this study, we examined the immune response in swine to single and dual *C. jejuni* and *T. suis* infections. Experiments were conducted in two ways. In the long-term experiment, 4-5-week old conventionally reared pigs were orally inoculated with *T. suis* (2500 eggs). After 21 days, half were orally inoculated with *C. jejuni*, and half with milk. At this time, additional groups, received either *C. jejuni*, *E. coli* DH5α or milk. In the short-term experiment, pigs were inoculated concurrently with either *C. jejuni*, *T. suis* or with both. Control pigs received *E. coli* DH5α or milk. Feces were collected daily, and fecal supernatants were analyzed for IL-4, IL-6 and IL-10 cytokines by ELISA. In the long-term experiment, 24 hours after infection with *C. jejuni*, pigs previously infected with *T. suis* showed elevated levels of IL-4 and IL-10 compared to pigs receiving milk alone. IL-10, but not IL-4 was elevated in pigs singly infected with *T. suis*. Pigs singly infected with *C. jejuni* did not exhibit significantly elevated IL-4 and IL-10 responses by our evaluation methods. Also, there were no significant differences in IL-6 cytokine levels in any of the groups. Conversely, in the short-term experiment, pigs dually infected with *C. jejuni* and *T. suis* and pigs

singly infected with *T. suis* or *C. jejuni,* showed elevated IL-6 responses but not IL-4 or IL-10 responses. However, pigs that received milk also showed elevated IL-6 responses in addition to a significant IL-4 response, suggesting that IL-6 elevations in the dual infected pigs are not important. Pigs that received *E. coli* DH5α had elevated IL-4 responses in their feces. Hence, no cytokines were considered significant during the short-term infections for the dual infection pigs. Because in natural infections disease is seen 21 days after introduction of *T. suis* in the lot, cytokine responses in the long-term infections are likely more reflective of the disease state. Hence this study suggests that IL-4 and possibly IL-10 play a role in progression of *T. suis* and *C. jejuni* induced disease and mucohemorrhagic diarrhea. Also, we show that measurement of adaptive cytokines like IL-4 and IL-10 from feces is a non-invasive tool to study anti-inflammatory cytokine changes in the host.

INTRODUCTION

Trichuris suis are parasites of the swine intestinal tract and cause a clinical syndrome called trichuriasis in pigs marked by anemia, anorexia, pronounced weight loss, bloody diarrhea and colitis (3). In his pioneering work on the life cycle of *T. suis*, Beer suggested that the burrowing activities of *T. suis* into the cecum and colon might enable secondary bacterial infections to occur, thereby enhancing the pathology (4). Antibiotic experiments conducted in conventionally reared weaned piglets suggested that a bacterial component might be involved in the pathology. 7–8 week old pigs given a low dose of *T. suis* (3000 eggs) along with antibiotics did not develop disease symptoms (18). On the other hand, swine that received *T. suis* eggs alone developed lesions and bloody diarrhea similar to that seen in natural infections. *Campylobacter jejuni*, a gram-negative spiral rod, was subsequently isolated from the distal colon of these pigs. In follow-up experiments, gnotobiotic germ-free piglets infected with both *T. suis* (2500 embryonated eggs) or *C. jejuni* (10^6 cfu) developed bloody diarrhea and colonic pathology, while piglets infected with either agent singly did not. Numerous bacteria were seen around the sites of worm attachment in the proximal colon and also in the lymphoglandular complexes (LCGs) in the distal colon of dual infected pigs. LGCs are secondary lymphoid organs present in the distal colon of swine, humans, rats, cattle, and other animals (13, 15, 20, 22) . They are structurally similar to the Peyer's patches in the small intestine and in the sensitized host are composed of a follicle-associated epithelium (FAE) overlying B-cell-dependent germinal centers. LGCs appear to be an important route for *C. jejuni* invasion.

We hypothesized that cytokine dysregulation might be a contributing factor to this polymicrobial disease phenomenon. Th-2 cytokines like IL-4, IL-5, and IL-10 are induced in the host in response to helminth infections (9). Bacterial infections typically induce proinflammatory cytokines marked by IL-12, TNF-α, IL-1β, IFN-γ and others, including IL-6 (12). In the dual infection model there is both a helminth component and a bacterial component. Also, manifestations of disease with clinical signs occur only in the presence of bacteria (17, 18). Therefore we posed the following hypotheses. 1) Resistance to *T. suis* infection involves a predominantly Th-2 immune response while resistance to *C. jejuni* infection involves a predominantly Th-1 immune response. However, the host resistance to *C. jejuni* also has a Th-2 component, since IgA is generated in the LGCs during *C. jejuni* infections (19). 2) The immune response is shifted towards a predominantly Th-2 type during polymicrobial infections with *T. suis* and *C. jejuni*, contributing to the clinical symptoms observed.

To evaluate these hypotheses, two different experiments were conducted. In short-term experiments 4–5 week old conventionally reared weaned piglets were infected with either *T. suis* (2500 embryonated eggs) or *C. jejuni* (2×10^8 cfu) or both. In long-term experiments, pigs were infected with *T. suis* eggs for 21 days prior to infection with either *C. jejuni* or milk. Additional pigs received *C. jejuni* alone, and IL-4, IL-6 and IL-10 cytokine responses were measured in the feces. Control pigs received *Escherichia coli* DH5α or milk in both experiments. IL-4 and IL-10 have been shown to inhibit Th-1 cytokine responses, while dendritic IL-6 has been recently shown to shift cytokine responses to a Th-2 type (6). Additionally, IL-6 is a switch factor for IgA production. The results from this study show that Th-2 cytokines, IL-4 and IL-10, are likely to play a role

during long-term dual infection of *T. suis* and *C. jejuni* in swine, while no significant cytokine responses were seen during short-term dual infections.

MATERIALS AND METHODS

Bacterial cultures

C. jejuni strain ATCC 33292 and the non-invasive control *E. coli* DH5α were used for the experimental procedures. *C. jejuni* strain 33292 was initially obtained from a human with enteritis, and passaged through a pig prior to experimental procedures. The *C. jejuni* strain was grown at 37°C, 5% CO_2 on Bolton agar supplemented with 5% Sheep's blood. The *E. coli* strain was grown on Bolton agar plates at 37°C without Sheep's blood. Both bacteria were grown for 18–20 hours, and resuspended in sterile milk to a final concentration of 1 x 10^8 cfu/ml.

T. suis embryonated eggs

T. suis eggs were collected from adult worms according to previously published methods (10), centrifuged 2–3 times at 10,000 X g for 5 minutes to remove the debris. The eggs were then suspended in deionized distilled water and incubated at 34°C, 5% CO_2 for nineteen days. On day 19, 10 mg/ml of porcine bile extract (Sigma Chemical Co., St. Louis, MO) was added and the eggs were incubated for an additional 3–5 days. Through this additional incubation process, the eggs were monitored for movement inside the eggs indicative of embyronation. The embryonated eggs were centrifuged for 10 minutes at 10,000 X g, resuspended in sterile water and stored at 4°C until required.

Animals

Four-to-five-week-old weaned, conventionally reared Duroc/Yorkshire/Landrace piglets were obtained from MSU swine farm, housed in groups of 5 or 6 pigs each in 5

separate rooms at the University Research Containment Facility, and provided with feed and water *ad libitum*. The rooms were sterilized with bleach prior to housing of pigs. The handling and management of the animals were performed in accordance with University and NIH guidelines.

Identification of *T. suis* eggs and *C. jejuni* from the feces prior to inoculations

To ensure that swine were specific pathogen free, prior to inoculations feces were collected from the pigs and analyzed for *T. suis* eggs by fecal floats. Briefly, ~5 g of feces were collected from each animal and transported in Cary-Blair medium (Difco Laboratories, Detroit, MI) on ice. In the laboratory, the feces was mixed with ~ 10ml of water and centrifuged in a 13 X 100 mm test tube for 4-5 minutes at 2000 rpm. The supernatant was discarded and the fecal pellet was resuspended in 1.2% sucrose solution containing 0.5% formaldehyde. The tube was centrifuged again at 2000 rpm for 4-5 minutes, filled with sucrose solution with a cover slip placed on top. After 5 minutes, the coverslip was removed and observed under a compound microscope at 40X magnification for the presence of *T. suis* eggs. Presence, or absence of *C. jejuni* was analyzed by *flaA* gene PCR (21) and Taq Man® assays (25). Briefly, fecal swabs obtained from pigs were plated on Bolton agar plates containing 5% sheep's blood, and incubated at 37° C, and 5% CO_2. DNA was obtained from bacterial colonies using Easy-DNA® kit from Invitrogen (Carlsbad, CA) and PCR assays were carried out as described (21, 25).

Animal Inoculation

The animals were inoculated orally with the different treatment groups as indicated in Table 3. 1A and Table 3.1B. All the treatments were suspended in sterile milk reconstituted from dry powder.

Long-term experiment

Thirty pigs were divided into 5 treatment groups with 6 pigs in each group. The *T. suis* group and the dual infection group received *T. suis* embryonated eggs on day 0. On day 21 after *T. suis* infection, the rest of the treatment groups received their respective infective doses (Table 3. 1A). Fecal samples were collected from all the pigs on day 0 and day 21 prior to inoculation. Feces were also collected on day 22 and day 23 post- *T. suis* inoculation (or 24 hours and 48 hours post *C. jejuni* or *E. coli* inoculation).

The experiment was repeated once more with 5 pigs in each group. Rectal swabs were also taken for *C. jejuni* isolation at each time point and transported in Cary–Blair medium (Difco Laboratories, Detroit, MI) on ice.

Short- term experiment

Six pigs in each group were inoculated with their respective treatments on day 0 and fecal samples were collected from each pig prior to inoculation (Table 3. 1B). Feces were also collected twenty-four hours and forty-eight hours post-infection from all the experimental animals. Feces were stored on ice in sterile containers during transportation to the laboratory for processing. The experiment was repeated once more with 4 pigs in each group.

Fecal Analysis

Identification of *C. jejuni* from feces-post inoculation

Bacterial DNA was extracted from the fecal samples using the DNA isolation kit specific for fecal samples (Qiagen Inc. Valencia, CA). The DNA was then used to detect the presence or absence of *C. jejuni* 33292 using Taq Man® assays and 23S rRNA gene Random Fragment Length Polymorphism (RFLP) as described previously (11, 25).

Extraction of supernatants for ELISA

5–10 g of each fecal sample was diluted 1:3 in sterile Phosphate Buffered Saline (PBS) containing trypsin inhibitors (1 mg/ml) and phenylmethylsulfonyl fluoride (PMSF, 1mg/ml), and centrifuged for 15–30 minutes at 16,500 rpm, 4°C. Supernatants were collected, aliquoted and stored at –80°C until analysis by ELISA. PBS was obtained from Gibco BRL, Rockville, MD, and the trypsin inhibitor and PMSF were obtained from Sigma, St. Louis, MO.

ELISA assays

The supernatants from fecal samples were analyzed for IL-4, and IL-10 cytokines using swine ELISA kits according to the manufacturer's protocol (Biosource International Inc. Camarillo, CA). An IL-6 ELISA assay was developed in the laboratory using swine reagents purchased from Biosource International, and carried out as described previously in chapter 2.

Data analysis

The cytokine values obtained from individual pigs were normalized to day 0 values for each pig. Fold changes in cytokine levels over time were obtained with the following formula: Fold change = (time x levels/ time 0 levels), where x = day 0, day 21,

22 or 23 for long-term experiments and 0 hr, 24 hr or 48 hr for short-term experiments. The results were graphed as mean +/- SEM. The Student's t test was performed on the results obtained to determine if increases in cytokines were significant. P values ≤ 0.05 were considered acceptable levels of statistical significance.

RESULTS

Fecal analysis

Identification of *T. suis* eggs and *C. jejuni* in the feces-preinoculation

We found no evidence of *T. suis* eggs or *C. jejuni* in the feces of pigs prior to inoculations, and hence the animals were deemed free of these specific organisms (data not shown).

ELISA–long-term experiment

IL-4 secretion

The changes in IL-4 levels, in response to the different treatments, are shown in Figure 3. 1. The dual infection group showed a statistically significant increase in IL-4 secretion by day 22 compared to day 0 ($p < 0.05$). There was a 2-fold increase in IL-4, 24 hr after *C. jejuni* inoculation in the dual infection group. On the other hand, there were no statistical increases in IL-4 secretion in pigs infected/inoculated with other treatments ($p > 0.05$). 55% of pigs infected with both *T. suis* eggs and *C. jejuni* showed an increase in IL-4 (Table 3. 2). A similar result was obtained in pigs infected with *T. suis* alone. On the other hand, only 36% of pigs infected with *C. jejuni* and 10% of pigs given milk showed any increase in IL-4. None of the pigs that received *E. coli* DH5α showed any increase in IL-4 after inoculation with this bacterium.

IL-6 secretion

None of the pig groups inoculated with any treatment showed a significant increase in IL-6 in their feces (Figure 3. 2). Pigs that received *T. suis* eggs and the pigs

that received milk showed some moderate increase by day 21, however, there were no statistically significant increases in IL-6 for any of the treatment groups ($p > 0.05$). On an individual basis, 55% of pigs that received *T. suis* eggs showed an increase in IL-6 in their feces; however, 64% of the pigs that received milk alone also showed an increase in IL-6. The rest of the groups had less than 50% of the pigs showing an increase in IL-6 in their feces (Table 3. 2).

IL-10 secretion

Pigs singly infected with *C. jejuni*, *T. suis,* or both showed a significant increase in IL-10 by day 22 ($p < 0.05$). There was nearly a 3-fold increase in IL-10 levels in the feces of pigs in all the three treatment groups. The groups that received milk or *E.coli* did not show any significant increase in this cytokine (Figure 3. 3, $p > 0.05$). 64% of the pigs that received both pathogens, and 73% of the pigs that received *T. suis* alone showed an increase in IL-10 while only 45% of the pigs given *C. jejuni* alone showed an increase (Table 3. 2). In the control groups, pigs that received milk or *E. coli*, 36% and 33% of the pigs respectively, showed an increase in IL-10.

ELISA–short-term experiments

IL-4 secretion

None of the groups that received *C. jejuni*, *T. suis,* or both showed any significant increase in IL-4 by 48 hr. However, pigs in both the control groups, milk and *E. coli*, showed a significant increase in IL-4 by 48 hr ($p < 0.05$, Figure 3. 4). Concurring with this result, 70% of the pigs that received milk and 60% of the pigs that received *E. coli*,

showed an increase in IL-4 by 48 hr. However, only 30% of the pigs given *C. jejuni*, *T. suis*, or both showed an increase in IL-4 (Table 3. 3).

IL-6 secretion

The pigs that received *C. jejuni* alone, milk alone, and both *C. jejuni* and *T. suis* showed a significant increase in IL-6 by 48 hr ($p < 0.05$). The pigs that received *T. suis* alone showed a similar statistically significant increase in IL-6 by 24 hr, while the group that received *E. coli* did not (Figure 3. 5). The dual infection group showed a 2-fold increase in IL-6 by 48 hr, while all the other groups except the *E. coli* group showed 1.5 fold increase in IL-6. On an individual basis, 70% of the pigs that received *T. suis* alone or both *C. jejuni* and *T. suis* showed an increase in IL-6 in their feces by 48 hr, while 50% of the pigs that received *C. jejuni* alone showed a similar result. In both the control groups, only 40% of the pigs showed an increase in IL-6 (Table 3. 3).

IL-10 secretion

None of the pigs given any of the treatments showed a statistically significant increase in IL-10 by 48 hr (Figure 3. 6, $p > 0.05$). Also, only 40% of the pigs that received both *C. jejuni* and *T. suis* showed an increase in IL-10, while 50% of the pigs that received *T. suis* had an increase in this cytokine (Table 3. 3). The other three groups, given *C. jejuni, E. coli,* or milk had ≤ 30% of the population showing an increase in IL-10 in their feces.

Identification of *C. jejuni* in the feces- post inoculation

In the groups that received *C. jejuni*, analysis of DNA obtained from feces did not show the presence of the bacteria by 48 hr post-inoculation; this was true for both the short-term and long-term experiments. However, proximal colon biopsies from the dual infection pigs in the long-term experiment, obtained 48 hr post-inoculation showed the presence of the bacteria in these tissues as determined by 23S rRNA RFLP. In addition, hematoxylin eosin stain of the proximal colon sections showed the presence of *T. suis* larvae (L4) from all the pigs tested (Dr. Kathryn Jones, personal communication).

DISCUSSION

In previous studies, differential development of pathology in swine infected with *T. suis* eggs with or without antibiotics suggested the involvement of a bacterium such as *C. jejuni* in clinical trichuriasis (18). This was borne out in subsequent studies, in which dual infection of germ-free piglets with both *T. suis* and *C. jejuni* resulted in bloody diarrhea and pathology but not single infections with *C. jejuni* or *T. suis* (17). In this study we show that dual infection of swine with *C. jejuni* and *T. suis* results in increased levels of IL-4 and IL-10 in the feces during long-term infections and of IL-6 during short-term infections. Since in previous studies, pathology is seen 13–14 days after infection with *T. suis* eggs, and since trichuriasis is known as a "21 day scour", we believe that long-term infections are more reflective of natural infections than short-term infections (3, 4, 18).

We hypothesized that during dual infection with *T. suis* and *C. jejuni*, the cytokine response could be shifted to a Th-2 type, down-regulating the response to the bacteria resulting in clinical disease and pathology. Since the animals were free of *T. suis* eggs and *C. jejuni* prior to inoculations, the responses were considered to be due to these organisms alone. We saw up-regulation of IL-4 and IL-10 but not IL-6, following long-term infection with *C. jejuni* and *T. suis*. IL-6 was up-regulated during short-term dual infections alone. However, since the control pigs also showed increases in this cytokine, it might not be a significant effect. Another interesting observation was that the cytokine responses in individual pigs during dual infection (Tables 3. 2 & 3. 3) mirrored that from pigs receiving only *T. suis*, suggesting that the responses are more helminth driven. This was also confirmed by the presence of worms in the proximal colon biopsies post-

infection. This suggested that innate responses might play a role during initial infections with *T. suis* (and *C. jejuni)*, while adaptive responses such as IL-4 come into play during long-term infections.

T. suis infections alone induced a significant IL-10 response during long-term infection and IL-6 responses during short-term infections. Although IL-4 responses were increased in 55% of the pigs during long-term infections (Table 3. 2), it wasn't a significant increase on average (Figure 3. 1). This is probably due to genetic variability between pigs. *C. jejuni* infections did not significantly induce any cytokines during either short-term or long-term infections. This was a little surprising, since we expected IL-6 responses to be up-regulated following infection with this bacterium. However, one reason could be that although all pigs were given the same infective dose, different doses might be required to establish infection and immune responses in individual pigs, since the pigs are out-bred. Also, measurement of cytokines beyond 48 hr might have been required to see significant response in some pigs.

IL-4 was significantly up-regulated in pigs given milk and *E. coli* during the short-term infections. The reasons are unknown at this point. An evaluation of other cytokines like IFN-γ or IL-12 might provide a clue as to whether or not this effect seen is simply a general stress response. Testing additional pigs might also help explain this result.

Although we failed to detect *C. jejuni* in the feces 48 hr after infection, we did detect it in proximal colon tissue samples obtained at the end of the study (day 23; data not shown). Since *C. jejuni* has been detected in fecal material only 4–5 days after

infection in other studies (24), and since this study was conducted for only 2 days after infection, we might have failed to detect this pathogen in the feces.

Recovery of cytokines from feces through artificial spiking showed that IL-4, IL-6 and IL-10 levels seen in our results are 10%, 20% and 10% respectively, of the actual cytokine values present (data not shown). This suggests that, when extrapolated, enormous amounts of cytokines are produced in response to infections at tissue level, and that the low recovery in feces might be due to dilution effects through the intestinal tract, as well as due to the short half-life of these cytokines. However, inspite of these low recovery levels, we were still able to see statistically significant differences with in groups. Hence, cytokine measurements from feces might provide a safe, non-invasive alternative for the diagnosis of immune status of the host. However, that said, it is still incumbent on the researcher to evaluate these results depending on the host and specific cytokines measured.

IL-4 and IL-10 are anti-inflammatory cytokines and are induced during a variety of helminth infections. IL-4 has been shown to increase epithelial cell permeability, which in turn is thought to loosen the worms from the mucosa. IL-4 also stimulates peristalsis in the gut for rapid worm expulsion. It also induces IgE, IgG1 and IgG4 class switching and increases fluid accumulation in the intestinal lumen (16). IL-4 has been shown to regulate enteropathy in *Trichinella spiralis* infections (8, 14), in IgE-mediated hypersensitivity reactions (5), in asthma in humans and other pathologies (2, 7). IL-10 is an anti-inflammatory cytokine, with a role in downregulating proinflammatory cytokines such as IFN-γ, IL-12, and others (1). The presence of IL-4 and IL-10 in the dually infected pigs suggested that the pathology could be Th-2 mediated. However, other

studies in the laboratory on these same samples indicated the presence of IL-1β and TNFα as well, during dual infections of swine with *T. suis* and *C. jejuni* (Cunningham L.D and L.S. Mansfield, unpublished data). Nevertheless, the production of IL-4 and IL-10 suggests that these two cytokines have a role during the pathology mediated by dual infection of *T. suis* and *C. jejuni* in pigs. The specific roles of these cytokines in pathology will be the subject of subsequent studies.

Millions of people worldwide are infected with the human equivalent of *T. suis*, *T. trichiura*, and *C. jejuni* is a well-known human pathogen. Hence this study has clinical significance for both humans and pigs. Recently, a patient with severe colitis was shown to have both *C. jejuni* and *T. suis* ova in the feces (23) – a clinical correlate of our experimental findings. With increasing awareness of the potential health hazard posed by helminth infections, more studies on helminth–bacterial interactions should be forthcoming.

ACKNOWLEDGEMENTS

This work was supported by the NIH-grant, 61-0954. We thank Dr. Pawin Padungtod, and Dr. Jeanie Gaymer for their help with the animal experiments. Thanks are also due to all the staff at the Containment Facility, Michigan State University. We thank Dr. Julia Bell for the critical review of this article.

REFERENCES

1. **Aggarwal, B. a. G., JU.** 1992. Other cytokines, p. 427-428, Human Cytokines, Handbook for basic and clinical research. Blackwell Scientific Publications, Cambridge, MA.

2. **Ajuebor, M. N., C. M. Hogaboam, T. Le, and M. G. Swain.** 2003. C-C chemokine ligand 2/monocyte chemoattractant protein-1 directly inhibits NKT cell IL-4 production and is hepatoprotective in T cell-mediated hepatitis in the mouse. J Immunol **170:**5252-9.

3. **Batte, E. G., R. D. McLamb, K. E. Muse, S. D. Tally, and T. J. Vestal.** 1977. Pathophysiology of swine trichuriasis. Am J Vet Res **38:**1075-9.

4. **Beer, R. J.** 1973. Studies on the biology of the life-cycle of *Trichuris suis* Schrank, 1788. Parasitology **67:**253-62.

5. **Cooper, E. S., J. Spencer, C. A. Whyte-Alleng, O. Cromwell, P. Whitney, S. Venugopal, D. A. Bundy, B. Haynes, and T. T. MacDonald.** 1991. Immediate hypersensitivity in colon of children with chronic *Trichuris trichiura* dysentery. Lancet **338:**1104-7.

6. **Dodge, I. L., M. W. Carr, M. Cernadas, and M. B. Brenner.** 2003. IL-6 production by pulmonary dendritic cells impedes Th1 immune responses. J Immunol **170:**4457-64.

7. **Fireman, P.** 2003. Understanding asthma pathophysiology. Allergy Asthma Proc **24:**79-83.

8. **Garside, P., M. W. Kennedy, D. Wakelin, and C. E. Lawrence.** 2000. Immunopathology of intestinal helminth infection. Parasite Immunol **22:**605-12.

9. **Gause, W. C., J. F. Urban, Jr., and M. J. Stadecker.** 2003. The immune response to parasitic helminths: insights from murine models. Trends Immunol **24:**269-77.

10. **Hill, D. E., H. R. Gamble, M. L. Rhoads, R. H. Fetterer, and J. F. Urban, Jr.** 1993. *Trichuris suis*: a zinc metalloprotease from culture fluids of adult parasites. Exp Parasitol **77:**170-8.

11. **Iriarte, P., and R. J. Owen.** 1996. PCR-RFLP analysis of the large subunit (23S) ribosomal RNA genes of *Campylobacter jejuni*. Lett Appl Microbiol **23:**163-6.

12. **Janeway, C., Travers P., Walport M, and Capra JD.** 1999. Host defense against infection, p. 363-416. *In* A. P. a. L. E (ed.), Immunobiology,The immune

system in health and disease, Fourth ed. Current Biology Publications and Garland Publishing, New York.

13. **Kealy, W. F.** 1976. Colonic lymphoid-glandular complex (microbursa): nature and morphology. J Clin Pathol **29:**241-4.

14. **Lawrence, C. E., J. C. Paterson, L. M. Higgins, T. T. MacDonald, M. W. Kennedy, and P. Garside.** 1998. IL-4-regulated enteropathy in an intestinal nematode infection. Eur J Immunol **28:**2672-84.

15. **Liebler, E. M., J. F. Pohlenz, and N. F. Cheville.** 1988. Gut-associated lymphoid tissue in the large intestine of calves. II. Electron microscopy. Vet Pathol **25:**509-15.

16. **Mansfield, L. S., Gause, W.C.,Finkelman, F.D., and J.F. Urban, Jr.** 2000. Gastrointestinal nematodes and the immune system, p. 555-567. *In* M. W. Cunningham, and Fujinami, R.S. (ed.), Effects of microbes on the immune system. Lippincott, Williams and Wilkins, Philadelphia.

17. **Mansfield, L. S., D. T. Gauthier, S. R. Abner, K. M. Jones, S. R. Wilder, and J. F. Urban.** 2003. Enhancement of disease and pathology by synergy of *Trichuris suis* and *Campylobacter jejuni* in the colon of immunologically naive swine. Am J Trop Med Hyg **68:**70-80.

18. **Mansfield, L. S., and J. F. Urban, Jr.** 1996. The pathogenesis of necrotic proliferative colitis in swine is linked to whipworm induced suppression of mucosal immunity to resident bacteria. Vet Immunol Immunopathol **50:**1-17.

19. **Mansfield, L. S. a. D. G.** 2004. Lymphoglandular complexes are important colonic sites for IgA induction against *Campylobacter jejuni* in a swine disease model. Comparative medicine.

20. **Morfitt, D. C., and J. F. Pohlenz.** 1989. Porcine colonic lymphoglandular complex: distribution, structure, and epithelium. Am J Anat **184:**41-51.

21. **Nachamkin, I., K. Bohachick, and C. M. Patton.** 1993. Flagellin gene typing of *Campylobacter jejuni* by restriction fragment length polymorphism analysis. J Clin Microbiol **31:**1531-6.

22. **O'Leary, A. D., and E. C. Sweeney.** 1986. Lymphoglandular complexes of the colon: structure and distribution. Histopathology **10:**267-83.

23. **Shin, J. L., G. W. Gardiner, W. Deitel, and G. Kandel.** 2004. Does whipworm increase the pathogenicity of *Campylobacter jejuni*? A clinical correlate of an experimental observation. Can J Gastroenterol **18:**175-7.

24. **Skirrow, M., and Blaser, M.J.** 2000. Clinical aspects of Campylobacter infection, p. 69-88. *In* I. Nachamkin, and Blaser, M.J (ed.), Campylobacter, Second ed. American Society for Microbiology, Washington, DC.

25. **Wilson, D. L., S. R. Abner, T. C. Newman, L. S. Mansfield, and J. E. Linz.** 2000. Identification of ciprofloxacin-resistant *Campylobacter jejuni* by use of a fluorogenic PCR assay. J Clin Microbiol **38:**3971-8.

Table 3. 1A: Inoculations for long-term experiment (2 ml volumes)

Group / Time	***T. suis* and *C. jejuni***	***T. suis***	***C. jejuni***	**Milk**	***E. coli* DH5α**
Day 0	*T. suis* (2500 embryonated eggs)	*T. suis* (2500 embryonated eggs)	Milk	Milk	Milk
Day 21	*C. jejuni* (2 x 10^8 cfu)	Milk	*C. jejuni* (2 x 10^8 cfu)	Milk	*E. coli* (2 x 10^8 **cfu**)

Table 3. 1B: Inoculations for short-term experiment (2 ml volumes)

Group / Time	***T. suis* and *C. jejuni***	***T. suis***	***C. jejuni***	**Milk**	***E. coli* DH5α**
Day 0	*T. suis* (2500 embryonated eggs) and *C. jejuni* (2 x 10^8 cfu)	*T. suis* (2500 embryonated eggs)	*C. jejuni* (2 x 10^8 cfu)	Milk	*E. coli* DH5α (2 x 10^8 cfu)

Table 3. 2: Percentage of pigs showing an increase in a given cytokine by day 22 after respective inoculations – long-term experiments

Group / Cytokine	Percentage of pigs showing an increase in a given cytokine				
	T. suis and *C. jejuni*	*T. suis*	*C. jejuni*	Milk	*E. coli* DH5α
IL-4	**55%**	**55%**	36%	10%	0%
1L-6	45%	**55%**	36%	**64%**	33%
1L-10	**64%**	**73%**	45%	36%	33%

Table 3. 3: Percentage of Pigs showing an increase in a given cytokine by 24 to 48 hr after respective inoculations – short-term experiments

Group / Cytokine	Percentage of Pigs showing an increase in a given cytokine				
	T. suis and *C. jejuni*	*T. suis*	*C. jejuni*	Milk	*E. coli* DH5α
IL-4	30%	30%	30%	**70%**	60%
1L-6	**70%**	**70%**	50%	40%	40%
1L-10	**40%**	50%	30%	**30%**	20%

Figure 3. 1. Changes in IL-4 in feces of pigs inoculated with different groups during the long-term experiment. Four-to-five-week-old piglets were inoculated with *T. suis* eggs on day 0. Twenty-one days later, half of the pigs received *C. jejuni*, while the others received milk. Additional groups received *E. coli* DH5α or *C. jejuni* at this point. Control pigs received milk alone. Values shown are means of two identical experiments with a total of 11 pigs in each group (n=6 for the *E. coli* group). Mean +/- SEM is shown. (*, $p < 0.05$).

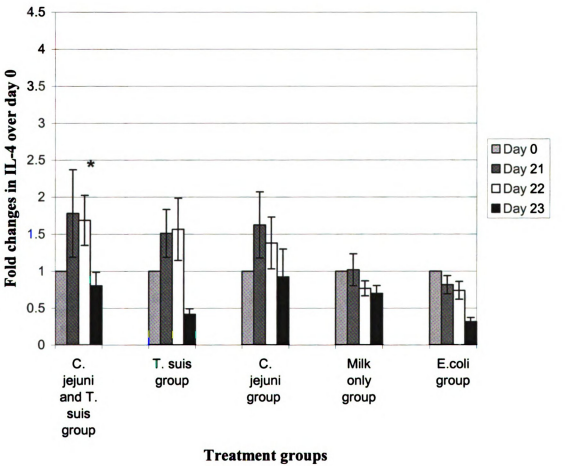


Figure 3. 2. Swine inoculated with the different treatment groups were analyzed for IL-6 cytokine changes in the feces of pigs from respective groups (n = 11; n=6 for *E. coli* group). Mean fold changes in IL-6 in different groups during the long-term experiment is shown.

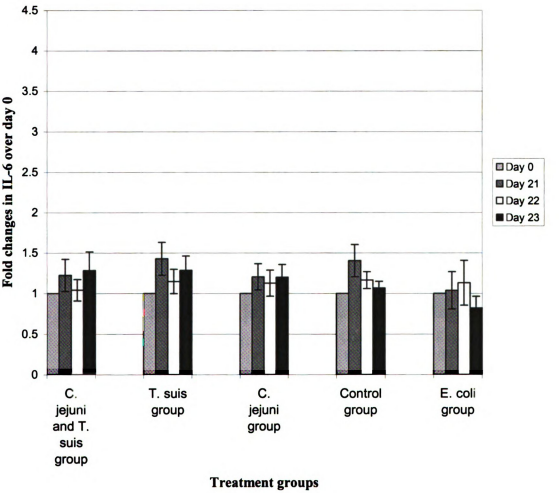


Figure 3. 3. Fold changes in IL-10 in the feces of pigs from long-term experiments. Mean of two identical experiments is shown with a total of 11 pigs in each group (n= 6 for the *E. coli* group). Values shown are mean +/- SEM. (*, $p < 0.05$).

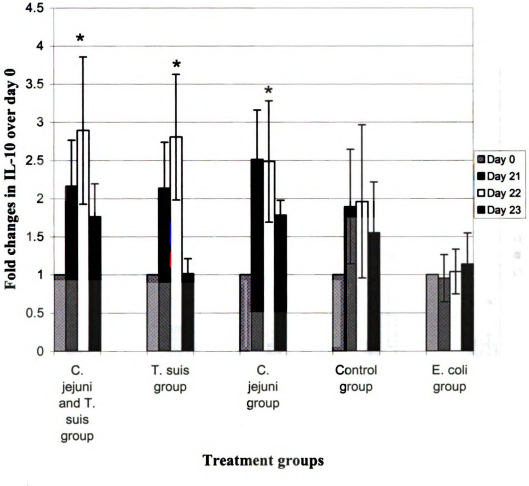


Figure 3. 4. Mean fold changes in IL-4 cytokine in the feces of pigs from the short-term experiments. Mean of two identical experiments is shown, with 10 pigs in each group. Values are mean +/- SEM. (*, $p < 0.05$).

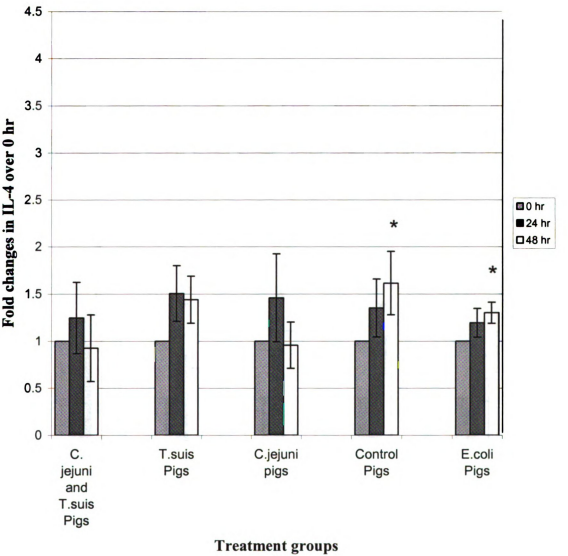


Figure 3. 5. Four to five week-old conventionally reared weaned piglets were inoculated with the different treatments orally for 48 hr. Feces were collected prior to inoculation (0 hr), 24 hr and 48 hr after inoculations. Mean of two identical experiments with 10 pigs in each group is shown. Values shown are mean +/- SEM. (*, $p < 0.05$).

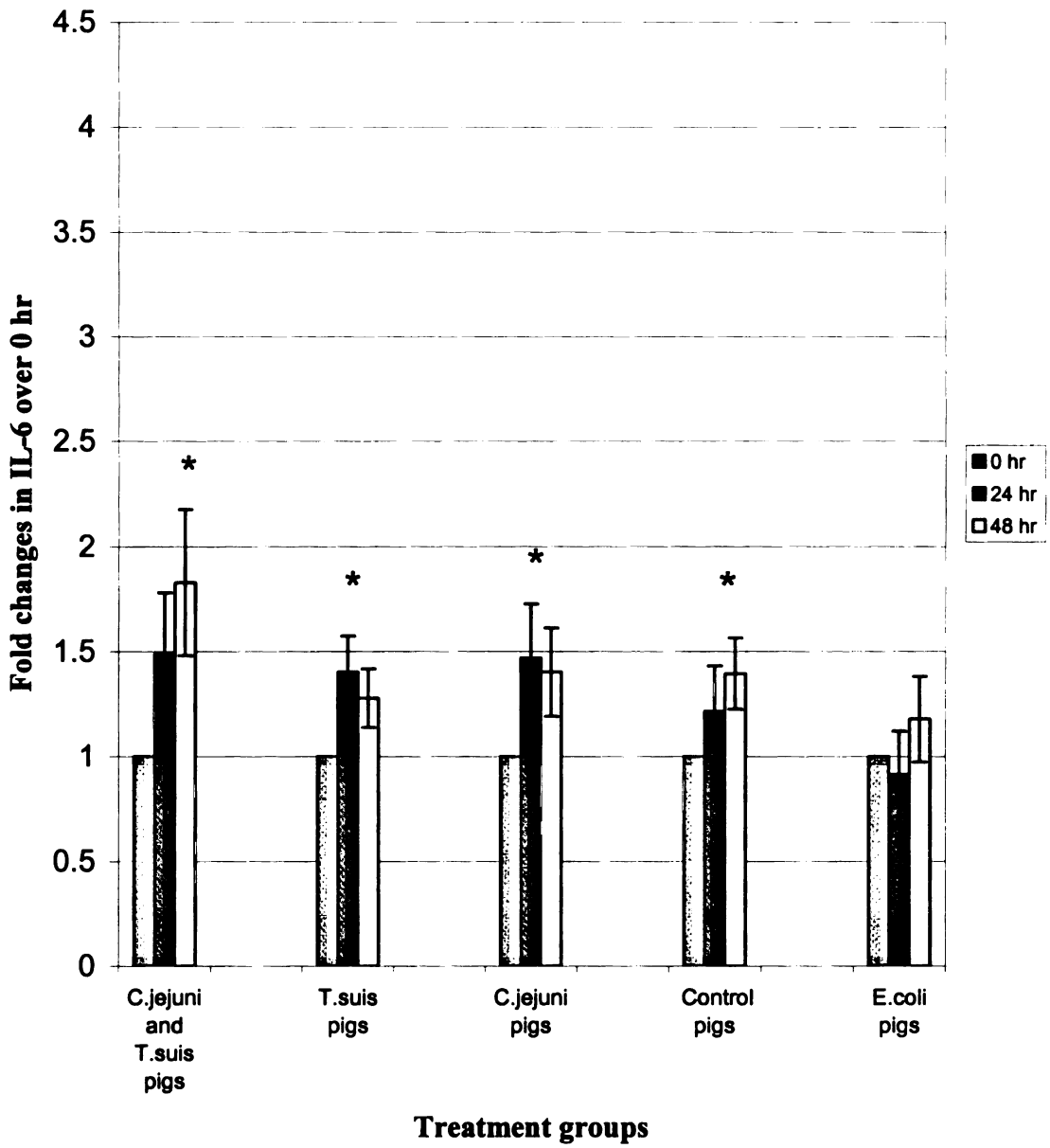
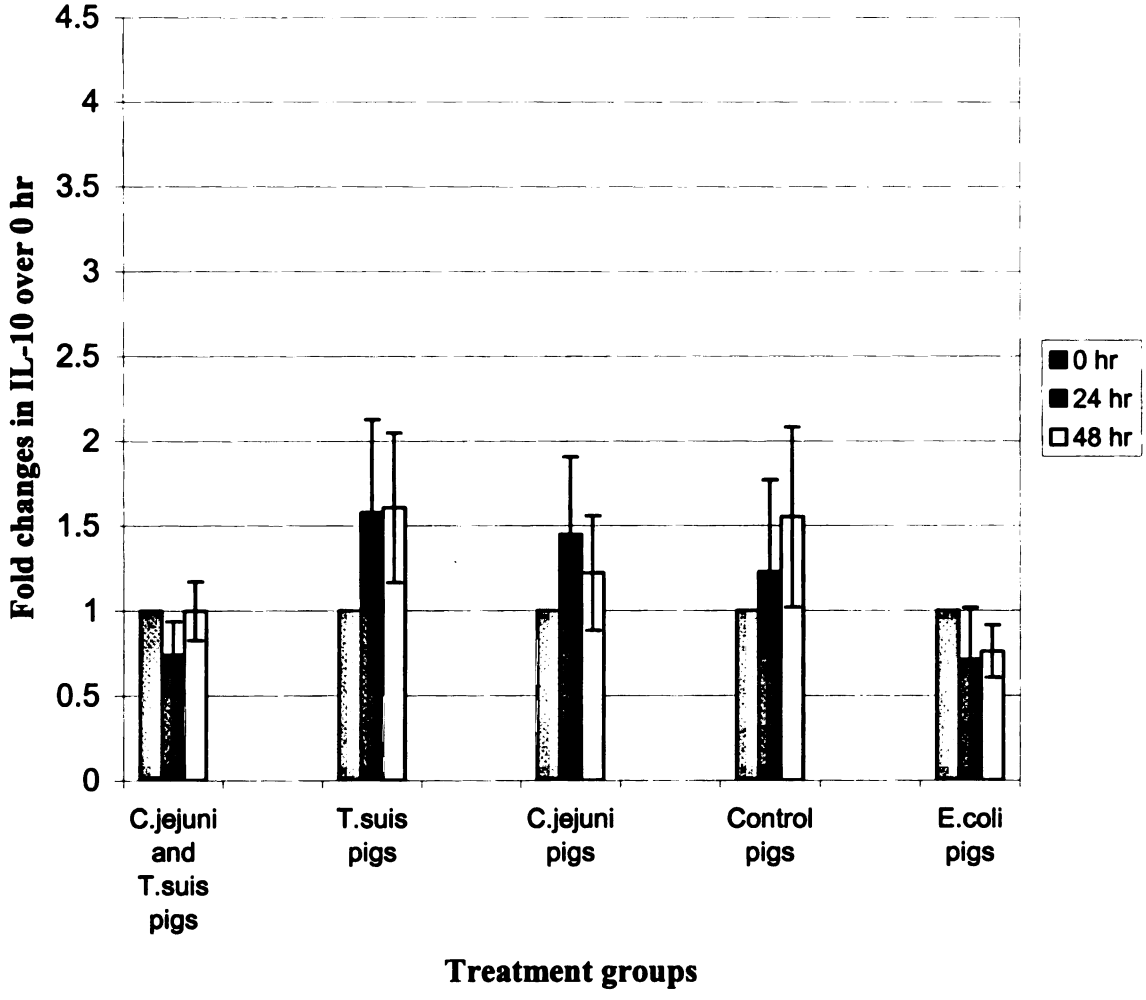


Figure 3. 6. IL-10 cytokine changes in pigs inoculated with different treatments during the short-term experiment. Values are means from 2 identical experiments. Mean +/- SEM is shown.



CHAPTER 4

RECOMBINANT IL-4 (rIL-4) ENHANCES *CAMPYLOBACTER JEJUNI* INVASION OF PORCINE INTESTINAL EPITHELIAL CELLS (IPEC-1)

ABSTRACT

Campylobacter jejuni (*C. jejuni*) is a food borne bacterial pathogen and is one of the leading causes of bacterial enteritis in humans. It is commonly acquired through ingestion of contaminated meat products or raw milk, and its pathogenesis is not completely known. Helminth parasites such as *Trichuris suis* (*T. suis*) have been documented to modulate immune responses to intracellular pathogens. In previous studies, germ-free piglets infected with either *C. jejuni* or *T. suis* alone display few clinical signs of disease. In contrast, dual infection results in bloody diarrhea and severe pathology with multifocal lesions, infiltration of inflammatory cells, excessive mucus secretion, and bacterial invasion into epithelial cells and macrophages. In follow-up studies, swine infected with both *T. suis* (2500 eggs) and *C. jejuni* (10^8 cfu) had elevated IL-4 (and IL-10) responses in the feces. In this study, we hypothesized that IL-4 (or IL-10) enhances invasion of *C. jejuni* in intestinal pig epithelial cells (IPEC). To test this hypothesis, IPEC-1 cells were pretreated with recombinant IL-4 (rIL-4) or rIL-10 for 5 hours followed by invasion assays with *C. jejuni*. Cells pretreated with rIL-4 showed ~ 6 fold increases in *C. jejuni* internalization, compared to cells that received no pretreatment. Addition of anti-IL-4 antibody reversed this effect. Preincubation with rIL-10 did not significantly alter the internalization of the bacteria. Also, the transepithelial electrical resistance (TER) was significantly reduced following rIL-4 treatment but not following rIL-10 treatment. Invasion increased with increasing doses of rIL-4. Light microscopy showed more vacuoles within IPEC-1 cells after rIL-4 treatment, while transmission electron microscopy showed multiple bacteria within these cells. Most of the bacteria were in the cytoplasm, and some were adjacent to vacuoles. We conclude

that rIL-4 alters the physiology of these epithelial cells allowing increased invasion of *C. jejuni*.

INTRODUCTION

Campylobacteriosis caused by *C. jejuni* and *C. coli* is a serious illness in humans characterized by a wide range of symptoms from acute diarrhea, fever, blood in stools, vomiting and abdominal cramps- to a non-inflammatory secretory diarrhea (25). Although rare, one of the serious complications is a neurological disorder termed Guillain Barré Syndrome which occurs in roughly 1/1058 infected individuals (22). Deaths due to *Campylobacter* infections are estimated to be in the hundreds in the United States alone (8). The mechanisms by which *Campylobacter* species cause disease are not well understood. Some of the virulence factors identified include toxins, adhesins, motility, and invasion. Invasive strains of *C. jejuni* have been more commonly associated with the inflammatory type of diarrhea and non-invasive strains with the secretory type of diarrhea. Also, flagellated, motile strains are considered more invasive than non-flagellated, non-motile strains (12). Recently, a campylobacter invasion antigen (Cia B) has been identified that is thought to play a vital role in invasion (23). CiaB is thought to be secreted into the host cells through a Type III secretion apparatus, where it triggers a series of signal transduction events, resulting in *C. jejuni* uptake. However, the host factors that contribute to invasion have not been studied very extensively. In a swine animal model to study campylobacter pathogenesis, *C. jejuni* acted synergistically with a whip-worm parasite *Trichuris suis* in the colon of gnotobiotic piglets to produce severe pathology marked by focal lesions, infiltration of inflammatory cells, excessive mucus secretion, bloody diarrhea, and bacterial invasion into epithelial cells and macrophages, similar to campylobacteriosis in humans (19). *C. jejuni* invasion into epithelial cells occurred in the undifferentiated crypt epithelial cells surrounding the worms in the

proximal colon as well as in the differentiated follicle-associated epithelium lining the secondary lymphoid organs in the distal colon (lymphoglandular complexes). Conversely, pigs infected with either *C. jejuni* (1 x 10^6 cfu) or *T. suis* (3000 infective larvae) alone did not show clinical signs of disease.

In similar studies with conventionally reared, weaned piglets, swine that received *T. suis* (2500 infective larvae) followed 21 days later by *C. jejuni* (2 x 10^8 cfu) showed enhanced IL-4 and IL-10 cytokine secretion in their feces compared to control pigs (Parthasarathy, G, L. D. Cunningham and L.S. Mansfield, unpublished data). IL-4 and IL-10 are anti-inflammatory cytokines that are normally induced during helminth infections (9). IL-4 has been shown to down-regulate IL-1 α, IL-1β, TNFα and nitric oxide *in vitro* (3). Most importantly, IL-4 has also been shown to play a critical role in the enteropathology associated with *Trichinella spiralis* infection in mice (17), asthma in humans (6), T-cell mediated hepatitis (2) and other pathologies (5). IL-10 has been shown to down-modulate Th-1 cytokine responses in macrophages (1). In this study, we hypothesized that IL-4 or IL-10 induced in response to dual infection enhances the invasion of *C. jejuni*, and, hence, accounts for some of the pathology. To test this hypothesis differentiated intestinal pig epithelial cells (IPEC-1) derived from a neonatal piglet (4) were used to determine the effect of rIL-4 and rIL-10 on *C. jejuni* invasion. The data from this study suggests that IL-4 is likely to play a dominant role in mediating pathology in *T. suis* and *C. jejuni* infected swine.

MATERIALS AND METHODS

Bacterial strains and culture

C. jejuni strains ATCC 33292 and 81-176 were used for the invasion studies. Both were initially isolated from humans with enteritis. *C. jejuni* 81-176 was kindly donated by Dr. Carol Pickett, University of Kentucky. Low passage bacterial colonies were grown for isolation on Bolton Agar supplemented with 5% sheep's blood (Cleveland Scientific, Bath, OH) for a period of 48–72 hr. The control strains *Escherichia coli* DH5α and *E. coli* O157:H43 (DEC7A) were grown similarly on Nutrient agar (Difco Laboratories, Detroit, MI). DEC7A was initially isolated from swine and was a kind contribution from Dr. Thomas Whittam, Michigan State University. An isolated colony was further grown as a lawn on corresponding medium for 20 hr. Bacteria were swabbed from plates and resuspended in RPMI 1640 supplemented with phenol red and 5% heat inactivated fetal bovine serum (Gibco BRL, Rockville, MD). The optical density (OD_{560}) was adjusted to 0.1 to achieve ~ 10^8 CFU/ml. Bacterial suspensions were used immediately after preparation.

Cell culture

Intestinal cells obtained from swine (IPEC-1) were used to elucidate the effect of rIL-4 (or rIL-10) on *C. jejuni* invasion of epithelial cells. IPEC-1 is a non-immortalized and undifferentiated neonatal small intestinal epithelial cell line that can be induced to differentiate by growth on porous substrates for 10–14 days (10, 28). All media and supplements for cell culture were obtained from Gibco BRL, Rockville, MD, unless otherwise stated. The transwells plates were obtained from Corning Costar, Corning, NY.

The IPEC-1 cells were cultured routinely in DMEM/F-12 medium supplemented with 5% Fetal Bovine Serum (FBS) and 1% Insulin-Transferrin-Selenium in cell culture flasks. After 5–6 days of growth, the cells were washed in versene and trypsinized. They were then cultured at a density of $3x10^5$/ well on transwell inserts (6.5 mm diameter; 3 μm pore size) that had been previously coated with fibronectin (20 μg/ml; Sigma, St. Louis, MO). Cells were allowed to differentiate for 10 – 14 days in RPMI 1640 medium containing phenol red and supplemented with 5% Fetal Bovine Serum (FBS). Transepithelial electrical resistance (TER) across the monolayer was measured to determine confluency and tight junction formation (EVOMX, World Precision Instruments, Sarasota, FL). TER values between 150-600 Ωcm^2 were considered as evidence of confluent monolayer.

Gentamicin killing assays

10–12 day old IPEC-1 cells on transwell membranes were treated with either 500 pg of rIL-4 or 1000 pg of rIL-10 in 100 μl of RPMI 1640 supplemented with 5% FBS (complete medium; CM) and incubated at 37° C, 5% CO_2. 100 μl of CM alone was included as a negative control for the assay. The working concentrations of cytokines were selected from optimal concentrations determined in previous studies in the swine model, and the cytokine reagents were obtained from swine ELISA kits purchased from Biosource International, Camarillo, CA. After 5 hr, the treatment solutions were removed as completely as possible. The time point was chosen based on previously published studies (29). IPEC-1 cells were not washed in order to avoid damage to the monolayers and were infected with 5 x 10^7 cfu of bacteria in 100 μl of CM. Invasion was

allowed to proceed for 3 hr at 37°C, 5% CO_2. The bacteria were then removed and adherent bacteria on both sides of the membrane were killed with 100 μg/ml of gentamicin (Gibco BRL, MD) suspended in CM, for 1 hr. The transwell membranes were excised and the epithelial cells were lysed with a final concentration of 0.1% sodium deoxycholate (Sigma Chemical Co., St. Louis, MO) in 1X Phosphate Buffered Saline (0.01M). The internalized bacteria were enumerated by plating on Bolton agar plates. All treatments were performed in triplicate.

Subsequently, gentamicin killing assays were also carried out 1) to determine the effect of anti-IL-4 antibody on invasion, 2) to establish the dose response of rIL-4 to *C. jejuni* internalization, and 3) to determine the effect of rIL-4 pretreatment on non-invasive *E. coli* DH5α. In addition, the number of *C. jejuni* that had translocated to the baso-lateral chamber also was determined. IPEC-1 cells were pretreated with either rIL-4 (500 pg/well) or media for 5 hr, followed by incubation with *C. jejuni* 81176 for 3 hr. Media in the lower chamber were sampled after the three-hours of incubation with *C. jejuni,* plated on Bolton agar and bacterial colonies were enumerated.

Light microscopy

12-day old IPEC-1 cells on transwells were treated with rIL-4 (500 pg/well) or medium for 5 hr. The treatments were removed, the membranes were excised with a sterile scalpel, and examined by light microscopy using a laser scanning microscope at 63x aperture (LSM Pascal 5, Zeiss International, Jana, Germany).

Adherence assays

Adherence assays were carried out similarly to the invasion assays. The control strain, *E. coli* O157:H43 DEC 7A, exhibiting adherence characteristics was included to determine the effect of IL-4 on adherence. After pretreatment with 500 pg of rIL-4 in CM or CM alone for 5 hr, the treatments were removed and the bacteria (5×10^7 cfu in CM) were allowed to adhere for 1 hr. The supernatants containing bacteria which did not adhere was then removed and the transwell membranes were excised with a sterile scalpel. The epithelial cells were then lysed as described in the gentamicin assays, and the cell associated (adherent) bacteria were serially diluted and enumerated on Bolton agar plates for *C. jejuni* (or nutrient agar plates for *E. coli*). Invasion assays were conducted for the *C. jejuni* strains concurrently as internal controls. However, invasion assays were not done for the *E. coli* strain since its invasion potential is not known.

TEM

IPEC-1 cells were subjected to invasion assays as described previously. After the gentamicin treatment, medium was removed, the membranes were excised and fixed in 0.1M phosphate buffer containing 2.5% glutaraldehyde and 2.0% paraformaldehyde (pH 7.4). Membranes were washed three times in 0.1 M phosphate buffer (pH 7.4) for twenty minutes each. Following the washes, the membranes were fixed in 2% osmium tetroxide and dehydrated in increasing concentrations of acetone. The dehydrated membranes were subsequently infiltrated with epoxy resin, embedded in silicone molds, polymerized for 2 days at 60° C, and then examined using the transmission electron microscope (JEOL 100CX, Japan). Complete gentamicin killing assays (plate assays) as described

previously, were conducted concurrently as internal controls; bacteria were identified by colony morphology, microscopic examination and colonies enumerated. Seven samples each for rIL-4 pretreatment and media pretreatment were processed for TEM. For each treatment sample ~30 frames were examined at multiple magnifications. Cells were scored for morphological changes and for location of *C. jejuni* (whether they were adherent, intracellular, intravacuolar or intracytoplasmic).

MTT assays

MTT assays were performed to determine the cytotoxic effect of rIL-4, if any, on IPEC-1 cells. Briefly, IPEC-1 cells on tranwells pretreated with rIL-4 or media were incubated with 0.5mg/ml of MTT (suspended in RPMI media with 5% FBS), for 3 hr at 37° C, 5% CO_2 in 50 µl volumes. The treatment was removed, and cells were incubated with 0.1% Triton-X-100 for 10 minutes at 37° C and 5% CO_2. 100 µl of acidic isopropanol (0.1N hydrochloric acid in isopropanol) was then added, mixed, and absorbance measured at 570nm. All treatments were performed in triplicate.

Data analysis

Data are presented as mean ± standard error of the mean (SEM). Student's t-test was performed on the results obtained using Microsoft Excel, unless otherwise stated. P values ≤ 0.05 were considered significant. Regression analysis was also performed when required using Microsoft Excel, with confidence set at 95% level.

RESULTS

rIL-4 pretreatment of IPEC-1 caused a significant increase in the numbers of *C. jejuni* invading IPEC-1 cells (Figure 4. 1). There was a 6-10-fold increase in the number of bacteria that invaded compared to IPEC-1 cells pretreated with media alone ($p < 0.05$). Both *C. jejuni* strains used invaded IPEC-1 efficiently. There were no significant differences in the ability to invade between these strains based on comparisons of intracellular bacteria. rIL-10 pretreatment, unlike rIL-4, had no effect on invasion of *C. jejuni* (Figure 4. 1).

To determine if rIL-4 treatment was necessary and sufficient for increased invasion of IPEC-1 by *C. jejuni,* cells were incubated with rIL-4 and anti-IL-4 antibody. The results are shown in Figure 4. 2. When IPEC-1 cells were pretreated with a mixture of rIL-4 and anti-IL-4 antibody, the number of bacteria that invaded was similar to the control levels, while pretreatment with rIL-4 alone showed a significant increase in invasion ($p < 0.01$). The same results were seen for both *C. jejuni* strains tested. Pretreatment with anti-IL-4 antibody alone had no effect on invasion (data not shown).

rIL-4 pretreatment had a dose dependent effect on *C. jejuni* invasion (Figure 4. 3). There was a progressive increase in the number of intracellular bacteria with increasing amounts of rIL-4 pretreatment. Regression analysis on the results obtained showed a significant dose response effect for both *C. jejuni* strains ($p < 0.05$).

To determine if rIL-4 facilitated uptake without active participation by the bacteria, a non-invasive strain of *E. coli* (DH5α) was used in the invasion assay, both with and without rIL-4 pretreatment of IPEC-1 cells (Figure 4. 4). Unlike its effect on *C. jejuni*, rIL-4 pretreatment had no effect on uptake of *E. coli* DH5α ($p > 0.05$).

rIL-4 treatment had a significant effect on the trans-epithelial electrical resistance (TER) across the IPEC-1 monolayer (Figure 4. 5). The TER fell to ~50% of its initial values after treatment with rIL-4 ($p < 0.01$) while cells treated with rIL-10 and medium alone showed no significant decrease in TER. This was corroborated by light microscopy, which showed IPEC-1 cells no longer adjoined after treatment with rIL-4 for 5 hr (Figure 4. 6C), unlike cells treated with medium alone which were confluent and closely packed (Figure 4. 6A and 4. 6B). Increased vacuolation also was seen in the cells that received rIL-4 treatment compared to cells that were treated with medium alone. The presence of vacuoles in rIL-4 treated cells was not due to toxicity. MTT treatment assays showed that these cells were viable, and not significantly different from medium alone treated cells (Figure 4. 10). Also, translocation experiments were performed to determine if there was an increase in the traffic of *C. jejuni* to the baso-lateral chamber after rIL-4 pretreatment. As seen in Table 4.1, there was no significant increase in the number of bacteria to the baso-lateral surface when compared to media pretreated cells.

The effect of rIL-4 on *C. jejuni* adherence to IPEC-1 cells was different than that seen for invasion (Figure 4. 7). *C. jejuni* strain 81176 adhered equally to IPEC-1 with or without pretreatment of rIL-4. The other two bacteria, *C. jejuni* 33292 and *E. coli* O157:H43 DEC7A, a pig pathogen with adherent capabilities, showed statistically significant decrease in adherence after pretreatment of IPEC-1 with rIL-4. Based on data from the invasion assays and adherence assays, the percentages of *C. jejuni* that invaded as a percentage of bacteria that adhered were ~0.2% (strain 81176) and 0.06% (strain 33292) for media pretreatment, and 0.8% (strain 81176) and 1.06% (strain 33292) for rIL-4 pretreatment.

Transmission electron micrographs of IPEC-1 cells showed bacteria inside cells following rIL-4 pretreatment and after gentamicin-killing assays (Figure 4. 8A) but not after medium pretreatment (Figure 4. 8B). The bacteria were seen predominantly in the cytoplasm just under the plasma membrane or adjacent to vacuoles (Figure 4. 9, upper row). This was the case in almost all the ~ 30 fields analyzed; bacteria were seen largely near the apical surface with a few on the baso-lateral side (Figure 4. 9 panels 3 and 4), and in ~ 95% of the time free in the cytoplasm. Electron micrographic appearance of intracellular *C. jejuni* were similar to those observed by Kiehlbauch *et al.*,(15) and showed a dense cytoplasm surrounded by a lumen (Figure 4. 9, lower row, panels 1, 2, and 4). Coccoid structures also were seen, similar to results seen by Mansfield *et al.*, (19) *in vivo* in pigs (Figure 4. 9, lower row, panel 3).

DISCUSSION

Host factors studied to date for *C. jejuni* invasion of epithelial cells have been focused on signal transduction pathways and host cell surface receptors required for binding. In this study, we show that a soluble factor produced by host cells can also play a role in bacterial invasion. IL-4 is an anti-inflammatory cytokine normally induced in response to helminth infections. Although pro-inflammatory cytokines like TNF-α and IFN-γ have been traditionally associated with the production of inflammation and pathology (13, 27), recently, IL-4 has been implicated as a source of pathology. In the swine intestinal epithelial cells used in our study, rIL-4 caused an ~ 6-fold increase in invasion by both the pathogenic strains of *C. jejuni* tested (Figure 4. 1). This effect was seen only when IPEC-1 cells were pretreated with rIL-4 for 5 hrs; simultaneous addition of both rIL-4 and *C. jejuni* to IPEC-1 cells had no effect on invasion (data not shown). These results suggest that the effect of rIL-4 on *C. jejuni* invasion required additional events to take place within the eukaryotic cells before invasion was enhanced. Also, antibody to rIL-4 had a neutralizing effect on rIL-4 enhanced invasion of the bacterial strains, presumably because the IL-4 antibody prevented rIL-4 from binding its receptor (Figure 4. 2). Together, these data suggest that rIL-4 might mediate its effect through binding to its receptor on the IPEC-1 cell surface, triggering signal cascades in the host cell and eventually leading to enhanced internalization of *C. jejuni*.

rIL-4 mediated enhancement of bacterial invasion was dose-dependent (Figure 4. 3). Increasing concentrations of rIL-4 led to increased internalization of bacteria. This suggests an increased cross-linking of IL-4 receptors, leading to increased signal strength,

(or increased recruitment of mediators in signaling pathways) leading to enhanced invasion.

The effect of rIL-4 on invasion was observed only for pathogenic bacteria like *C. jejuni* and not for a laboratory selected non- pathogenic species like *E. coli* DH5α (Figure 4. 4). It is possible that in live hosts, IL-4 may not influence uptake of non- invasive commensals, rather only invasive bacteria. However, more non-invasive strains should be tested before this conclusion can be drawn.

In previous studies with the nematode parasite, *Heligmosomoides polygyrus*, Shea-Donohue *et al.,* (24) demonstrated increased permeability in mouse intestinal epithelial cells treated with IL-4. They suggested that the role of IL-4 might be to loosen the junctions between cells so that the worms are no longer tightly embedded and hence can be washed away by the intestinal fluids. Similarly, we found that pretreatment with rIL-4 increased the permeability of the monolayer (Figure 4. 5 and 4. 6C). Also, Monteville and Konkel concluded from their translocation experiments on T84 monolayers that *C. jejuni* might invade through the baso-lateral surface (21). We performed translocation experiments, in which medium in the lower chambers were sampled after incubation with bacteria for 3 hr to determine whether the increased invasion seen was due to increased translocation to the baso-lateral surface. However, we found no significant increase in translocation of bacteria to the lower chamber after pretreatment with rIL-4 compared to translocation after media pretreatment (Table 4. 1). Additionally, TEM analysis showed more bacteria just under the apical surface, suggesting that invasion occurred largely through apical surfaces rather than through baso-lateral surfaces (Figure 4. 8A).

Although *C. jejuni* has been reported to reside inside vacuoles in other studies (12), and although increased vacuolation was seen within the epithelial cells after rIL-4 pretreatment (Figure 4. 6C), in these experiments more bacteria were seen in the cytoplasm and adjacent to vacuoles than within them (Figures 4. 8 and 4. 9). However, this does not negate the fact that *C. jejuni* might have escaped the vacuoles, since TEM at earlier time points were not conducted. In addition, cytotoxicity assays with MTT showed that this increased vacuolation after rIL-4 treatment was not due to a cytotoxic effect on IPEC-1cells as there was no significant decrease in viability compared to medium-alone treated cells (Figure 4. 10).

rIL-4 had differential effects on adherence of the two *C. jejuni* strains and the *E. coli* strain O157:H43 DEC7A (Figure 4. 7). The known adhesins for *C. jejuni* include the CadF protein that binds fibronectin and the outer membrane protein PEB1(14, 16). Other putative *C. jejuni* adhesins include the flagella and lipopoysaccharides (20). For the ETEC strain the fimbriae K88 have been known to mediate adherence in pigs (7). *C. jejuni* 33292 and *E. coli* O157: H43 showed decreased adherence after pretreatment of IPEC-1 cells with rIL-4, while rIL-4 pretreatment had no effect on adherence of *C. jejuni* strain 81176. This suggests that the binding sites on the host cell for *C. jejuni* 33292 and *E. coli* O157: H43 might be similar, but different from *C. jejuni* 81176 receptor, and that rIL-4 might selectively down-regulate these receptors. One of the host receptors implicated in K88 binding *in vivo* in pigs is an intestinal mucin-type glycoprotein (7), and is a likely candidate for *C. jejuni* 33292 binding. Other explanations for the differential binding of the two *C. jejuni* strains might be that these strains differentially express certain adhesins or bind through different adhesins. However, even though adherence was

reduced by rIL-4 pretreatment, strain 33292 still showed enhanced invasion compared to the cells that received media alone, since 1.06% of the adhered population invaded after rIL-4 pretreatment, while only 0.06% invaded after medium pretreatment. Thus the invasion potential of 33292 was higher after rIL-4 pretreatment, since the proportion of the adhered population that invaded was higher. Also many of the cell-associated bacteria could actually be internal, compared to media-treated cells. Results of an invasion assay conducted at 1 hr with *C. jejuni* 33292 support this conclusion (data not shown).

Since this study was conducted in part to explore possible pathogenic mechanisms in the dual infection model, we believe that the IL-4 previously shown to be induced in response to both *C. jejuni* and *T. suis* in pigs might cause the enhanced invasion of bacteria seen in the swine model (19). This is corroborated by the fact that Dr. Helene Kringel at USDA found 3–6 fold elevation in IL-4 mRNA in swine infected with *T. suis* in the proximal colon and the LGCs (personal communication)-the same areas where enhanced invasion of *C. jejuni* was seen in dually infected pigs (19).

Our results are unlike those obtained by Hess *et al.*, (11), who found no effect of IL-4 pretreatment on internalization of *Listeria monocytogenes* or *Salmonella typhimurium* into differentiated Caco-2 cells or HT-29 cells. However, Hess *et al.*, pretreated cells with IL-4 for 48 hr or 72 hr, and the events we describe here occur much earlier. In addition, the different results described here might be due to the different cell line and bacteria used.

Previous studies have shown that IL-4 can downregulate TNF-α expression in porcine macrophages by 6 hr after exposure and in murine mast cells by 24 hr after exposure (26, 29). IL-4 mediated down-regulation of TNF-α in the mast cells was

through STAT-6, a transcriptional activator, and occurred by destabilization of TNF-α mRNA. Recent studies show that alteration of intestinal barrier permeability is also mediated by STAT-6 (18). In addition, it is generally accepted that STAT-6 activation occurs through IL-4 receptors (26). Therefore, we propose the following model for the mechanism by which rIL-4 causes enhanced invasion of *C. jejuni*. rIL-4 binds to a receptor, presumably IL-4Rα, and causes intracellular signal transduction events leading to increased vacuolation and perhaps downregulation of TNF-α or other proinflammatory cytokines, via STAT-6. These events are accompanied by changes to extracellular surfaces marked by breakdown of tight junctions (and differential expression or exposure of *C. jejuni* host receptors), facilitating increased invasion by *C. jejuni*.

rIL-10, unlike rIL-4, did not have a similar effect on *C. jejuni* invasion or on the TER across the monolayer. Hence further studies were not conducted with this cytokine. Also, corroborating our result, Dr. Kringel did not find any increase in IL-10 mRNA after *T. suis* infection at the sites where increased bacterial invasion was seen. Thus the role of IL-10 in this system remains to be determined.

ACKNOWLEDGEMENTS

This work was supported by the National Institute of Health grant 61-0954 and by the Center for Emerging Infectious Diseases grant 61-6387, Michigan State University. We sincerely thank Dr. Julia Bell for critical review of this article and valuable advice.

REFERENCES

1. **Aggarwal, B. and G., JU.** 1992. Other cytokines, p. 427-428, Human Cytokines, Handbook for basic and clinical research. Blackwell Scientific Publications, Cambridge, MA.

2. **Ajuebor, M. N., C. M. Hogaboam, T. Le, and M. G. Swain.** 2003. C-C chemokine ligand 2/monocyte chemoattractant protein-1 directly inhibits NKT cell IL-4 production and is hepatoprotective in T cell-mediated hepatitis in the mouse. J Immunol **170:**5252-9.

3. **Banchereau, J. and M.E. Rybak.** 1994. Interleukin 4, p. 99-126. *In* A. Thomson (ed.), The Cytokine Handbook, Second ed. The Academic Press.

4. **Berschneider, H. M.** 1989. Development of normal cultured small intestinal epithelial cell lines which transport Na and Cl. Gastroenterology **96:**A41.

5. **Fallon, P. G., P. Smith, E. J. Richardson, F. J. Jones, H. C. Faulkner, J. Van Snick, J. C. Renauld, R. K. Grencis, and D. W. Dunne.** 2000. Expression of interleukin-9 leads to Th2 cytokine-dominated responses and fatal enteropathy in mice with chronic *Schistosoma mansoni* infections. Infect Immun **68:**6005-11.

6. **Fireman, P.** 2003. Understanding asthma pathophysiology. Allergy Asthma Proc **24:**79-83.

7. **Francis, D. H., P. A. Grange, D. H. Zeman, D. R. Baker, R. Sun, and A. K. Erickson.** 1998. Expression of mucin-type glycoprotein K88 receptors strongly correlates with piglet susceptibility to K88(+) enterotoxigenic *Escherichia coli*, but adhesion of this bacterium to brush borders does not. Infect Immun **66:**4050-5.

8. **Friedman, C. R., Neimann, J., Wegener, H.C., and R.V. Tauxe.** 2000. Epidemiology of Campylobacter jejuni infections in the United States and other industrialized nations, p. 121-138. *In* I. Nachamkin, and Blaser, M.J (ed.), Campylobacter, Second ed. American Society for Microbiology, Washington, DC.

9. **Gause, W. C., J. F. Urban, Jr., and M. J. Stadecker.** 2003. The immune response to parasitic helminths: insights from murine models. Trends Immunol **24:**269-77.

10. **Gonzalez-Vallina, R., H. Wang, R. Zhan, H. M. Berschneider, R. M. Lee, N. O. Davidson, and D. D. Black.** 1996. Lipoprotein and apolipoprotein secretion by a newborn piglet intestinal cell line (IPEC-1). Am J Physiol **271:**G249-59.

11. **Hess, D. J., M. J. Henry-Stanley, E. A. Erickson, and C. L. Wells.** 2002. Effect of tumor necrosis factor alpha, interferon gamma, and interleukin-4 on bacteria-enterocyte interactions. J Surg Res **104:**88-94.

12. **Hu, L., and Kopecko, D.J.** 2000. Interactions of Campylobacter with eukaryotic cells: Gut luminal colonization and mucosal invasion, p. 191-216. *In* I. Nachamkin, and Blaser, M.J (ed.), Campylobacter, Second ed. American Society for Microbiology, Washington, DC.

13. **Ishida, Y., T. Maegawa, T. Kondo, A. Kimura, Y. Iwakura, S. Nakamura, and N. Mukaida.** 2004. Essential involvement of IFN-gamma in *Clostridium difficile* toxin A-induced enteritis. J Immunol **172:**3018-25.

14. **Kervella, M., J. M. Pages, Z. Pei, G. Grollier, M. J. Blaser, and J. L. Fauchere.** 1993. Isolation and characterization of two *Campylobacter* glycine-extracted proteins that bind to HeLa cell membranes. Infect Immun **61:**3440-8.

15. **Kiehlbauch, J. A., R. A. Albach, L. L. Baum, and K. P. Chang.** 1985. Phagocytosis of *Campylobacter jejuni* and its intracellular survival in mononuclear phagocytes. Infect Immun **48:**446-51.

16. **Konkel, M. E., S. G. Garvis, S. L. Tipton, D. E. Anderson, Jr., and W. Cieplak, Jr.** 1997. Identification and molecular cloning of a gene encoding a fibronectin-binding protein (CadF) from *Campylobacter jejuni.* Mol Microbiol **24:**953-63.

17. **Lawrence, C. E., J. C. Paterson, L. M. Higgins, T. T. MacDonald, M. W. Kennedy, and P. Garside.** 1998. IL-4-regulated enteropathy in an intestinal nematode infection. Eur J Immunol **28:**2672-84.

18. **Madden, K. B., K. A. Yeung, A. Zhao, W. C. Gause, F. D. Finkelman, I. M. Katona, J. F. Urban, Jr., and T. Shea-Donohue.** 2004. Enteric nematodes induce stereotypic STAT6-dependent alterations in intestinal epithelial cell function. J Immunol **172:**5616-21.

19. **Mansfield, L. S., D. T. Gauthier, S. R. Abner, K. M. Jones, S. R. Wilder, and J. F. Urban.** 2003. Enhancement of disease and pathology by synergy of *Trichuris suis* and *Campylobacter jejuni* in the colon of immunologically naive swine. Am J Trop Med Hyg **68:**70-80.

20. **McSweegan, E., and R. I. Walker.** 1986. Identification and characterization of two *Campylobacter jejuni* adhesins for cellular and mucous substrates. Infect Immun **53:**141-8.

21. **Monteville, M. R., and M. E. Konkel.** 2002. Fibronectin-facilitated invasion of T84 eukaryotic cells by *Campylobacter jejuni* occurs preferentially at the basolateral cell surface. Infect Immun **70:**6665-71.

22. **Nachamkin, I., Allos, B.M and Ho, T.W.** 2000. *Campylobacter jejuni* infection and the association with Guillain-Barre Syndrome, p. 155-178, Campylobacter, Second ed. ASM press, Washington DC.

23. **Rivera-Amill, V., B. J. Kim, J. Seshu, and M. E. Konkel.** 2001. Secretion of the virulence-associated Campylobacter invasion antigens from *Campylobacter jejuni* requires a stimulatory signal. J Infect Dis **183:**1607-16.

24. **Shea-Donohue, T., C. Sullivan, F. D. Finkelman, K. B. Madden, S. C. Morris, J. Goldhill, V. Pineiro-Carrero, and J. F. Urban, Jr.** 2001. The role of IL-4 in *Heligmosomoides polygyrus*-induced alterations in murine intestinal epithelial cell function. J Immunol **167:**2234-9.

25. **Skirrow, M., and Blaser, M.J.** 2000. Clinical aspects of Campylobacter infection, p. 69-88. *In* I. Nachamkin, and Blaser, M.J (ed.), Campylobacter, Second ed. American Society for Microbiology, Washington, DC.

26. **Suzuki, K., H. Nakajima, K. Ikeda, Y. Maezawa, A. Suto, H. Takatori, Y. Saito, and I. Iwamoto.** 2003. IL-4-Stat6 signaling induces tristetraprolin expression and inhibits TNF-alpha production in mast cells. J Exp Med **198:**1717-27.

27. **Tracey, K.** 1994. Tumour Necrosis Factor-Alpha, p. 289-304. *In* A. Thomson (ed.), The cytokine handbook, Second ed. Academic Press, San Diego.

28. **Yao, Y., J. K. Eshun, S. Lu, H. M. Berschneider, and D. D. Black.** 2002. Regulation of triacylglycerol and phospholipid trafficking by fatty acids in newborn swine enterocytes. Am J Physiol Gastrointest Liver Physiol **282:**G817-24.

29. **Zhou, Y., G. Lin, M. J. Baarsch, R. W. Scamurra, and M. P. Murtaugh.** 1994. Interleukin-4 suppresses inflammatory cytokine gene transcription in porcine macrophages. J Leukoc Biol **56:**507-13.

Table 4. 1. Translocation of *C. jejuni* to the baso-lateral surface after 3 hr incubation with IPEC-1 cells pretreated with either rIL-4 (500 pg/well) or RPMI medium for 5 hr. Results shown are from a single experiment.

	Number of bacteria translocated to the baso-lateral chamber after 3 hours incubation (CFU)	
	rIL-4 pretreatment (500pg/well) for 5 hr	Medium pretreatment for 5 hr
C. jejuni 81176	2	0

Figure 4. 1. The effect of pretreatment of rIL-4 (500 pg/well) and rIL-10 (1000 pg/well) on *C. jejuni* invasion of IPEC-1 cells. Results shown are representative of five to seven independent experiments. All treatments were in triplicate. Values shown are mean +/- SEM. (*, $p < 0.05$).

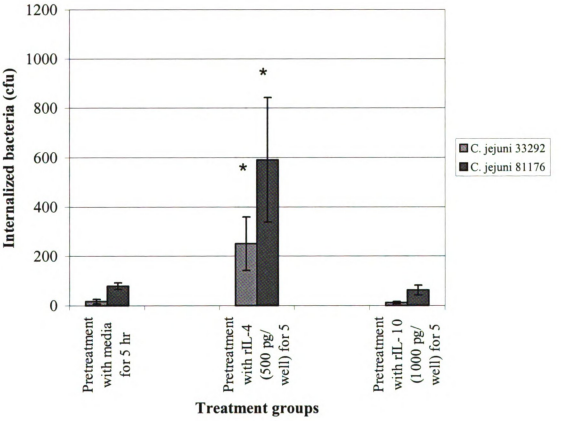


Figure 4. 2. The effect of rIL-4 antibody on invasion of *C. jejuni* of IPEC-1 cells. 12 day-old IPEC-1 cells on transwells were treated with rIL-4 +/- anti-IL-4 antibody or medium alone for 5 hrs. Gentamicin assay was carried out as described in Materials and Methods. All treatments were in triplicate. Results shown are representative of two identical, independent experiments. (**, $p < 0.01$).

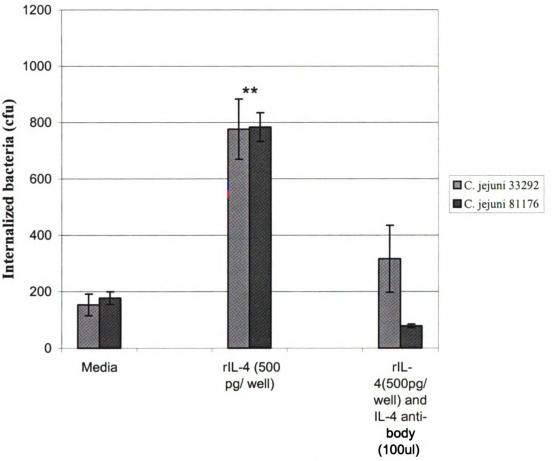


Figure 4. 3. Effect of increasing amounts of rIL-4 on *C. jejuni* invasion of epithelial cells. The IPEC-1 cells were treated with 100 pg/well, 200 pg/well, 300 pg/well, 400 pg/well, and 500 pg/well of rIL-4 for 5 hrs. Medium alone treatment was used as a control. Gentamicin assay was carried out as described in the Materials and Methods. Values shown are mean +/- SEM. All treatments were in triplicate. Results shown are representative of two identical experiments.

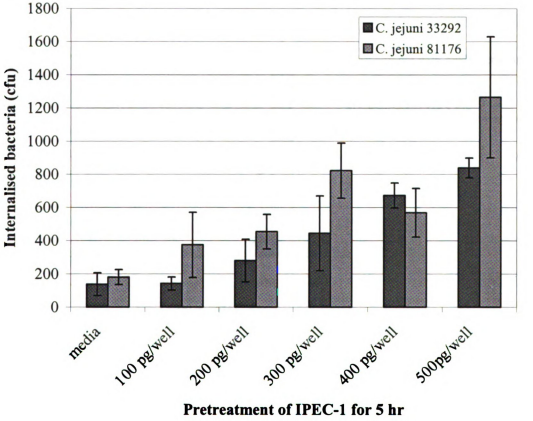


Figure 4. 4. Differential effect of rIL-4 on *E. coli* DH5α and *C. jejuni* invasion of IPEC-1 cells. Gentamicin killing assays were carried out as described in Materials and Methods. All treatments were in triplicate. Values shown are mean +/- SEM. (*, $p < 0.05$). Results are representative of two identical experiments.

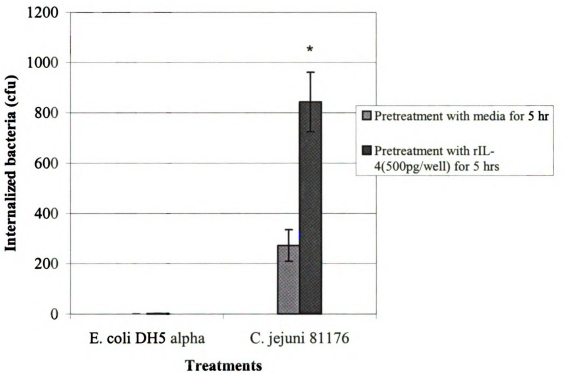


Figure 4. 5. Transepithelial electrical resistance (TER) across the 12 day-old IPEC-1 monolayer before and after treatment with rIL-4, rIL-10 or medium for 5 hrs. All treatments were in triplicate. Results shown are representative of three to four experiments. Values shown are mean +/- SEM. (**, $p < 0.01$).

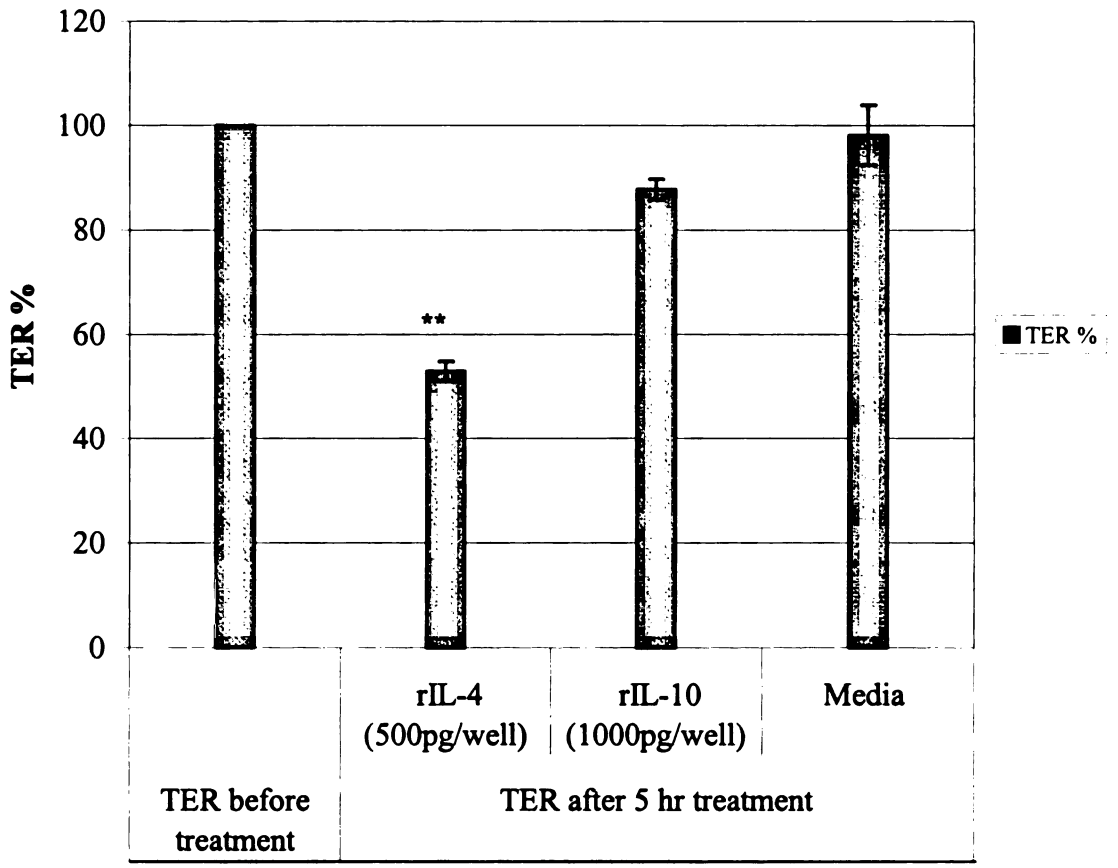


Figure 4. 6. Light microscopy of IPEC-1 cells on transwell membranes as seen through the baso-lateral surface. A) before any treatment, in medium, B) after treatment with fresh medium for 5 hr, and C) after treatment with rIL-4 (500 pg/well) for 5 hours. Bars represent 5 μm. Micrographs are representative of two to three samples analyzed for each panel.

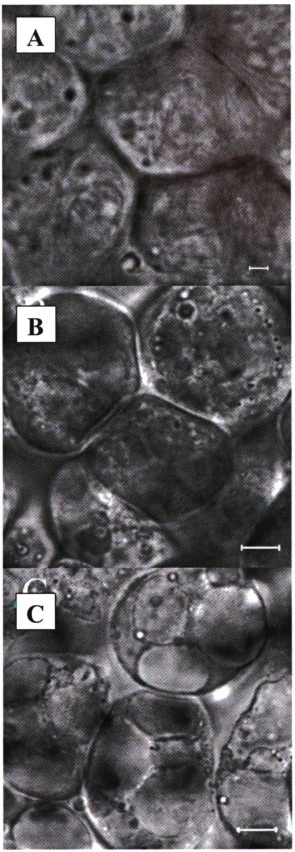

A
B
C

Figure 4. 7. Effect of rIL-4 pretreatment on adherence of bacteria to IPEC-1. Adherence assays were carried out as described in Materials and Methods. Values shown are mean +/- SEM. Results are representative of two identical, independent experiments. (**, $p < 0.01$; ***, $p < 0.001$).

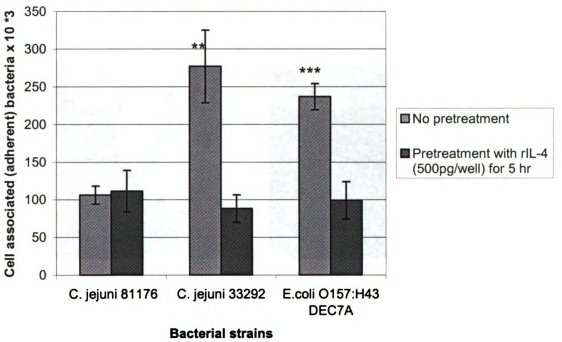


Figure 4. 8. Transmission electron micrographs of IPEC-1 cells pretreated with rIL-4 (8A) or medium (8B), followed by gentamicin killing invasion assays with *C. jejuni* strain 33292. Multiple bacteria were seen just beneath the plasma membrane (arrows) following rIL-4 pretreatment but not in the medium pretreated cells. Bars represent 1 μm. Panels are representative of micrographs from seven samples each from two identical experiments.

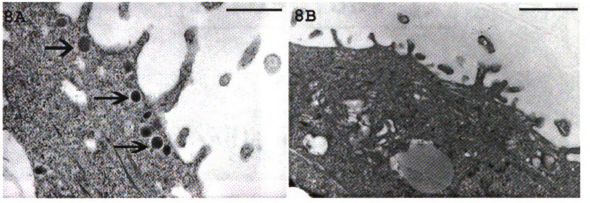


Figure 4. 9. Transmission electron micrographs of IPEC-1 cells pretreated with rIL-4, followed by gentamicin killing assays with *C. jejuni* 33292. Micrographs in the upper row show bacteria in the cytoplasm or adjacent to vacuoles (arrows); bars represent 500 nm. Micrographs in the lower row are magnifications of the ones directly above; bars represent 100 nm. Panels are from micrographs representative of seven samples.

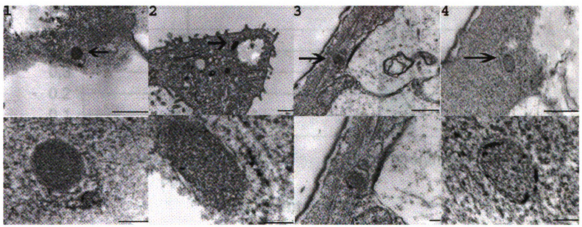
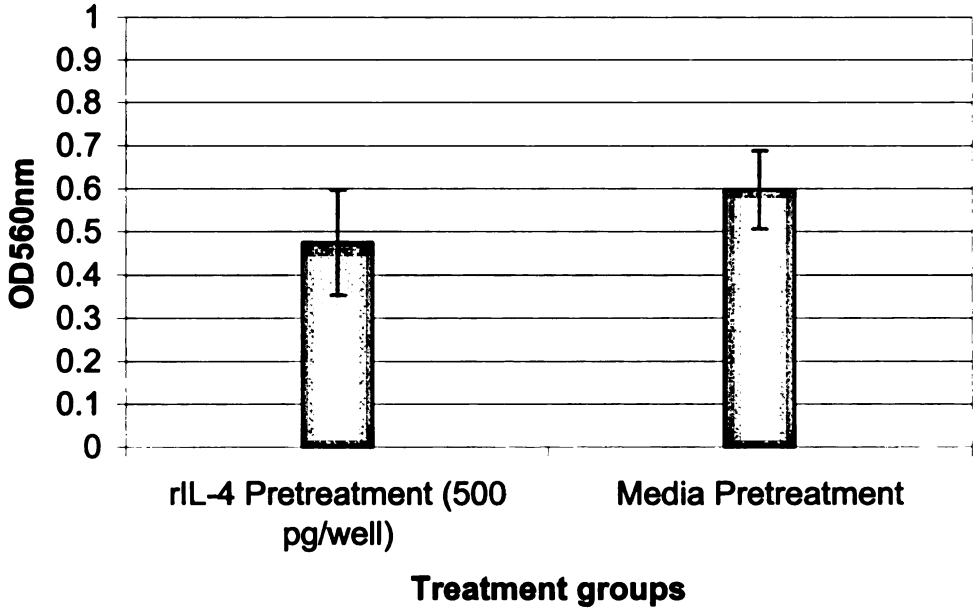


Figure 4. 10. MTT assays after IL-4 treatment and medium treatment. Assays were carried out as described in the Materials and Methods. Values shown are mean +/- SEM. All treatments were in triplicate and are representative of two independent experiments.



CHAPTER 5

SUMMARY AND CONCLUSIONS

The long-term goals of the laboratory are to determine the mechanisms of pathogenesis in the dual infection of swine mediated by *C. jejuni* and *T. suis*. We hypothesized that one of the mechanisms of pathogenesis could be an immune system dysregulation in cytokine responses to *C. jejuni* and *T. suis* infections, resulting in the pathology seen. Under this hypothesis, *C. jejuni* stimulates a predominantly Th-1 cytokine response accompanied by a moderate Th-2 component, while *T. suis* stimulates a predominantly Th-2 response from the porcine host. The Th-2 responses due to stimulation with both *C. jejuni* and *T. suis* act synergistically, causing a shift to a predominantly Th-2 profile and downregulation of Th-1 mediated immunity to the bacteria. Since *C. jejuni* is a known opportunistic pathogen (12, 23), down-regulation of the Th-1 response could facilitate *C. jejuni*-mediated pathology. A primary swine intestinal epithelial cell line, IPEC-1, was utilized to test these hypotheses, since epithelial cells form the first barrier to this primarily intestinal disease.

The secretion of IL-4, IL-6 and IL-10 Th-2 cytokines from IPEC-1 was tested in response to *C. jejuni* and *T. suis* Excretory Secretory Products (ESP). Since *T. suis* ESP has anti-microbicidal properties against *C. jejuni* (1), the cytokine responses to both these challenges were tested separately. Both differentiated and undifferentiated epithelial cells were tested for cytokine production to these pathogens to mimic the natural forms of these eukaryotic cells that exist *in vivo*. IL-6 and IL-10 were both secreted in response to *C. jejuni* infection. IL-6 was secreted within 24 hr in response to *C. jejuni*, while IL-10 induction took > 24 hr. In addition, IL-6 was secreted from both differentiated and undifferentiated IPEC-1 cells, while IL-10 was secreted predominantly from differentiated cells compared to negative controls. In all cases, secretion of IL-6 and IL-

10 occurred on the cell surface exposed to the bacteria. Also, *C. jejuni* strain 33292 induced baso-lateral induction of IL-10 while strain 81176 did not. *T. suis* ESP, on the other hand, induced IL-6 and IL-10 within 24 hr, and both differentiated and undifferentiated cells secreted these cytokines in response to a low level ESP (0.3 mg/ml) challenge. There was no secretion of IL-4 from IPEC-1 cells. These data suggest that IPEC-1 cells are a significant source of IL-6 and IL-10 innate immune responses to enteric infections, but not adaptive responses like IL-4. It is also likely that *C. jejuni* LPS, a major outer surface component, and an LPS-like substance in ESP can serve as inducer of these cytokines. LPS has been shown to act through Toll-like receptors (TLR), particularly TLR 4 and upregulate cytokine expression through the transcription factor NFκB (10). Exposure of IPEC-1 cells to purified LPS from *C. jejuni* and subsequent measurement of cytokines could test this hypothesis. Also, nuclear extracts from these treated IPEC-1 cells could be made and immuno-blot analysis for NFκB conducted to determine if this transcription factor is recruited for induction of these cytokines. A study to determine the presence of a LPS-like substance in ESP has been attempted, and revealed an LPS-like substance capable of recruiting NFκB in Chinese Hamster Ovary cells (CHO), and inducing TNFα secretion from RAW 264. 7, a mouse macrophage cell line. In addition, *T. suis* ESP also potentiated LPS mediated endotoxic shock response in mice, suggesting a role in immunomodulation similar to LPS (26).

The aim of these *in vitro* studies was to determine if there could be potential synergy in any of these cytokines. The data suggests that there may be synergy in IL-6 (and perhaps IL-10) innate cytokine responses *in vivo* during dual infections. The data also suggests that intestinal epithelial cells could contribute substantially to the cytokine

responses *in vivo*, since they form a large continuum throughout the gut. The presence of preformed IL-6 in IPEC-1 cells also was demonstrated, suggesting that IL-6 secretion could be a primary immune response to these enteric infections.

IL-4, IL-6 and IL-10 responses were tested during *in vivo* infections of swine with *C. jejuni* and *T. suis*. Experiments were conducted in two different ways. Short-term experiments involved simultaneous oral infection of swine with *C. jejuni* and *T. suis* eggs. In long-term experiments swine were infected orally with *T. suis* eggs 21 days prior to infection with *C. jejuni*. IL-4 and IL-10 cytokine concentrations in feces were significantly increased during the long-term infections in the dual infection, while IL-6 concentrations in feces increased in the short-term dual infections. Also, IL-4 and IL-10 levels were elevated 2-fold and 3-fold, respectively, during the long-term dual infections, while IL-6 levels were elevated 2-fold during the short-term dual infections. Because pathology is seen 21 days after introduction of *T. suis* during natural infections of swine, it is reasonable to assume that long-term experiments are more reflective of the natural infections that result in the observed disease and pathology than the short-term experiments. Hence, IL-4 and IL-10, which were upregulated during the long-term experiments, were hypothesized to play a role in pathology mediated by these two organisms together. In addition, based on the analysis of singly and dually infected pigs, the pattern of secretion was mainly reflective of *T. suis*-induced responses, suggesting that the whipworm was the predominant inducer of these cytokines. Also, as intestinal epithelial cells predominantly secreted IL-6 *in vitro*, accompanied by a moderate IL-10 component, upregulation of IL-4 during long-term infections suggests that other cell types like macrophages and lymphocytes likely secreted this cytokine. A previous

hypothesis from the *in vitro* experiments was that there might be synergy in innate IL-6 responses *in vivo* during dual infections with *C. jejuni* and *T. suis*. IL-6 responses were elevated 2 fold in 70% of pigs, compared to a slight induction in 40% of controls during short-term concurrent infections. It is likely that intestinal epithelial cells contributed significantly to this innate response.

In previous studies with swine, increased invasion of *C. jejuni* was seen in both the follicle associated epithelium (FAE) of the lymphoglandular complexes (LGCs) and in the crypt epithelial cells in the proximal colon around the worms (15). Since IL-4 and IL-10 cytokine responses were elevated during long-term dual infections in swine, the possibility that IL-4 or IL-10 might play a role in enhanced invasion of *C. jejuni* was tested. Differentiated IPEC-1 cells grown on transwell membranes to mimic the FAE were pretreated with rIL-4 or rIL-10, and infection assays with *C. jejuni* were carried out. Pre-treatment with rIL-4 enhanced *C. jejuni* invasion ~6 fold, while pretreatment with IL-10 had no effect. The rIL-4 mediated effect was dose dependent, and invasion increased with increasing doses. Thus, although only moderate increases (2-fold) in IL-4 levels were seen in pigs dually infected with *C. jejuni* and *T. suis* (chapter 3), this data suggests that this moderate increase might be sufficient to enhance invasion of *C. jejuni in vivo*. Because invasive strains of *C. jejuni* have been shown to be associated with inflammatory diarrhea (13, 18), this enhanced invasion during dual infection might contribute to the inflammatory diarrhea seen (15). rIL-4 also decreased the transepithelial resistance across the IPEC-1 monolayer by 5 hr. It has been suggested in other studies that IL-4 mediated increased permeability might loosen worms from the mucosa and enable worms to be washed away (21). A similar result here suggests that IL-4 might have a similar role

in loosening *T. suis* from the intestines *in vivo*. A study conducted at USDA by Dr. Helene Kringel and others also corroborated these results. They found IL-4 mRNA to be increased 3–6 fold, 21 days after *T. suis* infection in swine (personal communication). This increase was seen in the proximal colon and the LGCs, but not in the distal colon. Since the proximal colon and LGCs are the areas where enhanced *C. jejuni* invasion was seen in previous studies (15), and since cytokine responses *in vivo* seem more closely associated with *T. suis* infection, this suggests that *T. suis* induces IL-4 during natural infections, enabling enhanced invasion of *C. jejuni*. rIL-4 also enhanced vacuole formation in IPEC-1 cells. In previous studies, *C. jejuni* has been shown to be located inside vacuoles, as well as demonstrated to invade through the baso-lateral surface of human intestinal epithelial cells (9, 16). The presence of numerous vacuoles after rIL-4 pretreatment together with the increase in permeability, suggested that *C. jejuni* might increasingly translocate to the baso-lateral surface, and reside inside these vacuoles after rIL-4 treatment. However, translocation experiments did not reveal increased translocation of the *C. jejuni* strain 81176 to the baso-lateral side. In addition, transmission electron microscopy (TEM) showed that bacteria were largely seen just under the apical surface rather than near the baso-lateral suface. Also, bacteria were predominantly seen in the cytoplasm and adjacent to vacuoles rather than within them. However, *C. jejuni* might have escaped the vacuoles. Also, it is possible that cell type influenced this phenomenon since IPEC-1 cells are of non-human origin, and the cell lines used in other published studies were human cell lines, *C. jejuni* invasion mechanisms might differ according to species. To test these hypotheses, TEM examination of IPEC-1 cells at more time points is needed. Also, invasion assays with or

without rIL-4 pretreatment in a few human and non-human epithelial cells need to be done to compare and correlate invasion mechanisms by *C. jejuni*.

Since IL-6 is induced almost instantaneously with *C. jejuni* infection (Chapter 2), its role in IL-4 enhanced invasion was also investigated. IPEC-1 cells pretreated with rIL-4 were subjected to invasion assays with *C. jejuni*, with the simultaneous addition of anti-IL-6 antibody along with the bacteria. This was done to determine if concurrently produced IL-6 potentiates or decreases invasion. However, mixed results were obtained *in vitro*. In one experiment, addition of IL-6 antibody enhanced invasion of *C. jejuni* into IPEC-1 cells 2-fold, suggesting that IL-6 behaved in a pro-inflammatory manner, keeping invasion levels low (data not shown). However, a repeat of this experiment showed no change in invasion levels. Hence the role of IL-6 in invasion is inconclusive at this point, and can be made clear through repeats of this experiment or with use of different cell lines including those derived from humans.

IPEC-1 is a primary epithelial cell line that we used for *in vitro* studies. The immune responses are more likely reflective of *in vivo* immune responses because these cells are a primary cell line rather than a transformed cell line. The data from this thesis study and concurrent studies in the laboratory suggests that the predominant cytokines secreted from this cell line are IL-6, IL-8 and IL-10, IL-1β, TNFα, but not IL-4 or IFNγ (L. D. Cunningham, personal communication). At the beginning of this study, almost nothing was known about innate cytokine secretion responses from IPEC-1 cells. At present, we have made some headway in understanding the cytokine repertoire of this cell line. However, other cytokine responses still need to be explored. Based on the current data, this primary cell line makes a good model for understanding the patterns and

regulation of innate cytokine responses in swine intestinal epithelium to multiple enteric infections.

At the beginning of this study, my hypothesis was that there is synergy of Th-2 cytokine responses induced in response to both *C. jejuni* and *T. suis*, and this overproduction of Th-2 cytokines down-regulates the Th-1 responses to the bacteria. Indeed, we did see upregulation of two primary Th-2 cytokines IL-4 and IL-10 in the long-term experiments in the dual infection. However, only part of this hypothesis was accepted. Other studies done concurrently indicate that there is upregulation of IL-1β and TNF-α as well during long-term infections (Cunningham, LD and L.S. Mansfield, unpublished data). Therefore, the pathology seen during dual infection with *C. jejuni* and *T. suis* may be due to simultaneous production of both proinflammatory and Th-2 cytokines like TNF-α, IL-1β, IL-4 and IL-10, with individual cytokines playing a role in clinical symptoms. IL-4 enhanced *C. jejuni* invasion of IPEC-1 cells, while IL-10 and TNF-α did not (Cunningham LD, personal communication). However, IL-10 has been shown to downregulate IFN-γ from macrophages (2), IL-1β in inflamed tissues and TNF-α in colitis models (5, 25). In addition, IL-1β has been shown to potentiate the effect of TNF-α in mice (5). Since dual-infection with *C. jejuni* and *T. suis* in swine is characterized by inflammation and colitis as well, this suggests that TNF-α and IL-1β may play a role in this aspect of pathology during dual-infection (Figure 5. 1).

In conclusion, a few novel results have been seen from this dissertation, that shed new light on *T. suis* and *C. jejuni* mediated pathogenesis in swine. We show here for the first time that rIL-4 enhances invasion of pathogenic bacteria such as *C. jejuni* into epithelial cells, which suggests that, in natural dual infections of swine, *T. suis* indirectly

contributes to *C. jejuni* pathogenesis by induction of IL-4. This has enormous impact, not only in the veterinary field with respect to trichuriasis, but also in humans. Millions of people worldwide, particularly in the developing world, harbor the human counterpart of *T. suis*, *T. trichiura* (19, 20). *T. trichiura* also has been shown to induce IgE, IgG4 and IgG1 responses, which are IL-4 mediated (14, 17). In addition, *T. suis* has been shown to produce patent infections in humans (3, 4). *C. jejuni* is a well known human pathogen. Although helminth infections are uncommon in the developed world, it might be prudent and prophylactic to analyze for helminth infections in feces in addition to bacteria, during enteritis. This was borne out in a recent case study in Canada, where *T. suis* ova was seen along with *C. jejuni* in the feces of patient with toxic mega-colon and renal failure (22). Also, asthma, and other allergic diseases, which have etiology in Th-2 cytokine responses, affect close to 100 million people worldwide (11). A recent study showed that incidence of *Chlamydia pneumoniae*, an intracellular pathogen, increased in patients with asthma. It was suggested that the increase in incidence rate might be due to "modified susceptibility to the organism, due to changes in host-cell physiology" (8). If we extrapolate results from our study, this suggests that induction of Th-2 cytokines like IL-4 during asthma might cause a similar secondary bacterial infection to occur. Other potential invasive pathogens that can cause respiratory infections and intestinal infections, respectively, include *Mycobacterium tuberculosis*, *Streptococcus pnuemoniae, Pseudomonas aeruginosa* and *Salmonella* spp., *Intracellularis* bacteria and others. Thus this IL-4 mediated enhancement of bacterial invasion has the potential to be extended as a predisposing mechanism to multiple pathogens as well. Also, it has been suggested that when the immune profile is dominated by a Th-1 response, the resistance to HIV and *M.*

tuberculosis is increased, and impaired when the profile is slanted toward a Th-2 phenotype (6).

Recently, *T. suis* and other helminths are being advocated as ameliorative agents for inflammatory bowel disease, including Crohn's disease and ulcerative colitis (7, 24). Since these diseases have an etiology based on a Th-1 response, and since helminths induce a predominant Th-2 response, helminth eggs were given to patients with these conditions to downregulate these responses. This treatment was effective for most of the patients. However, given the results in this thesis study, this avenue needs to be followed with caution. *T. suis* and other helminths might not have any adverse effects on their own. However, our work suggests that patients must be carefully scrutinized for any potential infection with enteric pathogens that might elicit adverse reactions.

There are several other novel results from these studies. We show for the first time in this study, that *T. suis* ESP induces innate responses in epithelial cells cultured *in vitro*, as well as the innate and adaptive cytokine responses in feces in swine challenged with *T. suis* and *C. jejuni* (chapter 2 and chapter 3). Another novel contribution to science from this research was the identification of pre-formed IL-6 protein in IPEC-1 cells. This is the first demonstration of stored IL-6 cytokine in intestinal epithelial cells. This reiterates the fact that primary cell lines are likely to be physiologically different from immortalized cell lines. It also opens up a new avenue for study of protein synthesis, storage and secretion of IL-6 and other cytokines in defined intestinal cells.

Future directions

Host-pathogen interactions play a vital role in the outcome of a disease state. The aim of most researchers worldwide in the field of infectious diseases is to understand the mechanisms underlying these interactions in order to develop new avenues for therapy or create awareness of potential health hazards. The goal of this study was to delineate some of the mechanisms underlying the pathogenesis of dual infection mediated by *C. jejuni* and *T. suis* in swine. Also, *C. jejuni* is a significant human pathogen. These studies also were designed to understand how the host controls this pathogen. Additionally, because *T. suis* is currently being advocated as therapy for intestinal diseases (7, 24), the immune responses to this helminth and its consequences need to be explored in detail before its widespread use. We have shown from our study, that IL-4 produced likely in reponse to *T. suis* infections can potentiate the invasion of *C. jejuni*. However, it is likely that other mechanisms also occur concurrently, that contributes to the disease process. There are multiple avenues open for follow up experiments, in order to understand the immuno-pathogenic or microbial pathogenic mechanisms mediated by these two organisms, either together or alone. Also, other hypotheses need to be addressed to refine our *in vitro* and *in vivo* models. Furthermore, while some of the immune responses from the IPEC-1 cell line were characterized from this study, very little is known of other physiological responses from these cells. As swine intestinal system mimics humans very closely, this primary cell line makes an effective system to study innate immunity of swine to other enteric infections.

In chapter 2, we showed that *C. jejuni* strains 33292 and 81176 differed in their ability to induce IL-10 responses from differentiated cells. Also, only differentiated cells

induced any significant IL-10 response over the negative controls while the undifferentiated cells did not. The mechanisms underlying the differences between cells and different strains of bacteria should be followed up, as this would inform us about the development of host immune response, and factors contributing to functional variations in *C. jejuni* respectively. Understanding the antigenic basis of host response to *Trichuris* is also important. The immune responses to *T. suis* ESP were tested, but not to constitutive products of the worm. Worm homogenates could provide a useful antigen for these studies. Also, only immune responses to epithelial cells were tested in our study. Cell types making up the intestinal tract are complex and varied. Macrophage or lymphocyte responses will be important components to study. They are likely to drive immunoregulation in the gastrointestinal tract. Another important finding is that pre-formed IL-6 was seen for the first time. Its intracellular location, synthesis, gene regulation and secretion are some important areas for follow-up study.

In chapter 3, IL-4, IL-6 and IL-10 cytokine responses were measured in the feces. In another concurrent study, IL-8, IL-1β and TNFα were measured. While measurement of these cytokines gave us some insight into the pathogenic mechanisms during the dual infection, other cytokines such as IL-5, TGFβ, IFNγ, GM-CSF, IL-13 and IL-12 also need to be measured to completely understand the immunoregulation of *T. suis* and *C. jejuni* infections. Also, comparison of these *in vivo* induced cytokines with similar *in vitro* responses can help us understand the cell types predominantly driving the immune responses. A similar *in vivo* study like the one just conducted can be undertaken, and mRNA responses over time in different tissues could be measured. This would tell us the specific tissues that contribute to immune responses, and help us correlate the findings

from this thesis study to areas of pathology seen during dual infection of swine with *C. jejuni* and *T. suis*, and also corroborate Dr. Kringel's Real time PCR cytokine data.

In chapter 4, rIL-4 enhanced *C. jejuni* invasion of epithelial cells and increased the permeability of the cells. One question here is whether the increase in intestinal permeability is necessary and sufficient for increased bacterial invasion or does it need to be accompanied by intracellular changes in the IPEC-1 cells?, This would inform us on the specific changes that need to occur in IPEC-1 cells that precede enhanced invasion of *C. jejuni*. Another avenue is to determine whether the action of rIL-4 is receptor mediated, which receptors are bound by rIL-4, and which signal transduction pathways are activated thereafter. This provides an important avenue for intervention strategies with the dual infection. The downstream cytokine responses in IPEC-1 cells following rIL-4 treatment can be pursued, as this would tell us the immunomodulation that needs to take place in IPEC-1 cells to facilitate invasion. Effect of rIL-4 and rIL-10 on other tissue types like macrophages also can be investigated, since *C. jejuni* invasion of macrophages also occurred during dual infection of swine with *T. suis* and *C. jejuni*. This would give information on differential requirement of cytokines, if any, on macrophage invasion when compared to epithelial cell invasion. Differential adherence of the *C. jejuni* strains to IPEC-1 was seen after rIL-4 pretreatment. The microbial and eukaryotic factors contributing to this difference need to be explored. Since adherence is an important colonization factor for C. jejuni, this avenue would inform the researcher on differential factors contributing to adherence in *C. jejuni* and hence form a basis for vaccine studies. Since the enhanced invasion of *C. jejuni* into IPEC-1 was shown *in vitro*, the *in vivo* effect of rIL-4 on *C. jejuni* invasion needs to be measured by use of removable intestinal

tie-adult rabbit diarrhea (RITARD) models. This would validate our findings in a physiologically relevant setting.

In addition to host-pathogen interactions, interactions between *T. suis* and *C. jejuni* also need to be studied, especially as numerous *C. jejuni* were seen around the worms in the proximal colon during dual infection (15). The possible role of the worm or its homogenates in the regulation of bacterial virulence genes is one area for study, which provides another dimension to this complicated phenomenon.

Other relevant studies extending beyond the dual infection of swine are epidemiological studies on human patients with enteric bacterial infections to determine if these patients harbor helminths. This study would help explain if complications seen during enteric infections in humans could arise from other concurrent infections like the one seen in our study. A similar study could be conducted in swine farms as well, to determine the rate of *C. jejuni* infections in clinical trichuriasis, in order to correlate our experimental findings with those occurring naturally. Another epidemiological study would be to determine the susceptibility of patients with allergic diseases to bacterial infections. As mentioned before, close to 100 million people suffer from asthma (11), and such a study would help correlate our findings to extra-intestinal infections as well. Hence, numerous avenues are available for further study from the results obtained in this dissertation. However, the following avenues will be described in detail.

1) Modulation of cytokine responses in IPEC-1 cells following rIL-4 treatment.
2) Identification of IL-4 receptors on IPEC-1 cells, and signal transduction pathways mediated by rIL-4 leading to increased internalization.

3) Molecular basis for differential adherence of *C. jejuni* strains 33292 and 81176 on IPEC-1 surface.

Modulation of cytokine responses in IPEC-1 following rIL-4 pretreatment.

The hypothesis in this study is that rIL-4 treatment causes down-regulation of certain cytokines such as TNF-α, IL-6, IL-8, IL-1β, or IFNγ, leading to increased invasion of *C. jejuni*. To evaluate this hypothesis multiple techniques can be employed. **Real time RT-PCR:** 12-day old IPEC-1 cells on transwells will be exposed to rIL-4 or media for 5 hr, then cells will be challenged with *C. jejuni*, and the treatment removed. RNA will be extracted from the IPEC-1 cells using Trizol® reagent or Qiagen RNAeasy kit. cDNA will be constructed using first strand Pro-Star RT-PCR kit (Stratagene) and PCR carried out for TNF-α, IL-6, IL-8, IL-1β, IFNγ and IL-10 using real-time porcine primers. The control in this study will be cells exposed to media alone. Gentamicin assays will be conducted concurrently as additional controls. Another technique is **ELISA:** 12-day old IPEC-1 cells on transwells will be exposed to rIL-4 for 5 hr, then cells will be challenged with *C. jejuni* and the treatment removed. RPMI media will be applied in 400μl volumes and incubated for 10 minutes, 30 minutes, 1 hr, 2 hr and 3 hr. The media will be removed after regular intervals, centrifuged at 6000 rpm for 10 minutes, at 4° C, transferred to fresh centrifuge tubes, and analyzed for TNF-α, IL-6, IL-8, IL-1β, IFNγ and IL-10 by ELISA using kits from Biosource International, Camarillo, CA. Controls in this experiment will be cells exposed to media alone, rIL-4 and rIL-4 antibody. Another alternative technique is **Western blot analysis** for proteins. Whole cell extracts will be made, blotted on to PVDF or NCP membranes, along with standards, and probed with antibody to each cytokine. However, since the transwell size might not be

suitable for whole cell extracts (6.4 mm diameter), a bigger transwell size will be needed, or alternatively, IPEC-1 cells could be exposed to rIL-4 growing in tissue culture flasks. A valid control would be cells exposed to media alone.

This study would inform the researcher on cytokine responses being altered following rIL-4 treatment, and would give information on the cytokines that are potential targets for immunomodulation by IL-4 during dual infection of swine with *C. jejuni* and *T. suis*.

Identification of receptors bound by rIL-4.

Here, the hypothesis is that rIL-4 mediated enhancement of invasion occurs through IL-4Rα. Some of the experimental techniques that can be used to validate this hypothesis are 1) **Application of IL-4Rα antibody**: Antibody to IL-4Rα will be applied along with rIL-4 to the IPEC-1 monolayer, and gentamicin killing assays carried out as described previously. Cross-reactivity to rIL-4 needs to be ruled out by ELISA prior to use. If rIL-4 mediated enhancement of *C. jejuni* invasion is through IL-4Rα, then application of antibody to the receptor will not enhance invasion, and the bacterial numbers will be lower than in rIL-4 treated positive controls. If so, then further experiments can be done to verify this. A) Membrane preparations can be made, and incubated with rIL-4 beads for 5 hr. The bound proteins are then dissolved, resolved on an SDS-PAGE gel and analyzed by western blot using antibody to IL-4Rα. B) Knock down of the IL-4Rα gene using siRNA technology and subsequent invasion assays with rIL-4. 2) **Chemical cross-linking assays and affinity columns:** Whole cells or membrane preparations will be incubated with radiolabelled and unlabelled rIL-4 or with

radiolabelled rIL-4 alone, washed and crosslinked using disuccimidyl substrate (crosslinking agent), and gel shift assays performed. The protein bands will be excised from the gel, reverse cross-linked and mass spectrometry conducted on the radioactive complex, and the protein identified. Gel shift assays without crosslinking will be run concurrently. Membrane fractions will also be run through rIL-4 affinity columns so that the columns elute out the proteins bound by the cytokine. The protein band will be excised, and subjected to mass-spectrometry and protein identified.

Identification of the IL-4 receptor provides a potential target for intervention therapies, and also a route for studying signal pathways mediated by this receptor in IPEC-1 cells, of which little is known.

Molecular basis for differential adherence of *C. jejuni* strains 33292 and 81176 on IPEC-1 surface.

The hypothesis for this study is that *C. jejuni* strains 33292 and 81176 bind through different adhesins, or have differential concentrations of the same adhesins. In order to identify the adhesins on *C. jejuni* 33292 and 81176, again several techniques can be employed. 1) **Outer membrane protein (OMP) preparations**: OMPs will be extracted from individual *C. jejuni* strains using the EDTA buffer method. The preparations will be run on an SDS-PAGE gel and visualized using Coomassie stain. If the same adhesin is differentially expressed, the band intensity would be expected to be stronger in one strain than the other. If there are distinct band differences then, the protein bands will be excised, and partially sequenced by Edman degradation reaction, and partial N-terminal sequence obtained. This sequence can be compared against the

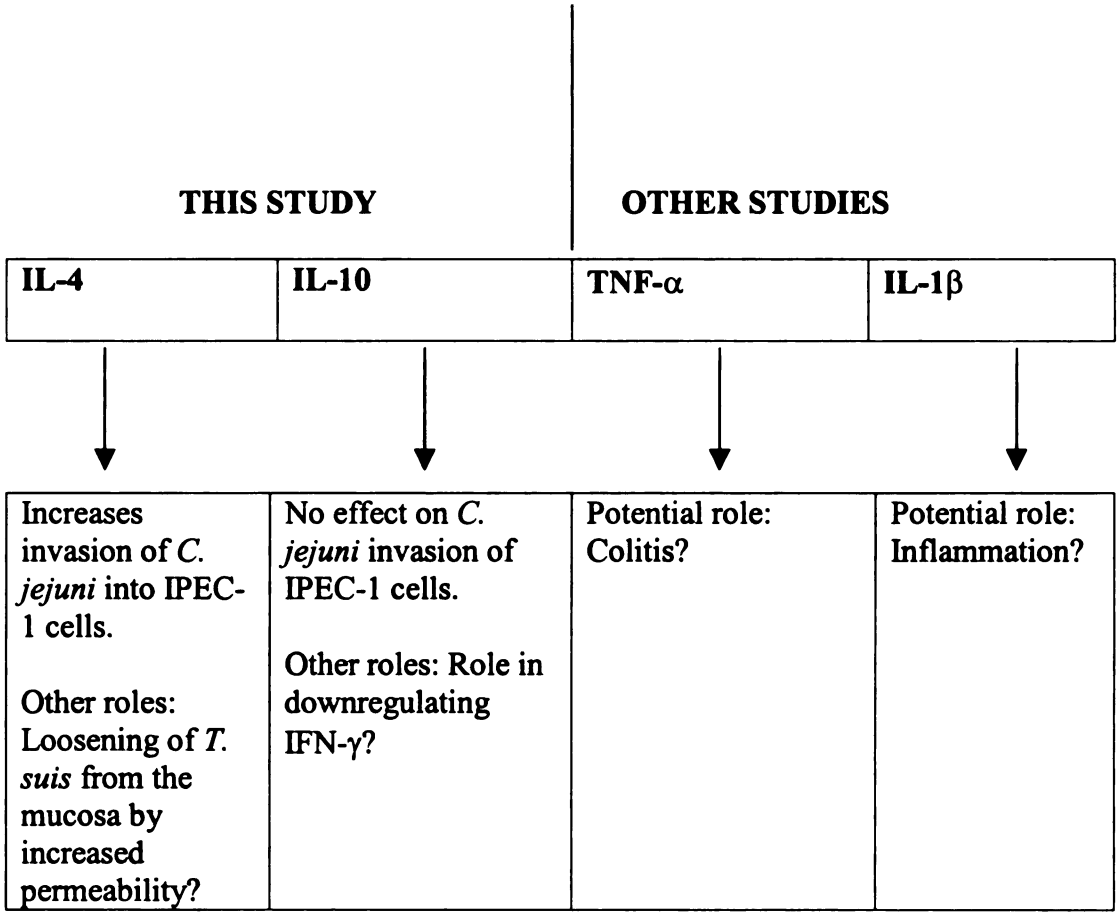
published *C. jejuni* genome sequence to identify the genes for adhesins. Alternatively, mass-spectrometry could be done on the excised protein band and the protein identified. 2) **Construction of a phage display library and immunoscreening**: A phage library for both strains will be constructed individually, with each phage containing a piece of bacterial genomic DNA. Anti-sera will be raised against individual bands in the OMPs and each serum will be screened for blocked adherence. The phage library will then be screened with sera that inhibited adherence, and phage colonies that react with sera further selected for analysis. The bacterial DNA in these colonies will be amplified by PCR, and sequenced, and compared against the published genome sequence to identify relevant genes. 3) **Random transposon mutagenesis**: Random transposon mutants could be constructed for each strain and screened for decreased adherence on IPEC-1 cells. The mutated gene would then be identified by PCR with primers made from the known transposon or antibiotic markers. 4) **Targeted gene mutation**: Since *peb1* and *CadF* are known adhesins for *C. jejuni*, these genes could be mutated individually, and screened for decreased adherence on IPEC-1. 5) **LPS analysis**: As LPS is also a known adhesin for *C. jejuni*, the LPS of each strain could be analyzed to determine if the difference in adherence is LPS mediated. For this study, LPS will be extracted by phenol-water extraction, and anti-sera generated in rabbits. The anti-sera could then be used in adherence assays to determine if LPS antibodies have an effect on adherence. Controls will be adherence assays using a non-specific antibody isotype control. If an effect is seen, the extracted LPS could be run on tricine SDS-PAGE gel and stained with silver stain and the subsequent pattern profiles analyzed for differences. In addition, serotypes

of the strains could be determined, and additional strains of similar serotypes analyzed for correlation in adherence patterns.

Campylobacter strains show enormous strain-to-strain variation. Identification of genetic or molecular factors that contribute to functional and phenotypic variations in *Campylobacter* infections will help the researcher understand the multiple mechanisms employed by this versatile bacterium in colonization and enable suitable intervention strategies for the control of its pathogenesis.

Figure 5. 1. The potential role(s) for each cytokine *in vivo* during long-term dual infection of swine with *C. jejuni* and *T. suis*.

POTENTIAL ROLE(S) OF SIGNIFICANTLY INDUCED CYTOKINES IN LONG-TERM DUAL INFECTION *IN VIVO*



REFERENCES

1. **Abner, S. R., G. Parthasarathy, D. E. Hill, and L. S. Mansfield.** 2001. *Trichuris suis*: detection of antibacterial activity in excretory-secretory products from adults. Exp Parasitol **99:**26-36.

2. **Aggarwal, B. a. G., JU.** 1992. Other cytokines, p. 427-428, Human Cytokines, Handbook for basic and clinical research. Blackwell Scientific Publications, Cambridge, MA.

3. **Beer, R. J.** 1971. Experimental infection of man with pig whipworm. Br Med J **2:**44.

4. **Beer, R. J.** 1976. The relationship between *Trichuris trichiura* (Linnaeus 1758) of man and *Trichuris suis* (Schrank 1788) of the pig. Res Vet Sci **20:**47-54.

5. **Dinarello, C.** 1994. Interleukin-1, p. 31-56. *In* A. Thomson (ed.), The cytokine handbook, Second ed. Academic Press Ltd., San Diego,CA.

6. **Fincham, J. E., and M. B. Markus.** 2001. Human immune response to Trichuris trichiura. Trends Parasitol **17:**121.

7. **Fox, J. G., P. Beck, C. A. Dangler, M. T. Whary, T. C. Wang, H. N. Shi, and C. Nagler-Anderson.** 2000. Concurrent enteric helminth infection modulates inflammation and gastric immune responses and reduces helicobacter-induced gastric atrophy. Nat Med **6:**536-42.

8. **Gencay, M., and M. Roth.** 2003. *Chlamydia pneumoniae* infections in asthma: clinical implications. Am J Respir Med **2:**31-8.

9. **Hu, L., and D. J. Kopecko.** 1999. *Campylobacter jejuni* 81-176 associates with microtubules and dynein during invasion of human intestinal cells. Infect Immun **67:**4171-82.

10. **Janeway, C., Travers, P, Walport, M and JD, Capra.** 1999. Signaling through lymphocyte receptors, p. 163-194. *In* P. a. E. L. Austin (ed.), Immunobiology-The immune system in health and disease, Fourth ed. Elsevier Science Ltd and Garland Publishing, New York.

11. **Kemp, J. P.** 2003. Recent advances in the management of asthma using leukotriene modifiers. Am J Respir Med **2:**139-56.

12. **Kerstens, P. J., H. P. Endtz, J. F. Meis, W. J. Oyen, R. J. Koopman, P. J. van den Broek, and J. W. van der Meer.** 1992. Erysipelas-like skin lesions associated with *Campylobacter jejuni* septicemia in patients with hypogammaglobulinemia. Eur J Clin Microbiol Infect Dis **11:**842-7.

13. **Konkel, M. E., and L. A. Joens.** 1989. Adhesion to and invasion of HEp-2 cells by Campylobacter spp. Infect Immun **57:**2984-90.

14. **Lillywhite, J. E., D. A. Bundy, J. M. Didier, E. S. Cooper, and A. E. Bianco.** 1991. Humoral immune responses in human infection with the whipworm *Trichuris trichiura*. Parasite Immunol **13:**491-507.

15. **Mansfield, L. S., D. T. Gauthier, S. R. Abner, K. M. Jones, S. R. Wilder, and J. F. Urban.** 2003. Enhancement of disease and pathology by synergy of *Trichuris suis* and *Campylobacter jejuni* in the colon of immunologically naive swine. Am J Trop Med Hyg **68:**70-80.

16. **Monteville, M. R., and M. E. Konkel.** 2002. Fibronectin-facilitated invasion of T84 eukaryotic cells by *Campylobacter jejuni* occurs preferentially at the basolateral cell surface. Infect Immun **70:**6665-71.

17. **Needham, C. S., D. A. Bundy, J. E. Lillywhite, J. M. Didier, I. Simmons, and A. E. Bianco.** 1992. The relationship between *Trichuris trichiura* transmission intensity and the age-profiles of parasite-specific antibody isotypes in two endemic communities. Parasitology **105 (Pt 2):**273-83.

18. **Newell, D. G., H. McBride, F. Saunders, Y. Dehele, and A. D. Pearson.** 1985. The virulence of clinical and environmental isolates of *Campylobacter jejuni*. J Hyg (Lond) **94:**45-54.

19. **Pedersen, S., and I. Saeed.** 2001. Acquired immunity to *Trichuris suis* infection in pigs. Parasitology **123:**95-101.

20. **Pedersen, S., and I. Saeed.** 2000. Experimental infection of pigs with three dose levels of *Trichuris suis*. Parasite **7:**275-81.

21. **Shea-Donohue, T., C. Sullivan, F. D. Finkelman, K. B. Madden, S. C. Morris, J. Goldhill, V. Pineiro-Carrero, and J. F. Urban, Jr.** 2001. The role of IL-4 in *Heligmosomoides polygyrus*-induced alterations in murine intestinal epithelial cell function. J Immunol **167:**2234-9.

22. **Shin, J. L., G. W. Gardiner, W. Deitel, and G. Kandel.** 2004. Does whipworm increase the pathogenicity of *Campylobacter jejuni*? A clinical correlate of an experimental observation. Can J Gastroenterol **18:**175-7.

23. **Sorvillo, F. J., L. E. Lieb, and S. H. Waterman.** 1991. Incidence of campylobacteriosis among patients with AIDS in Los Angeles County. J Acquir Immune Defic Syndr **4:**598-602.

24. **Summers, R. W., D. E. Elliott, K. Qadir, J. F. Urban, Jr., R. Thompson, and J. V. Weinstock.** 2003. *Trichuris suis* seems to be safe and possibly effective in the treatment of inflammatory bowel disease. Am J Gastroenterol **98:**2034-41.

25. **Tracey, K.** 1994. Tumour Necrosis Factor-Alpha, p. 289-304. *In* A. Thomson (ed.), The cytokine handbook, Second ed. Academic Press, San Diego.

26. **Wilder, S. R.** 2003. *Trichuris suis* excretory secretory products modulate host immune responses. Masters. Michigan State University, East Lansing.