INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.
Pathogenicity of *Campylobacter jejuni*: Virulence associated factors and development of *in vivo* campylobacteriosis model

Babakhani, Farah Kondori, Ph.D.

The University of Arizona, 1992
PATHOGENICITY OF *Campylobacter jejuni*: VIRULENCE ASSOCIATED FACTORS AND DEVELOPMENT OF IN VIVO CAMPYLOBACTERIOSIS MODEL

by

Farah Kondori Babakhani

A Dissertation Submitted to the Faculty of the Department of Microbiology and Immunology In Partial fulfillment of the Requirements For the Degree of DOCTOR OF PHILOSOPHY In the Graduate College THE UNIVERSITY OF ARIZONA

1992
As members of the Final Examination Committee, we certify that we have read the dissertation prepared by FARAH K. BABAKHANI entitled PATHOGENICITY OF Campylobacter jejuni: VIRULENCE ASSOCIATED FACTORS AND DEVELOPMENT OF IN VIVO CAMPYLOBACTERIOSIS MODEL and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

Date 11/25/92

Date 11/25/92

Date 11/25/92

Date Nov 18, 1992

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

Dissertation Director 11/18/92
STATEMENT BY AUTHOR

This dissertation has been submitted in partial fulfillment of requirements for an advanced degree at The University of Arizona and is deposited in the University Library to be made available to borrowers under rules of the library.

Brief quotations from this dissertation are allowable without special permission, provided that accurate acknowledgment of source is made. Requests for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the head of the major department or the Dean of the Graduate College when in his or her judgment the proposed use of the material is in the interests of scholarship. In all other instances, however, permission must be obtained from the author.

SIGNED: Forch Balbala
Acknowledgment

A very special thanks to Dr. Lynn Joens for his guidance, support and patience. Also, to my parents Akram and Hossein for their love, support and encouragement.
Table of Contents

List of figures .................................................................................. 7
List of tables ................................................................................... 9
Abstract ...................................................................................... 11
Significance and objective of the research ....................................... 13
Introduction ................................................................................ 16
  Attachment ................................................................................ 18
  Invasion ................................................................................... 22
  In vivo model system for Campylobacter enteritis ....................... 26
I. Association of C. jejuni with HEp-2 cells ..................................... 31
  Introduction ............................................................................ 31
  Materials and Methods ........................................................ 33
  Results ............................................................................... 35
  Discussion ............................................................................ 45
II. Invasion related antigens of C. jejuni ........................................ 50
  Introduction ........................................................................... 50
  Materials and Methods ........................................................ 51
  Results ............................................................................... 57
  Discussion ............................................................................ 70
III. Invasion of porcine intestinal cells by C. jejuni ....................... 74
  Introduction .......................................................................... 74
List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Invasion related antigens of <em>C. jejuni</em></td>
<td></td>
</tr>
<tr>
<td>1. Western blot analysis of monoclonal antibodies with homologous <em>C. jejuni</em> lysates</td>
<td>62</td>
</tr>
<tr>
<td>2. Antigenic recognition of <em>Campylobacter</em> whole cell lysates with 1B4 and 5D1 monoclonal antibodies</td>
<td>65</td>
</tr>
<tr>
<td>3. Chemical treatment of <em>Campylobacter</em> whole cell lysate and their separation by SDS-PAGE</td>
<td>66</td>
</tr>
<tr>
<td>4. Antigenic recognition of <em>C. jejuni</em> M96 whole cell lysates before and after affinity purification with 1B4 monoclonal antibody or with anti-<em>C. jejuni</em> antisera</td>
<td>67</td>
</tr>
<tr>
<td>5. Antigenic recognition of <em>C. jejuni</em> whole cell lysates with 1B4 monoclonal antibody, monospecific antisera prepared against 1B4 affinity purified antigens and polyclonal anti-<em>C. jejuni</em> antisera</td>
<td>69</td>
</tr>
<tr>
<td>II. Invasion of porcine intestinal cells by <em>C. jejuni</em></td>
<td></td>
</tr>
<tr>
<td>1. Colony morphology of <em>C. jejuni</em> before and after passage in swine intestinal cells</td>
<td>84</td>
</tr>
<tr>
<td>III. <em>In vivo</em> model system of Campylobacteriosis</td>
<td></td>
</tr>
<tr>
<td>1. Photograph of large intestine from <em>C. jejuni</em> and <em>E. coli</em> infected piglets</td>
<td>103</td>
</tr>
<tr>
<td>2. Light micrograph of large intestine from infected neonatal piglets</td>
<td>104</td>
</tr>
<tr>
<td>3. Immunoperoxidase staining of large intestine from <em>C. jejuni</em> infected piglets</td>
<td>105</td>
</tr>
<tr>
<td>4. Scanning electron micrograph of large intestine from <em>C. jejuni</em> or <em>E. coli</em> infected neonatal piglet</td>
<td>106</td>
</tr>
</tbody>
</table>
5. Scanning electron micrograph of large intestine from *C. jejuni* infected neonatal piglet..............................107

6. TEM micrographs of large intestine from *C. jejuni* or *E. coli* infected neonatal piglets........................................108

7. TEM micrographs showing association of bacteria with microvilli of newborn piglet infected with *C. jejuni*..............109

8. TEM micrographs showing damaged tissue from the large intestine of *C. jejuni* infected piglet..............................110

9. TEM micrograph representing damaged epithelial cells from large intestine of *C. jejuni* infected piglets...............111

10. TEM micrograph of large intestine from *C. jejuni* infected piglet showing the presence of internalized bacteria ....112
List of Tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Association of <em>C. jejuni</em> with HEp-2 cells</td>
<td></td>
</tr>
<tr>
<td>1. Kinetics of <em>C. jejuni</em> adhesion to HEp-2 cells</td>
<td>39</td>
</tr>
<tr>
<td>2. Effect of <em>C. jejuni</em> outer membrane extracts on adhesion to HEp-2 cells by <em>C. jejuni</em></td>
<td>40</td>
</tr>
<tr>
<td>3. Effect of <em>C. jejuni</em> whole cell lysates on adhesion to HEp-2 cells by <em>C. jejuni</em></td>
<td>41</td>
</tr>
<tr>
<td>4. Effect of collagen and fibronectin on adhesion of <em>C. jejuni</em> to HEp-2 cells</td>
<td>42</td>
</tr>
<tr>
<td>5. Effect of NaCl on adhesion of <em>C. jejuni</em> to HEp-2 cells</td>
<td>43</td>
</tr>
<tr>
<td>6. Effect of NaCl on viability of <em>C. jejuni</em> M129 and HEp-2 cells</td>
<td>44</td>
</tr>
<tr>
<td>II. Invasion related antigens of <em>C. jejuni</em></td>
<td></td>
</tr>
<tr>
<td>1. Comparison of invasiveness of low and high passage <em>C. jejuni</em> M96 for HEp-2 cells</td>
<td>63</td>
</tr>
<tr>
<td>2. Effect of 1B4 monoclonal antibody on invasiveness of <em>C. jejuni</em> for HEp-2 cells</td>
<td>64</td>
</tr>
<tr>
<td>3. Effect of specific antisera, prepared against 1B4 affinity purified antigens, on invasiveness of <em>C. jejuni</em> for HEp-2 cells</td>
<td>68</td>
</tr>
<tr>
<td>III. Invasion of porcine intestinal cells by <em>C. jejuni</em></td>
<td></td>
</tr>
<tr>
<td>1. Yield and viability of enterocytes after treatment of intestine with EDTA or Trypsin</td>
<td>82</td>
</tr>
<tr>
<td>2. Invasion of swine intestinal cells by bacteria</td>
<td>83</td>
</tr>
<tr>
<td>3. Invasiveness of <em>C. jejuni</em> to HEp-2 cells after passage through swine intestinal cells</td>
<td>85</td>
</tr>
</tbody>
</table>
Table

4. Invasiveness of *C. jejuni* to INT 407 cells after passage through swine intestinal cells ........................................ 86

IV. *In-vivo* model system of Campylobacteriosis

1. Clinical symptoms of neonatal pigs infected with bacteria ..... 100

2. Gross examination of neonatal piglet intestines after oral inoculation with bacteria ............................... 101

3. Microscopic examination of piglet intestinal tissue after oral challenge with bacteria ................................. 102
ABSTRACT

Competitive inhibition assays were performed to study the effect of various bacterial and host derived factors on the attachment and invasion of *Campylobacter jejuni* to HEp-2 cell lines. *Campylobacter jejuni* outer membrane or mammalian extracellular matrix components did not inhibit attachment of the bacteria to HEp-2 cells. Conversely, sodium chloride inhibited the attachment of *C. jejuni* to HEp-2 cells. This inhibition increased with higher salt concentrations, indicating that the attachment may have been mediated through charge interactions.

Penetration of *C. jejuni* into the HEp-2 epithelial cells was significantly inhibited by 1B4 monoclonal antibody in competitive inhibition assays. Western blot analysis with 1B4 monoclonal antibody identified an epitope on antigens of 64-44 KDa in lysates prepared from invasive and non-invasive isolates and in antigens of 42-38 KDa in lysates prepared from only invasive *C. jejuni* strains. Proteinase K and sodium *meta*-periodate treatment of *C. jejuni* lysate changed the mobility of antigens recognized by 1B4 monoclonal antibody, suggesting that the antigens required for HEp-2 invasion may be glycoprotein in nature. Specific antisera, prepared in rabbits against the 1B4-affinity purified antigens, were able to block the invasion of *C. jejuni* into HEp-2 cells, demonstrating the specificity of 1B4 monoclonal antibody to the invasin antigen(s). Western blot analysis revealed that this specific antisera identified distinct antigens at 62, 47 and between 42-35 KDa in the lysates.
prepared from invasive strains.

A primary intestinal cell line was developed using swine enterocytes to investigate and examine the validity of in vitro findings in relation to invasiveness of *C. jejuni*. One of the *C. jejuni* isolates (F1474) invaded swine enterocytes in significantly higher numbers. However, the invasive ability of *C. jejuni* T13192 increased dramatically during the third, fourth and fifth experimental trials. Recovered colonies from *C. jejuni* T13192 appeared highly mucoid and invaded tissue culture cells in higher numbers than the original isolate. The data not only support the previous in vitro findings regarding the invasiveness of *C. jejuni*, but also suggest that invasiveness of *C. jejuni* may be an inducible event.

An *in vivo* model system was developed by inoculating colostrum deprived newborn piglets orally with an invasive strain of *C. jejuni*. Lesions similar to those in humans with campylobacteriosis were observed mainly in the large intestine. Damage to the epithelial cells and the presence of intracellular bacteria were the salient features of the lesion.
SIGNIFICANCE AND OBJECTIVES OF THE RESEARCH

_Campylobacter spp._ were first identified at the beginning of this century (McFadyean and Stockman, 1913). Nonetheless, the significance of _Campylobacter jejuni_ as one of the leading causes of human enteritis was not realized until the 1970’s, when proper culture techniques were employed (Skirrow, 1977). Two decades of extensive research on _C. jejuni_ has not clarified the pathogenic mechanisms that the organism uses to cause disease. The lack of progress in understanding the pathogenicity of _C. jejuni_ is partly due to absence of a suitable animal model that mimics human campylobacteriosis, and the lack of a suitable nucleic acid exchange system.

In humans the clinical expression of _Campylobacter_ enteritis can vary from mild gastroenteritis to febrile illness with bloody diarrhea and abdominal pain (Blaser and Reller, 1981). The wide spectrum of clinical symptoms suggests that the pathogenicity of strains is due to a variety of virulence factors such as enterotoxins, cytotoxins and invasins. It is reported that _C. jejuni_ produces an enterotoxin, which is functionally and immunologically similar to heat-labile toxin of _Eschericia coli_ (LT) and _Vibrio cholerae_ (CT) (Ruiz-Palacios et al., 1983). The binding subunit of all three toxins interacts specifically with the GM1 gangliosides. By activating the host adenylate cyclase levels, via ADPR- of the G regulatory protein, these toxins raise the intracellular cyclic AMP levels. The increase cyclic AMP level is known to cause efflux of chloride and sodium ions and therefore loss of
water into the intestinal lumen, which results in dehydration (Klipstein et al., 1985).

Although the role of cytotoxins is not conclusive, rectal biopsies of patients with campylobacteriosis have demonstrated acute inflammatory infiltrates in the lamina propria, along with crypt abscesses. Internalized \textit{Campylobacter} \textit{spp.} have also been demonstrated in human gut epithelial cells (Mandal and Butzler, 1984), and in gut epithelial cells of pigs and hamsters with acute ileitis (Rowland and Lawson, 1981; Stills et al., 1987). Whether these conditions are caused by the same \textit{Campylobacter} \textit{spp.} is still unknown, but it is assumed that the lesions associated with each disease syndrome are due to the presence of an internalized \textit{Campylobacter} \textit{spp.}-like organism.

The mechanism of invasion by \textit{C. jejuni} is unknown. \textit{In vitro} tissue culture studies have suggested the presence of separate molecules for attachment and for invasion of the bacteria into epithelial cells (Konkel and Joens, 1989). In addition to flagella and lipopolysaccharide, outer membrane proteins of \textit{C. jejuni} have been proposed as mediators of the attachment of \textit{C. jejuni} to tissue culture cells (Mc Sweegan et al., 1986; Fauchere et al., 1989 and De Malo and Pechere, 1990). However, these results are controversial and the nature of the adhesin molecule(s), have not been identified. It is also unclear which molecule(s) on \textit{Campylobacter} \textit{spp.} mediates internalization of the bacteria.
The objective of the present study is to identify factors involved in the association and invasion of *C. jejuni* into cultured epithelial cell lines and primary gut epithelial cells. Studies were also done to develop an *in vivo* model system that would aid in determining the actual role of these factors in disease production.

**Specifically the research will:**

1. Produce monoclonal antibodies against antigens associated with virulence of *C. jejuni* and purify the virulence associated antigen(s).
2. Evaluate the attachment mechanisms that *C. jejuni* could employ during pathogenicity.
3. Examine the use of primary cells as an *in vitro* model system of adherence and invasion.
4. Develop an *in vivo* model system for campylobacteriosis.
INTRODUCTION

The genus name *Campylobacter* comes from the Greek word *Campylo* meaning curved, which refers to the morphology of the bacteria. Members of the genus *Campylobacter*, however, are highly pleomorphic and appear not only as curved rods but also as spiral, sea gull, coccoid or S shapes. The size of the organisms range anywhere from 0.2 to 0.5 μm in width and 0.5 to 5 μm in length. They are Gram negative, microaerophilic organisms which are motile by means of a single polar flagellum at one or both ends. Motility of the organisms is characterized by corkscrew-darting motion that is best observed by phase-contrast microscopy (Karmali and Skirrow, 1986).

The recognition of *Campylobacter* infection as one of the leading causes of diarrheal diseases was not feasible until the advancement of isolation techniques. The introduction of various selective media, such as Skirrow's media in the 1970's, allowed for the isolation of the organism from the stool specimens (Butzler et al., 1973 and Skirrow, 1977). Presently, *Campylobacter* infections and more specifically *C. jejuni* infections are being reported as one of the common causes of enteric infections throughout the world (Mandel et al., 1984).

In developing countries, *Campylobacter* infection is hyper-endemic and is a major cause of morbidity during the first two years of life. After this period, the disease-to-infection ratio declines. Asymptomatic infections are very common in these countries (Blaser et al., 1980; Rajan and Mathan,
1982 and Taylor et al., 1988) and may relate to the host response to continuous infection with the organism. In the industrialized nations, on the other hand, Campylobacter infections are usually sporadic with asymptomatic infections being rare (Blaser and Reller, 1981). The clinical signs of Campylobacter enteritis include fever, bloody diarrhea and the shedding of leukocytes in feces (Blaser et al., 1979; Blaser et al., 1983 and Taylor et al., 1988). Although this disease appears in all age groups, children appear to be the most susceptible.

The transmission of C. jejuni is most often associated with ingestion of contaminated water and food products, such as milk or poultry (Blaser et al., 1986). Animals can also serve as reservoirs of infection, since Campylobacter spp. is part of intestinal flora of many animals (Blaser et al., 1986). Contact with infected puppies has been shown to precede campylobacter enteritis in humans (Blaser et al., 1978). Although person-to-person transmission appears to be rare, nosocomial infections within neonatal units have been reported which have lead to serious complications (Butzler and Goosens, 1988). The infective dose appears to have a wide range. Illness has resulted from ingestion of as few as 500 cells to as many as $10^6$ bacteria (Walker et al., 1986). The variation in the infective dose could be due to either individual susceptibility to the organism (Taylor et al., 1988) or to differences in the virulence of organisms (Klipstein et al., 1985).

The vast array of clinical symptoms also denotes the presence of
different pathogenic mechanisms within different strains. Pathogenicity of some strains is believed to be due to enterotoxin production, in which case the infection manifests itself in the form of a watery diarrhea (Klipstein et al., 1985; Klipstein and Engret, 1985; Calva et al., 1989; Ruiz-Palacios, 1983;), while other strains produce cytotoxin and/or invade the epithelial cells of the intestinal tract (Klipstein et al., 1985; Moore et al., 1988). Clinical symptoms which indicate the organism might be invasive, include presence of blood and leukocytes in feces along with congestion, edema, ulcerative lesions of mucosal epithelium, and inflammatory infiltrates in the lamina propria (Blaser et al., 1979; Blaser et al., 1980; Duffy et al., 1980; King 1957; Lambert et al., 1979 and Van Spreeuwel et al., 1985). Within the past few years abundant work has been done, using tissue culture system models, to define the pathogenesis of C. jejuni infections. These reports are controversial and may not mimic the in vivo conditions. Certainly, the lack of a suitable animal model has hindered progress in clarifying the pathogenicity of campylobacter enteritis as well as the development of a sound and feasible preventive measures.

ATTACHMENT

In order for an enteric pathogen to exert its harmful effects on the host, it is necessary for the pathogen to first interact with the host intestinal mucosa. For some enteric pathogens, such as enterotoxigenic Eschericia coli, this interaction is primarily mediated through specific receptor-ligand
interactions (Gonzalez and Blanco, 1985). Other enterics, such as *Yersinia enterocolitica*, may associate with intestinal mucosa through hydrophobic or electrostatic interactions (Lachica et al., 1984). In the case of *C. jejuni*, various outer membrane structures of the bacteria have been implicated as being adhesin molecules. However, these results are controversial and differ from one laboratory to the next.

It has been suggested that the attachment of *C. jejuni* to cells might be mediated through proteins. McSweegan and Walker (1986) observed reduction in the attachment of *C. jejuni* to INT 407 cells after treatment of bacteria with various proteases. Treatment of *C. jejuni* with pepsin, trypsin and protease significantly inhibited attachment of bacteria to INT 407 cells. Fixation of *C. jejuni* with formaldehyde and glutaraldehyde also reduced adhesion of bacteria to INT 407 cells, indicating that the surface proteins of *C. jejuni* might be involved in adhesion to INT 407 cells (McSweegan and Walker, 1986). Reduction in the attachment of *C. jejuni* to porcine intestinal brush border membranes was observed after treatment of bacteria with proteolytic enzymes or after boiling of the bacteria (Ness et al., 1988).

The flagellar structure of *C. jejuni* has been implicated as a possible adhesin. *In vitro* studies by Newel et al. (1985) have shown better attachment of wild type or non-motile, flagellated strains of *C. jejuni* to INT 407 cells than aflagellated variants. McSweegan and Walker also demonstrated that flagella are involved in attachment (1986). By removing
flagella, they were able to reduce adhesion of bacteria to INT 407 epithelial cells. However, in a competitive inhibition assay, purified flagella were unable to inhibit adherence of *C. jejuni* to INT 407 cells (McSweegan and Walker, 1986). Transmission electron microscopy studies by De Melo *et al.* (1989) failed to show any apposition between flagella and the plasma membrane of HEp-2 cells incubated with *C. jejuni*.

Although the role of flagella as adhesins is controversial, the motility generated by flagella appears to be significant for colonization of *Campylobacter*. Newel *et al.* (1983) reported better colonization of the gastrointestinal (GI) tract of 5 day old Balb/c mice with a wild type strain of *C. jejuni* than with an aflagellated strain. Later work by Marooka *et al.* (1980) confirmed Newel’s observations and further suggested motility to be an important factor in colonization of the intestinal tract by *C. jejuni*. They observed that non-motile strains with complete flagella were less effective in colonizing the GI tract of suckling mice than wild type strains. Although none of the above *in vivo* studies specifically model human campylobacteriosis, motility produced appears to be an important virulence factor for the colonization of intestinal mucosa by *C. jejuni* (Lee *et al.*, 1986).

Other outer membrane components of *C. jejuni* have also been implicated as adhesin molecules. Ligand binding assays by De Melo *et al.* have identified in *C. jejuni* outer membrane antigens, with molecular weights of 28, 32, 36 and 42 KDa, that bind to HEp-2 cells (De Melo and Pechere,
1990). Fauchere et al. incubated outer membrane extracts of C. jejuni with HeLa cells, in a similar type of ligand binding assay, and identified antigens ranging from 26 to 30 KDa that bound to HeLa cells (Fauchere et al., 1989).

The lipopolysaccharide (LPS) portion of C. jejuni has also been identified as an adhesin molecule. McSweegan and Walker (1986) reported that LPS could bind to INT 407 cells and block the attachment of whole bacteria to these epithelial cells. Although in their studies LPS was able to block adherence of bacteria to the cells, it was unclear whether the LPS levels used were toxic to the tissue culture cells.

To understand the nature of the interaction of C. jejuni adhesins with enterocytes, various investigators have used biochemical studies. Competetive inhibition studies with individual sugars have given controversial results. Newel et al. (1985) observed inhibition of attachment of C. jejuni to the INT 407 cells by various sugars (mannose, galactose, glucose, fucose, N-acetyl glucosamine and N-acetyl galactosamine). Cinco et al. (1984) showed partial inhibition of adhesion of C. jejuni to INT 407 cells by L-fucose and mannose. These results are consistent with the findings of McSweegan and Walker (1986), who also observed reduction in attachment of C. jejuni to INT 407 cells by fucose and mannose. However, the same results were not observed by Naess et al. (1988) who tried to inhibit C. jejuni adhesion to intestinal brush border tissue by addition of various sugars (including fucose) to the assay. In their study, only L-rhamnose inhibited attachment
of the bacteria to intestinal brush borders. This inhibitory effect, however, did not show any clear dose-response relationship. The above biochemical studies imply that \textit{C. jejuni} might partially interact with specific host cell receptors, which contain fucose and mannose moieties. These results, however, are inconsistent and vary from one laboratory to the next.

There is also some suggestion that \textit{C. jejuni} might interact with extracellular matrix components of mammalian cells. Kuusela \textit{et al} (1989) reported the binding of two strains of \textit{C. jejuni} to coverslips coated with fibronectin, laminin or collagen. It was unclear whether these interactions were mediated through a specific receptor-ligand interaction or through other non-specific interactions (such as hydrophobic or electrostatic). Walan and Kihlstrom (1988) have studied surface charge and hydrophobicity of clinical strains of \textit{C. jejuni} and have observed some correlation between surface charge and the ability of the organism to associate with HT-29 cells.

The attachment mechanism of \textit{C. jejuni} to epithelial cells at the molecular level is poorly understood. Inconclusive results have been obtained on the role and nature of specific adhesin molecules on \textit{C. jejuni}. Although different structures of \textit{C. jejuni} have been implicated as containing adhesin molecules, competitive inhibition experiments with purified fractions have been unsuccessful.

\textbf{INVASION}

Clinical signs of \textit{Campylobacter} induced enteritis furnish evidence for
the invasive potential of *C. jejuni*. Blood and leukocytes in the stools, along
with mucosal ulceration and crypt abscesses in rectal biopsies of patients
with campylobacteriosis have been reported (Blaser *et al*., 1979; Blaser *et
al*., 1980; Duffy *et al*., 1980; King 1957; Lambert *et al*., 1979 and Van
Spreeuwel *et al*., 1985). *Campylobacter* inclusions have also been identified
by means of immunohistochemistry and electron microscopy in colonic
biopsy specimens of patients with campylobacteriosis (Newell, 1984 and

In addition to the clinical symptoms, studies using animal models have
established an invasive role for *C. jejuni*. Ruiz-Palacios *et al.* (1981) used
immunofluorescence and electron microscopy techniques to show
*Campylobacter* within the intestinal epithelial cells of young chickens after
oral administration of the microorganisms. When chicken embryos were
used by Field *et al.* (1986) to test for invasiveness of *C. jejuni*, variation in
invasion through the chorioallantoic membrane was observed between
strains. Although *C. jejuni* is negative in the Sereny test (Manninen *et al*.,
1982), studies with gnotobiotic mice and hamsters have demonstrated the
internalization of *C. jejuni* by intestinal cells (Fauchere *et al*., 1985 and
Humphery *et al*., 1985).

Most of the work in understanding the invasive properties of *C. jejuni*,
has come from *in vitro* cell culture systems. Using indirect
immunofluorescence and Giemsa staining techniques, Manninen *et al.*
(1982) observed the internalization of 14 *C. jejuni* isolates, in low numbers, into HeLa cells. In a more recent study, De Melo and Pechere (1988) observed that pre-treatment of HEp-2 cells with mucin increased the total number of cell associated colony forming units, and promoted internalization of *C. jejuni*. This appeared to be a strain-dependent event. Mucus significantly promoted internalization of freshly isolated *C. jejuni* strains, but had little effect on penetration of laboratory adapted strains.

The variation in the ability of different strains of *C. jejuni* to internalize has also been observed by other investigators. Newell *et al.* (1985) showed clinical isolates to be more invasive for HeLa cells than strains isolated from water. Konkel and Joens (1989) reported significant differences in invasion of HEp-2 cells between clinical isolates of *C. jejuni* and non-clinical isolates, the former group being highly invasive.

The mechanism of penetration by *C. jejuni* at the molecular level is poorly understood. It is suggested that, like *Yersinia enterocolitica* and non-typhoid *Salmonella* sp., *C. jejuni* crosses the epithelium and enters the lamina propria, where it multiplies and may spread to mesenteric lymph nodes (Kopecko *et al.*, 1989). The mechanism of uptake of *C. jejuni* by epithelial cells is thought to be mediated through induced phagocytosis. This process requires host cell energy production and microfilament function (De Melo *et al.*, 1989; Konkel and Joens, 1989). Cytochalasin B (inhibits phagocytosis by causing actin depolymerization), iodoacetate (blocks glycolysis) and
dinitrophenol (blocks Krebs cycle) have been shown to inhibit \textit{C. jejuni} invasion of HEP-2 cells (De Melo \textit{et al.}, 1989). These results indicate that the penetration mechanism is energy dependent and requires host cell microfilament involvement.

The role of surface molecules of \textit{C. jejuni} in the uptake mechanism is unclear. There is some evidence suggesting that the uptake of \textit{C. jejuni} might be mediated by a specific ligand on the surface of the bacterial cell. An enzyme-linked immunosorbent assay technique was used by Klipstein \textit{et al.} (1985) to demonstrate the invasive properties of \textit{C. jejuni} strains from patients with bloody diarrhea. Using an immunoglobulin fraction of antiserum produced against a formalin-killed invasive strain of \textit{C. jejuni}, they were able to differentiate between invasive and non-invasive strains of \textit{C. jejuni}. Additionally, Konkel and Joens (1989) showed that whole cell lysates from invasive strains competitively inhibited invasion of HEP-2 cells by \textit{C. jejuni}, and that oxidation of whole cell lysates of \textit{C. jejuni} with sodium metaperiodate eliminated this inhibitory effect. This led to the conclusion that the invasive ligand might contain a carbohydrate moiety.

The nature of bacterial surface components involved in invasion has been partially characterized in other enteric systems. In some of these studies, antibodies have been used to probe the nature of structures required for epithelial cell invasion. Schiemann and Nelson (1987) have found that antisera made against formalinized \textit{Yersinia enterocolitica} inhibited HeLa cell
invasion by the organism. Conversely, antisera against heat killed bacteria did not. Since heat denatures proteins, the authors suggested that protein structures may be involved in mediating invasion. Mills et al (1988) used monoclonal antibodies against IpaB and IpaC polypeptides (molecules involved in *Shigella flexneri* invasion of cells) to characterize and identify the location of the molecules on the bacteria.

**IN VIVO MODEL SYSTEM FOR CAMPYLOBACTER ENTERITIS**

The standard animal model systems used with other enteric organisms, such as the Sereny test or ligated rabbit ileal loops, have been found to be negative for *C. jejuni* (Manninen et al., 1982; Guerrant et al., 1978). Various other animals, such as monkeys, gnotobiotic beagle puppies, mice, rabbits, chicks, minks and ferrets have been used unsuccessfully to examine the pathogenicity of *Campylobacter* enteritis (Fitzgeorge et al., 1981; Prescott et al., 1981; Cober and Obi; 1991, Field et al., 1981; Bell et al., 1990, Fox et al., 1987; Ruiz-Palacios et al., 1981; Butzler and Skirrow, 1979; Manninen et al., 1982; Bell and Manning; 1990 and Prescott et al., 1970). These models have not been suitable either because of expenses, surgical procedures involved, or because of lack of clinical symptoms.

Fitzgeorge et al. (1981) orally inoculated young rhesus monkeys with human strains of *C. jejuni*, but only mild symptoms occurred. The animals had associated bacteremia and excreted the organism in the feces, but there were no histopathological lesions attributable to *Campylobacter* infection
identified in the gastrointestinal tracts of these monkeys.

A notable study was performed on gnotobiotic beagle puppies by Prescott et al. (1981). These workers orally inoculated the animals with C. jejuni of human and canine origin. The clinical manifestations of disease were milder in the gnotobiotically reared puppies than in humans. Whereas the disease in humans is accompanied with abdominal pain, fever and presence of blood and mucus in stools, the dogs were bacteremic and developed mild diarrhea. Histopathologically, the dogs showed mild colitis consisting of neutrophil infiltration of the lamina propria, loss of goblet cells with hypertrophy of glands, and exfoliation of the surface epithelium.

The cost associated with rearing of gnotobiotic animals has led investigators to look for other models for Campylobacter enteritis. Conventional mice, hamsters, rats and rabbits have been intragastrically inoculated with C. jejuni (Field et al., 1981; Fox et al., 1987; Stanfield, 1987). The experimental inoculation of these small mammals has resulted either in transient colonization and shedding of the bacteria in feces without any symptoms (Field et al., 1981) or it has produced mild symptoms with little histopathological damage to the intestine (Stanfield et al., 1987). One interesting study was done by Fox et al. (1987), who orally inoculated newborn ferrets in two different experiments with C. jejuni of either ferret origin or human origin. The authors reported presence of blood, mucus and leukocytes in feces of the infected ferrets. However, gross and microscopic
examinations revealed only mild lesions, which included small focal areas of neutrophil infiltration.

The association of *Campylobacter* infections with disease in poultry has led some investigators to experiment with young chickens or gnotobiotic chicks as models for human campylobacteriosis. The results from these studies have varied among laboratories. Studies by Ruiz-Palacios *et al.* (1981), showed that oral inoculation of 3 day old chicks, using low numbers of bacteria resulted in severe diarrhea, with disease symptoms in 90% of the chicks and a mortality rate of 32%. Other workers have been unable to produce any diarrhea in 3 day old chicks with various strains of *C. jejuni* (Manninen *et al.*, 1982). Oral inoculation of 8 day old chicks (Butzler and Skirrow, 1979) or 5 day old gnotobiotic chicks (Prescott *et al.*, 1970), with human isolates of *C. jejuni*, have not been fruitful either. In a recent study, Field and co-workers (Field *et al.*, 1991) reported the development of an *in*
has been associated with a specific type of lesion. The lesion is characterized by the proliferation of the intestinal crypt epithelial cells, loss of goblet cells, inflammation of the lamina propria and thickening of intestinal wall (Rowland and Lawson, 1981). These signs are always accompanied by the presence of intracellular *Campylobacter* spp.-like organisms within the apical cytoplasm of the crypt epithelial cells (Rowland and Lawson, 1981). The same type of lesion has also been observed in hamsters with proliferative enteritis (Stills *et al.*, 1987).

Although *Campylobacter* spp. have been isolated from animals with lesions of PPE, transmission studies with various combinations of *Campylobacter* has failed to reproduce the disease (Rowland and Lawson, 1981; Boosinger *et al.*, 1985). The only successful studies that have resulted in the experimental reproduction of the disease have been conducted with intestinal homogenates of infected animals (Lomax *et al.*, 1982a, 1982b; Mapother *et al.*, 1987a, 1987b; McOrist *et al.*, 1989b). The lack of success in reproducing ileitis with pure cultures of *Campylobacter* spp. has led some investigators to propose that the intracellular organism is an unidentified *Campylobacter* spp. (McOrist *et al.*, 1987a), and perhaps becomes attenuated by passage in culture media (Ogaard, *et al.*, 1985).

Despite experimentations with many varieties of animals, a suitable *in vivo* model system that would mimic human *Campylobacter* enteritis has not been identified. Partial success has only been obtained in germ free and
young animals (Prescott et al., 1981; Stanfield et al., 1987 and Fox et al., 1987) and, inconsistently, in chickens (Ruiz-Palacios et al., 1981). A suitable model system requires a host that is anatomically and physiologically similar to humans. This would allow the study of immune mechanisms and, therefore, development of potential vaccines in the model host. The cost associated with rearing and maintaining animals used as model systems is also a determinant factor in choosing such a system. The role of specific immunity in *Campylobacter* infection implies that a third factor namely the host defense system must be taken into consideration when searching for a suitable model system.
I. ASSOCIATION OF C. jejuni WITH HEP-2 CELLS

A. INTRODUCTION

Worldwide, Campylobacter jejuni is known as one of the leading causes of bacterial enteritis (Mandel et al., 1984). However, the mechanism by which it causes disease has not been clearly defined. Clinical and experimental evidence suggests that the pathogenicity of the strains could be due to a variety of virulence factors. These factors include enterotoxins (Klipstein et al., 1985; Ruiz-Palacios et al., 1983; Calva et al., 1989), cytotoxins (Johnson and Lior, 1986 and 1988 and Moore et al., 1988) and invasins. In order for C. jejuni to exert its harmful effects, it is necessary for it to first colonize the host intestinal mucosa. In this regard, flagella appear to be an important virulence factor. It has been proposed that, through chemotaxis, flagellar movements direct C. jejuni towards epithelium-associated mucus, therefore allowing its association with intestinal mucosa (Marooka et al., 1980).

Controversy exists as to whether this initial colonization step is followed by the attachment of specific bacterial adhesin molecules with host cell receptors. In vitro tissue culture studies have demonstrated internalization of C. jejuni into epithelial cells (Konkel and Joens, 1989). Furthermore, this internalization step is known to require both host energy and microfilament involvement (De Melo et al., 1989). The actual nature of initial host-bacterial interactions still remains to be characterized.
Several *C. jejuni* outer membrane molecules have been implicated as adhesins, including flagellar structures and lipopolysaccharides (LPS) (McSweegan and Walker, 1986). Other minor *C. jejuni* outer membrane antigens have been shown to bind to HEp-2 cells or HeLa cells. These antigens have molecular weights of 28, 32, 36 and 42 KDa (De Melo and Pechere, 1990; Fauchere et al., 1989). In limited studies, investigators have attempted to block *C. jejuni* adhesion with purified outer membrane fractions of bacteria or with antibodies prepared against these fractions. With the exception of Fauchere et al. (1989), results from these studies remain controversial or have failed to block the attachment process.

Some investigators have used biochemical studies to define the nature of *C. jejuni* interaction with mucosal epithelial cells. Competitive inhibition studies, with various sugars, have produced mixed results. These studies imply that *C. jejuni* partially interacts with specific host cell receptors which contain sugar moieties such as fucose or mannose (Newell et al., 1985; Cinco et al., 1984 Naess et al., 1988). These results, however, are inconsistent and vary from laboratory to laboratory. It has also been suggested that *C. jejuni* interacts with extracellular matrix components of mammalian cells. Kuusela et al. (1989) reported the binding of two *C. jejuni* strains to coverslips coated with either fibronectin, laminin or collagen. It was unclear as to whether these interactions were mediated through a specific receptor-ligand interaction or through other non-specific attachment
mechanisms (such as hydrophobic or electrostatic). Walan and Kihlstrom (1988) studied both surface charge and hydrophobicity of clinical strains of \textit{C. jejuni} and have proposed a correlation between surface charge and the ability of the organism to associate with HT-29 cells.

In order to determine the nature and specificity of \textit{C. jejuni} interaction with epithelial cells, competitive inhibition studies have been performed with purified fractions of \textit{C. jejuni}. This study has also examined the association of \textit{C. jejuni} with extracellular matrix proteins and the role that surface charge may play in the interaction of \textit{C. jejuni} with epithelial cells.

\textbf{B. MATERIALS AND METHODS}

\textbf{Bacteria.} \textit{C. jejuni} M129 was kindly provided by Dr. Kenneth Ryan (University Medical Center, University of Az, Tucson, Az.). This isolate was obtained from a patient with clinical signs of campylobacteriosis. The isolate was suspended in bovine blood and maintained frozen in liquid nitrogen. For experimental assays, a 24 hr culture of the strain, under ten passages, was used. Bacteria were grown microaerophilically (80:10:10, N₂:CO₂:H₂) at 37°C on Mueller-Hinton (MH) plate containing 4% citrated bovine blood.

\textbf{HEp-2 cell adhesion assay.} Human laryngeal carcinoma cells (HEp-2, ATCC CCL 23) were maintained in MEM supplemented with 10% FCS. For all experimental assays, 24-well tissue culture plates (Falcon, Becton Dickinson Labware, Oxnard, CA) were seeded with 8 X 10^4 cells/well. Plates were incubated for 18 h at 37°C in a humidified atmosphere with 5%
CO₂. Prior to the assay, semi-confluent monolayers were washed with cold MEM containing 1% fetal bovine serum (FBS). After washing, 0.5 ml of approximately 1 X 10⁸ CFU of C. jejuni were added to each well. The plates were then incubated on a rocking platform at 4°C for 40 min. At the end of the incubation period the wells were washed with MEM containing 1% FBS to remove non-adherent bacteria and the HEp-2 cells were lysed with 0.5% sodium deoxycholate. The suspensions were diluted and number of viable bacteria were determined by counting the colony forming units (CFU) on MH plates.

**Glycine-acid extraction.** Glycine-acid extraction was performed by the method of McCoy et al. (1975). C. jejuni M129 was grown, as described above, and was harvested into distilled water. After centrifugation, the pellet was washed 2 X with distilled water, and resuspended in 0.2 M glycine hydrochloride, pH 2.2 (3 g of cells per 100 ml), and stirred for 30 min at room temperature. Whole cells were removed by centrifugation at 12,000 X g for 15 min and the resulting supernatant neutralized with NaOH and dialyzed against distilled water. One ml of the glycine extract (0.5 mg protein/ml) was added to each well containing HEp-2 cells for 1 hr at 4°C on a rocking platform. Monolayers were washed with cold MEM containing 1% FBS, followed by inoculation with 1 ml of a suspension containing an equal volume of bacteria and glycine-acid extract (0.5 mg protein/well). The infected monolayer was incubated for an additional 40 min.
Whole cell lysates. *C. jejuni* M129 was harvested from MH plates with phosphate buffered saline (0.1 M) (PBS), and disrupted in a french pressure cell (10,000 lbs/in²). The cellular debris was removed by centrifugation at 6000 X g at 4°C for 25 min. The resulting supernatant was collected, aliquoted and stored at -70°C. One ml of the M129 lysate (0.5 mg protein/ml) was added to HEp-2 cells for 1 hr at 4°C on a rocking platform. The HEp-2 cell monolayer was then washed with cold MEM containing 1% FBS, and inoculated with a 1 ml suspension containing an equal volume of bacteria and whole cell lysate (0.5 mg protein/well) and incubated an additional 40 min.

Extracellular matrix components. Type I collagen, human plasma fibronectin (Sigma; St. Louis, Mo.), or albumin (100 μg/ml) were added to approximately 1 X 10⁸ CFU of *C. jejuni* for 2 hr at 37°C. A confluent HEp-2 cell monolayer was washed with cold MEM containing 1% FBS, and inoculated with a 0.5 ml suspension of 1 X 10⁸ CFU *C. jejuni* and 50 μg of either collagen, fibronectin or albumin, and incubated an additional 40 min.

Statistical analysis. Results were reported as mean ± standard deviation for four trials. Significance between control and treatment groups were evaluated by Student’s *t* test. P values ≤0.05 were considered significant.

C. Results

Kinetics of *C. jejuni* adhesion. An isolate of *C. jejuni* (M129) from a
patient with clinical signs of campylobacteriosis was tested for its ability to adhere to HEp-2 cells for a period of up to 2 hr. The study was performed at 4°C to prevent internalization of bacteria and, therefore, to strictly study the attachment mechanisms. Within the first 5 min, $10^4$ bacteria attached to HEp-2 cells (Table 1). The number of attached bacteria increased in a linear fashion for the first 40 min to a peak level of $4.5 \times 10^5$, and thereafter remained essentially constant.

**Inhibition of attachment with bacterial components.** The role of surface proteins of *C. jejuni* in its attachment to cells were examined in a competitive inhibition assay. Surface proteins were prepared using a glycine-acid extraction procedure. This extraction procedure harvested several outer membrane proteins of *C. jejuni*, including proteins at 28, 32, 38 and 42 KDa (data not shown), which are known to bind *in vitro* to tissue culture cells (De Melo and Pechere, 1990; Fauchere et al., 1989). To examine the specificity of this binding, the glycine-acid extract was used to block the attachment of *C. jejuni* to HEp-2 cells. It was demonstrated that 0.5 mg of glycine-acid extract had no inhibitory effect on the attachment of the bacteria to HEp-2 cells (Table 2).

The glycine extraction procedure employs acid-dissociation steps, which could denature a proteinacious adhesin molecule. Therefore, instead of using glycine extract, untreated whole cell lysates of bacteria were used in a competitive inhibition assay to examine the role of bacterial components
in its attachment to HEP-2 cells. The whole cell lysates (0.5 mg per well) were able to significantly (P < 0.05) inhibit attachment of *C. jejuni* to the HEP-2 cells (Table 3).

**Inhibition of attachment with extracellular matrix proteins.** A report by Kuusela et. al. (1989) indicate that *C. jejuni* adheres to several extracellular matrix proteins. Therefore, the specificity of *C. jejuni* adhering to extracellular matrix proteins were examined in our assay system. Type I collagen and fibronectin at 100 µg/ml were pre-incubated with bacteria to saturate the ligands on *C. jejuni*, then the mixture was added to HEP-2 cells and the number of attached bacteria determined. The specificity of the reactions was controlled by using a non-extracellular matrix protein, albumin. Unlike fibronectin, 100 µg of collagen was able to significantly inhibit attachment of *C. jejuni* M129 to HEP-2 cells (Table 4). However, this inhibition was comparable to that observed by albumin.

**Inhibition of attachment with salt.** The role of ionic interactions in attachment was investigated by inoculating HEP-2 cells with bacteria in presence of different concentrations of sodium chloride. Salt was able to inhibit attachment of *C. jejuni* M129 to HEP-2 cells (Table 5). The inhibition in attachment increased with higher concentrations of salt, which was significant at 0.5 and 1.0 M salt concentrations. This concentration of salt was not lethal to either *C. jejuni* or to HEP-2 cells during the assay period of 40 min. The number of viable bacteria present after incubation of bacteria
in 1.0 M salt was comparable to that in the absence of salt (Table 6). Trypan blue exclusion dye and light microscopic examination of HEP-2 monolayers in the presence of 1.0 M salt revealed no detectable damage to the cells (Table 6).
Table 1. Kinetics of *C. jejuni* adhesion to HEp-2 cells:

<table>
<thead>
<tr>
<th>Time (minutes)$^*$</th>
<th>Viable bacteria associated with HEp-2 cells$^\circ$</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>(1.5 ± 0.06) x $10^4$</td>
</tr>
<tr>
<td>30</td>
<td>(3.0 ± 0.00) x $10^5$</td>
</tr>
<tr>
<td>45</td>
<td>(4.5 ± 0.71) x $10^6$</td>
</tr>
<tr>
<td>60</td>
<td>(4.5 ± 0.71) x $10^6$</td>
</tr>
<tr>
<td>120</td>
<td>(7.0 ± 4.24) x $10^5$</td>
</tr>
</tbody>
</table>

$^*$3 x $10^8$ viable bacteria inoculated per well of a 24-well plate for period of up to 120 minutes.

$^\circ$Results ± standard deviation are expressed from three experiments done in duplicate.
Table 2. Effect of *C. jejuni* outer membrane extracts on adhesion to HEp-2 cells by *C. jejuni*.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Viable bacteria associated with HEp-2 cells&lt;sup&gt;°&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control&lt;sup&gt;†&lt;/sup&gt;</td>
<td>(3.33 ± 2.55) X 10&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td>Glycine-acid extract&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>(2.78 ± 2.03) X 10&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>†</sup>4-5 X 10<sup>8</sup> viable bacteria were inoculated per well of a 24-well plate.

<sup>‡</sup>HEp-2 cell monolayer inoculated with *C. jejuni* in the absence of glycine-acid extract.

<sup>§</sup>HEp-2 cell monolayer inoculated with *C. jejuni* in the presence of 0.5 mg glycine-acid extract.

<sup>°</sup>Results ± standard deviation are expressed from three experiments done in duplicate.
Table 3. Effect of *C. jejuni* whole cell lysates on adhesion to HEp-2 cells by *C. jejuni*.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Viable bacteria associated with HEp-2 cells&lt;sup&gt;°&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control&lt;sup&gt;†&lt;/sup&gt;</td>
<td>(5.5 ± 0.58) X 10&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cell lysates&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>(1.24 ± 1.06) X 10&lt;sup&gt;6&lt;/sup&gt;&lt;sup&gt;♂&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>†</sup>5 X 10<sup>8</sup> viable bacteria were inoculated per well of a 24-well plate.

<sup>‡</sup>HEp-2 cell monolayer inoculated with *C. jejuni* in the absence of cell lysates.

<sup>‡</sup>HEp-2 cell monolayer inoculated with *C. jejuni* in the presence of 0.5 mg cell lysates.

<sup>°</sup>Results ± standard deviation are expressed from three experiments done in duplicate.

<sup>♂</sup>Significant inhibition of attachment relative to control, P < 0.05.
Table 4. Effect of collagen and fibronectin on adhesion of *C. jejuni* to HEp-2 cells.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Viable bacteria associated with HEp-2 cells&lt;sup&gt;o&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control&lt;sup&gt;+&lt;/sup&gt;</td>
<td>(4.75 ± 2.21) X 10&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td>Fibronectin&lt;sup&gt;+&lt;/sup&gt;</td>
<td>(2.50 ± 2.38) X 10&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td>Collagen&lt;sup&gt;+&lt;/sup&gt;</td>
<td>(1.75 ± 0.96) X 10&lt;sup&gt;6&lt;/sup&gt;&lt;sup&gt;#&lt;/sup&gt;</td>
</tr>
<tr>
<td>Fibronectin + Collagen&lt;sup&gt;+&lt;/sup&gt;</td>
<td>(1.55 ± 0.97) X 10&lt;sup&gt;6&lt;/sup&gt;&lt;sup&gt;#&lt;/sup&gt;</td>
</tr>
<tr>
<td>Albumin&lt;sup&gt;+&lt;/sup&gt;</td>
<td>(2.73 ± 0.84) X 10&lt;sup&gt;4&lt;/sup&gt;&lt;sup&gt;#&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>*4-6 X 10<sup>8</sup> viable bacteria were inoculated per well of a 24-well plate.</sup>

<sup>+HEp-2 cell monolayer inoculated with *C. jejuni* in the absence of collagen, fibronectin or albumin.</sup>

<sup>+</sup>HEp-2 cell monolayer inoculated with *C. jejuni* in the presence of either collagen, fibronectin, collagen + fibronectin or albumin.

<sup>°Results ± standard deviation are expressed from three experiments done in duplicate.</sup>

<sup>#Significant inhibition of attachment relative to control, P < 0.05</sup>
Table 5. Effect of NaCl on adhesion of *C. jejuni* to HEp-2 cells.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Viable bacteria associated with HEp-2 cells ( ^{\circ} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control(^{+})</td>
<td>( (3.48 \pm 1.25) \times 10^6 )</td>
</tr>
<tr>
<td>0.05 M NaCl(^{#})</td>
<td>( (2.25 \pm 1.26) \times 10^6 )</td>
</tr>
<tr>
<td>0.1 M NaCl(^{##})</td>
<td>( (2.20 \pm 0.54) \times 10^6 )</td>
</tr>
<tr>
<td>0.4 M NaCl(^{##})</td>
<td>( (1.83 \pm 0.99) \times 10^6 )</td>
</tr>
<tr>
<td>0.5 M NaCl(^{##})</td>
<td>( (1.28 \pm 0.22) \times 10^6 )</td>
</tr>
<tr>
<td>1.0 M NaCl(^{##})</td>
<td>( (5.43 \pm 4.75) \times 10^4 )</td>
</tr>
</tbody>
</table>

\(^{+}\)HEp-2 cell monolayer inoculated with *C. jejuni* in the absence of NaCl.

\(^{\#}\)HEp-2 cell monolayer inoculated with *C. jejuni* in the presence of NaCl.

\(^{\#\#}\)5-6 \( \times 10^8 \) viable bacteria were inoculated per well of a 24-well plate.

\(^{\circ}\)Results ± standard deviation are expressed from three experiments done in duplicate.

\( ^{*}\)Significant inhibition of attachment relative to control, \( P < 0.05 \).
Table 6. The effect of NaCl on viability of *C. jejuni* M129\(^*\) and HEp-2 cells\(^o\).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Viable bacteria</th>
<th>Viable HEp-2 cells/well</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0 M NaCl</td>
<td>((1.8 \pm 0.28) \times 10^8)</td>
<td>((1.9 \pm 0.42) \times 10^6)</td>
</tr>
<tr>
<td>0.5 M NaCl</td>
<td>((1.7 \pm 0.28) \times 10^8)</td>
<td>ND</td>
</tr>
<tr>
<td>1.0 M NaCl</td>
<td>((1.65 \pm 0.21) \times 10^8)</td>
<td>((1.8 \pm 0.28) \times 10^6)</td>
</tr>
</tbody>
</table>

\(^*\)4 X 10\(^8\) viable *C. jejuni* M129 were incubated either in the presence or absence of salt for a period of 40 min.

\(^o\)HEp-2 cells were treated with salt for a period of 40 min.

\(^*\)Results ± standard deviations are expressed from three experiments done in duplicates.
D. DISCUSSION

Several investigators have examined the association of *C. jejuni* with tissue culture cells in the hope of delineating those molecules responsible for cellular adhesion. These studies have identified several distinct bacterial components that bind to tissue culture cells (Fauchere *et al.*, 1989 and De Melo and Pechere, 1990). However, the specificity of these interactions and their relevance during the actual disease process remains to be understood. In the present study we examined the specificity of *C. jejuni* attachment to tissue culture cells. This was achieved by conducting competitive inhibition assays with various bacterial and non-bacterial products. Previous reports have suggested that antigen(s) involved in attachment might be different from those which initiate internalization of bacteria (Konkel and Joens, 1989). Consequently, we chose an assay system which only examined the attachment process. This was achieved by performing attachment assays for 40 min at 4°C. The use of an incubation period of less than one hr, the minimal amount of time required for internalization of *C. jejuni* (De Melo and Paucher, 1989), and low temperature should have prohibited cellular internalization of *C. jejuni*.

Our results demonstrate that whole cell lysates of *C. jejuni* blocked attachment of the organism to the HEp-2 cells. However, the bacterial outer membrane proteins, in the form of glycine-acid extract, did not. It is possible that blockage of attachment by whole cell lysate is non-specific and is due
to interference with charge interactions between bacteria and the host cells. On the other hand, whole cell lysates may contain outer-membrane adhesins that become nonfunctional during the glycine-acid extraction procedure, which involves a 30 min incubation time at low pH. Reports by other investigators do not support the latter hypothesis. De Melo and Pechere (1990) identified antigens at 28, 32, 36 and 42 KDa in glycine extracts of invasive *C. jejuni* strains which bind to HEp-2 cells. Fauchere *et al.* (1989), reported blockage of *C. jejuni* attachment to HeLa cells by antisera prepared against the 26-30 KDa outer membrane fraction of *C. jejuni*. Although the glycine-extract of *C. jejuni* prepared in our laboratory contained the same antigens, the extract did not block the attachment of the organism to the HEp-2 cells. This discrepancy could be attributed to interstrain differences of organisms used in these experiments. Alternatively, different methodologies used in examining the role of antigens in attachment could explain the various observed outcomes.

Previous reports have pointed out differences that may exist among *C. jejuni* strains. Konkel and Joens (1989) observed differences in the virulence, noting the presence of strains that adhere to HEp-2 cells but do not invade the cells. This has led them to propose that different antigens are involved in attachment and invasion. Since De Melo and Pechere (1990) reported attachment of the outer membrane antigens only from the more invasive strains, it is possible that their proposed "adhesin" antigens are
exclusively involved in the invasion process. It may be that the antigens in the glycine extracts are involved in the internalization steps and not in the attachment process. Thus, glycine-acid extracts may lack specific "adhesin" molecules and therefore are undetectable in our assay system.

De Melo and Pauchere (1990) reported the binding of _C. jejuni_ outer membrane antigens to HEp-2 cells in an _in vitro_ ligand binding assay. Their technique involved resolution of the HEp-2 cell proteins on SDS-PAGE. The use of SDS may have caused a conformational change in the cell receptor thereby resulting in non-specific binding. Non-specific binding attributed to surface charge and hydrophobicity has been reported to play a role in the attachment of other bacteria, such as _E. coli_, _Salmonella_ and _Neisseria gonorrhoeae_, to cells (Magnusson _et al._, 1980).

We performed the adherence assay in the presence of different salt concentrations to examine the role of electrostatic interactions in the association of _C. jejuni_ with HEp-2 cells. Indeed, salt blocked attachment of the bacteria to the HEp-2 cells without being lethal to either HEp-2 cells or to the bacteria, as assessed by trypan blue viability and counting of CFU. Inhibition of attachment increased with higher salt concentrations, indicating that attachment may have been mediated through charge interactions. Our results are supported by the findings of Walan and Kihlstrom (1988) who have shown greater attachment of _C. jejuni_ strains with high negative surface charge and weak hydrophobic surfaces to HT-29 cells than strains
with less negative charge and more hydrophobic surfaces.

There is increasing evidence that microbial pathogens may adhere to extracellular matrix proteins such as fibronectin or collagen. A fibronectin-binding protein in *Staphylococcus aureus* has been cloned and characterized (Flock *et al.*, 1987). Similarly, a specific interaction between type IV collagen and O75X adhesin molecule of uropathogenic *Escherichia coli* has been reported (Westerlund *et al.*, 1989). A recent study by Kuusela *et al.* (1989) has shown that *C. jejuni* binds to coverslips coated with fibronectin or collagen. Using competitive inhibition assays, we examined the specificity of these interactions. In contrast to collagen, fibronectin did not block the attachment of the organism to the HEp-2 cells in our assay system. Furthermore, albumin, which was used to control the specificity of the assay, also inhibited the attachment of *C. jejuni* to the HEp-2 cells. Therefore, it is possible that the binding of extracellular matrix proteins to *C. jejuni* is not via a specific receptor-ligand interaction. These interactions may be mediated through non-specific mechanisms, such as hydrophobic or electrostatic forces.

The present study suggests that the attachment of *C. jejuni* to the HEp-2 cells is not mediated through specific outer membrane proteins. It appears that non-specific charge interactions play a role in the association process. Studies with *Yersinia enterocolitica* have identified a highly anionic, hydrophobic fibrillae structure on the organism which mediates its
attachment to human epithelial cells (Lachica et al., 1984 and Kappeerud et al., 1985). Although our study suggests that electrostatic interactions may play a role in the attachment of *C. jejuni* with cells, it is unclear as to whether electrostatic and hydrophobic interactions result from specific membrane structures as seen with *Y. enterocolitica*. Alternatively, *C. jejuni* may express a net negative charge and hydrophobicity that facilitates cellular attachment. Further studies with other *C. jejuni* strains are needed in order to resolve the mechanism of cellular association and its relevance to the disease process.
II. INVASION RELATED ANTIGENS OF C. jejuni

A. INTRODUCTION

The pathogenicity of C. jejuni has not been clearly defined. The wide range of clinical symptoms plus variation observed during in-vitro studies, has led to the conclusion that different mechanisms may operate among different strains of C. jejuni. Some strains may produce enterotoxins which would manifest clinically in the production of watery diarrhea (Klipstein et al., 1985). Other strains of C. jejuni may damage the epithelial cells resulting in the production of bloody diarrhea with leukocytes present (Mandal and Butzler, 1984). The latter scenario is characteristic of infection by an invasive organism.

In vitro studies suggest that C. jejuni is internalized by epithelial cells through phagocytic induction (Konkel and Joens, 1989). This type of phagocytosis requires both host cell energy expenditure and microfilament involvement (De Melo et al., 1989). The nature of those bacterial molecules that participate in the invasion process remains unclear. The present research will attempt to take advantage of monoclonal antibody specificity in identifying surface ligands which are instrumental in C. jejuni invasiveness. Using competitive inhibition studies, blockage of epithelial cell invasion by monoclonal antibodies should help to confirm the proposed mechanism of C. jejuni invasiveness.
B. MATERIALS AND METHODS

Bacteria. In examining those antigens required for epithelial cell penetration by *C. jejuni*, the following isolates were used: *C. jejuni* M96 and M125 were kindly provided by Dr. Kenneth Ryan (University Medical Center, University of Az, Tucson, Az.). *C. jejuni* SJ was obtained from Dr. P. Flynt (St. Joseph’s Hospital, Tucson, Az.). All three isolates had been isolated from patients with bloody diarrhea. *C. jejuni* Flan, a non-invasive isolate was obtained from swine with enteritis. *C. jejuni* 35925 a non-invasive isolate was obtained from the ATCC. *Campylobacter hyointestinalis* (35217) and *C. mucosalis* (43264), used as non-invasive controls, were obtained from the ATCC. All the isolates were maintained as described previously.

HEp-2 cells. Human laryngeal carcinoma (HEp-2, ATCC CCL 23) cells were grown in Eagle’s minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) without antibiotics. For all experimental assays, 24-well tissue culture trays (Falcon, Becton Dickinson Labware, Oxnard, CA) were seeded with 8 X 10^4 HEp-2 cells/well. Plates were incubated for 18 hr at 37°C in a humidified atmosphere with 5% CO₂. Prior to the assays, semiconfluent monolayers were washed with fresh MEM containing 1% FBS.

Whole cell lysates. *C. jejuni* isolates were harvested from MH plates with phosphate buffered saline (0.1 M) (PBS), and disrupted in a french pressure cell (10,000 lbs/in^2). The cellular debris was removed by
centrifugation at 6,000 X g at 4°C for 25 min. The resulting supernatant was collected, aliquoted and stored at -70°C.

**Outer membrane enriched fractions.** *C. jejuni* outer membrane fractions were prepared by the method of Rapp *et al.* (1986). Whole cell lysates from *C. jejuni* were pelleted by centrifugation at 100,000 X g for 60 min. Each pellet was resuspended in 1% sodium N-lauroyl sarcosinate and incubated at room temperature for 30 min to selectively solubilize the inner membrane while leaving the outer membrane intact. The outer membrane enriched fraction was pelleted by centrifugation at 100,000 X g for 60 min. The pellet was resuspended in PBS and aliquoted. Aliquots were stored at -70°C.

**Monoclonal antibody production.** A panel of monoclonal antibodies (Mab) were produced against *C. jejuni* isolates, including an invasive strain of human origin (SJ), a non-invasive animal isolate (Flan) and a non-clinical, non-invasive isolate (35925). These three strains provided us with *C. jejuni* antigens from two different mammalian hosts and of invasive and non-invasive potential. In three separate experiments Balb/c mice were immunized with either whole cell lysates or outer membrane enriched fractions of the three *C. jejuni* isolates. Sensitized spleen cells from these mice were harvested and hybridized with either SP/2 or P2X63-Ag 8.653 myeloma cells by the method of Kohler and Milstein (1975). Culture supernatants were screened for activity against *C. jejuni* lysates, using
enzyme-linked immunosorbent assay (ELISA). Antibody secreting cells were cloned by limiting dilution (Goding, 1986). Mab isotypes were identified by the ELISA technique using peroxidase-labelled anti-mouse IgG\(_1\), IgG\(_{2a}\), IgG\(_3\) and IgM.

**ELISA.** This test procedure was performed utilizing Immunlon 1 microtitration plates (Dynatech Ltd.) The working volume of each well was 200 \(\mu\)l. Microtiter plates were incubated overnight at 4°C with a 1:50 dilution of *C. jejuni* lysates in carbonate buffer (pH 9.6). Antigen coated plates were washed three times at 5 min per wash with washing buffer (PBS containing 1% Tween 20, 0.5% bovine serum albumin) and incubated with hybridoma supernatants at 37°C. After 2 hr unbound antibody was removed by washing three times and incubated with a 1:400 dilution of peroxidase-labelled goat anti-mouse IgG + IgA + IgM (Kirkegaard and Perry) for 90 min at 37°C. Antibodies were detected with 2,2'-azinobis-3-ethylbenzthiazoline sulfonic acid:H\(_2\)O\(_2\). Plates were read after 10 min at 410 nm on a microtiter plate reader (Dynatech Ltd).

**SDA-PAGE and Immunobloting.** These procedures were performed as previously described (Laemmli, 1970 and Towbin *et al.*, 1979) to detect *C. jejuni* antigens recognized by hybridoma supernatants and by rabbit antisera.

**Ascites fluid production.** Adult Balb/c mice were injected intraperitoneally (IP) with 0.5 ml of Pristane (Sigma). After 10 days, the mice were injected IP with 2 \(\times\) 10\(^6\) hybridoma cells (2D1 and 1B4 Mab
producing cells) in 0.5 ml of RPMI-base medium (Gibco, Grand Island, N.Y.). Ascites fluid was collected and either used immediately or stored at -70°C. The Ig fraction was precipitated with polyethylene glycol. Briefly, the ascites fluid was mixed with a 12% solution of PEG, prepared by dissolving PEG 8000 in Vernol-buffered saline (Oxoid U.S.A., Inc, Columbia, Md.). After 15 min incubation on ice, the preparation was centrifuged at 22,000 X g for 20 min, decanted and filtered through a 0.8-μm-pore-size membrane syringe filter. The ascites fluid was used in invasion assays to examine its effect on blockage of *C. jejuni* invasion to HEp-2 cells. This was achieved by first incubating the ascites fluid at 60°C for 30 min in a water bath to heat inactivate the complement fraction. The ascites fluid was then diluted 1:10 in MEM supplemented with 1% FBS and mixed with an equal volume of MEM containing 1 X 10⁸ cells/ml. One ml of this suspension was used to inoculate duplicate wells containing semiconfluent monolayers of HEp-2 cells.

**Invasion assay.** Internalization of bacteria into HEp-2 cells was determined using the method of Konkel and Joens (1989). Briefly, duplicate wells containing semi-confluent monolayers of HEp-2 cells were inoculated with approximately 5 X 10⁷ colony forming units (CFU) of the desired organism and incubated for 3 hr at 37°C under 5% CO₂ to allow bacterial adherence to HEp-2 cells. Infected monolayers were then washed 5 times with fresh MEM containing 1% FBS and incubated for 3 hr under the same
conditions to allow bacterial invasion. Medium containing 250 μg/ml of gentamicin (Gibco Laboratories, Grand Island, N.Y.) was added to one well to kill the extracellular bacteria and allow enumeration of intracellular organisms. Medium without antibiotic was added to the other well to allow enumeration of both intracellular and extracellular organisms. Following the 3 hr incubation, HEp-2 cell monolayers were washed 3 times with MEM and lysed by adding 0.5% sodium deoxycholate (Difco Laboratories, Detroit, Mich.) to each well. Suspensions were diluted and viable bacteria determined by counting CFU on MH agar plates containing 4% citrated blood.

**Purification of invasive antigen(s) with monoclonal antibody.** Ten mg of 1B4-S antibody (dissolved in 0.1 M NaHCO₃, pH 7.2, containing 0.5 M NaCl) were coupled to one ml of CNBR-activated Sepharose beads in an end-over-end fashion overnight at 4°C. The remaining active groups of the Sepharose were blocked with 0.2 M glycine followed by a washing step to remove excess adsorbed protein. Whole cell lysates of invasive strains were resuspended in PBS buffer containing 1% NP40, 1% BSA and 0.5% Tween 80. The resulting antigen preparation was added to the antibody-Sepharose immunoadsorbent. Gel contents were transferred to a chromatography column and non-specifically adsorbed material (unbound fraction) was removed from the gel by a wash with PBS containing 0.25% NP40. Elution of bound antigens was accomplished by using a low pH buffer (0.25% NP40
in 0.2 M glycine-HCl, pH 2.3). The procedure was performed twice with each resulting fraction (#1 and #2) neutralized with Tris base, dialyzed and concentrated by ultrafiltration with a YM 5 membrane (Amicon). These fractions were later assayed by SDS-PAGE and western blots.

**Preparation of antiserum.** Two New Zealand white rabbits were immunized subcutaneously and intramuscularly with the 1B4-affinity purified antigens. The eluted fraction #1 (0.5 ml) and eluted fraction #2 (0.25 ml) were each separately mixed with equal concentrations of Freund’s complete adjuvant and injected into two rabbits on day 1 and day 14. Twenty eight days after the first immunization, the animals were bled by cardiac puncture. Antisera were collected and stored at -20°C. The antisera were used in invasion assays to examine their effect on blockage of *C. jejuni* invasion to HEp-2 cells. The antisera were incubated at 60°C for 30 min in a water bath. Antisera were then diluted 1:10, 1:50 and 1:100 in MEM supplemented with 1% FBS and mixed with an equal volume of MEM containing 1 X 10^8 bacterial cells/ml. One ml of this suspension was used to inoculate duplicate wells containing semiconfluent monolayers of HEp-2 cells.

**Chemical treatment of lysates.** Whole cell lysate was prepared as described previously and treated with sodium *meta*-periodate (Sigma) or proteinase K (Sigma). Briefly, *C. jejuni* lysate was treated with 10 mM sodium *meta*-periodate at pH 4.5 for 15 min in the dark at room
temperature. Following chemical treatment, free aldehyde groups were then blocked with 1% glycine. After a 15 min incubation, the sample was extensively dialyzed against PBS. Additionally, lysate was treated with 100 \( \mu g/ml \) of proteinase K for 30 min at 37°C. Phenylmethylsulfonyl fluoride (PMSF) was then added to the sample at a 10:1 molar ratio of inhibitor to enzyme. After incubating for 5 min, the sample was extensively dialyzed against PBS. Following dialysis, the treated lysates were concentrated to their original volumes by passage through an ultrafiltration membrane (YM-10; Amicon Corp., Danvers, Mass.)

C. Results

Antigenic recognition of \textit{C. jejuni} by monoclonal antibodies. A series of monoclonal antibodies were produced against \textit{C. jejuni} isolates including an invasive strain of human origin (SJ), a non-invasive animal isolate (Flan) and a non-clinical, non-invasive isolate (35925). Eight different Mabs were cloned. Two of the Mabs did not react with antigens using the western blot method. The other six Mabs recognized antigens ranging in molecular weight from 62-12 KDa (Fig 1). These included:

1. 5D1-S, which recognized a 14 KDa antigen from LPS extract of SJ \textit{C. jejuni}.
2. 1A4-F, which recognized a 14 KDa antigen from LPS extract of Flan \textit{C. jejuni}.
3. 4B2-F, which recognized an 18 KDa antigen in all \textit{C. jejuni} lysates.
4. 2D1-A, which recognized flagella antigens of 62-52 KDa in all *C. jejuni* lysates.

5. 3C6-A, which recognized antigens of 50-42 KDa in all *Campylobacter* spp. lysates except *C. mucosalis*.

6. 1B4-S, which recognized antigens of 66-50 KDa in all *Campylobacter* spp. lysates and additional antigens between 50-35 KDa.

**Expression of C. jejuni virulence factors.** *C. jejuni* M96 was extensively subcultured in the laboratory to examine the effect of serial passage on invasion. Organisms subcultured fewer than twelve times were considered to be of low passage, whereas those subcultured more than thirty times were referred to as high passage. Significant differences were seen to exist between low and high passage *C. jejuni* M96 (Table 1). Low passage strains, which produced highly mucoid colonies, were highly invasive. On the other hand, high passage *C. jejuni* no longer invaded HEp-2 cells.

**Effect of monoclonal antibody 1B4 on C. jejuni infection of HEp-2 cells.** A monoclonal antibody, derived from a mouse hyperimmunized with *C. jejuni*, was determined by the Elisa technique to be of the IgM isotype. The 1B4 Mab was used in an attempt to inhibit the invasiveness of *C. jejuni* for HEp-2 cells. Control levels for internalized bacteria were established by infecting HEp-2 cell monolayers in the absence of the antibody. To examine
whether the ascites fluid containing the 1B4 Mab had a non-specific effect on internalization processes, HEp-2 cell monolayers were infected with a suspension of *S. typhimurium* in the absence and presence of the ascites fluid. Results are presented in Table 2. The invasiveness of *C. jejuni* was significantly (*P* < 0.05) reduced (approximately 50% inhibition) by ascites fluid (1:10) containing the 1B4 Mab. The 1B4 Mab had no effect on the ability of *S. typhimurium* to invade HEp-2 cells. The other monoclonal antibodies (5D1, 1A4, 4B2, 2D1 and 3C6) were also tested for their ability in inhibiting invasiveness of *C. jejuni* for HEp-2 cells. These Mabs, however, did not inhibit invasion of *C. jejuni* to HEp-2 cells.

**Western blot analysis of Campylobacter lysates with Mab 1B4.** The 1B4 Mab was reacted with *Campylobacter* whole cell lysates which had been separated by SDS-PAGE and electroblotted to immobilon paper (Fig. 2). Lysates were prepared from two invasive isolates, *C. jejuni* M125 and low passage M96, and three non-invasive isolates, *C. jejuni* M96 (high passage), *C. hyointestinalis*, and *C. mucosalis*. The 1B4 Mab recognized an epitope on antigens ranging from 64 to 44 KDa from both invasive and non-invasive isolates. In addition, the 1B4 Mab identified antigens ranging from 42 to 38 KDa in lysates prepared only from invasive isolates.

**Effect of chemical treatment of C. jejuni M96 lysate on Mab 1B4 reactivity.** *C. jejuni* M96 whole cell lysate was treated with 10 mM sodium *meta*-periodate and 100 μg of proteinase K in an attempt to identify the
chemical nature of *Campylobacter* invasion related antigens (Fig. 3). *C. jejuni* epitopes recognized by the 1B4 Mab had increased mobility after treatment with sodium *meta*-periodate or proteinase K. The increase in mobility reflects a decrease in size of antigens. This suggests that the Mab reacts with an epitope on an antigen of both carbohydrate and protein composition.

**Western blot analysis of 1B4-affinity purified antigens.** Mab-1B4, which inhibits invasiveness of *C. jejuni* for HEp-2 cells, was used to purify invasion-related antigens from whole cell lysates of M96 *C. jejuni*. The unbound fraction and eluted fractions (#1 and #2) were separated by SDS-PAGE and electroblotted onto immobilon paper, followed by reaction with 1B4 Mab.

Antigens that ranged in molecular weight between 64-54, 40-38 and at 25 KDa were recognized in fractions #1 (Fig. 4A). Antigens at 64, 22 and 10K Da were identified in fraction #2. To examine the purity of fractions, samples were also reacted with anti-*C. jejuni* antisera (Fig. 4B). This antisera, which was prepared in rabbits against formalin-killed *C. jejuni*, recognized similar antigens as 1B4-Mab. In contrast to the eluted fractions #1 and #2, the unbound fraction contained no antigens that reacted with the 1B4-Mab. As expected, anti-*C. jejuni* antisera identified numerous antigens in the unbound fraction.

**Antisera to the eluted fractions #1 and #2.** Antisera to the eluted
fractions (#1 and #2) were prepared in rabbits. These antisera were used, in a competitive inhibition assay, to block invasion of *C. jejuni* to HEp-2 cells. Only antisera prepared against fraction #1 was able to significantly block invasion of *C. jejuni* to HEp-2 cells (approximately 90 % blockage) (Table 3).

Antigenic recognition pattern of antisera to both eluted fractions were compared to identify specific antigens that participate in the invasion process. Lysates from invasive *C. jejuni* M 96 strain were electrophoretically separated, followed by electroblotting, as previously described. These antigens were then reacted with three different types of antibody preparations: 1, ascites fluid containing 1B4-Mab (1:50); 2, specific antisera prepared against both eluted fraction #1 and #2 (1:100) and 3, polyclonal antisera prepared against formalin-killed *C. jejuni* (1:100) (Fig. 5). Distinct antigens at 62 and 47 K Da and also between 42-35 KDa were recognized by monospecific antisera prepared against fraction #1 and by 1B4-Mab. These antigens were not recognized by antisera prepared against fraction #2.
Fig 1. Western blot analysis of monoclonal antibodies with homologous *C. jejuni* whole cell lysates. Lanes: 1, 5D1-S; 2, 1A4-F; 3, 4B2-F; 4, 3C6-A; 5, 1B4-S; 6, 2D1-A and 7, polyclonal antisera.
Table 1. Comparison of invasiveness of low and high passage *C. jejuni* M96 for HEp-2 cells°.

<table>
<thead>
<tr>
<th><em>C. jejuni</em> (M96)</th>
<th>Inoculum*</th>
<th>Intracellular†</th>
<th>Extracellular‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>low passage▼</td>
<td>(1.4 ± 1.0) X 10⁷</td>
<td>(1.0 ± 0.5) X 10³</td>
<td>(4.0 ± 3.4) X 10⁴</td>
</tr>
<tr>
<td>high passage*</td>
<td>(2.5 ± 2.4) X 10⁷</td>
<td>0.0</td>
<td>(3.5 ± 1.3) X 10²</td>
</tr>
</tbody>
</table>

°Results ± standard deviation are expressed from three experiments done in duplicate.

*Viable bacteria inoculated/well of a 24-well plate.

†Viable bacteria invading cells/well of a 24-well plate.

‡Viable bacteria invading cells and adhering to cells/well of a 24-well plate.

▼Bacteria subcultured fewer than twelve times in the laboratory.

*Bacteria subcultured more than thirty times in the laboratory.
Table 2. Effect of the 1B4 monoclonal antibody on invasiveness of *C. jejuni* M96 and *Salmonella typhimurium* for Hep-2 cells.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Inoculum'</th>
<th>Intracellular†</th>
<th>Extracellular +</th>
<th>Intracellular*</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. jejuni</em></td>
<td>(4.0 ± 2.4) X 10^7</td>
<td>(5.0 ± 3.2) X 10^4</td>
<td>(2.9 ± 1.3) X 10^4</td>
<td></td>
</tr>
<tr>
<td><em>C. jejuni</em> control</td>
<td>(4.0 ± 2.4) X 10^7</td>
<td>(1.6 ± 1.2) X 10^3</td>
<td>(3.4 ± 1.9) X 10^4</td>
<td></td>
</tr>
<tr>
<td><em>S. typhimurium</em></td>
<td>(1.6 ± 0.1) X 10^8</td>
<td>(6.2 ± 1.1) X 10^6</td>
<td>(2.0 ± 1.0) X 10^7</td>
<td></td>
</tr>
<tr>
<td><em>S. typhimurium</em> control</td>
<td>(1.6 ± 0.1) X 10^8</td>
<td>(6.3 ± 1.0) X 10^6</td>
<td>(2.1 ± 1.0) X 10^7</td>
<td></td>
</tr>
</tbody>
</table>

'Viable bacteria inoculated/well of a 24-well plate.
†Viable bacteria invading cells/well of a 24-well plate.
*Viable bacteria invading cells and adhering to cells/well of a 24-well plate.

Results ± standard deviation are expressed from three experiments done in duplicate.

Significant inhibition of infection relative to the control, *P* < 0.05.
Fig. 2. Antigenic recognition of *Campylobacter* whole cell lysates with 1B4 (lanes 1-5) monoclonal antibody. Lanes: 1, invasive *C. jejuni* M125; 2, invasive (low passage) *C. jejuni* M96; 3, non-invasive (high passage) *C. jejuni* M96; 4, non-invasive *C. mucosalis*; 5, non-invasive *C. hyointestinalis*.
Fig. 3. Chemical treatment of Campylobacter whole cell lysates. Lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, electroblotted to immobilon paper, and bands visualized by the western blot technique using 1B4 monoclonal antibody. Lanes: 1, untreated lysate; 2, sodium meta-periodate treated lysate; 3, proteinase K treated lysate.
Fig 4. Antigenic recognition of *C. jejuni* M96 whole cell lysates before (lane 1) and after (lane 2 and 3) affinity purification with 1B4 monoclonal antibody (A) or with anti-*C. jejuni* antisera (B). Lanes: 1, untreated whole cell lysate; 2, 1B4-affinity purified fraction #1; 3, 1B4-affinity purified fraction #2.
Table 3. Effect of rabbit anti-fraction #1 and #2 antisera on invasiveness of *C. jejuni* for HEp-2 cells.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Inoculum</th>
<th>Intracellular+</th>
<th>Extracellular +</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10⁸</td>
<td>10⁴</td>
<td>10⁵</td>
</tr>
<tr>
<td>Control</td>
<td>(4.0 ± 0.0)</td>
<td>(4.9 ± 0.1)</td>
<td>(2.3 ± 1.1)</td>
</tr>
<tr>
<td>Anti-fraction #1 antisera (1:10)</td>
<td>(4.0 ± 0.0)</td>
<td>(6.0 ± 0.3)</td>
<td>(9.7 ± 0.6)</td>
</tr>
<tr>
<td>Anti-fraction #1 antisera (1:50)</td>
<td>(4.0 ± 0.0)</td>
<td>(3.3 ± 2.3)</td>
<td>(9.3 ± 1.2)</td>
</tr>
<tr>
<td>Anti-fraction #1 antisera (1:100)</td>
<td>(4.0 ± 0.0)</td>
<td>(5.3 ± 4.0)</td>
<td>(1.0 ± 0.0)</td>
</tr>
<tr>
<td>Control</td>
<td>(4.0 ± 0.0)</td>
<td>(4.67 ± 2.3)</td>
<td>(2.33 ± 1.15)</td>
</tr>
<tr>
<td>Anti-fraction #2 antisera (1:10)</td>
<td>(4.0 ± 0.0)</td>
<td>(2.33 ± 1.1)</td>
<td>(1.06 ± 1.16)</td>
</tr>
<tr>
<td>Anti-fraction #2 antisera (1:50)</td>
<td>(4.0 ± 0.0)</td>
<td>(3.00 ± 1.7)</td>
<td>(1.57 ± 0.75)</td>
</tr>
<tr>
<td>Anti-fraction #2 antisera (1:100)</td>
<td>(4.0 ± 0.0)</td>
<td>(9.00 ± 2.6)</td>
<td>(2.00 ± 1.73)</td>
</tr>
<tr>
<td>Control</td>
<td>(4.5 ± 1.5)</td>
<td>(3.2 ± 0.5)</td>
<td>(1.7 ± 0.7)</td>
</tr>
<tr>
<td>Normal rabbit antisera (1:25)</td>
<td>(4.5 ± 1.5)</td>
<td>(2.6 ± 0.1)</td>
<td>(1.4 ± 0.5)</td>
</tr>
<tr>
<td>Normal rabbit antisera (1:50)</td>
<td>(4.5 ± 1.5)</td>
<td>(4.0 ± 0.9)</td>
<td>(1.1 ± 0.1)</td>
</tr>
</tbody>
</table>

*Results ± standard deviation are expressed from three experiments done in duplicate.

*Number of viable bacteria inoculated/well of a 24-well plate.

+Viable bacteria invading cells/well of a 24-well plate.

*Viable bacteria invading cells and adhering to cells/well of a 24-well plate.

*Significant inhibition of infection relative to the control, P < 0.05.
Fig. 5. Antigenic recognition of *C. jejuni* M96 whole cell lysates with: 1B4 Mab (lane 1), monospecific anti-fraction #1 antisera (lane 2), specific anti-fraction #2 antisera (lane 3) and polyclonal anti-*C. jejuni* antisera (lane 4)
D. DISCUSSION

Clinical and experimental evidence increasingly suggest that invasion may be one of the many pathogenic mechanisms that *C. jejuni* employs during the disease process. However, this is still a controversial subject and the nature of antigen(s) involved in this process have not been identified. Studies by Klipstein et al. have found that invasive *C. jejuni* could be differentiated from noninvasive strains, by ELISA, using antisera prepared against invasive *C. jejuni*. This implies that invasion-related antigens may exist which are conserved among invasive strains. In the present study, by using monoclonal antibodies, we have attempted to identify antigens that may participate during the invasion process.

A 1B4 Mab, produced in our laboratory, partially blocked invasion of *C. jejuni* to HEp-2 cells. This monoclonal antibody recognized antigens ranging in molecular weight between 64-44 KDa in both high and low passage *C. jejuni* M96 strain. It also recognized distinct antigens between 42-38 KDa only in the low passage M96 strain. Furthermore, immunogold labelling revealed staining of flagella and bacterial surface in low passage invasive strains as opposed to staining of only flagella in the noninvasive high passage strain (data not shown). It appears that both flagella and the bacterial surface share a common epitope. Experimental evidence supporting this hypothesis was provided by Newell (1986), who demonstrated that a Mab to purified flagella, 8immunogold labelled the bacterial surface of *C. jejuni*.
Investigators working with *Vibrio cholerae* have reported a similar finding using the same techniques (Fuerst and Perry, 1988). Both the flagella and bacterial surface of *V. cholerae* share common antigens.

A shift in the antigenic mobility was observed after treatment of the electroblotted lysates with both proteinase K and sodium *meta*-periodate, suggesting that epitopes recognized by the 1B4 Mab reside on a glycoprotein molecule. Evidence from studies by Konkel and Joens (1988) suggests that the functional site, or binding region of the invasive antigen, is dependent on a carbohydrate moiety. Additionally, the invasive potential appears to be associated with mucoid and spreading colonies rather than isolated individual colonies. The latter colonies appear in cultures that have been passed several times, indicating that the invasiveness of *C. jejuni* is an unstable characteristic. An antigenic response to this carbohydrate containing moiety may require an IgM response.

The inability of ascites fluid containing Mab 1B4 to totally block the invasiveness of low passage *C. jejuni* could be explained in at least two ways. It is possible that *C. jejuni* expresses more than one antigen that mediates its invasion into epithelial cells, as has been reported with other invasive bacteria. Alternatively, partial blockage of invasion could be due to steric hinderance rather than specific blockage of binding of the invasin antigen with its receptor. To rule out the latter possibility, monospecific antisera prepared against the 1B4 affinity purified antigens was used to
block the invasiveness of *C. jejuni* into the HEp-2 cells. The ability of the monospecific antisera, prepared against one of the eluted fractions (fraction #1), to block the invasiveness of *C. jejuni* into HEp-2 cells, invalidates the latter proposal.

Distinct antigens at 62, 47 KDa and also between 42-35 KDa were identified by the monospecific antisera prepared against the eluted fraction #1. Antisera to some of these antigens have also been reported in patients infected with *C. jejuni*. Although patient’s antibody response is usually directed against major antigens at 62, 42, 31, 21 and 14 KDa they occasionally recognize antigens in the clinical strains that are between 38-37 and 36-22 KDa as determined by western blot analysis (Mills and Bradbury, 1984). Furthermore, studies of De Melo and Pechere (1990) have identified distinct antigens at 42, 32 and 28 KDa only in *C. jejuni* invasive strains, which bind to HEp-2 and HeLa cells. These reports confirm our observations and point to the presence of distinct antigens between 42-35 KDa, which may participate in the invasion process.

The process of internalization by other invasive enterics is thought to be mediated by induced phagocytosis, in which several bacterial polypeptides are involved. For example, in *Shigella flexneri* expression of three polypeptides (*ipaB, ipaC and ipaD*) are thought to elicit a transmembrane signal which leads to polymerization of actin and accumulation of myosin (Sansonetti, 1991). *Yersinia enterocolitica* harbors
two chromosomally encoded genes, *inv* and *ail*, whose products mediate attachment to and invasion of tissue culture cells (Miller and Falkow, 1988). *In vitro* cell culture studies indicate that *C. jejuni* internalization is mediated through a phagocytic-like mechanism which requires microfilament production (De Melo and Pechere, 1988). Whether the antigens between 42-35 KDa induce phagocytosis or play another role in the invasiveness of *C. jejuni* requires further study.
III. INVASION OF PORCINE INTESTINAL CELLS BY *C. jejuni*

**A. INTRODUCTION**

*Campylobacter jejuni* is recognized as one of the leading causes of human enteritis throughout the world (Mandel *et al*., 1984). Pathogenic mechanisms which are required by the organism to establish an infection are not clearly understood. Various known virulent factors directly contribute to the pathogenicity of the organism. *C. jejuni* is known to produce enterotoxins that are functionally and immunologically similar to cholera toxin and heat labile toxin (LT) of *E. coli* (Calva *et al*., 1989; Ruiz-Palacios *et al*., 1983 and Klipstein and Engert, 1985). Although the role of cytotoxins in the disease process is not conclusive, evidence exists which demonstrates the invasive potential of *C. jejuni* (Mandai and Butzler, 1984).

Much of the work which has led to understanding *C. jejuni* mechanism of invasiveness has come from *in vitro* culture studies (Konkel and Joens, 1989; De Melo *et al*., 1989). These studies were accomplished by utilizing transformed cells lines which may have had altered receptors. Therefore, these results may not depict the actual pathogenic mechanisms employed during the disease process. In the present study a model system was developed, using primary epithelial cell lines, to investigate the invasive potential of *C. jejuni*. Primary epithelial cells were obtained from swine, which are anatomically and physiologically similar to humans.
B. MATERIALS AND METHODS

Bacteria. The following isolates were used to examine the invasive capabilities of *C. jejuni* with swine intestinal enterocytes: *C. jejuni* F1474 was obtained from a patient with clinical signs of campylobacteriosis (Saint Joseph Hospital, Tucson, Az.), *C. jejuni* T13193 was kindly provided by Dr. Kenneth Ryan (University Medical Center, University of Az, Tucson, Az.). *Salmonella typhimurium* (obtained from Harvey Olander, University of California at Davis), an invasive organism, was used as a positive control in the invasion assay. *E. coli* HB 101 (Bachmann, 1972), a non-invasive organism, was used as a negative control in the invasion assay.

Both *C. jejuni* strains were kept frozen at -70°C in bovine blood. The strains were grown microaerophilically on MH plates containing 4% citrated bovine blood and incubated at 37°C. For experimental assays, 24-48 hr cultures of *C. jejuni* strains under 10 passages were used.

Swine intestinal cells. Colostrum deprived newborn piglets were obtained from the sows at the time of farrowing. The piglets were rinsed with betadine (ACME United Corp., Fairfield, CT.) and transported to the laboratory in sterile boxes. Upon arrival, the piglets were sacrificed with two ml of Beuthanasia-D (Schering Corp., Kenilworth, NJ). Small intestines were removed and cut into 10-15 cm pieces. The lumen was rinsed with sterile PBS and each piece was then cut longitudinally. Intestinal pieces were incubated for 15 min in 0.25% trypsin and 1mM EDTA solution (Gibco,
Grand Island, N.Y.) at 37°C on a rotator and cells were washed 3 X by low speed centrifugation (200 x g, 10 min) with Eagle’s minimal essential medium (MEM) containing 1% fetal calf serum (FCS). The cell pellet was resuspended in 40 ml of MEM containing 15% FCS, 100 µg/ml gentamicin, 10,000 µg/ml penicillin G, 10,000 mcg/ml streptomycin and 25 µg/ml amphotericin B. The cells were then incubated at 37°C on a rotator for 45 min. Following a thorough wash (3 X) at low speed centrifugation (200 X g for 10 min), a viability cell count was performed using trypan blue.

To examine for the presence of any bacterial contaminants, cells were cultured on three different types of media: 1) tergitol 7 agar (Difco, Detroit, MI) for the presence of Salmonella spp. and E. coli, 2) trypticase soy agar (BBL, Cockeysville, MD) containing 5% citrated blood and spectinomycin (400 µg/ml) for the presence of Serpula hyodysenteriae and 3) MH agar containing 4% citrated bovine blood for presence of Campylobacter spp. Tergitol 7 agar was incubated aerobically at 37°C, trypticase soy agar plates anaerobically (50% hydrogen, 50% carbon dioxide) at 42°C and MH plates anaerobically at 37°C for the presence of C. mucosalis and microaerophilically (10% hydrogen, 10% carbon dioxide and 80% nitrogen) at 37°C for other species of Campylobacter.

HEp-2 and INT 407 cells. Human laryngeal carcinoma (HEp-2, ATCC CCL 23) and INT 407 cells (ATCC CCL 6) were maintained in MEM supplemented with 10 and 15% FBS respectively. For all experimental
assays, 24-well tissue culture trays (Falcon, Becton Dickinson Labware, Oxnard, CA) were seeded with $8 \times 10^4$ cells/well. Plates were incubated for 18 h at 37°C in a humidified atmosphere with 5% CO$_2$. Prior to the assay, the semiconfluent monolayers were washed with fresh MEM containing 1% FBS and the invasion assay performed.

**Invasion assay.** The invasion assay was performed using the method of Konkel and Joens (1989) with some modifications. Briefly, bacteria were harvested from plates with 3 ml of PBS and pelleted by centrifugation at 6000 X g for 10 min at 4°C. The pellets were resuspended in MEM containing 1% FBS to obtain approximately $1 \times 10^8$ bacteria/ml. A bacterial suspension (0.5 ml) was inoculated into duplicate tubes containing $1 \times 10^6$ swine epithelial cells. Test tubes containing swine cells and bacteria were rotated in an end-over-end fashion on a rotator for 3 hr at 37°C. Cells were then washed 3 X with MEM containing 1% FBS at 200 X g for 10 min. One ml of MEM containing 1% FBS and 250 $\mu$g gentamicin was added to one tube for the enumeration of the intracellular bacteria. To the other tube, medium without any antibiotic was added to enumerate the number of intracellular and extracellular bacteria. Following an additional 3 hr incubation at 37°C, cells were washed 3 X with MEM containing 1% FBS. Epithelial cells were then lysed with 0.5% sodium deoxycholate. The suspensions were diluted and viable bacteria were determined by counting the colony forming units (CFU) on MH plates.
Statistical analysis. To determine odds of invasion, data was analyzed using Proc Catmod in SAS (SAS User's Guide, 1985). The model fit used for each experiment was \( \ln \left( \frac{p}{1-p} \right) = \mu + t_i \), where \( \mu \) = overall mean, \( t_i \) = difference between overall mean and the mean in treatment \( i \) and \( p \) = the probability of internalized bacteria.

C. Results

Development of a suitable method for isolation of swine enterocytes.

Two cell isolation methods, mechanical vs. enzymatic, were compared. The mechanical method employed for isolation of enterocytes was a modification of the procedure described by Wilson and Hohmann (1974). Briefly, the small intestines were removed and immediately immersed in cold PBS. The intestinal lumen was rinsed with cold PBS, cut into small pieces (10-15 cm) and then each piece was everted onto a rod fastened to a motor drive. After the tissue was submerged into fresh PBS, epithelial cells were removed by vibration for 60 seconds, which was repeated three times. The suspension of dislodged cells were washed by centrifugation at 200 X g for 10 min. However, this procedure yielded low numbers of cells with less than 40% viability (data not shown).

In order to increase both the cellular yield and viability, another method was developed. Instead of mechanical vibration, a segment of small intestine (about 15 cm in length) was submerged in MEM and incubated at 4°C on a shaker. After 3 hrs, the segment was divided into three pieces.
Each piece was longitudinally opened and was treated either with 1 mM EDTA, 5 mM EDTA or with 0.25% trypsin and 1 mM EDTA solution for 15 min at 37°C. The use of EDTA at 1 mM concentration yielded low number of cells (Table 1). At a higher EDTA concentration (5 mM), more enterocytes were dislodged from the intestine, but viability of cells dramatically decreased (less than 15%). When 0.25% trypsin and 1 mM EDTA solution were used, more cells were recovered and the viability of cells was above 50% (Fig 1). Further studies indicated that it was not necessary to incubate the intestine at 4°C for a period of up to 3 hrs before enzymatic dislodging of cells. Therefore, for experimental studies, the small intestine was first cut into small pieces (10-15 cm), followed by rinsing of the lumen with cold sterile PBS. Then, after longitudinal cuts, the lumen was treated with 0.25% trypsin and 1 mM EDTA.

The enterocytes obtained by enzymatic treatment were washed thoroughly (3 X at 200 g for 10 min), followed by incubation in the presence of antibiotics for 45 min. The cells were washed, as previously described, and viability counts yielded approximately 1 X 10^7 cells from each piglet. For experimental assays, approximately 1 X 10^6 enterocytes, pooled from three piglets, were used.

**Invasion of swine intestinal cells by bacteria.** Two *C. jejuni* isolates were tested for their ability to invade swine intestinal cells. The experiments were performed on three separate occasions (Table 2) and, for each
experiment, enterocytes were pooled from three piglets. Prior to the assay, enterocytes were cultured for the presence of *Campylobacter spp.*, *Serpula hyodysenteriae*, *Salmonella spp.* and *E. coli*. Examination of enterocyte cultures on blood plates, prior to assay, revealed that the enterocytes were bacteria-free. Experiments were controlled by two different bacteria: *S. typhimurium*, which is an invasive organism and a non-invasive *E. coli* strain (HB 101).

*C. jejuni* strains and *Salmonella typhimurium* invaded the primary swine intestinal cells, whereas *E. coli* HB101 proved to be non-invasive in all the experiments. A non-invasive strain of *Campylobacter spp.* (*C. mucosalis*), was also used as a control and found not to invade the swine intestinal cells (data not shown). The odds of invasion varied among treatment groups and between experiments. This observation could be due to variation in inoculum size as well as variation in the host cells. Consistently, *C. jejuni* F1474 invaded swine enterocytes although in lower numbers (0.25%, 0.002% and 0.5%) than *Salmonella typhimurium* (0.75%, 0.03% and 2.2%). *C. jejuni* T13193, was not as invasive as the F1474 strain in the first two experiments. During the third experiment, and two additional experiments, for which data are not shown, *C. jejuni* T13193 invaded swine cells in significantly higher numbers. Interestingly the colony morphology of *C. jejuni* T13193 dramatically changed after invasion into swine cells. These colonies appeared highly mucoidy (Fig. 1).
Invasion of tissue culture cells by *C. jejuni* before and after swine intestinal cell passage. To examine the correlation between invasiveness and colony morphology, the invasion of tissue culture cells by *C. jejuni* T13193, before and after passage in swine cells, was compared. This organism was able to invade both HEp-2 (Table 3) cells and INT 407 cells (Table 4) in significantly (*P* < 0.05) higher numbers after it was passed through swine cells.
Table 1. Yield and viability of enterocytes after treatment of intestine with EDTA or Trypsin.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total numbers of viable cells/Total cells</th>
<th>% Viability</th>
</tr>
</thead>
<tbody>
<tr>
<td>1mM EDTA</td>
<td>3/14</td>
<td>21</td>
</tr>
<tr>
<td>5mM EDTA</td>
<td>6/48</td>
<td>12.5</td>
</tr>
<tr>
<td>0.25% Trypsin-1mM EDTA</td>
<td>25/47</td>
<td>53</td>
</tr>
</tbody>
</table>

*A section of small intestine was divided into three sections, cut open longitudinally and treated either with EDTA or Trypsin-EDTA.*

*Results are expressed from three experiments done in duplicate.*
<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Inoculum</th>
<th>Intracellular</th>
<th>Extracellular</th>
<th>Intracellular</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>(Trial)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>C. jejuni (F1474)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>(1.0 ± 0.00) X 10^7</td>
<td>(2.5 ± 0.71) X 10^4</td>
<td>(5.4 ± 0.00) X 10^6</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>(1.0 ± 0.00) X 10^8</td>
<td>(2.0 ± 0.00) X 10^3</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>(2.6 ± 0.00) X 10^9</td>
<td>(1.5 ± 0.71) X 10^6</td>
<td>(3.4 ± 0.91) X 10^6</td>
<td></td>
</tr>
<tr>
<td><strong>C. jejuni (T13193)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>(1.0 ± 0.00) X 10^6</td>
<td>(6.0 ± 0.00) X 10^3</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>(1.0 ± 0.00) X 10^8</td>
<td>(5.0 ± 7.00) X 10^1</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>(2.0 ± 0.00) X 10^6</td>
<td>(3.2 ± 4.0) X 10^6</td>
<td>(2.7 ± 0.88) X 10^6</td>
<td></td>
</tr>
<tr>
<td><strong>S. typhimurium</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>(2.0 ± 0.00) X 10^6</td>
<td>(1.5 ± 0.71) X 10^4</td>
<td>(1.4 ± 0.00) X 10^6</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>(4.0 ± 0.00) X 10^7</td>
<td>(1.3 ± 0.91) X 10^4</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>(2.0 ± 0.00) X 10^7</td>
<td>(4.5 ± 4.00) X 10^6</td>
<td>(1.0 ± 0.00) X 10^6</td>
<td></td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>(1.0 ± 0.00) X 10^7</td>
<td>(4.0 ± 0.00) X 10^1</td>
<td>(6.0 ± 0.00) X 10^4</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>(1.0 ± 0.00) X 10^6</td>
<td>(3.0 ± 4.20) X 10^1</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>(2.0 ± 0.00) X 10^6</td>
<td>0</td>
<td>(1.0 ± 0.00) X 10^6</td>
<td></td>
</tr>
</tbody>
</table>

¹Inoculation of approximately 1 X 10^6 swine epithelial cells with viable bacteria.

²Viable bacteria invading swine epithelial cells.

*Viable bacteria invading and adhering to swine epithelial cells.
Fig. 1. Colony morphology of *C. jejuni* T13193 before (a) and after (b) passage in swine intestinal cells.
Table 3. Invasiveness of *C. jejuni* (T13193) to HEp-2 cells after passage through swine intestinal cells.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Inoculum(^{+})</th>
<th>Intracellular(^{+})</th>
<th>Extracellular + Intracellular(^{+})</th>
</tr>
</thead>
<tbody>
<tr>
<td>T13193</td>
<td>(2.4 ± 1.7) X 10^8</td>
<td>(3.9 ± 4.0) X 10^3</td>
<td>(5.3 ± 0.2) X 10^4</td>
</tr>
<tr>
<td>T13193(^{#})</td>
<td>(3.7 ± 2.2) X 10^8</td>
<td>(2.2 ± 0.9) X 10^4(^{+})</td>
<td>(2.9 ± 2.1) X 10^6</td>
</tr>
</tbody>
</table>

\(^{\circ}\)Results ± standard deviation are expressed from three experiments done in duplicate.

\(^{+}\)Viable bacteria inoculated per well of a 24-well plate.

\(^{+}\)Viable bacteria invading cells per well of a 24-well plate.

\(^{+}\)Viable bacteria invading and adhering to cells per well of a 24-well plate.

\(^{\#}\)Invasiveness of *C. jejuni* strain T13193 tested after passage in swine intestinal cells

\(^{\circ}\)Significant increase in levels of infection relative to the control, *P* < 0.05.
Table 4. Invasiveness of *C. jejuni* (T13193) to INT 407 cells after passage through swine intestinal cells.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Inoculum(^*)</th>
<th>Intracellular(^+)</th>
<th>Extracellular ++</th>
</tr>
</thead>
<tbody>
<tr>
<td>T13193</td>
<td>(2.6 ± 2.2) X 10^8</td>
<td>(8.3 ± 7.9) X 10^3</td>
<td>(1.2 ± 0.8) X 10^6</td>
</tr>
<tr>
<td>T13193(^\circ)</td>
<td>(3.7 ± 2.2) X 10^8</td>
<td>(5.2 ± 2.1) X 10^4</td>
<td>(5.0 ± 4.0) X 10^6</td>
</tr>
</tbody>
</table>

\(^*\) Viable bacteria inoculated per well of a 24-well plate.

\(^+\) Viable bacteria invading cells per well of a 24-well plate.

\(^\circ\) Viable bacteria invading and adhering to cells per well of a 24-well plate.

\(^\circ\) Invasiveness of *C. jejuni* strain T13193 tested after passage in swine intestinal cells.

\(\circ\) Significant increase in levels of infection relative to the control, \(P < 0.05\).
D. DISCUSSION

Invasive characteristics of enteric pathogens have been extensively studied within the past few years. These studies have utilized a variety of tissue culture cell lines, which appear to influence the invasiveness of enteric bacteria. Finlay and Falkow (1988) have shown that strains of S. cholerae-suis, S. flexneri, and Y. enterocolitica invade Chinese Hamster Ovary cells (CHO) in higher numbers than HEp-2 or MDCK cell lines. It is not clear if these transformed cell lines are representative of typical intestinal mucosal epithelium. In fact, it is conceivable that transformed cell lines posses altered receptors that are absent from host intestinal epithelial cells. Therefore, the relevance of in vitro studies would be enhanced by using cells that more closely resemble the in vivo conditions.

Swine primary intestinal cells were used to study the invasive capabilities of C. jejuni. The intestinal cells were obtained from colostrum-deprived and relatively pathogen-free newborn piglets. After trypsinization, the cells were treated with gentamicin to inhibit growth of any pre-existing contaminant bacteria. To minimize destruction of the receptors on the surface of the cells, trypsinization was carried out only for a short period of time followed by extensive washing. Trypsinization, for short period of time, resulted in the removal of the epithelial crypts, therefore causing little damage to the individual cells. Cells were also extensively washed after treatment with gentamicin. Cell viability proved to be higher using this
method than with mechanical methods that have been suggested for the recovery of the cells (Wilson and Hohman, 1974; Isaacson et al., 1978).

The ability of *C. jejuni* F1474 to invade swine intestinal epithelial cells remained consistent in all experiments; however, the invasiveness was lower than that observed for the *S. typhimurium* control. On the other hand, the invasive capacity of *C. jejuni* T13193 changed. This strain was either non-invasive or invaded in very low numbers in the first two experiments. However, during the third experiment, and two subsequent experiments, for which data are not shown, *C. jejuni* T13193 invaded the swine cells in high numbers. Recovered colonies appeared as spreading and highly mucoid in texture, suggesting association of capsular type material with invasiveness of *C. jejuni*. Previous reports have associated the presence of a glycoprotein moiety with invasiveness of *C. jejuni* (Konkel and Joens, 1988 and Konkel et al., 1990).

The mucoid derivatives of *C. jejuni* T13193 also invaded tissue culture cells in higher numbers than the original isolate. The induction of invasion after passage through epithelial cells has also been reported in *Salmonella* spp (Finlay et al., 1989). It is postulated that some epithelial cells contain cell surfaces that induce expression of invasion-related proteins in *Salmonella*. These studies have shown that in order for *S. typhimurium* and *S. cholerasuis* to adhere and invade MDCK cells, they must synthesize RNA and proteins. However, proteins involved in the attachment and/or invasion
of *Salmonella* are not induced by CHO cells (Finlay *et al.*, 1989). Like *Salmonella*, invasion by *C. jejuni* requires not only host cell energy production but also viable bacterial cells (Konkel and Joens, 1989 and De Melo *et al.*, 1989). Therefore, it is possible that molecules involved in the invasion of intestinal epithelium were also induced in *C. jejuni* T13193 by the association with cultured swine epithelial cells.

In the present study a new *in vitro* model system, with swine intestinal cells, have been developed to study *C. jejuni* invasion. The data presented in this study supports previous *in vitro* findings related to *C. jejuni* invasiveness. Results also suggest role of a mucoid phenotype in the invasion process which is induced upon interaction with cultured swine intestinal cells.
IV. IN-VIVO MODEL SYSTEM OF CAMPYLOBACTERIOSIS

A. INTRODUCTION

The recognition of Campylobacter jejuni as one of the leading causes of human enteritis throughout the world (Mandel et al., 1984), was not possible until the advancement of proper culture techniques in the 1970's (Butzler et al., 1973 and Skirrow, 1977). Two decades of laboratory research on C. jejuni suggests that the virulence of the organism may be due to a variety of factors such as enterotoxins, cytotoxins and invasins. However, the biological significance of these virulence factors is unclear. The lack of progress, in clarifying the role of toxins and invasive properties of C. jejuni during the disease process, is partly due to the absence of a suitable animal model that would mimic human campylobacteriosis.

The standard animal model systems used with other enterics such as the Sereny test or ligated rabbit ileal loops have been found to be negative for C. jejuni (Manninen et al., 1982; Guerrant et al., 1978). Various other animals have been experimentally inoculated with C. jejuni in an attempt to reproduce the symptoms of Campylobacter enteritis (Fitzgeorge et al., 1981; Prescott et al., 1981; Field et al., 1981 and 1990; Fox et al., 1987; Ruiz-Palacios et al., 1981; Butzler and Skirrow, 1979; Manninen et al., 1982; Prescott et al., 1970; Bell and Manning, 1990; Kita et al. 1990 and Yrios and Balish, 1986). Some of these studies have investigated animal models that mimic complications of human Campylobacter infection, such as...
hepatitis or abortion (Kita et al., 1990 and Bell and Manning, 1990). Other studies have shown pathological changes to goblet cells and host death only after treating the host with iron-dextran or infecting them with *Cryptosporidium parvum* (Coker and Obi, 1991 and Vitovec et al., 1990). With the exception of few reports, the remainder of the studies have resulted either in transient colonization and fecal bacterial shedding without any symptoms or it has produced mild symptoms with little intestinal histopathological damage. An interesting study was done by Fox et al. (1987), who orally inoculated newborn ferrets with *C. jejuni* of either ferret origin or human origin. The authors reported presence of blood, mucus and leukocytes in feces of the infected ferrets. However gross and microscopic examinations revealed only mild lesions, which included small focal areas of neutrophil infiltration.

Only in a few reports, have investigators been able to reproduce the clinical symptoms of campylobacteriosis. These reports, have either used hosts that are not similar to humans (Stanfield et al., 1987) and are expensive to maintain (Prescott et al., 1981) or they have produced inconsistent results. A notable study was performed on gnotobiotic beagle puppies by Prescott et al. (1981). These workers orally inoculated the animals with *C. jejuni* of human and canine origin. The clinical manifestations of disease were milder in the gnotobiotically reared puppies than in humans. Whereas the disease in humans is accompanied with
abdominal pain, fever and presence of blood and mucus in stools, the dogs were bacteremic and developed mild diarrhea. Histopathologically, the dogs showed mild colitis consisting of neutrophil infiltration of the lamina propria, loss of goblet cells with hypertrophy of glands, and exfoliation of the surface epithelium. In a separate study, Ruiz-Palacios et al. (1981) have been able to produce clinical symptoms of Campylobacter infections in young chickens. However, attempts by other investigators have not produced the same results (Manninen et al., 1982; Butzler and Skirrow, 1979 and Prescott et al., 1970).

Despite experimentations with many varieties of animals, a suitable and inexpensive in vivo model system that would mimic human Campylobacter enteritis has not yet been identified. Partial success has only been obtained in germ-free and young animals (Prescott et al., 1981; Stanfield et al., 1987 and Fox et al., 1987) and, inconsistently, in chickens (Ruiz-Palacios et al., 1981). A suitable model system requires a host that is similar to humans. This would allow the study of immune mechanisms and therefore the development of potential vaccines in the model host. The cost associated with rearing and maintaining model systems is also a determinant factor in choosing a suitable model. The literature on Campylobacter infection implies that a third factor, host defense system, must be taken into consideration when searching for a suitable model system.

In the present study an attempt has been made to develop an in vivo
model system that would mimic human campylobacteriosis. Newborn, colostrum-deprived piglets have been used as hosts. The lack of competing intestinal flora and the absence of maternal antibodies as well as the similarities of swine to humans, makes the piglet, potentially, one of the preferred \textit{in vivo} model systems for human campylobacteriosis.

B. MATERIALS AND METHODS

\textbf{Inoculum.} \textit{C. jejuni} M129 was kindly provided by Dr. Kenneth Ryan from a patient with clinical signs of campylobacteriosis (University Medical Center, University of Az, Tucson, Az.). This isolate was kept frozen in liquid nitrogen in bovine blood. For experimental assays, a 24 hr culture of the strain (under nine passages) was used. The bacteria were grown microaerophilically at 37°C on MH plates containing 4% citrated bovine blood. \textit{E. coli} LE392 was grown aerobically on MH plates. Twenty-four hr cultures of the \textit{E. coli} strain were used to inoculate control piglets.

\textbf{Piglets.} Total of eleven newborn and colostrum deprived piglets (K \& K Pork, Chandler, Az) were used in the present study. These piglets were obtained on three separate occasions. Each time they were rinsed with betadine and were transported in sterile boxes to well-ventilated isolation units at the Campbell Ave. Farm in Tucson, Az. Experimental and control piglets were kept separate in different isolation units. Each piglet was fed three times a day with approximately 60 ml of similac (Ross Laboratories, Columbus, Ohio).
Animal inoculation. For experimental inoculation, 24 hr cultures of *C. jejuni* were harvested from plates with PBS. The suspensions were centrifuged at 6000 X g for 10 min at 4°C. The pellets were resuspended in similac and adjusted to yield between 1-7 X 10^8 viable organisms per ml. Prior to challenge, piglets were fasted for approximately 8 hrs. Three piglets per group were orally inoculated with approximately 40 ml of either *C. jejuni* M129 or *E. coli* LE392 (4-9 X 10^9 CFU).

Observations. All the piglets were observed daily for clinical signs (fecal consistency, presence of occult blood and mucus in stools, fever and general appearance) and the results recorded.

Isolation of bacteria. Fecal samples (before inoculation and daily after inoculation) and intestinal tissues (after necropsy) were cultured in three different kinds of media: 1) tergitol 7 agar (Difco, Detroit, MI) for the presence of *Salmonella* spp. and pathogenic *E. coli*, 2) trypticase soy agar (BBL, Cockeysville, MD)) containing 5% citrated bovine blood and spectinomycin (400 µg/ml) for the presence of *Serpula hyodysenteriae* and 3) on butzler media containing 4% citrated bovine blood for the presence of *Campylobacter* spp. Tergitol 7 agar was incubated aerobically at 37°C, trypticase soy agar plates anaerobically (50% hydrogen, 50% carbon dioxide) at 42°C and Butzler media microaerophilically (10% hydrogen, 10% carbon dioxide and 80% nitrogen) at 37°C.

Necropsy. Piglets were sacrificed on days 3, 4 and 6 with two ml of
Beuthanasia-D (Schering Corp., Kenilworth, NJ). Post-mortem observations of the small intestine, colon, caecum and other body organs were recorded. Portions of the small intestine, colon and cecum were fixed in 10% buffered-formalin. These fixed tissues were embedded in paraffin, sectioned (6 μm) and stained with hematoxylin and eosin or Warthin-Starry stains and observed by light microscopy.

**Electron microscopy.** Tissues were immersed in 3.5% glutaraldehyde overnight for fixation. After washing in 0.1 M cacodylate buffer, the tissues were postfixed with 1% osmium tetroxide for 90 min. After three washes in cacodylate buffer, the tissues were dehydrated in a graded series of ethanol (35-100%). For observation under scanning electron microscopy, a portion of the tissues were critical point dried in CO₂ followed by sputter-coating in palladium:gold (Modified Techniques Hummer I, Alexandria, VA). The tissues were examined with a DS-130 model scanning electron microscope from International Scientific Instruments (Milapitas, CA).

For observation via transmission electron microscopy, the remaining tissues were infiltrated and embedded in Epon followed by curing at 50, 75 and 100°C for 12, 12 and 1 hr respectively. The sections were then cut with a diamond knife, counterstained with Reynold’s lead citrate and uranyl acetate, mounted on copper grids and examined either with a Hitachi H500 (Scientific Instruments, Mountain View, CA) or Philips CM 12 (Mahwah, NJ) electron microscope.
C. Results

Daily observation. In three different trials, a total of eleven piglets were orally inoculated either with *C. jejuni* (7 piglets) or with *E. coli* LE392 (4 piglets). Each trial included both treatment groups. All the *C. jejuni* inoculated piglets showed clinical symptoms similar to those observed in humans infected with *C. jejuni*. These symptoms included presence of fever and diarrhea. Unlike the control piglets which had a transient watery diarrhea, the diarrhea in experimental piglets was characterized by the presence of visible or occult blood and mucus (Table 1).

Microbiology. Prior to inoculation, rectal swabs of all piglets were examined for the presence of *Salmonella* spp., *serpula hyodysenteriae* and *Campylobacter* spp. None of the above microorganisms were detected in the feces of piglets prior to inoculation. After inoculation, only the experimental piglets inoculated with *C. jejuni* were shedding the organism in their feces (Day 1-necropsy day). Neither *Salmonella* spp. nor *S. hyodysenteriae* were isolated from any of the piglets.

Macroscopic examination. Grossly, the small intestine from piglets inoculated with *C. jejuni* did not show any significant lesions, only in a few cases was there a presence of hyperemia or edema with occasional mucus. By contrast the large intestine from the piglets inoculated with *C. jejuni* contained gross lesions consisting of hyperemia with patches of petechial hemorrhage early in infection (Fig. 1). Later in infection, frank hemorrhage
was observed in some of the experimental piglets (Fig. 1). In other infected piglets, the large intestine was either hyperemic or edematous and mucus was present. Both the large and small intestine of control piglets appeared normal (Fig. 1). These results are summarized in Table 2.

**Microscopic examination.** With one exception, the large intestine proved to be the main site of injury for the piglets inoculated with *C. jejuni*. Damage to surface epithelial cells and the presence of intracellular bacteria were salient features of the lesions. Tips of villi showed increased eosinophilia of epithelial cells with exfoliation into the lumen. Erosion of the epithelial cells were observed in which the normally columnar cells were replaced by low cuboidal cells (Fig. 2). Damage to some cells was evident by the presence of pyknotic nuclear fragments in the epithelium. Mucosal and serosal blood vessels were congested and in some cases neutrophils were present in crypts and lamina propria.

The large intestinal tissue from the *C. jejuni* infected piglets was heavily colonized with bacteria. In addition to the lumen which was colonized with rod shaped bacteria, using immunoperoxidase staining techniques, intracellular bacteria were also detected (Fig. 3). The use of monoclonal anti-*C. jejuni* antibody, revealed diffuse cytoplasmic staining of the superficial mucosal epithelial cells.

The small intestinal tissues from five out of seven experimental piglets also showed some histopathological damage (data not shown). In some
cases the damage was characterized by erosion of epithelial cells with exfoliation into the lumen. In other cases, mucosal and serosal blood vessels were congested and neutrophils were present either in lamina propria, transmigrating between the cells, at tips of villi or in the lumen. Rod shaped bacteria were also detected either in the lumen or attached to the brush borders. By contrast, the control piglets had no significant lesions associated with their intestinal tissues (Fig. 2). These results are summarized in Table 3.

**Electron microscopic evaluation.** Using scanning electron microscopy, the large intestines from piglets inoculated with *C. jejuni* were compared to those from control piglets. The large intestine from *C. jejuni* infected piglets was characterized by the presence of heavy amounts of mucus (Fig. 4), red blood cells and spiral shape bacteria characteristic of *Campylobacter spp.* (Fig. 5). By contrast, tissue sections from the large intestine of control piglets did not reveal any of the above elements (Fig. 4).

The large intestine from experimental and control piglets were also compared using transmission electron microscopy. Whereas, cells from control piglets appeared normal with well preserved microvillous structures and organized goblet cells, experimental piglets had damaged cells with disrupted microvilli and an excessive amount of mucus (Fig. 6).

Bacteria that resembled *C. jejuni* M129 were present inside the epithelial cells of experimental piglets only. Although occasionally, they
were seen attached to the microvilli structures (Fig. 7), the majority were detected deep inside the intestinal tissue in the lamina propria (Fig. 8). The surface epithelial cells, in the infected area of the tissue, appeared damaged. This damage was characterized by the replacement of columnar cells with cuboidal cells in which pyknotic nuclear fragments were present (Fig. 9). Infected cells which contained intracellular bacteria had lost cellular integrity (Fig. 10), the endoplasmic reticulum were distorted and the swollen mitochondria lacked cristae. It was unclear as to whether bacteria were inside a vacuole or lying free in the cytoplasm. The majority of bacteria, however, were found in the lamina propria (Fig. 8). These bacteria were seen in close proximity with red blood cells, although not in a blood vessel. The lamina propria also was infiltrated with white blood cells (Fig. 8).
Table 1. Clinical symptoms of neonatal pigs infected with bacteria.

<table>
<thead>
<tr>
<th>Pig no.</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
<th>Day 6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D</td>
<td>B</td>
<td>M</td>
<td>D</td>
<td>B</td>
<td>M</td>
</tr>
</tbody>
</table>

A. *C. jejuni* infected pigs:

<table>
<thead>
<tr>
<th>Pig no.</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
<th>Day 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>necropsied</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>necropsied</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

B. *E. coli* infected pigs:

<table>
<thead>
<tr>
<th>Pig no.</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
<th>Day 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>necropsied</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>intestinal obstruction</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

'D indicates the presence of diarrhea in the infected pigs.

'B indicates the presence of blood in the stools of infected pigs.

'M indicates the presence of mucus in the stools of infected pigs.
<table>
<thead>
<tr>
<th>Bacteria pig no.</th>
<th>Significant lesions present in</th>
<th>Small intestine</th>
<th>Large intestine</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>C. jejuni</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>+ (R)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>+ (C)</td>
<td>+ (C,D)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>+ (N)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>+ (N)</td>
<td>+ (D,N,R)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>+ (D,N,R)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>+ (D,N,R)</td>
<td>+ (D,N,R)</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>+ (N,R)</td>
<td>+ (D,R)</td>
<td></td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>-(N)</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

*Congestion of mucosal and submucosal blood vessels.*
*Damage to intestinal surface epithelial cells.*
*Neutrophils present either in lumen or in lamina propria.*
*Small rod or comma shaped bacteria in crypts or in surface epithelial cells.*
Table 3: Microscopic examination of piglet intestinal tissue two or six days after oral challenge.

<table>
<thead>
<tr>
<th>Bacteria pig no.</th>
<th>Small intestine</th>
<th>Large intestine</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. jejuni</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>+ (R)</td>
</tr>
<tr>
<td>2</td>
<td>+ (C)</td>
<td>+ (C,D)</td>
</tr>
<tr>
<td>3</td>
<td>+ (N)</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>+ (N)</td>
<td>+ (D,N,R)</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>+ (D,N,R)</td>
</tr>
<tr>
<td>6</td>
<td>+ (D,N,R)</td>
<td>+ (D,N,R)</td>
</tr>
<tr>
<td>7</td>
<td>+ (N,R)</td>
<td>+ (D,R)</td>
</tr>
<tr>
<td>E. coli</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*Congestion of mucosal and submucosal blood vessels.
*Damage to intestinal surface epithelial cells.
*Neutrophils present either in lumen or in lamina propria.
*Small rod or comma shaped bacteria in crypts or in surface epithelial cells.
Fig. 1. Photograph of large intestine from neonatal pig. A) *C. jejuni* infected piglet: 1) early infection; 2) later infection. B) *E. coli* infected piglet.
Fig. 2. Light micrograph of large intestine from neonatal piglets (Haematoxyline and eosin). A) Colon from *C. jejuni* infected piglets showing damage to epithelial cells. B) Colon from *E. coli* infected piglets lacking damage to epithelial cells.
Fig. 3. Immunoperoxidase staining of large intestine from *C. jejuni* infected piglets showing cytoplasmic staining of the tissues with monoclonal anti-*C. jejuni* antibody.
Fig. 4. Scanning electron micrograph of large intestine from neonatal piglet.

A) Colon from the *C. jejuni* infected piglet is heavily covered with mucus.

B) The large intestine from *E. coli* infected piglet lacks mucus. Bar = 1 μm.
Fig. 5. Scanning electron micrograph of large intestine from neonatal piglet infected with *C. jejuni*. Note the presence of red blood cells (arrow heads) and spiral shape bacteria characteristic of *Campylobacter spp.* (arrows). Bar = 1 \( \mu m \).
Fig. 6. TEM micrographs of large intestine from neonatal piglets infected with A) *C. jejuni* or with B) *E. coli*. Note loss of microvilli structures and goblet cells from the intestinal tissues of *C. jejuni* infected piglets. Microvilli structures and goblet cells appear normal in the intestinal tissues of *E. coli* infected piglets. Bar = 1 μm.
Fig. 7. TEM micrographs shows association of bacteria with microvilli of the large intestine of newborn piglets infected with *C. jejuni*. Bar = 1 μm.
Fig. 8. TEM micrographs showing damaged tissue from the large intestine of *C. jejuni* infected piglet. A) Note the presence of internalized bacteria (arrow) in deeper layers of infected tissue. B) Represents intracellular bacteria (arrows) at higher magnification. Bar = 1 μm.
Fig. 9. TEM micrograph represents damaged epithelial cells from large intestine of neonatal piglets infected with *C. jejuni*. Bar = 1 μm.
Fig. 10. TEM micrograph of large intestine from neonatal piglet infected with *C. jejuni* showing the presence of internalized bacteria (arrow). The location of tight junctions are represented by arrow heads at higher magnification in panel B. Bar = 1 μm.
D. DISCUSSION

Piglets inoculated with an invasive strain of *C. jejuni* demonstrated both clinical and histopathological lesions which are similar to those observed in humans. All experimental piglets developed diarrhea that was characterized by the presence of mucus and blood, as noted in humans infected with *C. jejuni* (Duffy *et al.*, 1980; Mandal *et al.*, 1984). The results demonstrate that colostrum deprived newborn piglets are susceptible to oral inoculation with *C. jejuni*, and suggest this may be a suitable model to study human campylobacter infections.

Although, the small intestine has been reported as a site of infection in humans, rectal biopsies from significant proportions of patients have revealed damage to colonic tissue (Mandal *et al.*, 1984). This damage is characterized by abnormalities ranging from mucosal edema to hyperemia with or without petechial hemorrhage. In the present study, experimental oral infection also resulted in focal pathological damage in the piglets’ large intestine. Damage was characterized by the presence of edema, mucus and hyperemia. During the early stage of infection, large intestinal tissues of the experimental piglets demonstrated patches of petechial hemorrhage. Some experimental piglets, sacrificed at a later stage of infection, revealed large intestines that were characterized by the presence of frank hemorrhage.

Limited histopathological examination of human epithelial tissue infected with *C. jejuni* reveals, that in severe cases, damage to epithelial architecture
occurs along with mucus depletion and crypt abscess formation (Duffy et al., 1980). Similar histological findings, manifest as decreased intestinal mucus along with epithelial cell damage were noted in infected piglets. Although abscess formation was not the salient feature in this model, congestion of mucosal and serosal blood vessels along with the presence of intermittent neutrophil aggregations were occasionally observed in infected tissue sections.

*In vitro* tissue culture studies indicate that direct invasion of mucosal epithelium may be one of the pathogenic mechanisms employed by *C. jejuni* (Konkel and Joens, 1989; DeMelo et al., 1989). When compared with controls, histopathological examination of tissue sections from experimental piglets revealed the presence of intracellular bacteria that resembled *C. jejuni*. Immunofluorescence staining of these tissues with anti-*C. jejuni* antibody provided supportive evidence for the presence of *C. jejuni* within the intestinal cells.

High resolution electron microscopy revealed the presence of intracellular bacteria, morphologically similar to *C. jejuni*, only in intestinal cells of experimental piglets. When compared to controls, these intestinal cells appeared damaged which was characterized by the replacement of columnar cells with cuboidal cells containing pyknotic nuclear fragments. The majority of bacteria were found in deeper tissue layers in the lamina propria, and in close proximity to red blood cells.
The mechanism of entry of *C. jejuni* into the enterocytes could not be clearly defined in the present study. It was unclear as to whether bacteria were internalized through membrane-bound vacuoles or were lying free inside the cytoplasm. Occasionally, bacteria resembling *C. jejuni* were observed attached to microvilli structures of experimental piglets. In these piglets, disruption of microvilli was observed in the infected areas. This disruption has also been observed during internalization of other enteric organisms (Finlay and Falkow, 1987; Clerc and Sansonetti, 1987). Finlay and Falkow (1989) stated that such a disruption may be attributed to a rearrangement of actin filaments as seen during the *Salmonella*-induced phagocytosis process. *In vitro* tissue culture studies indicate that *C. jejuni* is internalized by a process which involves host cell microfilament function (Konkel and Joens, 1989; DeMelo et al., 1989). Possibly electron microscopy which employs a time table study may help in understanding the internalization process of *C. jejuni*. This study would be needed to rule out any chemical interactions between the mucosal epithelium and toxic products of *C. jejuni*.

In summary, the present study indicates that a colostrum-deprived, newborn piglet is a suitable model in studying the pathogenic mechanisms employed by *C. jejuni* during infection. The lack of protection from colostrum-derived anti-*C. jejuni* antibody and competing normal flora facilitates the establishing of an intestinal infection of the newborn piglet with virulent strains of *C. jejuni*. This model system has an additional
advantage which allows for the examination of the host immune response and the development of potential vaccines in treating severe cases of human campylobacteriosis.
Conclusion

Globally, campylobacteriosis has become one of the leading causes of food born bacterial enteritis (Butzler and Oosterom 1991). The highest incidence of infection have been observed in children under one year of age and in young adults (Blaser et al., 1983). However, the disease appears in all age groups and although usually a self limiting disease, it causes great discomfort to the host. Abdominal pain, fever and diarrhea are some of the clinical signs associated with the disease (Melby et al. 1990). Occasionally, complications in young children or in pregnant females may result in neonatal sepsis (Krishnaswamy et al., 1991) and abortion (Gribble et al., 1981). Understanding the pathogenic mechanisms of C. jejuni will be of significant value in control and prevention of disease.

A portion of this research attempted to identify some of the virulence factors associated with C. jejuni. Competitive inhibition studies indicated that ionic interactions play an important role during attachment of the organism to tissue culture cells. Invasion, which proceeds the attachment process, appears to involve distinct outer membrane antigens. One of these invasins was recognized by the 1B4 monoclonal antibody prepared in our laboratory. Enzymatic treatment studies suggest that the invasin antigen required for HEp-2 cell invasion by C. jejuni is most likely a glycoprotein moiety.
Much of the information on campylobacteriosis has come from \textit{in vitro} studies. Consequently, the relevance of these studies to the actual disease process remains unclear. Recognizing this need prompted us to look for a suitable \textit{in vivo} model. A primary swine cell line was developed to study the invasiveness of \textit{C. jejuni}. The success with this primary cell line led to the use of swine as a host and oral inoculation of them with virulent strains of \textit{C. jejuni}. The use of colostrum-deprived newborn-piglets, which are deficient in maternal antibodies or competing flora, proved useful in producing disease symptoms similar to those observed in infected humans. An additional advantage is that the model allows for the study of host immune response to \textit{C. jejuni} infection, which may lead to the development of a suitable campylobacteriosis vaccine.
References


23. Fauchere, J.L., M. Veron, A. Leilouch-Tubiana and A. Pfister. 1985. Experimental infection of gnotobiotic mice with *Campylobacter jejuni*: colonization of intestine and spread to lymphoid and reticuloendothelial


Pathogenesis. 4:115-126.


1980.


45:377-383.


