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Adhesion to and Invasion of HEp-2 Cells by *Campylobacter* spp.

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Twenty-one isolates were tested for their ability to adhere to and invade HEp-2 cells in vitro. Of the 21 organisms tested, 2 did not invade the HEp-2 cells, and 1 of these did not adhere to the epithelial cells. *Campylobacter jejuni* clinical isolates were more invasive than the nonclinical strains that were tested. When HEp-2 cells were treated with cytochalasin B, the invasiveness of *C. jejuni* was reduced, indicating active participation of the host cell in the uptake of these organisms. The number of intracellular *C. jejuni* isolates decreased when *Campylobacter* whole-cell lysates were adsorbed onto HEp-2 cell monolayers. Experiments were also conducted to identify the functional sites of the antigens responsible for expression of *Campylobacter* invasion. Oxidation of lysates with sodium *meta*-periodate significantly affected its inhibitory capacity. This implies that the *Campylobacter* invasive ligand appears to be dependent upon an intact carbohydrate moiety.

Members of the genus *Campylobacter* are gram-negative, microaerophilic, curved to spiral rods that have a single polar flagellum. They are recognized as causative agents of infectious disease in humans and animals throughout the world. *Campylobacter jejuni* is the third most common cause of diarrhea in developing countries and the third most common cause of enteritis in children residing in these same regions (4, 6, 10, 14). Knowledge of the mechanism(s) of *Campylobacter* pathogenesis is unclear. This lack of knowledge has confounded the prevention and treatment of campylobacteriosis. Potential virulence factors that may play a role in pathogenesis include enterotoxin production, cytotoxin production, and invasion.

At present, the least understood aspect of *Campylobacter* virulence is the interaction of these organisms with intestinal cells. *C. jejuni* has not been found to be invasive by the Sereny-Anton test, a standard reference test for bacterial invasion (28). However, both clinical and experimental evidence suggests that invasion plays a role in the disease process. Blood and leukocytes have frequently been observed in the feces of patients diagnosed or infected with *C. jejuni* (3, 11). Direct tissue invasion is further supported by in vivo models (13, 17, 35). Intracellular organisms have been observed by immunofluorescence and electron microscopy in infected chickens (35, 36).

Cell culture has been used to examine the invasiveness of many bacteria, including *Escherichia coli* (25), *Salmonella* spp. (31), *Yersinia* spp. (26), and *Aeromonas* spp. (8,27). Bacterial infection of cell cultures is a useful tool, since a uniform population of cells can be infected under defined conditions. These assays are simple and reproducible and allow for the quantitation of both adherent and internalized bacteria. Additionally, selective modification of either the bacterial inoculum or the host cell can be done.

Ascertainment of the mechanism by which campylobacters invade the intestinal epithelium is of primary importance in understanding the pathogenesis of the resulting disease and has provided the rationale for initiating these studies. Data are presented that show participation of the host cell in the infectious process and the nature of the invasive ligand.

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MATERIALS AND METHODS

Bacterial strains. Four *C. jejuni* strains (29428, 33560, 35919, and 35925), one *Campylobacter coli* strain, and one *Campylobacter fetus* strain were acquired from the American Type Culture Collection (ATCC; Rockville, Md.). Clinical isolates of *C. jejuni* (M95, M96, M97, M98, M125, M126, M128, M129, and M131) were kindly supplied by Kenneth Ryan (University Medical Center, University of Arizona, Tucson, Ariz.). These strains were isolated from patients with clinical signs of campylobacteriosis. *C. jejuni* SJ was obtained from Pat Flynt (St. Joseph's Hospital, Tucson, Ariz.). The experimental isolates *C. jejuni* SLA, *C. coli* FBM, and *Campylobacter hyointestinalis* Flan were isolated from pigs with clinical signs of bloody diarrhea, histological lesions of ileitis, and enterocytes with intracellular *Campylobacter* spp. *Salmonella typhimurium* was obtained from Harvey Olander (University of California at Davis). This strain was isolated from the gal bladder of an animal with clinical signs of diarrhea and vomiting. This organism was used as a positive control for the invasiveness of HEp-2 cells.

Cultivation of bacteria. Upon receipt in our laboratory, strains were passed only once or twice on Mueller-Hinton (MH) agar plates containing 4% citrated bovine before they were frozen at −70°C on bovine blood for use as stock cultures. *Campylobacter* isolates were cultured on MH plates and passed every 2 days. With the exception of *C. jejuni* SJ, all strains were incubated at 37°C. *C. jejuni* SJ was incubated at 42°C. Additionally, all *Campylobacter* strains, with the exception of *Campylobacter mucosalis*, were incubated under microaerophilic conditions (10.2% hydrogen, 10.2% carbon dioxide, and nitrogen balance). *C. mucosalis* was incubated under anaerobic conditions (50% carbon dioxide and 50% hydrogen). The purity of bacterial strains was monitored by phase-contrast microscopy and colony formation on culture plates.

Epithelial cells. HEp-2 cells (ATCC CCL 23) was maintained in Eagle minimal essential media (MEM) supplemented with 10% fetal bovine serum without the use of antibiotics. Cells were grown routinely in 75-cm^2 flasks at 37°C in a 5% CO_2, humidified atmosphere. Confluent stock cultures were trypsinized, and new stock cultures were seeded with 10^5 cells/ml. For all experimental assays, 24-well tissue culture trays (Falcon; Becton Dickinson Lab-

ware, Oxnard, Calif.) were seeded with 5.0×10^4 HEp-2 cells per ml. Plates were incubated for 18 h at 37°C in a humidified 5% CO_2 incubator. Prior to the assays, the semiconfluent monolayers were washed and incubated with MEM containing 1% fetal bovine serum.

Adherence and invasion assay. Bacteria were harvested from plates with phosphate-buffered saline (PBS) and pelleted by centrifugation at 6,000 × *g* for 10 min at 4°C. The pellets were suspended in MEM with 1% fetal bovine serum to approximately 10^8 bacteria per ml, and 0.5 ml of this suspension was inoculated into duplicate wells containing semiconfluent monolayers of HEp-2 cells. The titers of the bacterial suspensions were determined retrospectively on MH agar plates. Infected monolayers were incubated for 3 h at 37°C under a 5% CO_2 atmosphere to allow the bacteria to adhere to the cells. The monolayers were then washed five times with 1 ml of MEM with 1% fetal bovine serum and reincubated for 3 h under the same conditions to allow bacterial invasion of the cells. Medium containing 250 μg of gentamicin (GIBCO Laboratories, Grand Island, N.Y.) per ml was added to one of the wells for the enumeration of intracellular bacteria. In preliminary experiments, 250 μg of gentamicin per ml killed all *Campylobacter* isolates after a 3-h exposure. To the other well, medium without any antibiotic was added to enumerate the number of intracellular and extracellular bacteria. Following incubation, monolayers were washed three times with PBS and lysed with 0.5% sodium desoxycholate (Difco Laboratories, Detroit, Mich.) (12). The suspensions were diluted, and viable bacteria were determined by counting the CFU on MH agar plates. Results are expressed as the average number of bacteria adhering to and invading HEp-2 cells for three determinations.

Antiserum preparation. Bacterial antiserum was produced in New Zealand White rabbits. Approximately 10^9 formalized bacteria in PBS were mixed with Freund complete adjuvant (1:1), and 0.5 ml was inoculated into the rear footpad. At 14 days, a 1-ml bacterial suspension in Freund complete adjuvant (1:1) was split and inoculated intramuscularly and subcutaneously. Rabbits were exsanguinated at 28 days. Serum samples were prepared and frozen at −20°C.

Fluorescent-antibody test. Immunofluorescence techniques were used initially to determine the presence of intracellular and extracellular bacteria (23). This test is based on the fact that immunoglobulins do not cross the intact plasma membrane but diffuse freely into permeabilized cells (16). After infected monolayers were incubated with the bacterial suspensions for 3 h, duplicate wells were fixed with formaldehyde. To enumerate intracellular bacteria, one well was extracted with cold acetone. Cell monolayers were washed four times with PBS and inoculated with a 1:100 dilution of fluorescein isothiocyanate-labeled goat anti-rabbit immunoglobulin G (heavy and light chains) (Kirkegaard and Perry Laboratories, Inc.). Cell cultures were rinsed four times with PBS and mounted in glycerol-PBS (1:1) under a glass cover slip. Cells were observed under an epifluorescence microscope.

Treatment of HEp-2 cells with cytochalasin B. Cytochalasin B (Sigma Chemical Co., St. Louis, Mo.) was prepared as a 1-mg/ml stock solution in dimethyl sulfoxide (Sigma). This stock was diluted in MEM at concentrations ranging from 1 to 16 μg/ml and added to equal volumes of MEM containing approximately 10^8 CFU of *C. jejuni* 33560. One milliliter of the bacterial inoculum was suspended in grade concentrations of cytochalasin B and applied to each well containing a semiconfluent monolayer of HEp-2 cells. Plates were incubated for the standard 3-h infection period, and the number of intracellular and extracellular bacteria was determined as described above.

Whole-cell lysates. *C. jejuni* 33560 was harvested from MH plates with PBS and pelleted by centrifugation at 6,000 × *g* for 10 min at 4°C. The pellet was suspended in PBS, and the bacteria were disrupted by five passages through a French pressure cell (American Instrument Co., Inc.) at 16,000 lb/in^2. The preparation was then centrifuged at 6,000 × *g* for 30 min at 4°C to remove whole cells. The supernatant was harvested and dispensed into 1 ml fractions. These fractions were stored frozen at −70°C.

Chemical treatment of bacteria and lysates. All enzymes and chemicals used to pretreat *C. jejuni* 33560 and lysates were obtained from Sigma. *C. jejuni* was incubated with various amounts of trypsin (0.5 to 2.5 mg/ml) or proteinase K (10 to 100 μg/ml) for 30 min at 37°C (8, 9, 32). Pretreated bacteria were washed twice in PBS.

Lysates were treated with 10 mM sodium *meta*-periodate in 0.1 M sodium acetate buffer at pH 4.5 for 15 min at room temperature in the dark (19, 32). Free aldehyde groups were blocked with 1% glycine (37). Once treated with glycine, the sample was dialyzed extensively in PBS. Lysates were also treated with 100 μg of proteinase K per ml in

TABLE 1. Invasion of HEp-2 cells by *Campylobacter* spp.

Strain	No. of bacteria/well		
	Inoculum[a]	Intracellular[b]	Intracellular + extracellular[c]
S. typhimurium	$(7.8 \pm 2.8) \times 10^7$	$(6.8 \pm 3.0) \times 10^5$	$(5.1 \pm 1.9) \times 10^6$
C. jejuni SLA	$(9.3 \pm 1.2) \times 10^7$	$(2.7 \pm 2.1) \times 10^1$	$(1.2 \pm 1.0) \times 10^5$
C. jejuni 29428	$(1.4 \pm 1.2) \times 10^7$	0	$(5.1 \pm 4.9) \times 10^3$
C. jejuni 33560	$(8.9 \pm 6.7) \times 10^7$	$(2.7 \pm 1.7) \times 10^3$	$(1.5 \pm 0.8) \times 10^4$
C. jejuni 35919	$(1.2 \pm 1.1) \times 10^8$	$(1.1 \pm 0.9) \times 10^2$	$(6.8 \pm 5.6) \times 10^4$
C. jejuni 35925	$(7.2 \pm 6.9) \times 10^7$	$(1.4 \pm 1.3) \times 10^2$	$(2.2 \pm 0.9) \times 10^4$
C. coli ATCC	$(4.3 \pm 0.6) \times 10^7$	$(5.1 \pm 4.4) \times 10^2$	$(5.7 \pm 4.7) \times 10^5$
C. coli FBM	$(1.1 \pm 0.7) \times 10^8$	3.3 ± 5.8	$(2.2 \pm 1.2) \times 10^2$
C. hyointestinalis ATCC	$(1.4 \pm 1.0) \times 10^8$	0	$(1.4 \pm 1.3) \times 10^2$
C. hyointestinalis Flan	$(8.8 \pm 5.4) \times 10^7$	6.6 ± 11.5	$(4.3 \pm 4.9) \times 10^1$
C. fetus ATCC	$(1.7 \pm 0.3) \times 10^8$	6.6 ± 5.8	$(7.6 \pm 5.8) \times 10^2$
C. mucosalis	$(5.0 \pm 1.3) \times 10^7$	0	0

[a] Viable bacteria inoculated per well of a 24-well plate.
[b] Viable bacteria invading cells per well of a 24-well plate.
[c] Viable bacteria invading and adhering to cells per well of a 24-well plate.

TABLE 2. Invasion of HEp-2 cells by *C. jejuni* clinical isolates

C. jejuni strain	No. of bacteria/well		
	Inoculum[a]	Intracellular[b]	Intracellular + extracellular[c]
SJ	$(2.2 \pm 1.2) \times 10^7$	$(2.5 \pm 0.6) \times 10^2$	$(3.2 \pm 3.0) \times 10^3$
M95	$(1.3 \pm 1.2) \times 10^8$	$(5.7 \pm 5.4) \times 10^3$	$(1.3 \pm 1.0) \times 10^5$
M96	$(5.5 \pm 1.3) \times 10^7$	$(2.4 \pm 1.8) \times 10^4$	$(4.9 \pm 2.1) \times 10^5$
M97	$(9.0 \pm 3.1) \times 10^7$	$(3.3 \pm 3.0) \times 10^3$	$(3.4 \pm 2.5) \times 10^4$
M98	$(4.5 \pm 1.0) \times 10^7$	$(7.9 \pm 0.2) \times 10^3$	$(1.8 \pm 0.5) \times 10^4$
M125	$(1.5 \pm 0.4) \times 10^8$	$(2.9 \pm 1.6) \times 10^5$	$(3.7 \pm 3.4) \times 10^5$
M126	$(9.7 \pm 6.5) \times 10^7$	$(1.9 \pm 1.3) \times 10^4$	$(1.1 \pm 1.0) \times 10^5$
M128	$(1.0 \pm 0.6) \times 10^8$	$(7.3 \pm 1.3) \times 10^3$	$(3.4 \pm 1.4) \times 10^4$
M129	$(6.0 \pm 0.5) \times 10^7$	$(6.3 \pm 2.7) \times 10^4$	$(1.1 \pm 0.9) \times 10^5$
M131	$(3.3 \pm 2.1) \times 10^7$	$(2.3 \pm 1.4) \times 10^2$	$(5.2 \pm 4.3) \times 10^3$

[a] Viable bacteria inoculated per well of a 24-well plate.
[b] Viable bacteria invading cells per well of a 24-well plate.
[c] Viable bacteria invading and adhering to cells per well of a 24-well plate.

0.1 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer for 30 min at 37°C (34). A stock of phenylmethylsulfonyl fluoride was made in 100% 2-propanol. Phenylmethylsulfonyl fluoride was added to the sample at a 10 to 1 molar ratio of inhibitor to enzyme (20). After a 5-min incubation, the sample was dialyzed extensively in PBS. Sodium *meta*-periodate- and proteinase K-treated lysates were concentrated 10-fold by passage through an ultrafiltration membrane (YM-10; Amicon Corp., Danvers, Mass.). Additionally, lysates were heat treated at both 60 and 100°C in a water bath for 30 min. Samples were immediately placed in an ice bath after heat treatment (15).

Pretreated lysates were used in competitive inhibition studies in the following manner. Lysates (100 to 250 μg/ml) were adsorbed to HEp-2 cells for 1 h at 37°C in a humidified atmosphere. Monolayers were then washed with MEM, inoculated with a 1-ml suspension containing an equal volume of bacteria (10^8) and lysates (100 and 250 μg of protein per ml), and incubated for the standard 3-h infection period.

Gel electrophoresis. Protein concentrations were determined by the BCA assay (Pierce Laboratories). Samples containing 40.0 μg of total protein were heated for 4 min at 100°C in 40% distilled H_2O–20% glycerol–20% sodium dodecyl sulfate (10% [wt/vol]; Sigma)–2.5% 2-mercaptoethanol (Fisher Scientific Co., Pittsburgh, Pa.)–2.5% bromophenol blue (0.05% [wt/vol]; Fisher) in a final volume of 50 μl. Polyacrylamide gel electrophoresis was performed with 4% stacking and 10 to 20% exponential gradient separating gels. The acrylamide (Bio-Rad Laboratories, Richmond, Calif.) was cross-linked with 0.8% *N,N'*-methylenebisacrylamide (Bio-Rad). Slabs were electrophoresed at a constant current of 35 mA per slab at 12°C. Gels were fixed overnight with 8% acetic acid in 50% methanol. They were visualized by staining them with Coomassie brilliant blue dye (LKB Instruments, Inc., Rockville, Md.).

Statistical analysis. Standard deviations were calculated by the Student *t* test. Significance between sample and control groups was calculated by transforming the data with the following equation: $\log(1 + x)$. Values were then subjected to analysis of variance. *P* values exceeding 0.1 were considered not significant.

RESULTS

The ability of *C. jejuni* 33560 to adhere to and invade HEp-2 tissue culture cells was initially studied by using indirect immunofluorescence techniques. Intracellular and extracellular *C. jejuni* were easily identified. However, this method was inconvenient for screening a large number of isolates because of the time spent trying to count internalized bacteria. Thus, an assay was developed in which the number of intracellular and extracellular bacteria could be determined with ease and greater accuracy.

HEp-2 adherence and invasion assay. Eleven *Campylobacter* strains were tested for their ability to invade HEp-2 cells (Table 1). Four of five *C. jejuni* strains, two *C. coli* strains, one *C. hyointestinalis* strain, and one *C. fetus* strain were invasive for HEp-2 cells. However, only three of the four invasive *C. jejuni* strains and one *C. coli* strain (ATCC) were considered to be highly invasive, having at least 10^2 intracellular bacteria. The ability of *C. jejuni* clinical isolates to adhere to and invade HEp-2 cells was also addressed (Table 2). In general, the clinical isolates were found to be more invasive than ATCC strains and experimental *Campylobacter* strains. Each *C. jejuni* clinical isolate was considered to be highly invasive. *C. jejuni* M125 was the most invasive *Campylobacter* strain tested.

The total number of HEp-2 cell-associated bacteria (intracellular and extracellular) for each *Campylobacter* strain examined is reported in Tables 1 and 2. A significant variation in bacterial attachment to HEp-2 cells was observed. This occurred despite the source of the strain. *C. mucosalis* was the only isolate tested that did not adhere to the HEp-2 cells.

Experiments were done to determine whether a lack of adherence or invasiveness of isolates was related to a failure of bacteria to become associated with HEp-2 cells (Table 3).

TABLE 3. Invasiveness of *Campylobacter* spp. following centrifugation onto HEp-2 cell monolayers

Strain[a]	No. of bacteria/well		
	Inoculum[b]	Intracellular[c]	Intracellular + extracellular[d]
C. jejuni 29428[d]	$(1.4 \pm 0.4) \times 10^7$	$(4.2 \pm 0.7) \times 10^2$	$(2.8 \pm 0.8) \times 10^5$
C. coli FBM[d]	$(1.0 \pm 0.7) \times 10^8$	$(2.3 \pm 0.7) \times 10^2$	$(7.0 \pm 4.0) \times 10^4$
C. hyointestinalis ATCC[d]	$(1.0 \pm 0.2) \times 10^8$	0	$(1.0 \pm 0.4) \times 10^2$
C. hyointestinalis Flan[d]	$(2.1 \pm 0.9) \times 10^8$	$(8.0 \pm 4.0) \times 10^1$	$(3.0 \pm 2.0) \times 10^4$
C. fetus ATCC[d]	$(3.0 \pm 0.5) \times 10^8$	$(2.0 \pm 1.7) \times 10^2$	$(2.0 \pm 1.2) \times 10^4$
C. mucosalis	$(1.1 \pm 0.6) \times 10^8$	0	0

[a] Bacteria were centrifuged at 600 × *g* for 30 min onto the cells prior to incubation.
[b] Viable bacteria inoculated per well of a 24-well plate.
[c] Viable bacteria invading cells per well of a 24-well plate.
[d] Viable bacteria invading and adhering to cells per well of a 24-well plate.

TABLE 4. Effects of cytochalasin B on adhesion to and invasion of HEp-2 cells by *Campylobacter* spp.

Concn (μg/ml)	No. of bacteria/well		
	Inoculum[a]	Intracellular[b]	Intracellular + extracellular[c]
0.5	$(1.0 \pm 0.8) \times 10^8$	$(3.6 \pm 2.7) \times 10^3$	$(1.3 \pm 0.3) \times 10^5$
1.0	$(1.0 \pm 0.8) \times 10^8$	$(2.4 \pm 1.4) \times 10^3$	$(1.4 \pm 0.7) \times 10^5$
2.0	$(1.0 \pm 0.8) \times 10^8$	$(1.9 \pm 1.4) \times 10^3$	$(2.5 \pm 2.1) \times 10^5$
4.0	$(1.0 \pm 0.8) \times 10^8$	$(1.1 \pm 0.9) \times 10^3$	$(3.1 \pm 3.0) \times 10^5$[d]
8.0	$(1.0 \pm 0.8) \times 10^8$	$(9.8 \pm 6.7) \times 10^2$[d]	$(4.3 \pm 3.1) \times 10^5$[d]
Control	$(1.0 \pm 0.8) \times 10^8$	$(4.4 \pm 3.8) \times 10^3$	$(8.0 \pm 5.1) \times 10^4$

[a] Viable bacteria inoculated per well of a 24-well plate.
[b] Viable bacteria invading cells per well of a 24-well plate.
[c] Viable bacteria invading and adhering to cells per well of a 24-well plate.
[d] Significant at $P < 0.1$.

Isolates in which less than 10^1 internalized bacteria were previously found were centrifuged onto the HEp-2 cells following infection and prior to the first incubation (12). The invasiveness of *C. coli* FBM and *C. fetus* ATCC increased by more than 1 log unit. *C. jejuni* 29428, which was initially determined to be noninvasive, was found to be invasive. *C. hyointestinalis* ATCC and *C. mucosalis* did not invade HEp-2 cells, and *C. mucosalis* did not adhere to the cells, even though it was centrifuged onto the HEp-2 monolayer surface.

Effect of cytochalasin B on infection of HEp-2 cells. Cytochalasin B has been shown to inhibit particle engulfment by professional and nonprofessional phagocytic cells (1). Cytochalasin B inhibited the invasion of *C. jejuni* 33560 at concentrations of 1 μg/ml or greater (Table 4). In addition, the total number of cell-associated and extracellular adherent bacteria increased as the concentration of cytochalasin B increased.

Blockage of invasion. An attempt was made to block the invasion of *C. jejuni* and *S. typhimurium*. Monolayers were incubated with *C. jejuni* whole-cell lysates (100 to 250 μg of protein per ml) 1 h prior to and simultaneously with bacterial infection. The invasiveness of *C. jejuni* was significantly inhibited in comparison with that of the control in competitive inhibition assays with lysate (Table 5). *C. jejuni* lysates had no effect on the invasiveness of *S. typhimurium* for HEp-2 cells (Table 5).

Pretreatment of bacteria and lysates. To determine what type of *Campylobacter* surface antigens might be involved in the adherence and invasion of *C. jejuni* to HEp-2 cells, bacteria were pretreated with trypsin, proteinase K, and sodium *meta*-periodate prior to infection of HEp-2 monolayers. Pretreatment of bacteria with various concentrations of trypsin and proteinase K had no effect on the ability of the bacteria to adhere to or invade HEp-2 cells (data not shown). Sodium *meta*-periodate was toxic to the bacteria.

TABLE 5. Affect of *Campylobacter* lysates on the invasion of HEp-2 cells by *C. jejuni* and *S. typhimurium*

Treatment lysates	No. of bacteria/well		
	Inoculum[a]	Intracellular[b]	Intracellular + extracellular[c]
100 μg/ml[d]	$(8.0 \pm 0.3) \times 10^7$	$(9.0 \pm 1.0) \times 10^2$[e]	$(4.1 \pm 2.3) \times 10^4$
250 μg/ml[d]	$(8.0 \pm 0.3) \times 10^7$	$(2.0 \pm 0.5) \times 10^2$[e]	$(3.0 \pm 1.2) \times 10^4$
Control[d]	$(8.0 \pm 0.3) \times 10^7$	$(1.6 \pm 0.4) \times 10^3$	$(5.3 \pm 0.7) \times 10^4$
100 μg/ml[f]	$(1.1 \pm 0.3) \times 10^8$	$(2.0 \pm 0.8) \times 10^5$	$(5.0 \pm 1.2) \times 10^6$
250 μg/ml[f]	$(1.1 \pm 0.3) \times 10^8$	$(2.5 \pm 2.0) \times 10^5$	$(6.0 \pm 1.6) \times 10^6$
Control[f]	$(1.1 \pm 0.3) \times 10^8$	$(2.0 \pm 1.4) \times 10^5$	$(5.0 \pm 3.0) \times 10^6$

[a] Viable bacteria inoculated per well of a 24-well plate.
[b] Viable bacteria invading cells per well of a 24-well plate.
[c] Viable bacteria invading and adhering to cells per well of a 24-well plate.
[d] HEp-2 cells inoculated with *C. jejuni* 33560.
[e] Significant at $P < 0.1$.
[f] HEp-2 cells inoculated with *S. typhimurium*.

An attempt was then made to determine the functional sites of the antigens involved in adherence and invasiveness by treating *C. jejuni* lysates with proteinase K or sodium *meta*-periodate or by denaturation with heat at both 60 and 100°C for 30 min. Table 6 shows the competitive inhibition of *C. jejuni* with treated and untreated *Campylobacter* lysates and the noncompetitive (no lysate) control. The activity of proteinase K and sodium *meta*-periodate was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 1). Pretreatment of lysate with proteinase K or heat had no effect on the reduction of invasion of HEp-2 cells by *C. jejuni*. However, subjection of the lysate production to periodate oxidation significantly affected its inhibitory capacity.

DISCUSSION

In this study, we showed that *Campylobacter* strains adhere to and invade HEp-2 cells in culture. The assay was relatively simple, reproducible, and quantitative. Intracellular bacteria were measured by including gentamicin in the medium during the second incubation. The internalized bacteria were protected since the antibiotic could not penetrate HEp-2 cells (27). Occasionally, rounding of HEp-2 cells was observed during the 6-h infection period. Infection of HEp-2 cells with *C. jejuni* and *C. coli* strains has been reported to cause cytopathic effects as cell rounding, loss of adherence, and death after 24 to 48 h of incubation (24, 38).

Adherence of microbial pathogens to mucosal surfaces is a primary step in the pathogenesis of many intestinal infections (2). In most instances, adherence appears to be a prerequisite for invasion. Expression of both of these activities can be due to the presence of a single structure on the bacterial cell surface. Isberg et al. (18) have reported that a protein expressed on the surface of *Yersinia pseudotuberculosis* allows bacteria to bind to cell receptors. Organisms that do not express this protein do not bind to or invade cells. Results from our study indicate that the adherence of *Campylobacter* spp. to HEp-2 cells is necessary for invasion but that different antigens are responsible for the attachment to and invasion of HEp-2 cells by *Campylobacter* spp. *C. hyointestinalis* ATCC did not adhere to cells but was not found to be invasive. Furthermore, the number of adherent bacteria did not correlate with the number of intracellular bacteria. Four organisms, three *C. jejuni* strains (SLA, 35919, and 35925) and one *C. coli* strain (ATCC), adhered to HEp-2 cells at a greater extent than *C. jejuni* 33560 did. However, *C. jejuni* 33560 was more invasive than those four strains. Thus, at least two different antigens appear to be involved: one that is responsible for adhesion and another that is responsible for invasion.

The ability of *Campylobacter* isolates to invade HEp-2 cells varied considerably. *C. hyointestinalis* ATCC and *C. mucosalis* did not invade HEp-2 cells. *C. jejuni* clinical isolates, in general, were more invasive than the nonclinical strains that were tested. Eight clinical isolates (M95, M96, M97, M98, M125, M126, M128, and M129) were more invasive than *C. jejuni* 33560. Possibly, the host and environment for which an organism was isolated plays a role in its ability to invade epithelial cells in vitro. Newell et al. (33)

TABLE 6. Effect of pretreatment of lysates on its inhibitory capacity

Condition	No. of bacteria/well		
	Inoculum[a]	Intracellular[b]	Intracellular + extracellular[c]
60°C, 30 min	$(1.0 \pm 0.6) \times 10^8$	$(3.2 \pm 1.0) \times 10^3$	$(7.0 \pm 1.5) \times 10^4$
100°C, 30 min	$(1.0 \pm 0.6) \times 10^8$	$(1.6 \pm 0.5) \times 10^{3d}$	$(3.1 \pm 0.2) \times 10^4$
Proteinase K[e]	$(1.0 \pm 0.6) \times 10^8$	$(1.2 \pm 0.2) \times 10^{3d}$	$(5.0 \pm 2.1) \times 10^4$
Sodium *meta*-periodate[f]	$(1.0 \pm 0.6) \times 10^8$	$(7.0 \pm 2.4) \times 10^3$	$(2.8 \pm 1.4) \times 10^4$
250 μg/ml (no pretreatment)[g]	$(1.0 \pm 0.6) \times 10^8$	$(1.9 \pm 0.0) \times 10^{3d}$	$(3.0 \pm 1.0) \times 10^4$
Control[h]	$(1.0 \pm 0.6) \times 10^8$	$(7.8 \pm 1.4) \times 10^3$	$(3.0 \pm 1.0) \times 10^4$

[a] Viable bacteria inoculated per well of a 24-well plate.
[b] Viable bacteria invading cells per well of a 24-well plate.
[c] Viable bacteria invading and adhering to cells per well of a 24-well plate.
[d] Significant at $P < 0.1$.
[e] Lysate treated with 100 μg of proteinase K per ml for 30 min at 37°C (pH 7.5).
[f] Lysate treated with 10 mM sodium *meta*-periodate for 10 min at room temperature in the dark (pH 4.5). One percent glycine was added after treatment.
[g] Competitive inhibition of *C. jejuni* 33560 invasion with lysate.
[h] *C. jejuni* 33560 invasion (control) of HEp-2 cells.

have reported that *C. jejuni* and *C. coli* environmental isolates were less invasive compared with clinical isolates of *C. jejuni* and *C. coli*. We are currently studying whether the subculturing of bacteria for long periods of time has an affect on their invasiveness.

Cytochalasin B has been shown to inhibit the uptake of *Salmonella typhimurium* (22) and *Shigella flexneri* (16). It appears to act by disrupting subplasmalemma microfilaments, which play a role in the translocation of the plasma membrane during phagocytosis (1). Cytochalasin B was used in this study to determine whether the host cell is actively involved in the uptake of *C. jejuni*. Cytochalasin B affected both the number of internalized bacteria and the total number of cell-associated bacteria. These effects were dose dependent; as the concentration of cytochalasin B increased, the number of internalized bacteria decreased and the total number of cell-associated bacteria increased. Cytochalasin B-treated cells have a lower mean negative electrophoretic mobility than nontreated cells do (29). Kihlstrom and Nilsson (23) have reported an increase in cell-associated bacteria with *Salmonella typhimurium* infection of cytochalasin B-treated cells; they proposed that this was due to a reduction in electrostatic repulsion forces between the bacteria and host cells. Our results showed that cytochalasin B inhibits the uptake of *C. jejuni* by HEp-2 cells. This finding suggests that the uptake of these bacteria requires active participation of the host cell and proceeds by a process similar to phagocytosis.

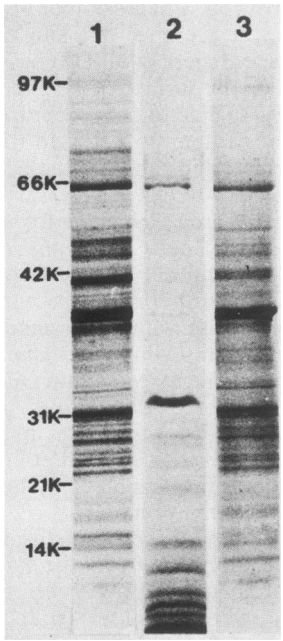

FIG. 1. Coomassie-stained sodium dodecyl sulfate-polyacrylamide gels of untreated and treated lysates of *C. jejuni*. Lanes: 1, Untreated lysate; 2, proteinase K-treated lysate; 3, sodium *meta*-periodate-treated lysate. K indicates molecular weight (in thousands).

C. jejuni lysates adsorbed to HEp-2 cells inhibited bacterial invasion. However, HEp-2 cell-adsorbed bacterial lysates had very little effect on the number of cell-associated bacteria. McSweegan and Walker (30) have reported that the adhesion of *Campylobacter* isolates to INT 407 cells is reversible. They proposed that irreversible binding of bacteria to the cells requires multiple points of attachment. Possibly, the adsorption of lysates to HEp-2 cells prevents the bacteria from establishing multiple bonds by saturation of cell receptor sites or steric hindrances. In our experiments, the bacteria might have detached from the cell receptor(s) prior to internalization. We considered the possibility that lysates inhibited bacterial invasion in a manner analogous to that of cytochalasin B, in that host cell participation was inhibited. This was examined by staining HEp-2 cells with trypan blue after incubation with lysates. Lysates were not toxic to HEp-2 cells, as determined by trypan blue viability staining. Additionally, preadsorption of lysates to HEp-2 cells had no effect on the invasiveness of *S. typhimurium*. Thus, the invasiveness of *Campylobacter* spp. which was demonstrated here in vitro, appears to be a result of specific physical-chemical interactions between ligands.

An attempt to determine the nature of the antigen(s) involved in adherence and invasiveness was then made by two methods. First, bacteria were pretreated with trypsin, proteinase K, and sodium *meta*-periodate. Neither trypsin nor proteinase K significantly affected the ability of the organism to adhere to or invade HEp-2 cells. Sodium *meta*-periodate was toxic to the bacteria. Second, lysates were subjected to various treatments. Lysates were treated at 60°C for 30 min to determine whether heat-labile protein antigens, such as flagella, were involved. A slight increase in *C. jejuni* adherence and invasion was found versus that in the untreated lysate sample. This finding is supported by the

work of McSweegan and Walker (30), who reported an increase in *C. coli* adherence to epithelial cells by using organisms which were treated with KCN to immobilize the flagella. The increase in adherence and invasion may be due to changes in electrostatic repulsion forces. Bacterial motility does appear to be important in facilitating the contact of the organisms with cells. The invasiveness of *C. jejuni* 29428, *C. coli* FBM, and *C. fetus* ATCC increased by more than 1 log unit in experiments that were used to determine whether a lack of invasiveness is related to a failure of the bacteria to become associated with HEp-2 cells. Jones et al. (21) have reported similar findings with *S. typhimurium* infection of HeLa cells in culture. The heating of lysates at 100°C for 30 min did not have an effect on the inhibition of invasion. This was done to test whether trace cytotoxin produced by *Campylobacter* spp. is heat labile at 100°C for 30 min but stable at 60°C for 30 min (38). Finally, proteinase K-treated lysates should have ruled out the possibility that a thermostable peptide is responsible. Regarding the nature of the receptor(s) involved, our results suggest that the antigen(s) responsible for invasiveness is dependent upon an intact carbohydrate moiety. Mild periodate oxidation at acidic pH has been shown to cleave carbohydrate vicinal hydroxyl groups without altering polypeptide chain structures (5). Subjection of lysates to periodate oxidation significantly affected its inhibitory capacity.

Naess et al. (32) have found that pretreatment of *C. coli* with trypsin, pronase, or periodate or by boiling reduced the adhesion of bacteria by 45%. However, the investigators were not able to show a reduction in adhesion by bacterial extracts made with heat, pronase, trypsin, and phenol-water-extracted lipopolysaccharide (LPS). Competitive inhibition results were not reported for extracts prepared with sodium *meta*-periodate. In contrast, McSweegan and Walker (30) have found that 250 to 1,000 μg of phenol-water-prepared LPS per ml completely abolishes the adherence of *C. jejuni* to INT 407 cells. However, they did not look at the viability of cells after they were subjected to such high levels of LPS. Furthermore, the method they used to determine the number of bacteria that adhered to monolayers determined the total number of cell-associated bacteria (intracellular and extracellular), not just the number of bacteria that adhered to the cells. Such high levels of LPS may have been toxic to the cultured cells, and the reduction of adherence may have been a reflection of fewer internalized bacteria. Toxic levels of LPS would inhibit bacterial internalization by inhibiting the cellular processes that are involved in phagocytosis. Izhar et al. (19) did show that the adherence of *Shigella flexneri* to intestinal cells was reduced by 50% with the addition of 10 μg of LPS per ml. In addition, subjection of *Shigella* LPS to periodate oxidation significantly affected its inhibitory capacity. Our results suggest that the antigen(s) involved in *Campylobacter* internalization is glycoprotein or carbohydrate in nature. This would mean that *Campylobacter* spp. possess one mechanism similar to that of *Shigella flexneri* for gaining entrance into host cells.

The data presented here suggest that infection of HEp-2 cell monolayers with *Campylobacter* spp. involves active participation of the host cell. We conclude that virulent *Campylobacter* spp. possess at least two distinct antigens, one that initiates interaction of the bacteria with host cells and one that initiates phagocytosis. In contrast to many bacteria which produce a proteinaceous surface antigen which promotes bacterial attachment and cell invasion (7, 18), the nature of the *Campylobacter* invasive ligand appears to be dependent upon an intact carbohydrate moiety. Our inability to reduce adhesion is reflective of the complexity of the interaction of the organisms with intestinal cells and suggests that it may be multifactorial. Considerable work is still needed to determine the nature of the antigen(s) responsible for the adhesion of *Campylobacter* spp. to cells, as well as to identify the invasive ligand.

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