INFECTION AND IMMUNITY, Sept. 1994, p. 3773–3779
0019-9567/94/$04.00+0

Vol. 62, No. 9

Cell Association and Invasion of Caco-2 Cells by *Campylobacter jejuni*

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Received 30 March 1994/Returned for modification 19 May 1994/Accepted 14 June 1994

Adherence and invasion studies were conducted in monolayers of Caco-2 cells. Three-day-old monolayers were inoculated with *Campylobacter jejuni* 81-176 at a bacterium/cell ratio of 1,000:1. Saturation studies demonstrated time- and dose-dependent saturation curves for *C. jejuni* cell association and invasion into Caco-2 cells. Electron microscopy revealed intracellular *C. jejuni* located within membrane-bound vacuoles. Cell association and invasion were inhibited by 0.3 and 0.5 M concentrations of various sugars, including D-glucose, D-mannose, and D-fucose. However, there was no inhibition with the corresponding L-sugars, indicating physiological specificity. The inhibition of cell association with phloridzin was less pronounced. There was no inhibition of bacterial entry with monodansylcadaverine or g-strophanthin, indicating that it was unlikely that coated-pit formation is important in the invasion of *C. jejuni* into Caco-2 cells. Furthermore, there was no inhibition with cytochalasin D, vincristine, or vinblastine. Inhibition of cell association was demonstrated at 4°C. Significantly decreased cell association and invasion were seen in potassium-depleted cells. Treatment of cells with bromelain also caused reduction in the number of *C. jejuni* binding to cells. A nonmotile aflagellate variant of *C. jejuni* also showed reduced invasion. The results of this study are consistent with energy-dependent invasion mechanisms. The results do not support an endocytic method of invasion for *C. jejuni* into Caco-2 cells.

Campylobacter jejuni is a common cause of diarrheal disease in humans (5), monkeys (35), and dogs (33). The organism causes colitis. The colon damage is characterized by necrosis of absorptive epithelial cells, erosion of the mucosa, crypt abscesses, and infiltration of predominately neutrophilic inflammatory cells in the mucosa (35). Some human patients have bloody diarrhea (5). Our recent studies in infant rhesus monkeys showed that the primary mechanism of colitis by strain 81-176 is associated with invasion of *C. jejuni* into the absorptive epithelial cells in the ascending and descending colon (36). The objective of this study was to further investigate the mechanisms of invasion by using an adherence and invasion assay in tissue culture cells. The assays were conducted with Caco-2 cells, a human colonic carcinoma cell line (34). We report the time course of cell association and the effects of inhibitors on the cell association and invasion of *C. jejuni* into Caco-2 cells.

MATERIALS AND METHODS

Bacteria. *C. jejuni* 81-176 was isolated from a milk-borne outbreak of diarrheal illness (24). This strain caused diarrhea in human volunteers (4) and infant monkeys (35). Stock cultures in 15% tryptic soy broth were stored at −70°C. All subcultures of *C. jejuni* were conducted under microaerophilic conditions at 37°C. An aflagellate variant of strain 81-176 was isolated during subculture in the laboratory. This variant was recognized by a small colony size (1 to 2 mm) compared with the large spreading colonies of the flagellated organism cultured on Columbia agar or *Brucella* anaerobic agar medium (Remel, Lenexa, Kans.). Electron microscopy showed that the variant lacked flagella. *Salmonella typhimurium* ATCC 14028 and *Citrobacter freundii* BR3447 were used as positive controls for cell association and invasion experiments in Caco-2 cells. The *C. freundii* isolate was selected from a patient with an indwelling catheter and was kindly provided by J. W. Warren (Division of Infectious Disease, Department of Medicine, University of Maryland at Baltimore).

Radioactive labeling of bacteria. Bacteria were subcultured from frozen stocks onto Columbia agar containing 5% sheep blood and then subcultured overnight in *Brucella* agar-broth biphasic medium with shaking at 37°C. For radiolabeling bacteria, an inoculum of 10^5 CFU of strain 81-176 per ml and 10 μCi of [^{3}H]acetic acid (specific activity, 75 to 150 mCi/mmol; New England Nuclear Corp.) per ml was added to 4 ml of the broth component of a biphasic culture. The bacteria were harvested by centrifugation of the broth and washed three times in Hanks–*N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES) buffer (Hanks balanced salts solution supplemented with 25 mM HEPES [Sigma Chemical Co., St. Louis, Mo.]).

Cell association and invasion assays. Human colonic carcinoma (Caco-2) cells were obtained from the American Type Culture Collection, Rockville, Md. Cells were grown in Dulbecco's minimum essential medium (Sigma) supplemented with 10% fetal calf serum, 200 mM L-glutamine, and penicillin (10,000 U/liter)-streptomycin (10 mg/liter) antibiotic solution. The cells were plated at a density of 2×10^5 cells per well into 24-well tissue culture plates (Corning Medical and Scientific, Corning, N.Y.) and grown for 72 h to confluency.

For assays of bacterial cell association with Caco-2 cells, confluent monolayers were washed twice with Hanks-HEPES buffer. The bacteria were diluted in buffer to a concentration of 3×10^9 bacteria per ml, determined by spectrophotometric analysis using turbidity measurements in the absorbance mode at a wavelength of 660 nm. In preliminary experiments, titration and culture of serial dilutions showed that an absor-

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bance of 0.860 corresponded to 3×10^9 CFU/ml. Caco-2 cell monolayers were inoculated with 100 μl of radioactively labeled bacteria (3×10^8 CFU) in a total volume of 300 μl Hanks-HEPES buffer per well and incubated at 37°C under aerobic conditions. After the incubation periods of specified duration, the supernatants were discarded and the wells were washed three times with Hanks-HEPES buffer. Radioactivity was measured by dissolving the washed cell monolayers in 0.5% sodium dodecyl sulfate for 30 min at 37°C. Total cell association of *C. jejuni* was quantified by counts per minute determined in a Beckman model L-9000 liquid scintillation counter.

The intracellular bacteria were measured in a gentamicin killing assay (18). After incubation of radiolabeled or unlabeled *C. jejuni* 81-176 (or *S. typhimurium*) with confluent monolayers of Caco-2 cells for periods of 0.5 to 2.5 h, the supernatants were discarded, the cells were washed twice with Hanks-HEPES buffer, and new buffer containing gentamicin (100 μg/ml; TechAmerica, Kansas City, Mo.) was added. After incubation for 30 min, the cells were washed three times and solubilized, and radioactivity was measured as described above. Control cells were handled similarly without antibiotic treatment. Percent invasion was calculated by dividing the counts per minute of the gentamicin-resistant bacteria by the counts per minute of the total associated bacteria and multiplying by 100. For viable counts of nonradioactively labeled intracellular bacteria, the Caco-2 cells were washed vigorously three times after the gentamicin treatment and then treated with 0.1% Triton X-100 for 20 min. Serial 10-fold dilutions of the cell suspension were prepared and inoculated onto Columbia agar plates. The number of intracellular CFU of bacteria per cell was calculated from the counts-per-minute data based upon the bacterium/counts per minute ratio obtained from counts per minute of standard samples with known bacterial CFU.

Inhibition assays were conducted by adding sugars dissolved in Hanks-HEPES buffer 5 min prior to the addition of the radioactively labeled bacteria. For all studies, inhibition was expressed as the percent decrease of control values. D- or L-glucose, D- or L-mannose, D-galactose, or D- or L-fucose (Sigma) was added to final concentrations of 0.3 and 0.5 M. Maltose was added to final concentrations of 0.2 and 0.3 M. Phloridzin (Sigma) was added to the cells at final concentrations of 0.5, 1, and 5 mM, monodansylcadaverine (Sigma) was added at final concentrations of 0.5 and 0.25 mM, cytochalasins B and D (Sigma) were used at 1 and 10 μg/ml during 30-min pretreatment of cell monolayers, vincristine and vinblastine (Sigma) were both tested at 5 μM, and g-strophanthin (Sigma) was assayed at 0.25, 0.5, and 1 mM. Potassium (K^+) depletion experiments were carried out by pretreating the washed Caco-2 cell monolayers with K^+-depleted Hanks-HEPES buffer for 30 min at 37°C. The inoculum was prepared in K^+-depleted Hanks-HEPES buffer and incubated with Caco-2 cells for the 2.5-h incubation period. Bromelain (Sigma) and trypsin (Sigma) proteases were used at a final concentration of 0.003% to pretreat cell monolayers for 5 min, and then the cells were washed three times with Hanks-HEPES buffer before addition of the inoculum. Bacteria were treated with chloramphenicol (30 μg/ml; Sigma) or rifampin (32 μg/ml; Sigma) for 30 min before addition, washed three times, and then inoculated onto Caco-2 cells for cell association and invasion assays. We also performed experiments in which bacteria were pretreated with chloramphenicol for 30 min prior to the assay and throughout the 2.5-h duration of the experiments. The MIC of chloramphenicol for *C. jejuni* in this study was 2.5 μg.

The cell viability after 3 h of incubation with each inhibitor and protease was evaluated by the trypan blue exclusion assay. Trypan blue is excluded from viable cells (40). Monolayers were stained with 0.4% trypan blue solution for 5 min.

Light and electron microscopy. For morphological studies of *C. jejuni* invasion, nonradioactive bacteria were incubated with Caco-2 cells as described above. After 2.5 h of incubation with *C. jejuni* 81-176, the cells for ultrastructural examination were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer overnight, pelleted, then counterstained with uranyl acetate, and examined by transmission electron microscopy in a JEOL microscope at 60 kV. Transmission electron microscopy was also used to confirm an aflagellate variant of *C. jejuni* 81-176 used in these studies.

Monolayers of Caco-2 cells in Lab-Tek chamber slides (Nunc, InterMed Corp., Naperville, Ill.) were washed three times after 2.5 h of incubation with nonradioactive *C. jejuni* 81-176. Cells were treated with gentamicin and incubated for 30 min. After the antibiotic-treated and control cells were washed three times, the monolayers were fixed with methanol and stained with Giemsa stain. They were examined by light microscopy, using computer enhancement to visualize extracellular and intracellular bacteria.

Statistical analysis. The radioactive counts following tests with inhibitors and the comparison of the number of intracellular bacteria by viable counts with that obtained by radioactive counts were analyzed by the paired Student's *t* test. The data reported are the average values (± standard deviations) of three individual assays. Each datum point was analyzed in triplicate within an assay, and the triplicates were averaged to obtain the assay value for the datum point. The datum point of each inhibitor tested was compared with that for the control (*C. jejuni* 81-176), which was included in each experiment.

RESULTS

The time-dependent kinetics of *C. jejuni* 81-176 cell association in 3-day-old monolayers is shown in Fig. 1. The cell association of *C. jejuni* with Caco-2 cells showed a hyperbolic saturation curve over a 2.5-h period. Quantitation of the number of viable bacteria at the completion of the assays showed no reduction in the number of *C. jejuni* in the supernatant fluid after 2.5 h of incubation compared with the inoculum. For preliminary experiments, the time course in 3-day-old cultures was similar to that in 11-day-old Caco-2 cell monolayers, and electron microscopy showed microvilli on 3-day-old monolayers of Caco-2 cells, similar to results for 11-day-old cultures (data not shown). *S. typhimurium* and *C. jejuni* showed similar time-dependent kinetics of binding to 3-day-old Caco-2 cells (data not shown). Incorporation of the [^{3}H]acetic acid radioactive label was substantially lower in *S. typhimurium* than in *C. jejuni*. There was twofold-higher cell association of *S. typhimurium* than of *C. jejuni* (Table 1).

The dose-dependent cell association of [^{3}H]acetate-labeled *C. jejuni* 81-176 in 3-day-old monolayers is summarized in Fig. 1. The saturation curve showed that maximum cell association was seen with doses of 1.5×10^8 to 1.5×10^9 CFU of *C. jejuni*. Given the results of time and dose-dependent experiments, all subsequent studies were conducted in 3-day-old monolayers with an inoculum of 3×10^8 CFU of radioactively labeled *C. jejuni* 81-176 and a duration of 2.5 h. This dose of inoculum is quantitated easily by spectrophotometric measurement.

Invasion assays (by gentamicin killing of extracellular *C. jejuni*) showed that approximately 77% of the total cell-associated *C. jejuni* and 50% of the total cell-associated *S. typhimurium* became internalized within 2 h of bacterial inoculation (Table 1). Light microscopy examination of Caco-2

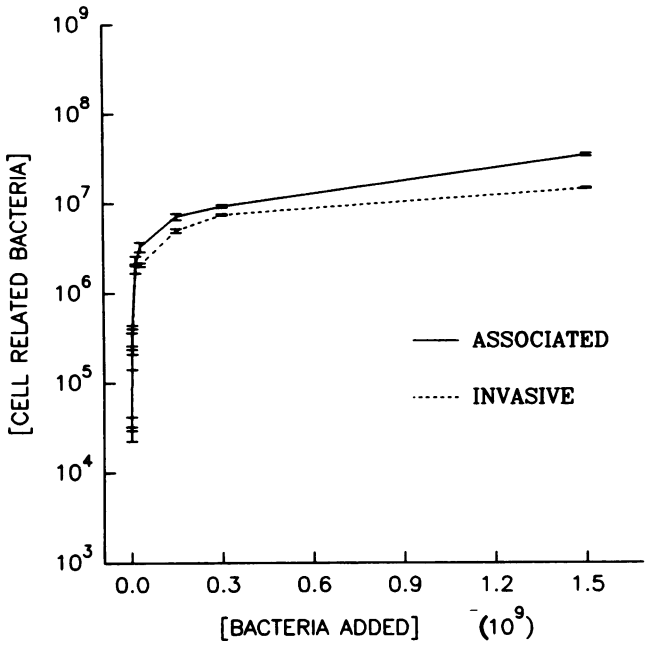

FIG. 1. Time course study showing the total cell-associated (——) and intracellular (– –) [^{3}H]acetate-radiolabeled *C. jejuni* in 3-day-old Caco-2 cells at various time intervals between 0.5 and 2.5 h postinoculation.

cells stained with Giemsa stain and electron microscopy confirmed extracellular bacteria in close association with the plasma membrane of Caco-2 cells and also showed intracellular *C. jejuni* within Caco-2 cells (Fig. 2). After gentamicin treatment, no extracellular *C. jejuni* was located adherent to or between Caco-2 cells by light or electron microscopic examination (data not shown). The validity of using radioactive counts to measure invasion of *C. jejuni* in gentamicin killing assays was also confirmed by direct quantitative counts. The number of intracellular bacteria measured by viable counts (11.4 ± 5.1 CFU per cell) was similar to the number of bacteria measured by radioactivity counts (19.9 ± 4.2 CFU per cell; P = 0.27). These comparisons validate the radioactivity assay for quantitating bacteria under the various experimental conditions used in this study.

Cell association assays conducted at 4°C and with an aflagellate variant of *C. jejuni* 81-176 showed significantly (P = 0.001 and 0.001, respectively) lower numbers of *C. jejuni* binding to Caco-2 cells (Fig. 3). Centrifugation of monolayers for 10 min at 500 or 1,000 × *g* after addition of the bacterial inoculum of aflagellate *C. jejuni* 81-176 caused significant increases in the numbers of cell-associated bacteria (increases of 42% [P = 0.005] and 83% [P = 0.003] cpm of cell-associated bacteria compared with monolayers that were not centrifuged). Centrifugation did not significantly increase the number of flagellated bacteria that were cell associated and invasive (P = 0.090 and 0.175 for the respective centrifugations). There was a significant increase in the number of intracellular aflagellate *C. jejuni* after centrifugation at 500 × *g* (19% increase in invasive bacteria; P = 0.043) and 1,000 × *g* (43% increase in invasive bacteria; P = 0.004) compared with uncentrifuged bacteria. However, the percentage of invasive aflagellate bacteria was significantly less (P = 0.002) than the percentage of invasive flagellated bacteria (59% versus 79%).

Assays were conducted to study the effect of inhibitors on cell association and invasion of *C. jejuni* 81-176 in Caco-2 cells. The cell association of *C. jejuni* 81-176 was significantly reduced in a dose-dependent manner by monosaccharides, including D-galactose (P = 0.006), D-mannose (P = 0.008), D-maltose (P = 0.004), and D-glucose (P = 0.001). The results are shown in Table 2. Inhibition of binding by D-fucose (Table 2) and other saccharides, including trehalose, fructose, and sucrose (data not shown), was not dose dependent. Sugars with L configurations, including L-fucose, L-glucose, and L-mannose did not cause a reduction in the number of cell-associated bacteria. Glucosamine and galactosamine were also tested at 0.3 and 0.5 M for inhibition of *C. jejuni* cell association with Caco-2 cells. No results were obtained because of the cytotoxicity observed with these sugars at the concentrations used. No other sugars exhibited this cytotoxicity, as determined by the trypan blue exclusion assay. The osmolarities of all of the sugar solutions were similar within the two molarities tested (930 mOsM for 0.3 M and 1,550 mOsM for 0.5 M).

Gentamicin killing assays to measure intracellular bacteria were conducted in experiments using 0.3 and 0.5 M D-glucose, D-mannose, and D-maltose inhibitors. Each of these sugars (0.5 M) caused a significant decrease (P = 0.001, 0.024, and 0.009, respectively) in the percentage of invasive organisms compared with untreated control cells (Table 2).

The cell association of *S. typhimurium* in the presence of 0.3 M mannose was similar to that for untreated controls (36.7 ± 1.9 and 40.0 ± 4.9 bacteria per cell, respectively; P = 0.136). However, there was a 25% inhibition (P = 0.007) of invasion of *S. typhimurium* in the presence of 0.3 M mannose (16.6 ± 0.5 bacteria per cell in treated cells and 22.1 ± 1.4 bacteria per cell in untreated control cells).

Competitive inhibition assays with phloridzin (1 and 5 mM) showed that *C. jejuni* cell association was decreased only at the higher concentration (P = 0.038; Table 3). At this dose (5 mM), there was a corresponding reduction of *C. jejuni* invasion (P = 0.045; Table 3). There was no inhibition of invasion at lower concentrations (0.5 and 1 mM) of phloridzin. There was inhibition of cell association by 1 mM g-strophanthin (P = 0.006; Table 3). Significant inhibition of invasion was not observed with concentrations of g-strophanthin below 1 mM (Table 3). The concentrations of g-strophanthin (1 mM) which caused reduced cell association and invasion also caused abnormal morphology of Caco-2 cells. The surface contour was

TABLE 1. Incorporation of [^{3}H]acetate in radioactively labeled bacteria and total numbers of cell-associated and invasive *C. jejuni* compared with *S. typhimurium* at 0.5 and 2.0 h postinoculation

Bacterium	[^{3}H]acetate uptake by radiolabeled bacteria[a] (no. of bacteria/cpm)	Total no. of Caco-2 cell-associated bacteria[a]		% of intracellular bacteria	
		0.5 h	2.0 h	0.5 h	2.0 h
C. jejuni	13,484 ± 11,358	12.2 ± 2.4	25.9 ± 5.4	37.2	77.2
S. typhimurium	213,334 ± 92,265	25.2 ± 2.5	60.2 ± 17.7	41.5	51.7

[a] Mean ± standard deviation of at least three experiments.

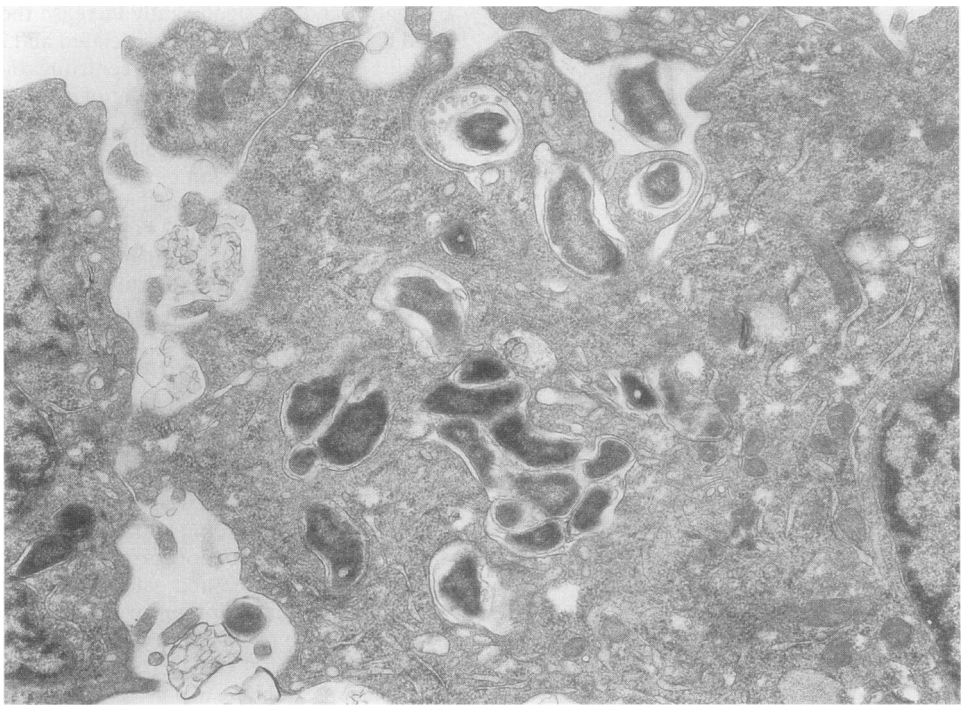

FIG. 2. Electron microscopy showing cell association and intracellular bacteria (magnification, ×18,000) after 2.5 h of incubation of *C. jejuni* 81-176 with Caco-2 cells.

irregular, and the cells exhibited increased spreading, indicating alterations in the cell membranes. The decreased cell association and invasion of *C. jejuni* at 1 mM g-strophanthin may have been nonspecific. Monodansylcadaverine, cytochalasin B, and cytochalasin D did not affect cell association or invasion (Table 3). Vincristine and vinblastine did not reduce the cell association or invasion of *C. jejuni* (Table 3).

Monodansylcadaverine (0.25 and 0.50 mM), g-strophanthin (1 mM), and vinblastine (5 μM) did not cause inhibition of cell association or invasion of *C. freundii*. This organism was used as a positive control because assays in INT-407 cells showed reduced invasiveness by these inhibitors (38).

Assays in potassium-depleted cells showed significantly impaired cell association ($P = 0.002$) and invasion ($P = 0.015$). Treatment of Caco-2 cells with bromelain (0.003%) caused a 37.0 ± 13.5% reduction ($P = 0.030$) in the numbers of bacteria binding to cells, whereas trypsin treatment (0.003%) did not reduce (<1.0% inhibition) the numbers of cell-associated bacteria. Trypan blue exclusion assays showed that there was no cytotoxic cell damage by any of the inhibitors or protease

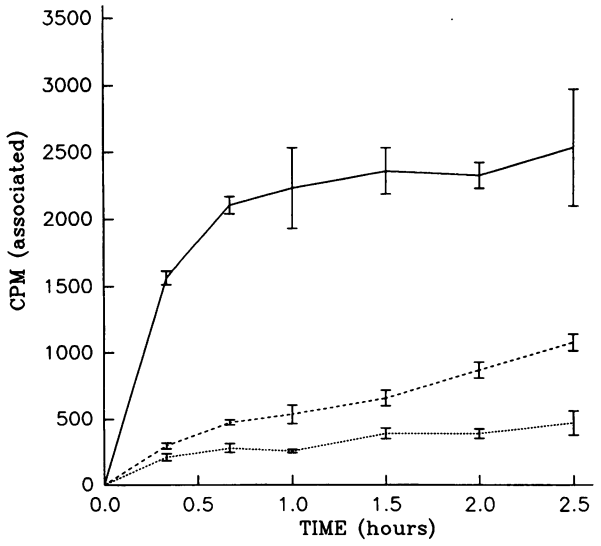

FIG. 3. Reduction in the number of cell-associated *C. jejuni* 81-176 at 4°C (. . .) and lack of binding by an aflagellate variant of *C. jejuni* 81-176 (– –), compared with the binding of control strain 81-176 (——) at 37°C.

TABLE 2. Inhibition of cell association and invasion of *C. jejuni* 81-176 with Caco-2 cells by mono- and disaccharides

Sugar	% Inhibition by[a]:	
	0.3 M	0.5 M
Cell association		
L-Glucose	<1.0	<1.0
D-Glucose	19.9 ± 4.9	25.9 ± 6.3
L-Mannose	<1.0	<1.0
D-Mannose	30.0 ± 1.4	34.5 ± 12.5
L-Fucose	<1.0	<1.0
D-Fucose	22.8 ± 14.7	3.7 ± 2.9
D-Galactose	21.9 ± 2.5	29.2 ± 7.8
D-Maltose	41.8 ± 1.5 (0.2 M)	48.4 ± 8.8 (0.3 M)
Invasion		
D-Glucose	23.8 ± 9.6	35.6 ± 8.8
D-Mannose	33.4 ± 16.1	41.3 ± 6.2
D-Maltose	15.0 ± 5.7 (0.2 M)	25.8 ± 12.0 (0.3 M)

[a] Mean ± standard deviation of three experiments. The numbers of cell-associated bacteria in control and treatment assays were quantitated by [^{3}H]acetate radioactive labeling of *C. jejuni*.

TABLE 3. Inhibition of cell association and invasion of *C. jejuni* 81-176 following treatment of cells with inhibitors of the Na^+/glucose transporter, microfilaments, microtubules, and endocytosis and depletion of intracellular potassium

Treatment	% Inhibition (*P*)[a]
Cell association	
C. jejuni	
Phloridzin	
1 mM	<1.0
5 mM	22.4 ± 14.0 (0.038)
Vincristine (5 μM)	<1.0
Vinblastine (5 μM)	<1.0
Cytochalasin B (1 μg/ml)	<1.0
Cytochalasin D (1 μg/ml)	<1.0
Monodansylcadaverine (0.5 mM)	<1.0
g-Strophanthin (1 mM)	11.9 ± 6.4 (0.006)
K^+ depletion	33.9 ± 8.8 (0.002)
C. freundii	
Vinblastine (5 μM)	<1.0
Monodansylcadaverine (0.5 mM)	<1.0
γ-Strophanthin (1 mM)	<1.0
Invasion	
C. jejuni	
Phloridzin	
0.5 mM	<1.0 (0.557)
1 mM	<1.0 (0.950)
5 mM	11.0 ± 4.6 (0.045)
Vincristine (5 μM)	<1.0
Vinblastine (5 μM)	<1.0
g-Strophanthin	
1 mM	11.2 ± 4.8 (0.038)
0.5 mM	10.6 ± 3.8 (0.100)
0.25 mM	9.8 ± 6.6 (0.248)
Monodansylcadaverine	
0.5 mM	<1.0
0.25 mM	<1.0
Cytochalasin B (1 μg/ml)	<1.0
Cytochalasin D (1 μg/ml)	<1.0
K^+ depletion	34.9 ± 3.6 (0.015)
C. freundii	
Vinblastine (5 μM)	<1.0
Monodansylcadaverine (0.5 mM)	<1.0
g-Strophanthin (1 mM)	5.1 ± 6.8 (0.630)

[a] Mean ± standard deviation of three experiments. The numbers of cell-associated bacteria in control and treatment assays were quantitated by [^{3}H]acetate radioactive labeling of *C. jejuni*.

treatments with the exceptions of the amino sugars glucosamine and galactosamine. Treatment of *C. jejuni* with chloramphenicol (30 μg/ml) and rifampin (32 μg/ml) for 30 min prior to the adherence assay had no effect (<1.0% inhibition) on *C. jejuni* cell association. Bacterial viability was not impaired by treatment with chloramphenicol, as determined by quantitative cultures.

DISCUSSION

Our studies in infant rhesus monkeys challenged with *C. jejuni* showed that invasion is the primary mechanism in the pathogenesis of diarrheal disease caused by *C. jejuni* 81-176. *C. jejuni* adhere to the microvillus border and enter the colonic epithelial cells, becoming enclosed in a membrane-bound vacuole within the cytoplasm (36).

In vitro binding assays with *C. jejuni* have been conducted in HeLa, HEp-2, and INT-407 cells (8, 12, 14, 15, 27–29). Time course studies in INT-407 cells were reported as being either linear over a 4-h incubation period (29) or reaching a maximum after 30 min of incubation, followed by a decline (8). The kinetics of cell association (i.e., total numbers of adherent and intracellular bacteria) with INT-407 cells contrasts with the hyperbolic curve of bacterial binding to Caco-2 cells that we observed in this study. However, closer examination of the data for INT-407 cells shows that the titers of cell-associated bacteria after 2.5 h of incubation were similar to the numbers that we measured in Caco-2 cells (approximately 3×10^9 CFU) in both studies. The L-fucose inhibition of binding to INT-407 cells (8) was not demonstrated in the Caco-2 cell binding assay in this study.

Invasion is an important virulence mechanism of several enteropathogenic bacteria, including *Salmonellae*, *Shigellae*, and *Yersinia* species (6, 9, 10, 16, 17, 19, 22, 30, 37).

There was no inhibition of invasion of *C. jejuni* 81-176 by cytochalasin D. Cytochalasins B and D cause inhibition of invasion by *Shigella flexneri*, *Salmonella typhimurium*, *Salmonella typhi*, and *Salmonella cholerae-suis* into epithelial cells, indicating that microfilaments are important for entry of these bacteria into monolayers of HEp-2 and MDCK cells (2, 7, 16, 17). However, no adherence studies have been conducted with these organisms in Caco-2 cells. In a previous study (32), invasion of *C. jejuni* 81-176 into INT-407 cells was microfilament independent. However, invasion of *C. jejuni* VC84 was reduced by 50% after microfilament depolymerization by cytochalasin D. The report that the invasion of *C. jejuni* in HEp-2 cells was inhibited by cytochalasin B (12) may be explained by the high concentrations used (10 μg/ml), which could cause inhibition by other mechanisms, such as inhibition of glucose transport proteins (3, 11, 21).

The results of this study indicate that the entry of *C. jejuni* into Caco-2 cells is partially dependent on a mechanism which is impaired by K^+ depletion. K^+ depletion arrests receptor-mediated endocytosis (10, 25). However, multiple cellular processes, including increased intracellular pH, cell volume, and membrane potential, are also affected by K^+ depletion (20, 25, 26, 42). Also, the regulation of pH in Caco-2 cells involves an Na^+/H^+ exchanger and H^+/K^+ ATPase which may be affected by K^+ depletion and the induction of a hypertonic medium state (1, 39).

The microtubule inhibitors vincristine and vinblastine did not reduce the invasive ability of *C. jejuni* 81-176 in Caco-2 cells (Table 3). This contrasts with observations in INT-407 cells, in which microtubule depolymerization reduced invasion by approximately 80% (32). Invasion of *C. freundii* into Caco-2 cells was used as a control of microtubule-dependent endocytosis because depolymerization of microtubules with inhibitors (including colchicine, demecolchicine, nocadazole, vincristine, and vinblastine) decreased entry into INT-407 cells. However, vincristine and vinblastine did not decrease the invasion of *C. freundii* BR3447 into Caco-2 cells, although a different strain of *C. freundii* was used in the studies by Oelschlaeger et al. (32). Microtubule-dependent endocytosis of *C. jejuni* may be cell line specific for INT-407 and possibly other cell lines but was not observed in Caco-2 cells in this study under the experimental conditions used.

The saturable time- and dose-dependent bacterial binding are consistent with a receptor-mediated or a transport-limiting mechanism of invasion of *C. jejuni* in Caco-2 cells. Receptor-mediated endocytosis is inhibited by hypertonic conditions (e.g., Hanks balanced salt solution containing sucrose), which block clathrin-coated pit formation (20). In this study, invasion was inhibited competitively by D-sugars, including the monosaccharides galactose, glucose, and mannose and the disaccharide maltose composed of glucose-[α1-4]-glucose. These solutions were hypertonic, but the metabolically inactive L-sugars

having the same osmolarity did not inhibit cell association or invasion.

There was no decrease in the numbers of intracellular *C. jejuni* following treatment with monodansylcadaverine, which inhibits transglutamase activity needed for coated-pit formation (20). Also, g-strophanthin (which inhibits potassium-dependent ATPase, resulting in depleted intracellular potassium and decreased uptake of coated pits) did not inhibit *C. jejuni* invasion. These observations suggest that entry of *C. jejuni* into Caco-2 cells is independent of clathrin-coated pits. The results contrast with a report that g-strophanthin and monesin inhibition of coated-pit formation inhibited endocytosis of *C. jejuni* 81-176 in INT-407 cells. Also, in contrast to the studies in INT-407 cells (32), we found that *C. freundii* invasion was not significantly impaired in Caco-2 cells by g-strophanthin and monodansylcadaverine. Differences in experimental conditions under which the assays were conducted, methods of quantitation, and the strains of *C. freundii* used may explain the different results, but the results could also be an indication of cell-line-specific mechanisms of bacterial invasion.

Since invasion of *C. jejuni* 81-176 was not markedly inhibited by phloridzin, which is a specific inhibitor of the Na^+/glucose transporter (41), the invasion mechanisms inhibited by the sugars appears to be unrelated to glucose transport. The lack of inhibition by cytochalasin B at high concentrations (1 μg/ml) also indicates that glucose transporters are probably not important in the adherence and invasion of *C. jejuni* into Caco-2 cells. Invasion of *C. jejuni* into HEp-2 cells is inhibited by iodoacetate and dinitrophenol, and cell association was markedly reduced at 4°C, indicating that entry into cells is an energy-dependent process (12).

C. jejuni association with Caco-2 cells was inhibited by treatment of cells with bromelain but not with trypsin. This finding indicates that invasion was inhibited by disruption of disulfide bonds in the plasma membrane (13). We do not precisely understand the mechanism of bromelain inhibition. Possible explanations are that *C. jejuni* utilizes a receptor that is independent of clathrin. Another possibility is that bromelain causes inhibition by an indirect mechanism such as perturbation of the plasma membrane, resulting in impairment of signal transduction mechanisms or other physiological responses needed for parasite-directed endocytosis. Alterations of the receptor maybe important in the binding of *C. jejuni* but may not be important in the internalization of *C. jejuni* into Caco-2 cells.

It was reported that following pretreatment of *C. jejuni* with 32 to 64 μg/ml, chloramphenicol decreased translocation of *C. jejuni* across polarized Caco-2 cells (23). Chloramphenicol treatment did not inhibit invasion of *C. jejuni* in this study, demonstrating that protein synthesis was not required for invasion of *C. jejuni* into monolayers of Caco-2 cells. This is consistent with the rapid invasion observed between 20 min and 1.5 h. The results of rifampin treatment of the bacteria (32 μg/ml, pretreated for 30 min) are also consistent with these observations.

Inhibition of cell association and invasion of *C. jejuni* into Caco-2 cells required concentrations of monosaccharides and disaccharides higher than those reported to inhibit cell association in INT-407 cells (8, 29). The mechanism of impaired entry by sugars may be indirect, e.g., nonphysiologic conditions associated with high levels of sugars in the supernatant.

The decrease in invasion corresponded to decreased adherence of the bacteria to the cell surface. Since this was observed with D- but not L-sugars, a nonspecific mechanism seems unlikely. McSweegan and Walker (29) reported that a 500-μg/ml concentration of lipopolysaccharide (LPS) extracted from the homologous strain caused inhibition of invasion of *C. jejuni* in INT-407 cells. Since LPS is composed of monosaccharides, and *C. jejuni* appears to lack known adhesins such as pili, the LPS of *C. jejuni* may interact with the cell surface, at least in cell culture monolayers, in a manner which is inhibited by sugars such as monosaccharides, disaccharides, or LPS.

The aflagellate variant of *C. jejuni* 81-176 showed low numbers of cell-associated bacteria as a result of impaired motility. However, centrifugation did not facilitate the entry of aflagellate *C. jejuni* into Caco-2 cells to the same extent that cell association was facilitated, suggesting that flagella are important for the entry of *C. jejuni* into Caco-2 cells. Wassenaar et al. (38) also demonstrated that motility is a major factor of *C. jejuni* invasion into INT-407 cells. Similarly, Morooka et al. (31) reported that nonmotile flagellar mutants were unable to colonize the intestine in suckling mice.

Flagella were spirally wrapped around the organisms during entry into colonic epithelial cells (36). This could be consistent with a drill-like rotation of the organism as it was propelled into the cell, utilizing the second bipolar flagellum. The importance of the functional flagella for invasion of *C. jejuni* into INT-407 cells has also been shown by molecular studies. Deletion of the *flaA* gene caused a marked decrease in the invasion, whereas the *flaB* gene was not essential for invasion of *C. jejuni* into INT-407 cells (38).

The results of this study suggest that *C. jejuni* may utilize mechanisms different from those described for other enteropathogenic bacteria and that the flagella are important for entry of *C. jejuni* into Caco-2 cells. Further studies are needed to elucidate the genetic loci responsible for invasion of *C. jejuni*, the precise events that occur during bacterial entry, and the mechanism of cell damage that results in colitis.

ACKNOWLEDGMENT

Financial support for this study was provided by National Institutes of Health grant RR03123.

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