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Identification and Characterization of Two *Campylobacter jejuni* Adhesins for Cellular and Mucous Substrates

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***Campylobacter jejuni* is able to colonize the human intestinal mucosa and cause disease. For this reason, it was important to investigate mechanisms by which *C. jejuni* adheres to epithelial cells and intestinal mucus gel. All strains of *C. jejuni* used were able to adhere to INT 407 epithelial cells and mucus, but high adherence to one substrate did not necessarily indicate comparable adherence to the other. The adherence of *C. jejuni* to cells was inhibited partially by treating the bacterial cells with proteases or glutaraldehyde or by adding a certain carbohydrate (fucose or mannose) to the medium. The flagellum of *C. jejuni* was identified as a potential adhesin by comparing adherence of flagellated and aflagellated variants. Shearing of the bacterial cells to remove the flagella reduced bacterial adhesion, whereas immobilization of the flagellum with KCN increased adhesion. Purified flagella showed specific, fucose-resistant binding to epithelial cells but not to intestinal mucus. The presence of a second, nonproteinaceous adhesin was suggested because no single treatment of the bacteria completely inhibited adhesion. Lipopolysaccharide (LPS) was identified as another *C. jejuni* adhesin. [^{3}H]LPS specifically bound to epithelial cells, and this phenomenon was inhibited by periodate oxidation of the LPS or glutaraldehyde fixation of the epithelial cells. LPS, unlike flagella, was fucose sensitive and inhibited binding of whole bacterial cells to INT 407 cells. LPS was also able to bind to intestinal mucus gel. These data indicate that both flagella and LPS are important in adhesion to the mucosal surface.**

Campylobacter jejuni is a frequent agent of diarrhea in humans (5), yet the interplay of its virulence factors in pathogenesis is poorly understood. The mechanisms by which *C. jejuni* interacts with the intestinal mucosa and initiates diarrhea are also unclear.

Adhesion of bacteria to mucosal surfaces is necessary for colonization and subsequent pathogenesis. This process is mediated by chemotactic factors (9) and bacterial appendages (adhesins) which can include pili, flagella, capsules, glycocalyces, lipopolysaccharides, and cell-associated lectins (1, 2, 7). Complete evaluation of adhesion mechanisms of many organisms, however, has only recently begun.

Newell and Pearson (24) used scanning electron microscopy to demonstrate in vitro adherence of *C. jejuni* to intestinal epithelial cells and saw morphologic evidence that this adhesion could be mediated by flagella. Later, flagellated organisms were shown to adhere in greater numbers to cells than did an aflagellated variant (23). Dijs and De Graaf (8) showed that *C. jejuni* cells do not possess fimbriae or pili but are able to agglutinate a variety of mammalian erythrocytes. Later, Naess et al. (22) were able to show in vitro adherence of *C. jejuni* strains to porcine small intestinal brush border preparations.

Cinco et al. (6) also studied *Campylobacter* adherence to intestinal epithelial cells. The adhesion was inhibited partially by L-fucose and D-mannose. This suggests the existence of a second adhesion. They also determined that one *Campylobacter* adhesin was probably heat stable and fucose sensitive (6).

Campylobacter interactions with the mucus component of the mucosal surface have not been studied. The intestinal mucus layer is a substantial structure, often 30- to 50-μm thick, and is continuous with the glycocalyx of the mucus (26). This viscous glycoprotein gel covering the epithelial cells of the intestinal tract is likely to be the initial point of contact between the host and *C. jejuni*.

Because *C. jejuni* adherence may be a complex, multicomponent process, this study was conducted to determine the ability of *C. jejuni* strains to adhere to epithelial cells as well as intestinal mucus gel. Adhesion parameters and the identification of two adhesins of *Campylobacter* are described.

MATERIALS AND METHODS

Bacteria. A clinical isolate of *C. jejuni* (WR-6) was obtained from Walter Reed Army Medical Center, Washington, D.C. Motile (M^+) and nonmotile (F^-) variants of *C. jejuni* 81116 were kindly provided by D. G. Newell (Center for Applied Microbiology, Salisbury, United Kingdom). Additional motile (strain BS) and nonmotile (strain B-NS) variants were obtained from Robert Black (Center for Vaccine Development, University of Maryland Medical School, Baltimore. *C. jejuni* HC, which was used in most experiments, was a blood culture isolate from a patient at the Bethesda Naval Hospital, Bethesda, Md. All of the other *C. jejuni* and *C. coli* strains were obtained from the Naval Medical Research Institute culture collection. The *Escherichia coli* K-12 strains were supplied by Richard Wilson, *E. coli* Reference Center, Pennsylvania State University, University Park.

Media and bacterial growth. *Campylobacter* strains were maintained in egg yolk in liquid nitrogen. Before use samples were quickly thawed and streaked onto commercial blood agar plates (England Laboratories, Beltsville, Md.). Blood agar plates were incubated at 42°C in polybags (Levin Brothers Paper Co., Chicago, Ill.) with an atmosphere of 85% N_2–10% CO_2–5% O_2. Growth from the blood agar plates was subsequently used to inoculate 25-cm^2 tissue culture flasks containing the biphasic culture system described by Rollins et al. (25).

Labeling of the bacteria. *Campylobacter* strains were

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grown in a biphasic brucella broth-brucella agar system (25) supplemented with 0.01 mCi of sodium [^{3}H]acetate per ml (specific activity, 90 mCi/mmol; New England Corp. Boston, Mass.). The inoculated tissue culture flasks were incubated at 37° for 18 to 20 h. Following incubation the cells were harvested, washed twice in *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-Hanks (HH) buffer (pH 7.4) and suspended in the same buffer. The optical density of the bacterial suspension was then determined at 600 nm and adjusted to an absorbance of 0.95. A fraction of the suspension was removed, and the number of viable *C. jejuni* cells present was determined by plating dilutions on brucella agar. A second fraction was taken to determine the level of radioactivity present. Typically, the level of [^{3}H]acetate incorporation was 5×10^{-5} to 5×10^{-4} cpm per bacterium.

INT 407 cell adhesion assay. INT 407 cells were routinely grown in Eagle minimal essential medium supplemented with 5% fetal calf serum. Twenty-four hours before the adhesion assay the cells were seeded into 24-well polystyrene tissue culture plates (Costar, Cambridge, Mass.) at a density of 1.8×10^5 cells per well. The following day the medium was aspirated from each well, and the cells were washed twice with 0.5 ml of HH buffer (pH 7.4). The plates were gently rocked back and forth a few times to ensure adequate washing.

After the INT 407 cell monolayers and blank control wells were washed, 0.25 ml of approximately 10^9 CFU of ^{3}H-labeled *C. jejuni* was added to each well. The plates were then incubated at 37°C for 3 h. At the end of the incubation period the individual wells were again washed twice with 0.5 ml of HH buffer to remove nonadherent bacteria. Adherent bacteria were recovered by adding 0.5 ml of 0.5% sodium dodecyl sulfate (SDS) to each well and reincubating the plates for 2 h. Samples (0.25 ml) were removed from each well, and the level of radioactivity was determined.

All INT 407 cell adhesion assays were performed in triplicate. In each assay the total level of radioactivity and the number of bacteria added to each set of wells were determined. In this way it was possible to calculate the number of bacteria (CFU) bound in each well.

The presence of adherent *C. jejuni* on INT 407 cells and mucus gel was confirmed by scanning electron microscopy and phase-contrast microscopy, respectively (data not shown). In addition, *C. jejuni* cells were examined for leakage of the radiolabel and were found to release only about 6.3% of the total [^{3}H]acetate label. Approximately 2.5% of this leaked material adhered to INT 407 cells or plastic control wells (189 to 202 cpm per well). Consequently, the majority of the radioactivity recovered from the wells could be attributed to intact, adherent bacteria.

Additionally, INT 407 cells were examined by the Evans blue dye exclusion method at the end of the 3-h incubation period and were found to remain viable. *Campylobacter* viability was also determined at the end of the incubation period by scraping bacteria-containing wells and plating the contents directly onto brucella agar.

Intestinal mucus adhesion assay. The ability of *Campylobacter* strains to adhere to crude preparations of intestinal mucus was also examined. A previously described mucus adhesion assay was employed (15). Briefly, mucus from the large and small intestines of 1-kg New Zealand rabbits (Dutchland Laboratories, Inc., Denver, Pa) was prepared in HH buffer. Mucus (1.0 mg of protein per ml) was added to 24-well tissue culture plates (0.25 ml per well) and incubated overnight at 4°C. Following incubation the wells were washed to remove unbound mucus, and the adhesion assay was conducted as described above. Rabbits were selected as a source of mucus because they are currently one of the few successful animal models of *Campylobacter*-induced diarrhea (5).

Rate of adhesion. *C. jejuni* HC was used to measure the rate of adhesion to INT 407 cells. The assay was initiated as described above. At regular intervals after the assay was begun, triplicate wells were rinsed and replaced with 0.5% SDS. The number of adherent bacteria at each time interval was then determined.

Reversibility of adhesion. At the end of the 3-h assay time, the wells were washed twice with HH buffer, and triplicate wells received either 0.5 ml of 0.5% SDS or 0.5 ml of HH buffer. At 10-min intervals triplicate wells containing HH buffer were washed and replaced with 0.5% SDS. The number of adherent bacteria in each set of wells was then determined.

Carbohydrates. The role of carbohydrates in the adherence of *Campylobacter* to epithelial cells was assessed by conducting the assays in the presence of various monosaccharides. The tested monosaccharides (50 mM) were prepared in HH buffer, and ^{3}H-labeled bacteria were suspended in the various sugar solutions. The INT 407 cell assay was then conducted as described above.

Proteases. The role of the *Campylobacter* surface protein in the adherence process was also examined. *Campylobacter* strains were treated with trypsin, pepsin, and *Streptomyces griseus* type VI protease (100 μg/ml) at 37°C for 30 min. The bacteria were then washed two times with HH buffer to remove the enzymes and digested proteins, and used immediately in the INT 407 cell adhesion assay.

Fixatives. *Campylobacter* cells were also fixed in fresh 1% formaldehyde or 2.5% glutaraldehyde at room temperature for 20 min. The bacteria were then washed two times to remove excess fixative, and used in the INT 407 cell adhesion assay.

Flagella. The contribution of the *Campylobacter* flagellum in mediating adherence to epithelial cells was examined by shearing the bacterial cells in an Omnimixer (Sorvall, Newton, Conn.) for two 30-s periods. The bacteria were washed twice with HH buffer and immediately used in the adhesion assay. Fractions of sheared *Campylobacter* cells were plated on brucella agar to determine viability and the number of CFU per milliliter. Additional *Campylobacter* cells were treated with 10 mM KCN for 30 min at room temperature to eliminate flagella-mediated motility.

Motility. Individual *Campylobacter* strains and isolates were assayed for motility by spotting them onto thioglycolate medium (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 0.33% agar.

Hydrophobicity. Retention of *Campylobacter* cells on phenyl-Sepharose columns was measured by the method of Jann et al. (12).

Labeling and extraction of flagella. Flagella were isolated from *C. jejuni* HC by a modification of the method of Logan and Trust (17), in which the acid dissociation step was omitted. The flagella were then concentrated by ultrafiltration through Centricon-30 microconcentrators (Amicon Corp., Lexington, Mass.).

Purified flagella were reductively methylated with [^{3}H] formaldehyde (2 μmol/mg of protein, 65 μCi/μmol) by the method of Jentoft and Dearborn (13). After methylation, the ^{3}H-labeled flagella were exhaustively dialyzed against HH buffer (pH 7.4). The flagella were then centrifuged at 100,000 × *g* for 1 h, suspended in HH buffer, and stored at −20°C.

TABLE 1. Specificity of *C. jejuni* HC attachment to INT 407 cells

Expt	10^6 CFU/well ± SE of[a]:		
	INT 407 cells	BSA[b]	Polystyrene
1	46.2 ± 1.0	11.0 ± 0.3	14.8 ± 0.1
2	20.0 ± 0.4	10.8 ± 0.6	7.7 ± 0.2
3	32.0 ± 1.6	9.1 ± 1.0	7.1 ± 0.9
4	40.0 ± 2.0	3.8 ± 0.2	7.8 ± 0.2

[a] Mean CFU per well was determined from triplicate sets of wells.
[b] 10 mg/ml; 0.25 ml per well.

Labeling and isolation of LPS. Lipopolysaccharide (LPS) was extracted from *C. jejuni* HC by the method of Westphal and Jann (27). *C. jejuni* HC was grown in 1-liter screw-cap flasks containing 400 ml of brain heart infusion broth supplemented with 1% yeast extract and 0.01 mCi of [^{3}H]sodium acetate. The flasks were shaken at 150 rpm for 12 to 18 h at 37°C.

Lyophilized LPS was suspended in HH buffer and in RNase (Sigma Chemical Co., St. Louis, Mo.) and DNase (Sigma) were added to 0.1 mg/ml. LPS samples were then incubated for 8 h at 37°C. Trypsin (Sigma) was added to 0.1 mg/ml, and the LPS was reincubated overnight at 37°C. Finally, the LPS was ultracentrifuged at 105,000 × *g* for 3 h. The pellets were suspended in distilled water and lyophilized.

2-Keto-3-deoxyoctulosonic acid was detected by the method of Karkhanis et al. (14). Protein was measured by the method of Lowry et al. (19). Samples of LPS were also oxidized with sodium *m*-periodate as described by Izhar et al. (11).

Calculations. All assays were run in triplicate. Results were expressed as the mean of triplicate sets of wells. Student's *t* test was used to determine significance. *P* values exceeding 0.05 were considered not significant. Numbers of adherent bacteria were calculated from the following formula: (cpm/well)/[cpm/ml × (ml/CFU)] = CFU/well.

RESULTS

Adherence to INT 407 cells. In Table 1 are presented the results of four separate adhesion experiments with *C. jejuni* HC. In all four experiments, adhesion of *C. jejuni* to INT 407 cells was significantly greater than adhesion to bovine serum albumin (BSA) or polystyrene controls ($P < 0.001$). The results in Table 1 are typical of the data generated by this assay. That is, for any given experimental group, the amount of radioactivity recovered from each set of triplicate wells was very consistent. However, the amount of [^{3}H]acetate incorporated by the bacteria, and the actual number of bacteria added per well, varied somewhat from experiment to experiment, as did the number of counts per minute per well and CFU per well that were recovered. Consequently, quantitative comparisons of actual numbers of adherent bacteria obtained from different experiments should be made with caution. In addition, the reversible expression of flagella (4) and the peculiar morphologic changes seen in *C. jejuni* strains (25) may have also contributed to interexperimental variation, although routine microscopic examination of the cultures for motile, spiral forms was done to reduce this possibility. The reproducibility of the assay, however, was very good in that it was never difficult to distinguish adherent *C. jejuni* strains bound to INT 407 cells or mucus from the BSA and polystyrene controls.

Rate of adhesion. *C. jejuni* HC was used to determine the rate at which *C. jejuni* bound to INT 407 cells. The adhesion rate was linear for the first 4 h (Fig. 1). The rate of adhesion was approximately 2.5 × 10^5 CFU/min. Beyond 4 h there was a rapid decline in the number of adherent bacteria. Therefore, to ensure assay reproducibility and convenience, the standard assay time was maintained at 3 h.

Reversibility of adhesion. *C. jejuni* adhesion to INT 407 cells was reversible (Fig. 2). Attempts to elute cell-adherent bacteria resulted in an 80% decrease in the number of bacteria attached to the cell substrate following a 60-min incubation in fresh, bacteria-free HH buffer. The greatest decrease in the number of adherent bacteria occurred within the first 10 min. Thereafter the rate of bacterial elution was greatly diminished. Light microscopy was used to show that loss of adherent *C. jejuni* was not due to a decrease in the extent of the INT 407 cell monolayers.

Hydrophobicity of *C. jejuni* HC. *C. jejuni* HC cells were not hydrophobic, as measured by retention on phenyl-Sepharose columns. *E. coli* K-12:K88ab and *E. coli* K-12 were used as positive and negative controls, respectively (7). *C. jejuni* HC cells that were fixed in glutaraldehyde, sheared, or treated with proteases showed only 13 to 18% retention. In contrast, 75% of *E. coli* K-12:K88ab and 20% of *E. coli* K-12 were retained.

Survey of strains. On the basis of the results obtained in Table 1, a survey was undertaken to assess the ability of

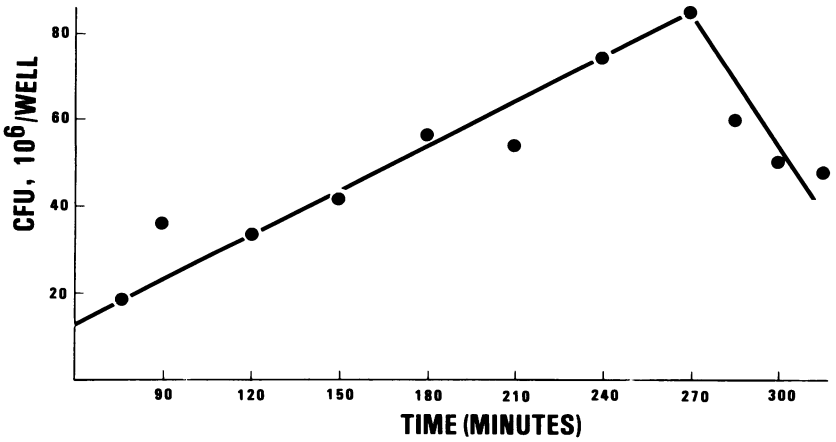

FIG. 1. Rate of adhesion of *C. jejuni* HC to INT 407 cells. Each point was determined from the mean of three wells.

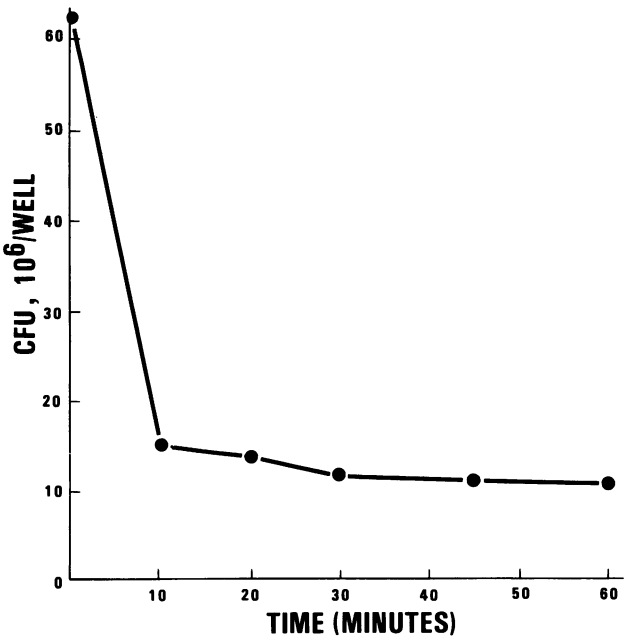

FIG. 2. Reversibility of *C. jejuni* HC adhesion to INT 407 cells. Values for polystyrene controls were subtracted from the experimental data before they were plotted.

other *Campylobacter* strains to adhere to INT 407 cells and to intestinal mucus (Table 2). A number of important points were evident. First, there was broad variation in the ability of various *C. jejuni* strains to adhere to INT 407 cells and intestinal mucus. Second, some strains adhered better to cells (e.g., HC, M^+, E23) than to mucus, while other adhered better to mucus than to cells (e.g., WR-6). Third, of the two sets of isogenic pairs (M^+–F^- and BS–B = NS), the motile variants M^+ and BS adhered better to cells than did the nonmotile variants F^- and B-NS.

Adhesion inhibition. The specificity of *Campylobacter* adherence to INT 407 cells was examined by attempting to inhibit the adhesion process with various monosaccharides (Table 3). Of the 14 sugars tested, fucose and mannose were the most inhibitory, but neither completely inhibited the adhesion.

TABLE 2. Attachment of *C. jejuni* and *C. coli* strains to INT 407 cells and rabbit intestinal mucus

Strain	10^6 CFU/well ± SE of[a]:		
	INT 407 cells	RLIM[b]	RSIM[c]
HC	83.4 ± 5.6	24.5 ± 1.0	45.7 ± 0.2
81116 (M^+)	11.2 ± 0.9	1.9 ± 0.1	2.9 ± 0.01
81116 (F^-)	1.7 ± 0.1	0.5 ± 0.05	0.8 ± 0.0
BS	30.4 ± 0.3	4.9 ± 0.9	9.3 ± 0.8
B-NS	10.4 ± 0.6	3.1 ± 0.5	5.4 ± 0.3
E15	11.1 ± 1.1	8.0 ± 0.1	8.3 ± 0.4
WR-6	27.4 ± 2.1	84.9 ± 7.0	98.2 ± 0.4
ATCJ	42.0 ± 2.7	6.6 ± 1.7	NT[d]
E8	9.6 ± 0.6	0.5 ± 0.05	NT
E23 (*C. coli*)	48.2 ± 2.1	11.4 ± 1.1	14.5 ± 0.2

[a] Nonspecific polystyrene and BSA background counts were subtracted from the INT 407 cell- and mucus-containing wells. In all experiments, triplicate cell- and mucus-containing wells were run, and all strains were tested simultaneously.
[b] Mucus from the rabbit large intestine (RLIM; 0.68 mg of protein per ml).
[c] Mucus from the rabbit small intestine (RSIM; 1.0 mg of protein per ml).
[d] NT, Not tested.

TABLE 3. Inhibition of *C. jejuni* HC adhesion to INT 407 cells with monosaccharide

Sugar (50 mM)	10^6 CFU/well ± SE[a]	% Reduction
Expt 1		
None	82.0 ± 2.2	
D-Mannose	43.0 ± 0.8	47
N-Acetylneuraminic acid	59.0 ± 1.8	28
Melibiose	64.0 ± 3.6	22
Rhamnose	60.0 ± 4.5	27
N-Acetylgalactosamine	66.0 ± 2.5	20
Raffinose	61.0 ± 2.9	26
Sucrose	74.0 ± 0.7	10
L-Fucose	43.0 ± 0.3	47
N-Acetylglucosamine	62.0 ± 2.5	24
N-Acetylmannose	82.0 ± 0.2	0
Xylose	67.0 ± 5.0	18
Mannan	68.0 ± 0.1	17
Expt 2		
None	61.5 ± 2.8	
L-Fucose	29.0 ± 2.1	53
D-Fucose	34.4 ± 0.3	44
D-Mannose	42.0 ± 1.2	32
L-Fucose + D-mannose	42.5 ± 3.2	31

[a] Sugar inhibition assays were done in triplicate. Polystyrene control cpms were subtracted from the data before the CFU per well was calculated.

Campylobacter surface protein appears to be involved in adhesion to INT 407 cells, because proteolysis with enzymes and fixation with gluteraldehyde or formaldehyde significantly reduced adhesion (Table 4). However, the various treatments failed to completely inhibit adhesion.

Flagellar protein was involved in the adhesion process, as indicated by comparisons of binding by motile and nonmotile strains of *C. jejuni*. In addition, when *Campylobacter* cells were sheared to remove the flagella, decreased adhesion to INT 407 cells was also seen (Table 5). However, if the bacteria were treated with KCN to inactivate the flagella, there was an increase in the level of adherence (Table 5).

***Campylobacter* receptors on INT 407 cells.** The ability of *Campylobacter* strains to specifically adhere to INT 407 cells over BSA or polystyrene controls indicates that specific receptors for *Campylobacter* adhesins exist on the INT 407 cells. Fixation of the INT 407 cells with glutaraldehyde significantly reduced the number of adherent *Campylobacter* cells (Table 6). This indicates that protein on the INT 407 cell

TABLE 4. Effect of treatment of *C. jejuni* surface protein on attachment to INT 407 cells

Treatment[a]	*C. jejuni* strains			
	HC		E8	
	10^6 CFU/well[b]	% Reduction	10^6 CFU/well	% Reduction
None	101 ± 9.7	—	85.7 ± 7.8	—
Pepsin	38 ± 2.4	62	11.5 ± 1.1	87
Protease	39 ± 2.1	61	23.0 ± 2.3	73
Trypsin	34 ± 0.9	66	26.2 ± 1.0	69
Glutaraldehyde	26 ± 1.1	74	23.4 ± 0.3	73
Formaldehyde	26 ± 0.2	74	NT[c]	

[a] See text for procedures.
[b] Polystyrene control counts per minute were subtracted from the experimental data before the CFU per well was calculated.
[c] NT, Not tested.

TABLE 5. Effect of shearing and immobilization of *Campylobacter* flagella on adhesion to INT 407 cells

Treatment[a]	*C. jejuni* strains			
	HC		E8	
	10^6 CFU/well	% Change	10^6 CFU/well	% Change
None	13.3 ± 0.6	—	85.7 ± 7.8	—
10 mM KCN	33.0 ± 4.0	180	127.5 ± 9.6	49
Shearing	4.7 ± 0.3	−60	32.3 ± 2.0	−62

[a] See text for procedures.

surface is involved in the binding of *C. jejuni* to the epithelial cell surface.

Fixation of the epithelial cells, in combination with proteolysis or fixation of the bacterial cells, effectively reduced *Campylobacter* adherence by 95 to 100% (Table 7). Previous attempts to eliminate bacterial adherence to the epithelial cells by treating the bacteria with combinations of proteases, fucose, and fixatives were unsuccessful (data not shown). The data presented in Table 7 indicate that complete inhibition of bacterial adherence required pretreatment of both the bacterial and the epithelial cells.

Binding of LPS and flagella to INT 407 cells. Tritiated LPS was extracted from *C. jejuni* HC and used in binding assays against the INT 407 cells. Each cell-containing and control well received 0.25 ml of a 2.0-mg (dry weight)/ml suspension of [^{3}H]LPS. After a 3-h incubation at 37°C the wells were washed two times with HH buffer, and the amount of radioactivity in each well was determined. In Table 8 are shown the results of three separate experiments with tritiated LPS from *C. jejuni* HC. The data indicate that (i) LPS specifically binds to INT 407 cells, (ii) protein on the INT 407 cell surface interacts with LPS because gluteraldehyde interferes with the binding of LPS, (iii) the binding of LPS to the INT 407 cells is inhibited by the sugar fucose, and (iv) periodate oxidation of the LPS reduces binding to INT 407 cells. This last finding suggests that binding of LPS is through the carbohydrate portion of the LPS molecule.

Similarly, isolated flagella (0.6 mg of protein per ml) from *C. jejuni* HC were also shown to bind specifically to the INT 407 cells (Table 9). Binding by flagella was found to be sensitive to glutaraldehyde fixation of the epithelial cells but, in contrast to the LPS, was not sensitive to fucose.

The LPS adhesin was also effective in blocking the attachment of whole *C. jejuni* HC cells to the epithelial cells (Fig. 3). Complete inhibition of *Campylobacter* attachment was obtained within the range of 250 to 1,000 μg of LPS per well. In contrast, the flagella adhesin showed no ability to block *Campylobacter* adherence (Fig. 3).

Binding of LPS and flagella to mucus. Both the LPS and the flagella from *C. jejuni* HC bound to epithelial cells. In contrast, only LPS bound to mucus from the rabbit small intestine (Table 10). Although flagella preparations consistently bound to the INT 407 cells, none of these same preparations showed any specificity for the intestinal mucus gel.

The data in Table 10 indicate that epithelial cells bind greater quantities of both LPS and flagella than does the intestinal mucus gel.

TABLE 6. *C. jejuni* adhesion to glutaraldehyde-fixed INT 407 cells

Strain	10^6 CFU/well ± SE of:		
	Cells	Fixed cells[a]	% Reduction
ATCJ	69.6 ± 4.8	16.0 ± 0.4	77
E8	85.7 ± 7.8	33.4 ± 0.6	61
HC	68.5 ± 6.0	12.3 ± 1.6	82

[a] Epithelial cells were fixed in 2.5% glutaraldehyde for 30 min at 37°C. The fixed cells were then washed twice with 0.5 ml of HH buffer (pH 7.4).

TABLE 7. Inhibition of *C. jejuni* HC adhesion to INT 407 cells by treatment of both bacterial and epithelial cells

Bacteria treated with:	10^6 CFU/well ± SE of[a]:	
	INT 407 cells	Glutaraldehyde-fixed INT 407 cells
Expt 1		
No treatment	39.0 ± 3.8	4.0 ± 0.8
Glutaraldehyde	15.0 ± 0.3	0.0
Expt 2		
No treatment	66.4 ± 2.5	14.5 ± 1.4
Protease	34.9 ± 4.6	11.0 ± 1.6
Glutaraldehyde	50.6 ± 3.3	3.8 ± 0.3

[a] Mean CFU per well was determined from triplicate sets of wells. Polystyrene controls were subtracted from the data.

DISCUSSION

Although results of previous studies have demonstrated adherence of *Campylobacter* to INT 407 cells, this is the first report of specific in vitro adherence to intestinal mucus. Furthermore, we have shown that there are two distinct adhesins which interact differently with these substrates.

The flagellum previously has been implicated in the adhesion process (20, 23). We were able to confirm the results of these studies by demonstration of reduced adherence after shearing the bacterial cells to remove the flagella. Likewise, F^- variants of *C. jejuni* bound in lower numbers to INT 407 cells than did M^+ variants. Conversely, adhesion was greatly increased by treating the bacteria with KCN to immobilize the flagella. These results suggest that flagella are important in facilitating attachment to the epithelial cells, but that once attached to the cells the rotary action of the flagella may

TABLE 8. Binding of *C. jejuni* HC LPS to INT 407 cells

LPS bound to:	μg of LPS/well ± SE[a]
Expt 1	
INT 407 cells	18.4 ± 0.8
Fixed INT 407 cells[b]	5.9 ± 0.1
Polystyrene	5.1 ± 0.5
Expt 2	
INT 407 cells	12.9 ± 0.2
INT 407 cells[c]	8.3 ± 0.6
Fixed INT 407 cells	6.4 ± 0.6
Polystyrene	5.1 ± 0.4
Expt 3	
INT 407 cells	17.3 ± 0.5
Fixed INT 407 cells	8.0 ± 0.7
INT 407 cells + 50 mM fucose	11.1 ± 1.6
Polystyrene	3.3 ± 0.1

[a] Mean micrograms were determined from triplicate wells.
[b] INT 407 cells were fixed in 2.5% glutaraldehyde for 30 min at 37°C.
[c] LPS (3 mg/ml) was oxidized with sodium *m*-periodate prior to use in the binding assay. See text for details.

TABLE 9. Binding of *C. jejuni* HC flagella to INT 407 cells

Target	μg of flagella/well ± SE[a]
Expt 1	
INT 407 cells	14.2 ± 0.2
Polystyrene	2.2 ± 0.2
Expt 2	
INT 407 cells	12.8 ± 0.3
INT 407 cells + 50 mM fucose	13.2 ± 0.6
Polystyrene	5.0 ± 0.5
Expt 3	
INT 407 cells	13.4 ± 0.7
INT 407 cells + 50 mM fucose	13.2 ± 0.8
Glutaraldehyde-fixed cells	3.8 ± 0.1
Polystyrene	3.5 ± 0.2

[a] Mean micrograms were determined from triplicate wells.

TABLE 10. Binding of *C. jejuni* HC LPS and flagella to INT 407 cells and rabbit small intestinal mucus

Adhesin	Adhesin concn (mg/ml)	μg of adhesin/well ± SE in:	
		Mucus	Cells
Expt 1			
LPS	1.00	1.3 ± 0.1	3.6 ± 0.2
Flagella	0.44	ND[a]	2.7 ± 0.1
Expt 2			
LPS	1.50	1.4 ± 0.2	8.4 ± 0.8
Flagella	0.56	ND	4.3 ± 0.3
Expt 3			
LPS	2.00	4.0 ± 0.1	NT[b]
Flagella	0.74	ND	12.0 ± 0.2

[a] ND, Not detected. The amount of bound flagella protein was less than or equal to the amount that bound to the BSA controls.
[b] NT, Not tested.

impede maximal, long-term adherence. Similar results were reported by McBride and Newell (20) with nonmotile and aflagellated variants of *C. jejuni*.

Purified flagella were found to specifically bind to the INT 407 cells. The binding of purified flagella to epithelial cells was inhibited by fixing the epithelial cells in glutaraldehyde. However, flagella were fucose resistant in contrast to binding by whole *Campylobacter* cells.

The difference in fucose sensitivity between whole cells and flagella substantiates the concept of multiple adhesins on *C. jejuni*. This has been suggested by the work of others (6, 8), as well as our own data which showed that binding of *C. jejuni* to epithelial cells could be partially inhibited by a number of factors. We found, as did Cinco et al. (6), that fucose and mannose reduce adherence by 50 to 60%. At least one adhesin appears to be a surface protein because proteases, glutaraldehyde, and formaldehyde also partially inhibit adhesion to epithelial cells.

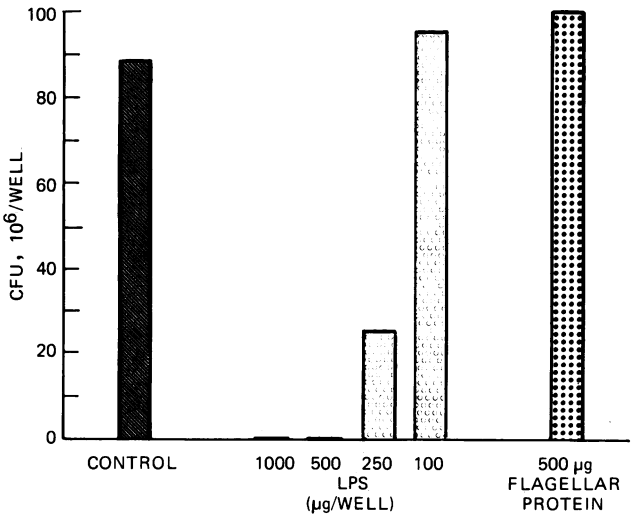

FIG. 3. Blocking adherence of *C. jejuni* HC to INT 407 cells. INT 407 cells were preincubated with the indicated quantities of LPS and flagella for 1.5 h at 37°C. The treated wells were then washed twice with HH buffer (0.5 ml). ^{3}H-labeled *C. jejuni* HC (0.25 ml) was added, and the adherence assay was conducted as described in the text.

Comparison of attachment of various strains of *Campylobacter* to mucus and cells also provided evidence for the complexity of the adherence process. Many strains attached in large numbers to cells but in relatively low numbers to the mucus substrate. With other strains, however, binding to mucus was relatively high. With one strain WR-6, binding to mucus was significantly greater than that to cells.

The rate of bacterial adhesion to the INT 407 cells was constant over a 4-h period, but the adhesion to cells and polystyrene was, surprisingly, reversible. Evidently, some of the adherent bacteria are not in a state of dynamic equilibrium because they could not be eluted from the INT 407 cells (Fig. 2). The tenacity of the cell-bacterium bond is probably due to multiple points of attachment between the receptors of the cell and the *Campylobacter* adhesins. A bacterial cell with numerous adhesins bound to receptor sites would show greater avidity than a similar bacterial cell with fewer adhesins bound to a single type of receptor site. Those bacteria that were readily eluted from the INT 407 cells were probably prevented from establishing multiple bonds or were sticking to other bacteria. Steric hindrances and receptor saturation by other adherent bacteria would limit the number of bacteria able to establish multiple bonds and irreversible adherence.

LPS may be a second *Campylobacter* adhesin. LPS was extracted from radiolabeled *C. jejuni* HC and used in the INT 407 cell adhesion assay. Tritiated LPS specifically bound to the cells. In addition, periodate oxidation of the LPS reduced the binding of LPS to cells, indicating that the short O side chains of *C. jejuni* LPS molecules are involved in adhesion. Because fixation of INT 407 cells with glutaraldehyde also reduced LPS binding, it is likely that a protein on the epithelial cell surface specifically recognizes the *Campylobacter* LPS. A similar adhesion mechanism involv-

TABLE 11. *C. jejuni* HC adhesin properties

Property	LPS	Flagella
Binds to INT 407 cells	+	+
Binds to mucus	+	−
Fucose-sensitive binding	+	−
Glutaraldehyde-sensitive INT 407 cell receptor	+	+
Blocks attachment of whole bacterial cells to INT 407 cells	+	−

ing LPS and epithelial cell protein receptors has been described for *S. flexneri* (11).

LPS was also effective in blocking the attachment of whole *C. jejuni* HC cells to the epithelial cells, whereas flagella were not. Nonspecific, hydrophobic interactions between the flagellar protein bound to the epithelial cells and the *Campylobacter* cell surface may actually encourage adhesion. A summary comparing properties of the LPS and flagella adhesins is presented in Table 11.

C. jejuni is an unusual enteric pathogen in that its LPS does not have an extensive O side chain (18). The presence of a rough LPS may be a significant factor in the ability of *C. jejuni* to colonize the epithelium. Myhal et al. (21) showed that fecal *E. coli* strains that were devoid of the O side chains of LPS were better able to colonize mice than fecal strains that had smooth-type LPS molecules. Later, Cohen et al. (7) showed that smooth-type LPS from fecal *E. coli* strains specifically bound to intestinal mucus glycoproteins. The lack of an extensive LPS O side chain may reduce the binding between *C. jejuni* and the mucus glycoproteins. Consequently, movement through the intestinal mucus to the underlying epithelium would be relatively unimpeded. Structural heterogeneity of LPS molecules has been reported (18) among strains of *C. jejuni* and may contribute to differences in cell and mucus adhesion.

The effectiveness of colonization of various sites within the intestine is probably due to the interplay among motility, chemotaxis (9), and adhesion characteristics of *C. jejuni*. Hugdahl and Doyle (10) found that mucus is chemotactic for *jejuni*; serine and fucose, both major components of mucus glycoproteins, are chemoattractants for *C. jejuni*. This finding is consistent with that of Lee et al. (16), who showed that *C. jejuni* colonizes the mucus-filled crypts of the intestine and suggested that this may be an important step in the pathogenesis of *Campylobacter* infection. Mucus penetration is undoubtedly facilitated by the spiral shape and darting motility of *Campylobacter*. It is important, therefore, that flagella do not show specific binding to the intestinal mucus gel. It would be advantageous for *C. jejuni* to be directed toward the mucus but not to have the flagellum interact with the fucose-bearing glycoproteins of the mucus layer.

In contrast to the intestinal mucus, the epithelial cell surface appears to be the preferred binding site of both the flagellum and the LPS. Greater quantities of bacterial cells, LPS, and flagellum protein were associated with the epithelial cells than with the mucus. This suggests that the epithelial cell surface contains more, or perhaps better, *Campylobacter* receptors than does the intestinal mucus. Whether a given strain of *C. jejuni* actually reaches the epithelium, however, could depend on its interactions with the mucus gel.

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