Enhanced Microscopic Definition of \textit{Campylobacter jejuni} 81-176 Adherence to, Invasion of, Translocation across, and Exocytosis from Polarized Human Intestinal Caco-2 Cells

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Received 18 October 2007/Returned for modification 21 November 2007/Accepted 26 August 2008

\textit{Campylobacter jejuni}-mediated pathogenesis involves gut adherence and translocation across intestinal cells. The current study was undertaken to examine the \textit{C. jejuni} interaction with and translocation across differentiated Caco-2 cells to better understand \textit{Campylobacter}’s pathogenesis. The efficiency of \textit{C. jejuni} 81-176 invasion of Caco-2 cells was two- to threefold less than the efficiency of invasion of INT407 cells. Adherence-invasion analyses indicated that \textit{C. jejuni} 81-176 adhered to most INT407 cells but invaded only about two-thirds of the host cells over 2 h (two bacteria/cell). In contrast, only 11 to 17\% of differentiated Caco-2 cells were observed to bind and internalize either \textit{C. jejuni} strain 81-176 or NCTC 11168, and a small percentage of infected Caco-2 cells contained 5 to 20 internalized bacteria per cell after 2 h. Electron microscopy revealed that individual \textit{C. jejuni} cells adhered to the tips of host cell microvilli via intimate flagellar contacts and by lateral bacterial binding to the sides of microvilli. Next, bacteria were observed to bind to the apical host membrane surface via presumed interactions at one pole of the bacterium and with host membrane protrusions located near intercellular junctions. The latter contacts apparently resulted in coordinated, localized plasma membrane invagination, causing simultaneous internalization of bacteria into an endosome. Passage of this \textit{Campylobacter} endosome intracellularly from the apical surface to the basolateral surface occurred over time, and bacterial release apparently resulted from endosome-basolateral membrane fusion (i.e., exocytosis). Bacteria were found intercellularly below tight junctions at 60 min postinfection, but not at earlier times. This study revealed unique host cell adherence contacts, early endocytosis-specific structures, and a presumptive exocytosis component of the transcellular transcytosis route.

\textit{Campylobacter jejuni} is a leading bacterial cause of human diarrheal disease. The U.S. Centers for Disease Control and Prevention estimates that \textit{C. jejuni} causes illness annually in 1\% of the population in the United States. \textit{Campylobacter} infection usually causes watery to bloody diarrhea with fever, abdominal cramps, and the presence of fecal leukocytes. The results of intestinal biopsies of patients, infected primates, and several other experimental model animals, as well as cell culture assays, have demonstrated that \textit{C. jejuni} is able to invade enterocytes and suggest that gut adherence, invasion, and mucosal translocation are important steps in pathogenesis (20, 29, 46).

The intestinal mucosa forms a barrier that protects against invasion of the host by nonpathogenic bacteria residing in the intestinal lumen. Some enteropathogens, such as \textit{Salmonella}, \textit{Shigella}, and \textit{Yersinia}, have specific tissue-invading capabilities and can physically breach the intestinal mucosal barrier (32). Pathogen-induced traversal of the human intestinal mucosa may involve crossing of absorptive enterocytes or passage through the specialized M cells (14–16). In addition to transcellular entry, some bacteria can disrupt the tight junctions between enterocytes and cross via paracellular mechanisms (47). \textit{Salmonella enterica} serovar Typhimurium may also cross the intestinal mucosa by sequestering itself within luminal CD18-positive mononuclear cells that shuttle the microbe paracellularly across the villus epithelium into the systemic circulation (48). \textit{Shigella} (45) and possibly \textit{Campylobacter} (49) may also breach the intestinal barrier by transcytosis across M cells. \textit{C. jejuni} has previously been reported to undergo transcellular translocation across the mucosa; there have also been reports of paracellular translocation of \textit{Campylobacter} (7, 9–12, 25, 28, 35, 47, 49).

Cultured mammalian cells are commonly used as a simple model for investigating the host epithelial cell-bacterium interaction that is more easily controlled than whole animals. Caco-2 cells were derived from transformed human colonic carcinoma cells. During 7 to 10 days in culture, these cells form polarized monolayers that have intercellular tight junctions and defined apical and basolateral surfaces. The differentiated apical surface has dense brush border microvilli containing normal intestinal brush border enzymes and antigens (13, 18, 43, 44). A key advantage of differentiated Caco-2 cells is that they provide a substrate monolayer similar to that for which \textit{Campylobacter} shows a natural tropism in vivo. This cell line allows assessment of the ability of \textit{C. jejuni} to penetrate and pass through a differentiated intestinal epithelial barrier, a process that appears to be important in causing overall colonic damage and the

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Published ahead of print on 2 September 2008.
occasional bacteremia associated with *Campylobacter* enterococlistis (9, 10, 26, 28).

*C. jejuni* 81-176 is a well-characterized strain which exhibits relatively high levels of epithelial cell invasion in cultured host cell assays and causes a bloody colitis, as demonstrated in human challenge studies (6; D. Tribe, unpublished data). Cell culture invasion assays conducted with inhibitors of cytoskeletal function initially demonstrated that this strain invades epithelial cells apically via a novel mechanism that is strongly microtubule (MT) dependent (38). Subsequent immunofluorescence studies of this 81-176 invasion process confirmed the involvement of MTs, demonstrated that the MT-associated molecular motor dynein is required for this uptake process, and defined the kinetics of INT407 cell invasion by *C. jejuni* 81-176 (19). Recent work has also shown that Ca$^{2+}$ release from host intracellular stores is essential for *C. jejuni* 81-176 internalization into host cells (21). In addition, host signal transduction studies have suggested that strain 81-176 interacts at filipin III-sensitive membrane microdomains of the host cell surface with G-protein-coupled receptors, a process which activates phosphatidylinositol 3-kinase and mitogen-activated protein kinases that appear to be intimately involved in events controlling 81-176 internalization (22). Very recently, the rho GTPases Rac1 and Cdc42 have been implicated in the 81-176 invasion process (31). It is also noteworthy that other researchers, using different invasion assay conditions, have observed mixed involvement of both MTs and microfilaments in 81-176 invasion (5, 36). Watson and Galan (51) recently demonstrated that following internalization into host cells *C. jejuni* 81-176 survives within modified endosomes that avoid fusion with lysosomes.

Based on previous *Campylobacter* pathogenesis studies with cultured cells, animals, and humans (4–8, 10, 12, 17, 19–22, 23–40, 46, 49–53), we hypothesize that during the first few hours of infection *C. jejuni* adheres to and crosses differentiated intestinal epithelial cells by a transcellular process which does not disrupt transepithelial electrical resistance (TER) (transcytosis is apical endocytosis followed by basolateral exocytosis). Thus, a kinetic examination of *C. jejuni* internalization and microscopic analyses of the events involving adherence, invasion, and transcytosis over a 4-h period were conducted with the goal of obtaining an enhanced understanding of *Campylobacter*-host cell interactions. Importantly, this study provided new information about entry kinetics and revealed new specific bacterium-host cell interactions involved in epithelial cell translocation that have not been observed in previous electron microscope (EM) studies of *Campylobacter* entry mechanisms (4, 10, 26, 28, 31).

**MATERIALS AND METHODS**

**Bacterial strains, cell lines, media, and culture conditions.** The experiments were conducted using *C. jejuni* 81-176, a well-studied strain that was originally obtained from a *Campylobacter* disease outbreak in Minnesota (30). A $\Delta cheY$ mutant of 81-176 designated RY213 (53), which is basically nonadherent and noninvasive, was used as a nonadherent control. Also, genome-sequenced strain NCTC 11168 was employed for adherence comparisons. *C. jejuni* was grown in Mueller-Hinton biphase medium and on Mueller-Hinton agar (Difco) under a *Campylobacter* microaerophilic atmosphere containing 10% O$_2$, 5% CO$_2$, and 85% N$_2$. Immortalized human colon cancer cells (Caco-2) and human embryonic intestinal epithelial cells (INT407), obtained from the American Type Culture Collection, were maintained in liquid nitrogen and cultured in minimal essential media with 20% (for Caco-2 cells) or 10% (for INT407 cells) heat-inactivated fetal calf serum (FCS) (Gibco), 2 mM l-glutamine, and 0.1 mM nonessential amino acids, as recommended by the American Type Culture Collection. The relative state of cell differentiation and maintenance of tight junctions were monitored by determining monolayer TER with a dual-voltage ohmmeter (Millicel-ERS; Millipore) (3), and brush border organization was verified by EM.

**Invasion assays.** An invasion assay was performed essentially as described previously (19). Epithelial cells at a concentration of $10^5$ cells/ml were added to each well of a 24-well plate and incubated for 1 to 15 days depending on the degree of differentiation desired. The cell culture medium was replaced daily. Bacteria in minimal essential medium were added to 1 ml of culture medium (containing heat-inactivated FCS as described above) in each well. The multicility of infection (MOI) was varied, as indicated below. Infected monolayers, which were not centrifuged, were typically incubated for 2 h at 37°C in a 5% CO$_2$/95% air atmosphere to allow invasion to occur. For time course analyses, the invasion period was varied from 0 to 120 min. Following the invasion period, the monolayer was washed three times with Earle’s balanced salt solution and incubated for another 2 h in fresh tissue culture medium with 10% FCS plus 100 µg/ml gentamicin to kill extracellular bacteria. After the gentamicin-killing period, the infected monolayers were washed as described above, and the host cells were lysed using 0.1% Triton X-100 in phosphate-buffered saline (PBS) for 15 min at room temperature on an orbital shaker. Following serial dilution in PBS, released intracellular bacteria were enumerated by colony counting on Mueller-Hinton agar incubated at 37°C under microaerophilic conditions. Each internalization assay was performed simultaneously in two separate wells and was repeated on at least three separate occasions. The results were expressed as means ± standard errors of the means. Control experiments confirmed that 100 µg/ml gentamicin killed essentially all (99.99%) extracellular bacteria within 2 h; any remaining bacteria after gentamicin killing had no appreciable effect on calculation of the invasion kinetics over the time period studied.

**Fluorescence microscopy.** To distinguish extracellular bacteria from internalized bacteria, an immunofluorescence procedure that rendered internalized bacteria red and noninternalized bacteria green was used. For these assays, host cells were grown as monolayers on 13-mm glass coverslips placed in 24-well culture plates and infected with *C. jejuni* for 2 h; any remaining bacteria after gentamicin killing had no appreciable effect on calculation of the invasion kinetics over the time period studied.

**Scanning and transmission EM.** The ability of *C. jejuni* to pass through confluent, polarized Caco-2 cell monolayers grown on Transwell filters was determined as a measure of bacterial translocation across the host epithelial cell barrier. Polarized Caco-2 monolayers were prepared by seeding $10^5$ cells on a Transwell clear polyester membrane (pore size, 3 µm; diameter, 6.5 mm; Millipore Corp.), followed by growth for $\geq$7 days. The integrity of tight junctions was monitored by measuring TER. Preliminary studies revealed that the Caco-2 cells differentiated faster in the presence of 20% FCS than in the presence of lower serum concentrations. Both 7- and 15-day-old Caco-2 monolayers displayed high TER (1,000 Ω/cm$^2$), developed dense microvilli, and showed identical *C. jejuni* invasion kinetics (Fig. 1). For scanning EM, filters carrying monolayers infected with *C. jejuni* 81-176 or NCTC 11168 were assayed three times in PBS and then immersed for 2 h at 4°C in primary fixative (pH 7.2) consisting of 4% paraformaldehyde, 1% glutaraldehyde, and 0.1 M sodium cacodylate. The specimens were rinsed three times in 0.1 M cacodylate buffer (pH 7.2), postfixed for 1.5 h in 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.2), rinsed three times in 0.1 M cacodylate buffer (pH 7.2), dehydrated gradually using 30, 50, and 70% ethanol, and brought to room
RESULTS

C. jejuni invasion efficiencies with undifferentiated and differentiated host cells. Caco-2 cells cultured in 24-well plates for 1, 7, or 15 days were infected with C. jejuni 81-176 at different MOIs, and the invasion was compared to invasion of young INT407 cells. Caco-2 cells differentiated significantly and formed tight junctions by 7 days in culture, as indicated by the development of TER typically greater than 1,000 Ω/cm². Host cells were incubated initially for 2 h in the presence of C. jejuni, which was followed by washing and then incubation for another 2 h in the presence of gentamicin prior to enumeration of internalized bacteria. The highest efficiency of invasion of Caco-2 cells (2%) was observed at the lowest MOI (0.02) in 1-day-old cells. It is noteworthy that this level of invasion is about 50% of the level observed with INT407 cells. C. jejuni entered 7- or 15-day-old differentiated Caco-2 cells at an efficiency that was even lower. The invasion efficiency decreased gradually, but not markedly, at higher MOIs, and eventually the efficiencies were equal for INT407 and Caco-2 cells (Fig. 1A).

Figure 1B shows the average number of internalized bacteria per cell, which was calculated by dividing the number of internalized bacteria by the total number of host cells per well at each MOI tested. It is noteworthy that not all host cells were infected, as discussed below. With undifferentiated host cells (INT407 cells or 1-day-old Caco-2 cells), the highest number of internalized bacteria was approximately 1.5 to 2.0 bacteria per host cell, which was observed only at MOIs of >100. Fewer bacteria entered differentiated 7- or 15-day-old Caco-2 cells (average, ~0.3 bacterium/cell), and the levels obtained required MOIs of >200 (Fig. 1B).

Distribution of bacteria interacting with differentiated Caco-2 cells. Differentiated Caco-2 monolayers on coverslips were infected with C. jejuni for times ranging from 10 to 120 min. Infected monolayers were then stained by using a fluorescence assay to quantify all cell-associated bacteria or to distinguish intracellular bacteria from extracellular bacteria. As shown in Table 1, entry of C. jejuni was observed as early as 10 min. The total number of internalized C. jejuni bacteria (Fig. 1B) and the number of infected host cells (Table 1) increased at each time point up to 2 h postinfection with either undifferentiated INT407 cells or differentiated Caco-2 cells. However, the distributions of internalized bacteria were dramatically different for INT407 and Caco-2 cells. Approximately two-thirds of INT407 cells were infected by C. jejuni after 2 h, and each infected host cell contained about two internalized C. jejuni bacteria. In contrast, only ~20% of differentiated Caco-2 cells were infected after 2 h, and ~10% of the infected Caco-2 cells (~2% of all host cells) appeared to be highly invasion susceptible and contained 7 to 20 internalized bacteria. Notably, the internalized bacteria were distributed unevenly in differentiated Caco-2 cells, compared with the more even distribution of two internalized C. jejuni bacteria per invasion-susceptible INT407 cell. Also, a much smaller percentage of differentiated Caco-2 cells than of INT407 cells were susceptible to infection after 2 h.

When the total bacteria associated with host cells over 2 h were analyzed, ~80% of INT407 cells contained surface-bound C. jejuni 81-176 bacteria (data not shown), but only 15
Undifferentiated 1-day-old INT407 cells

<table>
<thead>
<tr>
<th>Infection time (min)</th>
<th>% of host cells infected</th>
<th>No. of internalized bacteria/infected cell (range)</th>
<th>% of infected cells with 1 to 5 internalized bacteria</th>
<th>% of infected cells with ≥7 internalized bacteria</th>
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<tr>
<td>10</td>
<td>5 ± 2</td>
<td>1–2</td>
<td>100</td>
<td>0</td>
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<td>30</td>
<td>22 ± 4</td>
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<td>0</td>
</tr>
<tr>
<td>60</td>
<td>51 ± 6</td>
<td>1–3</td>
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<tr>
<td>120</td>
<td>68 ± 4</td>
<td>1–3</td>
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Differentiated 7-day-old Caco-2 cells

<table>
<thead>
<tr>
<th>Strain</th>
<th>% of host cells infected</th>
<th>No. of internalized bacteria/infected cell (range)</th>
<th>% of infected cells with 1 associated bacterium</th>
<th>% of infected cells with 2 associated bacteria</th>
<th>% of infected cells with 3 to 5 associated bacteria</th>
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<tr>
<td>81-176</td>
<td>28.02 ± 3.48</td>
<td>1–3</td>
<td>77</td>
<td>20</td>
<td>3</td>
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<tr>
<td>NCTC11168</td>
<td>13.36 ± 1.04</td>
<td>1–3</td>
<td>81</td>
<td>17</td>
<td>2</td>
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<tr>
<td>RY213</td>
<td>1.34 ± 0.30</td>
<td>≥1</td>
<td>100</td>
<td>0</td>
<td>0</td>
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</table>

Undifferentiated 1-day-old INT407 cells

<table>
<thead>
<tr>
<th>Strain</th>
<th>% of host cells infected</th>
<th>No. of internalized bacteria/infected cell (range)</th>
<th>% of infected cells with 1 associated bacterium</th>
<th>% of infected cells with 2 associated bacteria</th>
<th>% of infected cells with 3 to 5 associated bacteria</th>
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<td>1–10</td>
<td>50</td>
<td>20</td>
<td>30</td>
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<tr>
<td>NCTC11168</td>
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<td>1–7</td>
<td>62</td>
<td>20</td>
<td>18</td>
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<tr>
<td>RY213</td>
<td>1.94 ± 0.39</td>
<td>1–3</td>
<td>77</td>
<td>12</td>
<td>10</td>
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Differentiated 7-day-old Caco-2 cells

<table>
<thead>
<tr>
<th>Strain</th>
<th>% of host cells infected</th>
<th>No. of internalized bacteria/infected cell (range)</th>
<th>% of infected cells with 1 associated bacterium</th>
<th>% of infected cells with 2 associated bacteria</th>
<th>% of infected cells with 3 to 5 associated bacteria</th>
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<tr>
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<td>22</td>
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<td>0.92 ± 0.18</td>
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*Adherence assays were performed using an MOI of ~10. Caco-2 and INT407 cells were cultured for 1 to 7 days as described in Materials and Methods.*
bind via flagella to Caco-2 cell microvilli (Fig. 2). These events often occurred near a tight junction. Although the infected monolayers were washed thoroughly three times in PBS before fixatives were added, we occasionally observed *Campylobacter* bound perpendicularly to the host cell surface. Because of the considerable washing during preparation, we believe that this event was not happenstance and reflected specific binding at one pole of the bacterium to the plasma membrane (Fig. 3A). We presumed that the polar flagella may have been bent out of the plane of sectioning and that only the proximal tips of the flagella could be seen (Fig. 3A). Figure 3B shows several bacteria apparently in different stages of the internalization process. These bacteria are associated with an irregularly shaped membrane protrusion, which may represent shortened, coalesced microvilli at the Caco-2 cell surface. Several bacteria appear to be bound to microvilli or vestigial microvilli. One bacterium has apparently just been internalized and is in an endosome, as verified recently (51). At least two bacteria appear to have been internalized previously and are in endosomes which have moved to the base of a membrane protrusion. The membrane protrusions appear to be activated sites for the uptake of multiple *C. jejuni* bacteria. Transmission EM examination at 60 min revealed an occasional bacterium in the junctional space (Fig. 3C), while another bacterium was adjacent to this space but appeared to be transitioning from or to the junction.

At 1 h postinfection, *C. jejuni* 81-176 bacteria were still attached via flagellar contacts to microvilli, but the microvilli were thicker and shorter and many of the microvilli had collapsed, leaving smooth areas visible on the host cell surface (Fig. 2F). Some bacteria appeared to be engulfed by invagination of the plasma membrane (Fig. 2F). A highly enlarged transmission EM micrograph of *C. jejuni* NCTC 11168 revealed the previously unobserved, convoluted, brainlike, spiral surface of *C. jejuni* that may play a role in interaction with host cells (Fig. 2G).

In contrast to the observations made with Caco-2 infected cells, EM of INT407 cells during the initiation of *Campylobacter* invasion revealed a similar, but smaller, host cell membrane extension, which was about the size of *Campylobacter*. Thus, *Campylobacter* cells interacted at the INT407 cell surface and caused formation of a small membrane extension, a protrusion, or coalesced microvilli through which the *Campylobacter* cells were internalized by a membrane invagination event (Fig. 3D); also, there was an adjacent tight junction.

**C. jejuni 81-176 translocation involves a discrete exocytosis event.** By 60 min postinfection, the ultrastructural evidence revealed movement of internalized *Campylobacter* cells within endosomal vacuoles from the host cell apical domain to the basolateral host cell domain. Figure 4A shows three bacteria in separate endosomes, at least two of which are associated with the perinuclear region. Figure 4B shows a Caco-2 cell containing as many as 16 bacteria, and most of these bacteria were clearly within endosomes near the basolateral surface at 1 h postinfection. After moving within endosomes, first perinuclearly and then to the basolateral host surface (Fig. 4C and D), bacteria are released by an apparent exocytosis event at the basolateral host cell surface. Figure 4C shows two bacteria near the basolateral surface; one bacterium is outside the host cell but above the transwell filter surface, and one bacterium is apparently in the process of being released from a cell by exocytosis. Figure 4D is an enlarged image of the exocytosis event shown in Fig. 4C. The endosome containing *C. jejuni* has presumably fused with the basolateral host cell membrane, creating a pore through which the bacterium is being released to the extracellular space, completing a transcellular transcytosis process. This exocytosis event is not a simple reversal of the endocytosis mechanism shown in Fig. 3B and presumably occurs by a separate, unique process. However, although we consider it less likely based on our chronological studies, we cannot rule out the possibility that this event represented basolateral endocytosis of bacteria that entered paracellularly.

**DISCUSSION**

The current report describes an extension of our previous studies aimed at characterizing *C. jejuni* 81-176 invasion. The kinetics of *C. jejuni* 81-176 entry into differentiated Caco-2 cells showed a pattern with increasing MOIs similar to the pattern observed for INT407 cells, but the overall level of bacterial uptake was lower (Fig. 1). The invasion efficiency was highest at the lowest MOI (0.02), suggesting that single bacteria can trigger their own entry (29). Bacterial internalization into 7- or 15-day-old differentiated Caco-2 cells was reduced most notably (Fig. 1B). Bacterium-host cell association assays revealed that ~80% of INT407 cells contained adherent bacteria but that only 15 to 20% of differentiated Caco-2 cells contained bound bacteria. For example, entry into 1-day-old INT407 cells occurred as early as 10 min and increased over 2 h, after which 68% of the host cells were consistently infected with two internalized bacteria per host cell (Table 1). In striking contrast, only 20% of polarized Caco-2 cells had internalized *C. jejuni* bacteria after 2 h, and the number of internalized bacteria per host cell varied from 1 to 20. Thus, far fewer
differentiated Caco-2 cells than INT407 cells were infected over a 2-h period, suggesting that host “invasion receptor” availability may be decreased following differentiation. We speculate that this could be due to basolateral sequestering of receptors. In contrast to the even distribution of internalized \textit{C. jejuni} bacteria in infected undifferentiated INT407 cells, the distribution in differentiated Caco-2 cells was uneven, and \textasciitilde2\% of Caco-2 cells contained 7 to 20 intracellular bacteria per cell, indicating that a small percentage of differentiated cells are hypersusceptible to \textit{Campylobacter} invasion. These cells may represent M-like cells, the domes of which have been observed with differentiated Caco-2 cells and which collapse during \textit{C. jejuni} infection (33). Recently, coculture of Caco-2 cells with Raji B lymphocytes has been shown to result in the differentiation of M-like cells, defined by expression of surface galectin-9 (42). Unlike the situation for mouse M cells, there

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**FIG. 3.** Transmission EM micrographs showing apparent stages in \textit{C. jejuni} 81-176 invasion of Caco-2 and INT407 host cells. (A) \textit{C. jejuni} interacting end-first with a bald (microvillus-free) host cell apical surface, apparently penetrating the host cell with its polar flagellum (10 min postinfection; MOI, \textasciitilde100). The proximal end of each polar flagellum is indicated by an arrow; thin sectioning apparently removed most of the flagella. A nearby tight junction (TJ) at the bottom of the micrograph is indicated by an arrow. Bar = 0.5 μm. (B) Bacteria associated with an irregularly shaped, “invasion-activated” membrane protrusion of the Caco-2 apical cell surface. Three bacteria (asterisks) are interacting with apparent vestigial microvilli, one \textit{C. jejuni} cell has presumably just been engulfed by endocytosis (arrow), and two bacteria were apparently previously internalized (arrowheads) and have moved to the base of the membrane protrusion (10 min postinfection). Bar = 0.5 μm. (C) At later times after infection, \textit{C. jejuni} bacteria (arrow) are occasionally found in junctional spaces (TJ, tight junction); these bacteria may have entered paracellularly, followed by junctional readherence, or could have exited the host cell laterally after they were first endocytosed (60 min postinfection). Bar = 1 μm. (D) Small “invasion-activated” membrane protrusion of an INT407 host cell through which a \textit{C. jejuni} bacterium (arrow) has apparently just been internalized. This event occurred immediately adjacent to a cellular tight junction (10 min postinfection; MOI, \textasciitilde100). Bar = 0.2 μm.
are no commercially available antibodies to identify human M cells, which prevented confirmation of this interesting possibility.

Both scanning EM and transmission EM were used to examine the early *Campylobacter*–Caco-2 cell interactions in differentiated monolayers grown on Transwell filters. Scanning EM revealed unique torsional contacts between the sides of flagella and microvillus tips, in contrast to previous reports of flagellar tip adherence (26, 28), possibly due to the higher-resolution images obtained in the current study. Both *C. jejuni* 81-176 and NCTC 11168 were typically tethered at the host surface by each polar flagellum binding laterally to different microvillus tips (Fig. 2A, B, E, and G), an interaction which suggests that there is specific binding between the sides of flagella and components of the rounded tips of microvilli.

Transmission EM revealed additional contacts between the bacterial cell surface and the sides of microvilli (Fig. 2C and D). The latter contacts may involve capsular polysaccharide, Peb1, JlpA, or other outer membrane proteins which have been reported to be involved in adherence (2, 24, 39–41). *C. jejuni* has a brainlike convoluted surface (Fig. 2G), which may play a role in host cell-bacterium interactions. We speculate that ligands located at the apical surface (instead of deep in the folds) of the convolutions may be more active in host cell interactions. The bacterium-host cell interactions described above were readily observed early and throughout the invasion period. We presume that they represent different types of adherence, some of which may be reversible.

**FIG. 4.** Intracellular transport within endosomes and *C. jejuni* 81-176 exocytosis from Caco-2 cells. (A) Caco-2 cell containing three intracellular bacteria. One bacterium (arrow) close to apical surface appears to have just been internalized. The remaining two bacteria (asterisks) are in separate endosomes and have migrated to the perinuclear region (N, nucleus) (30 min postinfection; MOI, 100). Bar = 0.5 μm. (B) Multiple bacteria in endosomes at the basolateral host cell surface at 1 h postinfection. The membrane filter is below the cell. Bar = 1 μm. (C) At 45 to 90 min postinfection, *C. jejuni* endosomes are transported to the basolateral surface (membrane filter) and are released by endosome-plasma membrane fusion, resulting in bacterial exocytosis. Bar = 1 μm. (D) Enlargement of panel C, more clearly showing the exocytosis event at the basolateral surface of the host cell. The arrow indicates a pore created by membrane fusion of the endosome with the host basolateral membrane. The edge of the transwell filter is at bottom right of the micrograph.
Flagella have long been implicated as *Campylobacter* adhesins (34, 37). Flagellum-dependent motility and the flagellum itself appear to be essential for *C. jejuni* invasion of epithelial cells, but the mechanism(s) remains unknown (1, 12, 50, 52, 53). It seems unlikely that the *Campylobacter* flagellar adherence is casual because the infected monolayers were washed six times with Earle’s balanced salt solution prior to fixation for scanning EM, a process that involves an additional 10 washes and buffer changes. Although most EM studies were performed with an MOI of 100, using an MOI of 10 resulted in similar bacterium-host cell interactions, but the frequencies were much reduced. We speculate that *C. jejuni* flagellum-host cell interactions represent a primary adherence mechanism that augments the contact of “invasion-specific” bacterial ligands with host membrane receptors in lipid rafts, which result in signal transduction events that lead to bacterial internalization. Since ~10% of the invasion ability can be restored to nonflagellated *C. jejuni* cells by centrifugation onto a monolayer (50), we suggest that “invasion-specific” *C. jejuni* ligands may be located at the bacterial poles, which are normally adjacent to the polar flagella in wild-type *C. jejuni*. Strain NCTC 11168 is known to be less invasive than 81-176, possibly due to reduced numbers of “invasion-specific” ligands or expression of surface factors that interfere with these ligands.

*C. jejuni* cells were observed to interact typically with microvilli at the apical Caco-2 cell surface adjacent to intercellular junctions, as noted previously (28). Very infrequently, *Campylobacter* bacteria were observed bound perpendicular to the host cell, possibly with one polar flagellum interacting with the host cell (Fig. 3A); a similar observation of perpendicular bacterial interaction was recently reported (31). This is a relatively difficult event to observe, possibly because the organism is internalized rapidly, and it is technically difficult to find a thin section containing such a physically space-limited event. Figure 3A shows that the local host membrane surface lacks the typical dense microvilli shown in Fig. 2D, and this may reflect a differentiated M-like cell surface. We suggest that intimate bacterium-host cell contact, as shown in Fig. 3A, B, or D, may be “invasion specific” and that binding of bacterial ligands with specific host plasma membrane receptors may have triggered a localized collapse of the terminal F-actin web, leading to shortening and coalescence of microvilli. Previously, we and other workers have reported that pretreatment of monolayers with cytochalasin D actually enhances *Campylobacter* invasion (19, 38, 51), possibly by eliminating the terminal actin web. Thus, initial actin depolymerization and subsequent polymerization are probably involved in *Campylobacter* entry. In fact, a recent report showed that Rac1 and Cdc42 are involved and that there may be membrane ruffling prior to *C. jejuni* invasion (31). We have no evidence of effector secretion into host cells via the bacterium-host cell interaction shown in Fig. 3A, but we cannot exclude this interesting possibility. Konkel and coworkers (27) suggested that effector secretion is important in *Campylobacter* invasion.

An early membrane dynamic event observed during *Campylobacter* entry is the formation of an activated membrane protrusion, which may represent a coalescence of local microvilli. We speculate that invasion-specific bacterium-host cell contact may promote host signaling events that activate the membrane in a localized region, leading to bacterial internalization through this “activated” site (Fig. 3B). “Activation” may encompass host signaling events, as well as cytoskeletal and membrane alterations. In differentiated Caco-2 cells, this “activated” membrane protrusion is approximately the length of one bacterium wide and 2 to 3 lengths high and can apparently undergo multiple bacterial internalization events. In contrast, the comparable invasion-specific, “activated” membrane extension observed in undifferentiated INT407 cells during *C. jejuni* invasion is smaller and apparently engulfs a single organism (Fig. 3D). Regardless of the host membrane protrusion size, bacterial internalization apparently results from plasma membrane invagination that begins at the bacterium-host cell contact site (19, 26, 29, 31). As observed previously (28, 38), the bacteria are internalized into an endosome, which is transported over time from the apical host surface to the basolateral host surface (Fig. 4). Our previous confocal microscopic analyses suggested that *C. jejuni* 81-176 cells are transported in endosomes via the molecular motor dynein along MTs (19). Watson and Galan (51) recently reported that *Campylobacter* cells reside and transit within special endosomes that avoid fusion with lysosomes. At later infection times, an occasional bacterium is observed in the intercellular space below apical junctional adherence (Fig. 3C). This event has not been observed commonly or earlier in infection, leading us to suggest that this bacterium just exited the host cell laterally; however, the possibility of paracellular entry from the apical surface followed by junctional resealing cannot be ruled out (8). Based on the current study, we suggest that *C. jejuni* bacteria in endosomes are transported basolaterally through the cell and can undergo specific endosome-basolateral membrane fusion (Fig. 4D), an exocytosis process which releases *C. jejuni* subepithelially. This process does not visually appear to be a simple reversal of the apical endocytic mechanism. Although we consider a basolateral endocytosis event unlikely, this possibility cannot be ruled out by the results of EM. Previous reports indicated that the ability of *C. jejuni* to invade Caco-2 cells is not linked to translocation ability (7, 17), also suggesting that endocytosis and exocytosis are unique mechanistic events. Based on these observations, we speculate that *C. jejuni* bacteria proficient in transcytosis across the epithelium may be able to alter the *Campylobacter*-endosome membrane so that they traffic through the cell and can fuse with the host plasma membrane, resulting in basolateral exocytosis. Since monolayer TER is decreased at later infection times (33) and alterations in tight junction proteins have been observed at 24 to 48 h postinfection (8, 33), it seems reasonable to suggest that intercellular junction damage may occur at later times, following *C. jejuni* transcytosis across the intestinal epithelium.

In summary, this study revealed new specific *C. jejuni*-host cell interactions and structures involved in adherence to, invasion of, and translocation across differentiated Caco-2 cells that were not observed in previous studies. *C. jejuni* interactions with differentiated Caco-2 cells likely are more representative of events that actually occur in the intestine; nevertheless, INT407 cells have provided much useful information on *C. jejuni* adherence and invasion mechanisms. In contrast to the relatively consistent level of 2 internalized bacteria per undifferentiated INT407 cell, certain differentiated Caco-2 cells appeared to be hypersusceptible to invasion and contained as many as 20 internalized *C. jejuni*.
bacteria. Whether these hypersusceptible Caco-2 cells are human M-like cells could not be verified due to a lack of available reagents. Campylobacter transcytosis appears to involve discrete endocytic and exocytic events not previously observed by transmission EM. Together, these findings provide an improved concept for C. jejuni 81-176 adherence, endocytosis, and exocytosis mechanisms and indicate potentially important processes and structures that should be characterized further.

ACKNOWLEDGMENTS

We thank Timothy K. Maugel for technical help with the scanning EM and Michael Schmitt and Alain Debrabant for critical reviews of the manuscript.

REFERENCES


