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NOTES

Altered Synthetic Response of *Campylobacter jejuni* to Cocultivation with Human Epithelial Cells Is Associated with Enhanced Internalization

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***Campylobacter jejuni* has been shown to bind to and enter epithelial cells in culture. The interaction of *C. jejuni* with INT 407 epithelial cells was examined to determine whether bacterial protein synthesis is required for either binding or internalization. Chloramphenicol, a selective inhibitor of bacterial protein synthesis, significantly reduced the internalization, but not binding, of *C. jejuni* compared with untreated controls as determined by protection from gentamicin. Electrophoretic analysis of metabolically labeled proteins revealed that *C. jejuni* cultured with INT 407 cells synthesized 14 proteins that were not detected in organisms cultured in medium alone. The inhibitory effect of chloramphenicol on internalization was reduced by preincubation of *C. jejuni* with INT 407 cells. The results indicate that *C. jejuni*, like some other enteric pathogens, engages in a directed response to cocultivation with epithelial cells by synthesizing one or more proteins that facilitate internalization and suggest that this phenomenon is relevant to the pathogenesis of enteritis caused by *C. jejuni*.**

Campylobacter jejuni is a major cause of human enteritis; however, the mechanisms by which this organism cause disease are poorly understood (3). There is evidence that enterotoxins and cytotoxins may be involved in the pathogenesis of *Campylobacter* enteritis (5, 15, 29). However, it has also been proposed that disease involves translocation of the bacterium across the intestinal epithelium, with resultant tissue damage and inflammation (31). This latter proposal is based upon examinations of rectal biopsy specimens from *C. jejuni*-infected patients and, more recently, by the demonstration of the ability of *C. jejuni* to bind to and enter cultured epithelial cells (1, 2, 7, 9, 16, 17, 19, 21). Although the relevance of the internalization of *C. jejuni* by cultured epithelial cells to in vivo behavior is largely presumptive, some evidence does suggest that such in vitro models may be useful in the elucidation of pathogenic mechanisms associated with *C. jejuni* enteritis. For example, Fauchere et al. (9) observed that *C. jejuni* isolated from patients with febrile diarrhea associated more frequently with cultured epithelial (HeLa) cells than did *C. jejuni* isolated from patients without diarrhea or fever.

To be able to promote entry into eukaryotic cells, invasive microorganisms must express products that bind to host cell receptors and facilitate subsequent internalization in a process that has been referred to as parasite-directed endocytosis (14, 24). Epithelial cell culture models have been used with increasing frequency to examine the interactions of bacterial pathogens with host cells because they are convenient and permit a detailed quantitative analysis of the mechanics of the internalization process (8). While the details of the mechanisms used by most invasive bacteria to enter mammalian cells are not precisely known, such models have shown that bacterial metabolic processes are required for efficient internalization of *Haemophilus influenzae* (30), *Neisseria gonorrhoeae* (6, 28), *Rickettsia prowazekii* (32), *Salmonella typhimurium* (10), and *Shigella flexneri* (11). These studies suggest that various pathogenic bacteria engage in adaptive synthetic responses that facilitate internalization and/or intracellular survival. In contrast, *Yersinia* spp. (13) and *Chlamydia* spp. (24, 25) appear to express constitutively those components required for adherence to and internalization within mammalian cells. Accordingly, we sought to examine the role of metabolic activity in the interaction of *C. jejuni* with epithelial cells.

Immunofluorescence microscopy was used initially to determine whether metabolically inactive *C. jejuni* bind to INT 407 (Henle) epithelial cells. INT 407 cell cultures were inoculated with UV-irradiated, heat-killed, or untreated *C. jejuni* and examined by indirect immunofluorescence microscopy. Previous studies have demonstrated that UV-irradiated bacteria are capable of protein synthesis but not cell division (11, 12). No difference was apparent in the number of inactive (heat-killed) and active (UV-irradiated and untreated) *C. jejuni* bound to the cells (data not shown). A fluorescence staining technique using acridine orange and crystal violet was used to determine whether inactive *C. jejuni* enter INT 407 cells. The staining technique allows differentiation between extracellular and intracellular bacteria and between viable and nonviable bacteria (23). Viable bacteria stain green, whereas nonviable bacteria stain red. Spiral, green-fluorescent (viable) *C. jejuni* were visible within the epithelial cells that had been inoculated with both the UV-irradiated and untreated bacteria. However, no intracellular bacteria were observed in cultures that had been inoculated with heat-inactivated bacteria (data not shown). The results obtained from the acridine orange-crystal violet staining assays were confirmed by transmis-

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TABLE 1. Effect of chloramphenicol (CM) on adherence to and internalization within INT 407 cells by *C. jejuni* and *S. typhimurium*

Organism	Adherence[a]		Internalization[b]	
	− CM[c]	+ CM[d]	− CM	+ CM
C. jejuni	$(3.0 \pm 1.0) \times 10^5$	$(5.2 \pm 0.7) \times 10^5$	$(1.6 \pm 0.4) \times 10^4$	$(3.3 \pm 1.5) \times 10^{2e}$
S. typhimurium	$(2.3 \pm 0.7) \times 10^6$	$(2.1 \pm 1.7) \times 10^7$	$(3.9 \pm 1.5) \times 10^5$	$(1.0 \pm 0.0) \times 10^{4e}$

[a] Determined after a 30-min incubation period. After incubation, the monolayers were washed several times and lysed with a solution of deoxycholate. The number of viable bacteria associated with the cells was determined by direct plate counts. The values represent the number of bacteria per well of a 24-well tissue culture tray and are given as the means of triplicate determinations ± the standard deviations.

[b] Determined after a 3-h incubation period in the presence of gentamicin (250 μg/ml). The number of gentamicin-protected bacteria was determined by direct plate counts after lysing the monolayers with a solution of deoxycholate. The values represent the number of gentamicin-protected bacteria per well of a 24-well tissue culture tray and are given as the means of triplicate determinations ± the standard deviations.

[c] Bacteria not treated with chloramphenicol (control).

[d] Bacteria that were treated with chloramphenicol. *C. jejuni* and *S. typhimurium* were treated with 64 and 32 μg of chloramphenicol per ml, respectively, for 45 min prior to and during the assay.

[e] Mean value significantly different ($P < 0.01$) from the mean value derived from untreated (− Cm) cultures as judged by the two-tailed Student's *t* test. For statistical analyses, the values were logarithmically transformed to achieve equality of variances.

sion electron microscopy. Numerous bacteria were observed within the epithelial cells infected with untreated *C. jejuni*, many fewer bacteria were observed within cells infected with UV-irradiated *C. jejuni*, and no bacteria were visible within cells exposed to the heat-killed organisms (data not shown). Electron microscopic examination of the infected cell cultures also revealed that the UV-irradiated and heat-killed bacteria were morphologically abnormal, as judged by the presence of filamentous material protruding from the bacteria and by the lack of intact cell walls, respectively. Therefore, *C. jejuni* cells were also inactivated with sodium azide, a respiratory inhibitor, to minimize the potential modification of bacterial surface components associated with other treatments. No bacteria were observed within cells in cultures inoculated with the sodium azide-treated bacteria, as judged by transmission electron microscopy, again indicating that metabolically inactive *C. jejuni* cells fail to enter cultured epithelial cells.

Collectively, the microscopic findings indicated that the internalization of *C. jejuni* into INT 407 epithelial cells requires active bacterial processes and raised the possibility that *C. jejuni* synthesizes entry-promoting proteins in response to altered environmental conditions associated with cultivation or interaction with epithelial cells. Therefore, the bacteria were treated with chloramphenicol, an inhibitor of bacterial protein synthesis, to determine whether protein synthesis is required for internalization. *Salmonella typhimurium* was used as a control in these experiments because protein synthesis has been demonstrated to be required for this organism to efficiently enter epithelial cells (10). Control experiments revealed that the concentrations of chloramphenicol employed completely inhibited the synthesis of bacterial protein, but had no detectable quantitative or qualitative effect on INT 407 cell protein synthesis, as judged by determination of total incorporation into protein and by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography, respectively. In addition, chloramphenicol had no detectable effect on bacterial viability. No significant differences were observed in the numbers of treated and untreated *C. jejuni* bound to the epithelial cells (Table 1). However, significant decreases in the numbers of organisms internalized among both chloramphenicol-treated *C. jejuni* and *S. typhimurium* were observed as measured by gentamicin protection assays (Table 1).

To characterize the synthetic response of *C. jejuni* during interaction with cultured cells, the electrophoretic profile of proteins synthesized by bacteria in the presence and absence of INT 407 cells was examined by metabolic labeling with [^{35}S]methionine in the presence of 2.5 μg of emetine hydrochloride per ml to selectively suppress eukaryotic cell protein synthesis. Initial single-dimension electrophoretic analyses (18) revealed that the cell-associated *C. jejuni* (adherent and internalized) synthesized a number of proteins in greater amounts and several proteins in lesser amounts when compared with bacteria cultured in medium alone (data not shown). The analyses also demonstrated that *C. jejuni* cultured in the presence of INT 407 cells synthesized a number of proteins that were not produced by *C. jejuni* cultured in the absence of epithelial cells. Further analyses using two-dimensional electrophoresis (26) revealed that *C. jejuni* produced at least 14 new proteins in response to cocultivation with INT 407 cells (Fig. 1). In addition, a number of proteins were synthesized by *C. jejuni* in medium alone that were not detectable in bacteria cultivated with epithelial cells (Fig. 1). The molecular masses of the protein species whose syntheses are altered are summarized in Table 2. Experiments were done to determine whether viable eukaryotic cells are required for the expression of these newly synthesized bacterial proteins by inoculating glutaraldehyde-fixed INT 407 cell monolayers with *C. jejuni*. The synthetic profile of these bacteria was identical to that of bacteria cultured in the absence of the epithelial cells, indicating a requirement for viable or metabolically active eukaryotic cells in the induction phenomenon (data not shown). The synthetic profile of the bacteria present in the medium overlying the epithelial cell monolayers (i.e., nonadherent bacteria) was also examined and was found to be very similar to that observed for cell-associated bacteria, indicating that either transient association with INT 407 cells or exposure to the culture environment is sufficient to induce the altered synthetic response. To determine whether released INT 407 cell constituents or alteration of the growth medium is sufficient to induce altered synthesis, a conditioned medium was prepared by preincubating INT 407 cell monolayers with Eagle's minimal essential medium (EMEM) containing 1% fetal bovine serum for 3 h. Although bacteria incubated in conditioned medium and those incubated with INT 407 cell monolayers exhibited similar altered synthetic responses, the conditioned medium did not elicit the complete response associated with cultivation with cells. Specifically, conditioned medium failed to elicit the appearance of the high-molecular-mass proteins (>100 kDa) and several others as judged by single-dimension electrophoresis. These observations suggest that the total synthetic response is a function of both contact with factors or conditions present in

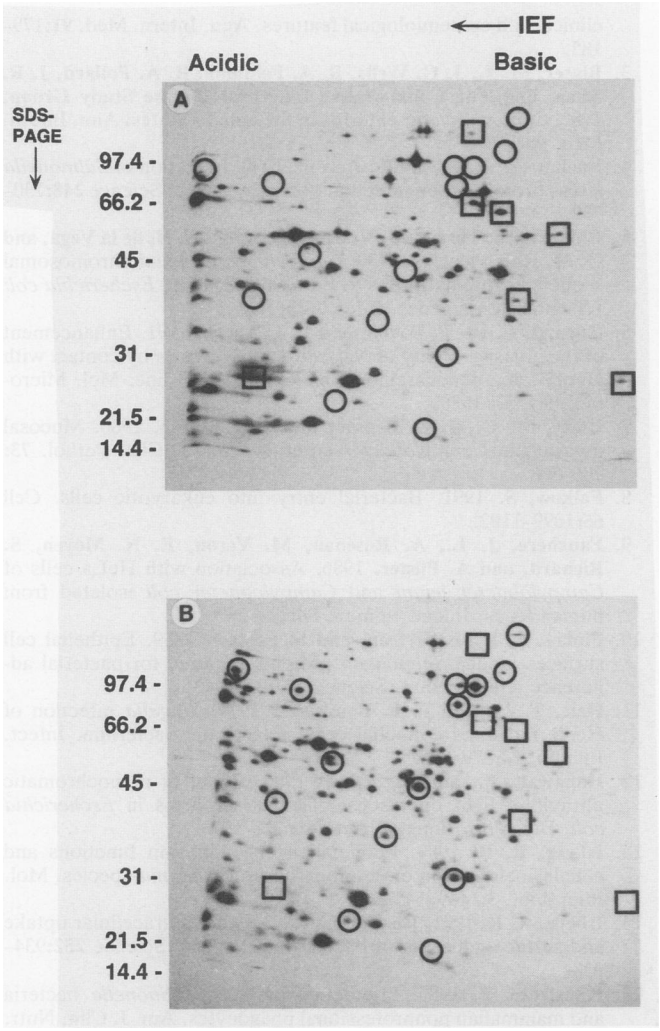

FIG. 1. Protein synthetic profiles of *C. jejuni* F38011 incubated with and without INT 407 epithelial cells. *C. jejuni* cells were initially incubated in the absence (A) or presence (B) of INT 407 cells for 1 h. Emetine hydrochloride (2.5 μg/ml) was then added to the cultures, and incubation was continued for an additional hour. [^{35}S]methionine (50 μCi/ml) was then added during the last hour of incubation. The monolayers were washed and lysed, and the labeled bacteria were recovered by differential centrifugation. The bacteria were lysed, and the labeled proteins were analyzed by using two-dimensional electrophoresis (26) using isoelectric focusing in a pH gradient of 3 to 10 in the first dimension and 10 to 15% gradient resolving gels in the second dimension. The positions of the molecular mass standards are given in kilodaltons. Equal amounts of trichloroacetic acid-precipitable radioactivity (5×10^6 cpm) were analyzed on each gel. The gels were dried and analyzed by fluorography. Circles indicate the position of labeled proteins detected only in extracts from *C. jejuni* incubated with INT 407 cells (panel B). Squares indicate positions of labeled proteins detected only in extracts from bacteria incubated in the absence of cells (medium alone; panel A).

the medium of the cultures and direct interaction with INT 407 cells.

Buchmeier and Hefron (4) have reported that *S. typhimurium* responds to internalization by macrophages by synthesizing a subset of stress response proteins (or heat shock proteins). To determine whether the newly synthesized proteins expressed by *C. jejuni* cultured in the presence of the epithelial cells were related to a stress response, bacterial proteins were radiolabeled with [^{35}S]methionine at an elevated temperature (43°C) for 5 min to induce a heat shock response. No apparent similarities between the electrophoretic profile of altered protein synthesis induced by cultivation with INT 407 cells and that induced by thermal stress were observed (data not shown).

TABLE 2. Altered protein synthetic profile of *C. jejuni* in response to cocultivation with INT 407 cells

Protein type	Molecular masses (Da)
Induced[a]	141,000, 126,000, 104,000, 95,500, 95,400, 85,100, 79,400, 53,700, 43,600, 38,900, 35,400, 27,500, 23,400, 18,000
Suppressed[b]	129,000, 71,000, 64,500, 57,500, 38,000, 25,700, 24,000

[a] Proteins synthesized by *C. jejuni* and detected only during cocultivation with INT 407 epithelial cell monolayers.

[b] Proteins synthesized by *C. jejuni* and detected only in the absence of INT 407 epithelial cells.

The induction of an altered protein synthetic response by exposure to INT 407 cells, coupled with the effect of chloramphenicol on internalization, suggested a direct relationship between the two phenomena. To examine this possibility, internalization assays were conducted by using organisms that had been preincubated with and without INT 407 cells for several hours. No difference in the ability of the two populations to enter the epithelial cells was observed; however, the internalization of *C. jejuni* preincubated with INT 407 cells appeared to be more refractory to subsequent inhibition by chloramphenicol than that of *C. jejuni* preincubated in medium alone (data not shown). When the bacteria were treated with chloramphenicol during and after preincubation with epithelial cells, internalization was attenuated, but chloramphenicol was most effective in reducing internalization when present during the preincubation phase of the assays (Table 3). Collectively, these observations suggest the occurrence of internalization-promoting responses during *C. jejuni*-INT 407 cell interaction that are sensitive to inhibition of protein synthesis.

Recently, Wassenaar et al. (33) also reported that chloramphenicol treatment reduces the entry of *C. jejuni* into INT 407 cells, although the details of the experimental conditions

TABLE 3. Effect of chloramphenicol (CM) on internalization of *C. jejuni* preincubated with INT 407 cells

Preincubation condition (inoculum size)[a]	Internalization[b]	
	− CM	+ CM
− CM (1.4×10^8)	$(1.1 \pm 0.4) \times 10^5$ (100)	$(2.5 \pm 0.4) \times 10^4$ (22.7)
+ CM (2.0×10^8)	$(1.7 \pm 0.6) \times 10^3$ (1.0)	$(3.4 \pm 0.4) \times 10^2$ (0.22)

[a] *C. jejuni* F38011 was incubated with (+ CM) or without (− CM) chloramphenicol (64 μg/ml) for 45 min at 37°C prior to addition to INT 407 cell monolayers. After incubation at 37°C for 3 h in a 5% CO_2 incubator, the bacteria were harvested and further incubated in the presence or absence of chloramphenicol prior to inoculation of fresh INT 407 cell monolayers and determination of internalization. The inoculum sizes used for the internalization assays are given in parentheses.

[b] Assays were performed in the presence or absence of chloramphenicol as indicated. The number of internalized bacteria was determined as described in Table 1, footnote *b*. The values represent the means of triplicate determinations ± the standard deviations. The numbers in parentheses represent the percentage of the control values (no CM at any time) derived from calculations of the ratio of the number internalized bacteria to the inoculum size multiplied by 100.

employed were not provided. It should be noted that we cannot formally exclude the possibility that the effects of chloramphenicol, as measured by the gentamicin protection assay, are due to impairment of intracellular bacterial survival or that the effects are due to suppression of constitutively produced and rapidly metabolized proteins. However, chloramphenicol had no detectable direct effect on the viability of *C. jejuni* when measured independently. Accordingly, we favor the interpretation that abrogation of protein synthesis predominantly results in impairment of internalization rather than intracellular survival. The fact that chloramphenicol treatment was unable to completely abrogate internalization may indicate either that a subpopulation of cells exists that is competent for internalization or that a certain proportion of bacteria retain sufficient amounts of those proteins important to internalization.

As noted above, certain pathogens (i.e., *Yersinia* spp. and *Chlamydia* spp.) appear to express constitutively the products that promote uptake by eukaryotic cells, while *S. typhimurium* synthesizes proteins that promote internalization as a result of contact with epithelial cells or culture conditions (10, 20). In the case of *S. typhimurium*, the response appears to be a function of anaerobiosis associated with cell culture conditions and of the growth phase of the organism (20). Other bacterial pathogens appear to synthesize virulence factors in response to intracellular conditions, as well as to alterations in the extracellular environment (for a review, see reference 22). It is noteworthy in this regard that the novel synthetic response of *C. jejuni* can be elicited in part by conditioned medium from INT 407 cell cultures. This observation indicates that *C. jejuni* responds to either physicochemical alterations of the medium or to cell-free products of the INT 407 cells themselves. Experiments are in progress to characterize those properties of conditioned medium which elicit the observed synthetic response.

An obvious prediction arising from our results is that *C. jejuni* engages in such novel synthetic responses in vivo. Indeed, a recent report by Panigrahi et al. (27) indicates that several *C. jejuni* proteins increase in abundance during incubation in rabbit ileal loops, and our initial inspection reveals that these proteins possess molecular masses similar to those of some of the newly synthesized proteins identified in this study. We believe that the findings presented herein support the proposal that enteritis caused by *C. jejuni* involves, as with some other enteroinvasive pathogens, a response to environmental conditions that facilitates penetration of the intestinal epithelium. Further analyses of the newly synthesized proteins using amino-terminal sequence analysis and molecular cloning may permit the identification of essential *C. jejuni* virulence determinants.

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