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Effect of Enteroviruses on Adherence to and Invasion of HEp-2 Cells by *Campylobacter* Isolates

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Coinfection of HEp-2 epithelial cells with coxsackievirus B3, echovirus 7, poliovirus (LSc type 1), porcine enterovirus, and *Campylobacter* isolates was performed to determine if a synergistic effect could be obtained. The invasiveness of *Campylobacter jejuni* ATCC 33560 was significantly increased for HEp-2 cells preinfected with echovirus 7, coxsackievirus B3, and UV-inactivated (noninfectious) coxsackievirus B3 particles. Additionally, the invasiveness of *C. jejuni* M96, a clinical isolate, was significantly increased for HEp-2 cells preinfected with coxsackievirus B3. Poliovirus and porcine enterovirus had no effect on *C. jejuni* ATCC 33560 adherence and invasiveness. Furthermore, poliovirus had no effect on the ability of *C. jejuni* M96 to adhere to and invade HEp-2 cells. *Campylobacter hyointestinalis* and *Campylobacter mucosalis*, two noninvasive isolates, did not invade virus-infected HEp-2 cells. The increase in the invasiveness of *C. jejuni* appeared to be the result of specific interactions between the virus and the HEp-2 cell membrane. The data suggest that the invasiveness of *Campylobacter* spp. is dependent upon the inherent properties of the organism. Virus-induced cell alterations can potentiate the invasiveness of virulent *Campylobacter* spp. but are not sufficient to allow internalization of noninvasive bacteria.

A synergistic relationship has been postulated to exist between *Campylobacter jejuni* and other enteropathogens, especially in developing countries. Melamed et al. (24) found a higher incidence of polymicrobial infections with *C. jejuni* than with other enteric pathogens. Additionally, Bukholm and Kapperud (3) demonstrated that noninvasive *C. jejuni* was able to penetrate cultured cells when challenged with a mixture of *Campylobacter* organisms and enteroinvasive *Salmonella typhimurium*, *Shigella flexneri*, *Shigella boydii*, *Shigella sonnei*, and *Escherichia coli*. Little is known about the mechanisms of synergistic infections. Furthermore, the effect of viruses on the adherence to and invasiveness for epithelial cells of *Campylobacter* organisms has not been examined.

Cellular alterations induced by viral infections may have a significant effect on the ability of enteric pathogens to adhere to and invade epithelial cells. Investigators have demonstrated that adsorption of certain viruses to cells results in an increase in bacterial adherence (8, 20, 28) and invasiveness (1, 2, 4). The mechanisms by which viruses enhance bacterial adherence and invasiveness are unclear. Adsorption of certain viruses to cells has been shown to increase membrane fluidity and permeability (5, 13, 21, 30). These changes may have a significant effect on secondary bacterial infections.

A variety of animal models have been used as experimental models to investigate the pathogenesis of *Campylobacter* organisms. However, no established animal models that mimic human disease without surgical or antibiotic pretreatment exist (31). The absence of a simple animal model has hampered investigations of the pathogenic mechanisms of the campylobacters. Cell culture models have been routinely used to examine the adherence and invasiveness of many bacteria, including *E. coli* (6, 15), *Salmonella* spp. (14, 25), *Shigella* spp. (12, 29), *Yersinia* spp. (18, 19), and *Campylobacter* spp. (3, 10, 11, 22, 23). Previously, we studied the adherence to and invasiveness for HEp-2 cells of *Campylobacter* organisms by two different models (17). Indirect immunofluorescence techniques were used initially to screen *Campylobacter* isolates for invasiveness potential. Although intracellular organisms could clearly be identified, it was difficult to enumerate them with this methodology. Thus, a HEp-2 model system which uses an antibiotic (gentamicin) was used to analyze the mechanisms of cellular penetration. This method allows for the enumeration of both adherent and invasive bacteria with ease and accuracy. This model was used to assay the effect of four enteroviruses on the ability of *Campylobacter* organisms to adhere to and invade HEp-2 cells. The organisms used in this study were chosen on the basis of their invasive properties (or lack of such), which were determined earlier in a study conducted in our laboratory. *C. jejuni* ATCC 33560 and *C. jejuni* M96, a clinical isolate, attached to and invaded HEp-2 cells, *Campylobacter hyointestinalis* attached to but did not invade HEp-2 cells, and *Campylobacter mucosalis* did not attach to or invade HEp-2 cells (17).

MATERIALS AND METHODS

Bacterial strains and maintenance of cultures. *C. jejuni* ATCC 33560, *C. hyointestinalis* ATCC 35217, and *C. mucosalis* ATCC 43264 were obtained from the American Type Culture Collection (Rockville, Md.). *C. jejuni* M96 was kindly supplied by Kenneth Ryan, University Medical Center, University of Arizona, Tucson. Upon receipt in our laboratory, bacterial strains were passed no more than twice on Mueller-Hinton agar plates containing 4% citrated bovine blood and frozen at −70°C in bovine blood for use as stock cultures. Inocula for experiments were prepared by quickly thawing a stock culture and placing 1 to 2 drops of the suspension onto a Mueller-Hinton agar plate with blood. The bacteria were subcultured every 2 days. *C. jejuni* ATCC 33560, *C. jejuni* M96, and *C. hyointestinalis* were incubated under microaerophilic conditions (10.2% hydrogen, 10.2% carbon dioxide, and nitrogen as the balance). *C. mucosalis* was incubated under anaerobic conditions (50% carbon dioxide and 50% hydrogen).

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Epithelial cells. Human laryngeal carcinoma (HEp-2; ATCC CCL 23) and Buffalo green monkey kidney (BGM; Microbiological Associates, Bethesda, Md.) cells were maintained in Eagle minimal essential medium (MEM) containing 10% fetal bovine serum without the use of antibiotics in 75-cm^2 screw-cap flasks (Falcon; Becton Dickinson Labware, Oxnard, Calif.). Both cell lines were incubated at 37°C in a 5% CO_2 incubator. Monolayers were trypsinized and split 1:2 upon reaching confluency. For coinfection assays, 24-well tissue culture trays were seeded with 5×10^4 HEp-2 cells per well. The seeded plates were incubated for 2 days at 37°C in a humidified CO_2 incubator. The semiconfluent monolayers were washed once with MEM containing 1% fetal bovine serum prior to the assays.

Viruses. Coxsackievirus B3, echovirus 7, poliovirus (LSc type 1), and porcine enterovirus (serotype 8) were used in coinfection assays. Porcine enterovirus was previously isolated in our laboratory from a pig with porcine proliferative enteritis and plaque purified (D. L. Finn, M.S. thesis, University of Arizona, Tucson, 1987). Porcine enterovirus was grown in BGM cells. Coxsackievirus B3, echovirus 7, and poliovirus were grown in HEp-2 cell monolayers. Viral stocks were prepared by infecting a 75-cm^2 tissue culture flask containing a confluent sheet of the appropriate cell line with 1.0 ml of virus. The cell cultures were frozen (−70°C) and thawed (42°C) when cytopathogenic effects were well developed. The supernatants were centrifuged at $600 \times g$ and filtered through a 0.22-μm-pore-size filter to remove cell debris. Supernatants were stored frozen at −70°C.

UV inactivation of virus. A virus suspension was inactivated by UV irradiation by the method of Bukholm et al. (2). Briefly, 5 ml of coxsackievirus B3 containing 2×10^7 PFU/ml was poured into a 60-mm petri dish (Falcon). The virus was inactivated by UV irradiation with a UV lamp (0.67 mW/cm^2) for 3 min at a distance of 140 mm. Virus infectivity was reduced to <1 PFU/ml. For coinfection assays, the suspension was diluted 10,000-fold and 0.5 ml was used to infect monolayers.

Plaque assay. For plaque assays, 6-well tissue culture plates (Falcon) were seeded with 5.0×10^5 HEp-2 cells per well. The seeded plates were incubated for 2 days at 37°C in a humidified CO_2 incubator. Confluent monolayers were washed once with phosphate-buffered saline and infected with 0.5 ml of serially diluted (10^{-1} to 10^{-6}) virus suspension. Inoculated cultures were agitated at 15-min intervals. After 1 h, the supernatant was aspirated and the infected monolayers were overlaid with 3 ml of an agar overlay. The agar overlay consisted of MEM supplemented with 2% fetal bovine serum and 1% Bacto-agar (Difco Laboratories, Detroit, Mich.). DEAE dextran (Sigma Chemical Co., St. Louis, Mo.) was added at a final concentration of 100 μg/ml to the agar overlay for both poliovirus and porcine enterovirus. Plates were incubated inverted in a 37°C humidified incubator for 3 days. Following incubation, the overlay medium was removed and the monolayers were stained with crystal violet. Virus concentrations were recorded as the number of PFU per milliliter of the original suspension.

Coinfection assay. Intracellular and HEp-2 cell-associated (intracellular and extracellular) campylobacters were determined with a previously described assay (17). Duplicate wells of semiconfluent HEp-2 cell monolayers were infected with 1,000 PFU of virus or MEM (control) and incubated for 2 h at 37°C in a 5% CO_2 incubator. Monolayers were washed once with MEM containing 1% fetal bovine serum, and each well was inoculated with 0.5 ml of a bacterial suspension. Bacteria had previously been harvested from Mueller-Hinton agar plates with phosphate-buffered saline and pelleted by centrifugation at $6,000 \times g$ for 10 min at 4°C. The pellets were suspended in MEM containing 1% fetal bovine serum to approximately 10^8 bacteria per ml. The titers of the bacterial suspensions were determined on Mueller-Hinton agar plates. The infected monolayers were incubated for 3 h to allow the bacteria to adhere to the virus-infected or noninfected (control) HEp-2 cell monolayers. The monolayers were washed five times with MEM containing 1% fetal bovine serum to remove the nonadherent bacteria. Medium containing 250 μg of gentamicin (GIBCO Laboratories, Grand Island, N.Y.) per ml was added to one well for the enumeration of intracellular bacteria. In preliminary experiments, the exposure of *Campylobacter* isolates to 250 μg of gentamicin per ml killed all the extracellular organisms after a 3-h exposure. To the other well, medium without antibiotic was added to enumerate the intracellular and extracellular bacteria. Following a 3-h incubation, the monolayers were washed three times with phosphate-buffered saline and lysed with 0.5% sodium deoxycholate (Difco) (11). The suspensions were diluted, and the numbers of viable bacteria were determined by colony counting.

Results were expressed as the mean ± the standard deviation of bacteria adhering to and invading HEp-2 cells for three or more determinations. The significance between treatment (noninfected versus virus-infected) groups was calculated by transforming the data with the function log (x). Values were then subjected to analysis of variance. P values exceeding 0.05 were considered not significant.

TABLE 1. Effect of enteroviruses on adherence to and invasion of HEp-2 cells by *C. jejuni* ATCC 33560

Enterovirus[a]	Inoculum[b]	Intracellular bacteria[c]	Intracellular and extracellular bacteria[d]
Coxsackievirus B3	$(6.8 \pm 3.2) \times 10^7$	$(6.9 \pm 0.8) \times 10^2$[e]	$(3.9 \pm 3.8) \times 10^4$
Control	$(6.8 \pm 3.2) \times 10^7$	$(2.3 \pm 0.6) \times 10^2$	$(1.6 \pm 0.8) \times 10^4$
Echovirus	$(4.0 \pm 2.5) \times 10^7$	$(7.0 \pm 2.4) \times 10^2$[e]	$(2.8 \pm 1.9) \times 10^4$
Control	$(4.0 \pm 2.5) \times 10^7$	$(2.9 \pm 0.3) \times 10^2$	$(1.2 \pm 0.9) \times 10^4$
Poliovirus (LSc type 1)	$(1.9 \pm 0.2) \times 10^8$	$(6.7 \pm 0.2) \times 10^2$	$(5.5 \pm 2.6) \times 10^4$
Control	$(1.9 \pm 0.2) \times 10^8$	$(6.9 \pm 1.0) \times 10^2$	$(4.7 \pm 2.5) \times 10^4$
Porcine enterovirus	$(4.5 \pm 1.3) \times 10^7$	$(3.5 \pm 1.6) \times 10^2$	$(1.9 \pm 1.8) \times 10^4$
Control	$(4.5 \pm 1.3) \times 10^7$	$(2.7 \pm 2.0) \times 10^2$	$(1.3 \pm 0.4) \times 10^4$

[a] For viruses, HEp-2 cell monolayers were infected with 10^3 PFU of virus. For controls, HEp-2 cell monolayers were not infected with virus.
[b] Number of viable bacteria inoculated per well of a 24-well plate.
[c] Number of viable bacteria invading cells per well of a 24-well plate.
[d] Number of viable bacteria invading cells and adhering to cells per well of a 24-well plate.
[e] Significant at $P < 0.05$.

TABLE 2. Effect of coxsackievirus B3 and poliovirus on adherence to and invasion of HEp-2 cells by *C. jejuni* M96

Enterovirus[a]	Inoculum[b]	Intracellular bacteria[c]	Intracellular and extracellular bacteria[d]
Coxsackievirus B3	$(2.6 \pm 0.1) \times 10^7$	$(1.3 \pm 0.2) \times 10^3$[e]	$(1.4 \pm 0.5) \times 10^4$
Control	$(2.6 \pm 0.1) \times 10^7$	$(8.1 \pm 0.8) \times 10^2$	$(1.0 \pm 0.1) \times 10^4$
Poliovirus LSc (type 1)	$(1.6 \pm 0.0) \times 10^8$	$(9.9 \pm 5.9) \times 10^2$	$(2.7 \pm 0.3) \times 10^4$
Control	$(1.6 \pm 0.0) \times 10^8$	$(1.1 \pm 0.6) \times 10^3$	$(1.7 \pm 0.6) \times 10^4$

[a] See Table 1, footnote *a*.
[b] See Table 1, footnote *b*.
[c] See Table 1, footnote *c*.
[d] See Table 1, footnote *d*.
[e] See Table 1, footnote *e*.

RESULTS

In preliminary studies, HEp-2 cell monolayers were seeded with 10^6 PFU of virus at 1, 2, 3, and 6 h prior to infection with invasive *C. jejuni* ATCC 33560 (data not shown). The greatest number of adherent and internalized bacteria was observed in cultures infected 2 h prior to bacterial inoculation. The number of internalized bacteria did not continue to increase 3 h postinfection with virus. Additionally, the invasiveness of *C. jejuni* for HEp-2 cells was essentially the same whether cells were incubated with 10^3 or 10^6 PFU of virus. Therefore, subsequent experiments were performed by infecting cultures with 10^3 PFU of each virus 2 h prior to bacterial inoculation.

Effect of enteroviruses on the invasiveness of *C. jejuni* for HEp-2 cells. HEp-2 cell monolayers were coinfected with 10^3 PFU of coxsackievirus B3, echovirus 7, poliovirus, or porcine enterovirus and with *C. jejuni* ATCC 33560 to determine if a synergistic effect could be obtained (Table 1). Coxsackievirus and echovirus significantly increased ($P < 0.05$) the number of intracellular *C. jejuni* but did not significantly increase the number of adherent *C. jejuni* relative to the controls. The number of adherent and internalized *C. jejuni* did not significantly increase for HEp-2 cells preinfected with porcine enterovirus. A slight reduction in the number of internalized organisms and an insignificant increase in the number of cell-associated (intracellular and extracellular) organisms was noted for HEp-2 cell monolayers coinfected with poliovirus and *C. jejuni* relative to the control.

To ascertain if the results obtained above with *C. jejuni* ATCC 33560 represented a strain-specific event, we coinfected HEp-2 cell monolayers with either coxsackievirus B3 or poliovirus and *C. jejuni* M96 (Table 2). Coinfection of HEp-2 cells with coxsackievirus B3 and *C. jejuni* M96 significantly increased ($P < 0.05$) the number of internalized bacteria but not cell-associated bacteria with respect to the control. In contrast, coinfection of HEp-2 cells with poliovirus and *C. jejuni* M96 resulted in a slight reduction in the number of internalized bacteria and a slight increase in the number of cell-associated bacteria relative to the control.

Effect of coxsackievirus B3 on adherence to and invasion of HEp-2 cells by *C. hyointestinalis* and *C. mucosalis*. We attempted to determine whether infection with virus would alter the ability of two noninvasive isolates, *C. hyointestinalis* and *C. mucosalis*, to invade HEp-2 cells (Table 3). Coxsackievirus B3 was used in the following assays because it was found to enhance the invasiveness of *C. jejuni* relative to the control. Infection of HEp-2 monolayers with coxsackievirus B3 did not significantly increase the ability of *C. hyointestinalis* to adhere to the epithelial cells and had no effect on the ability of *C. hyointestinalis* to invade the cells. *C. mucosalis* did not adhere to or invade either coxsackievirus B3-infected or noninfected (control) HEp-2 epithelial cells.

Effect of UV-inactivated (noninfectious) virus on adherence to and invasion of HEp-2 cells by *C. jejuni* ATCC 33560. UV-inactivated (noninfectious) viruses have been reported to enhance the invasiveness of bacteria to the same extent as do infectious virus particles (10). Therefore, experiments were performed to determine if infection of HEp-2 cell monolayers with noninfectious virus particles would increase the number of adherent and internalized *C. jejuni* to the same extent as does infection with infectious virus particles. A significant increase ($P < 0.05$) in the number of internalized *C. jejuni* was observed with HEp-2 cells infected with the same numbers of both UV-inactivated and noninactivated coxsackievirus particles relative to the controls (Table 4). Additionally, infection of HEp-2 cells with UV-inactivated coxsackievirus particles significantly increased the number of *C. jejuni* adhering to HEp-2 cells.

DISCUSSION

Combined infection with viral and bacterial agents often results in a more severe disease than does infection with either agent alone. One possible explanation is that the host becomes compromised and is therefore nonspecifically susceptible to bacterial disease (8). However, investigators have proposed that specific, rather than nonspecific, interactions may lead to bacterial colonization and penetration of virus-infected cells (9). Bacterial invasion of cells in culture has been previously shown to involve three stages: (i) reversible adherence, (ii) irreversible adherence, and (iii) invasion (14). Viral infection could influence all of these stages.

The number of internalized *C. jejuni* ATCC 33560 significantly increased with both coxsackievirus and echovirus-infected HEp-2 cells relative to the controls. Additionally, preinfection of HEp-2 cell monolayers with coxsackievirus

TABLE 3. Effect of coxsackievirus B3 on adherence to and invasion of HEp-2 cells by noninvasive *Campylobacter* isolates

Campylobacter isolate[a]	Inoculum[b]	Intracellular bacteria[c]	Intracellular and extracellular bacteria[d]
C. hyointestinalis	$(5.0 \pm 0.0) \times 10^7$	0	$(1.7 \pm 0.6) \times 10^2$
Control	$(5.0 \pm 0.0) \times 10^7$	0	$(1.3 \pm 0.6) \times 10^2$
C. mucosalis	$(1.4 \pm 1.0) \times 10^8$	0	0
Control	$(1.4 \pm 1.0) \times 10^8$	0	0

[a] See Table 1, footnote *a*.
[b] See Table 1, footnote *b*.
[c] See Table 1, footnote *c*.
[d] See Table 1, footnote *d*.

TABLE 4. Effect of UV-inactivated coxsackievirus B3 on adherence to and invasion of HEp-2 cells by *C. jejuni* ATCC 33560

Coxsackievirus B3[a]	Inoculum[b]	Intracellular bacteria[c]	Intracellular and extracellular bacteria[d]
UV-inactivated virus	$(4.8 \pm 0.6) \times 10^7$	$(7.3 \pm 1.2) \times 10^{2e}$	$(5.3 \pm 1.6) \times 10^{4e}$
Noninactivated virus	$(4.8 \pm 0.6) \times 10^7$	$(6.3 \pm 0.5) \times 10^{2e}$	$(4.7 \pm 2.9) \times 10^4$
Control	$(4.8 \pm 0.6) \times 10^7$	$(3.2 \pm 1.3) \times 10^2$	$(1.7 \pm 0.6) \times 10^4$

[a] See Table 1, footnote *a*.
[b] See Table 1, footnote *b*.
[c] See Table 1, footnote *c*.
[d] See Table 1, footnote *d*.
[e] See Table 1, footnote *e*.

significantly increased the number of internalized *C. jejuni* M96. The mechanism of this enhancement of *C. jejuni* invasiveness is not known. Bukholm et al. showed that the invasiveness of *S. typhimurium* was enhanced in HEp-2 cells preinfected with infectious and noninfectious coxsackievirus B1 (1, 2). They suggested that a change in the electrical potential in the medium, due to the increase in the number of particles in solution, may increase the probability of contact between the bacteria and the host cells. However, poliovirus and porcine enterovirus had no effect on bacterial invasiveness. Philipson and Bengtsson (27) demonstrated that coxsackievirus and echovirus competed for the same cell receptors. Adsorption of coxsackievirus and echovirus to HEp-2 cell receptors may either expose a membrane receptor (protein or phospholipid) so that it is more accessible to a *Campylobacter* invasive antigen(s) or trigger a cell-mediated event that causes the phagocytosis of invasive *Campylobacter* organisms. The increase in the invasiveness of *C. jejuni* for HEp-2 cells does not reflect the expression of virally synthesized membrane receptors on the epithelial cells because the enhancement was also demonstrated with UV-inactivated coxsackievirus relative to the control.

Preinfection of HEp-2 cell monolayers with porcine enterovirus did not significantly increase the adherence or invasiveness of *C. jejuni* relative to the control. Coinfection of HEp-2 cells with poliovirus and *C. jejuni* ATCC 33560 and M96 slightly reduced the number of internalized bacteria and slightly increased the number of adherent bacteria relative to the noninfected controls. The method of attachment interference (competition for receptors) was used to show that poliovirus receptors are distinct from coxsackievirus B receptors (7). Thus, poliovirus binds to receptors that are distinct from those of coxsackievirus and echovirus. We do not know if poliovirus and porcine enterovirus bind to the same receptors. However, Finn (M.S. thesis) showed that the viruses do cross-react; anti-poliovirus 1 sera reduced the cytopathogenic effect of porcine enterovirus 1,000-fold in a cell culture assay. The adsorption of poliovirus to cells has been reported to cause an influx of Ca^{2+} into the cells (26). The increase in the intracellular Ca^{2+} concentration induced by poliovirus adsorption results in the depolymerization of cellular microfilaments (16). Disruption of cellular microfilaments with cytochalasin B has been shown to inhibit the phagocytosis of *Campylobacter* organisms by HEp-2 cells (10, 17). The slight decrease in the numbers of internalized *C. jejuni* ATCC 33560 and M96 may have been a result of microfilament depolymerization in the cells with adsorbed poliovirus. The decrease in the number of internalized *C. jejuni* in poliovirus-infected cells was not due to a cytopathogenic effect, as measured by trypan blue viability staining.

Davison and Stanford (8) demonstrated that cells became susceptible to bacterial adherence as a result of virus infection. To determine whether infection of epithelial cells with virus would induce the adherence of an isolate previously found not to adhere to cells, we coinfected HEp-2 cells with *C. mucosalis* and coxsackievirus B3. *C. mucosalis* did not adhere to or invade either coxsackievirus-infected or noninfected (control) HEp-2 epithelial cells. We also examined whether coinfection of epithelial cells would induce internalization of a noninvasive isolate that does adhere to HEp-2 cells. Infection of HEp-2 monolayers with coxsackievirus did not significantly increase the adherence of *C. hyointestinalis* to cells and did not induce the invasiveness of *C. hyointestinalis* for HEp-2 cells. Similar results were reported by Bukholm and Degre (1), who found that noninvasive *E. coli* was not capable of invading virus-infected cells.

In summary, infection of cells with viruses may be an important contributing factor for *Campylobacter* colonization and penetration. The data indicate that enhancement of bacterial invasion is an event that occurs early in the infectious process and that it is a result of specific events rather than nonspecific events because the enteric viruses tested had different effects on *Campylobacter* invasiveness for HEp-2 cells. It is possible that virus-induced alterations in cell membrane receptors lead to this enhancement. Additionally, the lack of effect on adherence and invasiveness of noninvasive *C. hyointestinalis* and *C. mucosalis* isolates suggests that the adherence and invasiveness of *Campylobacter* organisms are dependent upon the inherent properties of the organisms and that virus-induced cell alterations are not sufficient to allow adherence or invasion by noninvasive bacteria.

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