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Adherence to and Invasion of Tissue Culture Cells by *Vibrio hollisae*

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The adherence to and invasion of cultured epithelial cells by *Vibrio hollisae* were examined by quantitative studies and by light, fluorescent, and electron microscopy. Condensed actin was observed around clustered adherent and intracellular bacteria. Bacteria multiplied intracellularly. Inhibitor studies indicated that internalization occurred by an integrated pleiotropic process involving eukaryotic and prokaryotic protein syntheses, microfilaments, microtubules, and receptor-mediated endocytosis.

Vibrio hollisae, a halophilic, gram-negative bacterium first described in 1982, is known to cause wound infections, gastroenteritis, and septicemia in humans (14, 16). It has been isolated from seafood such as oysters and coastal fish (10, 14, 18) and thus is a concern of the seafood industry and public health agencies. The only virulence factors published to date include the production of a thermostable direct hemolysin related to that of *Vibrio parahaemolyticus* (18) and a heat-labile enterotoxin that elongates Chinese hamster ovary cells and causes fluid accumulation in suckling mice (12).

The fact that *V. hollisae* infection can cause septicemia suggests an invasive disease. We therefore examined the invasive potential of this bacterium by infecting tissue culture cells with *V. hollisae*. Techniques including light microscopy, a previously described fluorescent acridine orange (AO) staining technique for visualization of internalized bacteria (15), indirect fluorescent-antibody staining, transmission electron microscopy (TEM), scanning electron microscopy (SEM), and lysis of infected cells after various infection times were used to detect and evaluate invasion.

The AO staining technique was used to screen eight *V. hollisae* clinical isolates for cell invasion. Strain 75-80 was further evaluated by studies involving lysis of infected cells, inhibitors, phalloidin-fluorescein isothiocyanate, indirect fluorescent-antibody staining to various cytoskeletal elements of the eukaryotic cell, TEM, and SEM. Preliminary studies were conducted by growing strain 75-80 on agar plates with colonization factor antigen (6) plus 1% NaCl (CFAS), Trypticase soy plus 1% NaCl (TSAS), and Columbia agar plus 0.5% NaCl for 18 h at 37°C prior to infection and adding different concentrations of bacteria to the cells. Since CFAS-grown *V. hollisae* at a concentration of 5×10^9 CFU/ml gave the best bacterium-cell association results (data not shown), *V. hollisae* strains, obtained from Mahendra Kothary, Food and Drug Administration, Washington, D.C., were grown on CFAS agar plates for all studies and diluted to a concentration of 5×10^9 CFU/ml. With the exception of EM and cell lysis studies, all assays were performed as described previously (15) on eight-chambered tissue culture slides (Nunc Inc., Naperville, Ill.) for 1 to 3 h, with 24-h HeLa, Henle 407 intestinal, and HCT-8 cell monolayers. Slides for light microscopy were stained with safranin O as described by Gander and LaRocco (7) and viewed by light microscopy. Compounds used in inhibitor studies included cycloheximide (10 mg/ml; Sigma Chemical Co., St. Louis, Mo.) (8), cytochalasin B (10 μg/ml; Sigma) (8), colchicine (10 mM; Sigma) (19), and ouabain (250 μM; Sigma) (20), and were applied to monolayers 1 h prior to initiation of the assay. Chloramphenicol (20 μg/ml; Sigma) was added at the time of infection. At 3 h after infection, the monolayers were stained with AO (15). Slides were examined blind with an Optiphot-2 microscope (Nikon Corp., Tokyo, Japan) equipped for epifluorescence. The number of cells with intracellular viable green fluorescent bacteria and the number of bacteria per cell in 10 fields were determined. Inhibitor studies were performed at least four times in quadruplicate. *Salmonella typhi* grown on Trypticase soy agar was used as an inhibitor control in all experiments. For phalloidin and antibody assays, 2.5-h infected monolayers were washed, fixed with 4% paraformaldehyde, and permeabilized as previously described (28). Monolayers were then washed and incubated for 1 h with a 1:50 dilution of either anti-tubulin, anti-actin, or anti-clathrin antibody (19). After being washed, antibody-treated monolayers were treated for 30 min with a 1:50 dilution of anti-rabbit, anti-goat, or anti-horse–fluorescein isothiocyanate antibody (11). For phalloidin treatment, monolayers were treated with phalloidin-fluorescein isothiocyanate (5 μg/ml) for 20 min (11). All slides were washed, and 1 drop of Vectashield (Vector Laboratories, Inc., Burlingame, Calif.) was added to each slide. A coverslip was mounted on each slide, and the edges were sealed with colorless nail polish. The slides were examined under fluorescence with an Optiphot-2 microscope (Nikon).

For intracellular survival and multiplication studies, infection was performed with HCT-8 cell monolayers in 12-cm-diameter tissue culture plates (Nunc) for 3 h. Both HeLa and Henle 407 cells were destroyed by the bacterial cytotoxin soon after 3 h of infection, making quantitative studies impossible. HCT-8 cells were more resistant and were therefore used for the longer infection times. Infected HCT-8 monolayers were washed, and fresh medium containing 50 μg of kanamycin (Sigma) per ml was added to each plate (17). *V. hollisae* was confirmed to be sensitive to kanamycin at 30 μg/ml. The plates were further incubated for an additional 2, 4, and 6 h and overnight to give total infection times of 5, 7, and 9 h and overnight. Monolayers were then washed and treated with 0.1% Triton X-100 for 15 min. Duplicate aliquots of 200 μl were spread on TSAS plates and incubated overnight at 37°C.

For EM studies, infection was performed for 1 to 3 h with

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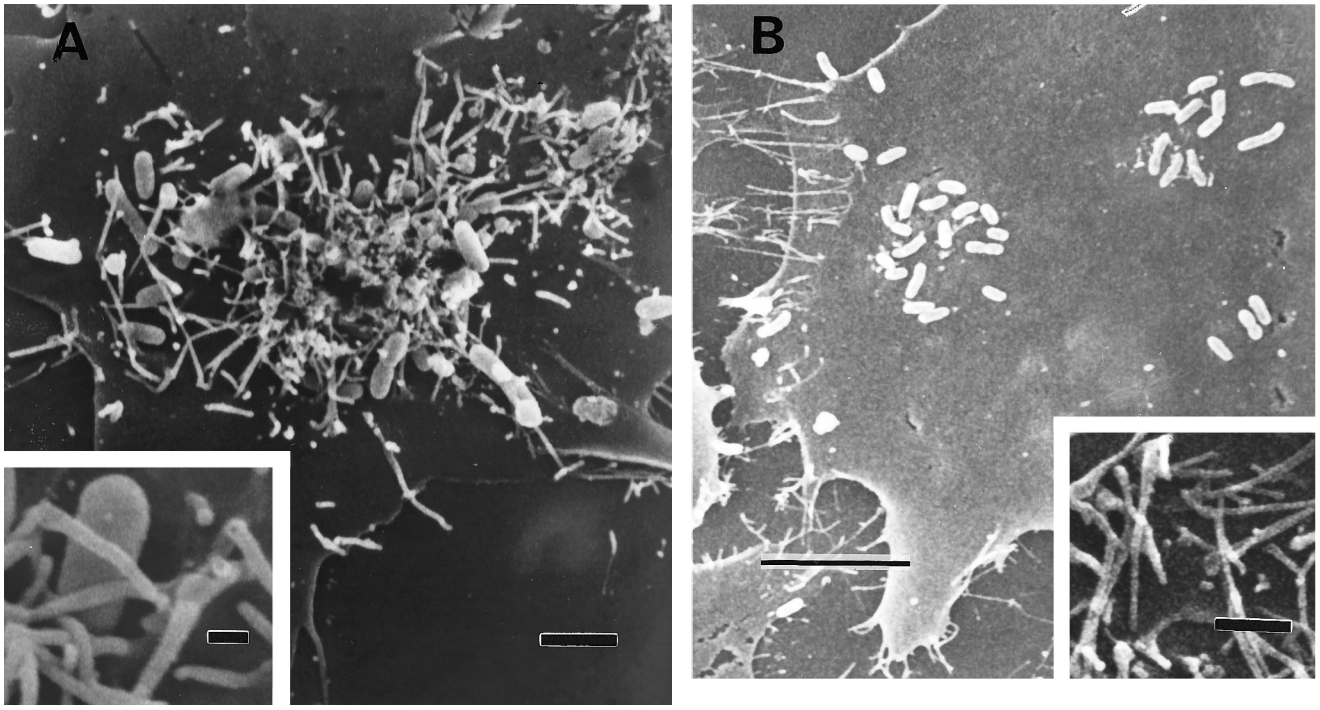

FIG. 1. Scanning electron photomicrographs of HeLa cells infected with *V. hollisae*. (A) elongated microvilli surrounding bacteria in clusters at 2 h. The inset shows a higher magnification of elongated microvilli around a bacterium. The bar markers each represent 1 μm. (B) Clustered adherence and denudation at 3 h. The bar marker represents 5 μm. The inset shows an uninfected HeLa cell showing normal microvilli. The bar marker represents 1 μm.

24-h HeLa, Henle 407, and HCT-8 cell monolayers in 12-cm-diameter tissue culture plates (Nunc) containing coverslips (Nunc). After each incubation time, monolayers were fixed with 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2). Coverslips were removed and processed for SEM by standard procedures and examined with an ISI Super IIIA SEM operating at an accelerating voltage of 15 kV (26). The remaining fixed cells on plates were harvested in 1 ml of 3% glutaraldehyde in sodium cacodylate buffer, centrifuged (8,000 × *g*, 5 min), resuspended in fixative, and processed for TEM (26). Samples were evaluated with a Philips 400 HM TEM operating at an accelerating voltage of 80 kV.

At 1 h, a few bacteria adhering to the surface of microvillus-laden HeLa cells were observed. Within 2 h, SEM showed two to four adherent bacteria in small clusters surrounded by elongated microvilli (Fig. 1A). By 3 h, light microscopy and SEM showed six to eight single bacterial cells colonizing the HeLa cell surface in clusters averaging 7 μm in diameter (range, 5.2 to 8.6 μm), similar to those observed by Yamamoto et al. (30), with a diffusely adherent *Escherichia coli* strain (Fig. 1B). Typically 1 to 3 clusters per epithelial cell were seen. At this time, microvilli were present only at sites of clustering (Fig. 1B). This denudation, a prominent morphological feature, suggests either a cytopathic effect of *V. hollisae* toxin(s) or microvillus rearrangement in response to bacterial adherence, possibly via membrane-associated cytoskeletal elements. TEM showed that colonization was associated with pedestals containing actin-like material (Fig. 2).

AO staining of HeLa and Henle 407 monolayers infected for 3 h with all eight *V. hollisae* strains revealed green viable fluorescent intracellular bacteria. Strain 75-80 was also examined at 1 and 2 h after infection. At 1 h, invasion was minimal: 7% of the cells were invaded, with an average of one bacterium per cell. By 2 h, 32% of the cells were invaded, with an average

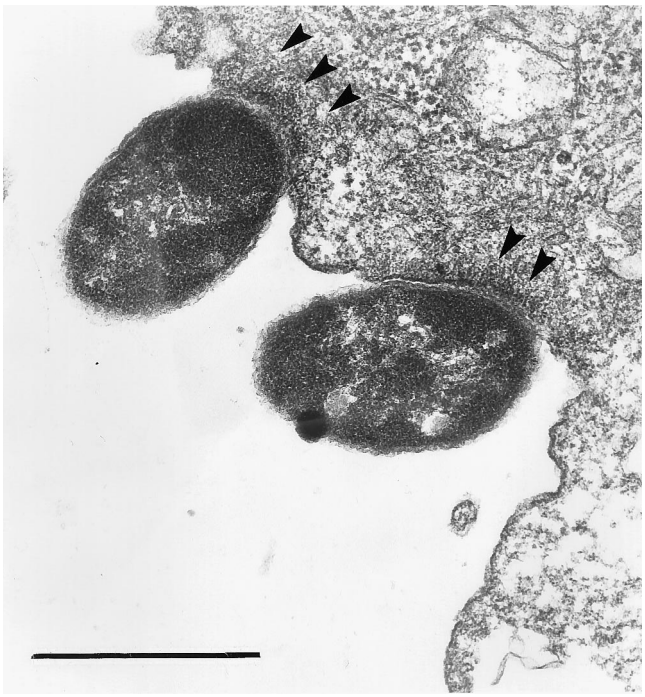

FIG. 2. Transmission electron photomicrograph of HeLa cells infected with *V. hollisae* at 2 h. Note pedestal formation and condensed actin-like material underneath the bacteria in the adjacent pedestal (arrowheads). The bar marker represents 0.5 μm.

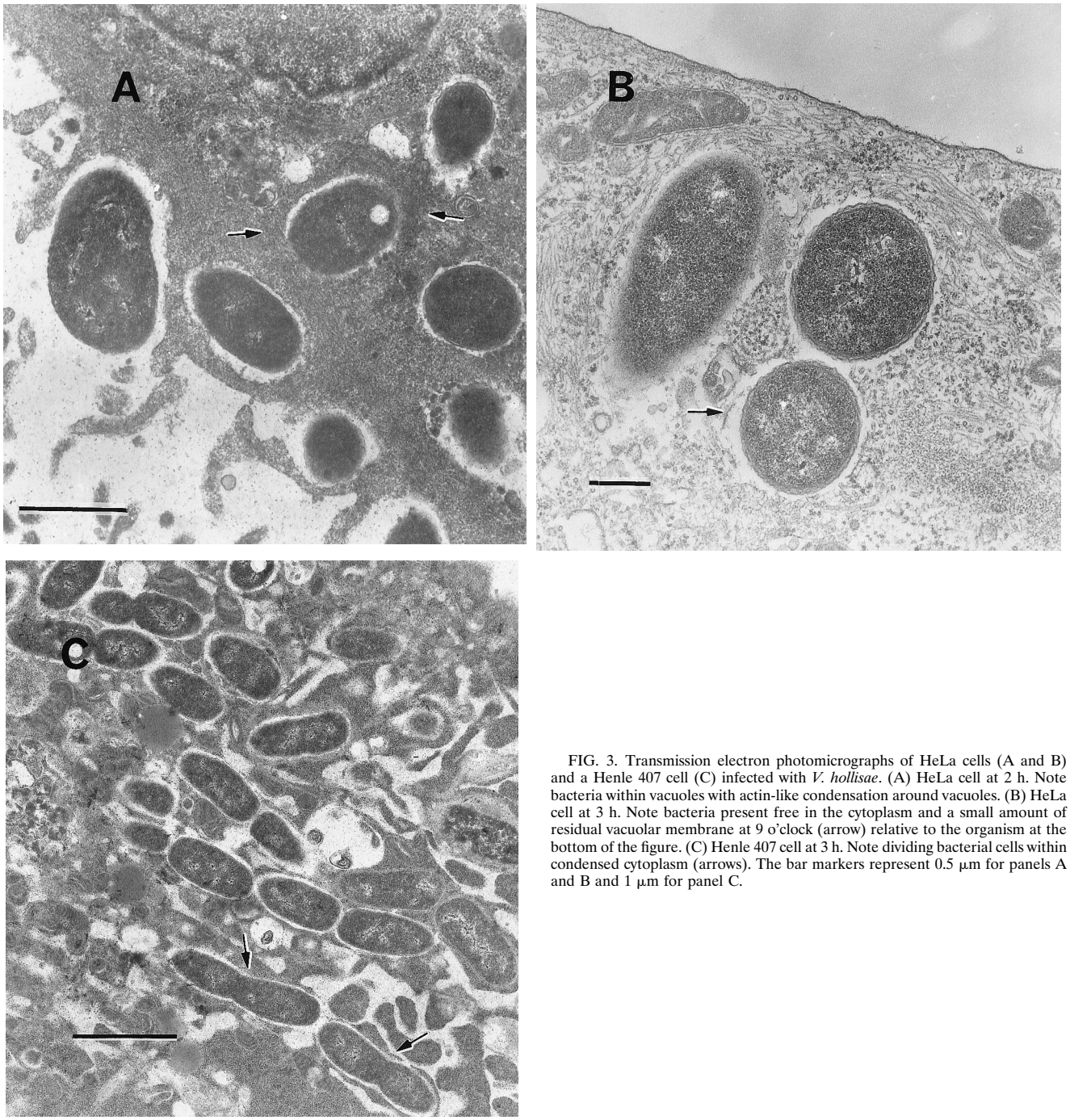

FIG. 3. Transmission electron photomicrographs of HeLa cells (A and B) and a Henle 407 cell (C) infected with *V. hollisae*. (A) HeLa cell at 2 h. Note bacteria within vacuoles with actin-like condensation around vacuoles. (B) HeLa cell at 3 h. Note bacteria present free in the cytoplasm and a small amount of residual vacuolar membrane at 9 o'clock (arrow) relative to the organism at the bottom of the figure. (C) Henle 407 cell at 3 h. Note dividing bacterial cells within condensed cytoplasm (arrows). The bar markers represent 0.5 μm for panels A and B and 1 μm for panel C.

of five to six bacteria per cell. At 3 h, a few more cells were invaded (38%), with the same number of bacteria per cell. Bacterial uptake was confirmed by TEM (Fig. 3). Microvilli appeared to be elongated and to engulf *V. hollisae* (Fig. 3A), similar to observations reported for diffusely adherent *E. coli* (30). The internalization of bacteria within vacuoles was evident 2 h after infection (Fig. 3A). The presence of intracytoplasmic bacteria by 3 h implied the breakdown of the endosomal membrane with release of bacteria into the cytoplasm (Fig. 3B). Dividing bacterial cells within Henle 407 cells were evident at this time, demonstrating that internalized cells were multiplying (Fig. 3C). The cytoplasm appeared condensed, as though the cell was becoming necrotic (Fig. 3C), preventing prolonged observation of intracellular multiplication with either HeLa or Henle 407 cells. Although invasion was minimal (<0.001% after 9 h of infection) with HCT-8 cells, intracellular multiplication was confirmed by infecting the cell line for longer time periods and performing quantitative plate counts. At 5 h postinfection, an average of 10 CFU/ml was isolated. By 7 h, bacteria had multiplied to an average of 75 CFU/ml, and by 9 h, they had increased by 1,000-fold. However, after overnight infection, no bacteria were recovered. Microscopic ex-

TABLE 1. Effects of biochemical inhibitors on HeLa cell invasion by *V. hollisae* and *S. typhi*[a]

Inhibitor[b]	*V. hollisae*		*S. typhi*	
	% HeLa cells invaded (*P*)[c]	No. of bacteria/cell (*P*)[d]	% HeLa cells invaded (*P*)[c]	No. of bacteria/cell (*P*)[d]
NI	45.2 ± 9.7	3.6 ± 0.9	40.2 ± 18.1	5.7 ± 1.5
CH	18.7 ± 12.7 (<0.05)	3.6 ± 1.2	15.0 ± 12.1 (<0.05)	3.8 ± 2.0
CB	9.7 ± 7.7 (<0.01)	1.3 ± 0.5 (<0.01)	28.1 ± 20.9	2.8 ± 2.1 (<0.05)
Col	19.6 ± 1.8 (<0.01)	2.1 ± 0.5 (<0.01)	30.5 ± 12.6	5.0 ± 2.8
Ouab	12.2 ± 9.6 (<0.01)	1.9 ± 0.8 (<0.01)	44.6 ± 15.1	6.24 ± 1.3
Cm	13.6 ± 8.4 (<0.01)	1.6 ± 0.3 (<0.01)	3.7 ± 2.5 (<0.01)	1.9 ± 0.8 (<0.01)

[a] Slides were examined blind. Data are the means ± standard deviations for four experiments performed in quadruplicate.
[b] NI, no inhibitor; CH, cycloheximide; CB, cytochalasin B; Col, colchicine; Ouab, ouabain; Cm, chloramphenicol.
[c] *P*, Level of significance of % HeLa cells invaded, as determined by Student's *t* test.
[d] *P*, Level of significance of number of intracellular bacteria per cell, as determined by Student's *t* test.

amination of HCT-8 cells infected with *V. hollisae* overnight revealed perforated cells devoid of bacteria. These observations suggest that multiplication did indeed occur, but that by 24 h, probably because of increased host cell membrane permeability caused by the bacterial cytotoxin, bacteria were released into the medium.

Association of actin condensation with internalized bacteria (Fig. 3A) intimated the involvement of cytoskeletal elements such as microfilaments (MFs) and/or microtubules (MTs) in the invasion process. To substantiate this, infection assays were performed in the presence of the inhibitors cytochalasin B, known to depolymerize MFs (20, 27), and colchicine, which depolymerizes MTs (20). Both inhibitors significantly ($P < 0.01$) reduced invasion of HeLa cells (Table 1). These results confirmed the positive roles of MFs and MTs in the internalization of *V. hollisae*. MFs and/or MTs have previously been found to be involved in entry of mammalian cells by members of other bacterial genera (3–5, 8, 9, 19, 20, 22, 25). *Citrobacter freundii* (20) and *Klebsiella pneumoniae* (unpublished data), like *V. hollisae*, require both MF and MT involvement. Treatment of infected monolayers with phalloidin-fluorescein isothiocyanate in the absence of cytochalasin B revealed fluorescent filamentous actin, with concentrated areas of fluorescence associated with *V. hollisae*, and in the presence of this inhibitor these monolayers demonstrated random fluorescence. Concentrated spots of fluorescence associated with *V. hollisae*, similar to those seen with phalloidin-treated cells, were also seen after indirect fluorescent staining with antibodies to actin (Fig. 4A) and tubulin, reconfirming the involvement of actin and tubulin in bacterial uptake. This fluorescence was absent when invasion was performed in the presence of cytochalasin B and colchicine, respectively.

However, a second system of internalization, receptor-mediated endocytosis, may be involved in *V. hollisae* uptake, as

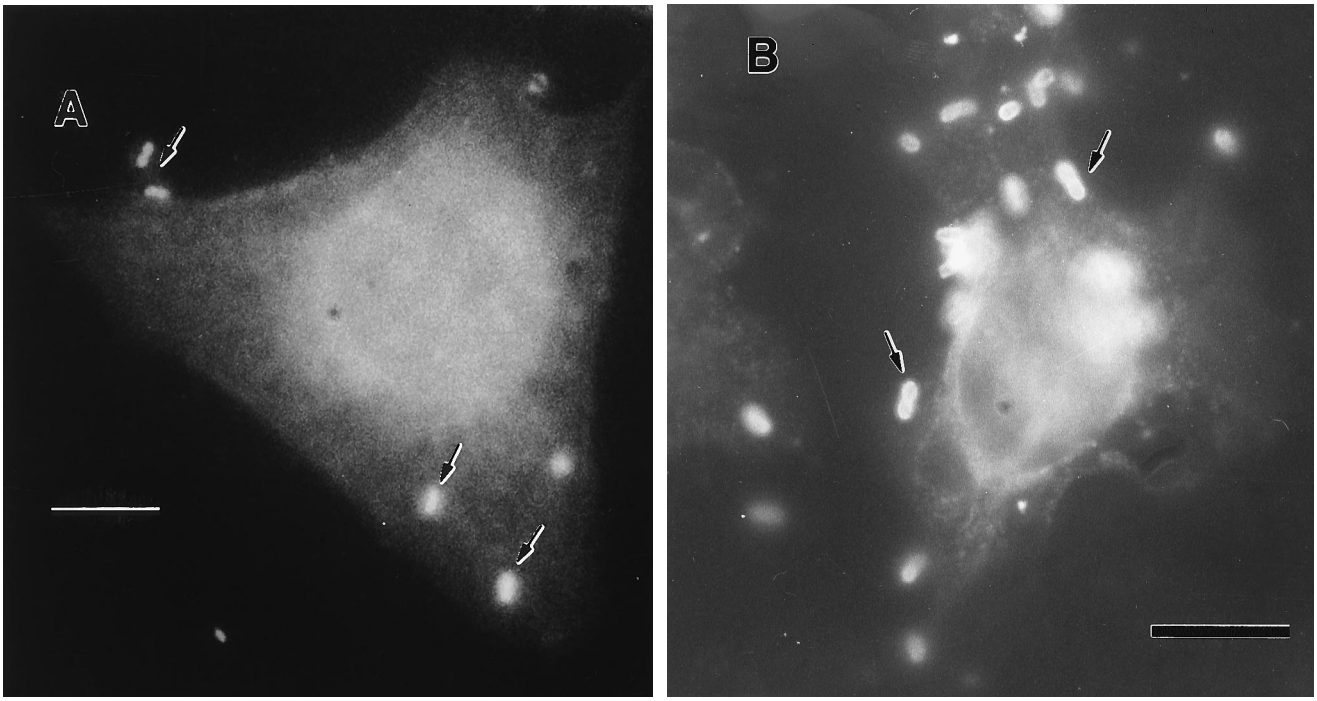

FIG. 4. Fluorescent micrographs of HeLa cells 2.5 h after infection with *V. hollisae* strain 75-80. (A) Cells stained with anti-actin antibody, showing concentrated areas of fluorescence associated with the bacteria (arrows). (B) Cells stained with anti-clathrin antibody, also revealing concentrated areas of fluorescence associated with the bacteria (arrows). The bar markers in each panel represent 10 μm.

indicated by a significant reduction of invasion ($P < 0.01$) in the presence of ouabain (G-strophanthin) (Table 1). Ouabain, known to inhibit K^+ uptake by arresting Na^+/K^+ ATPase activity, is thought to disrupt receptor-mediated endocytosis by decreasing the number of coated pits on the cell surface and possibly disturbing the cross-linkage (association) of clathrin, a major component of coated pits, with the plasma membrane (1, 13, 21, 25). The involvement of clathrin was confirmed by indirect fluorescent staining of infected monolayers with anti-clathrin antibody. Concentrated fluorescent spots associated with *V. hollisae* were evident in the absence of ouabain (Fig. 4B) but not in its presence. Initial entry into epithelial cells could be part of an integrated pleiotropic cell process involving intracellular regulation of K^+ ions, cytoskeletal elements, and cell membrane plasticity in response to *V. hollisae* adherence. Likewise, the internalization of other invasive bacteria is inhibited by interference with coated-pit formation and thus receptor-mediated endocytosis (1, 2, 5, 8, 19–23, 25).

Also, as with *Shigella flexneri* (8) and *S. typhi* (4), *V. hollisae* uptake was reduced by cycloheximide, an inhibitor of de novo eukaryotic cell protein synthesis (Table 1). This reaffirms the role of membrane-associated proteins in *V. hollisae* invasion. Invasion was also dramatically reduced in the presence of chloramphenicol (Table 1), demonstrating a requirement of de novo protein synthesis of the bacterial cell. Similar results were obtained with Henle 407 cell infectivity in the presence of the inhibitors, in that all the inhibitors used significantly reduced *V. hollisae* invasion (data not shown). Furthermore, as previously shown by other methods (4, 19, 20) and thereby justifying the more simple and rapid AO fluorescent staining, *S. typhi*-infected HeLa cells stained with AO also showed that chloramphenicol, cycloheximide, and cytochalasin significantly reduced invasion by *S. typhi* (Table 1). Neither colchicine nor ouabain had a significant effect on *S. typhi* invasion. The present study is the first published report of invasion of tissue culture cells by *V. hollisae*. The data presented here provide evidence that the pathogenicity of *V. hollisae* may be multifactorial in that besides producing toxins, *V. hollisae* also invades eukaryotic epithelial cells. This is in agreement with the septicemia, which indicates invasive disease, observed in some patients (14).

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