INFECTION AND IMMUNITY, Jan. 1993, p. 350–355
0019-9567/93/010350-06$02.00/0

Vol. 61, No. 1

Adherence of *Helicobacter pylori* to Cultured Human Gastric Epithelial Cells

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Received 20 May 1992/Accepted 19 October 1992

Experiments were performed to demonstrate that adherence of *Helicobacter pylori* to gastric epithelial cells causes alterations in the cell cytoskeleton. *H. pylori* intimately attached to cultured human gastric epithelial cells on small cellular projections, while there was no intimate association of *H. pylori* with cultured human esophageal epithelial cells. Fluorescein-conjugated phalloidin staining of gastric epithelial cells showed that *H. pylori* adherence stimulated actin polymerization; this stimulation was not observed with esophageal cells. Also, this organism's selectivity for gastric mucosa was supported by rare binding of bacteria to esophageal epithelial cells and gastric fibroblasts.

Helicobacter pylori infection of the gastric mucosa is very common in the United States, with more than 50% of adults over 60 years of age infected with this bacterium (5, 7, 8). Infection with this bacterium is localized to the gastric mucosa, with the esophagus and duodenum involved only when heterotopic or metaplastic gastric epithelium is present in these areas. Ultrastructural analysis of gastric mucosa infected with *H. pylori* shows that this bacterium causes effacement of normal gastric epithelial microvilli and closely adheres to the apical cell membrane (2, 3). *H. pylori* has also been shown to adhere to gastric epithelial cells on small cellular projections referred to as adherence pedestals (3). In

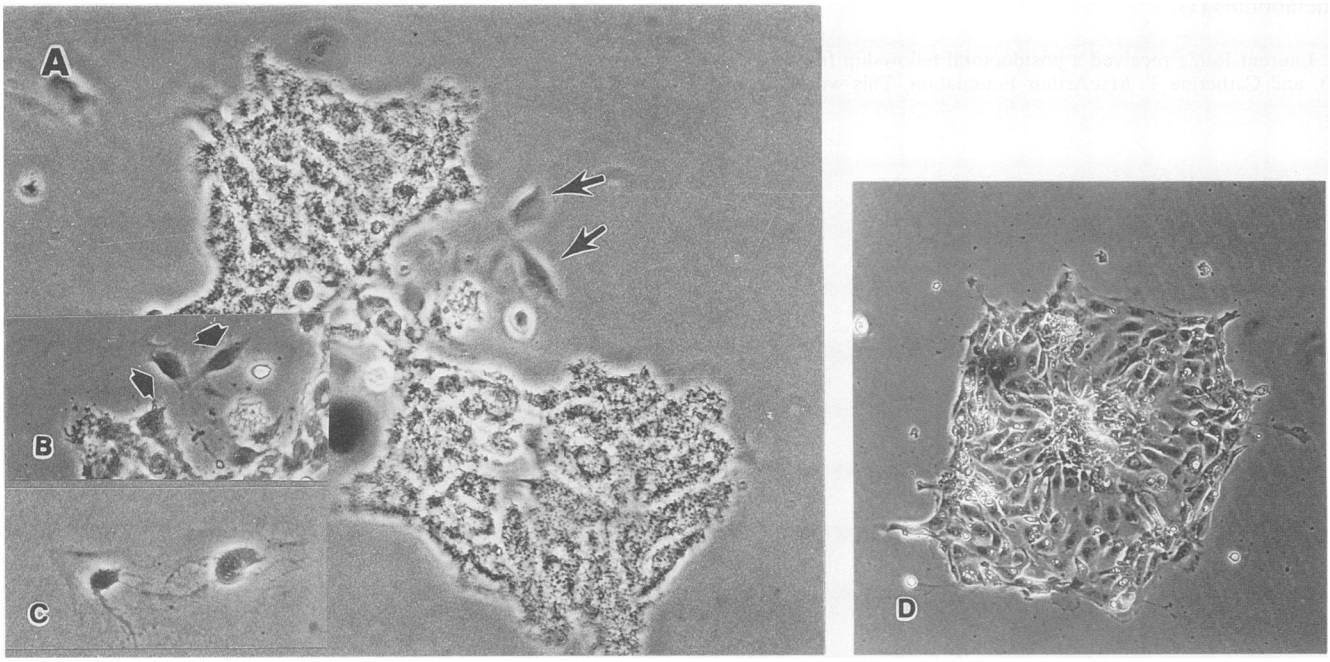

FIG. 1. Adherence of *H. pylori* to cultured primary human gastric epithelial cells. After 48 h in culture, primary cultures of human gastric epithelial cells were exposed to *H. pylori* suspended in culture medium for 3 h. (A) Phase-contrast microscopy demonstrates dense binding of *H. pylori* to gastric epithelial cells and rare binding of this organism to fibroblasts. (B) Rare binding of *H. pylori* to two fibroblasts indicated by the arrows (these are the same cells which are indicated by the arrows in panel A). (C) Fibroblasts to which *H. pylori* did not bind after 3 h of exposure to the bacteria. (D) Control primary human gastric epithelial cells not exposed to *H. pylori*.

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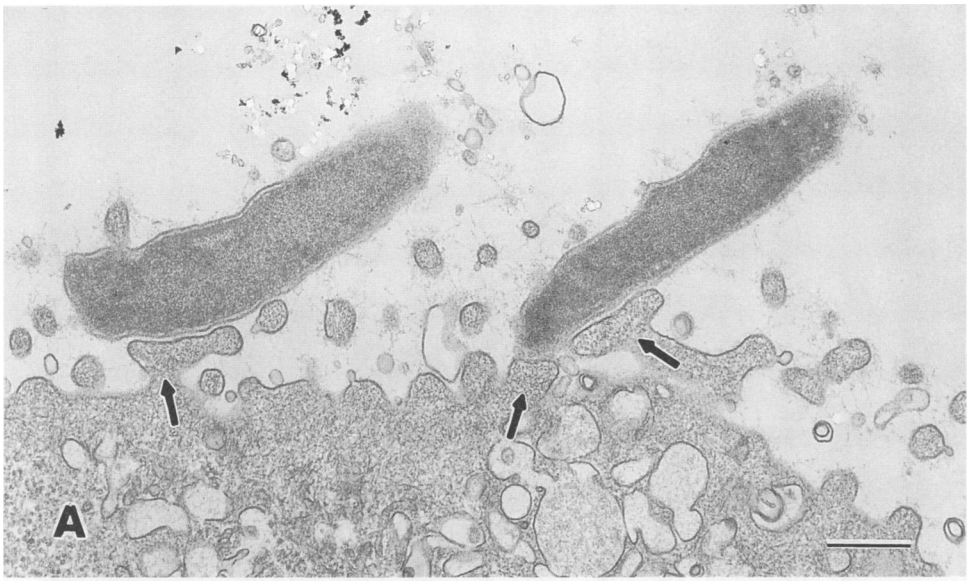


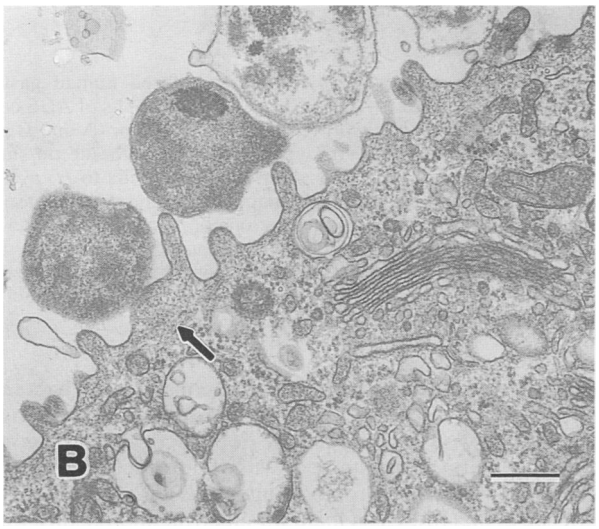
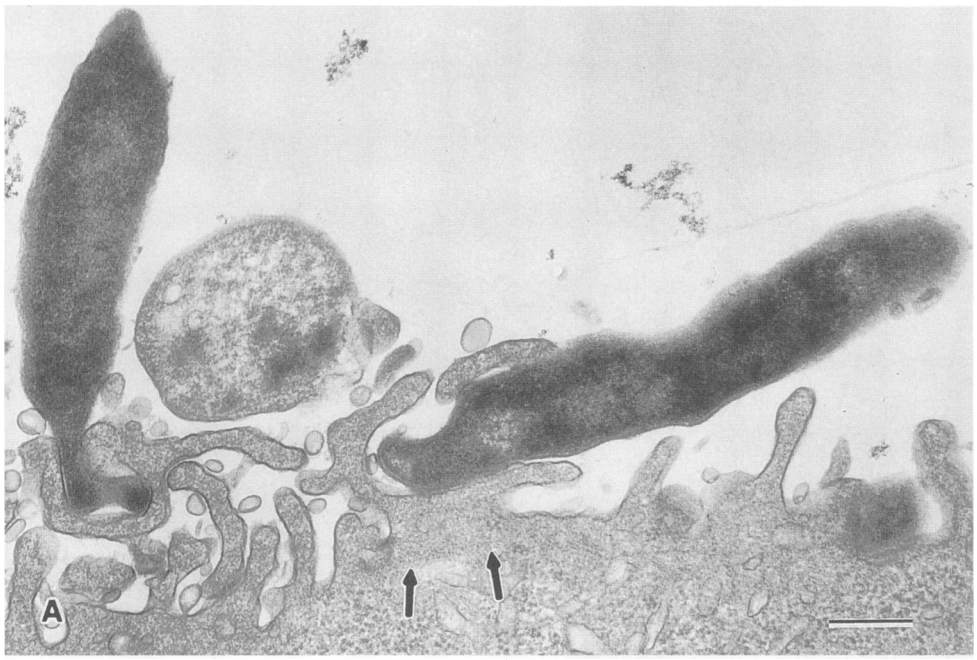


FIG. 2. Electron micrographs of primary human gastric epithelial cells exposed to *H. pylori* for 3 h. (A and B) The bacteria intimately associate with the cytoplasmic membrane, causing effacement of microvilli. In some cases, small projections appear to form from the base of damaged microvilli in contact with *H. pylori* (shown by the arrows). Bars = 0.5 μm.

this regard, *H. pylori* is similar to enteropathogenic *Escherichia coli* (EPEC), which causes effacement of normal enterocyte microvilli and closely adheres to the enterocyte apical membrane on cellular projections (12, 14). Knutton and colleagues (10) reported that the dense collection of microfilaments present in the cellular projections beneath adherent EPEC was actin and showed that adherence of EPEC stimulated actin polymerization within cultured small intestinal epithelial cells. The present study was designed to evaluate adherence of *H. pylori* to cultured human gastric and esophageal epithelial cells and to evaluate whether binding of *H. pylori* to cultured human gastric epithelial cells stimulated actin polymerization and adherence pedestal formation, as has been reported for EPEC.

H. pylori strains, obtained from endoscopic biopsy specimens of symptomatic patients seen at the University of Maryland Hospital and Veterans Administration Hospital (both in Baltimore, Md.) were isolated and characterized as previously described (13, 16). *H. pylori* strains (UMAB12, UMAB41, UMAB59, UMAB63, and UMAB69) were cultured on brucella agar (Difco Inc., Detroit, Mich.) containing 5% sheep blood, Skirrow's antibiotic supplement (Difco), and 0.25 μg of amphotericin B (Hazelton, Lenexa, Kans.) per ml or Trypticase soy agar with 5% sheep blood (Mathieson Scientific, Jessup, Md.) for 3 to 4 days at 37°C in an anaerobic jar with a palladium catalyst and activated Campypac (BBL Microbiology Systems, Cockeysville, Md.).

Primary human gastric epithelial cells were enzymatically isolated from gastric biopsies obtained during esophagogastroduodenoscopy. Institutional review board approval for this study is on file at Howard University. The biopsy tissue, after being minced with a scalpel, was incubated in a collagenase-dispase solution at 37°C for 60 min and then pelleted by centrifugation (9). The gastric cells were cultured in Ham's F12 medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% fetal bovine serum (GIBCO) and 0.25 mg of gentamicin (GIBCO) per ml and then plated onto 60-mm-diameter tissue culture dishes (Costar Corp., Cambridge, Mass.). The culture medium was changed after 24 h of culture. After 48 h of culture, cells were overlaid with suspensions of *H. pylori* (10^7 CFU/ml) in Ham's F12 medium without antibiotics and incubated at 37°C for 1 or 3 h, washed three times with phosphate-buffered saline to remove nonadherent bacteria, and then fixed in glutaraldehyde for electron microscopy. Only gastric cells from patients who were not infected with *H. pylori* were used in these studies. The absence of *H. pylori* in these patients was con-



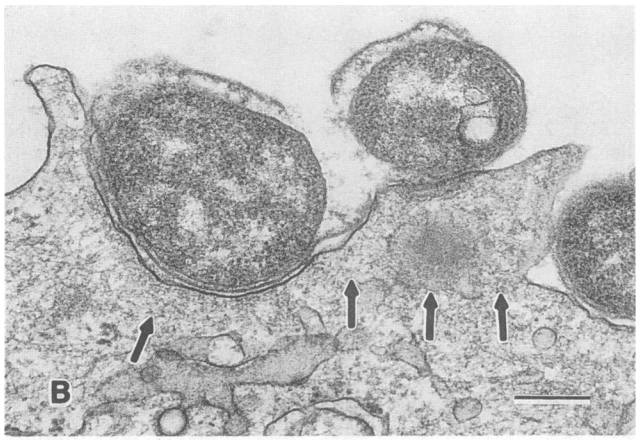


FIG. 3. Adherence of *H. pylori* to a cultured human gastric adenocarcinoma cell line (AGS). Electron micrographs of AGS cells that were exposed to *H. pylori* (10^7 CFU/ml) for 3 h. (A and B) *H. pylori* is adhering to the gastric epithelial cell membrane on small cellular projections after 3 h of exposure of the cells to *H. pylori*. The cellular projections (pedestals) appear to form from the base of damaged microvilli. Dense concentrations of microfilaments can be observed just below the epithelial cell cytoplasmic membrane beneath the adherent bacteria. Arrows show actin filaments collecting beneath adherent *H. pylori*. In panel A, bar = 0.5 μm; in panel B, bar = 0.25 μm.

firmed with a urea broth test and histological examination performed on additional biopsy tissue taken from each patient.

A human gastric adenocarcinoma cell line (AGS) CRL 1736 (American Type Culture Collection, Rockville, Md.) and a simian virus 40 T-antigen-transformed human esophageal epithelial cell line (HE457), derived from normal human esophageal epithelial cells were used in this study (1, 17). AGS cells were cultured in Ham's F12 medium supplemented with 10% fetal bovine serum. HE457 cells were cultured in keratinocyte serum-free medium (K-SFM [GIBCO]). All cultures were incubated at 37°C in a 5% carbon dioxide, 95% humidified, water-jacketed incubator (Forma Scientific, Marietta, Ohio). AGS and HE457 cells were plated onto glass Lab-TeK chamber slides (Nunc, Inc., Naperville, Ill.) or 60-mm-diameter tissue culture dishes and allowed to grow to approximately 80% confluency. For each experiment, *H. pylori* was suspended in cell culture medium (either Ham's F12 or K-SFM), warmed to 37°C, to a final concentration of 10^7 CFU/ml. The bacterial suspensions were immediately added to both cell lines and allowed to incubate at 37°C for 1, 3, and 6 h, washed three times with phosphate-buffered saline to remove nonadherent bacteria, and then fixed either in cold acetone for fluorescent light microscopy or in glutaraldehyde for electron microscopy. Actin filaments were stained with fluorescein-conjugated phalloidin (Molecular Probes, Eugene, Oreg.), as described by Resau et al. (15). Cells fixed for electron microscopy were scraped off the culture dishes with a spatula and pelleted by ultracentrifugation. Specimens were then prepared for electron microscopy as previously described (16).

Human gastric epithelial cells quickly form multiple small colonies of tightly packed cuboidal cells when placed in culture (Fig. 1). These small colonies of cuboidal cells were shown to be epithelial in origin by staining positively for cytokeratin and mucin (data not shown). Fibroblasts, which are characteristically spindle shaped, are scattered infrequently throughout these cultures and stain negatively for cytokeratin and mucin. *H. pylori* readily adhered to cultured primary human gastric epithelial cells, which had been in culture for 48 h. Phase-contrast microscopy demonstrated that *H. pylori* cells were densely concentrated on the surface of gastric epithelial cells after only 3 h of exposure to the bacteria, and the bacteria rarely adhered to fibroblasts (Fig. 1). Electron micrographs of primary human gastric epithelial cells exposed to *H. pylori* for 3 h demonstrated that this bacterium closely associates with the epithelial cell membrane, causing effacement of adjacent microvilli (Fig. 2). These bacteria also appear to stimulate formation of small cellular projections which develop beneath adherent organisms, from the base of damaged microvilli.

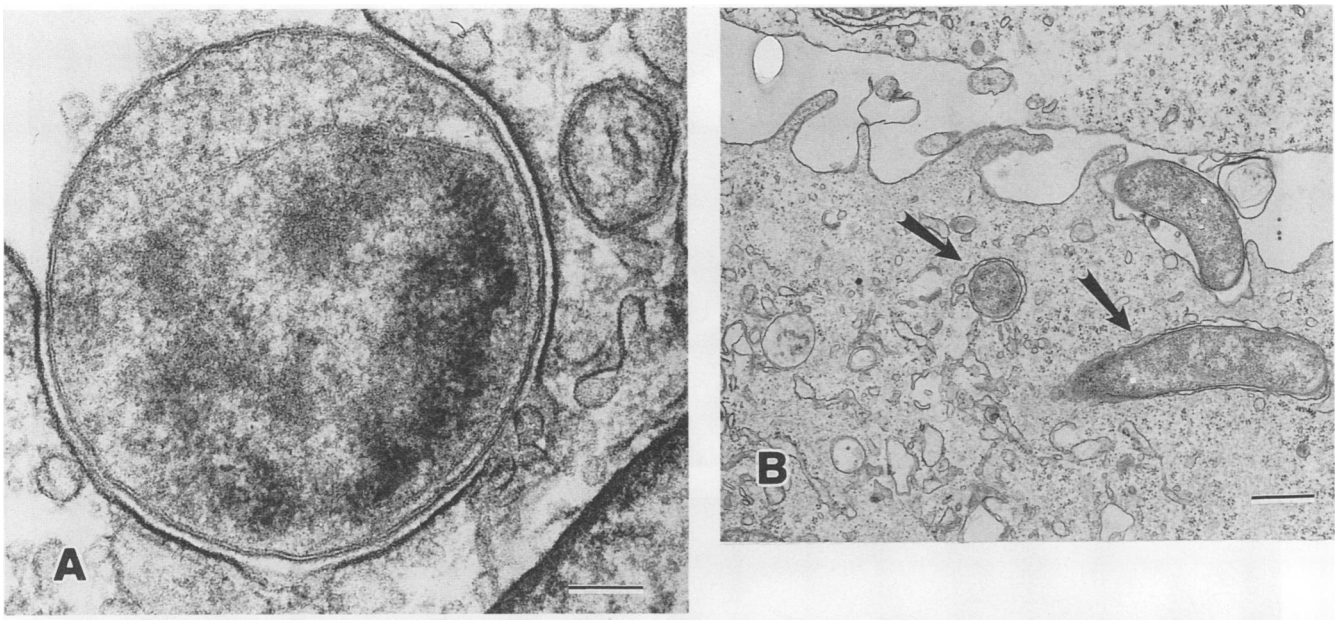


FIG. 4. Engulfment of *H. pylori* by human gastric adenocarcinoma cells (AGS). Electron micrographs show cultured gastric epithelial cells that have been exposed to *H. pylori* for 6 h. (A) *H. pylori* partially engulfed by the cytoplasmic membrane of an AGS cell. Bar = 0.1 μm. (B) *H. pylori* bacteria within an AGS cell, as shown by the arrows. Bar = 0.5 μm.

All five *H. pylori* strains tested by phase-contrast microscopy readily adhered to AGS cells. Electron micrographs demonstrated that *H. pylori* adherence to cultured AGS cells caused effacement of microvilli and stimulated the development of small cellular projections (Fig. 3). These small cellular projections appeared to form from the base of damaged microvilli and are predominantly composed of microfilaments (Fig. 3). However, *H. pylori* did not adhere to the surface of HE457 cells by phase-contrast microscopy. Transmission electron micrographs confirmed that there was no intimate association of *H. pylori* with the HE457 cellular membrane. Electron micrographs of AGS cells exposed to *H. pylori* for 6 h demonstrated that several of the AGS cells had engulfed this bacterium (Fig. 4). The engulfed bacteria showed evidence of deterioration and may not have been viable at the time of phagocytosis.

Control AGS cells stained for the presence of actin filaments with fluorescein-conjugated phalloidin demonstrated a loosely organized network of actin filaments and densely fluorescent borders (Fig. 5). AGS cells exposed to *H. pylori* for 1 h and stained with phalloidin were not significantly different from control cells. However, after 3 h of exposure to *H. pylori*, small foci of intense fluorescence were present in AGS cells, which suggested accumulation of actin filaments beneath adherent bacteria (Fig. 5). These changes were present with all five of the strains tested after 3 h of exposure to *H. pylori* and became more evident after 6 h of exposure to this bacterium. Phalloidin-stained HE457 cells exposed to *H. pylori* for 1, 3, and 6 h were not significantly changed from controls, showing no evidence of actin polymerization.

The close association of *H. pylori* with gastric epithelial cells stimulates not only morphological but also physiological changes within gastric epithelial cells. These in vitro studies confirm the specificity of *H. pylori* for human gastric epithelial cells and its lack of binding to human esophageal epithelial cells and human fibroblasts. The intimate associations of *H. pylori* with primary human gastric epithelial cells and with AGS cells appeared very similar by transmission electron microscopy. *H. pylori* caused effacement of microvilli and stimulated development of small cellular projections in both normal and malignant human gastric epithelial cells. Actin polymerization, demonstrated by phalloidin staining, occurred in AGS cells exposed to *H. pylori*, which correlated well with the development of cellular projections demonstrated on electron micrographs. Adherence pedestals, however, form only when bacteria attach to sites of the epithelial cell membrane adjacent to microvilli, suggesting that these projections form only from the base of microvilli and that this may be a specialized property of the apical cell membrane. The microfilaments within injured microvilli appeared to concentrate at the base of the microvilli as the cellular projections form, implying that polymerization of actin is also instrumental in the formation of these projections. The lack of adherence of *H. pylori* to esophageal epithelial cells (HE457), as demonstrated by both light and electron microscopy, is consistent with the predilection of this bacterium for gastric mucosa and suggests that this model will be useful in identifying receptors on the gastric epithelial cell to which this organism binds.

The actin filaments found to accumulate below adherent *H. pylori*, although similar, were less dense than those observed in association with adherence of EPEC to small intestinal epithelial cells (11). Actin polymerization is useful in distinguishing EPEC strains from other types of diarrheagenic *E. coli* which have different binding patterns, suggesting that this may become a useful diagnostic tool (11). However, each of the five strains of *H. pylori* tested in this study stimulated actin polymerization. Many more strains need to be tested to determine whether strain differences can be appreciated by staining cells for actin filaments.

In vitro studies have demonstrated that *H. pylori* produces a toxin which causes reversible cell injury (4, 11). In one study, broth culture supernatants of over one-half of 200 *H.*

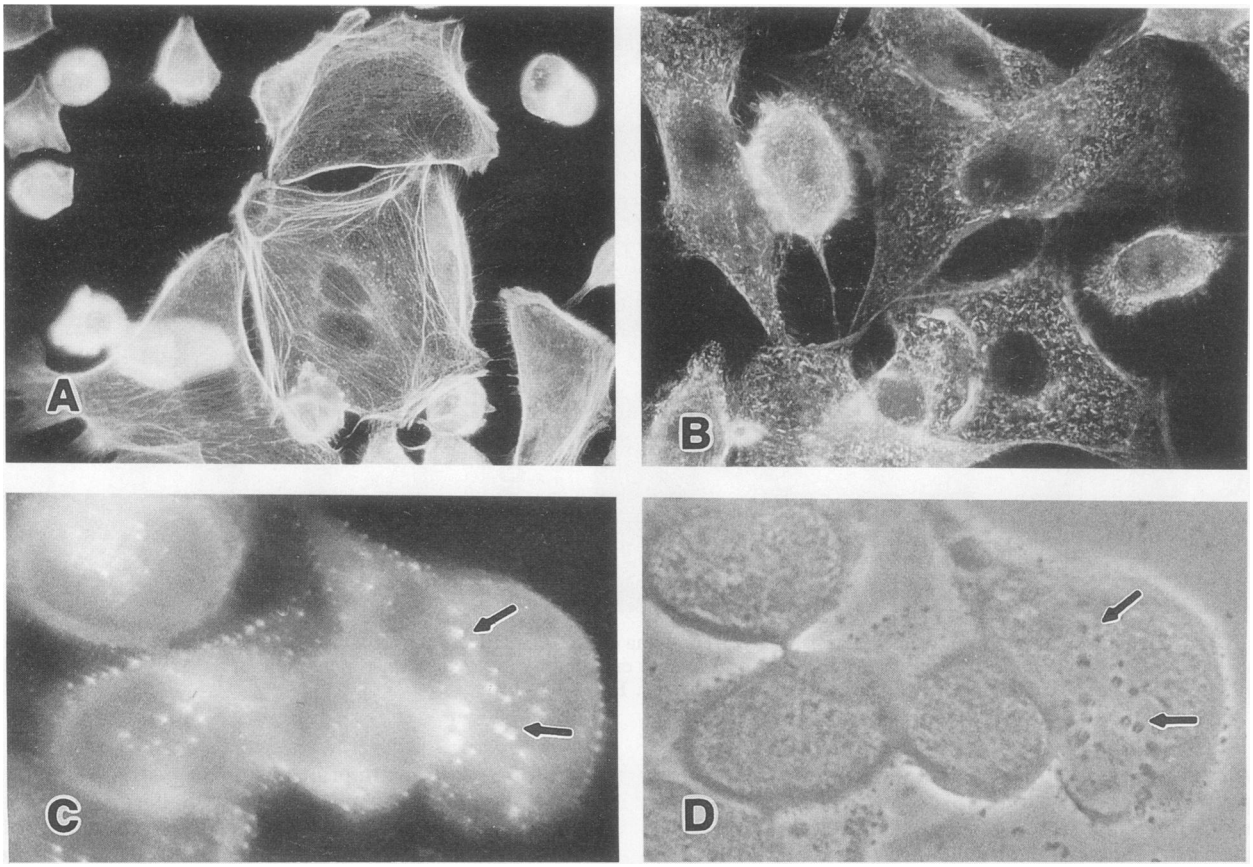


FIG. 5. Fluorescein-conjugated phalloidin staining of cultured human gastric adenocarcinoma cells (AGS) exposed or not exposed to *H. pylori*. (A) Control gastric cells (not exposed to *H. pylori*) stained with fluorescein-conjugated phalloidin show a loosely organized network of actin filaments with densely fluorescent cell borders. (B and C) AGS cells stained with fluorescein-conjugated phalloidin, after exposure to *H. pylori* (10^7 CFU/ml) for 3 h, demonstrate small foci of intense fluorescence, which correlate with sites of bacterial adhesion, as shown by phase-contrast microscopy (D) (the fields in panels C and D are the same). The arrows in panel C show actin filament polymerization in epithelial cells, and the arrows in panel D show bacteria above polymerized actin filaments.

pylori strains produced vacuoles in cultured cells (11). There is also evidence that this toxic activity is present in strains from patients with peptic ulcer disease more commonly than those from patients with gastritis alone (6). Virulence factors produced by some *H. pylori* isolates, such as a toxin, would likely influence the host cell response to the organism. Intimate attachment may represent an efficient delivery system for the toxin to its target cell. Cellular responses, such as actin polymerization, may allow bacteria to transfer toxins by direct contact with the host cell. Electron micrographs demonstrated that *H. pylori* was taken up by these gastric epithelial cells. Only on rare occasions has *H. pylori* been found within gastric epithelial cells in vivo. Further investigations are needed to determine whether *H. pylori* can become invasive and whether epithelial cells actively participate in eliminating this organism from the gastric lumen.

In summary, these studies demonstrate that *H. pylori* binds more readily to gastric epithelial cells than to esophageal epithelial cells or fibroblasts. *H. pylori* can induce changes in the gastric epithelial cell cytoskeleton, when adhering to the cell membrane adjacent to microvilli. The similarities between the attachment of *H. pylori* and EPEC to cultured epithelial cells suggests that adherence may play an important role in the pathogenesis of *H. pylori*.

This work was supported in part by Public Health Service grants GM-08244 and AI-25567 from the National Institutes of Health.

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