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Distinct Binding Properties of *eaeA*-Negative Verocytotoxin-Producing *Escherichia coli* of Serotype O113:H21

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Infection of humans with verotoxin-producing *Escherichia coli* (VTEC) O113:H21 is associated with clinical features comparable to those associated with infection with attaching and effacing VTEC strains including those of serotype O157:H7. We have shown previously that the adhesion phenotype of VTEC O157:H7 is influenced by the presence of a homolog of the chromosomal *eaeA* (for *E. coli* attaching and effacing) gene. In contrast, by colony blot hybridization, VTEC O113:H21 is negative for the *eaeA* gene. Therefore, the aim of this study was to define the adhesion phenotype of VTEC O113:H21 strain CL-15 to both cultured epithelial cells (HEp-2) and rabbit intestine in vivo. Under transmission electron microscopy, areas of microvillus effacement were observed in regions directly beneath the organism in CL-15-infected cells both in vitro and in vivo. However, F-actin adhesion pedestals on the host plasma membrane were absent. Failure of CL-15 to induce polymerization of actin was confirmed by using staining of F-actin with fluorescein-labeled phalloidin. Under indirect immunofluorescence microscopy, CL-15-infected HEp-2 cells also failed to demonstrate the recruitment of another cytoskeletal element, α-actinin, below foci of bacterial adhesion. In contrast, VTEC O157:H7 infection of HEp-2 cells was associated with increased α-actinin immunofluorescence. These findings suggest that bacterial factors distinct from those of EaeA are necessary for the adhesion phenotype of VTEC O113:H21.

Verocytoxin-producing *Escherichia coli* (VTEC) strains are associated in humans with sporadic cases and outbreaks of hemorrhagic colitis and with hemolytic-uremic syndrome (20). Although O157:H7 is the VTEC serotype most commonly isolated from humans, a number of other serotypes also cause human disease (20). In particular, the O113:H21 serotype of VTEC has been isolated from the stools of children with hemorrhagic colitis and hemolytic-uremic syndrome (21).

Multiple serotypes of VTEC demonstrate an attaching and effacing (A/E) form of bacterial binding both to tissue culture cells in vitro (33, 35) and to enterocytes and colonocytes in vivo (32, 34, 36). A homolog of the chromosomal *eaeA* gene, essential for A/E activity in enteropathogenic *E. coli* (19), was recently cloned and sequenced from two VTEC O157:H7 strains (6, 38). The presence of the *eaeA* gene in a variety of VTEC serotypes was then examined by using sequences based upon conserved central regions of the enteropathogenic *E. coli* (EPEC) and VTEC *eaeA* genes as a probe for colony hybridization and for primers in PCR assays (25). Although all 50 O157:H7 strains tested were *eaeA* positive, each of the six VTEC strains of serotype O113:H21 tested were *eaeA* probe negative. Therefore, the aim of this study was to define the adhesion phenotype of *eaeA*-negative VTEC O113:H21.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *E. coli* serotypes and strains used in this study are listed in Table 1. A VTEC strain, designated CL-15, of serotype O113:H21 was originally isolated from the stool specimen of a 44-month-old girl with hemorrhagic colitis and hemolytic-uremic syndrome (21) and kindly provided to us by M. Karmali (The Hospital for Sick Children, Toronto, Ontario, Canada). CL-15 is negative by colony blot hybridization with conserved regions of the *eaeA* gene (25). For comparisons, *eaeA*-positive *E. coli* was employed, including VTEC O157:H7 strains CL-56 (21) and 7785-5 (36) (kindly provided by T. Barrett, Centers for Disease Control, Atlanta, Ga.) and EPEC strain E2348, serotype O127:H6 (22). The *eaeA*-negative laboratory strain *E. coli* HB101 (O:rough) (25) was used as a negative control.

Bacterial strains were stored in Penassay broth (Difco Laboratories, Detroit, Mich.) plus 10% glycerol at −70°C. Prior to use, each bacterial strain was streaked onto Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) slants, grown overnight at 37°C, and stored at 4°C. For infection, *E. coli* strains were grown in static, nonaerated Penassay broth (Difco). After overnight growth at 37°C, bacteria were harvested by centrifugation at 2,500 × *g* for 15 min and resuspended in sterile phosphate-buffered saline (PBS; pH 7.4; ICN Biomedicals, Inc., Costa Mesa, Calif.) to a concentration of 10^{10} viable organisms per ml. Viable counts of bacteria were obtained by serial 10-fold dilutions that were plated onto bile salt agar plates (Oxoid Canada Inc., Nepean, Ontario).

Cell culture. The human epithelial cell line HEp-2 (American Type Culture Collection, Rockville, Md.) and the human embryonic lung fibroblast line HEL (American Type Culture Collection), both widely used as model systems for investigating bacterial adhesion profiles (22, 23), were grown in Eagle basal medium (ICN) supplemented with 15% decomplemented fetal calf serum (Bocknek, Rexdale, Ontario, Canada), 0.5% glutamine (ICN), 0.1% sodium bicarbonate (ICN), 2% penicillin-streptomycin (ICN), and 1% amphotericin B (ICN)

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TABLE 1. *E. coli* strains employed in this study

Strain	Serotype	*eaeA*[a]	Verotoxin production[a]	Source[b]
CL-15	O113:H21	−	+	HC, HUS
CL-56	O157:H7	+	+	HC, HUS
7785-5	O157:H7	+	+	HC
E2348	O127:H6	+	−	Infant diarrhea
HB101	O:rough	−	−	Laboratory strain

[a] +, presence; −, absence (21, 25).
[b] HC, hemorrhagic colitis; HUS, hemolytic-uremic syndrome (21, 35).

and cultivated on 75-cm^2 tissue culture flasks (Corning Glass Works, Corning, N.Y.) as monolayers at 37°C in 5% CO_2. Approximately 10^6 viable cells were used for each bacterial infection. The numbers of tissue culture cells were counted with a hemocytometer (Improved Neubauer; Reichert Scientific Instruments, Buffalo, N.Y.), and the viability of cells was determined by the percentage of cells excluding trypan blue (8).

In vitro bacterial adhesion. Tissue culture cells were washed with Hanks balanced salt solution and then infected with approximately 10^9 bacteria for 6 h at 37°C. The culture medium was changed midway through the incubation period. After 6 h, nonadherent bacteria were removed from the cells by six washes with PBS. To remove adherent bacteria and eukaryotic cells from plastic surfaces, the cells were gently scraped off with a rubber policeman.

Electron microscopy. The cells were then pelleted, fixed with 2% (vol/vol) glutaraldehyde (Sigma Chemical Co., St. Louis, Mo.) in 0.1 M phosphate buffer (pH 7.0), postfixed in 2% osmium tetroxide, and dehydrated through a series of graded acetone washes. Samples were embedded in Epon, and thin sections (0.5 to 1 mm) were cut in a microtome. After being mounted onto microscope slides, sections were stained with toluidine blue (Fisher Scientific Co., Toronto, Ontario, Canada) and then examined under bright-field illumination. To detect formation of A/E adhesion pedestals in infected epithelial cells, ultrathin sections (50 to 60 nm) were cut in a microtome, placed onto 300-mesh copper grids, and stained with uranyl acetate and lead salts as described previously (33). Grids were examined for evidence of A/E lesions by using a Philips 300 transmission electron microscope at an accelerating voltage of 60 kV.

Fluorescent actin staining (FAS). Assessment of the ability of infecting organisms to induce accumulation of F-actin in HEp-2 cells was performed by using the method of Knutton et al. (22). Briefly, an overnight broth culture of approximately 10^8 bacteria was added to approximately 10^6 HEp-2 cells grown on sterile chamber slides (Lab-Tek; Miles Scientific, Naperville, Ill.) and incubated for a total of 6 h at 37°C. After nonadherent bacteria were removed by six washes in PBS, the cells were fixed with 3% formalin (Sigma) and rendered membrane permeable by exposure to 0.1% Triton X-100 (Sigma). The cells were then washed again in PBS, and 0.5 ml of a 5-μg/ml solution of phalloidin conjugated to fluorescein isothiocyanate (Sigma) in PBS was added to each chamber for 30 min at 25°C. The cells were washed three times in PBS and then examined by alternating epifluorescence and phase-contrast microscopies (22). Since phalloidin binds specifically to F-actin (37), intense foci of fluorescence at sites of bacterial adhesion indicate the accumulation of F-actin in response to bacterial infection. EPEC strain E2348 and *E. coli* HB101 were employed as positive and negative controls, respectively.

Immunofluorescence detection of α-actinin. Accumulation of α-actinin in HEp-2 cells following bacterial infection was examined by using the method of Finlay et al. (18) with the minor modifications noted below. HEp-2 cells were grown to subconfluence, infected for either 3 or 6 h, and washed in the same manner as described for the FAS assay. Subsequently, the cells were fixed in cold methanol (100%, vol/vol) for 10 min at 25°C and washed three times with PBS. α-Actinin was then detected by incubation of the infected cells with a 1:100 dilution of murine monoclonal anti-α-actinin (Sigma) as the primary antibody for 1 h at 37°C. After six washes in PBS, cells were incubated with a 1:200 dilution of rabbit anti-mouse immunoglobulin G (heavy plus light chains) conjugated to fluorescein (Jackson ImmunoResearch Laboratories Inc., West Grove, Pa.) for 1 h at 37°C. After further washes in PBS and mounting of coverslips, the slides were examined by using alternating fluorescence and phase-contrast microscopies to detect increased foci of fluorescence in regions subjacent to areas of bacterial adhesion.

Immunofluorescence detection of phosphotyrosine. Accumulation of tyrosine-phosphorylated proteins in infected HEp-2 cells was evaluated by using the method described by Rosenshine et al. (29). Briefly, HEp-2 cells grown to subconfluence were infected for either 3 or 6 h and washed as described for the FAS assay. The cells were then fixed in 2% paraformaldehyde for 15 min at 25°C and washed three times with PBS. Prior to being stained for phosphotyrosine, the cells were permeabilized with 0.1% Triton X-100 for 5 min and washed six times with PBS. Accumulation of tyrosine-phosphorylated proteins was then detected by incubation of cells with a 1:50 dilution of murine monoclonal anti-phosphotyrosine immunoglobulin G (Upstate Biotechnology Incorporated, Lake Placid, N.Y.) as the primary antibody for 1 h at 37°C. After six washes in PBS, the cells were incubated with a 1:50 dilution of the secondary antibody in a manner identical to that described for the α-actinin assay. After further washes in PBS and mounting of coverslips, the slides were examined as described above for the α-actinin assay.

In vivo bacterial adhesion. Enteroadherence of VTEC strain CL-15 was evaluated by using both the ligated-loop model (31) and orogastric challenge of postweaning rabbits (32). For ligated loops, specific-pathogen-free, postweaning New Zealand White male rabbits (each weighing approximately 1.0 kg; Reimens, Guelph, Ontario, Canada) were fasted overnight, and six 10-cm ileal loops, with 2-cm intervening spaces between the loops, were surgically constructed while the rabbits were under general anesthesia. Intestinal segments were then inoculated with either 1.0 ml of PBS containing 5×10^8 *E. coli* bacteria or an equal volume of PBS alone. Rabbits were killed 24 h after inoculation with *E. coli*, and sections of intestine from the ligated loops were excised and fixed in 2% glutaraldehyde in phosphate buffer at pH 7.4. For evaluation of bacterial enteroadherence under transmission electron microscopy, sections were prepared as described above. Tissues to be examined by light microscopy were embedded in paraffin, and serial sections were cut in a microtome and stained with Giemsa (Fisher). A minimum of five well-oriented sections from each ileal loop were examined under bright-field illumination.

For orogastric infection, rabbits were housed individually in cages with wire mesh floors. After an overnight fast, the rabbits were sedated with an intramuscular injection of ketamine (Ketalar; Parke-Davis Co., Toronto, Ontario, Canada), and gastric acid was neutralized with 5 ml of 10% sodium bicarbonate (Sigma) delivered via an infant feeding tube. Subsequently, 2×10^8 *E. coli* bacteria suspended in 1 ml of sterile PBS were administered orogastrically. Uninfected rabbits and

rabbits challenged with the avirulent laboratory strain *E. coli* HB101 were used as negative controls. Rabbits were then allowed free access to food and water. Seven days after challenge, the animals were killed, and segments of the jejunum, ileum, cecum, and colon were excised and processed for electron and bright-field microscopies as described above.

RESULTS

Bacterial adhesion in vitro. Under both light microscopy and transmission electron microscopy, VTEC strain CL-15 (serotype O113:H21) was found to adhere to HEp-2 cells (Fig. 1). Areas of microvillus membrane effacement were evident underneath adherent organisms. However, CL-15-infected HEp-2 cells did not demonstrate the formation of electron-dense F-actin adhesion pedestals typical of A/E lesions. In contrast, VTEC strains of serotype O157:H7 and EPEC strain E2348 (serotype O127:H6) formed A/E adhesion pedestals on infected epithelial cells (data not shown).

By using methodology previously employed to characterize the surface properties of O157:H7 strains (33), VTEC strain CL-15 (O113:H21) demonstrated D-mannose-sensitive agglutination of erythrocytes of human type A, guinea pig, chicken, and African green monkey origins. Bovine erythrocytes were not agglutinated by CL-15. In contrast, most O157:H7 strains do not express type 1 pili as evidenced by mannose-sensitive agglutination of erythrocytes (33). Strain CL-15 adhered to rabbit ileal brush border membranes and, in a diffuse adherence pattern, HEp-2 cells and Intestine 407 cells both in the presence and absence of 1% D-mannose. In contrast with the majority of O157:H7 strains (33), pilus expression on the surface of strain CL-15 was evident following negative staining of the organisms and visualization under transmission electron microscopy (Fig. 2). As found previously with O157:H7 strains (33), the piliated CL-15 organisms were relatively hydrophobic as demonstrated by aggregation in 0.5 M ammonium sulfate and 36.0% ± 0.9% (mean ± standard error; $N = 4$) retention on substituted phenyl-Sepharose columns.

α-Actinin accumulation. Results from the immunofluorescence experiments demonstrated an increase in α-actinin fluorescence in HEp-2 cells infected with both VTEC O157:H7 strain CL-56 and EPEC strain E2348 (Fig. 3) for either 3 or 6 h. The foci of α-actinin fluorescence corresponded to areas of bacterial adhesion observed under phase-contrast microscopy. In contrast, CL-15 failed to induce α-actinin rearrangement in the epithelial cells (Fig. 3E). Findings were comparable to those obtained with cells infected with the negative control, *E. coli* HB101. Each of the bacterial strains tested alone did not demonstrate α-actinin fluorescence, indicating that the fluorescence observed originated from the epithelial cells.

Phosphotyrosine responses. Results from immunofluorescence microscopy showed foci of increased fluorescence in HEp-2 cells infected with EPEC strain E2348 (Fig. 4A). The foci of fluorescence corresponded to areas of bacterial adhesion observed under phase-contrast microscopy (data not shown). In contrast, the HEp-2 cells infected with VTEC strain CL-15 showed no phosphotyrosine response (Fig. 4B) at either 3 or 6 h of infection. Furthermore, the fluorescence intensity of VTEC strain CL-15-infected cells was comparable to that of cells infected with *E. coli* HB101 (data not shown). When tested alone for phosphotyrosine response, the EPEC strain E2348 did not demonstrate any fluorescence, indicating that the fluorescence observed was of epithelial cell origin and not derived from the infecting pathogen.

Bacterial adherence in vivo. The in vitro findings were confirmed in vivo by using a rabbit model of bacterial adhesion. After oral infection of rabbits, adherent organisms were noted mostly in the cecum sections, showing a binding morphology similar to that observed in infected HEp-2 cells with a lack of adhesion pedestals in CL-15-infected rabbit intestinal epithilium (Fig. 5). Adhesion pedestals were also not seen in rabbit ligated ileal loops infected with strain CL-15 (data not shown).

DISCUSSION

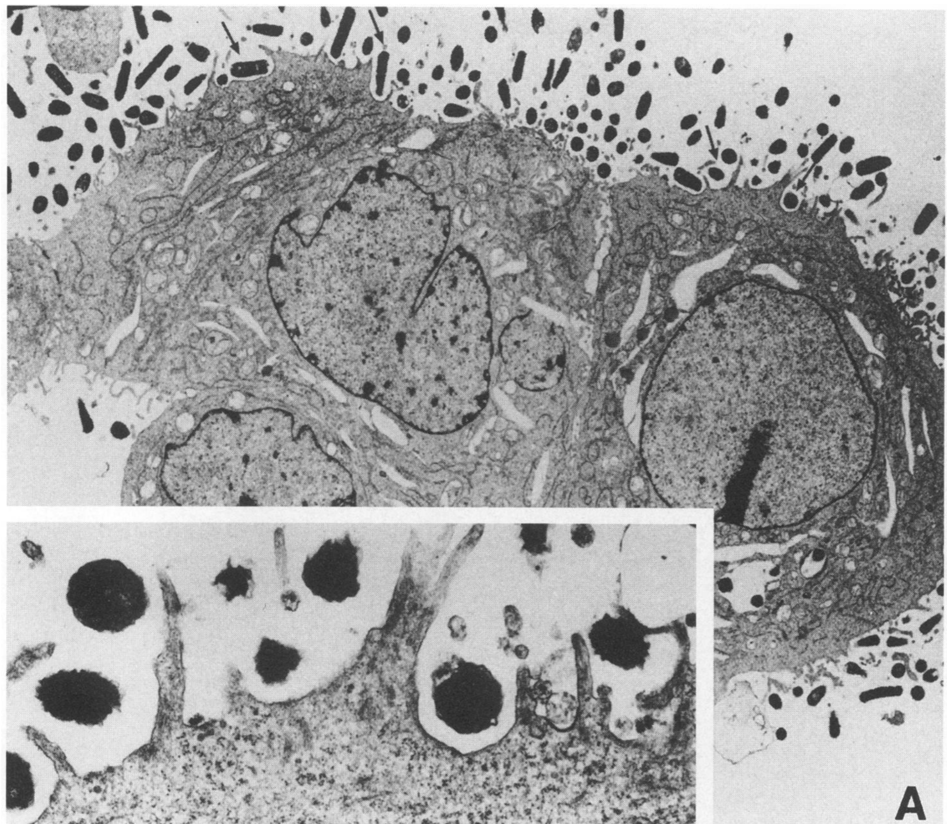
In this study, we have shown that VTEC of serotype O113:H21 exhibits a pattern of adhesion distinct from the classical A/E lesion previously described both for EPEC and VTEC O157:H7 strains. CL-15 adhered to microvillus-denuded regions but without detectable recruitment of underlying cytoskeletal elements including F-actin and α-actinin. These observations are comparable to our previous findings on the adhesion phenotype of the gastric pathogen *Helicobacter pylori* (15).

Similar to *H. pylori*, VTEC O113:H21 strains including CL-15 are negative by colony blot hybridization with an *eaeA* gene probe (15, 25). A/E pathogens, including certain EPEC strains (19), the lapine counterpart of EPEC strain RDEC-1 (19), VTEC O157:H7 (19, 25), *Hafnia alvei* (1, 2), and *Citrobacter freundii* biotype 4280 (30), are all *eaeA* positive. Therefore, the findings reported in the present study provide additional support for the role of the *eaeA* gene in encoding factors which result in cytoskeletal rearrangements in infected epithelial cells. The findings also indicate that bacterial factors distinct from the *eaeA* gene product are necessary for host microvillus effacement and intimate attachment of organisms to epithelial cells. Previous findings demonstrating that multiple bacterial determinants mediate both EPEC (9) and VTEC O157:H7 (16) attachment-effacement to eukaryotic cells provide additional support for this conclusion.

The specific virulence factors which mediate VTEC-associated diseases are not clearly defined. Similar to VTEC of other serotypes, verotoxin-producing O113:H21 strains do not produce classical heat-stable and heat-labile enterotoxins (21, 24) and are not enteroinvasive (33). In addition to the production of verocytotoxins (Shiga-like toxins), the adhesion of VTEC strains to epithelial surfaces in the intestine is a critical primary step in bacterial colonization of the host (5).

The A/E pattern of bacterial adhesion to epithelial cells was first described in certain EPEC serogroups by using both tissue culture cells (22) and animal models of human infection (28). Under transmission electron microscopy, the microvillus membrane of the eukaryotic cell underneath A/E bacteria is effaced and there is intimate bacterial contact with the host plasma membrane. Below the plasma membrane are adhesion pedestals derived from the recruitment of cytoskeletal elements, including filamentous actin (F-actin), α-actinin, ezrin, talin, and myosin (18, 22, 27). Recent reports indicate that both chromosomal and plasmid-encoded genes mediate A/E lesion formation by EPEC on infected host epithelial cells (9). These include the chromosomal *eaeA* (for *E. coli* attaching and effacing), *eaeB* and *cfm* (for class four mutants [29]), and the *perA* and *bfpA* loci present in the EPEC adherence factor 60-MDa plasmid (9).

The *eaeA* sequence in *E. coli* O157:H7 is 97% homologous to the EPEC *eaeA* gene for the first 2,200 bp and 59% homologous for the last 800 bp (6). The *eaeA* gene in *E. coli* O157:H7 encodes a 97-kDa outer membrane protein (26). Similar to findings with EPEC (19), an *eaeA*-insertional inactivation mutant of VTEC O157:H7 no longer causes A/E lesions in infected tissue culture cells or newborn piglets (11, 26), suggesting that the *eaeA* gene is essential for the A/E



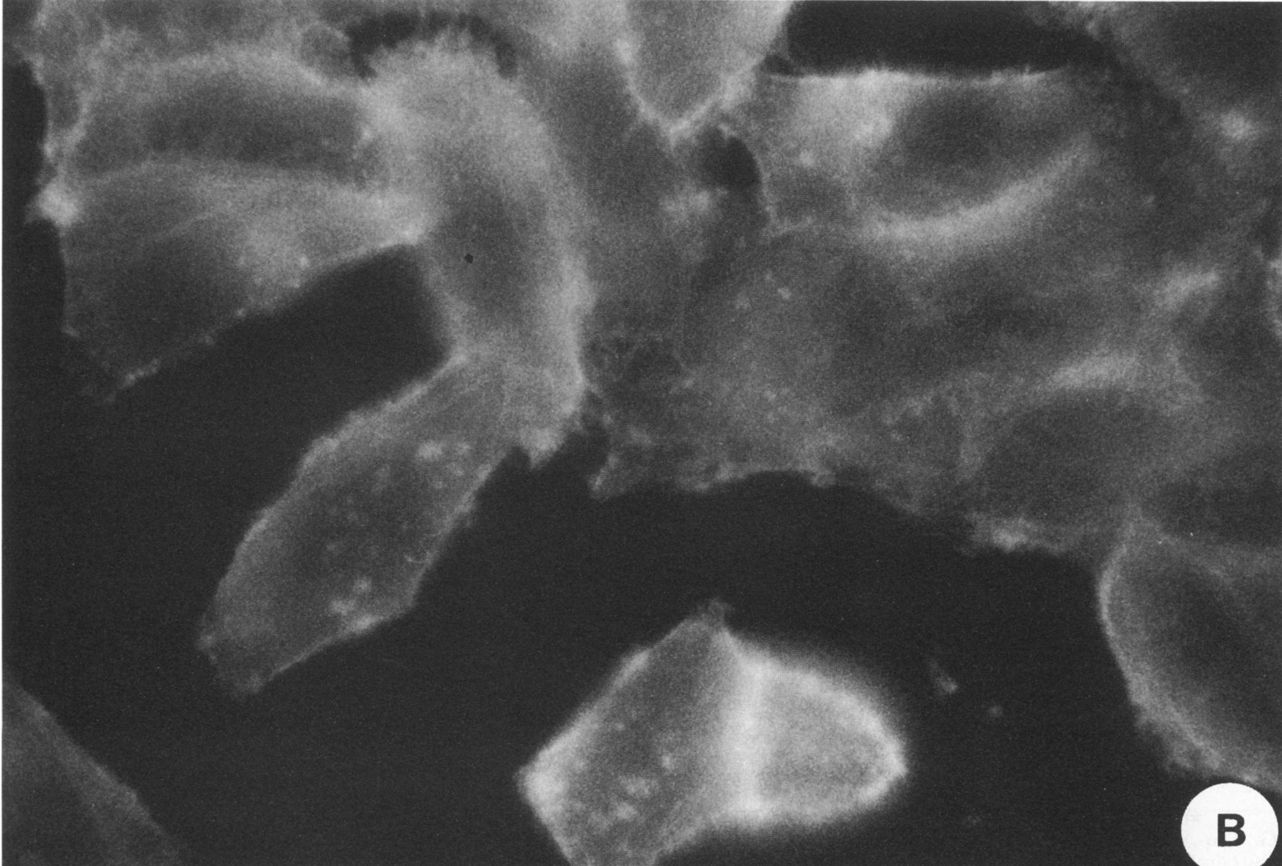


FIG. 1. Transmission electron (A) and fluorescence (B) micrographs of HEp-2 cells infected with VTEC O113:H21 strain CL-15. (A) Approximate magnification, ×7,000. Inset shows magnification of areas of bacterial attachment (approximate magnification, ×20,000). Organisms adhered to HEp-2 cells with areas of underlying microvillus effacement. (B) Negative FAS of CL-15-infected HEp-2 cells. Approximate magnification, ×1,000.

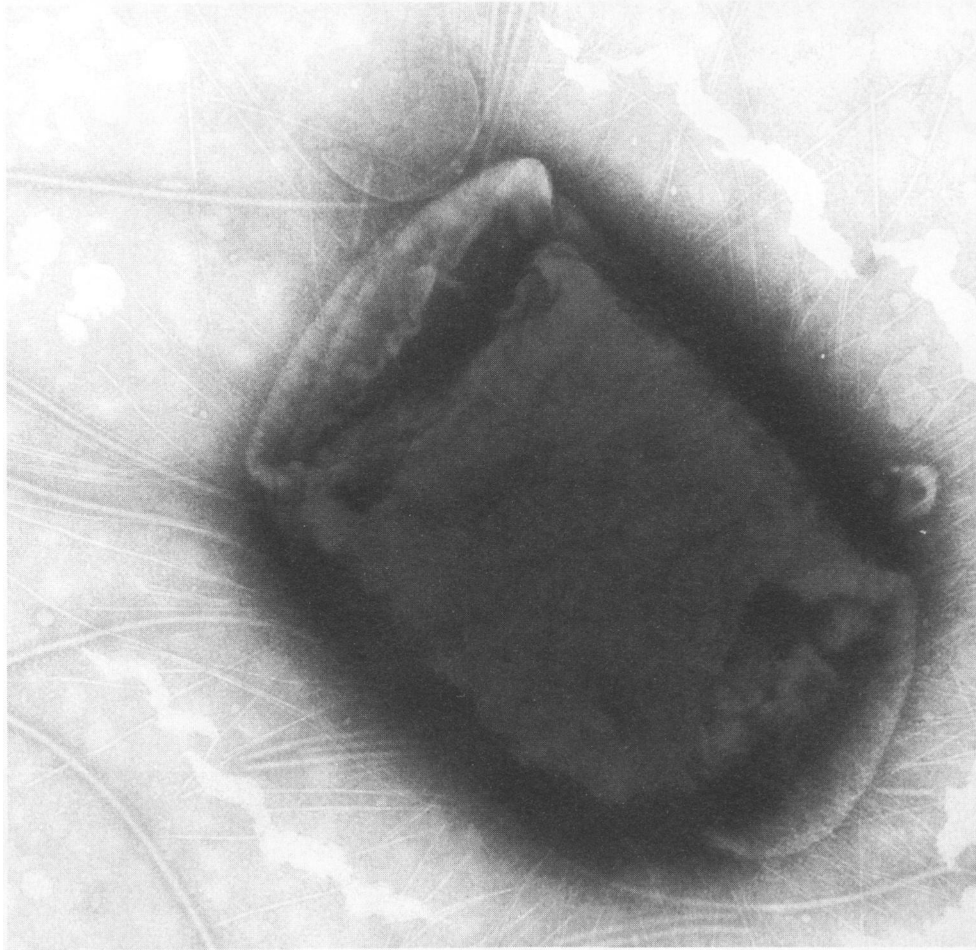


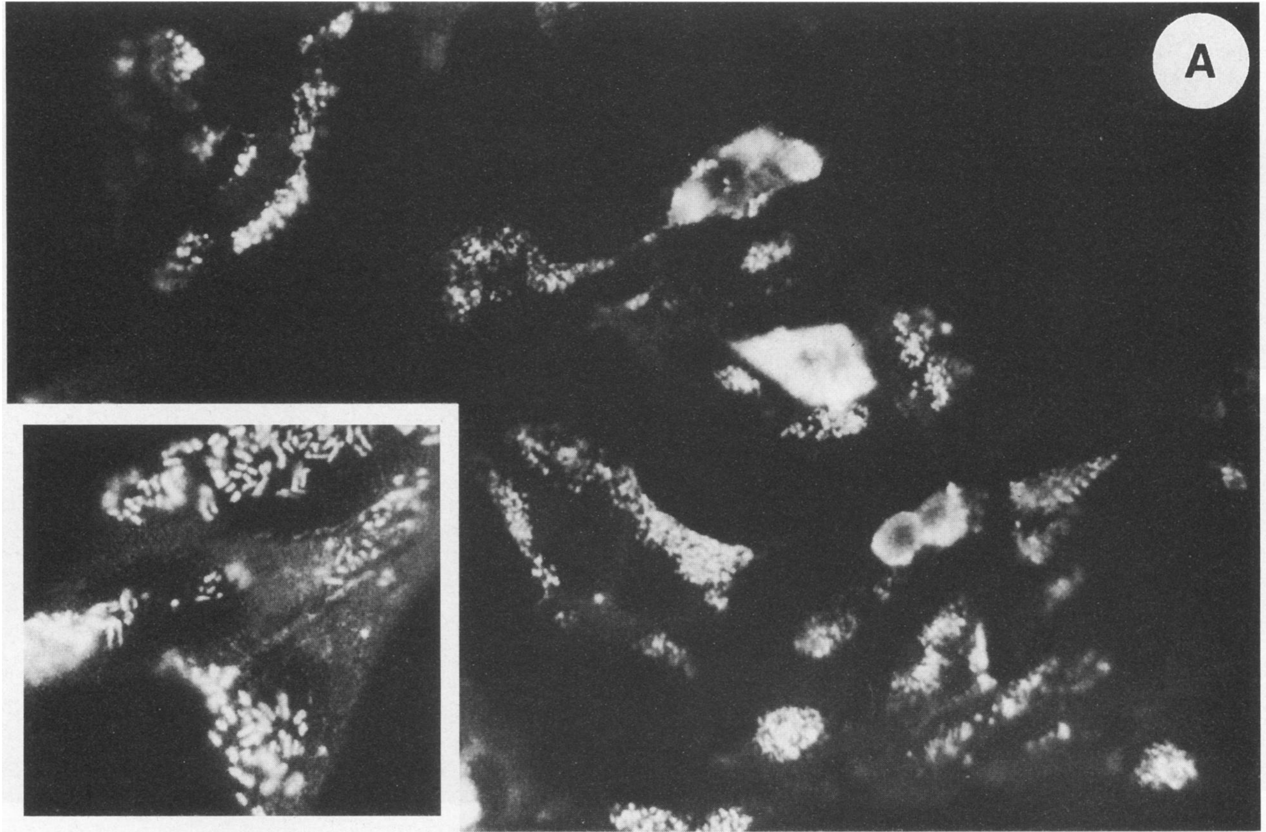
FIG. 2. Transmission electron photomicrograph of VTEC O113:H21 strain CL-15 after staining with uranyl acetate shows expression of pili on the bacterial cell surface (approximate magnification, ×70,000).

phenotype of O157:H7 both in vitro and during infection in vivo.

The ability of EPEC strains to form A/E adhesion pedestals on host cells is important for their diarrheagenic properties. Oral challenge with an *eaeA* deletion mutant of EPEC results in both a lower frequency and reduced severity of diarrhea in infected human volunteers than challenge with the wild-type parent strain (10). In gnotobiotic piglets orally challenged with *E. coli* O157:H7 strains, diarrhea correlates with A/E lesions and occurs in the absence of verotoxin production (36). These findings suggest that A/E adhesion contributes as a mechanism by which VTEC O157:H7 causes diarrhea in humans (20). However, VTEC O113:H21 strain CL-15, which is *eaeA* negative, is still associated with the hemolytic-uremic syndrome and the intestinal prodrome of hemorrhagic colitis (21). This strongly suggests that EaeA is not the sole virulence factor involved in the pathogenesis of VTEC-induced diarrhea. The findings that *eaeA*-negative VTEC O113:H21 strains are isolated from humans with diarrhea and are able to efface target microvilli provide support for this hypothesis. This study also confirms that probing for the presence of the *eaeA* gene will not be able to identify all VTEC strains. Similarly, screening by FAS, phosphotyrosine, and α-actinin assays will not identify VTEC strains, such as those of serotype O113:H21, that do not induce the A/E phenotype in infected eukaryotic cells.

The specific steps leading from bacterial attachment-effacement to fluid secretion and diarrhea are not known. Activation of the host phosphatidylinositol pathway (17) and elevations in intracellular second messengers such as cytosolic free calcium (4, 14) and protein kinase C (3) may signal both reduced sodium absorption and increased net chloride secretion in intestinal epithelia (12, 13). Alternatively, loss of microvillus membrane surface area could lead to malabsorption and osmotic diarrhea (9). Disruption of transcellular ion and water

FIG. 3. α-Actinin immunofluorescence (A, C, E, and G) and phase-contrast microscopy (B, D, F, and H) of HEp-2 cells infected for 3 h with EPEC strain E2348 (A and B), VTEC O157:H7 strain CL-56 (C and D), VTEC O113:H21 strain CL-15 (E and F), and *E. coli* laboratory strain HB101 (G and H). Approximate magnification, ×400. HEp-2 cells infected with both E2348 and CL-56 demonstrated bright spots of α-actinin fluorescence corresponding to areas of bacterial attachment. In contrast, similar to the response after HB101 infection, a negative α-actinin response was observed following infection of the cells with CL-15. Insets show magnifications (approximately ×1,000) confirming positive α-actinin responses to E2348 and CL-56 infection.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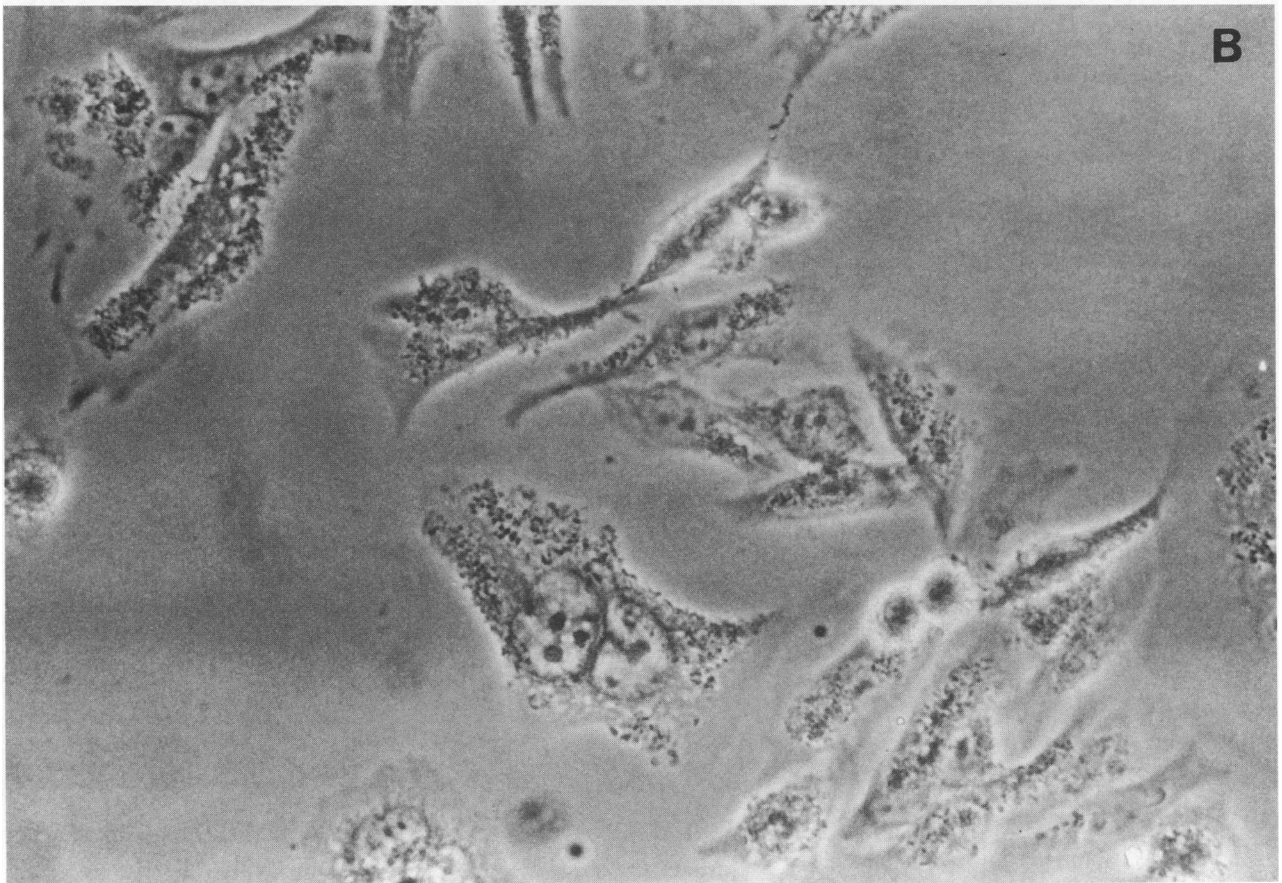
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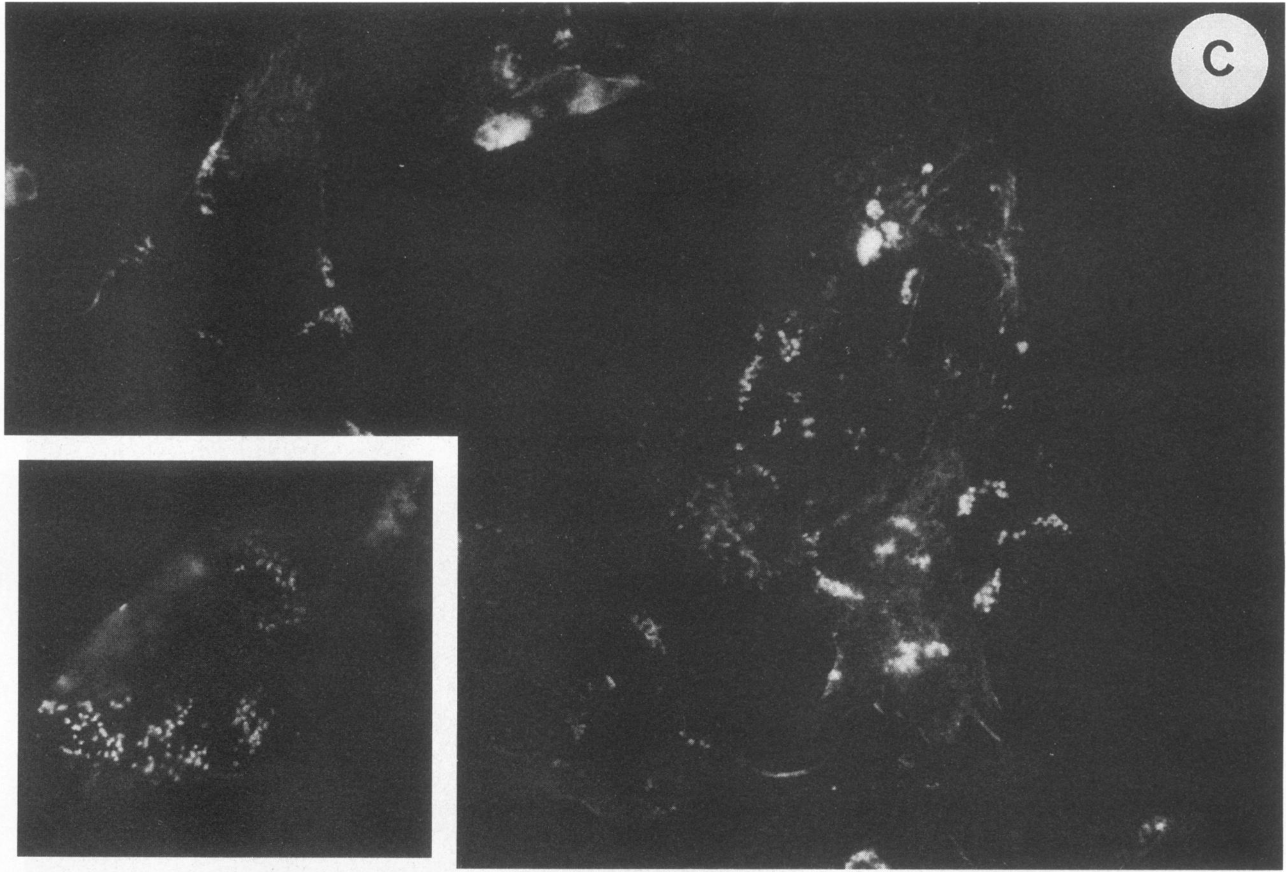

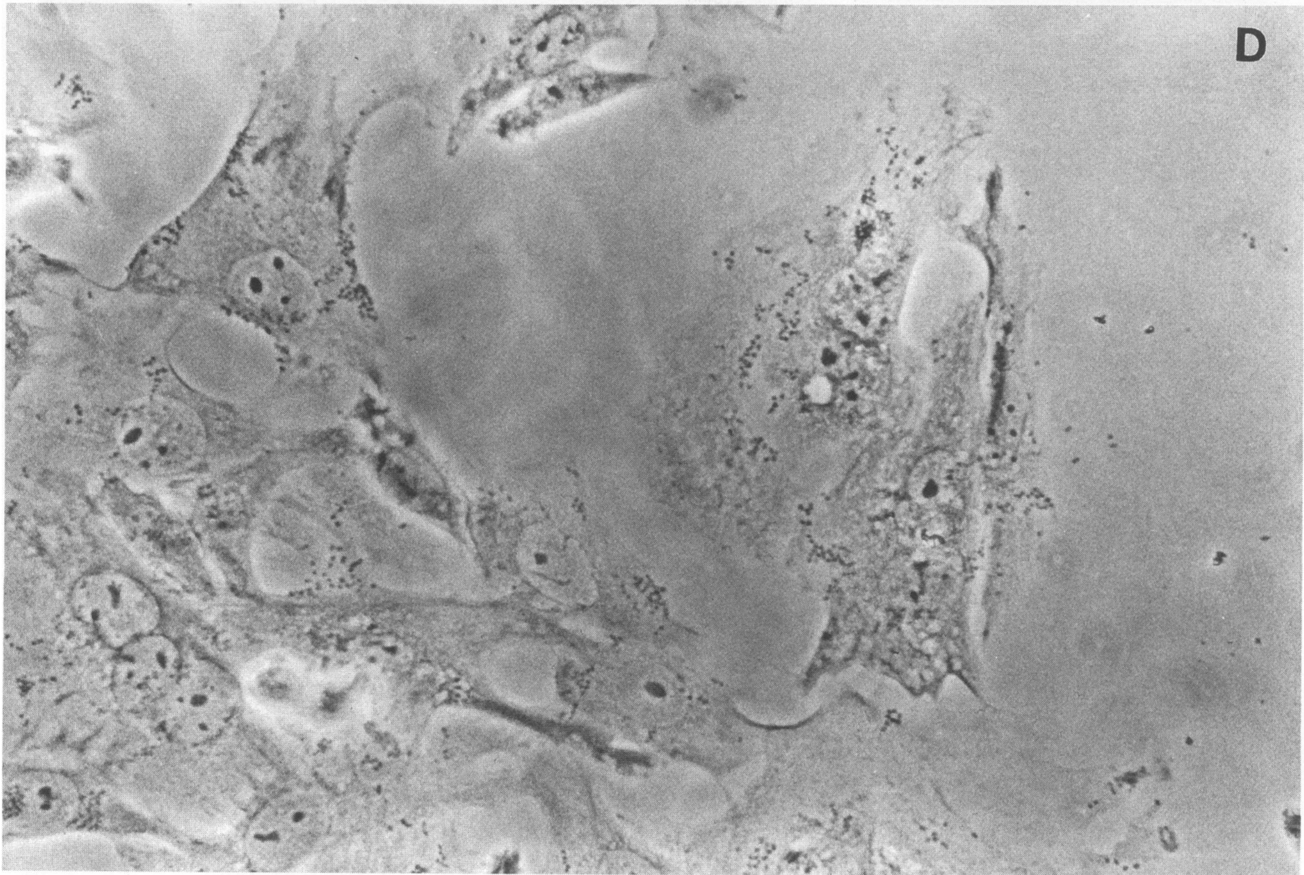

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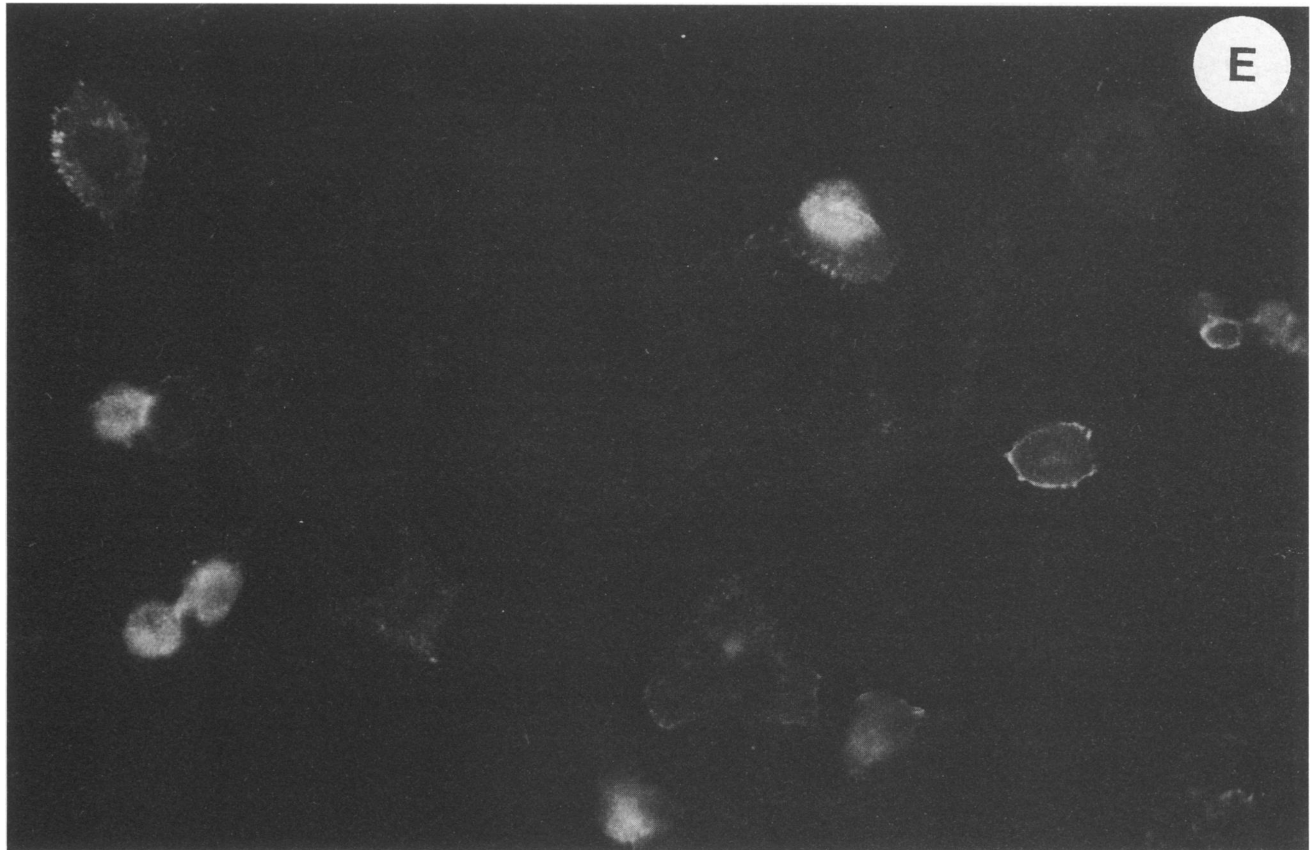

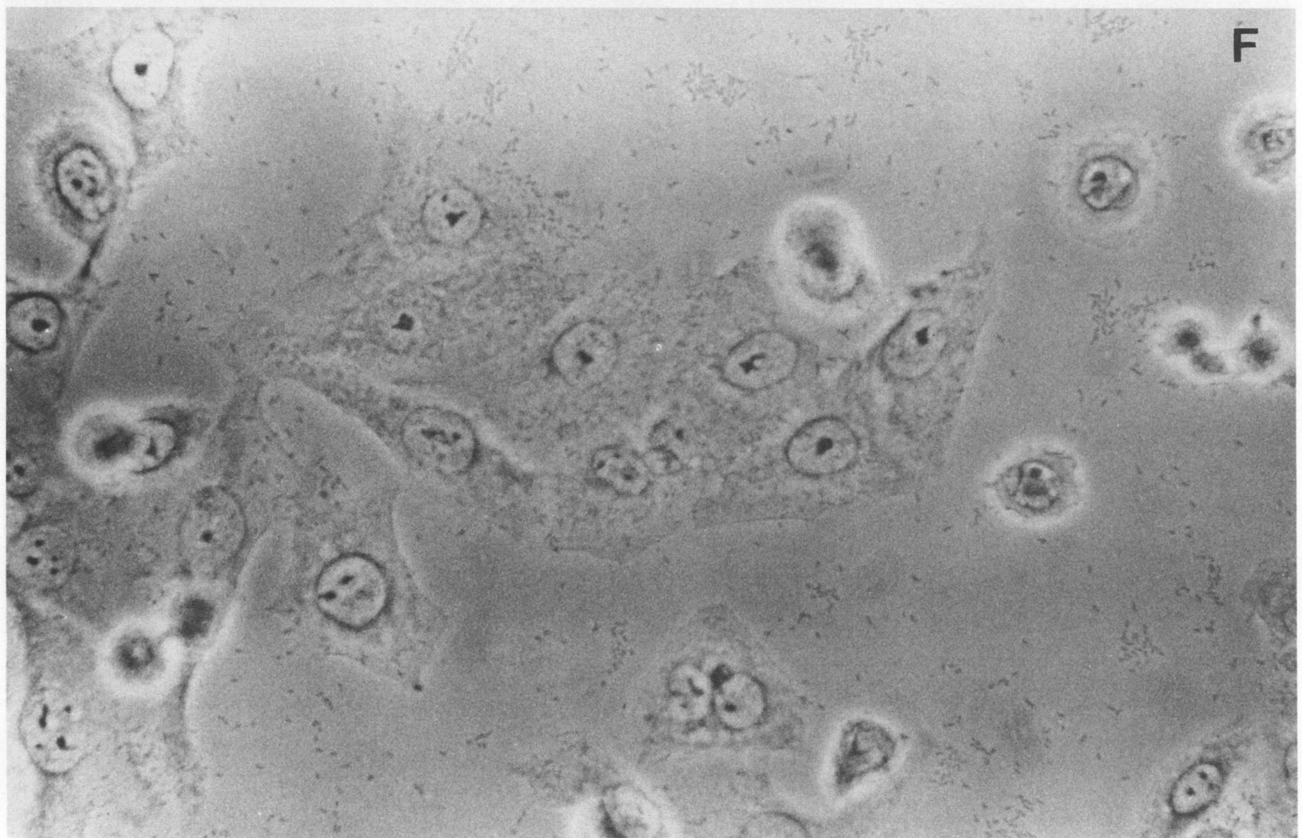

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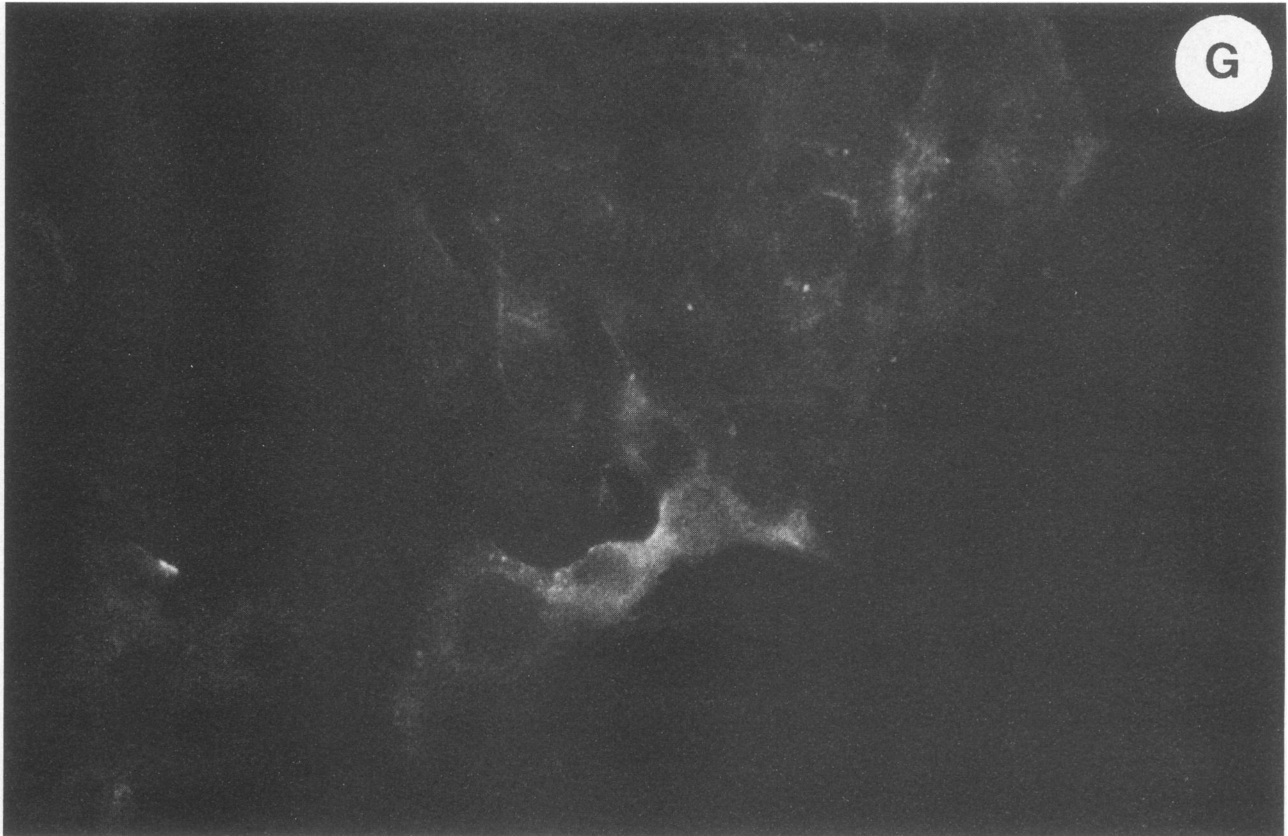

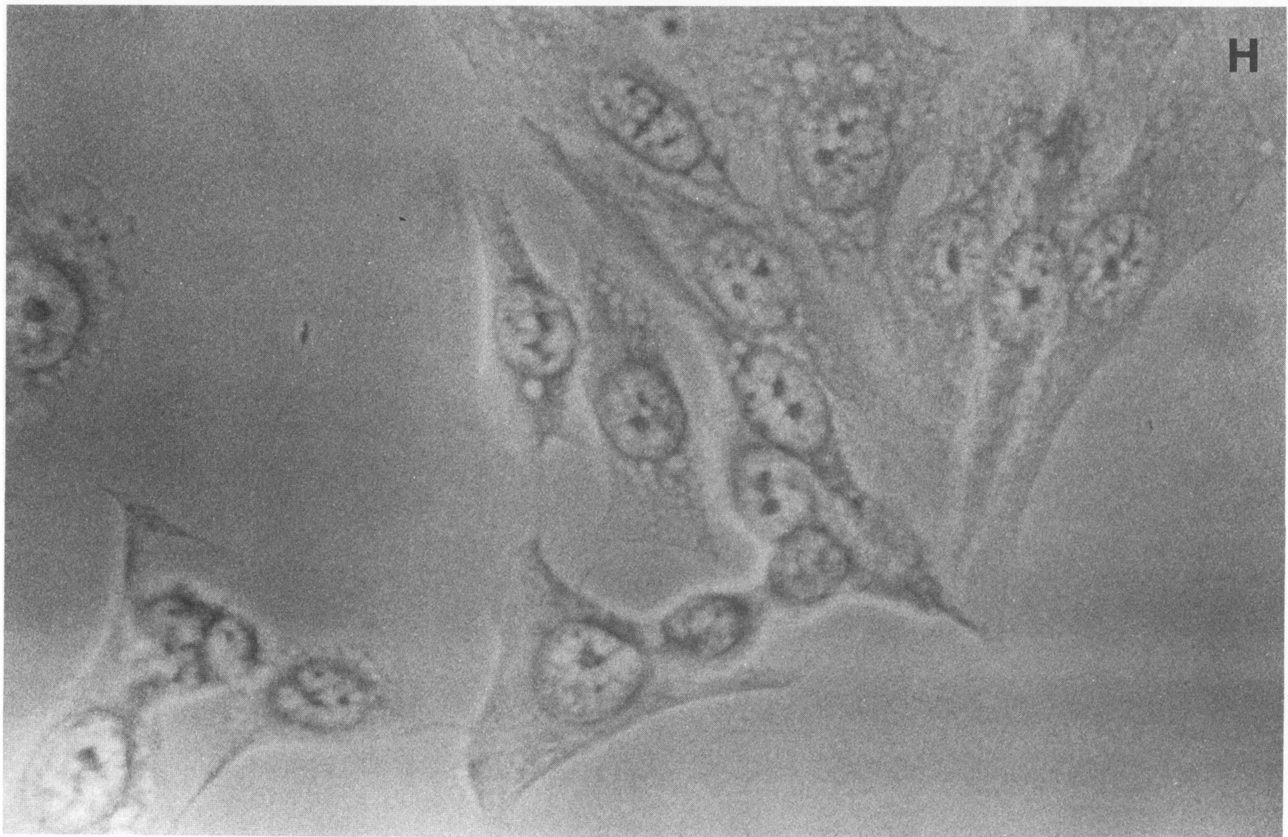

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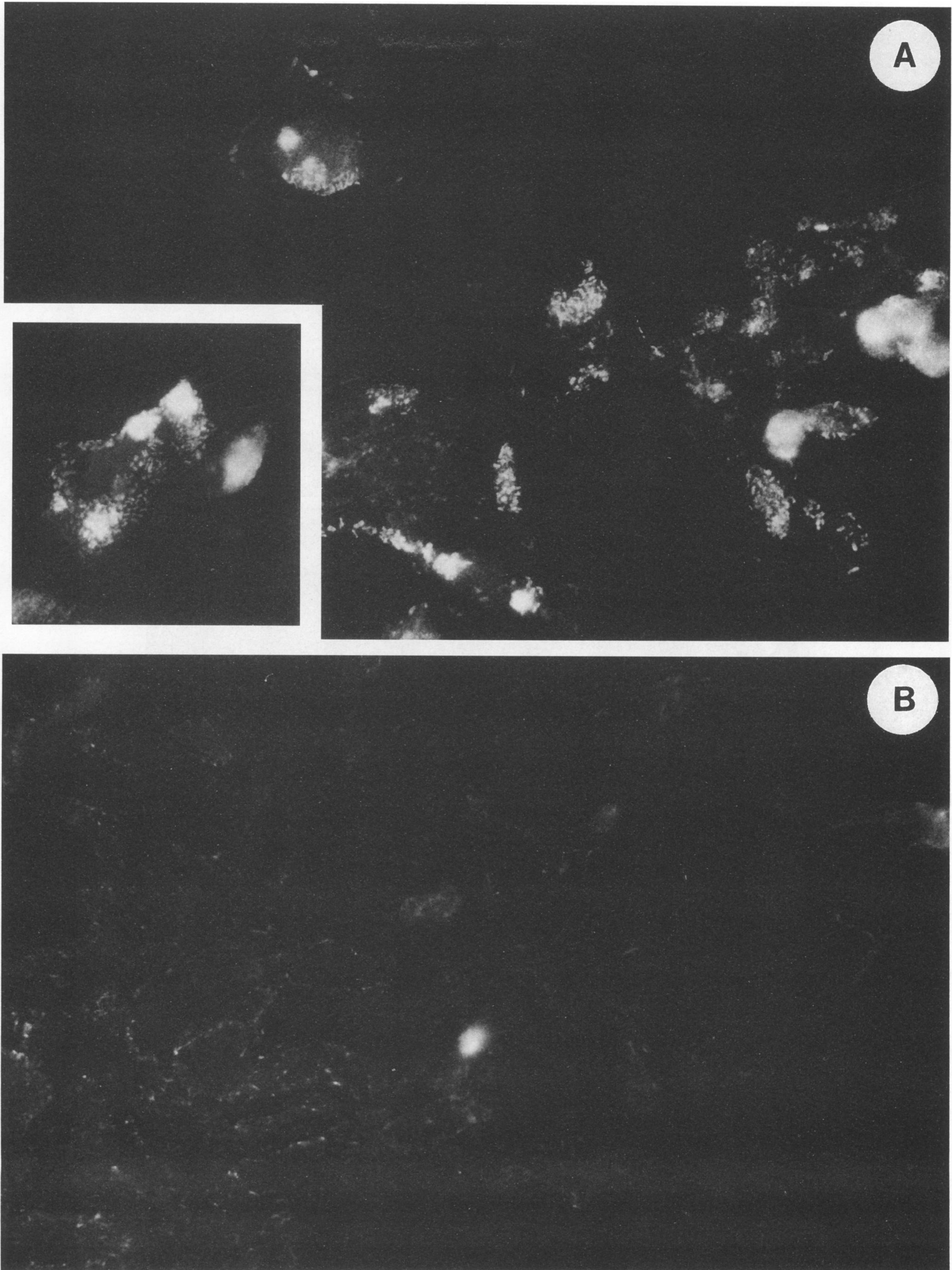

FIG. 4. Phosphotyrosine immunofluorescence of HEp-2 cells infected with EPEC strain E2348 (A) and VTEC O113:H21 strain CL-15 (B). HEp-2 cells infected with E2348 showed bright spots of phosphotyrosine fluorescence that corresponded to areas of bacterial attachment when observed under phase-contrast microscopy. In contrast, the HEp-2 cells infected with CL-15 showed no such areas of phosphotyrosine fluorescence. Approximate magnification, ×400. Inset shows magnification (approximately ×1,000) confirming a positive phosphotyrosine response to E2348 infection.

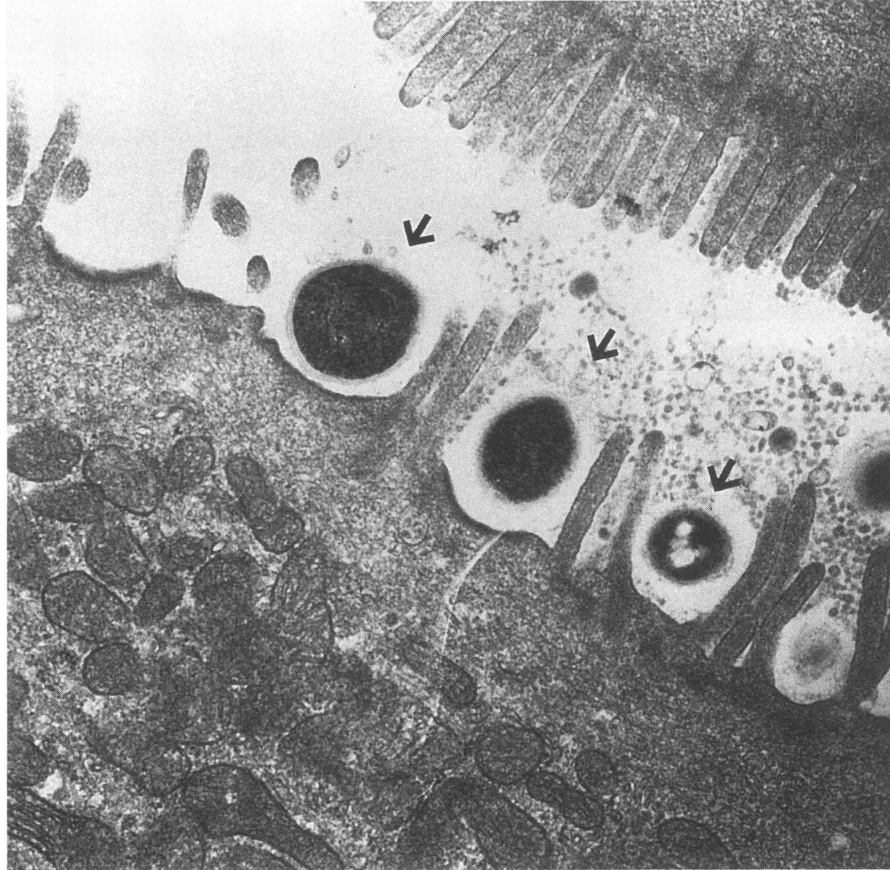

FIG. 5. Transmission electron micrograph of organisms (arrows) adherent to surface epithelial cells in the cecum of a rabbit challenged 10 days earlier with *E. coli* O113:H21 strain CL-15. Approximate magnification, ×20,000.

fluxes could also result in diarrhea (7). Whether effacement of host microvilli during VTEC infection mediates these host responses is not known.

We propose the use of VTEC strain CL-15, serotype O113:H21, to define the role of microvillus effacement in the absence of A/E lesions and cytoskeletal rearrangements in the pathophysiology of diarrhea following VTEC infection. Future studies will also characterize bacterial factors distinct from the *eaeA* gene that are essential for the virulence and adhesion properties of VTEC of serotype O113:H21.

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