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Attaching and Effacing Enteropathogenic *Escherichia coli* O18ab Invades Epithelial Cells and Causes Persistent Diarrhea

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A case of persistent diarrhea following *Escherichia coli* O18ab gastroenteritis is reported. Electron microscopy of a biopsy of the small intestine showed effacement of the brush border, attachment of bacteria to the epithelial cells with pedestal formation, and bacteria within the enterocytes. The bacterial isolate was an enteropathogenic *E. coli* isolate which did not contain the adherence factor (EAF) but possessed the attaching-effacing *eae* gene, was able to invade HeLa cells in a gentamicin invasion assay, and also invaded rabbit intestinal cells. Results suggest that *E. coli* organisms of the O18ab serotype may cause diarrhea by an as yet unknown pathogenic mechanism, involving attaching to and effacing of enterocytes followed by invasion of the epithelial cells.

Diarrheagenic *Escherichia coli* serotypes produce gastroenteritis by two well-established mechanisms, involving either the elaboration of enterotoxins or the direct invasion of the colonic mucosa (9, 21). A third mechanism was suggested by Cantey and Blake, following their study of rabbits infected with *E. coli* O15 (3). It is characterized by adherence of bacteria to the apical portion of the enterocyte, with cuplike pedestal formation and subsequent effacement of the brush border. This effect was first described by Staley et al. in 1969 (25) and was designated by Moon et al. in 1983 (17) as attaching and effacing. Confirmation that adherence of bacteria also occurs in human patients came in 1980, when Ulshen and Rollo described similar ultrastructural changes caused by *E. coli* O125 in an infant with chronic diarrhea (26). Rothbaum et al. (20) then reported on the adherence of *E. coli* O119:B14 associated with effacement of microvilli, leading to the formation of cuplike pedestals on enterocytes of infants with persistent diarrhea. The same pattern of focal bacterial adherence and inflammatory cell infiltration of jejunal mucosa following *E. coli* O111 (K58:H^-) infection was demonstrated by light microscopy (4). Intracellular bacteria were seen in other electron microscopy studies of biopsies from patients with enteropathogenic *E. coli* (EPEC) (11, 26). Recently, Fagundes-Neto et al. (7) also reported on the presence of bacteria within enterocytes from an infant with acute diarrhea caused by *E. coli* O111:H2.

We now describe a case of persistent diarrhea caused by an EPEC O18ab:H14 strain. The characteristic of this infection was the presence of bacteria within the enterocytes. The purpose of this study was to investigate bacterial properties in relation to mucosal invasion.

A 3-month-old infant with persistent diarrhea was admitted to the Brasilia University Hospital after 15 days of diarrhea. He had been well until the onset of profuse watery diarrhea, with a purging rate of six times daily, with no mucus or blood. The patient became moderately dehydrated and received oral rehydration therapy in the emergency room. As diarrhea worsened and dehydration persisted and was associated with metabolic acidosis (serum pH, 7.15; HCO_3, 12.5 mEq/liter), the patient was admitted to the ward for intravenous (i.v.) hydration and correction of the electrolyte imbalance. During rehydration therapy the patient fasted for a period of 4 h, and even with discontinuation of oral feedings, watery stools were still excreted. A sample of feces was examined for the usual enteric pathogens (*Salmonella* and *Shigella* spp., *Yersinia enterocolitica*, *Campylobacter* spp., and rotavirus) using standard techniques (6). Three to five colonies, biochemically identified as *E. coli*, were serotyped according to standard methods, using commercially available polyvalent and monovalent sera (PROBAC do Brasil, São Paulo, Brazil) against O antigens of EPEC, enteroinvasive *E. coli* (EIEC), enterotoxigenic *E. coli* (ETEC), and O157 (enterohemorrhagic *E. coli* [EHEC]) serogroups. H antigens were identified using H1 to H50 antisera prepared at the Centers for Disease Control and Prevention, Atlanta, Ga. (6). The EPEC O18ab:H14 strain was the only enteropathogenic microorganism identified in the stools of the patient. No other invasive enteropathogenic microorganism was isolated. On day 4 of hospitalization due to persistence of diarrhea, with parental consent, a small-bowel biopsy was performed with a 1.6-mm Twinport Watson intestinal biopsy capsule passed to the proximal jejunum. The course of the capsule was followed by fluoroscopy to the Treitz flexure. When the capsule reached the above-mentioned region, jejunal juice was carefully aspirated with a 2.5-ml syringe. The first 0.5 ml of the secretion was discarded, and another 1.0 ml was then obtained for bacterial culture. Once the intestinal secretion was collected, the capsule was fired and one fragment of jejunum was fixed in 2% glutaraldehyde buffered with 0.1 M cacodylate (pH 7.3) for electron microscopy.

The jejunal secretion revealed a pure culture of the identical EPEC O18ab:H14 strain at a concentration of 10^9 bacteria per ml. Observation of the mucosa showed several bacteria in intimate contact with the enterocyte membrane, causing effacement of the microvilli with pedestal formation, as well as bacteria within the enterocyte (Fig. 1). On day 5 a protein hydrolysate formula (Progestimil) was introduced, and after this dietary modification was established, diarrhea ceased and the patient started to gain weight. He was discharged from hospital 12 days after admission.

The strain was tested for virulence factors associated with EPEC. The following probes were used in a colony blot DNA

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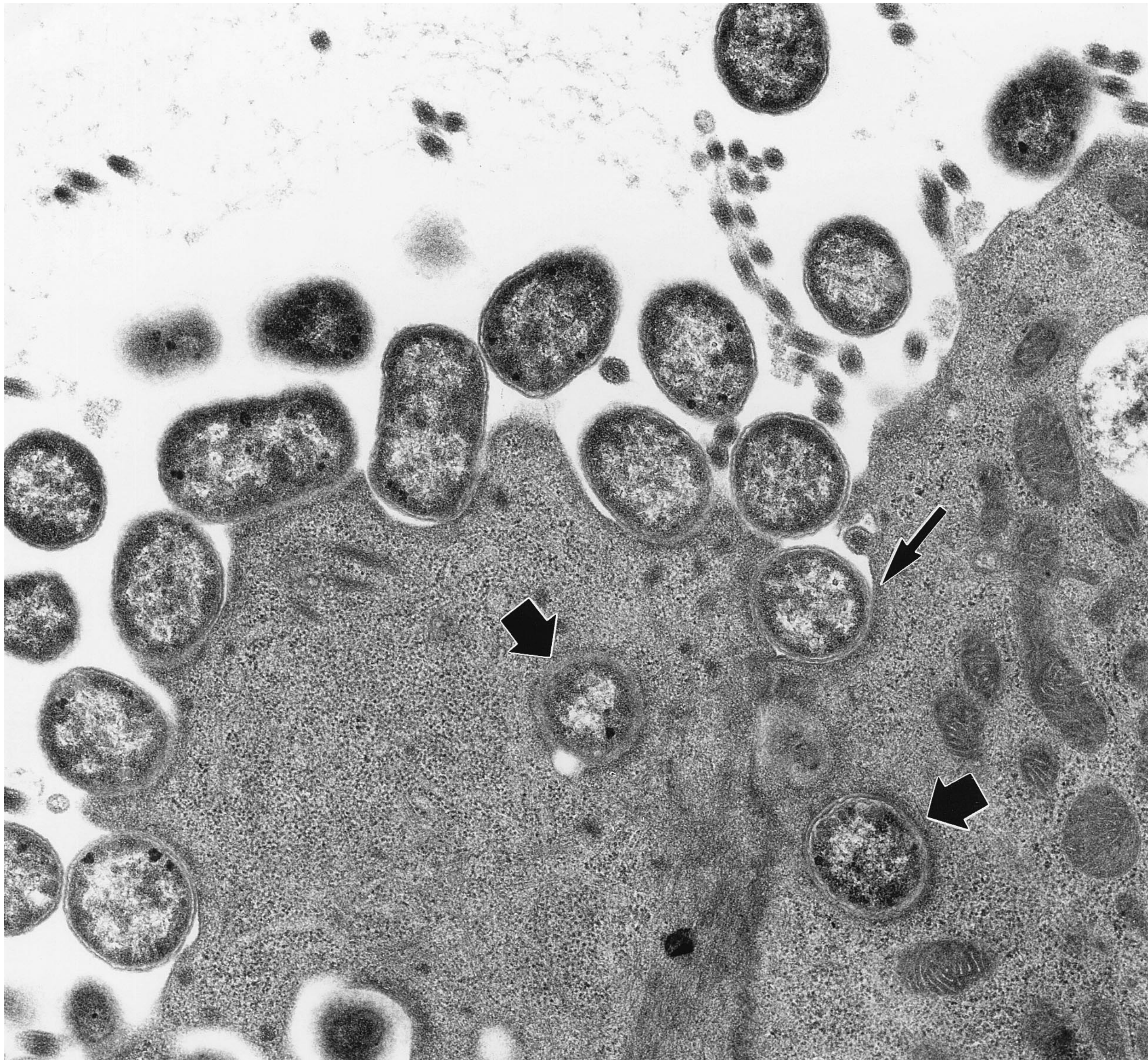

FIG. 1. Electron micrograph of small-bowel biopsy showing ultrastructural changes of microvilli, effacement of the microvilli, and pedestal formation at the sites of attachment. Bacteria are seen in enclosed in membrane-bound vacuoles (thick arrows). Bacteria penetrating the cell via invagination of the plasma membrane are also seen (thin arrow). Magnification, ×15,000.

hybridization assay (15): *eaeA* (for *E. coli* attaching and effacing [A/E] capability) (12), EAF (for enteropathogenic *E. coli* adherence factor) (18), and DA (for diffuse adherence factor) (1). Because the bacteria were found within the enterocyte, we also probed for EIEC (24). Probes were labeled by the random priming technique (15). The microorganism exhibited homology only with the probe for the *eae* gene.

Bacterial adhesion was studied with HeLa cell monolayers as previously described (22). Briefly, monolayers of 10^5 HeLa cells in Corning 35-mm-diameter tissue culture dishes with Dulbecco modified Eagle medium (GIBCO Laboratories, Grand Island, N.Y.) were infected with 3×10^7 bacteria. After incubation at 37°C for 3 h, the monolayers were washed with sterile phosphate-buffered saline (PBS), fixed with methanol, stained with May-Grunwald-Giemsa stain, and examined under a light microscope. The bacteria adhered to HeLa cells, showing an indefinite pattern of adherence (Fig. 2).

We investigated the ability of O18ab to enter epithelial cells by adding gentamicin to monolayers of HeLa cells exposed to the bacteria and measuring bacterial survival. After 3 h of infection, nonadherent bacteria were removed from the monolayers by washing 10 times with PBS, and then Dulbecco modified Eagle medium containing 100 μg/ml was added to each tissue culture dish. After incubation at 37°C for 1 h, the cell monolayers were washed three times and fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 1 h, gently scraped, and washed with the same buffer. Cell pellets were placed in agar, and small blocks were cut and prepared for transmission electron microscopy. The relative number of intracellular bacteria was expressed as the number of CFU of bacteria recovered from the lysed monolayer after 1 h of gentamicin treatment after 3 h of infection. All assays were performed in duplicate. Intracellular bacteria were released from the monolayer by addition of 1% Triton X-100, and dilutions

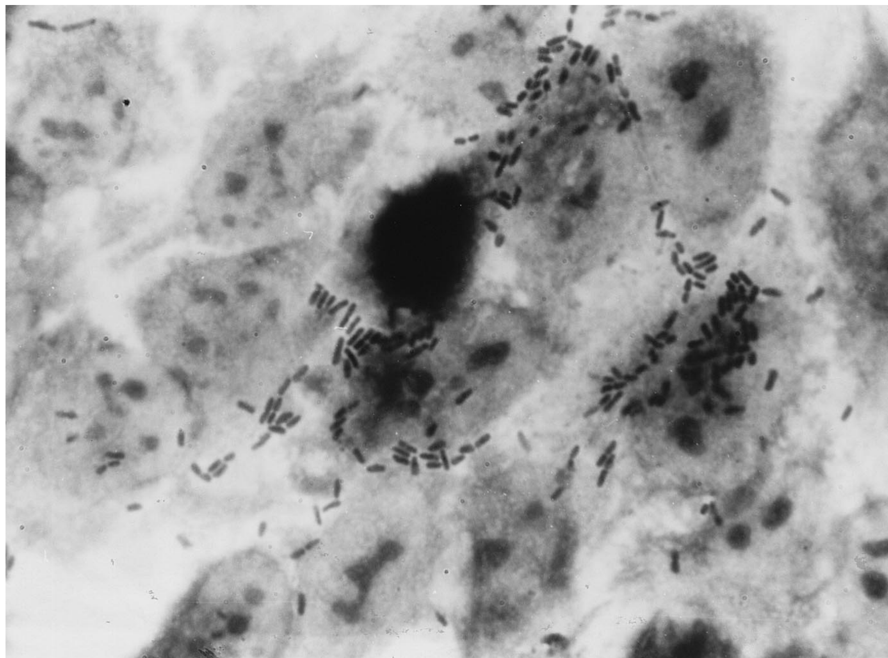

FIG. 2. *E. coli* O18ab showing an indefinite pattern of adherence to HeLa cells. Magnification, ×1,000.

of the suspension were plated on L-agar medium. EPEC O18ab:H14 was recovered from HeLa cells at a level significantly higher than that of the noninvasive strain HB101 (Table 1). The O18ab strain was, however, eightfold less invasive than an EIEC strain, only half as invasive as an EAF EPEC O111 strain, and as invasive as an another EAF-negative EPEC O55:H7 strain. Intracellular bacteria were typically seen within membrane-lined vacuoles (Fig. 3).

The strain was also examined by the fluorescent-actin staining test, an in vitro tissue culture assay which is diagnostic for the A/E lesions characteristic of EPEC infection (14). It was positive in this test, in accordance with the presence of the *eae* gene.

The invasive capacity of the strain was evaluated in vivo in a rabbit ileal loop assay system which is suitable for studying the A/E properties of EPEC serotypes (17). New Zealand White rabbits, 9 to 10 weeks old, were fasted for 48 h before ileal loop surgery. Six ligated loops, each approximately 5 cm long, separated by 2-cm interloops, were made in each rabbit. Each loop was inoculated with 0.5 ml of a suspension of 10^9 bacteria. The animals were sacrificed after 24 h, and tissue sections were prepared for histopathological inspection by electron microscopy. Examination of ultrathin sections of the loop inoculated with the strain showed typical lesions associated with bacterial attachment to the apical surface of the enterocytes, with loss of the microvillus border. There was cupping of the plasma membrane around the bacteria, pedestal formation, and increased electron density in the terminal web just beneath the site of bacterial attachment. Bacteria were seen inside the cell enclosed in membrane-bound vacuoles (Fig. 4).

TABLE 1. Invasive capacity of bacteria measured as recovery of bacteria in HeLa cell monolayers

Strain	No. of CFU recovered/35-mm-diameter coverslip (mean ± SD)
9/82 (EIEC)	$(1.3 \pm 0.1) \times 10^6$
O111 (EPEC)	$(2.2 \pm 0.1) \times 10^5$
O55:H7	$(1.4 \pm 0.2) \times 10^5$
O18ab	$(1.6 \pm 0.2) \times 10^5$
HB101	$(3.0 \pm 0.1) \times 10^2$

[a] Values are the results of duplicate assays performed at least twice.

EPEC organisms are a group of that causes infantile diarrhea by a unique mechanism involving attachment to the intestinal mucosa with effacement of the microvillus border. Studies to date have suggested that the critical virulence factors for full pathogenicity of traditional EPEC are the abilities to produce localized adherence (LA) and A/E lesions. Initially, EPEC bacteria adhere to epithelial cells in a characteristic LA pattern mediated by the EAF plasmid. Subsequently, an intimate interaction occurs, resulting in cytoskeletal rearrangement within the enterocyte. This is followed by EPEC invasion of the eukaryotic cell (5, 8). Although the pathogenicity of *E. coli* O18 has been questioned (13, 19), and strongly suggests that there is heterogeneity among O18 strains, in the present study the O18ab:H14 strain possessed the *eae* gene but did not have the EAF plasmid that encodes the bundle-forming pili, which mediate LA. This strain caused A/E lesions in experimental infections in the rabbit ileum and also showed intimate adherence (FAS^+). The EPEC O18ab strain also showed the capacity to invade HeLa cells and rabbit intestinal cells.

Donnenberg et al. (5) found that EAF^+ EPEC strains were generally more invasive in HEp-2 cells than EIEC strains, whereas EAF^- EPEC strains were less invasive. We showed that EPEC O18ab, which lacked the EAF plasmid, was less invasive than an EIEC strain and similar to an EPEC O111 strain in this respect. The invasiveness of the O18ab strain is similar to that of other EAF-negative EPEC.

The frequency of EAF^- eae^+ EPEC strains as a cause of diarrhea is unknown. More recently, Bratoeva et al. (2) reported on the occurrence of a non-EPEC EAF^- strain isolated from a patient with diarrhea and bacteremia that possessed the

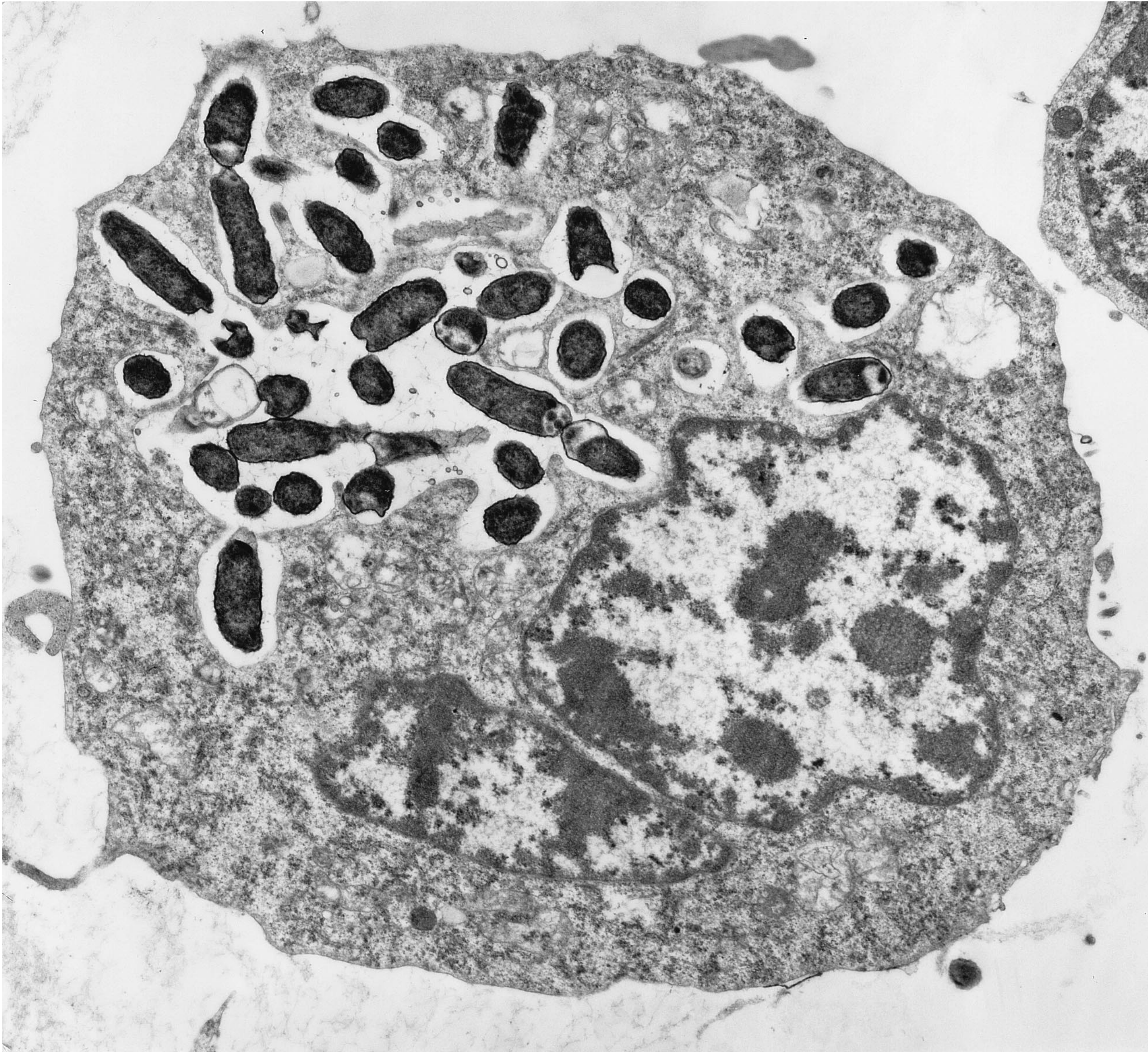

FIG. 3. Transmission electron micrograph of HeLa cells incubated with EPEC O18ab. Bacteria are seen within the cultured cells inside endocytic vesicles. Magnification, ×10,000.

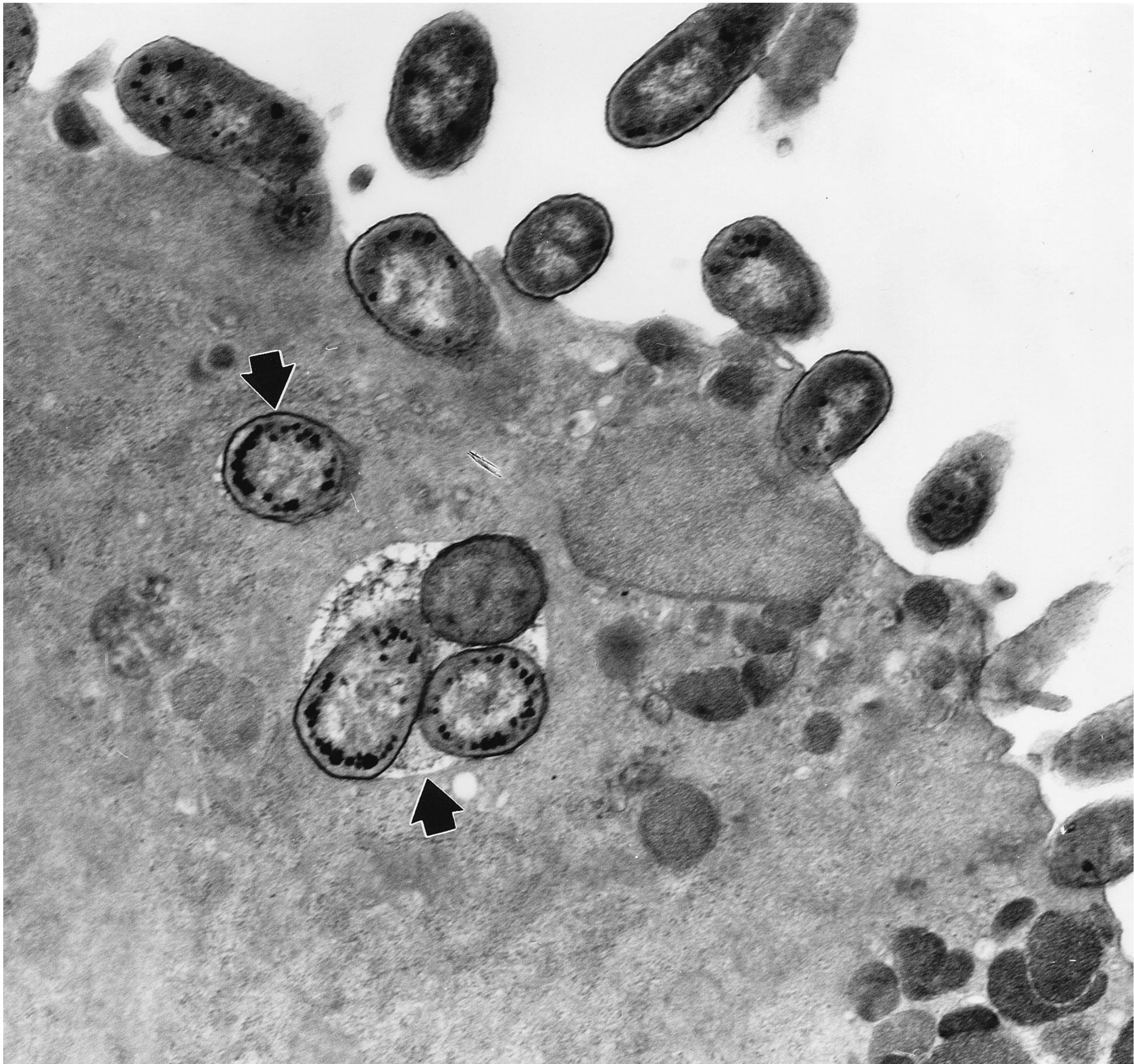

FIG. 4. Electron micrograph of section of rabbit ileal loop inoculated with *E. coli* O18ab. Effaced microvilli, pedestal formation, cupping of plasma membrane, and increased electron density of terminal web area associated with attached bacteria are evident. Bacteria are seen enclosed in membrane-bound vacuoles (arrows). Magnification, ×15,000.

eae gene. This strain was invasive in the gentamicin invasion assay and expressed two types of pili and K1 antigen.

Few studies have implicated *E. coli* O18 in infantile diarrhea (10). Recently, this serogroup has been considered invasive, causing sepsis in neonates and pyelonephritis and meningitis in infants (23). Since this microorganism is able to penetrate enterocytes as well as HeLa cells, it is likely that the intestine could be the natural route for the systemic dissemination of the infection.

In the present work we show that an *E. coli* O18ab strain can cause diarrhea by a pathogenic mechanism that has not been identified and was not reported before.

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