INFECTION AND IMMUNITY, Nov. 1996, p. 4761–4768
0019-9567/96/$04.00+0
Copyright © 1996, American Society for Microbiology
Vol. 64, No. 11

T84 Cells in Culture as a Model for Enteroaggregative *Escherichia coli* Pathogenesis

JAMES P. NATARO,[1]* SUSAN HICKS,[2] ALAN D. PHILLIPS,[2] PABLO A. VIAL,[1]† AND CYNTHIA L. SEARS[3]

Center for Vaccine Development, Department of Pediatrics, University of Maryland School of Medicine, Baltimore, Maryland 21201[1]; University Department of Pediatric Gastroenterology, Royal Free Hospital, London, England[2]; and Divisions of Infectious Diseases and Gastroenterology, Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205[3]

Received 2 May 1996/Returned for modification 24 June 1996/Accepted 15 August 1996

Enteroaggregative *Escherichia coli* (EAEC) is an important cause of persistent diarrhea in many developing parts of the world, yet the pathogenetic mechanisms of EAEC diarrhea are unknown. Experiments with animal models suggest that EAEC strains damage the intestinal mucosa, and a putative cytotoxin has been described. To characterize the mucosal effects of EAEC, we studied strain 042, which we have shown to cause diarrhea in adult volunteers. Strain 042 was incubated in an in vitro organ culture model with biopsy-derived normal intestinal mucosa from pediatric patients. Strain 042 adhered strongly to samples of jejunal, ileal, and colonic mucosa. In addition, scanning electron microscopic examination of in vitro-infected intestinal biopsies revealed cytotoxic effects marked by exfoliation of mucosal epithelial cells. To develop an in vitro model to study these effects, we incubated 042 with polarized monolayers of the human intestinal epithelial cell lines Caco-2 and T84. Strain 042 adhered strongly to T84 cells but not to Caco-2 cells. T84 cells infected with 042 displayed marked toxic effects, most prominently in areas where bacteria were adhering. The apical membrane of damaged cells exhibited vesiculation and shedding of microvilli. The cytoplasm of affected cells displayed subnuclear vacuolization, and in some cases, nuclei of affected cells became separated from the surrounding cytoplasm. Severely affected cells ruptured, releasing their nuclei. Vacuolated remnant cells were seen throughout the monolayer. Strain 042 was not internalized by T84 cells. We concluded that EAEC strain 042 alters intestinal cell morphology, ultimately leading to cell death. Although the factor(s) required for this effect remains to be elucidated, T84 cells may serve as a valuable model in EAEC pathogenesis studies.

Enteroaggregative *Escherichia coli* (EAEC) has been implicated as a cause of persistent diarrhea among children in developing parts of the world (3, 4, 8, 14, 36) and may be an emerging cause of diarrhea in developed parts of the world (7, 23). The mechanism of EAEC diarrheagenicity is uncharacterized, but several putative factors have been reported. Two distinct fimbrial antigens have been described, either of which may mediate the aggregative adherence (AA) phenotype in a particular strain (25, 26). The heat-stable-toxin-like enterotoxin EAST-1 (for enteroaggregative heat-stable toxin) has been well-characterized by Savarino et al. (30, 31). Although EAST-1 elicits self-limited short circuit current rises in rabbit intestinal mucosa mounted in Ussing chambers, an EAST-1-producing strain did not cause diarrhea in adult volunteers (25). Thus, an effect of EAST-1 in vivo is unsubstantiated. Moreover, any model of EAEC diarrheal pathogenesis must explain the characteristic persistence of EAEC diarrheal episodes.

Several studies with different systems have demonstrated that EAEC is capable of causing destruction of intestinal mucosa (34, 35). Vial et al. first showed that EAEC strains elicited severe mucosal damage in rabbit and rat ligated intestinal loops (35). Tzipori et al. subsequently reported that EAEC induced much milder, but significant, mucosal changes in the gnotobiotic piglet model (34). Several investigators have shown that such mucosal damage also can be demonstrated in human tissue. Eslava et al. have demonstrated mucosal damage in the ilea of children succumbing to EAEC diarrhea in Mexico (13). Those investigators have identified an immunogenic putative cytotoxin of 108 kDa which elicits mucosal toxicity when injected into rat ileal loops (13). Hicks et al. (16) have recently shown that some, but not all, EAEC strains are toxic to intestinal tissue obtained from patients at biopsy.

The present studies were undertaken to characterize the effects of EAEC on intestinal mucosa and to develop a model for EAEC mucosal toxicity. Since not all EAEC strains are proven human pathogens, we have focused our studies on a strain which elicited diarrhea in the majority of healthy adult volunteers studied at our institution (25).

MATERIALS AND METHODS

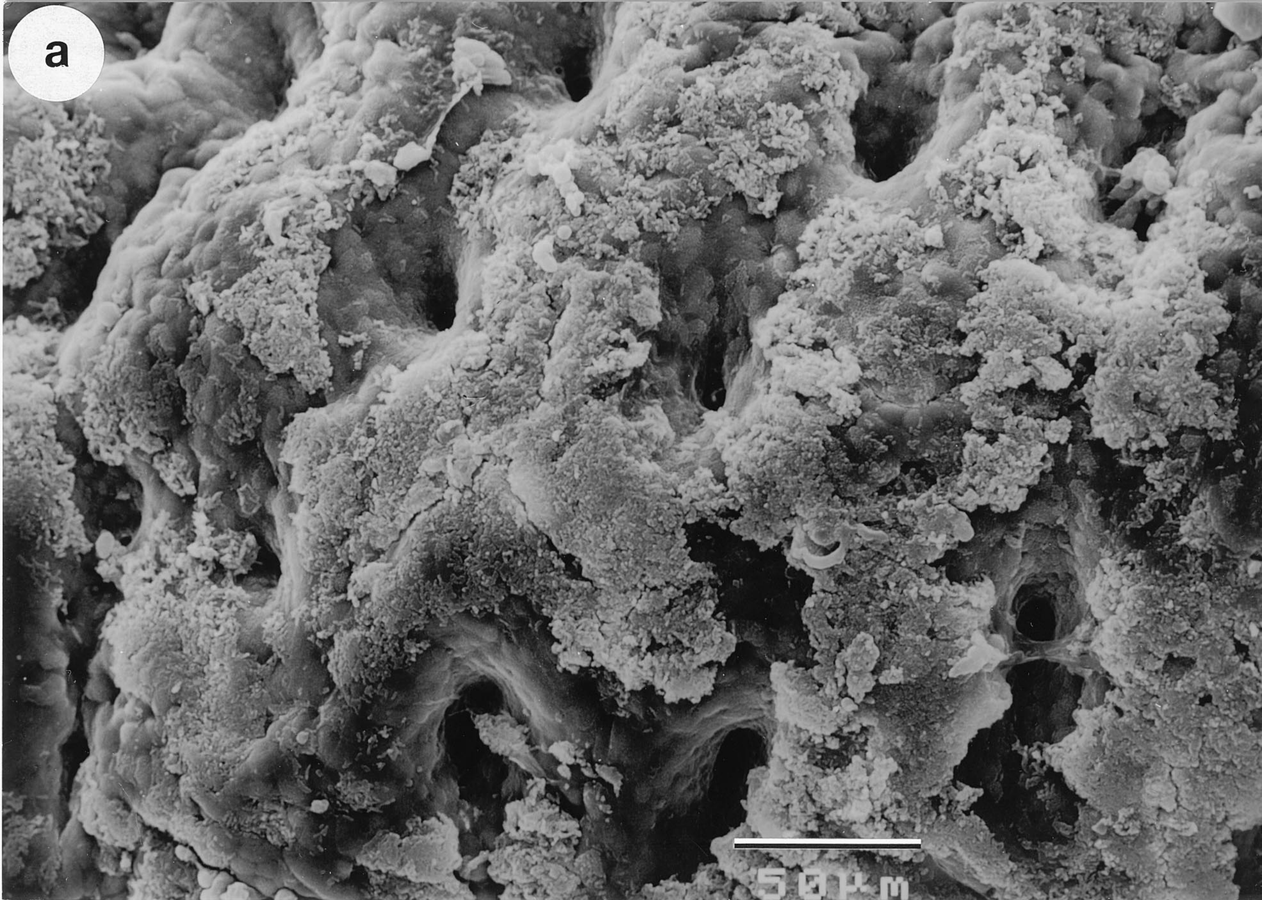
Bacterial strains. EAEC strain 042 (O44:H18) was isolated from an infant with diarrhea in Lima, Peru, in 1983. The organism adheres to HEp-2 cells in a "honeycomb" phenotype by virtue of the AAF/II adhesin (24). Volunteers fed 042 developed watery, nonbloody diarrhea without fever or fecal leukocytes (25). *E. coli* HS4, a commensal strain shown not to cause diarrhea in humans (20), was the negative control strain used in cell culture studies; *E. coli* HB101 (5) was used as a negative control strain in organ culture studies. All strains were routinely stored at −70°C in Trypticase soy broth with 15% glycerol.

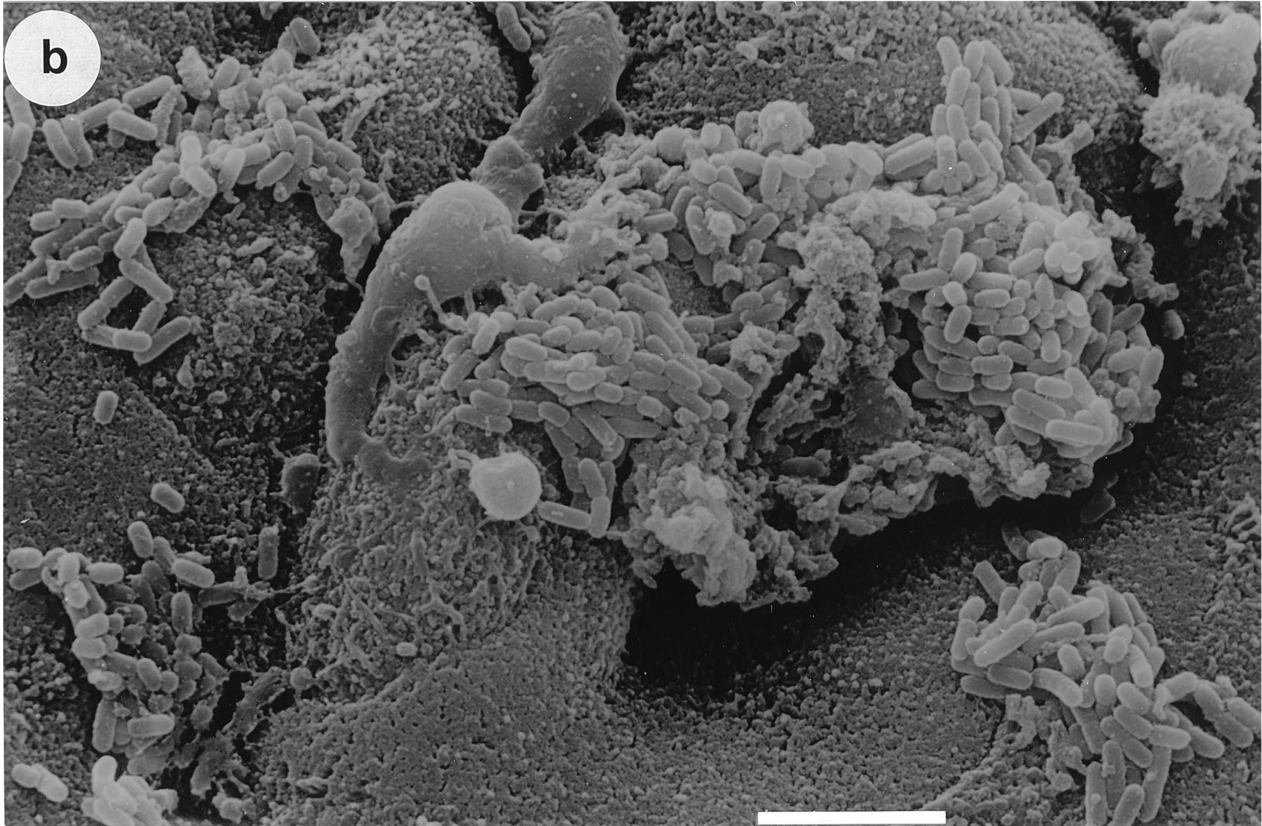
Strain 042 was cured of its 65-MDa adherence-encoding plasmid by the introduction of an incompatible temperature-sensitive plasmid, pMR5 (28), and subsequent cure of the latter plasmid at 44°C. The plasmidless 042 derivative strain [designated 042P(−)] is biochemically identical to the parent but does not adhere to HEp-2 cells. Antibiotic resistance markers of pMR5 (ampicillin and kanamycin resistance) are not expressed by 042P(−).

Tissue culture methods. T84 and Caco-2 cells were grown as polarized mono-

* Corresponding author. Mailing address: Center for Vaccine Development, University of Maryland School of Medicine, 685 W. Baltimore St., Baltimore, MD 21201. Phone: (410) 706-5328. Fax: (410) 706-6205. Electronic mail address: jnataro@umabnet.ab.umd.edu.

† Present address: Centro de Investigaciones Medicas, Escuela de Medicina, Pontifica Universidad de Chile, Santiago, Chile.


a
50μm


b

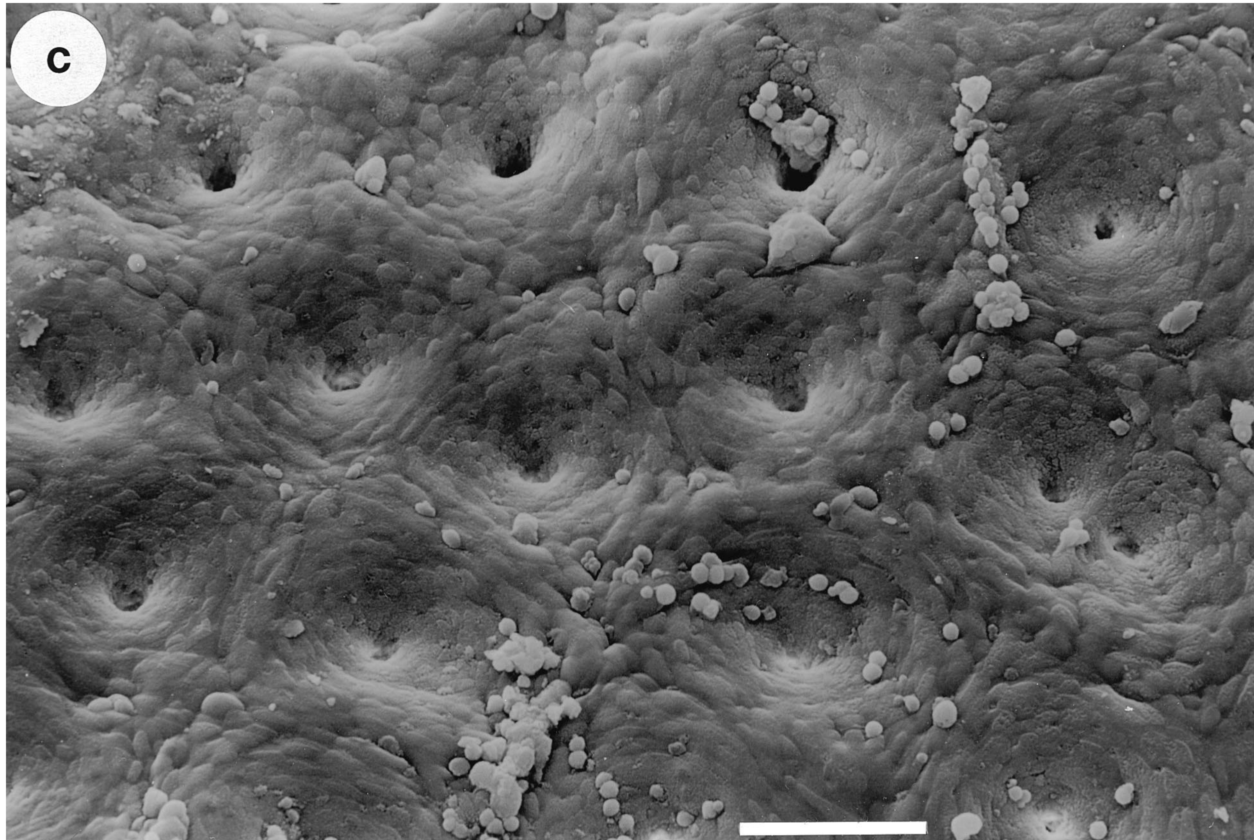


FIG. 1. Electron photomicrographs of in vitro-cultured human colonic tissue infected with EAEC strain 042 for 8 h. (a) SEM of 042-infected colon showing densely adherent bacterial growth on the mucosal surface. Bar, 50 μm. (b) Higher magnification of 042-infected colon showing dilated crypt opening and aggregating bacteria. Bar, 5 μm. (c) Uninfected colonic tissue. Bar, 50 μm.

layers on collagen-coated polycarbonate filters as previously described (32, 37). For time course experiments, bacteria were pelleted onto monolayers as described by Donnenberg et al. (11). Tissue culture monolayers were glutaraldehyde fixed and embedded as previously described (22) and examined on a JEOL JEM 1200EX11 transmission electron microscope and a JEOL JSM 35C scanning electron microscope.

In vitro organ culture (IVOC). For IVOC studies, test bacterial strains were subcultured into brain heart infusion broth and incubated at 37°C without agitation for 18 h. Endoscopically and histologically normal mucosal samples from the transverse colon were obtained from three pediatric patients (ages, 12 to 15 years) undergoing endoscopic investigation for possible inflammatory bowel disease. In the adhesion assay, done essentially as described by Knutton et al. (18, 19), intestinal mucosa was incubated with the *E. coli* strain for 8 h in 95% O_2–5% CO_2. The tissue culture medium was a 1:1 mixture of NCTC-135 medium (Sigma Chemical Co., St. Louis, Mo.) and Eagle's minimum essential medium (Sigma) containing 0.5% (wt/vol) D-mannose. After incubation with bacteria, tissue specimens were washed three times in fresh culture medium to remove any nonadherent bacteria and processed for scanning electron microscopy (SEM) and transmission electron microscopy (TEM). A JEOL JSM 5300 was used for SEM, and a JEOL JEM 1200EX11 was used for TEM.

Enumeration of adherent bacteria was performed by counting bacteria from 10 random fields at a fixed magnification of ×3,500. The median number of adhering bacteria per field was calculated, and data were compared by using the Mann-Whitney test, in which $P < 0.05$ was taken as significant.

RESULTS

Effect of EAEC 042 and 042P(−) on intestinal explants. Previous reports have suggested that EAEC strains are capable of eliciting destructive, cytotoxic effects in rat and rabbit models (35). Recently, Hicks et al. have shown that some EAEC strains bind to and damage human intestinal explants in culture (16). For this reason, we performed experiments to determine whether a known human pathogenic EAEC strain, 042 (not previously examined by Hicks et al.), is capable of causing damage to human intestinal mucosal tissue in culture and whether the adherence plasmid is necessary for this effect. Fresh jejunum, ileum, and colon mucosal explants obtained from three pediatric patients were harvested and incubated with strain 042 in tissue culture medium for 8 h. SEM of 042-infected tissue revealed the presence of bacteria heavily crusted over the mucosal surface of each intestinal specimen (Fig. 1a). The adherence was similar to that reported by Hicks et al. for other EAEC strains (16) in that the bacteria adhered in an aggregative pattern, but the density of colonic adherence appeared markedly greater for 042 than for other EAEC strains, despite identical methods. Enumeration of adherent bacteria by SEM revealed that the number of bacteria adhering to colonic specimens was significantly greater than that of bacteria adhering to specimens of small bowel mucosa (median numbers of adherent bacteria per field, 29.5 and 2.6; $P < 0.0001$).

In addition to adherence, disruption of the mucosal surface was also apparent by SEM (Fig. 1a and b). Toxic effects on colonic mucosa were particularly severe and were characterized by dilatation of the crypt openings, development of prominent intercrypt crevices, and exfoliation of colonic enterocytes. Rounded enterocytes were observed on the surface of the mucosa, most prominently around intercrypt crevices. The openings of goblet cells were found to be pitted and devoid of their apical mucus plugs. With the exception of cellular extru-

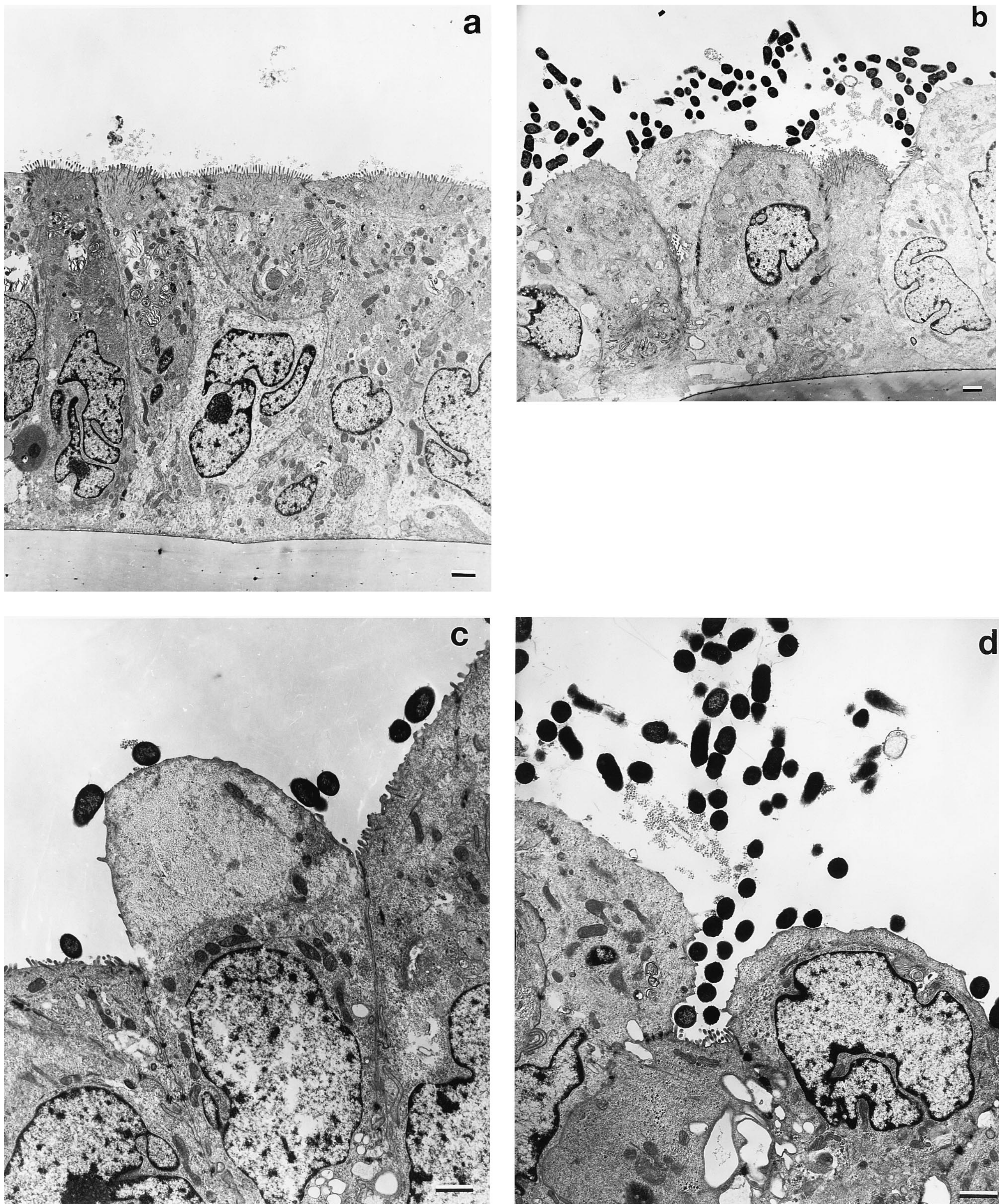


FIG. 2. TEM of T84 cell monolayers infected for 6 h. (a) Control strain *E. coli* HS4. The appearance of the cells is similar to that of uninfected T84 cells. (b to e) EAEC strain 042. Note the presence of adherent bacteria (b to e), loss of microvilli (c and d), ballooning of the apical cytoplasm (c), subnuclear vacuolization (c), and membrane vesiculation (b and d). Rupture of the cytoplasm in a severely affected cell is also visible (e). Bar, 1 μm.

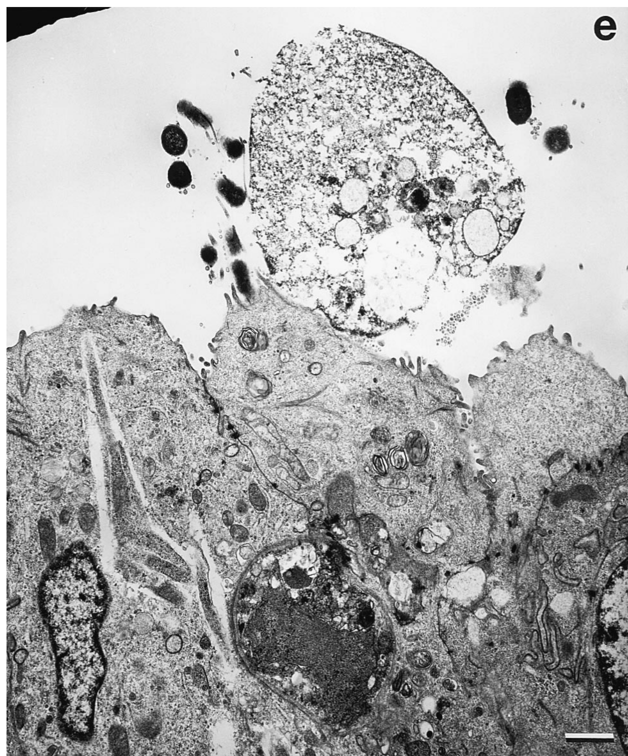


FIG. 2—*Continued.*

sion, these marked changes were not observed in small bowel specimens (data not shown).

Multiple putative virulence determinants have been associated with the 65-MDa plasmid of strain 042, including adherence to HEp-2 cells by virtue of the AAF/II adhesin and expression of EAST-1 (24). To assess the effect of the 65-MDa plasmid in mucosal damage, 042 cured of its adherence plasmid [designated 042P(−)] was also incubated with specimens of pediatric intestinal mucosa. By SEM, 042P(−) was found to elicit substantially less mucosal adherence than 042 (median numbers of adherent bacteria per field, 29.5 and 0.0; $P < 0.05$). In addition, the appearance of the colonic mucosa was indistinguishable from that of negative control and uninfected explants, lacking all toxic effects which were characteristic of the wild-type parent strain (data not shown).

Effect of 042 on Caco-2 and T84 cells. We sought to develop an in vitro system to characterize the effects observed in the IVOC model. Caco-2 and T84 cells were separately incubated with 042 for 6 h. After this time, the cells were fixed, stained, and examined by TEM (Fig. 2). Strain 042 adhered in loose aggregates to the surface of the T84 cells (Fig. 2b and d); most of the bacteria adhered to each other and not to the apical surfaces of the cells, apparently via flexible fimbria-like structures observed under high magnification to extend from the bacteria. The bacteria were not embedded within an obvious extracellular substance. High magnification of the apical membrane under adherent bacteria did not show intimate adherence (Fig. 2c), as is characteristic of enteropathogenic *E. coli* (EPEC). There was no discernible condensation of filamentous actin beneath the adherent bacteria, and internalization of bacteria was not observed. In contrast to T84 cells, 042 did not adhere to the surface of the Caco-2 cells.

Whereas Caco-2 cells infected with 042 appeared normal, infected T84 cell monolayers displayed marked cytotoxic effects. These effects were characterized by vesiculation of the apical brush border (Fig. 2d); in many cells, this resulted in total absence of the microvilli. Aggregates of vesicles often adhered to bacteria. Vesiculation and all other toxic effects were most severe in areas where bacteria adhered to the cells.

The cytoplasm of affected T84 cells showed marked changes. A typical affected cell exhibited a dome-shaped morphology in which the apical plasma membrane was denuded of microvilli and appeared to be "ballooning" out from the normally flat brush border (Fig. 2c and e). The apical cytoplasm appeared hypodense and devoid of organelles; such cells also typically developed increased vacuolization of the basolateral cytoplasm (Fig. 2c and d). Tight junctions were morphologically intact (Fig. 2c). In severely affected cells, the nuclei were separated from the surrounding cytoplasm (Fig. 2b). Upward migration of nuclei toward the apical cytoplasmic membrane was a characteristic feature, and in some cells the nuclei were observed to be expelled through the cytoplasmic membrane (Fig. 2d). The cell nuclei themselves were relatively unaffected, as were the mitochondria. There was no morphologic evidence of apoptosis. The monolayer also featured remnant cells, which appeared to be much smaller than normal T84 cells and were anucleate and vacuolated (Fig. 2d). The position of remnant cells, or of areas where the cells themselves were exfoliated, was generally indicated by the presence of a cleft in the monolayer.

SEM of 042-infected T84 cell monolayers revealed less obvious changes than those revealed by TEM (Fig. 3). Aggregates of bacteria were found to adhere to the monolayer, and there was thinning of the normally dense microvillar lawn. Infection with control strain HS4 yielded a normal microvillar layer.

Time course of cytotoxic effect on T84 cells. To characterize further the cytotoxic effects induced by 042, we repeated the above experiments but observed the monolayer after 1, 3, or 6 h of infection with 042 or control strain HS4. In this series of experiments, the bacteria were pelleted onto the monolayer to obtain uniform contact between the bacterial inoculum and the T84 cells. One hour after infection, the monolayer was apparently unaffected and was indistinguishable from HS4-infected or uninfected monolayers. After 3 h, the 042-infected monolayer revealed adherent bacteria and mild toxic effects (Fig. 4a). The most obvious effects were seen where bacteria adhered and consisted of vesiculation of the microvilli, which were generally fewer in number. In addition, some ballooning of cells was observed, with the result being an uneven apical brush border on the monolayer; less than 50% of the cells in any given monolayer were abnormal. There were no discernible cytoplasmic changes at 3 h. None of these changes were observed in HS4-infected or uninfected monolayers.

After 6 h of incubation, the brush border of 042-infected monolayers was markedly uneven, with many bulging cells and extensive loss of microvilli (Fig. 4b). Most cells in the monolayer were affected. At this time point, the cytoplasm of affected cells was severely disrupted, as evidenced by apical cytoplasmic clearing and vacuolization (Fig. 2). There was exfoliation and rupture of cells, and nuclear extrusion was observed. Several nuclei were separated from the surrounding cytoplasm. Remnant cells were heavily vacuolated.

Effect of the AA plasmid on T84 toxicity. IVOC studies suggested that the EAEC plasmid is required for induction of mucosal cytotoxicity, as well as adherence. We therefore incubated T84 cells with the plasmid-cured strain and compared the effects with those induced by the 042 parent. After 6 h of incubation, T84 monolayers infected with the plasmid-cured strain failed to demonstrate adherent bacteria and exhibited no cytotoxic effects. Strain 042P(−)-infected monolayers were in-

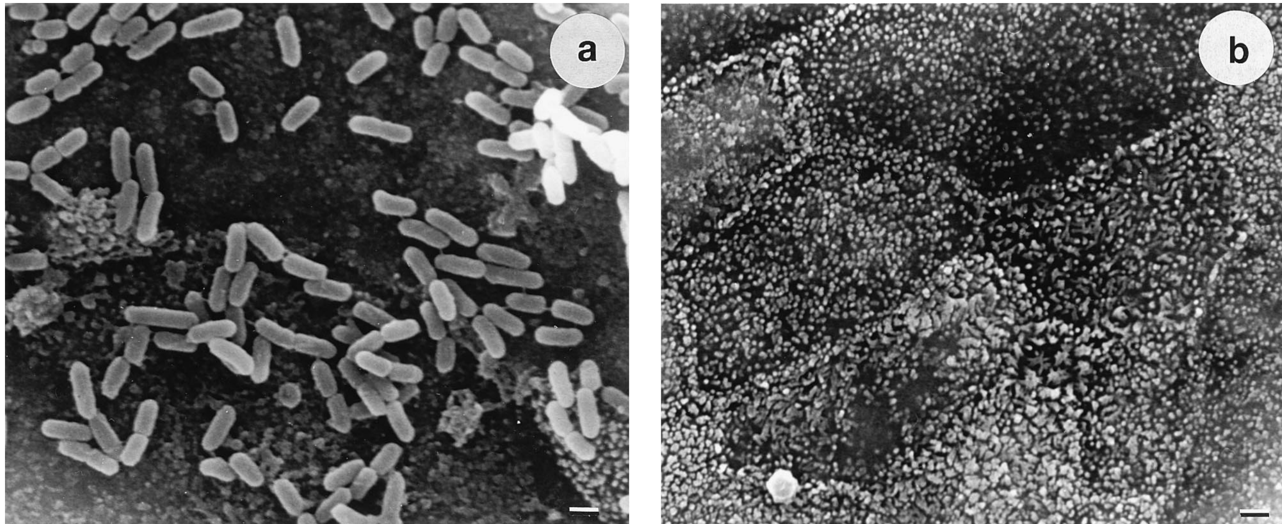


FIG. 3. SEM of T84 cell monolayers. (a) Monolayer infected with 042 for 6 h. Note adherent bacteria and the paucity of microvilli compared with uninfected monolayer (b). Bars, 1 μm.

distinguishable from monolayers infected with strain HS4 (data not shown).

DISCUSSION

The pathogenetic mechanism of EAEC diarrhea is unknown. The most plausible explanation for the persistent nature of EAEC disease involves intestinal mucosal damage elicited by these organisms, as observed by other investigators in animal models (34, 35). More recently, such effects have been observed in autopsy specimens from infected patients (13) and upon infection of intestinal mucosae cultured in vitro (16). However, the cause of this mucosal damage is not known.

As a first step in the study of EAEC cytotoxicity, we have demonstrated toxic effects of a proven human EAEC pathogen in organ culture of intestinal explants from children. EAEC strain 042 was found to elicit epithelial cell extrusion in pediatric jejunum, ileum, and colonic specimens. Furthermore, crypt dilatation and microvillous vesiculation were seen in colonic specimens. These findings were similar to those observed with other EAEC strains (16). Our observations were extended by incubating 042 with T84 and Caco-2 cells in vitro. We found that 042 adhered substantially more to T84 cells and that this adherence was followed by a time-dependent toxic effect on the T84 cells but not the Caco-2 cells. The toxic effect apparently began with disruption of the microvillar layer, with shedding of the microvilli by vesiculation, followed by clearing of the apical cytoplasm. Subsequently, subnuclear vacuolization developed and the nuclei assumed a more apical position within the cells. Severely affected cells ruptured and exfoliated from the monolayer.

The reason why T84 cells are susceptible to the toxic effects of 042, while Caco-2 cells are not, is unknown. Polarized T84 cells are phenotypically similar to colonic crypt cells, whereas polarized Caco-2 cells express brush border enzymes which suggest that they best represent a mid-villous small intestinal epithelial cell (9, 17, 27). The difference in susceptibility between these two cell lines parallels the observation that 042 exhibits greater toxicity to colonic specimens in organ culture than to those derived from the small bowel mucosa.

The effects exhibited by EAEC are reminiscent of, although not identical to, the effects induced by other bacteria and enteric toxins. The *Bacteroides fragilis* enterotoxin has been shown to induce denuding of the microvilli and ballooning of the apical membrane; however, the effects of the *B. fragilis* enterotoxin also include disruption of some tight junctions and surface blebbing, which are not induced by EAEC (6, 10, 33). Vesiculation of the microvilli is induced by other enteric bacteria, most notably, EPEC (12, 15). In fact, the effects of EAEC on T84 monolayers are similar to the attaching-and-effacing lesion of EPEC, with the prominent exception of intimate adherence, which EAEC does not exhibit; we have therefore termed the phenotype induced by 042 in T84 cells "nonintimate effacing." Extrusion of cells and apical cytoplasmic pallor are also seen with EPEC infection (29). However, the cytoskeletal changes induced by EPEC, featuring actin accumulation under the adhering bacteria, are not observed with EAEC, nor is invasion observed in the T84 model. Invasion has been reported for some EAEC strains (2), although our model suggests that invasiveness is not required to induce toxic effects.

Membrane vesiculation has been reported to be due to a severing of microvillar actin by villin in the presence of elevated intracellular calcium concentrations (21). Indeed, Baldwin et al. have described elevated intracellular calcium concentrations in HEp-2 cells induced by strain 042 (termed strain JPN10 in their report) (1). We are currently investigating the role of intracellular calcium concentrations in the induction of the cytopathic effects we have observed.

We have shown here that the presence of the large AA plasmid of EAEC is required for expression of the cytotoxic effect. In a recent study, we found that this is not due strictly to the effect of adherence, as insertion mutants which are negative for AA still elicit toxic effects in the IVOC model, albeit to a lesser degree (24). These data suggest that a diffusible cytotoxin is either encoded by the plasmid itself or dependent upon a plasmid gene for expression.

We hypothesize that the putative cytotoxin of EAEC results in the cellular effects we have observed and may account for the characteristic persistence of EAEC diarrhea. According to our paradigm, EAEC infection may lead to damage to the absorptive epithelium of the small and large intestines, which may result in malabsorption of water and solutes. Diarrhea may be exacerbated by intestinal secretion stimulated by one or more enterotoxins. Epidemiologic studies suggest that EAEC-

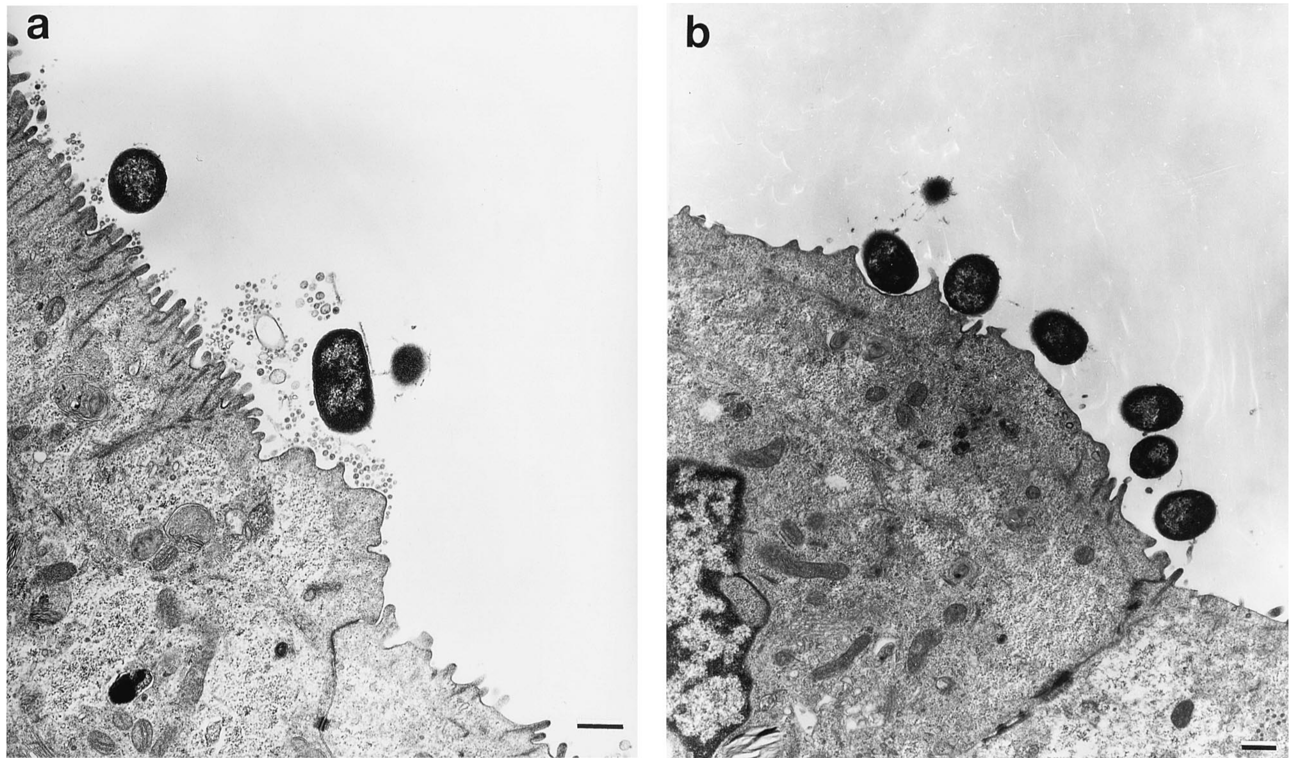


FIG. 4. Cytopathic effects of 042 on T84 cells over time. After 3 h of infection with 042 (a), some adherent bacteria are visible and membrane vesiculation is visible in proximity to the bacteria. After 6 h (b), extensive loss of microvilli is apparent. Tight junctions appear to be morphologically intact. Bars, 0.5 μm.

caused persistent diarrhea appears to be a greater problem in areas in which malnutrition is highly prevalent (3, 4, 14). Indeed, Cravioto et al. have observed a highly virulent, lethal outbreak of EAEC diarrhea in the malnutrition ward of a Mexico City hospital (13). In addition, EAEC has recently been implicated as a cause of diarrhea in human immunodeficiency virus-infected patients (23). The persistent nature of symptoms may be due to delay in the repair of the damaged mucosa manifested by malnourished or otherwise compromised hosts. In addition, the dense AA exhibited by EAEC isolates may also contribute to persistent colonization and damage. Studies are under way to test this paradigm, as well as to characterize further the cytotoxic effects of EAEC.

ACKNOWLEDGMENTS

This work was supported by U.S. Public Health Service grants AI33096 to J.P.N. and DK45496 to C.L.S. S.H. was supported by Kings College Joint Research Council grant JRC 159.

REFERENCES

1. **Baldwin, T. J., S. Knutton, L. Sellers, H. A. M. Hernandez, A. Aitken, and P. H. Williams.** 1992. Enteroaggregative *Escherichia coli* strains secrete a heat-labile toxin antigenically related to *E. coli* hemolysin. Infect. Immun. **60:**2092–2095.
2. **Benjamin, P., M. Federman, and C. A. Wanke.** 1995. Characterization of an invasive phenotype associated with enteroaggregative *Escherichia coli*. Infect. Immun. **63:**3417–3421.
3. **Bhan, M. K., V. Khoshoo, H. Sommerfelt, P. Raj, S. Sazawal, and R. Srivastava.** 1989. Enteroaggregative *Escherichia coli* and *Salmonella* associated with nondysenteric persistent diarrhea. Pediatr. Infect. Dis. J. **8:**499–502.
4. **Bhan, M. K., P. Raj, M. M. Levine, J. B. Kaper, N. Bhandari, R. Srivastava, R. Kumar, and S. Sazawal.** 1989. Enteroaggregative *Escherichia coli* associated with persistent diarrhea in a cohort of rural children in India. J. Infect. Dis. **159:**1061–1064.
5. **Boyer, H. W., and R. Roulland-Dussoix.** 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. **41:**459.
6. **Chambers, F. G., S. S. Koshy, I. Eromar, R. F. Saidi, D. P. Clark, and C. L. Sears.** Unpublished data.
7. **Chan, K. N., A. D. Phillips, S. Knutton, H. R. Smith, and J. A. Walker-Smith.** 1994. Enteroaggregative *Escherichia coli*: another cause of acute and chronic diarrhoea in England? J. Pediatr. Gastroenterol. Nutr. **18:**87–91.
8. **Cravioto, A., A. Tello, A. Navarro, J. Ruiz, H. Villafan, F. Uribe, and C. Eslava.** 1991. Association of *Escherichia coli* HEp-2 adherence patterns with type and duration of diarrhoea. Lancet **337:**262–264.
9. **Dharmasathaphorn, K., and J. L. Madara.** 1990. Established intestinal cell lines as model systems for electrolyte transport studies. Methods Enzymol. **192:**354–389.
10. **Donelli, G., A. Fabbri, and C. Fiorentini.** 1996. *Bacteroides fragilis* enterotoxin induces cytoskeletal changes and surface blebbing in HT-29 cells. Infect. Immun. **64:**113–119.
11. **Donnenberg, M. S., A. Donohue-Rolfe, and G. T. Keusch.** 1989. Epithelial cell invasion: an overlooked property of enteropathogenic *Escherichia coli* (EPEC) associated with the EPEC adherence factor. J. Infect. Dis. **160:**452–459.
12. **Donnenberg, M. S., and J. B. Kaper.** 1992. Enteropathogenic *Escherichia coli*. Infect. Immun. **60:**3953–3961.
13. **Eslava, C., J. Villaseca, R. Morales, A. Navarro, and A. T. Cravioto.** 1993. Identification of a protein with toxigenic activity produced by enteroaggregative *Escherichia coli*, abstr. B-105, p. 44. *In* Abstracts of the 93rd General Meeting of the American Society for Microbiology 1993. American Society for Microbiology, Washington, D.C.
14. **Fang, G. D., A. M. Lima, C. C. Martin, L. J. Barrett, J. P. Nataro, and R. L. Guerrant.** 1992. Aggregative HEp-2 cell-adherent (AA) *E. coli* and *Cryptosporidium*: important pathogens in hospitalized children with persistent diarrhea (PD) in northeast Brazil, abstr. 686, p. 225. *In* Program and abstracts of the 32nd Interscience Conference on Antimicrobial Agents and Chemotherapy. American Society for Microbiology, Washington, D.C.
15. **Formal, S. B., T. L. Hale, and E. C. Boedeker.** 1983. Interactions of enteric pathogens and the intestinal mucosa. Philos. Trans. R. Soc. Lond. **303:**65–73.
16. **Hicks, S., D. C. A. Candy, and A. D. Phillips.** 1996. Adhesion of enteroaggregative *Escherichia coli* to pediatric intestinal mucosa in vitro. Infect. Immun. **64:**4751–4760.
17. **Jacewicz, M. S., D. W. K. Acheson, M. Mobassaleh, A. Donohue-Rolfe, K. A. Balasubramanian, and G. T. Keusch.** 1995. Maturational regulation of globotriaosylceramide, the Shiga-like toxin 1 receptor, in cultured human gut epithelial cells. J. Clin. Invest. **96:**1328–1335.
18. **Knutton, S., D. R. Lloyd, and A. S. McNeish.** 1987. Adhesion of entero-

pathogenic *Escherichia coli* to human intestinal enterocytes and cultured human intestinal mucosa. Infect. Immun. **55:**69–77.
19. **Knutton, S., R. K. Shaw, M. K. Bhan, H. R. Smith, M. M. McConnell, T. Cheasty, P. H. Williams, and T. J. Baldwin.** 1992. Ability of enteroaggregative *Escherichia coli* strains to adhere in vitro to human intestinal mucosa. Infect. Immun. **60:**2083–2091.
20. **Levine, M. M., E. J. Bergquist, D. R. Nalin, D. H. Waterman, R. B. Hornick, C. R. Young, S. Sotman, and B. Rowe.** 1978. Escherichia coli strains that cause diarrhoea but do not produce heat-labile or heat-stable enterotoxins and are non-invasive. Lancet **i:**1119–1122.
21. **Matsudaira, P., and P. Jammey.** 1988. Pieces in the actin-severing puzzle. Cell **54:**139–140.
22. **Maunsbach, A. B.** 1994. Fixation of cells and tissues for transmission electron microscopy, p. 105–116. *In* J. E. Celis (ed.), Cell biology: a laboratory handbook. Academic Press, San Diego, Calif.
23. **Mayer, H. B., and C. A. Wanke.** 1995. Enteroaggregative *Escherichia coli* as a possible cause of diarrhea in an HIV-infected patient. N. Engl. J. Med. **332:**273–274.
24. **Nataro, J. P., S. Balepur, S. Hicks, and A. D. Phillips.** 1996. Unpublished data.
25. **Nataro, J. P., Y. Deng, S. Cookson, A. Cravioto, S. J. Savarino, L. D. Guers, M. M. Levine, and C. O. Tacket.** 1995. Heterogeneity of enteroaggregative *Escherichia coli* virulence demonstrated in volunteers. J. Infect. Dis. **171:** 465–468.
26. **Nataro, J. P., Y. Deng, D. R. Maneval, A. L. German, W. C. Martin, and M. M. Levine.** 1992. Aggregative adherence fimbriae I of enteroaggregative *Escherichia coli* mediate adherence to HEp-2 cells and hemagglutination of human erythrocytes. Infect. Immun. **60:**2297–2304.
27. **Pinto, M., S. Robine-Leon, M. D. Appay, M. Kedinger, N. Triadou, E. Dussaulx, B. Lacroix, P. Simon-Assman, K. Haffen, J. Fogh, and A. Zweibaum.** 1983. Enterocyte-like differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture. Cell **47:**323–330.
28. **Robinson, M. K., P. M. Bennet, S. Falkow, and H. M. Dodd.** 1980. Isolation of a temperature-sensitive derivative of RP1. Plasmid **3:**343–347.
29. **Rothbaum, R. J., J. C. Partin, K. Saalfield, and A. J. McAdams.** 1983. An ultrastructural study of enteropathogenic *Escherichia coli* infection in human infants. Ultrastruct. Pathol. **4:**291–304.
30. **Savarino, S. J., A. Fasano, D. C. Robertson, and M. M. Levine.** 1991. Enteroaggregative *Escherichia coli* elaborate a heat-stable enterotoxin demonstrable in an in vitro rabbit intestinal model. J. Clin. Invest. **87:**1450–1455.
31. **Savarino, S. J., A. Fasano, J. Watson, B. M. Martin, M. M. Levine, S. Guandalini, and P. Guerry.** 1993. Enteroaggregative *Escherichia coli* heat-stable enterotoxin 1 represents another subfamily of *E. coli* heat-stable toxin. Proc. Natl. Acad. Sci. USA **90:**3093–3097.
32. **Sears, C. L., F. Firoozmand, A. Mellander, F. G. Chambers, I. G. Eromar, A. G. M. Bot, B. Scholte, H. R. deJonge, and M. Donowitz.** 1995. Genistein and tyrophastin 47 stimulate CFTR-mediated Cl-secretion in T84 cell monolayers. Am. J. Physiol. **269:**G874–G882.
33. **Sears, C. L., L. L. Myers, A. Lazenby, and R. L. Van Tassell.** 1995. Enterotoxigenic *Bacteroides fragilis*. Clin. Infect. Dis. **20**(Suppl. 2)**:**S142–S148.
34. **Tzipori, S., J. Montanaro, R. M. Robins-Browne, P. Vial, R. Gibson, and M. M. Levine.** 1992. Studies with enteroaggregative *Escherichia coli* in the gnotobiotic piglet gastroenteritis model. Infect. Immun. **60:**5302–5306.
35. **Vial, P. A., R. Robins Browne, H. Lior, V. Prado, J. B. Kaper, J. P. Nataro, D. Maneval, A. Elsayed, and M. M. Levine.** 1988. Characterization of enteroadherent-aggregative *Escherichia coli*, a putative agent of diarrheal disease. J. Infect. Dis. **158:**70–79.
36. **Wanke, C. A., J. B. Schorling, L. J. Barrett, M. A. Desouza, and R. L. Guerrant.** 1991. Potential role of adherence traits of *Escherichia coli* in persistent diarrhea in an urban Brazilian slum. Pediatr. Infect. Dis. J. **10:** 746–751.
37. **Watson, A. J. M., S. Levine, M. Donowitz, and M. H. Montrose.** 1991. Kinetics and regulation of polarized Na^+-H^+ exchanger from Caco-2 cells, a human intestinal cell line. Am. J. Physiol. **261:**G229–G238.

Editor: P. E. Orndorff