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Adhesion of Enteroaggregative *Escherichia coli* to Pediatric Intestinal Mucosa In Vitro

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Organ cultures of small- and large-intestinal mucosa from children were used to examine the interactions of enteroaggregative *Escherichia coli* (EAEC) with human intestine. Mucosae from patients aged between 3 and 190 months were cultured with five EAEC strains isolated from infants with diarrhea in the United Kingdom and with two well-described prototype EAEC strains, 17-2 and 221. The prototype strains adhered to jejunal, ileal, and colonic mucosae. The wild-type strains also adhered to this tissue but showed a variable pattern of adhesion: two adhered to all intestinal levels, one adhered to jejunum and ileum, one adhered to ileum only, and one adhered to ileum and colon. Adherence was in an aggregative or stacked-brick pattern, resembling that seen on HEp-2 cells. Electron microscopy of infected small intestinal mucosa revealed bacteria in association with a thick mucus layer above an intact enterocyte brush border, which contained extruded cell fragments. This mucus layer was not present on controls. EAEC adherence to colonic mucosa was associated with cytotoxic effects including microvillous vesiculation (but without evidence of an attaching/effacing lesion), enlarged crypt openings, the presence of intercrypt crevices, and increased epithelial cell extrusion. These results demonstrate that in vitro organ culture of intestinal mucosa from children can be used to investigate EAEC pathogenesis in childhood directly. EAEC strains appear able to colonize many regions of the gastrointestinal tract, without overt changes to small intestinal mucosa but with cytotoxic effects on colonic mucosa.

Enteroaggregative *Escherichia coli* (EAEC) strains are the most recently described group of pathogenic *E. coli* and are recognized by a characteristic aggregative or stacked-brick pattern of adherence to human epithelial (HEp-2) cells cultured in vitro (29). Currently, this is the "gold standard" assay by which EAEC strains are distinguished; it also differentiates them from the other HEp-2 cell-adherent groups, i.e., enteropathogenic (EPEC) and diffuse adhering *E. coli* strains, which form localized and diffuse patterns, respectively (24). DNA gene probes to identify EAEC which recognize regions of the 60-MDa EAEC virulence plasmid (4, 11), but with variable sensitivity and specificity, have also been developed.

EAEC strains have been implicated as a cause of childhood diarrhea, particularly persistent episodes, in developing (5, 15, 36) and developed (8) countries. Animal models have demonstrated diarrhea in association with mild histopathological changes following EAEC infection (34, 35), and human challenge studies have shown the ability of some strains to cause diarrhea in adults (26, 30). A partially heat stable enterotoxin (EAST1) (32), an EAEC hemolysin (1), and a 108-kDa cytotoxin (12) have been suggested as putative toxigenic virulence factors for EAEC.

In an attempt to overcome appropriate ethical limitations on experimentation in pediatrics, formalin-fixed pediatric intestinal tissue was used to examine the adhesive ability of EAEC strains (16). These studies indicated that EAEC adhered to proximal small intestine as well as to distal ileum and colon (16). The fixed nature of the tissue means that such experiments have the disadvantage of allowing only the initial stages in adhesion to be examined. As the extent of colonization along the intestine may be an important factor in the production of malabsorption and chronic diarrhea, these studies were repeated in a system which was closer to the in vivo situation, i.e., in vitro organ culture. This system offers a means of maintaining intestinal mucosal specimens for 24 to 48 h, thus allowing more complex bacterium-host interactions to be studied. It has already proved to be a useful technique for such studies in adults (20, 21, 23). Pediatric intestinal mucosal organ culture has not been used previously to study bacterial adhesion, although it has been used in studies of celiac disease (13, 18).

Age-related differences in the host may be an important determinant of bacterial adhesion, and it is therefore logical to study a childhood disease by using pediatric tissue. Such differences may help explain the conflicting results obtained to date regarding EAEC intestinal adhesion. Studies have variously reported preferential adhesion to distal ileum (37) and proximal small intestine (16), using pediatric tissue, and colon (23) and duodenum (31), using adult tissue. Other factors include strain variability. Yamamoto et al. (37) studied only one strain, whereas the other studies included several (16, 23, 31). The investigative technique used is another variable, with formalin-fixed tissue (16, 37), organ culture (23), and isolated cells (31) being used in the different studies.

This study examined EAEC interactions with both small- and large-intestinal mucosae from children, using in vitro organ culture (20). We can confirm that EAEC strains possess the ability to adhere to proximal small intestine in childhood and further demonstrate a cytotoxic effect on colonic mucosa.

(Parts of this report were presented at the 3rd Commonwealth Conference on Diarrhoea and Malnutrition [15a] and at the 29th Annual Meeting of the European Society of Paediatric Gastroenterology and Nutrition [16a].)

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MATERIALS AND METHODS

Bacterial strains. Five wild-type EAEC strains (3862 [O85:H1], WJ19/10 [O126:H27], AN11/13 [O55:H4], HR15/6 [O126:H−], and LC9/6 [O111ab:H21])

TABLE 1. Characteristics of *E. coli* strains used[a]

Strain	Serotype	HEp-2 assay[a]	Hybridization[b]		Intestinal adhesion[c]		
			AA gene probe	EAST1 probe	Jejunum	Ileum	Colon
EAEC							
17-2	O3:H2	AA	+	+	✓	✓	✓
221	O92:H33	AA	+	−	✓	✓	✓
3862	O92:H33	AA	+	−	✓	✓	NA
LC9/6	O111:H21	AA	+	+	✓	✓	✓
AN11/13	O55:H4	AA	+	−	✓	✓	✓
WJ19/10	O126:H27	AA	+	+	NA	✓	✓
HR15/6	O126:H−	AA	+	−	NA	✓	NA
KH1/8 (EPEC)	O114:H2	LA	−	ND	✓	✓	NA
Normal flora							
SC13	O1:H−	NA	−	ND	NA	NA	NA
HB101	ND	NA	−	ND	NA	X	NA

[a] AA, aggregative adherence; LA, localized adherence.
[b] +, hybridization with DNA gene probe; −, no hybridization; ND, not done.
[c] ✓, significant adhesion above uninoculated control levels; X, insignificant adhesion; NA, no adhesion seen.

were examined in this study and have been described elsewhere (16). All had been isolated from children with acute or chronic diarrhea at Queen Elizabeth Hospital for Children, London, England; these strains had been detected by their aggregative pattern in the HEp-2 cell assay and were aggregative adherence gene probe positive (8). All five strains showed significant adhesion to formalin-fixed jejunal mucosa from children (16). Two further EAEC strains, 17-2 and 221, which have been well described elsewhere and used as prototype EAEC, were included. Strain 17-2 (serotype O3:H2) was isolated from a Chilean infant with diarrhea (24), and strain 221 (serotype O92:H33) was originally isolated from a student returning to the United States from Mexico with traveler's diarrhea (25). The latter strain was subsequently used to challenge adult volunteers and found to cause diarrhea (26), although strain 17-2 failed to elicit such a response (30). An enteropathogenic *E. coli* strain, KH1/8 (O114:H2), also isolated from a child admitted to Queen Elizabeth Hospital for Children, was used as a positive control. This strain showed an attaching/effacing lesion in vivo on the proximal small intestinal biopsy from the patient with chronic diarrhea from whom it was isolated (30a); it also caused an attaching/effacing lesion in vitro in adult human small-intestinal organ culture (19a) and showed a localized pattern of adhesion in the HEp-2 cell assay (22). Two *E. coli* strains were included as negative controls: normal flora strain SC13 (O1:K1) (kindly provided by S. Knutton, Birmingham, England) and laboratory strain HB101. Neither strain adhered to HEp-2 cells in vitro (Table 1 provides further characteristics of each *E. coli* strain).

All bacterial strains were stored routinely at −70°C in a Microbank (ProLab Diagnostics, Neston, England).

Prior to the adhesion studies, all bacterial strains were subcultured into brain heart infusion broth and incubated aerobically overnight without agitation for 18 h at 37°C.

HEp-2 cell assay. All bacterial strains were examined for adhesion to cultured human epithelial cells in the 3-h HEp-2 cell assay (9) prior to use in organ culture experiments. HEp-2 cells were grown to 50 to 70% confluency on 10-mm-diameter glass coverslips in 24-well tissue culture plates. Twenty-five microliters of overnight *E. coli* brain heart infusion broth culture and 1 ml of Dulbecco's minimum essential medium with 0.5% [wt/vol] D-mannose) were added to each well, and the plates were incubated for 3 h at 37°C. Coverslips were washed thoroughly with sterile phosphate-buffered saline to remove nonadherent bacteria, fixed with 70% methanol, stained with 10% Giemsa stain, and examined by light microscopy.

Tissue samples. Jejunal tissue was obtained, with fully informed parental consent, from pediatric patients undergoing routine proximal small-intestinal mucosal biopsy for gastrointestinal disorders (n = 20, age range = 5 to 109 months, median age = 29 months), using a double-port modification of the Crosby capsule (19). Macroscopically normal jejunal tissue which showed tall, intact villi under a dissecting microscope was selected. Distal ileal and transverse colonic samples were taken during routine lower gastrointestinal tract endoscopic investigation of inflammatory bowel disease (n = 9, age range = 3 to 168 months, median age = 114 months; and n = 8, age range = 64 to 190 months, median age = 132 months, respectively), using an Olympus PCF pediatric colonoscope. Ileal and colonic biopsies were taken from areas showing no endoscopic abnormalities. Data included in this report concern only those patients whose intestinal histology was subsequently reported as normal.

Mucosal tissue was divided into two groups, uncultured and cultured. Three specimens (from different patients) from each intestinal region were fixed immediately after excision, to act as uncultured controls. The cultured specimens were divided further into the following groups: specimens incubated with tissue culture medium alone without the addition of bacteria (uninoculated controls), tissue incubated with *E. coli* HB101 and SC13 (negative controls), tissue incubated with EPEC strain KH1/8 (positive controls), and tissue incubated with EAEC strains (experimental cultures).

An uninoculated control was included with each incubation procedure.

Organ culture adhesion assay. The assay was essentially that described by Knutton et al. (20). Briefly, 2- to 3-mm^2 biopsy specimens were oriented mucosal surface upward on sterile foam squares placed in 50-mm-diameter petri dishes. The foam was saturated with a bicarbonate-buffered culture medium consisting of Dulbecco's minimum essential medium and NCTC-135 medium (1:1) with 10% newborn calf serum plus 0.5% (wt/vol) D-mannose (all chemicals supplied by Sigma Chemical Co., Poole, England). The level was adjusted to cover the biopsy specimen with a thin film of medium by capillary action.

Twenty-five microliters of the overnight bacterial broth culture was added to the mucosal surface of the biopsy specimens, and the petri dishes were gassed with 95% O_2–5% CO_2 inside an airtight container and maintained on a rocking platform for 8 h at 37°C. The culture medium was changed completely every 2 h to maintain pH and nutrient levels, without reinoculation with the bacterial culture. Petri dishes were regassed after each medium change.

Incubations were repeated at least three times for each bacterial strain and mucosal region, using tissue from different patients on repeat occasions.

Tissue processing. After the culture period, tissue specimens were washed three times with fresh culture medium to remove any nonadherent bacteria, fixed in 3% phosphate-buffered glutaraldehyde, and postfixed in 1% aqueous osmium tetroxide. For scanning electron microscopy (SEM), specimens were taken through a graduated series of ethanol and critical point dried in liquid CO_2, using a Polaron E3000 critical point drying apparatus. Samples were sputter coated with gold-palladium in a Polaron E5100 series II coating system and examined in a JEOL JSM-5300 scanning electron microscope. Counts of mucosally adherent bacteria were taken from 10 random fields of view for each specimen at a fixed magnification of ×3,500.

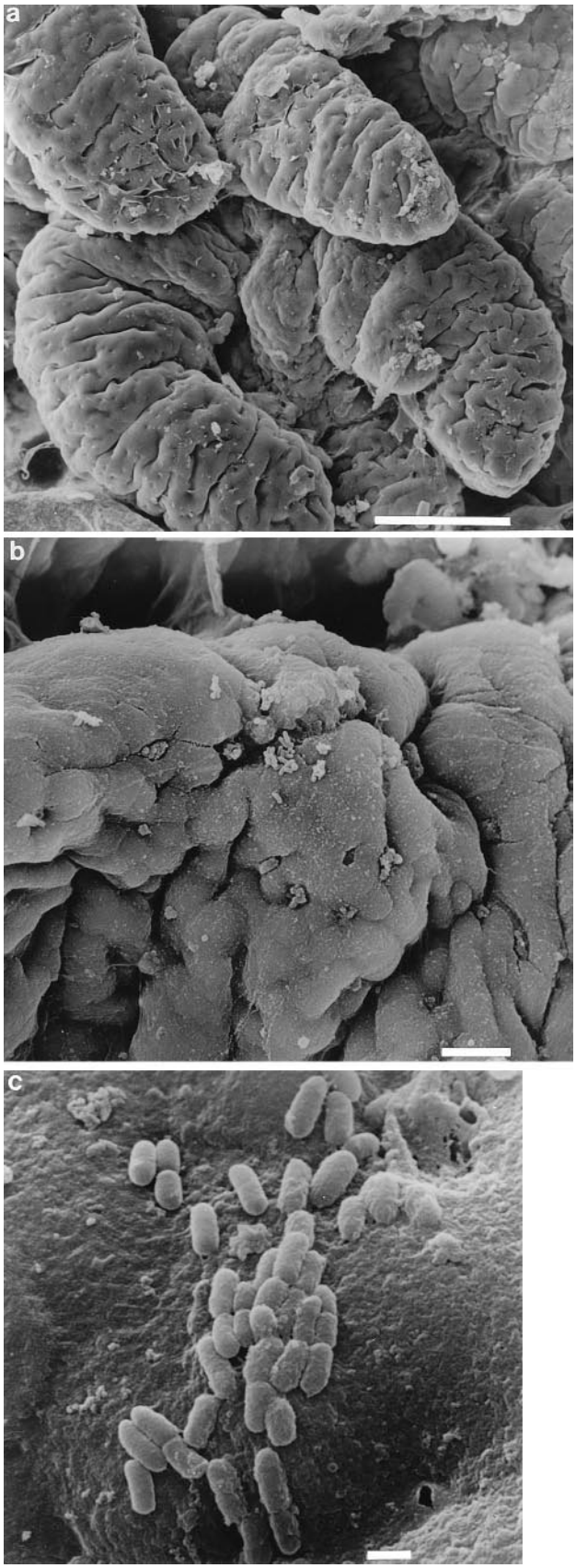
For transmission electron microscopy (TEM), postfixed specimens were dehydrated in 2,2-dimethoxypropane and embedded in TAAB resin (TAAB Laboratories, Ltd., Reading, England). Thick (0.5-μm) sections were stained with 1% toluidine blue for light microscopy. Ultrathin (0.1-μm) sections were double stained with 2% aqueous uranyl acetate and lead citrate and examined in a JEOL JEM 1200-EX II transmission electron microscope at an accelerating voltage of 80 kV.

Statistical analysis of adhesion. Statistical comparisons were performed by nonparametric analysis using a Mann-Whitney test, with a P value of <0.05 taken as significant adhesion. Values were recorded as a median number of bacteria adhering per field.

RESULTS

Adhesion to jejunum (i) SEM. Jejunal explants, fixed immediately (uncultured control) and those incubated in the absence of bacteria (uninoculated control), appeared similar. There was no evidence of excess cell loss, and extruding cells were observed only in the extrusion zone at the tips of villi. No bacteria were found adhering to any of the specimens observed (Fig. 1a).

Tissue incubated with the negative control strains HB101 and SC13 maintained an intact glycocalyx, but more extruding



cells were present than with the control specimens described above. No excess mucus or adhering bacteria were found.

SEM observations suggested that the positive control EPEC strain KH1/8 adhered to jejunal mucosa and caused characteristic elongation of microvilli only on those cells with adherent bacteria. This appeared to be a distinct cellular event, as neighboring uncolonized cells were normal with intact glycocalyx. KH1/8 adhered to jejunum in significantly large numbers (median = 0, range = 0 to 150 bacteria per SEM field; $P < 0.0001$ compared with uninoculated control values).

Both prototype EAEC strains 17-2 and 221 (Fig. 1b) adhered to jejunum (median values of 0.5 (range = 0 to 150) and 0.5 (range = 0 to 33) bacteria per SEM field, respectively). Three of the five wild-type EAEC strains showed similar levels of adhesion (3862 [median = 0, range = 0 to 150], LC9/6 [median = 0, range = 0 to 24], and AN11/13 [median = 2, range = 0 to 49]). Each of these results was highly significant compared with controls, with $P < 0.0001$.

In all cases in which EAEC strains adhered to the mucosa in significant numbers, they did so in aggregates with a stacked-brick pattern similar to that seen on HEp-2 cells in culture (Fig. 1c). However, there was increased rounding up and extrusion of cells throughout the length of the villi on specimens, with many large aggregates of adherent bacteria (17-2, 3862, and AN11/13). Bacteria also adhered to mucus from goblet cells.

The experimental specimens with no adhering bacteria (EAEC WJ19/10 and HR15/6) all maintained an intact glycocalyx, but more extruding cells were present than in uninoculated and negative controls.

(ii) TEM. Uninoculated control jejunal specimens showed epithelial cells with an intact glycocalyx overlying tightly packed, tall microvilli with small primary lysosomal bodies present; other organelles showed no obvious signs of abnormality.

All incubated specimens showed evidence of mild lamina propria edema in the villus region, regardless of the degree of bacterial colonization.

Tissue incubated with the negative controls appeared similar to uninoculated controls with no cellular abnormalities, although the lysosomal bodies were more electron dense than those previously described. No adherent bacteria were seen.

EPEC strain KH1/8 produced extensive enterocyte loss and the characteristic attaching/effacing lesions on intact cells. Bacteria were also associated with extruded cell debris and mucus above the mucosa.

Specimens with adherent EAEC showed some areas of epithelial cell extrusion. A layer of mucus containing cellular debris overlying the epithelial surface was noted, and many epithelial cells had a normal brush border appearance. Bacterial groups were found in association with this mucus-debris layer (Fig. 2a), and few were observed in close proximity with microvilli, despite appearing to do so on SEM. Tissue inoculated with EAEC strains 3862 and 17-2 showed bacteria adhering intimately to microvilli, although no underlying epithelial lesion was noted. Intracellular ultrastructural changes, i.e., an increase in size and electron density of lysosomal bodies in the apical region of the enterocytes (Fig. 2b) and mitochon-

FIG. 1. Scanning electron micrographs of 8-h in vitro organ culture of pediatric jejunum showing the normal appearance of an uninoculated control (a; bar = 100 μm), the appearance of jejunal mucosa inoculated with EAEC strain 221 (b; bar = 10 μm), and aggregative adherence of EAEC strain 221 (c; bar = 1 μm).

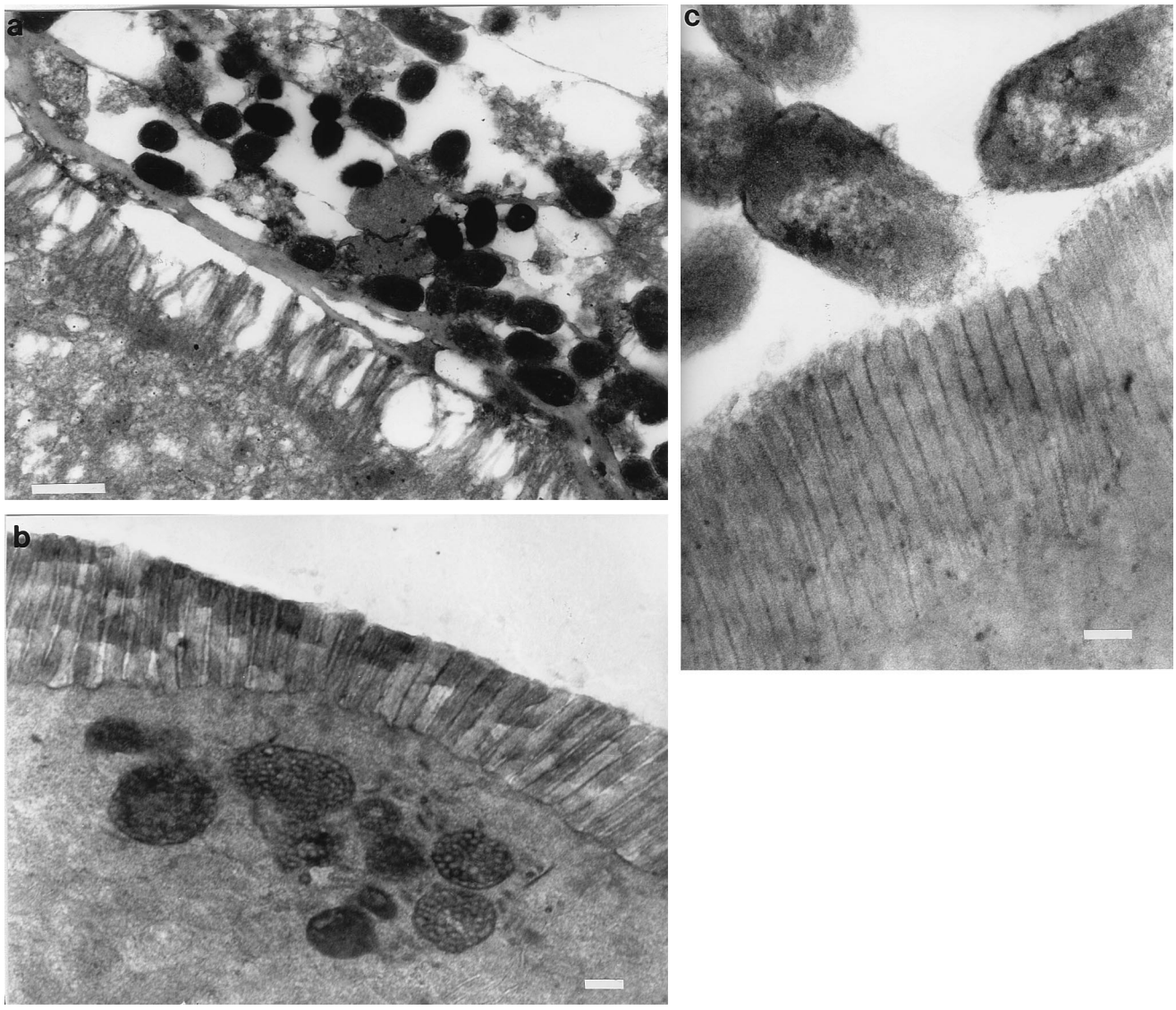


FIG. 2. TEM. (a) EAEC strain 17-2 associated with mucus and cell debris above the jejunal mucosal surface (bar = 1 μm); (b) dense lysosomal bodies in jejunal mucosa incubated with EAEC strain WJ19/10 (bar = 20 nm); (c) EAEC strain 221 adhering to the microvilli of an ileal specimen (bar = 200 nm).

drial swelling, were seen, and these changes were more pronounced in heavily colonized specimens.

Adhesion to ileum. (i) SEM. Tall finger-like villi were evident on all uninoculated ileal control specimens, and in contrast to jejunum, small numbers of scattered individual bacteria were found adhering to the mucosal surface despite thorough washing after incubation. However, there were no obvious associated pathological changes. More mucus was present on ileal samples than on jejunal controls; the visible mucosal surface showed an intact glycocalyx.

Bacterial counts were taken from nine uninoculated ileal control specimens to obtain indigenous levels of adhering bacteria over a total of 90 fields of view. The median number of bacteria per field was 0; the range was 0 to 5.

Specimens with significant numbers of adherent bacteria showed excess rounding up and extrusion of cells, with a layer of mucus over the tips of many villi. EPEC strain KH1/8 was associated with an effect on ileum similar to that on jejunum; i.e., microvillous elongation was associated with bacterial adhesion. Tissue incubated with the negative control strains showed bacteria adhering to mucus at the villus tips but with an otherwise normal appearance.

Both of the prototype EAEC and all five wild-type EAEC strains, as well as the EPEC strain, showed significant adhesion to ileal mucosa ($P < 0.001$) (Fig. 3). No significant adhesion was seen with the negative control strains SC13 and HB101.

(ii) TEM. Ileal tissue incubated with control strains SC13 and HB101 showed more evidence of extruding cells than uninoculated controls, and a layer of mucus was present over some areas. No organelle or brush border abnormalities were seen.

Positive control EPEC KH1/8 showed a typical attaching/effacing lesion.

The TEM appearance of ileal specimens with adherent EAEC was similar to that of jejunal tissue. A layer of mucus and cell debris with adherent bacterial groups was evident above an intact brush border. EAEC strains 3862 and 221 appeared to be the only strains adhering to the microvillus

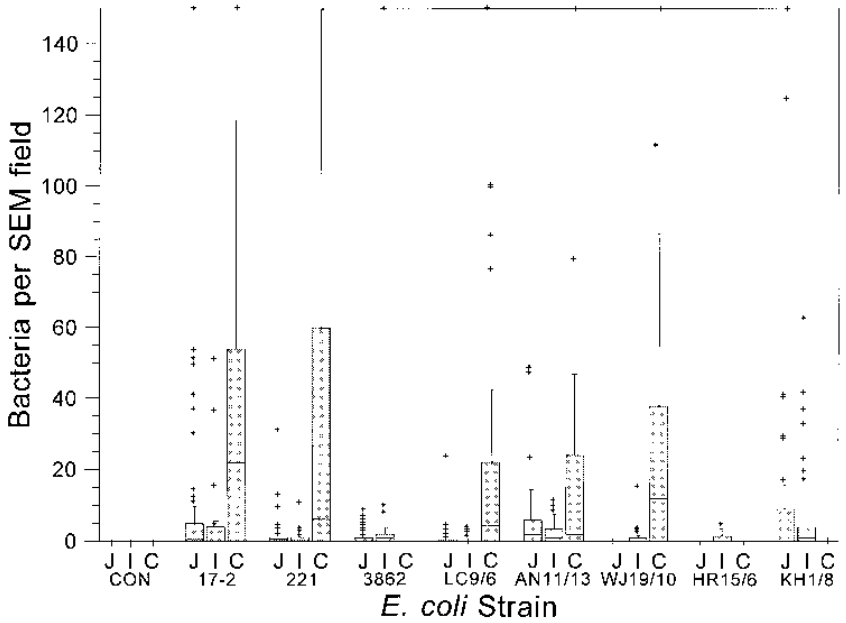


FIG. 3. Box and whisker plot showing levels of bacterial adhesion to small- and large-intestinal mucosa. J, jejunum; I, ileum; C, colon; CON, uninoculated control.

membrane (Fig. 2c), although no obvious brush border lesion was noted on any specimen.

Adhesion to colon. (i) SEM. There was good preservation of glycocalyx and crypt openings were small in all uninoculated colonic control specimens (i.e., incubated in the absence of bacteria) (Fig. 4a). Mucus discharge from goblet cells and some individual rod-like bacteria were seen on each specimen. Colonocytes distant from the crypt openings showed evidence of rounding up but with no obvious extrusion. Counts of adhering bacteria were taken from eight uninoculated control specimens, i.e., 80 fields of view, with a median number of bacteria per field of 0 and a range of 0 to 1.

Tissue incubated with negative controls SC13 and HB101 appeared similar to the tissue described above but with some cell rounding and mucus loss from goblet cells. SC13 and HB101 did not adhere to colon.

Positive control EPEC KH1/8 did not adhere to colonic mucosa, but loss of glycocalyx, extrusion of cells, and deep intercrypt crevices were evident on specimens incubated with the organism.

All experimental cultures showed rounding of the colonocytes distant from the crypt openings (Fig. 4b), and specimens incubated with strains 17-2, LC9/6, and WJ19/10 showed additional surface abnormalities. These included widened crypt openings, a rounded appearance of all cells, excess cell extrusion, and the presence of intercrypt crevices (Fig. 4c). Prototype EAEC strains 17-2 and 221 showed highly significant adhesion (median = 22, range = 0 to 150; and median = 6, range = 0 to 150, respectively; $P < 0.0001$) to colonic mucosa. Three of the five wild-type aggregative strains also showed similar levels of adhesion: LC9/6 (median = 4.5, range = 0 to 150), AN11/13 (median = 2, range = 0 to 150), and WJ19/10 (median = 12, range = 0 to 150). EAEC adhered to the mucosal surface in large, dense aggregates (Fig. 5a). There was no evidence of adhesion to surface mucus.

(ii) TEM. Uninoculated colonic control specimens showed good preservation, with many goblet cells retaining a brush border at the luminal surface. Internal epithelial cell organelle structure was normal, and crypt lumina were free from debris or mucus.

There was good correlation between abnormalities seen on SEM and the TEM appearance of experimental specimens. Rounded cells created an uneven epithelial surface, and deep crevices were readily distinguishable from crypts on TEM. True crypts had large openings at the luminal surface and often contained extruded cell fragments and mucus. There was a distinct lack of goblet cells, but there was no evidence of a thick mucus layer above the mucosa. With three EAEC strains (17-2, LC9/6, and WJ19/10), bacterial attachment was closely associated with vesiculated microvilli (Fig. 5b) but without any evidence of an attaching/effacing lesion such as that seen with EPEC; swollen mitochondria, electron-dense lysosomal bodies, and convoluted basolateral membranes were present in all specimens, although nuclei appeared normal.

Comparison of individual strain adhesion to different intestinal regions. Strains 17-2, 221, LC9/6, and WJ19/10 showed greater adhesion to colon ($P < 0.0001$) than to any other intestinal region. Strain AN11/13 adhered equally to all gut regions. The remaining strains (3862 and EPEC KH1/8) adhered to jejunum and ileum with equal significance. EAEC strain HR15/6 adhered to ileal tissue only. Figure 3 details these results, and a summary of the adhesion results is included in Table 1.

DISCUSSION

The ability to perform in vitro organ culture with pediatric intestinal mucosa has allowed the direct investigation of the interaction between EAEC and the mucosa in childhood. This technique has used animal (2, 3) and adult human (20, 21, 23) intestine and is extended here to use pediatric mucosa. In vitro organ culture lends itself well to microscopic studies, as intestinal explants remain structurally intact over the 8-h culture period. Previous studies have shown that human small intestine can be well maintained for up to 24 h with continued cell turnover (7) and protein synthesis (27).

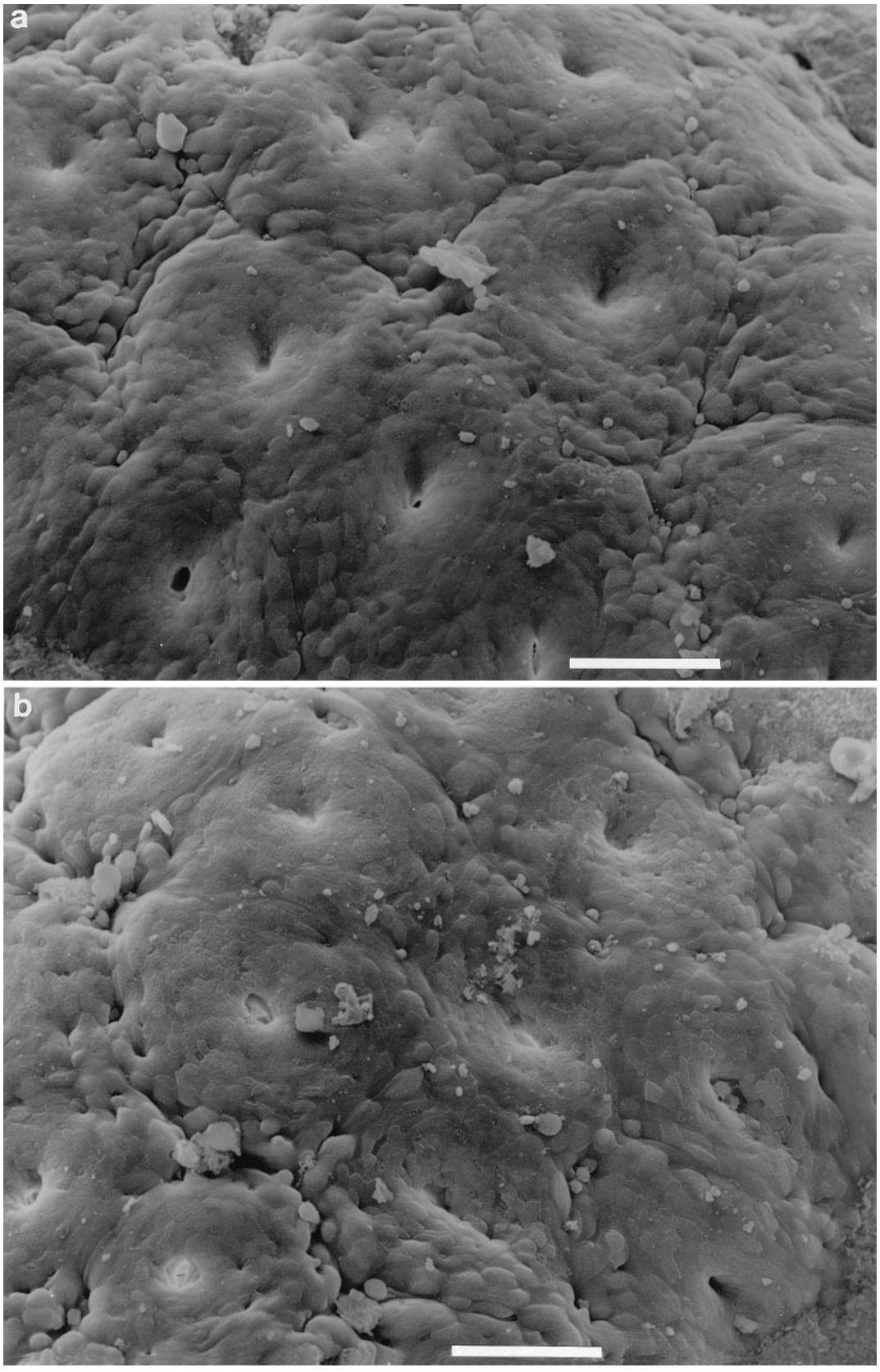


FIG. 4. SEM appearance of 8-h in vitro organ culture of colonic mucosa. (a) Uninoculated control; (b) EAEC strain AN11/13; (c) EAEC strain WJ19/10. Bars = 50 μm.

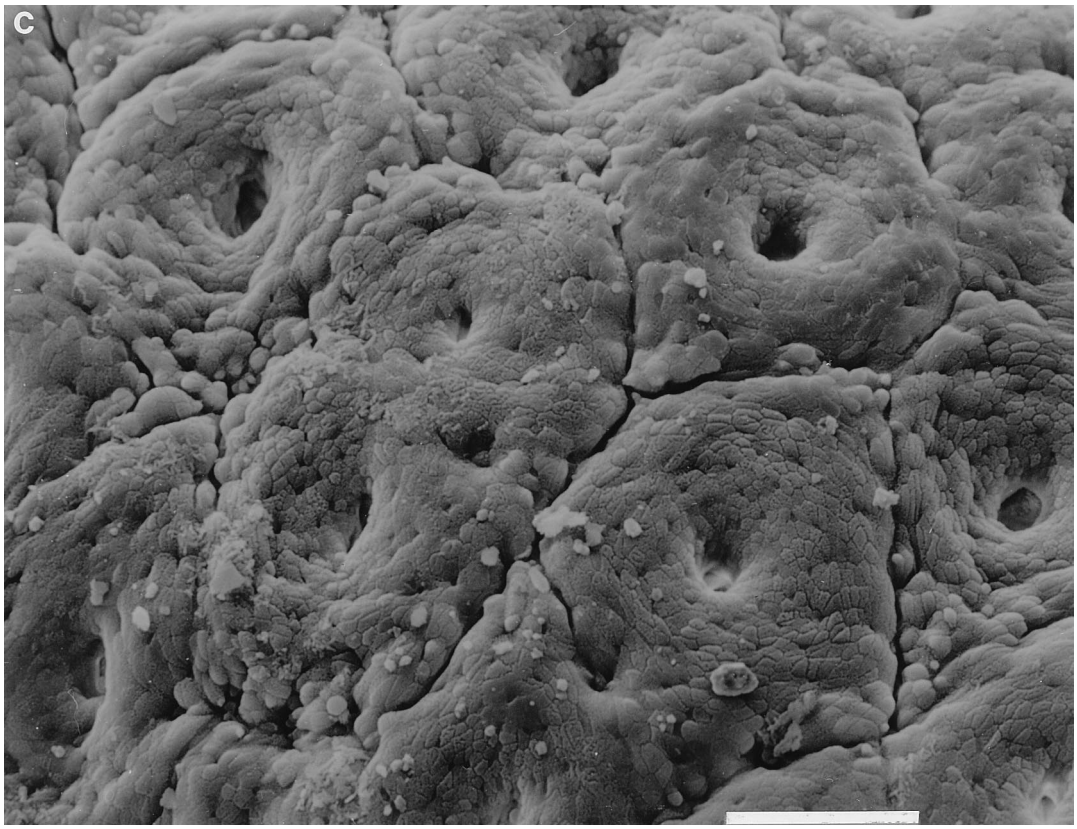


FIG. 4—*Continued.*

Data from SEM demonstrate that some EAEC strains are capable of adhering to many regions of the gastrointestinal tract from children and are not restricted to the distal small and large bowel as suggested previously (23, 37). In this study, five of seven strains adhered in significant numbers to jejunum, all seven adhered to ileum, and five adhered to colon. Greater numbers of bacteria adhered to colonic mucosa, suggesting that factors promote adhesion of some strains to this mucosal region although these strains possess the ability to adhere to small bowel. However, one strain (3862) readily adhered to small intestine alone.

There was evidence of excess cellular extrusion in all small- and large-bowel specimens with adherent EAEC. Enlarged crypt openings, intercrypt crevices, and microvillous vesiculation were features particular to EAEC-infected colonic specimens. Intercrypt crevice formation may be the result of excess extrusion of the more mature colonocytes distant from the crypt regions. The excess cell extrusion is suggestive of a cytotoxic effect and was more pronounced in those tissues which had been incubated with EAST1 DNA-positive EAEC strains. EAST1 is an enterotoxin without reported cytotoxic activity, and therefore it would be surprising if it brought about the observed changes. However, two other toxins have been described for EAEC, a hemolysin (1) and a 108-kDa cytotoxin (12), both of which have cytotoxic activity. The EAEC strains described here may harbor genes encoding one or more of these toxins, although this was not tested. Expression of these genes could account for the cytological damage observed. Coexpression of an enterotoxin and a cytotoxin have been described previously for *Salmonella* species (10) and *Campylobacter jejuni* (17). *C. jejuni* colonizes the small intestine initially, followed by the large bowel (6). EAEC strains also have the ability to adhere to small and large intestine, and an enterotoxin acting on the small bowel would lead to a watery diarrheal state followed by colonization of the large bowel, with elaboration of a cytotoxin which could lead to mucosal damage and possibly explain the bloody diarrhea observed in some EAEC-infected children (5). These cytotoxic effects have also been demonstrated with a well-characterized EAEC strain, O42 (O44:H18), which is known to cause diarrhea in adult volunteers and will be reported elsewhere (28).

The cytotoxic changes noted in vitro occurred within 8 h of EAEC inoculation, which may not be the case in vivo. In this organ culture model, the mucosal specimens were directly exposed to a high dose of bacteria (approximately 10^7/ml) without the dilution effects of intestinal or gastric secretions which may play a role in vivo.

In vivo, bacteria must penetrate the mucus barrier overlying the mucosa in order to reach the unstirred layer (a region of approximately 50-μm width above the mucosal surface [33]) and the epithelium itself. The organ culture system permits direct access to the mucosa without the presence of a thick mucus barrier, which suggests that the excess mucus production noted with EAEC-inoculated specimens may be a mucosal response to infection. It is unlikely to be an artifact of organ culture, as the negative and positive control incubations were not associated with mucus release. EAEC strains have been directly associated with mucus release in the gnotobiotic piglet model, in which bacterial aggregates were found within a "gel-like" matrix above an intact mucosal surface (35). Indirect evidence for EAEC producing mucus release in humans has

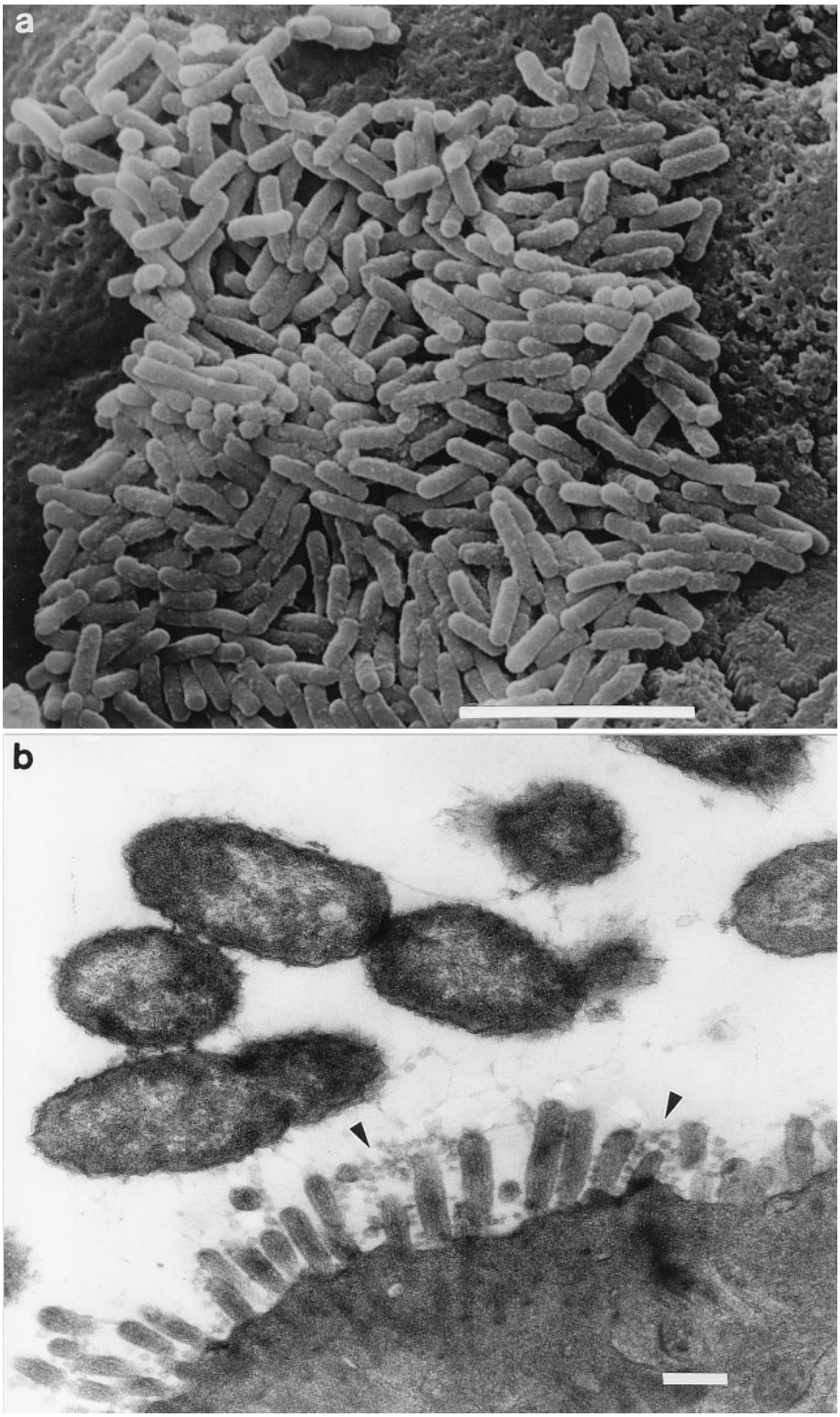


FIG. 5. (a) Aggregative adherence of EAEC strain WJ19/10 to colonic mucosa in vitro (bar = 5 μm); (b) transmission electron micrograph of adherence of EAEC strain 17-2 in association with vesiculation of colonic microvilli (bar = 200 nm).

been found in volunteer studies in which challenge with O42 produced mucoid stools (30).

On SEM, EAEC were observed adhering to both the mucosal surface and mucus exuded from goblet cells. However, on TEM, most bacteria were associated with mucus above the mucosa and few were found in close association with the mucosal surface. This apparent discrepancy may be explained by the aggregative nature of adherence seen on SEM. EAEC

adhered in large groups which obscured the view of the underlying epithelial surface. It is possible that some EAEC cells adhered to a hidden mucus layer and others adhered directly to the mucosa.

Unlike the case for EPEC, no attachment and effacement of the enterocyte brush border was associated with EAEC in organ culture. Only two strains were found in close association with microvilli of the jejunal enterocytes (other strains were associated with mucus above the mucosa), and no damage to the underlying brush border was seen. Microvillus vesicle formation was noted on pediatric colonic specimens with adherent EAEC and has been noted previously with adult colon incubated with EAEC in organ culture (23). Loss of the microvillous surface combined with other cytotoxic effects in the colon may be particular features of EAEC pathogenesis.

Other ultrastructural changes observed on TEM, such as mitochondrial swelling and the appearance of large secondary lysosomal bodies within cells of specimens inoculated with EAEC, may indicate elevated metabolic activity within the tissue (14); however, no tertiary lysosomes containing organelle fragments were seen, suggesting that there was no increase in organelle breakdown.

One observation which was consistent with all tissue, cultured control, and experimental specimens was a mild degree of lamina propria edema. This appeared to be an artifact associated with the in vitro organ culture technique whereby fluid absorption continues without an intact blood capillary supply.

All of the EAEC strains described here (except EAEC strain 17-2) were previously examined for adhesion to formalin-fixed small- and large-intestinal tissue from children (16). Although the use of fixed tissue and in vitro organ culture has demonstrated that EAEC can adhere to many regions of the gastrointestinal tract in children, discrepancies exist between the EAEC strain pattern of adhesion to different intestinal regions between the two methods. The use of formalin-fixed tissue allows the study of initial bacterial adhesion, and the nature of fixation preserves carbohydrate receptors. However, surface mucus is also fixed, and as in vitro organ culture has demonstrated, adhesion to mucus may be an important feature of EAEC pathogenesis. Fixation may alter mucus binding sites and the protein domains of mucosal receptors which may be important in EAEC adhesion. In vitro organ culture is advantageous in allowing more complex interactions between unfixed tissue and EAEC to be observed.

Thus, this in vitro system has indicated that EAEC can colonize both small- and large-intestinal mucosa, and the autoaggregative nature of adhesion mirrors that seen on HEp-2 cells in culture (28). This, together with the ability to adhere to mucus, may provide a way of increasing intestinal colonization and result in high concentrations of secreted toxin at the mucosal surface. Although excess cell extrusion was noted on small-intestinal specimens, cytotoxic changes were particularly evident with colonic mucosa. This could be explained by coexpression of an enterotoxin, acting on the small bowel, and a cytotoxin with activity in the large bowel. Autoaggregation, coexpression of two or more toxins, and the ability to cause microvillous vesiculation appear to be virulence characteristics contributing to the pathogenesis of EAEC infection.

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