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Ability of Enteroaggregative *Escherichia coli* Strains To Adhere In Vitro to Human Intestinal Mucosa

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A collection of 44 enteroaggregative *Escherichia coli* (EAggEC) strains isolated from infants with diarrhea in India and the United Kingdom were examined for their ability to adhere in vitro to human intestinal mucosa and by electron microscopy for production of putative adherence factors. None of the strains adhered to human duodenal mucosa, and six strains tested did not adhere to ileal mucosa; all 44 strains, however, adhered to human colonic mucosa in localized aggregates. Electron microscopy of infected colonic mucosa indicated fimbrially mediated adhesion of the EAggEC strains. Four morphologically distinct kinds of fimbriae, including a new morphological type of *E. coli* fimbriae consisting of bundles of fine filaments, were identified among the EAggEC strains; this new type of fimbria was observed in 43 of the 44 EAggEC strains. Forty-three of the 44 EAggEC strains were positive with a DNA probe developed to identify EAggEC, and most of the strains belonged to serotypes unrelated to the other major classes of diarrheic *E. coli*. These results suggest that EAggEC may be a large-bowel pathogen and colonize the colon by a fimbrially mediated adhesion mechanism.

Four major classes of *Escherichia coli* are recognized as causes of diarrheal disease: enteropathogenic (EPEC), enterotoxigenic (ETEC), enteroinvasive (EIEC), and enterohemorrhagic (EHEC) (16). The use of tissue culture (HeLa and HEp-2) cell adhesion assays has led to the identification of further putative classes of diarrheic *E. coli*. Three distinct patterns of adhesion, termed localized, diffuse, and aggregative, have been described previously (19). Localized, diffuse, and aggregative adherence patterns are characterized by localized microcolonies of adherent bacteria, by diffuse adherence of individual bacteria and by characteristic aggregates of bacteria frequently giving a "stacked-brick" appearance, respectively (19). Localized adhesion is characteristic of attaching and effacing EPEC strains (10, 13); *E. coli* strains exhibiting the diffuse (diffuse adhering *E. coli*) and aggregative (enteroaggregative *E. coli*) adherence patterns are two new classes of adherent *E. coli* which have been associated with diarrhea (7, 25). Enteroaggregative *E. coli* (EAggEC) strains may be especially important because of their epidemiological association with persistent diarrhea in infants in developing countries (3, 5).

Characterization of a collection of EAggEC strains (originally called enteroadherent-aggregative *E. coli*) from Chile showed them to be negative in tests for ETEC, EPEC, EIEC, and EHEC, nor did they fit these categories by serotyping. Many of the strains produced mannose-resistant fimbriae, and preliminary data suggested fimbrially mediated adhesion of EAggEC to rabbit mucosa. EAggEC caused characteristic lesions in rabbit and rat ileal loops, suggesting toxin involvement (25). A putative heat-stable enterotoxin produced by EAggEC has since been described (22). A 1-kb fragment from a plasmid of one EAggEC strain (17-2) was found to be a highly specific DNA probe for identifying EAggEC (2).

There is little information about human enteroadherence properties of EAggEC. Enteroadherence of an EAggEC strain (O127a:H2) isolated from a child with diarrhea in Thailand has been examined in native and formalin-fixed mucosa. This one strain showed little adhesion to small-bowel mucosa but good adhesion to formalin-fixed large-bowel mucosa (26). In this paper, we report human enteroadherence properties of a collection of 44 EAggEC strains isolated from infants with diarrhea in India and the United Kingdom.

MATERIALS AND METHODS

Bacterial strains. Forty-four *E. coli* strains isolated from infants with acute and persistent diarrhea in India (3) and in the United Kingdom (15) which showed aggregative adhesion to HEp-2 cells were examined. Also included in the study were strain 221(O92:H33), which displays aggregative adhesion and has been shown to cause diarrhea in human volunteers (18), and strain 17-2(O3:H2), from which the EAggEC DNA probe was prepared (2). For adhesion and electron microscopic studies, stock cultures of the strains were subcultured into Mueller-Hinton broth and incubated aerobically for 18 h at 37°C.

Serotyping. O and H antigens were determined by standard agglutination methods (8).

EAggEC probe tests. The EAggEC probe was that described by Baudry et al. (2). Strains were tested by colony hybridization as described by Maniatis et al. (17).

Hemagglutination tests. The strains were tested for agglutination of bovine, rat, and human type O erythrocytes in the presence of 0.5% mannose and for agglutination of guinea pig erythrocytes in the presence and absence of mannose. For the tests, the strains were subcultured twice in Mueller-

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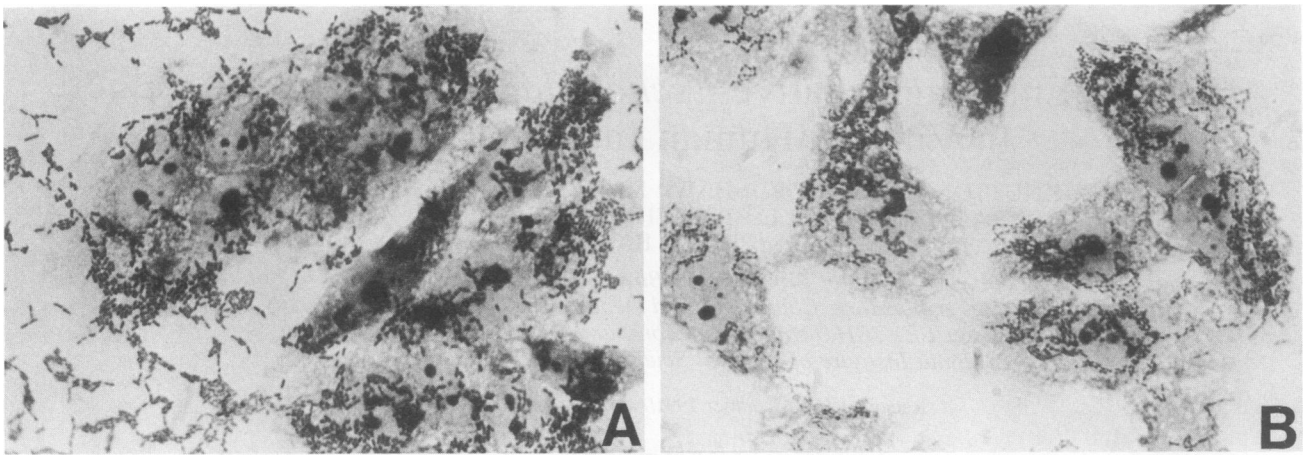

FIG. 1. HEp-2 cell adhesion assay preparations showing aggregative adhesion of bacteria to cells and background (A) and to cells only (B). Magnification, ×500.

Hinton or nutrient broth (Difco) and incubated statically each time for 48 h (23).

Tissue culture cell adhesion. Adhesion to HEp-2 cells was tested according to the method of Cravioto et al. (4) by using 3- and 6-h incubations of cells and bacteria. An identical protocol was used to assess Caco-2 cell adhesion although, in this case, adhesion was assessed by scanning electron microscopy.

Adhesion to human intestinal mucosa. Normal duodenal, ileal, and colonic mucosal biopsies obtained with informed consent from adults were maintained in organ culture and infected with EAggEC for 8 h as previously described (13). Mucosal adhesion was assessed by scanning electron microscopy (13).

Electron microscopy. Strains were monitored for fimbria production by negative-staining electron microscopy (11). Biopsies with adherent bacteria were processed for transmission electron microscopy by standard methods as previously described (11).

RESULTS

Aggregative adhesion. The 44 strains examined in the study were selected because they displayed the characteristic aggregative pattern of HEp-2-cell adherence as defined by Nataro et al. (19). Nineteen of the 44 strains showed aggregation of bacteria and the formation of a characteristic stacked-brick pattern both on the surface of and between HEp-2 cells (Fig. 1A); the remaining 25 strains showed aggregative adhesion to HEp-2 cells only (Fig. 1B). In general, aggregative adhesion of strains to cells only or to cells plus background was serotype specific (Table 1). Several strains were rejected from the study because they displayed aggregative adhesion to the background between cells but showed no cell adhesion. Seven of the Indian strains (F246A, F510A, F278A, F23A, F54A, F55B, and H505B) were to varying degrees cytotoxic for HEp-2 cells in a 3-h incubation assay; i.e., they caused rounding and death of cells.

All 44 strains exhibited aggregative adhesion to the brush border surface of differentiated Caco-2 cells as assessed by scanning electron microscopy (Fig. 2).

Serotypes. The serotypes of the EAggEC strains are shown in Table 1. Three serotypes (O51:H11, O77:H18, and O92:H33) accounted for 45% of the strains. Eight of the 44 strains (18%) were O-antigen untypeable.

EAggEC probe. Forty-three of the 44 strains were positive with the EAggEC DNA probe, the exception being strain F51A (Table 1).

Hemagglutination. Approximately half of the strains showed mannose-sensitive hemagglutination (MSHA), whereas all but 1 of the 44 strains (F417B) showed mannose-resistant hemagglutination (MRHA) of one or more of the four species of erythrocyte tested (Table 1); strains of the same serotype generally showed the same pattern of MRHA (data not shown).

Enterocyte adhesion. Isolated enterocyte adhesion assays (12), which we would normally use to screen collections of strains for enteroadherence properties, were unsuitable for studies of EAggEC adherence because the autoaggregative property of these strains made it impossible to separate enterocytes from aggregates of nonadherent bacteria. We therefore used cultured intestinal mucosa and assessed adhesion by scanning electron microscopy. None of the strains adhered to cultured duodenal mucosa (Table 1), whereas all

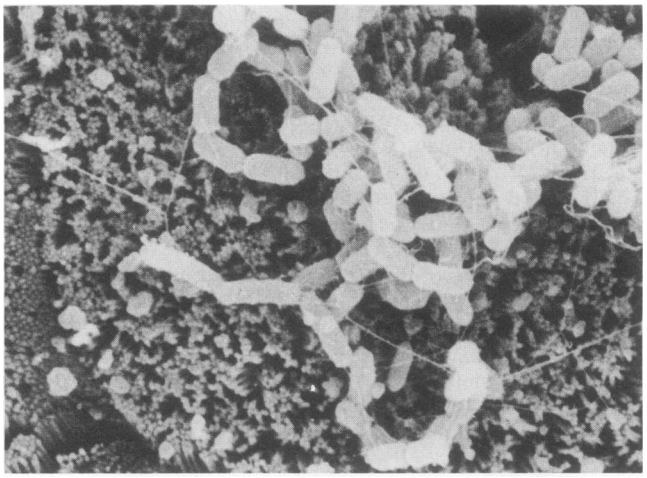

FIG. 2. Scanning electron micrograph showing aggregative adhesion of bacteria to the apical brush border surface of Caco-2 cells. Magnification, ×5,000.

TABLE 1. Characteristics of EAggEC strains

Strain	Serotype	Origin	HEp-2 cell adhesion[a]	MRHA[b]	MSHA[c]	Intestinal adhesion[d]			Fimbrial type[e]:				EAggEC probe
						Duodenum	Ileum	Colon	HR	R	F	FB	
F246A	O4:H7	India	AA[f,g]	+	+	−	ND[h]	+				+	+
F510A	O7:H-	India	AA[f,g]	+	−	−	ND	+				+	+
F417B	O9ab:H18	India	AA[f]	−	−	−	ND	+				+	+
F476A	O15:H-	India	AA[f]	+	−	−	ND	+				+	+
F27A	O51:H11	India	AA[f]	+	+	−	ND	+	+		+	+	+
F63II	O51:H11	India	AA[f]	+	+	−	−	+	+			+	+
F278A	O51:H11	India	AA[f,g]	+	+	−	ND	+	+			+	+
F356B	O51:H11	India	AA[f]	+	+	−	ND	+			+	+	+
F480A	O51:H11	India	AA[f]	+	+	−	ND	+	+		+	+	+
H769C	O51:H11	India	AA	+	+	−	ND	+	+		+	+	+
H766C	O59:H-	India	AA	+	−	−	ND	+	+		+	+	+
F6A	O77:H18	India	AA[f]	+	−	−	ND	+	+			+	+
F9I	O77:H18	India	AA[f]	+	−	−	−	+				+	+
F15A	O77:H18	India	AA[f]	+	−	−	ND	+				+	+
F17A	O77:H18	India	AA[f]	+	−	−	ND	+				+	+
F23A	O77:H18	India	AA[f,g]	+	−	−	ND	+	+			+	+
F25A	O77:H18	India	AA[f]	+	−	−	ND	+				+	+
F26A	O77:H18	India	AA[f]	+	−	−	ND	+				+	+
F28A	O77:H18	India	AA[f]	+	−	−	ND	+			+	+	+
F38A	O77:H18	India	AA[f]	+	−	−	ND	+			+	+	+
F39A	O77:H18	India	AA[f]	+	−	−	ND	+			+	+	+
F54A	O77:H18	India	AA[f,g]	+	−	−	ND	+			+	+	+
F482A	O86:H6	India	AA[f]	+	+	−	ND	+	+		+	+	+
F574A	O86:H18	India	AA[f]	+	−	−	ND	+			+	+	+
F425B	O91:H1	India	AA[f]	+	+	−	ND	+	+		+	+	+
F51A	O92:H33	India	AA	+	+	−	ND	+	+		+	−	−
F55B	O92:H33	India	AA[g]	+	+	−	−	+			+	+	+
F579A	O106:H24	India	AA	+	+	−	ND	+	+	+	+	+	+
H520C	O?:H-	India	AA	+	+	−	ND	+	+	+	+	+	+
H769A	O?:H-	India	AA	+	−	−	−	+	+	+	+	+	+
H769B	O?:H-	India	AA	+	−	−	ND	+	+	+	+	+	+
F597A	O?:H7	India	AA	+	−	−	−	+			+	+	+
H505A	O?:H21	India	AA	+	−	−	ND	+			+	+	+
H505B	O?:H21	India	AA[g]	+	−	−	ND	+			+	+	+
F535A	O?:H30	India	AA[f]	+	+	−	ND	+	+		+	+	+
H505C	OR:H21	India	AA[f]	+	−	−	−	+			+	+	+
3836	O21:H2	UK[i]	AA	+	+	−	ND	+	+	+	+	+	+
AN11/3	O55:H4	UK	AA	+	−	−	ND	+	+		−	+	+
3863	O92:H33	UK	AA	+	+	−	ND	+	+		+	+	+
3950	O92:H33	UK	AA	+	+	−	ND	+	+		+	+	+
LC9/6	O111ac:H21	UK	AA	+	+	−	ND	+	+		+	+	+
3864	O126:H-	UK	AA	+	+	−	ND	+	+	+	+	+	+
WJ19/10	O126:H27	UK	AA	+	+	−	ND	+			+	+	+
3865	O?:H33	UK	AA	+	−	−	ND	+		+	+	+	+
17-2	O3:H2	Chile	AA	+	+	−	ND	+	+		+	+	+
221	O92:H33	Mexico	AA	+	+	−	ND	+	+		+	+	+

[a] AA, aggregate adhesion to cells and between cells.
[b] MRHA to one or more of human, bovine, rat or guinea pig erythrocytes.
[c] MSHA to guinea pig erythrocytes.
[d] Adhesion to cultured human intestinal mucosa assessed by scanning electron microscopy.
[e] Fimbrial type assessed by electron microscopy: HR, hollow rod; R, rod; F, fine fibrillar; FB, fine fibrillar bundles.
[f] Aggregate adhesion to cells only.
[g] Strains cytotoxic to HEp-2 cells.
[h] ND, not determined.
[i] UK, United Kingdom.

44 EAggEC strains adhered to colonic mucosa (Fig. 3; Table 1). There was variation in the level of adhesion from strain to strain (Fig. 3), although all strains showed significant levels of adhesion. Bacteria adhered in localized aggregates to the luminal, but not crypt, mucosa, and there was no apparent mucosal damage caused by adherent bacteria. Because of the limited availability of human ileal tissue, it was not possible to assess adhesion of all 44 strains to this tissue. However, six strains that showed good adhesion to colonic mucosa did not adhere to ileal mucosa obtained from one donor (Table 1).

Presence of fimbriae. Four morphologically distinct kinds of fimbriae were identified among the 44 strains: (i) 6- to 7-nm-diameter hollow cylindrical rodlike fimbriae (Fig. 4A), (ii) 5- to 6-nm-diameter rodlike fimbriae (Fig. 4B), (iii) 2- to 3-nm-diameter fibrillar fimbriae (Fig. 4C), and (iv) bundles of fine 2- to 3-nm-diameter fibrils (Fig. 4D) (Table 1). These characteristic bundles of fine filaments were observed in 43

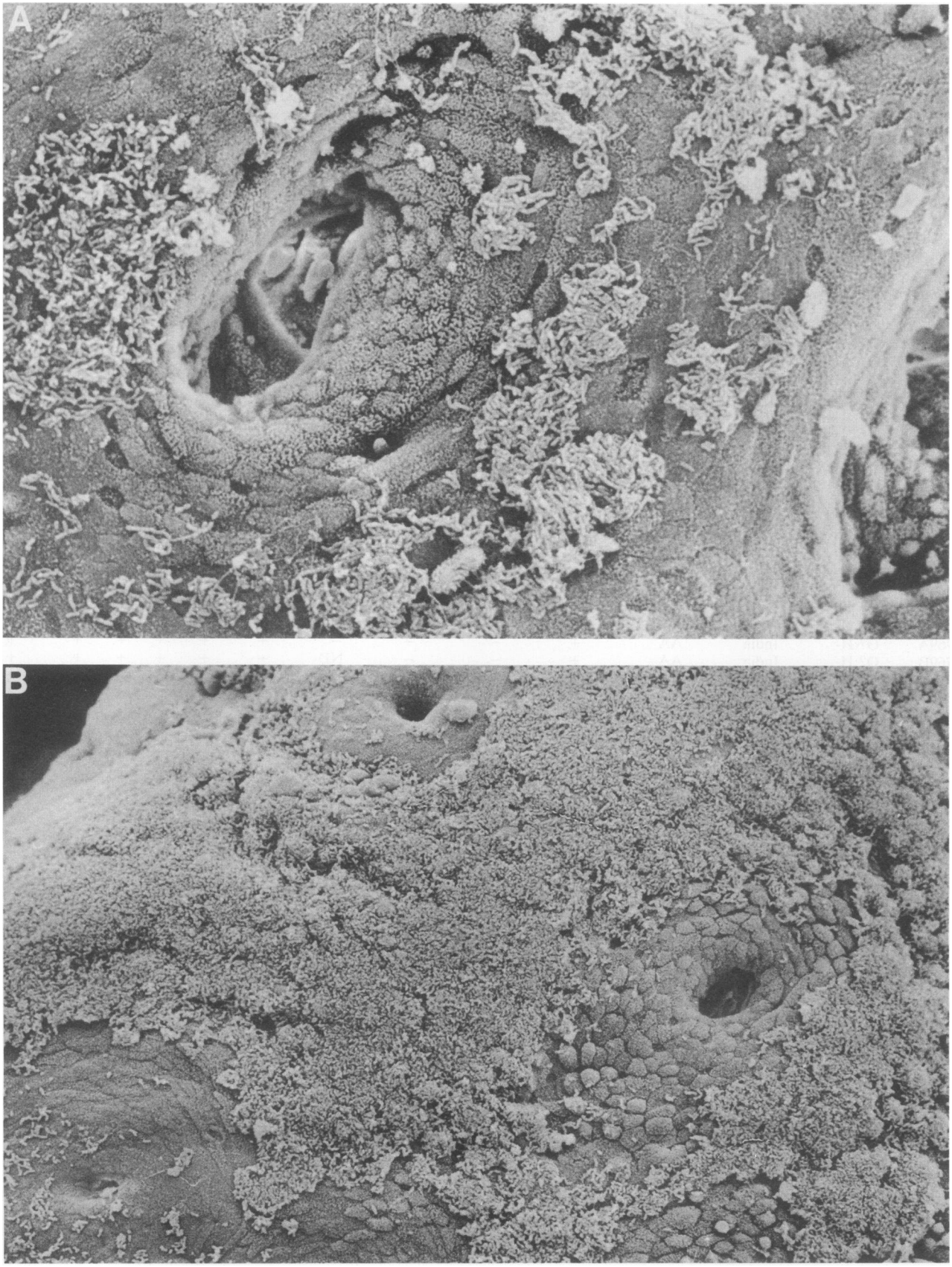

FIG. 3. Scanning electron micrographs showing typical adhesion of EAggEC strains to cultured human colonic mucosa. Bacteria adhered in localized aggregates to luminal but not crypt mucosa (A). After 8 h, some strains showed virtually confluent colonization of the mucosal surface except for distinct areas around the crypts (B). Magnifications, ×1,000 (A) and ×500 (B).

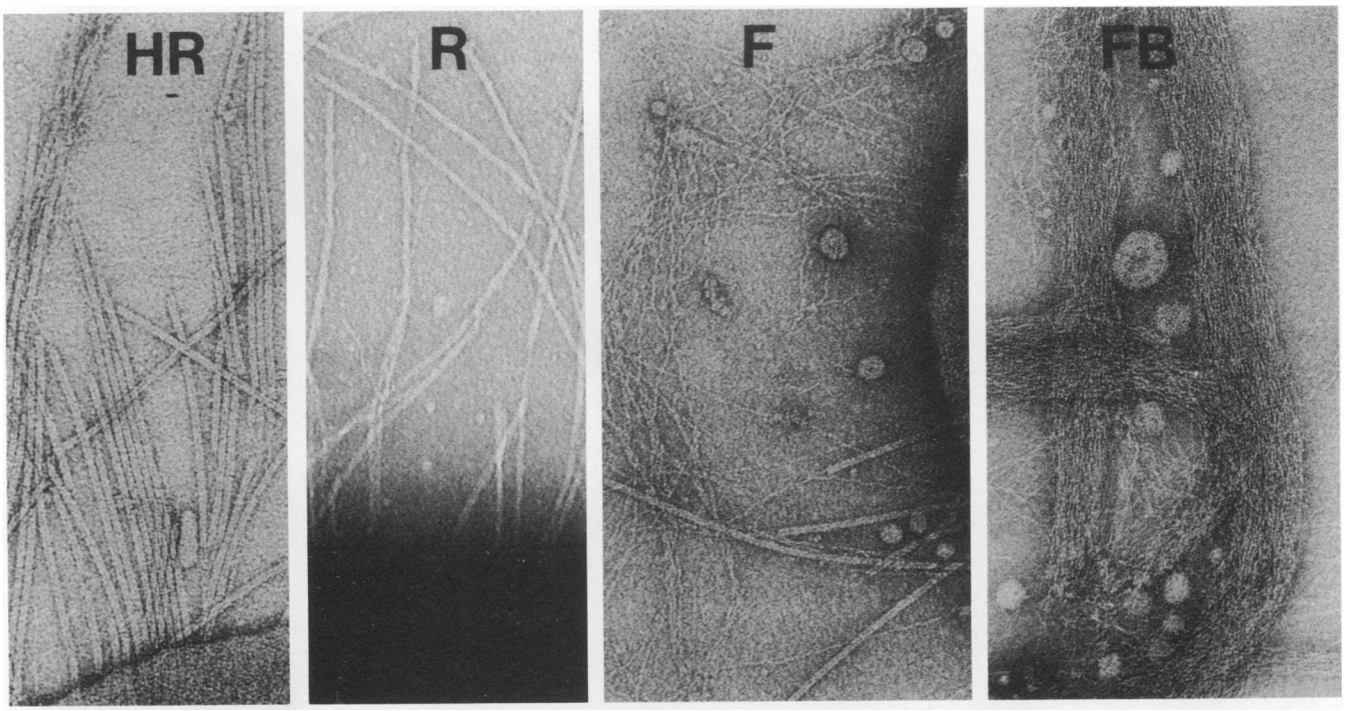

FIG. 4. Electron micrographs showing the four morphologically distinct types of fimbriae produced by the EAggEC strains: HR, hollow rod; R, rod; F, fibrillar; FB, fibrillar bundles. Magnification, ×150,000.

of 44 EAggEC strains, and 20 of the 44 strains (45%) were the sole fimbrial type detected (Fig. 5A). Bacteria produced one or a small number of filament bundles per cell, and at high magnification, a 4- to 4.5-nm repeating periodicity was frequently seen along the length of the bundles (Fig. 5B). Fibrillar bundles were also produced by strains 221 and 17-2 (Table 1).

Mechanism of adhesion. By electron microscopy, fimbrially mediated adhesion of bacteria is generally characterized by the presence of a distinct space between the bacterium and cell surface. The presence of this distinct space was the case with several EAggEC strains examined. When fimbriae of strain H769A were visualized by ruthenium red staining (11), rodlike fimbriae produced by this strain appeared to be mediating attachment of bacteria to HEp-2 (Fig. 6A) and Caco-2 (Fig. 6B) cell surfaces. Fibrillar surface structures produced by strain H505C also appeared to promote attachment of this strain to human colonic mucosa, although these structures were too indistinct to be confirmed as the fibrillar bundles produced by this strain and as seen by negative staining (Fig. 6C).

DISCUSSION

The fact that 43 of the 44 strains designated as EAggEC on the basis of tissue culture cell adhesion were positive with a DNA probe developed to identify EAggEC (2) not only confirms the accuracy of HEp-2 cell adhesion assays using the Cravioto protocol (4, 26) but also confirms the excellent sensitivity of this probe for identifying *E. coli* with the aggregative adherence property. It is not clear why strain F51A was EAggEC-probe negative since this strain also showed a clearly defined aggregative pattern of adhesion to HEp-2 cells, belongs to a serotype characteristic of other EAggEC, and possesses adherence properties and fimbriae characteristic of other EAggEC strains. It should be pointed out, however, that the EAggEC probe does not necessarily code for adhesive properties.

In contrast to the Chilean study (25), most of the Indian and United Kingdom EAggEC strains were serotypeable. Three O:H serotypes accounted for nearly 50% of the strains, suggesting that EAggEC, like the other classes of diarrheic *E. coli*, may be restricted to a limited number of specific *E. coli* serotypes. H antigen type 33 was common among the Chilean EAggEC (25) although these strains were O-antigen untypeable. Five H33-type EAggEC strains were identified in this study, and four belonged to serotype O92. Five strains belonged to serogroups O86, O111, and O126, serogroups which have been associated with infantile diarrhea and considered by some to be EPEC serogroups (21). Some strains belonging to these serogroups clearly are EPEC because they show localized adhesion to HEp-2 cells, produce the characteristic attaching and effacing intestinal lesion (13), and are positive by the fluorescence actin staining test, which is diagnostic for this lesion (10). However, the strains belonging to the O86, O111, and O126 serogroups examined in this study possessed none of the properties of EPEC and all of the properties of EAggEC and hence must be EAggEC. Scotland et al. (23) have also recently shown that strains belonging to serotypes O111:H21 and O126:H27 show aggregative adhesion and hybridize with the EAggEC probe. Pal and Ghose (20) described adherence factors in two diarrheic *E. coli* strains belonging to serogroup O86. It is quite clear from the HeLa cell adherence patterns reported in their study that the two strains in question are also EAggEC and not EPEC. Yamamoto et al. (26) examined an EAggEC strain belonging to serogroup O127. Thus O86, O111, O126, and O127 appear to be serogroups which include both EPEC and EAggEC strains.

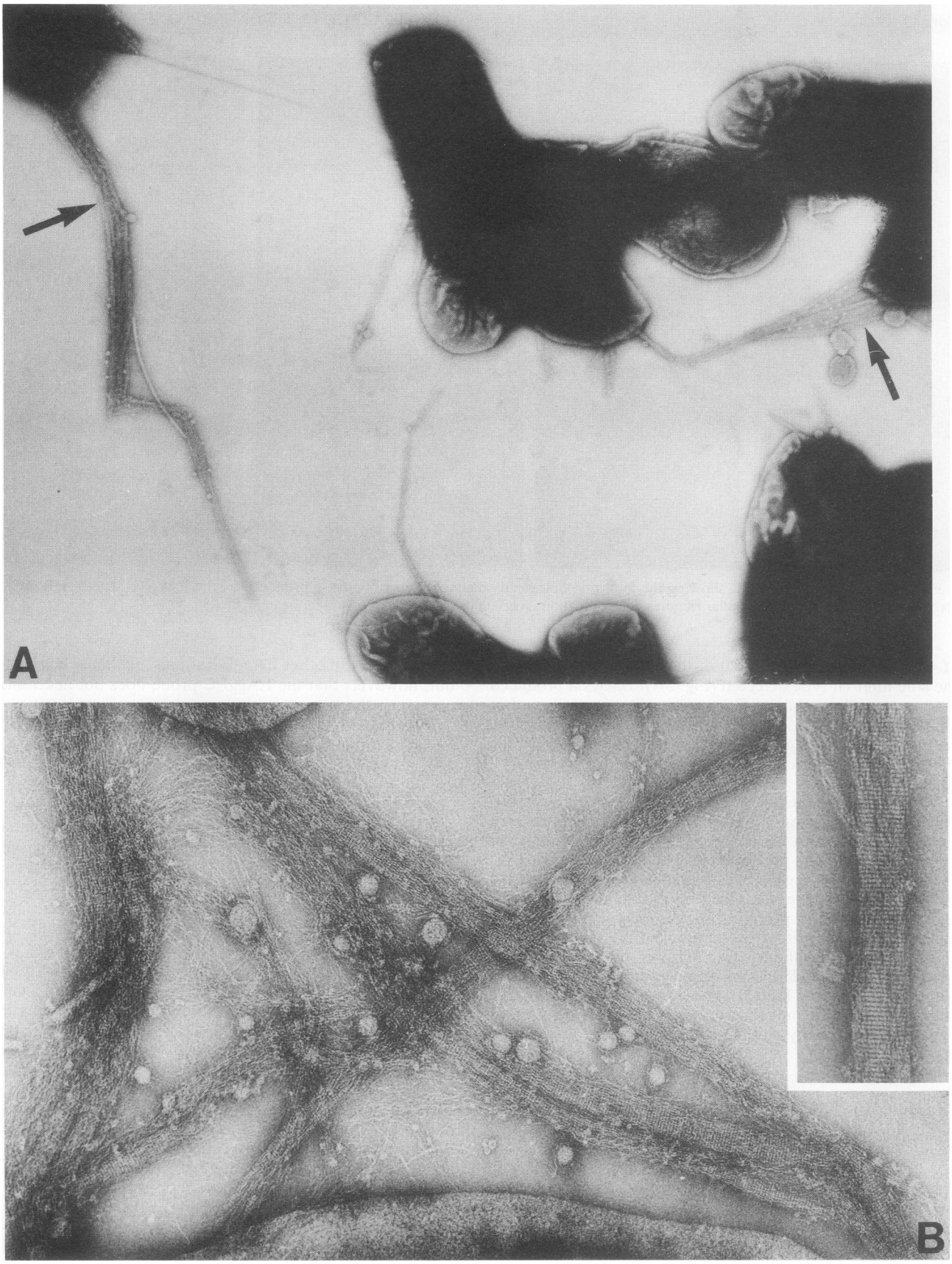

FIG. 5. Electron micrographs showing the fibrillar bundle type of fimbriae produced by all the EAggEC strains. (A) One or a small number of fibrillar bundles were produced by each EAggEC bacterium (arrows). (B) At high magnification, a regularly repeating periodicity consisting of 4- to 4.5-nm-wide striations was frequently seen along the length of the fibrillar bundles. Magnifications: ×30,000 (A), ×130,000 (B), and ×170,000 (inset [panel B]).

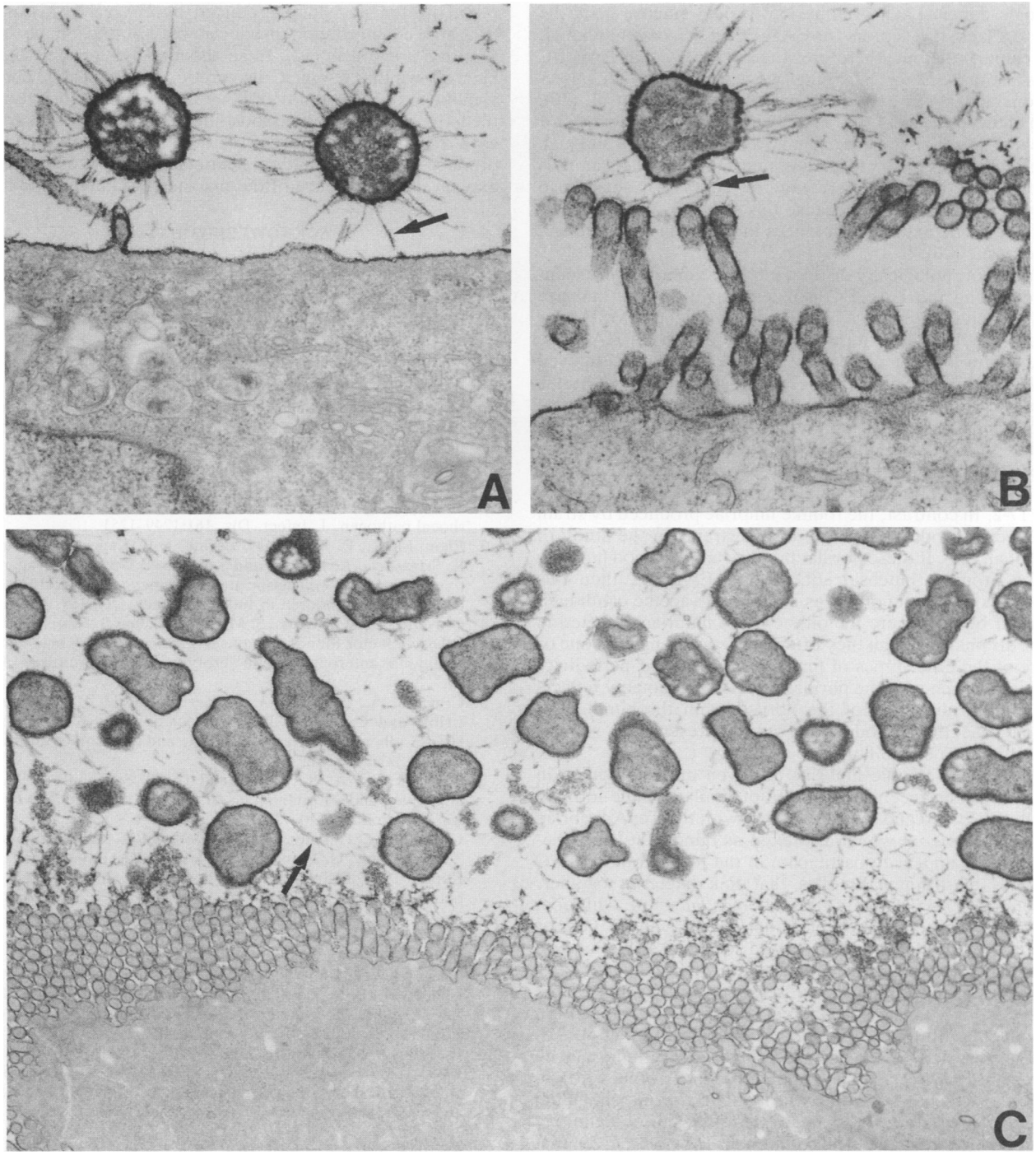

FIG. 6. Transmission electron micrographs showing rodlike fimbrially mediated adhesion of EAggEC strain H769C to HEp-2 (A) and Caco-2 (B) cells. Indistinct surface structures produced by strain H505C, possibly fibrillar bundles, appear to promote adhesion of this strain to colonic mucosa (C). Magnifications, ×30,000 (A and B) and ×18,000 (C).

The different classes of diarrheic *E. coli* colonize different parts of the gut: ETEC and EPEC colonize the small bowel, while EHEC and EIEC colonize the large bowel. The property of colonization is thought to be determined by the specificity of adherence factors for particular mucosal receptors. We have previously demonstrated these different specificities by using cultured human intestinal mucosa infected with ETEC, EPEC, EIEC, and EHEC (12–14). Adhesion of EAggEC to human large-bowel, but not small-bowel, mucosa not only shows that EAggEC possesses human enteroadherence properties but also suggests that this may be the site of colonization of this class of diarrheic *E. coli* in humans. Although we were able to test the ability of six strains to adhere to ileal mucosa, all this material came from

a single donor. Lack of adhesion to ileal mucosa could be due to a lack of receptors for EAggEC in this one individual. We cannot rule out, therefore, the possibility that EAggEC may also adhere to distal small-bowel mucosa. Our observations are consistent with those of Yamamoto et al. (26), who showed that an O127:H2 EAggEC strain exhibited low levels of adhesion to native jejunal and ileal mucosa and high levels of adhesion to formalin-fixed colonic mucosa. It is unfortunate that data were not available for adhesion to native colonic mucosa, because this study also showed that there could be marked differences in adhesion to native and fixed tissue (26).

Four morphologically distinct kinds of fimbriae were identified among the EAggEC isolates. Rigid rodlike fimbriae may be either type 1 fimbriae responsible for MSHA or putative adhesion fimbriae responsible for MRHA. The absence of such fimbriae in four strains which showed MSHA probably reflects the different culture conditions used for the hemagglutination and negative-staining studies. Of particular interest is the fibrillar type that exists as tightly packed bundles, not only because of their distinctive structure but also because this fimbrial type was observed in 43 of the 44 EAggEC strains examined, as well as in strains 221 and 17-2. In contrast, the rodlike fimbriae produced by strain 17-2 were found in only 17% of the strains in the study of Vial et al. (25). If these fibrillar bundles are adhesion fimbriae which promote mucosal adhesion of EAggEC, then this antigen could be important as a potential vaccine candidate. Fibrillar bundles were the only type of fimbriae detected in some strains, although they often occurred along with one or more of the other types of fimbriae. The regular periodicity frequently seen with the fibrillar bundles presumably reflects the regular alignment of the fibrils within the bundles, in which case the periodicity should represent the subunit repeat along a single fibril.

The fibrillar bundle fimbriae are even more intriguing in the light of the recent description of similar bundle-forming pili by EPEC (6) and their homology with the TcpA fimbriae of *Vibrio cholerae* which also exist as bundles (9). Bundle-forming pili appear to participate in the formation of EPEC colonies by forming bundles that link bacteria together. Given that EAggEC also forms colonies, albeit of a different pattern in tissue culture cell adhesion assays, the fibrillar bundles reported in this study could serve the same function and participate in the formation of EAggEC colonies.

The results of preliminary ultrastructural studies of HEp-2 and Caco-2 cells with adherent EAggEC strains are in agreement with the results of the studies by Vial et al. (25) and suggest that rodlike EAggEC fimbriae are important in adhesion. However, since EAggEC produces other types of fimbriae, which are not necessarily stained with ruthenium red, it is premature to conclude that adhesion is definitely being promoted by the rodlike fimbriae. However, given that EAggEC which adheres to human intestinal mucosa does produce several different morphological types of fimbriae, it seems likely that EAggEC strains of different serotypes, like ETEC, will produce more than one type of fimbrial adhesin.

EAggEC is associated with persistent diarrhea (3, 5). Unlike EPEC, which causes a brush border membrane lesion that could lead to persistent diarrhea, EAggEC did not appear to cause specific brush border damage, although it should be pointed out that scanning electron microscopy would not necessarily reveal damage beneath attached bacteria; preliminary transmission electron microscopy did not reveal mucosal damage. This would suggest a toxin-mediated mechanism of disease. A hemorrhagic lesion in the rabbit ligated ileal loop produced by EAggEC (25) and a heat-stable enterotoxin produced by EAggEC have been reported previously (22). In an accompanying paper (1), we have shown that EAggEC also produces a heat-labile hemolysinlike toxin. The ability of EAggEC to survive long term in the large bowel in close association with the mucosal surface as a result of specific fimbrially mediated adhesion and produce heat-labile and/or heat-stable enterotoxins could explain the persistence of the diarrhea in infected infants.

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