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Adhesion and Ultrastructural Properties of Human Enterotoxigenic *Escherichia coli* Producing Colonization Factor Antigens III and IV

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Human enterotoxigenic *Escherichia coli* (ETEC) producing colonization factor antigen III (CFA/III) and coli surface antigens 4, 5, and 6 (CS4, CS5, and CS6) of CFA/IV were examined ultrastructurally and for ability to adhere to human small intestinal enterocytes and to cultured human intestinal mucosa. Strains of serotypes O25:H−, O25:H42, and O167:H5 producing CFA/III plus CS6, CS4 plus CS6, and CS5 plus CS6, respectively, showed good adhesion to human enterocytes (1.8 to 4.2 bacteria per brush border) and cultured human intestinal mucosa, whereas variants lacking these antigens or producing only CS6 were nonadherent (0 to 0.03 bacterium per brush border). By electron microscopy, CFA/III, CS4, and CS5 appeared as morphologically distinct rodlike fimbriae: CFA/III was 7 to 8 nm in diameter, CS4 was 6 to 7 nm in diameter, and CS5 was 5 to 6 nm in diameter. CS5 was unusual in that it appeared to be composed of two fine fibrils arranged in a double-helical structure. CS6 was difficult to characterize morphologically but possibly has a very fine fibrillar structure. By specific fimbrial staining and immunoelectron microscopy, CS4 and CS5 were shown to promote mucosal adhesion of ETEC; a similar adhesion role for the CS6 antigen could not be confirmed. ETEC strains of serotypes O27:H7, O27:H20, O148:H28, and O159:H20 which produced CS6 showed good adhesion to human enterocytes (1.6 to 3.0 bacteria per brush border), whereas variants which lacked CS6 were nonadherent (0 to 0.01 bacterium per brush border). These strains, however, also produced fimbrial or fibrillar surface antigens, in addition to CS6, which probably represent additional coli surface antigens responsible for the observed adhesive properties of these ETEC serotypes.

Enterotoxigenic *Escherichia coli* (ETEC) bacteria are an important cause of infant diarrheal disease in less-developed countries (1) and of travellers' diarrhea (16). Adhesion of ETEC to the small intestinal mucosa is now recognized as an important early event in colonization and the development of diarrheal disease. Special classes of protein fimbriae which promote mucosal adhesion of ETEC have been identified in some ETEC strains of human origin (7). Termed colonization factor antigens (CFAs), these now include CFA/I (6), CFA/II (4), CFA/III (8), and CFA/IV (formerly PCF8775) (15, 21). In this report, CFA/III refers to that described by Honda et al. (8), since the CFA/III reported by Darfeuille et al. (3) was subsequently shown to be CFA/I (15). A putative human ETEC colonization factor (PCF0159:H4) has been described in ETEC serotype O159:H4 (19), and fibrillar structures which promote adhesion to human intestinal mucosa are thought to represent a colonization factor in ETEC serotype O148:H28 (11). A factor mediating attachment of strains of serogroup O27 to intestinal 407 cells has also been described (L. V. Thomas, Ph.D. thesis, C.N.A.A., London, United Kingdom, 1985).

CFA/I, CFA/III, and PCF0159 are rodlike fimbrial antigens with a diameter of 6 to 8 nm (6, 8, 19). CFA/II is heterogeneous and consists of three distinct coli surface (CS) antigens termed CS1, CS2, and CS3 (2, 17). CS1 and CS2 are morphologically similar to CFA/I, whereas CS3 consists of fine, 2- to 3-nm-diameter fibrils (12). CFA/IV also exhibits heterogeneity and currently consists of three distinct coli surface antigens, CS4, CS5, and CS6; CS4 and CS5 are rodlike fimbriae, whereas a structure has not been reported for CS6 (21, 23).

Human ETEC colonization factors are restricted to a limited number of ETEC serogroups. The prototype CFA/III strain of Honda et al. (8), which also produces CS6 (15), belongs to O25:H−. CS4 and CS6 occur together on strains of serotype O25:H42; CS5 and CS6 occur together on strains of serogroups O6, O92, O115, and O167. CS6 is also found on strains of serogroups O27, O92, O148, O153, O159, and O169 (15).

The colonizing ability of ETEC serotype O25:H42 carrying CS4 plus CS6 or CS6 only and ETEC serotype O167:H5 carrying CS5 plus CS6 has been demonstrated recently in rabbits, using the Ritard model (18). ETEC expressing CFA/IV, however, have not been examined in humans. In this study, we have examined ETEC producing CFA/III and CFA/IV for fimbrial production and for ability to adhere in vitro to human small intestinal enterocytes and to cultured human intestinal mucosa.

MATERIALS AND METHODS

Bacterial strains. The characteristics of the strains used in this study are shown in Table 1. They have been identified previously as possessing CFA/III or CFA/IV. Some of the strains had been tested for ability to adhere to intestinal 407 cells. Isolation of CS-negative variants has been described previously (15, 21). Stock cultures of strains were subcultured either onto modified Casamino Acids-yeast extract agar (CFA agar) (5) or in Mueller-Hinton broth and incubated aerobically for 18 h at 37°C.

Preparation of antisera. Antisera which reacted only against CS4, CS5, or CS6 were prepared as described previously (15). Formalin-fixed suspensions of the vaccine strains E17374A (O25:H42; CS4 and CS6) and E17018A (Table 1) were injected into rabbits intraperitoneally. The antisera were absorbed first with the mannose-resistant hemagglutination-negative variant of the same strains that

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TABLE 1. Characteristics and adhesive properties of ETEC strains

Strain	Serotype	CFA/IV component			Other putative colonization factor(s)[a]	MRHA[b]	Adhesion to human enterocytes (no. of bacteria per brush border)[c]	Adhesion to cultured human intestinal mucosa[d]
		CS4	CS5	CS6				
31-10	O25:H−			CS6	CFA/III	−	1.8	ND
E8775A	O25:H42	CS4		CS6		+	2.0	+
E8775B	O25:H42					−	0.02	−
E11881A	O25:H42	CS4		CS6		+	2.6	+
E11881C	O25:H42			CS6		−	0	−
E11881D	O25:H42					−	0.03	ND
E14341/4	O25:H42			CS6		−	0.02	−
E3135A[e]	O27:H7			CS6	2-nm fibrils	−	1.9	+
E3135B[e]	O27:H7					−	0.01	ND
E24133A[e]	O27:H20			CS6	4-nm fimbriae	−	1.0	ND
E24133B[e]	O27:H20					−	0	ND
E32018A[e]	O27:H20			CS6	2-nm fimbrils	−	2.3	ND
E32018B[e]	O27:H20					−	0	ND
E519/66A	O148:H28			CS6	3-nm fimbrils	−	2.4	ND
E519/66B	O148:H28					−	0	ND
E25117A	O148:H28			CS6	Fine fibrils	−	2.3	ND
E25117B	O148:H28					−	0	ND
E18519A	O159:H20			CS6	2-nm fimbrils	−	3.0	ND
E18519B	O159:H20					−	0	ND
E17018A	O167:H5		CS5	CS6		+	4.2	+
E17018B	O167:H5					−	0	−

[a] Detected by negative stain electron microscopy; CFA/III is an accepted ETEC colonization factor (8).
[b] MRHA, Mannose-resistant hemagglution of human erythrocytes.
[c] Data for bacteria grown on CFA agar; mean of at least two determinations. <0.05, Nonadherent; >0.5, adherent (10).
[d] Assessed by scanning electron microscopy. ND, Not determined.
[e] Strains E3135A and E32018A adhere to intestinal 407 cells; E3135B, E24133A and B, and E32018B did not adhere (Scotland, personal communication).

lacked CS4 plus CS6 or CS5 plus CS6, strains E17374B and E17018B, respectively. These antisera were reabsorbed with a CS6-producing strain of a different serotype (E17018A or E17374A) to give antisera that reacted only against CS4 or CS5. An antiserum for CS6 was raised against E11881C, a derivative of a strain of serotype O25:H42 which had lost the ability to produce CS4 (Table 1). The antiserum was absorbed with the CS6-negative strain E11881D (Table 1) to make it specific for CS6. An antiserum specific for CFA/III was produced by raising an antiserum to vaccine strain 31-10 (Table 1) and absorbing with a negative variant of the same strain that produced CS6 but lacked CFA/III (31-10D).

Hemagglutination. Mannose-sensitive and mannose-resistant hemagglutination was tested at room temperature on rocked glass slides, using equal volumes of a bacterial suspension (~10^9/ml) and a washed 3% suspension of human group A erythrocytes with or without 1% D-mannose.

Enterocyte adhesion. Brush border adhesion of ETEC was assessed as described previously (10). Enterocytes were isolated from human duodenal mucosal biopsies, using an EDTA chelation method, and suspended in modified Eagle medium containing 0.5% D-mannose at a concentration of ~10^6/ml. For each adhesion assay, 1 ml of the enterocyte suspension was added to 2 ml of a washed suspension of ~10^9 or ~10^6 bacteria per ml in a Bijoux bottle, and the contents were incubated for 1 or 3 h, respectively, on a rotary mixer at 37°C. Nonadherent bacteria were removed by washing, and adhesion was quantitatively assessed by counting bacteria adhering to the brush borders of 50 enterocytes selected at random (10).

Adhesion to intestinal mucosa. Human duodenal mucosal biopsies were maintained in culture as described previously (11). For adhesion studies, a bacterial suspension was added to the tissue culture medium (NCTC 135-Dulbecco modified Eagle medium [1:1] plus 10% newborn calf serum) and culturing was continued for up to 12 h at 37°C. At the end of the incubation period, biopsy samples were thoroughly washed prior to fixation for microscopy.

Electron microscopy. For negative staining of bacteria, a 10-μl sample of a washed bacterial suspension was mixed with an equal volume of ammonium molybdate (2%; pH 7). A 10-μl portion was applied to carbon-coated grids for 2 min, and the excess liquid was removed with filter paper.

Immunolabeling and electron microscopy of CFA/III, CS4, CS5, and CS6 were carried out as described previously (12). A 10-μl amount of a washed bacterial suspension was applied to a carbon-coated grid for 2 min, excess liquid was removed, and the grid was immediately placed face down on a drop of a suitable dilution of antiserum for 15 min. After washing, grids were placed on a drop of gold-labeled goat anti-rabbit serum for 15 min. After a further washing, grids were negatively stained with 1% ammonium molybdate.

Immunogold labeling of bacteria adhering to human intestinal mucosa was carried out as described previously (10). Biopsy samples were briefly fixed in 0.1% glutaraldehyde, washed, and incubated with a suitable dilution of antiserum for 1 h at 4°C. Tissue was washed and incubated with gold-labeled goat anti-rabbit serum for 12 h at 4°C. After a further thorough washing, tissue was fixed in 3% buffered glutaraldehyde and processed for thin-section electron microscopy, using standard procedures.

Ruthenium red staining of bacterial fimbriae was performed by adding ruthenium red (0.075%) to fixative and wash solutions during the processing of biopsy samples for thin-section electron microscopy (9). Tissue sections and negative stain specimens were examined in a Philips 301 electron microscope.

For scanning electron microscopy, fixed biopsy samples

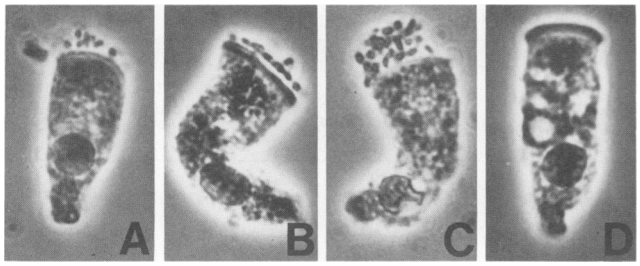

FIG. 1. Phase-contrast micographs illustrating adhesion of ETEC strains 31-10 (CFA/III plus CS6) (A) and E8775A (CS4 plus CS6) (B) grown on CFA agar and E17018A (CS5 plus CS6) grown in Mueller-Hinton broth (C) to isolated human duodenal enterocytes. CS6-only strain E11881C grown on CFA agar was nonadherent (D). Magnification, ×800.

were dehydrated and critical-point dried. Mounted samples were coated with gold and examined in a Hitachi S-2300 scanning electron microscope.

RESULTS

Enterocyte adhesion. The ability of ETEC strains grown on CFA agar to adhere to the brush border of isolated human duodenal enterocytes is shown in Table 1 and illustrated in Fig. 1. ETEC grown on CFA agar and producing CFA/III plus CS6, CS4 plus CS6, and CS5 plus CS6 all showed good adhesion (mean, 1.8 to 4.2 bacteria per brush border), whereas variants which lacked CS4 plus CS6 and CS5 plus CS6 were nonadherent (0 to 0.03 bacterium per brush border). There was some variability in the expression of CS4 and CS5 antigens when bacteria were grown on CFA agar, but good adhesion always correlated with good expression of these antigens, indicated by mannose-resistant hemagglutination or electron microscopic examination or both. Good adhesion and expression of CS4 by strain E11881A and of CS5 by strain E17018A were also observed when assays were carried out with broth cultures of bacteria (mean, 2.7 and 3.0 bacteria per brush border, respectively). Under these conditions, adhesion of bacteria to each other as well as to the enterocyte brush border surface was routinely observed (Fig. 1C); this probably reflects the additional production of type 1 fimbriae by bacteria grown in broth culture. The other CS4-carrying strain (E8775) did not express CS4 when grown in broth culture and was nonadherent (0 bacteria per brush border). CS6-only variants of CS4-plus-CS6 strains E11881A and E14341/4 were also nonadherent (0 and 0.02 bacterium per brush border).

ETEC strains of serotypes O27:H7, O27:H20, O148:H28, and O159:H20 which produced CS6 showed good enterocyte adhesion (mean, 1 to 3 bacteria per brush border), whereas variants which lacked CS6 were nonadherent (0 to 0.01 bacterium per brush border) (Table 1).

Good mucosal adhesion of strains producing CS4 plus CS6 and CS5 plus CS6 was observed when duodenal mucosal biopsies were cultured with ETEC strains for up to 10 h and examined histologically or by scanning electron microscopy (Table 1; Fig. 2).

ETEC ultrastructure. To understand why some CS6-carrying strains were adherent and others were nonadherent, we examined all strains by negative staining and by immunoelectron microscopy for expression of CFA/III, CS4, CS5, CS6, and other putative colonization factors. Electron microscopy was performed on bacteria taken from the enterocyte adhesion and mucosal adhesion assays (i.e., bacteria grown in tissue culture medium) since these were conditions under which bacteria were known to be adherent and thus expressing adhesion factors.

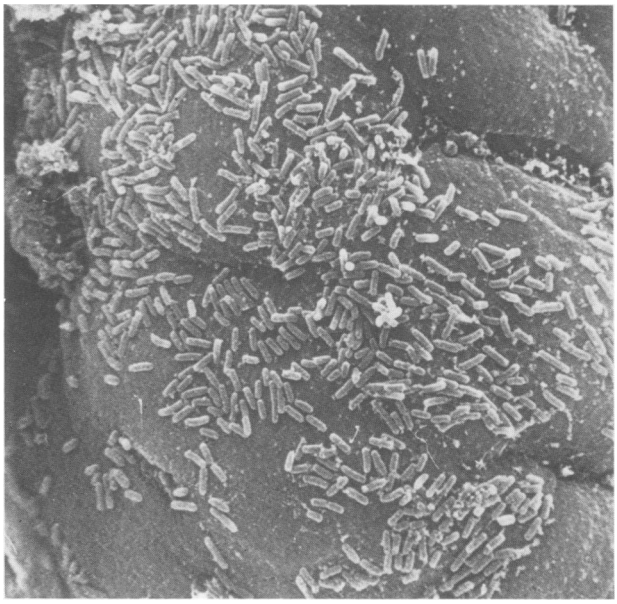

FIG. 2. Scanning electron micrograph showing in vitro adhesion to cultured human duodenal mucosa of ETEC strain E17018A producing CS5+CS6. Magnification, ×1,750.

Cultures of the CFA/III-plus-CS6-carrying strain 31-10 produced 7- to 8-nm-diameter rodlike fimbriae (Fig. 3A) which were specifically labeled with the CFA/III antiserum (data not shown). CFA/III fimbriae were somewhat flexible and frequently seen folded back on themselves. Strains E8775 and E11881A produced 6- to 7-nm-diameter, rigid, rodlike fimbriae (Fig. 3B) which were specifically labeled with the CS4 antiserum (Fig. 3C).

Rodlike fimbriae of ~7 and 5 to 6 nm in diameter were produced by strain E17018A, and these were sometimes coexpressed on the same bacterium (Fig. 4A); most bacteria, however, produced only the 5- to 6-nm-diameter fimbriae (Fig. 4B), and these fimbriae were labeled specifically with the CS5 antiserum (Fig. 4C). On the basis of mannose-sensitive and mannose-resistant hemagglutination titers, the ~7-nm rodlike fimbriae were concluded to be type 1 fimbriae. The structure of the CS5 fimbriae was unusual in that the diameter varied periodically along their length. At high magnification, CS5 fimbriae were seen to have a clearly defined helical structure and appeared to be composed of two fine fibrils arranged in a double helix. The criss-cross pattern of the two strands comprising the double helix is most clearly seen by viewing micrographs obliquely along the length of fimbriae (Fig. 4D). The apparent splitting of CS5 fimbriae into the two-component fine fibrils was also occasionally observed (Fig. 4E).

All of the CFA/III-, CS4-, and CS5-carrying ETEC which also carried CS6 plus variants E11881C and E14341/4 (which carried only CS6) were observed to express CS6 when examined by immunoelectron microscopy, using the CS6 antiserum (Fig. 5A and B). However, no surface fimbriae or fibrillar structures which correlated directly with expression of CS6 were routinely identified by negative staining. Very fine fibrils were sometimes seen projecting from the surface of bacteria expressing CS6 (Fig. 5C and D), but this was not

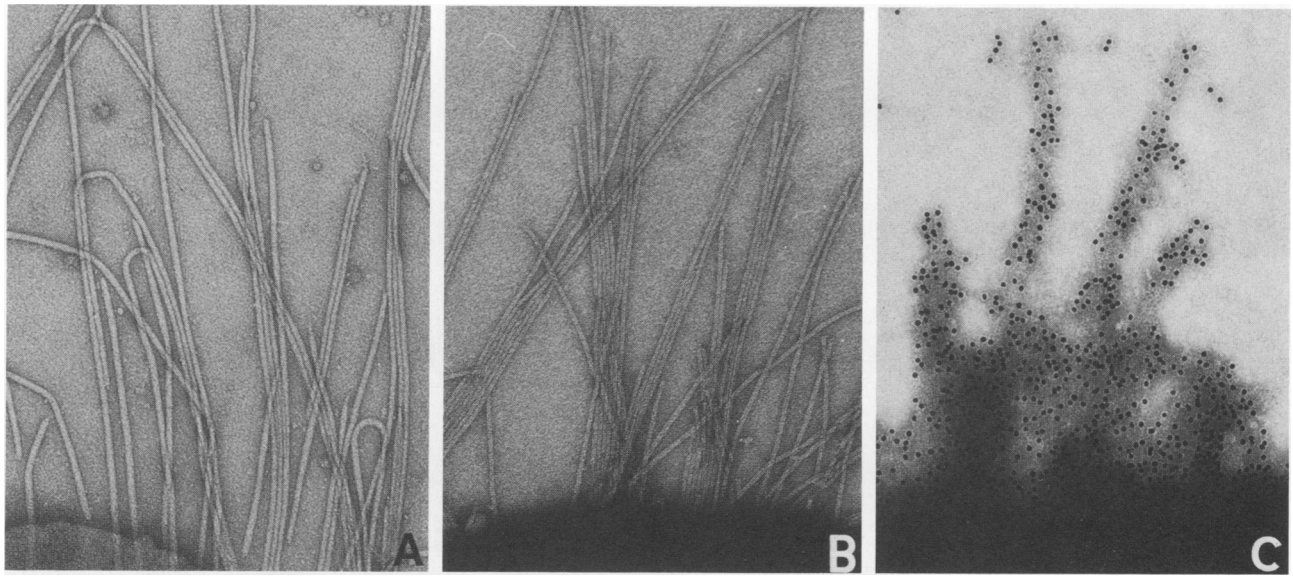

FIG. 3. Negative stain electron micrographs showing 7- to 8- and 6- to 7-nm-diameter fimbriae produced by strains carrying CFA/III plus CS6 (31-10) (A) and CS4 plus CS6 (E11881A) (B). The 6- to 7-nm fimbriae shown in panel B were specifically labeled with CS4 antiserum (C). Magnification: A and B, ×100,000; C, ×70,000.

a consistent observation with all CS6-producing strains. Such fibrils were not, however, seen at the surface of CS6-negative strains.

To identify which CS antigens of CFA/IV were promoting brush border adhesion, mucosal biopsies infected with ETEC strains producing CS4 plus CS6 and CS5 plus CS6 were examined following staining of CS4 and CS5 fimbriae with ruthenium red and labeling of CS4, CS5, and CS6 antigens with gold-labeled antibody. Brush border adhesion promoted by CS4 fimbriae of strain E11881A (not shown) and CS5 fimbriae of strain E17018A (Fig. 6A, arrows) was clearly apparent following visualization of these fimbriae by ruthenium red staining. Similarly, CS4- and CS5-mediated brush border adhesion was observed following staining of CS4 (Fig. 6B, arrows) and CS5 fimbriae with gold-labeled antibody. The same strains labeled with CS6 antiserum revealed only small amounts of CS6 antigen, and there was no apparent binding of CS6 to the brush border membrane (Fig. 6C, arrows).

By immunoelectron microscopy, strains of serogroups O27:H7, O27:H20, O148:H28, and O159:H20 all expressed CS6. However, additional fimbrial and fibrillar surface structures produced by these strains were revealed by negative stain electron microscopy. Strains E3135A (O27:H7) (Fig. 7A), E32018A (O27:H20) (not shown), and E18519A (O159:H20) (Fig. 7B) produced fine ~2-nm-diameter fibrils. In contrast to strain E32018A, the other O27:H20 serotype ETEC strain, E24133A, produced distinct ~4-nm-diameter rigid fimbriae (Fig. 7D). ETEC strain E519/66A (O148:H28) produced ~3-nm-diameter fibrils (not shown) similar to those described previously in ETEC strain B7A of the same serotype (11), whereas strain E25117A (O148:H28) produced much finer fibrils which were difficult to resolve because of their tendency to aggregate (Fig. 7C). In each case, strains grown at 20°C lacked both CS6 and these other surface fimbriae or fibrillar structures (data not shown).

DISCUSSION

Recent studies in an animal model demonstrated the colonizing ability of human ETEC producing the CS4, CS5, and CS6 antigens of CFA/IV (18). The present in vitro study has also demonstrated the ability of human ETEC producing CS4 and CS5 to colonize cultured human small intestinal mucosa, and complementary ultrastructural studies confirmed these fimbrial antigens as colonization factors which promote mucosal adhesion of ETEC. We were, however, unable to confirm such a role for the CS6 antigen of CFA/IV, since the variants of strains E11881A and E14341 which produced only CS6 were nonadherent to both isolated human enterocytes and cultured intestinal mucosa. Strains of serotypes O27:H7, O27:H20, and O159:H20 were adhesive and previously thought to carry only CS6 (15). However, electron microscopic examination of cultures known to be adhesive to human enterocytes revealed, in addition to CS6, more prominant surface fimbriae or fibrillar structures which, given the inability of the CS6-only strains to adhere to isolated human enterocytes, probably account for the adhesive properties of these particular ETEC serotypes. Adherence properties of ETEC serogroup O27 have been demonstrated previously with intestinal 407 cells (15). Adhesion to intestinal 407 cells of strains E3135A and E32018A but not of strain E24133A (S. M. Scotland, personal communication) could possibly now be explained on the basis of the different fimbrial and fibrillar structures, in addition to CS6, produced by these strains. The production of CFA/III, in addition to CS6, by the O25:H− strain 31-10 can account for the good adhesion of this strain. Similarly, the production of fibrillar surface structures, in addition to CS6, by the O148:H28 serotype strains could account for the adhesive properties of these strains. A putative colonization factor consisting of ~3-nm-diameter fibrils has been reported previously in ETEC serotype O148:H28 (11), and similar fibrils were seen in one of the two O148:H28 strains examined in this study. Morphologically different fibrils were seen with strain E25117A, and these may be related to a 19-kilodalton polypeptide seen in extracts of this strain (15). Studies are in progress to characterize further the surface antigens produced by ETEC serotypes O27:H7, O27:H20, O148:H28, and O159:H20, which may be new CS antigens.

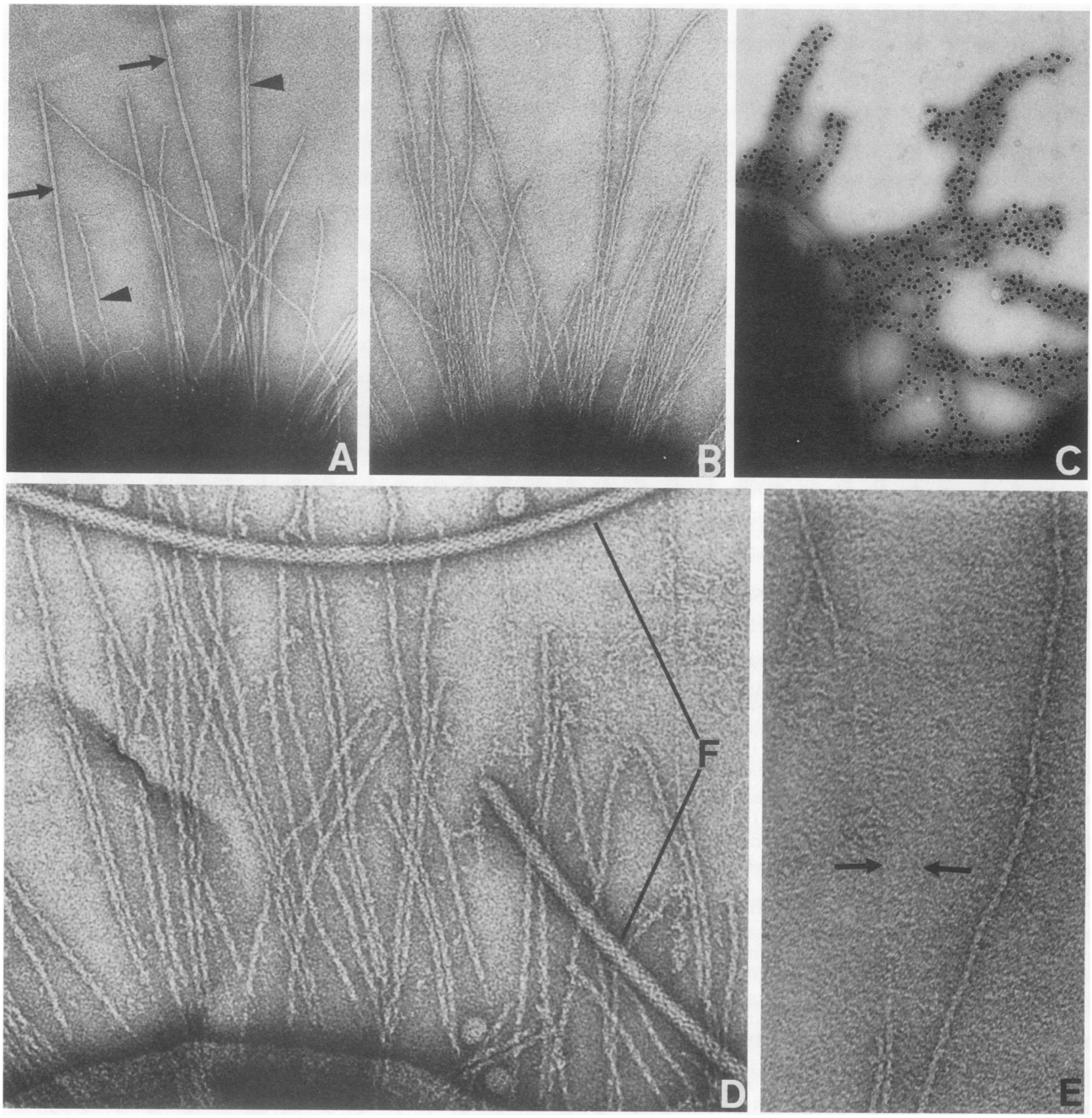

FIG. 4. Negative stain electron micrographs showing fimbriae produced by the strain carrying CS5 plus CS6 (E17018A). Broth cultures produced both ~7-nm-diameter, rigid, rodlike fimbriae (A, arrows) and 5- to 6-nm-diameter helical fimbriae (A, arrowheads), whereas only the 5- to 6-nm helical fimbriae were expressed on CFA agar (B). The helical fimbriae were specifically labeled with CS5 antiserum (C). Viewed obliquely, the helical CS5 fimbriae were seen to have a distinct criss-cross pattern (D) and occasionally to be split into two fine fibrils (E, arrows). F, Flagella. Magnification: A and B, ×100,000; C, ×65,000; D and E, ×200,000.

The diversity of ETEC serotypes carrying CS6 plasmids, which also encode LT and ST enterotoxins and other colonization factors (15, 22), and the ability of CS6-only strains to colonize rabbit intestinal mucosa in the Ritard model (18) suggest that CS6 is an important human ETEC colonization factor. It is surprising, therefore, that the CS6-only strains did not adhere to human small intestinal enterocytes in this study since all other known and putative human ETEC colonization factors, including CFA/I, CFA/II (CS1, CS2, and CS3), CFA/III, CFA/IV (CS4 and CS5), and PCF0159: H4 (S. Knutton, unpublished observations), have been found to promote adhesion of ETEC to human duodenal enterocyte brush borders (10, 11). It may be significant that all CS6-producing ETEC strains examined thus far have been found to produce additional surface structures. However, in other instances in which multiple adhesins are found (e.g., CS1-plus-CS3- and CS2-plus-CS3-producing CFA/II ETEC), each adhesin alone has been found to promote adhesion to human duodenal enterocytes (10, 12).

Nonadherence of the CS6-only strains in this study could

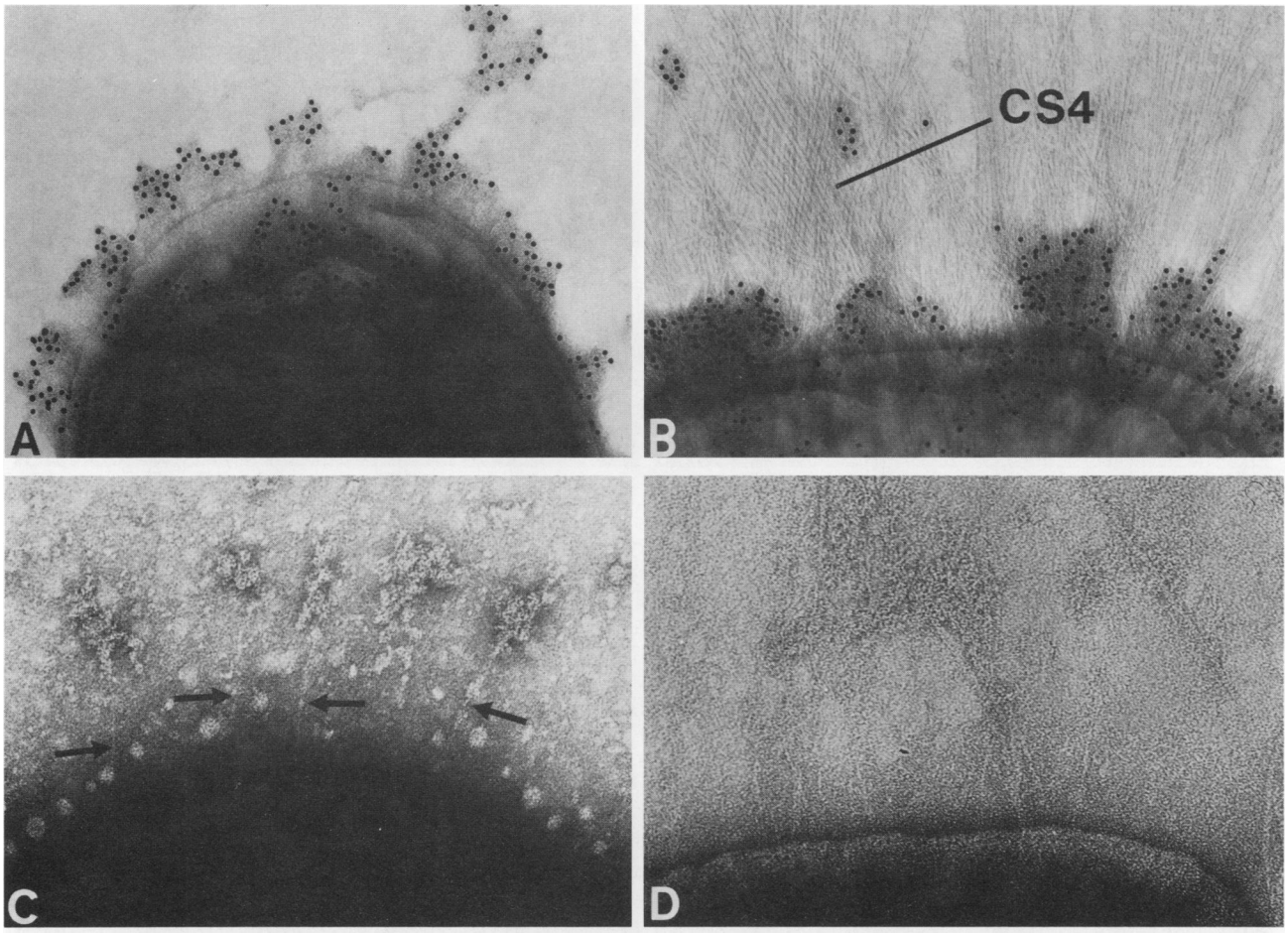

FIG. 5. Negative stain electron micrographs showing immunogold-labeled CS6 antigen on CS6-only (A) and CS4-plus-CS6-producing (B) ETEC. Fine ~2-nm-diameter fibrils (arrows) were occasionally seen projecting from the surface of CS6-only (C), CS5-plus-CS6-carrying (D), and other CS6-carrying ETEC. Magnification: A and B, ×85,000; C and D, ×160,000.

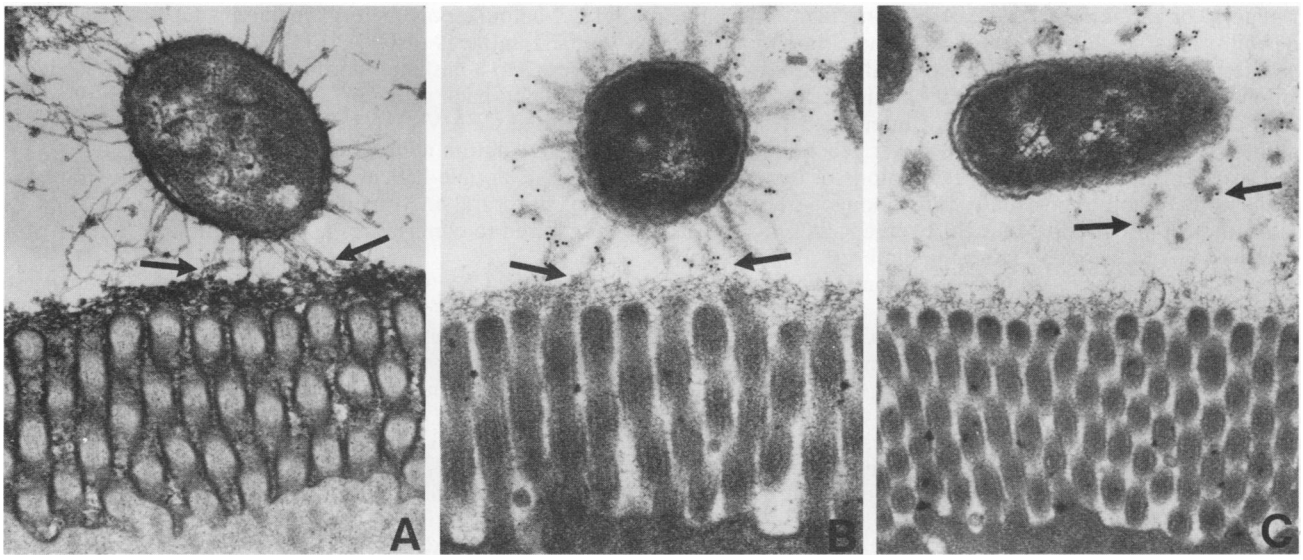

FIG. 6. Transmission electron micrographs showing fimbrially mediated adhesion of ETEC to cultured human duodenal mucosa. CS5 fimbriae of strain E17018A visualized by ruthenium red staining (A) and CS4 fimbriae of strain E11881A visualized with gold-labeled antibody (B) are seen to promote brush border attachment of bacteria (A and B, arrows). No evidence of CS6-mediated adhesion was apparent when the CS6 antigen of strain E11881A was visualized with gold-labeled antibody (C, arrows).

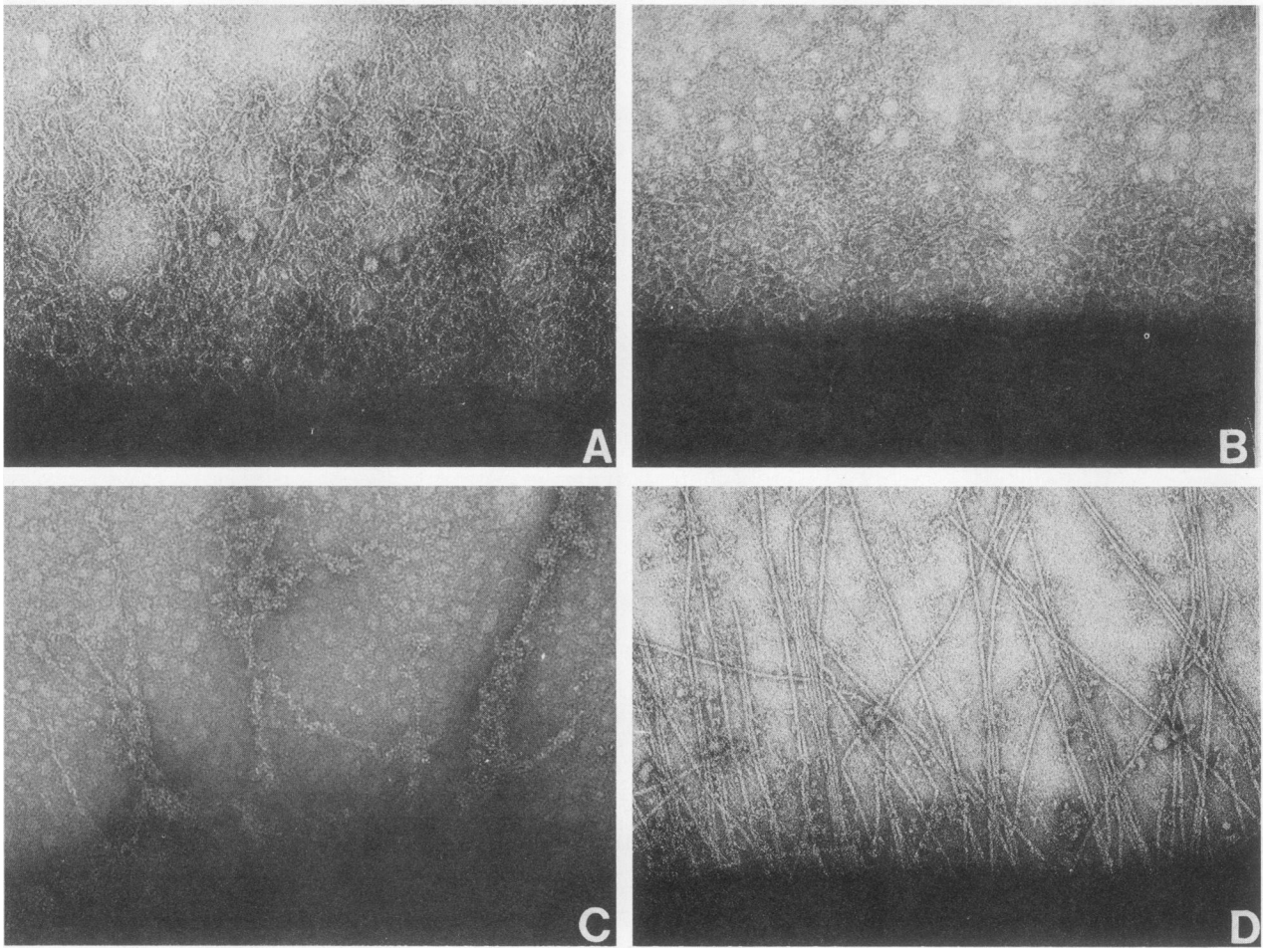

FIG. 7. Negative stain preparations of bacteria showing structures produced by ETEC serotypes O27:H7, O27:H20, O148:H28, and O159:H20 in addition to CS6. Strains E3135A (O27:H7) (A), E18519A (O159:H20) (B), and E25117A (O148:H28) (C) produced fine fibrillar surface structures, whereas strain E24133A (O27:H20) (D) produced distinct rodlike fimbriae. Magnification, ×100,000.

be due to a lack of receptors on human duodenal enterocytes in general or on some individuals in particular. The latter possibility is unlikely, however, since the CS6-only strains were tested with cells from at least 12 different individuals. Colonization of rabbits by CS6-only strains occurred in the ileum (18); it would therefore be of interest to test human ileum (or jejunum) to determine whether receptors for CS6 are present on enterocytes in these regions of the gastrointestinal tract. Another possibility is that nonadherence is due to insufficient production of CS6 by the CS6-only strains examined. Expression of adhesion fimbriae is frequently dependent on bacterial growth conditions. Indeed, in this study, we found that expression of CS4 and CS5 was very variable when bacteria were grown on CFA agar and that good adhesion to human enterocytes only occurred when there was good expression of these antigens. It was noticeable in the antibody-labeling experiments that CS4, CS5, and other putative fimbrial and fibrillar adhesion antigens were produced in much greater amounts than the CS6 antigen. Bacterial growth in rabbit ileum may favor good production of CS6 in contrast to the CFA agar and tissue culture medium used in this study, which could explain the successful colonization of rabbit intestines by CS6-only ETEC and their failure to colonize cultured human intestinal mucosa.

The ultrastructural studies confirmed previous reports that CFA/III, CS4, and CS5 are rodlike fimbrial structures (8, 20, 21). CS4 is morphologically similar to several other human ETEC colonization factors, including CFA/I and the CS1 and CS2 antigens of CFA/II (10, 12). CFA/III is somewhat thicker (7 to 8 nm) and more flexible since it was frequently seen folded back on itself. A helical structure in CS5 (also designated CFA/V [13]) has been noted previously, but the higher-resolution micrographs obtained in this study clearly suggest a double-helical structure for CS5 fimbriae; additional structural analysis of the micrographs will, of course, be required to confirm this apparently unique fimbrial structure.

Although CS6 was identified immunologically at the surface of all CS6-positive ETEC, it was not possible to correlate routinely expression of CS6 with a particular surface fimbrial or fibrillar structure. For similar reasons, CS6 previously has been designated a nonfimbrial antigen (21). Fine fibrils, which could be CS6, were occasionally seen extending from the bacterial surface of some CS6-producing ETEC, but there was not a direct correlation between CS6 detected by immunolabeling and such fibrils detected by negative staining. Nevertheless, the appearance of immunolabeled CS6 extending a considerable distance from the bacterial surface is similar to that seen for CS3, another fine fibrillar colonization factor (12), and suggests a similar fine fibrillar structure for CS6. Wolf et al. recently came to a similar conclusion (23). It is our experience that

fine fibrillar colonization factors are particularly difficult to demonstrate by negative stain electron microscopy, and it may be that, without stabilization by antibody, CS6 fibrils collapse onto the bacterial surface during preparation for electron microscopy, thus making their demonstration even more difficult.

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