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Use of Purified F1845 Fimbrial Adhesin To Study Localization and Expression of Receptors for Diffusely Adhering *Escherichia coli* during Enterocytic Differentiation of Human Colon Carcinoma Cell Lines HT-29 and Caco-2 in Culture

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Whole diffusely adhering *Escherichia coli* (DAEC) C1845 cells bearing the F1845 adhesive factor bind diffusely to differentiated human colon carcinoma cell lines HT-29 and Caco-2. By using antibodies directed against the purified fimbrial adhesin F1845 factor, the expression of the DAEC F1845-specific brush border receptors in the polarized human intestinal HT-29 and Caco-2 epithelial cells was studied by indirect immunofluorescence. A low level of DAEC F1845 receptors in undifferentiated intestinal cells was detected; they were localized in a cluster of cells. DAEC F1845 receptors were expressed at a high level in differentiated HT-29 and Caco-2 cells. DAEC F1845 receptors were expressed at a strikingly high level in the apical domains of the cells and developed during enterocytic differentiation in culture, in parallel with the apical expression of the intestinal brush border hydrolase, sucrase-isomaltase.

Escherichia coli strains responsible for human diarrheal illness may be divided into at least five groups on the basis of serotype and the proposed mechanism by which the disease is induced: enterotoxigenic *E. coli* (ETEC), attaching and effacing enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli*, enterohemorrhagic *E. coli*, and enteroadherent *E. coli*. *E. coli* causes diarrhea by the elaboration of cytotoxic or cytotonic toxins after attachment to target cells, a prerequisite for further colonization. Bacteria adhere to specific membrane receptors by means of specific adhesive factors. Numerous adhesive factors have been characterized and purified for ETEC (for a review, see reference 8). Adhesion of ETEC to isolated human enterocytes (4, 22, 24) and cultured human intestinal epithelial cells such as the HT-29 (28, 44) and Caco-2 (6) cell lines has been described elsewhere. Adhesion of EHEC in Henle-407 intestinal cells (41) and HEp-2 cells (38) has also been observed. The adherence of enterohemorrhagic *E. coli* O157:H7 to eucaryotic cells appears to be controlled by plasmid p0157 (41). There have been numerous reports on attachment of EPEC cells to isolated human enterocytes (23) and cultured HEp-2 and HeLa cells (for a review, see reference 25). Recently, attachment and effacement lesions and actin mobilization in Caco-2 cells have been described (21). Moreover, EPEC cells have been shown to invade HEp-2 cells in vitro (1, 9) and to multiply within Henle-407 cells (26). Two patterns of adhesion have been observed with EPEC. Bacteria attach by localized adherence, in which bacteria form clusters or microcolonies, and by diffuse adherence, in which the bacteria adhere over the entire surface of the cell (36). These different attachment patterns strongly suggest recognition of different receptors on the epithelial cell surface. Currently, three EPEC adhesive factors have been characterized. One, responsible for the localized adherence of EPEC O111:H on HeLa cells, is composed of two outer membrane components with approximate molecular sizes of 29 and 32 kDa (38). A second, named adhesin involved in diffuse adherence (AIDA-I) (2) is responsible for diffuse adherence to HeLa cells, and recently a chromosomal gene, which is thought to encode an adhesin, has been shown to be necessary for the attaching and effacing activity of EPEC (19). Another adhesin, F1845, of non-EPEC C1845 has also been shown to be responsible for the diffuse adherence of bacteria to HEp-2 and HI cells (3). Recently, diffusely adhering *E. coli* (DAEC) has been identified as a putative cause of diarrhea in children (16).

In this paper, we describe the tissue distribution and the cellular localization of the DAEC fimbrial adhesin F1845 receptors for the human intestinal cell lines HT-29 and Caco-2 in culture by using purified F1845 adhesive factor and rabbit polyclonal antiserum raised against it. Since the differentiation of Caco-2 cells, which form tight monolayers in culture, is growth related (34) and since the differentiation process of HT-29 cells is influenced by metabolic stress, such as glucose deprivation (33), we investigated the expression of apical proteins bearing the specific DAEC F1845 human intestinal receptor in relation to the state of differentiation.

MATERIALS AND METHODS

Cell culture. Caco-2 and HT-29 cells were obtained from Jorgen Fogh (Sloan Kettering Memorial Cancer Center, Rye, N.Y.) (13) and were used between 30 and 50 passages for the HT-29 cell line and between 65 and 90 passages for the Caco-2 cell line. Permanently differentiated HT-29 cells were obtained from Alain Zweibaum (Institut National de la Santé et de la Recherche Médicale U 178, Villejuif, France). Cells were routinely grown in Dulbecco's modified Eagle's minimal essential medium (25 mM glucose) (Eurobio, Paris, France) supplemented with 5% (HT-29) or 20% (Caco-2)

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inactivated (30 min, 56°C) fetal calf serum (Boehringer GmbH, Mannheim, Germany) and 1% nonessential amino acids. Monolayers of cells were prepared on glass coverslips, which were placed in six-well tissue culture plates (Corning Glass Works, Corning, N.Y.). Cells were seeded at concentrations of 10^5 (HT-29) and 7×10^4 (Caco-2) cells per ml. All experiments and maintenance of cells were carried out at 37°C in a 10% CO_2–90% air atmosphere. The culture medium was changed daily. Unless otherwise stated, cultures were used at postconfluence after 15 days (Caco-2) and 20 days (HT-29) of growth.

Radiolabeling of DAEC C1845. DAEC C1845 bacteria were stored on colonization factor antigen (CFA)-glycerol at −80°C. Before adherence assays, the strains were grown on CFA agar containing 1% Casamino Acids (Difco Laboratories, Detroit, Mich.), 0.15% yeast extract, 0.005% magnesium sulfate, and 0.0005% manganese chloride in 2% agar for 18 h at 37°C. For radiolabeling *E. coli* C1845, bacteria were subcultured twice at 37°C for 24 h in CFA broth and were metabolically labeled by the addition of [^{14}C]acetic acid (Amersham) (94 mCi/mmol; 100 μCi/10-ml tube) (28).

Purified DAEC fimbrial adhesin and specific antibodies. DAEC F1845 fimbrial adhesin was extracted and purified from *E. coli* C1845 of serotype O75:NM, as previously described, and antiserum against F1845 was produced in rabbits (3).

Adherence assay. The method used for the determination of adherence of DAEC C1845 to Caco-2 cells has been described elsewhere for ETEC binding to Caco-2 cells (6). Briefly, the Caco-2 monolayers were washed twice with phosphate-buffered saline (PBS). ^{14}C-radiolabeled *E. coli* cells were suspended in the culture medium, and a total of 2 ml (10^8 CFU/ml) of this suspension was added to each well of the tissue culture plate. The plates were incubated at 37°C in 10% CO_2–90% air for 1 h. The monolayers were then washed five times with sterile PBS. Adhering bacteria and intestinal cells were dissolved in a 0.2 N NaOH solution. The level of bacterial adhesion was evaluated by liquid scintillation counting. Each adherence assay was conducted in triplicate in three successive Caco-2 cell passages. To test for adhesion inhibition by antiserum raised against purified F1845, a suitable dilution of rabbit antiserum was added to 10^8 *E. coli* cells in PBS, and the mixture was allowed to incubate at room temperature for 20 min. Then it was mixed with the Caco-2 cell line and the adhesion test was performed as described above. To test for adhesion inhibition by using a competing protein, a suitable concentration of purified F1845 protein was added to the Caco-2 cell line and the mixture was incubated at room temperature for 20 min, and then 10^8 *E. coli* cells were added. The adhesion test was performed as described above.

Indirect immunofluorescence. Indirect immunofluorescence was performed both on unpermeabilized Caco-2 or HT-29 cell layers and on frozen cryostat sections (6 μm thick). Frozen cryostat sections were prepared after culture was grown in T-flasks. Two-thirds of the cell layer was detached with a rubber policeman, rolled up, snap frozen in liquid nitrogen, and used for cryostat sections. This method has the double advantage of visualizing, on the same section, almost the totality of the cell layer and of allowing the detection of apical, basolateral, and intracellular proteins. After three washes with PBS, the cell monolayers were fixed for 10 min at room temperature in 3.5% paraformaldehyde in PBS. Immunoreactivity of sucrase-isomaltase, a brush border-associated hydrolase characteristic of enterocytes (39), was detected by using rabbit polyclonal antibodies against sucrase-isomaltase purified from Caco-2 cells (42), obtained from Alain Zweibaum (INSERM U 178). Anti-rabbit fluorescein-coupled goat antiglobulins were from Institut Pasteur Productions (Paris, France). For detection of the DAEC F1845 receptors, cell monolayers were first incubated with purified F1845 (1 mg/ml) for 60 min at room temperature, washed twice with PBS, and then incubated again (30 min) with rabbit polyclonal anti-F1845 serum (diluted 1:10 in PBS) and washed and incubated for a further 30 min with fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G (diluted 1:20 in PBS). For detection of sucrase-isomaltase, cell monolayers were incubated with rabbit polyclonal anti-sucrase-isomaltase (diluted 1:200 in PBS) for 30 min at room temperature and then washed and incubated with fluorescein isothiocyanate-conjugated goat immunoglobulin G. Preimmune rabbit serum was used as a control. Immunolabeling was determined by using a Zeiss Axiofot microscope with epifluorescence. All photographs were taken on Kodak T-MAX 400 black and white film (Eastman Kodak Co., Rochester, N.Y.).

RESULTS

Whole DAEC cells bearing the F1845 fimbrial adhesive factor bind diffusely to differentiated Caco-2 cells (Fig. 1). To quantitate the DAEC adhesion to differentiated HT-29 and Caco-2 cells, we have developed an in vitro radiometric assay by using metabolically ^{14}C-labeled *E. coli* C1845 cells. Bacterial adhesion was expressed as the proportion of the total radioactivity in suspension remaining with the adhering microorganisms and the cells after washing. Each experiment was made in triplicate, with a reproducibility of better than 5%. The data were expressed as mean values of the experiments with three successive passages of Caco-2 cells, ± standard deviations. Polarized epithelia of both HT-29 and Caco-2 cells were able to retain between 3 and 5% of the incubated bacterial populations. A similar level of adhesion was observed with the diffusely adhering CFA/II-positive ETEC cells and differentiated HT-29 cells (28). To study adhesion inhibition by specific antisera, radiolabeled C1845 bacteria were incubated with polyclonal antibody raised against purified F1845 adhesin. The adhesion of the C1845 strain (3% ± 0.5%) was dramatically decreased by pretreatment of the bacteria with F1845 antiserum (0.04% ± 0.01%), and this inhibition appeared to be specific, since preimmune serum did not affect C1845 binding (3% ± 0.9%). Adhesion inhibition was also determined by using purified F1845 adhesive factor as an inhibitor. The adhesion of the radiolabeled C1845 strain no longer occurred when the Caco-2 monolayer was preincubated with purified F1845 adhesive factor (0.5% ± 0.04%). This result indicated that purified F1845 adhesin competitively inhibits the adhesion of the C1845 strain by blockading the apical intestinal receptor.

The purified F1845 fimbrial adhesin bound strongly to the cell surfaces of HT-29 and Caco-2 monolayers (Fig. 2 and 3). In HT-29 cells, no immunoreactivity was observed in the controls with preimmune rabbit antiserum (Fig. 2A to D) or with F1845 antiserum, alone, as a control (data not shown). The expression of F1845 receptors depended on the duration of culture of the HT-29 cells. The F1845 receptors appeared by day 5 in culture (Fig. 2E). The number of positively stained cells varied with the phase of growth. In actively dividing cultures, only a few cells were positive (<10%), but the proportion increased to more than 90% in late-stationary-phase cultures. In cultures grown for a short time, localized clusters of cells expressed F1845 receptors (Fig. 2E). In this

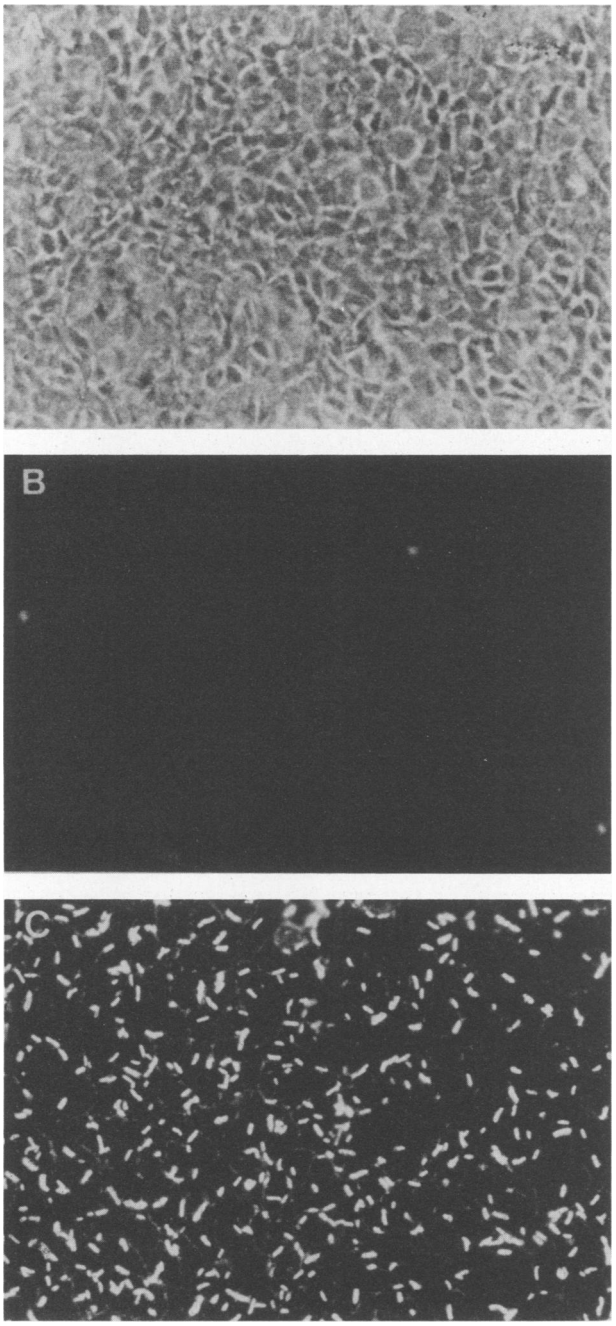

FIG. 1. Patterns of bacterial adherence to differentiated Caco-2 cells by DAEC C1845. (A) Phase-contrast observation of confluent differentiated Caco-2 cells; (B) preimmune rabbit serum; (C) immunofluorescence labeling of *E. coli* C1845 with antibody directed against purified F1845 fimbrial adhesive factor. Diffuse adherence was characterized by uniform distribution of the bacteria on the cell surfaces.

state of culture, the cells were nonpolarized and did not possess brush borders. Punctate labeling associated with an intensely immunoreactive cell rim was observed at the cell surfaces. During the phase of active growth, the positive immunoreactive cell rim disappeared, whereas the punctate labeling remained (Fig. 2F and H). At confluence, when the HT-29 cells were differentiated, large, nearly homogeneous patches of positive immunoreactivity were observed (Fig. 2G and H). The labeling pattern of F1845 receptors in differentiated HT-29 cells resembled the diffuse pattern of binding of DAEC C1845 to HEp-2 cells (3).

The expression of F1845 receptors in Caco-2 cells, which differentiate spontaneously in culture, was also studied. Fine-punctate positive immunoreactivity associated with intense staining of the cell-cell contact zones was observed at the cell surfaces (Fig. 3A to C). The appearance of F1845 receptors in Caco-2 cells, was growth related and evolved in step with the immunostaining of the specific apical hydrolase, sucrase-isomaltase, which indicates the differentiation state of the cells (Fig. 3D to F). The F1845 receptors were more widely distributed in the HT-29 cells than in the Caco-2 cells. This was particularly clear in frozen cryostat sections of differentiated Caco-2 cells, in which the positive immunostaining of F1845 receptors was localized in the apical domains of numbered cells (Fig. 4A), whereas sucrase-isomaltase immunostaining was seen in the apical domains of nearly all the cells (Fig. 4B). The lack of positive immunostaining in the basolateral domains of the intestinal epithelial cells when F1845 was used as a ligand strongly indicates the specific apical localization of the DAEC F1845 receptors.

DISCUSSION

Pathogenic bacteria have to develop adhesion mechanisms to be able to grow efficiently and colonize cell surfaces. The binding of bacteria to mucosal cells involves the interaction of specific ligands and receptors. Bacterial ligands are usually expressed as pili or fimbriae, or outer membrane proteins (8). For example, the invasin of *Yersinia pseudotuberculosis* and the filamentous hemagglutinin of *Bordetella pertussis* bind to multiple β_1-chain and β_2-chain integrins, respectively (18, 35). For ETEC, there appear to be different receptors (6, 28, 44) for each CFA (5, 7, 10, 11).

The intestinal epithelial cell lines HT-29 and Caco-2 were cultured carcinoma cell lines established by Fogh et al. (13). They are widely used to study enterocytic differentiation (for a review, see reference 45), have enterocytelike morphology, express brush border enzymes (17), and exhibit other characteristics of the ileum. Moreover, they display two clearly distinguishable plasma membrane domains: an apical membrane facing the exterior and a basolateral membrane facing the internal milieu. These two structurally different domains are separated by tight junctions (40). These cell lines closely mimic in vivo infection and have recently been used to study the pathogenic processes of several bacteria, such as adhesion of EPEC (21), ETEC (4, 6, 22, 28), and *Vibrio cholerae* (32) and invasion by *Listeria monocytogenes* (15, 27) and *Salmonella typhimurium* (12, 14).

Our results demonstrate that purified DAEC F1845 fimbriae can be used to localize the human intestinal receptors responsible for the diffuse adhesion of DAEC C1845. With differentiated HT-29 and Caco-2 cells, we observed a distribution of F1845 receptors on the cell surfaces which resembled the diffuse binding of DAEC C1845 to HEp-2 cells (3). However, we observed that DAEC F1845 receptors were also localized in cell-cell contact zones, particularly in HT-29 and Caco-2 cells cultured for a short time. In this state of culture, cells are undifferentiated and without an organized brush border. This particular localization was not reported for the diffuse binding of DAEC C1845 to HEp-2 cells, although similar cell-cell binding sites have been observed for the EPEC adhesion associated with attachment-effacement lesions and actin mobilization in Caco-2

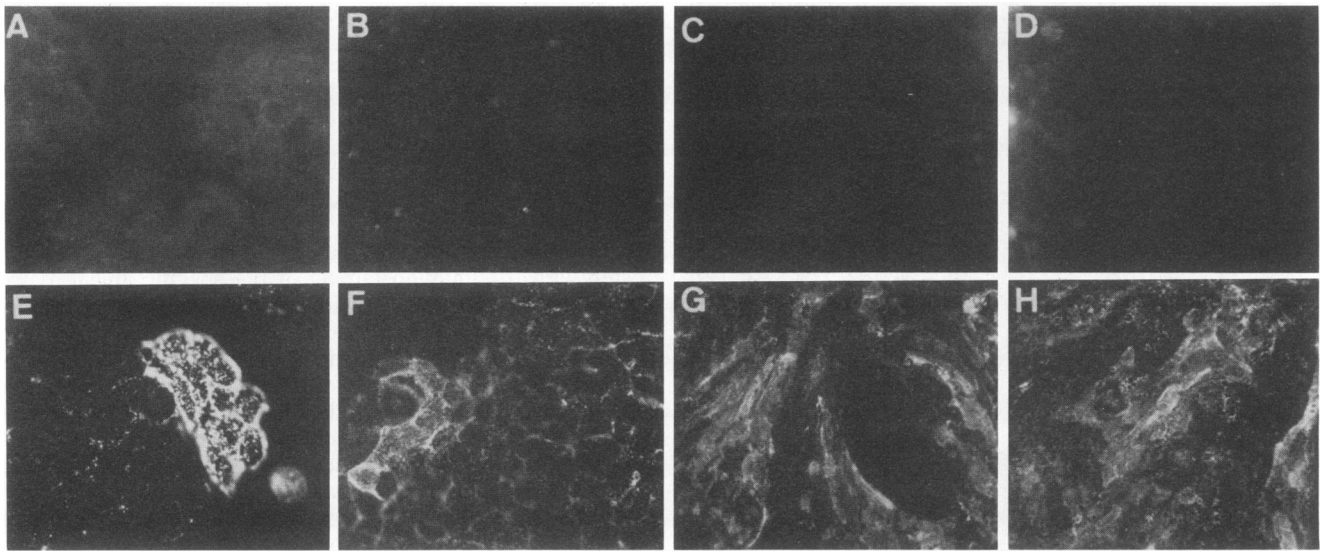

FIG. 2. Growth-related expression of DAEC F1845 fimbrial adhesin receptors in polarized human intestinal HT-29 epithelial cells. Results are shown for controls with preimmune rabbit serum (A to D) and immunolabeling of DAEC F1845 receptors (E to H) after 5 (A and E), 10 (B and F), 15 (C and G), and 20 (D and H) days in culture. Only the anti-F1845 serum shows the punctate labeling pattern. The number of positively stained cells increased with the phases of growth.

cells (21). The binding of DAEC F1845 fimbrial adhesin in cell-cell contact zones of undifferentiated intestinal cells in culture and the binding to the cell surfaces of differentiated intestinal cells suggest that DAEC cells may bind to both differentiated and undifferentiated human intestinal cells, such as crypt cells and mature enterocytes. The growth-related differentiation of Caco-2 cells is thought to resemble the situation in the normal intestine, in which dividing crypt

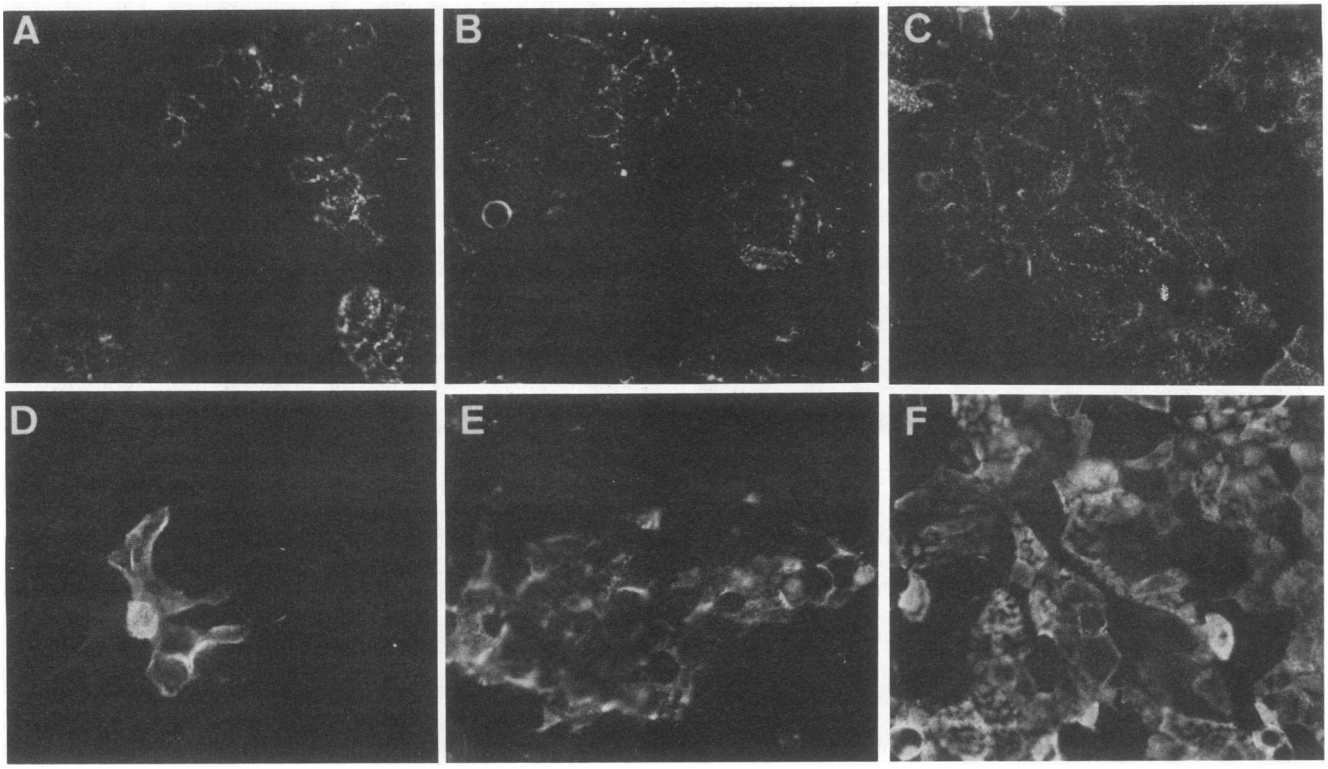

FIG. 3. Growth-related expression of DAEC F1845 fimbrial adhesin receptors in polarized human intestinal Caco-2 epithelial cells. Results are shown for immunolabeling of DAEC F1845 receptors (A to C) and indirect immunofluorescence staining of brush border sucrase-isomaltase (D to F) after 5 (A and D), 10 (B and E), and 15 (C and F) days in culture. For sucrase-isomaltase and DAEC F1845 receptors, the number of positively stained cells increased with the phases of growth.

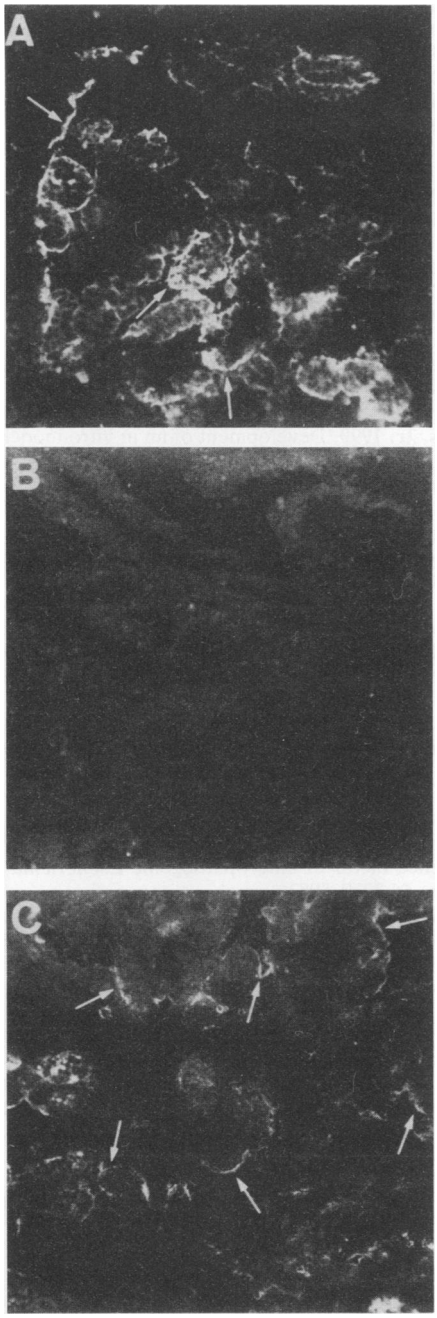

FIG. 4. Immunohistochemical localization of DAEC F1845 fimbrial adhesin receptors and differentiation-associated protein in a cryostat section of polarized human intestinal Caco-2 epithelial cell monolayers. (A) Expression of sucrase-isomaltase, showing a zone of apical staining (arrow); (B) absence of immunodetectable DAEC F1845 receptors with preimmune rabbit serum; (C) positive immunostaining of DAEC F1845 receptors, with antibody directed against purified F1845 fimbrial adhesive factor, in the apical domains of differentiated Caco-2 cells. Arrows, apical domains.

cells are undifferentiated but undergo differentiation when they migrate to the villi and stop dividing (45). Our results indicate that DAEC cells have the ability to colonize all human intestinal cells, whereas ETEC cells bind only to differentiated intestinal cells (20, 28) and invasive bacteria such as *L. monocytogenes* bind only to undifferentiated intestinal cells (15, 27). However, since the HT-29 and the Caco-2 cells are cultured carcinoma cell lines, which differ from normal human enterocytes, the distribution of F1845 receptors in these cells may not necessarily reflect the distribution in normal cells of the human ileum.

The nature of the human intestinal apical brush border component involved in the binding of F1845 fimbriae remains unknown. However, recent studies have shown F1845 to belong to a family of hemagglutinins including the afimbrial adhesins AFA-I and AFA-III and the Dr hemagglutinin of uropathogenic *E. coli*. All four of these hemagglutinins recognize as their receptor different epitopes on the Dr blood group antigen (30). The Dr receptor is thought to be a portion of the decay-accelerating factor, a membrane protein involved in regulating the complement cascade and in protecting erythrocytes from being lysed by autologous complement (29). Moreover, it has been shown that there is a high density of Dr receptors lining colonic glands and the urinary tract (31). Such receptor distribution has been suggested to facilitate colonization of the colon and the urinary tract by *E. coli* strains expressing these hemagglutinins (30). The human intestinal cell lines HT-29 and Caco-2 used in this study were established by Fogh et al. from colon adenocarcinomas (13). These cell lines are able to differentiate and form polarized monolayers and well-defined brush borders, mimicking a human intestinal epithelium (45). The fact that F1845 receptors were expressed on both differentiated and undifferentiated forms of these cell lines strongly suggests that strain C1845 can colonize the human intestinal tract. It remains to be determined whether these receptors are distinct from the Dr blood group antigen.

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