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Expression of Receptors for Enterotoxigenic *Escherichia coli* during Enterocytic Differentiation of Human Polarized Intestinal Epithelial Cells in Culture

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To study the expression of human intestinal receptors for enterotoxigenic *Escherichia coli* (ETEC), the human polarized intestinal epithelial cell line Caco-2 in culture and several subpopulations of HT-29 cells in culture—parental (mainly undifferentiated) HT-29 cells (HT-29 Std), an enterocytelike subpopulation obtained by selection through glucose deprivation (HT-29 Glc$^-$), and an enterocytelike subpopulation obtained by selection through glucose deprivation which maintains its differentiation characteristics when switched back to standard glucose-containing medium (HT-29 Glc$^{-/+}$)—were used. Since Caco-2 spontaneously differentiated in culture under standard culture conditions (in the presence of glucose) and HT-29 cells were undifferentiated when cultured under standard conditions (HT-29 Std) and differentiated when grown in a glucose-free medium (HT-29 Glc$^-$), we studied the expression of the receptors for colonization factor antigens (CFA) I, II, and III and the 2230 antigen of ETEC in relation to enterocytic differentiation. We provide evidence that expression of ETEC CFA receptors develops in parallel with other differentiation functions of the cultured cells. The expression of ETEC-specific brush border receptors was studied by indirect immunofluorescence using antibodies raised against purified ETEC CFA. No ETEC receptors were detected in HT-29 Std or short-term-cultured Caco-2 cells. However, among the population of HT-29 Std cells, 2 to 4% of the cells were found to bind ETEC, and these cells expressed positive carcinoembryonic antigen immunoreactivity. This indicated that among the population of undifferentiated HT-29 cells, clusters of differentiated cells were present. ETEC CFA receptors were expressed in the apical and basolateral domains of differentiated HT-29 cells, whereas in differentiated Caco-2 cells only apical expression was observed. Both in HT-29 cells (HT-29 Glc$^{-/+}$) and in Caco-2 cells cultured under standard conditions, ETEC CFA receptors develop as a function of day in culture. This indicated that the expression of the ETEC CFA receptors was a growth-related event. Indeed, ETEC CFA receptors developed in step with the apical expression of differentiation-associated proteins.

The human intestinal epithelial cell lines HT-29 and Caco-2, established from a human colonic adenocarcinoma by Fogh et al. (20), have been shown to undergo morphological and functional differentiation in vitro, characteristic of mature enterocytes (57, 58). These cell lines are now widely used in the study of human intestinal cell function (for a review, see reference 77). These cells form two clearly distinguishable domains, an apical membrane and a basolateral membrane, separated by tight junctions. These domains have strikingly different ultrastructures, with different protein and lipid compositions (for a review, see reference 65). For example, the apical surface, i.e., the brush border, contains peptidases and disaccharidases (28, 29, 57, 58, 62–64, 78), whereas the basolateral domain contains Na^+K^+ATPase (66) and peptide receptors (for a review, see reference 40). Specific proteins, ZO-1 and cingulin, are expressed in the tight junctions (2, 7). Since the differentiation of Caco-2 cells, which form tight monolayers, occurs spontaneously in culture (58) and the differentiation process of the HT-29 cells can be modulated by the culture conditions, such as glucose deprivation (78), these cells represent attractive models for study of the relationship between the state of differentiation and the expression of enterotoxigenic *Escherichia coli* (ETEC) receptors.

Binding of bacteria to mucosal cells and entry into the cells involve the interaction of specific ligands and receptors (15, 16, 31). Many adhesive ligands and invasive factors have been characterized recently. The use of cultured epithelial cells as an in vitro model to study bacterial attachment or invasion has begun to yield important information on the biochemical and genetic basis of cell attachment or invasion by a variety of pathogenic bacteria. In the absence of cultured polarized cell lines that closely mimic the intestinal interaction in vivo, various cell lines, such as those that have been employed to investigate the mechanisms of attachment to and invasion of the intestine by bacteria, for example, HeLa (16), HEp-2 (46), and Madin-Darby canine kidney cell lines, have been used to study the attachment and invasion characteristics of several pathogens. However, these cell lines do not form either an epithelial barrier or a well-defined brush border. To circumvent this problem, various workers have started to use the human colonic adenocarcinoma cell line Caco-2. Indeed, this cell line has been used to study the adherence of human pathogenic agents such as ETEC (3, 9) and diffusely adhering *E. coli* (36), *Vibrio cholerae* (56), *Cryptosporidium parvum* (5, 19), *Entamoeba histolytica* (61), astrovirus (74), reovirus (1), and rotavirus (67). Inter-

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action, invasion, and cell damage due to *Salmonella* spp. have been described (17, 24). In comprehensive and elegant microscopic studies, the morphological stages during the spread of *Listeria monocytogenes* from cell to cell (25, 26, 39, 51) and the interaction of *Shigella flexneri* with the intestinal cell tight junction (52, 70) have been analyzed in detail. Actin mobilization by enteropathogenic *E. coli* (EPEC) (37) and the role of the plasmid-mediated EPEC adherence factor and the chromosomally encoded *E. coli* attachment and effacement factor in adherence to and invasion of human intestinal cells by EPEC have been investigated (25, 34, 35). Moreover, the other differentiated human intestinal cell line, HT-29, in culture has also been used to study the attachment of ETEC expressing colonization factor antigen II (CFA/II) (53) and mannose-sensitive pili (75).

In a previous study, we showed that Caco-2 cells can be used to study the adhesion of ETEC expressing CFA/I, CFA/II, CFA/III, and antigen 2230 (9). This cell line was used instead of human enterocytes, which are difficult to isolate and tend to give rather variable results, depending on the particular donor. We found that whole-cell ETEC adhesion to Caco-2 cells resembled that observed with human enterocytes for all the ETEC strains examined. The lack of binding of ETEC strains to cultured cells other than human intestinal cells, even to polarized Madin-Darby canine kidney cells, which possess a poorly defined brush border, clearly indicates that ETEC have a high tissue specificity. Moreover, we have observed that whole-cell ETEC binding increases as a function of days in culture of Caco-2 cells. This result strongly suggests that the ETEC receptor could be expressed at the apical domain of Caco-2 cells during the enterocytic differentiation process of these cells.

In this study, we used radiolabeled whole-cell ETEC, purified CFAs, and polyclonal rabbit antibodies raised against purified CFAs to examine the expression of specific ETEC receptors in relation to the enterocytic differentiation. For this purpose, we used Caco-2 cells and three subpopulations of HT-29 cells: parental, mainly undifferentiated HT-29 cells (20); a differentiated HT-29 cell line obtained by selection through glucose deprivation (78); and an enterocytelike subpopulation obtained by selection through glucose deprivation which maintains its differentiation characteristics when switched back to standard glucose-containing medium (43, 44). We present evidence that the expression of apical proteins bearing the specific ETEC binding sites on enterocytes is related to the differentiation process of these human intestinal cells in tissue culture.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and radiolabeling. The ETEC strains used were the following: H10407 for CFA/I (14), Pb176 for CFA/II (Cs1 and Cs3) (13), 1373 for CFA/III (8), and 2230 for the nonfimbrial 2230 antigen (10, 22). The bacteria were stored in CFA-glycerin broth at −80°C. Before being used in the adherence assay, all 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, the bacteria were subcultured twice at 37°C for 18 h in CFA broth. They were metabolically labeled by the addition of [^{14}C]acetic acid (94 mCi/mmol; 100 μCi per 10-ml tube) (Amersham, Les Ulis, France), as previously described (53).

Cell culture. Four populations of cultured human intestinal cells were used. Caco-2 cells were obtained from J. Fogh (Sloan-Kettering Memorial Cancer Center, Rye, N.Y.) (20, 21). Parental, mainly undifferentiated, HT-29 cells (20) were referred to as HT-29 Std. Differentiated HT-29 cells, obtained by selection through glucose deprivation (78), were referred to as HT-29 Glc$^-$. An enterocytelike subpopulation obtained by selection through glucose deprivation that maintains its differentiation characteristics when switched back to standard glucose-containing medium (43, 44), referred to as HT-29 Glc$^{-/+}$, was obtained from A. Zweibaum (Institut National de la Santé et de la Recherche Médicale, Unité 178, Villejuif, France).

Caco-2 (58), HT-29 Std (57), and HT-29 Glc$^{-/+}$ (43, 44) cells were grown under standard conditions in Dulbecco modified Eagle's minimal essential medium (25 mM glucose) (Eurobio, Paris, France) supplemented with 10% (HT-29) or 20% (Caco-2) inactivated (30 min, 56°C) fetal calf serum (Boehringer, Mannheim, Germany) and 1% nonessential amino acids (Caco-2). Differentiated HT-29 cells (HT-29 Glc$^-$) were adapted to grow in a glucose-free medium (78). Monolayers of cells were prepared on glass coverslips that were placed in six-well tissue culture plates (Corning Glass Works, Corning, N.Y.). Cells were seeded at a concentration of 10^5 (HT-29) and 7×10^4 (Caco-2) cells per cm^2. All experiments and maintenance of cells were conducted 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 (Caco-2) and 20 days (HT-29) of culture.

Purified ETEC adhesive factors and specific antibodies. Overnight bacterial cultures of ETEC on CFA agar were harvested in 0.1 M phosphate buffer (pH 7.2). The bacterial adhesion antigens were extracted by heating the cells at 60°C for 20 min with gentle agitation. Cells were sedimented at 12,000 × *g* for 15 min. The supernatant was brought to pH 4.0 and stored overnight at 4°C. The precipitated proteins were collected by ultracentrifugation at 20,000 × *g* for 30 min and suspended in 0.1 M phosphate buffer (pH 7.2). They were partially purified by two ultracentrifugation steps (115,000 × *g* for 10 min and 200 min). The final sediment, representing the crude adhesion antigens, was suspended in 0.1 M phosphate buffer (pH 7.2). Adhesion antigens were purified on a Sepharose CL-6B column equilibrated in 100 mM sodium phosphate with 6 M guanidine hydrochloride. Antibodies against purified CFA were raised in rabbits. CFA were administered intravenously four times at 4- to 5-day intervals with increasing doses of purified protein (175, 350, 700, and 700 μg, respectively). The rabbits were bled 7 days after the last injection. To prepare CFA-specific antisera, each crude antiserum was absorbed with a negative variant of the corresponding CFA-producing strain.

Adherence assay. The method for determination of adherence of ETEC to Caco-2 and HT-29 cells has been described elsewhere (9). Briefly, the Caco-2 and HT-29 monolayers were washed twice with phosphate-buffered saline (PBS). ETEC were suspended in the culture medium without any antibiotic, and a total of 1 ml (10^8 ^{14}C-labeled cells per 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 three 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 duplicate with three successive Caco-2 or HT-29 cell passages.

Indirect immunofluorescence. The state of cell differentiation was evaluated by immunofluorescence staining of the

TABLE 1. Adhesion of ETEC harboring CFA/I, CFA/II, CFA/III, and 2230 adhesive factors in relation to expression of CFA receptors to cell lines HT-29 Std, HT-29 Glc$^-$, and Caco-2

Cell populations	Differentiation characteristics	ETEC adhesion[a] (immunostaining of CFA receptors[b])			
		CFA/I	CFA/II	CFA/III	Antigen 2230
HT29 Std	Undifferentiated	0.02 (Negative)	0.03 (Negative)	0.02 (Negative)	0.02 (Negative)
HT-29 Glc$^-$	Enterocytelike	0.02 (Negative)	1.21 (Positive)	1.46 (Positive)	1.59 (Positive)
Caco-2	Enterocytelike	2.40 (Positive)	2.75 (Positive)	2.19 (Positive)	2.12 (Positive)

[a] The binding assay was conducted with ^{14}C-labeled ETEC. Bacterial adhesion was expressed as the proportion of the total radioactivity in suspension remaining with the adhering microorganisms and the cells after the cells were washed. Each experiment was performed in triplicate (standard deviation, not shown, was less than 5%). The data represent mean values of experiments with three successive passages of Caco-2 and HT-29 cells.

[b] CFA receptors were revealed by indirect immunofluorescence staining using purified CFAs and rabbit polyclonal antibodies directed against CFA/I, CFA/II, CFA/III, and 2230 adhesive factors.

cells with antibodies directed against brush border-associated markers sucrase-isomaltase (64) and carcinoembryonic antigen (CEA) (42).

Indirect immunofluorescence was performed on frozen cryostat sections (6 μm thick) or nonpermeabilized Caco-2 or HT-29 cell layers. Frozen cryostat sections were prepared after cultures were 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 entire cell layer and of allowing the detection of apical, basolateral, and intracellular proteins (43, 62). Nonpermeabilized cell layers were prepared after cells were grown on glass coverslips, and monolayers were washed three times with PBS. Both types of preparations were fixed for 10 min at room temperature in 3.5% paraformaldehyde in PBS. Polyclonal rabbit antibodies raised against purified antigens CFA/I, CFA/II, CFA/III, and 2230 were used for the immunofluorescence labeling of ETEC. Immunoreactivity of sucrase-isomaltase was detected by using polyclonal rabbit antibodies against sucrase-isomaltase purified from Caco-2 cells (39), obtained from A. Zweibaum. Immunoreactivity of CEA was detected by using a monoclonal antibody obtained from A. Le Bivic (URA 179, Centre National de la Recherche Scientifique, Marseille, France). Anti-rabbit and anti-mouse fluorescein- and rhodamine-coupled goat immunoglobulins were from Institut Pasteur Productions (Paris, France). After the binding assay, the slides were fixed (10 min at room temperature with 3.5% paraformaldehyde in PBS), washed, incubated for 30 min at room temperature with rabbit polyclonal anti-CFA serum (diluted 1:10 in PBS), washed, and then incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin G (diluted 1:20 in PBS). For detection of the ETEC CFA receptors, cell monolayers were first incubated with purified CFAs (1 mg/ml) for 60 min at room temperature, washed twice with PBS, and then incubated again with rabbit polyclonal anti-CFA serum (diluted 1:10 in PBS), washed, and incubated with FITC-conjugated goat anti-rabbit immunoglobulin G (diluted 1:20 in PBS). For detection of sucrase-isomaltase and CEA, cell monolayers were incubated with rabbit polyclonal anti-sucrase-isomaltase (diluted 1:200 in PBS) or monoclonal anti-CEA (diluted 1:100 in PBS) for 30 min at room temperature, washed, and then incubated with FITC-conjugated goat immunoglobulin G or rhodamine isothiocyanate (RITC)-conjugated goat anti-mouse immunoglobulin G. Preimmune rabbit serum was used as a control. Immunolabeling was examined by using a Leitz Diaplan microscope with epifluorescence. All photographs were taken on T-MAX 400 black and white film (Eastman Kodak Co., Rochester, N.Y.).

RESULTS

Expression of ETEC CFA receptors on Caco-2 cells. Indirect immunofluorescence studies using nonpermeabilized or frozen sections of Caco-2 cells and antibodies against purified CFAs were conducted to examine the distribution of CFA receptors on Caco-2 cells. Positive immunoreactivities using CFA/I, CFA/II, CFA/III, and antigen 2230 as ligands and positive binding of ^{14}C-labeled ETEC harboring CFA/I, CFA/II, CFA/III, and antigen 2230 adhesive factors were observed on differentiated Caco-2 cells (Table 1), whereas no immunoreactivity was observed when preimmune rabbit sera were used (data not shown). In differentiated Caco-2 cells, which form domes randomly distributed over the monolayer, indicating an active vectorial transport of fluid and electrolytes across the cell monolayer (Fig. 1A and 2B), we observed high ETEC whole-cell binding to numerous domes (Fig. 1C), correlating with a high density of CFA receptors. In many cases, positive immunoreactivity formed a continuous cell rim (Fig. 1D). This result suggests that the cells present in the domes possess a high degree of maturation.

By using cryostat sections of differentiated Caco-2 cells (Fig. 2) that visualize, on the same section, almost the entire cell layer and allow the detection of apical, basolateral, and intracellular proteins, no CFA immunoreactivity was detected by indirect immunofluorescence using preimmune rabbit serum (Fig. 2D). ETEC CFA/III receptors were located essentially in the apical domain of the cells (Fig. 2F). The fluorescence staining was intense but sparse compared with the continuous and intense staining of sucrase-isomaltase in the brush border membranes of differentiated Caco-2 cells (Fig. 2B).

These results show that the ETEC binding was closely correlated with the expression of CFA receptors at the apical domain of the Caco-2 cells. Moreover, we have previously observed that the extent of adhesion of the major types of ETEC strains increases with the age of the cell culture (9). Altogether, these results strongly suggest that ETEC act with intestinal receptors expressed at the apical domain of the intestinal cells during the enterocytic differentiation process. To further dissect the relationship between the expression of the ETEC CFA receptors and the enterocytic differentiation process, we used three subpopulations of HT-29 cells that are derived from the pluripotent HT-29 cell line: the parental HT-29, consisting of mainly undifferentiated cells (HT-29 Std); the differentiated enterocytelike HT-29 cells (HT-29 Glc$^-$), obtained by selection through glucose deprivation; and the enterocytelike HT-29 subpopulation (HT-29 Glc$^{-/+}$), also obtained by selection through glucose deprivation, which maintains its differentiation char-

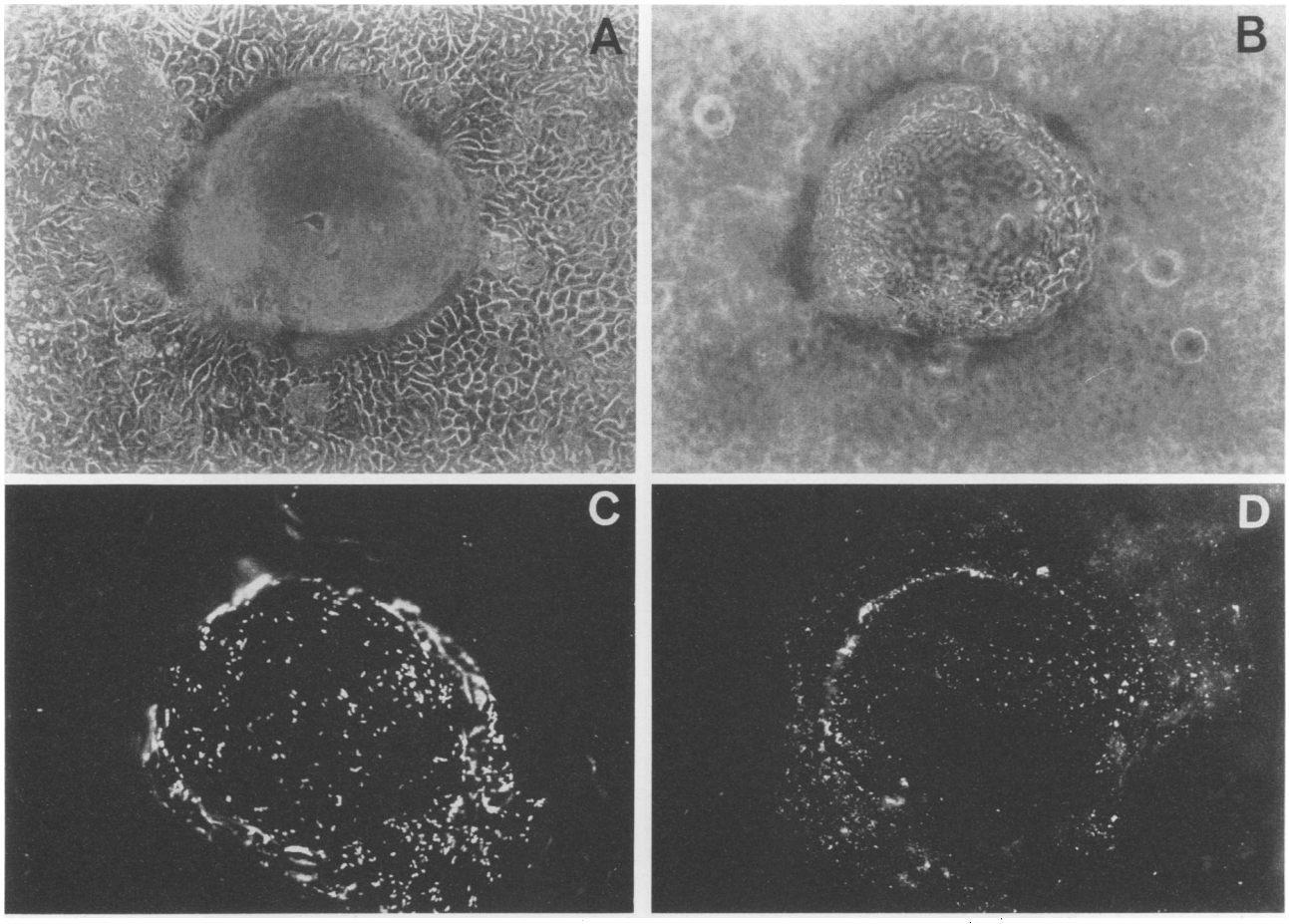

FIG. 1. Expression of CFA/III receptors on the domes of Caco-2 cells. (A and B) Phase-contrast micrographs of domes formed on the monolayer of confluent Caco-2 cells; (C) immunofluorescence labeling of adhering CFA/III-positive bacteria; (D) immunofluorescence labeling of CFA/III receptors by using purified CFA/III as ligand. Note that binding of CFA/III-positive bacteria colocalizes with the punctate labeling pattern of CFA/III receptors. The results were similar with other CFA-positive bacteria and other purified CFAs.

acteristics when switched back to standard glucose-containing medium. This last cell subpopulation spontaneously differentiates in culture (43, 44), as do Caco-2 cells (58).

Expression of ETEC CFA receptors on HT-29 cells in relation to the enterocytic differentiation process. Adhesion of whole-cell ETEC to the different subpopulations of HT-29 cells was assayed with ^{14}C-labeled ETEC bearing CFA/I, CFA/II, CFA/III, and 2230 antigen adhesive factors (Table 1).

^{14}C-labeled ETEC did not adhere to undifferentiated HT-29 cells (HT-29 Std). However, we have observed positive binding of whole-cell ETEC (Fig. 3A) and positive immunofluorescence staining of CFA/III receptors (Fig. 3B) on clusters of cells (2 to 5% of the cell population). In these cell clusters, positive immunostaining of the differentiation marker CEA was observed (Fig. 3C). These results strongly indicate that among the undifferentiated cell population of the parental HT-29 cell line (HT-29 Std), isolated differentiated cells and clusters of differentiated cells were present.

ETEC expressing CFA/II, CFA/III, and antigen 2230 adhered to differentiated HT-29 cells adapted to grow in a glucose-free medium (HT-29 Glc$^-$), whereas ETEC expressing CFA/I did not. These results are in agreement with those of Neeser et al. (53), who reported adhesion of CFA/II-positive ETEC, but not CFA/I-positive ETEC, to differentiated HT-29 cells. On cryostat sections of permanently differentiated HT-29 cells (HT-29 Glc$^{-/+}$), a subpopulation of HT-29 cells selected under conditions of metabolic stress, i.e., glucose deprivation, and switched back to standard glucose-containing medium, intense immunolabeling (apical domain) and slight immunolabeling (basolateral domain) were observed when CFA/III was used as the ligand (Fig. 2E), whereas no immunofluorescence staining was observed with preimmune serum (Fig. 2C). Expression of CFA receptors as a function of the state of differentiation was studied with the permanently differentiated HT-29 Glc$^{-/+}$ cell line. The state of differentiation was evaluated by indirect immunofluorescence labeling of the differentiation marker CEA. No immunofluorescence labeling of CFA receptors with preimmune rabbit sera was observed at any time (Fig. 4E to H). No immunolabeling of CFA receptors was observed before day 5; immunostaining appeared on day 10 and increased afterwards (Fig. 4I to L). Immunofluorescence staining for CEA (Fig. 4A to D), which labels apical domains of epithelial cells (51), developed in parallel with the CFA receptor immunolabeling. Similar results were obtained with Caco-2 cells in that CFA receptors develop in parallel with the differentiation marker sucrase-isomaltase (data not shown).

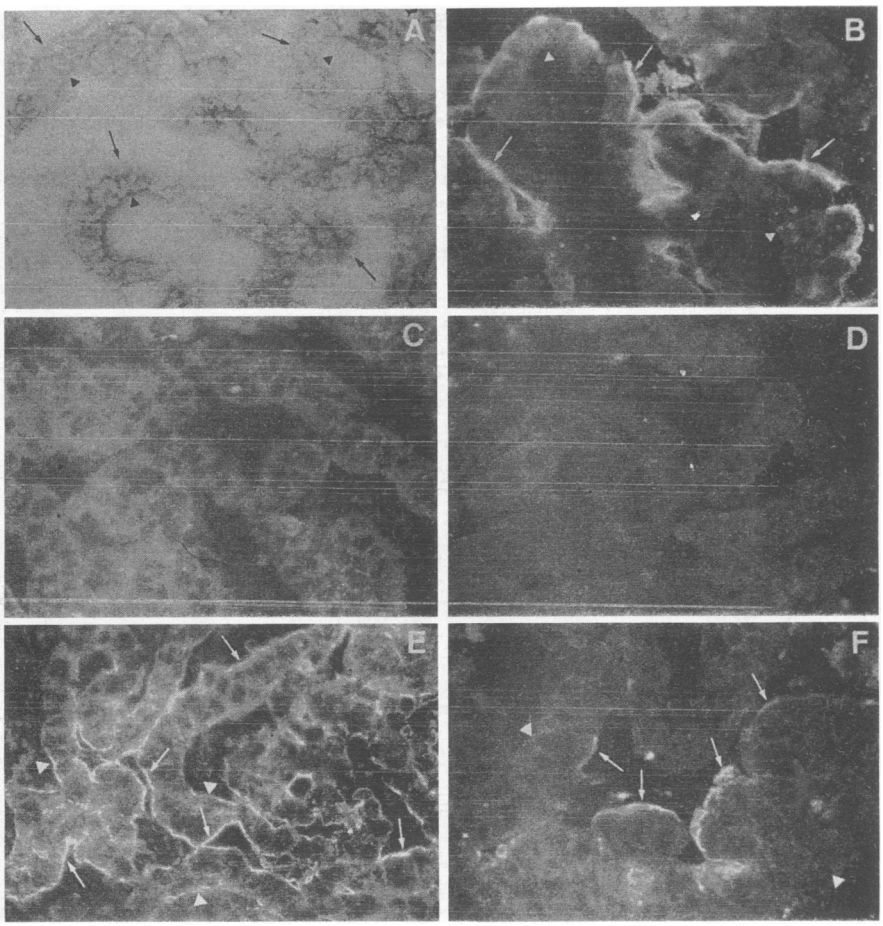

FIG. 2. Immunohistochemical localization of CFA/III receptors on cryostat sections of differentiated HT-29 $Glc^{-/+}$ and Caco-2 cell monolayers. (A) Phase-contrast micrograph of cryostat section of Caco-2 cells; (B) expression of the differentiation-associated protein sucrase-isomaltase with intense apical staining; (C and D) no immunodetectable CFA/III receptors by using rabbit preimmune sera in differentiated HT-29 $Glc^{-/+}$ (C) and Caco-2 (D) cells; (E) CFA/III receptors localized at both the apical and basolateral domains of differentiated HT-29 $Glc^{-/+}$ cells; (F) CFA/III receptors strikingly localized at the apical domain of differentiated Caco-2 cells. Arrows indicate apical domains; the arrowheads indicate basal domains. The results with other purified CFAs were similar.

DISCUSSION

ETEC are a major cause of infant diarrhea in developing areas and are the principal agent of traveler's diarrhea. ETEC develop their pathogenicity by cell attachment (11, 38), which is a prerequisite for intestinal colonization, and by production of cytotonic toxins (50). It has been noticed that the toxins produced by adherent ETEC are targeted more efficiently than those produced by nonadherent ETEC (54). Our understanding of the primary events in the pathogenicity of ETEC has been impeded by the lack of an appropriate cellular model for investigating attachment of CFA-positive ETEC strains. Due to the high tissue and species specificity, study of ETEC attachment has so far been possible only by using fresh human small intestine enterocytes and human cultured mucosa (6, 41). There are no suitable animal models, and ETEC strains unfortunately do not adhere to cell lines such as HeLa, HEp-2, HRT18 (human rectal teratoma), Hutu 80 (human duodenal carcinoma), and Madin-Darby canine kidney. In a previous study, we showed that Caco-2 cells could replace human enterocytes in the studies of the mechanism of attachment of CFA-positive ETEC (9). Other studies have shown that another differentiated intestinal epithelial cell line, HT-29, grown under conditions promoting enterocytic differentiation appears to bind CFA/II-positive but not CFA/I-positive ETEC strains (53), whereas undifferentiated HT-29 cells bind mannose-sensitive *E. coli* (75).

In the light of the results presented here, the human adenocarcinoma cell lines appear to be of value for the investigation of the adhesion process and for characterization, at the molecular level, of the interaction of CFA-positive ETEC with the brush border. Indeed, our results clearly demonstrate that attachment of ETEC to the enterocytic brush border was due to the expression of CFA-specific receptors during the cell differentiation process of human intestinal cells. Both in HT-29 and Caco-2 cells, the time course of the differentiation process, with exponentially dividing cells being undifferentiated and with the differentiation occurring when the cells stop dividing, closely mimics the situation found in the small intestine, with proliferating crypt cells being undifferentiated and with the differentiation occurring during the crypt-to-villus migration of nondividing cells (for a review, see reference 77).

Interaction of several pathogenic bacteria with Caco-2 cells was recently described. *Listeria monocytogenes* cells bind to undifferentiated intestinal cells (25, 26, 51). *Salmo-*

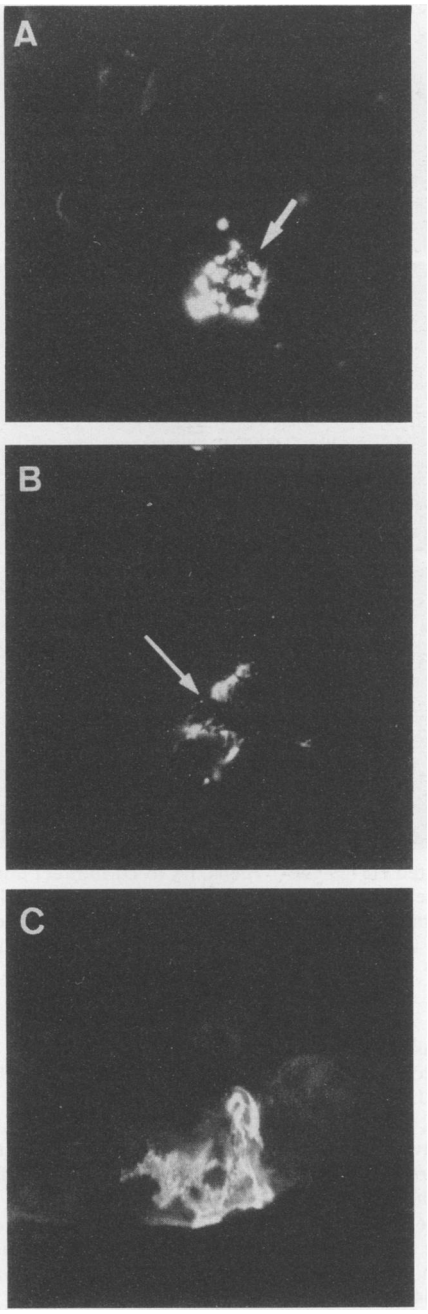

FIG. 3. Expression of CFA/III receptors to clusters of cells in the cell population of the parental HT-29 cell line (HT-29 Std). HT-29 Std cells were mainly undifferentiated (20, 57). Clusters of cells (arrows, panels A and B) (2 to 4% of total cell layer surface) expressed binding of CFA/III-positive bacteria (A), CFA/III receptors by using purified CFA/III as ligand (B), and expression of the differentiation marker CEA (C). Binding of CFA/III-positive bacteria (panel a) (FITC labeling) and positive immunoreactivity of CEA (panel c) (rhodamine isothiocyanate labeling) were observed in the same differentiated HT-29 cell cluster. Similar colocalization was observed with CEA and CFA/III receptors (data not shown). These results indicate that the parental, mainly undifferentiated, HT-29 Std cell line contains a small proportion of differentiated cells. The results were similar with other purified CFA and CFA-positive ETEC strains.

nella spp. (17), *Vibrio cholerae* (56), EPEC (23, 34, 35, 37), and ETEC (9, 53) bind to the brush border of differentiated intestinal cells, whereas *Shigella flexneri* interacts with the tight junction of differentiated intestinal cells (52, 70). These results strongly suggest adhesion and invasion of crypt cells by *Listeria monocytogenes*, whereas *Salmonella* spp., *Vibrio cholerae*, EPEC, and ETEC interact with bacterial receptors in the tips of the intestinal microvilli. In a fashion similar to the functional and enzymic differentiation of Caco-2 (58, 62, 63) and HT-29 cells (57, 68, 69, 78), the accumulation of ETEC receptors in the apical domain of Caco-2 and HT-29 cells is a growth-related phenomenon. It is known that HT-29 cells subjected to metabolic stresses tend to adapt to the new conditions (for a review, see reference 77). After an initial phase of mortality, differentiated cell populations such as absorptive (30, 57, 72, 78) or mucus-secreting cells (4, 30, 43, 44) emerge. Although the exact mechanisms involved in metabolic adaptation and differentiation are still unknown, it has been suggested that the differentiated populations evolve from a small population of cells present in the original cell line (43, 44). Our results are consistent with this theory. Indeed, in the undifferentiated HT-29 cells cultured in presence of glucose, we observed that 2 to 4% of the cells bound ETEC strains or expressed CFA-positive immunoreactivity. This proportion of differentiated cells is in agreement with the results reported by Lesuffleur et al. (43, 44). The results presented here show that whole-cell ETEC binding occurred in step with the expression of ETEC CFA receptors, increasing with the duration of culture. Apical expression of many intestinal proteins, such as brush border glycoproteins sucrase-isomaltase, dipeptidylpeptidase IV, and aminopeptidase N (27–29, 47, 48, 62, 63, 66, 68, 78), as well as the posttranslational processing of glycoprotein carbohydrate chains (55, 69, 73), has been shown to depend on the state of differentiation of human colon cancer cells in culture.

Currently, an important goal in microbiology and cellular biology is to identify the human enterocytic receptors involved in cell attachment (3, 9, 23, 31, 34–37, 45, 53, 59, 60, 72) and in cell invasion (12, 15, 17, 18, 24–26, 31–33, 39, 49, 51, 52, 70, 76). It remains to be determined what the receptors of ETEC are. Recent reports using HT-29 cells indicate that a subcomponent of CFA/II, i.e., the *E. coli* surface CS3 antigen, binds to three protein bands of about 30, 20, and 13 kDa (53), whereas Cs1 antigen alone or antigens Cs2 and Cs3 bind to proteins with molecular masses in the range 30 to 35 kDa (72). Moreover, the Cs3 antigen binding site appears to be expressed solely on brush border membranes of differentiated HT-29 cells. In this case, cell-cell recognition is of the lectin-carbohydrate type, involving a brush border membrane glycoprotein (53). However, conflicting results were obtained concerning the CFA/I-binding protein. Indeed, as was previously observed (53), we have obtained no adhesion with *E. coli* bearing CFA/I adhesive factor. Other reports described binding of CFA/I to crude membranes of differentiated HT-29 cells (72). Caco-2 cells are a suitable model to identify and characterize on a molecular level the human intestinal receptors of ETEC, since these cells expressed binding sites for all ETEC that we studied. However, the Caco-2 cells are cultured carcinoma cells, which differ from normal human enterocytes. The expression and distribution of the ETEC receptors in these cells may not necessary reflect the expression and distribution on normal cells of the human ileum. Biochemical characterization of the CFA receptors could be performed by using brush border membrane preparations of Caco-2

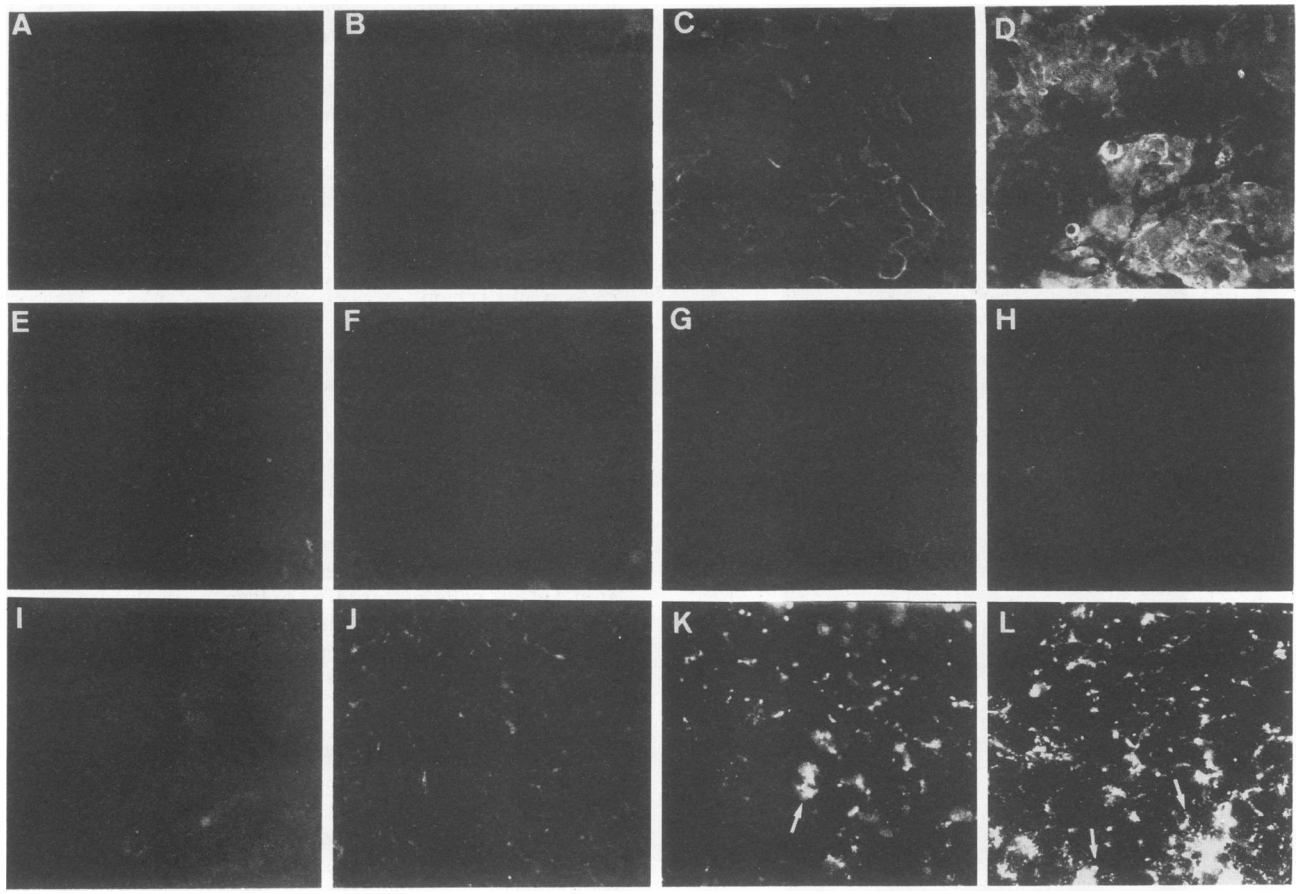

FIG. 4. Growth-related expression of CFA receptors in permanently differentiated HT-29 $Glc^{-/+}$ cells. (A to D) Indirect immunofluorescence staining of brush border CEA; (E to H) controls with preimmune CFA/III serum; and (I to L) immunolabeling of CFA/III receptors. Days in culture: panels 5; panels 10; panels 15; panels D, H, and L, 20. Only the immune sera of CFA/III show the punctate labeling pattern. For CEA and CFA/III receptors, the number of positively stained cells varied with the phases of growth. Arrows indicate patches of intense staining. The results were similar with other purified CFA.

cells and freshly isolated human enterocytes. Lectins and drugs which affect both the delivery of apical membrane proteins to the cell surface and glycosylation of apical intestinal glycoproteins could be used to identify the receptor membrane glycoproteins for ETEC. This should throw more light on the mechanism of human intestinal cell recognition by pathogenic bacteria.

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