INFECTION AND IMMUNITY, Apr. 1990, p. 893–902
0019-9567/90/040893-10$02.00/0

Vol. 58, No. 4

Adhesion of Enterotoxigenic *Escherichia coli* to the Human Colon Carcinoma Cell Line Caco-2 in Culture

ARLETTE DARFEUILLE-MICHAUD,[1]* DOMINIQUE AUBEL,[1] GILLES CHAUVIERE,[2] CHANTAL RICH,[1] MICHEL BOURGES,[3] ALAIN SERVIN,[2] AND BERNARD JOLY[1]

Laboratoire de Bactériologie[1] and Service de Microscopie Electronique,[3] Facultés de Pharmacie et de Médecine, 63001 Clermont-Ferrand Cedex, and Unité Institut National de la Santé et de la Recherche Médicale 178, 94807 Villejuif,[2] France

Received 12 September 1989/Accepted 19 December 1989

Enterotoxigenic *Escherichia coli* (ETEC) strains possessing colonization factor antigen I (CFA/I), CFA/II, CFA/III, and antigen 2230 were tested for their ability to adhere to the following cell lines: HeLa, HEp-2, HRT 18, Hutu 80, MDBK, MDCK, Vero, and Caco-2. ETEC strains adhered only to the Caco-2 cell line. Irrespective of the known adhesive factors, the ETEC strains that adhered to the brush border of human enterocytes also adhered to the Caco-2 cell line. The negative variants, which were cured of the plasmid encoding the adhesive factor, did not adhere. Adhesion of ETEC strains no longer occurred when the Caco-2 cells were pretreated with the homologous colonization factor antigen or when the bacterial cells were pretreated with homologous antibodies raised against the adhesive factors. This indicates that this adhesion is specific and that a different receptor exists for each type of adhesion factor. Electron micrographs of cross sections of the monolayer showed that the adhesion of ETEC strains to the brush border microvilli does not induce any lesion. Therefore, the Caco-2 cell line behaves in the same way as human enterocytes do.

Five pathogenic groups of *Escherichia coli* that are involved in diarrhea in humans have been described: enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli*, enterohemorrhagic *E. coli*, and enteroadherent *E. coli*. EPEC strains adhere to HEp-2 and HeLa cells in two different patterns; in one, the bacteria cover the whole surface of the cell (diffuse adherence), and in the other, attachment is limited to one or a few sites of the cell surface (localized adherence) (2, 23). Recently, Knutton et al. (12) showed adhesion of EPEC to HEL and Caco-2 cells. Enteroinvasive *E. coli* strains adhere to HEp-2 and HeLa cells before invasion (15). Enterohemorrhagic *E. coli* strains of serogroup O157:H7 are found to adhere to the intestine 407 or HEp-2 cells (24). Lastly, enteroadherent *E. coli* strains adhere to HEp-2 cells in a particular pattern that is distinguishable by both localized and diffuse adherence (aggregative adhesion) (17, 19). To date, no cell line has been described that would enable the study of the adhesion of ETEC strains. We looked for a continuous cell line which would allow ETEC strains to adhere specifically. Thus, in our studies we could replace human intestinal biopsy specimens, which are difficult to acquire and yield fluctuating results because of the different enterocyte donors (A. Darfeuille-Michaud, B. Lafeuille, C. Forestier, C. Rich, and B. Joly, Rev. Inst. Pasteur Lyon, in press), with a continuous cell line.

ETEC strains are involved in many cases of acute diarrhea in both humans and animals. They possess two pathogenic traits: adhesion to the small intestine and production of thermolabile and thermostable enterotoxins. Adhesion to the intestinal epithelium has been correlated with the production of specific antigens. The antigens are specific for the host. Antigens K88, K99, F41, and 987P are found on strains of animal origin (10, 11, 18, 20). In human ETEC strains, two important adhesive factors termed colonization factor antigens I and II (CFA/I and CFA/II, respectively) are well known (5, 6). Further distinct antigens produced by human ETEC strains have also been described, such as CFA/III (3, 9), PCF8775 or CFA/IV (26), antigen 2230 (4), and many others (14, 25). There are reports of two different CFA/III's (3, 9). We described a CFA/III that is produced by ETEC strain 1373, which shares certain common epitopes with CFA/I but which differs in its recognition of enterocyte receptors. Although the amino acid compositions of CFA/I and CFA/III are different, they share identical N-terminal amino acid sequences (A. Darfeuille-Michaud, Doctorat d'Etat thesis, Clermont II, France, 1987).

We report here the results of a study of eight cell lines that were tested for their ability to specifically bind four ETEC strains that produced CFA/I, CFA/II, CFA/III, and antigen 2230, respectively. Among the cell lines tested, we included the Caco-2 cell line. This cell line was established by Fogh et al. (7) and was derived from a carcinoma of the colon. It exhibits structural and functional differentiation patterns characteristic of mature enterocytes in postconfluent cultures. This maturity has been shown by immunoreactivity with anti-brush border enzyme antibodies and by the high levels of alkaline phosphatase, sucrase-isomaltase, and aminopeptidases (8, 22).

MATERIALS AND METHODS

Bacterial strains and culture conditions. The following enterotoxigenic *E. coli* strains were used: H10407 with CFA/I (6), Pb176 with CFA/II (CS1 and CS3) (5), 1373 with CFA/III (3), and 2230 with the nonfimbrial antigen 2230 (4). Negative variants that lacked each adhesive factor were designated H10407p, Pb176p, 1373p, and 2230p, respectively. Unless otherwise stated, all tests were performed with 18-h cultures of *E. coli* grown at 37°C on CFA-agar (5) containing 1% Casamino Acids (Difco Laboratories, Detroit, Mich.), 0.15% yeast extract, 0.005% magnesium sulfate, and 0.0005% manganese chloride in 2% agar.

Tissue cultures. The following cell lines were tested: HeLa; HEp-2; MDBK; MDCK; Vero (Flow Laboratories,

* Corresponding author.

Inc., McLean, Va.); HRT 18 (human rectal terratoma); and Hutu 80 (human duodenal carcinoma), which was kindly provided by M. Laporte (Institut National de la Recherche Agronomique, Thiverval Grignon, France); and Caco-2 (7).

Monolayers of HeLa, HEp-2, HRT, Hutu 80, MDBK, MDCK, and Vero cells were prepared in Leighton tubes containing glass cover slips and incubated for 48 h at 37°C in Eagle medium (Flow Laboratories) with 10% fetal bovine serum and 200 U of penicillin per liter, 50 mg of streptomycin per liter, and 2 mg of amphotericin B per liter.

Monolayers of differentiated Caco-2 cells were prepared in six-well Falcon tissue culture plates (Becton Dickinson Labware, Oxnard, Calif.). The cells were seeded at 4×10^4 cells per cm^2 in Dulbecco modified Eagle medium (Flow Laboratories) at 37°C in a 10% CO_2–90% air atmosphere for 15 days. The medium was supplemented with 20% fetal bovine serum and 1% nonessential amino acids. The culture medium was changed daily. Unless otherwise stated, cultures were used at postconfluence after 15 days of culture.

In vitro enterocyte adhesion assay. The in vitro adhesion assay was conducted as described previously (16). Briefly, human duodenal enterocytes were mixed with 10^8 *E. coli* cells in phosphate-buffered saline. This mixture was allowed to incubate at room temperature for 20 min with slow shaking. Bacterial adhesion was quantified by examination under a phase-contrast microscope at a magnification of ×1,000. The number of *E. coli* cells that adhered to each of 20 enterocytes was counted in duplicate. The adhesion index was expressed as the number of bacteria that attached to the brush border of one enterocyte.

Test of adhesion to continuous cell lines. The adherence of *E. coli* to HeLa, HEp-2, HRT 18, Hutu 80, MDBK, MDCK, Vero, and Caco-2 cells was examined by a modification of the HEp-2 cell assay of Cravioto et al. (2). Bacterial strains were grown for 18 h at 37°C either on CFA-agar (5) or in 1% tryptone water (23). A suspension of approximately 10^8 bacteria per ml was made in the cell line culture medium with 0.5% D-mannose. A total of 3 ml of this suspension was added to the tissue culture tube and was incubated for 1 or 3 h at 37°C. The cells were then washed three times in sterile phosphate buffer, stained with 20% Giemsa stain, and examined microscopically under oil immersion. An adhesion index was determined by examining 100 cells; it corresponded to the mean number of bacteria per cell. We also noted the percentage of positive cells with at least one adhering bacterium.

Adhesion inhibition tests. To test for adhesion inhibition by antiserum raised against CFA, rabbit antisera were prepared with purified antigens CFA/I, CFA/II, CFA/III, and 2230 as described previously (4). Briefly, rabbits were immunized intravenously four times at 4- to 5-day intervals with 175, 350, 700, and 700 μg of purified protein, respectively. The rabbits were bled on day 7 after the last injection. To prepare CFA-specific antisera, each crude antiserum was absorbed with a negative variant of the corresponding CFA-producing strain. A suitable dilution of antiserum was added to 10^8 *E. coli* cells in phosphate-buffered saline, 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 ensure that any decrease in adherence was not due to the agglutination of bacteria, we also performed inhibition assays with papain-treated antibodies.

Portions of each antiserum were treated with papain (Sigma Chemical Co., St. Louis, Mo.) to cleave the antibody molecules into Fc and Fab fragments before adherence testing. Portions of 1.0 ml were treated with 0.1 ml of papain (25 mg/ml) for 4 h at 37°C, after which 0.001 g of *p*-chloromercuribenzoic acid was added to each portion to stop the reaction. Each antiserum was then added to the bacteria as described above. As controls, papain and *p*-chloromercuribenzoic acid in saline and *p*-chloromercuribenzoic acid alone in saline were also incubated with bacteria.

To test for adhesion inhibition by competing protein, CFAs were extracted and purified as described previously (4). Overnight cultures on 10 Roux flasks with CFA-agar were harvested in 0.1 M phosphate buffer (pH 7.2). The adhesion antigens were separated from bacterial cells by heating the suspension at 60°C for 20 min with gentle agitation. Cells and bacterial debris were sedimented at 10,000 × *g* for 10 min. The supernatant was brought to pH 4.0 and stored overnight at 4°C. The precipitated proteins were collected by ultracentrifugation at 10,000 × *g* for 30 min and suspended in 0.1 M phosphate buffer (pH 7.2). Partial purification was performed by two ultracentrifugations; the sediment from the first centrifugation at 115,000 × *g* for 10 min was discarded, and the supernatant was submitted to a second centrifugation at 115,000 × *g* for 200 min. The sediment that was suspended in 0.1 M phosphate buffer (pH 7.2) represented the crude adhesion antigen extract. Purified adhesion antigens were obtained by chromatography on a Sepharose CL-6B column equilibrated in 100 mM sodium phosphate with 6 M guanidine hydrochloride. A suitable concentration of surface protein was added to the Caco-2 cell line, the mixture was incubated at room temperature for 20 min, and then 10^8 *E. coli* cells were added.

Statistical analysis. The results were expressed as means ± standard deviations of the means. Each mean number of bacteria per cell represents the results of six separate experiments.

Statistical significance was determined by analysis of variance by using the F distribution of Fisher.

Transmission electron microscopy. The cell culture medium was removed from the flasks and immediately replaced by 3% glutaraldehyde in 0.2 M cacodylate buffer (pH 7.4). Fixation was done at 4°C for 2 h. The cells were rinsed three times in cacodylate buffer. They were postfixed in 1% OsO_4 in cacodylate buffer at 4°C for 1 h. After dehydration in a graded series of ethanol without propylene oxide, the cultures were embedded in a 2-mm-thick Epon coating in the tissue culture well and polymerized for 3 days at 60°C. Suitable areas were reoriented perpendicular to the cell layer surface on Epon blocks with an Epon mixture. Ultrathin sections were contrasted with uranyl acetate and lead citrate and examined with an electron microscope (HU 12A; Hitachi).

Scanning electron microscopy. For scanning electron microscopy, cells were grown on glass cover slips. After the bacterial adhesion assay, the cells were fixed with 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at 4°C for 2 h. The samples were dehydrated in a graded series of ethanol and acetone, dried, and coated with gold. The specimens were then examined with a scanning electron microscope (Stereoscan 600; Cambridge Instruments Co., Cambridge, England).

RESULTS

Adhesion of the ETEC strains to the different cell lines. When cultured on a solid medium such as CFA-agar, ETEC H10407, Pb176, 1373, and 2230 strains adhered to the human

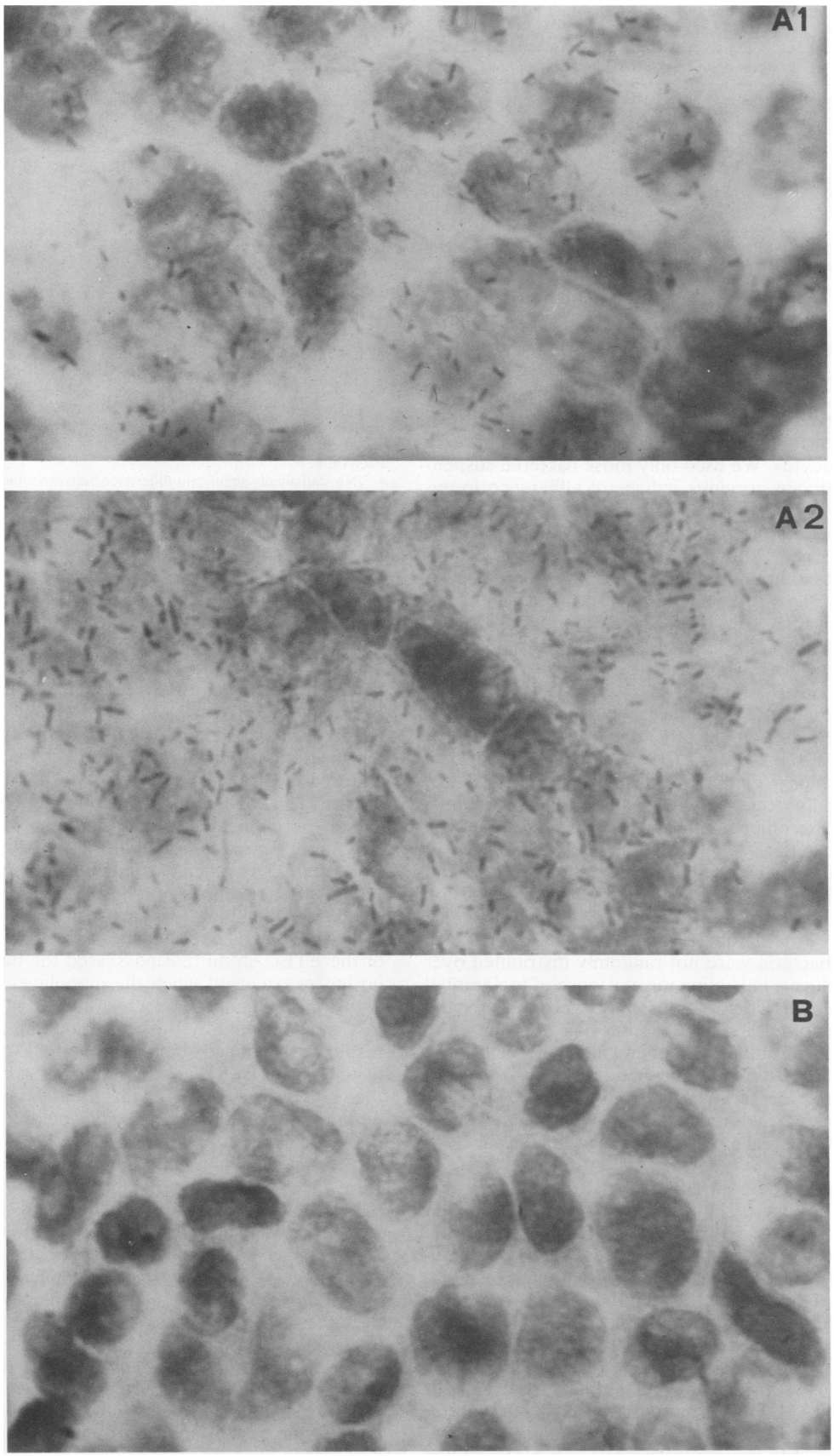

FIG. 1. Micrograph showing the adhesion of ETEC strain H10407 (A1 and A2) and its negative variant H10407p (B) to the Caco-2 cell line. The incubation of Caco-2 cells with 10^8 bacteria was performed at 37°C for 1 h (A1) and 3 h (A2 and B) in the presence of the cell line culture medium and 0.5% D-mannose. Cells and bacteria that were stained with 20% Giemsa stain were observed at ×1,000 magnification.

TABLE 1. Adhesion indices of ETEC strains to different cell lines in culture

ETEC strain (antigen)	Adhesion indices to the following cell lines[a]							
	Hep-2	HeLa	Vero	HRT 18	Hutu 80	MDBK	MDCK	Caco-2
H10407 (CFA/I)	0.01	0	0	0.01	0	0	0	2.39
Pb176 (CFA/II)	0	0	0.04	0	0	0	0	1.85
1373 (CFA/III)	0	0	0	0	0	0	0	1.90
2230 (2230)	0	0	0	0	0	0	0	1.81

[a] Adhesion index is the mean number of bacteria per cell, as determined by light microscopic examination of 100 cells.

enterocytes by the in vitro adhesion assay. These ETEC strains were tested for their ability to adhere to the following cell lines: HeLa, HEp-2, HRT 18, Vero, MDBK, MDCK, Hutu 80, and Caco-2. Because ETEC strains rapidly lose their adhesive properties, adhesion to cell lines was always performed concurrently with adhesion to the brush borders of human enterocytes. We used only those bacterial suspensions which matched previous indices of adherence to enterocytes as a control against the occasional loss of the adherence properties of our ETEC strains. The results are summarized in Table 1. Incubation of 10^8 bacteria per ml with a confluent culture of the different cell lines for 1 or 3 h at 37°C and in the presence of 0.5% D-mannose, which prevents nonspecific type 1-mediated adhesion, did not result in any adhesion of ETEC strains to the HeLa, HEp-2, HRT 18, Vero, MDBK, MDCK, or Hutu 80 cell lines. With bacteria grown on CFA-agar or in 1% tryptone water, the mean number of bacteria that adhered to one cell was always found to be less than 1. On the other hand, ETEC strains that possessed CFA/I, CFA/II, CFA/III, or antigen 2230 adhered to the human colon carcinoma cell line Caco-2 (Fig. 1A1). This adhesion only occurred with bacteria that were grown on CFA-agar. The number of bacteria that adhered to the cells was greatest after 3 h of incubation (Fig. 1A2). Thus, all tests were further performed by incubating bacteria and Caco-2 cells for 3 h at 37°C. Furthermore, this adhesion occurred even in the presence of 0.5% D-mannose. The adhesion index varied from 1.81 to 2.39 bacteria per cell (Table 2). The bacteria were not randomly distributed over the entire monolayer, indicating the existence of preferential areas where the cells could possess more receptors for the ETEC adhesive factors (Fig. 2). Adhesion was therefore quantitated by determining the percentage of cells with adherent bacteria. After 3 h of incubation, the percentage of cells with adherent bacteria varied from 72 to 83%, according to the adhesive factor considered, and the maximum number of positive cells was obtained with strain 1373 possessing CFA/III.

We investigated bacterial adherence to Caco-2 cell line cultures of various ages. The results are illustrated in Fig. 3 and 4. Until day 6, we noticed that ETEC strains did not adhere to the cells; the adhesion indices were not significantly different from the adhesion indices of the corresponding negative variants. Receptors for the ETEC strains appeared at day 9, and the ability of the cells to bind ETEC strains increased greatly until day 15. In the same way, the percentage of positive cells with at least one adhering bacterium increased during culture. Until day 6, only 20% of the cells could bind ETEC strains. After 15 days of culture, nearly 80% of the cells allowed bacteria to adhere to them.

Specificity of ETEC strain adhesion to Caco-2 cells. When cured of the plasmid involved in the synthesis of the adhesion factor, the negative variants H10407p, Pb176p, 1373p, and 2230p no longer adhered to the brush border of human enterocytes (Table 2) or to Caco-2 cells (Fig. 1B). Their adhesion indices to Caco-2 cells varied from 0.07 to 0.29 bacteria per cell, and the percentage of cells with adherent bacteria varied from 6.8 to 20.8% (Table 2). The number of positive cells appeared to be high, but often there was only one bacterium per cell. We noticed that after 15 days of culture, the Caco-2 cells were postconfluent and formed domes. Thus, on this irregular monolayer, some bacteria cannot be eliminated by the different washes, even when they do not possess any ability to adhere. Inhibition adhesion studies were then carried out to verify the specificity of ETEC strain adhesion to the Caco-2 cell line.

TABLE 2. Adhesion indices of ETEC strains and their negative variants to the brush borders of human enterocytes and to Caco-2 cells

ETEC strain (antigen)	Adhesion index[a] to:	
	Brush border of human enterocytes	Caco-2 cells (% of positive cells)[b]
H10407 (CFA/I)	1.8 ± 0.65	2.39 ± 0.38[c] (73.0 ± 9.9)
H10407p	0	0.17 ± 0.18[d] (14.3 ± 12.0)
Pb176 (CFA/II)	2.3 ± 0.70	1.85 ± 0.54 (72.0 ± 11.4)
Pb176p	0	0.29 ± 0.14 (20.3 ± 14.6)
1373 (CFA/III)	2.9 ± 0.82	1.90 ± 0.44 (81.0 ± 12.6)
1373p	0	0.07 ± 0.03 (6.6 ± 2.3)
2230 (2230)	2.8 ± 0.53	1.81 ± 0.32 (83.6 ± 10.0)
2230p	0	0.19 ± 0.14 (15.0 ± 11.2)

[a] Values are means ± standard deviations. Each mean number of bacteria per cell represents the results of six separate experiments.
[b] Mean percent ± standard deviation of cells with at least one adhering bacterium.
[c] No statistically significant difference between adhesion indices of positive ETEC strains ($P > 0.05$).
[d] No statistically significant difference between adhesion indices of negative variants ($P > 0.05$).

Adhesion inhibition was performed by using purified adhesive factors. The results are shown in Fig. 5. The adhesion of the ETEC strain that possessed the homologous antigen no longer occurred when the monolayer was preincubated with a purified adhesive factor, indicating that all the receptors were blocked by the corresponding adhesin. A similar result was obtained with the four ETEC strains that were tested. Some ETEC adhesion indices were decreased when the cells were treated with a nonhomologous adhesive factor, but statistical analysis showed that the difference was not significant ($P > 0.05$).

To study adhesion inhibition by specific antisera, bacteria were incubated with polyclonal antibodies raised against purified CFA/I, CFA/II, CFA/III, and antigen 2230. The results are shown in Fig. 6. The adhesion of each adhesive ETEC strain was inhibited by pretreatment of the bacteria with its corresponding specific antiserum. This inhibition appeared to be specific since preimmune sera did not affect the adhesion (Table 3). To ensure that this inhibition was not due to the agglutination of bacteria, we performed these assays with Fab fragments. As shown in Table 3, similar results were obtained. Furthermore, we verified that this decrease in adherence was due to Fab fragments and not to the enzyme papain or *p*-chloromercuribenzoic acid. A low decrease in the adhesion indices was obtained for these two controls, but it was not significantly different ($P > 0.05$).

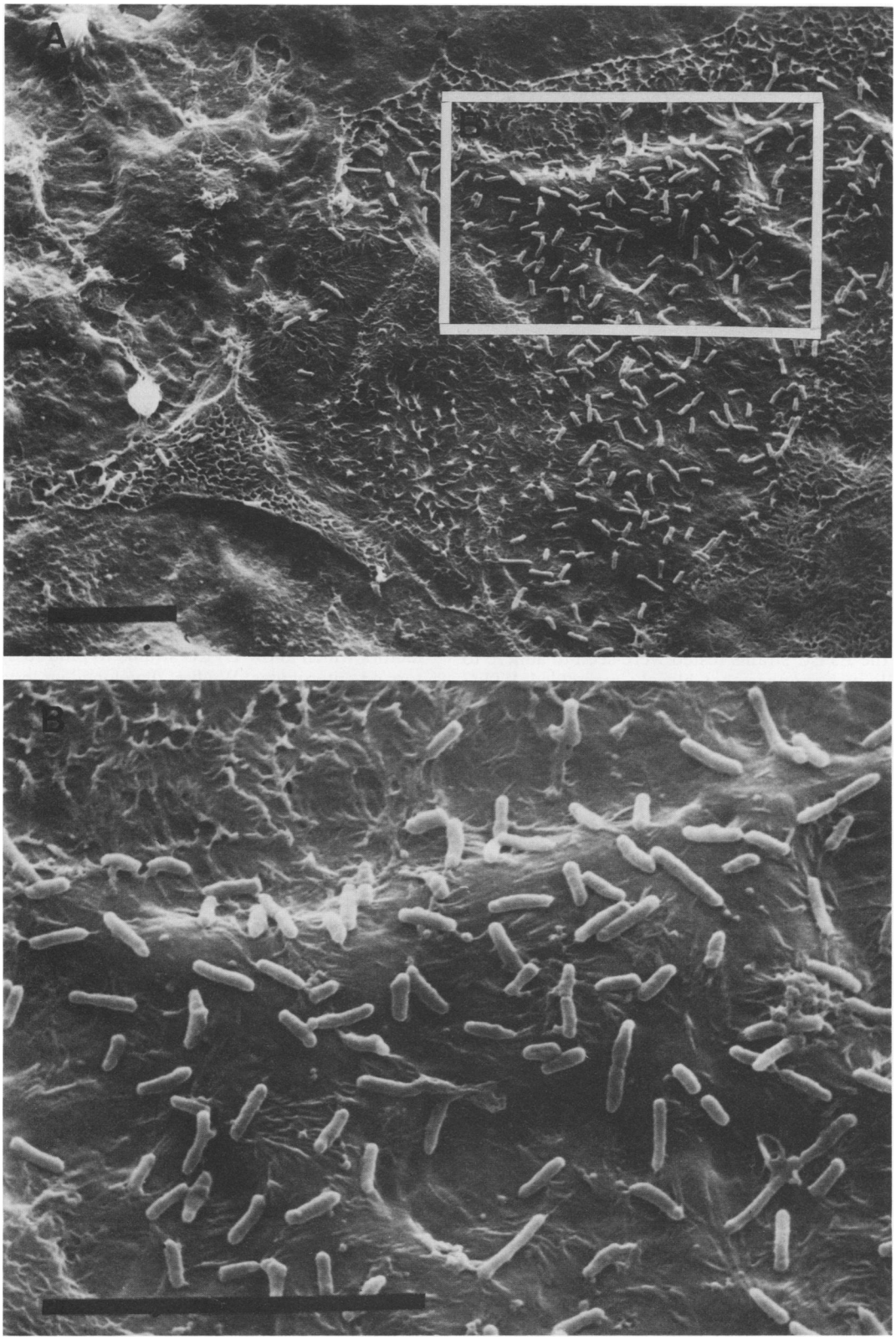

FIG. 2. Scanning electron micrographs of the Caco-2 cell line after 15 days of culture and infected with ETEC strain H10407. Note that the surface monolayer is irregular and that the cells form domes where bacteria preferentially adhere. Bars, 10 μm.

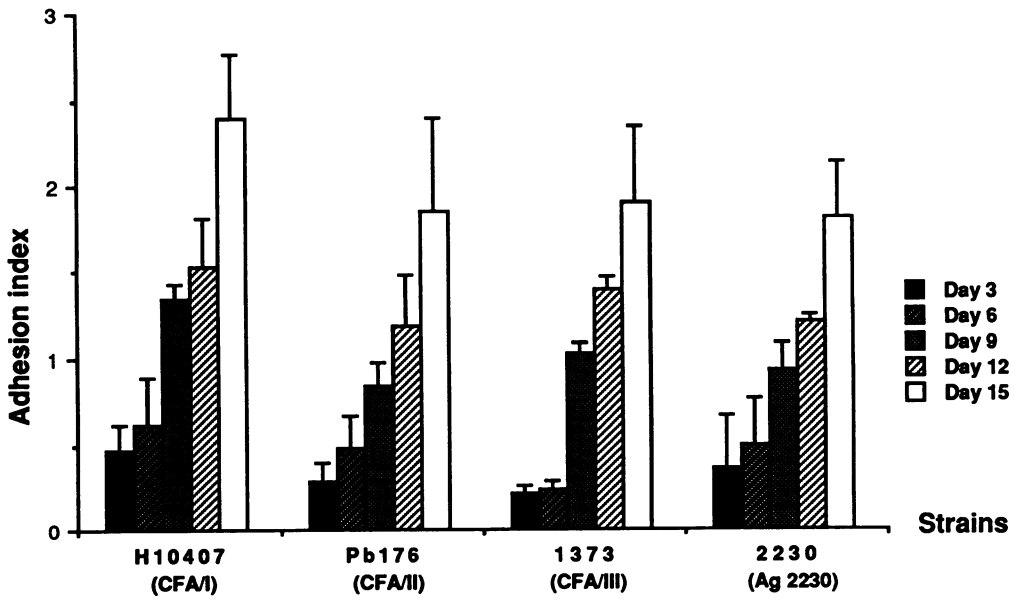

FIG. 3. Adhesion of ETEC strains to Caco-2 cells according to the age of the cell line culture. The adhesion index is the mean number of bacteria per cell determined in six separate experiments. Horizontal bars indicate standard deviations.

Electron microscopy. Transmission electron microscopy of cross sections of the Caco-2 cell monolayer showed that 15-day-old confluent cultures were covered by typical brush border microvilli that projected out perpendicular to the cell surface. All the ETEC strains tested were seen to adhere to the microvilli in a manner that closely resembled ETEC adhesion to human intestinal enterocytes (Fig. 7A). Depending on the morphology of the adhesive factor tested, the bacteria were found to be very close to the microvilli in the case of the nonfimbrial adhesive factor antigen 2230 (Fig. 7C) or at a greater distance in the case of fimbrial adhesive factors, such as CFA/I (Fig. 7B). In any case, the adhesion of ETEC strains to the Caco-2 cell line did not generate any modification of the microvilli.

DISCUSSION

The adhesion of ETEC strains to the intestinal epithelium has been directly correlated with the presence of adhesive factors which allow ETEC strains to bind to specific receptors on enterocytes. We first developed an in vitro adhesion assay using human small intestinal enterocytes (16). This adhesion test is specific for ETEC strains, but there is considerable variation in the adhesion of ETEC strains, depending on the intestinal cell donors (A. Darfeuille-Michaud et al., in press). To circumvent this problem, we looked for a continuous cell line to which ETEC strains could bind specifically. We tested eight cell lines, including HEp-2 and HeLa cells, which allow adhesion of EPEC,

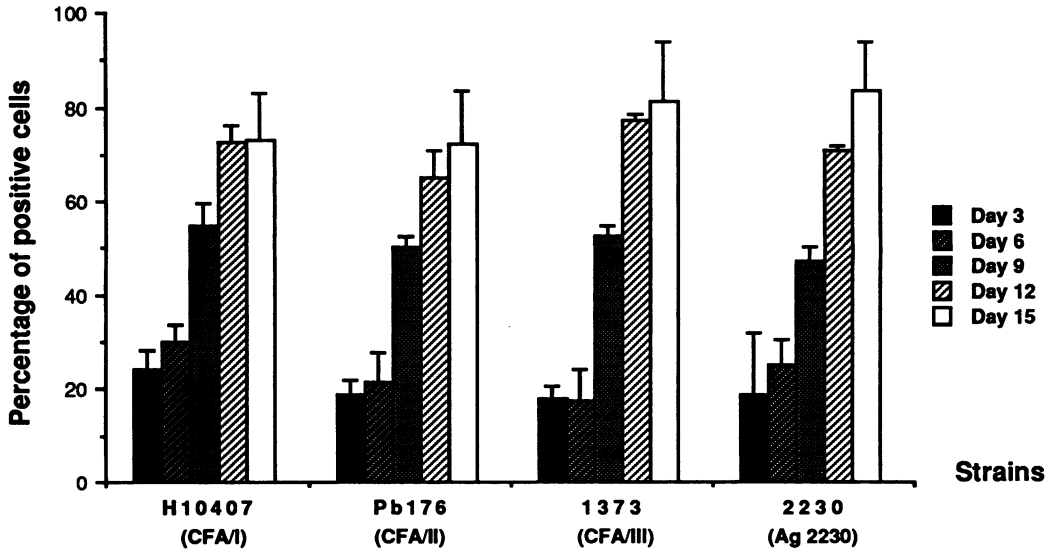

FIG. 4. Percentage of Caco-2 cells that bound ETEC strains according to the age of the cell line culture. The mean percentage of cells with at least one adhering bacterium was determined in six separate experiments. Horizontal bars indicate standard deviations.

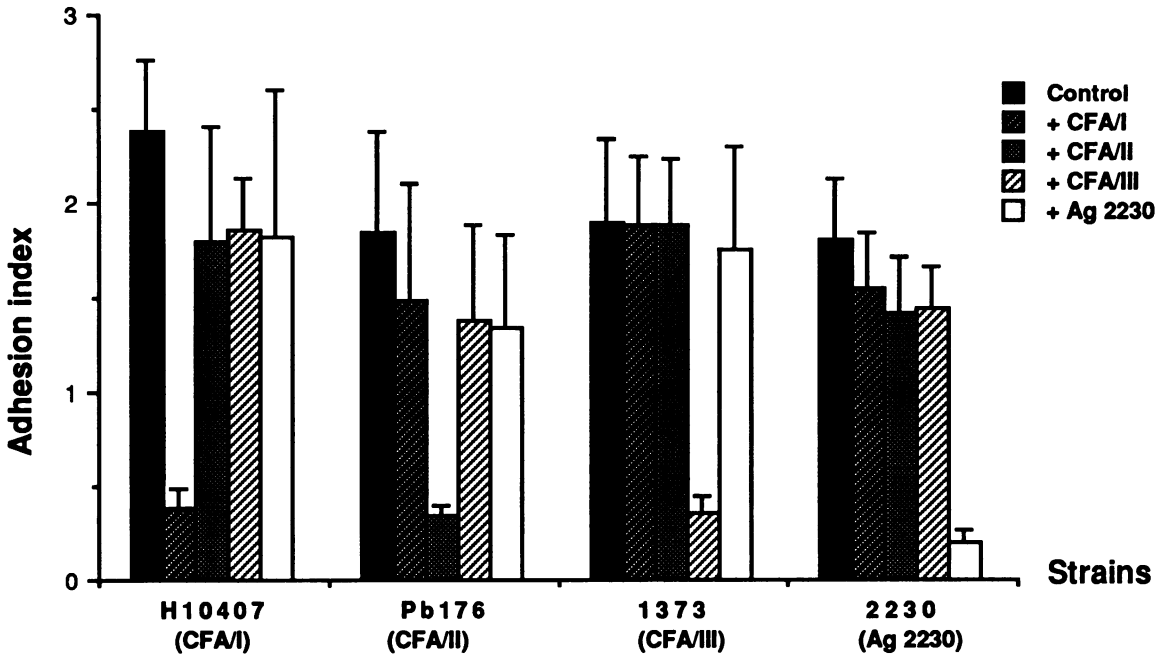

FIG. 5. Adhesion inhibition of ETEC strains to Caco-2 cells by purified adhesive factors. The adhesion index is the mean number of bacteria per cell determined in six separate experiments. Control indicates the adhesion index without pretreatment of Caco-2 cells with purified adhesive factors. Caco-2 cells were pretreated for 20 min at room temperature with the purified adhesive factors CFA/I, CFA/II, CFA/III, and antigen (Ag) 2230. Horizontal bars indicate standard deviations. Statistical analysis of adhesion indices was as follows: after pretreatment with nonhomologous adhesive factors, $P > 0.05$; after pretreatment with homologous adhesive factors, $P < 0.001$.

enteroinvasive *E. coli*, enterohemorrhagic *E. coli*, and enteroadherent *E. coli* strains (2, 15, 17, 23, 24) or K99-positive animal ETEC strains (J. P. Girardeau, personal communication). We performed adhesion assays with bacteria that were grown either in a tryptone broth, as described previously (2) for the adhesion assay for EPEC strains, or on CFA-agar, which is necessary for the adhesion of ETEC strains (16). Of the eight cell lines tested, ETEC strains only adhered to the Caco-2 cell line. This adhesion only occurred when bacteria were grown on CFA-agar. This was a very encouraging

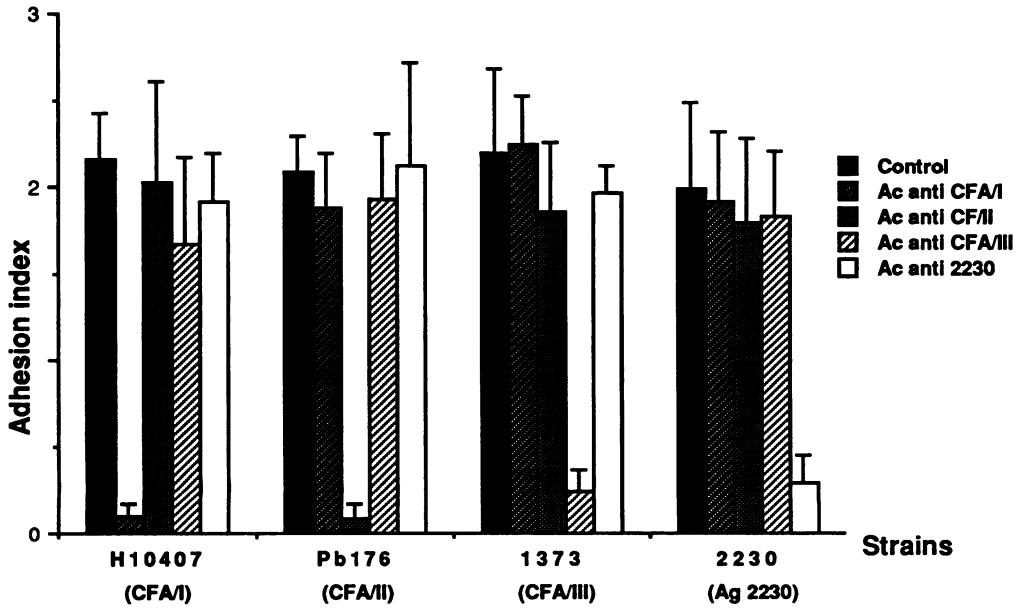

FIG. 6. Adhesion inhibition of ETEC strains to Caco-2 cells by antibodies raised against the adhesive factors. The adhesion index is the mean number of bacteria per cell determined in six separate experiments. Control indicates the adhesion index without pretreatment of Caco-2 cells with specific antibodies. Bacteria were pretreated for 20 min at room temperature with the specific antibodies anti-CFA/I, anti-CFA/II, anti-CFA/III, and anti-2230. Horizontal bars indicate standard deviations. Statistical analysis of adhesion indices was as follows: after pretreatment with nonhomologous antibodies, $P > 0.05$; after pretreatment with homologous antibodies, $P < 0.001$.

TABLE 3. Adhesion inhibition of ETEC strains to Caco-2 cells by homologous papain-treated antibodies

ETEC strain (antigen)	Adhesion index[a]		Adhesion index[a] of ETEC strains preincubated with:		
	Control[b]	Corresponding preimmune serum	Homologous papain-treated antibodies[c]	Papain + *p*-chloromercuribenzoic acid in PBS[d]	*p*-Chloromercuribenzoic acid in PBS
H10407 (CFA/I)	1.56 ± 0.36	1.67 ± 0.37	0.07 ± 0.05[e]	1.24 ± 0.33[f]	1.19 ± 0.10[f]
Pb176 (CFA/II)	1.84 ± 0.55	1.53 ± 0.43	0.11 ± 0.10	1.23 ± 0.18	1.35 ± 0.21
1373 (CFA/III)	1.72 ± 0.11	1.85 ± 0.40	0.22 ± 0.07	1.27 ± 0.36	1.32 ± 0.18
2230 (2230)	2.06 ± 0.54	1.79 ± 0.27	0.06 ± 0.03	1.70 ± 0.23	1.59 ± 0.33

[a] Values are means ± standard deviations.
[b] Adhesion index without pretreatment of bacteria.
[c] Antisera raised against ETEC adhesive factors were treated with papain for 4 h at 37°C; the reaction was stopped by the addition of *p*-chloromercuribenzoic acid.
[d] PBS, Phosphate-buffered saline.
[e] Statistical analysis, $P < 0.01$.
[f] No statistically significant difference between controls; for experiments with papain and *p*-chloromercuribenzoic acid and *p*-chloromercuribenzoic acid alone, $P > 0.05$.

result, because previously we have shown that ETEC adhesive factors are only expressed when bacteria are grown on a solid medium (A. Darfeuille-Michaud et al., in press). We thought that ETEC adhesive factor receptors might exist on Hutu 80 cells, because those cells come from a carcinoma of human duodenal epithelium. The absence of such receptors may be due to the lack of differentiation or the donor source of the Hutu 80 cells.

Caco-2 cells appear to be suitable for the study of the adhesive capacity of ETEC strains, even though this cell line is derived from a carcinoma of the epithelium of the colon instead of the small intestine. In postconfluent cultures, this monolayer exhibits structural and functional differentiation patterns characteristic of mature enterocytes in which the cell layer is covered by brush border microvilli. The adherence of ETEC strains to Caco-2 cells did not occur in the first 6 days of culture. However, clear adherence occurred on 15-day-old cultures, when the monolayers were postconfluent. Despite the colonic epithelial origin of this cell line, it acquires many small intestinal cell phenotypes, such as levels of alkaline phosphatase, sucrase-isomaltase, and aminopeptidase activities similar to those of small intestine enterocytes (22). Irrespective of the adhesion factor considered, the adhesion indices obtained with the Caco-2 cell line were close to those obtained in the in vitro adhesion assay with human enterocytes. The negative variants, which lost their ability to adhere to human enterocytes, did not adhere to the Caco-2 cell line. Thus, this adhesion seems to be specific and related to the presence of ETEC adhesive factors.

Adhesion of ETEC to Caco-2 cells closely resembles the diffuse adhesion pattern described for EPEC strains that adhere to HEp-2 or HeLa cells (23). In contrast to the EPEC strains, ETEC strain adhesion to Caco-2 cells only occurred when bacteria were grown on a solid medium. We demonstrated that, similar to the EPEC adhesion, there were a greater number of ETEC strains that adhered to Caco-2 cells after 3 h of incubation. Analysis of the distribution of the bacteria on the cell layer showed that there were some cell surface areas where the number of adhering bacteria was higher. This may have been due to differences in the maturation process of the cells and the subsequent density of ETEC receptors.

Electron microscopy of cross sections of the monolayer showed that the adhesion of ETEC strains to the brush border microvilli does not induce any lesion even after 3 h of incubation. In their study of EPEC adhesion, Knutton et al. (12, 13) showed that EPEC strains induce identical lesions of the microvilli of Caco-2 cells and human enterocytes, resulting in localized destruction of microvilli.

This work shows that pretreatment of Caco-2 cells with the purified adhesive factors competitively inhibits the adhesion of ETEC strains that possess the corresponding adhesive factor. The adhesion of ETEC strains was completely inhibited only when the cells were pretreated with their homologous adhesive factors. These results were confirmed by the adhesion inhibition assays with antibodies raised against each adhesive factor. Each antiserum only inhibited the adhesion of the ETEC strain that possessed the corresponding adhesive factor. It would seem, therefore, that there is a different receptor for each type of ETEC adhesive factor that was studied in this report. This result is in accordance with those obtained with the adhesive factors of uropathogenic *E. coli* that bind uroepithelial cells. Among the adhesive factors that have been described so far, they do not bind to the same receptor. For example, only P pili and Pap pili recognize the same receptor containing a galactose-α-(1-4)-digalactoside (1). S pili and M pili bind to neuraminidase acetyl-α-(2-3)-galactose-containing glycoconjugates and an amino acid sequence of glycophorine A, respectively (21, 28).

It would be interesting to test the binding ability to Caco-2 cells of the other adhesive factors that have been described, such as CFA/III of Honda et al. (9), PCF 8775 (27), PCF O159:H4 (25), and O148:H28 antigen (14), to determine whether there is a different cell receptor for each adhesive factor.

In conclusion, the Caco-2 cell line is suitable for use in the detection of ETEC strain adhesion. Furthermore, study of the receptors for the adhesive factors of ETEC strains requires a stable cell line. Previously, it was difficult to study these receptors with human or animal enterocytes. First, not all human enterocytes allow ETEC strains to adhere to them. Second, of the animal enterocytes, only those from rabbits can be used to detect the adhesion of ETEC strains. Furthermore, we have observed (A. Darfeuille-Michaud et al., in press) that the indices are highly significant by our in vitro enterocyte adhesion assay only when bacteria possess CFA/II.

In further studies on the Caco-2 cell line, we plan to determine whether all the different adhesive factors so far described in human ETEC strains bind to Caco-2 cells. Moreover, we propose to use this model to study the cell receptors that are involved in the binding of ETEC adhesive factors.

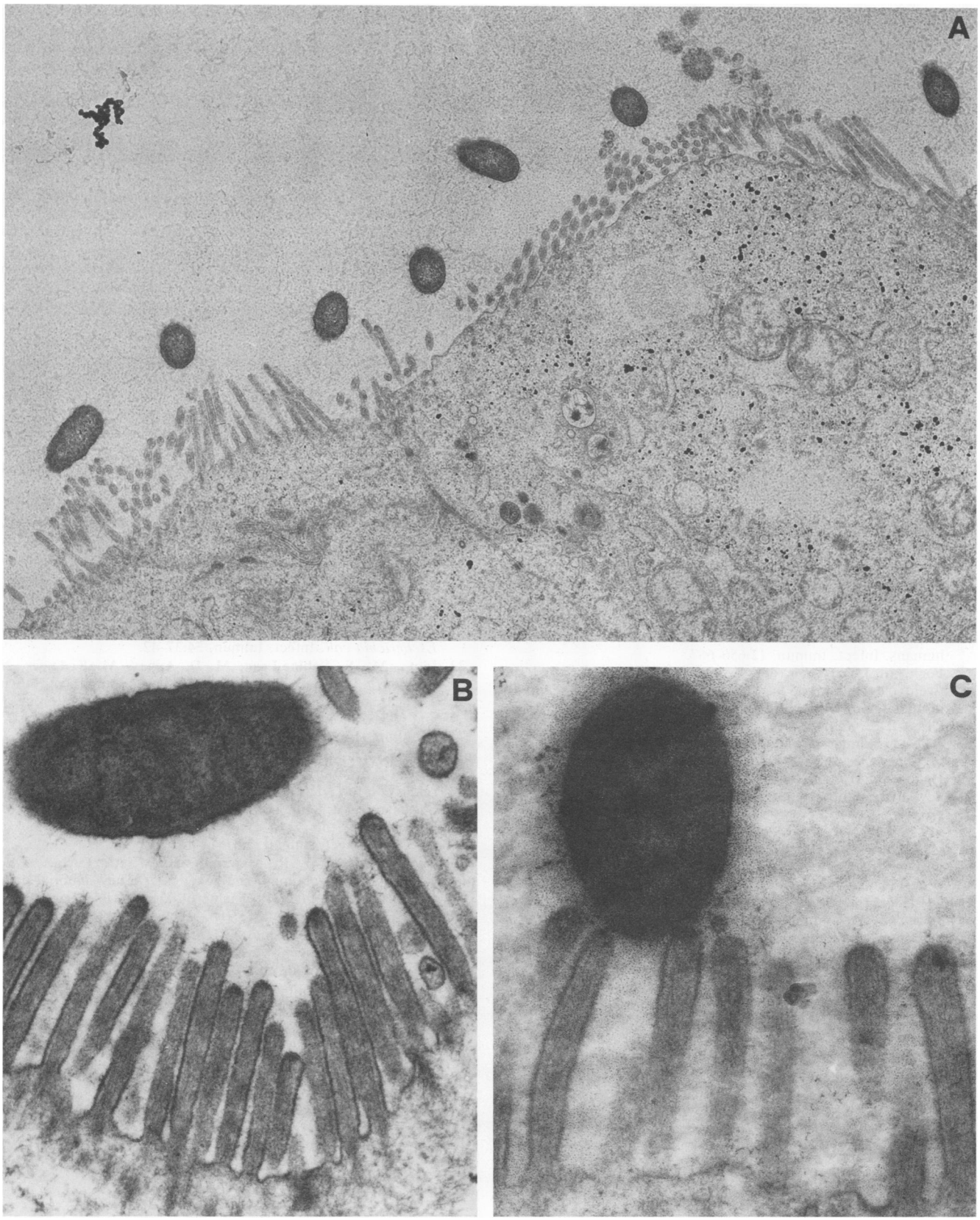

FIG. 7. Transmission electron micrographs showing the brush border-like microvilli of the Caco-2 cell line. When infected with ETEC strains H10407 (A and B) and 2230 (C), ETEC bacteria adhered to the Caco-2 cells and did not produce any lesion of the microvilli. Notice the difference in the adhesion between piliated and nonpiliated strains. The piliated strain (strain H10407) adhered to the microvilli probably by the tip of the fimbriae (B). The nonpiliated strain (strain 2230) was very close to the microvilli (C). Magnifications: ×11,500 (A); ×37,800 (B); ×54,000 (C).

ACKNOWLEDGMENTS

We thank the Virology Department and Henri Laveran for providing most of the tissue culture cells and Elisabeth Dussaulx from Unité Institut National de la Santé et de la Recherche Médicale 178 of Alain Zweibaum for providing the Caco-2 cell line. The technical assistance of Annie Fraisse, Monique Oron, and Josiane Payen in the electron microscopy assays is gratefully acknowledged. We also thank Rod Welch for kindly suggesting corrections in this manuscript.

LITERATURE CITED

1. **Bock, K., M. E. Breimer, A. Brignole, G. G. Hansson, K.-A. Karlsson, G. Larson, H. Leffler, B.-E. Samuelsson, N. Strömberg, C. Svanborg-Edén, and J. Thurin.** 1985. Specificity of binding of a strain of uropathogenic *Escherichia coli* to Galα(1-4)Gal-containing glycosphingolipids. J. Biol. Chem. **260**:8545–8551.
2. **Cravioto, A., R. J. Gross, S. M. Scotland, and B. Rowe.** 1979. An adhesive factor found in strains of *Escherichia coli* belonging to the traditional infantile enteropathogenic serotypes. Curr. Microbiol. **3**:95–99.
3. **Darfeuille, A., B. Lafeuille, B. Joly, and R. Cluzel.** 1983. A new colonization factor antigen (CFA/III) produced by enterotoxigenic *Escherichia coli* O128:B12. Ann. Microbiol. (Paris) **134A**: 53–64.
4. **Darfeuille-Michaud, A., C. Forestier, B. Joly, and R. Cluzel.** 1986. Identification of a nonfimbrial adhesive factor of an enterotoxigenic *Escherichia coli* strain. Infect. Immun. **52**: 468–475.
5. **Evans, D. G., and D. J. Evans.** 1978. New surface-associated heat-labile colonization factor antigen (CFA/II) produced by enterotoxigenic *Escherichia coli* of serogroups O6 and O8. Infect. Immun. **21**:638–647.
6. **Evans, D. G., R. P. Silver, D. J. Evans, D. G. Chase, and S. L. Gorbach.** 1975. Plasmid-controlled colonization factor associated with virulence in *Escherichia coli* enterotoxigenic for humans. Infect. Immun. **12**:656–667.
7. **Fogh, J., J. M. Fogh, and T. Orfeo.** 1977. One hundred and twenty seven cultured human tumor cell lines producing tumors in nude mice. J. Natl. Cancer Inst. **59**:221–226.
8. **Grasset, E., J. Bernabeu, and M. Pinto.** 1985. Epithelial properties of human colonic carcinoma cell line Caco-2: effect of secretagogues. Am. J. Physiol. **248**(5, Part 1):C410–C418.
9. **Honda, T., M. Arita, and T. Miwatani.** 1984. Characterization of new hydrophobic pili of human enterotoxigenic *Escherichia coli*: a possible new colonization factor. Infect. Immun. **43**: 959–965.
10. **Isaacson, R. E., B. Nagy, and H. W. Moon.** 1977. Colonization of porcine small intestine by *Escherichia coli*: colonization and adhesion factors of pig enteropathogens that lack K88. J. Infect. Dis. **135**:531–539.
11. **Jones, G. W., and J. M. Rutter.** 1974. The association of K88 with hemagglutinating activity in porcine strains of *Escherichia coli*. J. Gen. Microbiol. **84**:135–144.
12. **Knutton, S., T. Baldwin, P. H. Williams, and A. S. McNeish.** 1989. Actin accumulation at sites of bacterial adhesion to tissue culture cells: basis of a new diagnostic test for enteropathogenic and enterohemorrhagic *Escherichia coli*. Infect. Immun. **57**: 1290–1298.
13. **Knutton, S., D. R. Lloyd, and A. S. McNeish.** 1987. Adhesion of enteropathogenic *Escherichia coli* to human intestinal enterocytes and cultured human intestinal mucosa. Infect. Immun. **55**:69–77.
14. **Knutton, S., D. R. Lloyd, and A. S. McNeish.** 1987. Identification of a new fimbrial structure in enterotoxigenic *Escherichia coli* (ETEC) serotype O148:H28 which adheres to human intestinal mucosa: a potentially new human ETEC colonization factor. Infect. Immun. **55**:86–92.
15. **Knutton, S., P. H. Williams, D. R. Lloyd, D. C. A. Candy, and A. S. McNeish.** 1984. Ultrastructural study of adherence to and penetration of cultured cells by two invasive *Escherichia coli* strains isolated from infants with enteritis. Infect. Immun. **44**:599–608.
16. **Lafeuille, B., A. Darfeuille, S. Petit, B. Joly, and R. Cluzel.** 1981. Etude de l'attachement aux entérocytes humains in vitro de *Escherichia coli* isolés de selles diarrhéiques chez l'enfant. Ann. Microbiol. (Paris) **132B**:57–67.
17. **Mathewson, J. J., P. C. Johnson, H. L. Dupond, D. R. Morgan, S. A. Thornton, L. V. Wood, and C. D. Ericsson.** 1985. A newly recognised cause of traveler's diarrhea: enteroadherent *Escherichia coli*. J. Infect. Dis. **151**:471–475.
18. **Morris, J. A., C. Thorns, A. C. Scott, W. J. Sojka, and G. A. Wells.** 1982. Adhesion in vitro and in vivo associated with an adhesive antigen (F41) produced by a K99 mutant of the reference strain *Escherichia coli* B41. Infect. Immun. **36**:1146–1153.
19. **Nataro, J. P., J. B. Kaper, R. Robins-Browner, V. Prado, P. Vial, and M. M. Levine.** 1987. Patterns of adherence of diarrheagenic *Escherichia coli* to Hep-2 cells. Pediatr. Infect. Dis. J. **6**:829–831.
20. **Orskov, I., F. Orskov, H. W. Smith, and W. J. Sojka.** 1975. The establishment of K99, a thermolabile, transmissible *Escherichia coli* K antigen, previously called "Kco", possessed by calf and lamb enteropathogenic strains. Acta Pathol. Microbiol. Scand. Sect. B **83**:31–36.
21. **Parkkinen, J., G. N. Rogers, T. K. Korhonen, W. Dahr, and J. Finne.** 1986. Identification of the O-linked sialyloligosaccharides of glycophorin A as the erythrocyte receptors for S-fimbriated *Escherichia coli*. Infect. Immun. **54**:37–42.
22. **Pinto, M., S. Robine-Leon, M. D. Appay, M. Kedinger, N. Triadou, E. Dussaulx, B. Lacroix, P. Simon-Assmann, K. Haffen, J. Fogh, and A. Zweibaum.** 1983. Enterocyte-like differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture. Biol. Cell. **47**:323–330.
23. **Scaletsky, I. C., M. L. M. Silva, and L. R. Trabulsi.** 1984. Distinctive patterns of adherence of enteropathogenic *Escherichia coli* to Hela cells. Infect. Immun. **45**:534–536.
24. **Sherman, P., R. Soni, M. Petric, and M. Karmali.** 1987. Surface properties of the Vero cytotoxin-producing *Escherichia coli* O157:H7. Infect. Immun. **55**:1824–1829.
25. **Tacket, C. O., D. R. Maneval, and M. M. Levine.** 1987. Purification, morphology, and genetics of a new fimbrial putative colonization factor of enterotoxigenic *Escherichia coli* O159:H4. Infect. Immun. **55**:1063–1069.
26. **Thomas, L. V., A. Cravioto, S. M. Scotland, and B. Rowe.** 1982. New fimbrial antigenic type (E8775) that may represent a colonization factor in enterotoxigenic *Escherichia coli* in humans. Infect. Immun. **35**:1119–1124.
27. **Thomas, L. V., M. M. McConnell, B. Rowe, and A. M. Field.** 1985. The possession of three novel coli surface antigens by enterotoxigenic *Escherichia coli* strains positive for the putative colonization factor PCF8775. J. Gen. Microbiol. **131**:2319–2326.
28. **Väisänen, V., T. K. Korhonen, M. Jokinen, C. G. Gahmberg, and C. Ehnholm.** 1982. Blood group M specific haemagglutinin in pyelonephritic *Escherichia coli*. Lancet **i**:1192–1194.