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Attachment of *Escherichia coli* via Mannose- or Galα1→4Galβ-Containing Receptors to Human Colonic Epithelial Cells

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The role of bacterial adhesion for the maintenance of the large-intestinal microflora has not been established. In this study, colonic cells from the adenocarcinoma cell line HT-29 or from surgical specimens were tested for the ability to bind *Escherichia coli*. The *E. coli* strains were manipulated by transformation or by mutagenesis to express either mannose-specific type 1 fimbriae (strains 506 MS and HU742) or Galα1→4Galβ-specific P fimbriae (506 MR and HU824). Binding to HT-29 cells was seen with strains of either receptor specificity and was inhibited by α-methyl mannoside or globotetraosylceramide (GalNAcβ1→3Galα1→4Galβ1→4Glc-ceramide), respectively. The Galα1→4Galβ-specific strains interacted with a loosely surface-associated substance, which was sensitive to mechanical treatment and incubation at 37°C, while the mannose-specific strains bound both directly to the cell and to the loosely associated substance. Isolated colonic epithelial cells bound the mannose-specific bacteria in high numbers, while the attachment of the Galα1→4Galβ-specific strains depended on the elution method. Cells eluted sequentially with magnetic stirring were unable to bind the Galα1→4Galβ-specific bacteria, while elution by a more gentle method resulted in binding of these strains to material loosely associated with the epithelial cells. Thus, the binding pattern of isolated colonic epithelial cells paralleled that of the HT-29 cell line. Conceivably, binding to mannose- and Galα1→4Galβ-containing receptors could contribute to the maintenance of *E. coli* in the human large intestine.

Pathogenic *Escherichia coli* strains frequently express adhesins specific for host cell receptors. Thus, adhesins recognizing the Galα1→4Galβ moiety of the globoseries glycolipids are expressed by pyelonephritic isolates (10), while S fimbriae recognizing terminal sialyl residues are common among strains causing neonatal sepsis or meningitis (12). For enterotoxigenic *E. coli* strains, attachment to epithelial cell receptors is a prerequisite for the colonization of the small intestine as well as for the diarrhogenic effect (13).

The role of bacterial adhesion for the maintenance of the commensal large-intestinal microflora is, in contrast, less well studied. Biopsy specimens from human colonic mucosa contain stably associated *E. coli*, suggesting a role for adhesion to enterocytes or mucus in colonization (7). A majority of *E. coli* strains of fecal origin express mannose-specific adhesins in culture (6), and about 10% bind to Galα1→4Galβ-containing receptors (16). These specificities may, conceivably, mediate attachment to the human colonic epithelium or to mucus.

The human colonic carcinoma cell line HT-29 is morphologically well differentiated (11) and displays markers of mature colonic enterocytes, including the ability to express secretory component (8). This cell line was used to study the adherence of *E. coli* strains genetically manipulated to express adhesins with mannose or Galα1→4Galβ receptor specificity. The relevance of attachment to the cell line was verified by comparison with attachment to human colonic epithelial cells eluted from surgical specimens.

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MATERIALS AND METHODS

Bacteria. Two sets of *E. coli* strains with defined adhesins were used: transformants of a nonattaching strain and mutants of a wild-type strain.

(i) Transformants. The *E. coli* strains of the 506 series were derived by transformation of the nonadherent fecal isolate *E. coli* 506 (5). 506 MS had received a plasmid containing an 8-megadalton chromosomal restriction fragment inserted into the tetracycline site of the vector pACYC184. The insert contained the *pil* region coding for the synthesis and assembly of mannose-specific type 1 fimbriae (9). 506 MR had received pACYC184, with a 6.7-megadalton chromosomal restriction fragment inserted into the chloramphenicol site. This fragment contained the *pap* region encoding synthesis and assembly of P fimbriae specific for Galα1→4Galβ-containing receptors. The control strain 506 P received the vector without insertions. In order to select for plasmid-containing clones, 506 P and 506 MS were maintained on tryptic soy agar containing chloramphenicol (20 μg/ml) and 506 MR was maintained on agar containing tetracycline (10 μg/ml). All strains of the 506 family shared the O19,22K5H− serotype, and a number of other genetical markers (17).

(ii) Mutants. *E. coli* 824 and 742 are mutants of the pyelonephritic isolate GR-12, which expresses adhesins for mannose as well as for Galα1→4Galβ (5). Mutations were induced by treatment with nitrous acid and *N*-methyl-*N*-nitro-*N*-nitrosoguanidine, and mutants with either adhesin specificity were selected. The mutant HU742 expressed type 1 fimbriae specific for mannosides, while the mutant HU824 expressed P fimbriae specific for the Galα1→4Galβ oligosaccharide sequence. Apart from the difference in adhesive specificity, all other markers tested were shared by the mutants, including the serotype O75K5H− (17). To maxi-

mize fimbrial expression, HU824 was cultured on tryptic soy agar and HU742 was passaged on static Luria broth.

Hemagglutination properties of the bacteria. In parallel with each adhesion experiment, the receptor specificities of the bacteria were confirmed by hemagglutination. A drop of each bacterial suspension was mixed on a microscope slide with a 3% suspension of human AP_1 or guinea pig erythrocytes. The erythrocytes were suspended in either phosphate-buffered saline (PBS) or 2.5% α-methyl mannoside. Agglutination was read by the naked eye after gentle tilting of the slide for 3 min. Agglutination of guinea pig erythrocytes in the absence but not in the presence of α-methyl-mannoside was defined as a mannose-sensitive hemagglutination, while agglutination both in the presence and in the absence of mannose was defined as mannose resistant.

Intestinal epithelial cell line. The human adenocarcinoma cell line HT-29 (3) (kindly supplied by J. Mestecky, University of Alabama, Birmingham) was cultured in flat-bottom 80-cm^2 tissue culture flasks in Eagle medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 50 μg of gentamicin per ml. Cell cultures were split 1:4 weekly after detachment of the cells with trypsin (1 g of Difco 1:250 trypsin [Difco Laboratories, Detroit, Mich.] per liter) in buffered EDTA solution (2 g of disodium EDTA [Titriplex III; Kebo, Göteborg, Sweden] per liter in PBS).

The HT-29 cells were used in the adhesion assay when the cell layer had reached confluence (at least 5 days after the last passage). In the standard procedure, the cells were detached by EDTA treatment. The monolayer was gently rinsed twice with buffered EDTA solution, and the flasks were incubated over a 37°C water bath until the cells began to detach. The cells were disaggregated by blowing Hanks balanced salt solution (HBSS) forcefully with a pipette over the monolayer. The suspension of detached cells was spun down at 300 × *g* for 5 min and resuspended in HBSS.

Alternatively, the cells were removed by mechanical detachment by scraping the bottom of the flask with a rubber policeman. This method yielded large aggregates which were removed by sedimentation for 10 min. The cell suspension was washed and resuspended in HBSS as described above.

Adhesion assay. The bacteria were harvested from the agar plates with PBS or spun down in the broth and then washed once. The dose-response relationship between bacterial concentration and attachment was tested by using bacterial concentrations ranging from 10^7 to 10^{10}/ml. Binding was recorded when the cells were incubated with bacteria in concentrations from approximately 4×10^8 bacteria per ml (data not shown). For most experiments, the concentration of bacteria was adjusted to 5×10^9/ml in HBSS (2× optical density 1.5 at 597 nm) (Vitatron; Hugo Tillqvist, Göteborg, Sweden).

The epithelial cell suspension was adjusted to 5×10^6 cells per ml. For adherence, 0.1 ml of the cell suspension was mixed with 0.1 ml of bacteria and 0.3 ml of HBSS. After the incubation, 10 ml of cold PBS was added, and the cells were spun down at 300 × *g* for 5 min. The supernatant which contained unattached bacteria was discarded, and the cells were suspended in a minimal volume of HBSS. Two drops of neutral buffered Formalin (Histofix; Histolab, Göteborg, Sweden) were added, and the cells were examined in an interference-contrast microscope (Ortolux II, with interference-contrast equipment T; Leitz, Federal Republic of Germany) at 500× magnification. The number of attached bacteria to each of at least 40 cells was determined. The adherence value for one experiment was defined as the mean number of adhering bacteria per cell and was thus based on the observation of 40 epithelial cells.

Different incubation times (1, 5, 10, and 30 min) and temperatures (4, 22, or 37°C) were tested. Incubation at 4°C during 30 min with end-over-end rotation gave good binding and was used as the standard procedure.

For adhesion inhibition, 0.3 ml of either 2.5 mM α-methyl mannoside or 200 μg of globotetraosylceramide (GalNAcβ1→3Galα1→4Galβ1→4Glc-ceramide) per ml, both dissolved in HBSS, replaced the 0.3 ml of HBSS in the adhesion mixture. The globotetraosylceramide was isolated from human erythrocytes as previously described (10).

Isolation of human colonic epithelial cells. Colon specimens were obtained from patients undergoing colon resection because of carcinoma. A piece of colon distant from the dysplastic site was selected. Fat and remaining mesenterium were removed by dissection, and the mucosa was carefully freed from the muscular layer and cut into pieces of approximately 0.5 by 1 cm by using a scalpel blade.

Two different methods were used to remove the epithelial cells from the underlying mucosal tissue. The sequential elution method was used for two specimens. In this method, all incubations were performed at room temperature with magnetic stirring in a sterile flask. To remove mucus, the mucosal pieces were first incubated twice for 5 min with a 10 mM solution of dithiothreitol (Sigma Chemical Co., St. Louis, Mo.) in HBSS and the supernatants were discarded. Subsequently, the epithelium was removed by incubation with Weiser B solution containing 137 mM NaCl, 3 mM KCl, 1.5 mM KH_2PO_4, 6.5 mM Na_2HPO_4, and 1.5 mM EDTA but excluding the dithiothreitol included in the original buffer (19). The first two 5-min fractions were discarded; thereafter, two 30-min fractions were collected. The cells were pooled, washed twice with HBSS, and adjusted to 5×10^6 epithelial cells per ml. Adhesion was performed with these cells as with the detached HT-29 cells (see above).

A milder one-step preparation method was applied on the remaining surgical specimens. The dissected mucosa was cut with a scalpel blade into pieces of equal size, which were then put into 10-ml tubes. Bacteria were suspended at 2×10^9/ml in cold Weiser B solution containing 1.5 mM dithiothreitol. The bacterial suspension was added to the tubes, and these were incubated with end-over-end rotation at 4°C for 1 h. Detached cells, with or without adhering bacteria, were spun down, washed once with cold HBSS, and fixed with a few drops of buffered Formalin.

Statistical method. The mean adherence to 40 epithelial cells from one individual experiment constituted the unit of observation. Means and standard errors (as presented in the text and in Table 1) were calculated on the basis of repeated experiments where n = the number of experiments performed under identical conditions.

For inhibition testing (Table 2), the level of inhibition in one individual experiment constituted the unit of observation. Student's t test for paired values was used for significance testing.

RESULTS

Attachment to HT-29 cells. *E. coli* strains with either Galα1→4Galβ or mannose receptor specificity were able to attach to the HT-29 epithelial cell line as shown in Table 1. The specificity of this attachment was shown in two ways. (i) Transformation with the DNA sequence required for expression of Galα1→4Galβ- or mannose-specific adhesins conferred adhesive capacity. The parental strain transformed

TABLE 1. Adhesion to HT-29 cells and hemagglutination pattern of *E. coli* mutant and transformant strains

Bacterial strain	Receptor specificity	Hemagglutination pattern[a]		Mean no. of bacteria/cell (SEM)
		Human	Guinea pig	
Transformants				
506 MR	Galα1→4Galβ	MR		20 (3.7)[b]
506 MS	Mannose		MS	39 (6.4)[b]
506 P				0.8 (0.25)[c]
Mutants				
HU824	Galα1→4Galβ	MR		22 (8.5)[c]
HU742	Mannose		MS	43 (7.8)[c]

[a] MR, mannose-resistant hemagglutination (agglutination in the presence of 2.5 mM α-methyl mannoside); MS, mannose-sensitive hemagglutination (agglutination in the absence but not in the presence of 2.5 mM α-methyl mannoside).
[b] Result based on seven experiments.
[c] Result based on three experiments.

with the vector only, 506 P, did not attach to the HT-29 cells despite its hydrophobic surface properties (18). The pattern of attachment of the transformants was parallel to that of the mutants with Galα1→4Galβ- or mannose-specific adhesins. (ii) Adhesion to the HT-29 cells was specifically inhibited by soluble receptor analogs. Mannose practically abolished the binding of 506 MS and HU742, while globoside reduced the attachment of the Galα1→4Galβ-specific strains 506 MR and HU824 (Table 2). Conversely, mannose had no effect on the attachment of 506 MR and HU824 (102 and 97% of control, respectively), and globoside did not influence binding of the mannose-specific strains 506 MS and HU742 (108 and 100% of control, respectively).

The morphological patterns of adhesion showed interesting differences depending on the adhesin specificity of the bacterial strain. The strains with mannose-specific adhesins, 506 MS and HU742, attached strongly and uniformly to all epithelial cells in the suspension (Fig. 1A). In contrast, the attachment of the *E. coli* strains with Galα1→4Galβ-specific adhesins, 506 MR and HU824, varied drastically between individual epithelial cells, cells with 100 bacteria attached being found adjacent to cells free from bacteria (Fig. 1B). The Galα1→4Galβ-containing receptor thus seemed to be unevenly distributed among cells. Moreover, the distribution of bacteria on the surface of the individual cell varied depending on adhesive specificity. The Galα1→4Galβ-specific bacteria were found in clusters that often reached out distantly from the epithelial cell border, while the cell surface could be free of bacteria. This pattern gave the impression that the bacteria bound to receptors that were not located in the cell membrane but rather in an extracellular substance loosely associated with the HT-29 cells. Bacterial clusters reaching out from the cell were also found with the mannose-specific bacteria 506 MR and HU742, but in this case also, the cell surface was covered with bacteria. This pattern can be compared with the Galα1→4Galβ-specific adhesion of *E. coli* to urinary tract epithelial cells, in which no binding to material outside the cell border is observed (Fig. 1C).

In the standard procedure, the HT-29 cells were detached from the tissue culture flasks by EDTA treatment. If, instead, the HT-29 cells were mechanically removed, binding of the Galα1→4Gal-specific strains was reduced by 73% (standard error of the mean, 13%; three experiments; $P < 0.05$), while no effect was noted for the mannose-specific strains.

The bacterial attachment was dependent on the temperature at which the adherence assay was performed. Thus, at 4°C both 506 MR and 506 MS attached in high numbers, while at 22°C a lower degree of binding was recorded, and at 37°C the binding of 506 MR was essentially abolished (Fig. 2A), while that of 506 MS was further reduced compared with binding at 22°C (Fig. 2B).

Attachment of bacteria to epithelial cells isolated from surgical colon specimens. The two methods used to elute epithelial cells from colonic mucosal specimens gave different results with regard to bacterial adhesion. In the first method, cells were eluted sequentially with magnetic stirring in EDTA-containing buffer and washed repeatedly before the addition of bacteria. Both mature enterocytes and crypt epithelium prepared by this method bound the mannose-specific strains 506 MS and HU742 in high numbers (Fig. 3A). In contrast, the Galα1→4Galβ-specific bacteria 506 MR and HU824 did not adhere (Fig. 3B). Also, no binding was recorded by using the parental strain 506 P (data not shown).

In view of the degradation of HT-29 cell receptor substance by mechanical treatment and elevated temperature, a more gentle method was applied to the following specimens. Elution and bacterial adherence were performed simultaneously at 4°C, avoding magnetic stirring. The mannose-specific strains 506 MS and HU742 bound in high numbers to cells eluted in this way (Fig. 3C). Cells eluted by the one-step method also showed binding of the Galα1→4Galβ-specific strains 506 MR and HU824 (Fig. 3D). The attachment resembled that to the HT-29 cells morphologically, in that the bacteria appeared to bind to a substance loosely associated with the cell surface. The parental strain 506 P did not adhere under the same conditions (Fig. 3E).

TABLE 2. Inhibition of adhesion to HT-29 cells by receptor analogs

Bacterial strain	No. of bacteria/cell in indicated expt without (−) or with (+) inhibitor[a]								% Reduction in expt.				*P*
	−				+								
	1	2	3	4	1	2	3	4	1	2	3	4	
Galα1→4Galβ-specific													
506 MR	12	24	22	32	2.5	19	13	15	79	21	41	53	<0.05
HU824	42	4.9	15	28	9	2.1	4.2	11	79	57	72	61	<0.001
Mannose-specific													
506 MS	25	35	29	42	1	2	1	1	98	94	97	98	<0.001
HU742	32	23	35	21	1	3	1	1	98	87	97	95	<0.001

[a] HT-29 cells (5×10^6/ml) and bacteria (5×10^9/ml) were incubated for 30 min at 4°C in the presence of 120 μg of globoside per ml (for the Galα1→4Galβ-specific strains) or 15 mg of α-methyl mannoside per ml (for the mannose-specific strains).

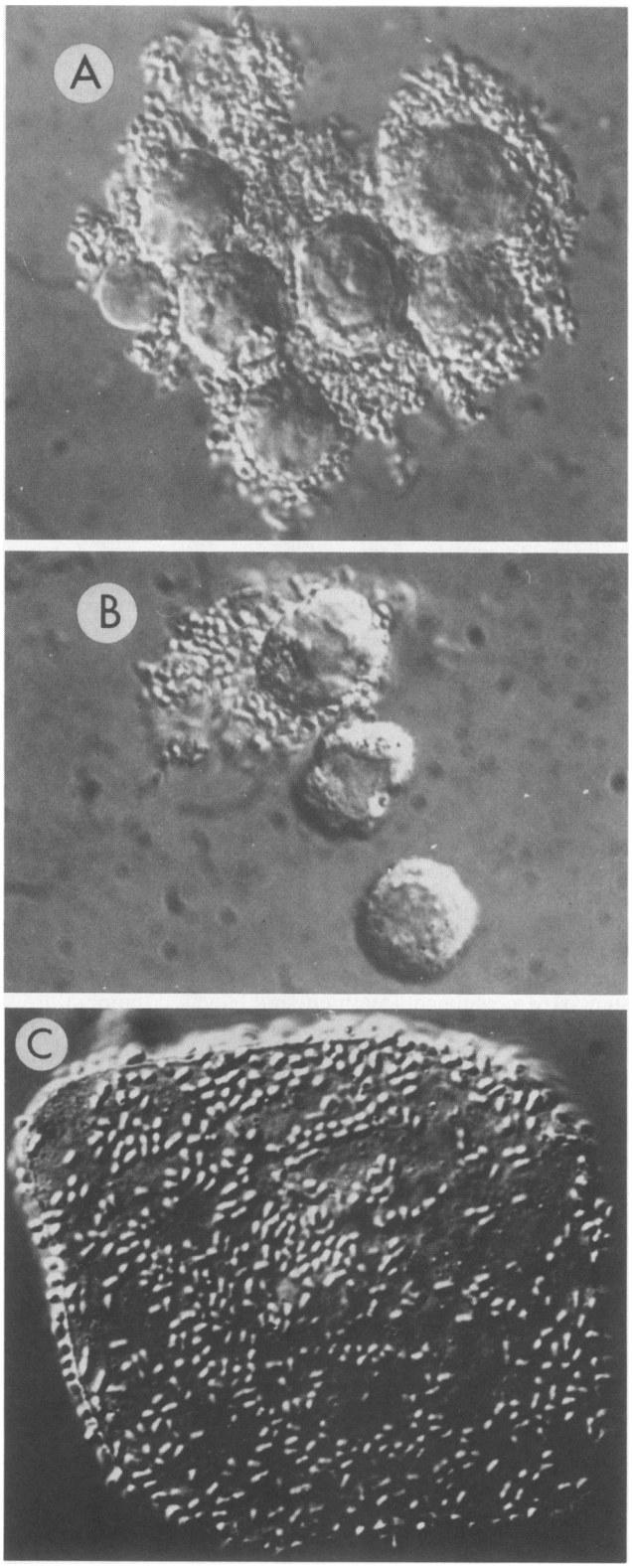

FIG. 1. Patterns of adhesion of *E. coli* strains to HT-29 cells and urinary tract epithelial cell. Bacteria attached to top surface of the HT-29 cells cannot be seen since they are not in the plane of focus. (A) 506 MS binding strongly to all HT-29 cells present. (B) 506 MR binding in high numbers to the upper but not to the two lower HT-29 cells. In panels A and B, bacteria adhere in clusters reaching out from cell borders. Cell concentration, 5×10^6; bacteria, 5×10^9; incubation, 30 min at 4°C; magnification, ×1,250. (C) Urinary tract epithelial cell with Galα1→4Galβ-specific *E. coli* 36692 adhering uniformly to the surface. No bacterial clusters reaching out from cell borders are present. The adherence assay was performed as previously described (15). Magnification, ×1,088. A Leitz interference-contrast microscope was used.

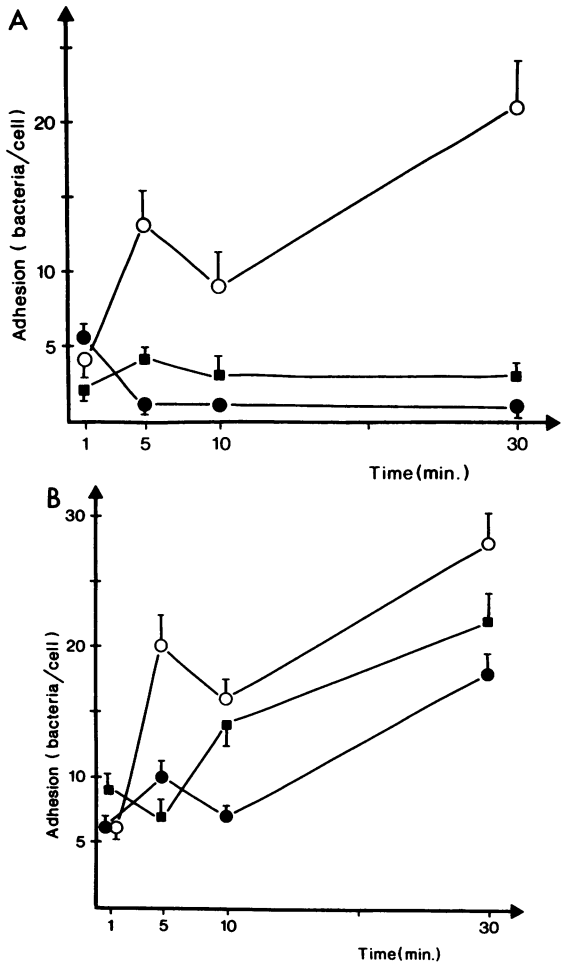

FIG. 2. Influence of incubation time and temperature on the adhesion to HT-29 cells by 506 MR (A) and 506 MS (B). The cell concentration was 5×10^6, and the bacterial concentration was 5×10^9. The figure depicts one experiment in which 40 cells were examined for the number of attached bacteria. The means and standard errors (indicated by bars) for each incubation time and temperature were calculated by using $n = 40$. Symbols: ○, 4°C; ■, 22°C; ●, 37°C.

The influence on bacterial attachment of dithiothreitol, which was used to remove mucus from the mucosal specimens, was tested by using HT-29 cells. The presence of 10 mM dithiothreitol during the whole attachment procedure did not decrease bacterial attachment (506 MR, 129% of control; 506 MS, 116% of control).

DISCUSSION

The human colonic adenocarcinoma cell line HT-29 as well as colonic epithelial cells isolated from surgical speci-

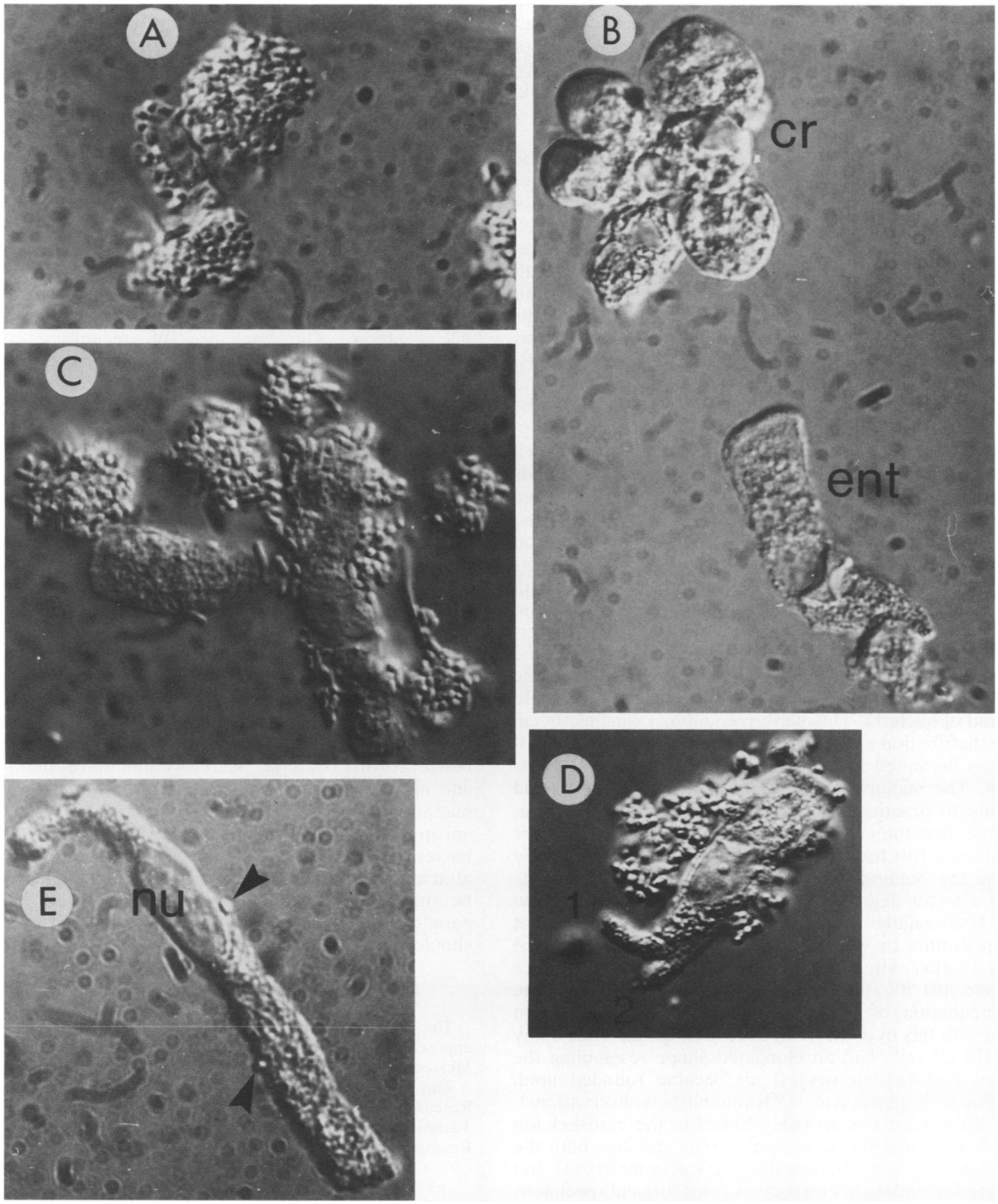

FIG. 3. Pattern of attachment of *E. coli* strains to enterocytes isolated from surgical specimens. (A) 506 MS attaching strongly to a mature enterocyte (folded) and to a crypt epithelial cell obtained by sequential elution. (B) Lack of binding of 506 MR to enterocyte (ent) and crypt epithelium (cr) obtained by the sequential elution method. (C) Binding of HU742 to a mature enterocyte and to crypt cells eluted by the one-step method. (D) Binding of 506 MR to material associated with enterocytes eluted by the one-step method. The micrograph shows one enterocyte (labeled 1) placed on the top of another (labeled 2). (E) Negligible binding of 506 P to mature enterocytes eluted by the one-step method. Two bacteria are attached (arrowheads), one on the left cell border near the nucleus (nu) and one on the right border between the nucleus and the brush border. Magnifications, ×1,250 (A, B, C, and E) and ×1,000 (D).

mens was shown to bind *E. coli* with mannose- or Galα1→4Galβ-specific adhesins. The specificity of the attachment was supported by parallel binding of *E. coli* mutants and transformants sharing receptor specificity but differing in other surface properties and by the absence of binding of the parental strain 506 P that lacks any known adhesins. Furthermore, attachment to the HT-29 cells could be blocked by the soluble receptor analogs globoside and α-methyl mannoside.

The Galα1→4Galβ-containing receptors seemed to be located exclusively in a surface-associated substance rather than in the cell membrane. The mannose-containing receptors, on the other hand, seemed to be present both in the cell membrane and in extracellular substance. This interpretation was based on the following observations. (i) The evaluation of bacterial attachment by interference-contrast microscopy permitted visualization of different aspects of cells by changing the plane of focus. Bacteria of both specificities were seen to adhere in clusters protruding from the cells, implying bacterial binding to material anchored to and reaching out from the cell membrane. In addition, the mannose-specific bacteria covered the cell surface evenly, while the Galα1→4Galβ-specific strains often left the cell surface clean of bacteria. (ii) Harvesting the HT-29 cells from tissue culture by mechanical means sharply reduced the binding of the Galα1→4Gal-specific strains despite the presence of morphologically intact cells in the suspension that were able to bind the mannose-specific strains. This suggests that extracellular substance produced by the cells in tissue culture was recovered together with the cells if these were removed by EDTA treatment but that most of this material remained attached to the tissue culture flask if the cells were scraped off. (iii) The Galα1→4Galβ-specific strains bound avidly to some cells, while adjacent cells could be devoid of bacteria. This pattern could be explained by an uneven distribution of extracellular material when the monolayer was disrupted in the preparation of a single-cell suspension. The mannose-specific strains, in contrast, bound uniformly to practically all of the cells in the suspension, suggesting that the cell surface too contained receptors for these strains. (iv) Incubation at 37°C was shown to greatly decrease the binding of the Galα1→4Galβ-specific strains and, to a lesser degree, of the mannose-specific strains to HT-29. Extracellular substance could have been degraded at this temperature by enzymes from bacteria or from injured epithelial cells. Alternatively, the extracellular substance may have lost its anchorage points in the cell membrane upon incubation of the cells in suspension at 37°C. In keeping with this hypothesis was the finding that while many of the HT-29 cells had an elongated shape resembling the shape of mature enterocytes, all became rounded upon incubation in suspension at 37°C (unpublished observations). Extracellular matrix is probably linked to the cytoskeleton by cell-bound proteoglycans, which thus stabilize both the cell morphology and organize the glycocalyx matrix (4). (iv) The bacterial binding to enterocytes from surgical specimens paralleled that to HT-29 cells in sensitivity to mechanical treatment. While the mannose-specific strains were able to attach avidly to cells eluted sequentially from the surgical specimens, cells eluted by this method showed no binding of the Galα1→4Galβ-specific strains. Binding of Galα1→4Gal β-specific strains to colonic epithelial cells could, however, be demonstrated by using a more gentle one-step method with all incubations carried out in the cold and all mechanical treatment during elution being avoided.

In comparison, binding of Galα1→4Galβ-specific *E. coli* to receptors in the urinary tract epithelium showed quite a different pattern. Bacterial clusters reaching out from the cells were not seen, and attachment was increased at 37°C as compared with that at 4°C and not decreased by repeated washes (15). While the Galα1→4Galβ-containing receptors of urinary epithelial cells belong to the globoseries of glycolipids, the chemical nature of the Galα1→4Galβ-containing receptors in colonic mucosa remains to be defined. Trace amounts of glycolipids migrating in thin-layer chromatography as globotetraosylceramide and globotriaosylceramide are present in colonic epithelial cells (J. Holgersson, N. Strömberg, and M. Breimer, Biochimie, in press), but whether these function as receptors for Galα1→4Galβ-specific *E. coli* is not known.

If extracellular material from colonic epithelial cells is continuously produced and shed into the bowel lumen, it could form the ecological niche of many commensal intestinal bacteria. The presence of receptors containing Galα1→4Galβ in this material might thus increase intestinal colonization with strains expressing the corresponding adhesins. These strains could secondarily colonize the urinary tract where Galα1→4Galβ-containing receptors are also present.

Binding of mannose-specific strains to guinea pig and human colonic epithelial cells obtained by scraping of the mucosa was shown already in 1957 in the pioneering work of Duguid and Gillies (1). A receptor for mannose-specific *E. coli* appearing both in the epithelial brush border membrane and in crude intestinal mucus from mice has recently been described (18a). Mannose-specific type 1 fimbriae are expressed by a wide range of enteric bacteria, both by commensal bacteria such as *E. coli*, *Klebsiella* spp., and *Enterobacter* spp. and by pathogens such as *Salmonella* spp. and *Shigella* spp., indicating an advantage for the bacteria to possess these adhesins (2). However, the ability to attach to mannose-containing receptors may not be of unequivocal benefit for the bacteria. Secretory immunoglobulin A binds and agglutinates mannose-specific *E. coli* by virtue of its mannose-containing oligosaccharide chains (14, 20), a mechanism which should reduce the survival of type 1 fimbriated bacteria. Owing to this complex balance, correlation of attachment with persistence of commensal bacteria needs to be studied epidemiologically. The HT-29 cell line, which parallels human colonic cells for two binding specificities, should prove useful for such studies.

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