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Adherence of an Enterotoxigenic *Escherichia coli* Strain, Serotype O78:H11, to Purified Human Intestinal Brush Borders

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The human enterotoxigenic *Escherichia coli* pathogen designated H10407 expresses two different types of surface pili, one designated type 1 pili and the other designated colonization factor antigen I (CFA/I). CFA/I pili are thought to promote the adherence of H10407 to the mucosa of the human small bowel. H10407 was grown under conditions which promoted the expression of either type 1 pili or CFA/I pili, and in each case, the adherence of H10407 to purified human intestinal brush borders was quantitated. The adherence assays revealed that H10407 adhered to human brush borders only when it expressed CFA/I pili. It appears that in vitro adherence of H10407 to human intestinal epithelial cells is dependent on the expression of CFA/I.

The human *Escherichia coli* pathogen designated H10407 (serotype O78:H11), which was originally isolated from a case of adult traveler's diarrhea, is a well-characterized enterotoxigenic organism. Oral challenge of adult volunteers with H10407 has been shown to lead to colonization of the host small bowel and subsequent elicitation of clinical diarrhea (7, 14). The diarrhea is thought to be a result of the ability of the organism to synthesize and secrete both heat-labile and heat-stable enterotoxins.

By analogy to many well-defined animal models of *E. coli* diarrhea (1–3, 10), it has been presumed that bacterial surface pilus antigens promote adherence of H10407 to the human intestinal mucosa, facilitate its colonization, and help H10407 resist the natural clearing mechanisms of the host. However, two biochemically and immunologically distinct types of pili can be detected on H10407. Evans et al. have reported that, when H10407 is grown on a defined agar medium, it expresses specific surface pili, called colonization factor antigen I (CFA/I) pili (7). The other class of pili, designated type 1 pili, can also be detected on H10407 when the organism is grown under conditions which promote type 1 pilus expression. The determination of which type of pili promoted the adherence of H10407 to human intestinal epithelial cell membranes has yet to be documented because of the difficulty in obtaining sufficient quantities of human mucosal epithelial cells.

In the present study, the adherence of CFA/I-piliated H10407 and type I-piliated H10407, together with other piliated *E. coli* strains to an enriched preparation of human brush borders (HBBs), was examined, and the adherence was correlated with the type of pili possessed by each strain.

MATERIALS AND METHODS

HBB preparation. A specimen of human terminal ileum (4 by 6 cm) was obtained from a patient undergoing surgery for removal of an adenocarcinoma of the cecum. Before surgery, the patient gave informed consent for the use of his resected tissue after it was reviewed and released by the surgical pathologist. No additional tissue was removed for these studies beyond that considered necessary by the surgeon for an adequate resection. The tissue was placed in normal saline within 5 to 10 min after removal from the patient, and all further procedures were conducted at 4°C. After the mucosal layer was carefully dissected away from the serosal layer, an HBB fraction was prepared from the mucosal epithelial cells by the procedure described by Houghton and McCarthy (8). Protein was estimated by the method of Lowry et al. (11), and the HBBs were diluted to a final concentration of 1 mg/ml with phosphate-buffered saline (145 mM NaCl–10 mM NaH_2PO_4-Na_2HPO_4, pH 7.0). Purity of the HBB fraction was assessed by examination under phase microscopy and by following the enrichment of maltase (4) and alkaline phosphatase activity (13) over the original mucosal homogenate. The number of HBBs per mg of protein, determined by counting on a hemacytometer under phase microscopy, was found to be 3.1×10^7.

Bacterial strains and culture conditions. *E. coli* strains to be tested for their adherence ability to HBBs included the documented human pathogen H10407 (serotype O78:H11); a nonpathogenic, piliated human fecal isolate, HS (O undetermined:H4); and the rabbit pathogen designated RDEC-1 (O15:NM). These orga-

nisms were grown in medium which promoted the expression of pili. The media used included Penassay broth (PAB; Difco Laboratories, Detroit, Mich.) for HS and RDEC-1 and Casamino Acids-yeast extract agar for H10407 (7). H10407 was also grown on PAB and brain heart infusion (BHI) medium (Difco). Each culture was grown overnight at 37°C. The bacteria were harvested directly from tube cultures by pelleting at 2,400 × *g* for 5 min or by pelleting saline suspensions from the agar plates. Each culture was washed twice and resuspended in phosphate-buffered saline. The final concentration of bacteria ranged from 2×10^9 to 3×10^9 per ml. A sample of each test *E. coli* strain was negatively stained with 1% phosphotungstic acid and examined under an electron microscope to document the presence or absence of pili.

Hemagglutination assays. The type of pili expressed on each test *E. coli* strain was documented with standard slide hemagglutination assays (6). Briefly, a single drop of a 3% suspension of guinea pig, bovine, or human type A erythrocytes in 0.9% saline with or without 125 mM D-mannose was mixed with a drop of test bacterial cells. The strength of the agglutination reaction which occurred within 30 s was graded from 0 to +4. Type 1 pili were identified by their ability to hemagglutinate guinea pig erythrocytes in a D-mannose-sensitive fashion, whereas CFA/I pili were identified by their ability to agglutinate human type A erythrocytes in a D-mannose-resistant fashion.

Bacterial adherence assays. The conditions utilized for quantitating the adherence of *E. coli* to HBBs were those previously determined to be optimal for the adherence of RDEC-1 to rabbit brush borders (2). In brief, these conditions included incubating 25 μl (10^5) of HBBs with 10 μl of test *E. coli* (10^7 bacteria) together with 15 μl of phosphate-buffered saline. After 15 min of shaking at 37°C, the reaction was terminated by vigorous vortexing, and samples were placed onto a hemacytometer. Adherence was quantitated by counting the number of bacteria adhering to 40 HBBs under phase microscopy at a magnification of ×600. The results were reported as the mean number of adherent organisms per HBB ± 1 standard error.

RESULTS

The preparation of HBBs utilized in this experiment was enriched over the whole mucosal homogenate with respect to the specific activities of alkaline phosphatase (17-fold) and maltase (5.6-fold). Examination of HBBs by phase microscopy revealed that the preparation possessed the characteristic morphological appearance associated with intestinal brush border preparations (Fig. 1A).

To determine whether CFA/I or type 1 pili promoted the adherence of H10407 to HBBs, we grew the organism in medium that would promote the expression of each class of pili. These media included Casamino Acids-yeast extract agar for CFA/I pili and PAB and BHI medium for type 1 pili. Table 1 shows the results of the in vitro assays for adherence to HBBs, together with the type of pili expressed on each suspension of test organisms as determined by hemagglutination assay (CFA/I, type I, or nontypeable).

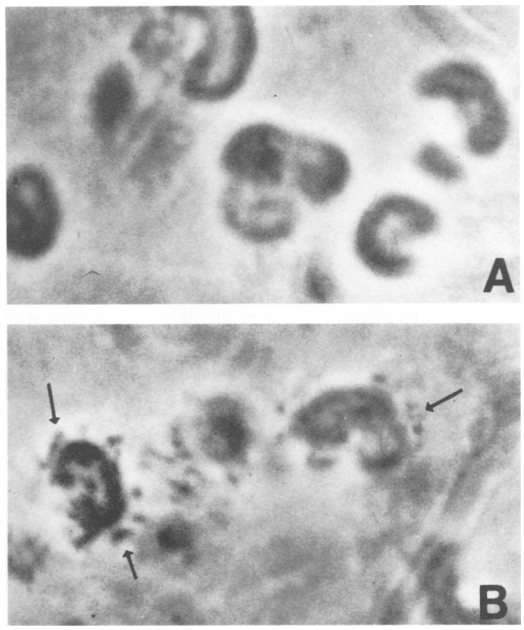

FIG. 1. Phase micrograph of purified human intestinal brush borders. (A) Sample of HBBs incubated alone in phosphate-buffered saline; (B) sample from the reaction mixture of 8×10^5 HBBs with 2×10^7 CFA/I-piliated H10407 organisms incubated at 23°C for 15 min. The black arrows indicate some of the adherent H10407 organisms. Magnification, ×600.

The greatest degree of adherence to HBBs was detected with H10407 grown on Casamino Acids-yeast extract agar. The ability of these organisms to hemagglutinate human type A erythrocytes (+4 agglutination) in a D-mannose-resistant fashion suggests the expression of CFA/I pili on their surface. Their inability to hemagglutinate guinea pig erythrocytes indicates the absence of type 1 pili. The number of CFA/I-piliated H10407 organisms adhering to HBBs ranged from 0 to 21 (mean, 4.4) per HBB. These organisms were localized to the microvillar surface of each HBB; rarely were any organisms seen adhering to the cytoplasmic side of the HBBs (Fig. 1B). Of the HBBs incubated with CFA/I-piliated H10407, 75% had at least one adhering organism. In contrast, when H10407 was grown in BHI broth, the mean adherence value to HBBs was significantly lower ($P = 0.01$) at 0.9 H10407 per HBB, and the range of organisms adhering was reduced to 0 to 5 organisms per HBB. Although growth of H10407 in BHI broth did not promote adherence, it did promote pilus production. However, hemagglutination assays revealed that only type

TABLE 1. Adherence of *E. coli* strains to a human intestinal brush border preparation and the type of pili expressed by each organism

Test strain	Type of pili[a]	Mean no. (± SE) of *E. coli* adhering per HBB	Range	*P*[b]	Growth medium
H10407	+4 CFA/I	4.4 ± 0.7	0–21		CAYE[c]
	+4 Type 1	0.9 ± 0.2	0–3	<0.01	BHI
	+4 Type 1, +1 CFA/I	0.8 ± 0.2	0–5	<0.01	PAB
RDEC-1	NT[d]	0.2 ± 0.1	0–1	<0.01	PAB
HS	+4 Type 1	0.4 ± 0.1	0–3	<0.01	PAB

[a] The type of pili was determined by a standard slide hemagglutination assay. The degree of hemagglutination was expressed from 0 to +4.
[b] A paired Student *t* test was used to compare the adherence results obtained with Casamino Acids-yeast extract agar-grown H10407 to those obtained adherence results using either PAB or BHI grown H10407 organisms.
[c] CAYE, Casamino Acids-yeast extract agar.
[d] NT, Not typable. Did not agglutinate any of the erythrocytes tested.

1 pili were present on the organisms grown in BHI broth.

Growth of H10407 in PAB led to a reduction in the range and mean number ($P < 0.01$) of H10407 adhering per HBB. Under these growth conditions, the organisms appeared to be primarily expressing type 1 pili, as judged by the strength (+4) of the hemagglutination reaction when guinea pig erythrocytes were used. The hemagglutination results obtained when human type A erythrocytes were used was decreased to a +1 reaction, indicating a marked reduction in the expression of CFA/I pili.

As a control, the rabbit pathogen RDEC-1 was grown under conditions which have been shown to promote its avid adherence to a similarly enriched preparation of rabbit brush borders. However, under these growth conditions, RDEC-1 did not adhere to the HBBs. The pili on RDEC-1 which promote their adherence to rabbit brush borders did not agglutinate any of the erythrocytes tested, indicating that they are in a class distinct from both type 1 and CFA/I pili.

The second control organism tested, the nonpathogenic human fecal isolate HS, also did not adhere to HBBs. The strength (+4) of the hemagglutination reaction of guinea pig erythrocytes with HS after its growth in PAB revealed that the organism possessed a high density of type 1 pili. Growth of HS in other media, including BHI broth and Casamino Acids-yeast extract agar, led only to the expression of type 1 pili as determined by hemagglutination reactions.

DISCUSSION

Recent studies have focused on the important role of the bacterial pilus antigens in the pathogenesis of *E. coli* diarrhea. Experiments in which animal diarrheal models, specifically the porcine (10, 15) and the bovine (1) *E. coli* models, are used have clearly shown that the virulence of these pathogens was closely associated with the expression of either K88 pili or K99 pili on the respective pathogens. In vitro adherence assays demonstrated that these pili promoted the adherence of each pathogen to its respective host intestinal epithelial cells (9, 10).

In part because of the general unavailability of sufficient quantities of human tissue to examine the in vitro adherence ability to many human pathogenic *E. coli* isolates, Evans et al. developed hemagglutination assays to screen for the presence of pili that could possibly confer the ability to adhere to human intestinal mucosa in vitro. These assays attempt to detect and differentiate among different classes of bacterial pili by the hemagglutination patterns of test *E. coli* strains with animal and human erythrocytes (6). These hemagglutination assays have helped to identify three immunologically and biochemically distinct classes of pili: CFA/I, CFA/II, and type 1 pili (5). CFA/I and CFA/II pili have been found on only human enterotoxigenic *E. coli* isolates, whereas type 1 pili appear to be ubiquitous pilus antigens in that they are found on *E. coli* isolates of both animals and humans.

By analogy to the swine and bovine *E. coli* diarrheal models, it has been postulated that the expression of CFA/I and CFA/II confers on human *E. coli* isolates the ability to adhere to the human intestinal mucosa, whereas type 1 pili are not generally thought to promote intestinal mucosal adherence. The studies reported herein confirm this hypothesis in that the presence of CFA/I pili on H10407 is associated with the adherence of this organism to human intestinal brush borders. Growth of H10407 on Casamino Acids-yeast extract agar promoted the expression of CFA/I pili, as indicated by +4 hemagglutination of human type A erythrocytes, and promoted the adherence of this organism to HBBs (Table 1). In contrast, growth of H10407

in BHI broth appeared to promote the expression of type 1 pili but did not promote the adherence of H10407 to HBBs. Similarly, growth of H10407 in PAB also promoted production of type 1 pili on H10407 but not adherence to HBBs. The hemagglutination assays of H10407 grown on PAB revealed both a +4 D-mannose-sensitive agglutination of guinea pig erythrocytes and a +1 D-mannose-resistant agglutination of human type A erythrocytes. We interpret this result to indicate that two classes of pili were present, but, based on the strength of the agglutination reactions, the majority of the pili functionally expressed on the bacterial surface of H10407 grown on PAB were type 1 pili. Hence, the failure of these organisms to adhere significantly to HBBs may have been due to a lack of sufficient CFA/I pili to promote adherence. The results of all these assays are based on the largely quantitative hemagglutination assays rather than on direct quantitative methods for determining the amounts of pili of the different classes. Nevertheless, the assays were at least semiquantitative (1+ to 4+), and, more importantly, they provided a measure of the functional expression of the adhesive properties of the test organisms for mammalian cells. One must bear in mind that, under the different culture conditions used, the expression of surface structures other than pili may also have been altered. Thus, the final results observed, in terms of hemagglutination and brush border adherence, may have resulted from the appearance or disappearance of the several types of pili or from more complex interactions of other molecules expressed at the bacterial surface with existing pili.

The adherence of CFA/I-piliated H10407 to human intestinal cells appears to be a receptor-specific event. This was suggested by the inability of the well-characterized, enteroadherent, piliated rabbit *E. coli* pathogen, RDEC-1, to adhere to the same preparation of HBBs. Additionally, the adherence of H10407 also appears to be species specific, because our laboratory has never been able to detect the ability of H10407 grown on Casamino Acids-yeast extract agar to adhere to an enriched preparation of rabbit intestinal brush borders (personal observation). This evidence suggests that pathogenic *E. coli* of both humans and animals recognize and attach only to specific receptors present on the intestinal mucosal surfaces of their respective hosts.

Our results complement those of other groups who have studied the adherence ability of human *E. coli* pathogens to human tissue. McNeish et al. (12), using human fetal tissue, were able to detect a significantly greater degree of D-mannose-resistant adherence of three human enterotoxigenic *E. coli* strains to disks of fetal intestinal tissue. Since classification of CFA/I and CFA/II pili had not been established at that time, they did not determine whether CFA/I or CFA/II pili were involved. However, they were able to exclude a role for type 1 pili because their adherent strains did not agglutinate guinea pig erythrocytes. Wadstrom and coworkers (16) also examined the adherence of *E. coli* possessing CFA/I and CFA/II to a preparation of isolated adult intestinal epithelial cells. They found that *E. coli* possessing CFA/I and CFA/II pili adhered to human gut cells with a range in means of between 23 to 29 organisms per cell. In contrast to the results reported in this paper, their control *E. coli* strains, which possessed type 1 pili, also adhered to the intestinal cells with a range in means of 18 to 21 *E. coli* organisms adhering per cell.

In summary, this study indicates that H10407 adheres to receptors on human intestinal cells and that this adherence occurred when CFA/I pili were expressed on H10407. Presence of type 1 pili on H10407 or on another *E. coli* control strain did not correlate with the ability to adhere to human ileal brush borders. Finally, the receptors on the HBBs showed specificity for CFA/I pili in that they did not support the adherence of the rabbit pathogen RDEC-1, which was grown under conditions that consistently promoted pilus expression and adherence to rabbit brush borders.

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