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Outer Membranes Are Competitive Inhibitors of *Escherichia coli* O157:H7 Adherence to Epithelial Cells

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***Escherichia coli* of serotype O157:H7 are Vero cytotoxin-producing enteric pathogens that have been associated recently with sporadic cases and outbreaks of hemorrhagic colitis and with the hemolytic-uremic syndrome. Adherence of many enteropathogenic bacteria to mucosal surfaces is a critical step in the pathogenesis of diarrheal disease. We showed previously that adherence of *E. coli* O157:H7 strain CL-56 to epithelial cells in vitro is inhibited by outer membranes. In this study we examined whether outer membranes from a series of *E. coli* O157:H7 strains mediated competitive inhibition of bacterial binding to epithelial cells grown in tissue culture. We also determined which constituents of the outer membrane mediated inhibition of CL-56 adherence. Binding of six O157:H7 strains to HEp-2 cells was determined by quantitating the number of adherent bacteria in the presence and absence of outer membranes which were extracted from each strain with *N*-lauroyl sarcosinate (1.7%, wt/vol). After separation of outer membranes by gel electrophoresis, four bands (94, 40, 36, and 30 kDa) were collected by electroelution. Immune sera were raised in rabbits to each of the four eluted bands. Outer membrane extracts from each of the six O157:H7 strains inhibited binding of homologous organisms to the HEp-2 cells. At dilutions which did not cause bacterial agglutination, antiserum raised against the 94-kDa outer membrane protein showed maximal inhibition of bacterial adherence (17.0 ± 7.3% adherence of control levels). Growth of bacteria in iron-depleted broth did not affect their binding to HEp-2 cells, suggesting that iron-regulated outer membranes were not involved. Fluid accumulation in ileal ligated loops of rabbits in response to *E. coli* O157:H7 challenge was diminished following both parenteral immunization with outer membranes extracted from the homologous strain and coincubation of organisms with immune serum which contained antibodies to outer membrane extracts. These data indicate that outer membranes are competitive inhibitors of *E. coli* O157:H7 adherence. Specific constituents of the outer membrane may function as bacterial attachment factors (i.e., adhesins) for *E. coli* O157:H7 adherence to epithelial cell surfaces.**

Escherichia coli strains of the serotype O157:H7 were first associated with human disease in two community outbreaks of hemorrhagic colitis (36). Subsequently, the organisms have also been associated with a spectrum of sporadic and endemic human illnesses ranging from nonbloody diarrhea to the hemolytic-uremic syndrome (24).

E. coli O157:H7 are sorbitol-negative strains that produce cytotoxins variously referred to as Vero cytotoxins (24) and Shiga-like toxins (31). *E. coli* O157:H7 demonstrate intimate adherence to enterocytes in animal models of human disease (42, 50). At sites of bacterial adherence to the apical plasma membrane, there is actin accumulation and disruption of normal microvillus membrane architecture (25). Binding of bacteria is similar both to other serotypes of *E. coli* that produce Vero cytotoxins (42, 51) and to enteropathogenic *E. coli* strains (28) but contrasts with normal enterocyte microvillus membranes in enterotoxigenic *E. coli* infections (8).

Previously, we (43) and other investigators (25, 33, 48) showed that *E. coli* O157:H7 also adhere to epithelial cell lines in tissue culture. *E. coli* O157:H7 strains do not, however, demonstrate either invasive properties (43) or localized internalization (15). In addition, *E. coli* O157:H7 do not adhere to CaCo-2 cells as is the case for classical enteropathogenic *E. coli* strains (25). We have also provided preliminary evidence showing that outer membranes, rather than H7 flagellin and O157 lipopolysaccharide, are surface constituents that function as bacterial colonization factors in vitro (46). In this report, we provide additional evidence indicating that outer membrane proteins are bacterial adhesins which mediate attachment of *E. coli* O157:H7 strains.

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MATERIALS AND METHODS

Bacteria and growth conditions. Six *E. coli* O157:H7 strains from children with hemorrhagic colitis and the hemolytic-uremic syndrome were provided by M. Karmali, The Hospital for Sick Children, Toronto, Ontario, Canada, and held in storage at −70°C in Penassay broth with 10% glycerol. Each strain demonstrates attaching and effacing adherence to rabbit intestinal epithelium in vivo (42). We have also shown that the six isolates adhere to epithelial cells in vitro (46) and that they have comparable outer membrane profiles on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (46). E2348 (serotype O127:H6), an attaching and effacing *E. coli* strain that does not elaborate Vero cytotoxins (1), was kindly provided to us by E. Boedeker, Walter Reed Army Institute of Research, Washington, D.C.

Each of the bacterial strains was grown overnight at 37°C in static, nonaerated broth cultures of Penassay (Difco

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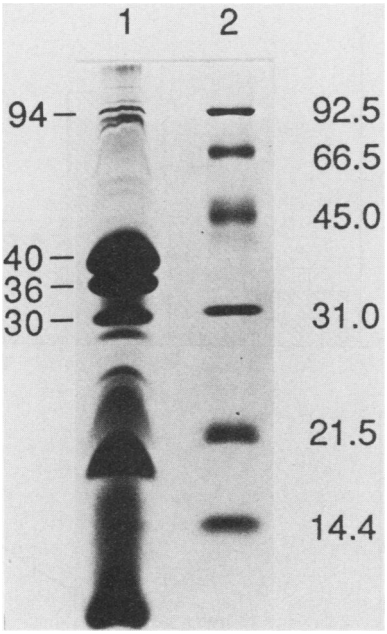

FIG. 1. Coomassie blue stain of outer membrane extracts from *E. coli* O157:H7 strain CL-56 following SDS-PAGE (lane 1). Arrows point to the four protein bands removed by electroelution (20) and used to raise immune sera in rabbits. Lane 2 contains reference proteins as molecular weight (10^3) standards.

Laboratories, Detroit, Mich.) without serial passage. In some experiments *E. coli* O157:H7 strain CL-56 was grown in Penassay broth with addition of 0.5 mg of the iron chelator ovotransferrin per ml (47).

Outer membrane preparations and antisera. Outer membranes were extracted from each strain by precipitation of bacterial sonicates in *N*-lauroyl sarcosine, 1.7% (wt/vol) (Sarkosyl; Sigma Chemical Co., St. Louis, Mo.), as described previously (46). Detergent-insoluble outer membranes from strain CL-56 were separated by electrophoresis in 12.5% polyacrylamide separating cells, in the presence of 0.1% SDS and in parallel with proteins of known molecular weight as reference standards (Bio-Rad Laboratories, Richmond, Calif.). Gels were stained with Coomassie brilliant blue to visualize proteins (44). Four proteins, of apparent molecular weights 94,000, 40,000, 36,000 and 30,000 (Fig. 1), were extracted from polyacrylamide gels by electroelution (20).

Antiserum to each of the outer membrane extracts and each of the four eluted outer membrane proteins from strain CL-56 was prepared by three biweekly subcutaneous injections of male New Zealand White rabbits, as described before (46). The presence of relevant antibodies in immune serum was confirmed by dot immunoblotting with antigen, as described previously (46). When tested by incubation with Vero cells, outer membrane preparations did not contain cytotoxic activity.

Adherence assays. Complementary binding assays were used to determine whether outer membranes functioned as colonization factors for *E. coli* O157:H7. First, we quantitated bacterial binding to HEp-2 cells, as described previously (43, 46), in culture medium alone and in the presence of various subagglutinating dilutions of preimmune sera and immune antisera. Results are expressed as percentage of adherence compared with binding assays run in the absence of putative inhibitors. The inoculum used in assays for quantitation of adherence was 10^8 bacteria in 0.1 ml. In some experiments, a series of sugars purchased commercially (Sigma) were preincubated with HEp-2 cells or bacteria for 30 min at 37°C at equal concentrations (0.01 M) to determine whether outer membranes were functioning as bacterial lectins.

The influence of coincubation of *E. coli* O157:H7 strain CL-56 with antibiotics on the adherence of organisms to epithelial cells was examined by using the quantitative HEp-2 cell adherence assay. Nalidixic acid (to inhibit DNA replication), rifampin (to inhibit RNA synthesis), and chloramphenicol and gentamicin (both used to inhibit bacterial protein synthesis) were each tested at MICs and one-fourth MICs of antibiotics. MICs of the antibiotics were determined by the broth dilution method, as described before (53). For strain CL-56, MICs were as follows: nalidixic acid, 4 μg/ml; rifampin, 8 μg/ml; chloramphenicol, 4 μg/ml; gentamicin, 0.25 μg/ml.

In other experiments, 500 μg of outer membranes from *E. coli* O157:H7 strain CL-56 was incubated with the proteases trypsin (50 μg; Sigma) and proteinase K (10 μg; Sigma) overnight at 37°C (30). Treated outer membranes were then used as inhibitors in the HEp-2 cell assay for quantitation of bacterial adherence.

Second, attaching and effacing binding of the bacterial strains to both HEp-2 and HEL (human embryonic lung) cells (American Type Culture Collection, Rockville, Md.) was semiquantitated by visual estimation, using the method first described by Knutton et al. (25). Fluorescein-labeled phalloidin (Sigma) was used to detect foci of actin accumulation on the plasma membrane of the eukaryotic cells. Briefly, tissue culture cells were grown overnight on 13-mm sterile coverslips in multiwell tissue culture plates (Costar) in 5% CO_2 at 37°C. Coverslips were then washed three times in Hanks balanced salt solution (without supplemental calcium or magnesium) and once in sterile phosphate-buffered saline (PBS; Dulbecco formulation). Bacteria from overnight broth culture, 0.020 ml, were added to 0.98 ml of tissue culture medium supplemented with 1% fetal bovine serum (GIBCO) but without antibiotics. The 1.0-ml mixture was then incubated with epithelial cells on the coverslips for 6 h in 5% CO_2 at 37°C. At 3 h of incubation, the tissue culture medium containing the bacterial inoculum was removed, the coverslips were washed once with sterile PBS, and 1.0 ml of fresh tissue culture medium was added. After a further 3 h of incubation at 37°C, the tissue culture medium was removed and coverslips were washed three times in sterile PBS. Cells were then fixed by incubation of coverslips with 3% Formalin for 10 min. After three washes in PBS, cell membranes were permeabilized by the addition of the detergent SDS (1%). After three additional PBS washes, 0.5 ml of a 5-mg/ml PBS solution of fluorescein isothiocyanate conjugated to phalloidin (Sigma) was added to each coverslip for 1 min. Coverslips were washed three times in PBS and mounted onto glass microscope slides for visualization under fluorescence microscopy. Phase-contrast microscopy was used in conjunction to examine the same slides, ensuring that fluorescence occurred at sites of bacterial adhesion.

To determine whether outer membranes functioned as bacterial adhesins during in vivo infection, fluid responses in rabbit ileal ligated loops were examined in immunized animals and following coincubation of organisms with antibody to outer membrane extracts. Four to six 10-cm ligated loops were surgically fashioned proximal to the appendix while 1-kg male New Zealand White rabbits were placed under

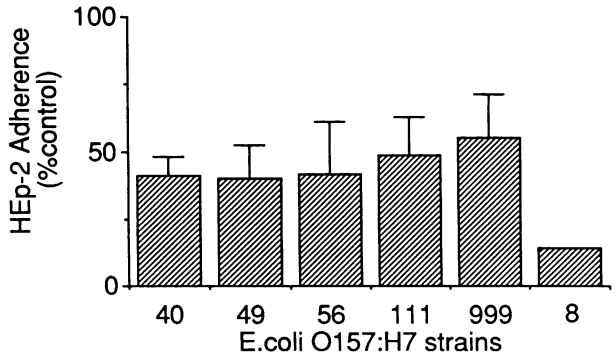

FIG. 2. Inhibition of *E. coli* O157:H7 binding to HEp-2 cells by 0.5 mg of sarcosinate-extracted outer membranes from homologous bacterial strains. Each assay was run in duplicate on four occasions except for strain CL-8, with which the assay was performed on two occasions.

general anesthesia. Each loop was separated by a 2-cm segment of bowel. Loops were then inoculated with 5×10^8 CFU of *E. coli* O157:H7 CL-56 resuspended in 1.0 ml of sterile saline. In some loops the bacteria were suspended in either 1.0 ml of a 1:100 dilution of preimmune serum or an equal volume of anti-outer membrane antiserum diluted 100-fold. In other experiments, the rabbits were parenterally immunized with either whole-cell antigen or outer membrane extracts of CL-56 by sequential subcutaneous injections, as described previously (43, 46). Six weeks later, the rabbits were used in ligated loop experiments, but only after dot immunoblotting of postimmunization serum demonstrated the presence of antibody specific to the challenge antigen.

In each rabbit, at least one of the 10-cm ligated loops was injected with 1.0 ml of sterile PBS to serve as a negative control. Purified verotoxin-1 (100 μg in 1.0 ml of PBS) was used as a positive control (VT-1 kindly provided to us by Susan Head, The Hospital for Sick Children, Toronto, Ontario, Canada). Following challenge of each loop, the peritoneal cavity was closed and the animals were held in an incubator for 18 h, allowing free access to water but not food. The rabbits were then sedated with an intramuscular injection of ketamine-xylazine and sacrificed with an intracardiac overdose of pentobarbitol. The peritoneal cavity was then reopened and the ligated loops were excised in toto. Fluid from the lumen of each of the loops was then extracted and quantitated as an indirect marker of bacterial enteroadherence.

Statistics. Results are expressed as means ± standard error. Differences between multiple groups were determined by one-way analysis of variance, and differences between two groups were determined by using the two-tailed, unpaired Student *t* test (38).

RESULTS

The quantitative bacterial adherence assay showed that outer membranes from six O157:H7 strains inhibited binding of homologous organisms to HEp-2 cells (Fig. 2). We demonstrated previously that this finding is specific because equal amounts of outer membranes from a fecal commensal *E. coli*, H7 flagellin, and O157 lipopolysaccharide do not inhibit binding of *E. coli* O157:H7 to HEp-2 cells (46).

To determine whether a specific constituent of the outer membrane served as the bacterial adhesin, we eluted four

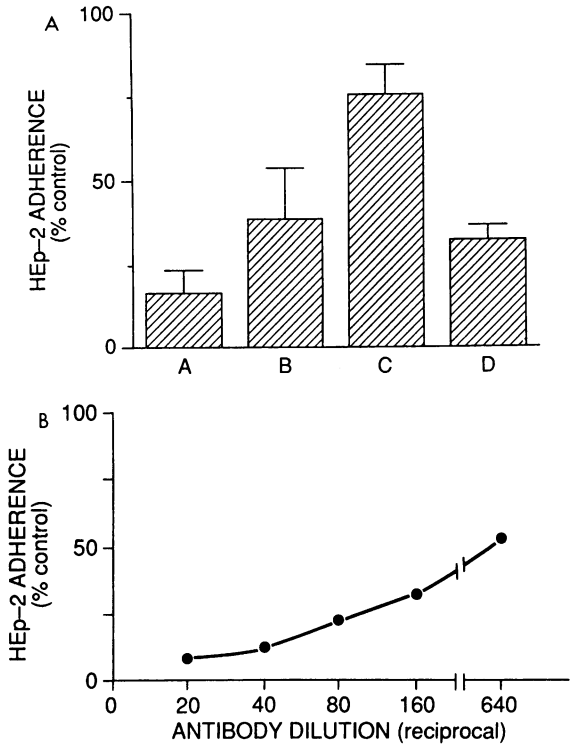

FIG. 3. Binding of *E. coli* O157:H7 strain CL-56 to HEp-2 cells in the presence of (A) 1:20 dilutions of antiserum raised to each of the four eluted outer membranes (A, 94-kDa protein; B, 40 kDa; C, 36 kDa; and D, 30 kDa) or (B) increasing dilutions of immune serum raised to the 94-kDa outer membrane protein band of the homologous bacterial strain. For panel A, each assay was performed in duplicate on 4 separate days.

bands from outer membranes of strain CL-56 that were separated in polyacrylamide by gel electrophoresis (Fig. 1). Small amounts of antigen precluded their use as competitive inhibitors in the in vitro binding assay. Therefore, we raised immune sera to both outer membrane extracts and each of the four eluted protein bands from the outer membrane preparation of strain CL-56. Dot immunoblotting demonstrated that each of the antisera recognized the antigen with which the rabbit was challenged and that antisera to the four eluted proteins did not cross-react (data not shown). Figure 3A shows that at subagglutinating concentrations (i.e., a 1:20 dilution of antiserum) the antisera demonstrated varying abilities to inhibit binding of strain CL-56 to HEp-2 cells. In the presence of antiserum to the 94-kDa outer membrane band, adherence of strain CL-56 was reduced to 17.0 ± 7.3% of control levels. Inhibition was concentration dependent because increasing dilutions of antiserum showed progressively less inhibition of binding (Fig. 3B). In contrast, antisera to the 40- and 36-kDa outer membrane bands, presumably porins (27), demonstrated less inhibition of bacterial attachment (Fig. 3A).

As expression of some outer membranes is regulated by the presence or absence of iron in the growth medium (12), we examined HEp-2 cell binding of *E. coli* O157:H7 strain CL-56 grown either in iron-replete Penassay broth or in the presence of an iron chelator, ovotransferrin (47). Figure 4 shows that growth of bacteria in iron-deficient broth resulted in changes in the profile of outer membrane extracts. For example, there was increased production of three proteins of

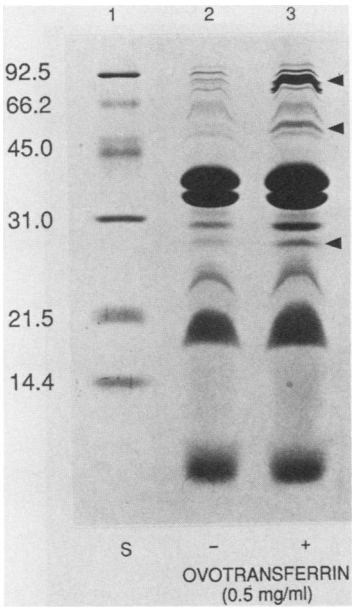

FIG. 4. SDS-PAGE of sarcosinate extracts from *E. coli* O157:H7 strain CL-56 following growth of organisms in Penassay broth in the absence (lane 2) and presence (0.5 mg/ml; lane 3) of the iron chelator ovotransferrin. Iron restriction induced the expression of outer membrane proteins (arrowheads in lane 3) but did not inhibit binding of bacteria to HEp-2 cells. Lane 1 contained reference proteins of known molecular weight (10^3).

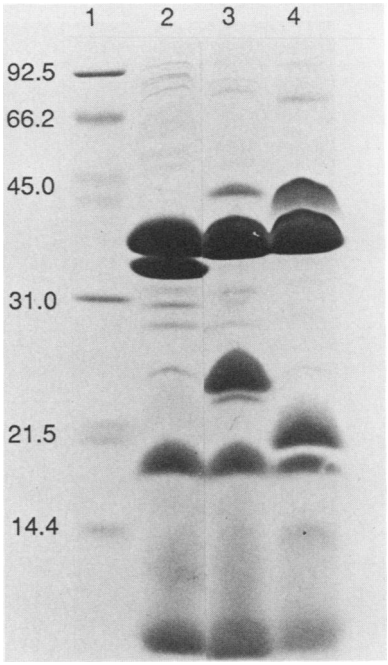

FIG. 5. SDS-PAGE of sarcosinate extracts of *E. coli* O157:H7 strain CL-56 following incubation of outer membrane extracts overnight at 37°C with saline (lane 2), trypsin (lane 3), and proteinase K (lane 4). Lane 1 contains known reference proteins as molecular weight standards (10^3).

apparent molecular weights of approximately 85,000, 52,000, and 28,000. The 94-kDa outer membrane protein was not affected by growth of organisms in the presence of the iron chelator. There was no reduction or enhancement of binding of these organisms to HEp-2 cells in the quantitative adherence assay (data not shown). This finding suggested that iron-regulated outer membrane proteins are not important bacterial attachment factors for *E. coli* O157:H7. Growth of strain CL-56 under strictly anaerobic conditions also did not alter the number of organisms adherent to HEp-2 cells (data not shown).

A series of sugars were tested at equal molar concentrations as competitive inhibitors of *E. coli* O157:H7 binding to HEp-2 cells. The sugars tested included D-mannose, L-mannose, mannosamine, D-glucose, glucosamine, *N*-actetylglucosamine, D-galactose, galactosamine, *N*-acetylgalactosamine, L-fucose, and D-lactose. At 0.01 M, none of the sugars demonstrated any inhibition of *E. coli* O157:H7 strain CL-56 adherence to HEp-2 cells (data not shown). At higher concentrations (0.5 M), sugars mediated a 90% reduction in binding of CL-56. However, this finding was nonspecific because multiple sugars (D-glucose, D-mannose, L-fucose, D-galactose, and glucosamine) demonstrated a comparable inhibitory effect (data not shown). Proteases resulted in altered outer membrane protein profiles on SDS-PAGE. There was apparent loss in the 94-, 36-, and 30-kDa constituents, whereas the 40-kDa porin was resistant to proteolytic degradation (Fig. 5). Outer membranes of *E. coli* O157:H7 strain CL-56 treated with proteinases showed less inhibition of bacterial binding in the quantitative adherence assay (trypsin, 31.2% of untreated outer membranes; pronase, 31.0% of inhibition produced by untreated outer membrane extracts).

Coincubation of *E. coli* O157:H7 strain CL-56 with nalidixic acid did not markedly inhibit binding of organisms to HEp-2 cells (9.2 ± 6.4% of bacterial binding following growth of organisms in the absence of antibiotic) (Table 1). In contrast, an antibiotic which inhibits protein synthesis (i.e., chloramphenicol [6]) showed greater inhibition of bacterial adherence to tissue culture cells (59.7 ± 4.7%; $P < 0.05$). Coincubation of bacteria with rifampin (23.5 ± 8.2%) and chloramphenicol (26.3 ± 13.3%) gave intermediate results. Although subinhibitory concentrations of antibiotics inhibit the binding of other bacterial pathogens by blocking synthesis of bacterial adhesins (39, 40), the antibiotics tested in this study did not demonstrate greater inhibition of binding of *E. coli* O157:H7 to HEp-2 cells (Table 1).

The fluorescein isothiocyanate-labeled phalloidin assay (25) demonstrated actin accumulation at sites of *E. coli* O157:H7 strain CL-56 binding to HEL cells (Fig. 6). Similar findings were observed with HEp-2 cells as substrate (data not shown). When preincubated with bacteria, antiserum to the 94-kDa outer membrane protein band completely abol-

TABLE 1. Effect of antibiotics on binding of *E. coli* O157:H7 strain CL-56 to HEp-2 cells

Concn	% Inhibition of binding[a]			
	Nalidixic acid	Rifampin	Gentamicin	Choramphenicol
At MIC	9.2 ± 6.4	23.5 ± 8.2	26.3 ± 13.3	59.7 ± 4.7
At 1/4 MIC	4.0 ± 4.7	23.9 ± 16.8	8.0 ± 9.8	27.4 ± 14.8

[a] Relative to adherence of bacteria that were grown in broth cultures in the absence of antibiotics. Mechanisms of antibiotic action (6) were as follows: nalidixic acid inhibits DNA replication (DNA gyrase); rifampin blocks RNA synthesis (RNA polymerase); gentamicin blocks protein synthesis (30S ribosome); and chloramphenicol blocks protein synthesis (50S ribosome).

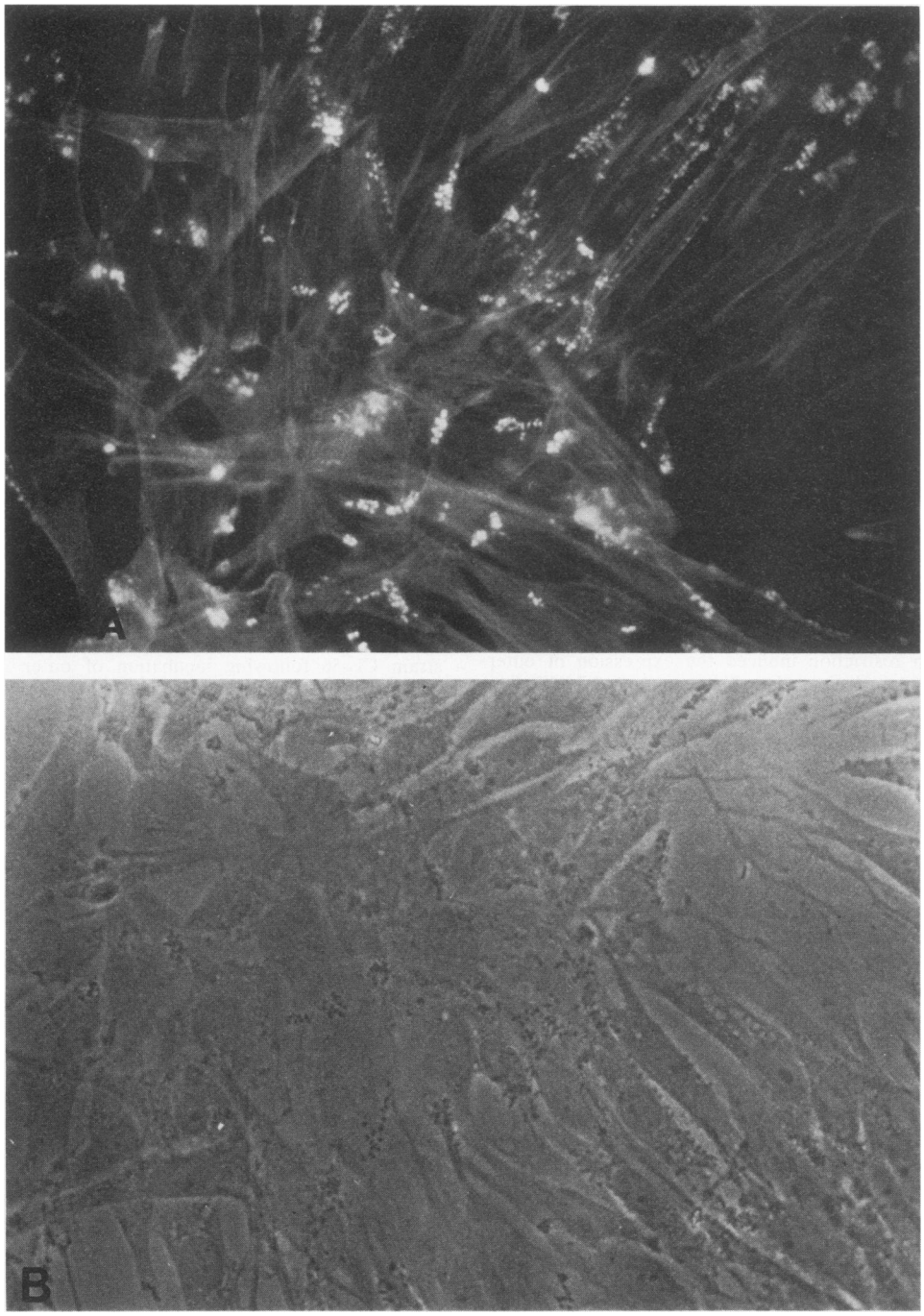

FIG. 6. Fluorescein-labeled phalloidin localized to site of actin accumulation (A) in areas of the surface of HEL cells which contained clusters of adherent *E. coli* O157:H7 strain CL-56 by phase-contrast microscopy (B). Fluorescence was inhibited by coincubation of bacteria with a 1:20 dilution of immune serum raised to the 94-kDa outer membrane protein of *E. coli* O157:H7 strain CL-56 (C) but not by equal dilutions of preimmune serum (D) and O157 lipopolysaccharide antiserum (E).

ished actin accumulation, as shown by the absence of positive fluorescence (Fig. 6). Preimmune serum and heterologous antiserum did not block adherence because fluorescence at sites of phalloidin binding to accumulated actin was still observed.

E. coli E2348, which also demonstrates attaching and effacing enteroadherence but does not produce Vero cytotoxins, showed identical actin accumulation in the fluorescein isothiocyanate-phalloidin adherence assay. However, in this case binding of bacteria and foci of actin accumulation were not inhibited by preincubation of bacteria with the 94-kDa antiserum (Fig. 7). These data indicate that there may be differences in attachment mechanisms among attaching and effacing *E. coli* strains (25).

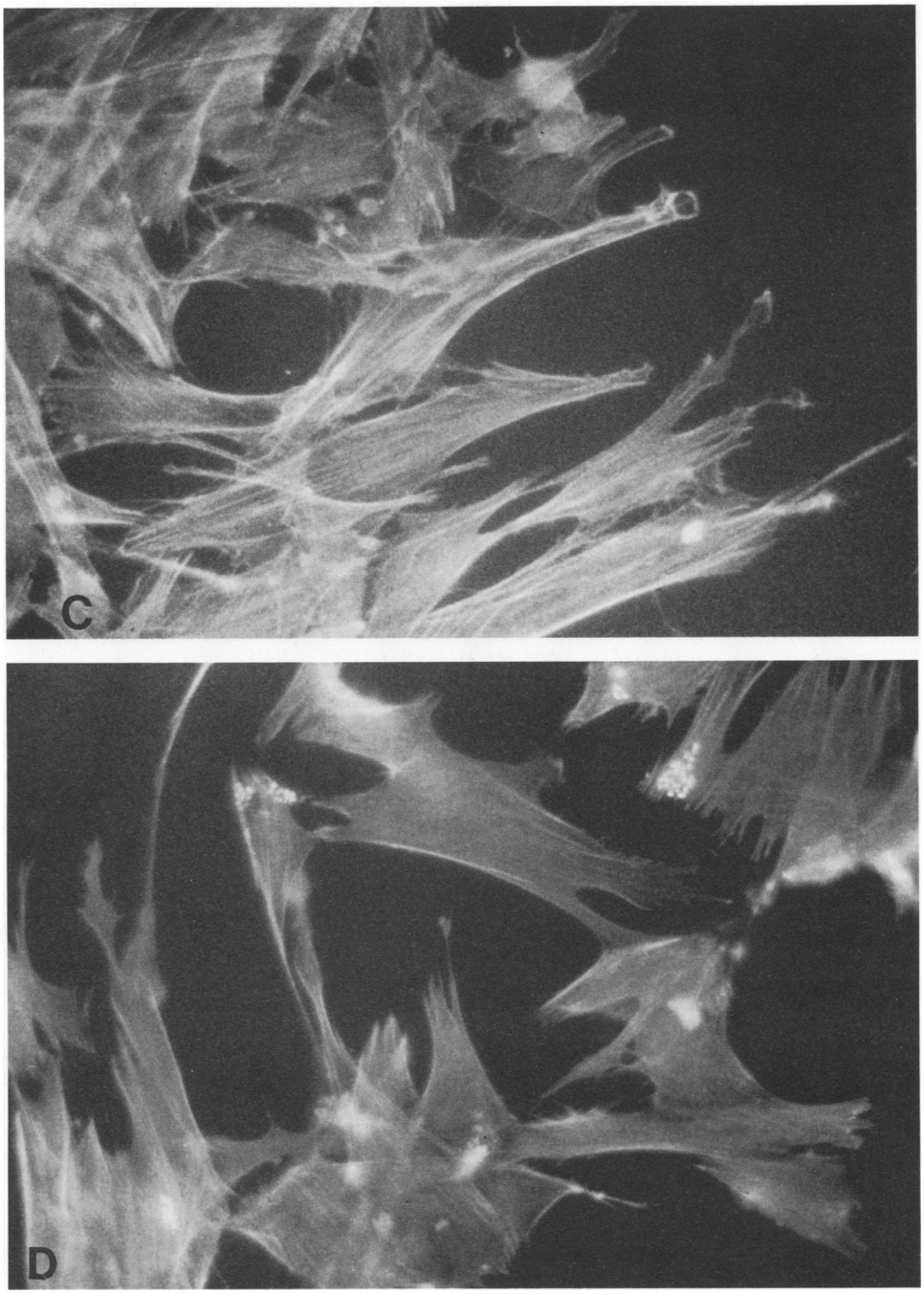

FIG. 6—*Continued.*

Inoculation of 5×10^8 CFU of *E. coli* O157:H7 strain CL-56 into ileal ligated loops for 18 h resulted in fluid accumulation (6.8 ± 3.0 ml/10 cm) that was slightly less than with 100 μg of purified verotoxin-1 (10.4 ml/10 cm) (Table 2). More importantly, however, immune serum raised to outer membranes of CL-56 blocked fluid accumulation in ligated loops (1.9 ± 0.5 ml/10 cm), whereas preimmune serum did not (5.6 ± 2.5 ml; $P < 0.05$). Compared with naive rabbits, parenteral immunization of animals with both whole-cell antigens and outer membrane extract as antigen blocked fluid accumulation in ligated loops following challenge with viable *E. coli* O157:H7 strain CL-56 (Table 2).

DISCUSSION

Binding of bacterial pathogens is a primary step in the pathogenesis of disease because it promotes internalization and invasion of invasive organisms (17). Adherence also promotes the delivery of bacterial exoproducts, including enterotoxins, to eukaryotic cell surfaces (55). Attachment to

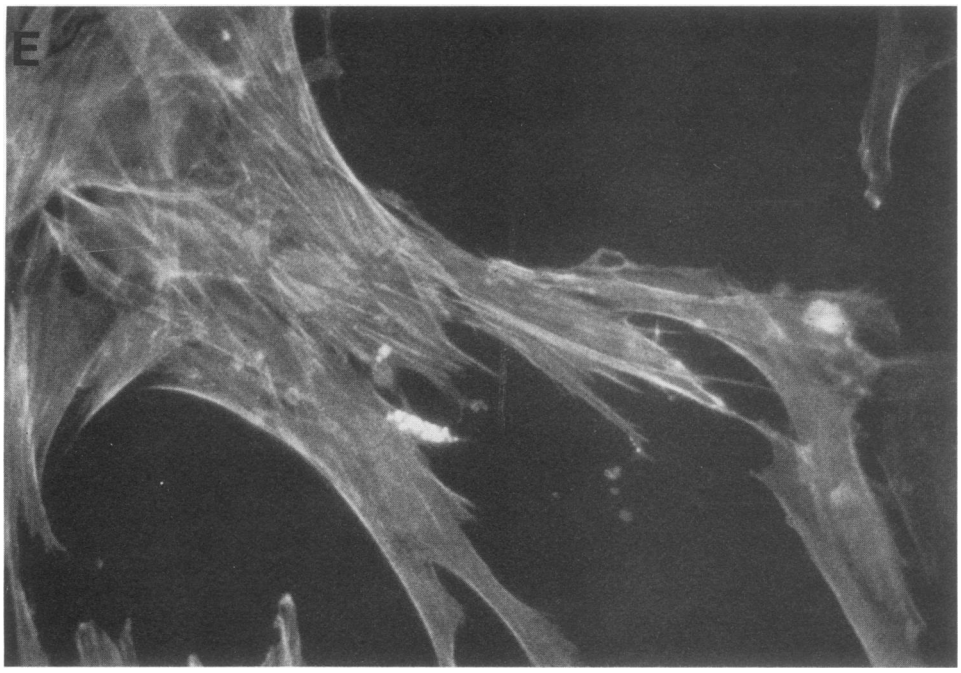

FIG. 6—*Continued.*

mucosal surfaces also promotes colonization of bacteria by preventing their loss into the environment away from the host (3).

Although adherence of *E. coli* O157:H7 to gastrointestinal epithelial cells has not been demonstrated in human disease, the organisms demonstrate adherence to the ileum, cecum, and colon of orally infected animals such as gnotobiotic piglets (18, 50), rabbits (42), and chickens (4). In addition, Vero cytotoxin-producing *E. coli* of other serotypes demonstrate enteroadherence both during natural infection of calves and after experimental challenge (9, 18, 29). Vero cytotoxin-producing *E. coli*, including those of the O157:H7 serotype, demonstrate intimate binding to the apical plasma membrane at sites where the normal microvillus membrane is disrupted (42, 51). At these sites the eukaryotic cell plasma membrane is raised to form a cuplike projection or pedestal. Actin accumulates at the site of pedestal formation (13, 25), perhaps in response to phosphorylation of signal peptides (2).

Intimate binding of bacteria at sites of pedestal formation has been observed previously for both human and other animal *E. coli* enteric pathogens which do not produce either enterotoxins or Vero cytotoxins (28). Three groups have proposed that there are two distinct stages in the intimate attaching and effacing binding of *E. coli* to eukaryotic cell surfaces (7, 26, 54). Bacterial lectins, including fimbriae, are reported to mediate the initial or early attachment of organisms to intact microvilli (24, 54). The mechanism of microvillus disruption and actin accumulation as pedestals has not been defined, although changes in permeability for calcium have been proposed as a possible intracellular signal.

Adherence of *E. coli* O157:H7 may itself be sufficient to cause disease (34). For example, Tzipori et al. (49) showed that gnotobiotic piglets develop diarrhea following colonization and adherence by isogenic mutants of *E. coli* O157:H7 in which the bacteriophages encoding for production of Vero cytotoxins were deleted.

Outer membranes were examined as a bacterial adhesin because we showed previously that Sarkosyl extracts competitively inhibit in vitro binding of *E. coli* O157:H7 strain CL-56 in contrast to equal amounts of H7 flagellin and O157 lipopolysaccharide which do not inhibit attachment of the organisms (46). Recent studies also indicate that constituents of the outer membrane on other gram-negative enteric pathogens, including *Vibrio cholerae* (41), *Campylobacter jejuni* (14), *C. coli* (16), and *Salmonella typhimurium* (52), are virulence factors that promote bacterial enteroadherence (32).

Although fimbriae (23) and lipopolysaccharide (35) have both been proposed previously as attachment factors for attaching and effacing *E. coli*, other studies also suggest a role for outer membranes as adhesins. For example, Levine et al. (27) showed that a 94-kDa outer membrane protein was immunogenic following oral challenge of human volunteers with a well-characterized attaching and effacing *E. coli* strain. The outer membrane component might be an important virulence factor because systemic antibody was present before challenge in the one volunteer who did not develop diarrhea. Scaletsky et al. (37) reported that outer membrane extracts from an enteropathogenic *E. coli* of serotype O111:H− inhibit in vitro binding of homologous organisms to HeLa cells. Their study indicated that a 32-kDa protein band was a likely candidate as the adhesin for localized attaching and effacing adherence of this enteropathogenic *E. coli* strain. However, Chart et al. (10, 11) were not able to confirm either of these observations.

Recently, Jerse et al. (22) reported the identification of a gene designated *eae* whose 102-kDa product is an outer membrane protein which is necessary but not sufficient to mediate the attaching and effacing lesion caused by the human enteropathogenic *E. coli* E2348. The *eae* gene product has 31% amino acid identity with the invasin protein of *Yersinia pseudotuberculosis* (21, 22), suggesting that there may well be functional homology. Moreover, an intragenic

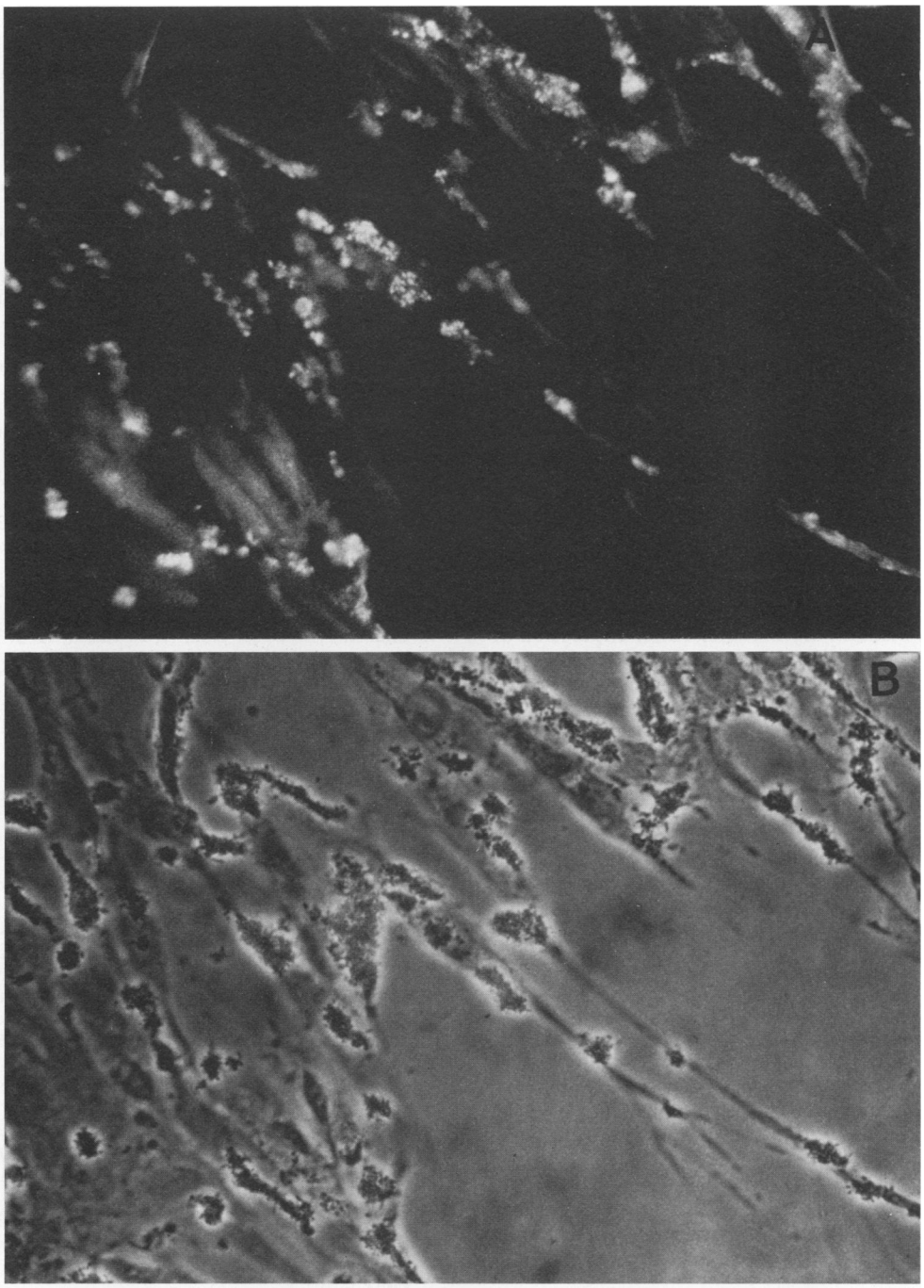

FIG. 7. Phalloidin assay showed fluorescence (A) at sites of actin accumulation and enteropathogenic *E. coli* E2348 adherence to HEL cells (B) in the presence of immune serum raised to outer membranes of *E. coli* O157:H7 strain CL-56.

eae probe hybridized with *E. coli* O157:H7. The size of the 94-kDa outer membrane protein examined in this study is similar to that of the *eae* gene product (102K). Therefore, one might ask what differences there may be between the *eae* gene of enteropathogenic *E. coli* and the putative homolog of enterohemorrhagic *E. coli*. First, our studies suggest that the two are not immunologically cross-reactive. Second, it is likely that the putative receptors for the two proteins differ. For example, *E. coli* O157:H7 strains do not produce attaching and effacing lesions on CaCo-2 cells, whereas enteropathogenic *E. coli* strains do (25). Furthermore, *E. coli* O157:H7 colonize and adhere to mucosa in the cecum and colon, whereas enteropathogenic *E. coli* primarily colonize the duodenum and upper small bowel (24, 34).

In the present study, we examined whether constituents of the outer membrane functioned as adhesive factors for *E. coli* O157:H7 strain CL-56. Bands at apparent molecular weights of 94,000 and 30,000 were eluted to raise immune

TABLE 2. Fluid response in rabbit ileal ligated loops

Inoculum	Fluid accumulation (ml/10 cm)	n
E. coli O157:H7 CL-56 (5×10^8)		
In naive rabbits	6.8 ± 3.0	8
Plus preimmune serum (1:100)	5.6 ± 2.5	4
Plus outer membrane protein immune serum (1:100)	1.9 ± 0.5	4
Immunized with whole-cell antigen	1.3 ± 0.2	4
Immunized with outer membrane protein antigen	1.1 ± 1.1	4
Verotoxin-1 (100 μg)	10.4	1
Saline	0.4 ± 0.2	9

sera because outer membrane proteins of comparable size were evaluated previously as adhesins for other *E. coli* enteric pathogens (27, 37). Proteins of apparent molecular weights 36,000 and 40,000 were eluted because they correspond to the size of porins contained in the outer membrane of *E. coli* (27, 32).

Our data indicate that the high-molecular-weight protein band was most effective as a competitive inhibitor of *E. coli* O157:H7 strain CL-56 attachment to HEp-2 cells. Iron content of growth medium did not alter either expression of this outer membrane band or binding of the organisms in vitro. Fluorescence microscopy showed that immune serum raised to the 94-kDa protein band abolished attaching and effacing binding of *E. coli* O157:H7 to HEL cells. These findings confirm those of Knutton et al. (25) showing that the fluorescein isothiocyanate-phalloidin assay for actin accumulation correlates extremely well with both sites of bacterial attachment and attaching and effacing adherence. Similar findings were also observed with HEp-2 cells but, like Knutton et al. (25), we observed that the plane-of-focus for visualization was optimal with HEL cells.

The phalloidin binding assay also showed that attaching and effacing adherence of *E. coli* strains might be the end result of at least two distinct mechanisms. In our study antiserum to the 94-kDa outer membrane inhibited binding and actin accumulation for both homologous and heterologous *E. coli* O157:H7 strains. However, the same dilution of antiserum did not affect attaching and effacing adherence of the *E. coli* enteropathogen strain E2348 that does not elaborate Vero cytotoxins. Knutton et al. (25) showed that *E. coli* E2348 binds to HEp-2, HEL, and CaCo cell lines whereas O157:H7 strains adhere to HEp-2 and HEL cells but not CaCo cells. These findings also suggest that distinct binding processes ultimately result in morphologic evidence of attaching and effacing adherence of bacteria to eukaryotic cell surfaces.

In summary, these data provide additional support for the role of specific constituents of the outer membrane as bacterial adhesins in the binding of *E. coli* O157:H7 to epithelial cell surfaces. Future work will use genetic manipulation of organisms to confirm the role of outer membranes as adhesins that mediate *E. coli* O157:H7 binding to the intestinal epithelium.

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