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Adherence Characteristics of Attaching and Effacing Strains of *Escherichia coli* from Rabbits

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Twelve strains of *Escherichia coli* previously reported to cause diarrhea in rabbits were examined for properties associated with virulence. Ten strains met the criteria for classification as enteropathogenic *E. coli* in that they were diarrheagenic strains that evoked attaching-effacing lesions in the small intestine and did not produce detectable enterotoxins or cytotoxins. These bacteria exhibited a variety of patterns when investigated for adherence to HEp-2 epithelial cells. Although several strains displayed localized and/or diffuse adherence to epithelial cells, they did not hybridize with DNA probes that recognize the genes responsible for these phenotypes in diarrheagenic *E. coli* from humans. The bacteria also varied in their ability to bind to erythrocytes and intestinal brush borders from various animal species. Six strains adhered to rabbit brush borders; two of these also adhered to brush borders from other animals. Two strains that did not adhere to rabbit brush borders adhered to those from guinea pigs or sheep. Only one of the strains investigated carried AF/R1 fimbriae, which are believed to govern the host specificity of this category of diarrheagenic *E. coli*. This strain was *E. coli* RDEC-1, which remains the only *E. coli* strain to date that is known to carry fimbriae of this type. The results indicate that although diarrheagenic *E. coli* strains from rabbits may have common properties associated with the ability to produce attaching-effacing lesions, they differ from each other and from enteropathogenic *E. coli* of humans in terms of some of the adhesins that mediate binding to eukaryotic cells.

Certain strains of *Escherichia coli* are able to cause diarrhea in humans or animals. These strains can be divided into subgroups comprising strains of clonal origin which belong to a restricted number of serotypes, biotypes, and electropherotypes (44). Each subgroup carries a characteristic set of virulence determinants (adhesins and/or protein exotoxins) that enable them to colonize the intestine and produce diarrhea (21). The nature of these virulence factors determines the clinical, pathological, and epidemiological features of the disease caused by the bacteria in a particular subgroup (21).

One such subgroup of diarrheagenic *E. coli* is enteropathogenic *E. coli* (EPEC). Bacteria in this group are characterized by the possession of adhesins, which are associated with the ability to cause distinctive attaching-effacing lesions in the intestine (9, 21, 25, 33, 40, 42). Although similar lesions are found in association with infections caused by another category of diarrheagenic *E. coli*, namely, enterohemorrhagic *E. coli* (EHEC), the latter can be distinguished from enteropathogenic strains by their ability to produce substantial quantities of Shiga-like cytotoxins (9, 21, 40). Furthermore, these two categories of bacteria appear to differ in their affinity for different regions of the intestine in that EPEC are found throughout the intestine, whereas EHEC are restricted mainly to the large intestine (41).

The pathogenic mechanisms of two varieties of EPEC have been well studied. These are the traditional EPEC from humans and an analogous group of bacteria from rabbits, which we have termed rabbit enteropathogenic *E. coli* (REPEC) (3, 9, 28). Both varieties are thought to adhere to the intestine by two distinct but complementary mechanisms, both of which are required for the full expression of virulence (5, 9, 10, 14, 17, 45). These adhesins are believed to act sequentially in two stages, the first of which typically involves a plasmid-encoded fimbrial structure that promotes initial contact between bacteria and susceptible target cells. The second stage may be mediated in part by intimin, the 94-kDa outer membrane protein product of the *eaeA* gene, which is essential (but not sufficient) for the production of the attaching-effacing lesions (9, 18). Bacteria lacking either adhesin show reduced adherence in vitro and a concomitant reduction in virulence for their natural host (1, 4, 10, 22, 45).

In EPEC, initial adhesion appears to be mediated by a recently described bundle-forming pilus, which promotes binding of the bacteria to tissue culture cells in a characteristic pattern termed localized adherence (16, 26, 39). As most isolates of EPEC associated with epidemic diarrhea in humans hybridize with a DNA probe derived from a gene for bundle-forming pili and exhibit localized adherence to tissue culture cells, these pili are thought to occur in the majority of these strains (26, 39). The analog of the bundle-forming pilus in REPEC is a plasmid-encoded fimbrial adhesin, termed AF/R1, which was identified in a prototype REPEC strain, named RDEC-1 (serotype O15:H−) (3, 4, 45, 46). By promoting initial contact between *E. coli* RDEC-1 and the host, AF/R1 fimbriae are believed to be responsible for the host, age, and tissue specificity of this strain. Thus, susceptibility to infection with RDEC-1 correlates with the number and distribution of receptors for AF/R1 in the rabbit intestine (6, 8). Recently, however, Pohl et al. (32) showed that only 1 of 41 attaching-effacing *E. coli* strains that caused diarrhea in rabbits carried the genes for AF/R1. By contrast, all of their strains hybridized with a probe derived from the *eaeA* gene of a human EPEC strain. These findings suggest that although REPEC strains have the adhesin(s) required for the production of attachment-

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TABLE 1. *E. coli* strains investigated in this study

Strain	Serotype	Biotype[a]	Pathogenic category	Source	Reference(s)
84/177	O2:K1:H6	3+	REPEC	Suckling rabbit	29–31
82/215/2	O8:K?:H?	1−	REPEC	Weanling rabbit	29–31
RDEC-1	O15:H−	3−	REPEC	Weanling rabbit	3, 8, 45
M34	O15:H−	3−	REPEC	Nonfimbriated mutant derived from RDEC-1	45
83/39	O15:H−	3−	REPEC	Weanling rabbit	29–31
82/260	O20:K−:H7	1+	REPEC	Weanling rabbit	29–31
84/110/1	O103:K−:H2	8+	REPEC	Weanling rabbit	29–31
82/123	O109:K−:H2	1+	REPEC	Suckling rabbit	29–31
82/183	O128:K−:H2	2+	REPEC	Weanling rabbit	29–31
82/90	O132:K−:H2	2+	REPEC	Weanling rabbit	29–31
83/146	O153:K−:H7	1+	REPEC	Weanling rabbit	29–31
83/64	Rough:H26	6+	REPEC	Weanling rabbit	29–31
E2348/69	O127:H6	NA	EPEC	Human infant	22
6/84	O144:H−	NA	EIEC[b]	Human infant	34
HS	O9:H4	NA	None	Healthy human adult	34

[a] Biotyping scheme of Peeters et al. (30). NA, not applicable.
[b] EIEC, enteroinvasive *E. coli*.

effacement lesions, which are largely conserved among all attaching-effacing strains of *E. coli*, they may carry colonization factors other than AF/R1 which determine initial adherence and possibly host specificity. Additional evidence for this suggestion stems from the observation that the susceptibility of rabbits to infection with different REPEC strains varies with age. Thus, rabbits are most susceptible to infection with RDEC-1 around the age of weaning, whereas some other REPEC strains are especially virulent for suckling rabbits (29). These observations suggest that some REPEC strains bind to receptors on the intestinal mucosa other than those recognized by the AF/R1 fimbriae of RDEC-1. To investigate this possibility further, we examined several REPEC strains of different serotypes and biotypes for their ability to adhere to eukaryotic cells in a number of in vitro assays of adhesion.

MATERIALS AND METHODS

Bacteria. The bacterial strains used in this investigation are listed in Table 1. They included 10 serologically distinct REPEC strains of seven different biotypes, which were isolated from diarrheic rabbits on commercial rabbit-fattening farms in Belgium and the Netherlands (30, 31). These strains were kindly supplied by J. E. Peeters, National Institute of Veterinary Research, Brussels, Belgium. *E. coli* RDEC-1 was provided by J. R. Cantey, Medical University of South Carolina, Charleston (3). *E. coli* M34, a nonfimbriate derivative of RDEC-1, derived by transposon mutagenesis of the 130-kb plasmid that encodes production of AF/R1, was provided by E. C. Boedeker, Walter Reed Army Institute of Research, Washington, D.C. (45). The pathogenicity of all of these strains for rabbits has been reported (3, 30, 31, 45). The other bacteria used in this study have been described previously (34). Before testing, all bacteria were stored in 30% (vol/vol) glycerol broth at −70°C. Unless otherwise stated, they were grown in 10 ml of Penassay broth (PAB; Difco Laboratories, Detroit, Mich.) without shaking at 37°C overnight.

Assays for enterotoxins and cytotoxins. (i) Enterotoxin assays. Sterile filtrates of bacterial cultures grown in casamino acid-yeast extract medium (12) (Difco) and Trypticase soy broth plus 0.6% yeast extract (BBL, Cockeysville, Md.) at 37°C overnight were assayed for the presence of heat-labile and heat-stable enterotoxins in ligated ileal loops of rabbits, Y-1 mouse adrenal cell culture, and 2- to 3-day old BALB/c mice as described previously (35).

(ii) Assay for cytotoxins. Culture filtrates were examined for the presence of Shiga-like cytotoxins in HeLa and Vero cell cultures in a quantitative microtiter assay (15).

Assays for adhesive and invasive capacity. (i) Assay for attaching and effacing capacity. The ability of *E. coli* to produce attachment-effacement lesions was determined in ligated loops of rabbit ileum as described previously (37).

(ii) Bacterial interaction with HEp-2 cells. The assay used to determine the pattern of bacterial adherence to HEp-2 epithelial cells was the CVD method reported previously (43). Bacteria were designated nonadherent if fewer than 10 of 200 cells examined had five or more attached bacteria. The ability of bacteria to adhere to and invade HEp-2 epithelial cells was assessed quantitatively as described previously (34). The fluorescent-actin staining (FAS) assay, a test which reflects the ability of *E. coli* to produce attachment and effacement lesions in the intestine, was performed with a 6-h incubation period as described by Knutton et al. (19, 20). At the completion of the assay, cells were examined by fluorescence microscopy and then by phase-contrast microscopy to confirm that fluorescent areas corresponded to attached bacteria.

(iii) Hemagglutination assay. Bacteria were grown in PAB or on CFA agar (11) at 37°C overnight. They were washed once and resuspended in phosphate-buffered saline (PBS), pH 7.4, at a final concentration of approximately 10^8 CFU/ml. Human group A, rabbit, calf, horse, sheep, and chicken erythrocytes, obtained from heparinized blood, were washed and used to prepare 5% (vol/vol) suspensions in PBS with and without 1% (wt/vol) D-mannose. One drop of erythrocytes was mixed with an equal volume of bacterial suspension, rocked gently on a glass slide for 2 min, and observed for clumping.

(iv) Adhesion to intestinal brush borders. The method used to quantify the ability of *E. coli* to adhere to intestinal brush borders was adapted from that described by Cheney et al. (7). Ileal brush borders were prepared from weaned New Zealand rabbits, BALB/c mice, outbred guinea pigs, sheep, calves, and pigs. Rabbits and guinea pigs were killed by a lethal injection of sodium pentobarbitone; mice were killed by CO_2 inhalation. Intestines from sheep, pigs, and calves were obtained from freshly killed animals at a local abattoir. In every case, the small intestine was removed, immediately placed on ice, in-

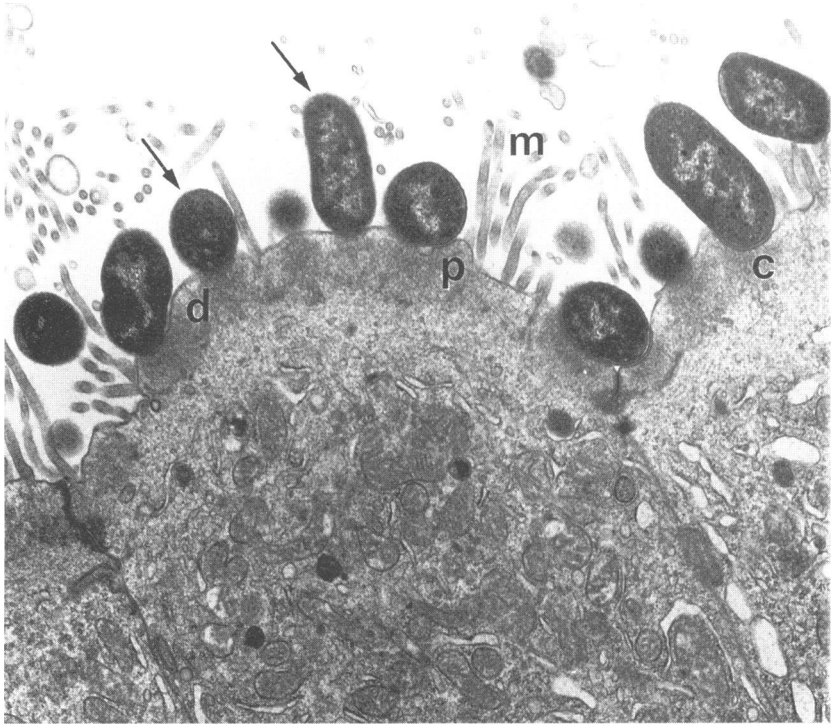

FIG. 1. Transmission electron micrograph of a section through a rabbit ileum 16 h after inoculation of *E. coli* M34 into a ligated ileal loop. Note the attachment-effacement lesions comprising closely attached bacteria (arrows) accompanied by alterations to the enterocyte cytoskeleton, evidenced by cupping (c), pedestal formation (p), accumulation of electron-dense material (d), and the absence of microvilli (m) at sites of bacterial attachment. Magnification, ×12,500.

cised along its mesenteric border, and washed repeatedly in an ice-cold solution of EDTA buffer containing 5 mM disodium EDTA and 5 mM Na_2HPO_4-NaH_2PO_4, pH 7.4. Portions of intestine with a surface area of 2 to 5 cm^2 were placed mucosal side uppermost on a glass slide in a shallow ice bath. Intestinal villi were removed by gentle scraping with a glass slide and suspended in EDTA buffer. The cells were then disrupted in a Teflon glass tissue grinder. Brush borders, separated by centrifugation at 180 × *g* for 20 min, were counted in a hemacytometer chamber and suspended in PBS at approximately 2 × 10^7 brush borders per ml.

TABLE 2. Interaction of *E. coli* strains with HEp-2 cells

Strain	Adherence pattern[a] (3-h assay)	Adherent bacteria[b] (%)	Invasive bacteria[c] (%)	Fluorescence pattern in 6-h FAS assay[a]
82/215/2	LA	10.3	0.002	LA
RDEC-1	NA	0.4	0.001	LA
M34	NA	0.5	0.002	LA
83/39	DA	40.1	0.001	DA
82/260	NA	0.9	0.001	LA
84/110/1	MA	19.5	0.005	MA
82/123	LA	16.7	0.008	LA
82/183	NA	0.3	0.001	LA
82/90	DA	14.2	0.003	DA
83/146	MA	15.2	0.003	MA
E2348/69	LA	17.0	0.067	LA
6/84	ND	1.1	1.270	ND
HS	NA	0.9	0.005	NA

[a] DA, diffuse; LA, localized; MA, mixed; NA, not adherent; ND, not determined.

[b] Bacteria (2×10^7) were incubated with HEp-2 cells for 3 h; nonadherent bacteria were removed by washing. HEp-2 cells were lysed, and the associated intra- and extracellular bacteria were counted. Results are the percentage of the original inoculum recovered and are the mean of two separate determinations.

[c] A parallel assay to the adherence assay was performed in which gentamicin (100 μg/ml) was added to each mixture after nonadherent bacteria had been removed by washing. Ninety minutes later, the gentamicin was removed by washing, the HEp-2 cells were lysed, and the surviving bacteria were enumerated. Results are expressed as a percentage of the bacteria recovered from the parallel adherence assay (34).

The adhesion assay was performed with bacteria grown in PAB overnight, washed twice in PBS, and resuspended at a concentration of 2×10^9 CFU/ml in PBS containing 1% D-mannose. Equal volumes of bacteria and brush borders, at a final ratio of 100 bacteria per brush border, were incubated together with gentle shaking for 20 min at ambient temperature. The suspension was then shaken vigorously in a Vortex mixer for 1 min to disperse any loose aggregates of bacteria and brush borders. Two drops of this suspension were examined by phase-contrast microscopy at a magnification of ×500. The number of bacteria adhering to 50 brush borders was counted and used to determine the mean number of adherent organisms per brush border. Each determination was performed on at least three separate occasions. Bacterial strains which adhered at a ratio of three or more bacteria per brush border were regarded to be adherent, whereas those which gave a result of less than one bacterium per brush border were considered nonadherent. Strains which gave intermediate values were classified as weakly adherent.

Preparation of antisera. Antisera to REPEC strains were prepared in New Zealand rabbits by a series of intravenous inoculations with increasing doses (from 10^4 to 10^8) of Formalin-killed bacteria twice weekly followed by a second series of inoculations with an equivalent number of live bacteria. For all of these inoculations, bacteria were grown in PAB without shaking at 37°C overnight. The antibody titer of each serum

was determined in a tube agglutination assay with heat-killed bacteria as the antigen (38). These sera were then absorbed at least three times with the homologous bacterial strain grown on CFA agar at 20°C (after preliminary studies had shown that bacteria grown under these circumstances failed to adhere to intestinal brush borders from any of the animal species tested). The completeness of the absorption procedure was demonstrated by a reduction in agglutinin titer to undetectable levels.

Inhibition assay. The ability of antisera to inhibit the binding of REPEC to rabbit ileal brush borders was examined by incubating 100 μl of absorbed serum (diluted 1:250 in PBS) with an equal volume of a suspension of test bacteria (4×10^9 CFU/ml) for 30 min at 37°C. Fifty microliters of this mixture was then added to a brush border suspension and tested as described above.

Hybridization with DNA probes. Selected DNA probes were investigated for their ability to hybridize with the test bacteria at high stringency (incubation at 68°C and inclusion of a final wash in low-salt buffer at 68°C) by the colony and Southern blotting techniques described previously (36, 37). The DNA probes used for this investigation were derived from the following recombinant plasmids (the corresponding virulence-associated property is given in parentheses): pCVD403 (heat-labile enterotoxin type I of enterotoxigenic *E. coli* [ETEC]), pCVD402 (heat-stable enterotoxin of ETEC, human subtype), pCVD404 (heat-stable enterotoxin of ETEC, porcine subtype), pCVD419 (adhesion-associated plasmid of EHEC), pCVD432 (adhesion-associated plasmid of enteroaggregative *E. coli*), pCVD434 (*eaeA* gene, required by EPEC to produce attaching-effacing lesions), pJPN16 (EPEC adhesion factor, associated with a plasmid that encodes the bundle-forming pili and the localized-adherence phenotype of EPEC), pJN37-19 (Shiga-like toxin type I of EHEC), pNN110-18 (Shiga-like toxin type II of EHEC), pRM17 (enteroinvasive capacity of enteroinvasive *E. coli*), and pSLM852 (fimbrial antigen, F1845, that mediates diffuse adherence of a human diarrheagenic *E. coli* strain to HEp-2 cells) (2, 23, 27). DNA probes were also prepared from the 2.4-kb *Eco*RI fragment of the 130-kb plasmid that carries the gene for the structural subunit of AF/R1 fimbriae of *E. coli* RDEC-1 (45, 46) and from the 0.6-kb PCR product of a reaction that was used to amplify a portion of the gene specifying the structural subunit of the bundle-forming pilus of the human EPEC strain E2348/69 (39).

Electron microscopic examination of bacteria. Bacteria were grown in 10 ml of PAB without shaking at 37°C overnight. Cultures were washed once in PBS and gently resuspended in PBS in one-fifth the original volume. A drop of this suspension was placed on a 3-mm carbon-coated copper grid (Alltech, Melbourne, Australia). After 10 to 20 s, the excess fluid was removed, and the sample was negatively stained for 30 s with a 1:10 solution of saturated ammonium molybdate. The excess fluid was removed, and the grids were examined in a Phillips 400 electron microscope at an accelerating voltage of 60 kV.

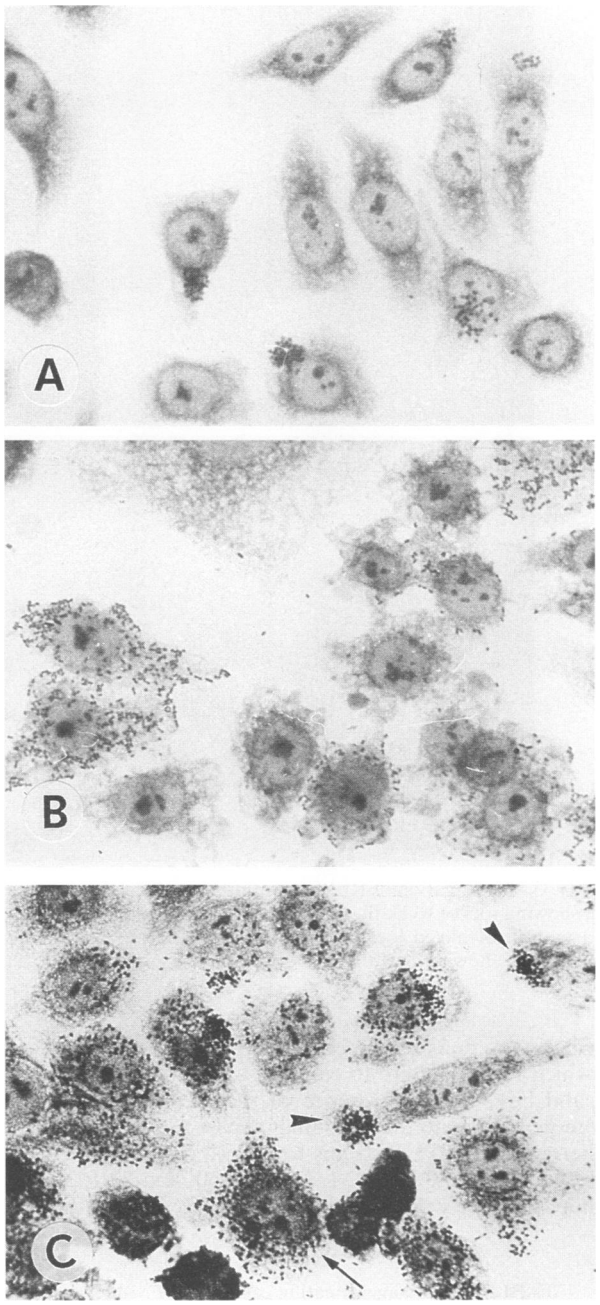

FIG. 2. Adherence patterns of REPEC strains to HEp-2 epithelial cells. (A) *E. coli* 82/123, showing localized adherence. (B) *E. coli* 83/39, showing diffuse adherence. (C) *E. coli* 84/110/1, showing a pattern of mixed localized (arrowheads) and diffuse (arrows) adherence. Giemsa stain; magnification, ×500.

RESULTS

Assays for enterotoxins and cytotoxins. All 12 putative REPEC strains were negative in the assays for enterotoxin and cytotoxins. Furthermore, none of these bacteria hybridized with the DNA probes for the heat-labile or heat-stable enterotoxins of ETEC or for the Shiga-like toxin types I and II of EHEC.

Assays for adhesive and invasive capacity. (i) Assays for attachment-effacement. Ten of the 12 putative REPEC strains produced characteristic attachment-effacement lesions in rabbit ileal loops (Fig. 1). All 10 of these strains hybridized with a DNA probe derived from the *eaeA* gene of a human EPEC strain and were positive in the FAS assay (Table 2). Southern blotting demonstrated that the *eaeA* gene was located on the bacterial chromosome in each strain (data not shown). Two strains, 84/177 and 83/64, did not adhere to the intestinal mucosa or react with the *eaeA* gene probe. These strains, which were also negative in the FAS assay, did not meet the criteria for classification as EPEC and were not investigated further.

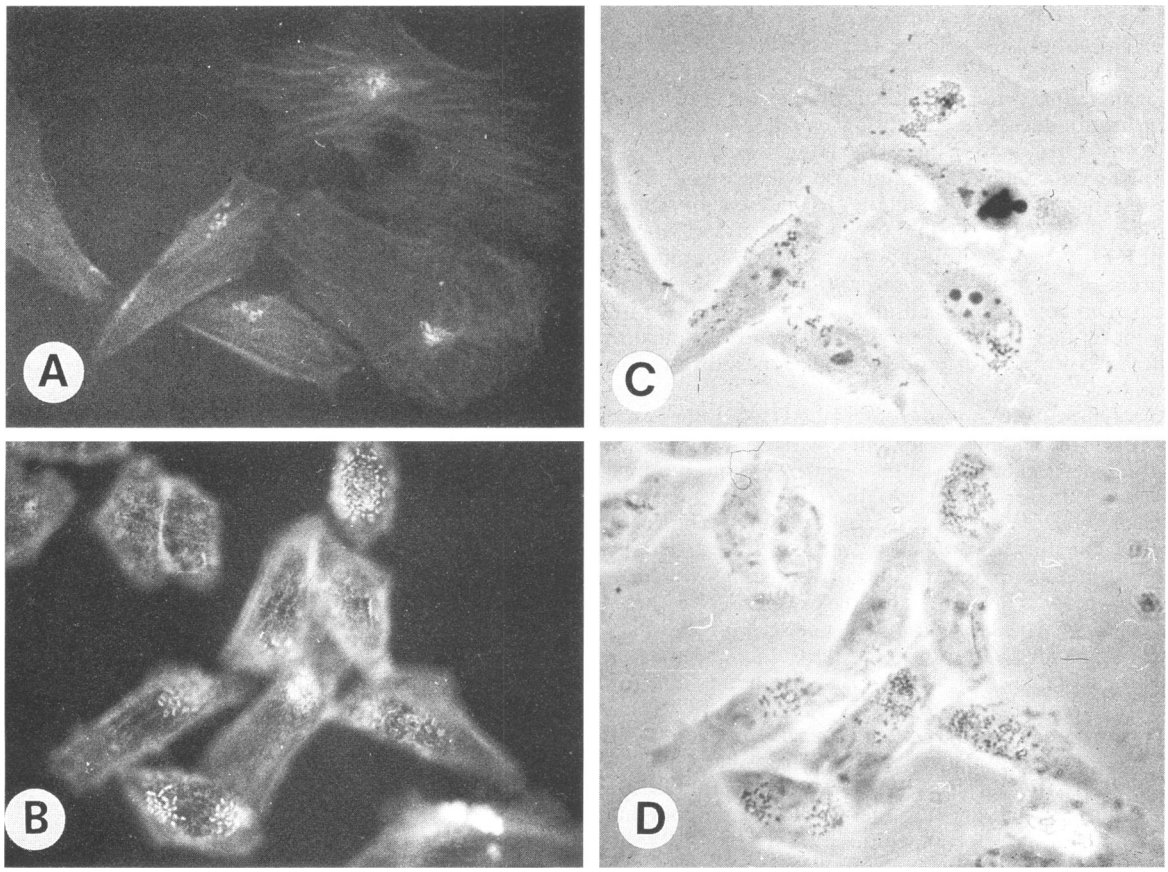

FIG. 3. Investigation of REPEC strains in a FAS assay (19, 20). (A) Fluorescent micrograph of HEp-2 cells incubated with *E. coli* RDEC-1 for 6 h, showing foci of weak fluorescence in a localized pattern. (B) HEp-2 cells incubated with *E. coli* 84/110/1 for 6 h, showing bright fluorescence in a pattern of mixed localized and diffuse adherence. (C and D) Phase-contrast micrographs of the same fields shown in panels A and B, respectively, showing that the foci of fluorescence correspond to the locations of attached bacteria. Magnification, ×500.

(ii) Interactions of bacteria with HEp-2 cells. The patterns in which EPEC adhere to cultured epithelial cells correspond by and large to the presence of specific adhesins that may promote binding to the intestinal mucosa (26). The patterns of adherence of REPEC strains to HEp-2 cells varied considerably. Four strains, RDEC-1, M34, 82/60, and 82/183, did not adhere to these cells, with fewer than 5% of the HEp-2 cells showing attached bacteria. Of the remaining strains, two, 82/215/2 and 82/123, showed localized adherence; two, 83/39 and 82/90, showed diffuse adherence; and two, 84/110/1 and 83/146, demonstrated a pattern that was designated mixed adherence, because it included areas of localized and diffuse adherence (Table 2, Fig. 2).

The results of the quantitative assay of adherence corresponded with the adherence patterns in that fewer than 1% of

TABLE 3. Hemagglutinating capacity of *E. coli* strains

Strain	Hemagglutination[a] of erythrocytes from:							
	Guinea pig	Human	Mouse	Sheep	Calf	Pig	Chicken	Rabbit
82/215/2	+	−	−	−	−	−	−	−
RDEC-1	−	−	−	−	−	−	−	−
M34	−	−	−	−	−	−	−	−
83/39	−	−	−	−	−	−	−	−
82/260	+	−	−	−	−	−	−	−
84/110/1	−	+	+	+	+	+	−	−
82/123	+	+	+	−	−	−	−	−
82/183	−	−	−	−	−	−	−	−
82/90	+	−	−	−	−	−	−	−
83/146	+	−	−	−	−	−	−	−

[a] +, hemagglutination; −, no hemagglutination. Hemagglutination of guinea pig erythrocytes was invariably inhibited by 1% D-mannose. Hemagglutination assays with erythrocytes from other species were performed in the presence of D-mannose only.

TABLE 4. Adherence of *E. coli* to intestinal brush borders

Strain	Adherence[a] (mean no. of bacteria/brush border)					
	Rabbit	Guinea pig	Mouse	Sheep	Calf	Pig
82/215/2	2.4	0.2	0.0	0.0	0.1	0.0
RDEC-1	8.7	0.0	0.0	0.0	0.0	0.0
M34	0.2	0.0	0.0	0.0	0.0	0.0
83/39	0.8	12.4	0.0	0.7	0.2	0.0
82/260	9.6	0.2	0.0	0.0	0.0	0.0
84/110/1	6.1	5.1	0.0	15.2	7.1	0.1
82/123	3.6	1.0	5.0	4.2	0.2	0.1
82/183	0.3	0.1	0.0	0.0	0.0	0.0
82/90	4.9	0.0	0.0	0.5	0.8	0.0
83/146	0.4	0.6	0.1	1.1	0.3	0.0

[a] Data are the mean number of bacteria per brush border, determined by counting 50 brush borders from at least three different animals of each species.

the inoculum was recovered from strains designated nonadherent, whereas at least 10% was recovered from bacteria regarded as adherent. There was, however, no direct correlation between the pattern of adherence observed (diffuse, localized, or mixed) and the number of bacteria recovered (Table 2).

All 10 REPEC strains which produced attaching-effacing lesions were positive in the FAS assay. The four strains designated nonadherent in the 3-h HEp-2 cell assay produced a pattern of localized weak fluorescence in the FAS assay (Fig. 3A). Adherent strains, by contrast, demonstrated patterns of bright fluorescence which corresponded to their pattern of adherence to HEp-2 cells (Table 2, Fig. 3B).

None of the REPEC strains invaded HEp-2 cells, giving results in the quantitative invasion assay similar to those for the negative control strain, *E. coli* HS. By contrast, the human EPEC strain E2348/69 invaded these cells to a limited extent (Table 2).

(iii) Hemagglutination. As the hemagglutinating properties of diarrheagenic *E. coli*, in particular enterotoxin-producing strains, correspond to the presence of specific colonization factors (11), we examined the REPEC strains for their ability to agglutinate erythrocytes from various animals. Five of the strains agglutinated guinea pig erythrocytes in a reaction that was inhibited by D-mannose, indicating the presence of type 1 fimbriae. One of these strains, 82/123, also agglutinated erythrocytes from humans (group A) and mice, but in this case, agglutination was mannose resistant (Table 3). One strain, 84/110/1, showed mannose-resistant hemagglutination of human, mouse, sheep, calf, and pig erythrocytes. None of the strains caused mannose-resistant hemagglutination of erythrocytes from chickens or rabbits (Table 3).

(iv) Adherence to intestinal brush borders. The adherence of RDEC-1 to intestinal brush borders from rabbits corresponds with the presence of AF/R1 fimbriae and reflects the species specificity of this strain (6, 8, 45). This strain and four other REPEC strains investigated here adhered to brush borders prepared from rabbits. One strain, 83/39, which belongs to the same serotype (O15:H−) and biotype (3−) as RDEC-1, did not adhere to rabbit brush borders but adhered avidly to brush borders from guinea pigs (Table 4, Fig. 4).

E. coli 84/110/1 adhered to brush borders from rabbits, guinea pigs, sheep, and calves but not to those from mice or pigs. Strain 82/123, which was originally isolated from a suckling rabbit with diarrhea, adhered to brush borders from weaned rabbits and to those from sheep and mice. This strain also adhered weakly to brush borders from guinea pigs (one bacterium per brush border). No other strain adhered to brush borders from mice (Table 4).

(v) Effect of antisera. Hyperimmune rabbit antisera were prepared against strains RDEC-1, 84/110/1, 82/215/2, 82/260, 82/123, and 83/146 and then absorbed with the homologous strain grown under conditions that discourage production of adhesins. The absorbed sera were then examined for their ability to reduce the capacity of RDEC-1, 84/110/1, 82/215/2, and 82/260 to adhere to rabbit brush borders. The results showed that in every case, adherence was completely inhibited by the homologous serum but not significantly reduced by any of the heterologous antisera (data not shown).

Hybridization with DNA probes. None of the REPEC strains hybridized with DNA probes prepared from a plasmid associated with adherence of human EHEC to cell culture or with probes prepared from the plasmids that encode localized, diffuse, or aggregative adherence of human diarrheagenic *E. coli* to HEp-2 cells. These bacteria also were not recognized by a DNA probe prepared from the structural gene for bundle-forming pili of a human EPEC strain. Apart from *E. coli* RDEC-1 and M34, moreover, none of the REPEC strains hybridized with the probe derived from the structural gene for AF/R1.

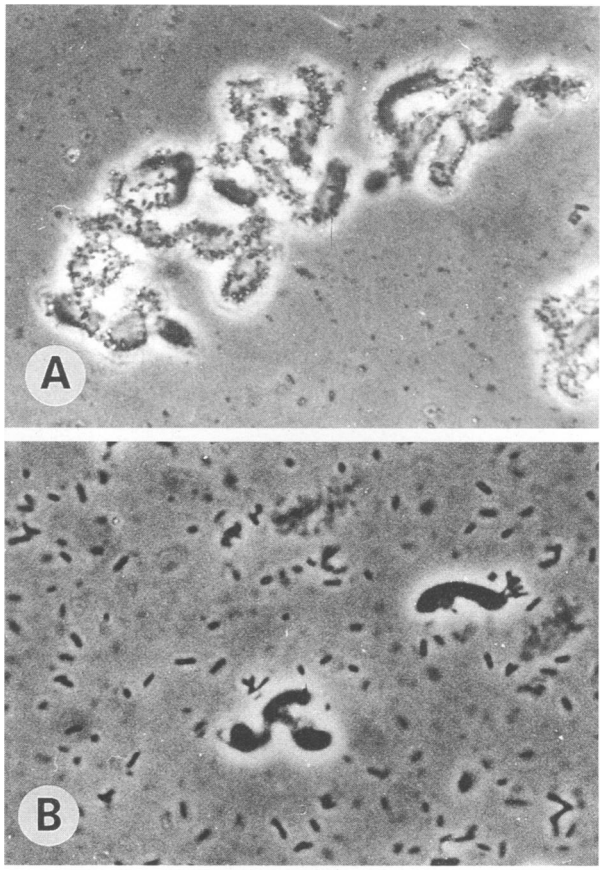

FIG. 4. Phase-contrast micrographs showing the interaction between isolated brush borders prepared from guinea pig ileum and REPEC. (A) *E. coli* 83/39 bound to brush borders, causing them to agglutinate. Note the almost complete absence of bacteria in the background. (B) Brush border preparation incubated with *E. coli* RDEC-1, showing numerous bacteria in the background, with none attached to the brush borders. Magnification, ×1,000.

Electron microscopy. Electron microscopic examination of the 10 REPEC strains revealed distinct fimbriae on 7, the exceptions being 82/183, 83/39, and M34. The fimbriae from six of these strains were similar, appearing as numerous rigid structures with a diameter of approximately 7 nm (Fig. 5A). With only one exception (*E. coli* 84/110/1), the presence of these structures corresponded with the capacity of the bacteria to cause mannose-sensitive hemagglutination of guinea pig erythrocytes, suggesting that they were type I fimbriae. Fimbriae from RDEC-1, by contrast, appeared finer and less rigid than the others and were approximately 3 nm in diameter (Fig. 5B).

DISCUSSION

Ten of the 12 putative REPEC strains investigated in this study fulfilled the criteria for classification as EPEC in that they were diarrheagenic attaching-effacing *E. coli* strains that did not produce detectable enterotoxins or cytotoxins. Two strains which had previously been reported to be attaching-effacing *E. coli* (30) did not display this phenotype in rabbit

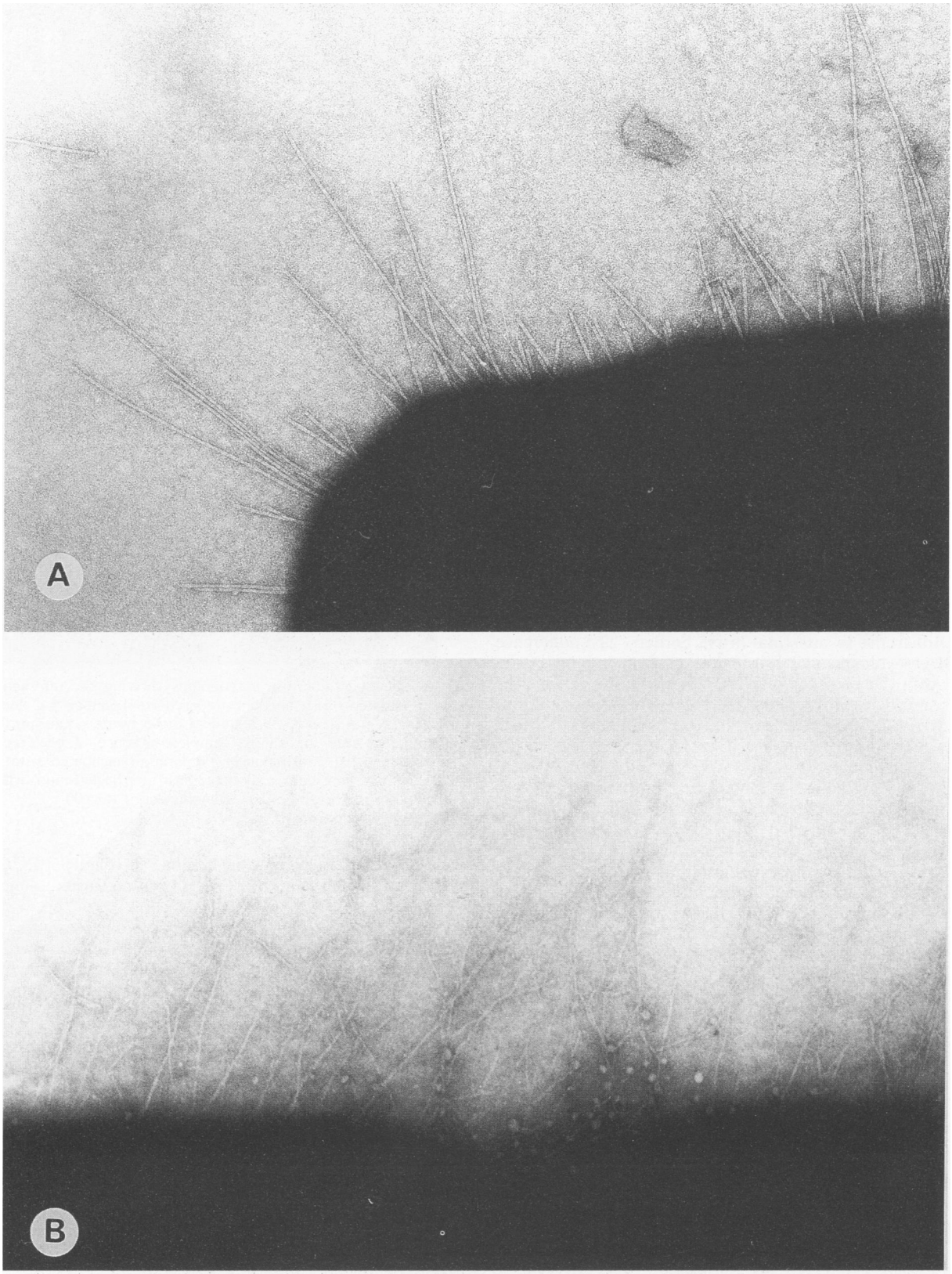

FIG. 5. Transmission electron micrographs of REPEC strains. (A) *E. coli* 83/123 carries numerous rigid fimbriae approximately 7 nm in diameter. Fimbriae with similar morphology were detected on strains 82/215/2, 82/260, 84/110/1, 82/123, 82/90, and 83/146. (B) *E. coli* RDEC-1, showing comparatively slender, slightly wavy fimbriae. Negative staining with ammonium molybdate; magnification, ×88,000.

ileum. These strains were also not recognized by the *eaeA* gene probe, which in our experience hybridizes with all attaching-effacing strains of *E. coli* regardless of origin (1). Possibly these strains carried the genes required for expression of the attaching-effacing phenotype on one or more plasmids that were lost during storage. Although plasmid-mediated expression of attachment and effacement has been reported (13), in most cases this locus is chromosomal (1, 18).

All of the attaching-effacing REPEC strains examined in this study were recognized by the *eaeA* gene probe, indicating that they carried virulence determinants involved in the production of the attaching-effacing phenotype, which is believed to reflect a comparatively late stage of adherence. In addition, all of the attaching-effacing *E. coli* strains were positive in the FAS assay. The differences that we observed in the 3- and 6-h FAS assays were comparable to those reported by Knutton et al. (20) for EPEC of human origin. Thus, the 3-h FAS assay was positive when used to examine bacteria which carried adhesins that bound directly to HEp-2 cells in an event analogous to stage 1 adherence of EPEC (9, 20, 45). The 6-h assay allowed detection of the attachment-effacement phenotype in bacteria which lacked the stage 1 adhesin.

Despite the obvious relatedness of the bacteria in terms of their attaching-effacing phenotype, they behaved differently in assays used to determine the nature of the initial attachment to eukaryotic cells. This was exemplified in the HEp-2 cell assay, in which four strains were not adherent and two strains each showed localized, diffuse, and mixed adherence. These findings extend the observations of Milon et al. (24), who demonstrated that some REPEC strains associated with outbreaks of diarrhea in rabbits adhered to HeLa cells in a diffuse pattern. These workers suggested that the adhesin responsible for this phenotype in highly virulent strains of serotype O103:H2 was a 32-kDa outer membrane protein. The serotype O103:H2 strain (84/110/1) which was examined in this study adhered to HEp-2 cells in a mixed pattern. This strain also agglutinated erythrocytes from humans, mice, sheep, calves, and pigs and adhered to ileal brush borders from rabbits, guinea pigs, sheep, and calves. Transposon mutagenesis of this strain with Tn*phoA* has shown that all of these properties can be ablated by insertional inactivation of a single gene locus for a secreted protein, suggesting that this phenotype is mediated by a common adhesin (1).

Strain 82/123 (O128:H2) also adhered to a variety of eukaryotic cells. This strain adhered to HEp-2 cells in a localized pattern, agglutinated erythrocytes from guinea pigs, humans, and mice, and adhered to ileal brush borders from rabbits, guinea pigs, mice, and sheep. Strain 83/39, which belongs to the same serotype and biotype as RDEC-1, the prototype REPEC strain, differed considerably from the latter in that it adhered to HEp-2 cells in a diffuse pattern and to brush borders from guinea pigs but not to those from rabbits. This strain also differed from RDEC-1 in having no detectable fimbriae on its surface. Three other REPEC strains did not adhere to rabbit brush borders, including M34, the nonfimbriated derivative of RDEC-1. One of these strains adhered weakly (approximately one bacterium per brush border) to brush borders from sheep.

Taken together, these results suggest that initial adherence by REPEC strains to eukaryotic cells involves a number of diverse mechanisms which appear to vary from strain to strain and which differ from the mechanisms employed by diarrheagenic *E. coli* from humans. One clear indication of the differences in the adhesins of REPEC stemmed from the observation that antisera prepared against putative REPEC adhesins inhibited adhesion to rabbit brush borders by the homologous strain only. This suggests that, at the very least, there are major antigenic differences between these adhesins. We also confirmed the finding of Pohl et al. (32) that only RDEC-1 carries AF/R1 fimbriae. The significance of this observation lies in the fact that AF/R1 is believed to enhance the virulence and govern the species specificity of RDEC-1 (4, 8, 45). The results of this study therefore raise questions as to the role in pathogenicity, and more particularly the species specificity, of adhesins which purportedly mediate the attachment of REPEC strains (other than RDEC-1) to host cells during the early stages of infection. These questions will be addressed in a separate study.

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