

Adhesion of *Escherichia coli* Strains Isolated from Diarrheic Weaned Rabbits to Intestinal Villi and HeLa Cells

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Thirty-eight strains, representative of 575 *Escherichia coli* isolates from weaned diarrheic rabbits, were tested for their ability to adhere in vitro to rabbit intestinal villi and to HeLa 229 cells. The O103 rhamnose-negative, highly pathogenic strains, which are epidemiologically predominant in France, attached to intestinal villi prepared from 8-day-old as well as 6-week-old rabbits and gave a diffuse adhesion pattern with HeLa cells. These adhesion properties were associated with the presence of a protein with a molecular weight of 32,000 in surface extracts of the strains. The expression of the adhesin was dependent on culture medium and temperature, and the adhesion was D-mannose resistant. Antisera raised against the 32,000-molecular-weight protein inhibited adhesion. This adhesin was not expressed in two nonpathogenic O103 strains, indicating its implication in virulence. However, the same adhesin was expressed by two O128 non- or moderately pathogenic strains. Therefore, adhesion to enterocytes is not the only factor involved in the pathogenicity of O103 strains.

In France, epizootics of life-threatening enteric diseases occur frequently in industrial rabbit-fattening farms. These diseases involve weaned animals and take place during the fattening period, and they have severe economic implications, due to weight loss in diarrheic rabbits and to high mortality rates (usually approximately 30%). They are associated with the colonization of the distal ileum and cecum by *Escherichia coli* strains. However, *E. coli* is frequently part of the normal intestinal flora of the rabbit (11, 14). A series of *E. coli* strains isolated from rabbits in farms affected by the disease was recently tested for serogrouping, biotyping, and pathogenicity (4). Of 575 strains isolated from rabbits in 119 French industrial farms with diarrheal problems, 53.6% belonged to the O103 serogroup (4). This group of strains could be further divided into rhamnose-negative biotypes, which were always highly pathogenic as judged by experimental reproduction of the disease, and rhamnose-positive biotypes, which were slightly pathogenic or not pathogenic at all (4). As few as 10⁴ live bacteria of the type strain B10 (O103, rhamnose negative) given per os to experimental rabbits are sufficient to produce disease and to kill approximately 50% of animals (3). This type strain adheres in vivo to the ileal epithelium and seems to be responsible for superficial lesions as judged by histology (3). An analogous strain, called GV (O103, rhamnose negative), has been shown, by transmission and scanning electron microscopy, to induce attaching-effacing lesions of the microvilli of rabbit enterocytes in vivo (9). This group of O103 strains may be provisionally considered as enteropathogenic *E. coli* (EPEC) animal analogs, as defined by Levine (13).

An EPEC analog isolated from a rabbit by Cantey and Blake, strain RDEC-1 (5), has been extensively studied. This O15:H- strain adheres to the enterocytes of rabbits older than 3 weeks (6), by means of fimbriae called AF/R1 with proteic subunits a molecular weight of 19,000 (1, 7). In rabbits which are being fattened, strains analogous to RDEC-1 seem to be frequently involved in field infection in Belgium or Holland (17, 20) but are rare in France.

The 38 strains used throughout this study (Table 1) were isolated from feces or cecal contents of weaned rabbits reared on farms experiencing episodes of *E. coli* diarrheas. These strains are representative of 575 strains isolated from 1984 to 1987 and have already been described for serogroups, simplified biotypes, (as described by Okerman and Devriese [17]), antibiotypes, and experimental pathogenicity, when administered per os to weaned rabbits (4). Briefly, 15 strains are O103, rhamnose negative, and highly pathogenic for weaned rabbits (i.e., inducing weight loss, dehydration, profuse watery diarrhea with patent or occult blood in the feces, and death of experimental animals 5 to 14 days after oral administration). Another four strains with the same pathogenicity are rhamnose negative and were previously described as O68 (4), a serogroup which gives cross-reactions with other serogroups (18). For that reason, we asked F. and I. Ørskov from the International *Escherichia* and *Klebsiella* Centre of Copenhagen to serotype them. These four isolates were found to be O26:H11 (isolates C102, C230, and D145) or rough:H11 (isolate C110). O26:H11 is a serotype which is also encountered in human EPEC (22). All the other bacterial strains are nonpathogenic or give only mild diarrheas, with no mortality after experimental oral administration. All of them are rhamnose positive. The RDEC-1 strain (O15:H-) was a kind gift of L. Okerman (Ghent University, Ghent, Belgium).

Adhesion tests. Adhesion to rabbit intestinal villi was carried out by using the technique described by Girardeau (10) with minor modification. Villi were prepared from 8-day-old and 6-week-old rabbits. After scraping of the mucosa, the villi were washed several times, treated with 4 ppt (4 ml/liter) Formalin for 2 h, and then washed again carefully. They were stored at -80°C, as described (10). In the adhesion test, 30 to 40 villi were incubated with Penassay broth (Difco Laboratories)-grown bacterial suspensions containing at least 5 × 10⁸ cells per ml in phosphate-buffered saline (PBS) (10 mM Na₂HPO₄, 10 mM KH₂PO₄, 0.15 M NaCl [pH 6.4]) supplemented with 2.5 µg of amphotericin B (GIBCO, Paisley, United Kingdom) per ml. The incubation was performed in the wells of round-bottomed microtiter

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TABLE 1. Adhesive properties in vitro of a set of 38 *E. coli* strains isolated from weaned rabbits from farms with episodes of diarrhea^a

Strain(s)	O sero-group ^b	Rhamnose reaction ^c	Pathogenicity ^d	Adhesion to intestinal villi		Adhesion to HeLa cells ^e
				8-day-old rabbit	6-week-old rabbit	
B10	103	-	+++	+	+	DA
14 others ^f	103	-	+++	+	+	DA
C102	26	-	+++	-	-	-
2 others	26	-	+++	-	NT ^g	-
C110	Rough	-	+++	-	+	LA
RDEC-1	15	+	+	-	+	-
C6	128	+	-	+	+	DA
C104	128	+	+/-	+	+	DA
C124	103	+	+/-	-	+	-
C127	103	+	-	-	+	-
C142	25, 26	+	-	-	-	-
B76	102	+	-	-	NT	-
D85	(68)	+	-	-	NT	-
C121	4	+	-	-	NT	-
E40	132	+	-	+	-	DA
3 others	132	+	- or +/-	+	-	DA
D100	2	+	-	-	-	-
4 others	2	+	- or +/-	-	-	-
D28	15	+	-	-	-	-
A155	85	+	-	-	-	DA

^a Strains were cultured in Penassay broth. Strain D85 gave only a mild reaction with serum anti-O68.

^b O serogroups were tested by slide agglutination (4).

^c Rhamnose-negative reactions are characteristic of strains highly pathogenic for weaned rabbits in France (4).

^d Pathogenicity was determined after oral inoculation of weaned rabbits with approximately 8×10^9 live bacteria. +++, Severe diarrhea, weight loss, dehydration, and mortality >50%; +, diarrhea with mortality <20%; +/-, mild diarrhea, no dehydration, growth retardation without weight loss, no mortality; -, no clinical signs (4).

^e DA, Diffuse adhesion, with an adhesion index ≥ 20 ; LA, localized adhesion; -, no adhesion, with an adhesion index <2.

^f Includes strains E22 and C55.

^g NT, Not tested.

plates for 20 min at 37°C on a Kline stirrer. The villi were then observed by phase-contrast microscopy. Bacterial adhesion to HeLa cells was tested by the technique of Scaletsky et al. (23). Cultures of HeLa cells on eight-chamber Lab-Tek slides (Miles Scientific, Div. Miles Laboratories, Inc., Naperville, Ill.) were washed, and 360 μ l of Eagle minimal essential medium containing 2% fetal calf serum and 1% D-mannose were added to each chamber. Overnight bacterial culture in Penassay broth (40 μ l) was added to each chamber. The Lab-Tek slides were then incubated for 30 min at 37°C, washed, incubated again for 3 h, washed again, fixed with methanol, stained with Giemsa stain, and observed under light microscopy. Adhesion indexes were calculated by counting the mean number of bacteria attached to 50 randomly chosen cells.

All the highly pathogenic strains of serogroup O103 presented a characteristic adhesion profile when grown in Penassay broth (Table 1): they could adhere to the brush border of intestinal villi prepared from 8-day-old or 6-week-old rabbits (Fig. 1) and showed a diffuse adhesion to HeLa cells, with adhesion indexes of at least 20 bacteria per cell (Fig. 2). The expression of the attachment phenotype depended on the medium used to cultivate the strains: no adhesion was seen when they were grown in brain heart infusion broth, Minca agar plus Polyvitex, Trypticase soy agar (BBL Microbiology Systems) with or without 1% glu-

cose, or Mueller-Hinton agar with or without 1% glucose; adhesion occurred irregularly when Trypticase soy broth (BBL) was used. The expression of the adhesion phenotype was influenced by the incubation temperature of the cultures: no adhesion occurred when the strains were cultivated overnight in Penassay broth at 20°C. In the adhesion test using rabbit villi, the results were not modified by the pH of the buffer: pHs ranging from 6.0 to 7.2 gave the same results. We therefore used in the routine test a buffer with a pH of 6.4 to approximate the usual pH found in distal ileum and in ceca of weaned rabbits (5.8 to 6.6) (3). This pH is compatible with good conservation of the villus structure. The origin of the villi did not seem to influence the results: we used villi from six different 8-day-old rabbits and from three different 6-week-old animals without noting any differences in the adhesion pictures. The presence of D-mannose (1% in the HeLa test and 0.2 M in the villus test) did not impair adhesion.

As compared with the adhesion profile of the O103 group, the following must be noted (Table 1). (i) The less or nonpathogenic O103 strains (C124 and C127) adhered only to villi from 6-week-old rabbits, a profile seen in strain RDEC-1 too. (ii) The highly pathogenic O26 strains did not adhere to any system, except for strain C110. This strain is probably a rough variant of O26:H11 strains (since it has the same H antigen and the same biotype). It adhered to 6-week-old villi and showed localized adhesion to HeLa cells. (iii) Among the less or nonpathogenic strains, two O128 strains (C6 and C104 [Table 1]) had the same adhesion profile as the O103 pathogenic strains.

Adhesin characterization. Crude surface bacterial extracts were prepared as described by de Graaf et al. (8). Bacteria were pelleted by centrifugation at $1,800 \times g$ at 4°C for 30 min and suspended to an optical density of 100 U at 660 nm in phosphate-buffered urea (2 M urea in 50 mM $\text{Na}_2\text{HPO}_4 \cdot \text{NaH}_2\text{PO}_4$, pH 7.2). The suspensions were heated at 60°C for 20 min and centrifuged at $35,000 \times g$ at 4°C for 20 min. The supernatants were submitted to precipitation by ammonium sulfate (60% of saturation, 4°C, 2 h). The precipitates were collected by centrifugation ($35,000 \times g$, 4°C, 20 min), solubilized in phosphate buffer without urea, and dialyzed for 24 h against this buffer. Samples of the extracts (approximately 10 μ g of proteins) were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in the presence of dithiothreitol, with linear 15% resolution gels and 4.5% stacking gels, by the method of Laemmli (12). The gels were stained with silver nitrate or Coomassie blue R-250, as described by Roberts et al. (21).

When applied to the RDEC-1 strain cultivated in Penassay broth, this technique gave an extract which showed a prominent component with a molecular weight of 19,000 (Fig. 3). This protein has been described as the subunit of the adhesive fimbriae, AF/R1, which characterize this strain (1). It was absent in an extract prepared from RDEC-1 cultivated in brain heart infusion broth, i.e., in conditions of nonexpression of the adhesin AF/R1 (1). Extracts were prepared from a set of strains cultured in Penassay broth at 37°C. This set included strains B10, C55, and E22 (O103, rhamnose negative), C127 (O103, rhamnose positive), C102 (O26, rhamnose negative), C110 (rough, rhamnose negative), C6 and C104 (O128, rhamnose positive), and E40 (O132, rhamnose positive). An extract was also prepared from the B10 strain cultivated at 20°C in Penassay broth, a condition in which the adhesion phenotype is not expressed. The extracts of the strains which adhered to both types of villi and to HeLa cells contained a component with a molecular weight

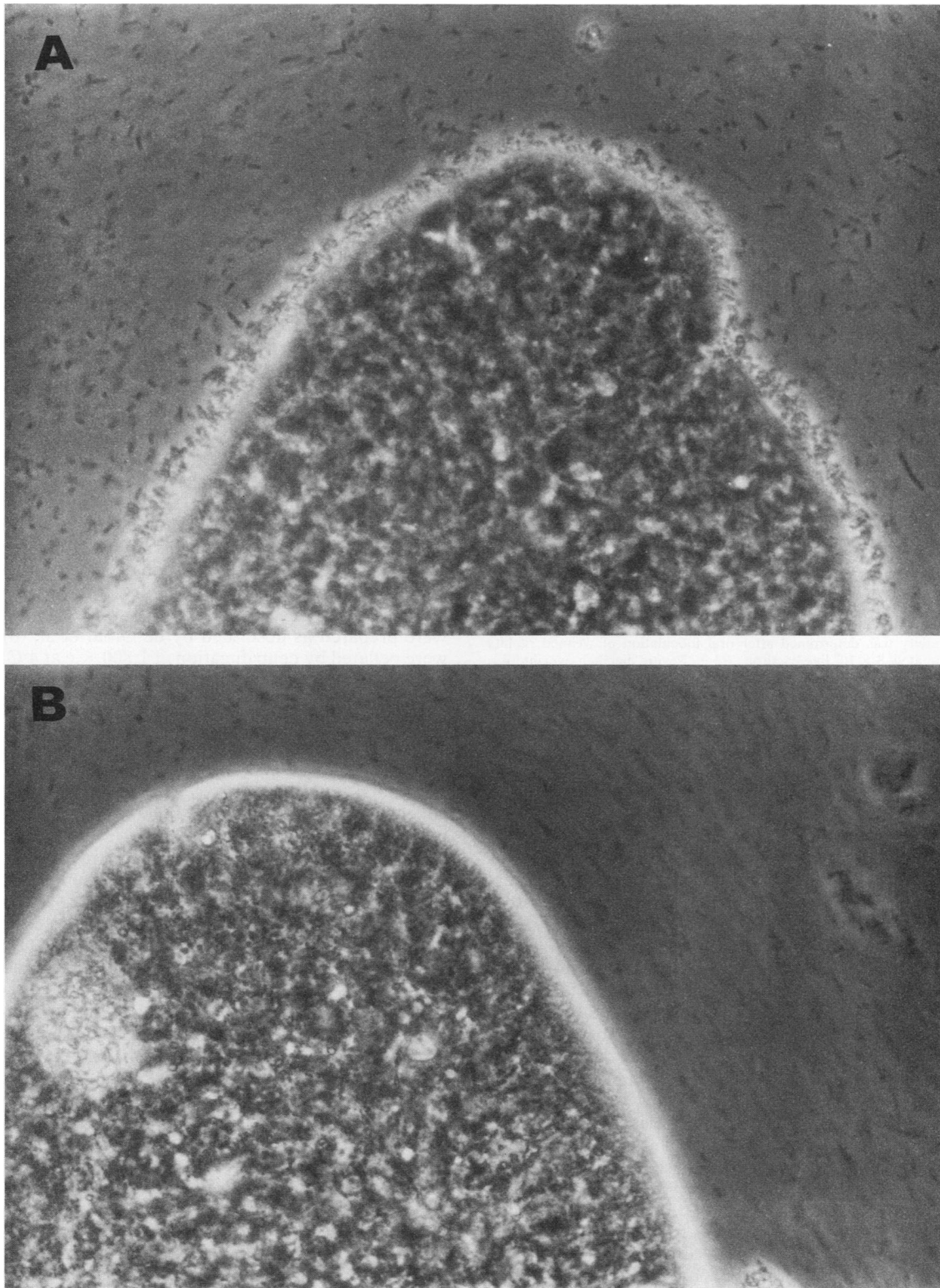


FIG. 1. Adhesion of *E. coli* strains isolated from weaned rabbits to 8-day-old rabbit intestinal villi. (A) Strain B10 (O103, rhamnose negative), showing prominent adhesion to the brush border of enterocytes; (B) strain D28 (O15, rhamnose positive), showing no adhesion.

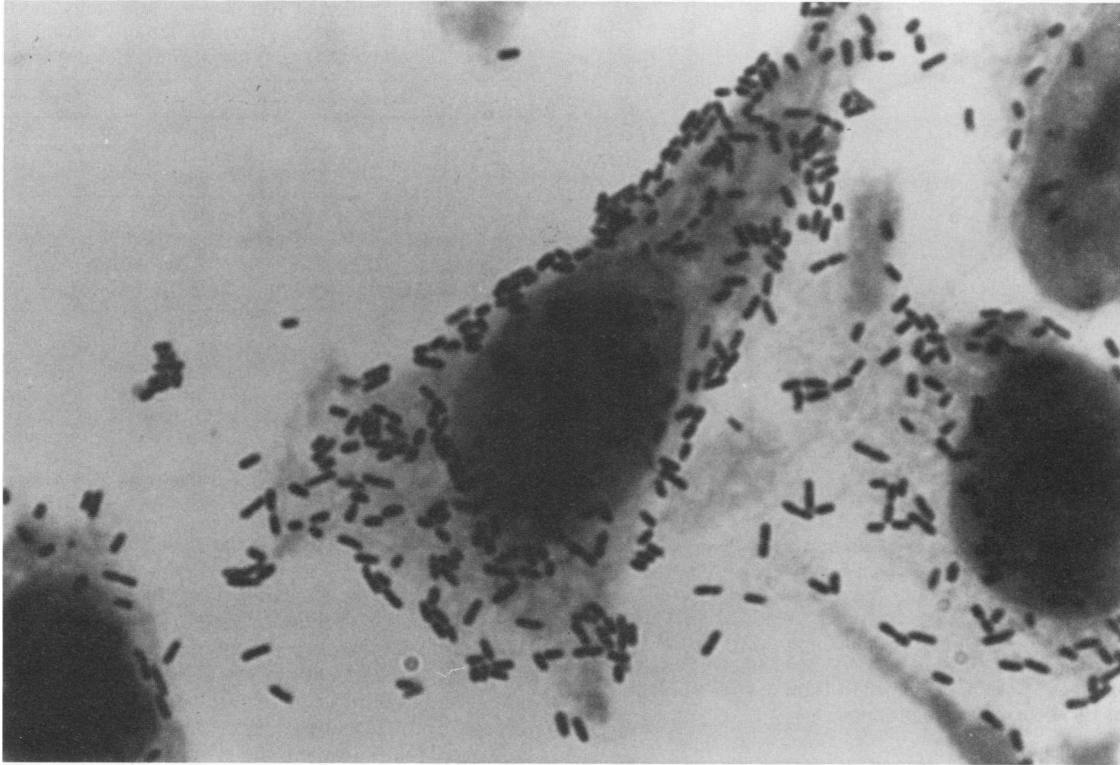


FIG. 2. Diffuse adhesion of rabbit *E. coli* B10 to HeLa cells.

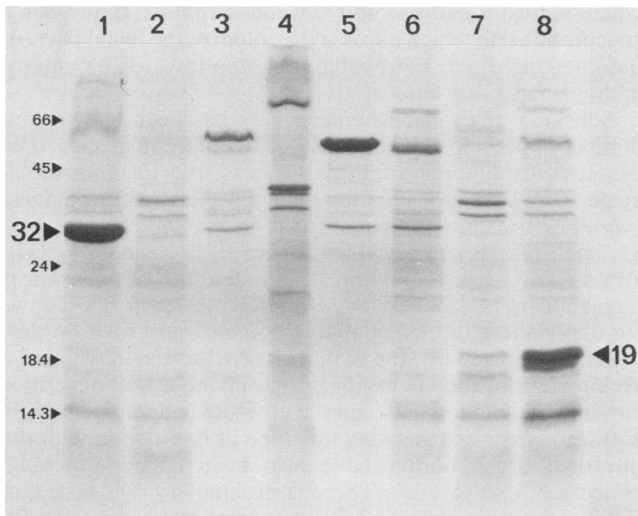


FIG. 3. SDS-PAGE (15% polyacrylamide) of surface extracts prepared from *E. coli* strains isolated from weaned rabbits. O103 rhamnose-negative strains which adhere to intestinal villi from neonates and weaned rabbits as well as to HeLa cells show a 32-kDa protein. This protein is present in two nonpathogenic *E. coli* O128 strains which have the same adhesion profile. The extract prepared from strain RDEC-1 shows the 19-kDa AF/R1 subunit. All strains were grown in Penassay broth at 37°C except B10 (20°C) in lane 2. Lanes: 1 and 2, B10; 3, E22; 4, C127; 5, C104; 6, C6; 7, C102; 8, RDEC-1. Strain characteristics are described in Table 1. Molecular mass markers (kilodaltons) are shown to the left of the gel.

of 32,000, apparently correlated with this adhesive profile (Fig. 3). This protein was absent in strain B10 after culture at 20°C and was encountered only in the O103 pathogenic strains and in the O128 nonpathogenic ones. Adhesion inhibition tests by the B10 crude extract were run by preincubating HeLa cells or villi with the crude extract at 37°C for 30 min. The tests were then run as described above. The extract prepared from strain B10 grown in Penassay broth at 37°C inhibited adhesion of the O103 rhamnose-negative strains to HeLa cells, as well as adhesion of strains C6 and C104 (Table 2). On the other hand, the extract prepared from the same strain grown at 20°C (i.e., in nonadhesive conditions) did not inhibit adhesion to HeLa cells.

The protein with a molecular weight of 32,000 identified in the crude extract of strain B10 was further purified by low-pressure liquid chromatography. Portions (approximately 5 mg) of undialyzed crude extract were first gel filtered on Bio-Gel A-5M (Bio-Rad Laboratories, Richmond, Calif.) in a K9-30 column (Pharmacia, Inc.) equilibrated with phosphate-buffered urea. Fractions (1 ml) were eluted at a flow rate of 4 cm/h. The 32,000-molecular-weight protein was identified in the eluted fractions by SDS-PAGE. Pooled fractions containing the 32,000-molecular-weight protein were further chromatographed on Ultrogel Aca 44 (LKB-IBF Pharmindustrial) in a K9-60 column with the same elution conditions. The fractions of a peak observed at an optical density of 280 nm eluting in the middle of the chromatogram showed nearly pure 32-kilodalton (kDa) protein, as judged by silver-stained SDS-PAGE analysis.

Antisera against the 32-kDa protein were raised in rabbits by multiple intradermal inoculations of dialyzed, chromatography-purified protein (0.1 mg in 0.5 ml of PBS mixed with

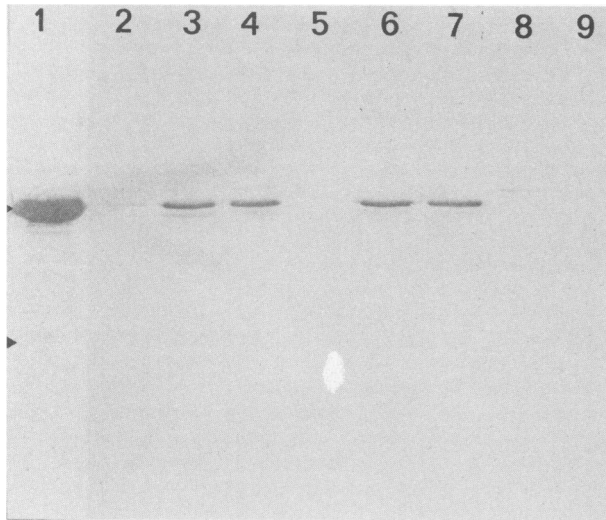


FIG. 4. Immunoblots of surface extracts of Penassay broth-grown strains, tested with serum raised against the chromatography-purified 32-kDa protein associated with adhesion. The serum recognizes the protein in strain B10 grown at 37°C (lane 1), E22 and C55 (lanes 3 and 4), and C104 and C6 (lanes 6 and 7), but not in B10 grown at 20°C (lane 2), C127 (lane 5), C102 (lane 8), or RDEC-1 (lane 9). Strain characteristics are described in Table 1. Arrowheads show the positions of 32- and 19-kDa proteins.

0.5 ml of complete Freund adjuvant). Two inoculations were done, and the animals were bled 10 days after the second inoculation. The serum specificity was tested by immunoblotting (Fig. 4). Briefly, crude extracts of the strains were subjected to preparative SDS-PAGE and then transferred electrophoretically to nitrocellulose sheets by the method of Towbin et al. (25). The sheets were air dried and stored at 4°C until used. When used, the unabsorbed sites were saturated by two successive washes at room temperature in 10 mM PBS, pH 7.4, containing 0.05% Tween 20 (PBS-T), following by a 30-min incubation in PBS plus 5% (wt/vol) skim milk (PBS-M). The sera described above were then applied at 1/100 dilution in PBS-M for 2 h at room temperature. After three washes for 10 min each with PBS-T, the immune complexes were detected by adding goat antibodies against rabbit immunoglobulin G conjugated to peroxidase (Biosys, Compiègne, France) diluted 1/1,000 in PBS-M. Sheets were then washed three times in PBS-T and once in PBS, and 3-3'-diaminobenzidine tetrahydrochloride (0.04% [wt/vol]) in PBS and H₂O₂ (1 μl of 30% H₂O₂ per ml) were added as a substrate for the enzyme. The reaction was stopped by extensive washing of the sheets in distilled water. The sera recognized mainly the 32,000-molecular-weight protein (Fig. 4). They were tested for their ability to inhibit the adhesion of bacteria to HeLa cells and to 8-day-old rabbit villi. Bacteria were preincubated with dilutions of 32-kDa antisera for 30 min at 37°C. The tests were then run as described above. Inhibition of adhesion to HeLa cells was quantified by the measurement of adhesion indexes. For inhibition of adhesion to villi, only qualitative results given by nonagglutinating dilutions of sera were taken into account. The sera completely inhibited the adhesion of strain B10 to villi when preincubated at 1/10 dilution with bacteria, without giving significant agglutination, and also inhibited the adhesion to HeLa cells at up to 1:100 dilutions (Table 2).

Our results clearly show the existence of a particular adhesion phenotype in the O103 rhamnose-negative group of

TABLE 2. Inhibition of adhesion to HeLa cells and to 8-day-old rabbit villi by B10 surface extract and antisera against chromatography-purified 32-kDa protein

Strain ^a	Adhesion to HeLa cells ^b			Adhesion to villi		
	No inhibitor	B10 extract ^c	Anti-32-kDa protein ^d	No inhibitor	B10 extract ^c	Anti-32-kDa protein ^d
B10	29.1 (3.1)	4.4 (0.6)	1.0 (0.1)	+	-	-
E22	20.0 (2.0)	5.7 (1.3)	0.2 (0.05)	+	-	-
C104	23.2 (2.7)	2.4 (0.7)	1.5 (0.5)	+	-	-
C6	22.8 (3.0)	1.0 (0.5)	1.2 (0.4)	+	-	-

^a Strain characteristics are shown in Table 1.

^b Adhesion index is the mean number of bacteria attached per cell (calculated from 50 cells). Standard error of the mean is in parentheses.

^c B10 extract preincubated at 1/10 dilution of adhesion medium (~250 μg of protein per ml) with cells or villi for 30 min at 37°C. An analogous extract prepared from strain B10 after culture at 20°C (i.e., nonadhesive) did not inhibit adhesion.

^d Antiserum was preincubated at 1/10 dilution with bacteria in adhesion medium for 30 min at 37°C. See Fig. 4 for serum specificity in the immunoblot. Control preimmune serum did not inhibit adhesion.

E. coli pathogenic for weaned rabbits. All 15 strains of the group we tested could adhere to intestinal villi prepared from rabbit neonates or from weaned rabbits and showed diffuse adhesion to HeLa cells. This adhesion phenotype was resistant to the addition of D-mannose and thus could not be due to type 1 pili. It was not sensitive from a qualitative point of view to pH variations in the range of 6 to 7.2, which includes the normal pH found in the ilea and ceca of healthy or diarrheic young weaned rabbits (3, 14). The adhesion was dependent on culture medium and temperature: adhesion took place essentially when the strains were grown in Penassay broth, as described also for strain RDEC-1 (7), and was suppressed when bacteria were grown at 20°C. Taken as a whole, these results suggest that the O103 group of *E. coli*, which is highly pathogenic for weaned rabbits, expresses a specific adhesin which allows it to colonize the distal parts of the intestinal tract, a probable first step towards its enteropathogenicity.

Adhesion of *E. coli* to enterocytes has been shown in a number of pathologic situations and in different species to be mediated by fimbrial adhesins. This is true for enterotoxigenic *E. coli*, responsible for neonatal diarrhea in bovines, swine, or travelling humans (for reviews, see references 2, 13, and 16). This is also true for an EPEC analog (1, 24), strain RDEC-1 in rabbits, in which step one of adhesion is mediated by plasmid-encoded AF/R1 pili. Step one allows for a peripheral attachment to the intestinal brush border, which is followed by step two, a close adhesion in which the architecture of the microvilli is lost, giving a well-described cup-like association between the enterocyte and the bacteria (24). *E. coli* strains showing this type of interaction with the intestinal brush border have also been called attaching-effacing *E. coli* (15, 19). There is preliminary evidence that O103 pathogenic strains give this type of picture in vivo (9).

In the O103 rhamnose-negative group of *E. coli* strains, the adhesion profile is correlated with the presence in surface extracts of the strains of a protein with a molecular weight of 32,000, which is higher than the size of already known fimbrial subunits. We have only scarce and indirect evidence that the adhesin may be of the fimbrial type: (i) bacteria of the strain B10 showed pili when grown in Penassay broth but not after Minca medium culture, a condition in which they did not express their adhesion phenotype (data not shown); and (ii) the technique used to

prepare the extract is known to give enriched preparations of pili, such as K99, as described previously (8), and it gives enriched AF/R1 subunits with strain RDEC-1 (Fig. 3). However, more experiments are needed to characterize precisely the bacterial structure which carries the 32-kDa protein. Nevertheless, several results give evidence of the involvement of this protein in adhesion: (i) the protein is present in each O103 rhamnose-negative strain tested so far as well as in two O128 strains which share the same adhesion phenotype; (ii) it is absent in O103 rhamnose-positive strains, which show a different adhesion profile, as well as in other strains (including strain RDEC-1); (iii) it is absent when strain B10 is grown at 20°C, i.e., in conditions of nonexpression of the adhesion phenotype; (iv) it is recognized by sera raised against whole bacteria expressing the same adhesion phenotype as O103 rhamnose-negative strains and is able to inhibit adhesion (data not shown); (v) it is not recognized by sera directed against O103 rhamnose-negative strains grown in Minca medium, in which expression of the adhesin is phenotypically undetectable (data not shown); and (vi) enriched extract of this protein and antisera raised against the purified protein inhibit adhesion of O103 (rhamnose negative) and O128 strains to villi and to HeLa cells.

To our knowledge, this is the first description of this adhesion system. It is clearly different from the AF/R1 adhesin of strain RDEC-1, which confers different adhesion properties and has a subunit with a molecular weight of 19,000 (7).

In EPEC, adhesion is important for the expression of pathogenicity. The implication of the above-described adhesion system in the high degree of pathogenicity of O103 rhamnose-negative *E. coli* is probable. However, the adhesin is surely not the only pathogenic factor in the O103 rhamnose-negative group, since it is also recognized in O128 nonpathogenic strains. There is need for further characterization of the adhesin described here and for research into other pathogenic factors, such as toxin secretion.

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