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Plasmid-Mediated Factors Conferring Diffuse and Localized Adherence of Enteropathogenic *Escherichia coli*

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Histopathological evidence suggests that the adherence of enteropathogenic *Escherichia coli* (EPEC) to the mucosa of the small bowel is an important step in pathogenesis. Several reports have shown that many EPEC isolates adhere to HEp-2 and HeLa cells in tissue cultures. In the HeLa cell assay, there are at least two distinct patterns of adherence: localized adherence, which is characterized by the formation of bacterial microcolonies, and diffuse adherence, in which bacteria cover the cell uniformly. We have found that these two patterns can be demonstrated in HEp-2 cells as well as in HeLa cells and that the results of the two assays are closely correlated. Using a DNA probe which is sensitive and specific for localized adherence to HEp-2 cells, we provide evidence that localized adherence and diffuse adherence by EPEC are due to at least two genetically distinct adhesins which confer phenotypic differences in both the morphology of HEp-2 cell adherence and in surface hydrophobicity. The two factors are each encoded on plasmids which vary in size from 55 to 70 megadaltons; one strain exhibiting localized adherence carried these genes on the chromosome.

Enteropathogenic *Escherichia coli* (EPEC) strains were the first *E. coli* strains to be implicated in diarrheal disease and continue to be an important cause of infantile gastroenteritis in many parts of the world (12a, 27). In contrast to the well-characterized enterotoxigenic *E. coli* (ETEC), the pathogenic mechanisms of EPEC diarrhea are just beginning to be unravelled (6). Several histopathological studies of infants and animals with EPEC infections have suggested that the adherence of the bacteria to the small bowel mucosa is important in the induction of disease (4, 17, 19–21, 28). Cravioto et al. (5) reported that the majority of EPEC strains isolated from outbreaks of infantile gastroenteritis adhered to HEp-2 cells in cultures but that this adherence was rare in non-EPEC strains. Baldini et al. (1) subsequently demonstrated that HEp-2 cell adherence was encoded on a 60-megadalton (Md) plasmid (pMAR2) in EPEC strain E2348 (serotype O127:H6), a strain previously shown to be diarrheagenic in adult volunteers (13). Volunteer studies (M. M. Baldini, J. P. Nataro, M. M. Levine, R. E. Black, and M. L. Clements, Abstr. Annu. Meet. Am. Soc. Microbiol. 1984, B128, p. 39) showed that the presence of this plasmid correlated with the ability of E2348 to cause disease in healthy adults. The name EPEC adherence factor (EAF) has been applied to the adhesin which is specified by plasmid pMAR2 and which confers both adherence to HEp-2 cells and diarrheagenicity in volunteers (A. D. O'Brien and J. P. Nataro, *in* L. Leive, ed., *Microbiology—1985*, in press).

Genetic studies on plasmid pMAR2 have identified a region which carries genes essential for the adherence of E2348 to HEp-2 cells. A DNA fragment lying within this region has been utilized as a sensitive and specific probe to detect EAF in *E. coli* isolates (Baldini et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1984, B128, p. 39).

Recently, Scaletsky et al. (22) reported that some *E. coli* isolates adhere to HeLa cells and that there are at least two distinct patterns of such adherence. These patterns were designated localized adherence (LA), in which bacteria form microcolonies on the HeLa cell surface, and diffuse adherence (DA), in which bacteria cover the cell uniformly. In this study, we compare the patterns of adherence of *E. coli* to HEp-2 and HeLa cells, examine the genetic relationship between DA and LA by using the EAF DNA probe, and determine the role of plasmids in mediating the adherence of *E. coli* to HEp-2 and HeLa cells.

MATERIALS AND METHODS

Strains. *E. coli* strains from Brazil were isolated from the stools of infants with diarrhea. Serotyping was performed by standard methods (7).

EPEC strains isolated from outbreaks of infantile gastroenteritis in the United States were kindly provided by I. K. Wachsmuth of the Centers for Disease Control, Atlanta, Ga.

E. coli K-12 HB101 (F$^-$ *hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44*) was used as the recipient for EPEC plasmids during transformation.

Tissue cultures. The adherence of *E. coli* to HEp-2 cells was examined by a modification of the HEp-2 cell assay of Cravioto et al. (5). HEp-2 monolayers were prepared in 12-well Costar tissue culture dishes with glass cover slips. HEp-2 cells were washed three times with Hanks balanced salt solution and incubated in 1 ml of Eagle essential medium with 0.5% D-mannose and 20 μl of an overnight bacterial culture (1% tryptone water without mannose). The presence of mannose in the assay inhibits adherence by type 1 pili (5). After 3 h of incubation, the cells were fixed with methanol, stained with 10% Giemsa stain, and examined under oil-immersion light microscopy. The adherence of *E. coli* to HeLa cells was assayed by the method of Scaletsky et al. (22).

DNA extraction and transformation. Plasmids were extracted by the alkaline extraction method of Birnboim and Doly (2) or by the method of Kado and Liu (10) and analyzed by electrophoresis through 0.7% agarose gels. Chromosomal DNA was extracted by the method of Brenner et al. (3) and

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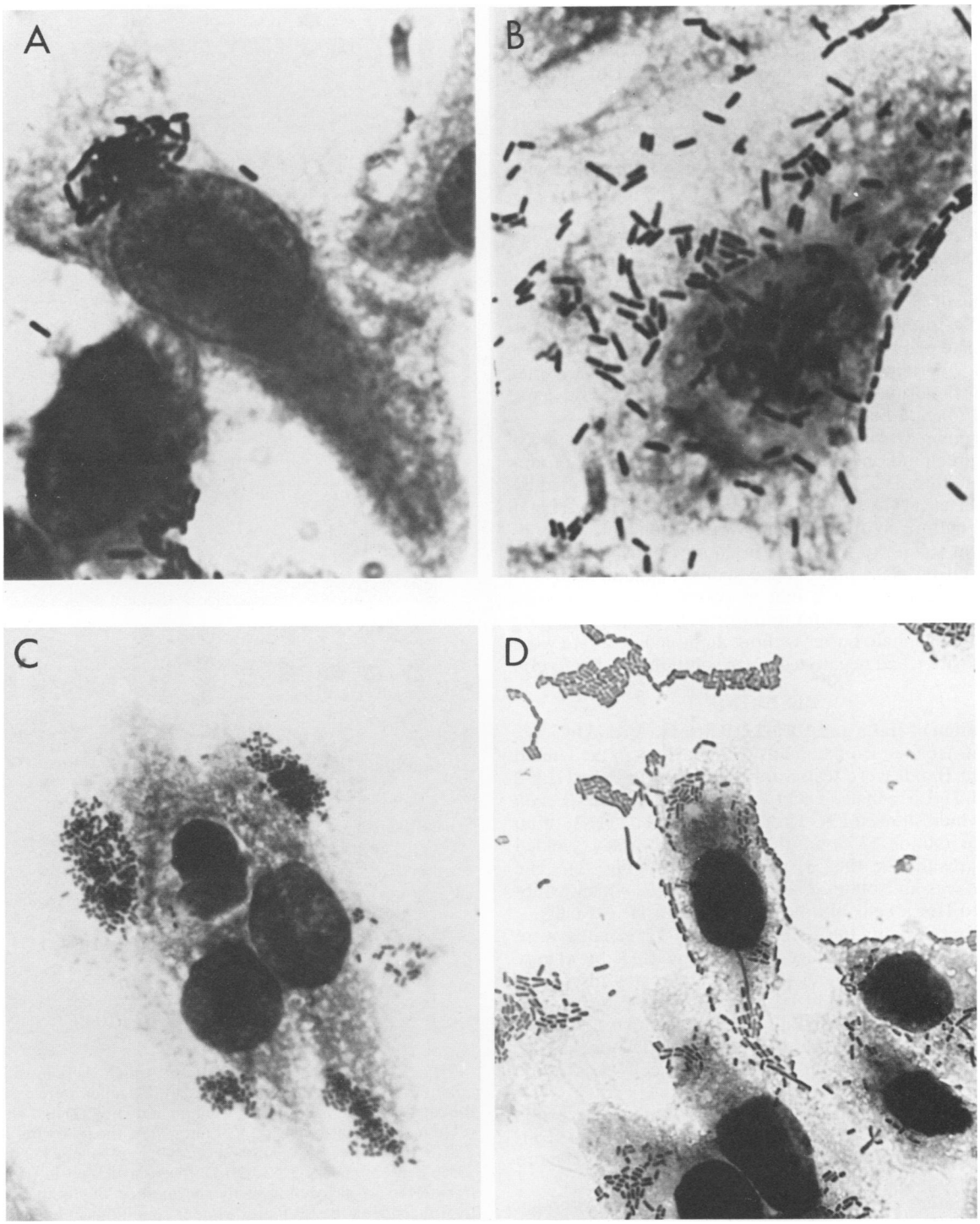

FIG. 1. LA and DA to HeLa and HEp-2 cells seen under oil-immersion light microscopy. (A) HeLa cells showing LA. Bacterial cells formed a characteristic microcolony. (B) HeLa cells showing DA. (C) HEp-2 cell showing LA. (D) HEp-2 cell showing DA; note the autoagglutination of bacteria free from the cell.

digested with restriction endonucleases in accordance with the instructions of the manufacturers.

Transformation was performed by the calcium chloride method of Mandel and Higa (15). Cotransformation was performed by the method of Kretschmer et al. (11) with pMR5 as the marker plasmid. After successful cotransformation, pMR5 was cured by several passages at 42°C.

DNA probe preparation. The EAF probe consists of a 1-kilobase *Bam*HI-*Sal*I fragment from plasmid pMAR2. Regions of this plasmid involved in adherence were localized by deletion and insertional inactivation of these genes as will be described elsewhere (M. M. Baldini, J. P. Nataro, and J. B. Kaper, manuscript in preparation). A derivative of pMAR2, pMAR22, was constructed by cloning a 13-kilobase

*Hin*dIII fragment from pMAR2 into the vector pACYC184. pMAR22 was digested with *Bam*HI and *Sal*I (Bethesda Research Laboratories, Inc., Gaithersburg, Md.), and the fragments were separated by polyacrylamide gel electrophoresis. The 1-kilobase *Bam*HI-*Sal*I fragment was removed from the polyacrylamide by electroelution and successively extracted with phenol, chloroform, and ether. After ethanol precipitation, the purified DNA was labeled in vitro with [^{32}P]dATP (New England Nuclear Corp., Boston, Mass.) by the nick translation method (16).

Hybridization. *E. coli* strains were examined for homology to the EAF probe by colony hybridization as described by Moseley et al. (18). Hybridizations were carried out under conditions of high stringency (50% formamide) or low stringency (25% formamide). Chromosomal restriction fragments were transferred to nitrocellulose by the method of Southern (26) and hybridized under stringent conditions. Plasmid DNA was depurinated for 30 min in 0.25 M HCl before being prepared for Southern blotting.

Hydrophobicity studies. Surface hydrophobicity was measured by precipitation with increasing concentrations of ammonium sulfate, i.e., the salting-out procedure described by Lindahl et al. (14). Briefly, equal volumes of an overnight bacterial culture in L-broth and various concentrations of ammonium sulfate (0 to 4.0 M) in 20 mM phosphate buffer were mixed on glass microscope slides. Agglutination was allowed to proceed for 2 min at room temperature with gentle rocking. Ammonium sulfate (4.0 M) in phosphate buffer and phosphate buffer without ammonium sulfate were used as positive and negative control solutions, respectively.

RESULTS

Correlation of HeLa and HEp-2 cell assays. A total of 75 *E. coli* strains isolated from humans with diarrhea in the United States and Brazil were tested for adherence to both HEp-2 cells and HeLa cells. Except for one isolate, all *E. coli* isolates which showed LA (27 or 36%), DA (8 or 11%), or no adherence (40 or 53%) in the HeLa cell assay yielded identical results in the HEp-2 cell assay (Fig. 1). The exception was an isolate of serotype O127:H$^-$ which exhibited LA to HeLa cells but did not adhere to HEp-2 cells.

Hybridization with the EAF probe. All 75 isolates were tested for the presence of genes encoding EAF by colony hybridization. It was found that all the isolates displaying LA to both HeLa cells and HEp-2 cells were positive with the EAF probe; conversely, all isolates negative for LA to both cell lines were negative with the probe. The one isolate adhering to HeLa cells but not to HEp-2 cells was probe positive.

All *E. coli* strains that displayed DA alone were probe negative upon colony blotting. When the hybridization stringency was reduced to allow the hybridization of more distantly related genes, there was still no homology.

Determination of the locations of EAF genes. Although nearly all HEp-2 cell-adherent *E. coli* isolates we have examined contain plasmids in the 55- to 70-Md range, no single plasmid species was conserved in isolates displaying the LA phenotype (Fig. 2a). We hypothesized, therefore, that the genes encoding adherence could be found on plasmids of various sizes and evaluated this possibility by using the EAF probe. Plasmids extracted from probe-positive *E. coli* strains were separated on agarose gels and transferred to nitrocellulose. Of the 27 isolates so analyzed, 25 were found to contain EAF genes encoded on single plasmids which varied in size from 55 to 70 Md (Fig. 2b).

One of the two probe-positive strains not carrying EAF genes on a plasmid was further analyzed to determine if EAF genes were encoded on the chromosome. Chromosomal DNA extracted from this strain (a diarrheal isolate of serotype O142:H$^-$) was digested with endonuclease *Bam*HI, *Sal*I, or *Hin*dIII, and the resulting fragments were separated by agarose gel electrophoresis. Southern blot analysis of this DNA revealed single discrete bands in each digest corresponding to chromosomal fragments which carried EAF-homologous sequences (Fig. 3).

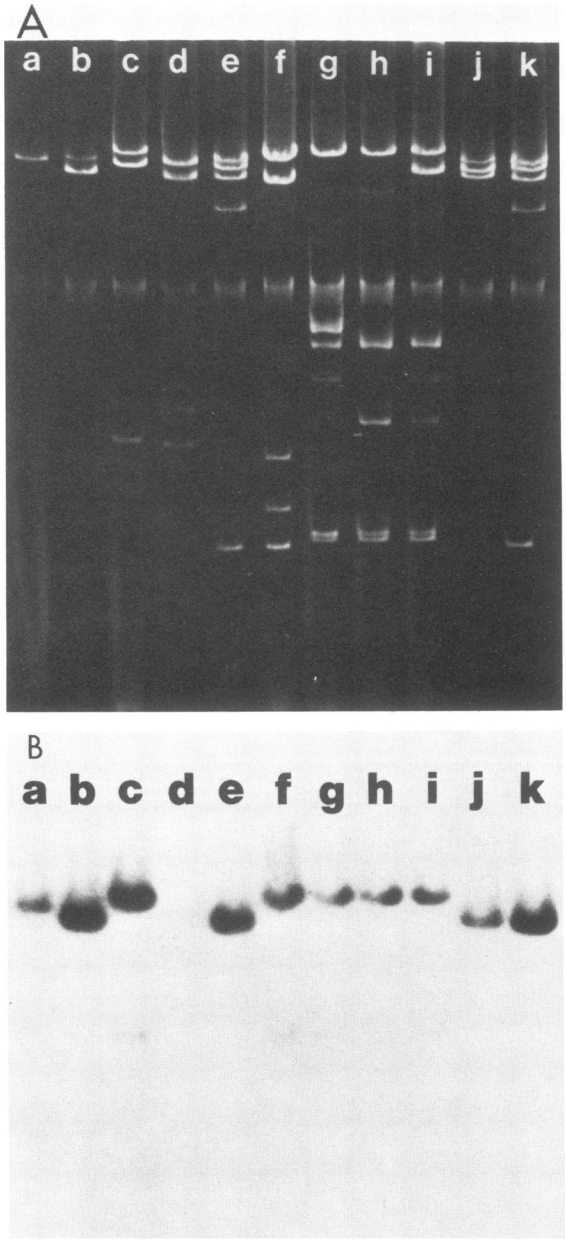

FIG. 2. Plasmid analysis of strains which were positive upon colony blotting with the EAF probe. (A) Plasmids were extracted by the rapid alkaline method of Birnboim and Doly (2) and electrophoresed in 0.7% agarose at 120 V. Lanes show the following strains: a, E2348; b, 2087; c, 1104; d, 1092; e, 2309; f, 2430; g, 2450; h, 0659; i, 2395; j, 2362; and k, 3252. (B) DNA from the gel in panel A was transferred to nitrocellulose by the method of Southern (26) and hybridized with the EAF probe under conditions of high stringency. Lanes are as in panel A.

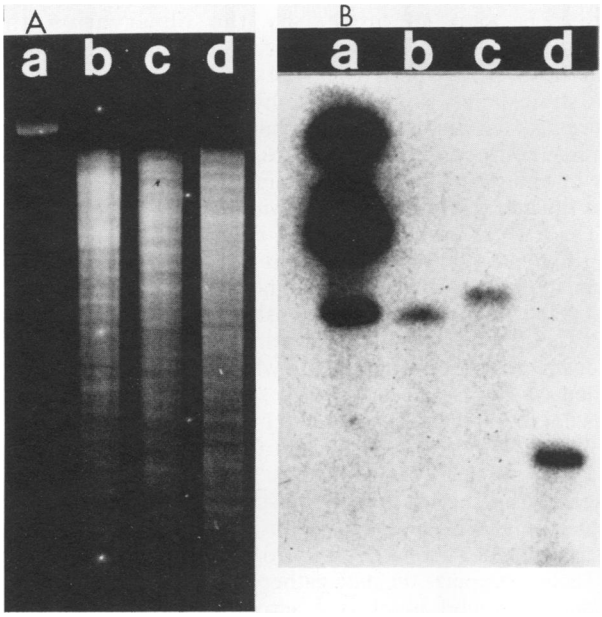

FIG. 3. Presence of EAF genes on the chromosome in LA isolate HC 21. (A) Chromosomal DNA was extracted by the method of Brenner et al. (3), digested with *Sal*I, *Bam*HI, or *Hin*dIII, and electrophoresed in 0.7% agarose at 80 V. Lanes: a, Three forms of plasmid pMAR22 containing cloned EAF genes; b, *Sal*I digest of HC 21; c, *Bam*HI digest of HC 21; and d, *Hin*dIII digest of HC 21. (B) Autoradiograph of the chromosomal blot in panel A hybridized with the EAF probe. DNA was transferred to nitrocellulose by the method of Southern (26) and hybridized with the EAF probe under conditions of high stringency. Lanes are as in panel A.

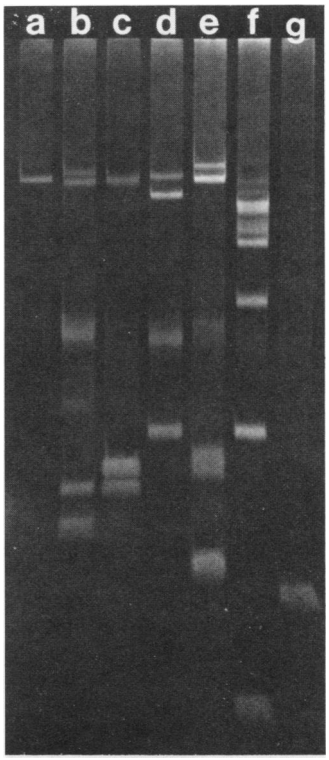

FIG. 4. Plasmids of DA strains. Plasmid DNA was extracted by the method of Kado and Liu (10) and electrophoresed in 0.7% agarose. Lanes show the following strains: a, 042; b, 051; c, 064; d, 764; e, 2262; f, 2322; and g, 5582.

Examination of plasmids in DA strains. Results of the EAF probe hybridization experiments suggested that the LA phenotype is due to the adhesin EAF and that the genes encoding this factor are usually plasmid encoded. Suspecting that the DA phenotype might also be plasmid mediated, we examined our DA strains for the presence of large plasmids (Fig. 4). We failed to find any single plasmid species which was conserved in most of the DA isolates. One isolate did not carry a 55- to 70-Md plasmid, as revealed by the procedures of Birnboim and Doly (2) or Kado and Liu (10).

To determine whether a plasmid was responsible for the DA phenotype, we screened DA isolates from this study and from another study in Peru (J. P. Nataro, M. M. Baldini, J. B. Kaper, R. E. Black, N. Bravo, and M. M. Levine, submitted for publication), looking for a strain carrying only a single plasmid. An isolate from Peru (serotype O44:H18) carried a single plasmid of 65 Md, designated pJPN8. Five micrograms of purified plasmid DNA from this strain was cotransformed with 0.2 μg of pMR5 DNA into *E. coli* K-12 HB101. Cotransformants acquiring pJPN8 exhibited DA to HEp-2 cells and HeLa cells. Subsequent curing of pMR5 by passage at 42°C did not affect DA.

Phenotypic differences between DA and LA K-12 transformants. In addition to a distinctly different pattern of adherence to cells in tissue cultures, DA strains exhibit significantly more autoagglutination in HEp-2 and HeLa cell assays than do LA strains. Because the property of autoagglutination can be evidence of high surface hydrophobicity (24), we compared the relative surface hydrophobicities of HB101, HB101 transformed with pJPN8 and HB101 containing pMAR7 (a derivative of pMAR2 with a Tn*1* insertion) by using the ammonium sulfate precipitation method. In this technique, highly hydrophobic bacterial cells will precipitate in the presence of lower concentrations of ammonium sulfate than will bacterial cells which are not as hydrophobic. Although HB101 carrying pMAR7 showed no detectable increase in surface hydrophobicity over the plasmidless HB101 (little aggregation even in 4.0 M ammonium sulfate), HB101 carrying pJPN8 aggregated in as low as 1.6 M ammonium sulfate.

Serogroups of LA and DA *E. coli*. Table 1 shows the distribution of LA and DA among EPEC serogroups. *E. coli* strains showing LA were found chiefly in certain sero-

TABLE 1. Distribution of LA and DA by serogroup

Serogroup	Total no. of strains	No. of strains showing:	
		LA	DA
O26	11	0	0
O44	1	0	1
O55	8	3	1[a]
O86	7	2	2
O111	14	9	0
O119	5	5	0
O125	1	0	1
O126	2	0	0
O127	8	5	1
O128	6	0	1
O142	6	3	0
O4, O15, O28, O50, O69, and rough	6	0	1

[a] This strain exhibited both LA and DA and is included in both columns.

groups, such as O111, O119, and O127. In contrast, DA was not found at a high frequency in these serogroups. One strain of serotype O55:H$^-$ displayed both DA and LA and carried two large plasmids.

DISCUSSION

Recent reports from several investigators have indicated that mucosal adhesion may be important in the pathogenesis of EPEC (4, 17, 19–21, 28). In this report, we further characterized two factors found in EPEC of human origin which confer adhesiveness to HeLa and HEp-2 cells in tissue cultures. The distinction between LA and DA of *E. coli* was originally described by Scaletsky et al. (22) with HeLa cells; we have extended this observation to HEp-2 cells and shown that there is a close correlation between the results of both assays. Among 75 *E. coli* strains isolated from humans with diarrhea in the United States and Brazil, all but 1 strain produced identical reactions in both HEp-2 and HeLa cells. These data not only substantiate the use of HeLa or HEp-2 cells interchangeably in the detection of these factors but also suggest that DA and LA receptors are not unique to a single cell line, lending plausibility to their presence on enterocytes.

The adhesin designated EAF has been shown to be important in the pathogenicity of some EPEC in both volunteer studies (Baldini et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1984, B128, p. 39) and clinical epidemiological studies (12). In this report, we used a DNA probe to show that EAF genes are responsible for the LA phenotype in EPEC and are usually encoded on plasmids varying in size from 55 to 70 Md. In 2 of the 27 EAF-positive isolates we tested, the genes were not found encoded on plasmids by the Birnboim-Doly extraction procedure (2). Southern blots (26) of chromosomal DNA extracted from one of these strains showed discrete restriction fragments homologous to the EAF probe, indicating that in this case, EAF genes may be encoded on the chromosome. These results raise the possibility that EAF genes may be part of a mobile genetic element which can become resident on plasmids or on the chromosome. A precedent for this hypothesis is the fact that *E. coli* heat-stable enterotoxin has been found on a transposon (25). We are currently investigating this possibility in the case of EAF.

Because DA often occurs in serotypes different from those in which EAF is commonly found (22; Nataro et al., submitted for publication), we sought to test whether the DA phenotype was actually due to a newly discovered adhesin or simply to differences in EAF expression. Using the EAF probe on colony blots of DA isolates, we showed that there is no detectable DNA homology between the EAF probe and DA bacteria even under conditions of low stringency. These data indicate that the two factors are genetically distinct.

By transforming *E. coli* HB101 with a 65-Md plasmid from a DA strain (serotype O44:H18) and demonstrating the acquisition of DA, we showed that, like LA genes, DA genes are encoded on a plasmid in at least one strain. Also, as in the case of LA strains, plasmid analysis of DA strains failed to reveal any plasmids which are conserved in a great number of isolates. One DA isolate did not contain any large plasmids and may carry the trait on the chromosome.

Using HB101 transformed with the DA plasmid pJPN8, we were able to demonstrate phenotypic differences between the factors conferring LA and DA. HB101(pJPN8) is significantly more hydrophobic than is HB101 or HB101 carrying the EAF plasmid pMAR7, as measured by the ammonium sulfate salting-out test. This greater hydrophobicity is also supported by the observation that HB101(pJPN8) shows a greater degree of autoagglutination than does HB101(pMAR7). The surface hydrophobicity of DA strains lends support to the hypothesis that the adhesin serves as an attachment mechanism in vivo. Increased surface hydrophobicity is a characteristic of several adhesins in enteric pathogens, including fimbriae of ETEC (29) and the adhesin of *Vibrio cholerae* (8, 9).

The EAF probe has previously been used on isolates from EPEC cases in Peru (O'Brien and Nataro, in press). Results from that study indicated that the adhesin was apparently restricted to the serogroups most frequently implicated in EPEC outbreaks worldwide (chiefly O55, O111, O119, O127, and O128). Those serogroups in which EAF was found were called class I EPEC, and those serogroups in which EAF was not found (O44, O86, and O114) were designated class II EPEC. EAF-positive class I EPEC and EAF-negative class II EPEC were each significantly associated with diarrheal disease. This subject is further discussed elsewhere (I. C. A. Scaletsky and L. Trabulsi, manuscript in preparation). The distribution of LA and DA by serogroup in this study as seen in Table 1 supports the data gathered in Peru. EAF was most frequently found in class I serogroups O111 and O119. Serogroup O86, however, in which none of 19 Peruvian isolates carried EAF, exhibited EAF in 2 of 7 isolates in this study.

Although EAF has been shown to contribute to the virulence of class I EPEC (12; Nataro et al., submitted for publication), we have yet to identify an important adhesin in class II EPEC. In this study, we found DA in class II isolates, raising the possibility that the adhesin could enhance virulence in class II isolates as EAF does in class I isolates. Further epidemiological studies and human volunteer studies with isogenic strains with and without DA are necessary to determine the role of the DA adhesin in pathogenesis.

The molecular nature of the adhesins conferring LA and DA have yet to be established. Scotland et al. (23) reported that LA is apparently not due to fimbriae demonstrable with electron microscopy. Such studies on the DA factor have not been performed. The increased surface hydrophobicity of strains expressing DA is also found in ETEC strains expressing colonization factor antigens I and II, which have been shown to be fimbrial (29). The DA adhesin, however, does not confer the hemagglutination patterns characteristic of ETEC adhesins (J. P. Nataro, unpublished observations). In contrast, recent work has indicated that autoagglutination in pathogenic *Yersinia* species is conferred by a hydrophobic outer membrane protein (24).

The factors conferring DA and LA to HEp-2 and HeLa cells represent two distinct and potentially important adhesins found in human EPEC isolates. Although the precise role of mucosal adherence factors has not been identified in EPEC, these factors have been shown to be important in the virulence of many human pathogens. Further work on these adhesins should help to elucidate the mechanisms of pathogenicity of EPEC diarrhea. In the past, diagnosis of EPEC infections has relied on the detection of *E. coli* belonging to certain recognized serotypes. By determining the essential EPEC virulence factors, diagnostic techniques such as that using the EAF probe will allow more precise identification and epidemiological study of these pathogens.

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