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HeLa Cell Adherence and Cytotoxin Production by Enteropathogenic *Escherichia coli* Isolated from Infants with Diarrhea in Thailand

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Enteropathogenic *Escherichia coli* (EPEC) strains isolated from hospitalized infants with diarrhea in Thailand were examined for HeLa cell adherence and cytotoxin production. Of 101 strains examined, 56 adhered to HeLa cells in a localized pattern (LA), 27 adhered in a diffuse pattern (DA), and 18 did not adhere. All 56 LA EPEC strains were O:K serotype O119:K69. A total of 20 (83%) of 24 EPEC O86:K61 strains and 7 (38%) of 19 EPEC strains belonging to six other O:K serotypes exhibited DA. All LA EPEC strains hybridized with a DNA probe for genes encoding EPEC adherence factor, whereas none of the 27 DA or 18 nonadherent EPEC strains hybridized with EPEC adherence factor probe. Sonic extracts of 57 (58%) of 98 EPEC strains tested at a dilution of 1:100 caused greater than 25% mortality of HeLa cell monolayers. A total of 50 (88%) of 57 cytotoxic sonic extracts were inhibited to various degrees by a 1:500 dilution of polyclonal rabbit antisera to purified Shiga toxin. The mean percent inhibition of cytotoxic sonic extracts by anti-Shiga toxin was 67% (range, 29 to 89%). Fifty percent (38 of 56) of LA EPEC strains, fifty-two percent (14 of 27) of DA EPEC strains, and fifty-three percent (8 of 15) of nonadherent EPEC strains produced Shiga-like toxins. Both adherence and low levels of cell-associated cytotoxins were identified in EPEC strains from Thailand, but there did not appear to be an association between these two factors.

In studies in Europe and the United States, certain serotypes of *Escherichia coli*, known as enteropathogenic *E. coli* (EPEC), have been epidemiologically associated with diarrheal disease (22, 29). EPEC strains of serogroups O55, O111, and O127 that were isolated from infants with gastroenteritis caused diarrhea when fed to adult volunteers (12, 16, 21, 29, 42). Most EPEC strains are not enterotoxigenic or enteroinvasive (10, 13, 14). In infants and animals, adherence of EPEC to the small-intestinal mucosa was necessary to induce diarrhea (26, 32, 33, 41). Cravioto et al. reported that 80% of EPEC strains adhered to HEp-2 cell monolayers and HEp-2 cell adherence was significantly more common among EPEC than among enterotoxigenic *E. coli* or normal *E. coli* flora (6). EPEC can adhere to HeLa or HEp-2 cells in a localized (LA) or diffuse (DA) pattern (27, 28, 36). Localized HEp-2 adherence in EPEC E2348 (serotype O127:H6) is encoded on a 60-megadalton plasmid (pMAR2) (1). The presence of this plasmid and the expression of LA correlated with the ability of E2348 to cause diarrhea in adult volunteers (23).

A DNA probe for genes coding for HEp-2 (or HeLa) cell adherence has been constructed and used to identify LA *E. coli* (27, 28). LA has been reported in EPEC serogroups O5, O111, O119, O127, O128, and O142 (27, 36). These serogroups, referred to as class I EPEC, are considered to be the most important causes of EPEC-associated diarrhea in both epidemic and endemic forms (27). In contrast, class II EPEC (serogroups O44, O86, and O114) is rarely associated with outbreaks, is a less important cause of sporadic diarrhea (27), and seldom adheres to epithelial cells in an LA pattern (27).

In addition to adherence, *E. coli* produces exoproteins distinct from *E. coli* heat-labile toxin and heat-stable toxin (19, 20, 30, 31, 37). Cytotoxins produced by EPEC that were cytotoxic to Vero cells were termed verotoxins (20). In the United Kingdom, 10% of 253 EPEC strains produced verotoxins (37). Subsequently, high levels of *Shigella dysenteriae* type 1-like toxin were found to be produced by EPEC, predominantly serotype O26:H11 and a previously unrecognized pathogenic serotype, O157:H7 (24, 31). Strains of *E. coli* serotype O157:H7 were isolated from cases of hemorrhagic colitis and hemolytic uremic syndrome (15, 17). Cytotoxins in strains which produce high levels of cytotoxins appear to play a role in enteropathogenicity, but the role of cytotoxins in EPEC that produce low levels of cytotoxin is uncertain (24). To determine the importance of these pathogenic mechanisms, 101 EPEC strains isolated from hospitalized infants with diarrhea in Thailand were examined for adherence to HeLa cells and production of Shiga-like toxins.

MATERIALS AND METHODS

EPEC strains. EPEC was isolated from infants under 1 year of age with diarrhea at Prapokklao Hospital, Chantaburi, Thailand, between 1 October 1984 and 31 March 1985. Fecal specimens were cultured on MacConkey agar, and five *E. coli* isolates from each child were tested by slide agglutination in three polyvalent pools of O:K antisera. Antisera were prepared by the Department of Medical Sciences of the Thai Ministry of Public Health by immuniz-

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TABLE 1. Mannose-resistant HeLa cell adherence and cytotoxin production by EPEC isolated from Thai children with diarrhea

OK serotype (no. of strains)[a]	Type of HeLa cell adherence[b] (no. of strains)			No. of sonic extracts	
	LA	DA	NA	Cytotoxic to HeLa cells	Inhibited by anti-Shiga toxin
O119:K69 (58)	56	0	2	34	28
O86:K61 (24)	0	20	4	12	11
O20ab:K84 (3)	0	2	1	1	2
O25:K11 (3)	0	1	2	2	2
O55:K59 (3)	0	0	3	2	2
O125:K70 (3)	0	1	2	2	2
O78:K80 (2)	0	1	1	2	2
O127:K71 (2)	0	1	1	1	1
O18ac:K77 (1)	0	1	0	1	1
O28:K70 (1)	0	0	1	0	0
O126:K71 (1)	0	0	1	NT[c]	NT

[a] Three isolates were not examined in the HeLa cell cytotoxicity assay: one isolate each of O25:K11, O55:K59, and O126:K71.

[b] LA, Mannose-resistant LA to HeLa cells; DA, mannose-resistant DA to HeLa cells; NA, nonadherence to HeLa cells in the presence of mannose. All isolates that adhered to HeLa cells in a localized pattern hybridized with the EAF probe.

[c] NT, Not tested.

ing rabbits with Formalin-killed *E. coli* of enteropathogenic serotypes obtained from the Robert Koch Institute, Berlin, Federal Republic of Germany, as described by Ewing (11). These pools contained antisera to 18 recognized EPEC O:K serotypes. Agglutinating colonies were tested by the Department of Medical Sciences, Ministry of Public Health in Bangkok, for agglutination in monovalent O:K antisera before and after heating at 100°C for 1 h. To confirm that these strains were of classical EPEC serogroups, isolates were reexamined by tube agglutination with O antisera obtained from the *Escherichia coli* Reference Center, Pennsylvania State University, University Park, after heating at 100°C. H antigen serotypes were not determined.

Assays for enteroadherence. Mannose-resistant adherence to HeLa cells was determined by the method of Scaletsky et al. (36), and genes encoding for EPEC adherence factor (EAF) were identified by colony hybridization with a DNA probe (27, 28).

Toxin assays. Enterotoxins and cytotoxins were identified by two methods. First, strains were inoculated into 1 ml of tryptic soy broth with 0.6% yeast extract (TSBY; Difco Laboratories, Detroit, Mich.) and incubated at 37°C for 24 h. A 0.1-ml sample of this culture was then inoculated into 5 ml of the same medium in a 125-ml Erlenmeyer flask and incubated with shaking at 200 rpm for 18 h. The original 1-ml culture was incubated for an additional 24 h (48-h total incubation) and tested in the Y-1 adrenal cell assay for heat-labile toxin (34). Sterile culture supernatants of the shaking cultures were tested for heat-stable toxin in the suckling mouse assay (7) and for cytotoxin by incubating twofold dilutions of sterile culture supernatants with monolayers of Vero cells (20).

EPEC isolates were also cultured on Mueller-Hinton agar at 37°C overnight. Single colonies were inoculated into 2 ml of modified syncase broth (MSB) (9) and incubated stationary for 6 h. A 0.1-ml sample of this starter culture was inoculated into 10 ml of MSB in 125-ml Erlenmeyer flasks and incubated with shaking at 200 rpm for 12 h. Bacteria were collected by centrifugation at 8,000 × *g* for 15 min, washed twice in 10 mM Tris hydrochloride (pH 7.4), and suspended in 20% sucrose–33 mM Tris–3 mM EDTA (pH 7.4). After 10 min at 25°C, the bacteria were centrifuged at 8,000 × *g* for 15 min, washed twice in 10 mM Tris hydrochloride, and suspended in 1 ml of 10 mM Tris hydrochloride. The optical density of the washed bacterial suspension was measured with a Spectronic 711 (Beckman Instruments, Inc., Fullerton, Calif.) at 260 nm. The bacterial suspension was then sonicated with a Sonic Dismemberator model 300 C with an intermediate probe (Fisher Scientific Co., Pittsburgh, Pa.) at 4°C in 30-s bursts at 60% amplification with 1-min pauses between sonications to avoid overheating. The bacterial suspensions were sonicated until there was ≥90% lysis as determined spectrophotometrically at 260 nm. The sonic extracts were then centrifuged at 10,000 × *g* for 20 min at 4°C, and the supernatant was removed and frozen at −70°C until tested.

Cytotoxic activities of sonic extracts were determined by the method of Keusch et al. (18). Shiga toxin-sensitive HeLa cell (CCL-2) monolayers were grown in microtiter plates in modified McCoy 5a medium (GIBCO Laboratories, Grand Island, N.Y.) with 10% fetal bovine serum to near confluency. Duplicate wells were then exposed to fresh tissue culture medium, a 1:100 dilution of the sonic extract in medium, and a 1:100 dilution of the sonic extract in tissue culture medium containing a 1:500 dilution (final concentration) of polyclonal rabbit anti-Shiga toxin (9). After 18 h at 37°C, the monolayers were washed to remove dead or loosely adherent cells. The remaining attached cells were removed with 0.025% trypsin, suspended in phosphate-buffered saline, and counted in duplicate in a hemacytometer chamber. HeLa cell mortality was calculated by comparison of control, sonic extract, and sonic extract–anti-Shiga toxin-exposed monolayers. Sonic extracts were also tested in a recently described enzyme-linked immunosorbent assay (ELISA) to identify Shiga-like toxin antigen (8).

EPEC strains were examined for colony hybridization with the α-^{32}P-labeled 17-kilobase *Eco*RI digestion fragment of pWR100 from *Shigella flexneri* 5 (M90T). This probe, originally described by Boileau et al. (2), is specific for enteroinvasive *E. coli* and *Shigella* spp.

RESULTS

None of 101 EPEC strains isolated from infants with diarrhea in Thailand produced heat-labile or -stable toxin or hybridized with the 17-kilobase DNA probe used to identify enteroinvasive *E. coli*. A total of 56 of 101 strains showed LA, 27 showed DA, and 18 showed no adherence (Table 1). All 56 *E. coli* strains that showed LA were O:K serotype O119:K69. Only 2 (4%) of the 58 *E. coli* O119:K69 strains did not show LA. A total of 20 (83%) of 24 EPEC O86:K61 strains and 7 (38%) of 19 *E. coli* strains belonging to 6 other EPEC O:K serotypes (O18ac:K77, O20ab:K84, O25:K11, O78:K80, O125:K70, and O127:K71) exhibited DA, and 18 *E. coli* strains belonging to 10 other EPEC serotypes did not adhere.

All 56 LA EPEC O119:K69 strains hybridized with the EAF probe, and the nonadherent *E. coli* O119:K69 strains did not hybridize with this probe. None of 27 EPEC strains that exhibited DA or the 18 EPEC strains that did not adhere to HeLa cells hybridized with the EAF probe.

None of the culture supernatants grown in TSBY were cytotoxic to Vero tissue culture cells. However, when EPEC strains were grown in MSB medium, sonicated, and tested at a dilution of 1:100, 57 (58%) of 98 sonic extracts caused greater than 25% mortality of HeLa cell monolayers. Of the

57 cytotoxic sonic extracts, 50 (88%) were variably inhibited by a 1:500 dilution of antisera to purified Shiga toxin (Table 1). The mean percent inhibition of cytotoxicity by EPEC sonic extracts by anti-Shiga toxin was 67% (range, 29 to 89%). A similar degree of inhibition of HeLa cell cytotoxicity by the same antibody occurred with culture supernatants of *S. dysenteriae* 1 60R and with 200 pg of purified Shiga toxin. When tested for Shiga toxin by ELISA, sonic extracts of 12 of 101 EPEC strains contained Shiga-like toxin. The amount of detectable Shiga-like toxin ranged from 45 to 759 pg/mg of protein. Eight ELISA-positive sonic extracts produced cytotoxins that were neutralized by anti-Shiga toxin; one strain, EPEC O119, that was cytotoxic to 100% of HeLa cells was not neutralized by anti-Shiga toxin; and three strains that were cytotoxic to less than 25% of HeLa cells were not tested for neutralization.

Fifty percent (28 of 56) of EPEC strains that adhered in a localized pattern, fifty-two percent (14 of 27) that adhered in a diffuse pattern, and fifty-three percent (8 of 15) that were nonadherent to HeLa cells produced Shiga-like toxins.

DISCUSSION

Mucosal adhesion is important in the pathogenesis of diarrhea caused by EPEC (4, 21, 23, 25, 32, 33, 41). Most EPEC isolated from infants with diarrhea in Thailand exhibited LA or DA. In this study, LA occurred only with *E. coli* O119; 97% of these strains adhered in an LA pattern. Serogroup O119 is defined as a class I serogroup, the majority of which have been shown to attach in an LA pattern (28). Five isolates of other class I EPEC, serotypes O55 and O127, were not locally adherent in this study. Most (83%) of 24 EPEC O86:K61 strains adhered in a DA pattern. Certain bioserotypes of *E. coli*, epidemiologically incriminated as causes of diarrheal disease, adhere to HeLa cells in an LA pattern, whereas no such disease association has been found with *E. coli* that adheres to HeLa cells in a DA pattern (35). Similarly, EAF probe-positive EPEC was isolated significantly more often from children with diarrhea than from controls in Peru (27). EAF-negative class II EPEC was also isolated significantly more often from children with diarrhea than from controls in the same study, suggesting that EPEC possesses other virulence traits.

None of the 101 EPEC strains examined in this study produced extracellular cytotoxins as measured by the Vero cell assay. Sonic extracts of 57 of 98 EPEC strains contained cell-associated HeLa cell cytotoxin activity at a dilution of 1:100. These cytotoxins were neutralized to different extents with high-titered rabbit Shiga antitoxin. Cleary et al. found that, although it was common for healthy adults and children to have cytotoxin-producing *E. coli* as part of their fecal flora, Shiga-like cytotoxin was detected more commonly and in greater quantities among EPEC than among other fecal *E. coli*, thus suggesting a role for Shiga-like toxin in the pathogenesis of EPEC diarrhea (5). Marques et al. examined 412 *E. coli* strains, 152 of which belonged to EPEC serogroups isolated from humans with diarrhea (24). Fifty-nine percent of 152 EPEC strains produced low levels and four percent produced high levels of cell-associated cytotoxins. Twenty percent of the EPEC strains that produced low levels of cell-associated cytotoxin and all of the six EPEC strains (five serogroup O26 and one O111) that produced high levels in that study were neutralized by Shiga antitoxin (24). Cell-associated cytotoxins not neutralized by antibody to Shiga toxin have been reported (5, 30). The extracellular cytotoxin produced by an EPEC serogroup O128 strain isolated from an infant with diarrhea was antigenically different from the cytotoxin of *E. coli* H30 (20). Antigenic variations in cytotoxins produced by *E. coli* strains isolated from pigs and humans have also been described (38, 39). Strockbine et al. recently reported that an enterohemorrhagic *E. coli* strain, 933 (O157:H7), produced two antigenically distinct cytotoxins that had the same biologic activities, which they designated Shiga-like toxins I and II (40).

O'Brien et al. previously observed that Shiga-like toxin and verotoxin were very similar if not identical (31). Although the Vero cells used in this study detected verotoxins in culture supernatants of *E. coli* 933 O157:H7, no cytotoxic activity was detected in culture supernatants of the EPEC we studied. Isolates were not grown in iron-free media, and it is possible that the Vero cells used in this study were less sensitive to verotoxins than the cells used by Scotland et al. (37). It did not appear, however, that the EPEC strains examined in this study produced verotoxins as reported in earlier studies (20, 37). This may be because of the EPEC serogroup examined. In this study, EPEC sonic extracts grown in iron-free media were variably inhibited by anti-Shiga toxin when tested on Shiga toxin-sensitive HeLa cells.

Of cytotoxic sonic extracts that were neutralized by anti-Shiga toxin, 89% (8 of 9) were identified in the Shiga toxin ELISA; one sonic extract of an EPEC serogroup O119 strain that was identified by the Shiga ELISA was not neutralized by polyclonal anti-Shiga toxin. This sonic extract presumably contained both Shiga-like toxin and another cytotoxin that was not neutralized by the polyclonal anti-Shiga toxin. Two different but related cytotoxins were found in *E. coli* O157 by Strockbine et al. (40) and Scotland et al. (38).

In this study, many EPEC strains produced cell-associated cytotoxins; however, based on neutralization of cytotoxicity to HeLa cells by anti-Shiga toxin, these cytotoxins appeared to be heterogeneous. Since the culture conditions, the method of preparing sonic extracts, and the cytotoxicity assay used may affect the level of cytotoxins detected, it may be easier and more specific to identify cytotoxic *E. coli* by constructing probes for genes that code for these cytotoxins. These probes are not yet available for use in clinical laboratories.

In the present investigation, low levels of cell-associated cytotoxins were as often associated with HeLa cell-adherent *E. coli* as with nonadherent *E. coli*. This is distinctly different from *E. coli* strains with colonization factors I and II, almost all of which are enterotoxigenic (3). Although high levels of Shiga-like toxin I may well be responsible for hemorrhagic colitis caused by *E. coli* O157:H7, the role of lower levels of cell-associated cytotoxins in EPEC remains unclear. Volunteers fed low-level-cytotoxin-producing EPEC (O127:H6) that adhered to HEp-2 cells in a localized pattern developed diarrhea, whereas others fed nonadherent but cytotoxic derivatives of this strain did not, suggesting that this EPEC strain causes disease by enteroadherence rather than by cytotoxin production (23).

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