Lack of Shiga-Like Cytotoxin Production by Enteroinvasive *Escherichia coli*

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Enteroinvasive *Escherichia coli* has not been extensively studied for cytotoxin production. We evaluated 30 well-characterized enteroinvasive *E. coli* strains of all the known invasive serogroups from several geographic regions for their ability to produce Shiga-like cytotoxic activity assayed in a HeLa cell system. None of these strains produced cytotoxic activity that was neutralizable with antibody to Shiga-like toxin I or II.

Enteroinvasive *Escherichia coli* (EIEC) comprises those *E. coli* strains that possess a high-molecular-weight virulence plasmid closely related to the virulence plasmid of shigellae (7). As a result, EIEC produces shigellalike, virulence-associated polypeptides and is capable of causing shigellalike invasive diarrhea (6, 13). A 17-kilobase *Eco*RI fragment of the virulence plasmid that has been shown to be essential for invasiveness (12) has been used to probe *E. coli* strains for this plasmid (16). We have recently shown that shigellae differ in the types and amounts of cytotoxins they produce. *Shigella dysenteriae* 1 is consistently a high-level Shiga toxin producer, while other shigellae produce little or no Shiga toxin (1). However, these other shigellae do produce low levels of a second cytotoxin not immunologically related to Shiga toxin. Shiga toxin is thought to be an important virulence determinant, which may account for the severity typical of *S. dysenteriae* 1 as well as for involvement of Shiga toxin in the pathogenesis of the *S. dysenteriae* 1-associated hemolytic uremic syndrome. Some *E. coli* strains make related toxins. Those strains that produce a cytotoxin neutralizable with antibody to Shiga toxin are said to make Shiga-like toxin I (SLT-I). Many strains of *E. coli* are high-level producers of a second cytotoxin (referred to as SLT-II) closely related to Shiga toxin but not neutralizable with antibody to Shiga toxin. Numerous serotypes of *E. coli* are now recognized as producers of either SLT-I or SLT-II or both. The most important of these serotypes are *E. coli* O157:H7 and *E. coli* O26:H11. There has been little investigation of EIEC strains to determine whether they produce shigellalike cytotoxins as well as possessing the shigella virulence plasmid. The purpose of this study was, therefore, to analyze all the usual EIEC serotypes from several different geographic regions to determine their capacities for cytotoxin production.

Bacterial strains. A total of 10 EIEC strains isolated from Thai patients and 9 EIEC strains from Vietnamese patients were obtained from Peter Echeverria. In addition, 10 strains of EIEC isolated from U.S. travelers to Guadalajara, Mexico, were obtained from Herbert DuPont. One EIEC strain associated with a foodborne outbreak of disease in Houston was provided by Gordon Reeve. For purposes of comparison, we chose, at random, colonies of *E. coli* from 3 patients who had traveler's diarrhea in Mexico and 10 well patients. These colonies of *E. coli* were analyzed for cytotoxin production. The enterotoxigenic strain H10407 was included

as a negative control, since it is known to be SLT-I and SLT-II negative. *S. dysenteriae* 1-60R and *E. coli* C600 (933W) were included as positive controls for SLT-I and SLT-II, respectively.

Virulence plasmid analysis. Lysates of each strain were prepared by the method of Kado and Liu (8). These lysates were then electrophoresed in 0.7% horizontal agarose gels, stained with ethidium bromide, and photographed under UV-light illumination. The sizes of plasmids were determined by using known molecular size standards.

Virulence probe analysis. The DNA probe used (5, 16) for these studies was the 17-kilobase *Eco*RI fragment originally isolated from the invasiveness plasmid of *Shigella flexneri* serotype 5 (2). The probe was isolated, nick translated with [α-^{32}P]dATP, and hybridized under conditions of high stringency (42°C in 50% formamide) (10) with filters containing colony lysates.

Cytotoxin characterization. Each strain was grown in iron-depleted syncase broth, sonicated, filter sterilized, and serially diluted in 96-well plates containing Eagle minimal essential medium and Earle salts (Hazleton Dutchland, Denver, Pa.), 10% fetal calf serum, and 2 mM L-glutamine and thymidine (2 μCi/ml; ICN Radiochemicals, Irvine, Calif.). HeLa cells ($\sim$50,000 cells per well) were added at the same time as the sonic extracts, incubated overnight at 37°C, washed to remove unattached cells and unincorporated label, lysed with 1 N KOH, and counted in a β-scintillation counter. The dilution of toxin that killed 50% of HeLa cells (CD$_{50}$/ml) was calculated and corrected for protein concentration (CD$_{50}$/mg) as described previously (4). Characterization of cytotoxin type (SLT-I, SLT-II, or nonneutralizable cytotoxin) was determined by using polyclonal rabbit antiserum to Shiga toxin prepared from *S. dysenteriae* 1-60R and rabbit antiserum to *E. coli* C600 (933W) kindly provided by A. D. O'Brien. Appropriate controls were used to ensure that the antibody dilutions used were adequate to neutralize each of the respective toxins. Each strain was assayed at least twice.

Table 1 lists the sources of EIEC strains and their serotypes, as well as their plasmid, probe, and cytotoxin characterizations. All EIEC strains contained large plasmids (120 to 140 megadaltons) that hybridized with the 17-kilobase probe for the invasiveness genes. Each EIEC strain had a different total plasmid profile, except for the four *E. coli* O143:H⁻ strains isolated in Mexico, which were identical to each other (A. Wanger, submitted for publication). Strain H10407, 3 diarrhea-associated fecal *E. coli* isolates, and 10

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TABLE 1. Relationship between invasiveness and cytotoxic activity

Strain and source	Serotype	Presence of:		Log_{10} CD_{50}/mg of protein	Neutralization by antibody to:	
		Plasmid	Probe		60R	933W
E. coli						
Thailand	O28:H⁻	+	+	2.36	−	−
	O28:H⁻	+	+	1.92	−	−
	O28:H⁻	+	+	1.67	−	−
	O29:H⁻	+	+	1.80	−	−
	O136:H⁻	+	+	1.71	−	−
	O136:H⁻	+	+	1.40	−	−
	O143:H⁻	+	+	1.65	−	−
	O144:H⁻	+	+	2.23	−	−
	O159:H⁻	+	+	1.87	−	−
	O164:H⁻	+	+	1.63	−	−
Vietnam	O28:H⁻	+	+	1.60	−	−
	O29:H⁻	+	+	1.42	−	−
	O112:H⁻	+	NDᵃ	2.14	−	−
	O124:H⁻	+	ND	1.63	−	−
	O136:H⁻	+	+	1.94	−	−
	O143:H⁻	+	+	1.96	−	−
	O144:H⁻	+	+	1.76	−	−
	O152:H⁻	+	+	2.10	−	−.
	O167:H⁻	+	+	1.74	−	−
United States	O143:H⁻	+	+	2.33	−	−
Mexico	O143:H⁻	+	+	1.88	−	−
	O143:H⁻	+	+	2.16	−	−
	O143:H⁻	+	+	2.09	−	−
	O143:H⁻	+	+	2.37	−	−
	NTᵇ	+	+	1.96	−	−
	NT	+	+	2.30	−	−
	NT	+	+	2.29	−	−
	NT	+	+	2.20	−	−
	NT	+	+	2.20	−	−
	NT	+	+	2.22	−	−
Control strains						
E. coli O78:H11(H10407)		−	−	2.35	−	−
S. dysenteriae 1-60R		ND	ND	4.7	+	−
E. coli C600 (933W)		ND	ND	3.1	−	+

ᵃ ND, Not done.
ᵇ NT, Not typeable.

E. coli isolates from the feces of 10 well persons were also studied. As expected, *E. coli* isolates that were probe negative were also plasmid negative. Each EIEC strain produced low levels of cytotoxic activity. Levels of cytotoxin produced were 100- to 1,000-fold lower than those produced by *S. dysenteriae* 1-60R. The amount of cytotoxic activity produced by EIEC ($10^{1.40}$ to $10^{2.37}$ CD_{50} per mg of bacterial protein) was similar to that produced by *E. coli* H10407 ($10^{2.35}$ CD_{50} per mg of bacterial protein), 3 diarrhea-associated probe- and plasmid-negative *E. coli* strains isolated in Mexico ($10^{2.10}$ to $10^{2.23}$ CD_{50} per mg of bacterial protein), and 10 normal flora fecal *E. coli* strains isolated in Mexico ($10^{1.72}$ to $10^{2.77}$ CD_{50} per mg of bacterial protein). The levels of nonneutralized cytotoxic activity were not different from those found in other *E. coli* strains or in shigellae other than *S. dysenteriae* serotype 1 (1, 4). There was no significant difference in the amount of cytotoxin produced by one serotype compared with that produced by the others, nor was there a significant difference in the amount of cytotoxic activity produced by strains isolated in different geographic regions. In no case was the cytotoxicity neutralized by antibody against SLT-I or SLT-II.

Studied here are typical EIEC strains representing all of the recognized EIEC serogroups. They were all well characterized in terms of serotypes, plasmids, and probes. Previous data on EIEC cytotoxin production is scant. The original description, by O'Brien et al., of Shiga-like cytotoxin production by *E. coli* included a single EIEC strain (14). That strain (*E. coli* O143 strain 4608-58-899) was said to produce 40 CD_{50} of SLT-I per mg of bacterial protein. Although this is the same range of total cytotoxin production we found, none of our isolates produced a neutralizable toxin. Since seven of our strains were *E. coli* O143 and none of them produced detectable Shiga-like toxins, we suspect that such toxin production by these strains is very uncommon. The same investigators have reported nine human EIEC isolates of undefined serotype that were low-level producers of nonneutralizable cytotoxin (11). The current study clearly demonstrates that all common EIEC serotypes are low-level cytotoxin producers. The toxin these strains produce is unrelated immunologically to SLT-I or SLT-II. Our data are consistent with those of Smith et al., who demonstrated that the genes for toxin production were absent in several EIEC isolates that they analyzed by toxin

probes (17). That all of the recognized serotypes of EIEC were evaluated is particularly important because for both *E. coli* and shigellae, serotype correlates well with the type of cytotoxin produced (9). That is, all *E. coli* O157:H7 are high-level SLT-I or SLT-II producers, while *S. dysenteriae* 1 is predictably a high-level Shiga toxin producer (1, 11).

On the basis of these data, it appears that although EIEC strains possess the shigella virulence plasmid, they do not necessarily have other shigella virulence factors. Virulence, at least in *S. flexneri*, is amplified by chromosomally encoded traits (15). EIEC strains are not well characterized in terms of shigellalike chromosomal traits, although they probably possess at least some shigella chromosomal genes. The lipopolysaccharides of EIEC strains are closely related to those of various shigellae (3). The O antigens of *E. coli* O124:H30 and O152:H⁻ appear to be identical to those of *S. dysenteriae* 3 and *Shigella* sp. serovar 3341:55, respectively. Although not identical, a number of other EIEC serotypes are closely related to shigellae: *E. coli* O112ac:H⁻ is related to *S. dysenteriae* 2, *Shigella boydii* 1, and *S. boydii* 15; and *E. coli* O136 and O164 are related to *S. dysenteriae* 3. None of the EIEC strains has been recognized to have lipopolysaccharide related to that of *S. dysenteriae* 1. This study demonstrated that EIEC strains are also unlike *S. dysenteriae* 1 in toxin production.

The significance of low-level cytotoxin production is uncertain. The amounts of nonneutralizable cytotoxin produced by EIEC were not different from those produced by the other *E. coli* and shigellae that were examined. Likewise, the levels are in the same range as those previously reported for other fecal *E. coli* (4, 11). That none of the serotypes of EIEC was found to produce either SLT-I or SLT-II is good evidence that these toxins are not involved in the pathogenesis of EIEC disease. From this evidence follows the prediction that as more *E. coli* that are associated with hemolytic uremic syndrome and hemorrhagic colitis are defined, EIEC will be found not to be associated with these illnesses.

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