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Shiga-Like Cytotoxin Production by Enteropathogenic *Escherichia coli* Serogroups

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The mechanism by which enteropathogenic *Escherichia coli* (EPEC) cause disease remains to be defined. We studied EPEC and non-EPEC strains of *E. coli* from stool specimens obtained from infants and adults for production of Shiga-like cytotoxin. Although it was common for healthy infants and adults to have cytotoxin-producing *E. coli* as part of the fecal flora, Shiga-like cytotoxin was detected more commonly and in greater amounts among EPEC than among other fecal *E. coli*. These results suggest a role for Shiga-like cytotoxin in the pathogenesis of EPEC-related gastroenteritis.

Enteropathogenic *Escherichia coli* (EPEC) are defined as *E. coli* belonging to somatic (O) serogroups epidemiologically incriminated as pathogens but whose pathogenic mechanism is not related to production of *E. coli* heat-stable enterotoxin or heat-labile enterotoxin or to enteroinvasion (1, 3, 10). It is not clear how EPEC cause disease or even whether defining them by serogroup is optimal. Some EPEC strains produce a cytotoxin which is detectable in Vero cells (4, 5, 11). O'Brien and associates have described the occurrence of Shiga-like cytotoxin in several EPEC and suggested that this is the same material previously called Vero toxin (6, 8). If Shiga-like cytotoxin is a virulence property among EPEC, it ought to be found with a high frequency in pathogenic strains. To determine whether Shiga-like cytotoxin production is common among EPEC, we evaluated the frequency of toxin production among EPEC and among fecal *E. coli* isolated from individuals who were healthy.

One hundred and twelve fecal *E. coli* strains were evaluated for Shiga-like cytotoxin production. Of these strains, 29 were from patients with diarrhea and were identified as EPEC on the basis of serogroup. All *E. coli* strains were serogrouped by using O antisera produced in rabbits by the methods of Edwards and Ewing (2). The EPEC strains studied included 11 serogroups: four O26 strains (strain 28956/0 from B. Rowe (Central Public Health Laboratory, London) and strains H19, HW1, and H30 from J. Konowalchuk, Bureau of Microbial Hazards, Ottawa, Canada); three O44 strains (one obtained from K. Gyr, Kantonsspital, Basel, Switzerland and two isolated from adult travelers in Mexico); three O55 strains (strain NCTC 8603 from an infant with diarrhea and two strains from adult travelers in Mexico); six O86 strains (one obtained from K. Gyr and five isolated from adults with travelers' diarrhea in Mexico); four O111 strains (strain NCTC 8008 from an infant with diarrhea, two strains from infants with diarrhea in Houston, Tex. and one strain from an adult with travelers' diarrhea in Mexico); one O114 strain (strain E12801 from M. Levine, Center for Vaccine Development, Baltimore, Md.); two O119 strains (strain JCP88 from R. Rothbaum, Washington University, St. Louis, Mo., and an isolate from an infant with diarrhea in Houston, Tex.); two O125 strains (one strain from an Egyptian infant with diarrhea and one strain from an adult traveling in Mexico); two O126 strains (one strain isolated from an infant with diarrhea in Houston, Tex., and strain HSC10 obtained from J. Konowalchuk); one O128 strain (strain E28690/0 from B. Rowe); and one O142 strain (strain E851/71 from M. Levine). All of these isolates were negative for heat-stable and heat-labile enterotoxins. Four of the EPEC strains from infants were among those originally reported by Konowalchuk et al. to produce Vero cytotoxin (4). Since many of the EPEC strains were obtained from other laboratories, the stool specimens from which they were isolated were not available to study multiple isolates; thus, a single EPEC from each patient was tested. To determine the frequency of Shiga-like toxin production by *E. coli* from stool specimens of healthy individuals, multiple isolates were studied. Eighty-three fecal *E. coli* strains from 30 healthy individuals were studied. These 83 isolates included 28 strains from stool specimens of 20 American college students who were studying in Mexico and 55 strains from stool specimens of 10 formula-fed infants living in Houston, Tex. Isolates were identified as *E. coli* by standard methods (2).

E. coli strains to be tested for Shiga-like toxin production were grown in Chelex 100-treated iron-depleted syncase broth for 48 h at 37°C (8). The cells were collected by centrifugation at 12,000 rpm for 15 min. The supernatant was discarded. The cell pellet was washed with 0.85% NaCl and suspended in phosphate-buffered saline (0.01 M; pH 7.4). The bacteria were lysed by sonication, and cell debris was removed by centrifugation. The supernatant was filtered through a 0.22-μm filter and tested in a HeLa cell assay. Dilutions of bacterial sonicates were incubated with rabbit anti-Shiga toxin serum at a 1:1,000 dilution, a concentration shown in preliminary experiments to neutralize crude Shiga toxin preparations. The serum, kindly provided by A. O'Brien, Uniformed Services University of the Health Sciences, Bethesda, Md., had been prepared in rabbits with purified Shiga toxin harvested from *Shigella dysenteriae* 1 strain 60R by a method previously described (7). Bacterial sonicate and antiserum were incubated for 1 h at 37°C and overnight at 4°C before use in the HeLa cell assay. Toxin preparations were incubated in parallel with nonimmune rabbit serum at a 1:1,000 dilution. HeLa cells were harvested after 7 days in culture by gentle trypsinization. Cells were suspended in media containing [^{3}H]thymidine (2 μCi/ml) and inoculated into 96-well plates at an initial concentration of 50,000 cells per well. Bacterial sonicates that had been

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preincubated with nonimmune serum and toxin preincubated with immune serum were inoculated into wells containing HeLa cells. Control wells containing HeLa cells and nonimmune serum were run simultaneously as were controls with Shiga toxin from *S. dysenteriae* 1 strain 60R. Each dilution was assayed in quadruplicate. After incubation in 5% CO_2 for 48 h at 37°C, the medium was removed, and the HeLa cells were washed with phosphate-buffered saline and lysed with 1N KOH. A sample from each well was placed in Liquiscint (National Diagnostics, Somerville, N.J.) and counted in a β-scintillation counter. The percent cell survival for each dilution with immune or nonimmune serum was determined by dividing the mean counts per minute by the mean counts per minute of the no-toxin control and multiplying by 100. Significant differences between immune- and nonimmune-treated toxin preparations were determined by comparison of means at each dilution by the Student *t* test. Shiga-like-cytotoxin-negative strains were defined as those which had no cytotoxicity that could be neutralized with antibody to the Shiga toxin. The dose of toxin which killed 50% of the HeLa cells (CD_{50}/mg of bacterial protein) was calculated from the linear regression of the logarithm of the precent HeLa cell survival versus the negative logarithm of the toxin dilution for the nonimmune serum control group. For comparison purposes, the CD_{50} of two high-level Shiga toxin producers, *S. dysenteriae* 1 strain 60R and *E. coli* O157:H7, were also determined. Data were expressed as the mean ± standard error of the mean.

Twenty-three of 29 (79%) EPEC strains were Shiga-like toxin producers. Two of the EPEC strains (E851/71 and H30) have previously been tested for Shiga-like cytotoxin (8). We confirmed that these strains are Shiga-like cytotoxin positive. No single serogroup was disproportionately represented among the toxin-negative EPEC. The toxin-negative strains included one of each of the following serogroups: O26, O44, O55, O86, O111, and O128. Among *E. coli* isolated from healthy individuals, only 20 of 83 (24%) strains were positive for Shiga-like cytotoxin production. This was significantly lower than the frequency of cytotoxin-producing EPEC strains (χ^2, 27.7; $P < 0.0000002$). Although most fecal *E. coli* were not toxin producers, it was common for healthy infants and adults to have a minority population of toxin-producing *E. coli* strains as part of their stool flora. Thirteen of 31 (42%) healthy individuals had at least one *E. coli* isolate which was a Shiga-like cytotoxin producer. Healthy individuals were less likely to have a Shiga-like-cytotoxin-producing strain than individuals with EPEC-associated diarrhea (χ^2, 8.72; $P < 0.004$).

The cytotoxicity of Shiga-like-cytotoxin-producing strains expressed as geometric mean CD_{50}/mg of protein was higher among EPEC than among non-EPEC ($P < 0.02$ by unpaired *t* test; Fig. 1). The ranges were from 3.1×10^1 to 4.4×10^3 CD_{50}/mg of protein for EPEC and from 6.6×10^{-2} to 1.2×10^3 CD_{50}/mg of protein for non EPEC. There was 9.5×10^4 CD_{50}/mg of bacterial protein for *E. coli* O157:H7 and 6.0×10^5 CD_{50}/mg of bacterial protein for *S. dysenteriae* 1 strain 60R. Since only presence or absence of Shiga-like cytotoxin was determined, no attempt was made to completely neutralize cytotoxicity. Thus, all of the cytotoxicity may not have been due to Shiga-like cytotoxin. By using the HeLa cell assay described above, all of the *E. coli* isolates had some cytotoxicity, but this toxicity could not be neutralized with antibody to Shiga toxin in most *E. coli* isolated from well individuals. The finding of cytotoxicity in non Shiga-like-toxin-producing strains has been reported previously by O'Brien (8).

The mechanism by which EPEC produce diarrhea is unclear. EPEC, particularly those of serogroup O26, produce a cytotoxin which can be demonstrated in Vero cells (4, 5, 11). O'Brien and associated reported that four of the five EPEC which they evaluated produced Shiga-like toxin when grown in iron-depleted medium (8). It appears that this toxin may be the same or closely related to the toxin previously identified in Vero cells (6). Our findings support this, since all of the Vero toxin-positive strains from Konowalchuk which we tested were Shiga-like cytotoxin producers.

From the current survey, it is apparent that EPEC produce Shiga-like cytotoxin more frequently than *E. coli* from healthy individuals. These strains were from widely separated areas, suggesting that Shiga-like cytotoxin production by EPEC is a widespread phenomenon. No particular serogroup of EPEC had a disproportionately higher or lower frequency of Shiga-like cytotoxin. Although the vast majority of *E. coli* strains do not make Shiga-like cytotoxin, both

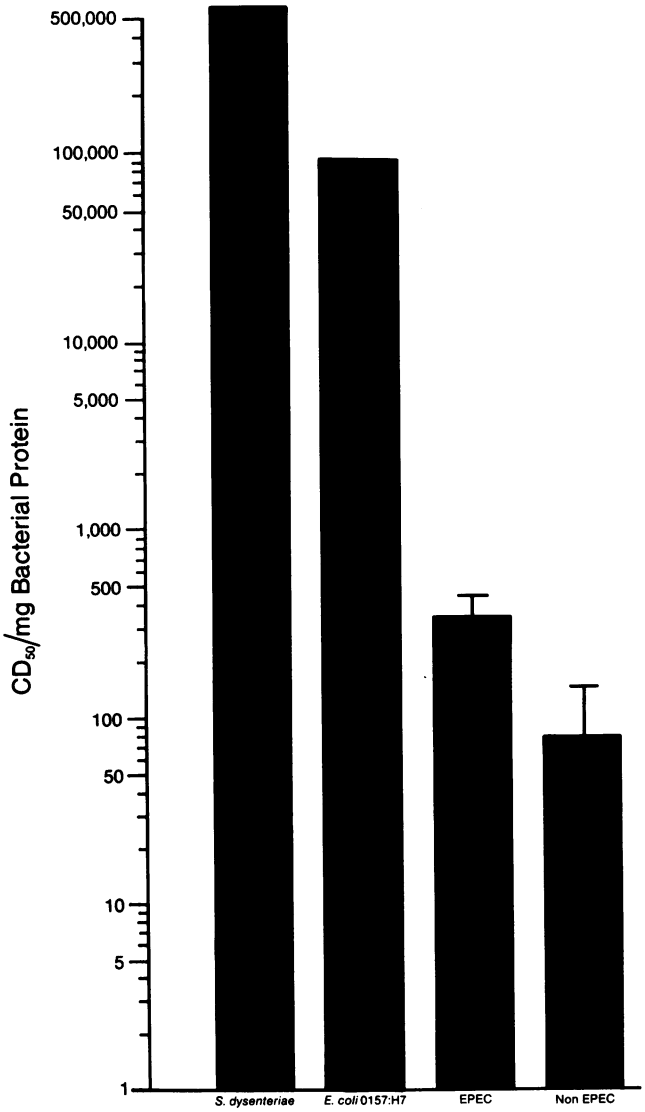

FIG. 1. Geometric mean CD_{50}/mg of bacterial protein for *S. dysenteriae* 1 strain 60R, *E. coli* O157:H7, Shiga-like-cytotoxin-producing EPEC, and Shiga-like-cytotoxin-producing non-EPEC.

infants and adults frequently have Shiga-like-cytotoxin-producing *E. coli* as part of normal fecal flora. Although the amount of cytotoxin produced by EPEC is statistically significantly higher than that produced by strains from healthy individuals, the biological significance is unclear since there is considerable overlap in the range of concentrations found. Obviously, a low level cytotoxin producer as part of stool flora was not in itself sufficient to cause illness in the formula-fed infants tested. It has not yet been proven that Shiga-like cytotoxin production by *E. coli* is sufficient to make an organism pathogenic, although data on *E. coli* O157:H7, a hemorrhagic colitis strain, suggest it may be (9). The amounts of cytotoxin produced in vitro by the EPEC reported here were 20- to 3,000-fold lower than those produced by *E. coli* O157:H7, which was about sixfold lower than that produced by *S. dysenteriae* 1 strain 60R.

At present, it is unclear whether serogrouping, serotyping, or biotyping best identifies which EPEC are true pathogens (1, 12). The relationship among these various phenotypes, adherence mechanisms, Shiga-like cytotoxin production, and disease needs further evaluation. This study shows that Shiga-like cytotoxin production by EPEC is sufficiently common to seriously consider it as a possible virulence factor in EPEC. The fact that exposure to this family of toxins is common early in life may explain the association of EPEC with infant diarrhea. The roles of gut immunity and of low-level cytotoxin producers in developing that immunity needs to be examined.

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