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Production of *Shigella dysenteriae* Type 1–Like Cytotoxin by *Escherichia coli*

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Strains of *Escherichia coli* previously implicated or proven to be causes of diarrhea were examined for production of a toxin similar to that of *Shigella dysenteriae* type 1 (Shiga). Organisms grown in an iron-depleted broth were lysed by pressure disruption followed by ultracentrifugation. Saline-dialyzed extracts were tested for cytotoxic effects on HeLa cells that were neutralizable with antiserum to Shiga toxin. Among the 13 *E. coli* strains so analyzed, 11 made a Shiga-like cytotoxin in levels ranging from trace (two avirulent isolates) to amounts equivalent to *S. dysenteriae* type 1 (two noninvasive strains that did not make *E. coli* heat-labile or -stable enterotoxins but were isolated from infants with diarrhea). As with extracts of Shiga toxin, lysates of these *E. coli* strains that produced high levels of Shiga-like toxin were enterotoxic for rabbits, paralytic and lethal for mice, and inhibited protein synthesis in HeLa cells. Thus, these data suggest that Shiga-like toxin may be another heretofore undiscovered factor in the pathogenesis of diarrhea caused by some *E. coli* strains.

Two mechanisms have been described by which *Escherichia coli* can cause diarrheal disease in humans and animals [1]. Some strains colonize the small bowel and cause fluid secretion by elaboration of heat-labile (LT) and/or heat-stable (ST) enterotoxins [2–4]. In contrast to these noninvasive, enterotoxic organisms, certain strains of *E. coli* penetrate and multiply within colonic epithelial cells [1, 5] and are able to produce keratoconjunctivitis in guinea pigs (the Séreny test) [6]. Infection with these enteroinvasive strains may cause symptoms that mimic those seen in patients with shigellosis [1, 5, 7]. That *E. coli* must be capable of mediating gastroenteritis by other means is indicated by the finding that some strains associated with diarrheal disease produce no detectable LT or ST and are Séreny test–negative [8–10]. Instead, such organisms have been shown by electron microscopic studies to displace the microvilli and adhere to the cell membrane of intestinal epithelial cells. Invasion of the epithelial cells occurs but apparently with less frequency and perhaps by a mechanism different from that of shigellae [10–12].

In a preliminary report [13] we indicated that some of these LT-negative, ST-negative, Séreny test–negative organisms can produce a toxin like that of *Shigella dysenteriae* type 1 (Shiga) — that is, the cytotoxicity is neutralizable by antiserum to Shiga toxin — and suggested that this toxin could be responsible for the diarrhea-evoking potential of these microbes. Subsequently, we had difficulty in consistently reproducing these findings. However, we recently observed [14] that the yield of Shiga toxin can be increased if the organisms are grown in alkaline medium or medium pretreated with the iron-chelating resin Chelex® (Bio-Rad, Richmond, Calif.). The purpose of the present investigation was to use these improved culture methods to verify that some *E. coli* isolates do indeed produce Shiga-like toxins. A second interdependent aim of the study was to determine the relationship between the pathogenic character (for example, enteroinvasive or enterotoxic) of a strain of *E. coli* and its capacity to elaborate Shiga-like toxin.

Materials and Methods

Bacterial strains. The various bacteria examined for production of Shiga-like toxin are described in table 1.

Table 1. Characteristics of bacterial strains examined in a study of production of *Shigella dysenteriae* type 1–like cytotoxin.

Organism, strain (serotype)	Source	Diarrhea in human volunteers	Culture supernatant				Séreny test[†]	Reference
			Rabbit ileal loop test	Heat-labile enterotoxin	Heat-stable enterotoxin	Water adsorption*		
Escherichia coli K12 strain 1133 (nontypable)	Laboratory strain, avirulent	NT	Negative	Negative	Negative	NT	Negative	15
HS (nontypable)	Healthy adult	Negative	Negative	Negative	Negative	NT	Negative	1, 9
E851/71 (O142: K86:H6)‡	Epidemic infant diarrhea	Positive	Negative	Negative	Negative	Positive	Negative	9, 16, 17
E74/68 (O128: K67:H2)‡	Epidemic infant diarrhea	Negative	Negative	Negative	Negative	Positive	Negative	9, 16, 17
12801/0 (O114)‡	Sporadic infant diarrhea	Positive	Negative	Negative	Negative	NT	Negative	§
NC (O125:H21)‡	Infant diarrhea	NT	Negative	Negative	Negative	NT	Negative	10
H30 (O26)‡	Infant diarrhea	NT	Negative	Negative	Negative	NT	Negative	18
S-22-1 (O103: H2:K?)	Infant diarrhea	NT	NT	Negative	Negative	NT	Negative	19
RDEC-1 (O15)	Rabbit diarrhea	NT	Negative	Negative	Negative	NT	Negative	8
H10407 (O78:H11:K2)	Adult diarrhea	NT	Positive	Positive	Positive	Positive	Negative	17, 20
E2531 (O25:K98)	Adult diarrhea	NT	NT	Positive	Negative	NT	Negative	21
TD 514C₁ (unknown)	Adult diarrhea	NT	NT	Negative	Positive	NT	Negative	22
4608-58-899 (O143)	Adult diarrhea	Positive	Negative	Negative	Negative	NT	Positive	1
S. dysenteriae type 1, 6OR	Rough avirulent laboratory strain	NT	Positive	Negative	Negative	Negative	Negative	14, 23, 24
Shigella flexneri type 2a, M4243	Adult dysentery, passaged in monkeys	Positive	Negative	NT	NT	NT	Positive	1, 25
Salmonella typhimurium, W118	Mouse typhoid, virulent in monkeys	NT	Negative	NT	NT	NT	Negative	26, 27
Pseudomonas aeruginosa	Burn patient	NT	NT	NT	NT	NT	NT	None

NOTE. NT = not tested.
* Inhibition of water adsorption in rat jejuna.
† Enteroinvasive (causes keratoconjunctivitis in guinea pigs) in Séreny test [6].
‡ Enteropathogenic *E. coli* of classical serotype (O26, O55, O86, O111, O114, O125, O126, O127, O128, or O142).
§ R. E. Black and M. M. Levine, personal communication.

Culture medium. Iron-depleted syncase broth, briefly described in a previous report [14], was prepared as follows. Ten grams of certified cas-amino acids (Difco Laboratories, Detroit), 1.17 g of NH_4Cl, 5 g of KH_2PO_4, 5 g of Na_2HPO_4, and 1 ml of trace salts (5% $MgSO_4$ and 0.5% $MnCl_2$ in 0.001 N H_2SO_4) were dissolved in 1 liter of quartz-distilled water, and the pH was adjusted to 8.0 with 2 N NaOH. Next, 20 g of Chelex 100 (100–200 mesh; sodium form; Bio-Rad) was added per liter of solution, the broth-resin mixture was incubated for 2 hr at 4 C with stirring, and the iron-chelating beads were removed by filtration through a large Büchner funnel lined with two sheets of filter

paper (Whatman no. 1; Arthur H. Thomas, Philadelphia). The medium was then distributed into flasks precleaned with cleaning solution (RBS-35; Pierce Chemical Co., Rockford, Ill.), a surface-active agent that removes iron and other contaminants from glassware. The broth was sterilized for 20 min at 121 C in an autoclave and then allowed to cool, and a sterile solution of 0.2% glucose, 0.004% L-tryptophan, and 0.002% nicotinic acid that had been treated with 2% Chelex was added.

To conserve Chelex, the resin was regenerated by the following method. The used beads were scraped from the Whatman filter paper, boiled in 10 volumes of 1 N HCl, and washed five times in distilled water. The acid-treated Chelex was then boiled in 10 volumes of 1 N NaOH and washed five times in distilled water, and the excess water was removed by suction through a fritted glass funnel.

Preparation of bacterial extracts. Organisms were incubated for 48 hr with shaking (260 rpm) at 37 C. The sample size was 3 liters, distributed as 2 liters and 1 liter in 4-liter and 2-liter flasks, respectively. The bacteria were harvested by centrifugation at 10,000 g at 4 C for 20 min and washed twice in 0.85% NaCl before the wet weight was determined. Weights ranged from 2 to 4.0 g/liter of medium, and larger bacterial yields generally indicated incomplete iron depletion of the broth and, consequently, reduced toxin production. The organisms were then resuspended in 20 ml of buffer (0.05 M KCl, 0.01 M MgCl₂, and 0.02 M Tris at pH 7.4) and disrupted by two passages through a French pressure cell at 15,000 psi. Lysates were then subjected to ultracentrifugation at 100,000 g for 70 min and supernatants were dialyzed against 0.85% NaCl at 4 C. The protein content of these extracts was estimated spectrophotometrically [28].

Biologic and immunologic assays. Cytotoxicity tests were performed with HeLa cells (Walter Reed Army Institute of Research, Washington, D.C.) by the method of Gentry and Dalrymple [29]. Neutralization tests were done by mixing equal volumes of serially diluted rabbit antiserum to Shiga toxin or preimmunization rabbit serum with 10 50% cytotoxic doses of toxin contained in filtered bacterial extracts and then incubating the mixtures for 1 hr at 37 C, followed by overnight incubation at 4 C. Rabbit antiserum to Shiga toxin was elicited against purified Shiga toxin [23] by a previously described immunization protocol [23].

A 0.1-ml aliquot of each toxin-serum mixture was then added to HeLa cells. All dilutions were prepared in Eagle's minimal essential medium with Earle's balanced salts (HEM Research, Rockville, Md.) plus 2 mM L-glutamine, 10% fetal bovine serum (Flow Laboratories, McLean, Va.), 100 units of penicillin G/ml, and 100 μg of gentamicin/ml.

Other analyses performed on some bacterial extracts included tests for enterotoxic activity by the rabbit ileal loop assay [30] and assessments of the paralytic and lethal activity for mice [25]. In addition, the technique of Brown et al. was used to quantitate the effects of certain cytotoxic extracts on protein synthesis in HeLa cells [31]. Finally, the antigenic relationships among some of the toxins contained in extracts were assessed by immunodiffusion against rabbit antiserum to Shiga toxin. Immunodiffusion plates were prepared as described [23].

Results

Bacterial cell extracts and culture supernatants from 13 strains of *E. coli* that had been grown in iron-depleted medium were tested for cytotoxic effects on HeLa cells that were neutralizable with antiserum to Shiga toxin. These particular isolates were selected because they were well-characterized and represented a pathogenic spectrum that ranged from avirulent to virulent by an established diarrhea-evoking mechanism (LT-positive, ST-positive, or enteroinvasive) or by undefined means (LT-negative, ST-negative, and Séreny test-negative). Positive controls for the study included lysates and culture supernatants of *S. dysenteriae* type 1 strain 60R, which produces high levels of Shiga toxin [14, 23, 24], as well as a strain of *Shigella flexneri* type 2a, which produces Shiga-like toxin [25]. Two other bacterial species were included in this investigation: *Salmonella typhimurium* (another Enterobacteriaceae) and the unrelated gram-negative microbe, *Pseudomonas aeruginosa*. Some pertinent characteristics of these microbes are listed in table 1.

The results of the cytotoxicity evaluations are shown in table 2. The organisms were arbitrarily grouped into four categories according to the effects of the bacterial extract on HeLa cells. Category 1 contained those isolates that made cell-associated cytotoxins that were not neutralized by rabbit antiserum to Shiga toxin, such as *P. aeru-*

Table 2. Production of cytotoxins, categorized by similarity to the toxin of *Shigella dysenteriae* type 1 (Shiga), by various bacterial strains.

Category	Toxin production	Bacteria	Cytotoxicity for HeLa cells		Cytotoxicity neutralized by rabbit antiserum to Shiga toxin (titer)*
			CD_{50}/ml of culture supernatant	CD_{50}/mg of protein in bacterial extract	
1	Cytotoxin not Shiga-like	*Escherichia coli*			
		E2531	ND	9	No
		NC	ND	15	No
		Pseudomonas aeruginosa	ND	60	No
2	Trace levels of Shiga-like toxin ($\leq$10 CD_{50}/mg of bacterial extract)	*E. coli*			
		K12 (strain 1133)	ND	2	Yes (1:3,200)
		HS	ND	10	Yes (1:6,400)
3	Low to moderate levels of Shiga-like toxin	*Salmonella typhimurium*			
		W118	ND	20	Yes (1:6,400)
		E. coli			
		TD 514C$_1$	ND	20	Yes (1:6,400)
		RDEC-1	ND	40	Yes (1:12,800)
		460-8-58-899	ND	40	Yes (1:6,400)
		E74/68	ND	60	Yes (1:6,400)
		E851/71	ND	100	Yes (1:6,400)
		H10407	ND	100	Yes (1:3,200)
		12801/0	ND	200	Yes (1:1,600)
		Shigella flexneri type 2a (strain M4243)	50	300	Yes (1:3,200)
4	High levels of Shiga or Shiga-like toxin	*E. coli*			
		H30	1×10^3	1×10^4	Yes (1:800)
		S-22-1	1×10^4	4×10^4	Yes (1:3,200)
		S. dysenteriae type 1 (strain 60R)	1×10^4	3×10^5	Yes (1:1,600-1:6,400)

NOTE. CD_{50} = cytotoxic doses required to kill 50% of HeLa cells; ND = not detected.
* Highest dilution of serum that completely protected HeLa cells from 10 CD_{50} of toxin.

ginosa. The microbes assigned to category 2 (trace levels of Shiga-like cell-associated cytotoxin) included the two avirulent *E. coli* strains 1133 [15] and HS [1, 9]. The agents listed in category 3 (producers of low to moderate levels of Shiga-like cytotoxins) included seven strains of *E. coli* ranging from an ST-positive strain that produced low levels of toxin (TD 514C) [22] to an enteropathogenic strain (12801/0) that made nearly as much toxin as *S. flexneri* type 2a. We found that *S. typhimurium* strain W118 [26, 27] also made low levels of a cytotoxin, an observation that supports the findings of others (F. C. W. Koo and J. Peterson, personal communication). The relationship between the *S. typhimurium* cytotoxin described by these investigators and the Shiga-like toxin described in the present investigation remains to be determined.

Category 4 included two strains of *E. coli* that made levels of Shiga-like toxin equivalent to the strain 60R positive control. One of the microbes, strain H30, was described by Konowalchuk et al.

[18]. They reported that culture filtrates of the bacterium were cytotoxic for Vero cells. The other organism, strain S-22-1, was obtained from M. Gurwith (Michigan State University, East Lansing). Gurwith and Williams [19] observed that a culture filtrate of the strain, which was isolated from the stool of a child with diarrhea, grown in brain-heart infusion broth was cytotoxic for HeLa cells. That the extracts from these two LT-negative, ST-negative, Séreny test–negative strains of *E. coli* isolated from infants with diarrheal disease [18, 19] do indeed make a toxin similar to Shiga toxin was verified by other biologic and immunologic tests. Lines of identity were seen when extracts of *E. coli* strain H30, *E. coli* strain S-22-1, and *S. dysenteriae* type 1 strain 60R were diffused against rabbit antiserum to Shiga toxin (figure 1). Furthermore, cell extracts of these two strains of *E. coli*, like *S. dysenteriae* type 1 strain 60R, paralyzed and killed mice, inhibited protein synthesis in HeLa cells, and caused fluid accumulation in ligated rabbit ileal loops (table 3), as did fil-

Figure 1. Results of immunodiffusion of rabbit antiserum to toxin produced by *Shigella dysenteriae* type 1 (center well) against crude cell extracts of *S. dysenteriae* type 1 strain 60R (well A), *Escherichia coli* strain H30 (well B), and *E. coli* strain S-22-1 (well C).
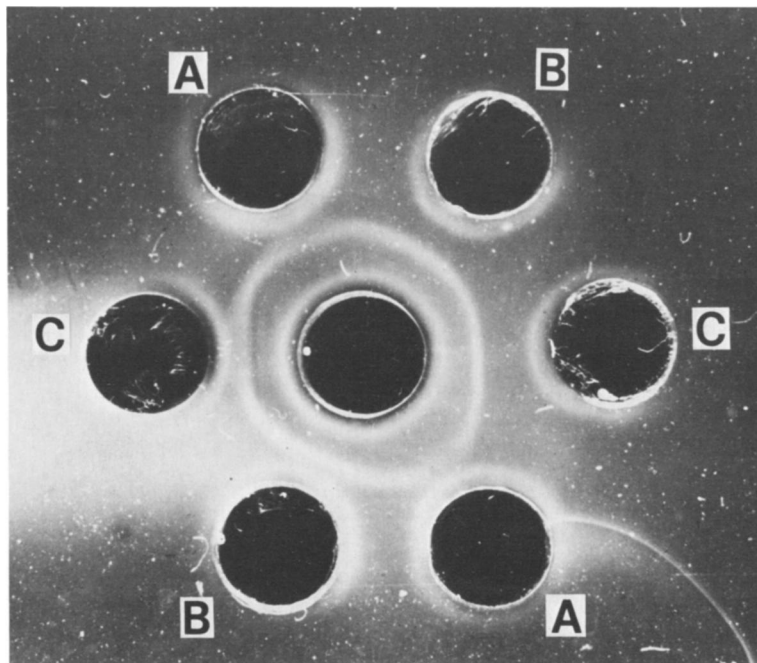
tered culture supernatants of the microbes (data not shown). By contrast, an extract of *E. coli* strain 12801/0 inhibited protein synthesis in HeLa cells but was not lethal for mice or enterotoxic for rabbits, and an extract of *E. coli* K12 strain 1133 was negative for all three activities. It should be noted that the mouse lethality and rabbit enterotoxicity assays for purified Shiga toxin are at least 10^4 times less sensitive than are tests for cytotoxicity or inhibition of protein synthesis [14, 32].

Discussion

The present study established that some strains of *E. coli* make a Shiga-like toxin and showed that

among such isolates toxic activity varied as much as 10,000-fold. The basis for the diversity in yields of cell-associated toxin activity was not examined but parallels the situation seen among species of shigellae—for example, *S. dysenteriae* makes 10^4–10^5 more cytotoxic material per milligram of cell extract protein than does *S. flexneri* [14, 25]. Several theories may be proposed to explain the *E. coli* strain-dependent differences in elaboration of Shiga-like toxin. (*1*) The regulatory mechanisms that control toxin production by the organisms may be quite distinct. For example, a category 4 (high-level producer of toxin) organism may make toxin constitutively, whereas toxin levels made by *E. coli* in category 2 or 3 (trace to

Table 3. Other biologic properties of extracts of representative bacteria in a study of bacterial cytotoxins.

Bacteria	Enterotoxicity for rabbits*	Lethality for mice ($LD_{50}/\mu g$ of protein)†	Inhibition of protein synthesis (ID_{50}/mg of protein)‡
Shigella dysenteriae type 1, strain 60R	Yes	Yes (50)	Yes (10^5)
Escherichia coli			
S-22-1	Yes	Yes (60)	Yes (10^5)
H30	Yes	Yes (90)	Yes (10^5)
12801/0	No	No	Yes (90)
K12, strain 1133	No	No	No (<10)

* Extract tested at a dilution of 1:20.
† Hind leg paralysis observed before death of mice.
‡ ID_{50} = dose required to inhibit protein synthesis in HeLa cells by 50%.

moderate levels of toxin production) may be determined by a repressible or inducible mechanism. (2) Toxin made by category 2 or 3 microbes may vary structurally from that produced by category 4 bacteria. Perhaps structural differences translate to variability in biologic activities. (3) Category 2 and 3 bacteria may manufacture large amounts of biologically inactive toxin that compete with active toxin in bioassays. If the reason for variability in toxicity among strains of *E. coli* is the same as that for shigellae, the last postulate is not likely because a previous investigation [14] showed that no biologically inactive toxin was found in extracts of *S. flexneri*. Genetic and biochemical experiments should address this issue of variability in the quantity of Shiga-like toxin made by isolates of *E. coli*.

Two lines of circumstantial evidence suggest that the Shiga-like toxin may play a role in the pathogenesis of some cases of *E. coli*-conferred diarrhea. (*1*) Two strains of *E. coli* implicated as mediators of gastroenteritis in children made high levels of Shiga-like toxin but did not elaborate LT or ST and were Séreny test-negative. (*2*) The avirulent *E. coli* strains 1133 and HS made only trace amounts of toxin. This finding suggests that if a Shiga-like toxin is in fact a virulence determinant, then either these isolates do not make enough toxin to be detrimental or another factor or factors may be required to confer pathogenicity. Such a factor could be a mechanism of adherence to the intestinal mucosa; indeed, Smith and Linggood [33] clearly established that enterotoxigenic strains of *E. coli* that lack the K88 colonizing factor fail to cause diarrheal disease in pigs. That some LT-positive, ST-positive, or enteroinvasive strains of *E. coli* also made low to moderate amounts of Shiga-like toxin (table 2) does not rule out a role for Shiga-like toxin in disease caused by *E. coli*. Rather, enterotoxic strains such as *E. coli* strain H10407 may colonize a region of the intestine where Shiga-like toxin is ineffective. Alternatively, Shiga-like toxin may actively contribute to the development of clinical manifestations in *E. coli* strain H14047–induced diarrhea. Obviously, further studies are required to define the role (if any) of Shiga-like toxin in the pathogenesis of diarrhea. These investigations could include an examination of whether the toxin functions in vivo as an enterotoxin or as a cytotoxin.

The structural relationship between Shiga and Shiga-like toxins was not examined in this investigation. The fact that Shiga-like toxic activity can be neutralized by antiserum to purified Shiga toxin suggests some degree of homology between the toxins. Other data that support the hypothesis that the molecules are physically similar include the range of biologic activities of the two moieties and the immunodiffusion results. Shiga toxin appears to be composed of two subunits: an enzymatically active A subunit (~30,500 daltons) that when nicked and reduced inactivates 60S ribosomes and inhibits peptide chain elongation and six or more copies of a B subunit (~5,000 daltons) that is presumed to function in the binding of toxin to the target cell [34, 35]. Thus, Shiga-like toxins probably contain one or both of these components. Purification of the toxic material from *E. coli* strain H30 should clarify this issue.

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