

Immunochemical and Cytotoxic Activities of *Shigella dysenteriae* 1 (Shiga) and Shiga-Like Toxins

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Toxins in culture supernatants and bacterial lysates of *S. dysenteriae* 1 and *S. flexneri* were quantitated by a cytotoxicity assay and a newly developed radioimmunoassay. Cytotoxin titers paralleled toxin antigen levels. Thus, variations in cytotoxicity among shigellae probably reflect differences in toxin yield rather than specific activity (cytotoxicity per microgram of toxin antigen).

Shigellosis is a gastrointestinal infection which may cause diarrhea, dysentery, or both. The primary virulence determinant of shigellae is the invasive capacity of the organisms; strains which cannot penetrate and multiply within colonic epithelial cells do not cause disease in humans (15) or monkeys (7). Nonetheless, Keusch et al. suggested a role for toxin in some of the clinical manifestations of shigellosis. This hypothesis was based on their observations that sterile culture filtrates of *Shigella dysenteriae* 1 are enterotoxic for ligated rabbit ileum (10) and produce pathological changes in rabbit intestine which mimic some of the histological alterations seen in experimental animals infected with the intact bacterium (11). McIver et al. further demonstrated that the enterotoxin comigrated in isofocused polyacrylamide gels with the Shiga lethal factor ("neurotoxin") and the Shiga cytotoxin (16). The direct relationship between these three biological activities was recently verified in this laboratory. Shiga toxin purified to homogeneity by antitoxin affinity chromatography was enterotoxic, cytotoxic for HeLa cells, and lethal for rabbits (17). The molecular basis for these diverse biological manifestations may be the capacity of Shiga toxin to inhibit protein synthesis in eucaryotic cells (2, 3, 19, 21).

S. dysenteriae is not the only species of *Shigella* which produces a toxin. *S. flexneri* and *S. sonnei* produce toxins which are biologically and antigenically related to Shiga toxin (12, 18). However, these Shiga-like toxins contain 10^4 - to 10^5 -fold less cytotoxic activity than do comparable preparations of Shiga toxin. The goals of this study were to examine the basis for the reduced toxic activity of Shiga-like toxins and to identify any differences in Shiga toxin production by different *S. dysenteriae* 1 strains. To achieve these aims, a radioimmunoassay (RIA) was developed with which to quantitate toxin protein. Toxic activity was also assessed by the sensitive

HeLa cell microcytotoxicity assay of Gentry and Dalrymple (8).

S. dysenteriae 1 strain 60R, a rough mutant originally described by Dubos and Geiger (6), was used as the toxinogenic control organism throughout this investigation. Other bacteria employed included: *S. dysenteriae* 1 strain 3818T, a smooth, high-toxin producer (7); *S. dysenteriae* 1 strain 725-78, a low-toxin-producing (13) derivative of 3818T (7); *S. flexneri* strain M4243, a producer of Shiga-like toxin (18). All strains except Shiga 60R are capable of epithelial cell invasion as determined by the Séreny test (20).

Several investigators have shown that the lethal activity of Shiga toxin is increased when organisms are cultured in a low-iron-containing medium (6, 22). Therefore, a modified syncase broth (18) was selected as the standard bacteriological medium because its iron content was below the level of detection ($<0.5 \mu\text{g}$ of ^{59}Fe /ml) in the bathophenanthroline assay (14). For some experiments the Fe^{3+} content of the syncase medium was further decreased by mixing the broth with Chelex 100 (100 to 200 mesh, sodium form; Bio-Rad Laboratories, Richmond, Calif.) for 2 h at 4°C . The resin was removed by filtration ($0.45 \mu\text{m}$ of Nalgene per 100-ml filter flask; Nalgene Co., Rochester, N.Y.) before inoculation of the broth with bacteria.

Cultures (50 ml of medium per 125-ml Erlenmeyer flask) were inoculated in duplicate with single bacterial colonies from tryptic soy agar (Difco Laboratories, Detroit, Mich.) plates and agitated (180 rpm) at 37°C for 48 h, the time at which maximum concentrations of cell-associated Shiga toxin are evident (18). On completion of incubation, 0.2-ml samples were obtained for the enumeration of viable bacteria by plate count. Organisms were harvested by centrifugation at $12,000 \times g$ for 20 min at 4°C , and samples of the supernatants were saved for toxin quanti-

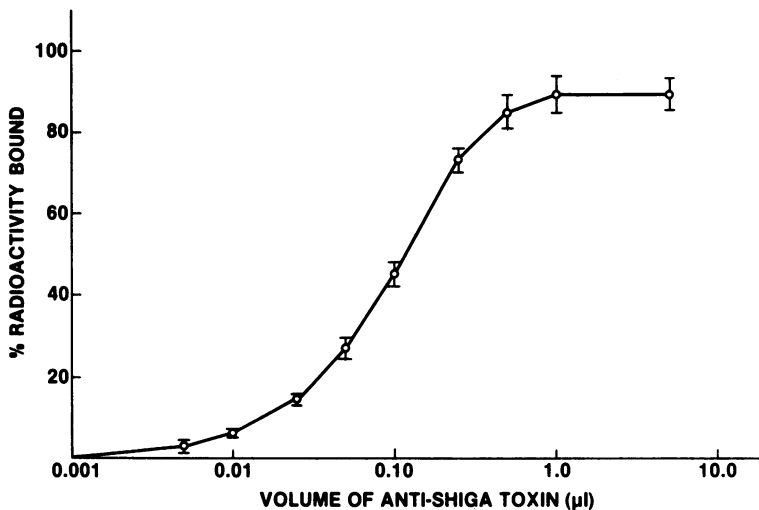


FIG. 1. Binding of ^{125}I -labeled Shiga toxin by rabbit anti-Shiga toxin. Various amounts of antiserum were mixed with purified radiolabeled toxin (50 ng; $0.1 \mu\text{Ci}/\mu\text{g}$), and the immune complexes were precipitated with staphylococci. Each point represents the mean (three separate experiments, six samples) percent radioactivity bound per serum volume. The bars depict \pm two standard errors of the mean.

tation. The bacterial pellets were suspended in 10 ml of sterile saline (0.85% NaCl) and subjected to centrifugation ($12,000 \times g$ for 15 min at 4°C). This washing procedure was repeated, and the cells were then suspended in 5 ml of sterile saline. The saline washes contained less than

0.1% of the cytotoxic activity associated with the bacterial pellets. The saline-washed bacterial samples were then placed in an ice water bath, and the cells were disrupted by 3 min of intermittent (15 s on, 10 s off) sonic oscillation at 50 W (Sonifer cell disrupter model W185 with microtip

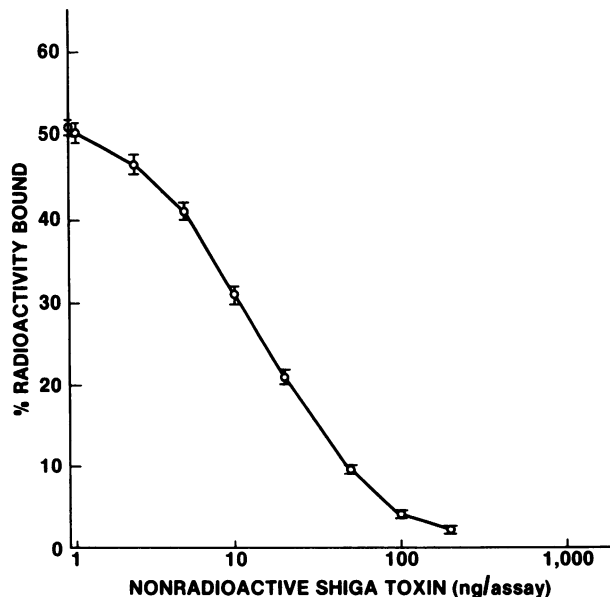


FIG. 2. Standard curve for the quantitation of Shiga toxin antigen by competitive binding RIA. Various amounts of purified unlabeled Shiga toxin were allowed to compete with purified ^{125}I -labeled toxin (50 ng; $0.1 \mu\text{Ci}/\mu\text{g}$) for binding to rabbit anti-Shiga toxin (0.1 to $0.2 \mu\text{l}$ as per 50% point, Fig. 1), and the immune complexes were precipitated with staphylococci. Each point represents the mean of 18 samples from nine separate experiments performed over a 6-month period. The bars depict \pm two standard errors of the mean.

probe; Heat Systems Ultrasonic Inc., Plainview, N.Y.). The sonic extracts were clarified by centrifugation ($12,000 \times g$, 20 min, 4°C), and the supernatants were saved. The protein concentrations of sonic lysates were quantitated by the Bio-Rad assay (Bio-Rad Laboratories), a procedure based on the method of Bradford (1). In addition, filtered samples of culture supernatants and sonic lysates were serially diluted (10-fold for Shiga toxin and 2-fold for Shiga-like toxins) and assessed for cytotoxic effects on HeLa cells (8) and for total toxin antigen by RIA.

The competitive-binding RIA for quantitation of Shiga toxin was developed by the technique used by Cryz et al. for immunochemical analysis of diphtherial toxin (4). Purified Shiga toxin for the RIA was prepared as previously described (17). The toxin contained 10^9 50% cytotoxic doses (CD_{50}), 10^4 50% mouse lethal doses, and 10^3 50% rabbit enterotoxic doses per mg of protein. A portion of this material was ^{125}I -labeled by the solid-state lactoperoxidase method (5). Rabbit anti-Shiga toxin was prepared as previously described (17).

Figure 1 depicts the curve used to determine the quantity of rabbit anti-Shiga toxin sufficient to bind 50% of radiolabeled toxin. The standard curve for quantitation of unlabeled toxin is shown in Fig. 2. Samples were diluted such that the percent radioactivity bound fell within the linear portion (10 to 40%) of the curve. Thus, as little as 5 ng of toxin in a 250- μl sample or 0.1 μg per 5 ml of bacterial sonic lysate could be detected by the RIA. Nevertheless, the procedure was less sensitive than the HeLa cell assay; 1 ng of toxin antigen was equivalent to 2×10^2 CD_{50} (99.9% confidence limits = 9×10^2 to 5×10^1 CD_{50} as calculated from the arithmetic mean of 45 separate analyses).

Both Shiga 60R and Shiga 3818T were used as the source of toxin for purification. As shown in Table 1 the invasive 3818T strain produced more cytotoxin and toxin antigen than did 60R under both standard iron and low-iron growth conditions. Specific toxicities of 60R and 3818T toxins were equivalent. That Chelex treatment did, in fact, remove iron from the media was confirmed by the reduction in total CD_{50} 's ($10^{6.8}$ to $10^{5.2}$) seen in a separate experiment when iron was added back to resin-treated Shiga 60R broth cultures. The finding that Shiga 3818T produced significantly more toxin antigen than did Shiga 60R under standard conditions was observed consistently in three separate experiments. Based on differences in the characteristics of these two strains, i.e., Shiga 60R is rough and noninvasive, whereas 3818T is smooth and invasive, one can propose several reasons for variations in toxin production. However, a more precise explanation for strain-dependent differences in toxin yields may be obtained from ongoing studies with isogenic pairs of *S. dysenteriae* 1.

The basis for the low toxic activity associated with Shiga 725/78 (13) and *S. flexneri* M4243 (18) was also examined (Table 1). Shiga 725/78 yielded low levels of cytotoxin under standard conditions, and the quantity of cytotoxin produced was slightly improved by iron depletion of the broth. Production of Shiga-like cytotoxin by *S. flexneri* M4243 was only observed in these small-volume cultures when the medium was pretreated with Chelex. Neither Shiga 725/78 nor *S. flexneri* M4243 made detectable amounts of toxin antigen. That the RIA should have detected toxin from these organisms when the materials were present in sufficient quantities is suggested by previous observations that rabbit anti-Shiga toxin can completely neutralize cyto-

TABLE 1. *Shigella* species and strain-dependent variation in toxin production

Organism ^a	Chelex-treated media ^b	Toxin			Specific toxicity
		Log_{10} CD_{50} ^c (% cell-associated)	Log_{10} CD_{50} /mg of protein	μg of toxin ^c antigen (% cell-associated)	Log_{10} CD_{50} / μg of toxin
Shiga 60R	-	6.7 (99)	6.2	30 (93)	5.2
	+	7.7 (10)	8.0	96 (10)	5.7
Shiga 3818T	-	7.7 (86)	7.1	87 (90)	5.8
	+	8.0 (50)	7.6	228 (73)	5.6
Shiga 725/78	-	2.1 (100)	1.3	<0.1	—
	+	2.4 (100)	1.8	<0.1	—
<i>S. flexneri</i> M4243	-	<2.0	—	<0.1	—
	+	3.0 (100)	2.5	<0.1	—

^a *Salmonella typhimurium* strain TML (9) was used as a control in this experiment. No toxicity was observed in sonic lysates or culture supernatants of TML.

^b Organisms were cultivated for 48 h at 37°C in modified syncase broth (pH 7.0, 0.2% glucose), which in some cases had been pretreated with 2% Chelex. All cultures contained an average total of between 1×10^{10} and 1×10^{11} viable bacteria.

^c The values represent totals per culture as calculated from geometric mean (CD_{50}) or arithmetic mean (micrograms of toxin antigen) of duplicate samples.

toxins of Shiga 725/78 (13) and *S. flexneri* M4243 (18). Toxins from these bacterial strains have not as yet been purified and, therefore, are not available for analysis by the RIA. Nonetheless, it appears that the reduced toxic activities of these bacteria also reflect decreased yields of toxin antigen rather than alterations in specific toxicity.

In summary, the RIA described herein permitted quantitative analyses of Shiga toxin antigen for the first time. Although the RIA was less sensitive than the HeLa cell assay, it detected low levels of toxin antigen (5 ng). The RIA was also specific; it detected toxin in crude lysates of *S. dysenteriae* but not in lysates of bacteria with similar (other *Shigella* species) or related (*Salmonella typhimurium*) nontoxigenic antigens. Finally, the combination of the RIA and the cytotoxin assay provided a means of assessing how various genetic and environmental factors affect toxicity.

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