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The pathogenesis of *Shigella* Diarrhea: *I. Enterotoxin production by Shigella dysenteriae 1*

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The previously described neurotoxic (mouse lethal) factor was also present and eluted from Sephadex G-150 with the enterotoxin. If these biological activities prove to be possessed by a single molecular species, it is suggested that it be renamed Shigella enterotoxin in recognition of the physiologically more relevant biological action.

The Pathogenesis of *Shigella* Diarrhea

I. ENTEROTOXIN PRODUCTION BY *SHIGELLA DYSENTERIAE* 1

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ABSTRACT A strain of *Shigella dysenteriae* 1, freshly isolated from a patient with dysentery in Guatemala in August 1969, was found to elaborate an enterotoxin into the liquid of broth cultures. Partial purification of the enterotoxin by ultrafiltration on graded polymeric membranes and Sephadex gel filtration (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) suggested an approximate molecular weight of 55,000–60,000. The partially purified toxin was heat-labile, pronase sensitive, and activated by alkaline pH, and it elicited fluid production in ligated rabbit ileal segments; it failed, however, to cause increased vascular permeability in skin. When the activities of equal weights of identically prepared *Vibrio cholerae* and *S. dysenteriae* enterotoxins were compared in the rabbit ileum the latter caused a significantly smaller volume response with increased concentrations of potassium, chloride, and protein.

The previously described neurotoxic (mouse lethal) factor was also present and eluted from Sephadex G-150 with the enterotoxin. If these biological activities prove to be possessed by a single molecular species, it is suggested that it be renamed Shigella enterotoxin in recognition of the physiologically more relevant biological action.

INTRODUCTION

A protein exotoxin elaborated by *Shigella dysenteriae* 1 (Shiga's bacillus) has been known since 1903 (1). The toxin was classified as a neurotoxin because parenteral inoculation in rabbits resulted in paralysis, cerebral and spinal cord hemorrhages, and death (1, 2). The protein neurotoxin was clearly distinguished from lipopolysaccharide endotoxin (LPS)[1] by heat lability, precipitation by trichloroacetic acid, suppression of synthesis under anaerobic conditions, and production by LPS-deficient rough strains (3–5). Van Heyningen and Gladstone obtained a highly purified preparation in 1953 (6), however with the exception of one negative study in monkeys (7), enterotoxicity was not tested by intraintestinal challenge.

The studies of Formal, LaBrec, and Schneider (8) clearly demonstrated the necessity for epithelial cell penetration and multiplication for virulence of *Shigella flexneri* 2a, but did not identify the nature of the resulting bacterial-host interaction. Enterotoxin production has recently been described in several bacterial species not previously thought to be enterotoxigenic, including *Escherichia coli* (9), *Clostridium perfringens* (10), and *Pseudomonas aeruginosa* (11). Earlier speculation that the exotoxin of *S. dysenteriae* 1 was not truly neurotropic (12–14) made this organism the logical choice to study first among the *shigellae*. The occurrence of a wide spread epidemic of dysentery due to Shiga's bacillus in Central America in 1969 (15) provided the opportunity for these studies, which document that *S. dysenteriae* 1 does produce enterotoxin in vitro which is distinct and separable from endotoxin, but not to date from neurotoxin.

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[1] *Abbreviations used in this paper*: K_{av}, fractional elution volume; LD_{50}, 50% lethal dose; LPS, lipopolysaccharide endotoxin; PF, permeability altering factor.

METHODS

Toxin production. A strain of *S. dysenteriae* 1, designated MK-102, was isolated at INCAP in Guatemala City from a young Guatemalan girl with dysentery in August 1969. It possessed an R factor for streptomycin, tetracycline, chloramphenicol, and mercuric chloride transferrable to *E. coli* K-12 (performed by Dr. David H. Smith). Culture media were prepared from 3% peptone broth (Difco Laboratories, Detroit, Mich.) fractionated by pressure dialysis through a graded polymeric membrane to retard passage of components above 10,000 mol wt (UM-10, Amicon Corp., Lexington, Mass.). 200 ml of the low molecular weight dialysate were placed in 2-liter Erlenmeyer flasks and inoculated with a log phase culture. After incubation for 18 hr at 37°C with vigorous aeration, organisms were removed by centrifugation at 16,300 *g* for 30 min (this and all subsequent operations were conducted at 4°C). Final sterilization was achieved by passage through a Millipore GS membrane (Millipore Corp., Bedford, Mass.).

Fractionation procedures. The resulting product, crude Shigella enterotoxin, was concentrated by pressure dialysis through Amicon UM-10 or XM-50 membranes (molecular weight cutoff 50,000). Approximately 3 ml of the concentrate (equivalent to 1000 ml of crude toxin) was applied to a 2.5 × 30 cm column of G-150 Sephadex (Pharmacia Fine Chemicals, Piscatway, N. J.) equilibrated with 0.02 M NH_4HCO_3. 4-ml portions were eluted and the six tubes encompassing peak enterotoxic activity were pooled and lyophilized.

Assay of biologic activity. Enterotoxic fractions were defined by titration in ligated segments of adult white New Zealand rabbit ileum as previously described (16). Active toxin solubilized in 0.1 M phosphate buffer (pH 8) and inoculated into the intestinal lumen resulted in fluid accumulation causing severe distention of the loop by 12 hr and pressure necrosis of the gut mucosa by 18 hr.

Neurotoxic activity was assayed by parenteral inoculation of the partially purified toxin in 20-g white mice (Charles River Laboratories, Cambridge, Mass.). Deaths within 6 days of injection were tabulated and the 50% lethal dose (LD_{50}) calculated by the formulae of Reed and Muench (17).

The presence of a permeability altering factor (PF) was sought by determining the response to intradermal injection in rabbit skin as described (16). Induration of 8 mm diameter or more at 24 hr was defined as a positive response. Erythema without induration was not considered a true response.

Endotoxin was assayed by the limulus amebocyte gelation test of Levin and Bang (18). Sodium and potassium concentrations were determined by flame photometry and chloride by the Cotlove electrode. Bicarbonate concentration was estimated as [Na + K] − [Cl]. Protein was measured by the method of Lowry, Rosebrough, Farr, and Randall (19) uisng bovine serum albumin as standard. Data were examined statistically by Student's *t* test.

RESULTS

Crude enterotoxin, diluted in 0.1 M phosphate buffer (pH 8) and inoculated into segments of rabbit ileum resulted in gross accumulation of fluid beginning after 4–6 hr. Heated toxin (90°C, 30 min) or media controls were inactive (Table I). 10 µl of the crude toxin suspended in 1–2 ml of phosphate buffer usually produced a maximum fluid response.

TABLE I

Effect of Crude Shigella Enterotoxin on Fluid Production by Ligated Rabbit Ileal Segments

Preparation	No. of tests	Fluid produced
		ml/cm
Active toxin*	42	1.8±0.2‡
Heated toxin (90°C, 30 min)	9	0.3±0.1
Uninoculated medium	13	0.1±0.1

* 1 ml of crude culture filtrate per 7 cm loop of rabbit ileum.
‡ ±1 SEM.

Fractionation of toxin and estimation of molecular weight. Simultaneous titration of enterotoxin activity of the filtrate and retentate obtained by membrane ultrafiltration allowed a preliminary estimation of molecular weight. Over 99% of the enterotoxicity was retained by the UM-10 membrane, while approximately 90% was present in the retentate from the XM-50 membrane. These data suggested a molecular weight somewhat in excess of 50,000.

Chromatography on a calibrated (16) Sephadex G-150 column supported this estimate. Three fractions were discernible (Fig. 1). Fraction A eluted at the void volume of the column and was distinctly opalescent. Previous experience with cholera enterotoxin indicated that anthrone-positive saccharide, presumably reflecting LPS, would be found principally in the fraction first eluted (16). In the current studies of shigella enterotoxin, LPS activity measured directly by the limulus assay was found to coincide with peak opalescence in fraction A (Table II). This material was essentially devoid of enterotoxicity (Fig. 1, middle panel). Rather, peak ileal loop reactive toxin was contained in fraction B, eluting at a fractional elution volume (K_{AV}) of 0.45–0.5, corresponding to a molecular weight of 55–60,000 (16). When crude toxin was washed with 3 vol of buffer through the Amicon XM-50 membrane before chromatography a sharper peak was obtained (Fig. 1, upper panel, solid line) although 50% of the toxin yield was thereby sacrified. Approximately 40% of the enterotoxin activity trailed into fraction C, a yellow-brown material also containing lower molecular weight media components. LPS activity did not parallel enterotoxicity; LPS per milligram dry weight in fraction B was less than 10% of that found in fraction A, while less than 1% was present in fraction C (Table II).

Properties of shigella enterotoxin. Consistently positive ileal loops were obtained with 0.5 µg of lyophilized fraction B; this material, partially purified Shigella enterotoxin (G-150 enterotoxin) was used for further study.

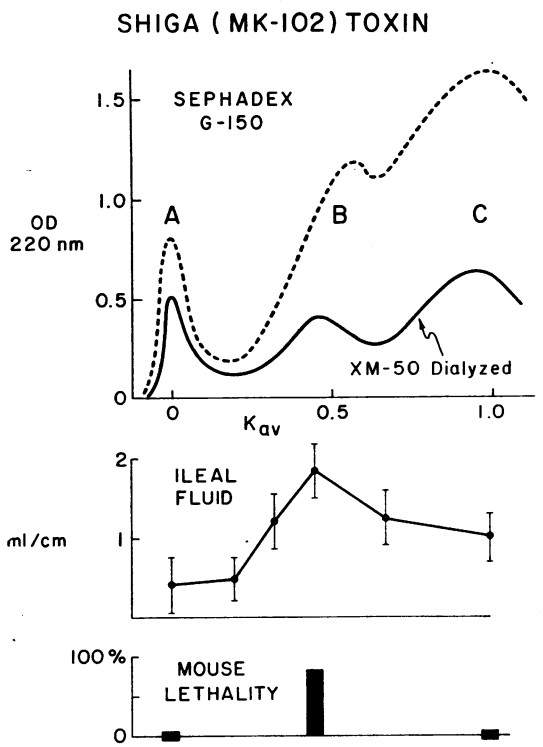

FIGURE 1 Upper panel: elution of *Shigella* enterotoxin from Sephadex G-150 without (dotted line) and with (solid line) prior diafiltration on an Amicon XM-50 membrane. Middle panel: fluid response of ligated rabbit ileal segments to various toxin fractions. Lower panel: assay of neurotoxin activity of the three major peaks obtained from Sephadex chromatography.

The effect of varying buffer pH on toxin induced ileal fluid accumulation is shown in Table III. Enterotoxicity was potentiated at alkaline pH and inhibited by acid conditions. While fluid production at pH 10 was increased over that at pH 8 at threshold doses of toxin, this was offset somewhat by more frequent occurrence of "nonspecific" positive loops. At a challenge dose of 5 μg, toxin in pH 8 buffer resulted in a maximum response, thus obscuring the increased sensitivity of the higher pH. Optimum conditions were defined on the basis of these data and toxin was solubilized as noted in 0.1 M phosphate, pH 8 for all other studies.

TABLE II

Endotoxin Activity (Limulus Lysate Gelation) in S. Dysenteriae 1 Culture Filtrate Chromatographed on Sephadex G-150

	Preparation tested				
	S. dysenteriae 1 filtrate			Endotoxin	
Weight	Peak A	Peak B	Peak C	*S. flexneri**	*E. coli**
μg					
10	+‡	+	+	+	+
1	+	+	−	+	+
0.1	+	−	−	+	+
0.01	±	−	−	+	+

* Difco Laboratories, Detroit, Mich.
‡ + = Gelation within 30 min at 37°C.

TABLE III

Effect of Varying Buffer pH on G-150 Shigella Enterotoxin Activity

	Ileal loop fluid, buffer pH		
Toxin dose	6	8	10
μg	ml/cm	ml/cm	ml/cm
0 (control)	ND*	0.3±0.2‡ (10)§	0.5±0.2 (11)
0.5	0.1±0.1 (7)	1.0±0.3 (7)	1.6±0.4 (7)
5.0	1.6±0.5 (6)	2.4±0.2 (8)	2.4±0.2 (6)

* Not done.
‡ ±1 SEM.
§ Number of segments inoculated.

Heat lability of G-150 enterotoxin was determined by assay of control and heated toxin preparations in 10 rabbits (Table IV). Partial inactivation was achieved at 60°C for 30 min, and approximately 90% inactivation at 90°C for 10 min. Virtually complete loss of enterotoxicity was obtained by heating at 90°C for 30 min.

The effect of proteolytic enzymes on biologic activity of the enterotoxin was studied using the mouse lethality (neurotoxin) assay. Incubation of 250 μg of toxin in 0.05 M PO_4 buffer (pH 7.5) with 20 μg of pronase (*Streptomyces griseus* protease, Grade B, Calbiochem, Los Angeles, Calif.) for 5 hr at 37°C before i.v. inoculation into mice completely destroyed toxicity (Table V). Similar incubation with 40 μg trypsin (2x crystalized, salt-free trypsin, Worthington Biochemical Corp., Freehold, N. J.) failed to reduce mouse mortality. Trypsin or pronase alone did not affect the results. Attempts to demonstrate pronase lability of

TABLE IV

Heat Lability of G-150 Shigella Enterotoxin

Heat treatment	Ileal fluid accumulation
	ml/cm
None (control)	2.3±0.1* (16)‡
60°C/10 min	2.4±0.2 (8)
60°C/30 min	1.8±0.3 (16)
90°C/10 min	1.1±0.3 (16)
90°C/30 min	0.1±0.2 (8)

* ±1 SEM.
‡ Number of segments tested.

TABLE V

Effect of Pretreatment with Proteolytic Enzymes on Biologic Activity of S. dysenteriae 1 Enterotoxin in Mice

Treatment	Toxin dose	Mouse lethality
		% mortality
None	50 μg	40
None	125 μg	80
None	250 μg	100
Trypsin	None	0
Trypsin	250 μg	100
Pronase	None	0
Pronase	250 μg	0

enterotoxin activity in the ileal loop, however, were inconclusive because the enzyme itself resulted in frequent positive loops.

The volume and composition of the evoked fluid were serially determined in another group of rabbits (Table VI). For the purpose of comparison, separate loops in each animal were inoculated with equivalent weights of lyophilized toxins similarly prepared and purified from *V. cholerae* or *S. dysenteriae* 1. Fluid composition was similar when 5 or 50 μg of toxin was inoculated, and these data have been pooled for this analysis. A marked increase in volume response of the intestinal mucosa to Shigella toxin was noted as the terminal ileum was approached. In some animals there was as much as a 100-fold change in threshold response over a span of 50–60 cm of intestine. The volume response to cholera toxin was significantly greater ($P < 0.05$) at 12 and 18 hr than that evoked by Shigella toxin (Table VI; Fig. 2). Na and K concentrations did not vary with time, whereas chloride steady rose (as HCO_3 and other anions presumably fell). The fluid response to Shigella toxin contained higher concentrations of K and Cl than found in response to cholera toxin ($P < 0.05$). Protein concentration in response to the latter remained characteristically low for the first 12 hr. In contrast, increased protein content in loops challenged with Shigella toxin was evident at 6 hr and reached statistical significance ($P < 0.01$) at 12 hr. Even after 18 hr, when the integrity of the mucosa was markedly altered, the protein concentration of the loop fluid remained well within the range of a transudate.

Other biologic activity. Neurotoxin activity was assayed in each of the three fractions obtained from Sephadex G-150 chromatography (Fig. 1, bottom panel) and it aligned with peak enterotoxin activity in frac-

TABLE VI

Composition and Volume of the Fluid Evoked by G-150 Shigella and Cholera Toxins

Determination	Toxin	Time			All values
		6 hr	12 hr	18 hr	
Volume, *ml/cm*	Shigella	0.8±0.1* (NS)‡	1.0±0.2 (<0.05)	1.8±0.2 (<0.02)	1.2±0.2 (<0.05)
	Cholera	1.0±0.1	1.6±0.2	2.7±0.3	1.8±0.2
Na^+, *mEq/liter*	Shigella	150 ±3 (NS)	153 ±3 (NS)	154 ±1 (NS)	152 ±2 (NS)
	Cholera	154 ±3	155 ±2	152 ±1	154 ±2
K^+, *mEq/liter*	Shigella	5.0±0.2 (NS)	5.4±0.7 (<0.05)	5.1±0.3 (<0.05)	5.2±0.3 (<0.05)
	Cholera	4.7±0.2	4.6±0.1	4.5±0.1	4.6±0.1
Cl^-, *mEq/liter*	Shigella	65 ±5 ($P < 0.02$)	75 ±2 ($P < 0.05$)	86 ±4 (NS)	72 ±3 (<0.05)
	Cholera	48 ±2	64 ±2	79 ±4	65 ±2
HCO_3^-§, *mEq/liter*	Shigella	90	83	73	85
	Cholera	111	96	77	94
Protein, *mg/100 ml*	Shigella	225 ±52 (NS)	363 ±61 (<0.01)	495 ±46 (<0.01)	388 ±36 (<0.01)
	Cholera	142 ±29	170 ±41	242 ±29‖	171 ±24

* Mean ±1 SEM of 6–12 determinations.

‡ P value; NS = not significant at the $P = 0.05$ level.

§ Calculated as $(Na^+ + K^+) - (Cl^-)$.

‖ Three determinations only.

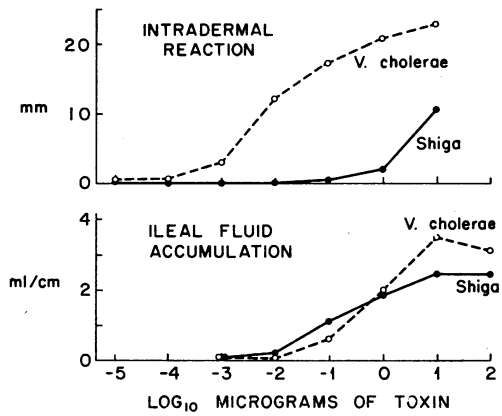

FIGURE 2 Simultaneous titration of identically purified enterotoxins from *V. cholerae* and *S. Shiga*. Upper panel: assay for permeability factor in rabbit skin (millimeters of palpable induration at 24 hr). Lower panel: enterotoxin activity assayed in ligated rabbit ileal segments.

tion B and was similarly destroyed by heating at 90°C for 30 min. When fractions B and C were simultaneously titrated to determined the i.v. LD_{50} the results paralleled their content of enterotoxic activity. The LD_{50} for fraction B was 68 μg, and for fraction C, 85 μg. On an absolute weight basis over 100 times more lyophilized toxin was required to kill a 20 g mouse than to induce fluid accumulation in a 7 cm ileal segment in the rabbit.

PF activity was not detected in the crude enterotoxin preparation. Similarly purified shigella and cholera toxins were then tested simultaneously in the skin of the same rabbit (Fig. 2). Although both toxins exhibited a comparable threshold response in the ileum, Shigella toxin contained 1/1000 as much PF activity. In contrast to cholera toxin, measurable induration occurred only at very high doses of Shigella toxin, at which levels heated toxin also resulted in marginal skin responses.

DISCUSSION

The present study demonstrates that we may now add *Shigella dysenteriae* 1 to the list of enterotoxin-producing bacteria. There has hardly been a pressing need to postulate the existence of such an enterotoxin because virulence of *Shigella* species has been clearly linked to mucosal penetration and intraepithelial multiplication (8). Moreover, attempts by others to find enterotoxin production by *S. dysenteriae* 1 (7) or *S. flexneri* 2a (20) had been uniformly unsuccessful. Arm, Floyd, Faber, and Hayes (20) suggested that diffusible cell-free products of *Shigellae* might play a role in the production of diarrhea (20). Their hypothesis was based on two observations: that living organisms were required for a positive response in ligated rabbit ileal segments, and the fact that inflammation of the bowel wall and accumulation of fluid in the lumen occurred in advance of significant bacterial proliferation. Takeuchi, Sprinz, La Brec, and Formal (21) reported a similar sequence following peroral challenge with living, virulent *Shigellae* in the opium treated guinea pig. At 8 hr after infection ileitis was present while only an occasional intraepithelial bacillus was found. By 24 hr, however, many invading bacilli were found, both by conventional histological and fluorescent antibody staining techniques. Degenerative changes were also noted in epithelial cells irrespective of the presence or absence of penetrating bacteria.

Whether enterotoxin is produced by all *Shigella* species or has a pathophysiological role in shigellosis is not clear at present. Proof of this hypothesis would not invalidate the current notions of the importance of epithelial penetration for virulence. The small bowel is rapidly cleared of bacteria by peristalsis (22), and avirulent nonpenetrating *Shigella* mutants quickly disappear (23). When this is prevented by ligation of the terminal ileum the in vivo growth curve of the mutant parallels that of the virulent strain (23). Epithelial penetration could, therefore, be seen as protecting the organism from the efficient clearance mechanisms of the bowel and thereby allowing establishment of propagating infection. Diarrhea would then follow subsequent in vivo enterotoxin production.

Because shigellosis is commonly termed bacillary dysentery and equated with colitis, one might question the clinical relevance of a toxin active in the small bowel. While the classical dysentery syndrome does include bloody mucoid stools and tenesmus related to large bowel involvement (22), the majority of humans with clinically manifest shigellosis develop only watery diarrhea (24, 25), which is usually considered a small bowel syndrome (22). In the monkey, the only natural host for Shigella other than man, colitis is the major manifestation of infection, although ileitis does occur in some of the animals (26). Ileitis is a prominent feature of laboratory infection of guinea pigs (21), and an acute small intestinal form has been described in man (27). In both naturally and laboratory acquired shigellosis, watery diarrhea precedes dysentery by 1–2 days. During this period of the induced laboratory disease with *S. flexneri* 2a, organisms in high concentration can be isolated from the small bowel fluids (H. L. Dupont, M.D., personal communication). The bacterial counts diminish in the ileum as colitis occurs and symptoms of dysentery become prominent. Thus the clinical manifestations of disease follow the progression of infection from distal small bowel to large intestine, and therefore appear dependent on duration of the infection. An identical progression has been noted in the guinea pig model (21, 28). The possible

role of enterotoxin in the colonic phase of shigellosis is open to speculation. Other enterotoxins are known to affect the large bowel. Severe colitis can result from large doses of staphylococcal enterotoxin (29). Prohaska and colleagues have suggested that pseudomembraneous enterocolitis due to the staphylococcus is a toxin-mediated disease (29, 30). Even cholera toxin, primarily active in the small bowel, has been reported to exert a measurable effect on the colon (31).

The current studies do not allow conclusions on the possible identity of *S. dysenteriae* 1 enterotoxin with the previously demonstrated Shiga neurotoxin. The latter has usually been isolated from bacterial autolysates (1) or extracted from the intact cells (6) whereas our preparation was isolated from cell-free growth medium. However the distinction is not clear because some autolysis is to be expected after 18 hr of incubation as indicated by the endotoxic fraction A demonstrated by Sephadex chromatography. Exotoxins may be present within the cells (where they have been referred to as protein endotoxins) as well as excreted into the extracellular medium (32). The highly purified neurotoxin produced by van Heyningen and Gladstone (6) contained 750 LD_{50}/mg (per kilogram mouse) whereas the material we isolated from a single passage through Sephadex G-150 contained only 12.5 mouse LD_{50}/mg. The latter figure is almost identical to that for the neurotoxic dialyzed autolysate from a culture of a rough strain of *S. dysenteriae* 1 studied by Howard (13). In the studies of Branham, Dack, and Riggs no effect was noted when 10,000–20,000 monkey i.v. LD_{50} doses of neurotoxin were inoculated intraluminally in isolated pouches of monkey small and large bowel (7). Several explanations are possible to account for this observation: a species difference in sensitivity of the gut to the toxin between monkey and rabbit, differential lability of enterotoxicity and neurotoxicity under the conditions of production utilized, or a lack of identity of neurotoxin and enterotoxin. We cannot resolve this question, but it is of interest that the strains of *S. dysenteriae* 1 utilized in Branham's study also failed to cause diarrheal disease when fed in enormous numbers to the monkeys.

The possible distinction between enterotoxin and neurotoxin is further blurred by the recent observation that purified cholera enterotoxin like Shigella neurotoxin kills mice when given i.v. (33). Since all manifestations of cholera are considered to be secondary to intestinal fluid loss (34), mouse lethality appears to be a laboratory phenomenon. This may also pertain to shigellosis since there has never been clinical evidence in man to suggest any particular nervous system involvement in disease due to the neurotoxin-producing Shiga bacillus. Central nervous system manifestations occur with equal frequency during infection with all Shigella species (35) and were, in fact, strikingly absent in the recent *S. dysenteriae* 1 epidemic (15). If Shiga enterotoxin is neurotoxin rediscovered, that is, a single exotoxin with two biological activities, it may be justified on physiological grounds to break historical tradition and reclassify it as an enterotoxin.

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