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Shigella dysenteriae 1 Cytotoxin: Periplasmic Protein Releasable by Polymyxin B and Osmotic Shock

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Treatment of *Shigella dysenteriae* 1 either with the antibiotic polymyxin B or by osmotic shock resulted in the release of 80 to 90% of the cytotoxin activity of the organism. Under the conditions employed, the release of toxin activity was accompanied by the appearance of a periplasmic enzyme, 5′-nucleotidase. There was no significant release of cytoplasmic contents, assessed by measurement of glucose-6-phosphate dehydrogenase activity. The release of cytotoxin and 5′-nucleotidase by polymyxin B were both dependent on the duration of incubation with, and the concentration of, the antibiotic. In terms of specific activity (cytotoxin activity per milligram of protein), the polymyxin B and osmotic shock extracts were 20- to 30-fold more active than crude toxin preparation derived from a whole-cell lysate. The data strongly support a periplasmic location for Shiga cytotoxin and the utility of the polymyxin B extraction to obtain starting material for toxin purification.

Since 1903, it has been known that strains of *Shigella dysenteriae* 1 produce a toxin (11). This toxin was originally designated Shiga neurotoxin because it resulted in a delayed-onset limb paralysis terminating in death when administered parenterally to sensitive species of animals. More recently, both tissue culture cytotoxic and enterotoxic activities have been detected in neurotoxin preparations from *S. dysenteriae* 1 (12). Other *Shigella* species have been shown to produce similar toxic activities (13, 20). Biochemical and genetic evidence indicate that the three biological activities are probably the properties of a single molecule (11).

It is apparent that under normal culture conditions, *Shigella* toxin (ShT) is a cell-associated protein (11). Little toxicity is present in cell-free culture supernatants during the exponential phase of growth. Only in the stationary growth phase do significant levels of toxic activity appear in the extracellular medium. McIver et al. (16) found only cell-associated toxin in the first 8 h of fermentor culture of *S. dysenteriae* 1. Thereafter, ShT was present in medium supernatant through four cycles of growth in the fermentor. Presumably, release of toxin during stationary phase is due to bacterial autolysis or to leakage of ShT from its cellular compartment.

Recent purification attempts have obtained crude ShT preparations by concentration of cell-free culture supernatants, by mechanical disruption of intact cells, or by alkaline extraction of heat-killed bacteria (11). All three methods are far from ideal. Although the yield of cytotoxin appears to be greatest from mechanical disruption, the background of contaminating proteins is enormous, and it is possible that the toxin may also be exposed to proteases not normally encountered in its cellular compartment. When compared with the cell lysate, the total yield of toxin from culture supernatants is low, and again the possibility of protease exposure exists. The precise nature of the alkaline extraction (23) is unclear. The actual recovery of ShT by alkaline extraction has not been reported, and one must wonder about the degree of inactivation of this heat- and alkali-sensitive toxin (11) by this procedure.

Griffin et al. (D. E. Griffin, P. Gemski, and B. P. Doctor, Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, B48, p. 21) reported that ShT is released from the intact bacterium by polymyxin B treatment. We report here that polymyxin B does in fact release toxin under conditions that remove periplasmic but not cytoplasmic proteins from the bacterial cell. Furthermore, a second procedure to release periplasmic proteins, osmotic shock, also results in the release of toxin. The release of toxin by both of these methods suggests that the intracellular localization of ShT is the periplasm.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *S. dysenteriae* 1 strain 60R was used for all studies reported. This

strain is a noninvasive rough mutant by Dubos and Geiger (6).

The culture medium employed was a modified syncase broth (8) containing 1% Casamino acids (Difco Laboratories) 0.004% tryptophan, and 0.2% glucose. To maximize the production of toxin, the medium was not supplemented with iron (11).

A 1% overnight bacterial inoculum was added to the medium, and the cultures were grown aerobically with shaking (300 rpm) at 37°C.

Polymyxin B treatment. After 5 h of growth at 37°C (optical density at 600 nm of 1 to 1.6), cells were harvested by centrifugation at 10,000 × *g* for 5 min. The cells were then washed two times by resuspension in 25 mM phosphate buffer (pH 7.3) containing 0.14 M NaCl followed by centrifugation. The final cell pellet was resuspended in the wash buffer to 1/25 of the original culture volume. The cell suspension was incubated at 37°C. Polymyxin B was dissolved in wash buffer at a concentration of 10 mg/ml. Polymyxin B was then added to the prewarmed cell suspension to the desired final concentration. After the indicated incubation time, the cells were removed by immediate centrifugation (12,000 × *g* for 5 min) at 4°C.

Osmotic shock procedure. The osmotic shock procedure of Neu and Chou (19) was employed. Early stationary phase (optical density at 600 nm, 3.5) cells were harvested by centrifugation at 10,000 × *g* for 5 min. The cells were washed two times by suspension in 0.01 M Tris-hydrochloride (pH 7.3)-0.03 M NaCl followed by centrifugation (10,000 × *g* for 5 min). The washed cell pellet was weighed and resuspended in 20% sucrose-0.03 M Tris-hydrochloride (pH 7.3) at 21°C to a ratio of 1 g (wet weight) to 80 ml of sucrose-Tris. After resuspension, EDTA was added to a final concentration of 1 mM, and the suspension was gently mixed for 10 min. The cells were harvested by centrifugation (10,000 × *g* for 5 min) at 4°C and resuspended in ice-cold water at a ratio of 1 g (wet weight) to 40 ml of water. The cell suspension was mixed for 10 min at 4°C, and the cells were removed by centrifugation. The supernatant represents the shock fluid.

Cells lysis procedure. Cells to be lysed were harvested by centrifugation at 10,000 × *g* for 5 min. The cells were washed two times by suspension in 0.01 M Tris-hydrochloride (pH 7.3) buffer followed by centrifugation (10,000 × *g* for 5 min). The washed cells were resuspended in 0.01 M Tris-hydrochloride (pH 7.3) buffer and passed through a French press at 10,000 lb/in^2. If lysis was less than 90% as measured by the optical density at 600 nm, a second passage through a French press was done. Unbroken cells were removed by centrifugation (5,000 × *g* for 15 min).

Enzyme assays. Glucose-6-phosphate dehydrogenase was assayed by the procedure of de Smet et al. (3). For the assay of 5′-nucleotidase, extracts in phosphate buffer were first dialyzed against 0.01 M Tris-hydrochloride (pH 7.4). The assay procedure described by Neu (18) was employed. The phosphate released in the procedure was assayed by the method of Chen et al. (2).

Cytotoxin assay. To assay for cytotoxin activity, a modification of the microassay procedure of Keusch et al. (14) was employed. HeLa cells were grown at 37°C in McCoy modified medium (GIBCO) with 10% fetal calf serum and 100 U of penicillin, 100 μg of streptomycin, and 50 U of nystatin per ml. Confluent monolayers were treated with trypsin (0.25%), and the detached cells were resuspended to a cell concentration of 100,000 cells per ml in McCoy modified medium containing 2 μCi of [^{3}H]thymidine per ml (New England Nuclear Corp.; 50 Ci/mmol). The cell suspension was added to 96-well flat-bottom microtiter wells (0.2 ml/well). After 24 h of incubation at 37°C, near confluent monolayers were formed in each well. The medium was removed, and the monolayer was washed three times with [^{3}H]thymidine-free medium. After the wash, 0.2 ml of new medium or medium containing serial dilutions of ShT was added to each microtiter well. The next day, the medium was removed, and the wells were washed extensively with phosphate-buffered saline. The washed adherent cells were then disrupted by the addition of 0.3 ml of 0.1 M KOH. From each well, 0.2 ml was added to 3 ml of scintillation fluid (Aquasol; New England Nuclear Corp.), and radioactivity was measured in a liquid scintillation spectrometer (Beckman Instruments, Inc.). In this assay, the amount of radioactivity remaining is a direct reflection of the number of cells surviving. The dose of toxin resulting in a 50% decrease in radioactivity as compared with the non-toxin-treated medium controls was calculated by the method of Reed and Muench (21).

Protein determination. Protein determinations were made with a Bio-Rad assay kit II, with bovine serum albumin as a standard. All samples containing polymyxin B were precipitated with 5% trichloroacetic acid before being assayed for protein.

Polyacrylamide gel electrophoresis. Electrophoresis was done in 15-cm slab gels, 1.5 mm thick. The sodium dodecyl sulfate gel system described by Dharmalingam and Goldberg (4) was used.

RESULTS

Polymyxin B treatment. The incubation of cell suspensions from *S. dysenteriae* 1 strain 60R with 2 mg of polymyxin B per ml for various lengths of time at 37°C led to the release of the majority of the cell-associated cytotoxin activity but only about 5% of the total cell protein (Table 1). The increase in specific activity, i.e., the dose of toxin resulting in a 50% decrease in radioactivity per milligram of protein, was between 20- and 30-fold greater than the crude toxin derived from a whole-cell lysate.

TABLE 1. Polymyxin B release of ShT

Duration of polymyxin B treatment[a] (min)	% Total cell protein[b]	% Cytotoxin recovery[b]	Cytotoxicity (TC_{50}/mg of protein)[c]
2	2.5	70	2.5×10^5
5	3.8	>90	2.6×10^5
10	5	>90	1.9×10^5
15	5.4	>90	1.8×10^5

[a] Polymyxin B concentration, 2 mg/ml.

[b] French press lysate, 100%.

[c] TC_{50}, Dose of toxin resulting in a 50% decrease in radioactivity.

TABLE 2. Effect of polymyxin B on release of protein, toxin, and enzymes from *S. dysenteriae* 1

Duration of polymyxin B treatment[a] (min)	Amt released as % of control[b]			
	Protein	Glu-6-P dehyd[c]	5′ Nucleotidase	Cytotoxin
0	0.5	<1	<5	<10
0.5	1.1	<1	51	20
1	2.1	<1	73	25
2	2.6	<1	97	45
4	4	<1	112	82
8	4.6	<1	105	>90
16	7.9	2	96	>90

[a] Concentration of polymyxin B, 2 mg/ml.
[b] French press lysate, 100%.
[c] Glu-6-P dehyd, Glucose-6-phosphate dehydrogenase.

Since under certain conditions polymyxin B treatment of bacterial cells is capable of releasing cytoplasmic contents as well as periplasmic proteins (1), we tested the release of the periplasmic enzyme, 5′-nucleotidase, and a cytoplasmic enzyme, glucose-6-phosphate dehydrogenase. The release of nucleotidase closely followed the release of cytotoxin activity (Table 2). Four minutes of polymyxin B (2 mg/ml) treatment yielded the maximal amount of both toxin and 5′-nucleotidase, with less than 1% of the glucose-6-phosphate dehydrogenase. The yield of 5′-nucleotidase activity at 4 and 8 min of polymyxin B treatment was greater than the total activity in the French press lysate and may reflect the absence of cytoplasmic 5′-nucleotidase inhibitor, which is present in both the whole-cell lysate and supernatants from cells treated with polymyxin B for longer than 4 min (17).

The release of both cholera (15) and *Escherichia coli* LT (7) toxins by polymyxin B has been shown to occur at an antibiotic concentration of 2 mg/ml. However, it has been shown by Cerny and Teuber (1) that treatment of *E. coli* cells with as little as 100 μg of polymyxin B per ml is sufficient to release periplasmic enzymes. The polymyxin dependence of protein, enzyme, and toxin release is shown in Table 3. Virtually all nucleotidase and cytotoxin activity was obtained with concentrations of 2 to 0.125 mg/ml. At concentrations of 0.063 mg/ml and below, there was a sharp decrease in the amount of toxin released and a concomitant decrease in the level of nucleotidase activity.

Osmotic shock release of toxin. A second method to preferentially release proteins in the periplasm is osmotic shock. Neu and Chou (19) have shown that osmotic shock treatment of *Shigella* species releases several periplasmic proteins, including 5′-nucleotidase. Treatment of *S. dysenteriae* 60R in stationary growth phase resulted in the release of a small percentage of the total protein but the majority of 5′-nucleotidase and cytotoxin activities. The amount of glucose-6-phosphate dehydrogenase released was 0.3 (expressed as percentage of activity in the French press lysate), the amount of 5′-nucleotidase was 107, the amount of cytotoxin was 70, and the amount of protein was 3.9.

Polyacrylamide gel electrophoresis. Figure 1 shows a comparative analysis by polyacrylamide gel electrophoresis of proteins derived from strain 60R by French press lysis, polymyxin B extraction, and an osmotic shock treatment.

TABLE 3. Dose response of polymyxin B extraction of *S. dysenteriae* 1

Polymyxin B[a] (mg/ml)	Amt released as % of control[b]			
	Protein	5′-Nucleotidase	Glu-6-P dehyd[c]	Cytotoxin
2	5.3	96	<1	75
1	5.6	118	<1	90
0.5	5.5	117	<1	90
0.25	5	73	<1	86
0.125	3.6	80	<1	82
0.063	0.4	33	<1	42
0.032	0.3	8	<1	<10
0	<0.1	<5	<1	<10

[a] Incubation for polymyxin B treatment, 4 min at 37°C.
[b] French press lysate, 100%.
[c] Glu-6-P dehyd, Glucose-6-phosphate dehydrogenase.

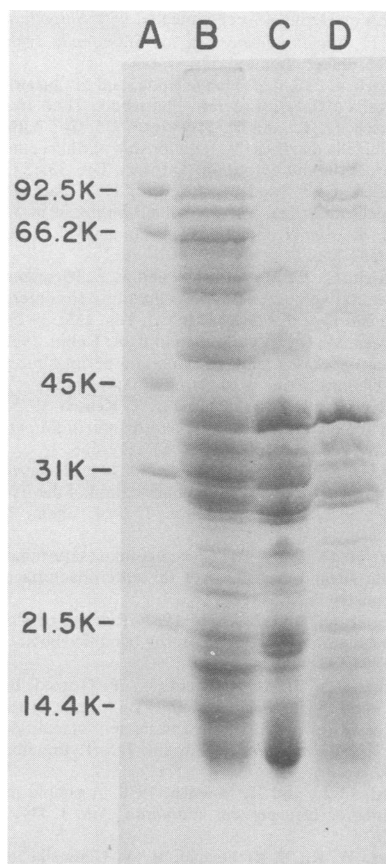

FIG. 1. Comparison of protein patterns by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Lane A, molecular weight standards: phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme, 92,500, 66,200, 45,000, 31,000, 21,500, and 14,400, respectively; lane B, proteins obtained from a whole-cell lysate; lane C, proteins released by polymyxin B treatment; lane D, proteins released by osmotic shock.

It is clear that the protein profiles of both the osmotic shock and polymyxin B extracts markedly differ from those of whole-cell lysate. It is also clear that, although similar in protein content, there are several proteins present in the osmotic shock preparation that are not in the polymyxin B extract and vice versa.

DISCUSSION

The envelope of gram-negative bacteria consists of three distinct compartments: the inner or cytoplasmic membrane, the outer membrane, and the periplasm, the space between the outer and the inner membranes. Within the periplasmic space is the peptidoglycan layer, containing a variety of proteins that may be classified into one of two general categories: binding proteins involved in the active transport of metabolites and degradative enzymes. The present studies demonstrate that conditions resulting in release of periplasmic proteins, i.e., polymyxin B treatment or osmotic shock, also release ShT activity. Although these findings do not completely eliminate the possibility that the toxin is an outer membrane constituent, the fact that two quite distinct methods to release periplasmic constituents also result in toxin release and that the polymyxin B release of toxin exhibits the same incubation time and concentration dependence as the release of the periplasmic enzyme 5'-nucleotidase favor an actual periplasmic location for ShT. It must be remembered that these data indicate that localization of intact biologically active holotoxin only, and not isolated A or B subunits or any possible inactive toxin precursors.

The disease shigellosis involves two anatomically distant regions of the intestine, the proximal small bowel and the colon, resulting in two distinct intestinal disease syndromes, watery diarrhea and dysentery, respectively (11). A working model for the role of toxin in pathogenesis is that both disease manifestations are a direct result of toxin action. The mechanism underlying the secretion of water and electrolytes into the lumen, resulting in the diarrhea syndrome, is unknown but may involve the binding of extracellular toxin to intestinal epithelial cells. The dysentery syndrome is clearly initiated by invasion of colonic epithelial cells by shigellae (9, 22). Colonic epithelium appears to be insensitive to exogenous toxin (5). However, if the events following bacterial invasion of HeLa cells or Henle 407 cells in vitro (10) are a model for events in the colonic cell, then intracellular production of toxin resulting in prompt inhibition of cellular protein synthesis could in turn lead to epithelial cell death and initiate the inflammatory colitis that follows invasion and multiplication of the organism. Such a model predicts that the toxin is or can easily become an extracellular protein. The finding that the majority of the cell-associated cytotoxin is situated in a region external to the cytoplasmic membrane, the periplasm, is consistent with this hypothesis. In the periplasm, ShT could be released from the organism without cell disruption, either by leakage through the outer membrane or perhaps by outer membrane bleb formation, as has been suggested for the delivery of *E. coli* LT toxin (24).

Toxin released by polymyxin B or osmotic shock has two major advantages as a starting source for further purification. First, the released cytotoxin represents an initial 20- to 30-fold purification in specific activity (Table 1)

over the activity in a cell lysate. Second, the released material is in a protein environment similar to its environment within the bacterium. This may minimize the possibility of alteration by either membrane or cytoplasmic-associated proteases, thereby improving both the yield and homogeneity of active toxin.

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