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Biological Properties of *Shigella flexneri* 2A Toxin and Its Serological Relationship to *Shigella dysenteriae* 1 Toxin

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A toxin extracted from heat-inactivated, alkaline-treated *Shigella flexneri* 2a showed biological properties similar to those of *Shigella dysenteriae* 1 toxin. The *S. flexneri* 2a toxin was lethal to mice, enterotoxic for ileal loops of rabbits, and cytotoxic for HeLa cells. A serological relationship between *S. flexneri* 2a and *S. dysenteriae* 1 toxin was shown with cross neutralization tests.

For over 70 years, it has been known that *Shigella dysenteriae* 1 (Shiga's bacillus) elaborates a neurotoxin, distinct from endotoxin (2), that causes peripheral paralysis and death when injected into mice and rabbits (10). Subsequent studies by Vicari et al. (14) demonstrated that *S. dysenteriae* 1 cell extracts are cytotoxic to various mammalian cell lines. More recently, Keusch et al. (7) have shown that sterile culture filtrates of *S. dysenteriae* 1 possess enterotoxin activity. Thompson et al. (12) have obtained a highly purified preparation of Shiga toxin from cell extracts possessing the three toxin activities and having an inhibitory effect on in vitro mammalian cell-free protein synthesis.

Although previous efforts failed to detect production of a similar toxin in species other than *S. dysenteriae* 1 (1), Keusch and Jacewicz (8) suggested that *Shigella flexneri* and *Shigella sonnei* may pro¹uce a toxin substance that is antigenically related to Shiga toxin. They observed that sera of patients with recent *S. flexneri* and *S. sonnei* infections could neutralize the cytotoxic activity of Shiga toxin (8). Recently, we established that *S. flexneri* 2a does indeed produce a Shiga-like toxin, although at very low levels as compared to those for Shiga toxin (M. R. Thompson, S. Formal, and P. Gemski, Fed. Proc. 35:1394, 1976). The purpose of this communication is to assess the biological activities of this toxin from *S. flexneri* 2a and to determine its serological relationship with *S. dysenteriae* 1 toxin.

MATERIALS AND METHODS

Bacterial strains. Studies were performed with *S. dysenteriae* 1 strain 60R and *S. flexneri* 2a strain M4243. Shiga strain 60R is a rough mutant originally described by Dubos and Geiger (3). Strain M4243 is a virulent strain used frequently in our previous studies (4).

Cultural conditions and purification. Cells were grown in modified syncase broth (1% Difco certified Casamino Acids, 2% glucose, 0.004% tryptophan, and 0.004% nicotinic acid) for 72 h at 37°C with aeration. Harvested heat-inactivated cells were alkaline extracted according to the method of van Heyningen (13). Neutralized alkaline extracts of strain 60R, designated crude Shiga toxin, were stored at 4°C. Strain M4243 extracts were subjected to further purification by 28 to 70% ammonium sulfate precipitation, followed by preparative chromatography on diethylaminoethyl-Sephadex A-50 to remove nucleic acids and, finally, gel filtration on Bio-Gel A-.5M. Fractions with cytotoxic activity in HeLa cells were pooled and concentrated and stored as aliquots in 0.05 M tris(hydroxymethyl)amino-methane-hydrochloride buffer, pH 7.8 at −70°C. This material was designated partially purified *S. flexneri* toxin. Crude Shiga toxin retained cytotoxicity with prolonged storage at 4°C, but partially purified *S. flexneri* toxin was stored at −70°C to maintain biological activity. Comparative studies using analytical gel electrophoresis indicated that the electrophoretic mobilities of partially purified *S. flexneri* toxin and pure *S. dysenteriae* toxin were similar (Thompson et al., Fed. Proc. 35:1394, 1976).

Tests for cytotoxicity, mouse lethality, and enterotoxicity. Cytotoxicity was assayed in HeLa cell monolayers according to the method of Vicari et al. (14). Toxin titers were taken as the highest dilution causing cytotoxic effects in cell monolayers. The rabbit ileal loop model was used to assess enterotoxicity (5). Mouse lethality was assayed by intraperitoneal injection of toxin diluted in normal saline into 15- to 20-g BALB/c mice (Jackson Laboratories). Deaths between days 2 and 7 inclusive were tabulated, and 50% lethal doses were calculated by using the method of Reed and Muench (11). Prior to death, mice injected with crude Shiga toxin usually exhibited hind-leg paralysis. However, no paralysis was observed in mice receiving lethal doses of partially purified *S. flexneri* toxin.

Preparation of antiserum. Graded doses of partially purified *S. flexneri* toxin in Freund incomplete

adjuvant were administered into footpads of rabbits at weekly intervals. Anti- S. dysenteriae 1 toxin was obtained after weekly subcutaneous or intravenous inoculation of crude Shiga toxin into rabbits.

Neutralization studies. The ability of rabbit antisera prepared against S. flexneri and Shiga toxins to neutralize cytotoxicity was tested. Twofold serial dilutions of serum were incubated at 37°C for 1 h in the presence of four times the minimal concentration of toxin required to produce cytotoxic effect in HeLa cells. All dilutions were performed in Earl balanced salt solution (Difco). After an additional overnight incubation at 4°C, 0.1 ml of the serum toxin mixture was assayed in HeLa cells.

RESULTS

Toxin production by S. dysenteriae 1 has been detected in a variety of preparations, including alkaline extracts of cells, sterile broth filtrates, and sterile filtrates of cells disrupted by sonication. In contrast, similar preparations of S. flexneri with equivalent protein concentrations were found to be consistently negative for toxin activity. Cell extracts of S. flexneri were found to have all three toxin activities only after partial purification (removal of nucleic acids and gel filtration).

Biological activity of toxins. S. flexneri toxin is lethal for mice, cytotoxic, and enterotoxic, as has been observed for toxin preparations from S. dysenteriae 1 (Table 1).

Neutralization studies. Table 2 demonstrates the capacity of rabbit anti-S. dysenteriae 1 and anti-S. flexneri toxin to neutralize cytotoxin action of the heterologous as well as the homologous toxin.

DISCUSSION

Our studies (Table 1) have revealed that S. flexneri 2a toxin has lethal, cytotoxic, and enterotoxic properties similar to those previously observed with S. dysenteriae 1 toxin. Although the molecular mechanism of toxin action has not yet been elucidated with respect to these three biological activities, previous studies of the Shiga toxin purified to near homogeneity have shown that it efficiently inhibits protein synthesis in a mammalian cell-free system (12).

As a consequence, studies have recently been initiated to determine whether the S. flexneri toxin likewise inhibits protein biosynthesis. In addition to these biological similarities, there is evidence of some structural and immunological relatedness between the S. flexneri and S. dysenteriae 1 toxins. Antiserum prepared against either toxin efficiently neutralizes the cytotoxin activity of both homologous and heterologous extracts (Table 2). Moreover, electrophoresis studies have demonstrated that S. flexneri cytotoxin has the same electrophoretic mobility and isoelectric point as highly purified Shiga toxin (Thompson, unpublished observations).

On the basis of our findings, it is apparent that S. flexneri has a structural gene(s) for production of a Shiga-like toxin. As revealed in Table 2, however, the specific toxin activity of crude Shiga extracts is significantly greater than that of partially purified toxin, a result that is in agreement with the previous conclusion that S. flexneri extracts contain at least 10^3-fold less toxin activity than similar Shiga extracts (Thompson et al., Fed. Proc. 35:1394, 1976). Several possibilites could account for this. It is conceivable that the regulatory mechanisms controlling the synthesis of toxin protein in S. flexneri differ from those of S. dysenteriae and that toxin production is repressed in species other than S. dysenteriae. Second, it is possible that S. flexneri produces a protoxin,

TABLE 2. Neutralization of Shigella cytotoxins by rabbit antisera

Antitoxin	Toxin dose (μg)[a]	Median serum dilution neutralizing cytotoxic activity[b]
S. flexneri 2a	S. flexneri 2a, 144	1:320
S. flexneri 2a	S. dysenteriae 1, 1.6	1:160
S. dysenteriae 1	S. dysenteriae 1, 1.6	1:1,280
S. dysenteriae 1	S. flexneri 2a, 144	1:640

[a] Dose expressed as protein determined by the method of Lowry et al. (9).
[b] Determinations performed in triplicate.

TABLE 1. Biological properties of S. dysenteriae 1 and S. flexneri 2a toxins

Toxin	Cytotoxicity in HeLa cells (μg)[a]	Enterotoxicity in rabbits (μg)	Lethality (LD$_{50}$, μg) in mice[b]
Crude S. dysenteriae 1	0.4[c]	+ (54)[d]	23
Partially purified S. flexneri 2a	36	+ 725	325

[a] Lowest concentration causing cytotoxic effect in HeLa cells.
[b] Three to five animals per dose. LD$_{50}$, 50% lethal dose.
[c] Dose expressed as protein determined by the method of Lowry et al. (9).
[d] Single dose tested.

which must be activated in order to express its toxic activity. Third, the low levels of toxin activity in *S. flexneri* could reflect the presence of some inhibitory substance that dramatically alters activity. Whatever the reason may be for the low toxin activity of *S. flexneri*, it is now evident that mutants of *S. dysenteriae* 1, which mimic *S. flexneri* in their low toxin levels, have been isolated (6). *S. dysenteriae* 1 mutant strain 725-78, previously considered to be non-toxigenic, has now been shown to produce very low levels of cytotoxin (Keusch and Thompson, unpublished data).

With the recognition that *S. flexneri* and *S. dysenteriae* 1 produce related toxins, it is reasonable to assume that both toxins play a similar role in shigellosis. Although it is not yet clear what this function is, it must be stressed that toxin production alone is not sufficient to confer virulence on shigellae. The ability of the organism to penetrate intestinal epithelia still remains a primary determinant of virulence.

ACKNOWLEDGMENTS

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