INFECTION AND IMMUNITY, Apr. 1983, p. 425–428
0019-9567/83/040425-04$02.00/0

Vol. 40, No. 1

Release of Shiga Toxin from *Shigella dysenteriae* 1 by Polymyxin B

DARRELL E. GRIFFIN AND P. GEMSKI*

Department of Biological Chemistry, Walter Reed Army Institute of Research, Washington, D.C. 20012

Received 9 December 1982/Accepted 29 December 1982

Release of Shiga toxin from *Shigella dysenteriae* 1 was found to occur after exposure to polymyxin B. The amount of toxin released was dependent on both the polymyxin concentration and time of incubation. An immunoblot characterization of the Shiga toxin released by polymyxin treatment demonstrated that it is electrophoretically similar to purified Shiga toxin and to Shiga toxin present in crude bacterial sonicates of *S. dysenteriae* 1 cells.

Shigella dysenteriae 1 produces a protein toxin which causes paralysis and death in mice (15), is cytotoxic to HeLa and other cultured cell lines (19), possesses enterotoxic activity as shown by fluid secretion in the ligated rabbit ileal loop (9), and inhibits protein synthesis in intact HeLa cells (2) and in cell-free extracts of mammalian and bacterial cells (3, 14, 17). Whereas the role of this toxin in the pathogenesis of shigellosis remains unknown (7), the discovery that an immunologically related toxin is produced in various amounts by *Shigella flexneri* 2a (13) and *Shigella sonnei* (10) suggests that this toxin may be important in the disease process.

Shiga toxin can be readily detected in cell-free alkaline extracts of *S. dysenteriae* (18) or in sterile filtrates of this organism after disruption of cells by sonication or a French press (1). Shiga toxin purification procedures have varied in their approaches and have included use of acid-treated chitin columns (16), antitoxin affinity chromatography (12), and a combination of ion-exchange chromatography, gel filtration chromatography, and preparative isoelectric focusing (1). We have sought to release Shiga toxin into supernatant fluids by other methods which may be useful alternatives to existing approaches. The use of the membrane-active antibiotic polymyxin B to facilitate release of enzymes and other proteins from *Escherichia coli* has been previously reported (4, 6). As a consequence, we have examined whether polymyxin B treatment of *S. dysenteriae* 1 results in the release of Shiga toxin from cells.

To determine whether polymyxin would cause the release of Shiga toxin, *S. dysenteriae* 1 strain 3818-0 (7) was grown in modified Syncase broth (1% Casamino Acids [certified grade; Difco Laboratories, Detroit, Mich.], 0.004% tryptophan, and 0.004% nicotinic acid) (13) at 37°C with aeration. The cells were then treated with polymyxin B sulfate (Calbiochem, La Jolla, Calif.) by the procedure of Cerny and Teuber (4). After incubation for various times with polymyxin at 37°C, the bacterial cultures were immediately chilled in an ice bath for subsequent analysis of toxin levels. Samples were assayed for cytotoxicity in HeLa cell monolayers according to the method of Gentry and Dalrymple (8) as modified by Brown et al. (1). The endpoint was defined as the dilution of toxin causing 50% cell detachment (CD_{50}) and was determined from a standard graph of the logarithm of the dye absorbance versus the logarithm of the toxin dilution.

Our initial studies with several *S. dysenteriae* 1 strains revealed that after incubation of bacterial cells overnight in the presence of polymyxin B, a substantial increase (>100-fold) in Shiga toxin in culture filtrates was detected. It had been established previously that both time of incubation and concentration of antibiotic were important determinants of the amount of protein released from bacteria by means of polymyxin B treatment (4). We therefore examined the effect of these variables on the release of Shiga toxin from *S. dysenteriae* 1 strain 3818-0 by polymyxin B. *S. dysenteriae* 1 strain 3818-0 was grown in Syncase broth overnight at 37°C. A fraction of the suspension was diluted 1:10 into prewarmed Syncase broth and allowed to incubate at 37°C for 3 h until the culture was in the logarithmic growth phase. Cells were harvested by centrifugation (15,000 × *g* for 15 min; yield, 1.0 g [wet weight]), washed twice, and suspended in 0.14 M NaCl. The suspended cells were divided and incubated for 60 min with polymyxin B at concentrations of 0.001, 0.010, 0.10, 1.0, 10, and 100 mg/ml. These cell suspensions were then centrifuged (15,000 × *g* for 20 min), filter sterilized (0.45-μm filter; Millipore Corp., Bedford, Mass.), and assayed for cytotoxicity to HeLa cells. Figure 1 shows the amount of cytotoxin

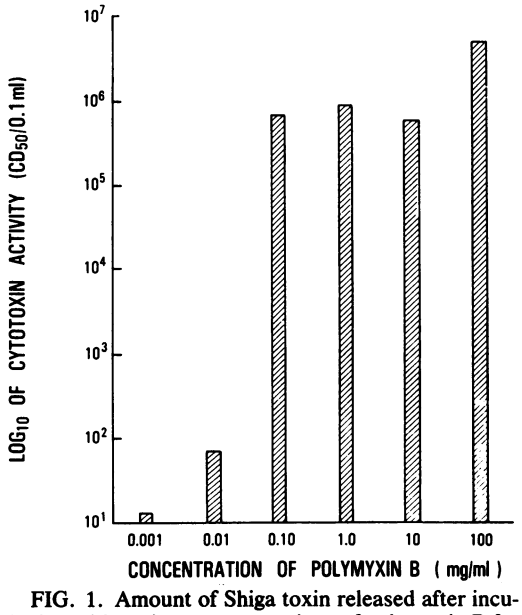

FIG. 1. Amount of Shiga toxin released after incubation with various concentrations of polymyxin B for 60 min as determined by measuring cytotoxic activity (see text). Control level was about 10 CD_{50}/0.1 ml.

activity in cell-free culture filtrates after treatment with various levels of polymyxin B. At concentrations of polymyxin B in the range of 0.10 to 100 mg/ml, amounts of cytotoxin activity released were similar. Toxin titers of about 10^6 CD_{50}/0.1 ml were routinely observed. Treatment of strain 3818-0 with polymyxin B levels of 0.001 and 0.010 mg/ml caused less cytotoxin to be released than was observed with levels of 0.10 mg/ml and above. At the lowest concentration studied (0.001 mg/ml), only minimal amounts of toxin were released, yielding titers of 10 CD_{50}/0.1 ml or less. Similar low levels were also detected in control experiments in which cells were suspended for up to 60 min in 0.14 M NaCl.

The effect of incubation time on polymyxin-induced toxin release from *S. dysenteriae* 3818-0 was also investigated. Cells (10 g [wet weight]) were prepared as described above, suspended in 100 ml of 0.14 M NaCl, and exposed to polymyxin B (final concentration, 1.0 mg/ml). Samples were removed after incubation at 37°C for 1, 2, 5, 10, 20, 30, and 60 min and chilled in ice. Each was then centrifuged, filtered, and assayed for cytotoxin activity (Fig. 2). Within 10 min of incubation in the presence of the antibiotic, cytotoxin levels in the filtrates had increased and plateaued at a level about 1,000-fold that at time 0. The relative amount of toxin released after 60 min of treatment with the antibiotic approached 90% of the total cellular toxin. Total cell-bound toxin was determined by assaying sonicates of untreated 3818-0 cells.

This increase in the toxin level most likely reflects lysis of strain 3818-0 cells caused by polymyxin B. Evidence for such lysis is provided in Fig. 3, with decrease in turbidity serving as the indication of lysis. A log-phase suspension (100 ml) of strain 3818-0 was prepared in 0.14 M NaCl (absorbance at 540 nm, approximately 0.800) and divided into two equal portions. Polymyxin B was added to one portion at a final concentration of 0.05 mg/ml; the other portion served as an untreated control. Samples were periodically removed during a 60-min incubation at 37°C, and turbidity of the cell suspension was determined by monitoring absorbance at 540 nm. Cell lysis was evident shortly after exposure to the antibiotic.

To assess the immunological nature of the material released by the antibiotic, samples from cells treated with polymyxin (2 mg/ml) for 60 min, crude bacterial sonicate, and purified Shiga toxin (1) were subjected to nondenaturing gel electrophoresis with 8% polyacrylamide gel slabs (5). These were analyzed for the presence of toxin by an immunoblot procedure (1) and for protein by staining with Coomassie blue R-250. After electrophoresis, the gel was divided into sections, each of which contained samples of the three preparations. To demonstrate the presence of toxin, the separated proteins in the gel were electrophoretically transferred onto nitrocellulose (BA 85; 0.45 μm; Schleicher & Schuell, Keene, N.H.) by using a gel destainer (E-C Apparatus Corp., St. Petersburg, Fla.) with a palladium anode at a constant voltage of 16 V. Each nitrocellulose sheet was incubated first

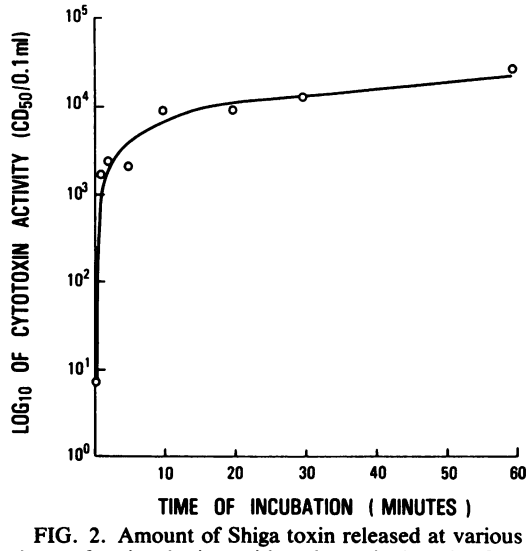

FIG. 2. Amount of Shiga toxin released at various times after incubation with polymyxin B. The final polymyxin B concentration was 1 mg/ml. Each data point is the average of two values.

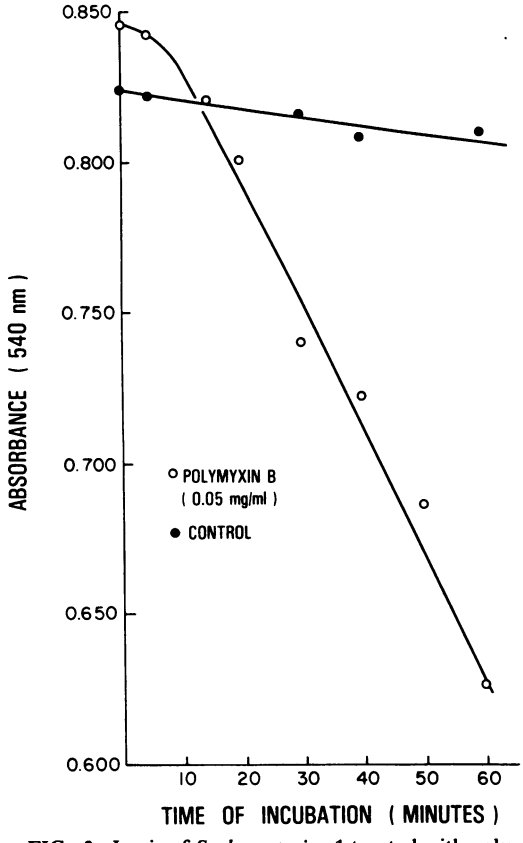

FIG. 3. Lysis of *S. dysenteriae* 1 treated with polymyxin B.

with rabbit antibody to Shiga toxin (1) or serum from rabbits which had not yet been immunized. Then incubation with ^{125}I-labeled protein A was performed. X-ray film (Kodak X-Omat AR film; Eastman Kodak Co., Rochester, N.Y.) was exposed to the nitrocellulose sheets at −70°C with an intensifying screen (Cronex Lightning Plus; Dupont, Wilmington, Del.). Figure 4A shows the autoradiographic pattern for serum from rabbits before immunization (lanes 1 to 3) and for hyperimmune antiserum (lanes 4 to 6). As we have previously observed, serum collected from rabbits before immunization contains antibodies to several *Shigella* proteins (1). A number of bands were detected in the lanes containing bacterial sonicate (Fig. 4A, lanes 1 and 4) and the polymyxin-treated cell extract (Fig. 4A, lanes 2 and 5). Except for four of these bands, all bands were also present in the autoradiograph of the samples when they were incubated with preimmune serum (Fig. 4A, lanes 1 and 2). The intense toxin band is readily detected at R_f = 0.26. Several closely migrating bands corresponding to the purified toxin were detected with the anti-Shiga toxin (Fig. 4A, lane 5). These bands represent the native toxin and proteolytically nicked toxin. A section of the gel was stained with Coomassie blue (Fig. 4B). The lanes with bacterial sonicate and the polymyxin-treated cells contained many protein species. The purified toxin could not be detected by staining, because the quantity of protein applied to the lane was below the level of detection (11). The pattern of bands in the section incubated with the immune serum indicated that Shiga toxin released by polymyxin treatment is similar to that released by sonication and to purified toxin.

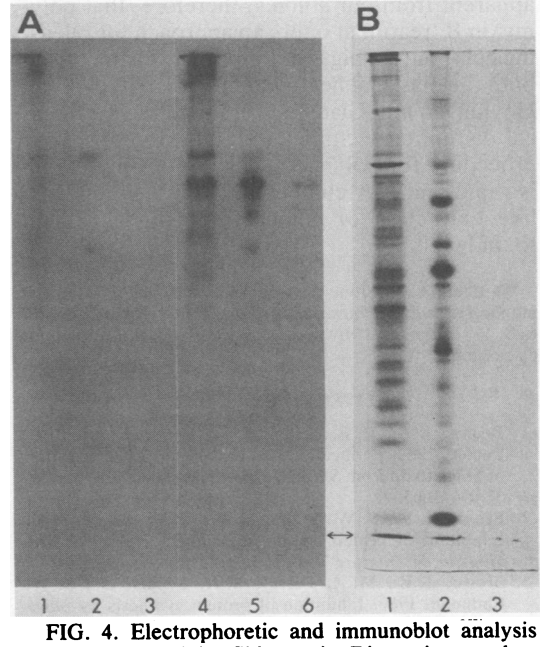

FIG. 4. Electrophoretic and immunoblot analysis of samples containing Shiga toxin. Discontinuous electrophoresis was performed in 8% polyacrylamide gel slabs until the dye front was within 1 cm of the bottom of the gel. After polyacrylamide gel electrophoresis, the gel was divided into three sections. Electrophoretic transfer of two of the three sections onto nitrocellulose was carried out for 11.5 h. The remaining section was not subjected to electrophoretic transfer. (A) Autoradiographs comparing preimmune and immune antiserum produced against Shiga toxoid. The nitrocellulose was divided into two sections and incubated with preimmune serum (lanes 1 to 3) or hyperimmune antiserum (lanes 4 to 6) before the addition of ^{125}I-labeled protein A. The autoradiograph was exposed for 17 h. The dye front of the gel is shown by the arrow. Lanes 1 and 4 each contained 15 μg of bacterial sonicate. Lanes 2 and 5 each contained 20 μg of the supernatant from cells treated with polymyxin for 60 min. Lanes 3 and 6 each contained 0.25 μg of purified toxin. (B) Coomassie blue-stained section of untransferred portion of the gel. The dye front was marked by injection of insoluble dye and is shown by the arrow. The samples are described above.

In this study, we have shown that polymyxin B causes the release of Shiga toxin, most likely by lysis of cells. As has been observed with other bacterial proteins (4), the polymyxin B-induced release of cytotoxin is dependent on both antibiotic concentration and the time of exposure to this agent (Fig. 1 and 2). At the highest polymyxin concentration, most of the cytotoxin present in the cells was released in as short a time as 10 min. By means of immunoblotting techniques, it was possible to demonstrate that polymyxin treatment results in the release of toxin protein with gross molecular properties identical to those of purified toxin and those of toxin present in crude whole cell sonicates. It is apparent from our studies, therefore, that polymyxin B treatment offers an approach for releasing cell-bound Shiga toxin which is distinct from both the classical heat-alkaline procedure of van Heyningen and Gladstone (18) and the mechanical disruption techniques (1, 9). Moreover, our procedure for polymyxin release of Shiga toxin is rapid and less cumbersome than either alkaline extraction or sonication and yields high toxin levels.

We thank A. B. Stone for technical assistance in maintaining the HeLa cell cultures used in this study. We are grateful to S. W. Rothman, J. E. Brown, and B. P. Doctor for helpful discussions.

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