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Column Chromatography and Cell Culture Assay of *Pseudomonas aeruginosa* Toxin Z Preparations

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Toxic material produced by *Pseudomonas aeruginosa* in cell culture was concentrated and partially purified. This toxic material, designated toxin Z, was produced during the growth of strain PA Z or PA 103 in HEp-2 monolayer cultures using Eagle minimal essential medium with 10% serum. Toxin Z, concentrated fourfold by Lyphogel or ultrafiltration, was used to produce antiserum in rabbits and also was fractionated by column chromatography. Twentyfold purification of toxin Z was obtained on a Sephadex G-200 column. Toxic column fractions were confirmed to have toxin Z by neutralization with specific antiserum. During concentration, purification, and neutralization procedures, the toxin was assayed exclusively by the cytopathic effect it produced in cell culture.

Pseudomonas aeruginosa is one of the leading causes of nosocomial infections, especially in burn patients (1). The pathogenicity of *P. aeruginosa* is attributed largely to various toxins it produces (5). Liu et al. (6) described a heat-labile exotoxin, designated exotoxin A, produced by a nonproteolytic strain of *P. aeruginosa*, PA 103, in dialysate from Trypticase soy broth. Exotoxin A produced a cytopathic effect (CPE) in cell culture (10). Meinke and Berk (8) described a heat-stable toxic fraction produced by *P. aeruginosa* strain E 2 in a tryptone-glucose medium. In our laboratory we previously described toxin Z (2), a toxic material produced by *P. aeruginosa* in cell culture, and its possible role in the formation of characteristic virus-like plaques.

In the present study toxin Z was concentrated and partially purified by column chromatography while using cell culture methods exclusively for its assay.

MATERIALS AND METHODS

Cell cultures. The established cell line used in this study was HEp-2 maintained in Eagle minimal essential medium (MEM) routinely supplemented with 10% calf serum and with 100 U of penicillin and 0.1 mg of streptomycin per ml; the term "supplemented MEM" hereafter refers to this complete medium. The stock HEp-2 culture was carried in an 8-oz (240 ml) bottle and routinely subcultured once or twice weekly, using 0.25% trypsin in calcium- and magnesium-free, phosphate-buffered saline at 37 C for 1 min to release the cells from the glass. The used medium obtained from the HEp-2 bottle cultures at the time of subculturing was collected and saved to provide nontoxic controls for toxin assays; this medium is hereafter designated as "used medium." Disposable plastic culture tubes were inoculated with 0.5 ml each of 5×10^4 HEp-2 cells suspended in supplemented MEM to provide tube cultures for toxin assays. The cell cultures were incubated at 37 C for 24 to 48 h prior to use for toxin assays.

Toxin production. Two strains of *P. aeruginosa* were used to produce toxin Z. One strain, a proteolytic strain designated PA Z, was previously isolated in this laboratory as a pure contaminant of HeLa S_3 and was traced to a technician whose infant child developed summer diarrhea (7). The other strain, PA 103 (4), was a nonproteolytic strain provided by P. V. Liu. Eight- to 32-oz (960 ml) prescription bottles, containing either used medium or else HEp-2 cells grown in supplemented MEM, were each inoculated with less than 1,000 cells of strain PA Z or PA 103, incubated at 37 C for periods ranging from 1 day to 1 week, and then placed in a freezer (0 C) for later harvesting. Strain PA Z was grown in the presence of HEp-2 until plaques, believed to be caused by toxin Z, appeared in the monolayer. Strain PA 103 was grown with or without HEp-2 until the cultures became turbid.

Toxin harvest. The frozen crude toxin was thawed in a 37 C water bath and then centrifuged at $12,100 \times g$ in a Sorvall superspeed RC2-B automatic refrigerated centrifuge with a type SS-34 head at 4 C for 10 min. The toxin-containing supernatant fluid was decanted, filtered twice through a 0.2-μm membrane, heated in a 70 C water bath for 1 h to inactivate any protease or exotoxin A that might be present, and stored at 4 C until used; the term "crude toxin Z" hereafter refers to this harvested toxin. Crude toxin Z was routinely assayed for protease activity by adding 0.5 ml of the toxin to 0.5 ml of 2%

skim milk; any toxin that showed protease activity, indicated by clearing of the milk, was discarded.

Toxin concentration. Routinely, a hollow fiber ultrafiltration device, a Bio-Fiber 80 unit (Bio-Rad), having a molecular weight cutoff of 30,000 was used to concentrate the crude toxin Z fourfold. Alternately, Lyphogel (Gelman) was allowed to swell in the crude toxin overnight at room temperature to obtain a fourfold concentration.

During the use of the Bio-Fiber ultrafiltration unit, the enzyme presoak (Biz), used to unplug the fibers, and formalin, used to store the unit, were found to cause nonspecific CPE if not thoroughly removed from the fibers and beaker by flushing with several liters of distilled water. These toxic agents were later avoided by relying on backflow with distilled water to keep the fibers unplugged and by storing the unit without formalin at 4 C. The unit was rinsed out with distilled water twice a month when not in use.

Column chromatography. Fractionation of toxin Z by column chromatography was accomplished with a column of Sephadex G-200 (40 by 2 cm) mounted on a fraction collector from the AO Instrument Company equipped with a drop counting unit. A sample of 2.5 ml of concentrated toxin was applied to the column, eluted with 0.01 M tris(hydroxymethyl)aminomethane buffer, pH 8.0, and collected in 2-ml fractions.

Toxicity assays. Crude toxin Z, concentrated toxin, and fractionated toxin were tested for toxicity on HEp-2 tube monolayers. The medium was removed from the tube cultures, and approximately 1 ml of toxin sample was added aseptically to each tube by filtration through a 0.2-μm membrane contained in a Swinex unit (Millipore). Cell destruction was indicated by rounding or flattening of cells regardless of whether or not the cells were released from the plastic surface of the tube; CPE was measured as 0, 1, 2, 3, and 4+, meaning 0, 25, 50, 75, and 100% monolayer destruction, respectively. Serial twofold dilutions of crude toxin and concentrated toxin were tested in HEp-2 tube cultures to obtain a toxicity titer; the reciprocal of the highest dilution of toxin showing 2+ CPE in 48 h was taken as the titer. Used medium provided nontoxic controls for toxicity assays; it was similar in composition to crude toxin Z except, having never been inoculated with *P. aeruginosa*, it lacked toxin Z and other bacterial products. Used medium undiluted or diluted did not cause any CPE in HEp-2 cultures in 48 h.

Neutralization tests. Antiserum against toxin Z was obtained from a rabbit given multiple injections of Lyphogel-concentrated toxin Z prepared by growing strain PA 103 in HEp-2 monolayers in the presence of rabbit serum instead of calf serum. One-half milliliter of this antiserum, diluted one-eighth with supplemented MEM, was added to 0.5 ml of toxin and was incubated at 37 C for 1 h before testing on HEp-2. Two controls were treated identically except one had normal rabbit serum and the other had plain supplemented MEM instead of antiserum.

Protein assays. The protein content of samples was measured by a modification of the Lowry method (9). Samples were read against a reagent blank in a Coleman model 6/20A junior 11A spectrophotometer at 660 nm. A standard solution of 100 μg of bovine serum albumin per ml of distilled water was routinely used as a quality control.

Mycoplasma assay. The established cell line HEp-2 used in this study was found free of *Mycoplasma* contamination using standard cultivation procedures.

RESULTS

Characteristics of cytotoxicity induced by toxin Z. *P. aeruginosa* strain PA 103 when inoculated with HEp-2 monolayer cultures produced the characteristic virus-like plaques seen previously with PA Z strain (2, 7). Plaque formation was used in the present study as in previous work to indicate the formation of toxin Z. The presence of serum in the culture medium as previously demonstrated for PA Z (2, 7) was necessary for the production of plaques; in the absence of serum a generalized cytotoxicity occurred before characteristic plaques appeared. Harvested cell-free crude toxin Z formed by PA 103 produced a generalized cytotoxicity of HEp-2 monolayers that was identical to that previously reported for PA Z (2). Characteristically, the CPE began with shrivelling of the cells at the periphery of the monolayer followed by progressive destruction of the entire cell sheet.

A new finding in the present study was the occasional observation that, when toxin Z from both PA strains was applied to HEp-2, random patches of round cells appeared throughout the monolayer. In time these patches of round cells formed plaques which were identical to those produced in HEp-2 monolayers inoculated with the organism itself. This is further evidence to support the suggestion that *P. aeruginosa* plaques in cell culture monolayers are caused by toxin Z being produced by the organism.

Higher concentrations of toxin Z per unit volume produced in the present study resulted in different types of cytopathogenicity appearing in HEp-2 cultures. For example, the cells became swollen and fixed to the tube rather than rounding up and detaching from the plastic. Such fixed monolayers lacked mitotic figures. Usually after 1 or 2 days these fixed cells began to shrivel up and detach from the sides of the tube; however, in the case of strongly potent preparations of toxin Z, the cells sometimes remained in their fixed state over the entire 3-day assay period. Table 1 shows how these types of CPE varied with dilution of toxin Z.

Ultrafiltration fractions. Table 2 shows that during filtration of toxin Z in a Bio-Fiber 80 unit almost all toxin Z activity was retained in the

TABLE 1. *Variation in types of CPE with dilution of concentrated toxin Z*

Dilution	CPE[a] in 72 h	
	Twofold toxin	Fourfold toxin
1/2	4L	4F
1/4	4L	4L
1/8	4L	4L
1/16	3R	4L
1/32	0	4R

[a] Types of CPE produced in HEp-2 monolayers: F, cells fixed and swollen; L, cells loose; R, cells round, but attached; 0, no effect.

TABLE 2. *Toxicity of fractions obtained by ultrafiltration of crude toxin Z in a Bio-Fiber 80 unit*

Sample	CPE[a]		
	Undiluted	1/2	1/4
Starting material	4+	1+	0
Residue	4+	3+	1+
Backwash with supplemented MEM	4+	4+	3+
Filtrate	1+	0	0

[a] At three concentrations in 48 h on HEp-2.

residue and backwash. During the filtration, material lodged in the fiber pores; the backwash collected from the Bio-Fiber 80 fibers was often more cytotoxic than the concentrated toxin residue. Used medium when filtered through the Bio-Fiber 80 unit never showed CPE on Hep-2 cells.

Column chromatography fractions. The eluant selected for the gel filtration of toxin Z was 0.01 M tris(hydroxymethyl)aminomethane buffer, pH 8.0. This buffer was nontoxic to HEp-2 cell cultures even at 0.2 M concentration. The presence of 0.02% sodium azide in the buffer to prevent bacterial growth in the column had no adverse effect on the HEp-2 cells. Nevertheless, the chromatographic bed was routinely washed with buffer without sodium azide before each chromatographic run, and toxin Z was eluted in the absence of sodium azide.

Toxin Z, concentrated by Lyphogel or ultrafiltration, was fractionated by Sephadex G-200 column chromatography. Concentrated used medium was also fractionated to serve as a nontoxic control. The protein content of each fraction, as determined by the Lowry method, was plotted. The protein distribution curve obtained for toxin Z was identical to that obtained for used medium. The fractions were assayed for toxicity on HEp-2.

Figure 1 shows the distribution of protein and toxin Z in fractions obtained by eluting concentrated crude toxin Z from a Sephadex G-200 column. The same protein distribution was obtained irrespective of the type of serum present, whether calf serum or rabbit serum. The elution curve had three definite peaks. The first peak was shown to contain material eluted in the void volume, such as globulin, by comparison with the elution of blue dextran from the same column. The second peak was shown to contain serum albumin by comparison with the elution of a pure solution of bovine serum albumin. The third peak contained phenol red and other materials eluted at the total bed volume.

Part of each 2-ml fraction obtained from the column was assayed for protein content, and another part was diluted one-half with supplemented MEM and tested for toxicity on HEp-2. Fraction 40 was the most toxic; the two adjacent fractions were slightly toxic, and the other fractions were free of toxicity. Toxin was eluted with the albumin and had a partition coefficient (K_{av}) of 0.37.

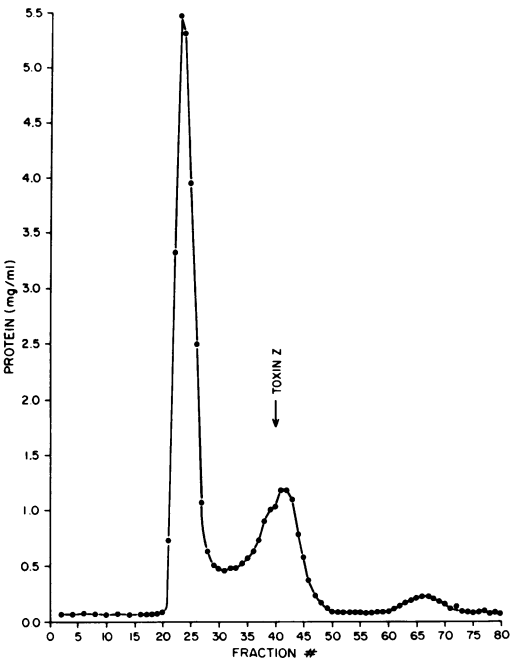

FIG. 1. *Elution of Pseudomonas aeruginosa-concentrated exotoxin Z from a Sephadex G-200 column with 0.01 M tris(hydroxymethyl)aminomethane buffer, pH 8.0; fractions were 2 ml each. Each fraction was assayed for protein content by a modification of the Lowry method as well as assayed for toxicity on HEp-2.*

Table 3 shows the results of a neutralization test on fraction 40. The presence of excess serum in the toxin control having the normal rabbit serum (15% total serum) had a partial protective effect permitting a 3+ (75% CPE) rather than 4+. Table 4 gives the recovery and purification data for toxin Z from the Sephadex G-200 column. A 20-fold purification as compared with total protein was obtained.

DISCUSSION

Toxin Z produced by *P. aeruginosa* may well be involved in the pathogenicity of this organism. We have previously shown a correlation between plaque efficiency and toxin Z potency in vitro as well as the cytopathogenicity of the toxin in vitro and its lethality in mice (2, 3). Continued subculturing of *P. aeruginosa* strains caused a decline in plaque efficiency and toxin Z formation, suggesting an apparent loss of virulence (3).

Most strains of *P. aeruginosa* are usually proteolytic (3); in this regard strain PA Z was typical. The nonproteolytic strain PA 103, however, also was found to produce toxin Z without the formation of the complicating proteases. This permitted the production of higher initial concentrations of toxin Z.

Sephadex G-200 column chromatography was used to prepare partially purified toxin Z from both *P. aeruginosa* strains. These purified toxins were confirmed to be toxin Z by the use of antiserum prepared against concentrated crude toxin Z produced with strain PA 103. The antiserum neutralized both toxin Z preparations produced by strain PA Z with HEp-2 as well as by strain PA 103 in used medium. This finding suggests that toxin Z is a monospecific toxin.

TABLE 3. *Neutralization of toxin Z in fraction 40 eluted from a Sephadex G-200 column*

Sample	CPE[a] in 48 h
Toxin plus supplemented MEM	4+
Toxin plus normal rabbit serum	3+
Toxin plus antitoxin Z	0

[a] The average of duplicate HEp-2 cultures.

The molecular weight of toxin Z appeared to be approximately 70,000 since it was eluted from Sephadex G-200 near the center of the serum albumin peak; the molecular weight of bovine serum albumin is 67,000. The stability of toxin Z to heat readily distinguishes it from Liu's heat-labile exotoxin A. Further purification and characterization of toxin Z and its relationship to Meinke and Berk's heat-stable E-2 toxin are presently under investigation.

In this study, we used cell culture exclusively for the production, detection, and titration of a toxin. Many toxins, including diphtheria toxin and staphylococcal alpha- and beta-hemolysins (11), are known to produce CPE in tissue culture. Other toxins might be detected and titered in tissue culture with greater efficiency and sensitivity, and with less expense, than in animals.

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TABLE 4. *Purification of toxin Z by Sephadex G-200 column chromatography*

Sample	Volume (ml)	Titer[a]	Total CPE units[b]	Recovery (%)	Total protein (mg)	CPE units per mg of protein	Purification (-fold)
Applied material	2.5	4	20	100	88	0.2	1
Fraction 40	2.0	2	8	40	2	4	20

[a] Titer, Reciprocal of highest dilution giving 2+ CPE in 48 h on HEp-2.
[b] Total CPE units, 2 CPE units × titer × volume.

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