

METHODS FOR THE PRODUCTION, MEASUREMENT, AND DETERMINATION
OF IMMUNOSPECIFICITY OF TOXIN Z BY SEVERAL
STRAINS OF PSEUDOMONAS AERUGINOSA

by

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ABSTRACT

Different methods were used to produce toxin Z by Pseudomonas aeruginosa. In the closed non-aerated system, toxins were produced with or without HEP-2 cells in different compositions of supplemented MEM medium. All these preparations were confirmed to be antigenically the same toxin Z by neutralization with antitoxin Z produced in a rabbit. The highest concentration of toxin Z was produced by PA 103 in serum-free MEM medium without HEP-2 cells. In the open aerated system, there was an increased production of fluorescein accompanied by a corresponding decrease in toxin Z production.

Toxin Z was shown to be different than Liu's exotoxin A on the basis of differences in protease susceptibility and heat-inactivation in vitro. Immunospecificity studies based on Ouchterlony agar diffusion assays confirmed the differences between toxin Z and exotoxin A.

The toxicity and neutralization of toxin Z were based on the qualitative CPE assay with HEP-2 cells and the quantitative ED₅₀ assay with Clone II cells.

Fourteen different Pseudomonas aeruginosa strains, including the seven Parke-Davis serologically-different strains, were typed under the Fisher schema prior to determining the immunospecificity of their toxin Z. By means of the qualitative and quantitative neutralization assays, toxin Z was found to be monospecific.

INTRODUCTION

Clinical Significance of Pseudomonas aeruginosa

Pseudomonas aeruginosa are gram-negative, polar monotrichously flagellated rods occurring singly, in pairs, or in short chains widely distributed in nature. On blood agar, the organism grows as a large, flat colony with a ground-glass appearance, and produces a zone of hemolysis. The colonies tend to spread and give off a characteristic grape-like odor. Most strains excrete pyocyanin and fluorescin, giving the colony a characteristic blue-green color.

Gessard first established Pseudomonas aeruginosa as a human pathogen in 1882. The importance of Pseudomonas aeruginosa as a human pathogen has increased considerably since the introduction of antibiotics for treatment of infectious diseases. Due to its antibiotic resistance this organism tends to proliferate in pathogenic processes where antibiotic-sensitive bacteria are eliminated, and infections caused by this organism are difficult to eradicate. Of particular significance is its increasing incidence in infections of patients already debilitated by other diseases. For these reasons it is a common cause of hospital acquired infection, especially in burn patients (1). It has been implicated in patients suffering from leukemia and lymphoma where it may cause septicemia and bacteremia (2). Frequently it is found as a pulmonary complication of cystic fibrosis (3). Chronic lung and heart disease have been shown to be predisposing

conditions that apparently increase susceptibility to infection with P. aeruginosa (4).

The organism also causes problems in wounds, tracheostomies, urinary tracts, skin abscesses, post surgical endocarditis, chronic middle ear infections, lesions of the eye and severe enteritis in infants (infant summer diarrhea). P. aeruginosa is a nosocomial organism which can survive and even multiply in moist environments with minimal amounts of organic matter. It has been incriminated in from 5 percent to 15 percent of all hospital-acquired infections (5).

Pathogenesis of Pseudomonas aeruginosa in Infections

At the present time, little is known about the mechanism by which this organism exerts its damaging effects on the host. Since the organism is a gram-negative bacillus, it may be speculated that the so-called endotoxin which is possessed by this type of bacteria may be responsible for its pathogenesis. However, it appears that the intact endotoxin complex of the cells does not play a particular important role in the disease process. This is supported by the relative refractoriness of normal laboratory animals to injections of large numbers of either living or dead organisms (6, 7). The cause of pathogenicity by P. aeruginosa is now attributed largely to its many exotoxins rather than to its endotoxin. For example, protease, lecithinase, hemolysin, elastase, pigments, hydrocyanic acid, phospholipase, enterotoxin and slime have been implicated as substances which may increase invasiveness of P. aeruginosa in the disease process

(8, 9, 10, 11, 12, 13). These products do contribute to the pathogenesis of the organism but they appear to play a minor role.

The most widely recognized work on pseudomonas lethal toxin has been done by Liu and his associates. Liu (in 14) described a heat labile exotoxin which was produced by a nonproteolytic strain of P. aeruginosa, PA 103, growing in trypticase soy broth. This toxin designated exotoxin A appeared to be a protein with molecular weight about 5×10^4 daltons. Injection of 5-10 LD₅₀ of exotoxin A by the intraperitoneal route into mice caused a rapid reduction of circulating leukocytes (13). The mice usually died within 24-48 hours, and prominent findings at autopsy included necrosis of the liver, edematous and hemorrhagic lungs, and tubular necrosis and hemorrhages in the kidneys (13). Antibody against toxin protected mice against infections with strains of P. aeruginosa whose somatic antigens were serologically unrelated to those of PA 103, provided the serotype of the exotoxin was identical (13).

Recently, Meinke and Berk (15) isolated a heat stable, non-proteolytic toxin produced by P. aeruginosa strain E₂ in a tryptone-glucose medium. This toxin was lethal for mice injected intravenously and intraperitoneally. However, the investigators reported no gross or microscopic pathological changes to internal organs.

The observation of Ludovici and Christian (16) in 1965 that P. aeruginosa produced virus-like plaques in cell monolayers led to the proposal of another exotoxin, subsequently designated toxin Z.

Coleman, Janssen and Ludovici (17) demonstrated that the addition of

the bacterial-free filtrate from such plaque forming cultures to fresh HeLa monolayers produced cell destruction. When calf serum was omitted from the cell culture medium, bacterial plaques failed to form in the cell monolayer but a generalized destruction of the cell sheet occurred. The bacterial-free filtrate from such cultures also was cytotoxic, but its cytotoxicity could be neutralized by the addition of 10 percent calf serum. On the other hand, toxin produced in the presence of serum was not neutralized by the addition of fresh serum and this toxin was designated toxin Z. Since bacterial plaques were formed only in the presence of serum, toxin Z was indirectly implicated as the causative factor responsible for plaques in HeLa monolayers (17). More recently (18), a positive correlation was demonstrated between efficiency of plaque formation and the potency of toxin Z as evaluated both in vitro on HEP-2 monolayers and by intraperitoneal injections in mice for several strains of P. aeruginosa isolated from clinical sources. However, LD₅₀ determinations of these P. aeruginosa strains in mice showed no significant difference. This finding was consistent with the results of others who have unsuccessfully attempted to utilize mouse LD₅₀ as an index of virulence.

Statement of the Problem

The present study involved the development of methods for large batch production of toxin Z to be used to produce antiserum in rabbits and thus permit the examination of the question whether different strains of P. aeruginosa produce a single or multiple type-specific toxin Z. The work required the development of a quantitative assay for

measuring the growth inhibition produced by toxin Z on cell cultures as well as the percentage protection of such cultures when specific antitoxin was used in neutralization tests.

METHODS AND MATERIALS

Tissue Culture Techniques

Culture Medium

The culture medium used in the study was Eagle's Minimum Essential Medium (MEM) supplemented with 10 percent calf serum (CS) and with 100 units of penicillin and 0.1 mg streptomycin per ml. This medium is hereafter referred to as 'regular growth medium'.

Cell Culture Maintenance

The cell cultures used in the study were HEp-2 and Clone II. Clone II is a HEp-2 modified established cell line in which a UV irradiated Herpes Simplex type 2 thymidine kinase coding gene has been integrated into the BUdR-FUDR treated HEp-2 cell genome (19).

Cells were stored in vials at -60 C in a Revco freezer and at -190 C in liquid nitrogen. Vials were quick-thawed at 37 C and cells transferred to an 8-oz prescription bottle. Then, 10 ml of 'regular growth medium' was added, and the culture incubated at 37 C in stationary position. 'Regular growth medium' was changed every other day.

The cultures were routinely subcultured once or twice weekly. Ten ml of 0.25 percent trypsin in calcium and magnesium-free, phosphate buffered saline at 37 C was added for 1 min to release the cells from the glass. Trypsin was decanted and 5 ml of 'regular growth medium' was added after 15 min to disrupt the released cells. Subculture was

completed by addition of 0.5 ml of this suspension plus 9.5 ml of 'regular growth medium' into 8-oz prescription bottles.

For toxin and neutralization assays, disposable plastic culture tubes were planted with 0.5 ml each of 5×10^4 cells suspended in 'regular growth medium'. The cell cultures were incubated at 37 C for twenty-four hours prior to use. HEp-2 cells were used for qualitative toxicity and neutralization assays while Clone II cells were used for quantitative toxicity and neutralization assays. Clone II cells were used because they dissociate to single cells more readily than HEp-2 and therefore permitted more consistent preparation of replicate tubes for quantitative assays.

Bacteriological Techniques

Collection and Storage of Strains

Two strains of P. aeruginosa, PA 103 and PA Z, were used routinely to produce toxin Z. PA Z was a proteolytic strain isolated in this laboratory as a pure contaminant of HeLa S₃, and traced to a technician whose infant child developed summer diarrhea (20). Strain PA 103, furnished by P. V. Liu, was modified by repeated growth in penicillin and streptomycin medium and it was used primarily because it is nonproteolytic (21).

In addition to PA Z and PA 103, the following strains were used for the determination of immunospecificity of toxin Z.

PA E₂ (22), furnished by R. S. Berk (in 22), was originally used by these investigators to produce a nonproteolytic toxin on tryptose-glucose agar. PA E₂ was isolated from a patient.

Of 342 cultures of P. aeruginosa isolated from clinical cases by M. W. Fisher (in 23), 95 percent could be placed in seven immunological groups. The seven Parke-Davis strains (PA 3198, PA 3201, PA 3202, PA 3203, PA 3200, PA 3223, and PA 3224) represented members of each of the seven serological groups and were kindly supplied by C. G. Druby of Schering Corporation.

Several other strains were obtained from frozen stock cultures of strains isolated by this laboratory from clinical cases. They included PA 408, PA 1381, PA 029, and PA 321-10 (18).

Cultures of P. aeruginosa were streaked onto nutrient agar slants (Difco). Since the production of toxin Z required the inoculation of a known small number of organisms, the following method was employed. Twenty-five ml of MEM was inoculated from the nutrient agar slants and incubated at 37 C for 6-8 hours. This suspension was transferred to 12 x 75 plastic tubes in 0.5 ml portions. These stock cultures were maintained in the frozen state, two tubes were quickly thawed, diluted, and plated in duplicate on nutrient agar to determine the average number of organisms per ml of frozen stock.

Serotyping

The P. aeruginosa strains were typed according to the schema of Fisher (in 23), who differentiated these organisms at the subspecies level on the basis of their protective antigens. This procedure consisted of inoculation of freshly poured Mueller-Hinton agar plates (Difco, Detroit, Mich.) and incubation for no more than 16-18 hours at 30 C. The resulting growth was removed and suspended in 0.5 ml of 0.85 percent

NaCl to achieve a dense, homogenous suspension. Nine individual drops of the suspension were placed on a glass slide, and one drop of each antisera prepared by C. G. Druby in rabbits immunized with the seven Parke-Davis serologically-different strains was added to the seven drops. Physiological saline and normal rabbit serum, which contains no antibody to *Pseudomonas*, was added to the two remaining drops as controls. The tests were then mixed with toothpicks, rocked gently for at least 1 min, and observed for macroscopic clumping.

Toxin Production

Closed Non-aerated System

Toxin Z Produced in 'Regular Growth Medium' with HEp-2 Cells.

After the formation of a complete HEp-2 cell monolayer in 8-oz glass prescription bottles (Brockway), the 'regular growth medium' was poured off and the cells were washed with 10 ml of 1X calcium-magnesium-free, phosphate buffered saline (CMF-PBS). One-tenth ml of bacterial suspension (containing 100-200 organisms) was then added and allowed to incubate for one hour at 37 C to permit adsorption of the bacteria. Ten ml of 'regular growth medium' was then added and the bottles returned to 37 C incubator. Incubation was stopped at 16-24 hours when plaques, which were believed to be caused by toxin Z, appeared in the monolayer. The bottles were then placed in a freezer (0 C) until later harvesting.

Sixteen and 32-oz glass prescription bottles were also used for large batches with a corresponding increase in the bacterial inoculum.

Toxin Z-1 Produced in Serum-free Medium without HEp-2 Cells.

For serum-free toxin production, 'regular growth medium' without calf serum and HEp-2 was inoculated with different strains of P. aeruginosa (about one million organisms in each bottle), incubated at 37 C for 1 to 2 weeks, and then placed in a freezer (0 C) for later harvesting. For small batches, the toxin was produced in capped, stationary, 16-oz prescription bottles with 25 ml of media in each bottle. For large batches, 32-oz prescription bottles were used with 50 ml of media in each bottle. This toxin was designated toxin Z-1. In one experiment toxin Z-1 was harvested daily and tested qualitatively for CPE.

Toxin Z-2 Produced in Serum-free Medium without HEp-2 Cells and Sodium Bicarbonate. The same procedure was followed as for the previously described toxin Z-1 production. 'Regular growth medium' without either calf serum or sodium bicarbonate was used; the pH was maintained at 7 with 1N sodium hydroxide. This toxin was designated toxin Z-2.

Toxin Z-3 Produced in Used 'Regular Growth Medium' without HEp-2 Cells. The same procedure was followed as for the previously described toxin Z-1 production except 'regular growth medium' previously used to grow HEp-2 was employed and designated as used. This toxin was designated toxin Z-3.

Toxin Z-4 Produced in Serum-free Medium with HEp-2 Cells. The same procedure was followed as for the previously described toxin Z production. In this case, 'regular growth medium' without calf serum was used. Plaques were not observed in the absence of calf serum.

These cultures were allowed to incubate at 37 C for 22-24 hours. At this point the cell culture medium was mildly turbid, and the monolayers showed 50 to 75 percent destruction. The bottles were placed in a freezer (0 C) for later harvesting. This toxin was designated toxin Z-4.

Open Aerated System

Toxin Z-1 Produced in Serum-free Medium without HEp-2 Cells and Sodium Bicarbonate in an Aerated System. The open system was designed to prepare large batches of toxin in one container instead of separate bottles. The toxin was produced in 2-liter spinner cultures in 5-liter Erylenmeyer flasks with cotton plugs. About five million organisms were inoculated into each flask. A large magnet bar was placed in the flask and agitation was achieved by placing the flask on a Bellco Bell-stir magnetic stirrer. The pH was adjusted daily with 1N sodium hydroxide to maintain the pH at 7, and the toxin was harvested after an incubation period of seven days at 37 C.

Toxin Z-5 Produced in 'Serum-free Enriched Medium' without HEp-2 Cells and Sodium Bicarbonate in an Aerated System. The same procedure was followed as for the previously described toxin Z-2 production in the open aerated system. The 'serum-free enriched medium' was 1X MEM contained fourfold concentration of amino acids, phosphate, and glutamine. The medium was used in an attempt to produce a stronger toxin. The pH was adjusted daily with 1N sodium hydroxide to maintain the pH at 7. In some batches phenol red was not added to the medium in order to detect fluorescin production by P. aeruginosa. In one batch

sodium bicarbonate instead of sodium hydroxide was added to the medium in order to compare the difference in fluorescein production.

Toxin Harvest

After thawing the crude toxins and removing the bacterial cells by centrifuging at 12,100 xg in a Sorvall Superspeed RC 2-B automatic refrigerated centrifuge with a type SS-34 head at 4C for 30 min, the toxin-containing supernatant fluids were filtered twice through 0.2 um Gelman membrane filters contained in Swinex units (Millipore). In early work the crude toxin was routinely heated in a 70 C water bath for 1 hour to inactivate any protease. The harvested toxin was then assayed for protease activity by adding 0.5 ml of the toxin to 0.5 ml of skim milk. Any toxin that showed protease activity, as indicated by clearing of the milk, was discarded. In later studies toxins were not heat-inactivated, and when protease activity was present it was neutralized by the addition of calf serum (10 percent).

Toxin Assays

Qualitative CPE

Plastic culture tubes were each planted with 5×10^4 HEp-2 cells in 0.5 ml of 'regular growth medium'. After 24 hours, the medium was poured off, and the tubes were each inoculated with 1 ml of sterile sample. The toxin was serially diluted twofold in 'regular growth medium' (undilute, 1:2, 1:4, 1:8, 1:16, and 1:32). Cell destruction was observed microscopically (Leiss inverted 5X) by rounding or flattening of cells regardless of whether the cells were released from the

plastic surface of the tube; CPE was measured as 0, 1, 2, 3, 4+, meaning 0, 25, 50, 75, and 100 percent of the cell monolayer affected, respectively. The titer of the toxin is defined as the reciprocal of the highest dilution causing 2+ (50 percent) CPE in HEp-2 cultures in 48 hours. For controls, the specific culture medium used in producing the different toxins was also assayed for toxicity.

Quantitative Assay

The toxicity of some crude toxins was assayed quantitatively by measuring the growth-inhibition of Clone II using a modification of the Oyama and Eagle method (24, 25). Plastic culture tubes were each inoculated with 5×10^4 Clone II cells in 0.5 ml of 'regular growth medium' and incubated 24 hours at 37 C. The medium was poured off, and the tubes were each inoculated with 1 ml of sterile sample in duplicate. The toxin was serially diluted twofold in 'regular growth medium'. Two sets of toxin-free control tubes were used to establish 0 percent and 100 percent growth of Clone II cells. The number of tubes included in each of these two sets of controls was the square root of the number of materials tested. After the initial 24-hour incubation period, the tubes used for 0 percent growth were rinsed twice with normal saline before adding 5 ml of Lowry alkaline copper reagent and storing at 4 C. The remaining tube cultures were incubated at 37 C for 24 hours and then assayed qualitatively and quantitatively for toxicity. For the qualitative assay, the tubes were examined microscopically for CPE; and then for the quantitative assay, the same tubes were rinsed twice with saline and Lowry alkaline copper reagent was added. Then all tubes,

including the 0 percent growth controls, were placed in a 56 C water bath for 5 min, mixed thoroughly on a Vortex mixer, and 1 ml of distilled water and 0.5 ml of properly diluted Phenol Reagent (Fisher) added. Then all the tubes were mixed thoroughly on the Vortex mixer. After 30 min, the samples were read against a distilled water reagent blank in a Spectronic 20 spectrophotometer (Bausch and Lomb) at 660 nm. Dose-response curves were plotted on two by one cycle logarithmic paper with dilution in decimal form versus optical density (OD). The point at which the linear dose-response curve crossed the 50 percent growth line was taken as the 50 percent effective dose (ED_{50}).

Immunization

Antitoxin Z was prepared in a rabbit by five weekly inoculations of toxin Z produced by PA 103 grown on HEp-2 monolayers with 10 percent rabbit serum replacing the 10 percent calf serum in 'regular growth medium'. The toxin was homogenized with an equal amount of Freund's incomplete adjuvant prior to injection. The first injection was equally divided between intramuscularly, intraperitoneally, and foot pad administration. Subsequent weekly injections were intramuscularly. Five days after the final injection the rabbit was bled by cardiac puncture and the blood processed for serum.

Neutralization Assays

Antitoxin Z Versus Z, Z-1, Z-2, Z-3,
and Z-4 Types of Toxin from PA 103 or
Z-1 Type Toxin from Different PA Strains

Qualitative Neutralization. The CPE titers were experimentally determined for each test toxin, and 2 ml of each toxin at the 50 per cent CPE end-point was mixed with 0.2 ml of undilute antitoxin Z. After incubation at 37 C for 1 hour, 1 ml of the mixture was added to a HEP-2 tube in duplicate and examined for inhibition of CPE for 48 hours. Toxin controls had normal rabbit serum instead of antiserum. All samples were tested in duplicate.

Quantitative Neutralization. The ED₅₀ dilutions were experimentally determined for each test toxin, and 2 ml of each toxin at the ED₅₀ was mixed with 0.2 ml of undilute antitoxin Z. After incubation at 37 C for 1 hour, 1 ml of the mixture was added to a Clone II tube in duplicate. Then the same procedure was followed as previously described for the quantitative assay. Toxin controls had normal rabbit serum instead of antiserum. All samples were tested in duplicate. Percentage protection was calculated according to the formula

$$\text{Percentage protection} = \frac{\text{Average OD (sample)} - \text{Average OD (0\% growth control)}}{\text{Average OD (100\% growth control)} - \text{Average OD (0\% growth control)}} \times 100\%.$$

Immunodiffusion Test

The immunodiffusion tests were performed by the Ouchterlony technique (26) with antitoxin Z at 1:4 dilution placed in the center well versus toxin Z (PA 103) at various dilutions, ranging 1:8 to 1:128.

Toxin Z-1 from different strains of P. aeruginosa and Liu's exotoxin A were also used to react with antitoxin Z, and antiexotoxin A tested against exotoxin A and toxin Z (PA 103).

RESULTS

Toxin Assays

Specific CPE Induced by Toxins Produced in the Closed Non-aerated System

After different toxins (toxin Z, Z-1, Z-2, Z-3, and Z-4) were separated from the organisms, they caused a generalized CPE when put on HEp-2 monolayers. The CPE began with the shrivelling of the cells at the periphery of the monolayer, followed by the appearance of random patches of round cells throughout the monolayer. Sometimes these patches of round cells formed a plaque in the center, which gradually enlarged, surrounded by a ring of round cells. Detachment of cells occurred readily. The generalized specific CPE characteristic of toxin Z is illustrated in Fig. 1 while a normal non-toxin treated control preparation is illustrated in Fig. 2.

Fluorescin CPE Induced by Toxins Produced in the Open Aerated System

Fluorescin CPE was encountered from the toxins produced in the open aerated system. The cells became flattened, granular, vacuolated, spindle-shaped and fixed to the glass rather than rounding up and detaching from the tube wall. Such fixed cells lacked mitotic figures. This fluorescin CPE is illustrated in Fig. 3. The normal non-toxin treated control in this experiment is illustrated in Fig. 4.

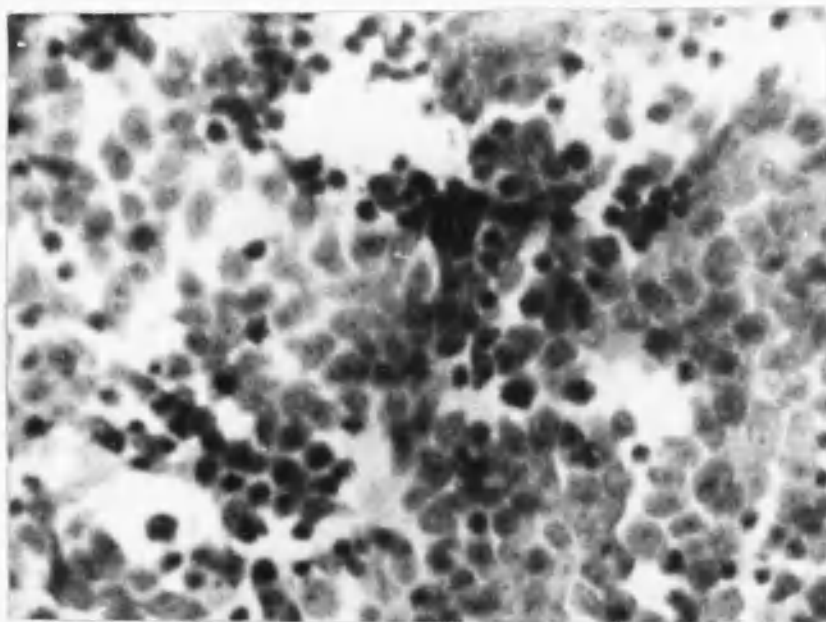


Fig. 1. Specific CPE of Toxin Z on HEp-2 Cells (70X)

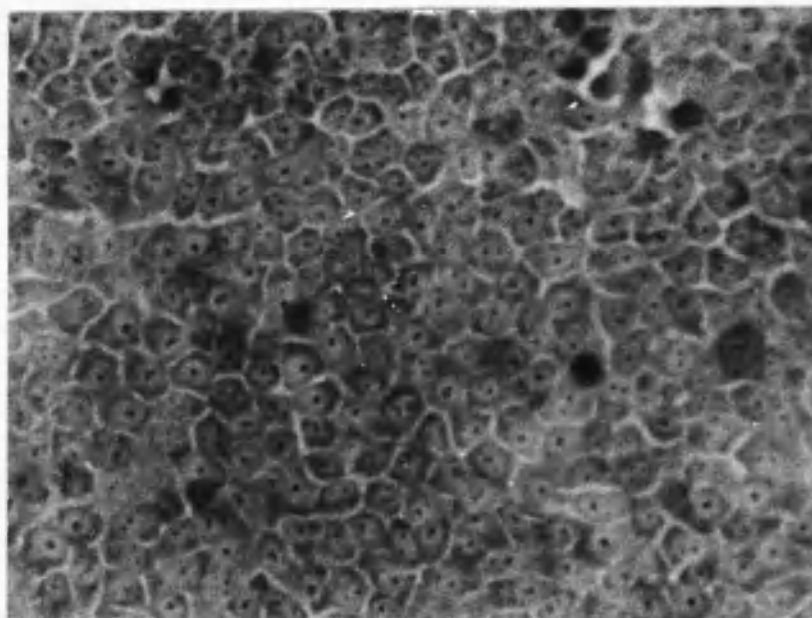


Fig. 2. Normal HEp-2 Cells
in Non-toxin Treated Control (70X)

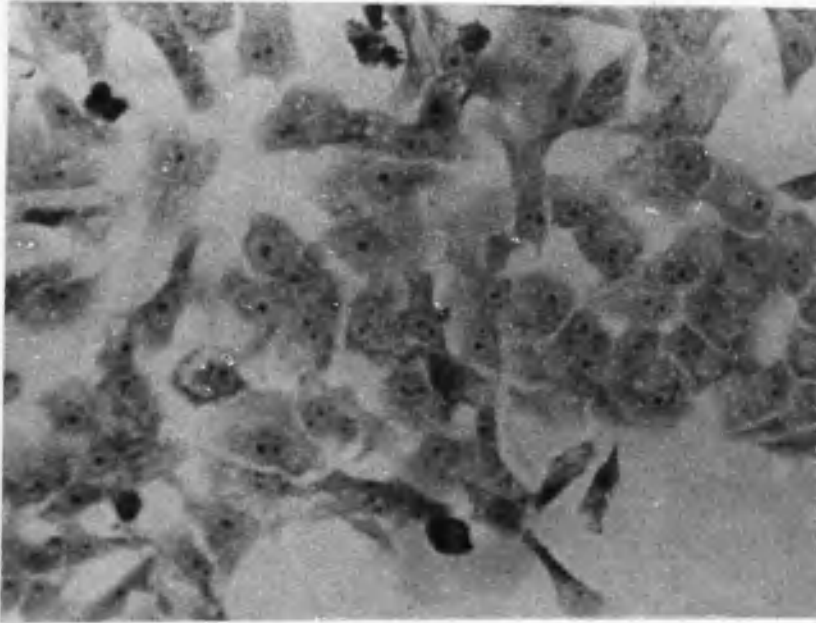


Fig. 3. Fluorescein CPE on HEp-2 Cells Detected in Toxin Z Prepared by the Open Aerated System (70X)

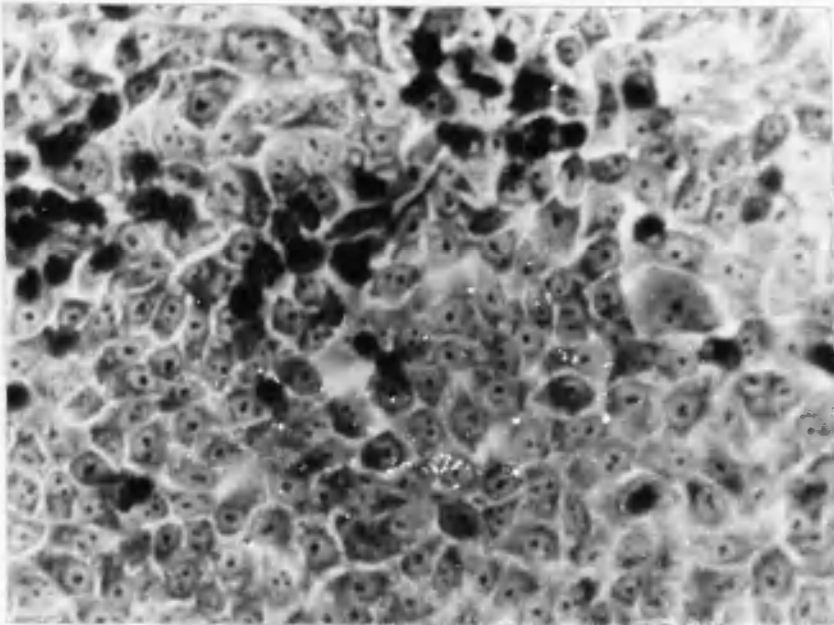


Fig. 4. Normal HEp-2 Cells in Non-toxin Treated Control (70X)

Quantitative Assay for Cytotoxicity

The cytotoxicity of the toxins could be quantitatively measured by a modification of Oyama and Eagle method. Clone II cells were used because they more readily dissociate into single cells than HEp-2 and so permitted more consistent preparation of the replicate tubes required for the quantitative assay. Fig. 5 illustrates a typical example of a dose-response curve obtained for toxin 49 (Table 8, p. 32). High toxin concentrations sometimes caused detachment of cells during the incubation period of the assay, and these cells were lost with saline washes. The ED_{50} obtained from the curve in this example was 0.255.

Toxin Z Production

Toxin Z Titer with Different Compositions of Medium

Table 1 shows the different compositions of medium used to produce toxin. Different medium compositions were studied in an attempt to produce a potent crude toxin. Obviously a simplified medium without serum would yield a more purified crude toxin. Toxin Z and toxin Z-4 were produced in the presence of HEp-2, however, calf serum was absent in toxin medium Z-4. Toxin Z-1, Z-2 and Z-3 were produced without HEp-2 cells. However, toxin medium Z-3 consisted of calf serum and the products of HEp-2 cells. Toxin medium Z-1 was found superior to toxin Z-2 because the presence of sodium bicarbonate in Z-2 maintained a pH of 7 while the pH of toxin medium Z-2 had to be adjusted daily with sodium hydroxide. This probably accounts for the twofold increase in CPE titer of Z-1 toxin. The high CPE titer of toxin Z-5 was due to non-specific cytotoxicity by fluorescein as illustrated in

Fig. 5. Dose-response Curve of a Typical Toxin Z

Toxin 49 (Toxin Z-1, PA 103, Table 8), the toxin was serially diluted in regular medium and 1 ml of each dilution was added to 24 hour Clone II tube cultures in duplicate. After 24 hours at 37 C, the cell protein in the tubes was measured by a modification of Oyama and Eagle method. Dilution of toxin was plotted against OD at 660 nm.

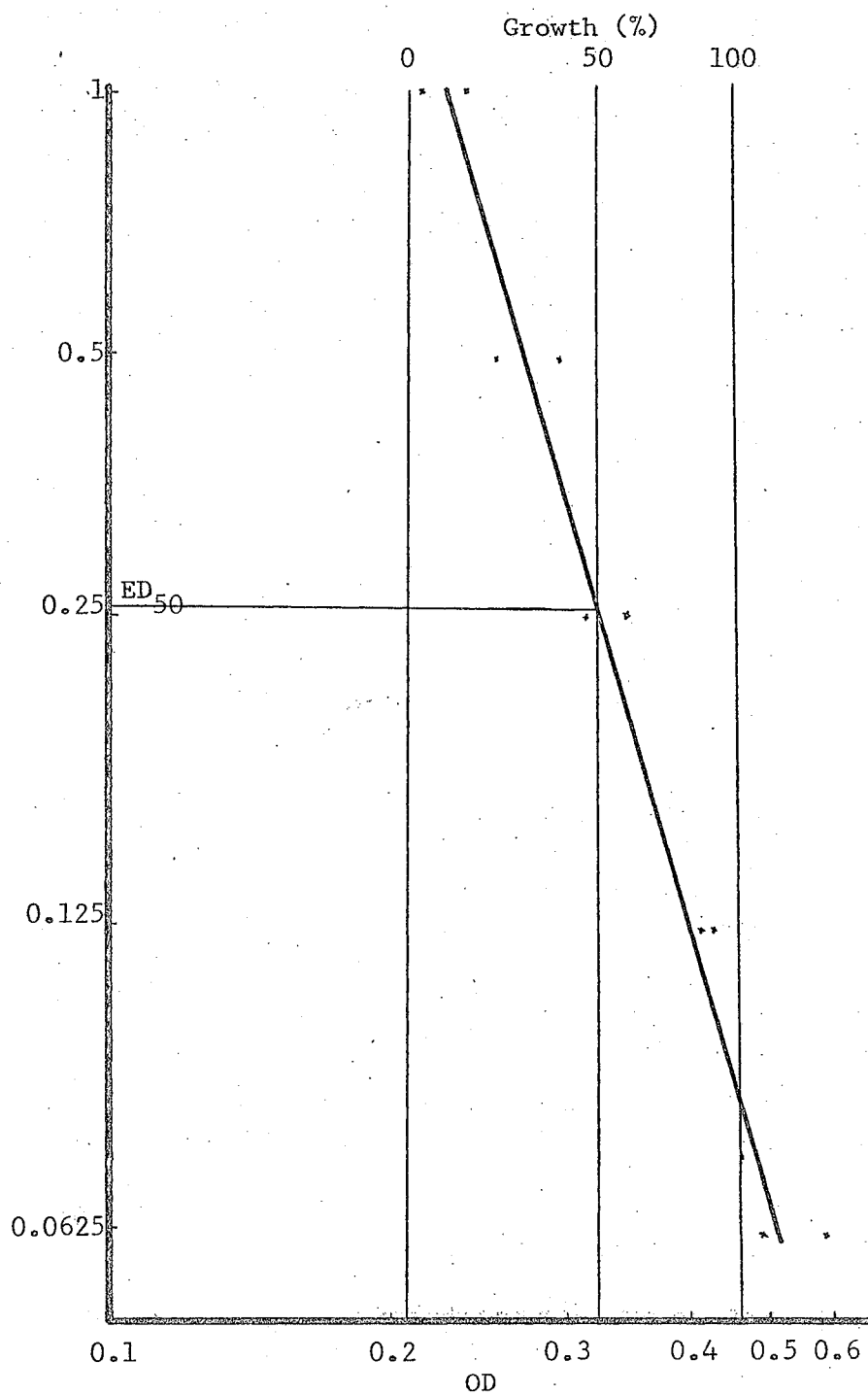


Fig. 5. Dose-response Curve of a Typical Toxin Z

Table 1. Toxin Z Titer with Different Compositions of Medium either with or without HEp-2 Cells

Toxin	HEp-2	Toxin Medium Compositions				CPE Titer
		Calf Serum	HCl	NaHCO ₃	MEM	
Z	+	+	+	+	+	4 ^a
Z-1	-	-	+	+	+	8
Z-2	-	-	-	-	+	4
Z-3 ^b	-	+	+	+	+	4
Z-4	+	-	+	+	+	4
Z-5 ^c	-	-	-	-	+	16

^aTiter is the reciprocal of the highest dilution causing 2+ (50 percent) CPE on HEp-2 at 48 hr. The average of duplicate tubes of HEp-2.

^bToxin Z-3 was produced in used 'regular growth medium', previously used to grow HEp-2.

^cToxin Z-5 was produced in 'serum-free enriched medium' consisted of 1X MEM, 4X amino acids, 4X phosphate, and 4X glutamine.

Fig. 3 and will be discussed later (Table 8). Therefore, Z-1, Z-2, Z-3, Z-4, and then Z would be the order of medium preference for toxin production, assuming that all preparations contained the same toxin.

Antitoxin Z Versus Toxin Z, Z-1, Z-2,
Z-3, and Z-4 (PA 103) as Measured
Qualitatively

Table 2 is a summary of the qualitative neutralization assay. Antitoxin Z neutralized toxin Z, Z-1, Z-2, Z-3, and Z-4 (PA 103) to an approximate equal degree indicating that PA 103 produced the same antigenic type of toxin Z in the different media. Therefore, toxin Z could be produced without calf serum and HEp-2 permitting the use of a completely defined medium for its production.

Antitoxin Z Versus Toxin Z, Z-1, Z-2,
Z-3, and Z-4 (PA 103) as Measured
Quantitatively

In order to confirm the findings of the qualitative neutralization assay, the quantitative neutralization assay was performed. These experiments also included the neutralization of the non heat-inactivated portions of the same toxins to determine whether antitoxin Z (made with heat-inactivated toxin) could neutralize toxins which were not heat-inactivated. Table 3 illustrates the results of these studies. The toxin control at ED₅₀ toxin dilution showed variations from an absolute 50.0 percent (the values showed a range from 48.9 percent to 58.9 percent). These variations could be caused by two factors. The exact toxin dilution at ED₅₀ was not used, for example, 1:3 was used instead of 1:3.28. Secondly, variations could be caused

Table 2. Monospecificity of Toxin Z, Z-1, Z-2, Z-3, and Z-4 as Measured by the Qualitative CPE Neutralization Method^a

Sample	Toxin				
	Z	Z-1	Z-2	Z-3	Z-4
Toxin Plus Supplemented MEM ^b	3+ ^c	3+	3+	3+	3+
Toxin Plus Antitoxin Z ^d	0	0	0	0	0
Toxin Plus Normal Rabbit Serum ^e	2+	2+	2+	2+ to 3+	2+ to 3+

^aThe average of duplicate tubes of HEp-2.

^bToxin was diluted with supplemented MEM to give the CPE titer listed in Table 1. The total volume of each sample was 1 ml.

^cCPE was measured as 0, 1, 2, 3, 4+, meaning 0, 25, 50, 75, and 100 percent of the cell monolayer affected, respectively.

^d0.1 ml of antitoxin Z was added to each sample.

^e0.1 ml of normal rabbit serum was added to each sample.

Table 3. Monospecificity of Toxin Z, Z-1, Z-2, Z-3, and Z-4 as Measured by the Quantitative ED₅₀ Neutralization Method Expressed in Terms of Percentage Protection^a

Sample	Toxin									
	Z		Z-1		Z-2		Z-3		Z-4	
	Heat	Non-heat	Heat	Non-heat	Heat	Non-heat	Heat	Non-heat	Heat	Non-heat
Toxin Plus Supplemented MEM ^b	58.6	48.9	56.6	55.5	58.9	55.4	55.7	55.0	55.1	55.4
Toxin Plus Antitoxin Z ^c	100	100	100	100	100	100	100	100	100	100
Toxin Plus Normal Rabbit Serum ^d	66.5	66.4	69.4	66.3	68.5	67.2	63.3	67.5	66.8	62.3

^aThe average of duplicate tubes of Clone II. Quantitative ED₅₀ neutralization was measured by percentage protection by the formula

$$\text{Percentage protection} = \frac{\text{Average OD (sample)} - \text{Average OD (0\% control)}}{\text{Average OD (100\% control)} - \text{Average OD (0\% control)}} \times 100\%$$

^bToxin was diluted with supplemented MEM to give the dilution at ED₅₀ as listed in Table 4. The final volume of each sample was 1 ml.

^c0.1 ml of antitoxin Z was added to each sample.

^d0.1 ml of normal rabbit serum was added to each sample.

by experimental errors. However, the data clearly indicate that anti-toxin Z offered the cells 100 percent protection from all five toxins, either heat-inactivated or non heat-inactivated. It showed that heat-inactivation did not significantly alter the antigenic structure of the toxins. It also showed that normal rabbit serum had a slight protective effect. The quantitative neutralization assay confirmed the previous qualitative finding that toxin Z, Z-1, Z-2, Z-3, and Z-4 (PA 103) were the same antigenically.

A comparison of the qualitative CPE titer with the related quantitative ED_{50} titer (Table 4) indicates that the quantitative method of measuring growth inhibition is more sensitive than the qualitative CPE methods. The CPE titers were about twofold lower than the corresponding ED_{50} values.

Factors Which Influence Toxin Z-1
Production in Closed Non-aerated System

Period of Incubation

In toxin Z-1 (PA 103) production, turbidity usually appeared on the second day of incubation and remained at approximately the same level after the third day. The titer of the toxin, however, increased with the length of the incubation period until it reached a maximum on the sixth day (Table 5). An incubation period of seven days was chosen assuming that the titer was at its maximum on the seventh day and would increase no further.

Table 4. Comparison of the Qualitative CPE Titer and the Quantitative ED₅₀ of Toxin Z, Z-1, Z-2, Z-3, and Z-4

Sample	Toxin									
	Z		Z-1		Z-2		Z-3		Z-4	
	Heat	Non-heat	Heat	Non-heat	Heat	Non-heat	Heat	Non-heat	Heat	Non-heat
CPE Titer at 24 hour	2 ^a	2	2	4	2	4	2	2	2	2
Toxin Dilution at ED ₅₀	3 ^b	6	4	8	4	8	4	8	4	8

^aTiter is the reciprocal of the highest dilution causing 2+ (50%) CPE on Clone II. The average of duplicate tubes of Clone II.

^bThe reciprocal of the estimated toxin dilution at the point the linear dose-response curve crossed the 50% growth line.

Table 5. Toxin Z-1 Titer over a Seven-day Incubation Period^a

Day	1	2	3	4	5	6	7
Titer	2 ^b	4	4	4	8	16	16

^aPA 103 in Z-1 medium (Table 1).

^bTiter is the reciprocal of the highest dilution causing 2+ (50%) CPE on HEp-2 at 48 hr.

Heat-inactivation

Toxin Z was reported to be heat stable, withstanding 70 C for one hour with little loss in CPE activity. That toxin Z is heat stable is a major difference between toxin Z and Liu's exotoxin A which is considered heat labile. In this study, it was found that toxin Z, Z-1, Z-2, Z-3, and Z-4 decreased in titer twofold after heat-inactivation, as illustrated in Table 6. The same type of CPE was observed in both heat-inactivated toxins and non heat-inactivated toxins. However, in a separate study (27), Liu's exotoxin A lost all of its CPE activity after heating at 70 C for one hour. Thus, as far as in vitro effects toxin Z appears to be different from exotoxin A.

In in vivo studies (27) heat-inactivated toxin Z did not produce lethality in mice while the non heat-inactivated toxin did produce lethality. Therefore, it is possible that toxin Z is made up of a heat stable component and a heat labile component. The latter being responsible for the toxin's lethal effect in mice. For these reasons, the

Table 6. Effect of Heat-inactivation on Toxins^a

Toxin	Titer before Heat-inactivation	Titer after Heat-inactivation
Z	4 ^b	2
Z-1	16	8
Z-2	8	4
Z-3	8	4
Z-4	8	4

^aToxins were heat-inactivated at 70 C for one hour. The average of duplicate tubes of HEp-2.

^bTiter is the reciprocal of the highest dilution causing 2+ (50%) on HEp-2 at 48 hr.

toxins produced at the late stages of this study were not subjected to heat.

Effect of Protease on Toxin Z Production

Another means of differentiating toxin Z from Liu's exotoxin A is based on the trypsin susceptibility of exotoxin A. This is the reason Liu used the PA 103 strain to produce exotoxin A because it is a nonproteolytic strain. Table 7 illustrates that toxin Z is not susceptible to protease since the titer of toxin Z increased fourfold from day 1 to 5 at the same time protease increased twentyfold. Most strains of P. aeruginosa are proteolytic strains. In the present study with the exception of PA 103 strain obtained from Liu and PA 3203, all the other strains were proteolytic.

The Open Aerated System

In an attempt to produce larger batches of toxin, the toxin was produced in 2-liter spinner cultures in 5-liter Erylenmeyer flasks with cotton plugs. Because the Erylenmeyer flask was equipped with a cotton plug, sodium bicarbonate was not used since it would easily escape and elevate the pH. Sodium hydroxide was used to adjust the pH daily. Because the first batch, toxin 31 (toxin Z-1, PA 103, Table 8) yielded a low titer (1:4), 'serum-free enriched medium' was used to produce toxin 32 (toxin Z-5, Table 8) in an attempt to produce a toxin with higher potency. The titer of toxin 32 was 16. However, the increased concentration of toxicity was due to a nonspecific CPE caused by the complicating pigment fluorescin (Fig. 3). Toxin 38B, 39B, 40B,

Table 7. Increase in Toxin Z and Protease Titer with Time (Toxin Z-1, PA 408)^a

Day	CPE Titer	Protease Titer
1	2 ^b	3 ^c
3	4	40
5	8	60
7	8	80

^aPA 408 strain grown in Z-1 medium.

^bTiter is the reciprocal of the highest dilution causing 2+ (50%) CPE on HEp-2 at 48 hr. Protease activity neutralized by the addition of 10 percent calf serum.

^cProtease titer is the reciprocal of the highest dilution hydrolysing skim milk.

Table 8. The List of Toxins Produced During the Period of This Research

Toxin No.	Toxin Class	<u>P. aeruginosa</u> Strain	Period of Incubation (days)	Heat-Inactivation (70 C, 1 hr)	CPE Titer	Aeration
18	Z-3	PA 103	7	+	8 ^a	-
19	Z-3	PA 103	7	+	2	-
20	Z-3	PA 103	7	+	4	-
21	Z	PA Z	1	+	2	-
23	Z-3	PA 103	10	+	4	-
24	Z-3	PA 103	15	+	8	-
26A	Z-4	PA 103	10	+	8	-
27	Z-3	PA 103	10	+	8	-
28	Z-1	PA 103	15	+	8	-
29	Z-1	PA 103	15	+	8	-
30	Z-1	PA 103	15	+	8	-
31	Z-2	PA 103	11	+	4	+
32	Z-5	PA 103	14	+	16	+
38 ^b	Z-2	PA Z	7	+	8	+
39 ^b	Z-2	PA Z	7	+	8	+
40 ^b	Z-2	PA 103	7	+	8	+
41 ^c	Z-1	PA 103	7	+	8	+
45	Z-1	PA 103	7	-	16	-

Table 8--Continued

47	Z-1	PA 103	7	-	16	-
48	Z-1	PA 103	7	-	16	-
49	Z-1	PA 103	7	-	16	-
50	Z-1	PA 103	7	-	16	-
51	Z-1	PA 103	7	-	16	-
52	Z-1	PA 103	7	-	16	-

^aTiter is the reciprocal of the highest dilution giving 2+ (50%) CPE at 48 hr on HEp-2.

^bNo phenol red in medium; strong fluorescin production was manifested in toxin 38B, 39B, 40B, and to a less extent in 41B.

^cNaHCO₃ instead of NaOH was used in 41B.

and 41B (Table 8) were produced with no phenol red in the medium in an attempt to detect the formation of yellow-green fluorescin. In these experiments fluorescin production was manifested as early as the second day of the incubation period, and increased in concentration in direct proportion to an increase in bacterial cell numbers as a result of the aerated culture system. Sodium bicarbonate instead of sodium hydroxide was used in toxin 41B, and it not only maintained the pH near 7 but reduced the amount of fluorescin produced. The overall data, however, indicated that the open aerated system could not be used for toxin production because it was complicated by fluorescin production at the expense of toxin Z.

Development of Methods for Toxin Z Production

Large batches of toxin (more than 200 ml in one batch) were each given a number and are listed in Table 8. This represents an assortment of the different toxins produced during the period of this study. Although several methods could be used to produce toxin Z, it is obvious that toxin Z-1 in the closed non-aerated system yielded the highest titer and is the most economical and reliable method available to date.

Immunodiffusion Assay

Antitoxin Z was diluted at 1:4 in the center well versus toxin Z (PA 103) at various dilutions, ranging from 1:8 to 1:128. Three lines of precipitate were clearly observed at all dilutions indicating there were at least three antibody-antigen reactions present. The

result suggested that the crude toxin used to immunize the rabbit contained other bacterial proteins besides toxin Z.

Similarly in immunodiffusion assays with antitoxin Z against various toxin Z-1 from different P. aeruginosa strains, an extremely diffused band was observed indicating a weak antibody-antigen reaction. Thus, future antiserum must be made with purified toxin before immunodiffusion assays will have any validity in studying the serological specificity of toxin Z preparation.

In the immunodiffusion assay of antitoxin Z against Liu's exotoxin A, there was no reaction. However, in the cross matching immunodiffusion assay, a single precipitin line was formed between antiexotoxin A and exotoxin A, but there was no reaction between antiexotoxin A and toxin Z. These results indicated that toxin Z and exotoxin A were antigenically different.

Serotyping of Strains

Table 9 shows the result of serotyping the P. aeruginosa strains used in this study using the schema of Fisher. Immunotyping of strains of Pseudomonas by the agglutination reaction was simple, rapid, and reproducible. The control Parke-Davis strains when mixed with their homologous type-specific antisera showed obvious macroscopic clumping, but there was no reaction when mixed with heterologous antisera. The P. aeruginosa strains from our laboratory were typed according to their agglutination with type-specific antisera and assigned the corresponding immunotype. This serotyping was done prior to studying

Table 9. Fisher Immunotypes of P. aeruginosa Strains

PA Strain	3198	3200	3201	3202	3203	3223	3224	E ₂	408	103	1381	Z	029	321-10
Fisher Immunotype	1	2	3	4	5	6	7	2	3	5	2	5	4	2

the immunospecificity of toxin Z from these same serologically-different P. aeruginosa strains.

Immunospecificity of Toxin Z from
Different Strains of Pseudomonas aeruginosa

Qualitative Neutralization Assay

Toxin Z-1 from different strains of P. aeruginosa was titered on HEp-2 and the qualitative neutralization assay of these toxins with antitoxin Z is illustrated in Table 10. It is clear from the data that antitoxin Z neutralized toxin Z-1 produced by all the P. aeruginosa strains used in this study to approximately the same degree. The fact that antitoxin Z neutralized toxin Z-1 of the seven Parke-Davis serologically-different strains (PA 3198, PA 3200, PA 3201, PA 3202, PA 3203, PA 3223, and PA 3224) as well as that of seven other strains suggests that the toxins produced by all these strains are antigenically the same. The data also show that normal rabbit serum offered a slight partial protective effect but this was clearly not equivalent to the antitoxin Z effect (Table 10).

Table 10. Qualitative Neutralization of Toxin Z Produced by 14 Different Strains of *P. aeruginosa* with Antitoxin Z^a

PA Strain	3198	3201	3202	3203	3200	3223	3224	103	Z	E ₂	1381	029	321-10	408
Fisher Immunotype	1	3	4	5	2	6	7	5	5	2	2	4	2	3
Toxin Plus Supplemented MEM ^b	3+ ^c	3+	3+	3+	3+	3+	3+	3+	3+	2+	4+	3+	3+	2+
Toxin Plus Antitoxin Z ^d	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Toxin Plus Normal Rabbit Serum ^e	2+	2+	3+	2+	2+	2+	2+	2+	2+	1+	3+	2+	2+	2+

^aThe average of duplicate HEp-2 tubes expressed as CPE.

^bToxin was diluted with supplemented MEM to give 2+ CPE at 48 hr on HEp-2. The total volume of each sample was 1 ml.

^cCPE was measured as 0, 1, 2, 3, 4+, meaning 0, 25, 50, 75, and 100 percent of the cell monolayer affected, respectively.

^d0.1 ml of antitoxin Z (PA 103) was added to each sample.

^e0.1 ml of normal rabbit serum was added to each sample.

Quantitative Neutralization Assay

Quantitative neutralization experiments were done to confirm the findings observed with the qualitative assays. Table 11 shows the data of the quantitative neutralization assays. It is obvious that antitoxin Z offered 100 percent protection to Clone II cell growth when it was added to the toxins. Thus, confirming the finding that serologically-different strains of P. aeruginosa produce the same antigenic type of toxin Z. Again normal rabbit serum had a partial protective effect which was clearly distinguishable from the antitoxin Z effect.

Table 11. Quantitative Neutralization of Toxin Z Produced by 14 Different Strains of P. aeruginosa with Antitoxin Z Expressed in Terms of Percentage Protection^a

PA Strain	3198	3201	3202	3203	3200	3223	3224	103	Z	E ₂	1381	029	321-10	408
Fisher Immunotype	1	3	4	5	2	6	7	5	5	2	2	4	2	3
Toxin Plus Supplemented MEM ^b	59.5	55.3	62.0	52.1	51.1	48.2	49.9	52.2	56.3	53.4	50.2	53.2	52.2	48.0
Toxin Plus Antitoxin Z ^c	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Toxin Plus Normal Rabbit Serum ^d	63.2	68.6	65.7	62.7	64.6	65.8	63.7	70.2	59.8	63.6	72.0	68.9	64.5	62.0

^aThe average of duplicate Clone II tubes.

$$\text{Percentage protection} = \frac{\text{Average OD (sample)} - \text{Average OD (0\% control)}}{\text{Average OD (100\% control)} - \text{Average OD (0\% control)}} \times 100\%$$

^bToxin was diluted with supplemented MEM to give the ED₅₀ (the estimated toxin dilution at the point the linear dose-response curve crossed the 50 percent growth line). The final volume of each sample was 1 ml.

^c0.1 ml of antitoxin Z (PA 103) was added to each sample.

^d0.1 ml of normal rabbit serum was added to each sample.

DISCUSSION

Toxin Z was originally detected during the growth of P. aeruginosa Z strain in HEP-2 or HeLa cell cultures in which the organism produced virus-like plaques in the presence of serum (17, 20). Recently, this laboratory (25) reported the production of toxin Z by PA 103 in used 'regular growth medium'. One of the objects of the present research was to find the most efficient way to produce crude toxin Z with high potency for the purpose of purification and immunization. Since used 'regular growth medium' could be used to produce the toxin, several other media with or without HEP-2 cells or calf serum (Table 1) were studied and found to be suitable for toxin production. Toxin Z, Z-1, Z-2, Z-3, and Z-4 were found to be the same antigenically using qualitative and quantitative neutralization assays with antitoxin Z. Since toxin Z-1 had the highest titer and since it did not require the presence of HEP-2 cells or calf serum, thus greatly reducing the extraneous protein content; it was the toxin medium of choice. Moreover, pH was maintained consistently around 7 by the bicarbonate buffer system present in MEM and this was considered the optimal pH for toxin production.

An attempt to use an open aerated system for the production of large batches of toxin Z led to nonspecific toxicity due to the production of large amounts of fluorescein. This was especially true when 'serum-free enriched medium' containing fourfold concentrations of amino acids, phosphate, and glutamine were used because the increase in

phosphate and nitrogen concentration increased bacterial growth with a corresponding increase in fluorescein and a reduction in toxin Z (toxin 32, Table 8).

Toxin Z-1 (PA 103) prepared in a closed non-aerated system was the best procedure to produce the toxin. The titer was consistently 16 for the last seven batches of toxin Z-1 (PA 103) prepared (Table 8). This method allowed the production of toxin Z on a relatively large scale.

Toxin Z is probably an exotoxin which is produced by P. aeruginosa in a medium containing only simple identifiable compounds. The toxicity appeared early in the bacterial culture before cell lysis would be expected, and was found in the filtrate after the bacterial cells were removed.

Toxin Z was previously reported to be relatively heat stable (17, 20). The fact that it is heat stable is a major difference between toxin Z and Liu's exotoxin A which is considered heat labile (13, 14). In the present study, the titer of toxin Z decreased in vitro about twofold after heat-inactivation (70 C, 1 hour). In a separate study (27) Liu's exotoxin A lost 100 percent of its CPE activity after similar heat-inactivation. Therefore, it would appear that at least in vitro toxin Z is different than Liu's exotoxin A. In vivo, however, the picture is still not clear since in recent experiments (27), it was observed that heat-inactivation caused toxin Z to lose its virulence for mice. For these reasons, the toxins produced at the late stages of this study were not heat-inactivated (Table 8).

One advantage of heat-inactivation is that it destroys any protease that might be present. The fact that the majority of P. aeruginosa strains used in this study were found to be proteolytic made heat-inactivation a very useful tool in inactivating protease without any damage to the in vitro activity of toxin Z. However, in the later stages of the study, since heat-inactivation was not done, protease activity was neutralized by the addition of 10 percent calf serum prior to testing for toxin on HEp-2 cells.

The fact that serum neutralizes protease rules out the possibility that proteases play a significant role in the pathogenicity of P. aeruginosa. However, the fact that toxin Z is not inactivated by protease (Table 7) or serum suggests the possibility that it may play a role in the pathogenicity of P. aeruginosa. Most strains of P. aeruginosa are proteolytic strains and therefore the complicating factor of protease formation must be considered. Conversely, the few nonproteolytic strains like PA 103 can produce toxin Z without the formation of protease. This is the reason Liu used the PA 103 strain to produce exotoxin A because it is a nonproteolytic strain and exotoxin A is inactivated by protease or trypsin. Therefore, the non-susceptibility of toxin Z to protease illustrated in Table 7 is further in vitro evidence that toxin Z is different than exotoxin A.

The toxicity assays of toxin Z have been based on the qualitative cytopathogenic assay. The correlation of the various conditions of CPE as well as cell detachment has been largely a subjective process. In order to obtain a more objective measurement, a

quantitative cell culture assay was desirable. The quantitative assay, which measured the growth-inhibition of Clone II cells, provided a rapid detection and titration of toxic material. In the quantitative assay, Clone II cells have an obvious advantage over HEp-2 cells because Clone II cells are able to dissociate more readily into individual cells and thus permit better standardization of cell preparation yielding more consistent data. This method permitted the performance of a large number of assays more rapidly, economically, and with greater sensitivity, than by the use of animals. Furthermore, this quantitative assay compared favorably with the qualitative assay (Table 4), although the cytopathic effect characterized by cell rounding was often less intense than the corresponding growth-inhibition. The quantitative method was more objective and precise, allowed the detection of Clone II growth-inhibition, and required only 48 hours from initiation of cultures to their fixation with Lowry reagent.

Antitoxin Z was produced against toxin Z formed by PA 103 grown in HEp-2 cells with rabbit serum. In both qualitative and quantitative neutralization assays, antitoxin Z neutralized toxin Z-1 produced by serologically-different strains of P. aeruginosa under the Fisher schema; this indicates that all these strains of P. aeruginosa produce the same antigenic type of toxin Z. The quantitative assay proved to be a very useful tool in confirming the immunospecificity of toxin Z because the Ouchterlony immunodiffusion technique could not be applied as antitoxin Z was produced against a crude toxin and at least three precipitin lines were observed in the preliminary assay. However, the

Ouchterlony assay was effective in differentiating Liu's exotoxin A from toxin Z since in cross matching, Liu's exotoxin A only reacted to antiexotoxin A and toxin Z only reacted to antitoxin Z.

Further work, including the concentration and purification of this toxin, as well as in vivo experiments with laboratory animals, is expected to eventually yield information on the role of toxin Z in pathogenesis of P. aeruginosa infections.

SUMMARY

The method of preparing toxin Z in a closed non-aerated system yielded the highest concentration of toxin and therefore was considered the most economical technique. Such toxin was produced without HEp-2 cells and calf serum, thus, greatly reducing the extraneous protein content. This method allowed the production of toxin Z on a relatively large scale which is essential for future work on concentration and purification.

Toxin Z was shown to be different than Liu's exotoxin A on the basis of differences in protease susceptibility and heat-inactivation in vitro. Further evidence that toxin Z differs from exotoxin A was shown by Ouchterlony agar immunodiffusion assays in which antitoxin A reacted with exotoxin A but not with toxin Z while antitoxin Z reacted with toxin Z but not with exotoxin A.

By using qualitative and quantitative neutralization assays, toxin Z was found to be monospecific suggesting that different strains of P. aeruginosa produce the same antigenic type of toxin Z.

The in vitro methods developed in this research provide additional approaches to the study of P. aeruginosa disease processes. The qualitative and quantitative assays can be adapted to the study of other toxins elaborated by P. aeruginosa which may be involved in the pathogenesis of infections. Such toxins can be assayed in vitro for CPE and quantitatively for growth-inhibition. The specificity of these reactions can be determined by neutralization tests using homologous

and heterologous antisera. Such studies using in vitro models will be considerably more economical in time and money than similar efforts in vivo.

The role of toxin Z in pathogenesis is still unknown. However, the fact that toxin Z is not inactivated by protease and serum suggests the possibility that it may play a role in the pathogenicity of P. aeruginosa.

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