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Possible Role of Toxin in the Formation of Virus-Like Plaques by *Pseudomonas aeruginosa** (33864)

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(Introduced by Wayburn S. Jeter)

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Pseduomonas aeruginosa produces various toxic substances *in vitro* including hemolysin, lecithinase, and protease (1). In the formation of these materials, specialized bacterial growth media are required which differ considerably from the nutrients and conditions the organisms might encounter *in vivo* (2). If such toxic substances are responsible for the lethal effects of Pseudomonas infections, then the organisms must be capable of producing these lethal materials from cells, tissues, and body fluids in the host. Rabbit or human serum, however, when used as the sole source of nutrient *in vitro,* did not permit the organism to produce hemolysin or lecithinase and only small amounts of protease (2). Recently, a new lethal toxin formed *in vivo* has been described which apparently is not associated with *in vitro* activity (3). It has yet to be fully characterized but it appears to be a protein with a molecular size in the range of serum globulin (3).

Another approach to the study of factors responsible for the lethal effects of Pseudomonas infections is the use of an *in vitro* system which incorporates host cells in tissue culture. In this respect, *P. aeruginosa* was reported previously to produce viral-like plaques in monolayer cultures of human cells (4). The present report describes the possible role of toxins in the formation of such plaques by *P. aeruginosa* in HeLa monolayers.

Materials and Methods. Cell culture. The HeLa S_3 stock was propagated in milk-dilution bottles according to the methods previously reported (5). The medium used throughout consisted of 10% newborn calf serum (NBCS), 90% Eagle's basal medium (EBM) with Earle's balanced salt solution (BSS). A 0.25% trypsin solution was used to harvest cells for subculture.

Bacteria. The *P. aeruginosa* strain used was

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a pure culture contaminant of HeLa S_3 which was isolated from a technician whose infant child developed summer diarrhea. The organism was grown in the above medium for 6 hr at 37°, placed in ampules in 1-ml portions, and frozen in liquid nitrogen at —196°. Prior to use, an ampule was rapidly thawed in a 37° water bath and the contents were diluted with the same medium to the proper concentration.

Bacterial assay. Numbers of bacteria were estimated by the standard pour plate method using MPH-standard plate count agar (BBL).

Standard bacterial plaque system. Test tubes (16 × 125 mm) were seeded with 1 ml of growth medium containing 10^5 cells and incubated horizontally in a stationary position at 37°. When the cell monolayers were confluent (3–4 days), the medium was decanted and the monolayer was washed with BSS. A known inoculum of bacteria (usually 100) was added in 0.1 ml of medium, and the organisms were permitted to adsorb to the HeLa monolayers for 1 hr at 37°. Then the monolayers were washed with BSS; 1 ml of growth medium was added per tube; and the cultures were incubated at 37°. Tubes were examined microscopically (15–60×) at hourly intervals to determine the time when plaques became visible. When necessary, plaque counts were made 18–20 hr after bacterial inoculation.

Preparation of bacterial-free toxins. Sixteen-oz prescription bottles were seeded with 30 ml of medium containing 3 × 10^6 cells and incubated in a stationary position at 37°. When the cell monolayers were confluent, the medium was decanted, and the bottles were inoculated with 1 ml of medium containing 2 × 10^3 bacteria. After the organisms were permitted to adsorb for 1 hr at 37°, the monolayers were washed with BSS, and 30 ml of medium were added/bottle. The bottle cultures were incubated at 37° and examined microscopically at periodic intervals to determine the time of plaque formation as well as the extent of general cell destruction. At 23 hr (early plaques) and 48 hr (complete cellular degeneration) the cultures were harvested for toxin production. This included scraping any degenerating HeLa cells still attached to the glass with a rubber "policeman" into the medium, homogenizing the mixture in Ten Broeck glass homogenizers, centrifuging at 5090*g* for 25 min, and filtering the supernatant fluids through 0.45-μ Millipore membranes to remove bacteria.

Toxin assays. The sterile filtrates were serially diluted at twofold concentrations with medium and tested immediately for cytopathogenicity (CPE) to HeLa. Monolayers in tube cultures were washed with BSS and inoculated in duplicate with 1 ml of each dilution of sterile filtrate. After incubation at 37°, the tube cultures were examined microscopically at hourly intervals for CPE. This was graded 1+ to 4+ according to 25 to 100% cell destruction.

Results. Plaque formation. As shown previously (4), plaques formed by *P. aeruginosa* in HeLa monolayers were indistinguishable from viral plaques. Also, such bacterial plaques were not produced when NBCS was omitted from the medium. These findings were confirmed in the present study.

Cytopathogenicity of toxins for HeLa. Table I illustrates the results of a typical toxin

TABLE I. Production of Bacterial-Free Toxin Cytopathogenic for HeLa by *P. aeruginosa.*

Time (hr)		Cytopathogenicity to HeLa monolayers;[b] dilution of membrane-filtered toxin[c]			
Effect recorded	Toxin harvested[a]	Undiluted	1:2	1:4	1:8
19	23	±	—	—	—
	48	2+	2+	—	—
24	23	2+	±	—	—
	48	3+	2+	—	—
28	23	3+	±	—	—
	48	4+	4+	1+	—

[a] Time after bacteria were inoculated into bottle cultures of HeLa in 10% NBCS–EBM.

[b] Cytopathogenicity in duplicate HeLa monolayer tubes graded 1+, 2+, 3+, or 4+ according to 25, 50, 75, and 100% cell destruction, respectively.

[c] Dilutions made in 10% NBCS–EBM and 1 ml was added per HeLa monolayer tube previously washed with BSS.

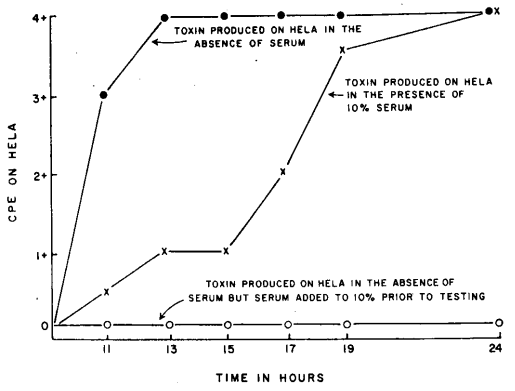

FIG. 1. Cytopathogenicity to HeLa monolayers of bacterial-free toxins produced by *P. aeruginosa* grown in HeLa in the presence or absence of serum.

experiment. The generalized toxic activity was not visible until 17 or 20 hr depending on whether the toxins were harvested at 48 or 23 hr after infection. In both cases, initiation of the CPE approximated the incubation time necessary for Pseudomonas to begin the induction of plaques in infected cultures. Futhermore, once the sterile filtrate initiated CPE, the HeLa monolayer was inevitably destroyed within 14 hr. This result also was characteristic of cultures infected with bacteria, for once plaques were first seen, approximately 14 hr were required for complete cellular degeneration. The fact that the time intervals for CPE coincided with approximately the same time intervals required for plaque formation suggested the possibility that toxin might be responsible for the actual formation of plaques.

Demonstration of more than one type of toxin. To examine the possibility that toxin might be responsible for bacterial plaque formation, *P. aeruginosa* toxins were prepared in HeLa monolayer bottles in the presence or absence of NBCS to take advantage of the fact that bacterial plaques are not formed in the absence of serum (4). Presumably, if a toxin for HeLa was not produced by *P. aeruginosa* when serum was omitted from the media, this would be indirect evidence that a toxin was responsible for plaques.

For this purpose 16-oz bottle cultures of HeLa were inoculated with *P. aeruginosa* using the medium described with or without NBCS. After 26 hr incubation at 37°, the cell–bacterial suspensions were harvested for toxin production, filtered, and assayed immediately for CPE in HeLa monolayer tubes. The results presented in Fig. 1 indicate that two types of toxin were produced. The most potent toxin was formed in the serum-free medium, producing 3+ CPE by 11 hr. However, this same toxin could be completely neutralized by addition of NBCS to a 10% concentration prior to testing. Conversely, the other toxin produced by *P. aeruginosa* in the presence of NBCS caused CPE which chronologically coincided well with the time plaques formed when the organism *per se* was added to HeLa monolayers. Furthermore, the requirement for serum in the production of such a toxin is in accord with the requirement for serum in the formation of plaques.

Discussion. The results of this study confirm the previous findings (4) that *P. aeruginosa* produces plaques in HeLa monolayers. The fact that NBCS is a medium requirement for plaque formation also was confirmed in the present work.

Indirect evidence is presented in this study to suggest that a toxin may be responsible for the formation of plaques by the bacteria. This evidence is based on the findings summarized in Fig. 2 which presents a tentative model of the possible relationship between toxin and plaques by *P. aeruginosa* in the mammalian cell system. The toxin aspect is presented on the left and the plaque aspect on the right. *P. aeruginosa*-infected HeLa cells maintained in the absence of serum produce one or more toxins designated here as Y. Toxin Y, when membrane filtered and added to HeLa produced rapid CPE (3+ in 11 hr). However, the addition of NBCS to a 10% level to this Y toxin completely neutralized the CPE.

Conversely, *P. aeruginosa* infected HeLa cells in the presence of NBCS formed a different type of toxin designated here as Z which after membrane filtration produced a slow CPE (3+ at 24 hr) on HeLa monolayers with or without the addition of NBCS. In this case, if Y toxin was produced, it was

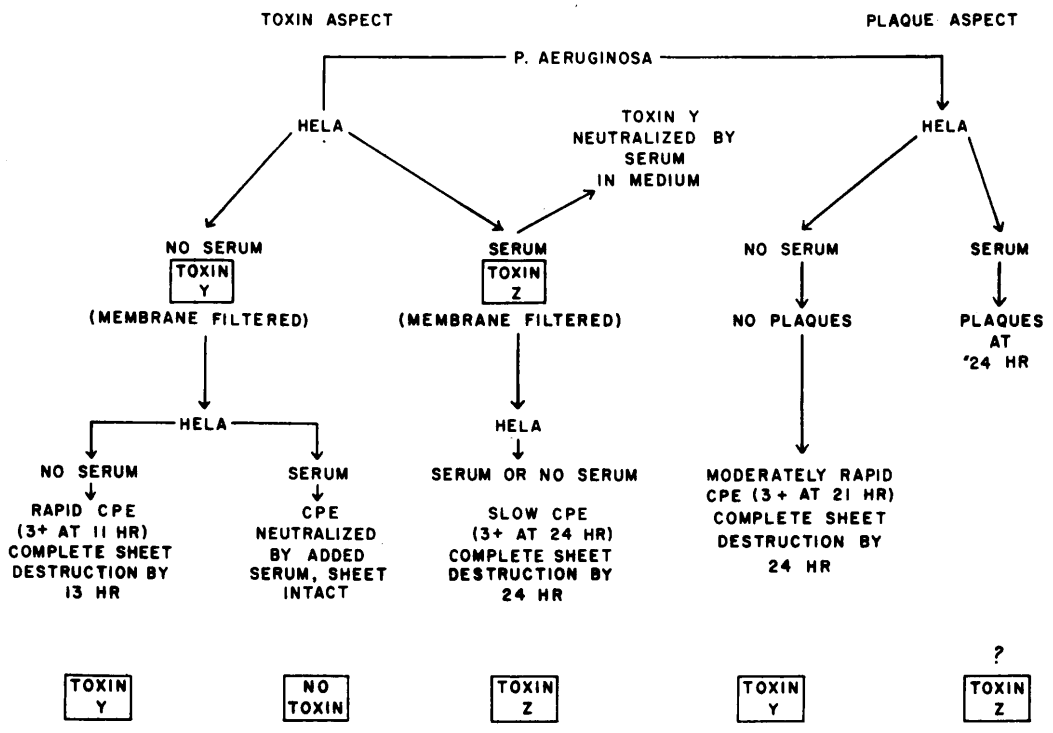

FIG. 2. Tentative model of the possible relationship between toxins and plaques produced by *P. aeruginosa* on HeLa monolayers.

apparently neutralized as it was formed by the serum in the medium.

The fact that toxin Z was produced in the presence of serum or required serum for its production coupled with the fact that *P. aeruginosa* formed plaques only in the presence of serum suggests that toxin Z may be implicated as the substance responsible for plaques in HeLa monolayers.

Liu (1–3, 6, 7) in a series of classic papers has described several toxic fractions of *P. aeruginosa* as assessed *in vivo*. In one of these reports (1), he studied the toxicity of various fractions of *P. aeruginosa* on HeLa cells. Fractions containing pyocyanin, slime, or dialyzable substances other than pyocyanin produced a delayed toxicity resulting in the rounding of HeLa cells and destruction after several hours. Fractions containing hemolysin or extracellular enzymes (proteases) produced instantaneous dissolution of the HeLa cells. More recently Liu (3) described a new lethal toxin formed *in vivo* which could not be correlated with any *in vitro* activity. This new toxin appeared to be a protein with a molecular size in the range of serum globulin (3). Our toxin Z has characteristics similar to Liu's lethal toxin. However, considerable work must be done to characterize both toxin Y and Z before any definite statements can be made as to their relationship to any of Liu's toxins.

It is hoped that the tissue culture approach to the study of the lethal effects of *P. aeruginosa* infections may lead to the development of methods to assess the virulence of various strains of this organism. Perhaps a clinically useful system based on the ability of the strains to produce plaque-forming toxin or plaques could be developed.

Summary. The possible mechanism involved in the production of virus-like plaques by *P. aeruginosa* in HeLa monolayers was investigated by testing the cytopathogenicity of sterile filtrates from the plaque-forming system on fresh HeLa monolayer cultures. The results indicated that two types of toxin were produced. One type designated Y was

produced in the absence of serum and could be neutralized by the addition of serum. Conversely, the second type of toxin designated Z required the presence of serum for its production and was not neutralized by the addition of fresh serum. Since bacterial plaques are formed only in the presence of serum, toxin Z is indirectly implicated as the causative factor responsible for plaques in HeLa monolayers.

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The Influence of Phenoxybenzamine (Dibenzyline) on the Pulmonary Veins of Intact Anesthetized Dogs* (33865)

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The pulmonary veins of intact dogs have been shown to be active in hemodynamic responses to acute changes in the circulating blood volume (1), immersion hyperthermia and hypothermia (2), and administration of histamine (3). The effects of phenoxybenzamine, a potent alpha-adrenergic blocking agent, on the systemic circulation are well known (4–6), whereas its effects on the pulmonary circulation, especially the veins, have received little attention. Studies were undertaken to observe the pulmonary venous response to phenoxybenzamine infusion in intact dogs anesthetized with urethane.

Material and Methods. Eleven adult mongrel dogs weighing 14.8–18.2 kg (mean, 16.5 kg) were lightly anesthized with urethane (1.5 g/kg). After intubation with an endotracheal tube, a mixture of 100% oxygen with room air was given at the rate of 2.5–3 liters/min to prevent the animal from becoming anoxic. Final oxygen concentration of the gas mixture at these flow rates probably ranged between 35 and 55% (7).

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Under fluoroscopic control, cardiac catheters were passed transeptally into a small pulmonary vein (i.d., 0.51 min; o.d., 0.91 mm) and left atrium (i.d., 1.5 mm; o.d., 2.8 mm) as described previously (1, 2). Separate catheters (i.d., 1.2 mm; o.d., 2.2 mm) were positioned in the main pulmonary artery just beyond the pulmonary valve and at the junction of the inferior vena cava and the right atrium. Polyethylene tubes were inserted into the right femoral artery, right femoral vein, and percutaneously into a small vein in the left hind leg. It has been shown (10) that the catheters in the pulmonary veins do not obstruct venous flow sufficiently to interfere with the experiment.

All catheters were connected by polyethylene tubing to strain gauge transducers[1] and mean pressures were recorded simultaneously on a multichannel oscillographic recorder.[2] Zero levels were determined 7–9 cm above the top of the fluoroscopic table.

Cardiac output and pulmonary blood volume were measured by the dye-dilution technique, using the Stewart–Hamilton formula (8). A known quantity of indocyanine dye

[1] Statham P23Db strain gauge transducers.

[2] Electronics for Medicine recorder.