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Pseudomonas aeruginosa Exotoxin: Effect on Cell Cultures

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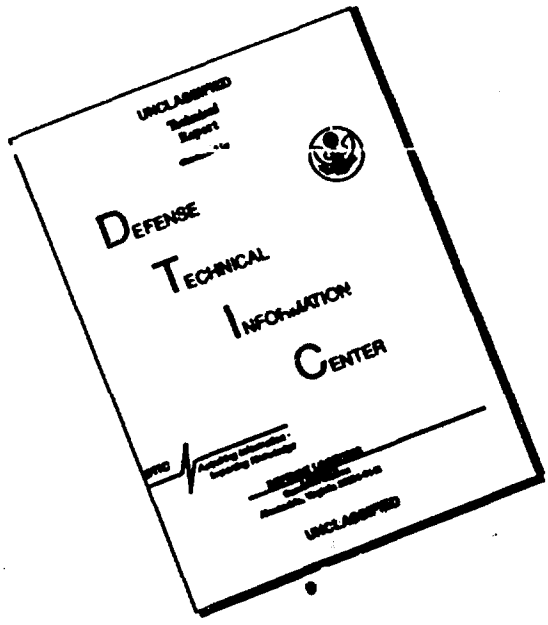

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13. ABSTRACT

An exotoxin, toxic to both mice and cultured cells, was isolated from cultures of Pseudomonas aeruginosa. Relatively small amounts of the exotoxin inhibited the uptake of uridine and amino acids by Vero cells. Within limits, this toxic action was reversible and could be inactivated by heating at 70 C or by proteolytic digestion, but it was not affected by nucleases. The inhibitory activity of the toxin could also be decreased by rabbit antiserum. Metabolic activities of Vero cells, generally associated with the production of energy, did not appear to be affected by the toxin.

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Pseudomonas aeruginosa Exotoxin: Effect on Cell Cultures

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An exotoxin, toxic to both mice and cultured cells, was isolated from cultures of *Pseudomonas aeruginosa*. Relatively small amounts of the exotoxin inhibited the uptake of uridine and amino acids by Vero cells. Within limits, this toxic action was reversible and could be inactivated by heating at 70 C or by proteolytic digestion, but it was not affected by nucleases. The inhibitory activity of the toxin could also be decreased by rabbit antiserum. Metabolic activities of Vero cells, generally associated with the production of energy, did not appear to be affected by the toxin.

Pseudomonas aeruginosa produces a number of toxic substances [1–10] that have been implicated, each to an unknown extent, in the pathogenicity of the organism. In addition, a relatively pure preparation of exotoxin was isolated [7] which, when infused into dogs, caused shock characterized by circulatory and biochemical changes similar to those of endotoxic shock [9]. Since it has been shown that the endotoxin of *P. aeruginosa*, unlike that of the other gram-negative organisms, does not contribute significantly to the pathogenicity of the organism [1, 11], the possibility has been suggested that the exotoxin is responsible for shock in cases where *P. aeruginosa* has not invaded the bloodstreams of patients [9]. At the present time, however, the mode of action of this exotoxin has not been determined. This report describes studies on the effect of the exotoxin on cells in tissue culture. We were encouraged in this study by a recent report that *P. aeruginosa* produces virus-like plaques in monolayers of tissue cultures [12], and that this phenomenon is due, at least indirectly, to the production of a toxin by the organism [13].

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The experiments reported herein were conducted according to the "Guide for Laboratory Animal Facilities and Care" of the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences —National Research Council.

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The opinions or assertions contained herein are those of the authors and are not to be construed as official or as reflections of the views of the Department of the Navy or of the Naval Service in general.

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Materials and Methods

P. aeruginosa. Strain PA-103 [8], originally isolated from human sputum and producing neither protease nor hemolysin, was kindly supplied by Dr. P. V. Liu.

Production of exotoxin. The procedure of Liu [7, 9] was slightly modified. Before precipitation with 50% saturated $(NH_4)_2SO_4$, the solution was dialyzed, and the nondialyzable material was fractionated by passage through an ultrafiltration cell with a membrane that retained substances of molecular weights greater than 30,000 (Diaflo PM 30, Amicon Corp.). The retained fraction was used. Finally, the exotoxin was filtered through a 0.45-μ membrane, tested for sterility, and frozen in aliquots until used.

Exotoxin labeled with ^{14}C was produced by growing the organism in the presence of a ^{14}C-labeled L-amino acid mixture (New England Nuclear) added to the standard growth medium. The exotoxin was dialyzed to remove the amino acids.

The final preparation was tested on a Sephadex G-15 column to determine whether or not any free amino acids were present. All of the radioactivity was found in the void volume with the toxin, and none of it was associated with the marker amino acids.

Tissue-culture cells. The results of experiments using cell cultures in vitro are expressed as averages of triplicate sets of cells.

African green monkey-kidney cells (Vero) were used. The growth medium was Eagle's minimal essential medium (MEM, Grand Island Biological Co.) supplemented with 5% fetal calf serum, L-glutamic acid, and MEM vitamins (Grand Island Biological Co.).

PS-Y15 (porcine kidney) cells were received from Y. Kanda Inoue [14]. The cells were grown on Eagle's MEM containing 10% calf serum, L-glutamate, and 5% lactose-albumin hydrolysate.

LLC-MK_2 cells (no. ccl 7.1, Rhesus monkey kidney) were received from the American Type Culture Collection Cell Repository and maintained on Eagle's medium with Earle's saline and 5% calf serum.

All growth media contained penicillin (100 units/ml) and streptomycin (100 μg/ml). The cells were grown in monolayers in either 2-oz prescription bottles or rolling bottles (no. 7000, Bellco Biological Glassware Co.).

Determination of cell viability. Trypsinized cells were suspended in 0.15% eosin-Y (Difco Laboratories, Detroit, Mich.) for approximately 3 min, and placed in a hemocytometer. Stained (nonviable) and unstained (viable) cells were enumerated.

Digestion of tissue-culture cells and counting procedure. The cells were collected and washed three times with 0.15 M Hepes buffer (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, *p*H 7.2, Calbiochem). The cells were then transferred to counting vials and incubated overnight with 1.5 ml of tissue solubilizer (Protosol, New England Nuclear) at 55 C. The turbidity of the digested suspension disappeared after the addition of 2.5 ml of methanol. Fifteen milliliters of scintillation fluid was added to the vials according to a procedure used in our laboratory [15], and the samples were counted in a liquid-scintillation counter.

Lactic-acid determination. Concentration of lactate was determined according to the UV method published by Boehringer Mannheim Corporation.[1] Lactic dehydrogenase, A-grade, was obtained from Calbiochem. The chemical-reagent solutions used in the test were prepared in our laboratory.

Manometric assay. Uptake of oxygen by tissue-culture cells was measured at 37 C with a conventional Warburg apparatus. The cells were suspended in twice concentrated MEM diluted with an equal volume of 0.15 M Hepes buffer (*p*H 7.2). Each flask contained 5 mg of glucose, 100 units/ml of penicillin, 100 μg/ml of streptomycin, 52 μg of exotoxin in the side arm, and 0.4 ml of 40% KOH in the center well. The total volume was 3 ml. After 10 min of equilibration, the contents were mixed.

Enzyme treatment of exotoxin. Pronase was obtained from Calbiochem and self-digested for 2 hr at 37 C. DNase, grade B, from Calbiochem, and RNase from Worthington Biochemical were used. The toxin was incubated in Hepes buffer (0.01 M, *p*H 7.5) with the respective enzyme for 24 hr at 37 C. The controls consisted of the inactivated enzyme incubated with the toxin and the enzyme suspension without toxin. The suspensions were sterilized by filtration before incubation. No attempts were made to inactivate the enzyme after incubation of the toxin.

Animals. Swiss white mice of either sex (NIH-NMRI inbred strain, weighing 20–25 g) were used. For titrations of toxin, only female mice were used. The LD_{50} was expressed as μg of protein/g of body weight.

Immunization procedure. Rabbits weighing 2,400–3,100 g were injected once a week for four weeks. The first injection of 130 μg of toxin was given iv, followed by an im injection a week later. The next two inoculations of 260 μg of toxin each were mixed with an equal volume of Freund's incomplete adjuvant and given sc. After two weeks another sc injection, similar to the previous two, was given. Two weeks later the rabbits were bled and the serum titered. The antiserum completely protected mice against the exotoxin when 0.25 ml of the serum, up to a 1:16 dilution, was preincubated with an equal volume of exotoxin (2 LD_{50}) before iv injection.

[1] Article no. 15972, 1963.

Results

Toxicity in mice. Initial studies were performed to determine the LD_{50} for mice of the various fractions precipitated with ammonium sulfate. Three fractions of different molecular weights were tested by iv injection: (*1*) the dialyzable material; (*2*) the nondialyzable material after passage through the ultrafiltration membrane PM 30 (mol wt, <30,000); and (*3*) material retained by the membrane (mol wt, >30,000). It was found that most of the toxicity resided in the 50% $(NH_4)_2SO_4$ precipitate of the fraction of mol wt >30,000, as determined by ultrafiltration. The LD_{50} for mice of different preparations of exotoxin was relatively uniform (table 1).

Like the exotoxin isolated by Liu, our toxin could be inactivated by heating at 70 C or by proteolytic digestion [7]. Nucleases did not affect its activity.

Cell toxicity. In order to select a cell line for study of the mode of action of the toxin, Vero, PS-Y15, and LLC-MK_2 cell lines were tested. Cell layers growing in 2-oz prescription bottles were exposed to graded amounts of exotoxin for 24 hr. After incubation the cells were washed with saline and overlaid with fresh medium containing 1% agarose and neutral red diluted 1:12,000. Periodic microscopic examination indicated that the Vero cells were more sensitive to the exotoxin than the other cell lines. Vero cells, at levels of exotoxin (1.0 μg) that did not affect PS-Y15 and LLC-MK_2 cells, showed a marked cytopathic effect and did not take up the vital dye.

When a ^{14}C-labeled preparation of toxin was added to a culture of Vero cells, it could be shown that, even though the cell count remained constant (controls, 2.4×10^6 cells per bottle; toxin treated, 2.7×10^6 cells per bottle), the toxin became associated with the cells at a rate which remained linear for at least 12 hr (figure 1).

Effect of toxin on uptake of uridine. The addition of various amounts of unlabeled toxin to cells growing in the presence of uridine-2-^{14}C resulted in a rapid dose-dependent decrease of ^{14}C uptake (figure 2). A direct relation appeared to exist between the amount of toxin added and the time required for significant inhibition of uridine uptake. Readings at 20 hr (not shown) indicated that, even at the lowest dose, 50% inhibition took place in about 24 hr. Similar results were obtained with ^{14}C amino acids. The decrease in uptake of ^{14}C could not be attributed either to lysis or to death of cells. The total cell count and the viable-cell count, as determined by dye exclusion (eosin-Y), remained approximately the same for both the exotoxin-treated and the untreated cells.

The three preparations of exotoxin were compared for lethal activity in vivo and were found to have similar activity in inhibition of uridine-

Table 1. Biological activity of different preparations of *Pseudomonas aeruginosa* exotoxin.

Toxin preparation	In vivo LD_{50} (μg of protein)	In vitro* LD_{50} used	In vitro* ^{14}C-uridine uptake inhibition (%)†
PS 5	1.21	2.1	30.8
PS 14	1.14	2.1	34.0
	1.29		
PS 24	1.07	2.3	36.0
	1.22		

* Vero cells (2.0×10^5 viable cells).
† 2.6 μg of exotoxin used.

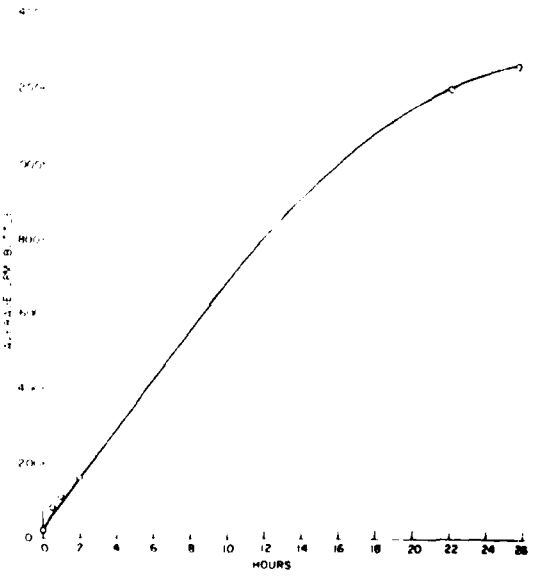

Figure 1. The uptake of *Pseudomonas aeruginosa* exotoxin labeled with ^{14}C by Vero cells (2.7×10^6 viable cells per bottle).

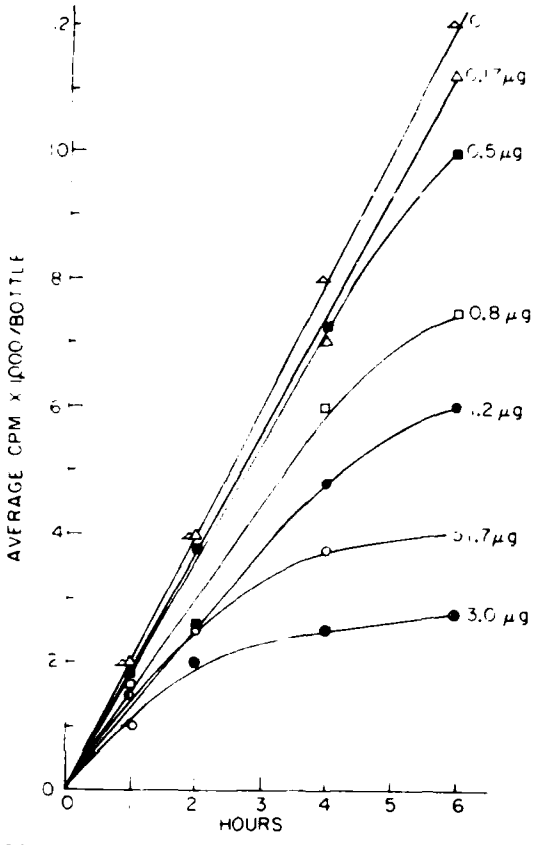

Figure 2. The effect of different amounts of *Pseudomonas aeruginosa* exotoxin on the uptake of uridine-2-^{14}C by Vero cells (2.6 × 10^6 viable cells per bottle).

2-^{14}C uptake by Vero cells (table 1). A dose that roughly corresponded to 2 LD_{50} inhibited about 35% of the uridine uptake.

The above described action of the toxin on Vero cells was reversible to certain extents (figure 3). The cells were grown in the presence of uridine-2-^{14}C, and the toxin was added 3 hr later. At various intervals, the cells were washed free, and fresh medium containing uridine-2-^{14}C was added. When the toxin was not removed, uptake of uridine ceased approximately 4 hr after the addition of toxin. If the cells were washed within this period of time, no reduction in uptake of uridine was demonstrable. After 4 hr, and as late as 20 hr, washing permitted a spurt of activity to take place, but the original rate of incorporation was not maintained.

A dose-dependent inactivation of the exotoxin

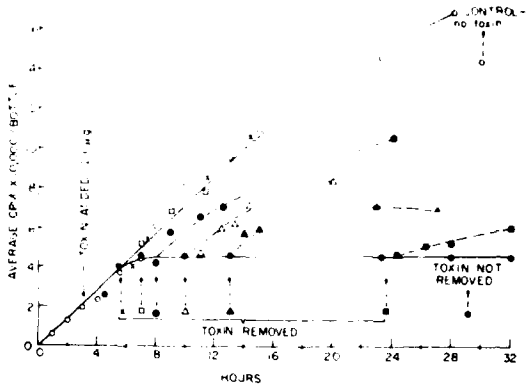

Figure 3. Uptake of uridine-2-^{14}C by Vero cells treated with *Pseudomonas aeruginosa* exotoxin (6.2 × 10^6 viable cells per bottle).

by antiserum was shown. A 0.1-ml sample of the exotoxin (2.6 μg) was added to various amounts of antiserum; the suspension was adjusted to a total volume of 0.4 ml with normal rabbit serum and was incubated for 30 min. The mixture of toxin and antitoxin was then added to Vero cells growing in the presence of uridine-2-^{14}C, and radioactive uptake was measured (figure 4).

Effect of toxin on metabolism of Vero cells. The occurrence of elevated levels of lactate in serum in both hemorrhagic and septic shock has been recognized [16–18]. Acidosis due to lactic acid has also been demonstrated in dogs in shock after the infusion of *P. aeruginosa* exotoxin [9].

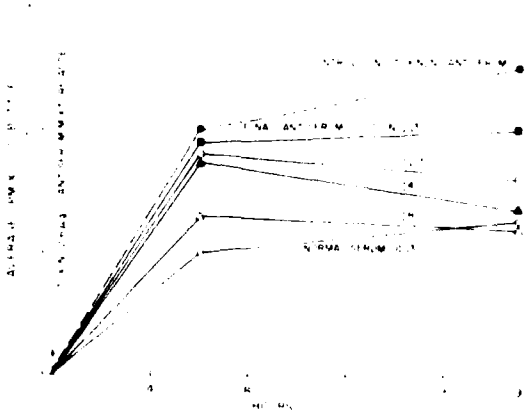

Figure 4. The effect of *Pseudomonas aeruginosa* exotoxin (preincubated with antiserum and normal serum) on the uptake of uridine-2-^{14}C by Vero cells (2.6 × 10^6 viable cells per bottle).

Therefore, it was of interest to investigate the anaerobic metabolism of tissue-culture cells in the presence of toxin. Actively growing cells in 2-oz prescription bottles were washed, fresh medium without phenol red containing uridine-2-^{14}C was added, and the cells were incubated. The exotoxin was added at zero-time. At given time periods, the medium from growing cells was collected from triplicate bottles, and the concentration of lactic acid was determined. The cells were harvested, and uptake of radioactivity was measured. The results (figure 5) show that even though the uptake of uridine decreased in the cells treated with toxin, the production of lactic acid proceeded at the same rate in treated as in untreated cells.

Since the anaerobic metabolism appeared not to be affected by the exotoxin, the possibility existed that the exotoxin may have altered the oxidative metabolism in a manner similar to that of endotoxin [19, 20]. To detect any changes in cellular respiration, uptake of oxygen by tissue-culture cells was measured, using the standard manometric assay. Vero and MK_2 cells were grown in roller bottles, harvested with trypsin, and washed. The cells were then suspended in the reaction solution containing uridine-2-^{14}C and 52 μg of exotoxin, and oxygen uptake was measured. Routine assays after the run showed that the suspensions were free of any significant bacterial contamination. The results showed that, although uptake of uridine decreased in the cells treated with toxin, as expected, no difference in uptake of oxygen was observed between the treated and untreated cells. After 180 min, the controls had consumed 78.5 μliters and the toxin-treated cells, 75.0 μliters of oxygen.

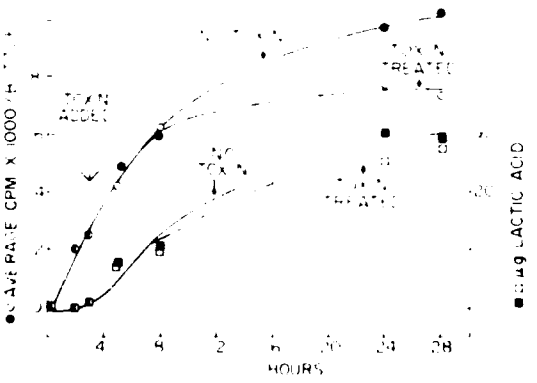

Figure 5. The effect of *Pseudomonas aeruginosa* exotoxin on the uptake of uridine-2-^{14}C (● and ○) and the production of lactic acid (■ and □) in Vero cells (2.6×10^5 viable cells per bottle).

Discussion

The purpose of this study was to investigate the action of *P. aeruginosa* exotoxin at the cellular level. The data indicate that the energy-generating systems in the cell are not affected by the exotoxin at levels that inhibit uptake of uridine and amino acids. Both in glycolysis (figure 5) and in respiration, there was a significant decrease in uptake of uridine before either production of lactic acid or uptake of oxygen changed.

A direct relation was found between the decrease in uptake of ^{14}C-uridine and the concentration of exotoxin (figure 2). Even though the ^{14}C-labeled exotoxin was associated immediately with the cells (figure 1), it seems that there is a lag period before the toxic activity of the toxin can be expressed, regardless of the amount of toxin used (figure 2). This lag period may be necessary for the irreversible adsorption of toxin (figure 3). The results shown in figure 3 indicate that for several hours after contact, the toxin apparently can be removed from the cells without any damage to them. There is irreversible damage only after 5 hr of exposure to the toxin.

The effects of the toxin demonstrated in cell cultures are not necessarily the only effects or the critical ones that occur in the intact animal and result in death. Nevertheless, when tested simultaneously (table 1), the several preparations of toxin possessed similar titers of in-vitro and in-vivo effects, indicating that the simpler and more rapid in-vitro test can be used as an estimation of the lethal titer (in mice) of preparations of toxin. Cells in culture also proved to be potentially useful for evaluating antiserum (figure 4).

Either one of two general mechanisms can be postulated to explain the above data. The first possibility may be that the toxin acts initially on the cell membrane, resulting in the secondary disruption of an internal process, such as synthesis of proteins or nucleic acids. The second possibility may be that the toxin acts directly on some intracellular mechanism. Although sufficient data are not available, the present information appears

to support the first postulate. The lag period as well as the reversibility of the toxic action can best be explained by the assumption that the membrane is affected before disruption of internal processes.

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