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Characterization of Exotoxin of *Pseudomonas aeruginosa*

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Heat-labile exotoxin of *Pseudomonas aeruginosa* has been purified and concentrated. Studies in mice have shown that the toxin affects protein synthesis in the liver within 3 hr of administration, whereas inhibition of protein synthesis in other organs occurs only during terminal stages. In short-term experiments in vitro, purified exotoxin did not inhibit ingestion or killing of bacteria by human polymorphonuclear leukocytes.

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Pseudomonas aeruginosa is known to elaborate various toxic fractions that are demonstrable in vitro and in experimental animals. A heat-labile exotoxin has been described [1, 2]. This toxin is lethal for mice [1] and is capable of eliciting hypotensive shock in stumptail rhesus monkeys (O. R. Pavlovskis and C. M. Herman, unpublished data) and in dogs [3]. We have successfully isolated and separated the toxin from contaminating materials and studied some of its biochemical properties [4]. We have also investigated the biological activity of the toxin at cellular and subcellular levels in vitro and in vivo [5–7]. Finally, a study was made to determine if exotoxin alters the ability of white cells to phagocytize bacteria, since this mechanism was shown to be an important factor in protection against and survival of infections due to *P. aeruginosa* [8, 9]. This report summarizes our more recent results.

The opinions or assertions contained herein are those of the authors and are not to be construed as official or as reflecting the view of the Department of the Navy or the naval service in general.

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The animals used in this study were handled in accordance with the provisions of Public Law 89-44 as amended by Public Law 91-579, the "Animal Welfare Act of 1970," and the principles outlined in the "Guide for the Care and Use of Laboratory Animals," U.S. Department of Health, Education and Welfare Publication No. (NIH) 72-23.

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Experimental Procedures and Results

Purification and characterization of the toxin. The purification of the exotoxin of *P. aeruginosa* strain PA 103 consisted of four major steps: membrane ultrafiltration, adsorption (hydroxylapatite), anion exchange (DEAE cellulose), and gel-permeation (Sephadex G-150) chromatography. The procedure resulted in the recovery of 48% of the exotoxin with a 40-fold increase in specific activity (μg of protein per LD_{50}). The LD_{50} of the purified preparation administered iv into mice weighing 20 g was approximately 6 μg of protein. The toxin preparation contained virtually no nucleic acid, pigment, or lipopolysaccharide (LPS). When assayed for purity by sodium dodecyl sulfate polyacrylamide gel electrophoresis, the toxin preparation was observed to contain several protein components, which migrated during electrophoresis at similar rates, and, therefore, appeared to have similar molecular weights. The estimated molecular weight of the toxin is 54,000, and its isoelectric point is 5.0.

Biological activity of the toxin. Previous work in this laboratory has demonstrated that the toxin inhibits uptake of amino acid and uridine by tissue culture cells [5]. Since tissue culture cells do not represent a true in vivo system, we studied the localization of the toxin in various organs of the mouse and its effect on protein synthesis [7].

The distribution of iv-administered 2-LD_{50} doses of ^{125}I-labeled toxin was studied and compared with the fate of ^{125}I-toxoid (toxin heated at 56 C for 10 min) [4] and ^{125}I-bovine serum albumin (BSA). Toxin appeared to be cleared from blood much more rapidly than BSA. For example, the proportions of ^{125}I recovered from the blood of

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mice treated with toxin and BSA were 5% and 30% after 2 hr, and 1% and 10% after 16 hr, respectively. The difference in the rates of clearance between toxin and toxoid was small, but in almost every instance the rate of clearance was greater for toxoid.

The highest concentration of radioactivity was observed consistently in the kidneys of toxin-treated mice. This may represent degradation of toxin, because about 30% of the radioactivity was recovered (as a component soluble in trichloroacetic acid) from the urine within 2 hr after injection. Lesser amounts of the label were recovered from the liver and spleen. Uptake in heart, pancreas, lung, and brain was very small. The fate of ^{125}I-toxoid was similar to that of the toxin. In contrast, in mice treated with ^{125}I-BSA, the label was distributed uniformly among the organs examined, but the concentration was low.

Since previous experiments in this laboratory had indicated that the toxin affected the respiration of mouse liver cells [6], the effect of toxin (2 LD_{50}) on protein synthesis in mouse liver was investigated. Incorporation of amino acid in the liver was reduced by more than 50% during the 2–4-hr interval after injection. At 16–18 hr after inoculation, protein synthesis was reduced to a very low level. No significant changes in protein synthesis were seen when either toxoid or 0.25 μg of LPS of *P. aeruginosa* strain PA 103 was used. The amount of LPS injected corresponded to 90 times the amount of LPS present in 2 LD_{50} of toxin. Thus it appears that inhibition of protein synthesis in the liver is closely associated with biological activity of the exotoxin.

Next, experiments were done in which liver, kidney, spleen, heart, pancreas, lung, and brain were studied simultaneously. Kidney and spleen displayed a slight reduction of protein synthesis 2–4 hr after inoculation. In the other organs, incorporation of amino acid decreased only when the animals approached the terminal stage (18 hr after inoculation).

Therefore, the highest concentration of toxin appears to occur in the kidney, where it is degraded, but the greatest toxic effect takes place in the liver.

Effect of exotoxin and endotoxin on polymorphonuclear leukocytes. LPS of *P. aeruginosa* has been shown to confer type-specific humoral immunity [10], and its administration as vaccine to patients with impaired host defenses results in modest but significant protection in reducing the mortality of infections with *P. aeruginosa* [9]. In addition, LPS of *P. aeruginosa* has been shown to inhibit phagocytosis of the organism by polymorphonuclear leukocytes [10]. In order to study the effect of exotoxin on white cells, it was necessary to determine the amount of LPS present in toxin preparations. The *Limulus polyphemus* assay for LPS was performed to determine the amount of LPS in preparations of exotoxin used. Limulus lysate was obtained from Sigma Chemicals (St. Louis, Missouri) or from bleedings of horseshoe crabs by the method of Levin and Bang [11]. Standardization of the assay was achieved by use of LPS from *Escherichia coli* O111-B4 (Difco, Detroit, Michigan) and *P. aeruginosa* (phenol-water extraction) in known concentrations in pyrogen-free glassware. The exotoxin of *P. aeruginosa*, purified by the four steps described above and containing 3.5×10^{-4} g of protein/ml, had no LPS demonstrable by limulus assay at a sensitivity of 10^{-9} g/ml. Fraction A derived from the second step in purification, hydroxylapatite adsorption chromatography [4], which contained the major portion of the pigment and no exotoxin, contained 10^{-6} g of LPS/ml. Fraction C from the same step [4], which contained the major portion of the nucleic acids and no exotoxin, contained 10^{-5} g of LPS/ml.

In order to study the possible interactions of the exotoxin and LPS, direct and indirect tests of phagocytosis were performed with normal human polymorphonuclear leukocytes according to the methods of Hirsch and Cohn [12] and of Young and Armstrong [8]. In addition to crude and purified preparations of exotoxin, amounts of purified LPS corresponding to the amounts found in fractions A and C or crude preparations were also tested. *Staphylococcus albus*, serum-sensitive *P. aeruginosa* PA 103 (Fisher type 2), and serum-resistant *P. aeruginosa* 2127 (Fisher type 2) were the test organisms. Crude fractions of exotoxin preparations were shown to inhibit killing, but not uptake, of bacteria ingested by polymorphonuclear leukocytes. More highly purified preparations of exotoxin, however, had no effect on ingestion or killing of bacteria when added to polymorphonuclear leukocytes either at the beginning of the

phagocytic test or after incubation with polymorphonuclear leukocytes for 1 hr at 37 C. Fractions A and C inhibited phagocytosis only to a degree corresponding to contaminating LPS as demonstrated by the limulus assay and did not contain any heat-labile inhibitors of phagocytosis.

Thus it appears that the exotoxin elicits no short-term effects on the phagocytic capabilities of polymorphonuclear leukocytes. In an attempt to study long-term effects, rabbits were injected iv with an estimated mouse LD_{50} of exotoxin, which was adjusted on the basis of the weight of the rabbit (0.5 mg protein). At 24, 48, and 96 hr after injection, neutrophilis from these rabbits were harvested and were found to ingest and kill *P. aeruginosa* normally.

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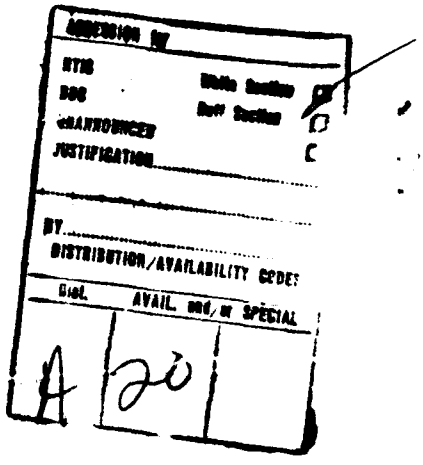

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13. ABSTRACT

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