In Vivo Studies with a Toxic Fraction of *Pseudomonas aeruginosa*[1] (35051)

GERALDINE MEINKE AND RICHARD S. BERK

Department of Microbiology, Wayne State University, School of Medicine, Detroit, Michigan 48207

Despite increasing recognition of *Pseudomonas aeruginosa* as a human pathogen, relatively little is known about the mechanism by which this organism exerts its damaging effects on the host. Suggestions have been made that the diffusion of toxic bacterial products from sites of massive bacterial invasion could affect circulatory and metabolic functions as well as host defenses in such a way that death could result (1–4). It has been observed that extracts from agar and broth cultures of *P. aeruginosa* contain toxic substances including a hemolysin, lecithinase, and protease. Some of these factors are capable of causing local or systemic lesions (5–7). The presence of these factors alone, however, does not appear to account totally for the virulence of *P. aeruginosa*. Other studies (8, 9) have also implicated a heat-labile protein toxin in the virulence of this organism. Recently, a nonproteolytic protein factor lethal for mice was isolated in our laboratory as a by-product of the purification of a protease exhibiting elastase activity (7). This factor was found to be heat stable; thereby, differentiating it from the toxin described by Liu (8). This paper describes some of the *in vivo* properties of this nonproteolytic toxic fraction.

Materials and Methods. A strain of P. aeruginosa (E2) isolated from a patient at Detroit General Hospital was employed in this study (7). Organisms were cultured on a medium composed of 2% tryptone (Difco), 1% glucose, 0.5% sodium chloride, and 1.5% agar. The entire surface of agar plates were swabbed with 18-hr cultures of E2 previously grown in 2% tryptone–glucose broth and then incubated for 3 days at 37°.

After the prescribed incubation period, the plates were stored at −10 to −20° overnight and then allowed to thaw at 4° so that proteases were eluted from the agar. The collected agar filtrates were freed of bacteria by centrifugation at 27,000*g* for 2 hr prior to fractionation steps. After several precipitation steps employing $MnCl_2$, $(NH_4)_2SO_4$, and acetone (7) the nonproteolytic fraction was isolated after separation from the protease by column chromatograpy on DEAE A-50 Sephadex (Pharmacia). The toxic nonproteolytic fraction failed to adsorb to the column material and traveled with the solvent front while the protease remained adsorbed to the column. The wash buffer consisted of 0.02 *M* Tris–HCl buffer, pH 8.6, at 4°.

Protein was determined by a modification of the Lowry method (10) with bovine serum albumin (Armour) used in the construction of a standard curve. The protease assay was based on the casein digestion method (11) as described previously (7). Filtrates were assayed for a relative concentration change in trichloroacetic acid-soluble protein components by the use of the modified Folin phenol method (10). Hemolysin titers were performed as described previously (12). The reciprocal of the highest dilution showing complete hemolysis was taken as an approximation of the hemolytic units per milliliter present in the preparations. An egg yolk assay previously described (5) was utilized for lecithinase while reducing sugars were detected by the modified ferricyanide method using glucose as a standard (13). Thiobarbiturate-reacting materials were quantitated by use of the modified 2-keto-3-deoxyoctonate determination (14, 15). Deoxyribonucleic acid

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was estimated by diphenylamine procedure (16), while the orcinol procedure (17) was used to quantitate ribonucleic acid.

Disc electrophoresis in polyacrylamide gel was carried out by the method of Davis (18) using 0.012 *M* Tris–HCl–0.08 *M* glycine buffer, pH 8.5. Gels were stained with 1% amido black in 7% acetic acid. A constant current of 4 mA/tube was used. Electrophoresis was allowed to proceed until the bromphenol blue marker reached the end of the gels. Samples were then destained electrophoretically.

Studies were performed *in vivo* with unheated and heated samples isolated from DEAE A-50 columns. Fractions were dialyzed against distilled water and then concentrated against Aquacide No. 2 (Calbiochem). Preparations were filter sterilized and tested for sterility by inoculating tryptone–glucose agar plates and broth with 0.1-ml samples. Animals used in these studies were white female mice (av wt, 20 g). The lethality of the nonproteolytic fraction was determined by intravenous, intraperitoneal, intranasal, and subcutaneous administration of twofold serial dilutions into groups of five mice each. The doses for the various routes were the following: intraperitoneal, 1.0 ml; intravenous, 0.5 ml; intranasal, 0.05 ml; and subcutaneous, 0.2 ml. Animals were observed at several time intervals over a period of 72 hr and the LD_{50} values were calculated according to the method of Reed and Muench (19). Control animals received equivalent amounts of sterile pyrogen-free distilled water. Groups of six mice each were injected both intraperitoneally and intravenously with samples of both unheated and heated toxic fractions. Samples were heated for 30 min at 100°. Control animals received an equivalent amount of sterile pyrogen-free distilled water. Animals were sacrificed by cervical detachment in groups of three at two predetermined time intervals (3 and 24 hr after injection). Sacrificed animals were autopsied immediately. After group postmortem observations were recorded, the liver, lungs, kidneys, spleen, heart, intestines, and stomach were removed and fixed in 0.1 *M* phosphate buffered formalin (4%), pH 7.0. Selected tissue specimens were then processed and stained with hematoxylin and eosin.

TABLE I. LD_{50} Values (μg of protein) for Mice Injected with Heated and Unheated Nonproteolytic Samples Calculated After 72 hr.

Nonproteolytic fraction (μg of protein)	LD_{50} (μg of protein); route of administration:			
	ip	iv	in[a]	sc[a]
Unheated	237	105	>100	>400
Heated	237	118	>100	>400

[a] No toxicity was observed in or sc when concentrations were administered up to 100 and 400 μg of protein/mouse, respectively. For the determination of the LD_{50}, sets of five mice were used with each dilution tested.

Results. Prior to *in vivo* studies, a preliminary *in vitro* characterization of the toxic fraction was initiated. No proteolytic, hemolytic, or lecithinase activity was detected in pooled, concentrated preparations, although crude agar extracts contained 8 protease units/ml and 16 hemolytic units/ml. The protein content consisted of 2 mg/ml. It was this unusually high concentration of protein which first suggested the presence of a fraction other than protease. In addition to protein, the toxic fraction contained 210 μg of RNA/ml, 10 μg of DNA/ml, 10.8 μg/ml of reducing sugar, and 57 μg of thiobarbiturate-reacting material. Disc electrophoretic studies indicated the presence of two bands as opposed to at least nine in the crude agar extracts.

The mouse LD_{50} values of *P. aeruginosa* toxin was investigated by utilizing four different routes. To avoid the possibility of denaturation, freeze–drying was avoided and fresh samples were utilized for all studies. Of the four routes, only the intraperitoneal and intravenous routes produced death in mice. The LD_{50} values were 237 μg of protein/mouse for the intraperitoneal route and 105 μg of protein/mouse by the intravenous route (Table I). After boiling the toxin for 30 min, the LD_{50} values were found to remain the same with the intraperitoneal route, while the values rose slightly to 118 μg of pro-

tein/mouse from 105 by the intravenous route. No deaths were obtained with the toxic fraction in mice injected subcutaneously with up to 400 μg of protein of this fraction. Intranasal instillation of samples containing up to 100 μg of protein/mouse proved nonlethal and did not produce any visible illness in the animals. However, solubility limitations prevented administration of higher doses in an instillation volume of 0.05 ml by this route.

The first signs of the toxic response when lethal doses were administered intraperitoneally or intravenously occurred between 3 to 4 hr after injection. The animals displayed reduced activity. After 6 to 8 hr their fur became ruffled and their eyes matted and encrusted. The mice tended to crouch in corners and when they moved they did so with a rolling, trembling gait. These signs gradually intensified. A few hours prior to death they showed a motor weakness of the hind legs with increasing severity of incoordination and ataxia. Finally, there was complete loss of control over caudal mobility and animals placed on their sides were unable to right themselves. A majority of the mice showed signs of diarrhea and respiratory difficulty. Most of the animals showing these severe symptoms died within 24 to 48 hr after administration of the toxic material.

All animals that succumbed to the toxic fraction were autopsied within 30 min after death. Animals that survived regardless of the route of administration were sacrificed at 72 hr. No gross pathological changes were consistently noted; however, an occasional petechial lung hemorrhage was noted in a few mice that had died within 48 hr from either intraperitoneal or intravenous injections. Histological studies confirmed the presence of occasional foci of alveolar hemorrhage which was absent in tissue sections of control animals.

Discussion. Studies explaining the virulence of *P. aeruginosa* are still incomplete although it appears that there may be a number of diverse factors potentally involved. The ability of these organisms to produce various extracellular products such as slime, elastase, other proteases, and hemolysin (5, 7) may explain in part some of the symptoms and lesions associated with *P. aeruginosa* infections. However, these factors do not fully account for the virulence of this organism. The current studies suggest the presence of a hitherto undescribed "extracellular" toxin in agar extracts of *P. aeruginosa* cultures. It appears that this substance cannot be easily separated from the protease (elastase) by chemical precipitation procedures. However, the two toxic agents are easily resolved by subsequent chromatographic technics and can also be differentiated on the basis of *in vivo* studies. Although the toxic fraction did not display protease, lecithinase, or hemolytic activity there is no evidence to indicate that some other enzymatic or enzyme-like activity is not associated with this fraction. However, since there were no significant differences in the LD_{50} values of both unheated and heated preparations the results suggest that enzymatic activity, if present, is not responsible for the observed toxicity unless it is remarkably heat stable. In the present studies the toxic fraction exhibited the greatest degree of toxicity when the intravenous route was employed and little or no significant differences were noted between the LD_{50} values of heated and unheated preparations. However, previous studies with the purified elastolytic protease (20) indicated that it exerted its greatest toxicity when administered intranasally, and lethality was dependent upon enzymatic activity. It would appear that lethality was dependent upon the enzymatic destruction of lung tissue. Thus, a concomitant loss in toxicity occurred when enzymatic activity was destroyed by heating. However, partially purified pre-column proteolytic preparations retained some lethality after heat denaturation of the protease thereby suggesting the presence of a nonenzymatic toxic factor (20) or heat stable enzyme.

Although the exact nature of the extracellular toxic material described herein remains unclear, the chemical and electrophoretic analyses suggest that this fraction is either composed of a heterogeneous complex or is contaminated with such materials. Since these

preparations appear to be composed primarily of protein and RNA with almost no reducing sugar present, it would suggest that the toxic fraction is a moiety other than endotoxin. However, the presence of endotoxin or related fractions cannot be totally discounted since a certain amount of autolysis could occur prior to the harvest of extracellular protease after a 72-hr incubation. Consequently, further study is necessary before the exact nature of this fraction is elucidated.

Summary. A nonproteolytic toxic fraction was found present in agar extracts of *Pseudomonas aeruginosa* grown on tryptone–glucose for 72 hr. The toxic fraction could not be separated by chemical means from a protease which exhibits elastase activity. However, the moieties were separable by use of column chromatography employing DEAE-Sephadex A-50. Mouse lethality was obtained by the intraperitoneal and intravenous routes. The mouse LD_{50} values were 237 and 105 μg of protein, respectively. The intranasal and subcutaneous routes were not lethal within the concentrations tested. No gross pathological changes were noted with the exception of occasional petechial lung hemorrhage.

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