CHAPTER 1

INTRODUCTION

All of the infections due to bacterial opportunists caused by Pseudomonas aeruginosa, which have long been of special concerned by the lack of susceptibility to most antibiotics together with a consequently high mortality rate, may attack a wide variety of hosts, ranging from higher animals to insects, or even plants (1, 2).

Pathogenesis-inducing factors associated with Pseudomonas aeruginosa infection are attracting to scientists during the recent years. Such factors as endotoxin, hemolysins, proteases, enterotoxin and exotoxin produced by this microorganism were revealed in relation with the disease (3).

The main objective of this research project is to study the pathogenic effects of exotoxin.

1.1 The characteristics of Pseudomonas aeruginosa

1.1.1 Taxonomy

Pseudomonas aeruginosa is classified according to Bergey (4) as :

Genus Pseudomonas

Family Pseudomonadaceae

Order Pseudomonadales

Class Schizomycetes

There are more than 140 species of bacteria in this genus, but Pseudomonas aeruginosa is the only one pathogenic to human-being (5).

Pseudomonas aeruginosa is gram negative, bacillary in shape, 0.5 to 0.6 by 1.5 microns (μ), usually motile by virtue of one or more polar flagella(e), non-sporing and non-capsulated. It is a stricted aerobe and grows on a wide variety of laboratory media, its culture will give a marked-order of trimethylamine. This organism which was formerly called "Bacillus pyocyaneous" is "the bacillus of blue green pus" (6, 7).

The pathogenicity of gram-negative bacteria has been ascribed to endotoxin the polysaccharides of the cell walls (2, 8) since Pseudomonas aeruginosa is a gram negative bacillus, its pathogenicity is then different, lipopolysaccharides isolated from the cell walls of Pseudomonas aeruginosa were not as toxic as those isolated from the enteric bacilli (3). Furthermore, Pseudomonas aeruginosa might elaborate many extracellular substances that are much more toxic (3,9).

The substances that have been implicated in the pathogenicity of Pseudomonas aeruginosa are as follows:

1.1.2 Phytotoxic factor

This factor was sufficiently small to pass through a cellophane sheet and was heat-stable. Its structure was similar to that of methionine with an only exception that the sulfur molecule of methionine was usually replaced by a carbon. This factor functioned as a metabolic analogue of methionine, blocking its metabolism, and appeared to be entirely nontoxic to animal tissue (9).

1.1.3 Pigments

Pseudomonas aeruginosa was known to produce a number of pigments (3, 4, 5). The best-knowns were:

- Pyocyanine ; a phenazine pigment.
- Fluorescein; the structure of this pigment was undetermined.

Some strains of Pseudomonas aeruginosa could produce other types of phenazine pigments, such as phenazine alpha carboxylic acid and chloroaphine while some produced a melanine-like brown pigment, described in the term "pyomelanin" (10). These pigments simply did not cause apparent deleterious effects but phenazine, like the alpha oxyphenazine pigment, which was usually used as an antibiotic to treat some bacterial infections particularly anthrax (3).

1.1.4 Hydrocyanic acid

Pseudomonas aeruginosa might produce hydrocyanic acid but there was no certain evidence to indicate that its sufficient quantity accounting for the symptom of infection and the autopsy findings in man and animals that died with infections, due to Pseudomonas aeruginosa,

were not those of cyanide poisoning (3).

1.1.5 Proteolytic enzymes

Most strains of Pseudomonas aeruginosa produced a mixture of proteolytic enzymes. These enzyme proteases generally liquefied gelatin, clean milk, dissolved elastin and fibrin but were negative in classical collagenase activity. They might attack terminal peptides of native collagens and liberated amino acids or peptides from these substrates (11).

Injection of the proteases of Pseudomonas aeruginosa into animal-skin induced hemorrhagic lesion within few minutes (12). This lesion was relatively non-specific that it could be produced by many different proteolytic enzymes including trypsin. These proteases were most likely the factors that were responsible for the destruction of corneal tissue when Pseudomonas aeruginosa infected eyes (13, 14).

However, the effects of proteases were usually quite localized, and proteolytic activity was not demonstrated in sera of moribund animals (15). Furthermore, autopsy findings of human-beings and animals that died of infection due to Pseudomonas aeruginosa were usually differed from those observed in animals that were killed experimentally by injections of large doses of proteases, therefore, they are not the lethal factor in the usual infections with Pseudomonas aeruginosa. The 50% Lethal Dose (LD_{50}) of protlases in mice is about 75 micrograms (mcg.) (16). It is not very potent when compared with other protein toxins of bacteria as shown in Table 1 page 7.

1.1.6 Hemolytic substances

Pseudomonas aeruginosa simply produced two hemolytic substances (16);

- A heat-labile phospholipase C that liberated phosphoryl choline from lecithin.

- A heat-resistant glycolipid that solubilized phospholipids.

A purified preparation of the glycolipid was not extremely toxic; about 5 milligrams (mg.) was required to kill one mouse. When a phospholipase preparation of Pseudomonas aeruginosa was injected into animal-skin, within 24 hours, it would produce a central abscess surrounded by an area of redness and induration (12).

The skin lesions produced by the injection of live cultures of Pseudomonas aeruginosa resembled in some features of the lesions produced by phospholipase that was not the important factor in the lethality (15).

1.1.7 Enterotoxin

Pseudomonas aeruginosa was associated with diarrheal condition, variously described as "five-days-fever" or "Shanghai fever" that was the result of enterotoxin which produce a necrotizing enteritis and outpouring of fluid and electrolytes into the lumens of intestine (17). This enterotoxin had not yet been characterized, but it is heat labiled and is probably protein in nature (18).

1.1.8 Lethal toxins

The lethal effect of Pseudomonas aeruginosa infections should be due to an exotoxin that was probably protein. Poor or non-proteolytic enzyme producing strains might also be the good producers of the exotoxin. The exotoxin produced by the strain designated PA 103 was exotoxin A. This toxin contained about 8,000 mouse LD_{50} per milligram of protein; it was calculated on a weight basis that the exotoxin would be more than 20,000 times as toxic as the endotoxin (19).

1.1.9 Surface slimes

The slimes on the cell surface of Pseudomonas aeruginosa were polysaccharides (20), which were probably the functional equivalent of the capsules of many gram negative bacilli (21, 22). The completely purified polysaccharide fractions were usually nontoxic (23, 24).

1.1.10 Endotoxin

An endotoxin is composed of two components separable by electrophoresis (8, 19);

- Component I : Consisted of a lipopolysaccharide protein complex.

- Component II: Consisted of deoxyribonucleic acid, ribonucleic acid and a polyribose complex.

Table 1 Toxicities of various products of Pseudomonas aeruginosa compared with other bacterial toxins (3).

Bacterial species	Toxin	Toxicity*
Clostridium botulinum	Neurotoxin	1.2×10^{6} (G)
Clostridium tetani	Neurotoxin	1.2×10^{6} (G)
Shigella dysenteriae	Neurotoxin	1.2×10^{6} (R)
Corynebacterium diphtheriae	Exotoxin	3.5×10^{3} (G)
Clostridium perfringens	Alpha-toxin	2.0×10^{2} (M)
P. aeruginosa	Exotoxin A	1.6×10^{2} (M)
Staphylococcus aureus	Alpha-toxin	5×10^{1} (M)
Pasteurella pestis	Plague toxin	2.5×10^{1} (M)
Streptococcus pyogenes	Streptolysin O	5×10^{-1} (M)
P. aeruginosa	Protease	2.6×10^{-1} (M)
P. aeruginosa	Glycolipid	4×10^{-3} (M)
P. aeruginosa	Exotoxin	10^{-2} (M)
P. aeruginosa	Phospholipase C	No data

*Number of LD_{50}/kg. body weight per milligram of toxin in various animal species.

G, R, and M indicate guinea pig, rabbit, and mouse, respectively.

1.2 The characteristic of exotoxins

Pseudomonas aeruginosa was known to elaborate various toxic fractions that were demonstrable in vitro and in the experimental animals. One of the elaborated toxic fractions, designated exotoxin A appeared to be excreted as an intact polypeptide chain and was relatively stable. It did not lose its toxicity after freezing and thawing, and could be concentrated by lyophilization. The other exotoxins were so labile that they lost their toxicities via freezing and thawing (15, 25). However all these toxicities could be destroyed by heating at 70°C for 30 min (26).

The estimated molecular weight of the exotoxin A was 66,000 and its isoelectric point was 5.1, with N-terminal arginine and four disulfide bridges (25). This toxin preparation contains virtually no nucleic acid, pigment or lipopolysaccharide (27). This toxin inhibited uptaking of amino acid and uridine by tissue culture cells (28) and it was lethal for mice (15), the medium lethal dose of this toxin preparation in mice weighing 20 gram was 0.1 mcg and it showed that the capability of eliciting hypotensive shock in stumptial rhesus monkeys and in dogs (29).

Intradermal injection of exotoxin A into rabbit did not elicit the effect that were observed with protease or phospholipase but merely produced edema without redness or necrosis (29).

Intraperitoneal injection of 5 to 10 LD_{50} of exotoxin A into mice caused a rapid reduction of circulating leukocytes, the leukocytes counts usually dropped within a few hours from 15,000 cells per cubic millimeter (mm^3) to the range of 3,000-5,000 cells/mm^3, and the mice

usually died within 24-48 hours (29).

Intravenous injection of exotoxin A into dogs initially produced a rapid rise in portal pressure that was followed by a decline of peri pheral blood pressure (29). The dogs eventually died in shock, with findings that included acidosis, elevating levels of circulating catecholamine, increasing of arterial-venous difference in oxygen saturation, leukopenia and circulatory collapse. These observations essentially mimiced the findings in animals dying of infection with Pseudomonas aeruginosa. This toxin was also cytocidal for a number of culture cell lines (30), and inhibited protein synthesis in those cells as well as in numerous organs of all animals (32). Kidney and spleen displayed a slight reduction of protein synthesis 2-4 hours after inoculation (29).

In other organs, incorporation of amino acid decreased only when the animals approached the terminal stage (18 hours after inoculation), therefore, the highest concentration of exotoxin appeared to occur in the kidney, where it was degraded, but the greatest toxic effect took place in the liver. This toxin inhibited mammalian protein synthesis by catalyzing the adenosine diphosphate (ADP) ribosylation of elongation factor 2 (EF 2), with nicotinamide adenosine dinucleotide (NAD) as the donor of ADP-ribose (32,33,34,35).

$$\text{EF-2} + \text{NAD} \rightleftharpoons \text{ADP-ribose EF-2} + \text{Nicotinamide} + H^{+}$$

1.3 Factors that influence the production of exotoxin

Most strains of Pseudomonas aeruginosa grow in simple salt medium that contains ammonium salts as the sole source of nitrogen and simple carbon sources, such as sodium succinate or lactate but no toxin was detectable. The combinations of amino acids and nucleotides are not able to produce good growth and production of toxin. The organism appears to require some factors in the dialysate of complex media for good growth and maximum production of toxin. Pure proteins, such as crystalline bovine albumin, enhance growth as well as production of toxin. Aeration of culture, the presence of glycerol as a carbon source, and an incubation temperature of around 32°C appears to be essential for maximal production of toxin (36) as shown in Table 2 page 11. Nucleic acids of various types, particularly after autoclaving, inhibited production of toxin but enhanced growth of the organism (36) as shown in Figure 1 page 12.

Table 2. Comparison between growth and production of toxin of *Pseudomonas aeruginosa* (strain PA-103), in a dialyzed medium of trypticase soy broth and in the original medium (36).

Medium	Growth mg (dry Weight)/ ml of cells	LD_{50}/ml of super-natant
Dialysate with 1% glycerol	6.2 - 7.8	48 - 72
Original trypticase soy broth with 1% glycerol	12.2 - 15.5	24 - 48

Note. All cultures were shaken at 32°C for 24 hours.

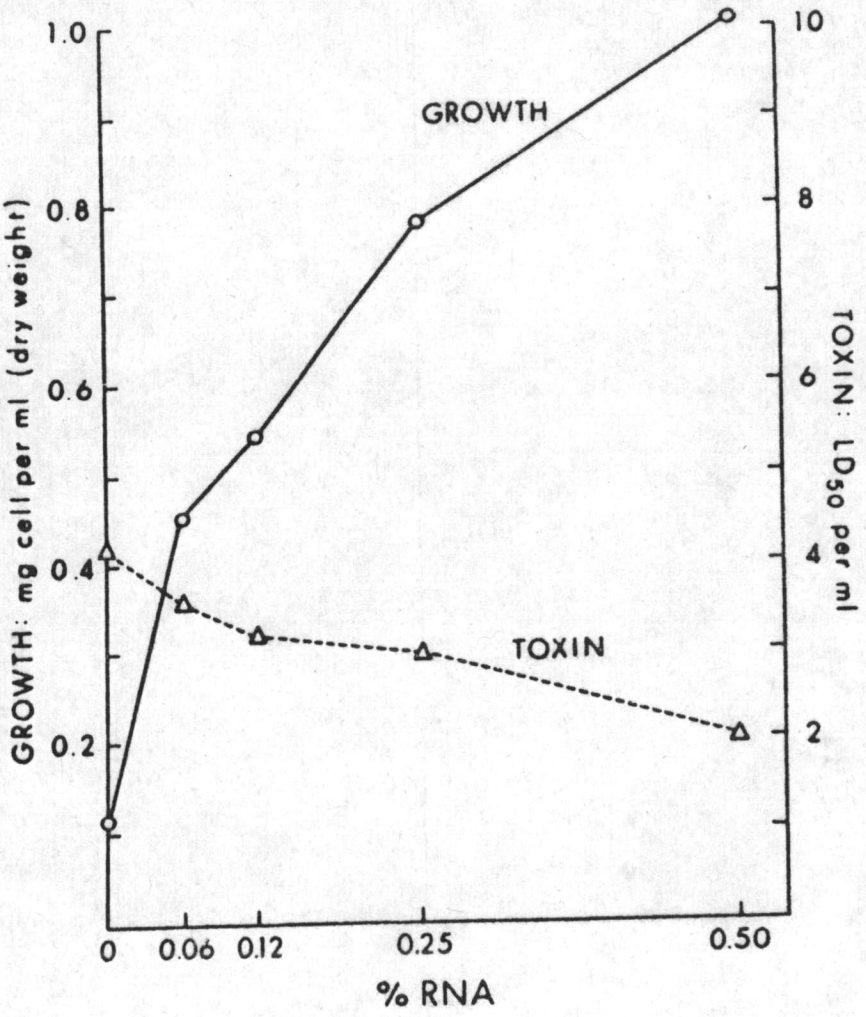

Figure 1. Effect of yeast RNA on growth and toxin production by Pseudomonas aeruginosa (strain PA-103), in a synthesis base medium containing ∝-alanine, aspartic acid, and glutamic acid as the nitrogen sources. The cultures were shaken at 32°C for 24 hours (36).