CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

Organism: Pseudomonas aeruginosa ATCC 27853 was obtained from the Department of Bacteriology and Mycology, SEATO Medical Research Laboratory, Bangkok, Thailand.

Laboratory animals

Mice: young, white, female Swiss mice, weighing 18 to 20 grams, were used in this study.

Rabbits: four adult rabbits weighed about 2.5 to 3.0 Kg. were used.

Chemicals

Acetic acid	(May & Baker)
Albumin bovine, Crystallized	(Sigma Chemical Company)
Ammonium sulfate	(Merck U.S.A.)
Barbitone sodium (Sodium diethyl barbiturate)	(Merck U.S.A.)
Black and white panchromatic films	(Kodak)
Charcoal activated, extra pure	(Merck U.S.A.)
Complete Freund's adjuvant	(Difco Laboratories)

Diethyl barbituric acid (5, 5 diethyl barbituric acid)	(Merck U.S.A.)
Disodium hydrogen phosphate	(Merck U.S.A.)
Eosin, yellowish	(E. Merck Co.)
Ethyl alcohol	(Mallinckrodt)
Fluorescein isothiocyanate No. F 7250 Isomer I	(Sigma Chemical Company)
Folin Ciocalteu's phenol reagent	(BDH Lab. Reagents)
Formalin	(Mallinckrodt)
Glycerin G.R.	(Merck U.S.A.)
Hematoxylin crystal	(E. Merck Co.)
Incomplete Freund's adjuvant	(Difco Laboratories)
Mercuric Oxide (red)	(Mallinckrodt)
Paraffin pellet (M.P. 56-58°C)	(Will Scientific)
Permount	(Fisher)
Picric acid	(E. Merck Co.)
Potassium aluminium sulfate	(Mallinckrodt)
Potassium dichromate	(E. Merck Co.)
Sephadex G-25	(Sigma Chemical Company)
Sephadex G-200	(Sigma Chemical Company).
Sodium chloride	(Merck U.S.A.)
Sodium dihydrogen phosphate	(Merck U.S.A.)
Sodium hydroxide	(Merch U.S.A)
Special Nobel Agar	(Difco Laboratories)
Tryptic Soy Agar	(Difco Laboratories)

Xylene	(Mallinckrodt)

Glass wares

Beaker	(Pyrex)
Burette	
Centrifuge tube	
Chromatography column	
5.0 cm. x 90.0 cm.	
1.0 cm. x 60.0 cm.	
Coplin jar	(Arthur H. Thomas)
Cover glasses	(Resistance)
Flask	(Pyrex)
Glass slides	(Clay Adams)
Measuring cylinder	
Pipette	
Staining jars	(Arthur H. Thomas)
Staining racks	(Arthur H. Thomas)
Stirring rod	

Instruments

Analytical balance	(Mettler B 5)
Autoclave	(Amsco International)
Centrifuge	(Clay Adams)
Cryo-Stat	(American Optical)
Deep freeze refrigerator	(Continental)
Dialysis tubing	(Union Carbide Corp.)

Fluorescent microscope	(American Optical)
Fracto Mette 400 Automatic Fraction Collector	(Buchler instruments)
Histokinette	(American optical Co.)
Hot plate	(Chromalox)
Humid Chamber	
Incubator, Precision model 6	(Thelco)
Lightfield microscope	(Americal Optical)
Microtome knife	(American optical Co.)
Millipore filter set	
Millipore membrance 0.45 μ	
Oven, Precision model 27	(Thelco)
Paraffin oven (58°C)	(Precision)
Pinted scissors	(A. Dumont & Films)
Refrigerator	(MARCO)
Rotary microtome	(American optical Co.)
Surgical blades	(Swann-Morton)
Tissue floatation bath	(Arthur H. Thomas)
Tooth forceps	(A. Dumont & Films)
U V spectrophotometer	(Unicam S.P. 1800)

2.2 Preparation of solutions

2.2.1 Phosphate buffered saline (0.1 M., pH 7.7)

This buffer was prepared from two stock solutions with the following formular:

Stock solution A (0.2 M Sodium acid phosphate solution)

Sodium acid phosphate, anhydrous	8.0	g.
Distilled water, q.s.	1000.0	ml.

Stock solution B (0.2 M Disodium phosphate solution)

Disodium phosphate, anhydrous	8.47	g.
Distilled water, q.s.	1000.0	ml.

Dissolved and stored each stock solution separately, then mixed 1 part of solution A and 9 parts of solution B, and diluted the mixture with equal volume of saline solution containing 1.7% weight by volume of sodium chloride in distilled water. The so prepared buffer finally contained 0.1 M phosphate and 0.85% weight by volume of sodium chloride at pH 7.7.

2.2.2 Barbitone buffer 0.05 M, pH 8.6

Diethyl barbituric acid	1.83	g.
Sodium barbitone	10.3	g.
20% Bovine serum albumin	0.5	ml.
Distilled water q.s. ad	1000.0	ml.

2.2.3 0.1 N HCl

Hydrochloric acid	8.3	ml.
Distilled water q.s. ad	1000.0	ml.

2.2.4 0.1 N NaOH

Sodium hydroxide	4.0	g.
Distilled water q.s. ad	1000.0	ml.

2.2.5 10% Formalin

Full-strength formalin (37-40%)	10.0	ml.
Distilled water	90.0	ml.

2.2.6 Harris alum hematoxylin

Hematoxylin crystals	5.0	g.
Ethyl alcohol, absolute	50.0	ml.
Potassium aluminium sulfate	100.0	g.
Mercuric oxide (red)	2.0	g.
Distilled water, q.s.	100.0	ml.

Dissolved the hematoxylin in alcohol and alum in water by the aid of heat. Added hematoxylin solution to potassium aluminium sulfate solution, brought to a boil as rapid as possible then removed from heat. Placed the flask of this solution in cold water and added mercuric oxide quickly, kept the mixture in cold water until the mixture developed dark purple colour. Then added 2 to 4 ml. of glacial acetic acid per 100 ml. solution, to increase selectivity of the stain for nucleus, filtered the solution before use.

2.2.7 Picro-eosin solution

Eosin yellowish, water soluble	10.0 g.
Potassium dichromate	5.0 g.
Picric acid, saturated aqueous	100.0 ml.
Ethyl alcohol, absolute	100.0 ml.
Distilled water	800.0 ml.

Dissolved the eosin Y and potassium dichromate in water, in separated portion. Mixed the two solutions then added saturated aqueous of picric acid to the mixture. Absolute alcohol and distilled water were added to the mixture and filtered before use.

2.2.8 Differentiating solution (Acid alcohol solution)

Dissolved hydrochloric acid concentrated, 10 ml. in ethyl alcohol 70% to make 1000 ml.

2.2.9 Ammonia water

Dissolved 3 ml. of strong ammonia water in 1000 ml. of tap water.

2.3 Method

2.3.1 Preparation of exotoxin

To grow the Pseudomonas aeruginosa, minor modifications of the culture condition described by Liu (36) was applied. The medium was Tryptic Soy Agar supplemented by addition of 1% volume by volume (V/V) glycerin and the final pH was adjusted to 7.4.

An inoculum of Pseudomonas aeruginosa was simply grown on this slant medium, containing 15 millilitre (ml.) medium in each 2.1 centimeter (cm.) diameter and 21 cm. length tube, by streaking method. After 18 hours of growth at 37°C, the exotoxin was extracted by using 5 ml. of 0.85% normal saline solution (NSS) per culture tube to wash out the culture and shook for 10 minutes, then, allowed to stand for one hour at room temperature and the fluid preparations from culture tubes were pooled together.

Bacterial cells were removed by centrifugation at 1500 revolutions per minute (rpm.) for 30 minutes. The collected supernatant fluid was recentrifuged at 1500 rpm. for 30 minutes and it was then filtered through the 0.45 μ millipore membrane. The filtrate was tested for sterility by streaking on the Tryptic Soy Agar slant and incubated at 37°C for 48 hours. The bacterial free filtrate obtained was used as the primary source of crude exotoxin.

2.3.2 Concentration of exotoxin

At this stage, the primary exotoxin preparation (bacterial-free filtrate) was precipitated by adding saturated ammonium sulfate solution pH 7.4 to make 60% final salt concentration. The precipitate was collected by centrifugation at 3,000 rpm. for 20 minutes and suspended in the 0.1 Molar (M) phosphate buffered saline (PBS) pH 7.7. The process of precipitation was repeated three times, and the final precipitate was dissolved in 0.1 M PBS pH 7.7 at the one-fifth of primary volume of crude exotoxin preparation. The concentrated meterial was dialyzed against 0.1 M PBS pH 7.7 at 4°C and changed the buffer twice daily for 3 days.

2.3.3 Purification of exotoxin

Ten ml. of the exotoxin was applied to a column, 5.0 x 90 cm. of Sephadex G-200, which was pre-equilibrated with 0.1 M PBS pH 7.7. This buffer was used for elution of exotoxin in the column. Successive 5 ml. aliquot fractions were collected with a fraction collector and the optical density (O.D.) at 280 nanometer (nm.) wave length were recorded. The protein peak was pooled together and concentrated with 60% final concentration of ammonium sulfate as mentioned before. The final protein precipitate was dissolved in 0.01 M PBS pH 7.7, at the same volume that was used to apply on to the column, and this buffer was also used to dialyzed the prepared protein in the same manner as described before.

2.3.4 Determination of the protein content

The protein content of the exotoxin at this stage was determined by the method described by Lowry et al (37).

2.3.5 Determination of the 50% Lethal Dose of exotoxin in mice

Mice were inoculated intraperitoneally with 0.5 ml. of two fold serial dilutions of exotoxin. Ten mice were used for each dilution. The animals were observed for 72 hours.

LD_{50} was calculated by the method of Litchfield et al (38).

2.3.6 Histopathological technique

To study the histopathology of exotoxin, mice were injected intraperitoneally with $2LD_{50}$ per mouse of exotoxin. Eight to twelve hours after injection they were exsanguinated and the livers, spleens and kidneys were isolated for sectioning. The mentioned organs from normal mice were used as control.

The isolated organs were sectioned and stained for histopathology observation as follows:

2.3.6.1 Fixation:

Each organ was cut into small blocks, ideally not more than 2 square centimeters (cm^2) area, 4-5 millimeter (mm.) thickness, was placed in 10% formaldehyde, 20 times of the volume of the specimen.

2.3.6.2 Dehydration:

After fixation, the specimens were placed in 80% ethyl alcohol for 1 hour, then transferred to 90% ethyl alcohol for 1 hour and finally to absolute ethyl alcohol for 1 hour.

2.3.6.3 Clearing:

The dehydrated specimens were immersed in xylene for 1 hour.

2.3.6.4 Infiltration:

After clearing, the tissues were infiltrated with melted paraffin wax.

The four steps described above were carried in Automatic Tissue Processor (Histokinette).

2.3.6.5 Embedding:

The tissues after infiltration, were embedded in paraffin wax.

2.3.6.6 Cutting

The paraffin embedded tissues were cut by a rotary microtome with a sharp clean microtome knife, 4 μ in thickness. The sectioned ribbons were floated on the surface of warm water at 45°C in tissue floatation bath. Albuminized slides were partially submerged in the water in order to bring up the sections on the surface of the slides. The slides were then incubated at 56°C for 30 minutes.

2.3.6.7 Staining:

The prepared slides were placed in xylene by 3 successive changes for 3 minutes each, they were then transferred into three changes of absolute ethyl alcohol for 2 minutes each, and finally placed in 95% ethyl alcohol for 2 minutes. Rinsed the slides in distilled water and stained in hematoxylin solution for 3 minutes then the excess dye was washed out with running tap water until the sections were blue in color.

Dipped the stained slides into acid alcohol for few seconds and returned to distilled water and then ammonia water. Rinsed the excess of ammonia by washing with running tap water for 10 minutes and counter stained with eosin solution for 30 seconds. Transferred the slides to water for cleaning, then dehydrated in 95% ethyl alcohol by 2 changes and absolute ethyl alcohol by 3 changes for about 2 minutes each. The sections were cleared in xylene by 3 changes for 2 minutes each (39, 40, 41).

The slides were mounted with 1 or 2 drops of per-mount, depending on the size of the tissue sections, then they were covered with cover glass and ready for examination under light microscope.

2.3.7 Production of rabbit antitoxin and immunodiffusion test

Four female rabbits weighting from 3 to 5 kilograms (Kg.) prebled for normal sera and then immunized with approximately 40 mcg. of exotoxin in equal volume of complete Freund's adjuvant.

Injections were given by dividing the immunizing material into two equal portions, one for subcutaneous injection and another

intramuscularly. The rabbits were boostered with 40 mcg. of exotoxin in equal volume of incomplete Freund's adjuvant at the same routes 2 to 3 weeks after the primary immunization. The rabbits were exsanguinated 2 to 3 weeks after the secondary immunization and the bloods were collected and pooled. The immune sera was separated from the blood and concentrated with 50% final concentration of ammonium sulfate. The final precipitate was dissolved in 0.01 M PBS pH 7.7 at the half volume of original serum, and dialyzed against this buffer.

The immune serum globulin obtained was tested by Ouchterlony's double gel diffusion technique against the prepared exotoxin antigen to confirm the specificity of this antiserum. Double immunodiffusion tests were performed on microscopic slides. The slides were precoated with 2% Nobel Agar in 0.15 M barbiturate buffer pH 8.0 as the modification of the procedure of Ouchterlony (42). The 4 mm. diameter holes were set on the slides at the distance of 1 cm. apart. The volume of 0.1 ml. of exotoxin antigen, immune globulin, and the normal rabbit serum control were filled into the corresponding holes. The slides were incubated in moist chamber at room temperature and the precipitin lines were observed 24-48 hours afterwards.

2.3.8 Preparation of fluorescent antibody:

Weighed 3.125 mg. of fluorescein isothiocyanate powder and added to 125 mg. of anti exotoxin globulin, the pH was adjusted to 8.0 with 0.1 N sodium hydroxide. Allowed the reaction to proceed overnight at 4°C with continuous stirring. Then the conjugated immune globulin was applied to a column 1 x 60 cm., of Sephadex G-25, which was

pre-equilibrated with 0.01 M PBS pH 7.7, the conjugate was eluted with 0.01 M PBS pH 7.7. The primary yellow eluted portion was collected and absorbed with 12.5 mg. of PBS-washed activated charcoal and centrifuged at 3,000 rpm for 20 minutes to remove the insoluble particles.

2.3.9 Application of immunofluorescent antibody

Liver, spleen and kidney from normal control mice as well as mice which were 8 to 12 hours preinjected with exotoxin were sliced at 4 μ thickness in a -4°C cryo-stat. The frozen were fixed with acetone for 10 minutes on the slides and then were flooded with appropriate dilution of the fluorescent conjugated globulin. After incubation at 37°C for 30 minutes in a humid chamber, the slides were immersed in a jar containing 0.01 M PBS pH 7.7 to wash out the excess unreacted conjugate. Washing process were repeated 3 times at 10 minutes each. After washing, the stained sections were let drying and then flooded with a mounting medium consisting of 9 parts of glycerin and 1 part of PBS and covered carefully with a coverslip and sealed with enamel. The prepared slides were examined under fluorescent microscope and photographed.

2.3.10 In vitro exotoxin neutralization and in vivo testing:

Dilutions of exotoxin in sterile normal saline containing 1 and 2 LD_{50} per ml. were prepared. Each dilution was divided into two parts, one was mixed with equal volume of immune globulin containing 7.13 mcg./ml. globulin and the other was mixed with 14.26 mcg./ml. globulin. The mixtures were agitated and allowed standing for 2 hours at room temperature and then were centrifuged at 3,000 rpm. for

20 minutes. The supernatant fluid obtained were injected 2 ml. intraperitoneally into each of 20 mice per group. The control group for 1 and 2 LD_{50} per ml. of 20 mice each were performed in the same manner excepted that normal saline solution (NSS) was used instead of immune globulin. The injected animals were observed and the mortality were recorded during 3 days period.