CHAPTER III

MATERIALS AND METHODS

1. Experimental Animals

Rabbits were chosen in this study of cobra venom on account of their susceptibility, ease of frequent blood collection and the availability in this Institute. Twenty-seven rabbits of both sexes (24 males and 3 females) weighing from 2.1-3.3 kg. (average 2.64±0.37 kg.), the age of which ranged from 4-12 months (average 7.77±2.39), were used.

2. Source of Venoms

Lyophilized snake venoms were kindly provided by the Queen Saovabha Memorial Institute of the Thai Red Cross Society, Bangkok. The cobra venom "milk" from Siamese cobra (*Naja naja kaouthia*) was used throughout the study. In addition, the following venoms were employed in the specificity test for ELISA:

King cobra (*Ophiophagus hannah*)
Banded krait (*Bungarus fasciatus*)
Green pit viper (*Trimeresurus* species)
Russell's viper (*Vipera russelli*)

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3. Administration of Venom

Lyophilized cobra venom was dissolved in saline (0.9% NaCl) at the concentration of 2 mg/ml and stored at 4°c in 1 ml aliquots. The venom solution, as calculated from the weight of the rabbits tested, was drawn into a one milliliter syringe fitted with a 26 gauge (5/8 inches long) needle and injected subcutaneously into the lower right leg of the rabbits. After injection, the rabbits were observed for neurotoxic signs and blood was collected at intervals.

4. Antivenines

4.1 For the treatment of envenomed rabbits

Equine monovalent anticobra venom, batch No. 36 from the Thai Pharmaceutical Organization was used throughout. The antivenine obtained were ammonium sulfate-purified globulin, refined and concentrated in 10 ml liquid. According to the producer, one millilitre of antivenom was able to neutralize at least 0.6 mg of cobra venom when injected intravenously into mice (= neutralizing dose). The antivenom was kept at 4-10° c until use, the dose of which was calculated as 6 times the neutralizing dose.

4.2 For laboratory testing

The anticobra venom used in immunodiffusion, indirect hemagglutination, and ELISA was produced in rabbits. Increasing doses of cobra venom as described in section 6 were

injected subcutaneously and the animals were bled two weeks after the last injection.

5. Study Design

Rabbits were divided into 2 groups ;

Group 1. Kinetic study : Various doses of crude cobra venom were injected subcutaneously into 10 rabbits at the lower right leg. Dosages of 80, 125, 150, 160 and 190 µg/kg were administered. At least 2 rabbits were included at each dose level. The rabbits were observed for neurotoxic signs and blood samples were taken from the middle ear vein at time 0, 15 minutes, 1, 2, 4, 8, 12 and 24 hours consecutively after venom injection.

Group 2. Effects of antivenine : Eleven rabbits were injected with crude cobra venom at a dose of 190 µg/kg, followed by a single dose of monovalent antivenine at either 0, 15 minutes or 2 hours after venom injection. The amount of antivenine administered was calculated as 6 times the neutralizing dose. Antivenine (equine monospecific anticobra venom) was infused into the rabbit via the ear vein with a sterile 26 G scalpvein infusion set. The animals were observed for neurotoxic signs and blood samples were taken at intervals as stated for the first group.

6. Preparation of Monospecific Anticobra Venom in Rabbits

Three rabbits weighing 2-3 kgs were immunized with crude

cobra venom (<u>Naja</u> <u>naja</u> <u>kaouthia</u>). The venom solution containing 2% bentonite as an adjuvant in the ratio of 1:1 was injected subcutaneously. After the initial injection of 0.0125 mg cobra venom /kg of body weight, the dosage increased gradually every 7 days for a period of 84 days.

The schedule of immunization was as follow :

Day	Concentration of cobra venom (mg/kg body weight)
0	0.0125
7	0.025
14	0.050
21	0.075
28	0.100
35	0.150
42	0.225
49	0.300
56	0.400
63	0.500
70	0.625
77	0.750
84	0.875

Rabbits were bled on day 70, 77, 84 and two weeks after the last injection. Antibody titer was measured by passive hemagglutination test. The specificity of the antisera was tested by gel diffusion against 1 mg/ml of venom from cobra, Russell's viper, green pit viper, king cobra and banded krait.

The antiserum was stored at -20°c until use.

7. Passive Hemagglutination Test for Cobra Venom Antibody

Rabbit anticobra venom antibody was semiquantitatively determined by passive hemagglutination test according to the method described by Khupulsup et al. (69). A two-fold serial dilution (50 µl/well) of rabbit anticobra venom was performed in a microtiter plate (U shape), using phosphate buffer saline, pH 7.2 containing 0.5% bovine serum albumin and 0.1% sodium azide as diluent. To each well, 50 µl of 0.5% cobra venom-coupled sheep red blood cells was added, the tray was gently shaken and incubated at 25°c for 2 hours. Controls of the test included uncoupled sheep red blood cells in 1:100 dilution of antiserum, and toxin-coupled or uncoupled sheep red blood cells in the presence of normal rabbit serum.

8. Rabbit Anticobra Venom Immunoglobulin G Preparation

Monospecific antisera to cobra venom was raised in rabbits as previously described. Immunoglobulin G (IgG) was obtained from hyperimmune sera by affinity chromatography on protein A sepharose CL-4B (Pharmacia-fine chemicals, Uppsala, Sweden) according to the method of Goding (98) with minor variation (88):

An 8-ml column of protein A sepharose CL-4B saturated in phosphate buffer saline (PBS), pH 7.4 was washed with 20 ml of PBS , pH 7.4, the flow rate of which was adjusted to 50 ml/hour. Six millilitres of hyperimmune rabbit serum was then slowly

applied to the edge (top) of the column and when the serum had penetrated the column, the sepharose was washed, first with 10 ml of 1 g/l Tween 20 in 0.05 mol/l phosphate buffered saline solution, pH 7.4, followed by 20 ml of PBS, pH 7.4. Immunoglobulin G was eluted with 20 ml of 0.1 mol/l glycine/HCl 1 mol/l NaCl, pH 3.0 and neutralized with 1 mol/l NaOH immediately after elution (1 mol/l NaOH was added drop by drop and the pH monitored by pH meter). The eluent (3 ml in each tube) was measured for protein at 280 nm by a spectrophotometer (Beckman ACTA III UV-Visible spectrophotometer, USA). The eluent with protein content in the same peak (peak 2) was pooled and dialyzed against 0.05 M PBS, pH 7.4 at 4°c overnight with several changes of buffer. The protein solution was concentrated by ultrafiltration (Amicon ultrafiltration stirred cell with PM 10 ultrafiltration membrane) to the original volume (6 ml).

The purity of rabbit antivenom IgG was tested by immunodiffusion, IgG antibody titer was measured by passive hemagglutination test and the protein concentration was determined by Biuret method (99).

9. <u>Preparation of Enzyme-Labeled Rabbit Anticobra Venom IgG</u>

Rabbit anticobra venom IgG was labeled with enzyme alkaline phosphatase according to the method of Voller et al. (100). Alkaline phosphatase (bovine intestine mucosa, Sigma type VII , Sigma Chemicals Co., St Louis, Mo, USA.) supplied as a precipitate in ammonium sulphate, specific activity 1100 units/mg

protein was used.

The appropiate volume of enzyme suspension was measured to contain 5 mg and centrifuged at 2,000 rpm for 5 minutes; the supernatant was discarded. To the precipitate, 2 mg of rabbit anticobra venom IgG in 1.0 ml of phosphate buffer saline, pH 7.4 was added , mixed at room temperature and the mixture was dialyzed extensively at 4°c with several changes of PBS . After dialysis, 2.5% glutaraldehyde (Sigma Chemicals Co, St.Louis, MO, USA) was added to give a final concentration of 0.2%. The solution was mixed , incubated at room temperature for 1-2 hours , and the reaction was stopped with the addition of 0.2 ml of 1M lysine, pH 7.0. After dialyzing at 4°c against several changes of PBS, pH 7.4, the dialysis tubing was transferred to 0.05M tris-(hydroxymethyl) aminomethane (Tris) buffer, pH 8.0 and dialysis continued extensively at 4°c with several buffer changes. The conjugate solution was diluted to 4.0 ml with Tris buffer containing 1.0 % bovine serum albumin, 0.02 % sodium azide and equal volume of glycerine and stored in the dark at 4°c until use.

10. Determination of Factors Affecting the ELISA System

10.1 Checkerboard titration to determine the optimal condition of the reagents for double-antibody sandwich ELISA test

The IgG fraction of rabbit anticobra venom was diluted 1:1,000, 1:2,000, 1:4,000 in coating buffer, pH 9.6, to

give final concentrations of 1, 0.5 and 0.25 µg of protein per ml respectively and 100 µl of each dilution was added to duplicate horizontal row of wells in a polystyrene microtiter plate (Nunc Immunoplate I, Denmark) and incubated at 37°C for 1 hour. The contents were then shaken out and the wells were filled with PBS Tween, pH 7.4 and allowed to stand for 5 minutes. This washing procedure was repeated twice. The plate was then divided into four sections, each containing three rows of six wells. Into the first and second vertical rows of each section, 100 µl of positive reference samples (50 ng and 1 ng/ml of cobra venom) were added. The third row of wells contained normal rabbit serum (1:5). Samples were incubated for 1 hour at 37°C followed by three rounds of washing. Enzyme-labeled rabbit anticobra venom IgG conjugate was diluted 1:50, 1:100, 1:200 and 1:400 in PBS-Tween albumin, 100 µl of each dilution were added to each section of the plate and incubated at 37°C for 1 hour. Washing was repeated as mentioned before and 100 µl of substrate solution (p-nitrophenyl phosphate in diethanolamine buffer, pH 9.8) was added to each well. After 1 hour incubation at 37°C, the reaction was stopped by adding 50 µl of 3 M NaOH and color change was determined by a micro-ELISA reader (Titertek Multiscan, Flow Laboratories, Helsinki, Finland) at an absorbance of 405 nm.

10.2 Temperature and time course for coating

To determine the optimal temperature and time course for rabbit anticobra venom IgG in coating plates, 100 µl of rabbit anticobra venom IgG was diluted 1:2,000 in coating

buffer, pH 9.6 were pipeted in duplicate into wells of 4 microtiter plates, and incubated at different conditions as follows :

Plate 1 : incubated at 4°C overnight (about 18 hours)

Plate 2 : incubated at 37°C for 1 hour

Plate 3 : incubated at 37°C for 1 hour and kept at 4°C overnight

Plate 4 : incubated at 37°C for 3 hours.

Into each plate, 100 µl of standard cobra venom containing 50, 25, 10, 5, 1 ng/ml and normal rabbit serum diluted 1:5 in PBS-Tween albumin as a negative control were added in duplicate.

The plates were then processed as in the routine assay.

10.3 Determination of time course for :

10.3.1 Anticobra venom and cobra venom reaction

10.3.2 Enzyme-labelled anticobra venom reaction

10.3.3 Color development

One hundred microlitres of standard cobra venom containing 50,25,10,5,1 ng/ml and normal rabbit serum (1:5) were pipeted in duplicate into wells of 4 microtiter plates precoated

with 1:2,000 dilution of anticobra venom as mentioned.

10.3.1 The plates were incubated at 37°C for a period varying between 15 and 120 minutes (15, 30, 60 and 120 minutes respectively) and processed as in the routine assay.

10.3.2 One hundred microlitres of appropriate dilution of anticobra venom alkaline phosphatase conjugate (from checkerboard titration) was added to each well and the plate was incubated at 37°C for a period varying from 15 to 120 minutes (15, 30, 60 and 120 minutes). The plates were then processed as in the routine assay.

10.3.3 One hundred microlitres of p-nitrophenyl phosphate substrate solution were added to each well and incubated at 37°C for a period verying from 30 to 120 minutes (30, 60, 90 and 120 minutes).

11. Standardization of the ELISA Test

11.1. Precision study

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A within-plate precision was determined using three QC pools of cobra venom prepared by diluting cobra venom solution in PBS-Tween albumin. Precision for each pool was calculated by running 10 duplicates in one microtiter plate.

Overall precision was estimated using three QC pools analysed on separate microtiter plates.

Mean and standard deviation were calculated.

Coefficient of variation (CV) in per cent was calculated by the following formula :

$$\% \text{ coefficient of variance} = \frac{\text{standard deviation}}{\text{mean}} \times 100$$

11.2. Specificity of anticobra venom in ELISA test

The specificity of rabbit anticobra venom was tested against different concentrations of 4 other snake venoms in Thailand as followed :

- Banded Krait venom (*Bungarus fasciatus*)
- King cobra venom (*Ophiophagus hannah*)
- Green pit viper venom (*Trimeresurus* species)
- Russell's viper venom (*Vipera russelli*)

All the venoms were diluted in PBS-Tween albumin to give final concentrations of 1,10,100,1000 ng/ml.

12. Detection and Quantitation of Cobra Venom

Cobra venom was detected and quantitated by micro-ELISA, double-antibody sandwich technique as described by Theakston et al.(85) with some modifications.

Polystyrene microtiter plate (Nunc Immunoplate 1) coated with 100 µl amount of the IgG fraction of rabbit anticobra venom dilution 1:2,000 (0.5 µg/ml) in coating buffer, pH 9.6 were incubated at 37°C for 1 hour, or followed by incubation at 4° C overnight and washed three times before use. After washing, 100 ul of reference cobra venom containing 50, 25, 10, 5, 1 ng/ml

were added to the wells in duplicate. Test sera from rabbits diluted 1:5 in PBS-Tween albumin were included. The plates were incubated, washed and reacted with 100 μl of enzyme labelled rabbit anticobra venom IgG conjugate after which the procedure followed the routine color development. The absorbance of the contents in each well was read at 405 nm in a micro-ELISA reader (Titertek Multiscan, Flow Laboratories, Helsinki, Finland), the machine being blanked with substrate solution.

Since the intensity of the color reaction is proportional to the amount of antigen present, the concentration of venom in each test serum could be determined from a standard curve plotting the average absorbance (OD) against venom concentrations of reference samples on semilogarithmic scales.

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