CHAPTER IV

RESULTS

1. Development of ELISA Method for the Detection of Cobra Venom in Serum

A micro-ELISA, double-antibody sandwich technique for the detection of venom as described by Theakston et al. (85) was developed for the detection and quantitation of Thai cobra (Naja naja kaouthia) venom with some modifications.

1.1 Preparation of monospecific anticobra venom in rabbits

Three rabbits were immunized with increasing doses of crude cobra venom from day 0 to 84 and the anticobra venom antibody titers were determined on day 70,77,84 and 98 by passive hemagglutination test. As shown in Table 1, high titers of antibodies were obtained in all rabbits on day 70 at titers of 12,800- 102,400, while non-immunized rabbits serum had antibody titers of less than 1:100. Immunized rabbits serum also gave a negative result (HA titer < 1:100) when tested with uncoupled sheep red blood cells. It is interesting to note that in spite of continued immunization with increasing doses of cobra venom, the antibody titer in all 3 rabbits decreased after day 70.

Specificity of the anticobra venom was tested by gel

diffusion against 1 mg/ml. of venom from cobra, Russell's viper, green pit viper, banded krait and king cobra. No cross reaction was observed as shown in Figure 2.

1.2 Isolation of rabbit anticobra venom IgG

Immunoglobulin G fraction was isolated from hyperimmune anticobra venom produced in rabbit No.1 (day 70) by affinity chromatography using protein A sepharose CL-4B. Three peaks of protein were obtained when measured at 280 nm (Figure 1, Appendix III). Since the protein A has the capacity to bind specifically and with high affinity to immunoglobulin chains of most species (101,102), most of the unbound serum proteins were passed directly through the column (peak 1) . The bound IgG was eluted with acid and obtained mostly in peak 2 (fraction 24-31). The fractions in peak 2 were found to be composed of only IgG as determined by a single line with swine anti-rabbit serum (Figure 3) and the antibody titer was estimated by PHA test to be 1:3200. However, peak 3 was also found to be composed of IgG fraction but with a much lower antibody titer (1:512). Peak 2 was chosen for the subsequent test and the optimal concentration for coating the microtiter plate in ELISA test was determined by checkerboard titration.

1.3 Determination of factors affecting the ELISA system.

Various concentrations of rabbit anticobra venom IgG (1, 0.5, 0.25 μg of protein/ml or 1:1,000, 1:2,000 and 1:4,000 dilutions respectively) and rabbit anticobra venom IgG-alkaline

phosphatase conjugate (1:50, 1:100, 1:200 and 1:400 dilutions) were titrated against known amounts of cobra venom (50 and 1 ng/ml), control negative serum (normal rabbit serum, 1:5 dilution) and PBS-Tween albumin (reagents control). As shown in Table 2, dilutions of 1:2,000 for rabbit anticobra venom IgG and of 1:50 for the enzyme-conjugated anticobra venom produced better results and were chosen for subsequent tests.

Optimal temperature and reaction times in each step of ELISA test were determined . As in the coating plate step, the best condition was found to be the incubation at 37°c for 1 hour , 37°c for 1 hour followed by keeping 4° c overnight or 37°c for 3 hours (Figure 2, Appendix III.). Thus, incubation at 37°c for 1 hour or 37°c for 1 hour followed by 4°c overnight were chosen for the sake of simplicity and time saving. The time course for venom and antivenom reaction, enzyme-labeled anticobra venom and color development were shown in Figure 3,4,5 (Appendix III.) respectively. Reaction time of 60 minutes at 37°c in each step gave the best distinction between negative control and each venom concentration, thus was chosen for subsequent test. The results were summarized in Table 3.

1.4 Standardization of the assay.

1.4.1 Specificity of anticobra venom in ELISA test

The specificity of rabbit anticobra venom IgG in ELISA was tested by reacting with different concentrations of four other snake venoms (banded krait, king cobra, green pit

viper and Russell's viper). As shown in Figure 4, no cross -reactivity was demonstrated.

1.4.2 Precision of ELISA test for cobra venom

Precision of ELISA test was shown in Table 4. In a within plate precision analysis, the coefficient of variation (CV) ranged from 2.87% to 9.4% for mean concentration of 2.4 ng/ml and 13.0 ng/ml respectively. Overall precision varied, with coefficient of variation from 4.4% to 15.2% (for mean concentration of 34 ng/ml and 12 ng/ml respectively).

2. Kinetics of Cobra Venom (Naja naja kaouthia) in Rabbit Serum and Envenomation

Rabbits were injected subcutaneously with graded doses of cobra venom after which symptoms and signs were observed and blood samples were taken at different intervals. At the sublethal doses of 80, 125 and 150 µg/kg, cobra venom could be detected in the serum within 15 minutes of injection in all rabbits. The venom remained in the circulation up to 12 hours or longer depending on the amount of venom injected (Figure 5,6,7). At the dose of 80 and 125 µg/kg, none of the animals developed any neurotoxic signs, while the two rabbits which received 150 µg/kg of cobra venom, one developed drowsiness, muscular weakness at 4-6 hours, followed slowly by complete recovery, and the other rabbit completely asymptomatic.

At lethal doses of 160 µg/kg and 190 µg/kg, the venom was also detectable in 15 minutes, reaching a maximum level in 30-60

minutes (Figure 8,9). All animals began to show neurotoxic signs which included drowsiness and muscular weakness in 1-2 hours, followed by paralysis, cyanosis and death in 90-370 minutes. It was observed that the rate of neurotoxicity and mortality development was also dose-dependent, since both rabbits receiving 190 µg/kg of venom died in 90 and 240 minutes while those receiving 160 µg/kg died in 250 and 370 minutes after venom injection.

Mean serum concentrations of cobra venom after subcutaneous injection of various doses of cobra venom are summarized in Table 5 and Figure 10.

It should be noted that no local necrosis was observed at the site of venom injection in all rabbits.

3. The Effects of Antivenine Treatment in Envenomed Rabbits

The efficacy of antivenine treatment in envenomed rabbits was measured by determining the serum cobra venom levels as well as the survival rate. Eleven rabbits were injected subcutaneously with a lethal dose of 190 µg/kg of cobra venom followed by a single injection of equine monovalent anticobra venom at either 0, or 15 minutes, or 2 hours.

3.1 Effects of antivenine on serum cobra venom levels.

When antivenine was given immediately (at time 0) in 3 rabbits, cobra venom could not be detected in sera from 15 minutes up to 24 hours after treatment (Figure 11). Likewise, in

the other two groups of animals with delayed treatment of antivenine (15 minutes or 2 hours after venom injection), cobra venom levels dropped in 15 minutes and remained undetectable up to 24 hours (Figure 11).

3.2 Effects of antivenine on survival rate

The efficacy of antivenine treatment on survival is summarized in Table 6. Of the two rabbits challenged with 150 ug/kg cobra venom, one received an early antivenine treatment at 15 minutes and no neurotoxic symptoms had ever been observed. The other rabbit developed severe muscular weakness and convulsion at 8 hours when antivenine was given followed by a slow recovery after 2 hours.

When antivenine was given immediately (at time 0) and at 15 minutes after challenge with lethal dose of 190 µg/kg cobra venom, all 6 rabbits survived without symptoms. The same result was also shown in another rabbit receiving only half dose of antivenine (3 times of neutralizing dose) at 15 minutes.

In the group with delayed antivenine treatment at 2 hours, three out of four survived. These rabbits had already shown some evidence of neurotoxicity when antivenine was administered but recovered within 4 hours. The fourth rabbit with signs of drowsiness and weakness at 1 hour after challenge progressed to paralysis, cyanosis and death within 5 minutes after antivenine treatment at 2 hours. (Another rabbit, to which antiveninen was given at 3 hours during the time that the

symptoms had progressed to complete paralysis , died within 45 minutes after antivenine injection.)

Thus, of the eleven rabbits receiving antivenine infusion at 0 minute, 15 minutes or 2 hours (excluding the rabbit which antivenine was given at 3 hours) after a lethal dose of cobra venom (190 µg/kg) administration, ten survived (overall survival rate 90.90 %). The single death was in that group receiving a delayed treatment.