CHAPTER V

DISCUSSION

In order to determine the kinetics of Thai cobra (Naja naja kaouthia) venom in serum and the effects of antivenine on serum venom levels, a double-antibody sandwich micro-ELISA technique has been developed to assay the venom levels. The sensitivity of the test was improved by the utilization of highly purified antisera. In this experiment, anticobra venom was produced in rabbits. Since most of the venom constituents are small molecular size and very poisonous, the immunization, therefore, was started with the lowest dose of venom and bentonite was used as adjuvant. According to Christensen (103), crude venoms absorbed onto bentonite lose some of their toxicity but retain their immunogenicity. The method of immunization was simple and high antibody titer was usually achieved 2-3 months after immunization. In the three rabbits tested, high titer of antibody was obtained on day 70. The decrease of antibody titer after day 70 in spite of continued immunization with increasing doses of the venom indicates the process of immune elimination in rabbits.

The IgG fraction was purified through protein A affinity chromatography. The method is a simple one-step procedure for preparation of IgG from whole serum. The protein A binds specifically to IgG with high affinity. This gives rise to IgG

al a higher degree of purity over that prepared from ion-exchange chromatography or from other conventional methods (98). The use of protein A purified rabbit IgG antivenom has been claimed to improve the sensitivity of a venom RIA assay by a factor of five over IgG prepared from DEAE cellulose column (88). However, the antibody titer of the prepared IgG fraction dropped to 1:3,200 as determined by hemagglutination test which was much lower than that of the original serum (1:25,600). This is most likely due to the binding ability of protein A for only some subclasses of IgG and the exclusion of IgM and IgA antibodies (104). Moreover, some of the IgG antibody was also lost in the peak 3 fractions (Figure 1 , Appendix III) which occurred because of improperly chromatographic column was used.

The prepared rabbit anticobra venom IgG gave good specificity since no cross-reaction was observed when tested against venoms from other terrestrial neurotoxic snakes, for instance, venoms from banded krait, king cobra or venoms from snakes in the family Crotalidae (green pit viper) and Viperidae (Russell's viper) at a concentration range of 1-1000 ng/ml (Figure 4). The technique was proven to be sensitive enough to detect 1-5 ng of cobra venom per ml of sera.

Because of the high sensitivity of the assay, greatest caution was taken to prevent non-specific reaction resulting from improper usage of the reagents and of the solid phase. For instance, the outer rows of the ELISA plate were omitted to avoid non-specific reaction due to edge effects probably caused by

illumination or temperature difference between the peripheral and the central wells (105). Non-specific reactions were also observed when commercial (unfractionated) cobra antisera were used in place of purified antivenom IgG .

It is clearly shown in this study that cobra venom is rapidly absorbed from the site of injection into the circulation. In all 10 rabbits injected with 80-190 µg/kg of cobra venom , the venom was readily detectable in sera within 15 minutes. The rapid absorption of cobra venom is attributed to the small molecular size of the constituents present in most of the venom toxins. For instance, neurotoxin of Thai cobra (M.W.7,800) (10) was assumed to be readily absorbed into the blood stream by passing directly through the capillary wall, whereas venoms of higher molecular weight (exceeding 20,000) such as those of black tiger snake and Russell's viper were absorbed via the lymph stream (36).

In this study, maximum venom levels were reached after 15-60 minutes of injection. After reaching the peak, the venom declined rapidly during the first 2 hours and somewhat slower thereafter. Similar observations were seen in the movement of Naja naja venom (53) and tiger snake venom (106) in which the venoms were rapidly absorbed from the site of injection, became detectable in the plasma of monkeys within 15 minutes and peaked about 30-60 minutes after a subcutaneous injection.

However, our results are in contrast to those of Thwin et al. (107) with Russell's viper venom. According to these worker, Russel's viper venom, when injected intramuscularly into mice,

peaked between 8-16 hours and remained detectable in the circulation even after 48 hours of venom administration. As for Echis carinatus, venom levels were low for the first 24 hours, reaching a maximum at 48 hours and remained detectable for 3 weeks following a subcutaneous injection into mice (85). The difference in the movement of each venom depends on the molecular weight as stated earlier and other contributing factors such as, the unique properties of each venom constituents, tissue affinity, dosage and route of administration, all of which require further study.

When sublethal dosages of cobra venom (80,125 and 150 µg/kg) were inoculated into rabbits, the venom disappeared from the circulation within 12-24 hours after injection. Rabbit receiving higher doses of 160 and 190 µg/kg developed neurotoxic signs in about one hour and died at 90 to 370 minutes. No assisted ventilation was given to these animals therefore it was not possible to follow the wane of the venom. It was assumed that by the time the venom level in serum decreased, most of the toxins especially neurotoxin, had already reached the target organs and fixed on the receptors of various tissues thereby causing paralysis and death.

The kinetics of cobra venom levels may partly depend on the size of the inoculum. According to recent studies on the kinetics of cobra venom in a group of non-antivenine treated patients, cobra venom was found in the serum at the concentration of 17-470 ng/ml. It rapidly declined with an average half life

of 7 1/2 hours in the first 20-30 hours after bite and inconsistently decreased thereafter. The venom remained in the serum until 13 to 65 hours after bite depending on the initial serum concentration which in turn reflects the amount of venom inoculated into each patient (108).

It is also interesting to note that no tissue necrosis was observed at the site of injection when administered subcutaneously. Nonetheless, most patients recovered from cobra snake bites were usually afflicted with necrotic wound (48) suggesting that certain consitituents in cobra venom such as cytotoxin, cardiotoxin might play the role of skin necrotizing factor in human. Rabbit skin may be unsusceptible to this factor. Moreover, the route and dose of envenoming may also play role in skin necrosis in rabbit.

In order to study the effect of antivenine treatment, 6 times the calculated in vitro antivenine requirement for the neutralization of venom was given to the envenomed rabbits in order to save as many rabbits as possible since it was suggested that the in vitro estimation of antivenine unitage fall far below that required in vivo (106). Christensen (109) found that more elapid antivenine was required when elapid venom was injected separately. Sutherland et al. (104) reported a slow recovery in monkey when 3 times the in vitro neutralization dose of antivenine was given after a challenge of tiger snake venom, therefore a minimum of 6 times the in vitro neutralization dose was recommended.

The effect of antivenine as reflected by the fall of the serum venom levels and the improved survival rate was demonstrated in rabbits receiving a lethal dose of 190 µg/kg of cobra venom. After specific antivenine was infused (at 0 or 15 minutes or 2 hours following venom inoculation), the concentration of venom in sera dropped to undetectable level within 15 minutes and 10 out of 11 envenomed rabbits survived (overall survival rate 90.90%). Early treatment of antivenine at 0 and 15 minutes completely abolished the venom toxicity whereas delayed treatment led to slow recovery and one death. These results emphasized the effectiveness of antivenine in neutralizing the venom in circulation and in reversing the systemic neurotoxicity in most rabbits. However, the clinical efficacy appeared to depend on the dosage of venom injected and on the timeliness of antivenine treatment. A rabbit , inoculated with 150 µg/kg of cobra venom causing severe weakness had already developed, was able to recover slowly within 2 hours after antivenine treatment. In the other group of animals afflicted with a lethal dose of 190 µg/kg, in which antivenine was given immediately or 15 minutes after venom injection, all rabbits survived without any neurotoxicity observed. When antivenine treatment was delayed to 2 hours, a time when neurotoxic signs began to manifest, the rabbits were slowly recovered and one death. This indicates that the ability to reverse the toxic effect depended on the stage and the degree of toxicity that had already developed.

This finding indicates that when severe neurotoxic signs

have evolved denoting the significant fixation of the toxin on target organ receptors, the antivenine is unable to reverse the binding of the toxins to the receptors. However, dissociation of neurotoxin-acetylcholine receptor complexes by antiserum or monoclonal antibodies has been demonstrated in the case of Naja nigricollis toxin (110). This might be inapplicable in the case of Thai cobra venom, of which the neurotoxin 3 has been shown to be a potent neuromuscular blocking agent acting irreversibly on the cholinergic receptors (5,25). In contrast, when given at the right time, specific immunoglobulin was able to bind free neurotoxin molecules in the circulation rendering it inactive thereby neutralizing the would-be toxicity. The observation that cobra venom became undetectable immediately after antivenine infusion was probably attributed to the fact that most of the antibody-combining sites on the venom molecules were blocked. Since the ELISA assay required at least 2 antibody-combining sites on the venom for its detection. This negative finding of cobra venom in sera reflects the binding ability of the antivenine following infusion.

Accordingly, specific antivenine is an effective therapeutic agent in cobra bites, particulary when no artificial ventilation was available to assist the envenomed victim. The effectiveness of antivenine treatment most likely depends on the timing of antivenine administration after the bite. In other words, the antivenine will be less effective if most of the toxins were already fixed on the cholinergic receptors. Other factors that may account for the recovery of neurotoxic symptoms

include : route of antivenine administration, dosage of venom and antivenine, etc.

Since this is an experimental model, the number of rabbits used might be considered too small and the dose of venom inoculated is probably much lower than the natural bite. It is yet too early to conclude right now for the complete kinetics of the venom and the effects of antivenine in many aspects. Nevertheless, it is evident in this study that an experimental model could be designed for the study of cobra venom movement in vivo. The development of a sensitive ELISA technique is invaluable, not only for the serial detection of cobra venom following venom inoculation in this study, but also as a potential diagnostic tool for cobra bites in the future. The study reveals the kinetics of cobra venom in sera, the peak concentration which might be used as a prognostic indicator in snake bite patients. The rate of neurotoxicity development and mortality were related to serum concentration which in turn reflected the size of the inoculum. It is also perceptible that a well-timed antivenine treatment is still an effective measure for the management of cobra bite victims.

Conclusions :

1. A micro-ELISA (double-antibidy sandwich technique) was developed for the detection and quantitation of Thai cobra (Naja naja kaouthia) venom in rabbit serum with high sensitivity and specificity.

2. Cobra venom was detected in serum within 15 minutes after a single subcutaneous injection of various doses of cobra venom, peaking at 15-60 minutes and disappearing from the circulation in 12-24 hours.

3. When high doses of cobra venom (160 or 190 μg/kg) were injected, most of the rabbits developed signs of neurotoxicity in about 1 hour and died at 90-370 minutes after venom injection.

4. Administration of specific antivenine was shown to neutralise the cobra venom in serum to undetectable level within 15 minutes. It was also effective in preventing and reversing the neurotoxic effects of cobra venom in envenomed animals. The efficacy was shown to be related to the timeliness of antivenine administration and the dose of venom injected.

Table 1. Passive hemagglutination titer of rabbit anticobra venom in three rabbits immunized with crude cobra venom (Naja naja kaouthia).

Days after immunization	PHA titer			Cells*
	rabbit 1	rabbit 2	rabbit 3	control
0	< 100	< 100	<100	< 100
70	25,600	102,400	12,800	< 100
77	12,800	51,200	ND	< 100
84	6,400	25,600	12,800	< 100
98	3,200	51,200	6,400	< 100

* Uncoupled sheep red blood cells.

Table 2. Checkerboard titration to determine the optimal concentration of reagents in ELISA test (double- antibody sandwich Technique). Optical density (OD) was measured at absorbance 405 nm.

anti-cobra venom	conjugate 1:50			conjugate 1:100			conjugate 1:200			conjugate 1:400		
	CV* (ng/ml)		Neg.@	CV (ng/ml)		Neg.	CV (ng/ml)		Neg.	CV (ng/ml)		Neg.
	50	1	serum	50	1	serum	50	1	serum	50	1	serum
1:1,000 (1µg/ml)	1.560	0.378	0.315	0.741	0.228	0.258	0.399	0.074	0.034	0.299	0.040	0.023
1:2,000 (.5 µg/ml)	1.276	0.226	0.105	0.622	0.107	0.063	0.473	0.082	0.038	0.221	0.039	0.026
1:4,000 (.25µg/ml)	0.861	0.159	0.088	0.482	0.073	0.046	0.273	0.033	0.000	0.182	0.036	0.023

* Cobra venom
@ Normal rabbit serum

Table 3. Optimal conditions of ELISA test for the detection of cobra venom

Steps of ELISA test	Concentration or dilution	Temperature (°c)	Reaction time (minute)
Coating of venom Ab	0.5 µg/ml	37°C	60
		or 37°C + 4°C	60+overnight
Reacting with venom	vary	37°C	60
Reacting with enzyme-labeled Ab	1:50	37°C	60
Color development	1 mg/ml*	37°C	60

*p-nitrophenyl phosphate substrate concentration

Table 4 Precision of ELISA test for cobra venom.

QC pool (ng/ml)	1	2	3
Within-plate(n=10)			
Mean	33.50	13.00	2.40
SD	2.10	1.22	0.07
CV%	6.27	9.40	2.87
Between-plate(overall)			
Mean	33.00	12.00	2.53
SD	1.51	1.82	0.23
CV%	4.44	15.20	9.20

Table 5. Mean serum concentrations of cobra venom detected after subcutaneous injection with various doses of cobra venom

Dose	No.	Time after cobra venom injection (hours)							
(μg/kg)	tested	0.25	0.5	1	2	4	8	12	24
80	2	70	55	40	29	20	12	0	0
125	2	98	90	66	56	52	15	8	0
150	2	128	118	90	67	38	18	15	0
160	2	111	135	116	90	82	ND *	ND	ND
190	2	118	141	137	95	65	ND	ND	ND

* Not done (due to death)

Table 6. Efficacy of antivenine treatment in envenomed rabbits.

Dose ug/kg	Number of survivals (%)	
	Without antivenine	With antivenine
80 (N=2)	2/2 (100)	ND@
125 (N=2)	2/2 (100)	ND
150 (N=4)	2/2 (100)	2/2 (100)*
160 (N=2)	0/2 (0)	ND
190 (N=13)	0/2 (0)	10/11 (90.90)**

@ Not done

*Antivenine was given at 15 min. & 8 hr. after envenoming

**Antivenine was given at 0 min. (n=3), 15 min.(n=4), 2 hr.(n=4) after envenoming

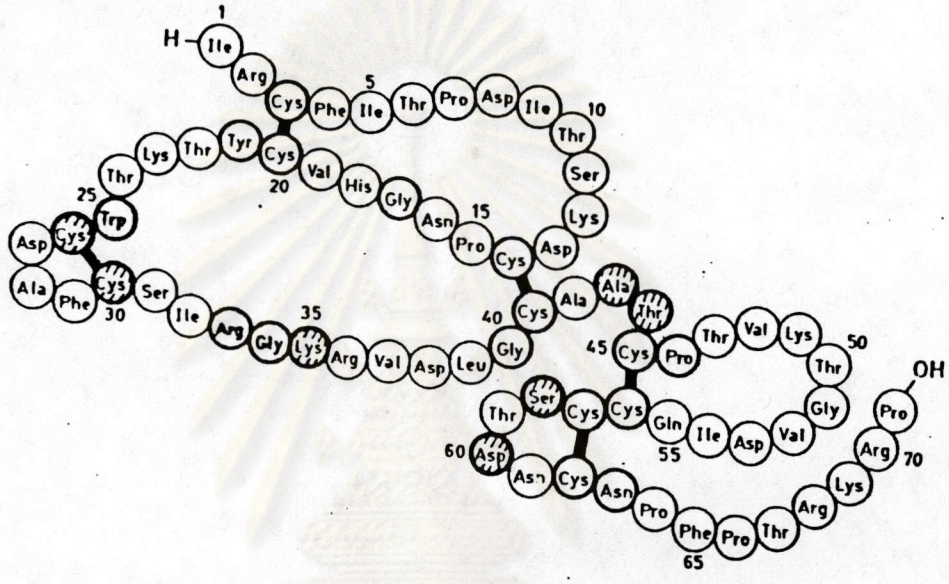

Figure 1. The covalent structure of a long postsynaptic neurotoxin, the siamensis toxin of Naja naja siamensis.

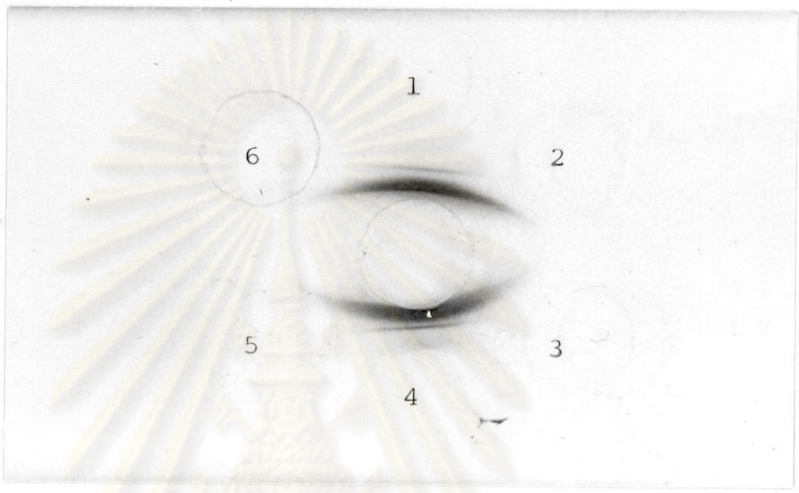

Figure 2. Immunodiffusion reaction of rabbit antiserum to cobra venom (central well)against 1 mg/ml of venom from ; cobra (well 1,4), russell's viper (well 2), green pit viper (well 3), banded krait (well 5), and king cobra (well 6).

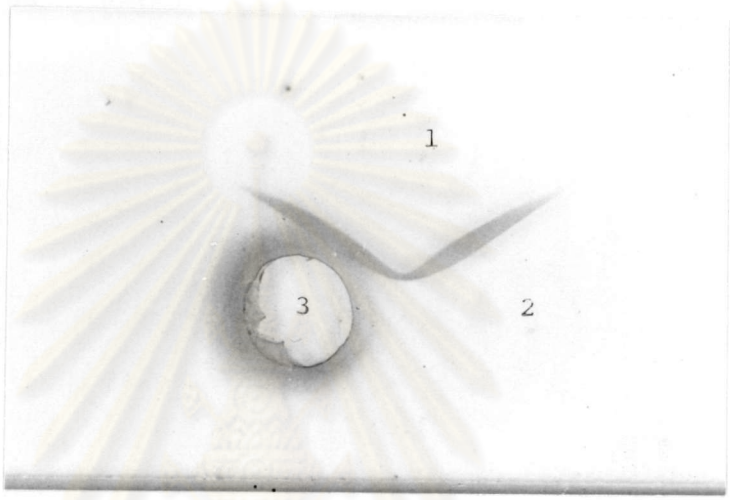

Figure 3. Immunodiffusion reaction of rabbit antiserum to cobra venom (well 1) against goat antiserum to rabbit IgG (well 2) and swine antiserum to rabbit serum (well 3).

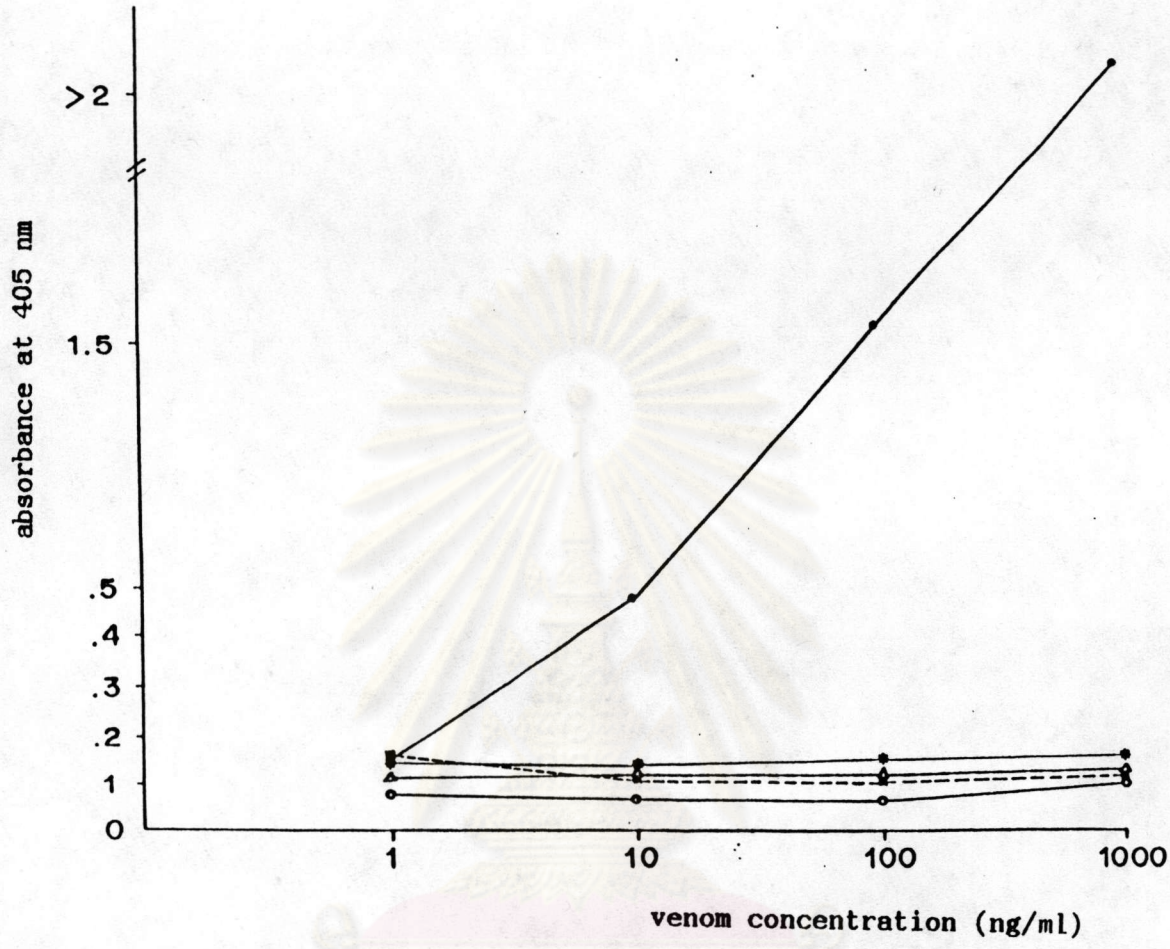

Figure 4. Specificity of ELISA test for the detection of cobra venom.

Cobra venom

Russell's viper venom

King cobra venom

Green pit viper venom

Banded krait venom

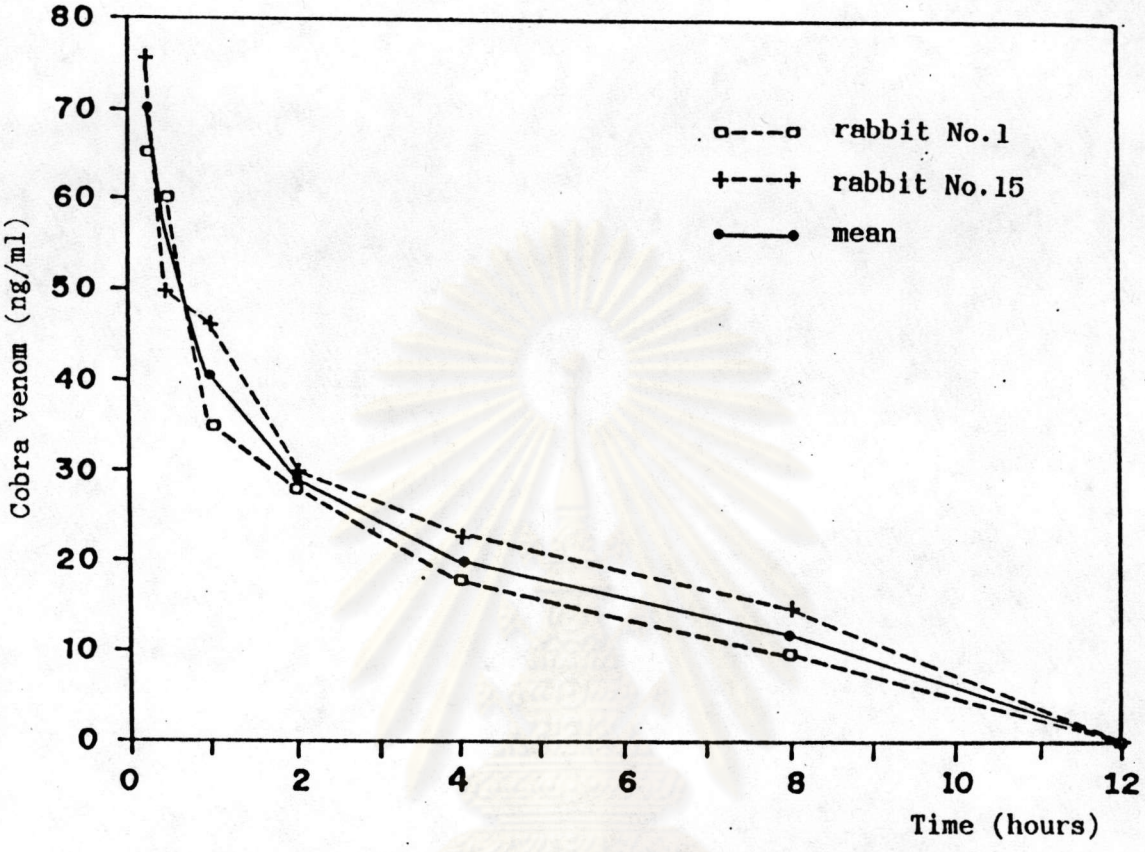

Figure 5. Levels of cobra venom in rabbit serum following a subcutaneous injection of 80 µg/kg cobra venom .

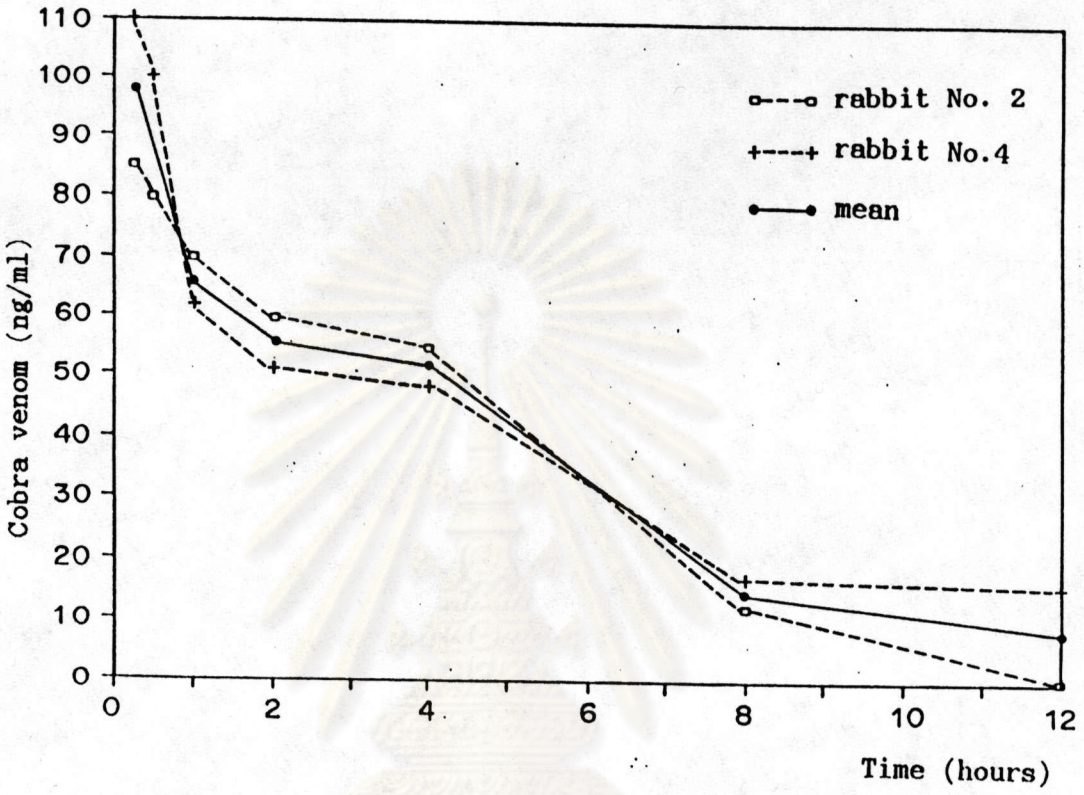

Figure 6. Levels of cobra venom in rabbit serum following a subcutaneous injection of 125 µg/kg cobra venom.

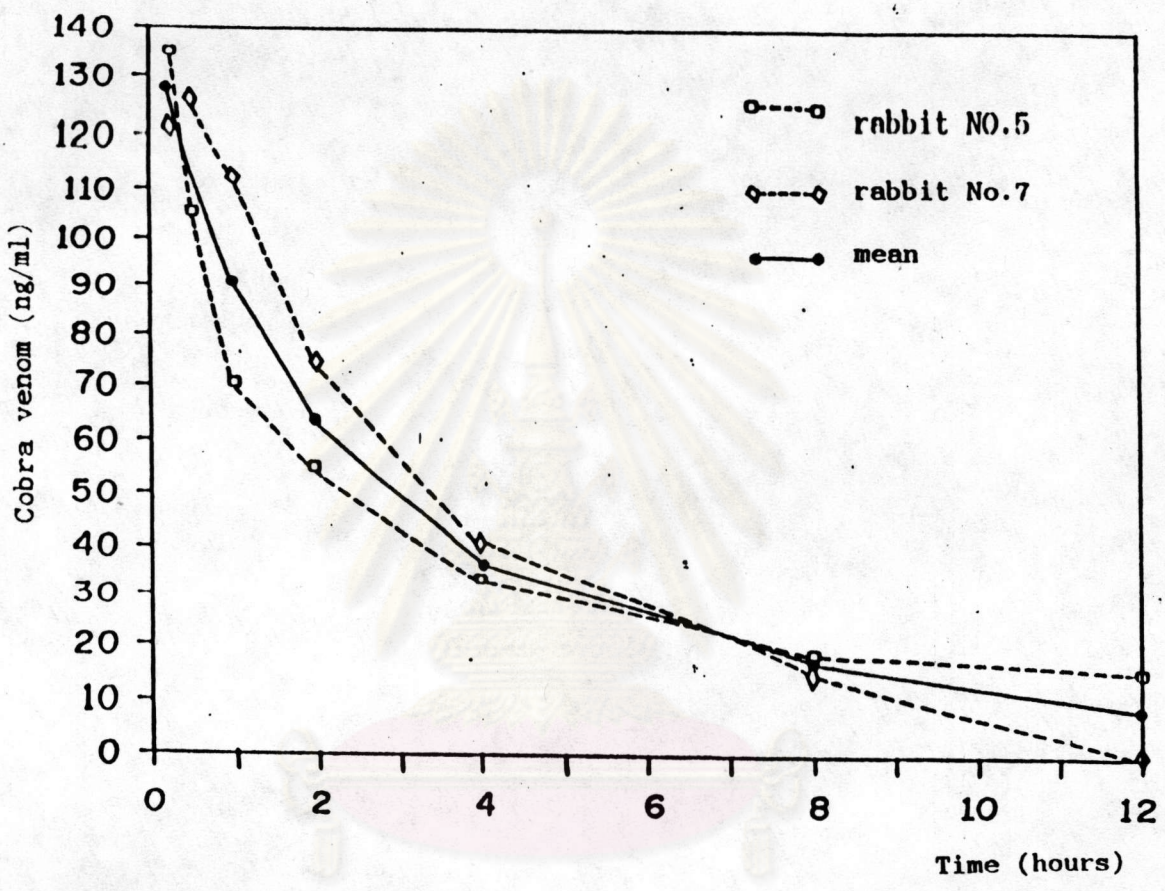

Figure 7. Levels of cobra venom in rabbit serum following a subcutaneous injection of 150 μg/kg cobra venom.

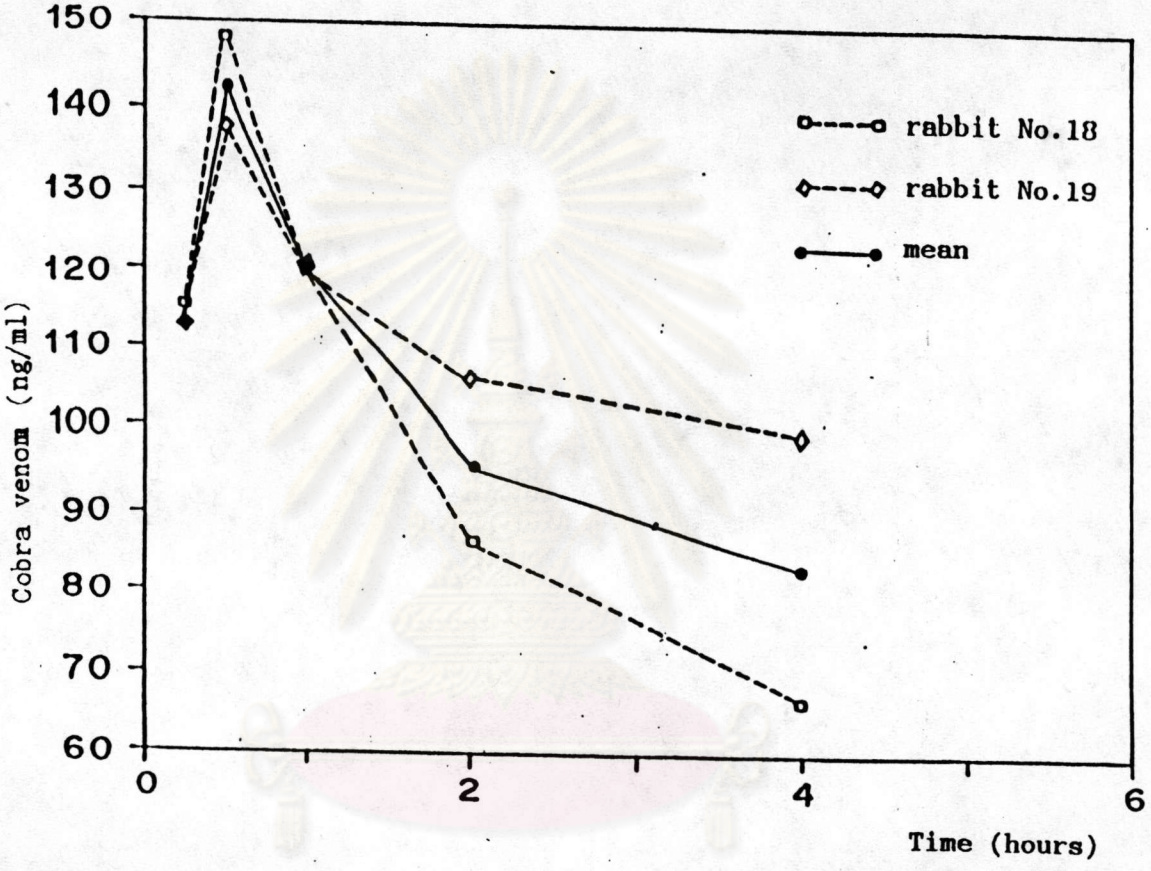

Figure 8. Levels of cobra venom in rabbit serum following a subcutaneous injection of 160 μg/kg cobra venom.

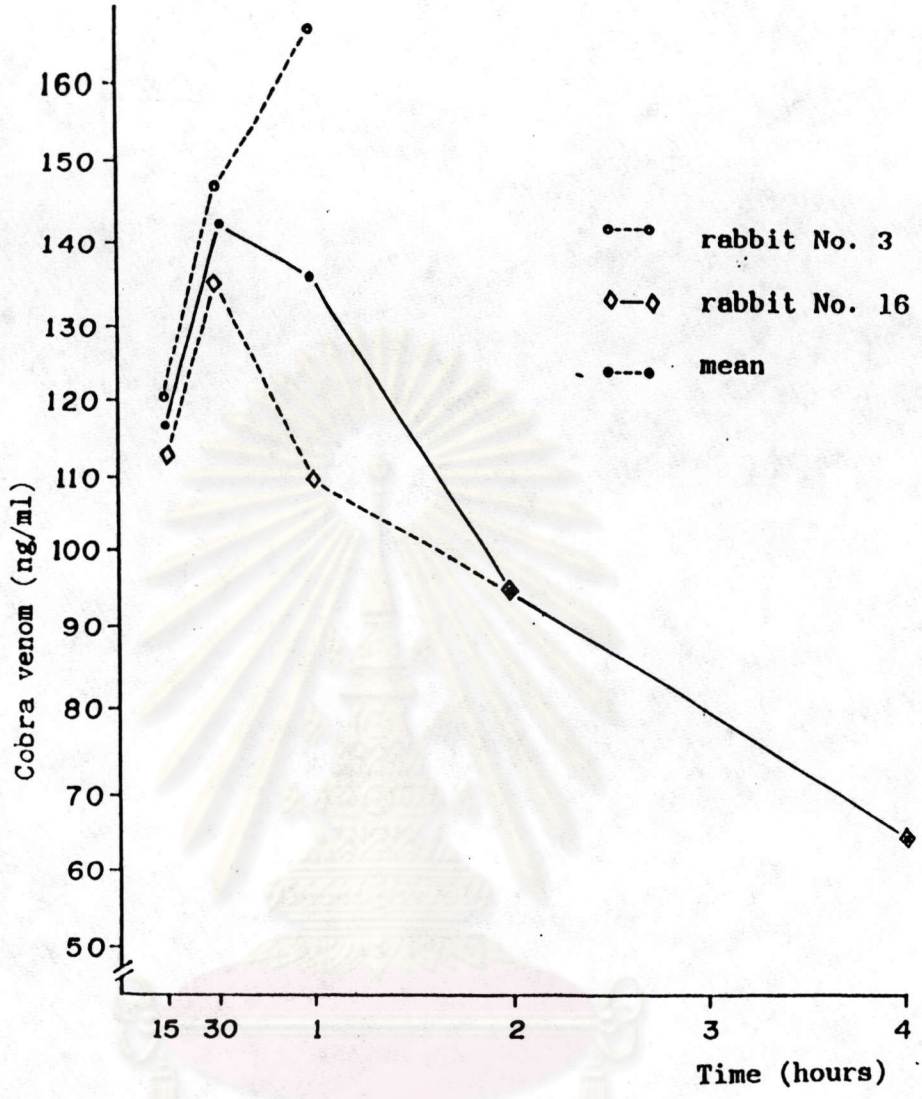

Figure 9. Levels of cobra venom in rabbit serum following a subcutaneous injection of 190 µg/kg cobra venom.

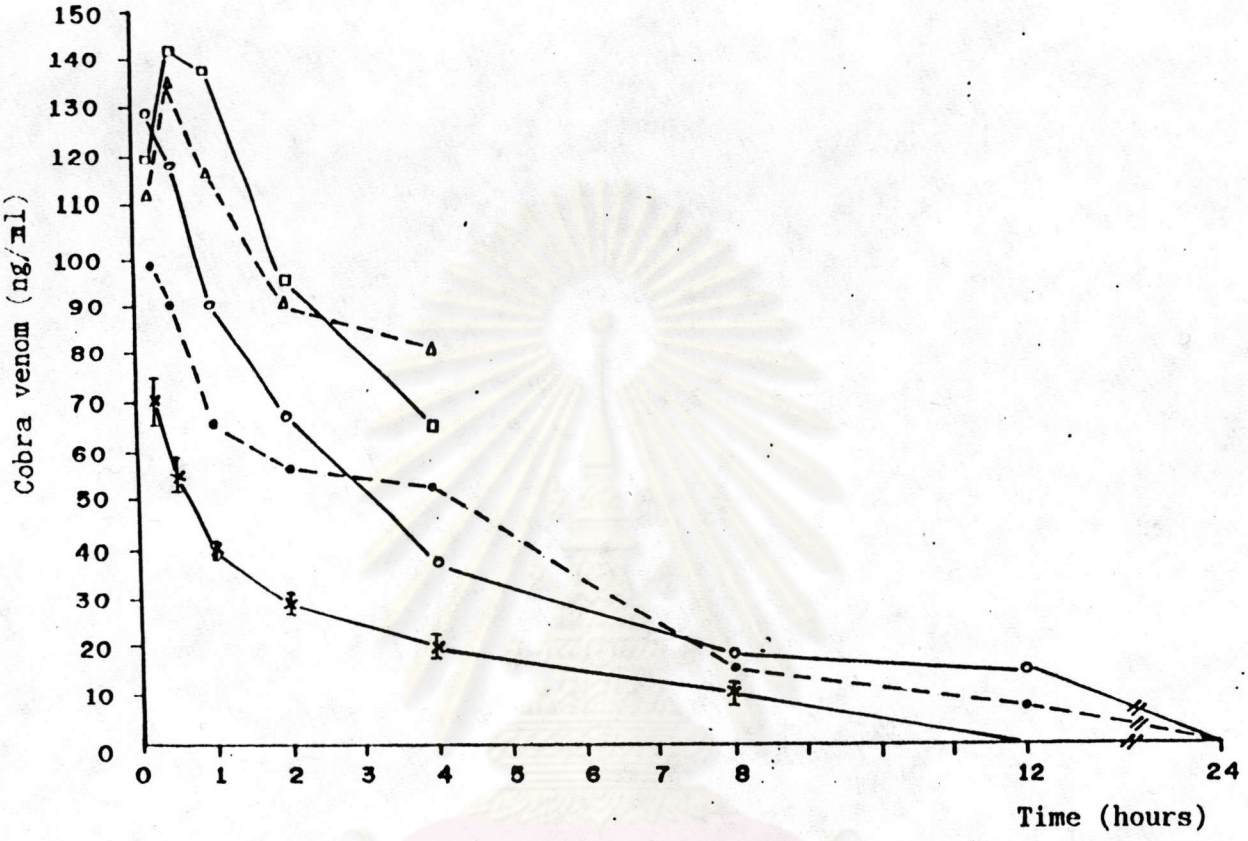

Figure 10. Mean serum concentrations of cobra venom in rabbit serum after subcutaneous injection with various doses of cobra venom.

×—× 80 µg/kg

•—• 125 µg/kg

o—o 150 µg/kg

Δ—Δ 160 µg/kg

□—□ 190 µg/kg

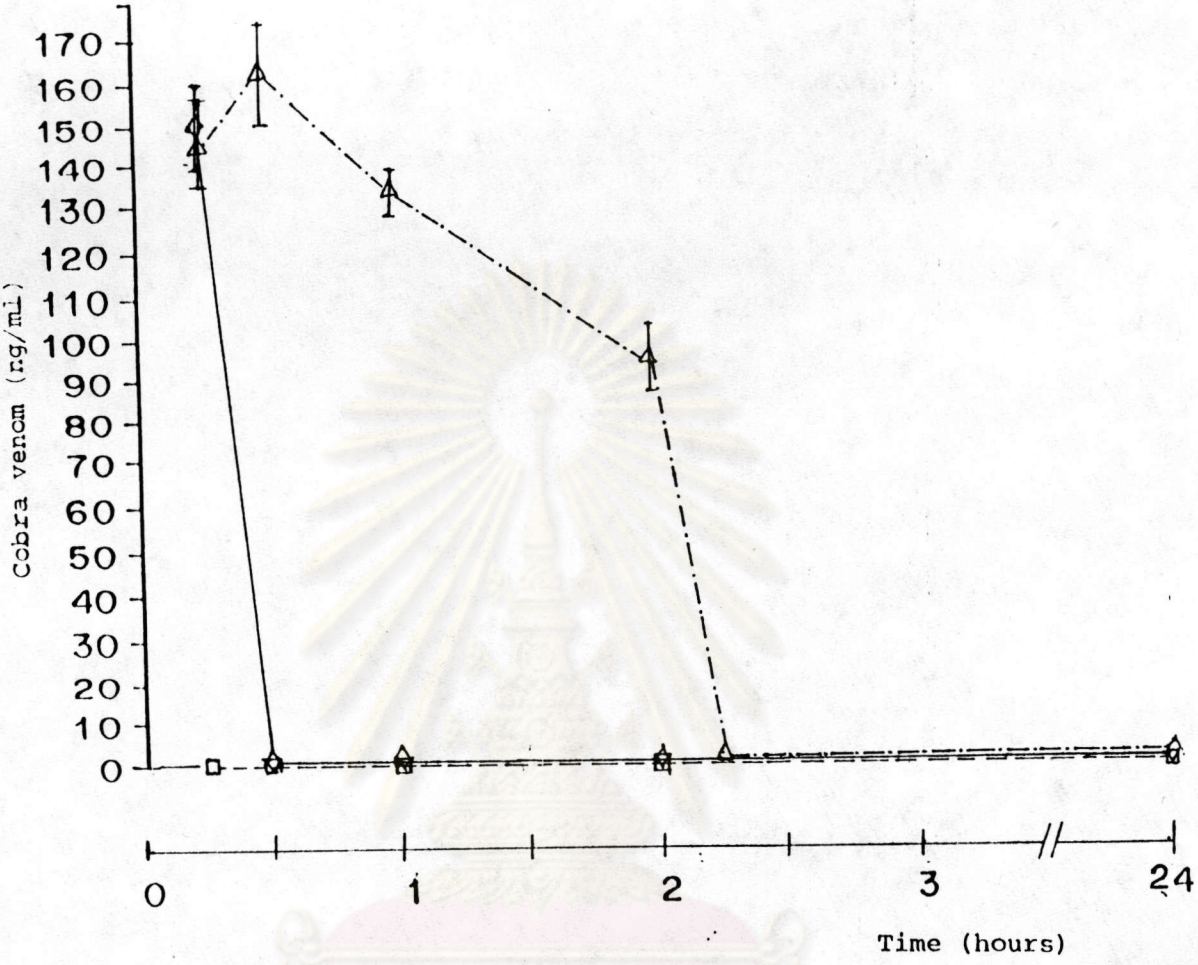

Figure 11. Mean serum concentrations (±SE) of cobra venom after subcutaneous injection with 190 μg/kg cobra venom, followed by a single injection of anticobra venom at 0 or 15 minutes or 2 hours.

□--□ 0 minutes

◇—◇ 15 minutes

△--△ 2 hours