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MEMORANDUM

TO: AID/PPC/CDIE/DI, room 209 SA-18

FROM: AID/SCI, Victoria Ose

SUBJECT: Transmittal of AID/SCI Progress Report(s)

Attached for permanent retention/proper disposition is the following:

AID/SCI Progress Report No. 5.221
PR #3

Attachment

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PN-ABA-558
5.221

PROGRESS REPORT NO. 3

Development of Immunodiagnosis and Improvement of Immunotherapy of Snake Venom Poisoning

A Research Project

USAID / PSTC PROGRAM

GRANT NO. 936-5542-G-00-5079-00

SUBMITTED BY

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Project profile

Country	:	Thailand
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Project title	:	Development of Immunodiagnosis and Improvement of Immunotherapy of snake venom poisoning.
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Table of content

Item	Topic	Page
# 1	Development of immunodiagnostic test for the detection of snake venoms	1
# 2	Development of ELISA as an *in vitro* assay of antivenom potency	3
# 3	Purification of *O.hannah* postsynaptic neurotoxins	5
# 4	Study on the improvement of immunogenicity of cobra postsynaptic neurotoxins and preparation of polyvalent antivenom	6
# 5	Refinement of anti-snake venom antiserum	8
# 6	Preparation of monoclonal antibodies against Siamensis toxin 3	11

1. Development of immunodiagnostic test for the detection of snake venoms.

Background in the previous progress reports, the study on two different immunoassays namely passive hemagglutination inhibition (PHAI) test and the reverse latex agglutination (RLA) test were described. The PHAI was shown to be rather insensitive and probably not useful for venom detection in actual cases. The RLA was found to be very sensitive, specific and rapid. As we mentioned in the previous report, two problems were encountered in RLA. First, N.n.siamensis venom cross-reacted with anti-B.fasciatus venom sensitised latex. Second, some non-specific interference was observed from the body-fluids of rural people. During this reporting period we found that the cross-reaction was the result of one peculiar rabbit serum ; no sera from other rabbits produced such cross-reaction. The problem with non-specific interference was partially solved by the inclusion of normal rabbit serum. However, the addition of normal rabbit serum resulted in dilution of the antigen sample and thus the reduction in the sensitivity of the test.

While trying to solve the problem encountered in RLA, we decided to concurrently explore another immunoassay -- reverse passive hemagglutination (RPHA) test pioneered by Coomb and his colleages. They have shown that RPHA in many cases is as sensitive as ELISA in the detection of antigen and yet it is simple to perform and does not require special expertise or equipment.

Since erythrocytes from different species behave

4

differently in RPHA eg., stability to venom enzymes, speed of sedimentation, interference from heterophile antibody etc., we decided to study erythrocytes from sheep, human and chicken and compare their properties in RPHA in the detection of the six snake venoms.

Materials and Methods.

The erythrocytes from various sources were sensitized with Protein A-purified rabbit IgG against each of the six snake venoms. The coupling agent used was chromic chloride. All other procedures were carried out similar to those previously described.

Coupling of purified rabbit IgG to trypsin-treated red blood cells (TT-RBC).

a. Trypsin treatment. Red blood cell was washed 6 times with 0.15 M PBS pH 7.2. A 10% suspension of the washed cell was mixed with an equal volume of 0.25% trypsin (Sigma) and incubated at 37 °C with gentle shaking for 30 minutes. The cell was then washed 3 times with PBS. A 10% suspension was incubated with an equal volume of 0.025% soybean trypsin inhibitor for 30 minutes at room temperature. Finally the cell was washed 3 times with normal saline solution.

b. Coupling of IgG to trypsin treated red blood cell (TT-RBC) using chromic chloride. An equal volume of 2-3 mg/ml rabbit IgG was added to packed TT-RBC with rigorous mixing. Two volume of 0.02% chromic chloride was added dropwise to the red cell suspension. The reaction mixture was rotatory mixed for 60' at room temperature.

Results. A comparison of the erythrocytes from these 3 sources in RPHA is show in Table 1.

In summary, all erythrocytes gave highly sensitive detection of venom antigens in RPHA. The minimal detection limits were between 2 ng/ml to 610 ng/ml which are comparable to those obtained from ELISA. Cross-reaction of heterologous antigens were observed at concentrations of several hundred or thousand folds higher than that of homologous antigens.

The rates of sedimentation of the erythrocytes differed greatly. Chick erythrocytes, being nucleated, sedimented rapidly and the result of RPHA could be read within 30 min. However, some interference from sera of rural people was again observed in the RPHA test using any kind of erythrocytes. The stability of the sensitized erythrocytes are also quite satisfactory.

Future plan

When all the data on this comparative study are available, selection will be made on the most suitable RPHA and study on serum samples obtained from victims of snakebite.

A total of about 40 samples of wound aspirates and sera from snakebite victims are currently available for study with the RPHA and RLA described previously.

2. Development of ELISA as an in vitro assay of antivenom potency.

As previously reported, there are several important reasons for the development of an in vitro assay of antivenom potency. The progress made was the success in the development of

TABLE I

Sensitivity

Sheep red blood cell

Antigen	NS	BF	OH	CR	VR	TA
ng/ml	4	[illegible]	312	610	[illegible]	[illegible]
ng/50ul	[illegible]	0.25	15.65	30.5	0.5	0.25

Human red blood cell

Antigen	NS	BF	OH	CR	VR	TA
ng/ml	2	4	312	610	10	2
ng/50ul	0.1	0.2	15.65	30.5	0.5	0.1

Chicken red blood cell

Antigen	NS	BF	OH	CR	VR	TA
ng/ml	2	4	625	610	20	-
ng/50ul	0.1	0.2	31.25	30.5	1.0	0.1

Specificity

Antibody \ Antigen ug/ml	NS	BF	OH	CR	VR	TA
Anti-NS	[illegible]	2250	1862	1650	812	308
Anti-BF	225	[illegible]	[illegible]	525	1625	625
Anti-OH	450	1150	[illegible]	525	812	625
Anti-CR	450	575	2300	[illegible]	406	308
Anti-VR	450	575	1862	131	[illegible]	625
Anti-TA	225	288	2300	262	812	[illegible]
Normal IgG	450	575	1862	262	812	[illegible]

Antibody \ Antigen ug/ml	NS	BF	OH	CR	VR	TA
Anti-NS	0.002	575	2300	66	1625	10
Anti-BF	56	[illegible]	1862	66	202	10
Anti-OH	112	575	[illegible]	262	812	38
Anti-CR	450	1150	2300	[illegible]	812	38
Anti-VR	900	575	2300	33	[illegible]	38
Anti-TA	225	288	2300	33	812	[illegible]
Normal IgG	900	4600	2300	262	1625	38

Antibody \ Antigen ug/ml	NS	BF	OH	CR	VR	TA
Anti-NS	[illegible]	2300	2300	131	1625	154
Anti-BF	112	[illegible]	1150	131	812	308
Anti-OH	225	1150	1625	262	812	154
Anti-CR	450	2300	1862	[illegible]	[illegible]	154
Anti-VR	112	2300	2300	525	[illegible]	308
Anti-TA	450	575	1862	262	812	[illegible]
Normal IgG	900	2300	2300	262	1625	1250

Absorption of RBC

Sample - absorbed by packed TT-BRBC and 50% TT-BRBC-nIgG in EDTA 10 mM
ratio of sample : packed TT-BRBC : 50% TT-BRBC-nIgG = 2:1:1
rotated 30 minutes -- centrifuged 5 minutes -- assay
supernate by serial two fold dilution (1:4)

Urine - absorbed by 50% TT-HRBC-nIgG
ratio of urine : 50% TT-HRBC-nIgG = 1:1
rotated 30 minutes -- centrifuged 5 minutes -- assay
supernate by serial two fold dilution (1:8)
or - add normal rabbit serum 50% -- assayed by serial two fold dilution (1:8)
Serum, Plasma - diluted serial two fold dilution (1:8)

Sample - absorbed by packed TT-CRBC and 50% TT-CRBC-nIgG in EDTA 10 mM
ratio of sample : packed TT-CRBC : 50% TT-CRBC-nIgG = 2:1:1
rotated 30 minutes -- centrifuged 5 minutes -- assay
supernate by serial two fold dilution (1:4)

Sedimentation time (min)	90	60	30
Total test time (min)	120	60	60
Stability			
No glutaldehyde, 4°C (days)	7	7	7
0.1% glutaldehyde, 4°C (month)	>4	>2	>1
Technique			
TT-RBC lysis	+++	+	++
Simplicity	+++	+	+
Reproducibility	+++	++	+

an ELISA using purified principal postsynaptic neurotoxin of N.n.siamensis as antigen in the estimation of antivenom potency. The Background, Materials and Methods, Results and Discussion of this study are described in the enclosed manuscript (ms #2) entitled "Selection of cobra venom antigens for use in an ELISA of horse antivenom potency" by Jarasporn Rungsiwongse et al.

This ELISA will be introduced to the Government Pharmaceutical Organization of Thailand and the Queen Soavabha Memorial Institute for use during the production of horse antivenom against Thai cobra. It will also be used in studies on the improvement of immunogenicity of cobra neurotoxin (#4) and studies on refinement of horse antivenom (#5) described below.

Under study on this aspect is a similar development of another ELISA for use in the estimation of potency of antivenom against Russell's viper. The Vipera russelli venom has been fractionated by chromatography on a Sephadex G-100 column (2.6 x 97 cm) into 2 major protein peaks (Fig 1). Each peak and also the crude venom will be used as antigens of ELISA in a comparative study to select the best ELISA which gives the highest correlation coefficient with the in vivo neutralization assay using mice.

A total of 10 batches of horse antivenom against V. russelli venom have been assayed by the in vivo neutralization method. The results are shown in Table 2.

Future plan

In progress is the assay of these 10 batches of antivenom using the ELISA's using each of the 3 antigen

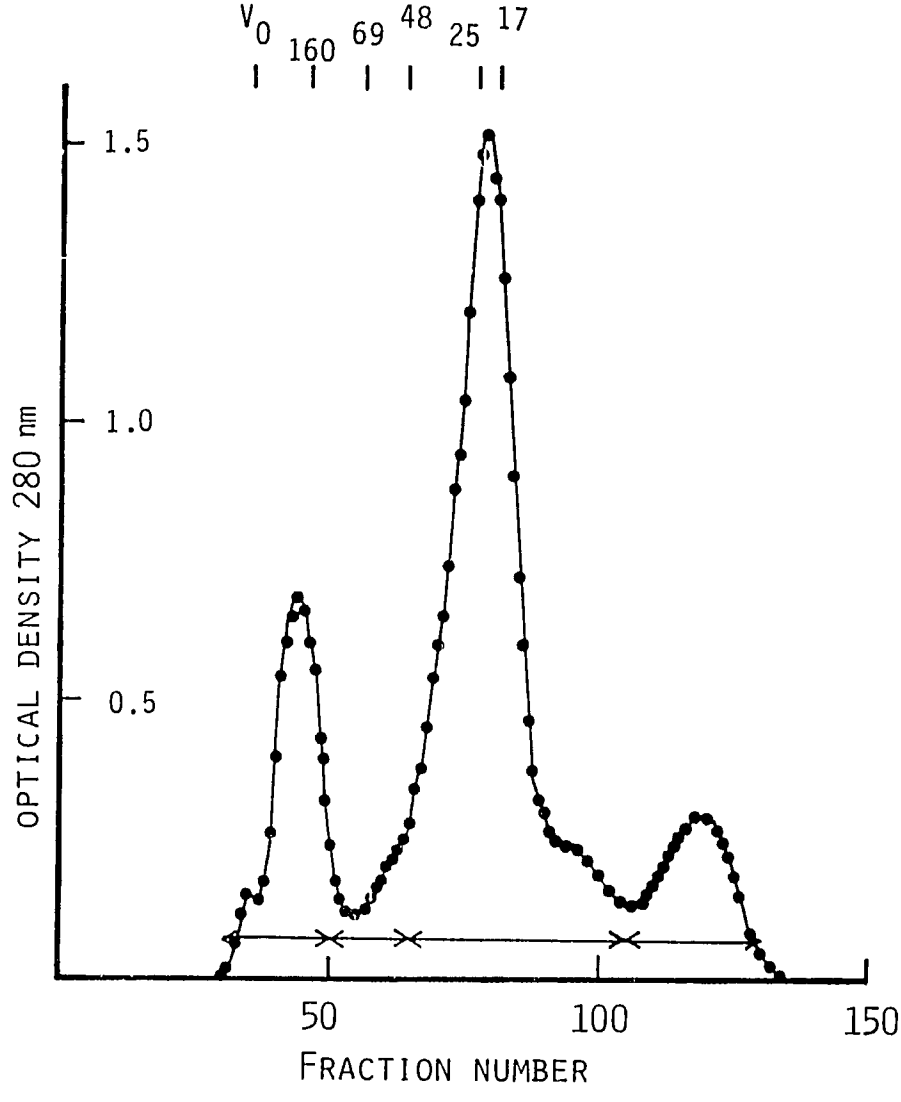


Fig 1. Chromatographic profile of V.russelli venom on a Sephadex G-100 column.

Table 2 Horse antivenom potencies determined by *in vivo* neutralization

Anti VR#	in vivo neutrolization activity (ug/ml)	
1	695.8	(667.8-724.8)*
2	630.8	(611.5-650.7)
3	580.8	(555.2-607.5)
4	390.8	(363.3-420.1)
5	470.5	(447.8-494.8)
6	860.8	(835.0-887.3)
7	720.8	(693.3-749.6)
8	512 8	(492.0-534.4)
9	840.8	(746.8-898.1)
10	536.8	(513.5-561.0)

* Numbers in parintheses are 95% fiducial limits.

preparations. Comparison will be made on the correlation coefficients of the ELISA's and the *in vivo* neutralization. The antigen which gives the highest correlation coefficients between the two assays will be used in the production and refinement of antivenom against *V.russelli*.

3. Purification of *O. hannah* postsynaptic neurotoxins.

Background.

As mentioned previously, the postsynaptic neurotoxin(s) of *O. hannah* are needed for

(a) basic study on the chemistry of the toxins

(b) study on the immunogenicity of the toxin and preparation of polyvalent antivenom

(c) in monoclonal antibody study of epitope

Meterials and Methods

The venom of *O.hannah* (3 gm) was fractionated by successive chromatography on a Sephadex G-50 followed by a Biorex-70 column using gradient elution. The chromatographic profile of Biorex-70 column is shown in the previous report.

Results and Discussion

Of the 4 peaks, only peaks #1 and #4 are lethal at 10 ug/ 25 gm mouse. A study on the amino acid composition of peak 1 give results shown in Table 3. The toxin contains 70 amino acid residues with 5 disulfide bridges. It contains one residue of tryptophan which is also present in *N.n.siamensis* toxin 3.

With the limited amount of toxin available, the study

on amino and carboxyl terminals have not yet been carried out. Unfortunately, the latest attempt to purify more of these neurotoxins of O.hannah gave results which are extremely puzzling. In stead of the 4 peaks of toxins obtained previously, a total of about 15 peaks of basic, small molecular weight peptides were obtained (Fig. 3). From the nature of the linear gradient generated, it is believed that all these peaks are bona fide protein peaks, not an artifact of the gradient. It is not known, however, how the venom obtained in lyophilised form from Queen Sowavabha Memorial Institute could contain so many protein components.

Meanwhile, for immunogenicity study, we have prepared a toxin fraction containing all the low molecular weight toxins (< 3,000 daltons). This toxin fraction will be used in the study on preparation of potent polyvalent antivenom against all 3 elapid snakes of Thailand.

Future plan. The major toxin peaks will be further purified by gel filtration. The homogeneous toxins will be studied for N and C terminals and also amino acid compositions. These results will be compared with those of toxins from other elapids. The information should be useful to the understanding on structures and functions of different elapid toxins.

4. Study on the improvement of immunogenicity of cobra postsynaptic neurotoxins and preparation of polyvalent antivenom.

Background. We have previously shown that postsynaptic

12

PROTEIN COMPOSITION (Toxin BR1T)

Amino Acid	Adjust 24A	Adjust 24B	Adjust 48A	Adjust 48B	Adjust 72A	Adjust 72B	Adjust 96A	Adjust 96B	Adjust P1	Adjust P2	Adjust M1	Adjust M2	Amino Acid	Optimum Nmoles	Mean Res Weight	ng AA	Residues per mol
Asx	58.60	76.66	59.38	59.96	43.39	56.54	58.11	57.77	57.73	58.69	53.10	52.48	Asx	57.701	115.1	6641.34	7.37
Thr	69.67	69.55	72.31	72.64	59.66	69.94	73.70	74.07	76.71	74.33	49.11	0.00	Thr	71.257	101.1	7204.08	9.10
Ser	32.78	28.40	30.09	31.73	16.84	30.66	28.75	28.11	28.00	30.53	31.85	0.00	Ser	28.589	87.1	2490.08	3.65
Glx	33.86	33.30	37.37	34.57	14.05	34.87	37.39	36.62	35.95	36.57	31.35	32.10	Glx	33.165	129.1	4281.59	4.23
Pro	49.64	46.19	45.91	42.84	59.34	49.20	45.20	36.76	46.89	48.30	46.62	39.74	Pro	46.384	97.1	4503.92	5.92
Gly	36.67	33.17	37.41	36.76	45.66	35.70	35.39	36.77	35.91	35.40	45.57	47.47	Gly	38.489	57.1	2197.73	4.91
Ala	33.67	29.45	33.57	33.63	42.55	34.39	34.35	35.00	34.25	33.45	32.42	31.98	Ala	34.059	71.1	2421.59	4.35
Val	35.44	33.70	35.80	38.07	36.43	38.77	41.41	39.70	37.26	34.04	33.60	34.20	Val	36.534	99.1	3620.54	4.66
Met	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	Met	0.000	131.2	0.00	0.00
Cys	30.22	60.21	66.93	30.82	16.81	33.20	57.64	71.19	75.04	75.72	0.00	0.00	Cys	75.383	102.2	7704.10	9.62
Ile	20.98	14.77	11.61	22.43	19.80	25.13	24.94	24.02	21.21	20.13	17.87	16.16	Ile	19.921	113.2	2255.94	2.54
Leu	9.84	2.43	9.71	8.93	8.63	10.59	10.27	9.59	9.74	10.16	8.32	8.48	Leu	8.891	113.2	1006.46	1.14
Tyr	12.15	5.74	12.69	12.03	3.43	12.09	13.51	12.61	7.61	7.41	13.26	12.36	Tyr	10.407	163.2	1698.48	1.33
Phe	7.09	8.53	7.72	7.28	7.61	1.57	6.66	6.54	6.47	8.54	7.30	7.37	Phe	6.889	147.2	1014.01	0.88
Lys	46.26	45.45	48.05	48.44	44.59	49.75	50.79	48.91	48.31	47.66	44.02	42.58	Lys	47.068	128.2	6034.07	6.01
His	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	His	0.000	137.2	0.00	0.00
Arg	30.98	28.04	29.69	30.48	29.52	32.53	31.63	30.62	32.12	32.05	30.34	29.34	Arg	30.610	156.2	4781.33	3.91
Trp	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	4.70	4.84	Trp	4.767	186.2	887.57	0.61
	169.75	169.75	169.75	169.75	169.75	169.75	169.75	169.75	169.75	169.75	169.75	169.75				TOTAL RESIDUES =	70.2368

Total Protein = 58741.94 nanograms

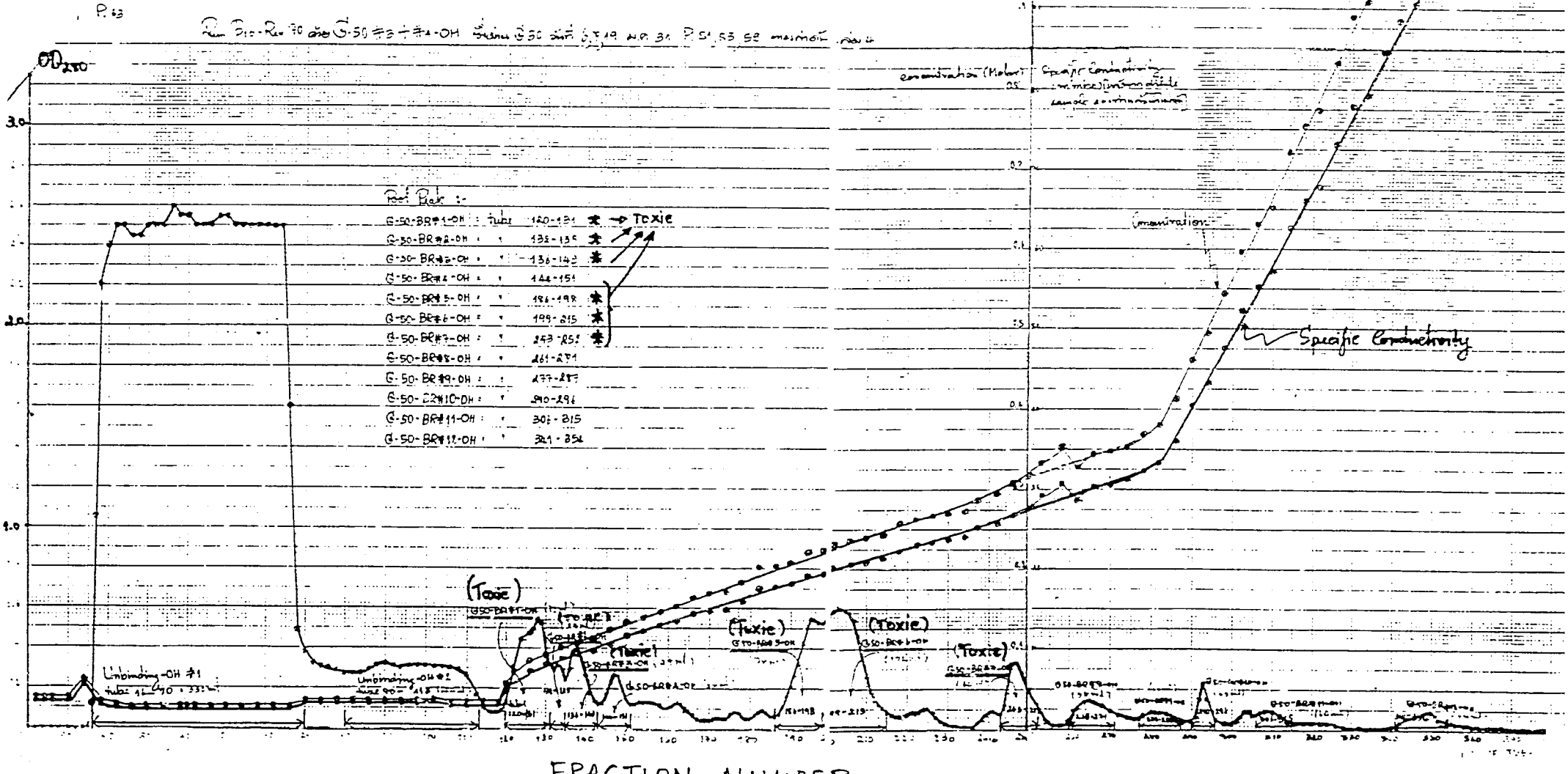
Molec. Weight = 7500

Protein Load = 7.832258 nanomoles

Table 3. Amino acid composition of a neurotoxin from O.hannah venom.

Figure 3

Fractionation of O. hannah venom on a Biorex-70 column.



neurotoxins of elapids i.e., N.n.siamensis, B.fasciatus and O.hannah all exhibit very low immunogenicity (enclosed ms #1). Furthermore, immunological cross-reaction between their antibodies were not significant. The purpose of this project is to study whether the immunogenicity can be increased using chemical coupling to give better immunogens and whether heteropolymer of postsynaptic neurotoxins of the 3 elapids could be successfully used to prepare potent polyvalent antivenom.

Materials and Methods

1. Polymerisation using 1-ethyl-3-(3 dimethylaminopropyl) carbodimide hydrochloride (ECDI) as coupling reagent. Ten mg of ECDI was added to 4 mg of toxin (concentration 18 mg/ml) with constant stirring at room temperature for varying period of time. The pH of the reaction was between 6.8-7.0. The reaction was terminated by dialysis using Spectapor membrane (MW cut off 3,500).

2. Polymerisation using glutaldehyde as coupling reagent. A solution of glutaldehyde at 1, 2, or 3% was added dropwise to a solution of toxin (18 mg/ml) with constant stirring. The volume of glutaldehyde added was 0.1 ml/mg toxin. The reaction time was varied from 1-3 hours and the reaction was terminated as described above.

3. Quantitation of neurotoxin incorporation was made using ^{125}I-toxin.

Results The following polymers based on N.n.siamensis toxin 3 have been prepared using ECDI and/or glutaldehyde as coupling agents.

Homopolymers

1. Homopolymers of NTx3 using ECDI. Under any conditions studied (varying time and concentration of ECDI), polymers (di , tri , tetra and pentamers) of the toxin were observed (Fig 2). Overnight polymerization resulted in particulate that did not enter the gel.

2. Homopolymer of NTx3 using glutaldehyde. Similar results as described above with ECDI were obtained but larger polymers were evident.

Heteropolymers

Heteropolymers of NTx3 and BSA,NTx3 and thyroglobulin have been prepared using ECDI as coupling agent. In addition, heteropolymers of N. n. siamensis toxin 3, postsynaptic toxins of B.fasciatus and postsynaptic toxins of O.hannah. have also been prepared. Quantitation of the incorporation of NTx3 in each polymer is carried out by SDS polyacrylamide gel electrophoresis followed by cutting the gel into 1 or 2 mm slices and count each slice for radioactivity. From this data, the amount of remaining free toxin can be substracted from the total protein in the reaction. From the specific radioactivity of ^{125}I-NTx3 used, one can calculated the mole of NTx3 incorporated in each polymer.

Future plan. If the incorporations of NTx3 into each type of polymer are found to give reasonably high toxin density, the polymerization reaction mixture will be used as immunogens in the study in rats. Comparison will be made between different types of polymers in the production of antibody against NTx3 Quantitation of antibody against NTx3 will be made using the

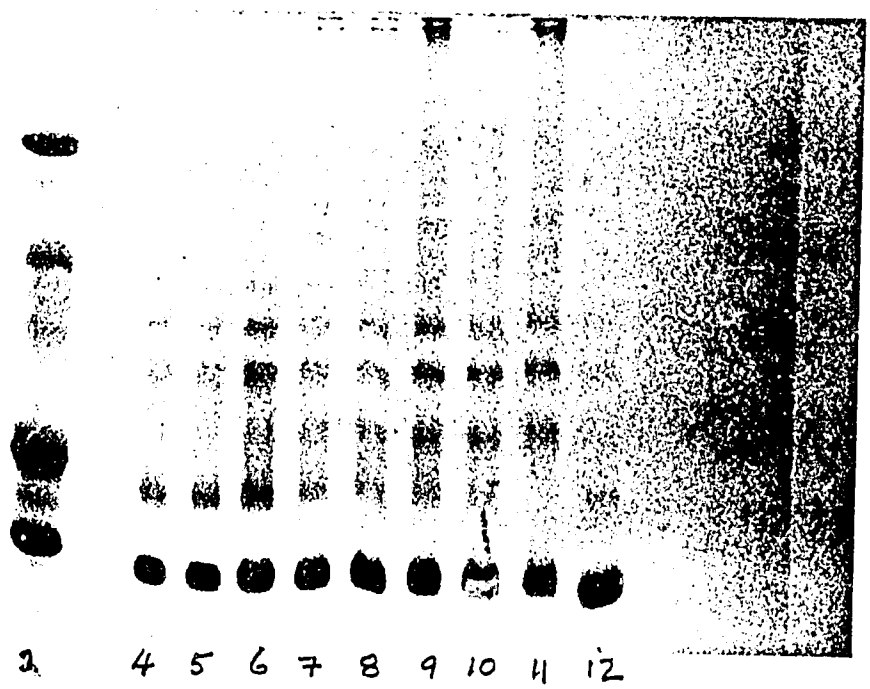


Fig 2. SDS-polyacrylamide gel electrophoresis of polymerization reaction of N.n.siamensis toxin 3 with ECDI.

Track #1 : Standard protein markers ; tracks #4, 5 and 6, toxin with 10 mg ECDI for 1, 2 and 3 hrs, respectively ; tracks #7, 8 and 9 ; toxin with 20 mg ECDI for 1, 2 and 3 hrs, respectively ; tracks #10, 11 and 12, polymerization for 2 hrs at 25 °C followed by overnight incubation at 4 °C. SDS-PAGE was under non-reducing conditions.

ELISA described in #2. The best 2-3 immunoogens will future be compared in the study using larger animals. It is hope that highly immunogenic polymers which could stimulate production of potent antitoxin antibody will be found.

5. Refinement of anti-snake venom antiserum.

Background. Anti-snake venom antisera available in Thailand are monospecific crude horse sera. The supply of the antivenoms are about half of the demand and thus increase of the potency or volume of the sera is needed. The crude horse sera also cause hypersensitirity reactions in about 50% of the cases while 'refined' horse globulin causes 30% reactions. The major obstrucle that hinders the much needed improvements so far is the lack of an accurate *in vitro* assay of the antivenom potency (see attached ms #2). With the success in the development of ELISA for the assay of horse antibody against the principal postsynaptic neurotoxin of Thai cobra (#2), accurate estimation of antivenom against cobra venom during immunization and refinement become possible. We therefore decide to improve the horse antivenom in terms of quantity and quality.

Since elapid postsynaptic toxins are very basic peptides, amino groups are likely to be part of the dominant epitopes on the toxins. The corresponding antigen-binding sites and the horse immunoglobulins should therefore be acidic, more so than the average horse antibody. Such characteristics have been previously observed with rabbit antibodies against human choriogonadotropin and *N.n.siamensis* toxin 3 (KR, unpublished).

Thus, the conventional procedure for the fractionation of horse antibody may not be optimal for the fractionation of anti-cobra antibody. A study towards optimization of conditions for the refinement in terms of yield and purity of horse antibody is the aim of this research project.

Materials and Methods

The ELISA for the quantitation of horse antibody against N.n.siamensis toxin X has previously described (attached ms #2).

Results. So far we have studied the yield and purity of the horse antibody under various conditions of ammonium sulfate precipitation. The yield of antibody was measured by protein concentration and antibody activity in the ELISA. The purity was studied by SDS-polyacrylamide gel electrophoresis. The conditions under study are : varying the concentration of ammonium sulfate, the rate of salt addition, the time and temperature of protein precipitation reaction, the dialysis time, for example.

Future plan. Development of another ELISA for the quantitation of horse $F(ab)_2'$ fraction against N.n.siamensis toxin is now under study. This involves the pepsin digestion of horse anti-cobra antibody followed by gel filtration on a Sephadex G-200 column and follow by ion-exchange chromatography on a DEAE-cellulose column. This assay is essential to the optimization of conditions during pepsin digestion and preparative ion-exchange chromatography of the horse $F(ab)_2'$.

6. Preparation of monoclonal antibodies against Siamensis toxin 3. The Background and Objectives of this study was described in details in the previous reports.

Materials and Methods

Production of Hybridomas. Several additional immunized mice were used in fusion experiments in an effort to produce hybridmas secreting anti-toxin monoclonal antibodies. Different strains of the same myeloma line were used in the fusions since evidence clearly demonstrated that our previous myeloma line was infected with mycoplasma. Use of this myeloma resulted in highly unpredictable and usually unsuccessful outgrowth of hyridomes from the fusion plating as the mycoplasma repidly destroyed the thymidine in HAT selection medium leaving all cells including hybrids susceptible to killing by aminopterin.

Of the successful fusions, one was made with a myeloma obtained from another local laboratory. The spleen cells for this fusion were from an animal immunized twice with a toxin-throglobulin conjugate emulsified in Freund's complete and incomplete adjuvants. The same material without adjuvent was adminsitered 3 days prior to fusion. The second successful fusion, was made between glutaraldehyde-toxoid immunized spleen cells and a mycoplasma-free myeloma line pruchased from Flow Laboratories Australia.

The fusions were made in the usual manner using a short exposure to 40% polyethytiene glycol. Culture supernatante were assayed for antibody using toxin-coated polyvinyl SE-well plates in an ELISA scored by (rabbit) anti-mouse Ig enzyme conjugate.

Cell from anti-toxin positive wells were transferred to 24-well cluster dishes. Only well which maintained moderately high secretion at the 24-well stage were cloned by limiting dilution. This cloning-selection was repeated at least twice for each hybrid line.

Scale up of Monoclonal Antibody Production. Two methods were used to obtain sufficient monoclonal antibody for characterization, especially the in vivo neutralizatikon tests in mice. Hybridoma supernatants from low-serum, high density cultures were concentrated by 50%-saturated ammonium sulfate precipitation. The pellet was resuspended in 1/20th volume of PBS dialyzed. The second procedure was to induce ascites tumors in Pristane-injected histocompatible mice by i.p. injectin of 1 - 10×10^5 washed hybridoma cells. Ascites fluid collected 2-3 weeks later was similarly partilally purified of serum protein by ammonium sulfate precipitation.

An indirect method was used to estimate the concentration of monoclonal antibody in the ascites fluid. The dot immunobinding assay was used to establish a standard curve, spotting DEAE-cellulose purified mouse IgG onto nitrocellulose. Hybridoma supernatants, where the only mouse Ig is monoclonal antibody, were spotted in parallel and detected by anti-mouse Ig-horseradish peroxidase (HAP) with 4-chloro-1-napthol as precipitating chromogen. The monoclonal antibody in ascites which contains additional mouse Ig's, was then related to the determined concentration in culture supernatant through end-point ELISA measurements.

Determination of antibody class/subclass for monoclonal

antibodies. Hybridoma supernatants were reacted with toxin-coated wells as in routine ELISA. Class and IgG subclass-specific (rabbit) anti-mouse Ig's were used in parallel on each hybridoma supernatant. Swine anti-rabbit Ig-HAP conjugate with 2-phenylenediamine was used to score all wells.

In vivo neutralization using monoclonal anti-toxin antibodies. A single preparation of pooled purified toxin of known concentration was used for all *in vivo* tests. Mice of similar weight were injected i.v. with varying concentrations of toxin in fixed volume, with 6 animals for each concentrations. This estimate of the LD_{50} (approx. 3.0 ug/ 20 gm mouse), was doubled in the neutralization test (i.e., 6 ug, where LD_{100} was about 4.0 ug/mouse).

Toxin mixed with ammonium sulfate fractionated monoclonal ascites fluid was preincubated at 37 ^{o}C for 1 hr. Then equal volumes containing 2 X LD_{50} (1.5 X LD_{100}) of toxin plus McAb were injected i.v. into 8 mice, along with toxin-only and McAb-only controls. Neutralization or protection was scored as the time until death, which in turn was converted to multiples of the mean survival time of toxin-only control animals from the some day in order to compare results from different days. Monoclonal antibody in the toxin-Ab mixture was determined to be at minimum, a 4-fold molar excess of antigen combining site. This was increased again several fold in later repeat experiments in attempts to obtain a more definitive (*i.e.*, greater than 40 hr) protection.

results and Discussion

Production of Monoclonal Antibodies against Siamensis toxin 3 :

Determination of the sourse of previous fusion difficulties. Many fusions have been made by persons working on this project or by others with different antigens, but using common equipment and highly similar procedures. Almost all fusions made until recently were of very limited success in terms of numbers of hybrids produced or surviving to the assay stage. Some fusions never evidenced clear signs of hybridoma growth, while others showed variable high, or low frequency growth, which suddenly ceased in all wells and was rapidly followed by nearly simultaneous death of almost all clones. The erratic appearance and timing of the problem combined with different persons performing fusions and tending hybrids with similar, but slightly different techniques and reagents, sent us searching in many different directions as to the cause(s) of these failures. Each component of HAT seleciton media (supplemented for low density growth) was checked and where possible, substituted. finally, we began using an established HAT-resistant hybridoma as a method of bioassay for our media. The most frequent observation was that media in which the HAT sensitive mycloma had died, had somehow become toxic to otherwise HAT-resistant hytbridomas. Then we found that normal myeloma culture supernatant added to the hybridoma rendered in susceptible to HAT at any subsequent point. However, filtration (0.1 um pore) or heat (56 ^{o}C, 30 min), conditions known to eliminate mycoplasma, pervented this HAT reversal. Death of infected hybridomas was delayed by increasing

the thymidine concentration, suggesting that the mechanism of HAT reversal is via the known mycoplasmal pyrimidine nucleoside phosphorylase activity, thus reducing the available T to levels insufficient to support growth by the Thymidine kinase salvage pathway. The variability in hybridoma growth and final yield, then probably reflect the levels of infection of the myeloma, which in turn are dependent upon the frozen stock, time in culture, and use of Gentamicen, as well as the use of myeloma-"conditioned" media for low density growth, number and timing of early HAT replacment or feedings.

The preferred solution is to dispose of all infected cultures and select an alternative myeloma line. One of the two successful fusions reported here was made with a myeloma obtained from another local hybridoma lab, and appears by this crude bioassay (ability to induce HAT reversal in hybrids) not to be infected with similar mycoplasma. However, the history of this line is largely unknown, it shows irregular morphology not alterable by antibiotics against mycoplasma, and has been grown in continuous culture for long periods at high density. The second fusion was made with a mycoplasma-free myeloma strain purchased from Flow-Australia.

Fusion Results Two fusion gave useful hybridomas. The first was made using spleen cells from a mouse immunized twice on day 0 and day 58 with toxin coupled to thyroglobulin, in Freund's complete and incomplete adjuvants. Three weeks later the animal received 70 ug of toxin-Tg without adjuvant, and spleen cells were fused 3 days later with the 653 myeloma from another lab.

Little is known of the history of this line, except that it had been cloned many times, kept in long term culture at high density and grown in antibiotics including Gentamicin. It passes the HAT reversal test, but shows poor growth at low density and gives a low but consistent hybrid yield. This fusion gave only 3 antigen-specific clones, one of which was lost. An additinal 7 wells originally scored as "positive" were equally reactive against the blocking/saturating protein and were not characterized further. Possibly these were stimulated by the carrier and cross-react with similar epitopes on ovalbumin used to block additional protein binding sites on plastic wells.

The second fusion used spleen cells from a mouse immunized with 40 ug of glutaraldehyde toxoid in Freund's complete adjuvant and was boostered 3 days before fusion with another 80 ug. These immune cells were fused with mycoplasma-free 653 myeloma cells purchased form Flow-Australia. This fusion gave 9 antigen-specific hybridomas which survived transfer and cloning. None of these hybrids appear to be "false positives" in the sense of reacting with the blocking/saturating protein rather than with toxin, unlike those where toxin-carrier was immunogen.

Characterization of Monoclonal Antibodies. Cloned hybridomas were scaled up and grown under standard conditions in order that end point dilution titers could be compared by ELISA. Titers ranged from about 1:100 to greater than $1:10^5$ for IgG-secreting clones. The 2 IgM secretors had very much lower titers (1;4 and 1:9). Of 11 different monoclonal antibodies, 2 were of the IgM class and one of the IgG_{2b} subclass; all others were

IgG_1.

To obtain sufficient monoclonal antibody for *in vivo* neutralization tests, ten of the hybridomas were grown in ascites tumor form in BALB/c mice primed with Pristane. All except one of these produced high titer ascites fulid; clone # 63 gave ascites fluid but with extremely low and unsuitable titer. One batch of purified toxin was used for all experiments. The i.v. LD_{50} for this batch was determined to be 3.0 ug/ 20 g mouse. At an estimated 4 fold excess of antigen-combining site over toxin, all of the partially purified ascites monoclonal antibody preparations preincubated with 2 x LD_{50} of toxin failed to provide an "absolute" protection (*i.e.*, no death or survival greater than 48 hr). Nonetheless, several evidenced neutralization through the prolongation of the interval before respiratory paralysis. These *in vivo* tests were repeated using much higher concentrations of ascites fluids, however with the same general result--prolongation of life without complete protections. The time before death relative to toxin controls without antibody was then taken as a measure of relative neutralization. The concentration of the single IgM monoclonal tested could not be estimated accurately by the procedure used, and the ascites titer was sufficiently low that it could not be further concentrated for the repeat analysis. This IgM clone showed no signficant protection. However, for all IgG antibodies the results are known to represent large antibody excessed despite the fact that the IgG concentrations are not equal. All of the IgG monoclonals exhibited neutralization with at least 3-

26

fold prolongation of life. and several showed much longer protection.

Fusion clone	End point ELISA titer	Class / subclass	Neutralization*
toxin-Tg			
2	> 1: 20,000	IgG1	5.9 ± 2.1
5	1: 9	IgM	1.2
Blutaraldehyde-toxoid			
20	> 1: 81	IgG20	9.1 ± 2.6
20	> 1: 6,500	IgG1	4.5 ± 0.7
30	1: 19,000	IgG1	5.3 ± 0.5
37	1:177,000	IgG1	10.1 ± 1.6
50	1: 8,000	IgG1	41.7 ± 3.3
56	1: 59,000	IgG1	7.2 ± 1.6
59	1: 65,000	IgG1	3.7 ± 0.9
63	N.D.	IgG1	N.D.
69	1: 4	IgM	N.D.

* number represents ratio of times until death with/without concentrated ascites monoclonal antibody ± SD (death followed an average of 28 (used with clones 20 and 50), 42, 44, or 48 min after injection of toxin alone in the series of tests shown)

Conclusion and woahplan

Monoclonal Antibodies against Venom Toxins Eleven anti-Siamensis toxin 3, monoclonal antibody-secreting hybridomas were produced from 2 fusions. For unknown reasons, the majority of these are of the IgG_1 subclass; possibly the commercial anti-mouse Ig-HAP scoring reagent favores identification of clones of this class. Two IgM secretors were of very low titer in culture supernatant and in ascites. All clones tested displayed toxin neutralization when tested by an in vivo assay, with the exception of the single IgM secretor, where because of the low titer we cannot be certain of antibody excess. Neutralization, as reflected by slower onset of envenomation symptoms and eventual death, appears to show no relationship to the original titer in culture or escites, provided the antibodies are in excess.

During the next period we shall complete our epitope competition studies to determine whether better neutralization is associated with a particular epitops. We will also rank monoclonal antibodies in terms of affinities to determine the extent to which the neutralization disparity is affected. We are troubled by the failure to find a clone which confers absolute protection. This could mean that none of the clones recognize the active site of the toxin with sufficient affinity or that precipitin or higher order complex formation is critical with a low MW toxin. Once we have completed the epitope mapping, we shall attempt monoclonal mixing experiments to assess the contribution of precipitation and complex formation. It would appear that these monoclonal antibodies would not be effective in

neutralizing the toxin in crude venom used as immunogen for antivenin production in horses. However they can be used to simplify our toxin purification procedure, and purified toxoid could be used to supplement horse immunogen. It should be of interest also to determine the residual toxicity of toxin-depleted Siamensis venom.

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SPECIAL ARTICLE

Immunodiagnosis of Snake Venom Poisoning

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SUMMARY Uncertainty as to the species diagnosis remains a serious problem in the management of snake venom poisoning. This is particularly so in areas inhabited by numerous species, the venoms of which elicit similar pharmacological effects and clinical symptoms and against which para-specific cross-neutralizing antivenom is not available. Attempts have been made to eliminate some of this ambiguity through the development of various immunodiagnostic tests. Tests based on ELISA are sensitive, specific and even quantitative and adaptable to field application. In the development of diagnostic tests for use in developing countries, however, practical consideration must be given to speed, cost, simplicity in terms of equipment and expertise, and stability to the climate and storage conditions. This may dictate further modification or selection of more suitable alternative methodologies. Furthermore, the test may have to allow more flexibility in accomodating local species distributions and to address probable complications of heterophile antibodies in test samples from rural people.

Snake venom poisoning and problem in management

Snake venom poisoning remains an important health problem in Thailand and many other developing countries in Asia and Africa. Although specific antivenoms are available in most of these countries, a significant fraction of the snakebite victims die and many who survive suffer some degree of permanent injury.

The current scheme of management of snake envenomization includes attempt at identification of snake species involved and the selection of the corresponding antivenom for therapy. The species diagnosis, almost always without catching the culprit snake, is based on configuration of the bite, differentiating clinical manifestations, knowledge of species commonly recognized in a given geographical area and information from the victim or witnesses. None of these provide the clinician with sufficient grounds to arrive at an accurate identification of the source of envenomization and often lead to failure to do so. Differentiating the culprit species on the basis of clinical manifestations is difficult simply because toxins contained in venoms of different species are often physico-chemically and pharmacologically similar. For example, venoms of *Naja naja siamensis*, *Ophiophagus hannah* and *Bungarus fasciatus* all contain postsynaptic neurotoxins inhibiting neuromuscular transmission leading to similar predominant clinical features, namely, paralysis, respiratory failure and eventually death.[1] The distribution of snakes is not clear cut and each locality is usually inhabited by several species. Higher frequency of inhabitation by a given species does not necessarily indicate higher incidence of bites by that species.

The Thai Ministry of Public Health recently estimated that the culprit species was identified in only around 60 percent of the reported bites. Until recently, however, once an identification was made, there was no way of confirming it. Some indication that a correct identification has been made is obtained when use of the corresponding antivenom effectively and dramatically improves conditions. Unfortunately, for a variety of reasons, this is not often the case; more frequently, correct antivenom merely arrests progressive deterioration without immediate

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confirmation of diagnosis. Thus the actual rate of correct diagnosis is likely to be even lower than estimated.

Accurate identification of the source of envenomization is crucial since there is very little, if any, paraspecific or cross-neutralization by the monovalent horse antivenoms available in Thailand.[2] Thus, only the appropriate specific antivenom will be effective; misdiagnosis is both wasteful of precious antivenom and dangerous to the patient, in that effective treatment may be omitted while the patient is nonetheless subjected to adverse hypersensitivity reactions. This is particularly disconcerning since in antivenom therapy, massive quantities in the range of 30-600 ml may be required in a given case.[3] The incidence of hypersensitivity reactions has been estimated to be as high as 30%-50% according to various reports.[4,5] The inability to achieve a prompt and unequivocal, accurate identification of the source of envenomization remains a major limiting factor in the management of snakebites.

Development of immunoassays and immunodiagnostic tests

As mentioned above, venoms of many snakes are similar physicochemically and pharmacologically yet each of them requires specific antivenom for neutralization. Thus, various assays and diagnostic tests based on the immunochemical differences of these venoms have been developed with different characteristics and degrees of success. The reagents and methods developed were reviewed recently.[6]

Immunodiffusion and immunoelectrophoretic assays for the detection of various venom proteins have been described.[7] Venom proteins were detected in wound aspirates, blister fluids, sera or urine by immunodiffusion assay in 40 out of 101 patients tested. Passive hemagglutination (PHA) was first studied for venom antibody detection by Boche and Russell,[8] who used venom coupled to bis-diazobenzidine treated sheep RBCs. The preparation could be used to detect anti-venom antibody at dilutions as high as 2×10^5, while the venom proteins could be detected by the corresponding inhibition test.

Radioimmunoassay has been developed by several groups of investigators for the detection of venom or anti-venom antibody.[7,9-10] It is by far the most sensitive technique, capable of detecting subnanogram quantities of venom.[9] However, the requirement for routine protein labeling with short-lived ^{125}I, the need for expensive detection equipment and well-trained personnel, the problems of radioactive waste disposal and the time required to complete the assay (24 hrs) preclude its use in diagnosis of venom poisoning especially in rural areas of developing countries.

Theakston *et al.*[11] were the first to use enzyme-linked immunosorbent assays (ELISA) performed in microtiter plates for specific detection of various snake venoms. Using specific antibodies (IgG fraction) immobilized on the plate and an alkaline phosphatase-antibody conjugate for scoring, they could detect 1-5 ng of venom per ml of sera from experimental animals. The time required for the test is about 3 hrs. Coulter *et al.*[12] using similar techniques could detect Australian snake venoms at about 6 ng/ml within 30 min. The improvement was attributed to the use of antibody purified on a protein A-Sepharose column.

Recently, Chandler and Hurrell[13] have developed a new test kit consisting of six glass capillary tubes joined by silicone rubber tubing and connected to a 1-ml syringe. Internally, each glass tube is coated covalently with specific rabbit antibody against one of five poisonous snake venoms. The test sample containing venom is draw up through the tubes resulting in venom attachment to one of the coated tubes. An enzyme conjugate (urease coupled to anti-venom IgG) is drawn up, followed by substrate (urea plus bromocresol purple as pH indicator), resulting in color development on the tube coated with homologous antibody. This test kit, which has a sensitivity of 5-10 ng/ml and a test time of 30-40 min, costs about US$15/test. The cost factor relative to per capita income likely will prevent direct application of this assay methodology or its wide-spread use in developing countries.

Of the various immunodiagnostic tests described, ELISA thus far has been the most widely used. However, Ho *et al.*[14] recently cautioned that various factors influencing the successful application of ELISA in snake venom detection have not yet received due consideration. These factors include nonspecific reactivity, the cross-reactivity and the quality of the reagents. Nonspecificity may be encountered if sera contain heterophile antibodies — especially common in sera of rural people, or rhematoid factors. These immunoglobulins elevate background ELISA values and frequently yield false positives. Cross-reactivity between heterologous venom antigens may give ambiguous ELISA results and thus equivocate the diagnosis. The quality of reagents, *e.g.*, the purity and affinity of the antibody influences the signal to noise ratio and reaction times and hence affect the sensitivity of the test. Ho *et al.*[14] also suggested various technical manipulations to avoid or minimize these problems.

Some possible future improvements

Two extremely simple and useful immunoassay systems, passive

hemagglutination (PHA) and latex agglutination (LA) have received limited or no attention thus far for the purpose of venom detection. PHA has been adapted as a laboratory assay for venom antigen and antibody as reviewed above but has not been adapted for immediate diagnosis. The sensitivity of this assay, calculated from the result of Boche and Russell,[8] should be at the level of detecting 2 ng/ml of antibody, in accord with the observations of Adler and Adler,[15] that PHA and reverse PHA are capable of detecting 1-10 ng/ml of antibody or antigen, respectively. Thus, the sensitivity of this assay should be 2-3 orders of magnitude greater than immunodiffusion or immunoelectrophoresis and comparable to that described by Treakston *et al.*[11] using an ELISA. Venom proteins present in body fluids should thus be detectable by reverse PHA. It should be mentioned that snake venom detection based upon hemagglutination tests may be prone to hemolysis (P. Boquet, personal comm.). However, in our experience, using glutaraldehyde treated sheep RBCs,[16] no hemolysis was observed in the presence of high concentrations of three Elapid venoms, known to contain direct lytic factor and phospholipase A_2. The advantages of PHA and reverse PHA and the corresponding inhibition assays include simplicity, sensitivity and economy.[25] In many cases the sensitized erythrocytes can be lyophilized and reconstituted with distilled water before use.[17,18] It has also been reported that with avian erythrocytes which are nucleated and settle faster, the agglutination result can be read within 30 min.[18]

Latex agglutination assays, although providing additional advantages, have not been adapted as yet for use in venom detection. In addition to enhanced stability and relative insensitivity of the particles to enzymes and toxins, the greatest advantage of this technique is the speed with which the result can be read. It also does not require any special laboratory equipment or expertise. The detection of human choriogonadotropin in the pregnancy test, for example, can be completed within a few minutes, using only medicine droppers and a microscope slide. The sensitivity of the test can be very high, for example, polyribose phosphate can be detected at a level of 0.2-0.5 ng/ml of serum, cerebrospinal fluid, joint fluid or pleural fluid.[19] For protein antigens, for example immunoglobulins, the detection limit is about 35-225 ng/ml.[20]

The successful adaptation of reverse PHA or LA for snake venom detection should offer several advantages over ELISA, in that both are relatively easy to perform and are considerably faster--especially in the case of latex agglutination. Sensitivities may be somewhat less. Preliminary results from our laboratory showed that a reverse PHA using sheep RBC and a reverse LA could detect 5-80 ng/ml and 160-1200 ng/ml, respectively, of various snake venoms (Unpublished observations). Considering the previously reported[21] concentrations of venom in wound aspirates (200 ng/ml) and in serum (100 ng/ml), reverse PHA and possibly, reverse LA with some improvements, should be adequate. They are about 10-20 times less expensive than ELISA to produce in Thailand (B. Petchelai, personal comm.). The stability of the reagents for each test may be an important factor in developing countries. Sensitized latex particles or red cells can usually be stored at 4°C for years[22,23] or in some cases, lyophilized and stored desiccated at room temperature for even longer periods of time.[24] Our preliminary experiments indicated that latex particles coated with rabbit IgG against snake venom are stable to lyophilization and to cycles of freezing and thawing (Unpublished observations). ELISA plates sensitized with anti-venom IgG deteriorated at 4°C after 16 weeks.[12]

Special requirements of immunodiagnostic assays for developing countries

People in the developing countries face a number of serious problems as far as snake venom poisonings are concerned. This is because a far greater variety and number of deadly snakes inhabit the tropics. The occupation and way of life of the people also make them more vulnerable to snakebites. The relatively poorer means of transportation and medical facilities and the resultant delay or shortage of specific antivenoms make treatment of venom poisonings even more difficult as a result.

Against this background, the immunodiagnostic tests for snake venom poisonings also must incorporate certain special features to be useful in developing countries. Not only must the tests be specific, sensitive and rapid enough for diagnostic purposes, but they also must be simple to perform and require minimal equipment or expertise. The tests must be reasonably cheap relative to the per capita income of the people. Lastly, they must be stable to the climate, and be able to withstand transportation and storage conditions which may be far from optimal.

Since the components of the immunodiagnostic tests are dictated by the species of the snakes in the geographic area, each country most likely will have to produce its own tests except where there is an overlap of snake habitats. The ideal test therefore should allow for regional customization to include additional species or to delete those unnecessary within a given area. The low commercial viability provides little incentive for pharmaceutical cor-

32

porations in developed countries to invest in the research and development of inexpensive venom diagnostic tests tailored to developing countries or regions thereof. Thus, it is essential that researchers in developing countries share whatever methodologies and reagents developed whenever possible.

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ANTIGENIC RELATIONSHIPS AND RELATIVE IMMUNOGENICITIES OF VENOM PROTEINS FROM SIX POISONOUS SNAKES OF THAILAND

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LEERA CHINONAVANIG, P.B. BILLINGS, P. MATANGKASOMBUT and K. RATANABANANGKOON. Antigenic relationships and relative immunogenicities of venom proteins from six poisonous snakes of Thailand. Toxicon_____, _____ _____, 19_____.

Venoms from *Naja naja siamensis*, *Ophiophagus hannah*, *Bungarus fasciatus*, *Vipera russelli*, *Calloselasma rhodostoma* and *Trimeresurus albolabris* have been studied by means of sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblotting. The immunoblots were stained with rabbit homologous and heterologous antibodies. In general, the higher the molecular weight the protein the higher the immunogenicity although two proteins with molecular weights of 23,000 and 25,000 from *O. hannah* venom are extraordinarily immunogenic. Cross reacting and species specific venom proteins were readily identified by the immunoblot techniques. It was revealed that only a small number of venom proteins were cross-reactive among the snake species tested while the remaining appeared to be species specific.

INTRODUCTION

Recently, a great deal of interest has been focused on the development of various immunoassays for studies of snake venoms and their respective antibodies (Theakston, 1983). For example, immunodiagnostic tests for snake venom proteins have been developed for rapid identification of the source of envenomation in victims of snakebite. Immunoassays for the detection of human antibody against snake venoms have also been studied for epidemiological surveys of snakebite incidence (Pugh and Theakston, 1980). In addition, *in vitro* assay of horse serum antivenom potency has been under study as a potential replacement for *in vivo* neutralization tests (Theakston and Reid, 1979).

These various immunoassays have been studied using crude venoms and their corresponding antibodies. However, complications could arise from the use of uncharacterized and unfractionated venoms in these assays. For example, immunological cross reactions between venom proteins of different species could lead to ambiguity in the species diagnosis (Fulde and Smith, 1984; Marshall and Herrmann, 1984; McCarthy, 1984). On the other hand, *in vitro* antivenom potency estimation would be meaningful only if it is primarily the antibodies against major lethal components in the venom which are being assayed (Christensen, 1979). Thus, knowledge of the properties of constituent venom proteins with regard to immunological cross-reactivities, relative immunogenicities, abundancies, toxicities, etc., would be useful in identifying and eliminating any complications that may arise during the development of these

immunoassays. It the present communication, a combination of sodium dodecyl suffate polyacrylamide gel electrophoresis and the Western- or immunoblot has been used to study the relative abundancies, immunogenicities and immunological cross-reactivities of the various venom proteins from the six major poisonous snakes of Thailand.

MATERIALS AND METHODS

Venoms and chemicals

Six major poisonous snake species, against which horse antivenoms are available, were studied. These were: *Naja naja siamensis* which is also known as *Naja kaouthia* (Viravan *et al.*, 1986), *Bungarus fasciatus* and *Ophiophagus hannah* of the Elapidae family and *Vipera russelli*, *Calloselasma rhodostoma*, *Trimeresurus albolabris* of the Viperidae family. The snakes were kept and milked at the Queen Saovabha Memmorial Institute, Bangkok. The venom milked from 3-8 snakes of the same species was pooled and immediately frozen in dry-ice. The venom was kept frozen at -20 ^{o}C until use. Extreme care was taken to avoid cross contamination of these venoms during milking and throughout this study. Chemicals were of reagent grade obtained from Sigma Chemical Co., St. Louis, Missouri, USA, except as indicated.

Immunization of rabbits and preparation of IgG from rabbit antiserum.

Venom of each snake species was used to immunize three or four rabbits. Primary immunization was with 40 ug venom protein (determined by Lowry method, see below) emulsified with Freund's complete adjuvant injected s.c. into the back. Subsequent injections were made in Freund's incomplete adjuvant at one month intervals. Rabbits were bled biweekly and the sera were prepared. The rabbit IgG was purified by Staphylococcal Protein A-Sepharose column chromatography (Goding, 1978). Elution of the IgG was made with ice cold 3 M sodium thiocyanate (Twining and Atassi, 1979).

Western Blot.

Proteins of each venom were fractionated by polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS) under non-reducing conditions and the protein patterns were visualized by direct staining with Coomassie blue. Identical gels with the same six venoms were transferred electrophoretically to nitrocellulose membranes (0.1 micron pore ; Schleicher and Schull) by the procedure of Towbin et al (1979) for simultaneous immunological reaction with rabbit antiserum generated against one of the six snake venoms. The transfer was carried out at 15 V/cm for 4 hr at room temperature. For direct visualization of protein bands, the nitrocellulose membrane was stained with amido schwartz. For immunological detection of protein bands on the nitrocellulose membrane, the membrane was first incubated for 1 hr in 50 mM Tris-saline (pH 7.4) containing 5 % non-fat dry powdered milk to saturate the remaining protein binding site. After washing, the membrane was incubated overnight with rabbit antiserum appropriately diluted in Tris-saline containing 5% bovine serum albumin (BSA). This was followed by incubation for 2 hr with horse-radish peroxidase conjugated anti-rabbit IgG (Dakopatts, Denmark) in Tris-saline and 5% BSA. The protein bands with the enzyme conjugate attached were then visualized by 20 min incubation in 25 ug/ml of o-dianisidine and 0.01 % hydrogen peroxide in 10 mM Tris.HCl pH 7.4.

Other methods.

SDS-PAGE was performed by the procedure described by Laemmli (1970) using 10% acrylamide. Molecular weights of venom proteins under SDS-PAGE were calculated from their electrophoretic mobilities by comparison to a curve of logarithm mol. wt. versus mobility of protein standards. Standard proteins used were : rabbit muscle phosphorylase B, mol. wt. 94,000 ; BSA, mol. wt. 68,000 ; ovalbumin, mol. wt. 45,000 ; soybean trypsin inhibitor, mol. wt. 20,100 ; N.n.siamensis toxin 3, mol. wt. 7,821. Protein was assayed by the method of Lowry et al. (1951) using BSA as standard.

RESULTS

Molecular sizes and relative abundancies of venom proteins from the six Thai poisonous snakes.

By SDS polyacrylamide gel electrophoresis, each venom exhibits a characteristic polypeptide size and abundance distribution pattern. The separation in pattern of the two families is not as apparent; however, the presence of abundant very low mol. wt. components of less than 8,000 mol. wt. together with several predominantly very high mol. wt. proteins appears characteristic of the elapid venoms. In contrast, the viper venoms show little stainable material of less than 10,000 mol. wt. and display several discrete polypeptides of intermediate mol. wt. Among the elapids, N. n. siamensis venom shows the greatest abundance of very low mol. wt. polypeptides, largely post-synaptically acting toxins and cardiotoxin (Karlsson et al., 1971) of mol. wt. ca 7,000 - 8,000 . O. hannah venom contains more high mol. wt. proteins, with a proportionate reduction of the toxins of mol. wt. less than 8,000, consistent with the comparatively lower lethality of this venom (Ganthavorn, 1969). The viperid venoms exhibit unexpectedly large quantities of protein(s) of mol. wt. about 10,000 even under the non-reducing electrophoretic conditions employed. No function(s) can be ascribed to these, since the known abundant toxins and enzymes of these venoms are of greater mol. wt. (Iwanaga and Suzuki, 1979).

Immunogenicity and antigenic similarity

The relative immunogenicities of individual constituent polypeptides from a single venom, and the extents and complexities of the immunological cross-reactions between venoms were studied using Western- or immunoblots. Since reducing SDS-polyacrylmide gel electrophoresis showed diminished antibody binding on blots, non-reducing conditions were used to minimize denaturation of protein antigenic determinants. In addition, 0.1 um nitrocellulose membrane was used in the blot to maximize recovery of low molecular weight venom proteins. The immobilized venom proteins were reacted with rabbit antiserum generated against the venom of one snake species. In this case, binding of antibody to the individual polypeptides in the lane with the corresponding or homologous venom provides a measure of the relative immunogenicity/abundance of each constituent. Similarly, antibody binding to resolved polypeptides in the lanes containing heterologous venoms reflects the immunological cross-reactivity. It is the extent of this cross-reaction relative to the identification of the homologous proteins which potentially limits the use of this antiserum in an immunodiagnostic application.

By comparing the immunological reaction of homologous antiserum with the abundancies of individual venom polypeptides, separated according to size, it is quite apparent that polypeptides differ considerably in terms of their abilities to stimulate antibody responses when immunized as a mixture in crude venom. Although, in general, the higher mol. wt. components are more immunogenic, a direct relationship between molecular size

and the response was not observed, nor did a higher abundance offset a poor immunogenicity. Of particular relevance in terms of immunotherapy, is the observation that the elapid toxins of 7,000 to 8,000 mol. wt. exhibited considerably reduced immunogenicities in comparison to other, less abundant proteins of the same venoms. In the case of O. hannah venom blot reacted with the homologous antiserum almost no antibody bound to the low mol. wt. region containing the postsynaptic neurotoxin (Fig. 2A), despite the fact that by chromatographic analysis, the toxin comprised about 8% of the total venom protein (K.R., unpublished observation). Homologous antisera against the other two elapid venoms showed low, but still detectable reactions with the low mol. wt. toxins. Of the higher mol. wt., generally more immunogenic polypeptides, immunological reactivity on blots from SDS gels was occasionally observed with polypeptide bands which were not readily apparent by protein stain, and the reverse, where moderately abundant bands of reasonably high mol. wt. are poorly identified. Fig. 2A provides examples of each: bands labeled #3 and #4 of mol. wt. 23,000 - 25,000 appear highly immunogenic but are undetectable by direct protein stain, whereas bands #1 and #2, which are of higher mol. wt. and more abundant, show almost no reactivity.

Reaction of a blot of a gel such as that in Fig. 1, in which proteins from all six venoms have been resolved in different lanes, enables direct visualization and comparison of the reaction of each single antiserum with homologous and heterologous venom constituents under identical conditions. With each of the six antisera analyzed in this manner, the reaction

with homologous antigen was, as expected, the most diverse and the most intense, often identifying an entire mol. wt. range of polypeptides (see Table 1 for summary of homologous and heterologous reactions). Cross-reactivity, where it was observed, was restricted to only a subset of polypeptides in the heterologous venom, which because of their lower reactivities, could now be resolved as discrete mol. wt. species. In terms of numbers of different cross-reacting polypeptides and the intensities with which heterologous bands were identified, cross-reaction was largely confined within the same family. Within a family, T. albolabris venom was considerably more cross-reactive than that of other species ; it is also more reactive with antibody against the V. russelli than that against C. rhodostoma (Table 1). None the less, some inter-family cross-reactivity which can not be attributable to cross-contamination (see Materials and Methods) was observed, such as that between a few polypeptides of Trimeresurus albolabris venom and antiserum raised against venom of the Thai cobra, N.n. siamensis. Reciprocal reactivity was not observed in this case, nor in several other cases. Representative blots containing the proteins of all six venoms reacted with antiserum against an elapid (O. hannah) or against a viper (V. russelli) are presented in Figs. 2B and 3B, respectively.

Since the elapid postsynaptic neurotoxins demonstrate such low immunogenicity by the immunoblot assay, it was not surprising that very little cross-reactivity was detected. Binding of heterologous antibodies in the 7,000 - 8,000 mol. wt.

region of blots of elapid venoms was not observed except for minimal reaction of anti-*N.n. siamensis* serum with *O. hannah* venom.

DISCUSSION

The elapid neurotoxins were generally less immunogenic compared with other venom proteins. This accounts for the well-recognized difficulty in the preparation of potent neutralizing hyperimmune sera against elapid venoms (Christensen, 1979). In contrast, certain venom proteins (e.g., bands #3 and #4 of the O. hannah venom) are extraordinarily immunogenic. It will be of interest to determine the extent to which chemical coupling of the postsynaptic toxins with these high mol. wt. constituents enhances their immunogenicities.

It is well known that horse antivenoms against snake venoms are quite specific and that cross-neutralization is not commonly observed (Ganthavorn, 1969). Nevertheless extensively cross-reacting precipitin bands between various venoms, even those belonging to different families, have been observed using immunodiffusion or immunoelectrophoresis (Minton, 1979). These observations have led to the conclusion that the cross-reaction was confined to non-toxic venom proteins. The alternative interpretation, i.e. that the toxins were involved in the antigen - antibody cross-reaction, but were not neutralized, was considered unlikely. Results from Western blots presented here clearly show that the failure of the heterologous antivenom to cross neutralize is due to the absence of immunological cross-reaction.

In the development of immunodiagnostic tests for rapid identification of source of envenomization, cross-reacting venom antigens could lead to ambiguity or even error in diagnosis (Marshall and Herrmann, 1984). The immunoblotting technique enables precise identification of the venom antigens involved in the cross-reaction. Where such cross-reaction presents a serious problem to the species diagnosis, species-specific venom antigens could be identified, isolated and used to prepare mono-specific antibody for immunodiagnostic tests.

ACKNOWLEDGEMENT

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LEGENDS TO FIGURES

Figure 1. SDS-polyacrylamide gel electrophoresis of the six Thai poisonous snake venoms. The positions of the protein standards with the molecular weights in kilodaltons (kd) are shown on the left. NS, N.n. siamensis ; BF, B. fasciatus ; OH, O. hannah ; CR, C.rhodostoma ; VR, V.russelli and TA. T. albolabris.

Figure 2. Reaction of antiserum against O. hannah with homologous and heterolgous venom antigens.

A. Western Blot of O. hannah venom on 0.1 micron nitrocellulose membrane. The right hand lane was stained with amido schwartz. The left hand lane was reacted with rabbit homologous antibody against O.hannah (a-OH) followed by enzyme conjugate and o-dianisidine.

B. Immunochemical cross-reactivity between antiserum against O. hannah and venom proteins from various snakes. A Western Blot of the six snake venoms was reacted with rabbit antibody raised against O.hannah venom. Venom abbreviations are as in Fig. 1.

Figure 3. Reaction of antiserum against V. russelli with homologous and heterologous venom antigens.

A. Western Blot of V.russelli venom on 0.1 micron nitrocellulose membrane. The right hand lane was stained with amido schwartz. The left hand lane was reacted with rabbit homologous antibody against V. russelli (a-VR) followed by enzyme conjugate and

o-dianisidine.

B. Immunochemical cross-reactivity between antiserum against *V.russelli* and venom proteins from various snakes. A Western Blot of the six snake venoms was reacted with rabbit antibody raised against *V. russelli* venom. Venom abbreviations are as in Fig. 1.

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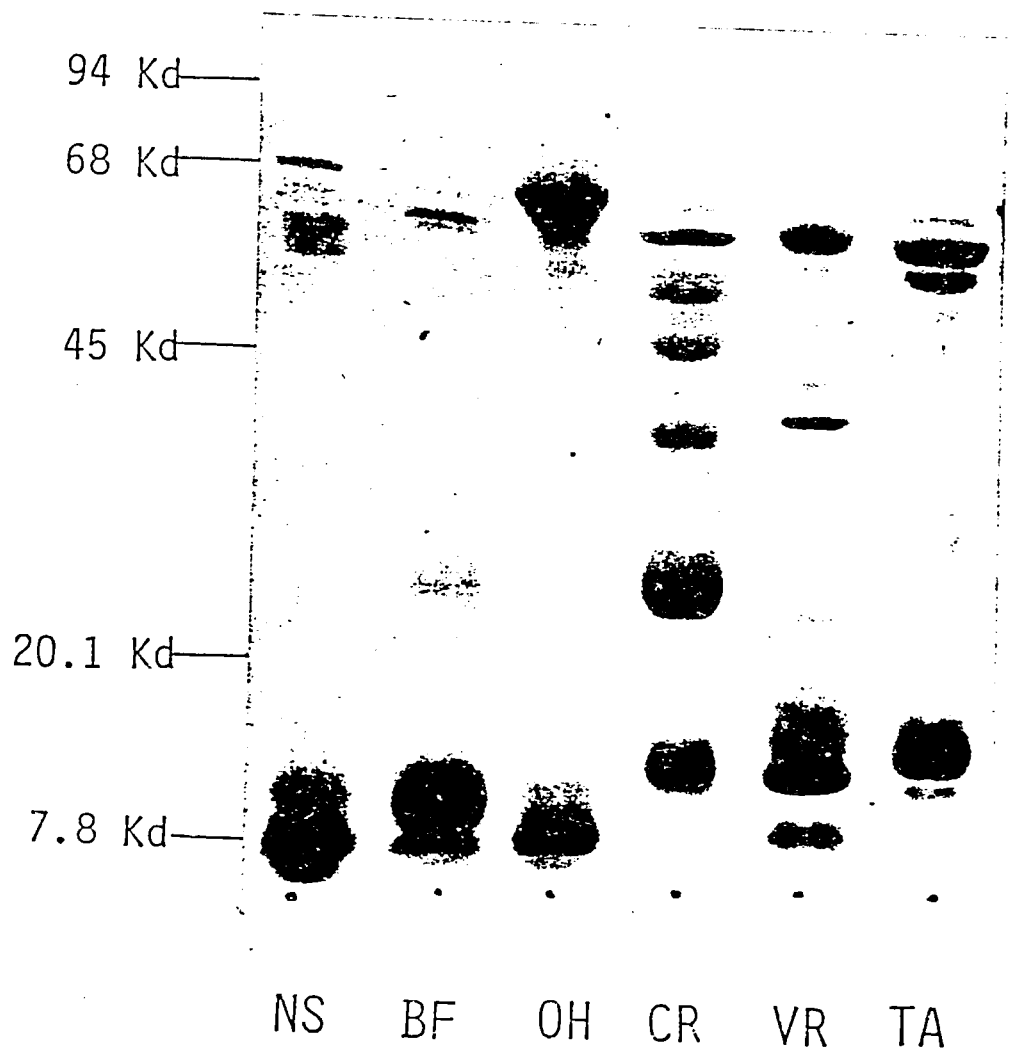
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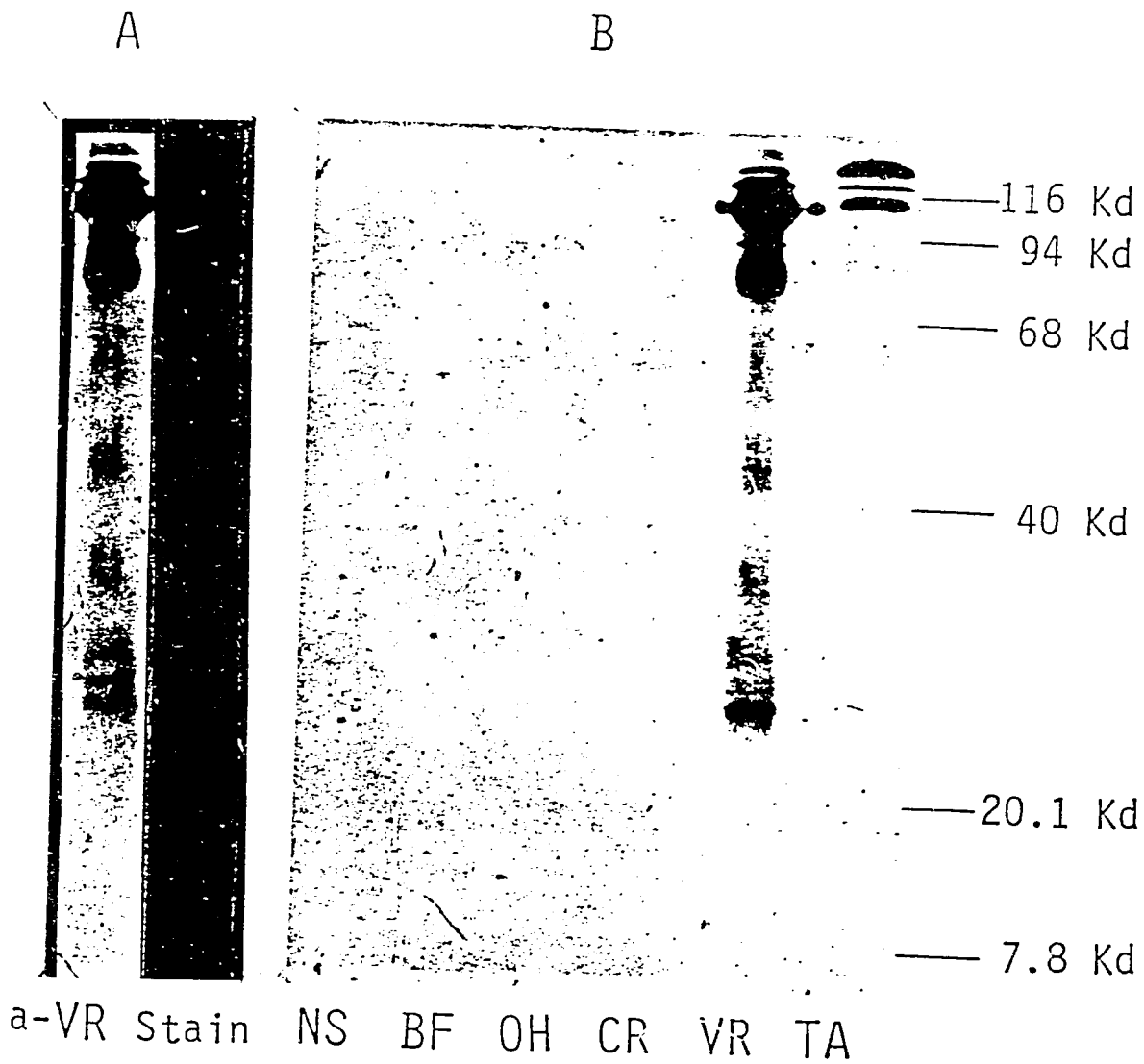
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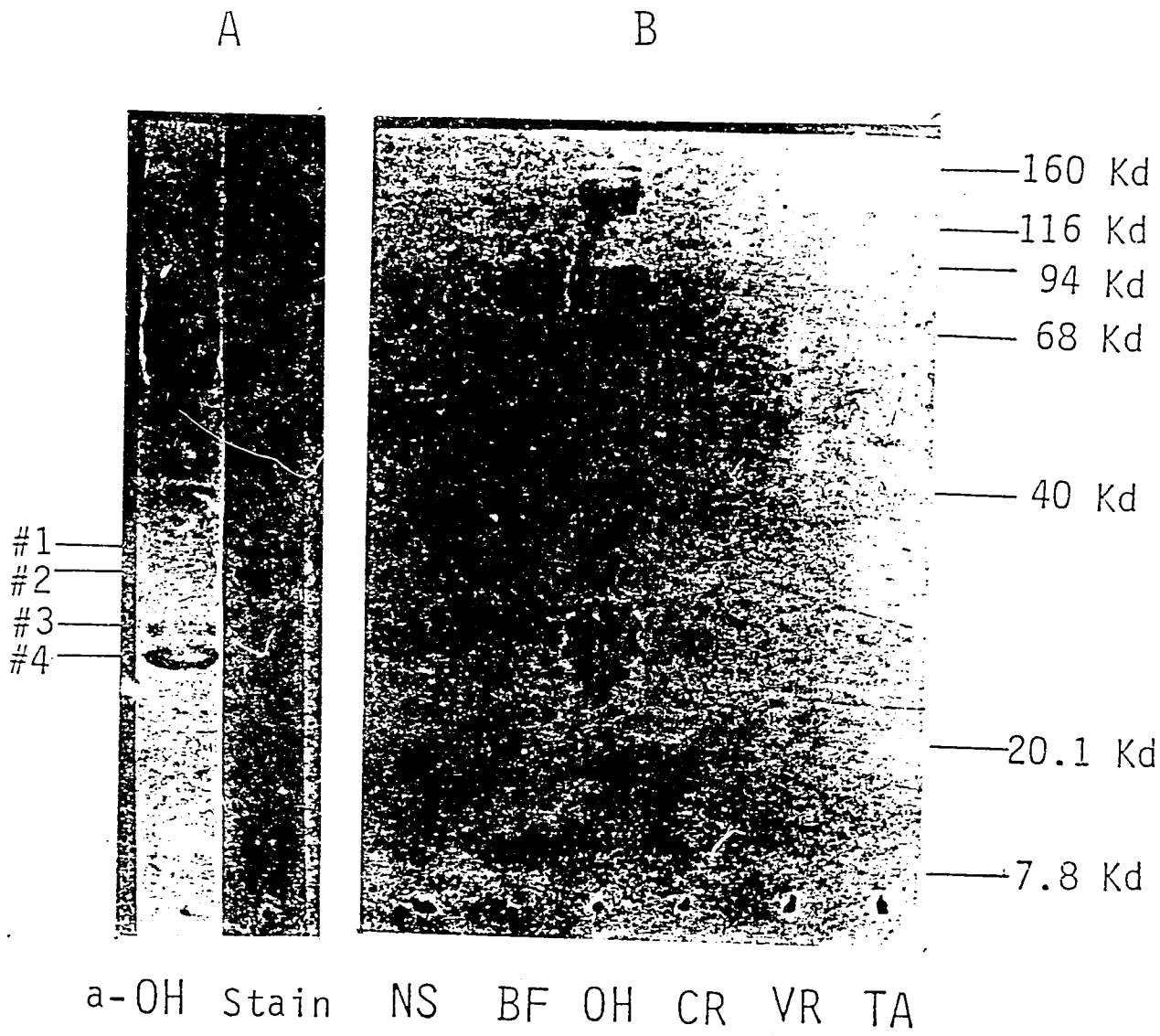
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a-OH Stain
NS BF OH CR VR TA

5d

Table I Summary of the reactions and cross-reactions of venom proteins from each of the six snakes with rabbit homologous and heterologous antisera. The data were compiled from a series of blots, as in Figs. 2 and 3, reacted in parallel with the various antisera. Molecular weights of the immunologically reactive polypeptides were calculated from their electrophoretic mobilities (see Materials and Methods). Venom abbreviations are as in Fig. 1.

Antigen / Antibody	NS	BF	OH	CR	VR	TA
Anti-NS	8K-22K 32K 58K 66K 87K 140K		8K,19K,22K 80K-105K			48K-54K 130K-170K
Anti-BF	58K	8K 38K 48K 58K 66K 74K	58K	66K 90K,110K	66K 84K-96K,110K	45K-54K 54K-170K
Anti-OH	22K 52K,58K,66K	48K,66K	19K 23K 25K 32K 39K 43K-100K 100K-155K	90K 110K		25K 48K 125K
Anti-CR				10K-18K 40K 66K 80K 90K 105K 110K		66K 80K 110K
Anti-VR					25K-35K 42K-50.5K 56K-74K 84K-96K 100K 103K-120K 125K 130K	45K 66K 90K 110K 125K 130K
Anti-TA			56K	90K 110K	90K-105K 110K	16.5K-19K 19.5K 22K-28K 34K-42K 45K-54K 54K-90K 110K-140K 150K-170K

MS # 2

SELECTION OF COBRA VENOM ANTIGENS FOR USE IN AN ELISA OF HORSE ANTIVENOM POTENCY

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ABSTRACT An ELISA for the quantitation of antibodies against *Naja naja siamensis* venom proteins has been developed for use as a possible replacement of the *in vivo* neutralization assay of antivenom potency. Comparison was made with 3 preparations of venom proteins as antigens of ELISA ; these were crude venom, toxin fraction and purified principal postsynaptic neurotoxin of the Thailand cobra. With one batch of horse monovalent anti-cobra antivenom as reference standard, seven other batches of antivenom were assayed by the ELISA and by the *in vivo* neutralization assay using mice. When crude venom, toxin fraction and pure neurotoxin were used as antigens in the ELISA, the correlation coefficients between the ELISA neutralization and *in vivo* neutralization were 0.76 ($P < 0.01$), 0.91 ($P < 0.001$) and 0.93 ($P < 0.001$), respectively. Thus, in the development of ELISA as an *in vitro* assay of potency of anti-snake venom antivenom, well-defined toxic component rather than the crude venom, should be used as antigen of the ELISA. The conclusion should be applicable to similar study on antivenoms against other elapid venoms whose lethal components are small, poorly immunogenic peptides.

INTRODUCTION

The current method for the estimation of antivenom potency involves *in vivo* neutralization assay using mice. The procedure is extremely laborious, expensive and gives results which are variable and difficult to reproduce. Not of the least concern is the killing of a large number of animals. Furthermore, the problems and difficulties of the *in vivo* neutralization assay are a major obstrucle that hinders the much needed improvements on potency and purity of antisera against snake venoms. In the preparation of potent hyperimmune sera against snake venoms, for example, experiments have to be carried out with different venom antigens and forms, immunization schedules, adjuvents etc., to establish optimal immunization conditions. In the fractionation of hyperimmune sera, conditions must be found so as to give the highest yield and purity of the product. Such studies are almost impossible with the currently used *in vivo* neutralization assay for the evaluation of the results.

Recently, *in vitro* assay based on enzyme-linked immunosorbent assay (ELISA) has been developed as an possible alternative to the *in vivo* neutralization test (Threakston and Reid, 1979). They studies horse antivenoms against two viperid and two elapid venoms using crude venoms as antigens. The correlation between the ELISA and the *in vivo* neutralization assay was good with the correlation coefficient of 0.92; $P<0.001$.

It has long been observed, however, that in antisera against snake venoms, the antibody contents, as estimated by the intensities and numbers of precipitin bands, do not usually correlate with the neutralizing capacities (Minton, Jr.,1979). Most of the antibody generated against the venom are directed against the non-toxic yet more immunogenic components of the venom. This is specially true with the elapids whose toxins are low molecular weight (about 60 to 74 amino acids) peptides and are extremely toxic yet barely immunogenic. Therefore, at least with elapid venoms, the use of fractionated toxin(s) rather than the crude venom as antigen in the ELISA should give better estimate of the antivenom potency.

In the present study, we compared three different preparations of antigen from Thai cobra venom in an ELISA of horse antivenom antibody. These preparations are crude cobra venom, partially purified toxin and purified principle postsynaptic toxin. The results obtained from the ELISA of 8 batches of horse antivenoms were compared with those from <u>in vivo</u> neutralization assay using mice. The use of purified toxin as antigen in the ELISA was found to give much higher correlation coefficient than that of crude cobra venom.

MATERIALS AND METHODS

1. Biochemicals and chemicles

Venom of Thailand cobra (*Naja naja siamensis*, also known as *Naja kaouthia*) and monospecific horse anti-cobra antivenom were purchased from Queen Soavabha Memorial Institute, Bangkok. Other chemicals were generally of reagent grade and were obtained from Sigma Chemical Co., St. Louis, Missouri, USA, except as indicated.

2. Preparation of a "toxin fraction" from N.n.siamensis venom.

Crude cobra venom (700 mg) was chromatographed on a Sephadex G-50 column (2.6 x 97 cm) using 25 mM ammonium acetate as eluent. The toxin fraction as indicated by (<--->) in Fig. 1 was pooled, lyophilized and used as an antigen in the ELISA. This "toxin fraction" was used also as starting material for the purification of *N.n.siamensis* postsynaptic neurotoxin 3.

3. Purification of N.n.siamensis postsynaptic neurotoxin 3.

The *N.n.siamensis* neurotoxin 3 which is the most abundant and most toxic component of the cobra venom was purified from the "toxin fraction" using ion-exchange chromatography on a carboxymethylated polystyrene (Biorex-70 from BioRad, Richmond, Calif.) as described by Karlsson *et al* (1971). The pure toxin was lyophilized free of the volatile buffer and used as an antigen in the ELISA.

4. Determinations of medium lethal dose (LD_{50}) of cobra venom and the in vivo neutralizing activity of horse antivenom.

Groups of six Swiss albino mice weighing 25 ± 2 gm were injected intraperitoneally with cobra venom at doses ranging from 0.16 to 0.61 ug/gm mouse. The venom was prepared in normal saline and the volume of injection was kept constant at 0.1 ml / 25 gm mouse. Control animals were inject i with normal saline only. The percent death of animals were recorded 24 hours after injection. The medium lethal dose (LD_{50}) of the venom was estimated by the method of Litchfield and Wilcoxon (1949).

The *in vivo* neutralizing activity of horse anticobra antivenom was determined by a procedure recommended by WHO (1983). The procedure is similar to that described above for LD_{50} determination but cobra venom at different concentration in normal saline (0.2 - 0.8 mg/ml) were incubated with an equal volume of horse anticobra antivenom for 1 hour at 37 °C before injection. The volume of injection was kept constant at 0.1 ml / 25 gm mouse. The LD_{50} of the venom in the antivenom-venom mixture was determined and the neutralizing activity was calculated as follows :

$$\text{Neutralizing activity} = (LD'_{50} - LD_{50}) \times \frac{\text{weight of mouse (gm)}}{\text{volume of antivenom (ml)}}$$

where - LD_{50} is the medium lethal dose of cobra venom alone (ug venom / gm mouse)

- LD'_{50} is the medium lethal dose of the venom and antivenom mixture.

5. Comparison of results of in vivo neutralization assay and ELISA of horse antivenoms.

One batch of horse antivenom was arbitarily selected as reference antivenom. Various dilutions of this reference antivenom were made and assayed for antibody by ELISA using each of the 3 different antigens. The reference curves of optical densities from ELISA using the three antigens were then plotted against the mouse *in vivo* neutralizing activity.

For a given antigen, the optical densities from of 7 other horse antivenoms were then compared with that of the reference antivenom to give the theoretical ELISA neutralizing activity. The results were then compared with those obtained from *in vivo* neutralizing activities of the antivenoms.

6. Other biochemical procedures.

Protein concentration was determined by a procedure described by Lowry *et al* (1951) using serum bovine albumin (BSA) as standard. Slab SDS-polyacrylamide gel electrophoresis was carried out as described by Laemmli (1970) using ten percent acrylamide and the following protein standards : rabbit muscle phosphorylase B, 94 Kd; BSA, 68 Kd; ovalbumin, 45 Kd; soybean trypsin inhibitor, 20.1 Kd and *N.n.siamensis* toxin 3, 7.8 Kd.

RESULTS

1. Molecular weight characterization of the antigens

The "toxin fraction" and the purified N.n.siamensis neurotoxin 3, prepared as described in Materials and Methods, together with the crude cobra venom were subjected to SDS-polyacrylamide gel electrophortesis. The results are shown in Fig. 2. In all these preparations of antigen, N.n.siamensis neurotoxin 3 which is the principal toxin of the venom (Karlsson et al, 1971) is obviously the most abundant component. The "toxin fraction" differs from the pure N.n.siamensis neurotoxin 3 by the presence of a minor protein of 12,400 daltons (Fig. 2.). It should be mentioned, however, that also present in the neurotoxin 3 band of the "toxin fraction" are some minor short postsynaptic neurotoxins and cardiotoxins which were not separated from the principal neurotoxin under these conditions (Karlsson et al, 1971).

2. Optimal conditions of ELISA of horse antisera against cobra venom.

Using checkerbroad titrations, it was established that the reagent concentrations and optimal conditions of ELISA for the determination of antibody in horse antivenoms are as follows:

a. plastic microtiter wells were coated with crude cobra venom (10 ug/ml) or "toxin fraction" or N.n.siamensis neurotoxin 3 (5 ug/ml) by overnight incubation at 4 ^{o}C

b. horse monovalent antivenoms at dilution of 1 : 6250 were allowed to bind the immobilized antigen on the well by incubation for 1 hour at 25 ^{o}C

c. to detect the bound horse antibody, sheep anti-horse IgG peroxidase conjugate at dilution of 1:30 was incubated for 1 hour at 25 ^{o}C

d. a substrate solution (10 mg O-phenylenediamine in 25 ml of 75 mM phosphate-citrate buffer pH 5.0 containing 0.003 % hydrogen peroxide) was incubated for 30 min. at 25 ^{o}C and the optical density at 490 nm was recorded.

3. In vivo neutralizing activity of horse antivenoms.

The ELISA of 8 batches of monospecific horse antisera against cobra venom, as carried out under optimal conditions described above, gave optical densities shown in Table 1 for each of the 3 antigens. In general, the optical densities obtained from the use of crude venom were higher than those using the toxins as antigens. The *in vivo* neutralizing activity of each antiserum was determined and the results are shown in Table 2. The average neutralizing activity was 311.3 ug/ml.

Fig. 3 shows a plot on the correlation between the *in vivo* neutralizing activities and the ELISA neutralizing activities of the 8 batches of horse antivenoms using purified *N.n.siamensis* toxin 3 as antigen. In this case the correlation coefficient is 0.93 ($P < 0.001$). The corresponding values when crude cobra venom and the "toxin fraction" were used as antigens in the ELISA are 0.76 ($P < 0.01$) and 0.91 ($P < 0.001$), respectively.

DISCUSSION

It is clear from various reasons that *in vitro* immunoassays for antivenom potency estimation are needed to replace the *in vivo* neutralization assay using life animals. Various immunoassays based on indirect hemagglutination (Peel, 1980), radioimmunoassay (Habermann *et al.*, 1977) and gel diffusion (Mouillot *et al.*, 1978) have been studied. However, one of the most promising assays seems to be that based on ELISA since the method can give quantitative and reproducible results and is simple to preform. Although there are some concern about the use of ELISA to measure neutralization by quantitating antibody of unknown affinity (Steward and Lew, 1985), several ELISA's have been developed for tetanus antitoxin (Melville-Smith *et al.*, 1983 ; Simonson *et al.*, 1986 ; gentili *et al* 1984) and for anti-snake venom (Theakston and Reid, 1979).

In the case of snake venoms especially those of the elapids, the lethal components are low molecular weight basic peptides called postsynaptic neurotoxins. These toxins bind specifically and 'irreversibly' to nicotinic acetylcholine receptor at the muscle end-plate. The binding results in blockade of neuromuscular transmission and causes death by respiratory paralysis. These neurotoxins, although in some case present as a minor constitnents (about 2 percent in *B. fasciatus* venom) have been shown to be the component that causes death in animal (Ratanabanangkoon *et al.*, 1978). Because of the low immunogenicity and in some cases, low abundancies of these toxins, antibodies against them are present in the serum in

disproportionately small amount as compared to those against larger non-toxic venom proteins. Thus, useful *in vitro* assays of antivenom potency should measure only antibodies against the venom toxic components (Christensen, 1979). The results present here clearly show that much better correlation with the *in vivo* neutralization assay was obtained when the toxin was used as antigen in the ELISA. Furthermore, this study shows that for the Thai cobra venom and most likely for other elapid venoms as well, the use of "toxin fraction" which is much easier to obtain, can serve as good antigen in the ELISA.

Thus, in the development of ELISA for potency determination of antisera against snake venoms, fractionation and experimentation with different venom components as possible antigens in the ELISA should be carried out. The venom fraction which gives result best correlated with the *in vivo* neutralization should then be used as antigen in an *in vitro* neutralization assay.

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LEGENDS TO FIGURES AND TABLES.

Fig. 1 The elution profile of cobra venom on a Sephadex G-50 column (2.6 x 97 cm). The eluent was 25 mM ammonium acetate, pH 7.4. The flow rate was 15 ml per hour, at 4 °C and fractions of 3 ml were collected. The dashed line is the elution profile of *N.n.siamensis* neurotoxin 3 in a separate chromatography on the same column under identical conditions.

Fig. 2 SDS-polyacrylamide gel electrophoresis of crude cobra venom, "toxin fraction" and purified *N.n.siamensis* neurotoxin 3. Molecular weights in Kilodaltons of stardard proteins are shown on the left.

Fig. 3 Correlation between the *in vivo* neutralizing activity and the ELISA neutralizing activity when purified *N.n.siamensis* neurotoxin 3 was used as antigen in the ELISA.

Table 1. ELISA optical densities of horse antivenoms using 3 different preparations of antigen.

Table 2. Horse antivenom potencies as determined by *in vivo* neutralization assay using mouse and by ELISA using 3 different preparations of antigen.

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Table 1 ELISA optical densities of horse antivenoms using 3 different preparations of antigen.

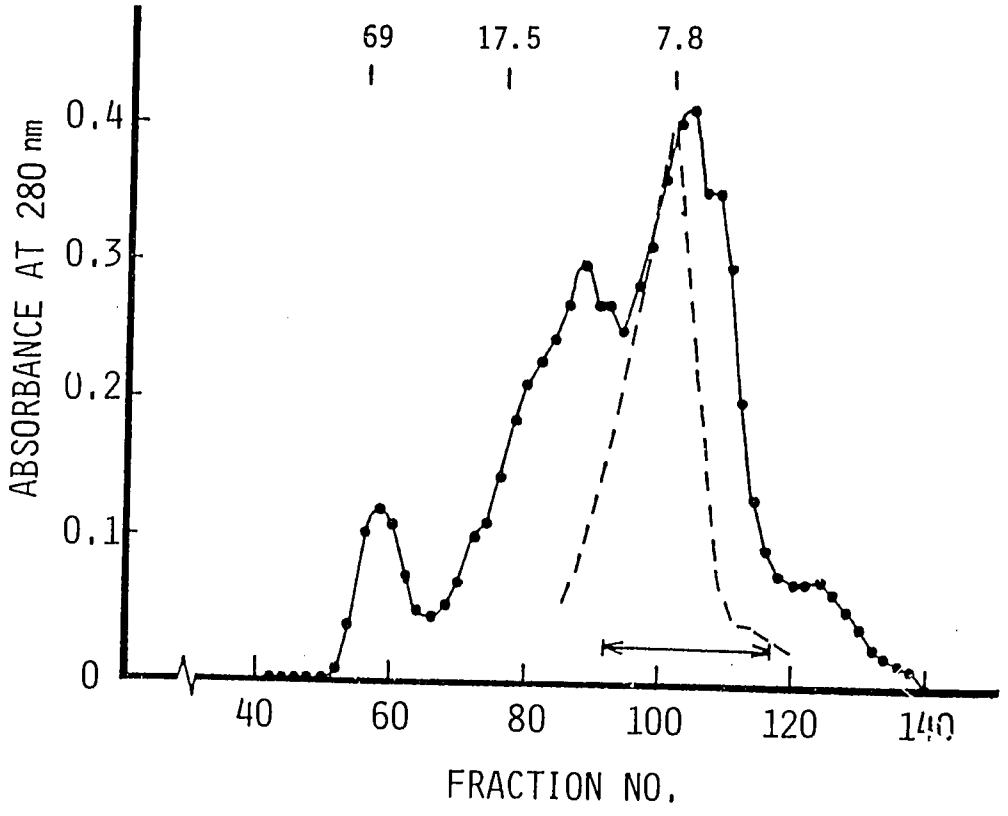
Ag of ELISA	ELISA optical density at 490 nm.+		
Horse antivenom	Crude venom	Toxin fraction	Pure toxin 3
1	0.869±0.156	0.570±0.077	0.591±0.049
2	0.976±0.142	0.869±0.138	0.750±0.064
3	0.760±0.110	0.743±0.122	0.680±0.041
4	0.940±0.091	0.844±0.167	0.931±0.040
5	0.717±0.209	0.537±0.074	0.488±0.067
6	0.876±0.134	0.744±0.129	0.714±0.174
7	0.776±0.096	0.621±0.058	0.519±0.063
8	0.920±0.108	0.751±0.103	0.735±0.152

The values represent means of 8 determinations ± SD.

Table 2 Horse antivenom potencies as determined by *in vivo* neutralization assay using mice and by ELISA using 3 different preparations of antigen.

Horse antivenom	*In vivo* neutralizing activity (ug/ml)	ELISA neutralizing activity (ug/ml)		
		Crude venom	Toxin fraction	Pure toxin3
1	252.5 (240.8-264.6)*	385.7	205.6	253.5
2	402.0 (364.3-443.5)	518.6	462.8	387.3
3	345.0 (315.3-376.6)	284.9	328.5	321.5
4	485.0 (455.9-515.5)	469.33	432.6	626.9
5	150.0 (128.1-173.6)	252.6	188.2	193.1
6	277.5 (255.5-300.7)	392.8	329.5	351.7
7	228.6 (201.4-256.6)	297.5	235.9	209.3
8	350.0 (323.2-378.4)	443.9	318.5	372.2

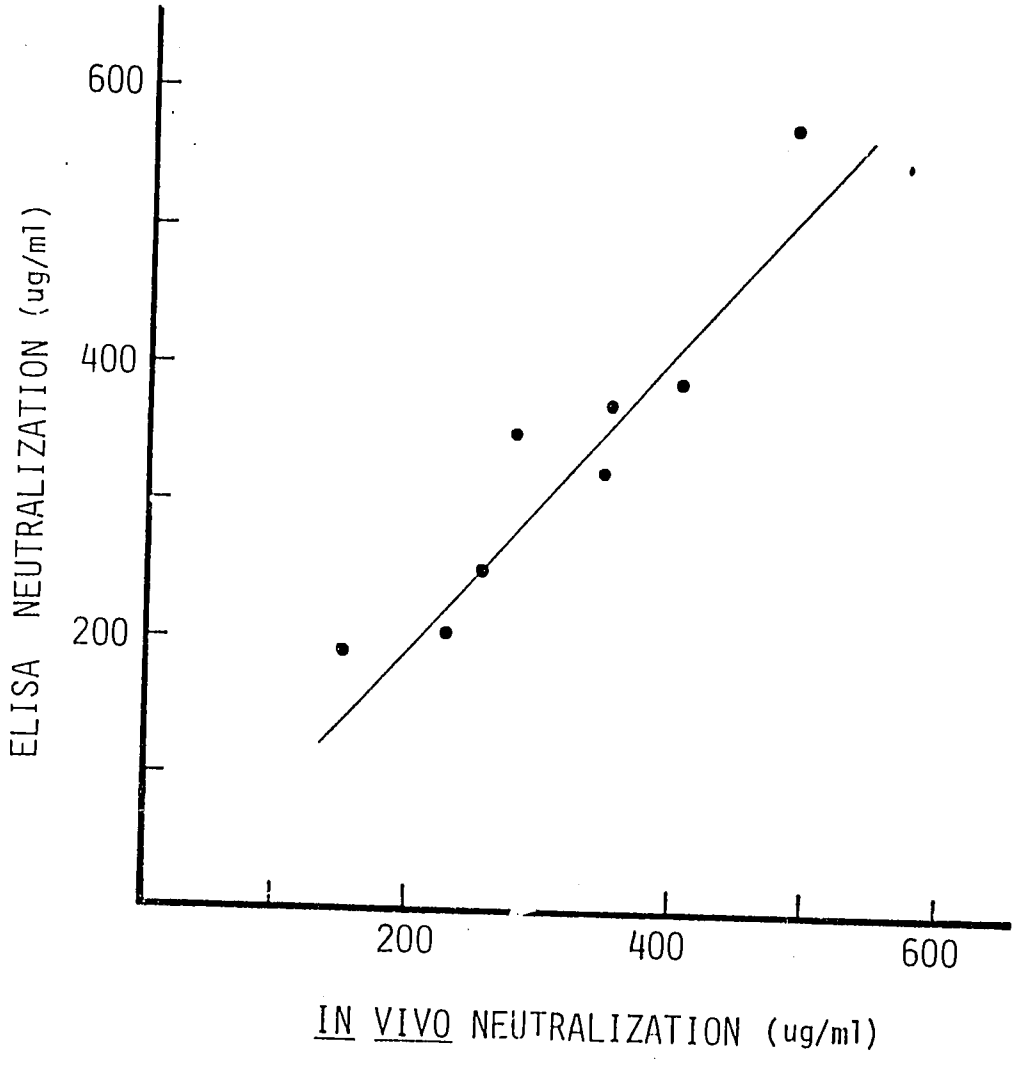
* Numbers in parentheses are 95% fiducial limits.


69
17.5
7.8
0.4
0.3
0.2
0.1
0
ABSORBANCE AT 280 nm
40
60
80
100
120
140
FRACTION NO.

— Crude cobra venom

— Toxin fraction

— Purified *N.n.siamensis* toxin 3


600
400
200
ELISA NEUTRALIZATION (ug/ml)
200
400
600
IN VIVO NEUTRALIZATION (ug/ml)