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PROGRESS REPORT NO. 4

Development of Immunodiagnosis and Improvement of Immunotherapy of Snake Venom Poisoning

A Research Project

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Project profile

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

Background In previous reports, the developments of two simple immunoassays for the detection of Thai snake venoms were described. These two assays are reverve latex agglutination (RLA) and reversed passive hemagglutination (RPHA). In the case of RPHA comparison of 3 species (sheep, human and chicken) of red blood cells were made with respect to the sensitivity, specificity and speed of venom detection, the stability and cost of reagents together with the interference from clinical samples (sera, plasma, urine). It was concluded that sheep red blood cells are the most suitable for use in the RPHA for snake vnom detection.

Described below is the result on the use of the RLA and RPHA in the detection of various snake venoms from clinical samples taken from 59 victims of snakebite.

Materials and Methods. The procedures for RLA and RPHA have been described in the previous report. Serum samples were collected from those patients treated at hospitals for snakebites where the culprit snakes were brought for identification. These patients were:

(1) 15 patients bitten by *Vipera russelli* at Chaopraya Aphaiphubet Hospital, Prajinburi Province.

(2) 17 patients bitten by *Naja naja siamensis*, *Calloselasma rhodostoma*, *Vipera russelli* or *Trimeresurus albolabris*. The sera from these patients were kindly provided by Dr. Sasithon Pukrittayakamee of Hospital for Tropical Diseases, Bangkok, where the ELISA on these samples were carried out.

(3) 29 patients bitten by Calloselasma rhodostoma from Sadao Hospital, Songkla Province; 24 paired serum and swab samples were collected from the same patients.

The samples (59 sera and 26 wound swabs) were kept frozen at -20 ^{o}C until used. The wound swab samples were soaked with 1.0 ml of 0.85% normal saline solution and the cotton swabs were removed before assay.

Comparison between the RLA, RPHA and ELISA tests in the detection of N. n. siamensis, C. rhodostoma, V. russelli and T. albolabris venoms in serum samples

ELISA versus RLA

The results of the ELISA and RLA tests in the detection of venoms were compared using serum samples (17 cases) from the Hospital for Tropical Diseases (Table 1a, b). The ELISA detected 12 cases of positive samples whereas 8 cases were positive by the RLA method. Two cases of negatives by the ELISA were positive when examined by the RLA test. The results from the RLA and ELISA methods were not statistically different (p-value > 0.05) by X^2 McNemar Test.

ELISA versus RPHA

The samples studied were the same as those described above. Table 2 shows that 10 samples assayed positive by both ELISA and RPHA while each method alone had 2 additional positive cases which assayed negative by the other. It is obvious that the RPHA results were not significantly different from those of the ELISA in the detection of various snake venoms (p-value > 0.05).

Comparison between the RLA and RPHA tests in the detection of N. n. siamensis, C. rhodostoma, V. russelli and T. albolabris venoms in sera

The species diagnosis assays were carried out on all 59 serum samples from various provinces using RLA and RPHA tests. There were 30 cases of positives by both methods, and 18 cases of positives by the RPHA but not by the RLA test. One positive case was detected only by the RLA method (Table 3a, b). The RLA and RPHA tests showed a significant difference (p-value < 0.05). However, this was not the case when the results (Table 4) on the wound swabs were compared. The results from the RLA and RPHA tests were not significantly different (p-value > 0.05).

Table 5 shows the comparison of venom detection in the sera and swab samples by the RLA test. Out of 24 paired serum and swab samples, 7 were dual positives, whereas swab samples from 3 patients with negative sera, and serum samples from 7 patients with negative swabs, gave discordant sensitivities. However, there was no significant difference between the two sample sources (p-value>0.05). When the same paired samples were assayed by the RPHA test, the number of dual sample positives increased to 12, with 8 serum-only positives and 2 swab-only positives (Table 6). These also showed no significant difference between sample types (p-value>0.05).

Discussion In this research project, a total of 4 immunoassays have been studied for possible use as rapid immunodiagnostic test of snake vneoms. These tests are passive hemagglutination inhibition (PHAI), latex agglutination inhibition (LAI), RLA and RPHA. Only the last two assays were found to be promising in terms of sensitivity,

specificity and speed of venom detection.

Studies using clinical samples have shown that RLA and RPHA could correctly identify snake venoms in these samples. RPHA was found to be slightly more sensitive than RLA. The results obtained by each of these two tests were not statistically different from those obtained by ELISA. However, RPHA and RLA exhibit several advantages over the ELISA in terms of simplicity, speed, and stability of reagents (Table 7).

There results indicate that either of these two immunoassays should be useful for rapid diagnosis of snake venoms. Attempt should be made to produce one of these tests in langer scale and distribute them to rural hospitals to study the accuracy of venom detection and acceptability by the physicians.

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

Background It is highly desirable to have an *in vitro* procedure for the assessment of antivenom potency. In the previous report, a successful development of an ELISA for this purpose using the principal postsynaptic neurotoxin of *N. n. siamensis* as antigen, was described. The procedure has been introduced to the Government Pharmaceutical Organization for use in antivenom standardization. It has also been used in our laboratory in a study on the improvement of snake toxin immunogens (# 3).

In this report, we describe an ELISA for the accurate assessment of potency of antivenom against *V. russelli* which is one of the most medically important vipers not only of Thailand but also of many other Asian countries.

Materials and Methods The V. russelli venom was fractionated on a Sephadex G-100 column (Fig 1) into 3 major protein peaks (VR#1, VR#2 and VR#3). The first two fractions (VR#1 and VR#2) together with the crude venom were used as antigens of the ELISA. Peak VR#3 corresponds to low molecular weight peptides and was therefore not included in this study.

Fifteen batches of horse antivenoms against V. russelli were assayed for their in vivo neutralizing activity using mice. These antivenoms were also assayed for their content of specific antibodies by ELISA using the venom protein fractions as antigens.

Results The in vivo neutralizing activities and the ELISA titers of these antivenoms using various antigens are shown in Table 8. The correlation coefficients of these assays using each of the antigens are shown in Table 9. It is evident that when VR#1 fraction was used as antigen of the ELISA, the ELISA titer significantly correlated with the in vivo assay. When the crude venom was used as antigen, lower correlation coefficient was obtained. This is not surprising since the principal toxin of the venom resides in the VR #1 fraction.

Discussion The high correlation coefficient obtained when VR#1 was used as ELISA antigen strongly indicates that this ELISA can be used as an in vitro assay of antivenom against V. russelli. This result again underscores the need to use venom toxin fraction as antigen since under these conditions, primarily the antitoxin antibodies are assayed.

Thus, we have accomplished our goal of developing in vitro assays based on ELISA for the assessment of antivenoms against two of the most medically important snakes of Thailand and of Southeast Asia.

The basic approach and procedures described should be applicable to similar study with antivenoms against other snakes. These *in vitro* assays should be very useful in antivenom standardization and in research on improvement of antivenom potency.

3. Purification and characterization of O. hannah postsynaptic neurotoxins.

Background In previous reports we described a successful purification of an *O. hannah* postsynaptic neurotoxin. The amino acid composition of this toxin was determined. However, not enough material was available for amino- and carboxyl- terminal analysis. Subsequent attempt was made with a large batch of *O. hannah* venom. However, the result obtained from the ion-exchange column was an extremely complicated protein profile. It is thought that the starting venom was most likely milked from dead *O. hannah* specimens (an usual practice of The Thai Red Cross Society snake farm). The various protein peaks are most likely antolytic products. Described in this report is another successful purification of the *O. hannah* postsynaptic neurotoxin.

Materials and Methods A combination of gel filtration on Sephadex G-50 and an ion-exchange chromatography on Biorex-70 was used as described previously. The same continuous gradient elution was also used.

Results and Discussion The postsynaptic neurotoxin of *O. hannah* was obtained in about 10 mg yield. It will be used for amino- and carboxyl- termini analysis and also for other studies eg. the

improvement of immunogenicity of elapid toxin (#4).

4. Study on the improvement of immunogenicities of cobra postsynaptic neurotoxins and preparation of polyvalent antivenoms.

Background In an attempt to prepare potent, polyvalent antivenoms, we previonsly described the synthesis and characterization of 7 monovalent and polyvalent toxin immunogens. These immunogens were prepared by limited polymerization or conjugation of elapid postsynaptic toxins. In this report, the results on the immunogenicities of these immunogens in rats are described.

Materials and Methods The 7 monovalent and polyvalent toxin immunogens were synthesized and characterized as described previously. Each immunogen was immunized into groups of 8 rats using Freunds' incomplete adjuvant. Crude cobra venom and purified N. n. siamensis toxin 3 were included for comparative purpose.

The rats were boostered every 6 weeks. Blood was collected from each raptrior to immunization. The in vitro neutralizing activity of each serum sample was determined by using the ELISA developed in our laboratory (#2) using N. n. siamensis toxin 3 as antigen.

Results The content of specific antitoxin antibody in each breeding from each group of rats is shown in Fig 2. It is evident that N. n. siamensis toxin-3 tetanus toxiod conjugate elicited the highest level of antitoxin antibody. Sinificantly, all 3 polyvalent immunogens were found to be as immunogenic or more so than the crude cobra venom alone. When these 'polyvalent' sera were assayed using neurotoxins of

B. fasciatus or O. hannah as ELISA antigens, comparable antitoxin antibody levels were obtained.

Discussion These results clearly show that the elapid toxins can be rendered more immunogenic by controlled covalent coupling of the toxins to highly immunogenic protein carriers. Moreover, these results also demonstrated that preparation of potent, truely polyvalent antisera is possible, at least in rats. It is thus interesting to see whether similar results can be obtained in horse. Should this proves to be the case, the avaible potent polyvalent horse antivenoms should drastically reduce the cost of therapeutic antivenoms, it should also save more lives of snakebite victims with severe venom poisoning or with ambiquious snake identification.

In progress is a similar study in which the effect of different adjuvants were compared in rats using some of the above toxin immunogens.

5. Refinement of anti- snake venom antiserum.

Background The therapeutic antivenoms currently available at the Thai Red Cross Society are crude horse sera. Hypersensitivity reactions of varying degrees are observed in about 50% of the patients receiving the horse sera. The Government Pharmaceutical Organization has begun to produce 'refined' antivenoms which are ammonium sulfate precipitated, pepsin-treated horse antibody. However, the precedures result in very low yield of antibody activity. Attempts are being made in our laboratory to fractionate the horse serum so as to produce antivenoms with higher purity with high recovery of antitoxin activity. To achieve this, optimization of the ammonium sulfate

precipitation and pepsin digestion is neccessary. It is therefore desirable to have assays which can quantitate the specific horse intact IgG antibody and the specific horse $F(ab)'_2$ fragment. ELISA for the horse antibody against N. n. siamensis toxin 3 has been described previously. In this report, we describe the preparation of reagents neccessary to the development of an ELISA. for horse $F(ab)'_2$ in the presence of intact IgG.

Materials and Methods The horse $F(ab)'_2$ was obtained by pepsin digestion of IgG. The $F(ab)'_2$ fragment was purify from the starting IgG by ion-exchange chromatography on Sepharose S FF. The $F(ab)'_2$ obtained was coupled to CNBr-activated Sepharose to prepare an affinity absorbent on which rabbit antibody against horse $F(ab)'_2$ was purified.

Results Fig 3 shows the chromatographic profile of horse $F(ab)'_2$ on Sepharose S FF column. The fractions were also assayed for their antitoxin (N. n. siamensis toxin 3) activity. The antibody activity coincides with the protein profile.

Discussion In order to quantitate the $F(ab)'_2$ in the presence of intact IgG, an assay which can measure the horse Fc fragment is also needed. Currently the rabit antibody against horse Fc is being prepared. When rabbit antibodies against horse $F(ab)'_2$ and against horse Fc are available, we will proceed to develop the ELISA's to quantitate the horse $F(ab)'_2$ and intact IgG when they are in mixtures. These ELISA's will enable us to optimize the pepsin degestion process used in the refinement of antivenom.

Table 1a Results on the detection of various snake venoms in serum samples from various provinces by the RPHA and the RLA as compared to th ELISA#

Serum number	Identification	ELISA ng/ml	Reciprocal RPHA Titers NS	BF	OH	CR	VR	TA	Reciprocal RLA Titers NS	EF	OH	CR	VR	TA
1	N. n. siamensis	62	8	-	-	-	-	-	2	-	-	-	-	-
2	N. n. siamensis	42	16	-	-	-	-	-	2	-	-	-	-	-
3	T. albolabris	0	-	-	-	-	-	-	-	-	-	-	-	-
4	T. albolabris	0	-	-	-	4*	-	-	-	-	-	-	2*	-
5	N. n. siamensis	0	2	-	-	-	-	-	-	-	-	-	-	-
6	V. russelli	70	-	-	-	-	4	-	-	-	-	-	-	-
7	C. rhodostoma	338	-	-	-	16	-	-	-	-	-	-	-	-
8	T. albolabris	110	-	-	-	-	-	-	-	-	-	-	-	-
9	V. russelli	0	-	-	-	-	8	-	-	-	-	-	2	-
10	V. russelli	640	2	-	-	-	4	-	-	-	-	-	2	-
11	V. russelli	145	-	-	-	-	2	-	-	-	-	-	2	-
12	T. albolabris	690	-	-	-	-	-	16	-	-	-	-	-	2
13	C. rhodostoma	72	4	4	2	8	-	-	-	-	-	-	-	-
14	T. albolabris	110	-	-	-	-	-	-	-	-	-	-	-	-
15	C. rhodostoma	0	-	-	-	-	-	-	-	-	-	2	-	-
16	C. rhodostoma	143	-	-	-	4	-	-	-	-	-	-	-	-
17	N. n. siamensis	+	2	-	-	-	-	-	2	-	-	-	-	-

These samples were kindly provided by Dr. Sasithon Pukrittayakamee of the Hospital for Tropical Diseases where the ELISA on these samples were carried out; see details about patients on APPENDIX.

* False positive cases

Table 1b Comparison between the ELISA and the RLA tests in the detection of *N. n. siamensis*, *C. rhodostoma*, *V. russelli* and *T. albolabris* venoms in 17 serum samples.

RLA	ELISA		
	Positive	Negative	Total
Positive	6	2	8
Negative	6	3	9
Total	12	5	17

p-value > 0.05 by χ^2 McNemar Test

Table 2 Comparison between the ELISA and the RPHA methods in the detection of *N. n. siamensis*, *C. rhodostoma*, *V. russelli* and *T. albolabris* venoms in 17 serum samples.

RPHA	ELISA		
	Positive	Negative	Total
Positive	10	2	12
Negative	2	3	5
Total	12	5	17

p-value >0.05 by χ^2 McNemar Test

Table 3a Comparison between the RPHA and the RLA tests in the detection of *N. n. siamensis*, *C. rhodostoma*, *V. russelli* and *T. albolabris* venoms in 59 serum samples.

RPHA	RLA		
	Positive	Negative	Total
Positive	30	18	48
Negative	1	10	11
Total	31	28	59

p-value < 0.05 by χ^2 McNemar Test

Table 3b Comparison of the sensitivities of ELISA, RPHA and RLA in the detection of various snake venoms.

Source of serum samples	No. of samples	No. of positive results by ELISA#	RPHA	RLA
Various Provinces†	17	12 (70.6)	12 (70.6)	8 (47.1)
Prajinburi Province	15	ND	13 (86.7)	7 (46.7)
Songkla Province;				
serum samples	27	ND	23 (85.2)	16 (59.3)
wound swab samples	26	ND	16 (61.5)	10 (38.5)

*Identification of the culprit snakes was considered as a reference test with which the three methods were compared. The percent positive results are shown in parentheses.

#ELISA was performed at the Hospital for Tropical Diseases and data together with samples were provided by Dr. Sasithon Pukrittayakamee.

†Clinical specimens were collected from the patients treated at Samutprakarn, Lopburi, Prajinburi, Chinat and Trang Provinces.

ND = Not determined.

Table 4 Comparison between the RPHA and the RLA methods in the detection of *C. rhodostoma* venom in 26 wound swab samples.

RPHA	RLA		
	Positive	Negative	Total
Positive	9	7	16
Negative	1	9	10
Total	10	16	26

p-value > 0.05 by χ^2 McNemar Test

Table 5 Comparison of 24 paired serum and swab samples from the same patients in the detection of *C. rhodostoma* venom by the RLA test.

Serum	Swab		
	Positive	Negative	Total
Positive	7	7	14
Negative	3	7	10
Total	10	14	24

p-value > 0.05 by χ^2 McNemar Test

le 6 Comparison of 24 paired serum and swab samples from the same patients in the detection of *C. rhodostoma* venom by the RPHA test.

Serum	Swab		
	Positive	Negative	Total
Positive	12	8	20
Negative	2	2	4
Total	14	10	24

p-value > 0.05 by χ^2 McNemar Test

Table 7 Comparison of three different methods.

	ELISA	RLA	RPHA
1. Nature of tests	quantitative	semiquantitative	semiquantitative
2. Method and equipment	immunoassay plate, spectrophotometer	microscopic slide	microtiter plate
3. End point reading	colorimetry	titer	titer
4. Total test time	$\geq$ 3 hr	40 min	1-2 hr
5. Relative cost per test (Baht)	ca. 12-13	ca. 2-4	ca. 1-2
6. Shelf-life of reagents	long	long	long
7. Health hazards for laboratory personel	minor	none	none
8. Sensitivity	high	low	high
9. Specificity	depends on antibody preparation	depends on antibody preparation	depends on antibody preparation
10. Reproducibility	acceptable	acceptable	acceptable

Figures are from Ramathibodi Hospital; the cost of antigen and antibody are excluded

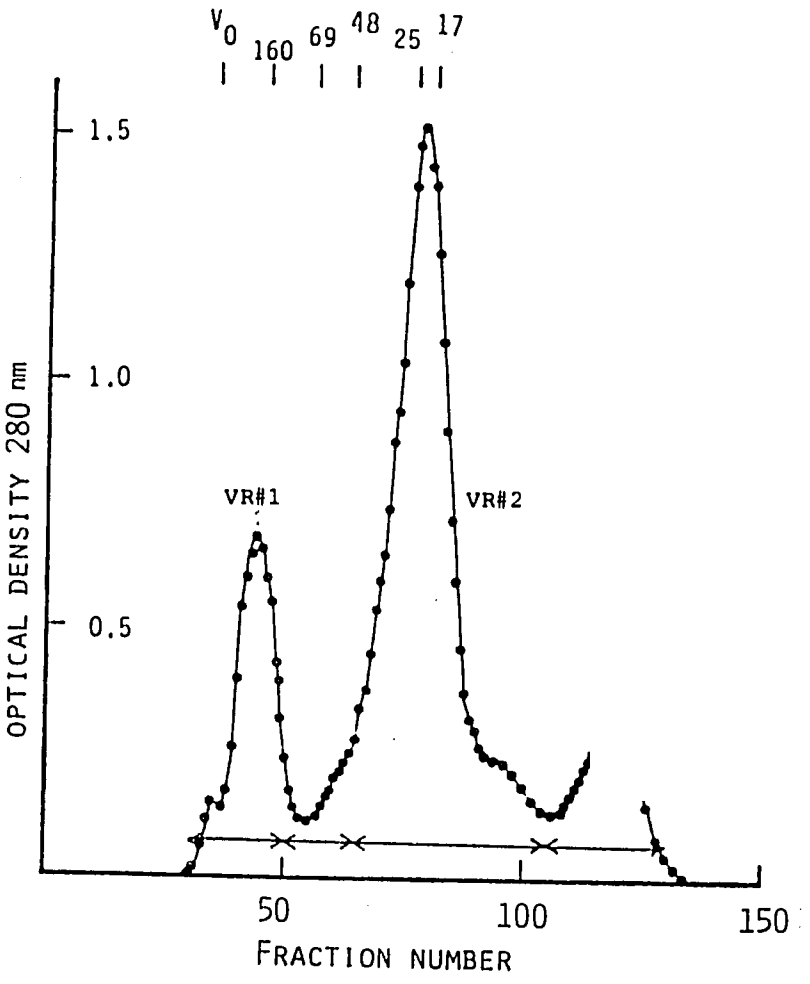
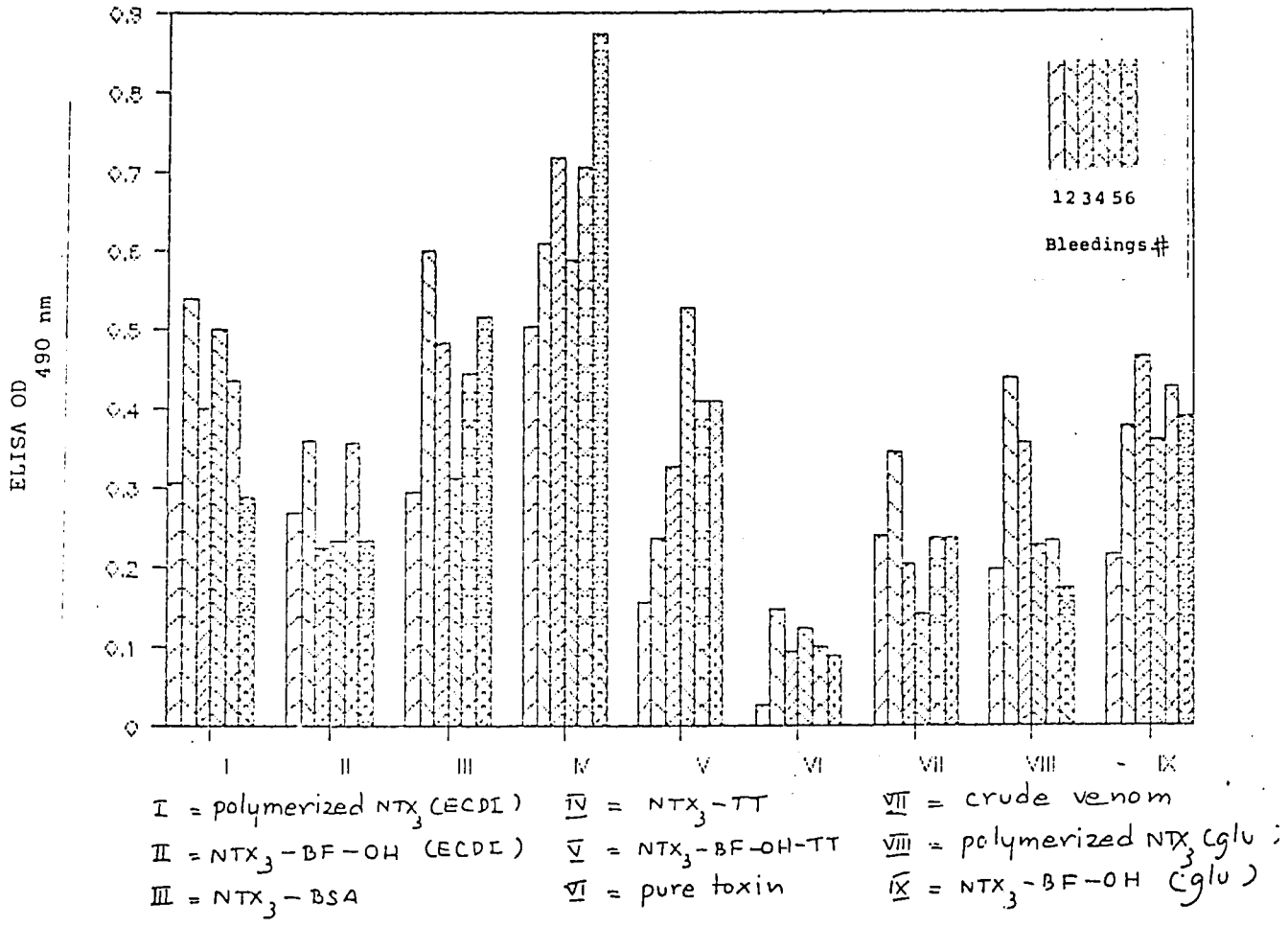


Fig 1. Chromatographic profile of V.russelli venom on a Sephadex G-10(column.



* **Abbreviations**

NTx 3	:	neurotoxin 3 of *N. n. siamensis*
BF	:	*B. fasciatus* neurotoxin
OH	:	*O. hannah* neurotoxin
BSA	:	bovine serum albumin
TT	:	tetanus toxiod
glu	:	glutaldehyde
ECDI	:	1-Ethyl-3-(3-demethyl aminopropyl) carbodiimide hydrochloride

Fig 2 Antibody response to various monovalent and polyvalent toxin immunogens in rats.

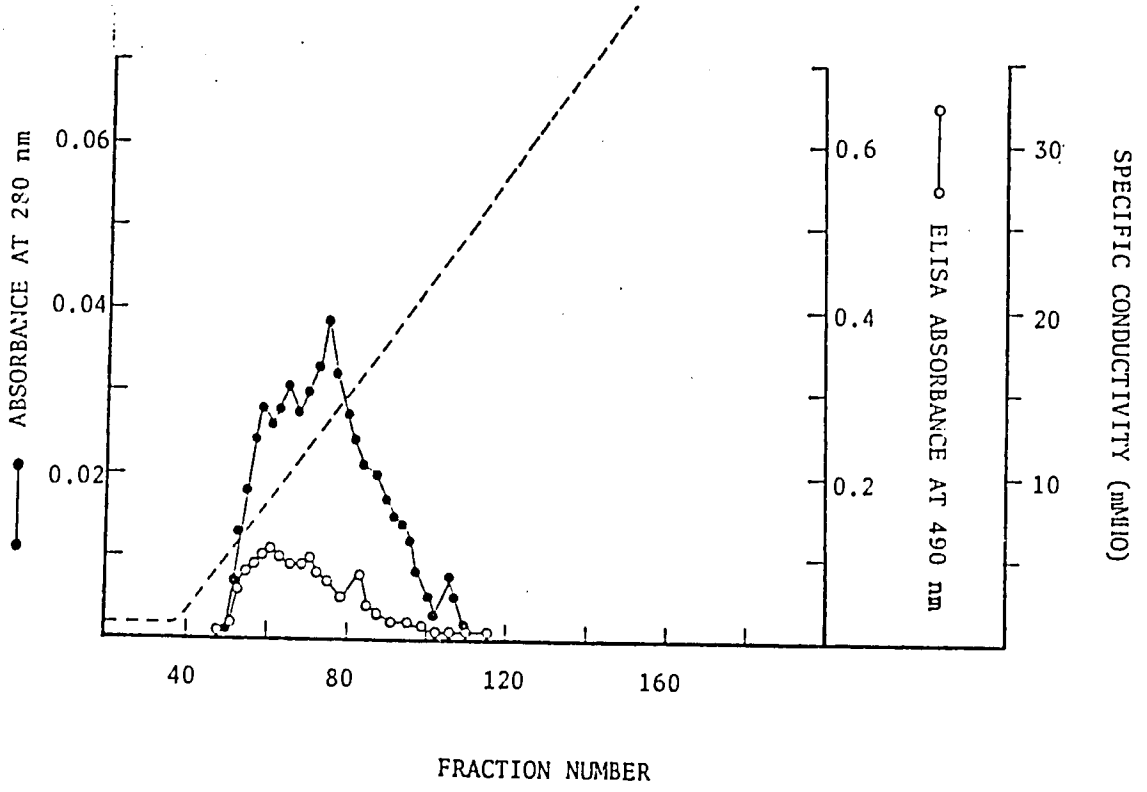


Fig 3 Chromatographic profile of horse $F(ab)'_2$ fragment in Sepharose S FF column.