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An *in vitro* Assay Method for "Mamushi" Antivenin Sera

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SUMMARY

The supernatants from flocculation reactions of "Mamushi" venom and its antiserum, were tested for excess antigen and toxin. The zone of excess antigen and that of excess toxin were identical. Only in the first two tubes of antigen excess only toxin was found (by the gel diffusion method).

Of about 10 precipitation lines given between the venom preparation and its antiserum, the line nearest to the antiserum well was shown to be formed by the toxin-antitoxin system.

An *in vitro* assay method using the gel diffusion technique and the supernatant of the flocculation test containing only toxin as antigen was devised.

The toxin was shown to be precipitated by demonstrating that toxin was recovered from the neutralized precipitates after inactivating the antitoxin with sodium bisulfite.

INTRODUCTION

Since Lamb (1904) first studied the precipitin reaction of *Indian cobra* venoms, several authors have attempted to find a reliable *in vitro* assay method for the potency of antivenin sera. Calmette and Massol (1909) suggested the possibility of assaying the potency of cobra antivenin sera by the precipitin reaction. However later Mallick (1935), by studies on the same venom, found this was not possible. Studies were also made on Ramon's flocculation test. Miyake (1952) found a good agreement between the potency of "Habu" (*Trimeresurus flavoviridis*) antivenin sera as assayed by the flocculation reaction and that as estimated in mice. However Schöttler (1952) failed to confirm this with *Bothrops jararaca* and *Crotalus terrificus* venoms, and Christensen (1953) could find no correlation between *in vivo* and *in vitro* units in his studies on *Bothrops arietans* and *Naja flava* venoms.

Unsuccessful attempts were also made to confirm this by Kaneko, Imai and Konzo (1954) and by the present authors using the venom of "Mamushi" (*Agkistrodon blomhoffii*), the only venomous snake in the main island of our country.

Therefore, further studies were made on the estimation of excess toxin and excess neutralizing antibodies in the supernatants of the flocculation reaction. When increasing concentrations of the crude venom were mixed with a constant amount

of the antiserum, the supernatant of the tube, next to that where the neutralization end-point was reached and which was shown to contain excess toxin by injection into mice, gave only one gel precipitation line against antiserum, although the crude toxin gave about ten gel precipitation lines against antivenin sera in Ouchterlony plates. The present studies deal with the identification of the precipitation line of the toxin-antitoxin system among the ten lines found and with a simple assay method for the potency of the antitoxin sera.

MATERIALS

1. *Antivenin sera*

Horses were highly immunized for about 5 months by injecting increasing amount of a mixture of "Mamushi" and "Habu" venoms. "Habu" venom was used as an adjuvant.

2. *"Mamushi" venom*

Preparations were obtained in liquid form and dried in a desiccator. The batches used were M 18 (intravenous mouse LD_{50}=0.028 mg) and M 29 (i.v. mouse LD_{50}=0.029 mg).

3. *In vivo assay of antivenin sera*

Aliquots of 1.0 ml of the venom solution of 2 to 3 LD_{50}, were added to a series of tubes containing 1.0 ml of varying dilutions of antivenin sera. The tubes were kept at room temperature for 1 hour and then 0.2 ml aliquots of each mixture were injected into the tail veins of mice. Mice were observed for 4 days. Intoxicated mice usually died within 2 days. The ED_{50} units of antisera were obtained by Reed and Muench's method (1938). No correction was made when the amount of toxin used was between 2 and 3 LD_{50}.

RESULTS

1. *Analytical studies on supernatants after flocculation*

As the "Mamushi" venom preparation used for hyperimmunization of horses in the present studies was pooled saliva of snakes and probably contained several antigenic substances in addition the venom, the neutralization end-point probably did not coincide with the most quickly flocculating tube ("indicator" tube) when varying amounts of the crude venom were added to a constant amount of the antiserum. This discrepancy can be explained as follows: if an antigen with most powerful antigenicity was a nontoxic substance and its antibodies were most abundant in the antiserum, flocculation of the system should appear most quickly at its optimal point. To reinvestigate the discrepancy between the neutralization end-point and the "indicator" tube of the flocculation reaction, the following experiments were performed.

Varying amounts (4.4, 6.6, 10.0, 15.0, 22.4, 36.0, 54.0, 91.0, 121.4 and 183.4 mg) of crude "Mamushi" venom preparation (Batch M 18), dissolved in 2.0 ml of saline, were added to a series of tubes (Nos. 1-10) containing 2.0 ml of "Mamushi" antivenin serum (Batch P 6). As a control, a tube containing 2.0 ml of serum and 2.0 ml of saline was also included. 2.0 ml samples from each tube were introduced into a series of empty tubes. Thus, two parallel experiments were set up. The first series was incubated in a water bath at 37°C for 8 hours with occasional shaking and the turbidity

measured. The time when the turbidity was first detected in each tube was recorded. After observation for 8 hours, two 0.2 ml aliquots of each reaction mixture were injected into the tail veins of two male mice (strain dd/O) of 4 weeks of age. The mice were observed for 4 days. The intoxicated mice usually died within 48 hours. The second series of tubes was incubated in a water bath at 37°C for 1 hour and then at 4°C for 96 hours. The tubes were centrifuged in the cold and the supernatants were tested for excess antigen and toxin. To test for excess antigen, each supernatant were mixed with 0.5 ml of antiserum. The tubes were incubated at 37°C for 1 hour and then over night at 4°C. To test for excess toxin two 0.2 ml aliquots of each supernatant were injected into the tail veins of two male mice as described above. Mice were also observed for 4 days. The results are presented in Table 1.

Table 1. Neutralization and Flocculation of the "Mamushi" venom with antivenin serum.

Tube No.			1	2	3	4	5	6	7	8	9	10
Antiserum (1:1) Batch P 6			1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Venom (Batch M 18) mg per ml			2.2	3.3	5.0	7.5	11.2	18.0	27.0	40.5	60.7	91.7
Neutralization	Tests on supernatants	Toxicity* test	0/2	0/2	0/2	0/2	2/2	2/2	2/2	2/2	2/2	2/2
		Antigen excess test	−	−	−	−	+	+	+	+	+	+
Flocculation	Kf in min.		−	−	250′	330′	330′	380′	30′	21′	43′	180′
	Toxicity*		0/2	0/2	0/2	0/2	2/2	2/2	2/2	2/2	2/2	2/2

* Numerators indicate number of mice died.
Denominators indicate number of mice injected.

As can be seen, tube No. 4 gave the end-point of neutralization. The supernatants of tubes No. 5-10 contained excess toxin together with excess antigen, though in tube No. 8 turbidity appeared most quickly.

The same results were obtained when different batches of antivenin sera were tested. From these results it was assumed that the supernatant of tube No. 5 might contain only one kind of antigen and that this antigen must be the toxin itself.

2. *Analytical studies on the "Mamushi" venom by the gel precipitation technique*

As the supernatant of tube No. 5 in Table 1 was expected to contain only toxin lethal to mice, an antigenic substance in the crude venom preparation, supernatants of all the tubes shown in Table 1 were tested against antivenin serum (Batch P 6) on Ouchterlony plates (Wilson *et al.*, 1954).

As shown in Fig. 1, 6 antigen wells were made along the upper line and 5 antiserum wells along the lower parallel line. To the leftmost antigen well was added 0.2 ml of the crude venom solution containing 100 mg of venom (Batch M 18) and in each of remaining antigen wells from left to right, 0.2 ml of the supernatants of tubes No. 3, 4, 5, 6 and 7. To each antiserum well, 0.2 ml of antivenin serum (Batch P 6) was added. The plate was incubated at 37°C for 5 days and then photographed (Fig. 1).

As can be seen, the supernatants (wells 3 and 4) of tubes No. 3 and 4 gave no precipitation lines. Those (wells 5 and 6) of tubes No. 5 and 6 gave only a single very

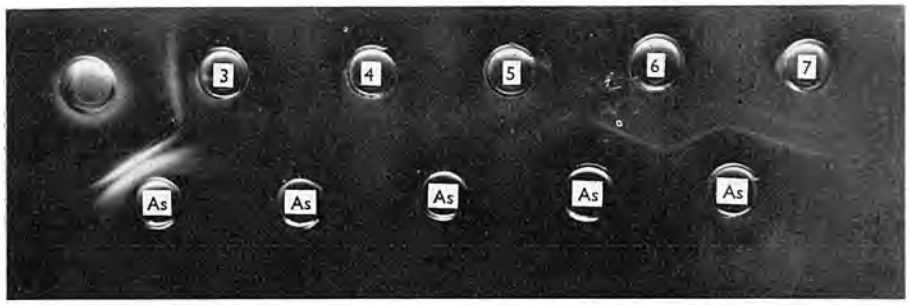

Fig. 1. Gel precipitation reactions between the flocculation supernatants and "Mamushi" antiserum.

faint line. That (well 6) of No. 6 gave two lines though the second one was very faint. No. 7 (well 7) gave three lines. These facts were in good accord with results obtained by analysis of the supernatants with regard to excess antigen and excess toxin. As the supernatant of tube No. 5 gave only one precipitation line and it had already been shown to contain excess toxin, it can be concluded that this was formed by the toxin-antitoxin system.

To confirm this, other batches of antivenin sera were studied using the same toxin (Batch M 18). Several series of precipitation tubes were set up using different batches of antiserum and the first toxin excess supernatants were obtained. Comparisons were made of the precipitation line given by each (well 2) of these supernatants against standard antiserum (Batch P 6, well As) with that produced between the supernatant of the tube No. 5 in Table 1 (well 1) and the well As.

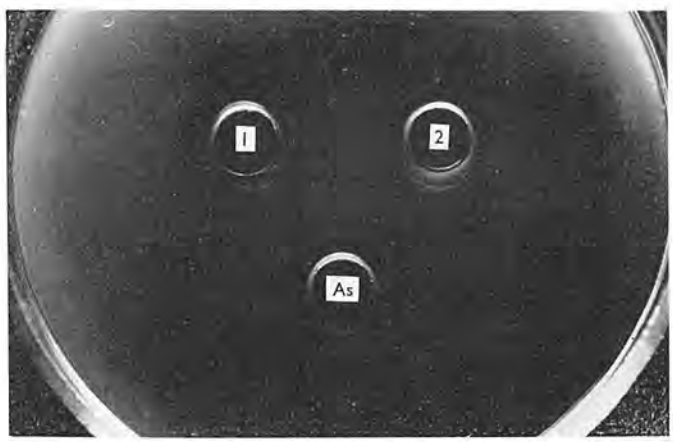

Fig. 2. Comparison of antigenicity of the flocculation supernatants containing only toxin.

An example of results is given in Fig. 2. The supernatant added to well 2 was prepared with venom and antiserum (Batch P 20). A single precipitation line was formed between each of antigen wells and the antiserum well (As). The two single lines fused with each other without forming a "spur".

Though different batches of venom were not studied in a same manner, the same results could be expected because the first antigen excess supernatants obtained in the experiments shown in Table 1 using different batches of venom preparation and antivenin sera, always contained excess toxin.

3. *Identification of the toxin-antitoxin line among the lines given by the crude venom preparation and its antiserum*

An investigation was made to identify the precipitation line due to the toxin-antitoxin system among the several lines given by the crude venom preparation and its antiserum.

As shown in Fig. 3, 0.2 ml of the antiserum (Batch P 6) were added to well A_1 and the same amount of the antiserum (Batch P 20) was added to well A_2. 0.2 ml aliquots of solution containing 10 mg of crude venom were added to the two wells V. To well 8 was added 0.2 ml of the supernatant of tube No. 5 shown in Table 1 and to well 9, 0.2 ml of the first toxin excess supernatant of the system of Batch P 20 antiserum and toxin (Batch M 18).

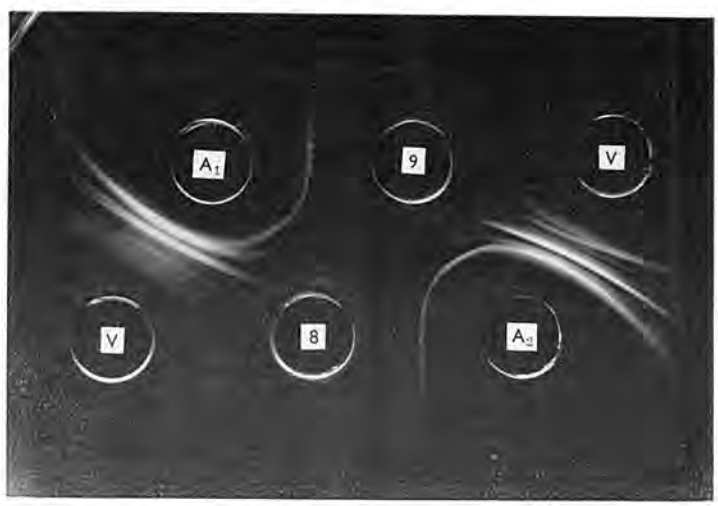

Fig. 3. Identification of the toxin-antitoxin line among the lines given by the venom preparation.

As can be seen from Fig. 3, about ten precipitation lines were given between wells V and each of the antiserum wells (A_1 and A_2). The antigen of wells 8 and 9 gave only one common precipitation line against the serum wells and the line fused with the line nearest to the serum well formed by the crude venom preparation. Thus the nearest line to the serum well, given between the crude venom preparation and the antisera, was formed by the toxin-antitoxin system. In addition, it can be assumed from the results that the concentration of the antitoxin was the lowest of the antibodies produced by the antigens in the crude venom, because the toxin-antitoxin line appeared nearest to the antiserum well and very little precipitate was formed in

the tubes No. 3 and 4 shown in Table 1.

The fact, that two or three lines were formed between well V and the first toxin excess supernatant (wells 8 and 9), indicated that the first toxin excess supernatant contained excess antibodies to nontoxic antigenic substances.

4. *A simple assay method for the potency of the antivenin sera*

Studies were made on the assay of the potency of the antivenin sera, using Ouchterlony plates. To the centre well was added 0.2 ml of the supernatant of tube No. 5 in Table 1 and to each of the six wells placed hexagonally around it were added 0.2 ml of various dilutions of antiserum. Fig. 4 shows results of one such experiment.

Fig. 4. Assay of the *in vitro* units of antiserum
Centre well, flocculation supernatant containing only toxin (0.2 ml).
well 10, 1:28 diluted antiserum (0.2 ml)
well 11, 1:32 diluted antiserum (0.2 ml)
well 12, 1:36 diluted antiserum (0.2 ml)
well 13, 1:40 diluted antiserum (0.2 ml)
well 14, 1:44 diluted antiserum (0.2 ml)
well 15, 1:48 diluted antiserum (0.2 ml)

0.2 ml of 1:28, 1:32, 1:36, 1:40, 1:44 and 1:48 dilutions of antiserum P 6 were added to wells 10, 11, 12, 13, 14 and 15, respectively. As can be seen from Fig. 4 the faintest line was given by the antigen of well 12. Thus the potency of serum P 6 was expressed as 36.

The potency of various batches of antisera were estimated in the same manner. The results, compared with the potency estimated by the *in vivo* assay method in mice, are shown in Table 2. The latter method is now adopted by the National Institute of Health of Japan. In the latter method, the ED_{50} units of antisera are measured using from 2 to 3 LD_{50} doses of each venom preparation without regard to the varying contents of "toxoid" in the preparations. Moreover the units thus obtained may fluctuate because, 1) there may be differences in the batches of toxin

Table 2. Comparison of the units assayed by the *in vivo* and *in vitro* methods on the same antisera.

Antitoxin	Units by *in vitro* assay	*In vitro/in vivo*	Units *in vivo* by assay	nLD_{50}*
Batch 1	18	1:26	467	2.94
2	32	1:23	727	
3	8	1:35	416	2.59
4	18	1:26	462	2.61
5	22	1:24	521	3.10
6	8	1:31	249	
7	8	1:38	301	

* Amount of toxin

used and 2) there may be fluctuations in the LD_{50} units in the toxin preparation caused by heterogenity in sensitivity of the mice, although the mice used in this assay are the best strain available in Japan. Fluctuations in the LD_{50} units of the toxin (Batch M 18) are also presented in the table. Therefore, no conclusion can be drawn as to which of the two methods is superior.

5. *Investigation of the precipitability of the toxin in the zone of complete neutralization*

As already mentioned, the first toxin excess supernatant gave a single precipitation line and it was concluded that the line was formed by the toxin-antitoxin system. However, if the toxin could not be precipitated by the antitoxin, the precipitation line might have been caused by another antigenic substance. Therefore the results presented in Table 1 had to be reconsidered.

As can be seen from Table 1, tubes No. 3 and 4 where the toxin was completely neutralized were turbid. However, the precipitate could not be assumed without proof to have been formed by the toxin-antitoxin system. To see whether the toxin was precipitated in the zone of complete neutralization, attempts were made of recovering the free toxin from the neutralized complexes. For this purpose it was necessary to find a protein denaturing agent which was selectively active to antibodies and not to toxin. After several unsuccessful attempts, sodium bisulfite was used.

First, the relative stability of the "Mamushi" venom preparation was studied.

Varying amounts (5, 25 and 50 mg) of sodium bisulfite, dissolved in 0.5 ml of saline, were pipetted into three tubes. 4.5 ml aliquots of a solution containing 50 mg of a crude venom preparation (Batch M 29) were added to each tube. The tubes were incubated at 20°C for 10 hours. 1.0 ml samples were removed at intervals (1, 3, 5, 7 and 10 hours) from the tubes and the remaining bisulfite was oxidized by shaking the tubes. The decrease of toxicity was assayed by estimating the amount corresponding to one mouse LD_{50} unit. The results are shown in Table 3.

It can be seen that sodium bisulfite in 1.0 per cent final concentration did not affect the toxin very much and half of the toxin remained after 10 hours. Therefore

Table 3. Effect of treatment of the venom with $NaHSO_3$.

Final concn. of $NaHSO_3$	Reaction time / pH	Amount of LD_{50} 1 hr	3 hr	5 hr	7 hr	10 hr	Toxin control
%		mg					
0.1	5.8	0.033	0.033	0.033	0.033	0.050	
0.5	5.4	0.033	0.033	0.050	0.050	0.050	0.029
1.0	5.2	0.033	0.050	0.067	0.067	0.067	

Venom: M 29 10 mg/ml.

a concentration of 1.0 per cent was used to inactivate the antitoxin.

Studies were made on the recovery of toxin from the supernatant and precipitates of the flocculation reaction by denaturing the antitoxin with one per cent bisulfite.

Varying amounts (15.4, 23.1, 35.0, 52.5, 78.4 and 126.0 mg) of venom preparation (Batch M 29), dissolved in 7.0 ml saline, were added to a series of large tubes (Nos. 1-6) containing 7.0 ml of antivenin serum (Batch P 6). The tubes were incubated at 37°C for 1 hour and then at 4°C for 96 hours. The reaction mixtures were centrifuged in the cold. The precipitates were washed three times with chilled saline and each suspended in 10.0 ml of saline. The pH of the suspensions was adjusted to 4.6 with dilute hydrochloric acid. After the precipitates had been dissolved and the pH of the tube contents had been readjusted to 5.2, the volume was adjusted to 14.4 ml. To each of the solutions 1.6 ml of 10 per cent sodium bisulfite solution were added. To 10 ml of each supernatant of the original reaction mixtures were added 0.8 ml of saline and 1.2 ml of 10 per cent sodium bisulfite solution. As a control, a tube containing 10.8 ml of saline together with 1.2 ml of 10 per cent sodium bisulfite solution was also included. Then the thirteen tubes were kept at room temperature (20°C) for three hours. After this the bisulfite was oxidized by aeration. After pH of the solutions had been adjusted to 7.0, the toxicity of the solutions was tested by injecting 0.2 ml aliquots of each solution into the tail veins of mice. The solution from the control tube had no harmful effect on mice. The results are presented in Table 4.

Table 4. Recovery of toxin from precipitates and supernatants after 3 hours treatment with $NaHSO_3$.

Tube No.	1	2	3	4	5	6
Antiserum (P 6) ml	7.0	7.0	7.0	7.0	7.0	7.0
Venom (M 29) mg	15.4	23.1	35.0	52.5	78.4	126.0
Toxicity of supernatant	0/2	0/2	0/2	0/2	2/2	2/2
Supernatant after 3 hours treatment with 1% $NaHSO_3$	0/2	1/2	2/2	2/2	2/2	2/2
Precipitates after 3 hours treatment with 1% $NaHSO_3$	0/2	1/2	2/2	2/2	2/2	2/2

It can be seen that the toxin could be recovered from the supernatants and precipitates of all the original reaction tubes except tube No. 1 though the yield was very low. These results show that the toxin was precipitated by the antitoxin and hence the precipitation line assumed to be formed by the toxin-antitoxin system described above was proved in fact to be so because the toxin was really precipitated.

DISCUSSION

The fact, that the neutralization end-point did not coincide with the most quickly flocculating tube ("indicator" tube) when varying amounts of crude venom were added to a constant amount of the antivenin serum, led us to make analytical studies on the flocculation reaction. On analysis of excess antigen and excess toxin in the supernatants of the tubes, a very interesting phenomenon was observed. In the supernatant of the tube next to that in which the neutralization end-point was achieved excess toxin and excess antigen were found and this tube was always the beginning of the series of tubes having excess antigen as well as excess toxin. Therefore the assumption was made that the excess antigen was the toxin itself. To prove this the supernatant was studied by the agar diffusion technique according to the method of Ouchterlony. It gave only one precipitation line when tested against antivenin sera. Therefore the above assumption was proved correct and the antigen found in the supernatant was shown to be the toxin itself. If the toxin had not been precipitated by antitoxin molecules at all, such an assumption would not be possible. However the toxin was proved to be precipitated by antitoxin molecules by the results of the last experiment. Thus it can be stated that the precipitation line between the supernatant and the antivenin serum was formed by the toxin-antitoxin system.

Of the 10 precipitation lines given between the crude venom preparation and antivenin serum, the line nearest to the antiserum well was found to be formed by the toxin-antitoxin system, because it fused with the single line of the toxin-antitoxin system without forming a "spur." There would seem to be two possible mechanisms to explain the fact that, of the 10 lines formed with the antitoxin preparation, only the one nearest the antitoxin well was due to a reaction with toxin. First, it could be that antitoxin is a minority component of the antivenin preparation or, perhaps the major component of the venom may be toxin itself. The latter explanation was incorrect, because such a concept cannot explain the scanty flocculation at the neutralization end-point in Table 1. If the latter explanation were correct, tube No. 4 in Table 1 should show the greatest and most rapid flocculation, so that a discordance would not have been observed between the neutralization end-point and the indicator tube of the flocculation.

The new *in vitro* assay method described in this paper seems to be useful, because the Ehrlich's method for *in vivo* assay of antitoxin has not yet been used for the assay of "Mamushi" antivenin sera. Fluctuations in the antivenin units, which were observed on testing from 2 to 3 LD_{50} units of every toxin preparation with dilutions of antisera to find the ED_{50} value of the antisera, are actually caused by different amounts of "toxoid" in the different batches of venom preparation. Even in one batch, after different periods of storage, the "toxoid" content may not be constant. In addition, it is indispensable to assay the amount of the venom corresponding to one LD_{50} unit. Even in the same batch of venom preparation, the estimated volume containing one LD_{50} unit may vary with heterogeneities in sensitivity of the mice

used, even when using the best strain in Japan. If one LD_{50} unit fluctuates, the antivenin titer fluctuates. Thus the present authors favour their new *in vitro* method to the *in vivo* ED_{50} method.

The enzymic nature of "Mamushi" venom is still unknown. Lecithinases and phosphodiesterase have been studied by us and no proof has been gained about the identity of these enzymes and mouse lethal toxin (Kubo *et al.*, 1960 a, b). Further purification will be made of the substance giving only one gel precipitation line with antivenin sera and forming a complete fusion with the line given by the first toxin excess supernatant and antivenin sera.

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