Mouse Foot Pad Test for Determination of Antiedema-forming and Antihemorrhagic Potencies of Snake Antivenom.

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Introduction

Two species of venomous snakes are a hazard on residents of Ryukyu Islands. One of them Habu (Trimeresurus flavoviridis) inhabits the main island of Okinawa and its neighboring off-island ; the other the Sakishima Habu (Trimeresurus elegans) inhabits the Yaeyama Islands, about 170 Km south of Okinawa Island.

Severe local lesions of swelling, hemorrhage and necrosis and fatal bites occurred not infrequently in Habubites whereas local lesion is less severe and fatal case is very rare in Sakishima Habu bites.

We had previously experimented on fractionation and characterization of the Sakishima Habu venom. From this experiment, we knew that the Sakishima Habu venom had many toxinological differences from that of Habu venom.

For the treatment of patients bitten by Sakishima Habu, Habu antivenom derived from horses hyperimmunized with Habu venom has been used. For the evalution of above facts, cross neutralization test of Habu and Sakishima Habu venom with its antivenom has carried out appling the mouse foot pad test also reported previously.

Another purpose of this stuy is establishment of a routine method for titration of antiedema forming and antihemorrhagic potency of Sakishima Habu antivenom.

Materials and Methods

1. Test toxins : a). The venom used was a dried and powdered pool of venom milkd from a species of Habu (T. flavoviridis) and Sakishima Habu (T. elegans) collected in Okinawa and Yaeyama Islands. These crude venom were used as test toxins for cross neutralization test with Habu and Sakishima Habu antivenoms.

b). Two fractions S_2 and S_3 separated from venom of Sakishima Habu by Sephadex G 150 column were used. Fraction S_2 which cause severe edema with intense hemorrhage in the mouse pad was used as test toxin for titration of anti-edema forming S_2 unit of antivenoms.

Fraction S_3 which show severe edema but little hemorrhage was also used as test toxin for titration of anti-edema forming S_3 unit of antivenoms.

2. Antivenoms : Nine lots of antivenoms were prepared from equine plasma hyperimmunized with crude Habu venom, crude Sakishima Habu venom and its fractions. Immunogen and protein content of each antivenom are shown in Table 1. These antivenoms were purified with salt -precipitate, digested with pepsin and lyophilized.

3. Methods : a). Mice in groups of five were given injection of 20 μl of a sample solution into right hind foot pads and saline into left foot pads. Four hours after injection, the mice were killed by inhalation with chloroform. Then both legs were cut off at the ankle joint with scissors and weighed in pool of legs.

Table 1. Immunogen and protein content of antivenoms.

Lot No:	Immunogen	Protein Content (mg/ml)	Year Prepared
Lot 17S	SC &S_3	75	1973
〃 18S*	SC	87	〃
〃 26S	〃	106	1979
〃 27S	S_2	75	〃
〃 28S	SC	90	〃
〃 31S	S_{3}-g	94	1980
〃 T81S	SC	32	1981
〃 12	HC	64	1974
〃 T78	〃	75	1978

HC: Crude Habu venom. SC: Crude Sakishima Habu venom.

S_2 & S_3: Fractionated from crude Sakishima-habu venom

S_3-g : purified from fraction S_3.

* Reference antivenom

The percentage of the weight of the injected legs to that of the healthy legs was calculated to express the severity of edema by

$$\text{Edema ratio (\%)} = \frac{\text{mg of (right) edematous legs}}{\text{mg of (left) healthy legs}} \times 100$$

b). On the other hand, mouse legs with hemorrhag were used to determine the extent of hemorrhagic activity by measuring hemoglobin content in the tissue.

First, mice legs were crushed in the porcelain mortar with 3.6 ml per leg of distilled water and this was passed through membrane filter (Millipore Co. Type HA) to make clear eluate of hemoglobin. Then, 3.6 ml of the hemoglobin solution thus obtained was allowed to react with 0.4 ml of potassium ferricyanide reagent.

The cyanmethohemoglobin content of a solution was estimated photometrically at 540 nm with distilled water as a control, and was calculated using the following formula;

$$\text{Hemoglobin content} = \frac{Es}{Est} \times C$$

where Es : Absorbency of sample solution.

Est: Absorbency of standard hemoglobin solution.

C : Hemoglobin content of standard solution.

In this method, commercial hemoglobin solution for clinical test was used as a standard which contained 2.864 mg/4 ml of hemoglobin.

c). Preparation of venom-antivenom mixture : One hundred test dose of each test toxins(containing 0.4 ml) was mixed with each serial dilutions of antivenom graded at 1.25 fold(for antihemorrhagic potency against crude venoms and anti S_2 units) or 1.6 fold(for antiedema potency against crude venom and anti-S_3 units) intervals as shown in Table 2 and Table 3. The mixture was kept at room temperature for about one hour, centrifuged at 3200 rpm for 10 minutes, and in volumes of 20 μl the right foot pads of mice were injected with supernatant flued. Four hours after injection, the edema ratio and hemoglobin content were estimated as mentioned above.

All of the experiment for determination of edema ratio and hemorrhage were repeated three times.

Table 2. Antivenom dilutions at 1.25 fold for estimation of antihemorrhagic potencies against crude venom and fraction S_2.

Tube No.	1	2	3	4	5
Dil. Antivenom	0.63	0.8	1.0	1.25	1.6
Test Toxin(100 TD)	0.4	0.4	0.4	0.4	0.4
PBS	0.97	0.8	0.6	0.35	0
Total	2.0	2.0	2.0	2.0	2.0

Table 3. Antivenom dilutions at 1.6 fold for estimation of atiedema potencies against crude venom and fraction S_3.

Tube No.	1	2	3	4	5
Dil. Antivenom	0.25	0.4	0.63	1.0	1.6
Test Toxin(100 TD)	0.4	0.4	0.4	0.4	0.4
PBS	1.35	1.2	0.97	0.6	0
Total	2.0	2.0	2.0	2.0	2.0

Results

A. Determination of Test Dose

1. Measurement of edema-forming activity of test toxins.

Mice, in groups of five, were injected with 20 μl of venom solution graded in 3.2 fold intervals. Four hours after injection, the edema ratio was measured as described previously. Tests for measurement of edema ratio were repeated three times. The average of data is plotted in Fig. 1.

The Minimum Edema Dose(MED) was defined as the least quantity of venom causing 130 % of the edema ratio. Thus, one MED of each test toxin can be read from Fig. 1.

There were little difference of edema forming activity between crude Habu venom(HC) with crude Sakishima Habu venom(SC).

Fraction S_2 which sparated from SC had highest activity in those venoms. Fraction S_3 which is also separated from SC showed lower swelling activity.

One Test Dose(TD) used for titration of the antiedema potency of antivenom are tabulated in Table 4

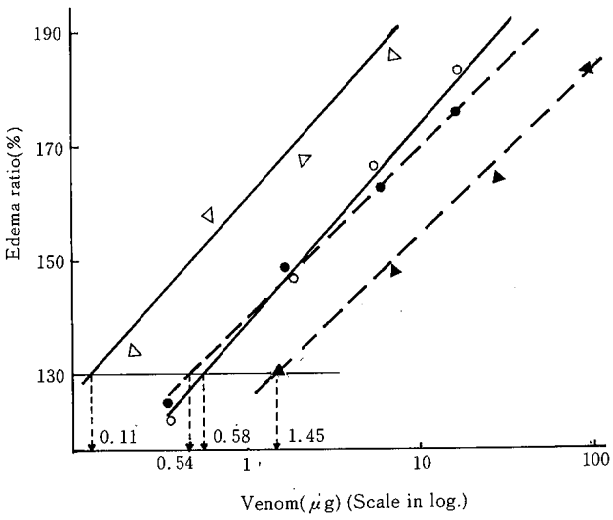

Fig. 1. Dosage-response curves of edema forming activities of test toxins. (See foot note of Table 1)
○—○ : HC, ●·····● : SC, △—△ : S_2, ▲·····▲ : S_3

Table 4. The Minimum Edema Dose and one Test Dose of test toxins.

Venom	MED	TD
HC	0.58 μg	10MED(5.8 μg)
SC	0.54 μg	10MED(5.4 μg)
S_2	0.11 μg	20MED(2.2 μg)
S_3	1.45 μg	8 MED(11.6 μg)

2. Measurement of hemorrhagic activity of test toxins.

The hemorrhagic activity of test toxin must be determined before assay of antihemorrhagic unit of antivenom by inoculation of venom into the foot pad of mouse.

The mouse leg with hemorrhage was used to determine the extent of hemorrhagic activity by extracting hemoglobin.

After having verified that the maximum photo-absobency of cyanmethohemoglobin was at 540nm, the hemoglobin content in tissue was then measured. (Fig. 2)

Mice, in groups of five, were injected with 20 μl of venom solution into the right hind-foot pads. The mouse legs with hemorrhage, caused by injection of venom were then cut off at the angle joint and crushed in porcelain mortar with 3.6 ml per leg of distilled water. The extracted fluid was passed through a membrane filter(Millipore Corp. HA filter) to produce clear eluate of hemoglobin. The hemoglobin content, thus obtained, from tissues of mouse legs was estimated photometrically at 540 nm by cyanmethohemoglobin method. Commercial hemoglobin solution(Wako Corp., Japan) for clinical tests was used as standard.

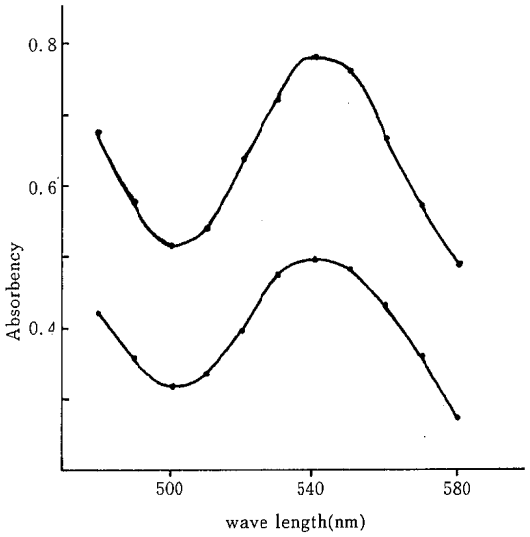

Fig. 2. Observation of maximum photo-absorbency of cyanmethohemoglobin.
▲ — ▲ : Sample hemoglobin solution which prepared from mouse leg with hemorrhage.
● — ● : Standard hemoglobin solution from horse.

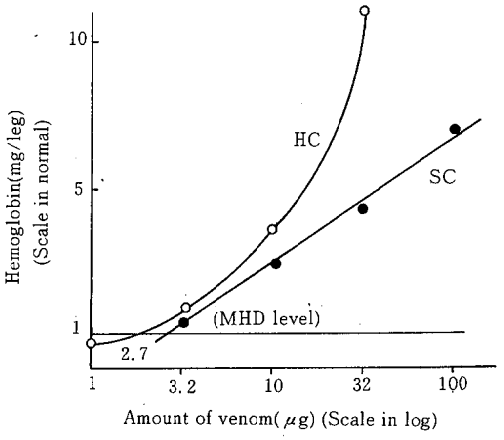

Fig. 3. Dosage response curves for hemorrhagic activity of crude Habu venom and crude Sakishima-habu venom.

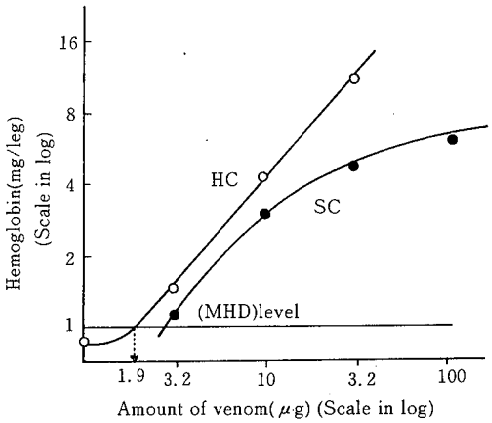

Fig. 4. Dosage response curves for hemorrhagic activity of crude Habu venom and crude Sakishima-habu venom.

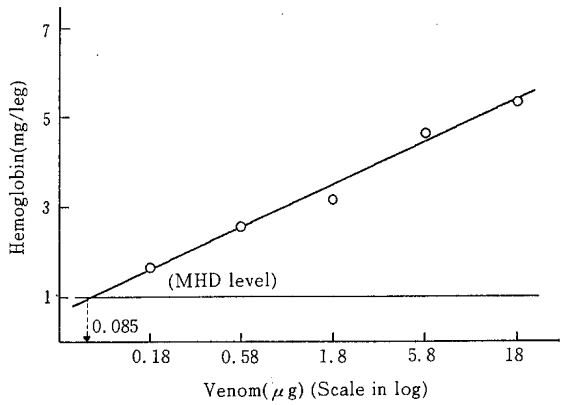

Fig. 5. Dosage-repponse curve of hemorrhagic activity of test toxin S_2.

The dosage-response curves at 4 hours observation between venom and hemoglobin content in mouse legs are shown in Fig. 3, Fig. 4 and Fig. 5. In Fig. 3, the hemoglobin content in a mouse leg against injected dose of crude Habu venom(HC) was increased in a logarithmic fashion. Whereas, crude Sakishima Habu venom(SC) showed nearly linearity between the log dose of the amount of venom injected and hemoglobin content.

The scale of hemoglobin content for crude Habu venom should be replaced by log scale so that the response curve is near linearity as shown in Fig. 4. Crude Sakishima Habu venom was not identifiable as linearity when hemoglobin content scaled in log.

In this experiment, the mice legs which injected with test toxin S_2 previously used for determination of edema forming effect were again used to determine the hemorrhagic activity, and the dosage response curve is shown in Fig. 5.

The Minimum Hemorrhagic Dose(MHD)is defined as minimal quantity of venom producing a release of one mg of hemoglobin into the tissue of mouse leg. Thus, one MHD of test toxins are readable in each corresponding figure. One Test Dose to titrate the antihemorrhagic potency of antivenom was arranged as indicated in Table 5.

Table 5. The Minimum Hemorrhagic Dose and one Test Dose of test toxins.

Venom	MHD	TD
HC	1.9 μg	10MHD(19 μg)
SC	2.7 μg	20MHD(54 μg)
S_2	0.085 μg	26MHD(2.2 μg)

B. Cross neutralization test of crude Habu venom and crude Sakishima Habu venom with its antivenom.

3. Test for antiedema effect of Habu and Sakishima Habu antivenom against crude venom(HC & SC).

Ten MED of crude Habu and Sakishima Habu venom were used as one test dose, and were mixed with each of serial dilutions of an antivenom. The preparations for titration of antiedema potency were prepared according to Table 3 and wes injected into the right foot pads of mice.

The percentage of the weight of the injected legs to that of the control legs was calculated as described previously. The results are shown in Fig. 6 and Fig. 7 by plotting of average data.

The Effective Dose(ED) was defined as the least quantity of antivenom neutralizing a test dose with 130 % of edema ratio as end point. Each ED of antivenom preparations shown in Table 6, were read from Figure 6 and Figure 7. Antiedema-forming units per 100 μl of antivenom was caluculated by

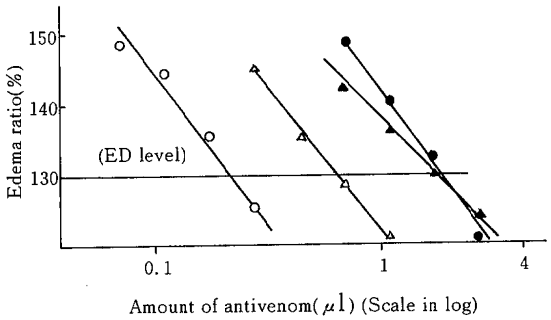

Fig. 6. Neutralization of antivenom preparations with crude Habu venom.
○—○ : Lot T78, △—△ : Lot 12, ●—● : Lot 26, ▲—▲ : Lot 17

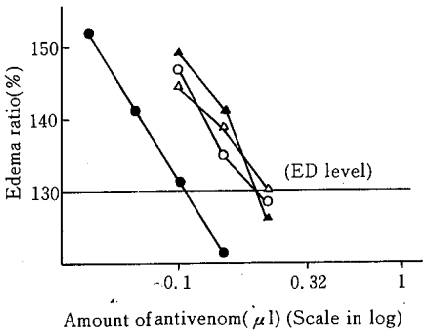

Fig. 7. Neutralization of antivenom preparations with crude Sakishima-habu. venom.(See foot note of Fig. 6)

100/ED.

According to the Table 6, there are marked difference of u/100 μl between Habu antivenoms(T78 & Lot 12) and Sakishima Habu antivenoms(Lot 26 & Lot 17) against HC. Sakishima Habu antivenoms whereas are more effective than Habu antivenoms against SC.

Table 6. Effective Dose and antiedemaforming units of antivenom preparations.

			vs HC		vs SC
			ED μl	u/100 μl	ED μl	u/100 μl
Lot	T78	0.195	513	0.22	455
Lot	12	0.58	172	0.23	435
Lot	26	1.7	59	0.11	909
Lot	17	1.6	63	0.21	476

4. Test for antihemorrhagic effect of Habu and Sakishima Habu antivenom against crude venom (HC & SC).

Ten MHD of crude Habu venom and twenty MHD of crude Sa ..ima Habu venom were used as one test dose. The venom-antivenom mixture were prepared as shown in Table 2 and was injected into the foot pads of mice. The hemoglobin content in mouse leg was estimated by the method as descibed previously. The results were indicated in Fig. 8 and Fig 9.

The Effective Dose(ED) is defined as minimal quantity of antivenom neutralizing a release of hemoglobin into the tissue of leg, caused by one TD of venom the end point of one mg of hemoglobin. The ED of each antivenom read from Fig. 8 and Fig. 9 were tabulated in Table 7 and antihemorrhagic units per 100 μl of antivenom was given by 100/ED. Habu antivenom gave almost equivalent potency of antihemorragic activity to neutralize with crude Sakishima Habu venom as compared with that of Sakishima Habu antivenom. However, Sakishima Habu antivenom had very poor neutralizing power against crude Habu venom.

This results showed that an antivenom gave a higher potency when titrated with a homologous venom than with a heterologous one.

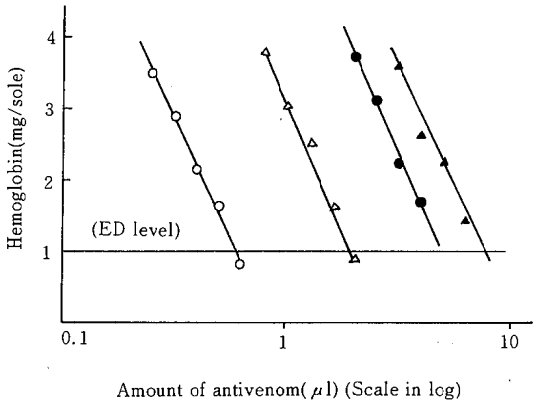

Fig. 8. Neutralization of antivenom preparations with crude Habu venom. ○—○ : Lot T78, △—△ : Lot 12, ●—● : Lot 26, ▲—▲ : Lot 17.

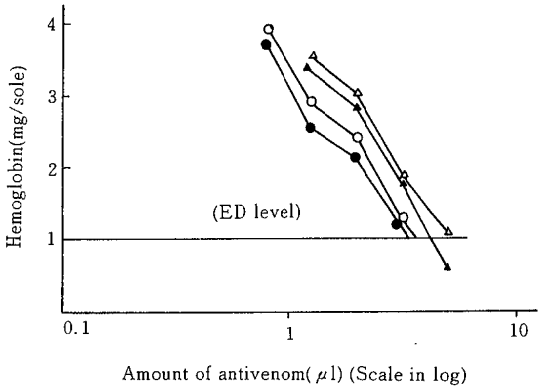

Fig. 9. Neutralization of antivenom preparations with crude Sakishima-habu venom. (See foot note of Fig. 8)

Table 7. Effective Dose and antihemorrhagic units of antivenom preparations.

			vs HC		vs SC	
		ED μl	u/100μl	ED μl	u/100μl
Lot	T78	0.58	172	3.9	26
Lot	12	1.99	50	5.1	20
Lot	26	5.0	20	3.2	31
Lot	17	7.7	13	4.2	24

C. Titration of potencies of antivenom using test toxin S_2 and S_3.

5. Determinatian of antiedema-S_2 units.

The preparation for titration of antiedema-S_2 potency is carried out by method 3 c) and Table 2. Tests were repeated three times. Recognizably there is linearity and parallelism between log dose of each antivenom and edema ratio.

The Effective Dose(ED) was defined as the least quantity of antivenom neutralizing a test dose with 130 % of edema ratio as end point. Each corresponding ED of antivenom is graphically readable from ED level in Fig. 10. Fig. 10 shows only the four lots of antivenoms, all the ED of antivenoms are revealed in Table 8. (Although ED is obtainable by statistical calculation, the graphic method is simpler.)

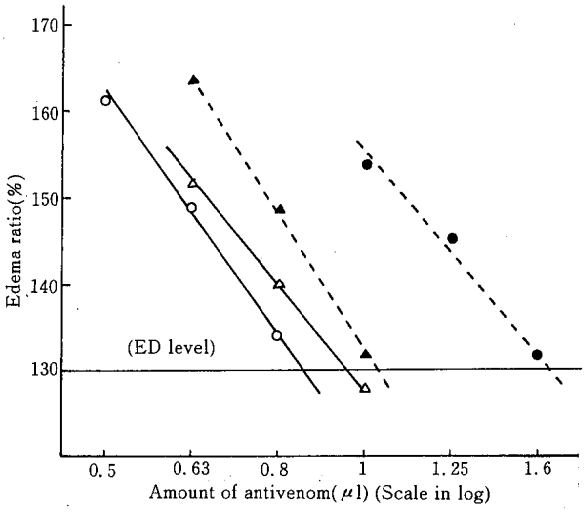

Fig. 10. Neutralizdtion curves of antivenoms with test toxin S_2 for antiedema potency.
○—○ : Lot 26S, ●···● : Lot 17S, △—△ : Lot 18S, ▲···▲ : Lot 27S.

Table 8. Antiedema-S_2 units expressed by Relative potency and Specific Activity of antivenom preparations.

Antivenom	Protein Content (mg/ml)	Effective Dose (μl)	Relative Potency (u/ml)	Specific Activity
Lot 56S	106	0.86	(100)	1.
Lot 17S	75	1.7	51	0.721
Lot 18S	87	0.96	90	1.145
Lot 27S	75	1.05	82	1.177
Lot 28S	90	1.16	74	0.954
Lot 31S	94	ND	ND	ND
Lot T81S	32	1.08	80	2.651

ND: Nondetectable level.

The Relative Potency of each antivenom preparation is expressed after providing an arbitrary 100 units/ml to Lot 26S. Lot 26S are adopted as the reference antivenom. The Specific Activity is calculated from units/mg of antivenom, based on the assumption that units per one mg of reference antivenom, Lot 26S, are 1.0.

In Table 8, the antiedema-S_2 units of antivenom, Lot 31S, are at nondetectable levels. The ND was caused by use of $S_{3\text{-}8}$, a different immunogen. The antiedema-S_2 units of Lot 27S are far from

satisfactory although immunized with fraction S_2. The antivenom Lot T81S, provided for clinical examination, has the highest specific activity among the antivenoms.

6. Determination of antiedema-S_3 units.

The preparation of venom-antivenom mixture for estimation of antiedema-S_3 units was carried out as shown in Table 3.

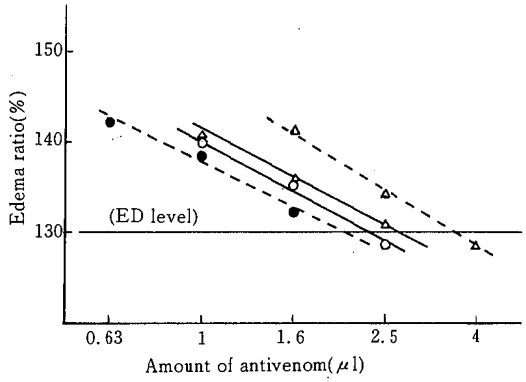

Fig. 11. Neutralization curves of antivenoms with test toxin S_3 for antiedema potency.
○—○ : Lot 26S, ●···● : Lot 17S, △—△ : Lot 18S, ▲···▲ : Lot 31S.

Table 9. Antiedema-S_3 units expressed by Relative potency and specific Activity of antivenom preparations.

Antivenom	Protein Content (mg/ml)	Effective Dose (μl)	Relative Potency (u/ml)	Specific Activity
Lot 26S	106	2.3	(100)	1
〃 17S	75	1.6	144	2.035
〃 18S	87	2.6	88	1.072
〃 27S	75	ND	ND	ND
〃 28S	90	2.2	105	1.237
〃 31S	94	3.3	70	0.789
〃 T81S	32	2.2	104	3.445

ND: Nondetectabla level.

The linearity and parallelism between log dose of each antivenom and edema ratio are also recognizable as shown in Fig. 11 and each ED of antivenoms was obtained by reading. Antivenom Lot 26S, addopted as reference antivenom, is provided an arbitrary 100 units/ml. The relative potency of antivenom preparations against reference antivenom Lot 26S was calculated from the various Effective Doses as the antiedema-S_3 units. In Table 9, the antivenom Lot 31S shows poor efficacy against test toxin S_3. Antivenom Lot T81S has about 3 times the specific activity than Lot 26S.

7. Determination of antihemorrhagic-S_2 units.

The same mice legs used previously estimate antiedema-S_2 potencies were again utilized for titration of antihemorrhagic potency of antivenom. The hemoglobin content in tissue was estimated using the method as previously described. Portions of those data were plotted in Fig. 12 as the neutralizing curves of antivenoms. These lines almost mutually corresponded in their linearity and parallelism.

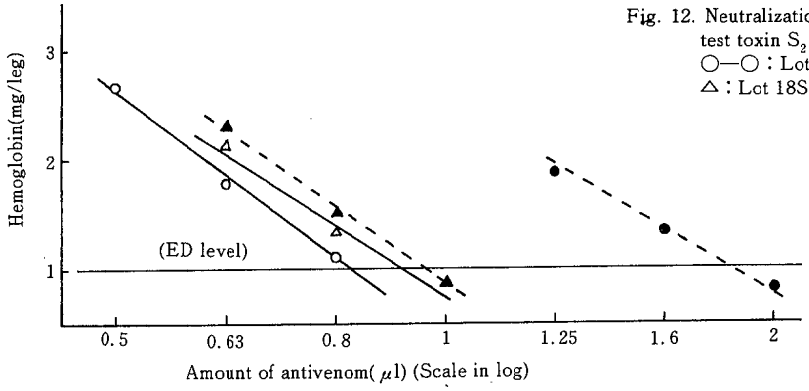

Fig. 12. Neutralization curves of antivenoms with test toxin S_2 for antihemorrhagic potency. ○—○ : Lot 26S, ●···● : Lot 17S, △—△ : Lot 18S, ▲···▲ : Lot 27S.

Table 10. Anti-hemorrhagic-S_2 units expressed by Relative Potency and Specific Activity of antivenom preparations.

Antivenom		Protein Content (mg/ml)	Effective Dose (μl) Relative Ptency (u/ml)	Specific Activity	
Lot	26S	106	0.83	(100)	1
〃	17S	75	1.71	49	0.692
〃	18S	87	0.91	91	1.109
〃	27S	75	0.95	87	1.230
〃	28S	90	1.05	79	0.931
〃	31S	94	ND	ND	ND
〃	T81S	32	1.02	81	2.684

ND: NT etectable level.

The Effective Dose(ED) is defined as minimal quantity of antivenom neutralizing a release of hemoglobin into the tissue of leg, caused by one TD of venom the end point of one mg of hemoglobin.

The ED of these antivenoms could be read from ED level in Fig. 12 and were tabulated in Table 10. The relative potency of antivenom against reference antivenom Lot 26S giving 100 units/ml arbitrarily is expressed as antihemorrhagic-S_2 units. Antivenom Lot 17S against S_2 showed poor effective level. Lot 31S was ND. Antivenom Lot T81S by specific activity is comparatively determined as best of the lot.

The result of this neutralizing test is almost similar with that of antiedema-S_2 potency, shown in Table 8, because the same measurement material was used.

Discussion

The expression of toxicity of snake venom in terms of lethal activity only is not sufficient, because the lethality of venom is the final consequence of the complicated reactions of various toxic components. The toxicity of snake venom should be expressed, therefore, not only in lethal activity but also in local effects, for instance, hemorragic, edema-forming and necrotizing activities.

We have previously proposed the quantitative methods for the determination of edema-forming and hemorrhagic activities of Habu and Sakishima Habu venom by injecting the venom into the foot pads of

mice. An attempt has ben made to apply these methods for the assay of antiedema-forming and antihemorrhagic potencies of snake antivenoms.

By the method of mouse foot pad test, there were little difference of edema forming activity between crude Habu venom with crude Sakishima Habu venom. Crude Habu venom showed intensive hemorrhagic effect into the mouse leg, while hemorrhagic activity of crudeSakishima Habu venom was insignificant as compared with that of crude Habu venom.

The results of cross neutralization tests of Habu and Sakishima Habu venom with its antivenoms showed that Habu and Sakishima Habu antivenoms were more effective to neutralize homologous venom than heterologous venom.

Crude Sakishima Habu venom was partialy neutralized by Habu antivenom, however, Sakishima Habu antivenom has very poor neutralizing power against crude Habu venom.

From these results, we concluded that homologous antivenom should be used in the treatment of Sakishima Habu bites, but Habu antivenom still could be used when Sakishima Habu antivenom could not be available.

In previous report, it was indicated that the Sakishima Habu venom could separated into three lethal fractions by gel filtration method ; S_1 , S_2 and S_3 of which the recovery ratio is 1 : 4 : 12. Fraction S_2 showed the main hemorrhagic activity with severe edema forming effect.

Anti-edema and anti-hemorrhagic potency of seven lots of Sakishima Habu antivenoms was determined by mouse pad test, using test toxins(S_2 and S_3) which were separated from the venom as main toxic principles of edema and hemorrhage. The test doses of S_2 and S_3 used for edema were decided as 20 MED(2.2 μg) and 8 MED(11.6 μg) respectively, whereas 26 MHD(2.2 μg) was the test dose of S_2 for hemorrhage.

Minimum effective dose(ED) of each antivenom for S_2 and S_3 were summarized in Table 8, Table 9 and Table 10 which were calculated from the neutralization curves shown in Fig. 9, Fig. 10 and Fig. 11. The effective dose of each antivenom varied according to the method of preparation. Thus, highest specific activity of anti-edema(S_2 and S_3) and anti-hemorrhagic potency was found in Lot T81S antivenom which was immunized with crude venom, and purified repeatedly by ammonium sulphate, peptic digestion, and zinc chloride. From potencies of antivenoms of Lot 27S and Lot 17S, it is suggested that the antigenicity of S_2 is better than that of S_3. These results suggested that the neutralizing potency of the antivenom against main toxic components of the vonom could be determined by the new method described above.

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マウス足蹠注射法による蛇毒抗毒素の抗腫脹、抗出血作用の定量的研究

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ま と め

1．マウス足蹠内注射法によってハブ粗毒及びサキシマハブ粗毒の腫脹活性及び出血活性を定量した。
2．この方法を利用して各抗毒素製品の抗腫脹、抗出血作用を測定し比較した。
3．その結果、ハブ粗毒とサキシマハブ粗毒の腫脹活性はほぼ同程度であった。
4．ハブ粗毒の出血活性はサキシマハブ粗毒に比べて著しく強かった。
5．ハブ抗毒素及びサキシマハブ抗毒素の抗腫脹作用及び抗出血作用は同種毒に対する中和効果の方が異種毒に対するより優れていた。
6．しかし、ハブ抗毒素がサキシマハブ粗毒を中和する効果の方がサキシマハブ抗毒素がハブ粗毒を中和する効果より優れていた。
7．各種抗毒素製品の抗S_2（腫脹）価及び抗S_2（出血）価の測定を試み、同抗毒素間の力価を比較することができた。
8．抗S_3（致死）価が測定レベル以下の抗毒素でも抗S_3（腫脹）価を測定することができた。
9．足蹠出血法による抗毒素の抗出血価とウサギ背皮法による抗出血価はほぼ一致していた。
10．ハブ毒に対して高い中和能を有するハブ抗毒素 Lot T 78 は標準サキシマハグ抗毒素 Lot 26 S の約60％程度の中和力しか示さなかった。