(Short Communication)

MOUSE FOOT PAD TEST FOR DETERMINATION OF HEMORRHAGIC ACTIVITY OF SNAKE VENOM

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The local effects of snake venoms, especially Crotalidae and Viperidae venoms, are often expressed by the extent of local bleeding, swelling and necrosis.

Several authors have tried to develop a method for determining local inflammatory activity. For instance, MINTON(1956) tried to measure the "local necrotizing effect" of various kinds of venoms by intradermal injection into the shaved belly skin of guinea pigs. JUST et al(1970) and T'ABORSKA(1971) reported the method for determining hemorrhagic activity by using radioactive isotope of ^{51}Cr-labeled erythrocytes which were administered in the blood vessel of animals, before venom injection. BONTA et al(1970) developed another method emploing dog open-chest preparations. KONDO et al(1960) devised a quantitative method for determining hemorrhagic activity by injecting snake venom into the clipped back skin of rabbits.

YAMAKAWA et al(1976) had previously proposed the quantitative method for determining edema-forming activity of Trimeresurus flavoviridis and Trimeresurus elegans venoms by injecting the venom into the hindfoot pads of mice. In this experiment, the mouse legs with edema changed to dark red due to internal bleeding.

We propose the usage of this phenomenon applying the quantitative method for determining the hemorrhagic activity of snake venom by extracting hemoglobin from mouse legs.

Six kinds of snake venom were used as test toxins. One, pooled lyophilized Mamushi(Agkistrodon halys blomhoffii) venom, was providid by Okinawa Branch of the Japan Snake Center.

Five, were milked in our laboratory from the following snakes ; Trimeresurus flavoviridis(Habu), Trimeresurus elegans(Sakishima Habu), Trimeresurus Tokalensis(Tokala Habu), Trimeresurus Okinavensis(Hime Habu) and Trimeresurus mucrosquamatus(Taiwan Habu).

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.

By time observation, the venom-caused hemorrhage in mouse lag reached a maximum about 2–4 hours after injection. It turned toward gradual recovery after 4–6 hours as shown in Fig. 1. From this observation, 2–4 hours are required for adaption in estimating hemorrhagic activity.

The dosage-response curves at 4 hours observation between venom and hemoglobin content in mouse legs are shown in Fig. 2. This proved that there are characteristic patterns of dosage-response curves.

For instance, the hemoglobin content in mouse leg against injected dose of Habu venom increased

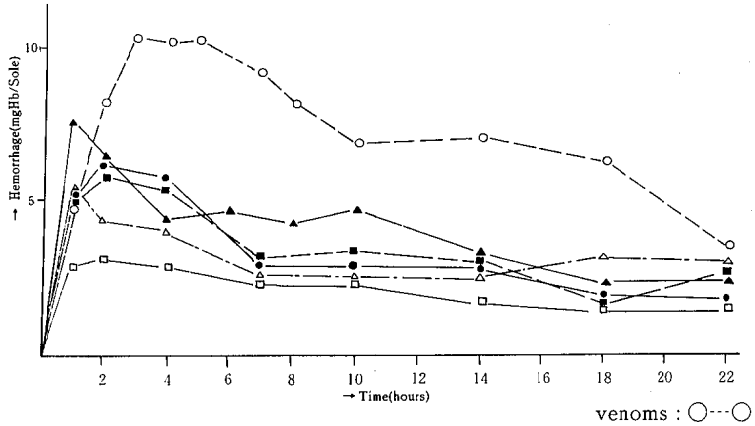

Fig. 1. Time course curves of hemorrhage caused by venom injected into mouse pads.

The mice were killed at times indicated in figure. Each injected legs were then cut off at angle joint and hemoglobin content measured in five pooled legs. Snake venoms : ○···○ ; T. flavoviridis (Habu) 30 μg, ●—● ; T. mucrosquamatus (Taiwan Habu) 30 μg, △···△ ; A, h. blomhoffii (Mamushi) 10 μg, ▲—▲ ; T. elegans (Sakishima Habu) 100 μg, □ — □ ; T. tokalensis (Tokala Habu) 100 μg, ■···■ ; T. okinavensis (Hime Habu) 30 μg.

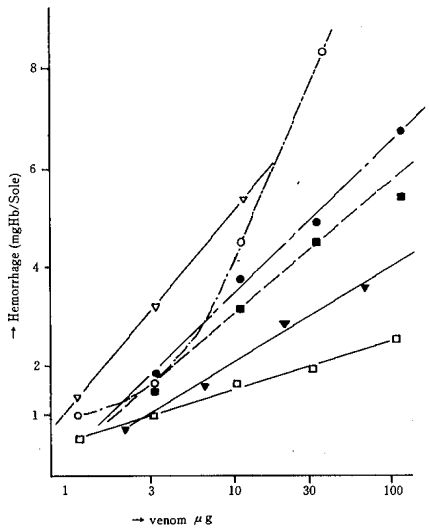

Fig. 2. Dosage-response curves of each snake venom.

Four hours after venom injection, the mice legs were individually crushed with 3.6 ml of water. Then, hemoglobin fluid was extracted from each mouse leg. Thus obtained, the 1.8 ml of hemoglobin solution was allowed to react with 0.2ml of potassium ferricyanide reagent, resulting in the estimated cyanmethohemoglobin content in solution. Snake venoms : See foot note of Fig. 1.

in a logarithmic fashion. The scale of hemoglobin content for Habu venom only should be replaced by log scale so that the response curve is near linearity as shown in Fig. 3.

The other venoms showed near linearity with individual charactersic slopes between log doses of venom and hemoglobin.

The dosage-response curve of Mamushi venom showed a comparatively higher slope. The venom of Tokala Habu and Sakishima Habu showed very low slopes.

Using this method, the hemorrhagic activity of Habu and Sakishima Habu venoms were compared with their partially purified venoms.

In Fig. 3, it was also noted that each fractionated venom had different slopes of dosage-response curves from that of crude venom, comparable to other crude snake venoms in Fig. 2. There was no parallelism in crude Habu venom with the fractionated venoms.

On the other hand, a parallelism was recognizable in crude Sakishima Habu venom and fraction S_2 because fraction S_2 possessed the main hemorrhagic activity of the crude Sakishima Habu venom as shown in Fig. 4.

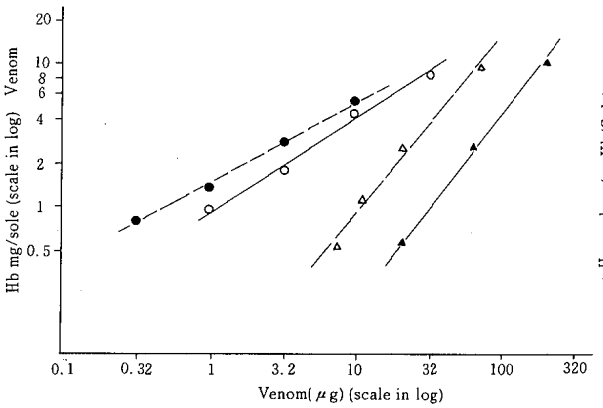

Fig. 3. Dosage response curves of crude Habu venom and its fractionated venoms at 4 hours observation.

The crude Habu venom (○－○) solution was passed through a Sephadex G 100 column to separate two fractions, HR 1 (●···●) and crude HR 2 (△－△). Then, crude HR 2 was futher separated by Amberlite CG 50 column chromatography into HR 2 and H_{2-0} (▲···▲) fractions.

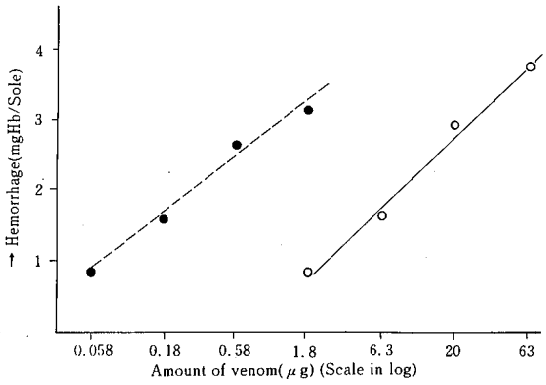

Fig. 4. Dosage response curves of Sakishima Habu venom and its fractionated venom at 4 hours observation.

Fraction S_2 (●···●) was separated from crude Sakishima Habu venom (○－○) by Sephadex G 150 column gel filtration.

For study of hemorrhagic actvity, there is the KONDO method using rabbit skin with higher sensitivity. Other methods, such as MINTON, MITSUHASHI et al(1959), and TABORSKA used animal skin.

Our method is an experiment in hemorrhagic activity without using animal skin for reasons of toxicity of snake venom and venom fractions exhibit different affinity against various tissues.

This method is convenient for the study in relationship between edema and hemorrhage by concomitant use of the method for determining edema forming activity (YAMAKAWA et al, 1976). For instance, it proved that there is no significant difference in edema forming activity between Habu and Sakishima Habu venoms in spite of marked differences in hemorrhagic activity between these two venoms.

Swelling by snake venom developed quite early and reached the maximum in about 30–60 minutes after mouse-pad injection. However, it took about 2–4 hours to reach maximal amount of hemorrhage in mouse leg.

We hope that this method will be employed to other snake venom as a simplified model for the study of snake envenomation.

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マウス足蹠注射法による蛇毒の出血活性の比較

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ま と め

1．サキシマハブ粗毒と他の5種類の蛇毒との出血活性を比較した結果、ハブ毒の出血活性が著しく強く次いでマムシ毒が強い出血活性を示した。サキシマハブ毒の出血活性はトカラハブ毒と共に可成り弱いことが認められた。

2．各蛇毒の注射毒量と出血量との関係を示す用量反応曲線は各々蛇毒特有のスロープが認められ

た。特にハブ粗毒の出血作用は対数的に増加するのに対してサキシマハブ粗毒はゆるやかなスロープを有する直線であった。

3．本法によるハブ粗毒とその分画毒の用量反応曲線は直線性は認められるが平行性は否定された。即ち、各分画毒にも固有のスロープがある。

4．サキシマハブ粗毒とその分画毒S_2の用量反応曲線は相互に平行でありかつ直線関係が認められた。

これはサキシマハブ粗毒の殆どの出血活性がS_2画分に含まれているからであると思われる。