Mem. Inst. Butantan
Simp. Internac.
33(1):331-337, 1966

37. PROBLEMS IN DETERMINATION OF ANTIHEMORRHAGIC POTENCY OF HABU (*TRIMERESURUS FLAVOVIRIDIS*) ANTIVENINE IN THE PRESENCE OF MULTIPLE HEMORRHAGIC PRINCIPLES AND THEIR ANTIBODIES

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Introduction

We demonstrated the presence of two hemorrhagic principles, HR1 and HR2 (1), in the venom of *Trimeresurus flavoviridis*, which is called "Habu" in Japanese. The two principles are not distinguishable by hemorrhagic action on the rabbit skin (2-4) but are distinguishable immunologically from each other (5). Consequently, preparations of Habu antivenine contain the corresponding two antibodies, anti-HR1 and anti-HR2, in varying proportions (5).

Should the potency of such an antivenine be titrated against the test toxin containing the two hemorrhagic principles, we must ask what the result of the titration indicates, the potency of exclusively anti-HR1 or anti-HR2, or some other implication (3, 5).

Recently we proposed a model for the mechanism involved in titration of the antihemorrhagic potency of an antivenine containing two antibodies with a test toxin containing two corresponding hemorrhagic principles (5).

The purpose of this presentation is to discuss the general implications of our model in standardizing antivenines and also bacterial antitoxins.

Definition of the terms

The minimum hemorrhagic dose (MHD) of venom is defined as the least quantity of venom in μg causing a hemorrhagic spot of 10 mm in diameter 24 hr after intracutaneous injection into the rabbits (2).

Effective dose (ED) is defined as a quantity of an antivenine in ml which, when mixed with a given dose of a test toxin, makes a mixture producing a hemorrhagic spot of 10 mm in diameter when 0.2 ml is injected intracutaneously into the rabbit (5).

One unit of antihemorrhagic activity is defined as the amount of antivenine containing one ED at the level of 100 MHD (5). The antihemorrhagic potency of an antivenine is expressed as units per ml.

The design for the determination of effective dose (ED) of an antivenine by the rabbit skin test

Table 1 shows an example of the design for determination of effective doses (EDs) of an antivenine by the rabbit skin test (5).

TABLE 1 — AN EXAMPLE OF THE DESIGN FOR DETERMINATION ON EFFECTIVE DOSES (EDs) OF AN ANTIVENINE BY THE RABBIT SKIN TEST

Test toxin : Crude venom (batch No. 48)
Antivenine preparation : Antivenine No. 23

Hemorrhagic activity of test toxin in MHD*	Rabbit No.	Cross-diameters of hemorrhagic spot in mm against varying amount of antivenine					
		0.00063	0.00050	0.00040	0.00032	0.00025	0.00020 ml*
30	1556	±	12 x 12	12 x 13	14 x 13	14 x 15	16 x 15
	1557	±	12 x 13	13 x 13	13 x 15	15 x 16	18 x 16
	1559	–	±	15 x 13	16 x 16	15 x 17	17 x 16
		0.00200	0.00160	0.00125	0.00100	0.00080	0.00063 ml*
100	1556	±	13 x 15	15 x 15	16 x 16	16 x 18	18 x 18
	1557	±	±	11 x 11	13 x 14	16 x 16	18 x 19
	1559	±	11 x 11	15 x 14	17 x 18	19 x 20	20 x 21
		0.00630	0.00500	0.00400	0.00320	0.00250	0.00200 ml*
300	1556	±	13 x 15	15 x 15	15 x 16	17 x 19	18 x 19
	1557	±	±	11 x 11	14 x 15	17 x 18	19 x 21
	1559	±	11 x 11	15 x 15	17 x 17	19 x 19	20 x 23

* Per injected dose

Test toxin solutions containing 30, 100 or 300 MHD per 0.1 ml were prepared. An aliquot from each of these solutions was mixed with the equal volume of each of serial dilutions of the antivenine graded with 1.25-fold intervals. The mixtures were kept standing for 1 hr at room temperature. Each was injected into the depilated back skin of three rabbits at a dose of 0.2 ml and hemorrhage developed was measured after 24 hr from the visceral side of the removed skin as described in the literature.

The ED of the antivenine against each level of test toxin was expressed in the volume of the antivenine reducing the size of the hemorrhagic spot to 10 mm in diameter, when 0.2 ml of the venom-antivenine mixture was injected into the rabbit. In practice, the amount of antivenine in mixture causing a hemorrhagic spot of 10 mm in diameter was determined by interpolation.

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Neutralization curves of antivenines with the crude venom or the partially purified hemorrhagic principles

Effective doses (EDs) of several antivenines were determined with a crude venom (batch No. 48), HR1 and HR2 as test toxins (5). The results were plotted to give the neutralization curves shown in Fig. 1 where the ordinate is ED of the antivenines and the abscissa hemorrhagic activity of the test toxins in MHD, both in logarithmic scale.

Statistical analyses proved that all the neutralization curves obtained with the crude venom as well as the venom fractions (HR1 and HR2) are linear and parallel to each other. This was shown also with a number of other antivenines. The common slope (b) of the neutralization curves was 1.12. (5)

Fig. 1 shows that: 1) the amount of each antivenine required to neutralize a given MHD dose of HR2 was larger than that needed to neutralize an equal dose of HR1; 2) the ED of the three antivenines varied depending on the test toxin used. The ED of antivenine No. 23 was the largest against HR2 and the smallest against HR1 among the three antivenines tested. This situation was reversed with antivenine No. 7. The ED of antivenine No. 9 was between those

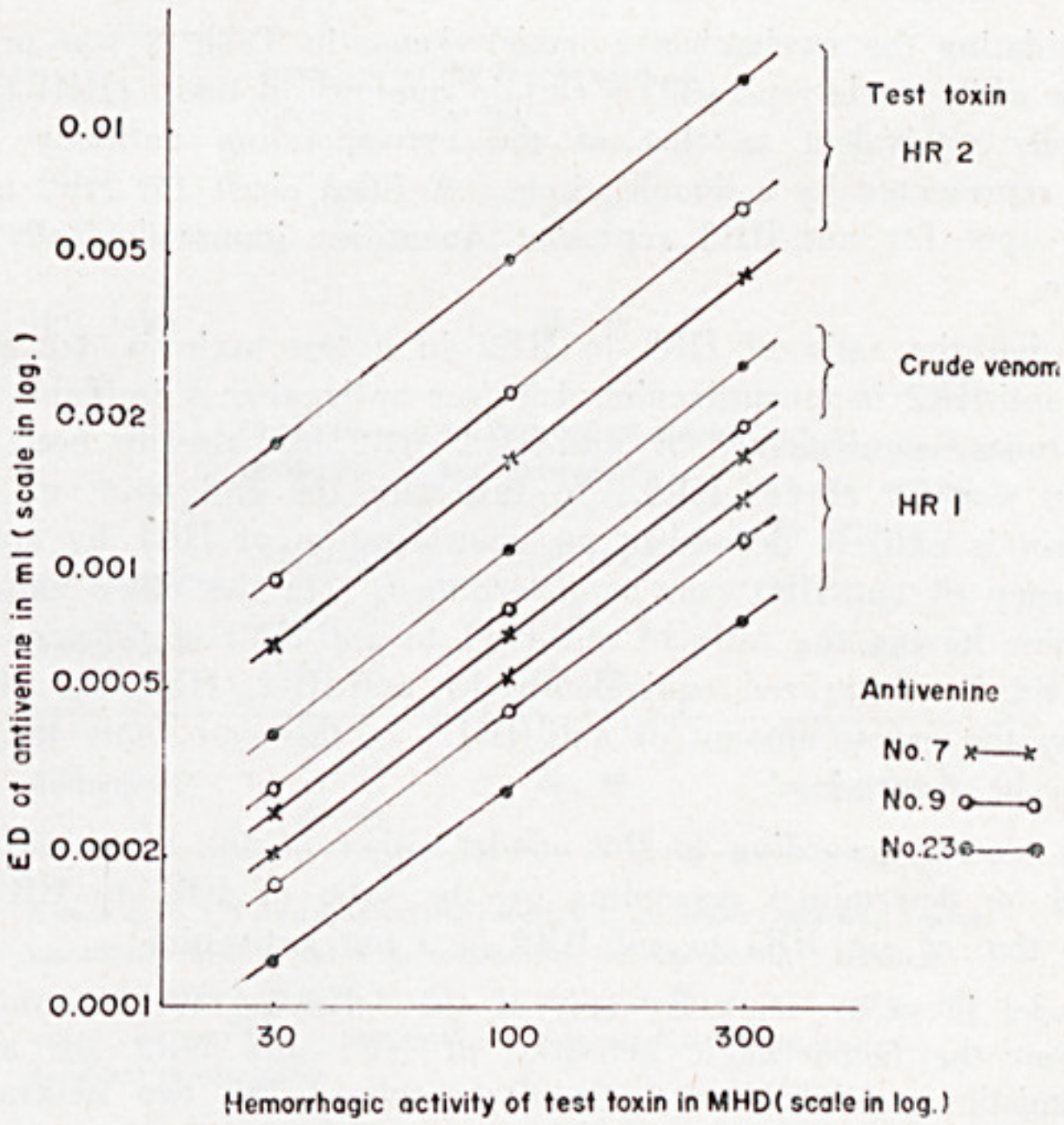

Fig. 1 — Neutralization of antivenine against three test toxins as determined by skin test. Test toxin: Crude venom (batch No. 48) and venom fractions (HR1 and HR2).

of the other two antivenines against either HR1 or HR2; 3) the EDs of the three antivenines against HR2 and against the crude venom were in the same order.

From these results we (5) postulate: 1) that HR1 and HR2 are not distinguishable in hemorrhagic action on the rabbit skin but are distinct immunologically from each other, since the amounts of an antivenine required to neutralize the two principles of an equal MHD dose were different; 2) that each antivenine contains two distinct antibodies corresponding to each of the two hemorrhagic principles; 3) that the ratio of the quantity of the two antibodies differed from one antivenine to another.

Should the potency of such an antivenine be titrated against the test toxin containing the two hemorrhagic principles, the question arises as to what the results of such titration indicate, the potency of only anti-HR1 or anti-HR2, or some other implication.

This question can not be answered unless the mechanism involved in titration of the antivenine potency in the presence of two hemorrhagic principles and the corresponding antibodies is elucidated.

Mechanism involved in titration of antivenine potency in the presence of two hemorrhagic principles and their antibodies

For elucidating the mechanism a model shown in Table 2 was proposed (5). In the table a white circle represents a certain quantity of toxin (HHR1) molecules immunologically equivalent to that of the corresponding antibody (anti-HR1) moleculos as represented by a double circle. A filled circle for HR2 and a circle with a dark spot for anti-HR2 represent quantities immunologically equivalent to each other.

Assume that the ratio of HR1 to HR2 in a test toxin is 4:2 and that of anti-HR1 to anti-HR2 in an antivenine 4:3 (see antivenine A in Table 2). When HR1 is neutralized equivalently by anti-HR1, HR2 has already been neutralized by the excess amount of anti-HR2. In this case the end point of titration in the rabbit skin is entirely dependent on neutralization of HR1 by anti-HR1 and only the potency of anti-HR1 can be determined. On the other hand, with another antivenine having the ratio of anti-HR1 to anti-HR2 of 6:2 (see antivenine B), when HR2 is neutralized equivalently by anti-HR2, HR1 has already been neutralized by the excess amount of anti-HR1. In this case, only the potency of anti-HR2 can be determined.

In other words, according to this model either of the two anti-hemorrhagic activities will be determined depending on the ratio of HR1 to HR2 in a test toxin and on that of anti-HR1 to anti-HR2 in a test antivenine.

This model is valid, however, only if the following four assumptions hold good: 1) that the hemorrhagic activities of HR1 and HR2 are additive but neither synergistic nor inhibitory; 2) that each of the two neutralization reaction systems, HR1 to anti-HR1 and HR2 to anti-HR2, has its own immunological specificity; 3) that formation of toxin-antitoxin complexes of the two systems occurs at the constant molecular ratio of toxin to antitoxin irrespective of the

concentration of the toxin; and 4) that the dissociation constants of the two neutralization reactions are relatively small and of approximately the same magnitude.

The first assumption has been verified by the results of our experiments (5). The results shown in Fig. 1 seem to support the second assumption. The third and fourth assumptions also seem to be valid since the two neutralization reactions followed essentially the "Law of Multiple Proportion" * as shown in Fig. 1, where the slope of each neutralization curve is approximately a unity.

For further verification of the model, an experiment was conducted. In Table 3, antivenines No. 7, 9 and 23 were titrated separately against HR1 and HR2. The ratios of anti-HR1 to anti-HR2 in these antivenines were calculated to be 2.73:1, 4.55:1 and 14.5:1, respectively. When the anti-hemorrhagic potencies of the three antivenines are titrated against the test toxins containing HR1 and HR2 at varying ratios, antihemorrhagic activity against the individual hemorrhagic principles should be determined as predicted in Table 3, if our model (Table 2) holds true.

TABLE 2 — A MODEL FOR THE MECHANISM INVOLVED IN TITRATION OF ANTIVENINE POTENCY IN THE RABBIT SKIN IN THE PRESENCE OF TWO HEMORRHAGIC PRINCIPLES AND THE CORRESPONDING ANTIBODIES

	Proportion of the two hemorrhagic principles in test toxin		
	HR 1	HR 2	
Test toxin	○ ○ ○ ○	● ●	
	Proportion of the two corresponding antibodies in test antivenine		Specific antihemorrhagic activity to be titrated
	Anti-HR 1	Anti-HR 2	
Antivenine A	◎ ◎ ◎ ◎	◉ ◉ ◉	Anti-HR 1
Antivenine B	◎ ◎ ◎ ◎ ◎ ◎	◉ ◉	Anti-HR 2

A white circle (○) represents a certain quantity of toxin (HR 1) molecules immunologically equivalent to that of the corresponding antibody (anti-HR 1) molecules as represented by a double circle (◎). A filled circle (●) for HR 2 and a circle with a dark spot (◉) for anti-HR 2 represent quantities immunologically equivalent to each other.

* The "Law of Multiple Proportion" implies that "if a certain amount of antitoxin neutralizes a certain quantity of toxin, then to neutralize a multiplum of the quantity of toxin the same multiplum of the amount of antitoxin is required" (Jerne, 1951 (6)).

The prediction shown in Table 3 was in exact accordance with the actual results of the experiment, except for antivenine No. 23 in Section 7. The experiment has been published elsewhere (5).

TABLE 3 — THE NEUTRALIZATION REACTIONS DETERMINING THE END POINTS OF TITRATIONS IN THE RABBIT SKIN PREDICTED FROM THE PROPOSED MODEL (TABLE 1)

Section	Test toxin	Antivenine (and the ratio of anti-HR1 to anti-HR2 in the antivenine)		
	The ratios of HR1 to HR2	No 7 (2.73:1)	No.9 (4.55:1)	No. 23 (14.5:1)
4	60:40(1.5:1)	HR 2*	HR2	HR2
5	75:25(3:1)	HR 1*	HR2	HR2
6	83:17(4.9:1)	HR 1	HR1	HR2
7	95: 5(19:1)	HR 1	HR1	HR1

* HR1 is the abbreviation for the neutralization reaction of HR1 to anti-HR1; HR2 is for that of HR2 to anti-HR2.

Conclusion

We conclude that the antihemorrhagic potency of Habu antivenine relative to a standard antivenine can only be determined when the two hemorrhagic principles (HR1 and HR2) are used separately as test toxins instead of a crude venom.

To generalize, all the toxic components in a venom indistinguishable in respect to a biological response should be separated and each component should be used as a test toxin (3, 5). This general conclusion has been fortified by our experiments on determination of antilethal potency of Habu antivenine. (7)

The reasoning thus confirmed is applicable to the assay of antitoxic potency of a polyvalent antivenine. Unless each toxic component in venoms is separated and available as a test toxin, monovalent antivenines should not be combined before determining the potencies of the individual antivenines, or immunization with mixed venoms should be avoided.

It is worth mentioning the suggestion made by Iguchi (8) in 1940 about the possible presence of multiple lethal toxic components in culture filtrate of *Cl. welchii* (*perfringens*) type A and the corresponding antibodies in different proportions in antitoxin preparations; he observed varying antilethal potencies for an antitoxin preparation depending on the test toxin used.

As pointed out by Llewellyn Smith (9) in 1938, the assay of the potency of tetanus antitoxin is also influenced by the test toxin used. Based on similar observations, Petrie (10) in 1943 suggested the multiplicity of composition of tetanus toxin and the consequent multiplicity of antibodies in the antitoxin preparations.

The reasoning described in this presentation should naturally be applicable to the assay of antitoxic activities of various bacterial antitoxins including *Cl. welchii* type A antitoxin and *Cl. tetani* antitoxin.

Acknowledgment — We wish to express our gratitude to the Division of Public Health, Kagoshima Prefecture, Japan, for their generous gifts of Habu venom.

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