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Development of simple standard assay procedures for the characterization of snake venoms*

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In accordance with the recommendations of the report of a WHO Coordination Meeting on Venoms and Antivenoms, methods have been developed for the assessment of lethal, defibrinogenating, procoagulant, haemorrhagic, and necrotizing properties of venoms, and used to study 53 venoms from 30 different species of snakes of medical importance throughout the world. The venoms studied included Echis carinatus *(Iran),* Naja naja kaouthia *(Thailand),* Notechis scutatus *(Australia),* Trimeresurus flavoviridis *(Japan),* Vipera russelli *(Thailand), and* Crotalus atrox *(USA), which comprise six of the eight venoms designated by WHO as international reference venoms (IRVs). (*C. atrox *venom replaced* C. adamanteus *venom, as an adequate supply of the latter was not available.) The tests used were simple and should be reproducible in other laboratories throughout the world. Procedures for assaying neuromuscular paralytic activity and systemic myotoxic activity have yet to be developed.*

The tests will be used to assay the neutralizing potency of both international standard antivenoms (raised using the IRVs) and new and currently available commercial antivenoms. Such studies should result in the production of more potent antivenoms for use in both developing and developed countries, and improve the understanding and management of snake bite throughout the world.

In 1979, a WHO Coordination Meeting on Venoms and Antivenoms was held to examine the work in progress throughout the world on the development and standardization of assay methods for assessing the biological activity of medically important venoms and the neutralizing capacity of antivenoms (*1*). It was concluded that methods for the measurement of lethal, defibrinogenating, procoagulant, haemorrhagic, and necrotizing activity should be standardized, so that the effect of antivenom on these activities could be assessed by different laboratories throughout the world using identical methods.

The present study, carried out at the WHO Collaborating Centre for the Control of Antivenoms (CCCA) at the Liverpool School of Tropical Medicine, evaluated two methods for assessing the defibrinogenating potency of snake venoms, as well as techniques for estimating lethal, procoagulant, haemorrhagic, and necrotizing effects of medically important venoms.

MATERIALS AND METHODS

Venoms

A total of 53 venoms were obtained from 30 different species of snake, 20 of which are maintained at the Liverpool School of Tropical Medicine. Six of the

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venoms were international reference venoms (IRVs), details of which are given in Table 1.

Median lethal dose (LD_{50})

For each venom, the lethal toxicity was assessed by injection of venom in 0.2 ml of physiological saline into the tail vein of 18–20-g male CFW-strain mice. Six mice were used at each venom dose. The LD_{50} was calculated by probit analysis (*2*) of deaths occurring within 24 hours of venom injection.

Minimum coagulant dose (MCD)

The MCD is defined as the least amount of venom (in mg dry weight per litre of test solution) that clots either a solution of bovine fibrinogen (2 g/litre) in 60 seconds at 37 °C (MCD-F) and/or a standard citrated solution of human plasma (fibrinogen content, 2.8 g/litre) in 60 seconds at 37 °C (MCD-P).

For measurement of the MCD-F, 50 µl of physiological saline with final venom concentrations ranging from 240 to 0.5 mg/litre was added to 0.2 ml of bovine fibrinogen solution (2 g/litre)[a] at 37 °C. The solutions were mixed thoroughly and the clotting time recorded. The MCD-P was estimated by adding the same venom concentrations to 0.2 ml of a standard human plasma solution under the same conditions and recording the clotting time. In each case, the MCD was calculated by plotting clotting time against venom concentration and reading off the level at the 60-second clotting time.

[a] Diagnostic Reagents Ltd, Thame, Oxfordshire, England.

Minimum haemorrhagic dose (MHD)

The MHD is defined as the least amount of venom (µg dry weight) which, when injected intradermally into rats, results in a haemorrhagic lesion of 10 mm diameter 24 hours later.

The method of Kondo et al. (*3*) was used in rats. Aliquots of 0.1 ml of physiological saline containing between 120 and 5 µg of venom were injected into the shaved dorsal skin of 250-g male Sprague Dawley rats under light ether anaesthesia. After 24 hours the animals were killed, the dorsal skin removed, and the diameter of the lesion measured on the inner surface of the skin, in two directions at right angles, using calipers and background illumination. Care was taken not to stretch the skin. The mean diameter of the haemorrhagic lesion was calculated for each venom dose and the MHD estimated by plotting mean lesion diameter against venom dose and reading off the dose corresponding to a 10-mm diameter.

Minimum necrotizing dose (MND)

The MND is defined as the least amount of venom (µg dry weight) which, when injected intradermally into rats, results in a necrotic lesion of 5 mm diameter 3 days later.

The method used was the same as that for the MHD, except that the dorsal skin was removed 3 days

Table 1. International reference venoms maintained at the WHO Collaborating Centre for the Control of Antivenoms[a]

Species	Source	Area collected	No. of samples	Length of snake (cm)	Amount of venom (g)
Crotalus adamanteus[b]	America	Georgia to southern tip of Florida	40	122–168	5
C. atrox	America	Arizona, Texas	58		30
Echis carinatus	Islamic Republic of Iran	Zabol (S.E. Iran)	3440	30–65 (adult) 20 (< 1 year)	10
Trimeresurus flavoviridis	Japan	Ryukyu Islands			10
Vipera russelli	Thailand	Saraburi	20	90–100	15
		Lopburi			
Notechis scutatus	Australia	Portsea (S. Victoria)	110	1000	10
		Lake George (Central New South Wales)			
Naja naja kaouthia	Thailand	Samutprakaan	15	90–100	5

[a] The remaining two IRVs, namely *Bothrops atrox asper* (Costa Rica) and *E. carinatus* (Nigeria), have not yet been received at the Collaborating Centre.

[b] *C. atrox* replaced *C. adamanteus* as IRV (see text).

after intradermal injection of venom. The MND was calculated by plotting mean lesion diameter against venom dose and reading off the dose corresponding to a diameter of 5 mm.

Minimum defibrinogenating dose (MDD)

For measurement of the MDD, a wide range of doses was selected for each venom tested and each dose was injected intravenously into 4 mice. One hour after injection, the mice were anaesthetized with ether and bled by cardiac puncture. The blood was pooled and divided into two equal portions, one of which was tested using the whole blood clotting method (MDD-WBC), while the other was mixed with citrate containing 50 g/litre aminocaproic acid to prevent fibrinolysis, and tested using the fibrin polymerization time test (MDD-FPT).

Whole blood clotting method. The blood was placed in a clotting tube and left at room temperature for 1-2 hours. The clot quality was then recorded using the "1-5" grading system (*4*). The MDD-WBC was defined as the minimum dose of venom that produced non-clotting blood within 60 minutes of intravenous injection.

Fibrin polymerization time (FPT) test

1. *Preparation of standard curve.* Blood was taken from 10 normal mice, mixed with citrate containing 50 g/litre aminocaproic acid, and pooled. The plasma was diluted with physiological saline, to give final plasma concentrations ranging from 100% to 10%. An aliquot of 0.4 ml of each plasma dilution was added to 0.9 ml of physiological saline, followed by 0.1 ml of thrombin (600 IU/ml).[b] The time taken for a web of fibrin to form on a gently agitated glass rod was then plotted against the log plasma concentration to give a standard curve (*5*). The actual amount of fibrinogen present in the sample was determined by carrying out a similar assay using known amounts of bovine fibrinogen.

2. *Assay of unknown samples.* Aliquots of 0.4 ml of each citrated test portion were treated as described above. Using the computed standard curve, the fibrinogen content of the test sample was determined one hour after injection of venom. The MDD-FPT was defined as the dose of venom resulting in a decrease in the fibrinogen level to 10% of that for the standard plasma fibrinogen solution.

RESULTS

The MCD-P and MCD-F for a range of medically important venoms are given in Table 2. Twelve venoms, including the IRV *Crotalus adamanteus*, had a direct thrombin-like action on fibrinogen and therefore also clotted plasma. Twelve viperine venoms, including the IRV *Echis carinatus* (Iran), 2 elapid venoms, including *Notechis scutatus* (IRV), and the venom of the colubrid, *Rhabdophis subminiatus*, exerted a thromboplastic-like action only; 2 venoms had a milder thromboplastic-like action on plasma and trace activity on fibrinogen. Trace plasma procoagulant activity was detected in the venoms of 5 viperine species and one elapine species; no detectable activity was found in the venoms of 15 viperine and 3 elapine venoms (including the IRVs, *Trimeresurus flavoviridis* and *Naja naja kaouthia*).

At the limits of the test, the venom of the Saudi Arabian *E. carinatus* was remarkable in that no significant action on plasma was recorded, unlike venoms of *E. carinatus* from six other geographical areas. (In fact, plasma was clotted by this venom, but only with venom doses of 3 times the maximum amount normally used in the test (MCD-P, 870 mg/litre).) Of 12 separate *Cerastes cerastes* specimens from north and south Oman, 1 had no detectable procoagulant activity, 10 had trace activity, and 1 had strong activity (MCD-P, 1.8 mg/litre). The results shown in Table 2 represent a pooled batch containing equal amounts of these venoms.

A close correlation was observed between the two estimates of defibrinogenating activity given by the whole blood clotting test and the fibrin polymerization time test ($r = 0.99$, $P < 0.001$). One of the most active venoms in this respect was the IRV, *V. russelli* (Thailand). Of 45 viperine venoms tested, 35 (78%) had defibrinogenating activity, whereas none of the elapid venoms had significant activity (Table 2).

The elapid venoms, with the exception of the IRV, *N. scutatus*, had no measurable haemorrhagic activity, whereas all the viperine venoms, except *T. wagleri*, exerted some activity (Table 2). The most haemorrhagic viperine venom was Burmese *V. russelli* and the least haemorrhagic the IRV, *T. flavoviridis*. Venom from immature *Bitis arietans* was twice as haemorrhagic as that from mature snakes, but it should be noted that the snakes originated in different geographical areas (Nigeria and Saudi Arabia).

Of the 53 venoms tested, 48 had necrotizing potential (Table 2), the most active being *V. russelli* (India), *E. carinatus* (Oman), and mature *C. viridis viridis* among the viperine group, and *N. nigricollis* in the elapine group. Venoms from mature specimens of *B. arietans*, *C. atrox*, and *C. viridis viridis* had almost double the necrotizing potential of those from immature snakes. There were also considerable differences among individual samples from the same species (*E. carinatus* and *V. russelli*) from different regions.

[b] Diagnostic Reagents Ltd, Thame, Oxfordshire, England.

Table 2. Lethal, procoagulant, haemorrhagic and necrotizing effects of 53 snake venoms[a]

Species	Source	No. of samples	LD_{50}[b] (μg/mouse)		MDD (μg/mouse)	MCD-P (mg/litre)	MCD-F (mg/litre)	MND (μg/rat)	MHD (μg/rat)
Colubrine:									
Rhabdophis subminiatus	Hong Kong	8	150.6	(136–172)	13.3	3.4	NA	NA	118.0
Viperine:									
Agkistrodon rhodostoma	Malaysia	50	107.5	(104–111)	0.9	18.4	32.6	48.0	60.0
Bitis arietans (immature)	Saudi Arabia	1	20.3	(16–24)	5.6	NA	NA	40.0	13.5
B. arietans (mature)	Nigeria	3	25.0	(20–29)	tr	NA	NA	25.5	26.5
B. gabonica	Nigeria	10	35.2	(29–40)	NA	tr	NA	39.0	20.4
Bothrops atrox asper	Costa Rica	?	43.0	(41–46)	7.8	1.4	18.0	40.0	70.0
B. nasutus	Costa Rica	?	135.1	(128–144)	NA	tr	NA	92.5	23.2
B. schlegellii	Costa Rica	?	39.0	(38–40)	12.2	13.2	153.0	60.0	28.2
Cerastes cerastes	Oman	12	10.0	(6–14)	5.8	152.0	NA	30.5	12.0
C. cerastes	Saudi Arabia	1	5.0	(3–7)	4.1	NA	NA	31.3	8.2
C. cerastes	Libyan Arab Jamahiriya	1	12.5	(8–15)	8.2	NA	NA	27.3	24.0
C. vipera	Israel	4	10.0	(6–12)	4.1	tr	NA	29.5	12.5
Crotalus adamanteus	N. America	2	50.4	(41–59)	27.8	73.0	84.0	45.0	40.0
C. adamanteus[c]	N. America	40	57.6	(54–63)	7.5	37.0	40.8	42.5	15.5
C. atrox (immature)	N. America	3	25.0	(21–28)	25.6	38.4	86.0	64.2	31.0
C. atrox (mature)	N. America	3	72.5	(69–76)	NA	NA	NA	30.5	25.5
C. atrox[d]	N. America	58	56.0	(42–65)	47.5	tr	tr	50.0	22.5
C. durissus	Costa Rica	3	28.1	(22–35)	11.6	59.0	55.0	37.5	29.0
C. viridis helleri	N. America	1	22.3	(20–25)	7.5	106.0	tr	NA	31.2
C. v. viridis (immature)	N. America	5	26.3	(24–28)	NA	NA	NA	28.0	77.5
C. v. viridis (mature)	N. America	5	25.0	(23–29)	NA	NA	NA	10.0	41.0
Echis carinatus	India	10+	19.0	(16–24)	3.0	14.0	NA	28.0	17.8
Echis carinatus	Islamic Republic of Iran	10+	12.2	(6–17)	1.6	2.0	NA	27.6	14.0
E. carinatus[d]	Islamic Republic of Iran	3440	17.9	(16–20)	2.5	2.2	NA	27.5	46.5
E. carinatus	Kenya	10	14.0	(12–15)	6.0	16.0	NA	26.0	18.2
E. carinatus	Kenya	61	12.0	(10–14)	1.2	14.0	NA	25.0	19.0
E. carinatus	Nigeria	13	8.1	(7–9)	5.3	3.0	NA	32.3	9.8
E. carinatus	Nigeria	4	17.3	(14–21)	2.9	4.0	NA	30.0	11.0
E. carinatus	Oman	6	15.7	(14–18)	11.0	14.0	NA	12.0	4.6

E. carinatus	Pakistan	?	38.0	(30–46)	1.8	7.6	NA	50.0	23.0
E. carinatus	Saudi Arabia	5	75.3	(69–82)	9.0	NA	NA	22.0	18.6
E. coloratus	Saudi Arabia	3	24.9	(21–30)	3.0	13.0	NA	43.0	16.4
Lachesis muta	Costa Rica	?	110.0	(61–139)	4.0	17.5	17.0	37.0	16.5
Trimeresurus albolabris	Thailand	14	12.5	(9–15)	15.0	58.0	77.0	85.0	70.0
T. flavoviridis	Ryukyu Islands	?	75.1	(65–80)	163.0	NA	NA	50.0	40.8
T. flavoviridis[d]	Ryukyu Islands	?	183.0	(175–191)	95.0	NA	NA	35.0	160.0
T. macrops	Thailand	5	175.0	(140–192)	40.0	146.0	NA	146.5	23.5
T. popeorum/stejnegeri	Thailand	5	30.0	(21–39)	22.5	58.0	98.0	38.5	26.5
T. purpureomaculatus	Thailand	2	25.0	(14–36)	22.5	59.0	98.0	71.0	18.0
T. stejnegeri	?	?	90.0	(67–121)	0.8	29.0	35.0	55.0	62.0
T. wagleri	Thailand	2	12.5	(8–15)	NA	NA	NA	NA	NA
T. wagleri	Malaysia	?	15.0	(9–12)	NA	NA	NA	NA	NA
Vipera palaestinae	Israel	4	15.0	(13–18)	NA	NA	NA	60.0	15.8
V. russelli	India	10+	5.2	(4–6)	8.7[e]	NA	NA	4.0	21.5
V. russelli[d]	Thailand	20	2.7	(2–3)	4.4	NA	NA	38.0	39.0
V. russelli	Burma	?	4.0	(3–5)	95.0	tr	NA	23.0	3.0
Elapine:									
Micrurus nigrocinctus	Costa Rica	?	9.8	(8–13)	NA	tr	NA	60.0	NA
Naja naja naja	Malaysia	30	15.2	(14–17)	NA	NA	NA	50.0	NA
N. n. kaouthia[d]	Thailand	15	3.5	(3–4)	NA	NA	NA	30.0	NA
N. nigricollis	Nigeria	1	25.4	(20–29)	NA	NA	NA	14.5	NA
Notechis scutatus	Australia	?	4.5	(4–6)	tr	95.0	tr	30.0	NA
N. scutatus[d]	Australia	110 approx	0.8	(0.7–0.8)	NA	16.5	NA	35.5	42.3
Oxyuranus scutellatus	Australia	?	3.1	(2–4)	tr	13.8	NA	NA[f]	NA[f]

[a] NA = No activity; tr = trace activity, i.e., 1.2–2.0 LD_{50} (MDD), 240 mg/litre (MCD), and 120 μg (MND, MHD).
[b] 95% confidence limits are given in parentheses.
[c] Previous international standard venom, now replaced by *C. atrox*.
[d] International reference venom.
[e] Result obtained by extrapolation (FPT test).
[f] No activity with 5-μg dose; rat died with 10 μg.

DISCUSSION

It has previously been reported that, in certain instances, the procoagulant properties of venoms change as the snakes age. For example, the venom of immature *C. atrox* specimens has the ability to clot fibrinogen directly; as the snakes age, the venom acts only on plasma and above the age of one year, no procoagulant activity can be detected (*6*, *7*). The MCD-Ps of the venoms of *E. carinatus* from different geographical regions varied considerably; of 16 specimens of *E. carinatus* of all ages and from a wide geographical distribution kept at the Liverpool School of Tropical Medicine, only venom from the Saudi Arabian snakes showed no significant procoagulant activity, although it should be noted that this venom caused defibrinogenation *in vivo*. Taborska (*8*) reported that, of venoms from 21 individual specimens of *E. carinatus* collected from Karachi, Pakistan, 8 showed no detectable clotting activity on human oxalated plasma. Similarly, of 12 specimens of *C. cerastes* from Oman, only one had strongly procoagulant venom. This implies a considerable amount of both inter- and intraregional variation in the clotting activity of venoms of these species. The age of the snake might also contribute to the variation in activity.

The tests used for MDD estimation appeared to be equally accurate. The advantages of the whole blood clotting method are that it is simpler, does not require expert supervision, and is much cheaper in terms of materials. The method has been used extensively in clinical studies on snake bite patients (*9*). Possible advantages of the FPT method are that it is quicker and more specific, as it gives a direct estimation of fibrinogen levels (F. Kornalik, personal communication, 1981). For example, non-coagulability may, in certain circumstances, be due to lack of procoagulant factors other than fibrinogen, and antithrombin may interfere (B. Blombäck, personal communication, 1980). However, for the majority of situations, we suggest that the MDD-WBC test is the method of preference.

Although snake bite defibrinogenation is remarkably benign in itself and is not, as a rule, a primary cause of spontaneous bleeding (*10*, *11*), our results suggest that if the MDD is near to or greater than the LD_{50}, then the observation of defibrinogenation in man can be considered to have serious clinical implications. (It should be remembered, however, that caution is needed when extrapolating observations in animals to the situation in man.) This would apply to the venoms of *V. russelli*, *E. carinatus* (Nigeria and Oman), immature *C. atrox*, *C. cerastes*, *T. flavoviridis* and *C. adamanteus*. The venoms of *Oxyuranus scutellatus*, mature *B. arietans* and *N. scutatus* possessed a trace of defibrinogenating activity; if defibrination were to be observed in persons bitten by these snakes, the prognosis would be extremely grave. Conversely, when the MDD is much less than the LD_{50}, defibrinogenation is unlikely to have serious clinical implications, as in the case of *Agkistrodon rhodostoma* venom. It should also be noted that the defibrinogenating capacity of some venoms may change as the snakes age, as in the case of *C. atrox* (*6*, *7*) and *B. arietans*.

According to Reid (*12*), haemorrhage is the outstanding feature of viperine poisoning and is caused by the action of certain powerful proteases on the vascular endothelium; it does not appear to be characteristic of elapid poisoning. The results of the MHD estimations support these observations. Although haemorrhage is frequently accompanied by a clotting defect in viperine poisoning, the two properties are by no means invariably related and in many cases the factors that cause coagulation defect can be separated from those responsible for haemorrhage (*13*). When clotting defect does accompany haemorrhage, it may be an aggravating factor, but it should be stressed that the coagulation defect does not in itself lead to haemorrhage. Seven venoms from 5 species had defibrinogenating activity but no detectable *in vitro* clotting activity as assessed by the MCD test. It seems likely either that, in some cases, other factors may be involved in defibrinogenation or that the MDD test is more sensitive than that used for the MCD.

Most of the venoms possessed the ability to cause local necrosis when introduced intradermally, and the MND estimation proved a reasonably reliable test for assessing this activity. The test should have applications for estimating the rather doubtful neutralizing ability of most commercial antivenoms against the local necrotizing activity of many medically important venoms. The results also demonstrated that local necrotizing activity is not necessarily related to local haemorrhagic activity.

The main aim of this study was to develop relatively simple and reliable methods for estimating *in vitro* coagulant activity and *in vivo* haemorrhagic, necrotizing, and defibrinogenating activities of snake venoms. Further studies will include the development of a test for assessing the neuromuscular paralytic activity of neurotoxic venoms and the systemic myotoxic activity of myotoxic venoms (D. Mebs, personal communication, 1981). The development of reliable tests is necessary if antivenoms are to be graded easily and uniformly, in terms of their venom neutralizing abilities, by different laboratories. This applies especially to the IRVs, which have been selected because of their medical importance worldwide.

The study also demonstrated the variability of snake venoms within the same species both from an age and distribution aspect, and it is hardly surprising

that conflicting reports of the severity of snake-bite poisoning by the same species frequently occur in the medical literature. However, as with all animal-based studies, we urge great caution in extrapolating the results of venom characterization to envenoming in man.

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RÉSUMÉ

MISE AU POINT D'ÉPREUVES NORMALISÉES SIMPLES DE CARACTÉRISATION DES VENINS DE SERPENTS

Lors de la présente étude, on a évalué les propriétés létales, défibrinogénantes, procoagulantes, hémorragiques et nécrosantes de 53 venins produits par 30 espèces différentes de serpents, dont 6 étaient des venins internationaux de référence désignés par l'OMS. La toxicité létale a été évaluée par injection intraveineuse de différentes quantités de venin à des souris, à raison de six souris par dose de venin. La dose létale médiane (DL_{50}) a été calculée par analyse des probits des décès survenant dans les 24 heures. La dose défibrinogénante minimale (MDD) a été évaluée à partir de l'épreuve de temps de polymérisation de la fibrine (FPT) et de l'épreuve de coagulation du sang total (WBC), une heure après injection intraveineuse de venin à des souris. La MDD a été définie comme étant la quantité de venin qui abaisse le taux de fibrinogène à 10% de celui d'une solution standard de fibrinogène plasmatique (épreuve FPT) ou comme étant la plus petite quantité de venin produisant un sang non coagulant (épreuve WBC). On n'a pas observé de différence significative entre les résultats obtenus par ces deux méthodes. On a estimé la dose coagulante minimale (MCD) en notant le temps de coagulation après avoir mélangé le venin avec un plasma humain standard (MCD-P) et une solution standard de fibrinogène bovin (MCD-F). La MCD-P a été définie comme étant la plus petite quantité de venin par ml de solution à éprouver qui coagule un plasma humain standard en 60 secondes à 37 °C; la MCD-F a été définie comme étant la plus petite quantité de venin qui coagule une solution standard de fibrinogène bovin dans les mêmes conditions.

La dose hémorragique minimale (MHD) et la dose nécrosante minimale (MND) ont été estimées à l'aide d'une épreuve intradermique chez le rat. La MHD a été définie comme étant la plus petite quantité de venin qui, injectée par voie intradermique à des rats, provoque une lésion hémorragique de 10 mm de diamètre à la surface interne de la peau 24 heures après l'injection. La MND a été estimée par la même méthode, à l'exception du fait que la peau du dos des rats a été prélevée trois jours après l'injection; la MND a été définie comme étant la plus petite quantité de venin qui entraîne une lésion nécrotique de 5 mm de diamètre trois jours après l'injection.

On a observé une absence manifeste de corrélation entre l'activité défibrinogénante *in vivo* et l'activité coagulante *in vitro*; de même, l'activité nécrosante locale n'est pas nécessairement corrélée à l'activité hémorrhagique locale. Il faut donc être très prudent lorsqu'on extrapole les résultats de ces épreuves de caractérisation à l'intoxication humaine.

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