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Synergism of procoagulation effect of thrombin-like enzymes from *Dienagkistrodon acutus* and *Agkistrodon halys* snake venoms

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KEY WORDS *Dienagkistrodon acutus*; *Agkistrodon halys*; snake venoms; thrombin; blood coagulation

AIM: To study the synergistic effect of thrombin-like enzymes (TLE) of *Dienagkistrodon acutus* (*DA*) and *Agkistrodon halys* (*AH*) venoms **METHODS**: TLE were isolated from venoms of *DA* and *AH* by successive column chromatography. Effects of combination of DATLE and AHTLE and related factors on the clotting time and clot quality were tested *in vitro* **RESULTS**: One coagulation unit was 2.7 μg for DATLE and 304.4 μg, for AHTLE with reference standard of thrombin (42.2 μg). TLE-induced clot was a fibrin monomer which was fragile and did not retract. Combination of AHTLE and DATLE shortened the clotting time and decreased the solubility of the clot in urea 5 mol · L^{-1}. When subthreshold concentration of thrombin or physiological concentration of Ca^{2+} was added, the clotting time was further shortened, the clot was no longer soluble in urea and retracted well, and the resistance of the clot to plasmin degradation was increased. **CONCLUSION**: A synergistic effect of DATLE and AHTLE accelerated hemocoagulation and improve clot quality.

Proteinases that exert effects on hemocoagulation have been isolated from *Crotalid* snake venoms, including thrombin-like enzymes (TLE), thrombocytin, RVV-V, and RVV-X. Since the late 1960s, preparations of TLE such as ancrod, reptilase, batroxobin and crotalase were introduced as defibrinating agents in clinical use for the treatment of thrombotic diseases[1]. But it was no better than heparin even in case of deep vein thrombosis[2]. In China, TLE has been isolated from venoms of *Dienagkistrodon acutus* (*DA*)[3], *Agkistrodon halys* (*AH*)[4], *Trimeresurus stejnegeri*, and *Trimeresurus mucrosquamatus*. But the local procoagulant effect of TLE was not appreciated because of the fragility of fibrin monomer clot they formed. The present study is aiming at improving the quality of the clot by TLE.

MATERIALS

Snake venoms *Dienagkistrodon acutus* venom collected in Anhui Province and *Agkistrodon halys* venom collected in Zhejiang Province were lyophilized and stored in desiccator.

Reagents DEAE-Sephadex A-50 and Sephadex G-75 were from Pharmacia, Sweden; acrylamide, N, N'-bis (acryloyl)cystamine and Coomassie brillant blue from Fluka, Switzerland; bovine fibrinogen from Sigma, USA; thrombin from the Biochemistry and Pharmaceutical Factory of Zhuhai Special Economic Zone (lot No 9310002/5); urokinase from Zhuhai Libao Biochemistry and Pharmaceutical (Factory) Co Ltd (lot No 930603); All materials were reagent grade.

Apparatuses UV-2100 uv-vis spectrophotometer (Shimadzu, Japan); 75034 Bench top freeze dryer (Labconco, Canada); BJQ-74-2 fractionation collector (Shanghai No 10 Medical Instruments Factory); DYY-III 2 electrophoresis apparatus (Beijing Liuyi Instruments Factory).

METHODS

Isolation of TLE from venoms of *DA* and *AH* by successive column chromatography (Tab 1).

Test for the purity of TLE By polyacrylamide slab gel electrophoresis (PAGE)[5]. The 12.5 % gel of high pH system was used.

Blood coagulation tests

1 Unitage of TLE activity[6] One coagulation unit was equivalent to the activity of the thrombin required to produce a clot of 0.4 mL of fibrinogen 2.5 g·L^{-1} at 28 ℃ in 15 s.

2 Clotting time of TLE and thrombin 0.1 mL of the TLE or thrombin solution was added to 0.4 mL of citrated dog plasma or fibrinogen 2.5 g·L^{-1} at 37 ℃, and the clotting time was determined. All the tests were done in triplicate samples and the average value was taken.

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Tab 1. Isolation of TLE by successive column chromatographies.

Fractions	Chromatography	Column size/cm	Elution/mol·L^{-1}
DA venom	DEAE-Sephadex A-50	2.6 × 100	CH_3COOH_4 0.05 (pH 8) to 0.6 (pH 6)
DA(6)	Sephadex G-75	1.6 × 100	$NaHCO_3$ 0.02 and NaCl 0.2
DA(7,8,9)	Sephadex G-75	1.6 × 100	$NaHCO_3$ 0.02 and NaCl 0.2
DA(12)	Sephadex G-75	1.6 × 100	$NaHCO_3$ 0.02 and NaCl 0.2
DA(6)-I	DEAE-Sephadex A-50	2.5 × 50	NaCl 0.1 to 0.6
DA(7,8,9)-I	DEAE-Sephadex A-50	2.5 × 50	NaCl 0.2 to 0.8
DA(12)-I	DEAE-Sephadex A-50	2.5 × 50	NaCl 0.3 to 1.0
DA(6)-I-III (DATLE-1)	Sephadex G-75	1.6 × 100	$NaHCO_3$ 0.02 and NaCl 0.2
DA(6)-I-V (DATLE-2)	Sephadex G-75	1.6 × 100	$NaHCO_3$ 0.02 and NaCl 0.2
DA(7,8,9)-I-II (DATLE-3)	Sephadex G-75	1.6 × 100	$NaHCO_3$ 0.02 and NaCl 0.2
DA(12)-I-I (DATLE-4)	Sephadex G-75	1.6 × 100	$NaHCO_3$ 0.02 and NaCl 0.2
AH venom	DEAE-Sephadex A-50	2.6 × 100	NaCl 0 to 0.2 and 0.2 to 1.0
AH(8,9)	Sephadex G-75	1.6 × 100	$NaHCO_3$ 0.02 and NaCl 0.2
AH(8,9)-II	DEAE-Sephadex A-50	2.5 × 50	NaCl 0.1 to 0.22
AH(8,9)-II-I (AHTLE)	Sephadex G-75	1.6 × 100	$NaHCO_3$ 0.02 and NaCl 0.2

3 Susceptibility of fibrin clot to plasmin digestion[7] Fibrin formed by TLE and thrombin were incubated at 37 ℃ for 1 h, then 0.5 mL of urokinase 10^6 IU·L^{-1} was added and the time required for complete clot lysis was recorded.

4 Blood coagulation tests were determined by the routine methods in hematology.

RESULTS

Isolation of TLE Five (VI – IX and XII) of the 15 fractions with TLE activity were obtained after initial DEAE-Sephadex A-50 column chromatography and only four fractions (DATLE-1, 2, 3, and 4) were finally purified after repeated chromatography. The relative amount of the above four TLE were 1:5:4:2 and total yield was 8.7 % of the crude DA venom. Similarly, two (VIII and IX) of the 14 fractions with TLE activity were obtained initially and only one AHTLE was purified. The yield was 1.2 % from crude *AH* venom. All these TLE were homogeneous as judged by PAGE. Using thrombin as a reference standard, the clotting activity of above TLE was determined (Tab 2).

Hematological characterization of TLE A mixture of DATLE-1, 2, 3, and 4 was used throughout the study and was defined as DATLE. The clots induced by DATLE and AHTLE were different from that induced by thrombin in solubility in urea, in susceptibility to plasmin degradation, and in retractility (Tab 3). The clotting activity of DATLE was more potent than thrombin, but the clot was fragile, unstable and inability to retract because factor XIII and platelets were not activated. This was the reason why TLE so far was not used as a local hemostatic agent.

Tab 2. Clotting activity of TLE.

	Clotting activity/μg·U^{-1}
Thrombin	42.2
DATLE-1	14.4
DATLE-2	6.6
DATLE-3	4.7
DATLE-4	1.9
DATLE	2.7
AHTLE	304.0

Synergism on clotting time and clot quality

1 Synergism of DATLE and AHTLE

1.1 The clotting time Combination of DATLE and AHTLE shortened the clotting time (Tab 4). Combinations of 2 : 1 and 1 : 1 were apparently better.

1.2 Clot quality The dissolution time in urea 5 mol·L^{-1} of the blood clot by either DATLE or AHTLE was about 0.5 h but prolonged to 2 – 4 h when they were combined at 2 : 1, 1 : 1, and 1 : 2 ratios, 1 : 1 of which defined as DA + AHTLE was found optimal for clotting time and clot quality.

Tab 3. Improvement in TLE clotting time and clot quality of dog plasma. $n = 6$, $\bar{x} \pm s$. $^cP < 0.01$ *vs* **DA + AHTLE.**

Drug/ mg·L^{-1}	Clotting time/s	Dissolution time in urea/h		Lysis time in urokinase/min	Clot retraction
		Plasma	Fibrinogen		
Thrombin (200)	14.3 ± 1.3	not dissolved	1	127 ± 5	yes
DATLE (25)	14.2 ± 1.8	0.5	0.2	52 ± 5	no
AHTLE (25)	125 ± 8	0.5	0.2	47 ± 7	no
DA + AHTLE (25)	16.5 ± 2.1	2 − 4	1	55 ± 5	no
DA + AHTLE (25) + Ca^{2+} (2.5 mmol·L^{-1})	10.8 ± 1.2^c	not dissolved	1	100 ± 6^c	yes
DA + AHTLE (25) + thrombin (1.5)	12.7 ± 0.9^c	not dissolved	1	126 ± 6^c	yes

Tab 4. Clotting time(s) of dog plasma. $n = 6$, $\bar{x} \pm s$, $^cP < 0.01$ *vs* **control.**

AHTLE mg·L^{-1}	DATLE/mg·L^{-1}				
	0	6.25	12.5	25.0	50.0
Control		61 ± 7	37 ± 5	24.8 ± 2.2	15.6 ± 1.9
6.25	442 ± 14	48 ± 6^c	31.5 ± 3.8^c	20.2 ± 2.1^c	14.8 ± 1.6^c
12.5	281 ± 11	46 ± 5^c	30.7 ± 3.9^c	19.8 ± 1.8^c	14.5 ± 1.1^c
25.0	123 ± 8	42 ± 5^c	29.5 ± 2.6^c	19.0 ± 1.9^c	13.9 ± 1.0^c
50.0	77 ± 7	40 ± 5^c	26.5 ± 2.7^c	17.8 ± 1.5^c	13.5 ± 0.9^c

When fibrinogen was used instead of plasma, the dissolution time of the clot by DA + AHTLE was equal to that by thrombin (Tab 3). The result suggests that the degree of polymerization of the fibrin by combined effect of DATLE and AHTLE might be as well as that by thrombin.

2 Thrombin Even in the presence of sub-threshold concentration of thrombin (1.5 mg·L^{-1}, the plasma remained unclotted after 10 min), the DA + AHTLE clotting time was shortened. This clot did not dissolve in urea 5 mol·L^{-1}, and well retracted (Tab 3).

3 Ca^{2+}

3.1 Influence of Ca^{2+} on clotting time

Taking citrated plasma (3.8 % sodium citrate to blood is 1:9) as decalcified plasma, the recalcification plasma (recalcification of citrated plasma with $CaCl_2$ 6.25 mmol·L^{-1}, the Ca^{2+} was about 2.4 mmol·L^{-1}) and the heparinized plasma (10^4 U·L^{-1} of heparin, the Ca^{2+} was about 2.5 mmol·L^{-1}) as normal calcium plasma, the values of the DATLE clotting time of the later two plasmas were shorter than the former, and no difference between the two normal calcium plasmas was found. When fibrinogen was used instead, the DATLE clotting time of calcified fibrinogen solution was shortened too (Tab 5).

It suggests that physiological concentration of Ca^{2+} has the ability to potentiate DATLE activity by accelerating the hydrolysis of fibrinogen. No more effect of the excess amount of Ca^{2+} on DATLE clotting time was found by increasing concentration

Tab 5. Effect of Ca^{2+} on DATLE clotting time(/s) of plasma and fibrinogen. $n = 6$, $\bar{x} \pm s$. $^cP < 0.01$ *vs* **citrated plasma;** $^dP > 0.05$ *vs* **recalcification plasma;** $^iP < 0.01$ *vs* **control.**

DATLE/ mg·L^{-1}	Plasma			Fibrinogen	
	Citrated	Recalcification	Heparinized	Control	Calcification
6.25	46 ± 4	32.6 ± 3.4^c	30.7 ± 2.8^{cd}	105 ± 10	45 ± 6^i
12.5	23.5 ± 3.2	16.5 ± 2.2^c	15.2 ± 1.7^{cd}	63 ± 7	31 ± 5^i
25.0	15.3 ± 2.1	10.6 ± 1.4^c	10.0 ± 1.0^{cd}	41 ± 5	19.8 ± 3.0^i
50.0	9.2 ± 1.4	6.0 ± 1.2^c	5.5 ± 0.7^{cd}	25.4 ± 3.4	10.5 ± 1.7^i
100.0	5.0 ± 1.0	3.0 ± 0.7^c	3.0 ± 0.5^{cd}	15.0 ± 2.0	8.2 ± 1.1^i

of Ca^{2+} to heparinized plasma. It indicates that just the physiological concentration of Ca^{2+} is the best.

3.2 Influence of Ca^{2+} on clot quality The clot of recalcified plasma of DA + AHTLE did not dissovled in urea 5 mol·L^{-1} and well retracted, and the resistance of clot to plasmin digestion was markedly increased (Tab 3). It indicated that factor XIII and platelets were activated to yield a cross-linked fibrin similar to that obtained with thrombin.

4 Rabbit brain thromboplastin The DATLE clotting time of plasma was shortened in the presence of 0.1 mL suspension of 150 mg rabbit brain thromboplastin in 2.5 mL NS (Tab 6).

Tab 6. Effect of rabbit brain thromboplastin on DATLE clotting time(s) of plasma.
***n* = 6, $\bar{x} \pm s$. $^cP < 0.01$ *vs* control.**

DATLE/mg·L^{-1}	Control	Rabbit brain thromboplastin
6.25	49 ± 6	28 ± 6^c
12.5	26 ± 5	15.3 ± 2.1^c
25.0	16.2 ± 1.9	9.8 ± 1.7^c
50.0	9.6 ± 1.2	6.7 ± 1.0^c
100.0	6.0 ± 0.8	4.5 ± 0.8^c

DISCUSSION

TLE was distinguished from thrombin by its action upon fibrinogen. Thrombin hydrolyzes fibrinogen and releases both fibrinopeptides A (FPA) and B (FPB). It releases FPA at a faster rate than FPB. The removal of FPB was necessary for reinforcing the α-polymers of fibrin by crosswise polymerization. Ancrod, TLE from *Agkistrodon contortrix* and *Bitis gabonica* exemplified three types of TLE that split FPA or FPB or both FPA and FPB, respectively[8]. DATLE released only FPA from fibrinogen while AHTLE split off FPB at a much faster rate than FPA[9]. Because release of FPA was crucial for the formation of a visible clot, DATLE showed a very high while AHTLE showed a very weak clotting activity.

Combination of DATLE and AHTLE made the degree of polymerization of fibrin to be the same as that by thrombin, and the clotting time was shortened. Release of both FPA and FPB was believed to be the main mechanism of the synergistic procoagulation effect. But combination of DATLE and AHTLE did not yiled a cross-linked fibrin clot because factor XIII was not activated. Ultimately, in the presence of subthreshold concentration of thrombin or physiological concentration of Ca^{2+}, the DA + AHTLE clotting time was further shortened, the clot quality was improved, ie, increase in clot firmness, increase in resistance to plasmin lysis, insolubility in urea and ability to retract. So that the clot was made perfect as well as a natural clot. At the side of bleeding, the local release of thromboplastin and thrombin, aggregation of platelets and exudation of plasma Ca^{2+}, together with extrinsically applied DATLE and AHTLE, could shorten the bleeding time and form a firm, retractile clot without doubt. Besides, TLE-induced coagulation was uninfluenced by heparin-antithrombin III and was more resistant to FDP than that induced by thrombin[10]. These made TLE more reliable in cases of heparinization and DIC. So, the high potency and high specificity of action made TLE potential for hemorrhages of whatever cause except deficiency of fibrinogen.

In conclusion, a synergism of procoagulation effect of DATLE and AHTLE was found. The combination of them makes TLE a potentially effective local hemostatic agent.

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尖吻蝮蛇毒和蝮蛇毒凝血酶样酶促凝的协同作用

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关键词 尖吻蝮蛇；蝮蛇；蛇毒；凝血酶类；血液凝固

目的：尖吻蝮蛇(*DA*)和蝮蛇(*AH*)蛇毒凝血酶样酶(TLE)对促凝作用的协同效果 **方法**：柱层析分离纯化 TLE 并进行体外凝血试验. **结果**：一个凝血单位 DATLE 为 2 7 μg，AHTLE 为 304 4 μg 它们单独作用形成的凝块很松脆，合用时凝血时间缩短，凝块的尿素溶解性下降. 再配伍阈下浓度的凝血酶或生理浓度的 Ca^{2+}，凝血时间进一步缩短，凝块能够回缩，不溶于尿素 5 mol·L^{-1}，对纤溶酶降解的抵抗性也增强. **结论**：DATLE 和 AHTLE 具有协同促凝作用，两者合用时凝血活性增强，凝块质量提高，可望成为一种有效的局部止血药.

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Benzylpenicillin induced specific non-IgE antibody response in mice

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KEY WORDS penicillins; IgM; IgG; IgA; enzyme-linked immunosorbent assay; haptens; cross reactions; drug hypersensitivity

AIM: To study whether or not the specific non-IgE antibody response in mice can be induced by benzylpenicillin *in vivo*. **METHODS**: Antibody response and antigenic cross reactions were determined by enzyme-linked immunosorbent assay (ELISA). Antigen molecules recognized by antibodies were tested by hapten inhibition assay. **RESULTS**: During d 1 - d 50 after immunization, positive % of specific IgM, IgG, and IgA to benzylpenicillin were 100 %, 50 % - 100 %, and 17 % - 100 %, respectively. IgM and IgG to benzylpenicillin also recognized ampicillin and piperacillin. The positive % of IgM and IgG to ampicillin were 23 % - 100 % and 50 % - 100 %, to piperacillin 43 % - 100 % and 50 % - 100 %, respectively. Aged benzylpenicillin showed an inhibitory effect on specific antibodies in a dose-dependent manner, and inhibitory % of specific IgM and IgG were 29 % - 87 % and 29 % - 71 %. However, freshly prepared benzylpenicillin had no effect. **CONCLUSION**: Specific non-IgE antibody response was successfully induced by benzylpenicillin in mice, in which the isotypes were mainly IgM and then IgG and IgA. Antibodies recognized degraded products, not benzylpenicillin molecule itself. Antigenic cross reactions occurred between benzylpenicillin, ampicillin, and piperacillin. Isotypes of antibodies responsible for cross reactions were mainly IgG and then IgM.

Many adverse drug reactions (ADR) are described as immunological mechanisms[1-3]. A large proportion of ADR to penicillin is mediated by the immune system, in which penicillin-reactive IgE antibody is considered to be responsible for acute

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