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Induction of platelet activation by cobra venom factor from *Naja naja atra* in rat

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KEY WORDS cobra venoms; complement; blood platelets; calcium; cyclic AMP; prothrombin; monoclonal antibodies; Fura-2

ABSTRACT

AIM: To study the effects of cobra venom factor (CVF) on platelets of rat platelet rich plasma (PRP) and to elucidate its cellular mechanism. **METHODS**: PRP was used to measure platelet aggregation and ATP-release simultaneously; prothrombinase, intracellular free calcium, and cAMP were assayed using chromogenic substrate, fluorophor Fura-2, and RIA respectively. **RESULTS**: From 19.5 nmol·L^{-1} to 617 nmol·L^{-1} CVF induced platelet aggregation and ATP release concentration-dependently. The ATP release induced by CVF 195 nmol·L^{-1} was independent of aggregation and was much weaker than that induced by thrombin 1 U/ml. CVF 195 nmol·L^{-1} increased the prothrombinase activity on platelet surface in a time-dependent manner. SZ-1, SZ-21, and SZ-22, which are monoclonal antibodies against glycoproteins Ⅰb/Ⅸ, Ⅲa, and Ⅱb located on platelet membranes, inhibited CVF-induced platelet aggregation. CVF 195 nmol·L^{-1} also elevated moderately intracellular free calcium ion from (141 ± 46) to (240 ± 64) nmol·L^{-1} in Fura-2 AM loaded platelets, and decreased intracellular cAMP. **CONCLUSION**: Complement activator CVF induced platelet aggregation and ATP release, and increased prothrombinase activity on platelet surface. These actions were dependent on fibrinogen receptors on the platelet surface, elevation in intracellular free calcium ion, and reduction in cAMP.

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INTRODUCTION

Tissue injuries induced by activation of complement system are often accompanied with significant intravascular coagulation that definitely involves platelets. In oleic acid-induced lung injury, complement depletion could prevent pulmonary arteriolar thrombosis, increase LD_{50} of thrombin, and inhibit platelet aggregation as well as ATP release[1,2]. These results indicate that platelet activation triggered by complement activation may play an important role in development of pathological intravascular coagulation.

Cobra venom factor (CVF), an acid glycoprotein existing in cobra venom, can interact with factor B (C3PA) in plasma to form CVFBb with stable C_3/C_5 convertase activity and thus activate complement cascade via alternative pathway[3,4]. Due to the lack of other direct biological activities, CVF is a good tool to be used as a complement activator. In our previous study, we found that CVF induced rat platelet aggregation following a marked metamorphosis in platelet rich plasma (PRP) and the aggregation was complement- and Ca^{2+}-dependent[5]. In addition, the aggregation induced the influx of Ca^{2+} and Na^{+} and was not inhibited by indomethacin[5]. In this study, we further investigated the effects of complement activator CVF on rat platelet and to elucidate its cellular mechanism to explore the role of complement system in platelet activation.

MATERIALS AND METHODS

Drugs and chemicals CVF was isolated and purified from the venom of *Naja naja atra* using two-step chromatography successively on DEAE-Sephadex A25 column (Ø3 cm, L80 cm) and Bio-Gel P200 column (Ø3 cm, L75 cm), as has been reported previously[6]. Purified CVF was identified to be homogeneous both by immuno-electrophoresis and by polyacrylamide gel electrophoresis. It consisted of three subunits with M_r 86 500, 74 000, and 65 500 respectively, which were

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shown in 10 % SDS polyacrylamide electrophoresis. One unit of anticomplement activity and direct hemolytic activity were assayed to be 20.7 μg and 67.2 μg respectively using a standard procedure[6].

Thrombin (Thr, lot № 961022) was purchased from Biochemistry Pharmaceutical Factory of Zhuhai, China; luciferin-luciferase, ATP, from Chrono-Log Co, USA; monoclonal antibodies directed against platelet membrane glycoprotein Ⅰb/Ⅸ, Ⅱb, and Ⅲa, from Thrombosis Institute, Suzhou Medical College, China; adenosine diphosphate (ADP), Fura-2 AM, Chromozym TH, prothrombin, and factor Ⅱ, from Sigma, USA; ^{125}I-RIA kit, from Department of Isotope, Shanghai University of Traditional Chinese Medicine, China.

Experimental animals Male Sprague-Dawley rats weighing 300 g ± 50 g were provided by Experimental Animal Center of Sun Yat-Sen University of Medical Sciences (Grade Ⅱ, Certificate № 26-98A001).

Preparation of platelet suspension PRP and platelet poor plasma (PPP) were prepared from rat artery blood using 3.8 % trisodium citrate as anticoagulant for determining platelet aggregation and ATP release. ACD (citric acid 7 mmol·L^{-1}, sodium citrate 100 mmol·L^{-1}, and dextrose 140 mmol·L^{-1}, pH 6.5) was used as anticoagulant for preparation of PRP for determining prothrombinase activity, intracellular free calcium level ($[Ca^{2+}]_i$) and cAMP[7].

Platelet aggregation Platelet aggregation was measured turbidimetrically using a lumi-aggregometer (Chrono-Log) connected to an IBM computer through Aggro/Link interface (Model 810). The aggregation curve was shown on a monitor and amplitude of aggregation (A) and maximal velocity of aggregation (V) were calculated automatically with a built-in computer procedure[7,8]. According to the aggregation curve shown on the monitor, platelet suspensions were smeared with Wright-stain to observe the shape change and aggregation under the light microscope at metamorphosis and aggregation phase, respectively.

Monoclonal antibody against membrane glycoprotein was incubated for 5 min before adding CVF to PRP for observing the role of glycoproteins in platelet aggregation.

Determination of ATP release The released ATP was also measured using the lumi-aggregometer. Luciferin-luciferase mixture was added into the prewarmed samples at 37 ℃ before adding aggregation inducers. Amount of released ATP was calculated by the formula given below:

ATP (nmol) = (Luminescence of test/Gain of test) × (Gain of ATP standard/Luminescence of ATP standard) × standard ATP amount

Measurements of prothrombinase activity The prothrombinase activity was quantified according to the thrombin activity induced in the presence of prothrombin according to the procedure described by Kawai *et al*[9] with minor modification. Aliquots of the reaction mixture were sampled and added to a Tris-NaCl solution containing EDTA-Na_2 and Chromozym TH at various time intervals. After centrifugation the absorbance was measured and the concentration of thrombin was estimated from the standard curve.

Determination of intracellular Ca^{2+} PRP was washed with pH 7.4 HEPES buffer containing 0.1 % BSA and 0.8 μmol·L^{-1} PGE_1 and the platelet pellets were loaded with Fura-2 AM 3 μmol·L^{-1} at 37 ℃ for 45 min. After washing out the extracellular Fura-2 AM with HEPES buffer containing PGE_1, the pellets were resuspended in HEPES buffer with platelet count adjusted to 10^{11}/L and Ca^{2+} corrected to 1.5 mmol·L^{-1}. Mixed with 1/3 volume of fresh PPP, the fluorescence of platelet suspension was measured with an RF-5000 spectrophotometer (Shimadzu, Japan). Emission wavelength used was 500 nm and the excitation wavelength was switched alternatively between 340 nm and 380 nm. Intracellular Ca^{2+} was estimated by the formula given below[10,11]:

$$[Ca^{2+}]_i = K_d(F_0/F_s)_{380}(R - R_{min})/(R_{max} - R)$$

cAMP measurements PRP was incubated with CVF or vehicle at 37 ℃ for 25 min followed by addition of EDTA-Na_2 240 mmol·L^{-1}. After washing with ACD and resuspending with distilled water, the samples were incubated in boiling water for 5 min and dried at 60 ℃. Redissolved in assay buffer, the concentration of cAMP was determined using commercially available ^{125}I-RIA kit.

Statistics The results are represented as $\bar{x} \pm s$. The significance was determined using the t-test or variance analysis unless otherwise indicated.

RESULTS

Platelet aggregation induced by CVF In the concentration range from 19.5 nmol·L^{-1} to 617 nmol·L^{-1} (the M_r of CVF used to calculate the molar concentration was 226 000), CVF induced rat platelet aggregation in a concentration-dependent manner (Fig 1). The

regression equation of A (amplitude of aggregation) against log concentration of CVF is $Y = 10.493 \lg X + 4.981$ ($r = 0.65$, $P < 0.01$, $n = 20$) and the regression equation of V (maximal velocity of aggregation) against log concentration of CVF is $Y = 6.269 \lg X - 5.168$ ($r = 0.917$, $P < 0.01$, $n = 20$).

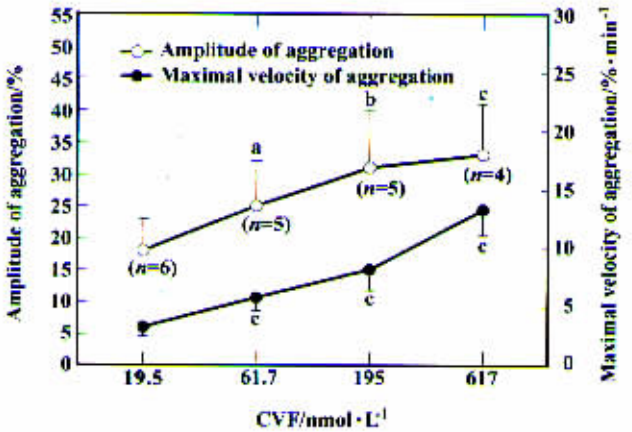

Fig 1. Dose-response curve of CVF-induced rat platelet aggregation. $\bar{x} \pm s$. $^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ *vs* 19.5 nmol·L^{-1} group.

Under the light microscope, platelets became enlarged and swollen at the phase of metamorphosis which lasted 7.7 min ± 2.0 min ($n = 7$) and was followed by aggregation (Fig 2).

ATP release induced by CVF ATP release began in the metamorphosis phase. Amounts of ATP released by CVF were far less than those induced by thrombin (Tab 1, Fig 3).

Tab 1. Rat platelet ATP release induced by CVF and Thr. n is the number of platelet suspensions. $\bar{x} \pm s$. $^cP < 0.01$ *vs* Thr group. $^fP < 0.01$ *vs* CVF 61.7 nmol·L^{-1} group.

		n	ATP/pmol
CVF	61.7 nmol·L^{-1}	4	15 ± 4^c
	195 nmol·L^{-1}	5	58 ± 16^{cf}
	617 nmol·L^{-1}	7	72 ± 26^{cf}
Thr	1 U·ml^{-1}	6	5900 ± 1700

CVF induced prothrombinase expression on the surface of platelets CVF-induce prothrombinase activity on the surface of platelets was time dependent. With CVF 195 nmol·L^{-1}, prothrombinase activities increased from basic value of (0.7 ± 0.4) U ($n = 5$) up to (3.4 ± 0.6) U at 5 min, (3.6 ± 0.6) U at 15 min, (3.1 ± 0.8) U at 30 min, and (2.7 ± 0.7) U at 45 min.

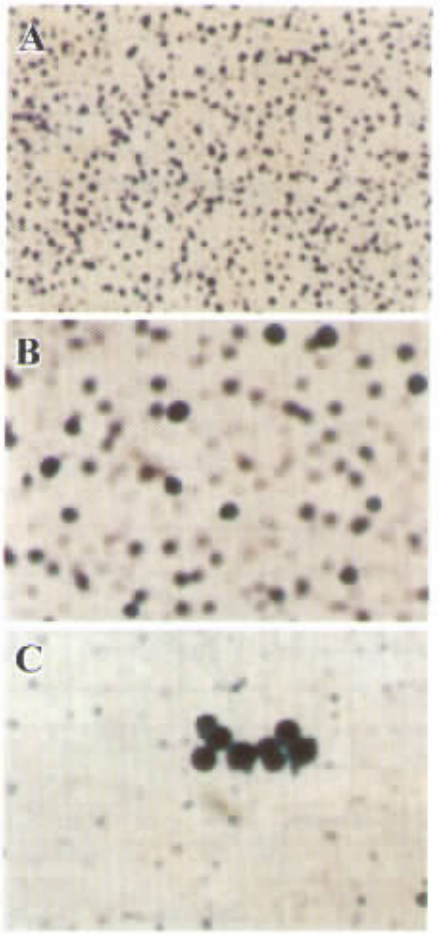

Fig 2. Microphotograph (×1000) of rat platelet metamorphosis and aggregation induced by CVF 195 nmol·L^{-1} (Wright stain). A: normal platelets; B: metamorphosed platelets; C: aggregated platelets.

Influence of monoclonal antibodies against platelet membrane glycoproteins on CVF-induced platelet aggregation SZ-1, SZ-21 and SZ-22 were monoclonal antibodies against GP Ⅰ b/Ⅸ, GP Ⅲ a, and GP Ⅱ b respectively. All of them inhibited platelet aggregation induced by CVF 195 nmol·L^{-1} (Tab 2).

Effects of CVF on intracellular free calcium Rat platelets were loaded with calcium-sensitive fluorophor Fura-2 AM and intracellular Ca^{2+} was detected using dual excitation wavelength fluorospectrophotometer. In resting platelets, $[Ca^{2+}]_i$ was (141 ± 46) nmol·L^{-1}. After addition of CVF 195 nmol·L^{-1}, $[Ca^{2+}]_i$ increased to (240 ± 64) nmol·L^{-1} ($n = 8$, $P < 0.01$, Fig 4).

Effects of CVF on cAMP CVF decreased the

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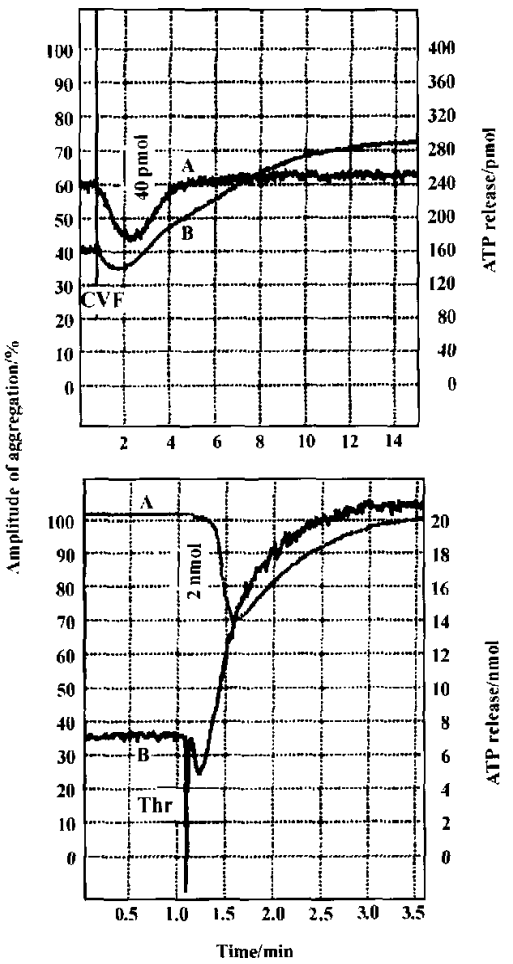

Fig 3. Synchronous tracing of rat platelet aggregation and ATP release induced by CVF 195 nmol·L^{-1} and Thr 1 U·ml^{-1}. A: ATP release; B: aggregation.

cAMP level in platelets. After incubation with CVF 61.7 nmol·L^{-1}, 195 nmol·L^{-1} and 617 nmol·L^{-1} for 25 min, cAMP decreased from (24 ± 7) pmol/10^9 platelets in control to (16 ± 6) pmol/10^9 platelets, (14 ± 6) pmol/10^9 platelets, and (13 ± 5) pmol/10^9 platelets ($P < 0.05$, $n = 6$ *vs* control), respectively.

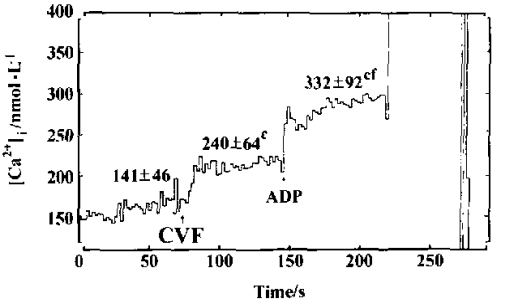

Fig 4. Intracellular free calcium elevation in rat platelets induced by CVF 195 nmol·L^{-1} and ADP 20 μmol·L^{-1}. $n = 8$. $\bar{x} \pm s$. $^cP < 0.01$ *vs* resting $[Ca^{2+}]_i$. $^fP < 0.01$ *vs* CVF group using two-way ANOVA.

DISCUSSION

Though platelet aggregation measurement described by Born[12] is used widely to evaluate platelet activation, sometimes the increase in light transmission does not always reflect platelet aggregation. Ando *et al* noticed that membrane attack complex of complement (MAC) could increase light transmission of platelet suspension, increase ATP release and prothrombinase expression, but platelet aggregates could not be seen under phase contrast microscope[13]. However, according to our observations *in CVF-treated rat PRP*, besides ATP release and prothrombinase expression, both the increase in light transmission and microaggregates as well as metamorphosis were observable, indicating that complement activator CVF separated from Chinese cobra indeed induced platelet aggregation, which might be induced by simultaneous activation of C_3 and C_5.

Tab 2. Influence of monoclonal antibodies against platelet membrane glycoproteins on CVF 195 nmol·L^{-1} induced platelet aggregation. n is the number of platelet suspensions. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* control using paired t-test. A: amplitude of aggregation. V: velocity of aggregation.

	Concentration	n	A/%		V/%·min^{-1}	
			control	after	control	after
SZ-1/μg·ml^{-1}	10	6	46 ± 13	23 ± 10^b	11 ± 4	5.3 ± 2.3^c
SZ-21/μg·ml^{-1}	20	5	42 ± 13	26 ± 7^b	9.8 ± 2.9	5.8 ± 1.8^b
SZ-22/μg·ml^{-1}	20	6	41 ± 12	19 ± 5^c	9.7 ± 2.6	5.0 ± 1.8^c

CVF-induced ATP release was much weaker than thrombin, but it appeared before the aggregation, indicating that CVF-induced mild ATP release response was independent of aggregation. The positive feedback mechanism of platelet aggregation resulted from the release response. The weaker ATP release induced by CVF might have contributed to its mild aggregating effect on platelets.

GP Ⅱ b/Ⅲ a is the receptor of fibrinogen on the platelet membrane. The binding of fibrinogen with the activated GP Ⅱ b/Ⅲ a is a common final pathway leading to platelet aggregation. CVF-induced platelet aggregation was suppressed by SZ-22 and SZ-21, monoclonal antibodies against GP Ⅱ b and GP Ⅲ a respectively. Therefore, CVF might have induced platelet aggregation via this common final pathway. GP Ⅰ b/Ⅸ is another important glycoprotein on the platelet membrane with functions similar to thrombin receptor. The binding of thrombin to GP Ⅰ b/Ⅸ can prompt the transformation of GP Ⅱ b/Ⅲ a to its activated form. GP Ⅰ b/Ⅸ is necessary for CVF-induced platelet aggregation, because CVF-induced platelet aggregation is inhibited by SZ-1, a monoclonal antibody against GP Ⅰ b/Ⅸ.

Wiedmer *et al* observed that MAC increased the prothrombinase activity on the platelet surface[14]. In this study we found that CVF, a complement activator, also increased the prothrombinase activity on the platelet surface, which might be mediated by MAC.

Intracellular free calcium ions play a critical role in platelet activation. CVF-induced platelet aggregation depended on extracellular calcium ion and could be inhibited partially by non-specific calcium channel blocker[5]. With Fura-2 AM loaded platelet preparations, we herein found that CVF elicited moderate rise in $[Ca^{2+}]_i$ that could be further potentiated by ADP. Therefore, CVF may trigger the activation of platelets through calcium influx. cAMP can decrease $[Ca^{2+}]_i$ by prompting the reentry of free Ca^{2+} into Ca^{2+} storage pool. CVF decreased levels of cAMP in platelets, which might have participated in the rise of $[Ca^{2+}]_i$ and the resulting platelet activation. The exact mechanism remains to be clarified.

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眼镜蛇毒因子对大鼠血小板的激活作用[1]

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关键词 眼镜蛇毒液类；补体；血小板；钙；环腺苷一磷酸；凝血酶原；单克隆抗体；Fura-2

目的：研究补体激活剂眼镜蛇毒因子（CVF）对大鼠血小板的作用及其细胞机制．**方法**：比浊法测富血小板血浆内血小板聚集并同步记录 ATP 释放；生色底物法测血小板表面凝血酶原酶活性；用钙离子荧光指示剂 Fura-2 AM 负载血小板测细胞内游离钙；放免法测细胞内 cAMP 含量．**结果**：CVF 在 19.5－617 nmol·L^{-1}范围内浓度依赖性地诱导血小板聚集和 ATP 释放，195 nmol·L^{-1}诱导的 ATP 释放不依赖于聚集，且明显弱于凝血酶 1 U/ml 的作用．CVF 195 nmol·L^{-1}时间依赖性地增加血小板表面凝血酶原酶活性．抗血小板膜糖蛋白Ⅰb/Ⅸ、Ⅲa、Ⅱb 的单克隆抗体 SZ-1、SZ-21、SZ-22 抑制 CVF 诱导的血小板聚集．CVF 195 nmol·L^{-1}使血小板内游离钙从静息态的（141 ± 46）nmol·L^{-1}升高到（240 ± 64）nmol·L^{-1}，在 61.7－617 nmol·L^{-1}范围内轻度降低血小板内的 cAMP．**结论**：补体激活剂 CVF 能诱导大鼠血小板聚集、ATP 释放，增加血小板表面凝血酶原酶活性，其激活血小板的作用与血小板表面纤维蛋白原受体及血小板内游离钙升高、cAMP 下降有关．

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