Preventive Effect of 2-Halogen-Substituted Derivatives of Cyclic Adenosine 3',5'-Monophosphate on Experimental Disseminated Intravascular Coagulation

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Cyclic adenosine 3',5'-monophosphate (cAMP) derivatives were tested for inhibitory effect on the development of experimental disseminated intravascular coagulation (DIC). Two compounds, 2-Cl–cAMP and 2-Br–cAMP, which inhibited the platelet aggregation induced by collagen in vitro, had a strong inhibitory effect on the development of experimental DIC in rats in vivo as judged by five diagnostic laboratory tests, prothrombin time (PT), activated partial thromboplastin time (aPTT), fibrin and fibrinogen degradation products (FDP), fibrinogen and platelet count. The effect of these derivatives was preventive rather than therapeutic, and the inhibition of rat platelet aggregation by these substances was marked when the stimulator was collagen or thrombin but less strong when it was calcium ionophore A23187 or adenosine 5'-diphosphate (ADP).

Introduction

Disseminated intravascular coagulation (DIC) is a pathologic process due to the injury of endothelial cells, tissues, red blood cells or platelets.[1] Damage to these cells caused by various etiologic stimuli result in a final common product, thrombin, which elicits formation of fibrin, consumption and loss of platelets,[2] and secondary activation of the fibrinolytic system.[3−5] The combination of these effects leads to the clinical manifestations of diffuse hemorrhage and fibrin thrombus formation.

Thrombin aggregates platelets irreversibly at concentrations much lower than those needed to form a fibrin clot,[2] and activated platelets release many procoagulants (factors V, VIII, XII and XIII) and phospholipids,[6−8] the components needed for proper functioning of both the intrinsic and extrinsic clotting systems. Therefore, it is probable that platelet aggregation plays an important role in the induction and development of DIC, and we attempted to evaluate the preventive effect of cyclic adenosine 3',5'-monophosphate (cAMP) derivatives, which inhibited platelet aggregation in vitro, on experimental DIC.

Intracellular cAMP is thought to be one of the second messengers,[9] which mediate various extracellular stimuli by regulating metabolic processes. Dibutyryl cyclic adenosine 3',5'-monophosphate (DBcAMP) is transferred into the cell interior and hydrolyzed therein to produce cAMP, and is used widely for increasing intracellular cAMP content directly.[10] It depresses responses (such as platelet aggregation) that are inhibited by an increase in intracellular cAMP concentration.[11] We examined some cAMP derivatives including DBcAMP for ability to inhibit platelet aggregation and to prevent the development of experimental DIC in rats.

Materials and Methods

Materials——All the derivatives of cAMP used in this work were generously supplied as the free acids by Seishin Pharmaceutical Co., (Tokyo), and cAMP was purchased from Sigma (St. Louis, Mo., U.S.A). These compounds were dissolved in 0.15 M NaCl solution (saline) and stored below $-30\,^\circ$C until use.

Experimental Model of DIC——Experimental DIC was produced by an endotoxin injection essentially according to the method of Schoendorf et al.[12] Endotoxin (Escherichia coli 026: B6 lipopolysaccharide, control No. 708944, Difco, Detroit, U.S.A.) was freshly dissolved in saline before every experiment. Endotoxin was injected intravenously into rats (Sprague-Dawley, male, 7—9 week old) at a dosage of 1.0 mg/kg. Individual rats were treated with a cAMP derivative intravenously 1 h before the endotoxin injection in order to estimate the preventive effect. Blood was obtained at various times by heart puncture, citrated (0.38% sodium citrate), and centrifuged at $3000 \times g$ for 10 min to obtain plasma, and PT (prothrombin time), aPTT (activated partial thromboplastin time), content of fibrinogen (Fbg) and FDP (fibrin and fibrinogen degradation products) were determined. The number of platelets in citrated whole blood was counted by using a platelet counter (model PL-100, Toa Medical Electr. Co., Ltd., Kobe).

PT and aPTT were measured by the standard methods as described elsewhere.[13—16] Fbg content was determined by the thrombin time method[17] using Data-Fi Fibrinogen Determination Reagents (Dade Diagnostic Inc., Aguada, Puerto Rico) by the procedure recommended by the manufacturer, and expressed as mg/dl. FDP was determined by means of the latex agglutination test[18] using FDPL Test Kit (Teikoku Hormone Mfg. Co., Tokyo).

Platelet Aggregometry——Blood was drawn from each rat heart and anticoagulated with 1/10 volume of 3.8% sodium citrate. Platelet aggregation was performed essentially according to the method of Born.[19] Citrated blood was centrifuged for 10 min at $150 \times g$ to obtain the platelet-rich plasma (PRP). The precipitate layer including the blood cells was centrifuged again for 20 min at $3000 \times g$, and platelet-poor plasma (PPP) was obtained as the supernatant. The number of platelets contained in PRP was counted, and PPP was added to PRP to adjust the number of the platelets in the plasma to $3.0 \times 10^5/\mu$l. Washed platelets were prepared by the gel filtration method[20] using a Sepharose 2B column (16×240 mm) with Ca^{2+}-free Tyrode's buffer (0.137 M NaCl, 0.42 mM NaH_2PO_4, 2.0 mM $MgCl_2$, 12 mM $NaHCO_3$, 2.0 mM glucose, pH 7.3) containing 3.0% bovine serum albumin as an elution buffer, and used for the study of thrombin-stimulated platelet aggregation. The number of cells in the washed platelet preparation was also adjusted to $3.0 \times 10^5/\mu$l. Collagen (Collagenreagent Horm, Hormon-Chemie, München, GmbH), adenosine 5'-diphosphate (ADP), thrombin (Green Cross Company, Osaka), and calcium ionophore A23187 were used as platelet aggregation-inducing reagents at final concentrations of 20 μg/ml, 1.0×10^{-4} M, 1.0 IU/ml, and 10 μM, respectively. In the case of aggregation induced by thrombin, washed platelets were used in place of PRP to avoid the coagulation of plasma. Platelet aggregation profile was recorded in terms of the increase in light transmission at 600 nm. Maximum aggregation (MA) was measured from the aggregation profile, and percent inhibition was calculated with the following equation.

$$\text{percent inhibition} = \frac{\text{MA (control)} - \text{MA (cAMP derivative)}}{\text{MA (control)}} \times 100$$

Results

Endotoxin-Induced Experimental DIC in Rats

Endotoxin was injected into rats intravenously at the dosage of 1 mg/kg, and blood was obtained by heart puncture at a required time. Five laboratory tests were performed with the blood obtained to make a diagnosis of DIC (Table I). Decrease in Fbg and increase in FDP were observed within 2 h after an endotoxin injection, and the deviations of these values from control values (Time 0 in Table I) became more marked at 4 h after the endotoxin injection. PT and aPTT were prolonged by 1 or 2 h after the endotoxin injection. Thus, rats revealed marked DIC at 4 h after the endotoxin injection as judged from the above five laboratory tests. Blood obtained 4 h after the endotoxin injection was used for the evaluation of DIC in this study.

Effects of cAMP and cAMP Derivatives on Experimental DIC

The DIC-preventive effect of cAMP derivatives which inhibited platelet aggregation were evaluated with endotoxin-induced experimental DIC in rats (Table II). Individual derivatives of cAMP were administered to rats intravenously at the dosage of 25 or 2.5 mg/kg 1 h before the endotoxin injection, and blood samples were obtained 4 h after the endotoxin injection.

TABLE I. Five Laboratory Tests Used as Parameters of Experimental DIC Induced by Endotoxin

Time after endotoxin administration (h)	PT[a] (s)	aPTT[a] (s)	Platelet count[a] ($\times 10^4/\mu l$)	Fbg[a] (mg/dl)	FDP[b] ($\mu g/ml$)
0	12.8 ± 0.5	20.6 ± 0.9	81.8 ± 6.0	158.3 ± 10.9	2.5
1	$15.3 \pm 0.4^{c)}$	21.5 ± 0.9	80.0 ± 5.3	153.3 ± 8.8	3
2	$18.6 \pm 0.5^{d)}$	$26.4 \pm 1.1^{c)}$	63.3 ± 4.4	$111.7 \pm 7.3^{c)}$	5
4	$25.1 \pm 0.5^{d)}$	$49.3 \pm 5.0^{d)}$	$39.8 \pm 3.9^{d)}$	$84.5 \pm 4.6^{d)}$	12

a) Values are means ± S.E.M. (n = 3). b) Values are means (n = 3). The values were significantly different (c) p < 0.05, d) p < 0.01) from control values (time 0).

TABLE II. Effects of cAMP Derivatives on Values of Five Laboratory Tests in Experimental DIC

Sample	Dose (mg/kg)	PT[a] (s)	aPTT[a] (s)	Platelet count[a] ($\times 10^4/\mu l$)	Fbg[a] (mg/dl)	FDP[b] ($\mu g/ml$)
Without endotoxin	—	$12.8 \pm 0.4^{d)}$	$19.8 \pm 0.8^{d)}$	$83.2 \pm 4.1^{d)}$	$176.0 \pm 6.5^{d)}$	2.5
Saline (control)	—	25.3 ± 0.5	51.3 ± 3.9	37.6 ± 3.6	87.0 ± 5.9	13.7
2-Cl–cAMP	25	$17.8 \pm 1.0^{d)}$	$32.0 \pm 2.2^{d)}$	$74.2 \pm 5.4^{d)}$	$156.3 \pm 18.7^{d)}$	2.5
	2.5	$15.5 \pm 2.2^{d)}$	$25.1 \pm 2.3^{d)}$	$58.4 \pm 5.6^{c)}$	$140.5 \pm 8.0^{d)}$	4.1
2-Br–cAMP	25	$18.5 \pm 1.1^{d)}$	$28.2 \pm 1.2^{d)}$	$86.3 \pm 5.9^{d)}$	$160.0 \pm 8.2^{d)}$	1.0
	2.5	$19.4 \pm 1.5^{d)}$	$25.6 \pm 1.7^{d)}$	$59.3 \pm 8.8^{c)}$	$133.1 \pm 6.9^{d)}$	9.3
cAMP	25	$21.9 \pm 1.6^{c)}$	$30.0 \pm 0.6^{d)}$	40.5 ± 2.0	82.6 ± 4.3	16.2
DBcAMP	25	$21.2 \pm 0.7^{d)}$	$31.0 \pm 0.8^{d)}$	41.2 ± 4.5	90.6 ± 4.6	25.4

a) Values are means ± S.E.M. (n = 4—6). b) Values are means. Each derivative was preadministered to rats 1 h before an endotoxin injection. Five laboratory tests were performed on blood which was obtained 4 h after the endotoxin injection. The values were significantly different (c) p < 0.05, d) p < 0.01) from control values.

The five laboratory tests mentioned above were performed. The rats that had received 2-Cl–cAMP or 2-Br–cAMP showed restored values in all five laboratory tests at the dosage of 2.5 mg/kg as well as 25 mg/kg, and these two compounds were thus proved to inhibit the development of experimental DIC potentially. On the other hand, cAMP and DBcAMP poorly suppressed the experimental DIC. Improved values were obtained in PT and aPTT as compared with those of the experimental DIC state, but little therapeutic effect was seen in terms of FDP, Fbg and platelet count. Neither 2-Cl–cAMP nor 2-Br–cAMP at the dosage of 25 mg/kg had any effect in any of the five laboratory tests when administered to rats 5 h before blood collection. Namely, the rats that received 2-halogen-substituted cAMPs without endotoxin did not have significantly different values from normal rats in diagnostic tests.

Effects of Administration Time of cAMP Derivatives

2-Cl–cAMP or 2-Br–cAMP was administered to individual rats at various times before or after the endotoxin injection, and the five laboratory tests were performed on blood samples obtained 4 h after the endotoxin injection (Table III). Pretreatment or simultaneous treatment with 2-Cl–cAMP or 2-Br–cAMP had a preventive effect on the endotoxin-induced experimental DIC. However, the rats that received one of these two compounds 1 or 2 h after the endotoxin injection showed only a small therapeutic effect.

Effects on Rat Platelet Aggregations in Vitro

These derivatives of cAMP were selected on the basis of their potent ability to inhibit the platelet aggregation induced by collagen, so we examined the inhibitory effect of 2-halogen-substituted cAMP on rat platelet aggregations induced by various agonists (Table IV).

TABLE III. Effect of Administration Time of 2-Halogen-Substituted cAMP

Administration time (h)	$PT^{a)}$ (s)	$aPTT^{a)}$ (s)	Platelet count$^{a)}$ ($\times 10^4/\mu l$)	$Fbg^{a)}$ (mg/dl)	$FDP^{b)}$ ($\mu g/ml$)
Without endotoxin	$12.8 \pm 0.4^{d)}$	$19.8 \pm 0.8^{d)}$	$83.2 \pm 4.1^{d)}$	$176.0 \pm 6.5^{d)}$	2.5
Saline (control)	25.3 ± 0.5	51.3 ± 3.9	37.6 ± 3.6	87.0 ± 5.9	13.7
2-Cl-cAMP -2	$15.1 \pm 0.3^{d)}$	$23.9 \pm 0.3^{d)}$	$84.0 \pm 1.0^{d)}$	$176.8 \pm 6.5^{d)}$	1.2
-1	$17.3 \pm 1.0^{d)}$	$32.1 \pm 6.0^{c)}$	$73.8 \pm 4.9^{d)}$	$169.4 \pm 17.7^{d)}$	2.4
0	26.9 ± 5.3	$35.2 \pm 0.3^{c)}$	$76.8 \pm 2.1^{d)}$	97.7 ± 6.6	4.8
1	$22.6 \pm 0.4^{d)}$	39.0 ± 8.8	$86.1 \pm 6.5^{d)}$	80.3 ± 28.4	11.2
2	$22.7 \pm 1.0^{c)}$	$33.3 \pm 3.1^{c)}$	50.3 ± 6.7	105.8 ± 16.8	10.0
2-Br-cAMP -2	$15.6 \pm 0.3^{d)}$	$21.6 \pm 0.3^{d)}$	$76.0 \pm 1.0^{d)}$	$179.2 \pm 6.5^{d)}$	2.4
-1	$14.8 \pm 1.4^{d)}$	$28.6 \pm 1.3^{d)}$	$85.9 \pm 6.3^{d)}$	$173.7 \pm 7.4^{d)}$	1.1
0	$19.3 \pm 1.9^{d)}$	$30.8 \pm 2.0^{d)}$	$81.7 \pm 11.9^{d)}$	$145.7 \pm 9.8^{d)}$	5.6
1	21.9 ± 4.0	38.7 ± 9.6	52.4 ± 13.6	118.0 ± 39.3	10.0
2	23.4 ± 3.0	39.8 ± 4.9	58.0 ± 11.6	106.2 ± 28.3	12.6

a) Values are means $\pm$ S.E.M. ($n = 4$—6). b) Values are means ($n = 4$—6). 2-Halogen-substituted cAMP was administered to rats before or after the endotoxin injection at the dosage of 25 mg/kg. The values were significantly different (c) $p < 0.05$, d) $p < 0.01$) from control values.

TABLE IV. Inhibitory Effects of cAMP Derivatives on Rat Platelet Aggregation

Inhibition (%)	Collagen	ADP	Thrombin	A23187
2-Cl-cAMP	94.7 ± 4.6	16.3 ± 1.6	48.0 ± 11.5	10.3 ± 6.5
2-Br-cAMP	86.4 ± 15.3	6.3 ± 8.5	45.8 ± 16.2	17.1 ± 7.2
DBcAMP	92.0 ± 9.5	30.4 ± 5.7	91.4 ± 15.4	17.5 ± 2.4

Each cAMP derivative was added to a platelet suspension at 1.0×10^{-3} M (final concentration) 5 min before the inducer was added. Values are means $\pm$ S.D. ($n = 4$).

Collagen, ADP, thrombin and calcium ionophore A23187 were used as the inducers of rat platelet aggregations. 2-Halogen-substituted derivatives of cAMP inhibited the rat platelet aggregation when either thrombin or collagen was used as the stimulator of the aggregation, but had little effect when either ADP or A23187 was used. DBcAMP had an inhibitory effect similar to that of 2-halogen-substituted cAMPs on rat platelet aggregation *in vitro*, although it was less able to prevent the endotoxin-induced experimental DIC *in vivo* (see Table II).

Discussion

In the DIC state, excessive activation of the clotting system occurs, producing thrombin and causing a deficiency of several clotting factors. Therefore, PT and aPTT are prolonged as compared with normal values. Thrombin irreversibly aggregates platelets at concentrations much lower than those needed to clot fibrinogen to remove platelets from the circulation, and the number of platelets decreases in the blood.[2] Moreover, substantial fibrinolysis occurs in DIC, probably by plasmin, and Fbg and FDP are very important in the hemorrhagic diathesis of DIC. Thus, these five laboratory tests are thought to be good diagnostic indicators of DIC, with reference to the considerations of Colman *et al.*[1] and Yahara *et al.*[21]

Experimental DIC used in this study represents endotoxemia (mostly in gram-negative septicemia), which causes endothelial cell injury. The damage induced by endotoxin activates Hageman factor and the intrinsic clotting system to elicit DIC.[1] In this experimental model of

DIC, 2-halogen-substituted cAMP suppressed the development of DIC. The two compounds also inhibited rat platelet aggregation, especially that induced by thrombin or collagen. Collagen comes into contact with platelets in blood vessels as a result of injury to the endothelial cells in gram-negative septicemia-induced DIC, and thrombin is probably a very important key factor in the generation of DIC. Therefore, the finding that 2-halogen-substituted cAMPs were inhibitory (mostly to the reactions induced by collagen and thrombin) may be important for the prevention of experimental DIC. DBcAMP, however, could not prevent ongoing experimental DIC notwithstanding its strong inhibitory effect on rat platelet aggregation *in vitro*. This discrepancy might be due to differences in bioavailability or half-life in the blood stream after administration. Alternatively, these two chemicals may have actions distinct from the inhibition of platelet aggregation.

Therapy of underlying disease is most important, but certainly the prevention of development of DIC is temporarily life-saving and may provide a chance to cure DIC, which is noteworthy. 8-Substituted derivatives of cAMP have been reported to be more effective than cAMP itself in inhibiting phosphodiesterase,[22] activating protein kinase[23] and stimulating the synthesis of β-galactosidase in *Escherichia coli*,[24] but little is known about the biological activities of 2-substituted derivatives of cAMP. The foregoing results show that 2-Cl-cAMP and 2-Br-cAMP have stronger preventive effects than cAMP itself or DBcAMP, although the mechanism of this phenomenon is not clear at present.

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