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The Effects of an Antimetastatic Agent, (±)-1,2-Bis(3,5-dioxopiperazin-1-yl)propane (ICRF 159), on Platelet Behavior

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SUMMARY

Some agents that inhibit platelet aggregation, *e.g.*, aspirin and dipyridamole, have been reported to prevent metastasis formation. To see whether inhibition of platelet aggregation could account for the antimetastatic action of (±)-1,2-bis(3,5-dioxopiperazin-1-yl)propane, this compound was investigated *in vivo* for any effects on platelet behavior and thrombogenesis. (±)-1,2-Bis(3,5-dioxopiperazin-1-yl)propane inhibited the formation of platelet thrombi in blood vessels on the surface of the rat brain and in the hamster cheek pouch. 1,2-Bis(dioxo-4-methylpiperazin-1-yl)ethane, a closely related analog of (±)-1,2-bis(3,5-dioxopiperazin-1-yl)propane, but without antimetastatic action, inhibited thrombus formation *in vivo* as effectively as (±)-1,2-bis(3,5-dioxopiperazin-1-yl)propane. It seems unlikely therefore that the antimetastatic action of (±)-1,2-bis(3,5-dioxopiperazine-1-yl)propane derives from its effects on thrombogenesis. Neither platelet numbers nor the ionized plasma calcium concentrations were changed after (±)-1,2-bis(3,5-dioxopiperazin-1-yl)propane administration.

INTRODUCTION

The adhesion of tumor cells to blood vessel endothelium is thought to be associated with the production of intravascular thrombi at the site of cell lodgement. This process is probably a necessary 1st step in the establishment of metastases from a tumor that disseminates via the blood stream. In rabbit ear chamber vessels Wood (14) observed the formation of such thrombi around rabbit V_2 carcinoma cells injected i.v. that stuck to the vessel endothelium. While enmeshed within these thrombi, the cells leave the vessel via the interendothelial cell junctions and subsequently divide to form new deposits. Warren and Vales (13) examined the ultrastructure of adherent tumor cells in damaged venules and also found them to be surrounded by a fibrin and platelet network.

These and other observations have led to attempts to prevent metastases by using agents that reduce the platelet count or inhibit their aggregation. Gasic *et al.* (6) reduced the number of TA_3 ascites metastases in the lungs of mice made thrombocytopenic by neuraminidase treatment. Aspirin, an inhibitor of platelet aggregation, reduces metatases after i.v. injections of BW 10232 cells (9) and after T241 and MCA2 ascites cells (5). Dipyridamole, another inhibitor of platelet aggregation, inhibits platelet adhesiveness to circulating tumor cells (7).

Treatment with ICRF 159[1] completely inhibits pulmonary metastases in mice implanted with Lewis lung carcinoma at doses that do not affect the growth rate of the primary implant (10). It also inhibits the appearance of spontaneous liver metastases from a s.c. implant of the hamster lymphoma (ML) at doses that do not decrease the growth rate of the primary, whereas a compound with a closely related chemical structure, ICRF 158, is ineffective at the same dose regimens.[2] The mechanism of this inhibition may be due to the prevention of the release of cells from the primary tumor as a result of the normalization of the developing tumor vasculature (10, 12).[2] It is possible, however, that other mechanisms of action may be involved. Since experimental metastases can apparently be inhibited by antithrombotic agents, it seemed of interest to find out whether ICRF 159 was able to prevent thrombus formation.

Tests for platelet function in hamsters were therefore carried out with doses of ICRF 159 known to prevent secondaries in this species. In addition, ICRF 158, a close analog of ICRF 159, but without antimetastatic action, was also tested for antithrombotic activity. The immediate effect of a single injection of ICRF 159 on the platelet count was determined, and because ICRF 159 is an *in vivo* and *in vitro* chelating agent the ionized calcium concentration in the blood was also measured.

MATERIALS AND METHODS

Drugs

ICRF 158 and ICRF 159 were suspended in a solution of 0.5% CMC. Each agent was ball-milled for 24 hr in the dry

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[1] The abbreviations used are: ICRF 159, (±)-1,2-bis(3,5-dioxopiperazin-1-yl)propane; ICRF 158, 1,2-bis(3,5-dioxo-4-methylpiperazin-1-yl)ethane; CMC, carboxymethylcellulose; MEC, minimum effective concentration.

[2] A. Atherton, unpublished data.

form before use. Hamsters received an i.p. injection of 7.5 to 60 mg of these drugs per kg in a volume of 4 ml per kg. The rats were given an i.m. injection of 12.5 to 50 mg/kg in a volume of 5 ml/kg body weight. Control animals were treated with the appropriate volume of CMC only.

Determination of Antithrombotic Activity

Thrombogenesis in the Hamster Cheek Pouch. The cheek pouches of cream hamsters weighing approximately 150 g were prepared for microscopic observation as previously described (1). Microthrombi were induced in venules by the iontophoretic application of ADP (10^{-2} M) via a micropipet. This method allows the repeated application of a constant amount of ADP at 1 site. The delay between switching on the iontophoretic current and the 1st appearance of platelets adhering to the vessel wall was timed every 5 min before and after i.p. injections of ICRF 159. This delay has previously been shown to be constant for 2 to 3 hr in untreated hamsters (2). In all experiments, vessels with diameters between 60 to 70 μm were used, and the ADP was applied with a current of 400 na for no longer than 60 sec every 5 min.

Venules were traumatized by pushing the tip of a glass micropipet 5 to 10 μm through the vessel wall. This damage caused repeated production of thrombi followed by their embolization. Every embolization from each damage site was recorded on an event marker. The length of time during which embolization occurred is defined as "activity time" of that site.

Thrombogenesis in the Rat Brain. Thrombogenesis was induced in blood vessels on the surface of the rat brain by the method of Honour and Mitchell (8).

The exposed vessels (72 to 200 μm in diameter) of male Charles River Fischer rats (60 to 120 g) anesthetized with urethan were compressed with forceps to produce a minor injury (*i.e.*, no bleeding). The brain surface was bathed with a warm (37°) solution of ADP in 0.9% sodium chloride solution and the MEC of ADP required to induce thrombus formation in the injured vessel was determined. The damaged site continues to produce embolizing thrombi for some time, and the activity time and rate of embolization were recorded also. These observations were repeated on different vessels at 15- to 20-min intervals for about 75 min after a single i.m. injection of ICRF 159 or ICRF 158.

Measurement of Ionized Plasma Calcium

The carotid arteries of 10 hamsters were cannulated and 3.0-ml samples of blood were taken under liquid paraffin 30 min before an i.p. injection of either ICRF 159, 60 mg/kg, or CMC. After a further 30 min another sample of blood was taken. The serum was analyzed for ionized calcium by the use of the Orion flowthrough electrode system (11).

Platelet Count

Samples of blood (0.1 ml) from the carotid arteries of 4 hamsters were collected into disodium EDTA (Sequestrene) tubes immediately after and 30, 40, 60, 120, and 180 min after cannulation. Injections of ICRF 159, 60 mg/kg, or CMC i.p., were made at 32 min after cannulation. Platelets were counted by the method of Brecher and Cronkite (3).

RESULTS

Effect of ICRF 159 and ICRF 158 on Thrombogenesis in the Hamster Cheek Pouch. In 22 experiments the delay preceding ADP-induced thrombus formation was measured before and after ICRF 159 injection. Within 5 min of an i.p. injection of ICRF 159, 15, 30, and 60 mg/kg, the delay before the 1st platelets were seen sticking to the venule wall increased greatly. CMC had no effect. For evaluation of the relative effectiveness of each dose, the average delay was calculated for readings taken during a period of 30 min before and during 3 consecutive 30-min periods after the injection of ICRF 159. The results for all experiments are shown in Table 1.

The percentage increase in mean delay to thrombus formation during each 30-min period before treatment was calculated for each dose of ICRF 159 (Chart 1). ICRF 159, 60 or 30 mg/kg, rapidly inhibited the onset of thrombus formation by over 200%, while the effects of 15 and 7.5 mg/kg on the onset of thrombus formation were slightly delayed and the inhibition was less marked. In a few cases the activity of ICRF 159 started to fall approximately 1 hr after injection, but in most experiments the drug was still active after 2 hr.

ICRF 159 and ICRF 158 (both at 60 mg/kg) were also effective in reducing the number of emboli leaving the site of injury while CMC was inactive (Table 2). The results of 1 such experiment with ICRF 159 are shown in Chart 2. Close observation of the injury site after treatment suggested that, although the thrombi were forming after an increased delay, their rate of formation was similar to their rate of disintegration whereas in control animals the rate of formation allowed the build-up of a compact thrombus which broke cleanly away from the vessel wall as a single embolus.

Effects of ICRF 159 and ICRF 158 on Thrombogenesis in the Rat Brain. Single i.m. injections of ICRF 159 or ICRF 158 inhibited thrombogenesis in damaged blood vessels on the surface of the rat brain. Doses of 50 or 25 mg of either compound per kg increased the MEC of ADP from a control value of 3 μg/ml to either 5 or 10 μg/ml and reduced the activity time of the site and the rate of embolization by over 50%. Inhibition developed rapidly after the injections and was still apparent 75 min later. A dose of 12.5 mg of either compound per kg did not affect the MEC of ADP, but it reduced the activity time and embolization rate by about 30 to 50%.

Effects of ICRF 159 on ionized Serum Calcium Concentration. In hamsters treated with both ICRF 159 and CMC, the total ionized calcium concentration in serum remained unaffected. The mean value for all animals before treatment was 5.35 ± 0.11 mg/100 ml serum, after treatment with CMC it was 5.42 ± 0.18 mg/100 ml, and

Table 1

Delay before appearance of ADP-induced platelet thrombi in hamster cheek pouch venules before and after treatment with ICRF 159

In hamster cheek pouch venules, the mean delay before thrombus formation was determined during a period of 30 min before and during 3 consecutive 30-min periods after a single i.p. injection of ICRF 159 or CMC. Since the ADP was applied for a maximum period of 60 sec during any 1 attempt to form a thrombus, a possible maximum value of 60.0 ± 0.0 would indicate total and immediate inhibition of thrombus formation.

Dose (mg/kg)	Hamster	Time (sec) before appearance of thrombi			
		30 min before injection	0-30 min after injection	30-60 min after injection	60-90 min after injection
CMC (control)	1	8.2 ± 0.8[a]	6.1 ± 0.3	8.3 ± 0.9	7.0 ± 0.5
	2	11.5 ± 2.2	9.3 ± 1.5	13.4 ± 2.5	13.2 ± 1.9
	3	8.6 ± 1.1	8.4 ± 0.2	10.5 ± 1.5	
	4	21.4 ± 2.5	20.6 ± 1.6	20.8 ± 3.1	
	5	7.0 ± 0.9	11.2 ± 1.1	23.5 ± 2.3	25.4 ± 4.1
	6	10.2 ± 2.0	9.2 ± 2.5	8.8 ± 0.9	
7.5	7	12.5 ± 0.9	16.1 ± 1.7	17.5 ± 2.0	21.0 ± 0.0
	8	7.1 ± 0.8	9.5 ± 1.4	5.5 ± 0.5	
	9	19.8 ± 1.2	20.1 ± 2.2	15.0 ± 4.2	25.3 ± 5.4
15	10	15.6 ± 2.7	23.3 ± 4.4	48.3 ± 6.2	
	11	13.7 ± 1.6	21.1 ± 2.7	36.1 ± 6.7	20.0 ± 0.0
	12	21.1 ± 1.4	24.3 ± 2.0	38.8 ± 7.1	54.2 ± 4.0
30	13	11.7 ± 1.2	33.4 ± 7.3	41.8 ± 8.5	33.0 ± 13.7
	14	12.3 ± 1.5	29.8 ± 5.7	29.5 ± 8.2	25.6 ± 8.8
	15	10.3 ± 1.3	25.8 ± 7.0	41.0 ± 19.0	
	16	8.6 ± 0.7	35.6 ± 5.6		
60	17	11.5 ± 1.9	37.5 ± 13.0	39.3 ± 11.1	
	18	11.2 ± 0.9	31.0 ± 8.1	39.2 ± 8.2	
	19	12.3 ± 1.7	23.5 ± 3.0	19.8 ± 3.1	32.6 ± 5.9
	20	10.6 ± 1.4	30.7 ± 7.3	37.8 ± 7.3	
	21	13.5 ± 1.6	38.8 ± 8.1	41.5 ± 5.6	
	22	9.0 ± 1.2	46.6 ± 8.3	50.4 ± 5.9	

[a] Mean ± S.E.

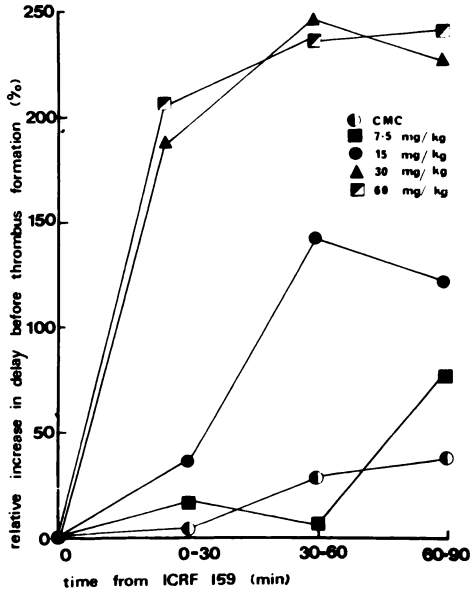

Chart 1. The relative increase in delay to thrombus formation after 4 different doses of ICRF 159 and CMC expressed as a percentage of the delay observed before treatment.

after ICRF 159 it was 5.49 ± 0.20 mg/100 ml. A Student *t* test revealed no significant difference in ionized calcium between control and treated animals after treatment.

Effect of ICRF 159 on Platelet Count. A single injection of ICRF 159 had no effect on the total platelet count in hamster blood within 2.5 hr of injection.

DISCUSSION

It is not clear whether tumor cells released from a primary tumor adhere only to endothelium that has been previously damaged or whether they are also capable of sticking to normal endothelium. Nor is it known whether tumor cell lodgement initiates thrombus formation, possibly by ADP release, or whether the malignant cells are entrapped within thrombi formed at damaged sites. Both ADP and injury were used as stimuli to thrombus formation *in vivo* in an effort to reconstruct such events. ICRF 159 was shown to be effective in inhibiting the formation, growth, and embolization of platelet thrombi induced by both these methods in hamsters and rats.

The minimum dose of ICRF 159 required to inhibit

Table 2

The influence of ICRF 159 and ICRF 158 on embolus formation after injury to hamster cheek pouch venules

Hamster cheek pouch venules were injured by piercing the endothelium with a sharp micropipet. The mean number of emboli leaving the injury sites during the 1st min were scored before and after the single i.p. injection of ICRF 159, ICRF 158, or CMC.

	No. of emboli formed in the min following injury	
	Before treatment	After treatment
ICRF 159, 60 mg/kg	8.2 ± 1.7[a]	1.5 ± 0.3
	6.7 ± 3.0	1.5 ± 0.6
	3.6 ± 0.9	1.3 ± 0.4
ICRF 158, 60 mg/kg	5.8 ± 0.6	1.0 ± 0.8
	3.6 ± 0.4	1.2 ± 0.4
	6.0 ± 1.2	2.4 ± 0.4
CMC	7.0 ± 3.0	5.0 ± 1.1
	5.0 ± 0.8	5.1 ± 1.3

[a] Mean ± S.E.

metastasis in hamsters lies between 7.5 and 15 mg/kg, and the lowest doses of the drug, which just inhibit thrombus formation in these animals, are in the same region.[2] This dose also inhibits lung metastases in mice (11). This fact and the consideration that some antithrombotic substances will reduce metastases (5, 7, 9) suggest that the action of ICRF 159 may be related to its ability to prevent thrombogenesis.

However, it has previously been suggested that the antimetastatic effect of ICRF 159 is due to its action in normalizing growth of tumor blood vessels in mice (10). This effect has now also been observed in the hamster lymphoma (ML) in which metastases are inhibited by ICRF 159.[2] It is possible, therefore, that either or both of these mechanisms are responsible for the antimetastatic action of ICRF 159. The fact that ICRF 158, while it is an equally potent inhibitor of thrombus formation, is inactive in preventing metastases points to the conclusion that the action of ICRF 159 is not mediated via its antithrombotic activity. To elucidate these points would require a study of the cumulative effect of these substances on the platelets of pretreated animals and of their effect on intravascular adhesion between tumor cells, platelets, and the endothelium. The close chemical relationship of ICRF 159 to EDTA made it possible that the effect of ICRF 159 on thrombus formation could have been due to the chelation of plasma calcium. This, however, was not found to be the case.

The absorption of ICRF 159 into the blood stream after an i.p. injection is immediate and corresponds with its rapid action as seen in the present experiments. The possible cessation of its action on thrombus formation after 1.5 hr agrees reasonably with its clearance from the blood stream (4).

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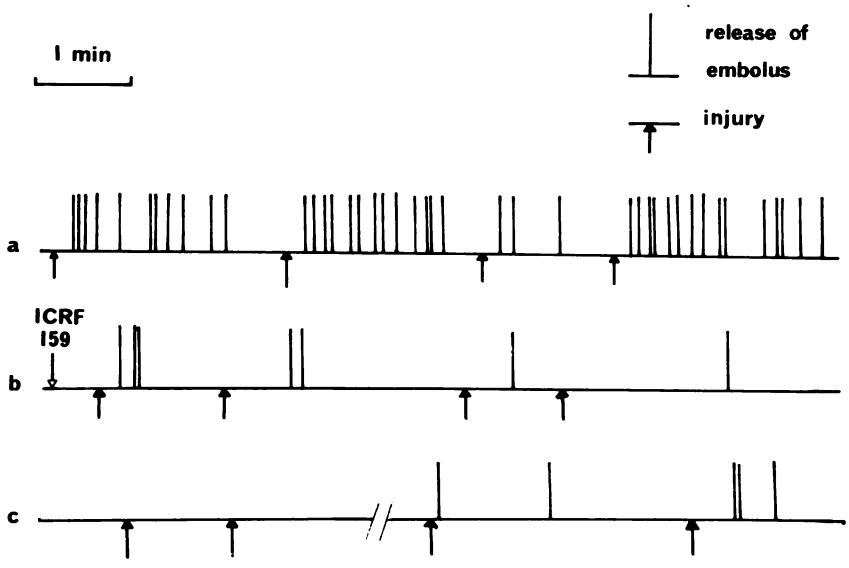

Chart 2. The release of emboli from sites of minor injury in a hamster cheek pouch venule, recorded during a single experiment. *a*, 20 to 27.5 min (before treatment); *b*, 30 to 40 min (ICRF 159, 60 mg/kg, injected at 30.25 min); *c*, 70 to 106 min.

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