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A STUDY OF INTRAVASCULAR PLATELET AGGREGATION BY CONTINUOUS PLATELET COUNTING

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ABSTRACT

Intravascular platelet aggregation has been studied by measuring the response to aggregating agents using continuous platelet counting. Dose-dependent falls in platelet count occurred after the injection of collagen, prostaglandin H_2 (PGH_2), arachidonic acid (AA) and four synthetic prostanoids in rats. Indomethacin partially inhibited collagen- and AA-induced aggregation in the rat and collagen-induced aggregation in the rabbit. Indomethacin and aspirin did not produce a dose-dependent inhibition of collagen-induced aggregation over the range of doses tested. 1-n-butylimidazole inhibited collagen- and PGH_2-induced aggregation in the rabbit but did not inhibit collagen- or AA-induced aggregation in the rat.

INTRODUCTION

The intravascular aggregation of blood platelets has been implicated in the formation of arterial thrombi. The experimental study of platelet aggregation has been hampered by the absence of quantitative *in vivo* methods that involve platelet-vessel wall interactions. Measurement of the circulating platelet count indicates the number of platelets that are not adhering and aggregating in the microvasculature when the count is taken.

Key words: Platelet aggregation, platelet count, collagen, arachidonic acid, prostaglandins.

Born and Cross (1) and Smith and Freuler (2) have shown that adenosine diphosphate (ADP) will cause a fall in the number of circulating platelets in anaesthetised experimental animals. Although collagen and arachidonic acid (AA) have been extensively studied *in vitro*, *in vivo* studies have been restricted to pathological studies (3,4). Also, the synthetic prostanoids, e.g. U44619, have not been the subject of *in vivo* investigation.

Since the continuous platelet count technique (2) can be used in different species of experimental animals, it has been possible to compare the activities of aggregating agents and inhibitors of aggregation in rabbits and rats.

Smith and Willis (5) showed that aspirin prevented the synthesis of prostaglandins in human platelets. It is now well established that these drugs inhibit the cyclo-oxygenase enzyme that converts AA to the cyclic endoperoxides PGG_2 and PGH_2. Holmes (6) showed that aspirin and proquazone, a potent cyclo-oxygenase inhibitor, partially inhibited collagen-induced aggregation in the rat.

Imidazole (7) and 1-n-butylimidazole (8) prevent the conversion of PGH_2 to TxA_2 and also inhibit platelet aggregation *in vitro*.

This paper examines the effects of aggregating agents *in vivo* and attempts to explain the mechanisms involved in intravascular platelet aggregation.

METHODS

Measurement of intravascular platelet aggregation

Male Sprague-Dawley rats (400-550 gm) were anaesthetised by an intraperitoneal injection of pentobarbitone sodium (60 mg/kg). New Zealand white rabbits (male or female, 2.75-4.5 kg) were anaesthetised by an intravenous injection of pentobarbitone sodium (40-60 mg/kg) or a mixture of urethane (420 mg/kg) and diallylbarbitone sodium (105 mg/kg). The trachea and a jugular vein were cannulated and in rabbits a femoral artery was cannulated for the measurement of blood pressure. The platelet count was recorded via a special double cannula inserted into a carotid artery (2). This enabled blood to be continuously sampled by the Technicon Autocounter^{R}. 3.8% ^{W}/v trisodium citrate was pumped to the tip of the cannula and the citrated blood removed at 0.1 ml/min. It was then diluted with a mixture of ammonium oxalate 1% and saponin 0.002% which lysed the erythrocytes. The platelets were counted optically in the counting flow-cell and the number of platelets continuously recorded on pre-calibrated chart paper. The counter was calibrated by previously published procedures (9).

Preparation of PGH_2

1.0 gm of ram seminal vesicle acetone-pentane powder was prepared according to the method of Wallach and Daniels (10)

and suspended in 50 ml of Tris HCl buffer pH 8.5 containing 1 mM phenol. After 30 min at 4°C the suspension was homogenised, p-hydroxymercuric benzoate (0.1 M in Tris HCl pH 8.5) added to the enzyme suspension to give a final concentration of 0.003 M and incubated at 37°C for 15 min. 5 mg of AA (Nu Chek) in 50 µl ethanol was added to the enzyme mixture and stirred vigorously for 2 min. The reaction was terminated by the addition of 7.5 ml of 0.5 M citric acid and the mixture immediately extracted with 2 x 50 ml of ether:methanol (8:1). 1 gm of NaCl was added to aid phase separation which was completed by centrifugation for 2 min. The organic phase was removed, dried over anhydrous magnesium sulphate and evaporated to dryness under vacuum at room temperature. The residue was dissolved in 5 ml of diethyl ether:petroleum ether (60°-80°) 20:80, applied to a column prepared from 1.5 g 100-200 mesh silica gel and developed as described by Ubatuba and Moncada (11). Each fraction collected from the column was subjected to TLC to locate the PGH_2 which was then stored at -20°C in dry acetone.

Drugs and Materials

ADP (Sigma) was infused intravenously for 2 min. Collagen (Diamid Diagnostics) was given as bolus injections to rats and by infusion for 2 min in rabbits. AA (Sigma) was converted to the sodium salt by adding a 1 M solution of sodium carbonate with a microlitre syringe until a clear solution was obtained and the volume adjusted to 1 mg/ml with normal saline. The sodium arachidonate (AA) was given as a bolus injection to rats and by infusion for 2 min in rabbits. PGH_2 was given as a bolus injection to both rats and rabbits. The synthetic prostanoids (Fig. 1) were given as bolus injections to rats. All aggregating agents were given at 15 min intervals and their effects quantitated by measuring the decrease in circulating platelet count that occurred after each injection expressed as a percentage of the platelet count obtained immediately preceding the induced fall. Aspirin (Macarthy Ltd.), indomethacin (MSD) and 1-n-butylimidazole (Koch-Light) were given as bolus injections and their inhibitory activity measured by comparing the mean of two consistent consecutive responses to an aggregating agent with the responses to an aggregating agent in the presence of an inhibitor and calculating the percentage inhibition.

STATISTICAL METHODS

Inhibitory responses were compared using Student's t-test and where this test could not be used due to an invalid F-test, the Welch test was used.

RESULTS

1. The effects of aggregating agents

(a) Rats

Three different doses of either collagen, PGH_2, AA, U44619, U44069, ICI86841, Wy18189 and Wy19068 were given in random order

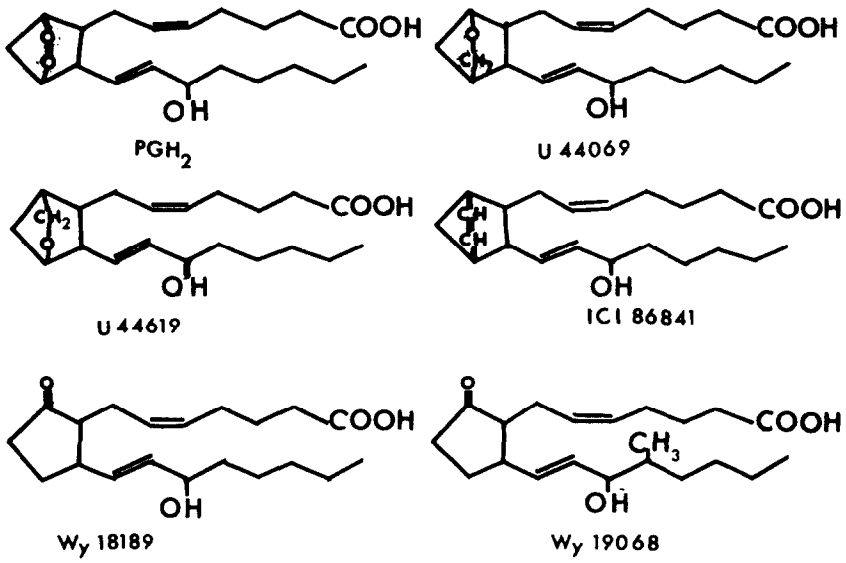

FIG. 1
Chemical structures of PGH_2, U44069, U44619, ICI86841, Wy18189 and Wy19068

to groups of five rats after a priming injection of collagen. As the first response to any aggregating agent has been found to be unreliable for quantitative work, an initial priming dose of an aggregating agent was given before the injection of the agents under investigation. Fig. 2 shows that all the agents produced dose-dependent falls of platelet numbers except U44619 which was inactive at non lethal doses (< 8 ng/kg). U44069 and PGH_2 were the most potent aggregating agents. To show the small 'between animal' variation of responses to aggregating agents used for inhibitory studies, standard errors are included in Fig. 2 for collagen, AA and PGH_2. Collagen and AA gave dose-dependent responses without any signs of respiratory distress. PGH_2 (4-10 µg/kg) produced a small but dose-dependent fall in platelet count. 10 µg/kg was the largest dose that could be given without the solvent affecting the response.

To test the reproducibility of the responses to collagen, 40 µg/kg was injected at 15 min intervals to a group of five rats over a period of 90 min. Fig. 3 shows that consistent responses were obtained but that the aggregation produced was only partially reversible.

(b) Rabbits

Table 2 shows that both collagen and PGH_2 gave consistent responses. Due to the toxicity of higher doses of collagen, a

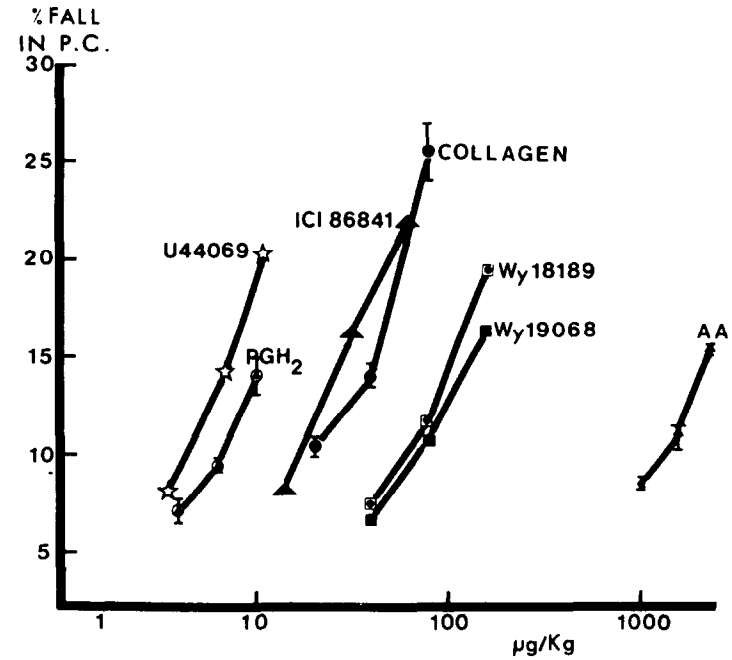

FIG. 2
The effect of PGH_2, collagen, AA and four synthetic prostanoids on intravascular platelet aggregation in the rat. Vertical lines indicate the SEM.

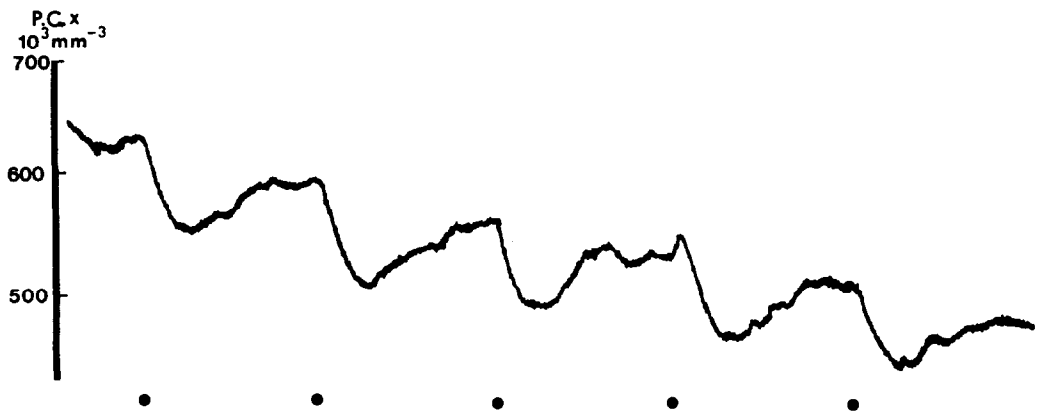

FIG. 3
Reproducibility of collagen-induced platelet aggregation in the rat. Collagen 40 µg/kg was given as shown, at 15 min intervals and the platelet count (PC) recorded over a period of 75 min.

dose-response curve could not be performed. It was also not possible to prepare sufficient PGH_2 for construction of dose-response curves. It was not possible to find a dose of AA that produced a fall in platelet count without the subsequent death of the animal.

TABLE 1
The Effect of Repeated Doses of Collagen and PGH_2 on Platelet Aggregation in the Rabbit
Four doses of 20 µg/kg/min for 2 min of collagen were infused into 4 rabbits at 15 min intervals. Four doses of 20.5 µg/kg of PGH_2 were injected into another 4 rabbits at 15 min intervals.

	% fall in PC mean ± SEM	
Rabbit	Collagen	PGH_2
1	12.3 ± 0.06	11.1 ± 0.69
2	18.4 ± 0.33	10.8 ± 0.38
3	13.7 ± 0.33	12.7 ± 0.78
4	16.4 ± 0.51	10.7 ± 1.58
5		13.0 ± 0.41

2. Effect of inhibitors

(a) Indomethacin

After two consistent consecutive responses to 40 µg/kg collagen, indomethacin (1-8 mg/kg) was injected intravenously into rats followed 5 min later by collagen. Further injections of collagen were given at 15 min intervals. The maximum inhibition was obtained 5 min after the injection of indomethacin and Fig. 4 shows that a dose-dependent inhibition of collagen-induced aggregation only occurred between 1 and 4 mg/kg. Inhibitory activity of 8 mg/kg was significantly less than that of 4 mg/kg ($P < 0.01$). Fig. 4 also shows that indomethacin gave a similar dose-response relationship when 1 mg/kg AA was used as the aggregating agent. Again the inhibitory activity of 8 mg/kg was significantly less than that of 4 mg/kg ($P < 0.001$). In rabbits, using the same experimental design, 4 mg/kg of indomethacin inhibited collagen-induced aggregation by 47.5% (Table 2).

(b) Aspirin

The effects of aspirin (10-80 mg/kg) on collagen-induced aggregation (40 µg/kg) was studied using the same experimental design as for indomethacin. The inhibitory activity of aspirin in rats was again only partial but the inhibition lasted until

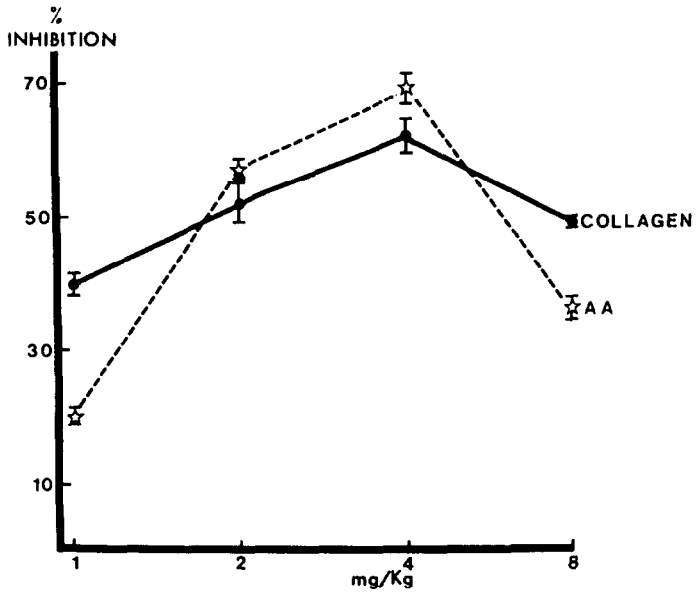

FIG. 4
The effect of indomethacin on aggregation produced by collagen and AA in the rat
After two consistent consecutive responses to 40 µg/kg collagen or 1 mg/kg AA (sodium salt), indomethacin 1-8 mg/kg IV was given, following in 5 min by the appropriate aggregating agent. Each point is the mean of five observations and the vertical lines indicate the SEM.

the end of the experiment (1 hour after administration). A shallow dose-response curve was obtained (Fig. 5) showing again a reduction of inhibition at the highest dose studied. In this experiment the difference was not statistically significant.

(c) 1-n-butylimidazole

50 mg/kg of 1-n-butylimidazole was injected intravenously into rats and its inhibitory activity studied using collagen, AA and PGH_2 as aggregating agents. This was the maximum tolerated dose as 75 mg/kg produced toxic reactions. Table 2 compares the inhibitory activity of indomethacin with that of 1-n-butyl-imidazole. Indomethacin, as shown earlier, partially inhibits collagen-induced aggregation in rats and rabbits and partially inhibits AA-induced aggregation in rats. Inhibition of PGH_2-induced aggregation in both rats and rabbits could not be obtained with indomethacin. 1-n-butylimidazole did not inhibit aggregation induced by collagen or AA in rats but did cause a small but reproducible inhibition of PGH_2-induced aggregation.

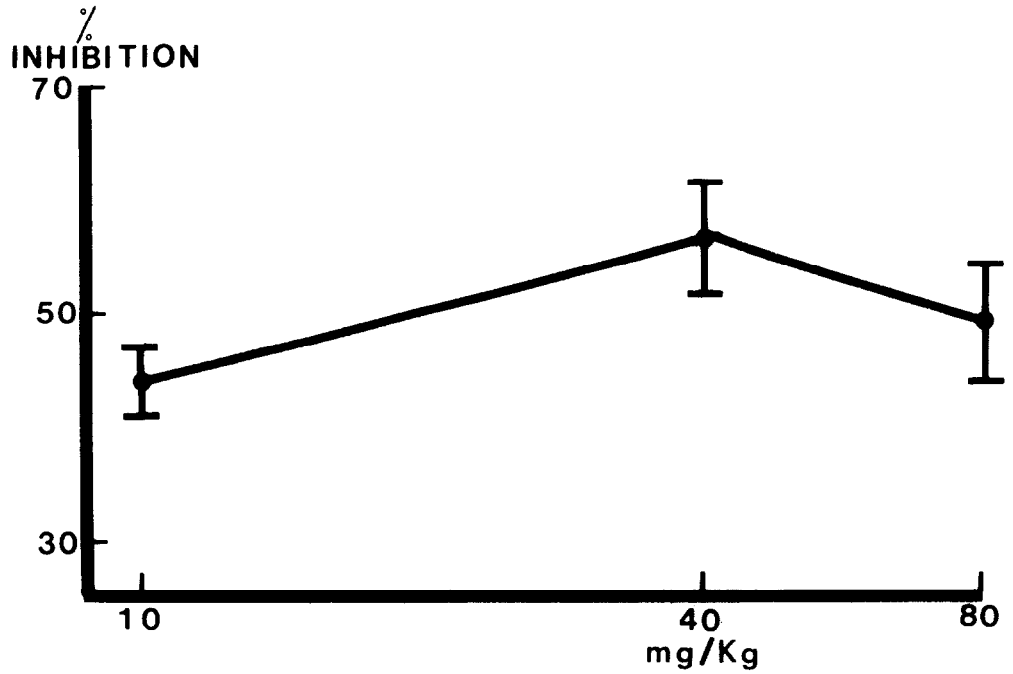

FIG. 5
The effect of aspirin on aggregation produced by collagen in the rat
After two consistent consecutive responses to 40 µg/kg collagen, aspirin, 10-80 mg/kg IV was given followed in 5 min by another dose of collagen. Each point is the mean of five observations and the vertical lines indicate the SEM.

In rabbits, 1-n-butylimidazole completely inhibited aggregation produced by collagen and PGH_2. 1-n-butylimidazole did not inhibit aggregation responses in rabbits induced by an infusion of 25 µg/kg/min of ADP for 2 min.

DISCUSSION

The continuous platelet counting technique has enabled platelet aggregation to be reproducibly quantitated *in vivo*. The bicyclic prostanoid U44069 was the most active aggregating agent, being slightly more active than the natural endoperoxide PGH_2. Nishizawa et al (12) reported that in the rat, PGH_2 did not cause platelet aggregation *in vitro* although TxA_2 is formed from AA. In this *in vivo* study, PGH_2 did produce a small dose-dependent aggregation in the rat. The significant difference between their *in vitro* study and the *in vivo* study described in this paper is the presence of an anti-coagulant in the *in vitro* study. Preliminary experiments in this laboratory have shown that trisodium citrate, 7.5 mg/kg completely inhibited collagen-induced aggregation in the rat. This indicates that either PGH_2

TABLE 2
Comparison of the Effects of 1-n-butylimidazole (1-n-B) and Indomethacin (Indo) on Aggregation produced by Collagen, PGH_2, AA, ADP in rabbits and rats

Inhibitor	Dose mg/kg	Aggregating Agent	Dose µg/kg	% inhibition of PA ± SE	
				Rabbits	Rats
Indo	4	Collagen	40*	47.5±0.7(4)	62±2.7(5)
		PGH_2	20.5[a] 12.5[b]	0(3)	0(1)
		AA	1000	Not tested	69±2.4(5)
1-n-B	25[c] 50[d]	Collagen	40*	100(3)	0(3)
		PGH_2	20.5[a] 12.5[b]	100(3)	23±1.6(5)
		AA	1000	Not tested	0(3)
		ADP	50*	0(3)	Not tested

No. of animals studied are indicated in brackets

* Doses of collagen and ADP in rabbits given as infusions of 20 µg/kg/min and 25 µg/kg/min for 2 min respectively

a - PGH_2 dose in rabbits, 20.5 µg/kg and b - in rats, 12.5 µg/kg.
c - 1-n-B dose in rabbits and d - dose in rats.

requires Ca^{++} ions for aggregation or that the *in vivo* study is just more sensitive to aggregating agents in the absence of citrate. The other bicyclic prostanoid, ICI86841, was about 4 times less active than the Upjohn compound. The two monocyclic compounds that resemble PGE_2 were about 3 times less active than the ICI compound. MacIntyre et al (13) showed that the order of potency of these compounds *in vitro* using rabbit PRP was U44069 > Wy19068 > ICI86841 > Wy18189 whilst in this *in vivo* study in the rat, the order of potency was U44069 > ICI86841 > Wy18189 > Wy19068. This shows that in the rat the compounds that resemble the endoperoxides were the most potent. The inability of U44619 to produce a fall in platelet count in non-toxic doses may indicate that the TxA_2 receptors in the bronchial smooth muscle of the rat are more sensitive to U44619 than those in the platelet.

This study confirms the previous observation (8) that collagen produces reproducible and dose-dependent falls in the circulating platelet count in the rat. Holmes and Freuler (14) showed that 5 doses of 160 µg/kg collagen at 15 min intervals

caused a fall in the basal platelet count in rats of nearly 50% in 75 min, whereas Smith and Freuler (2) and Holmes et al (9) showed that in rabbits and rats ADP even in high doses caused only about 25% fall over a 2 hour period. In fact they showed that doses up to 30 µg/kg/min for 2 min did not cause a fall in basal count at all. This suggests that low doses of ADP cause a brief and completely reversible adhesion and aggregation in the microcirculation equivalent to the first phase aggregation observed *in vitro* with an aggregometer. On the other hand, collagen, AA and higher doses of ADP produce aggregation that is only partially reversible which causes a progressive lowering of the basal platelet count. This is equivalent to the secondary phase of *in vitro* aggregation indicating that the platelet release reaction has occurred.

Using the continuous platelet count technique it was hoped to quantitatively study collagen and AA in rabbits. Silver et al (4) have shown that AA caused sudden death in rabbits. An approximate LD_{50} was 1 mg/kg and the cause of death was found to be due to the presence of platelet thrombi in the microvasculature of the lungs. It was hoped to find a dose of AA that produced a slight fall in platelet count without causing death. This proved to be remarkedly unsuccessful. 250 µg/kg/min for 2 min did not cause a fall in platelet count whereas 500 µg/kg/min for 2 min produced a 75% fall but the animals did not survive a subsequent injection of the same dose. It was concluded that barbiturate-anaesthetised rabbits were highly sensitive to microthrombi in the lungs. Collagen was not as lethal as AA but still great care had to be taken to avoid death when giving it by infusion to rabbits. 20 µg/kg/min for 2 min gave reproducible effects but some of the animals did not survive more than 3-4 doses of collagen.

Holmes (6) and Vargaftig et al (15) have shown that cyclo-oxygenase inhibitors partially inhibited collagen-induced aggregation in rats and guinea pigs respectively. In the guinea pig study (15), it was not possible to obtain a dose-dependent response with indomethacin or aspirin. Indomethacin was also found to be equiactive with aspirin. In the rat study reported in this paper, indomethacin has produced a dose-dependent response between 1 and 4 mg/kg whereas aspirin produced a very shallow dose-response relationship. The relative potency of indomethacin to aspirin was approximately 10. The difference between this figure and that obtained by Vargaftig et al (15) may be explained by their use of the lysine salt of aspirin. With both compounds the highest dose studied produced less inhibition than the penultimate dose, suggesting that at these doses cyclo-oxygenase in the blood vessel walls was also inhibited, thus reducing the formation of prostacyclin. The partial inhibition of collagen- and AA-induced aggregation by indomethacin confirms and extends similar previous observations (6 and 15).

Experiments *in vivo* to show the nature of the indomethacin-resistant component have not been successful. *In vitro* studies of platelet aggregation have established that collagen-induced

aggregation is mediated by ADP-release from the platelets (16) by the formation of PGG_2, PGH_2 and TxA_2 from platelet arachidonate (17) and by a third pathway independent of ADP and the arachidonate pathway (18). Preliminary experiments in this laboratory could not show any involvement of ADP or 5HT but further experiments are in progress.

Sun, Chapman and McGuire (19) showed that rat platelet microsomes did not produce TxA_2. Vincent and Zijlstra (20) found that rat platelets incubated with phospholipase A_2 did produce a TxA_2-like substance. Imidazole (7) and 1-n-butylimidazole (8) have been shown to inhibit platelet aggregation *in vitro* and to prevent the conversion of PGH_2 to TxA_2. Imidazole was too toxic to study *in vivo* but 1-n-butylimidazole could be given intravenously to rabbits in non-toxic doses that inhibited collagen-induced aggregation. The comparative study of 1-n-butylimidazole in rats and rabbits showed a different profile of activity in the two species. In contrast to the effect of indomethacin in rabbits, 1-n-butylimidazole completely inhibited collagen-induced aggregation. The observation that responses to ADP were not inhibited indicated that the effect is not due to a non-specific toxic reaction. It can be suggested that this effect may be due to the conversion of platelet endoperoxide to prostacyclin when thromboxane synthesis is inhibited (21). The effects of 1-n-butylimidazole on responses to PGH_2 in rats and rabbits also suggest this possibility but further work is required to substantiate these results. In rats, 1-n-butylimidazole did not inhibit collagen- or AA-induced aggregation. The relative inactivity of 1-n-butylimidazole in the rat compared with the rabbit suggests that conversion of the endoperoxide to TxA_2 is not necessary for aggregation in the rat. The existence of a specific receptor activated by TxA_2 has been proposed by Coleman et al (22) and recently an antagonist for TxA_2 at this receptor has been described (23). Its activity in the rat may explain the role of TxA_2 in this species.

The evidence presented in this paper indicates that cyclo-oxygenase inhibitors and a thromboxane synthetase inhibitor inhibit collagen-induced aggregation in the rabbit. Cyclo-oxygenase inhibitors also reduce collagen- and AA-induced aggregation in the rat but a thromboxane synthetase inhibitor did not affect aggregation produced by these agents in the rat. Confirmation of these results requires further study with more specific antagonists.

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