THE INTERACTION OF VARYING DOSES OF DIPYRIDAMOLE AND ACETYL SALICYLIC ACID ON THE INHIBITION OF PLATELET FUNCTIONS AND THEIR EFFECT ON BLEEDING TIME

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- 1 In normal volunteers maximum reductions in platelet functions, collagen aggregation, adhesion and PF4 availability, were achieved using combined doses of 50 mg three times daily dipyridamole + 180 mg ASA or 75 mg three times daily dipyridamole + 120 mg ASA daily.
- 2 These doses did not prolong the bleeding time.
- 3 A synergistic effect has been demonstrated with 25 mg dipyridamole three times daily and 60 mg ASA.
- 4 At higher doses the effects on platelet functions were additive up to the maximal response.
- 5 The effect of low doses of ASA on platelet function was cumulative.
- 6 As lower doses of ASA in the combination studied inhibit platelet functions maximally without altering the bleeding time and probably without inhibiting prostacyclin, we suggest that these combinations of dipyridamole and ASA merit consideration in future clinical trials.

Introduction

Antiplatelet drugs acetyl salicylic acid (ASA) and dipyridamole have been used in clinical trials alone and in combination to assess their value in preventing thrombo-embolism. These have been reviewed recently (Weiss, 1977; Verstraete, 1977). They have proved to be successful in the prevention of arterial thrombus formation particularly on prosthetic heart valves (Sullivan, Harken & Gorlin, 1969), arterial venous shunts (Evans, Packham, Nishizawa, Mustard & Murphy, 1968) and cannulae (Kricheff, Zucker, Ischopp & Kolodjie, 1973), but they have been found to be less successful than conventional anticoagulants in the prophylaxis of venous thrombosis. All the clinical trials so far carried out have used doses of ASA in excess of that required to reduce platelet functions. ASA in high doses is known to inhibit the production of prostaglandin I_2 (prostacyclin) which is a potent disaggregator of platelets (Burch, Stanford & Majerus, 1977; Baenziger, Dillender & Majerus, 1977; Moncada & Korbitt, 1978), and more significantly the presence of prostacyclin is necessary for the action of dipyridamole on platelets. In addition, conventional doses of ASA prolong the bleeding time and predispose to gastro-intestinal bleeding.

Dipyridamole in high doses is known to cause headaches and dyspepsia. By combining the two drugs in lower doses the side effects could be minimized and the clinical effectiveness may also be increased. In this study we examined the smallest doses of both drugs, alone and combined, which will reduce maximally the tests of platelet function. We also investigated the nature of the interaction, whether additive or synergistic, between ASA and dipyridamole and the effects of the combination on bleeding time.

Methods

Ten normal healthy volunteers aged 25–45 years (six female, four male) participated in the study. All had platelet counts and platelet functions within the normal range. They were given single doses of ASA (60, 120, 180, 240, 300, 360 and 1,000 mg) in random order at fortnightly intervals and blood was collected 1.5–2 h after each dose. The same volunteers, at fortnightly intervals, took dipyridamole 25, 50, 75 or 100 mg three times daily in random order for 3 days. On day 3, ASA 60, 120 or 180 mg was added to the morn-

ing dose on days 2 and 3 into a 30 ml plastic syringe using a 19g needle without venous stasis. Fifteen ml of the blood was mixed with 3.8% sodium citrate 9:1) and 1 ml with EDTA. The syringe was then immediately attached to the Adiplat S syringe pump for platelet adhesion measurement by a modification of Hellem's method (Hellem, 1960). Blood was pumped through the standardized column of glass beads at a constant rate of 1 ml every 18 s. The first 2 ml of blood passed through the column was discarded and the third ml was collected into EDTA. The blood remaining in the syringe was transferred to a plain tube for salicylate and dipyridamole measurement. Platelet aggregation was determined using a Medicon Aggregometer (Model No. MA2) based on Born's principle (Born, 1962). The aggregants used were Sigma bovine collagen (Sanderson, 1975) (aliquots being kept in liquid nitrogen and thawed out immediately before use) and ADP at a final concentration of 2 mmol/l. The percentage adhesion was calculated from the difference between the platelet count before and after passage through the column of glass beads. Heparin neutralizing activity (PF4 availability) was determined by the method of Niewiarowski & Thomas (1969). Platelet count and platelet factor 3 availability were measured using standard methods (Dacie & Lewis, 1968). Serum salicylate was measured using Trinder's method (Trinder, 1954) and dipyridamole was measured fluorometrically as previously described (Rajah, Crow, Penny, Ahmad & Watson, 1977b). The standardized Ivy bleeding time was measured by the template method (Mielke, Kaneshiro, Maker, Weiner & Rapaport, 1969). The paired *t*-test, the Wilcoxon sign rank test and rank sum test were used to compare the results of each dose with the control and with each other (Snedecor & Cockran, 1967).

Results

Platelet counts remained constant throughout the trial and PF3 was not affected by any dose of either drug.

The effect of low doses of ASA on platelet function

The effects on four platelet function tests following doses of ASA alone are shown in Table 1. Secondary wave aggregation was abolished by a single dose of 60 mg, collagen induced aggregation was reduced by 120 mg (Figure 1), adhesion was reduced by 180 mg (Figure 2) and PF4 was reduced by 240 mg (Figure 3). All four of these tests were maximally inhibited by a single dose of 300 mg ASA. Significant negative correlations were obtained for serum salicylate level 2 h after the dose with collagen aggregation ($r = 0.75$, $P < 0.01$, $n = 50$), with adhesion ($r = 0.48$, $P < 0.02$, $n = 50$) and with PF4 ($r = 0.39$, $P < 0.05$, $n = 50$). After 2 days on 60 mg three times daily ASA platelet functions were reduced by the same amount as with a single dose of 180 mg (Table 1). The effect of ASA on platelet functions was therefore cumulative.

The effect of varying doses of dipyridamole on platelet functions

The effect of dipyridamole alone is shown in Table 2. Collagen aggregation (Figure 1), adhesion (Figure 2) and PF4 availability (Figure 3) were reduced by 25 mg three times daily. Increased dosage gave further reductions in all functions and almost maximal reductions of adhesion and PF4 (but not aggregation) were produced by 100 mg three times daily. Significant negative correlations were obtained for serum dipyridamole level 2 h after the dose with collagen aggregation ($r = 0.75$, $P < 0.01$, $n = 40$),

Table 1 Effects of ASA alone on platelet functions. Results are expressed as mean $\pm$ s.e. mean.

Dose ASA (mg)	Collagen aggregation %	Adhesion %	PF4 % available	Frequency of second wave aggregation ADP 2 mmol/l
Control	88 $\pm$ 3.2	85 $\pm$ 2.2	81 $\pm$ 5.7	10/10
60	82 $\pm$ 3.9	85 $\pm$ 2.7	82 $\pm$ 4.0	0/10
120	57 $\pm$ 4.1**	83 $\pm$ 2.2	80 $\pm$ 4.4	0/10
180	39 $\pm$ 2.5**	65 $\pm$ 6.3*	66 $\pm$ 3.8	0/10
240	18 $\pm$ 3.8**	42 $\pm$ 3.8**	14 $\pm$ 6.6**	0/10
300	12 $\pm$ 2.2**	36 $\pm$ 3.8**	0	0**
360	15 $\pm$ 4.4**	33 $\pm$ 3.2**	0	0**
1000	13 $\pm$ 2.8**	30 $\pm$ 2.8**	0	0**
60 three times daily for 2 days	41 $\pm$ 3.1**	65 $\pm$ 4.6*	64 $\pm$ 4.1	

* $P < 0.01$ compared with control, $n = 10$;

** $P < 0.001$ compared with control, $n = 10$

Figure 1 The effects of varying oral doses of ASA and dipyridamole on collagen induced aggregation of platelets (mean $\pm$ 1 s.e. mean) for each dose of ASA alone ($\circ$), dipyridamole 25 mg three times daily+ASA ($\triangle$), dipyridamole 50 mg three times daily+ASA ($\bullet$), dipyridamole 75 mg three times daily+ASA ($\blacktriangle$) and dipyridamole 100 mg three times daily+ASA ($\blacksquare$).

Figure 2 The effects of varying oral doses of ASA and dipyridamole on adhesion of platelets to glass beads using the Adipat S system (mean $\pm$ 1 s.e. mean). ASA alone ($\circ$), dipyridamole 25 mg three times daily+ASA ($\triangle$), dipyridamole 50 mg three times daily+ASA ($\bullet$), dipyridamole 75 mg three times daily+ASA ($\blacktriangle$) and dipyridamole 100 mg three times daily+ASA ($\blacksquare$).

with adhesion ($r = 0.48$, $P < 0.02$, $n = 40$) and with PF4 ($r = 0.39$, $P < 0.05$, $n = 40$). All results were below the normal range with serum dipyridamole levels greater than $3.0 \mu\text{mol/l}$. These findings are similar to those reported previously on patients with mitral valve prostheses taking dipyridamole (Rajah *et al.*, 1977b).

The effect of combined dipyridamole and low doses of ASA on platelet functions

The effect of the combination of the two drugs is shown in Table 3. At the very low dose level of 25 mg three times daily dipyridamole + 60 mg ASA there was a definite synergistic effect between the two drugs

Table 2 Effects of dipyridamole alone on platelet functions. Results are expressed as mean $\pm$ s.e. mean

Dose dipyridamole (mg three times daily)	Collagen aggregation %	Adhesion %	PF4 % available	Frequency of second wave aggregation ADP 2 mmol/l
Control	88 $\pm$ 3.2	85 $\pm$ 2.2	81 $\pm$ 5.7	10/10
25	68 $\pm$ 7.0*	66 $\pm$ 6.0**	58 $\pm$ 7.6*	9/10
50	57 $\pm$ 7.0*	42 $\pm$ 6.6***	14 $\pm$ 4.4***	6/10
75	57 $\pm$ 8.9*	44 $\pm$ 7.0**	15 $\pm$ 4.7***	6/10
100	41 $\pm$ 6.0**	39 $\pm$ 5.0***	7 $\pm$ 3.2***	2/10

* $P < 0.05$ compared with control, $n = 10$;

** $P < 0.01$ compared with control, $n = 10$;

*** $P < 0.001$ compared with control, $n = 10$.

Figure 3 The effects of varying oral doses of ASA and dipyridamole on platelet factor 4 availability (heparin neutralising activity), (mean $\pm$ 1 s.e. mean). ASA alone (○), dipyridamole 25 mg three times daily + ASA (△), dipyridamole 50 mg three times daily + ASA (●), dipyridamole 75 mg three times daily + ASA (▲) and dipyridamole 100 mg three times daily + ASA (■).

on collagen aggregation (Figure 1), adhesion (Figure 2) and PF4 (Figure 3), the combined effect being greater than the sum of the individual effects ($P < 0.05$). At higher dose combinations the effects on all three were additive until maximal response was achieved. Using the combination all functions were

below normal with a serum dipyridamole level of only $1.0 \mu\text{mol/l}$.

The serum level of both drugs increased linearly with dosage and the combination did not significantly alter the serum concentrations of either drug 2 h after ingestion (Figures 4 and 5) so the absorption rates are probably not affected. However there may be some interaction involved upon drug binding or clearance rates.

The effect of dipyridamole and ASA on bleeding time

A single dose of ASA 1,000 mg prolonged the bleeding time by a mean of 94% ($P < 0.001$) and 300 mg by 50% ($P < 0.05$). The bleeding time was not significantly altered by 180 mg ASA, by 100 mg dipyridamole, by a combination of 50 mg dipyridamole + 180 mg ASA or by 75 mg dipyridamole + 120 mg ASA (Figure 6). These combinations therefore maximally inhibited platelet functions but did not prolong the bleeding time.

Discussion

ASA and dipyridamole are known to affect platelets by different mechanisms and a combination may therefore be more effective than either drug alone. ASA acts by acetylating and thus inactivating the membrane bound enzyme cyclo-oxygenase (Rome, Lands, Roth & Majerus, 1976). This enzyme in platelets converts arachidonic acid to the unstable prostaglandin endoperoxides PgG_2 and PgH_2 (Willis, Vane, Kuhn Scott & Petrin, 1974) which are then converted to the more unstable thromboxane A_2 (Hamberg, Svensson & Samuelsson, 1975), a potent platelet aggregating agent and vasoconstrictor. Cyclo-oxygenase in the vascular wall also produces endoperoxides. These are then converted to prostacyclin, a strong inhibitor of platelet aggregation and a vasodilator (Moncada &

Table 3 Effects of combined dipyridamole + ASA on platelet functions. Results are expressed as mean $\pm$ s.e. mean.

Dose dipyridamole ASA (mg three times daily)	Collagen aggregation %	Adhesion %	PF4 % available	Frequency of second wave aggregation ADP 2 mmol/l
Control	88 $\pm$ 3.2	85 $\pm$ 2.2	81 $\pm$ 5.7	10/10
25 60	40 $\pm$ 5.7*	55 $\pm$ 3.8*	26 $\pm$ 8.9*	0/10
50 120	26 $\pm$ 4.7*	40 $\pm$ 7.0*	1.5 $\pm$ 1.3*	0/10
50 180	21 $\pm$ 3.2*	28 $\pm$ 5.0*	1.5 $\pm$ 1.3*	0/10
75 120	24 $\pm$ 4.1*	34 $\pm$ 5.4*	4 $\pm$ 1.3*	0/10
75 180	23 $\pm$ 4.2*	33 $\pm$ 4.9*	3 $\pm$ 1.2*	0/10
100 120	19 $\pm$ 3.8*	31 $\pm$ 4.1*	0 0*	0/10

* $P < 0.001$ compared with control $n = 10$.

Figure 4 Serum salicylate concentration (mean $\pm$ 1 s.d.) 2 h after single doses of a) ASA alone (■) and b) ASA combined with dipyridamole (○).

Figure 5 Serum dipyridamole concentration (mean $\pm$ 1 s.d.) 2 h after doses of a) dipyridamole alone and b) dipyridamole combined with ASA.

Korbitt, 1978; Moncada, Higgs & Vane, 1977). The enzyme in platelets and vessel wall is inhibited by ASA, the platelet enzyme being nine times more sensitive (Baenziger *et al.*, 1977). ASA taken 20–650 mg daily inhibited the platelet enzyme by 61–95% (Burch *et al.*, 1977), but higher doses of ASA inhibit the production of prostacyclin in the vessel wall (Baenziger *et al.*, 1977; Moncada & Korbitt, 1978).

Figure 6 The effect of ASA and dipyridamole on bleeding time (mean $\pm$ 1 s.e. mean).

Dipyridamole is known to inhibit the enzyme phosphodiesterase in platelets (Mills & Smith, 1971) thus causing an accumulation of cyclic AMP which is claimed to inhibit platelet activity (Haslam, 1975). The anti-aggregating action of prostacyclin is effected through stimulation of adenylyl cyclase increasing the production of cAMP (Tateson, Moncada & Vane, 1977). It is also claimed that the presence of prostacyclin is necessary for dipyridamole to be effective. Therefore large doses of ASA which reduce prostacyclin production may neutralize the effect of dipyridamole. On the other hand doses of ASA which only inhibit platelet cyclo-oxygenase but not the endothelial enzyme have a potentiating effect on the disaggregating action of dipyridamole *in vivo* (Moncada & Korbitt, 1978).

Previous human studies have shown that dipyridamole 400 mg daily in four divided doses or 100 mg daily combined with 1 g ASA normalized the reduced platelet survival in patients with prosthetic heart valves (Harker & Slichter, 1970), whereas ASA 3 g daily failed to produce this effect despite reducing platelet function. The failure of 3 g ASA to normalize platelet survival may be due to the inhibition of prostacyclin production.

The results from this study show that in normal human volunteers, maximal inhibition of collagen induced aggregation, adhesion to glass beads and factor 4 availability were produced by ingestion of a single dose of 300 mg ASA and significant reductions were achieved with 180 and 240 mg ASA. These findings are in agreement with previous studies (O'Brien, Tulevski & Etherington, 1971; Stuart, 1970). Dipyridamole 50 mg three times daily also produced significant reduction in platelet functions which were further inhibited by increasing the dose to 100 mg three times daily. These results are consistent with our previous work on patients with prosthetic

heart valves (Rajah *et al.*, 1977b). A combination of 50 mg three times daily dipyridamole with 180 mg ASA or 75 mg three times daily dipyridamole with 120 mg ASA also gave maximum inhibition of collagen induced aggregation, adhesion to glass beads and factor 4 availability. At very low dose combinations the two drugs were synergistic but as the dose increased the effect became additive. Honour, Hockaday & Mann (1978) have shown a synergistic effect between ASA and dipyridamole in protecting rabbits against artificially induced thrombosis.

ASA at 180 mg partially inhibited platelet function but had no effect on bleeding time, whereas at 300 mg platelet functions were maximally inhibited and the bleeding time was prolonged. When 75 mg three times daily dipyridamole was added to 180 mg ASA, platelet functions were maximally inhibited without alteration in bleeding time. It seems difficult to explain the normal bleeding time when the platelet functions are maximally inhibited with the combined regime. We believe the inhibition of platelet function is important in the initiation of thrombosis in the arterial circulation, whilst numerous factors besides platelet function, such as capillary contractility, may be involved in capillary haemostasis.

We have shown that the effect of combined

dipyridamole and ASA on *ex vivo* platelet functions is synergistic with very low doses but additive as the doses increase. We have also shown (unpublished data) that a combined low dose of ASA and dipyridamole is effective in reducing platelet adhesion to dacron grafts in an *in vitro* model (Rajah, Crow, Hamlin & Kester, 1977a). These effects are produced at doses below those shown to normalize platelet survival (Harker & Slichter, 1970). The currently recommended combination of 75 mg three times daily dipyridamole + 330 mg three times daily ASA may carry a risk of gastro-intestinal bleeding because of the effect of continual ASA consumption at this level. We believe that the ASA in the combination could be reduced without loss of efficacy because of the cumulative effect of ASA. We therefore suggest that lower dose combination merits further evaluation in clinical trials.

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