

Sensitivity of platelets to aspirin in von Willebrand's disease

J. LESLIE¹ AND D. Y. MASON²

From the Department of Haematology, St Thomas' Hospital, London

SYNOPSIS The sensitivity of collagen-induced platelet aggregation to aspirin was measured *in vitro* on platelet-rich plasma from 20 normal subjects and five patients with von Willebrand's disease. Although aspirin tolerance tests performed *in vivo* at the same time showed an abnormal prolongation of the bleeding time in three of the patients studied, no evidence was found for an enhanced sensitivity to aspirin *in vitro*.

Quick (1966) showed that aspirin increases the bleeding time in normal subjects, and this increase is accentuated in patients with various bleeding disorders (Beaumont, Willie, and Lenègre, 1955a). Weiss and Aledort (1968) showed impaired interaction between platelets and connective tissue *in vivo* in man after the ingestion of aspirin. This inhibitory effect has also been demonstrated *in vitro* by Evans (1967). If platelet aggregation induced by collagen were abnormally sensitive to the inhibitory effect of aspirin in patients with bleeding disorders, it might suggest a mechanism by which this inordinate prolongation of the bleeding time occurs. This hypothesis has been tested in the following investigation.

Materials

'Collagen' was prepared by partly homogenizing human Achilles tendon and suspending the homogenate in 0.05 M Tris buffer, pH 7.2, at 21°C, with filtration through coarse nylon mesh. The suspension was centrifuged at 17,000g, the supernatant discarded, and the sediment resuspended in Tris. The process was repeated three times. The final suspension was stored at -20°C in 0.5 ml aliquots after rapid freezing at -78°C.

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¹Present address: Department of Haematology, Royal South Hants Hospital, Exmoor Road, Southampton SO2 0AS.

²Present address: Department of Haematology, Radcliffe Infirmary, Oxford.

Aspirin solutions for studies *in vitro* were prepared by dissolving the powdered compound in sodium bicarbonate solution at a stoichiometric concentration, as this was found to improve solubility. Further dilutions were made in Tris buffer and solutions were stored at 4°C until ready for use. Solutions were discarded if not used within six hours of preparation.

Platelet-rich plasma was prepared by adding venous blood to 3.2% disodium citrate solution (9:1 v/v). After gentle mixing, specimens were centrifuged at 125g for 15 minutes at 4°C.

Polystyrene tubes and syringes were used throughout, and all glassware was siliconized.

Subjects

Normal subjects consisted of laboratory and medical staff, none of whom had a history of bleeding disorder. All the patients with von Willebrand's disease had a clear history of haemostatic defect and affected close relatives of both sexes. In addition, each patient had on at least one occasion in the past been found to have a bleeding time of more than 10 minutes and a factor VIII level of less than 45%. Subjects were not tested until at least 10 days had elapsed since they last ingested aspirin. Platelet aggregation tests could not reproducibly be performed on subjects with peripheral blood platelet counts less than 150,000/cmm, so that these were excluded from the study.

Methods

BLEEDING TIME EXPERIMENTS

The effect of aspirin on the bleeding time was determined in a double-blind trial in which bleeding times on normal subjects were performed before, and two hours after, the ingestion of two gelatine-coated capsules containing either placebo (lactose) or 600 mg aspirin. Bleeding times were performed on five patients with von Willebrand's disease after aspirin ingestion, but these patients did not receive placebo on another occasion. However, blood for platelet aggregation studies *in vitro* was collected from the patients immediately before base-line bleeding times were obtained. Platelet aggregation studies were also carried out one on other occasion on each patient in the group. Bleeding times were determined by applying a cuff at 40 mm Hg to the upper arm, and making three punctures 3 mm deep and 1 mm long in quick succession in the skin of the forearm using a disposable lancet. The wounds were lightly blotted at 15-second intervals until bleeding had ceased. The mean of the three values was taken. The second bleeding time was performed on the opposite arm at the equivalent site. Punctures apparently entering veins, as shown by the blood immediately welling up, were not recorded.

PLATELET AGGREGATION STUDIES

These were carried out in the aggregometer designed by Born (1962). Platelet-rich plasma was dispensed in 0.48 ml aliquots into a series of tubes containing magnetic stirrers covered with silicone rubber. Either Tris buffer or aspirin solution (0.06 ml) was added to each tube and the mixtures were incubated for exactly 10 minutes at 37°C. Then 0.06 ml of collagen suspension was blown in and aggregation curves were recorded. For each subject, aggregation curves were obtained in response to two concentrations of collagen. At

each strength of collagen, the effect of pre-incubation of platelet-rich plasma with Tris buffer and two different concentrations of aspirin was observed. A value for the rate of aggregation of each mixture was obtained by measuring the change in light transmission per unit time when aggregation was in its most rapid phase. Tests were replicated following a balanced design to eliminate the effect of spontaneous changes in the activity of reagents as discussed by Cox (1951).

Results

BLEEDING TIME EXPERIMENTS

These were performed on 10 normal subjects and six patients with von Willebrand's disease. The ingestion of 600 mg of aspirin in normal subjects produced a significant mean rise in the bleeding time of 1.5 min (Table I). Placebo tablets produced no significant difference in the bleeding time. Table I also compares the effect of aspirin or placebo in normal subjects with that of aspirin in patients. As can be seen, three of the patients (nos. 1, 4, and 5) showed a response to aspirin markedly in excess of the normal range.

PLATELET AGGREGATION STUDIES

The Figure shows dose-response results obtained from a normal subject. It will be seen that the degree of inhibition was related to the concentration of aspirin in the reaction mixture. The degree of inhibition of the collagen activity may be calculated in such a parallel-line bioassay and expressed as the 'relative collagen activity' (Table II). Values for relative collagen activity were calculated for patients and normal subjects. Table III shows increases in bleeding times in response to aspirin beside the dose-response plots. No difference between the two groups was found, and three patients showing the greatest prolong-

	Normal Subjects (10)		von Willebrand Subjects (5)				
	Lactose	Aspirin (600 mg)	Aspirin (600 mg)				
	Mean	Mean	1	2	3	4	5
Before	4.26 (2.50 - 6.25)	4.43 (3.41 - 5.90)	11.70	8.90	7.00	7.40	12.80
After	4.51 (2.15 - 7.00)	6.02 (4.41 - 8.50)	33.25	9.60	8.70	21.00	20.00
Difference	+ 0.25 (- 1.00 - + 3.15)	+ 1.50 (- 0.25 - + 3.30)	+ 21.50	+ 0.70	+ 1.70	+ 13.60	+ 7.20

Table I Effect of ingestion of aspirin or placebo on the Ivy bleeding time (min) in 10 normal subjects and five patients with von Willebrand's disease

The mean difference in bleeding time after placebo was not significant (SE diff = 0.429, $t = 0.58$, $df = 18$, $P > 0.3$), but the mean difference two hr after the ingestion of aspirin was significant (SE diff = 0.155, $t = 10.16$, $df = 18$, $P < 0.001$). The difference between the mean aspirin and the mean placebo effect, ie, the difference between the differences, was also significant (SE diff between diffs = 0.356, $t = 3.47$, $df = 36$, $P > 0.0025$). These SEs are derived from the pooled between-subjects variance of bleeding time results before and after placebo or aspirin in the 10 normal subjects (homogeneous by Bartlett's test: $\chi^2 = 3.897$, $df = 3$, $0.3 > P > 0.2$), with the appropriate allowances for covariance.

Differences between von Willebrand patients and normal subjects were not calculated because of the large between-subjects scatter in the von Willebrand group.

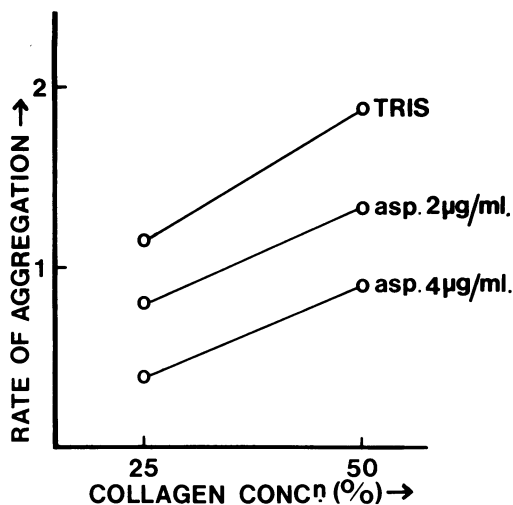


Fig. Effect of Tris buffer and two different concentrations of aspirin on the rate of aggregation of platelets using two different dilutions of an arbitrary suspension of collagen in a normal subject; aspirin concentrations are those of the reaction mixture, but collagen concentrations are those of the reagent suspension in Tris buffer. The units for the rate of platelet aggregation are arbitrary and refer to the slope of the aggregation curve in its most rapid phase. The rate of platelet aggregation is directly related to the collagen concentration and inversely to the aspirin concentration in the reaction mixture. The degree of inhibition of collagen activity may be calculated as a parallel-line bioassay to obtain 'relative collagen activity' for each concentration of collagen.

	Aspirin Concentration		Slope Index	Position Index
	2 µg/ml	4 µg/ml		
	A	B	A - B	A + B
Normal subjects (20)	0.68 (0.39 - 0.89)	0.47 (0.32 - 0.62)	0.21 (0.10 - 0.36)	1.16 (0.67 - 1.49)
von Willebrand subjects (5)	0.64 (0.40 - 0.74)	0.46 (0.26 - 0.68)	0.18 (0.14 - 0.30)	1.00 (0.66 - 1.44)

Table II Effect of aspirin on the interaction between platelets and collagen in vitro ('relative collagen activity')

The differences between the mean indices for slope or position obtained from the 'relative collagen activities' of normal subjects and von Willebrand patients were clearly not significant (for slopes, SE diff = 0.25, $t = 0.24$, $df = 23$, $P > 0.5$; for positions, SE diff = 0.22, $t = 31.8$, $df = 23$, $P > 0.5$).

	von Willebrand Subjects (5)					Normal Subjects (20)
	1	2	3	4	5	Mean
Bleeding time increase (min)	0.7	1.7	7.2	13.6	21.5	0.25 (- 1.00 - + 3.15)
Slope index	0.24	0.30	0.08	0.14	0.14	0.21 (0.10 - 0.36)
Position index	1.24	1.06	1.44	0.66	1.06	1.16 (0.67 - 1.49)

Table III Comparison of slope and/or position index with bleeding time increase after ingestion of aspirin in von Willebrand's disease

ation of bleeding time after aspirin ingestion could not be distinguished from normal using either the position- or slope-indices of their dose-response plots.

Discussion

Ashford and Freiman (1967) showed that haemostasis in small blood vessels partly involves adhesion of platelets to collagen fibres exposed at the time of injury. Adenosine diphosphate (ADP) is released locally by platelets (Spaet and Zucker, 1964) and probably also by lysed red cells (Rorvik, Holmsen, and Stormorken, 1968). Aspirin has been shown to inhibit the release of ADP by platelets (Weiss, Aledort, and Kochwa, 1968) rather than the response to platelets to ADP, and Beaumont *et al* (1955b) showed that aspirin may prolong the bleeding time in haemophilia. Platelet aggregation *in vivo* might therefore be inhibited by aspirin, thus exaggerating an existing haemostatic defect. The present study was carried out to determine whether the degree of inhibition of platelet aggregation in response to collagen in von Willebrand's disease was greater than normal. Although a platelet abnormality in this disease may have gone undetected in the design of the present experiment, the results show that in spite of an inordinate prolongation of the bleeding time in three of the patients studied, there is no evidence for enhanced sensitivity towards aspirin could be obtained in these subjects. Two of the subjects studied showed an increase in the bleeding time within the expected normal range, suggesting that a negative aspirin tolerance test is of little diagnostic value.

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