Defective platelet aggregation to the calcium ionophore A23187 in a patient with a lifelong bleeding disorder

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SUMMARY A patient with a lifelong bleeding disorder is presented with a prolonged bleeding time and abnormal aggregation and secretion responses to arachidonic acid, thromboxane A_2, PAF-acether and the divalent calcium ionophore A23187. Platelet α and dense granule contents and morphology appear normal. The proposed defect is due to an abnormality of a platelet intracellular calcium dependent process.

When platelets are activated, arachidonate is enzymatically released from platelet membrane phospholipids and subsequently converted via cyclo-oxygenase and thromboxane synthetase into the cylic endoperoxides, PGG_2 and PGH_2 and to thromboxane A_2 (TXA_2).[1] These compounds and especially TXA_2 are potent inducers of platelet aggregation and granule secretion. It has been suggested that TXA_2 may act as a calcium ionophore, releasing stored calcium from the dense tubular system and thus raising the level of free cytoplasmic calcium.[2]

Mobilisation of intracellular calcium is involved in the regulation of several platelet functions and secretory processes, suggesting a controlling trigger role for intracellular calcium flux and free cytoplasmic calcium in the platelet activation process.[3]

Recently, a few patients have been described with a lifelong bleeding tendency in whom arachidonate metabolism including TXA_2 formation was normal but whose platelets were unresponsive to TXA_2 showing defective aggregation and granule release.[4-8] Also a similar acquired defect has been reported in one patient with chronic myeloid leukaemia.[9]

We present here a patient with a lifelong bleeding tendency who has abnormal aggregation and secretion responses suggestng a defect in either the mobilisation of intracellular calcium, or a response to free cytoplasmic calcium.

Patient and methods

CASE REPORT

A 57-year-old Caucasian housewife presented with a lifelong bleeding disorder. As a child, she had frequent epistaxis lasting up to 24 h at a time until 16 years old. Since then, she has had widespread spontaneous non-traumatic purpura but not excessive menstrual loss or serious bleeding episodes. She had no operations in the past and had previously been told to avoid all drugs. She did not imbibe alcohol. A family history was relevant in that two brothers died in childhood from uncontrollable haemorrhage after minor trauma in one case and abdominal surgery in the other. Two other sisters and a brother were untroubled by bleeding, one sister having had an uncomplicated appendicectomy. Her parents had lived into old age without any bleeding problems. Physical examination was normal apart from some purpuric lesions on the lower limbs. Initial studies showed a normal blood and platelet count, normal renal and liver biochemical profiles and normal concentrations of immunoglobulin.

Methods

As anticoagulant, one volume of 3·13% sodium citrate was used for nine volumes of blood. Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were prepared by centrifugation at 164 g for 10 min and 1465 g for 15 min respectively at 22°C. Bleeding times were performed by the template method using a Simplate-II from General

Diagnostics. Platelet counts were performed using a Technicon Autocounter (Technicon Instruments). Coagulation parameters were performed by standard techniques.[10]

The patient had been examined on four occasions. On each occasion a healthy control subject, who had ingested no drugs for the previous 14 days, and a control subject who had ingested 900 mg of acetylsalicylic acid 12 h previously, were studied simultaneously.

Platelet-aggregation studies were performed with an Aggregraph dual-channel aggregometer at 37°C. Aliquots of PRP (500 μl) or washed platelet suspensions, with a final platelet count of 200–300 × 10⁹/l were placed in a cuvette in the aggregometer and aggregation induced with a final concentration of 2–16 μmol/l adenosine-5'-diphosphate (ADP Sigma), 1–2 μmol/l arachidonic acid, sodium salt (Sigma), 1·0–1·5 mg/ml ristocetin (H Lundbeck, Copenhagen), 1–6 μg/ml collagen (Hormon Chemie, Munchen), 0·5–2·5 mg/ml endoperoxide analogue U-44069 (kindly supplied by Dr GL Bundy, Upjohn, Kalamazoo, Michigan), 1–12·5 μmol/l ionophore A23187 (Calbiochem, La Jolla, California), 0·5–2·5 mg/ml. PAF-acether (1-0- alkyl -2- acetyl- glyceryl-3- phosphorylcholine; Calbiochem, La Jolla, California) and 0·2–1·0 U/ml human thrombin (Blood Products Laboratory, Elstree, UK). Platelets were washed by an albumin gradient separation technique[11] and resuspended in a calcium-free TRIS HCl buffered saline, pH 7·4.

Platelet electron microscopy, thromboxane B_2 (TXB$_2$) in serum and after release from platelets in response to various stimuli, malondialdehyde (MDA) formation after stimulation with 1 mmol/l N-ethylmaleimide (NEM), plasma 6-keto prostaglandin F1α and cyclic AMP levels in unstimulated PRP were assayed as previously described.[12] Platelet lysate was examined for antigenic material related to platelet PF4 and factor VIII:Rag.[13]

The concentrations of the platelet nucleotides ATP and ADP and their release after platelet stimulation by various agonists were determined by the firefly-luminescence method as described previously.[14] The PRP was corrected to a final platelet count of 300 × 10⁹/l in all experiments involving platelet nucleotide determinations.

Results

The patient consistently had a prolonged bleeding time of >30 minutes with a platelet count ranging between 223–357 × 10⁹/l. All the coagulation parameters including the thrombin time, prothrombin time, activated partial thromboplastin time, factor VIII:C, factor VIII:Rag and factor VIII:RiCoF gave normal results.

Aggregation tracings of the patients and normal control PRP in response to ADP, collagen, arachidonic acid, U44069, A23187 and PAF-acether are shown in Fig. 1. Aggregation was reversible with ADP (2–16 μmol/l), with the endoperoxide analogue U44069 (0·5–2·5 μg/ml) and with PAF-acether (0·05–0·5 μg/ml). Aggregation was delayed and reduced in response to arachidonic acid (0·5–2 mmol/l) collagen (1–6 μg/ml) and the calcium ionophore A23187 (1–12·5 μmol/l). Ristocetin (1·0–1·5 mg/ml) gave normal aggregation. Upon stimulation the patient's platelets underwent a normal shape change to all the above agonists. Aggregation of washed platelets with thrombin (0·2–1·0 U/ml) was normal. Normal washed platelets do not form platelet aggregates in response to A23187 (1–2 μmol/l),[15] but on the addition of 0·5 mmol/l exogenous calcium both control and the patient's washed platelets produced similar aggregation responses.

In a transfer experiment, PRP from the patient and control were stirred in the aggregometer for 30 s after the addition of 1 mmol/l arachidonic acid. After rapid centrifugation for 30 s, 100 μl of each platelet poor supernatant was added to 400 μl of PRP from the patient or from a normal subject who had ingested 900 mg of aspirin 12 hours previously. These mixtures were stirred in the aggregometer and aggregation recorded (Fig. 2). Supernatant from the control induced full aggregation of the aspirin-treated PRP but none with the patient's PRP. Similarly supernatant from the patient induced full aggregation of the aspirin-treated PRP but none with the patient's own PRP.

Malondialdehyde formation, serum thromboxane B_2 (TXB$_2$) production and the generation of thromboxane B_2 from PRP after stimulation with 2 μg/ml collagen and 1 mmol/l arachidonic acid, and plasma 6-keto prostaglandin F$_1\alpha$ levels are shown from the patient and a normal subject who had ingested 900 mg of aspirin 12 hours previously in Table 1.

The patient's total platelet ATP and ADP contents and ATP/ADP ratio were within our normal range as previously reported.[14] The percentage release of platelet ATP and ADP after stimulation with A23187, collagen and PAF-acether are also shown for the patient and a normal control (Table 2).

The total ATP and ADP contents and ATP/ADP ratio from washed platelets of the patient and a normal subject and the percentage release of platelet ATP and ADP after stimulation with A23187 are shown in Table 3.

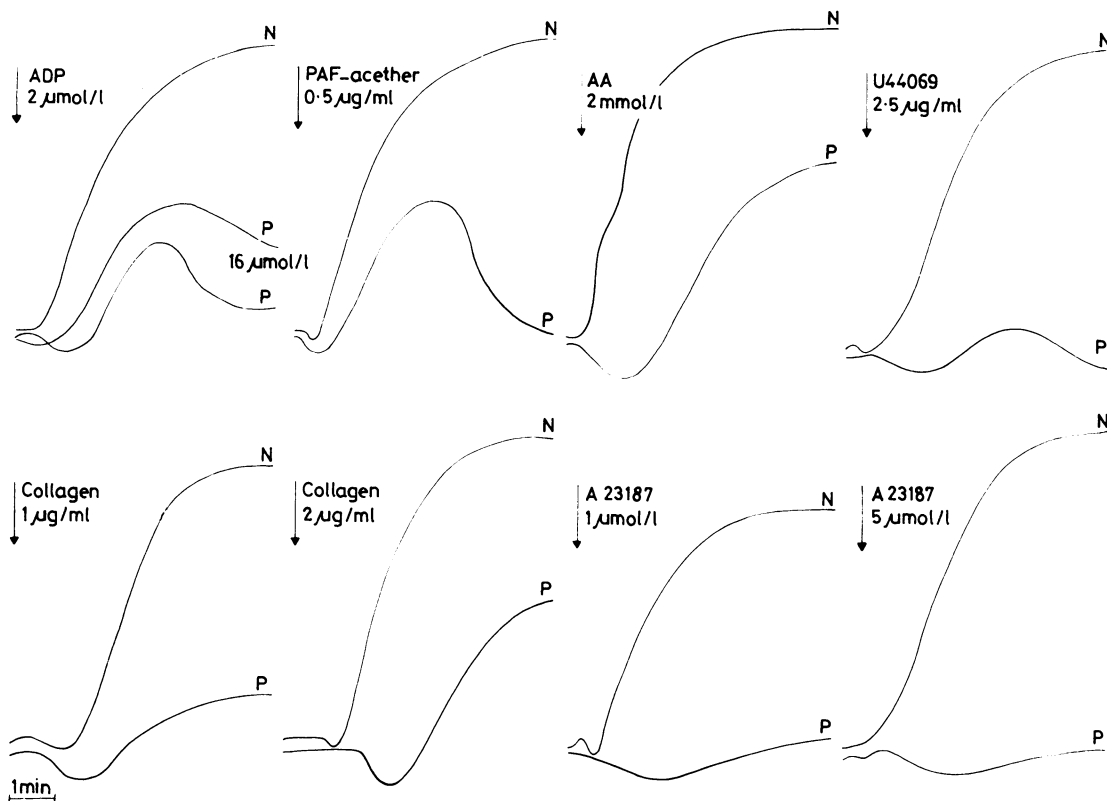By light and electron microscopy the patient's platelets showed normal morphology and granule

Fig 1   *Aggregation tracings of the patient (P) and normal control (N) to various agonists.*

content. Intra platelet PF4 and factor VIII:Rag content appear to be normal. The concentration of cyclic AMP in the patient's unstimulated PRP was 23·71 pmol/10^8 platelets (normal range 14·7–33·8 pmol/10^8 platelets).
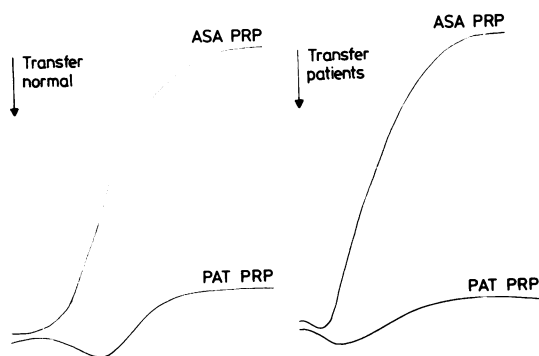

Fig. 2   *Aggregation tracings after transfer of an aliquot of normal and patient platelet poor supernatant to aspirin treated platelets (ASA PRP) and patient platelets (PAT PRP).*

Table 1   *Parameters of arachidonic acid metabolism in the patient and control subject who had ingested acetylsalicylic acid (ASA:control)*

	Patient	ASA:Control	Normal range
MDA after NEM Stimulation (nmol/10^9 platelets)	3·2	< 0·2	1·1–3·94
Serum TXB$_2$ (ng/ml)	409	< 10	200–900
TXB$_2$ generation* 2 μg/ml. Collagen (ng/ml)	272	< 10	163–297
TXB$_2$ generation* 1 mmol/l arachidonic acid (ng/ml)	303	< 10	251–394
Plasma 6-keto PGF$_{1\alpha}$ (pg/ml)	24	< 10	0–80

*The platelet counts of PRP were all corrected to 300 × 10^9/l and the stimulus applied for 4 min in the aggregometer.

Conclusions

This patient's platelets were normal in number and morphology and contained normal α and dense granule contents. However, they showed markedly

Table 2 *Percentage release of platelet nucleotides after stimulation of PRP for 4 min in the aggregometer with the agonists. Each value is the mean of two determinations.*

Agonists (final concentration)	Patient		Control subject	
	ATP	ADP	ATP	ADP
2·5 μmol/l A32187	3·2	5·9	14·7	6·0
6·25 μmol/l A23187	4·7	17·7	19·7	57·6
2 μg/ml collagen	3·7	9·9	35·6	77·6
6 μg/ml collagen	6·4	15·4	34·1	94·5
0·05 μg/ml PAF-acether	1·7	1·8	10·9	24·9
0·5 μg/ml PAF-acether	1·8	3·2	16·5	49·1

abnormal aggregation responses to ADP, arachidonate, low and high dose collagen, endoperoxide analogue, PAF-acether and A23187. This is suggestive of a defect in the prostaglandin pathway. We were able to exclude production of a biologically abnormal TXA_2 molecule, as has previously been described[16] by showing that TXA_2-like material from our patient's platelets produced normal aggregation of aspirin-treated normal platelets. In addition, platelet TXB_2 and malondialdehyde production were quantitatively normal. We also excluded the possibility that our patient's platelets did not respond to endogenous TXA_2-like material due to a TXA_2/PGH_2 receptor abnormality as has been reported.[5] That patient had normal aggregation and release to A23187. Similarly, A23187 induced aggregation is not inhibited by the TXA_2 receptor blocking compound AH 19437.[17] It has been reported that high dose collagen and PAF-acether[18] induce platelet aggregation and release independently of arachidonate metabolism and TXA_2 generation. Our patient's platelets showed defective aggregation and release in response to collagen, PAF-acether and A23187. Furthermore, it is now accepted that A23187 induced aggregation is dependent on the mobilisation and response to intracellular platelet calcium. This would suggest a defect in either calcium content, mobilisation or utilisation of intracellular calcium. This concept is supported by the fact that platelet aggregation induced by A23187 was corrected by the addition of exogenous calcium to the washed platelets. This

abnormality was not associated with any increase in the basal level of cyclic AMP which is believed to inhibit calcium mobilisation.[19]

Therefore, we propose that our patient has a defect in a calcium dependent process, such as a calcium dependent protease[20] or a calcium calmodulin binding defect[21] which is overcome in washed platelets by the presence of extracellular calcium.

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Table 3 *Platelet nucleotides levels of washed platelet suspension and the percentage nucleotide release following stimulation with A23187 in a calcium-free medium*

	Patient			Control subject		
	ATP	ADP	Ratio	ATP	ADP	Ratio
Total nucleotide levels (nmol/10⁹ platelets)	33·9	20·7	1·6:1	37·4	23·0	1·62:1
Percentage release to:						
0·25 μmol/l A23187	7·7%	28·0%		20·1%	43·3%	
2·0 μmol/l A23187	5·3%	36·7%		31·7%	73·3%	

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