New Data on Glanzmann's Thrombasthenia (35432)

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Glanzmann's thrombasthenia is a congenital qualitative platelet abnormality, characterized by a normal platelet number, long bleeding time, and defective clot retraction and platelet aggregation (1). Since the pathogenesis of this disease is not clear, any further findings related to it will be important not only in elucidating the platelet defect but also in developing a better understanding of the first steps of hemostasis. The present study was undertaken to examine the development of platelet factor 4 and acid phosphatase activity, and the release of ADP from the platelets of 3 thrombasthenic individuals by different release inducers, and to assess the aggregation of washed thrombasthenic platelets.

Materials and Methods. Patient material. Three previously described patients with thrombasthenia were studied (Table I).

1. J., female, 18 years old, case no. 10 in Caen *et al.* (1).

2. H., female, 31 years old, case no. 8 in Caen *et al.* (1).

3. B.J., female, 12 years old, case no. 2 in Cronberg *et al.* (2).

Methods. Citrated platelet-rich plasma (PRP) was obtained by centrifuging human blood containing 3.8% trisodium citrate in a ratio of 9:1, at 1000 rpm for 15 min and 8°. Platelet suspensions were prepared according to Cronberg and Caen (3). Blood was drawn in disodium-EDTA, and after differential centrifugation the platelet button was resuspended by gentle pipetting in 25 ml of a wash solution consisting of 2 parts of 0.077 *M* Na_2H_2 EDTA and 98 parts of 0.130 *M* NaCl, 0.005 *M* KCl, and 0.015 *M* Tris adjusted to pH 7.4 with 1 *N* HCl. The platelet suspension was recentrifuged at 1000*g* for 12 min at 4°, the washing was repeated once more, and the platelets were resuspended in the buffer solution used in the washing, omitting the EDTA. Platelet counts were performed by a modification of Piette's method (4). The number of platelets in the PRP varied between 300,000 and 400,000/mm^3.

Bovine thrombin (Topostasine, Hoffmann-La Roche, Basle), Heparin (Sobio, Paris), ADP (Boehringer, Mannheim), Adrenaline (Stago, 92-Asnières, France), as well as human and bovine fibrinogen (Kabi, Stockholm), were diluted to the required concentrations with Tris-buffered saline, pH 7.4. Purified calf-skin collagen prepared according to the method of Legrand and Caen (5) was used. Platelet aggregation in platelet-rich plasma was tested at 37° using an aggregometer provided by G. V. R. Born. This permitted constant magnetic stirring and continuous recording on a 400 M Vitatron recorder. The maximal slope and deflection were measured. Platelet aggregation of washed platelet suspensions were tested at 23° using an apparatus permitting continuous stirring without automatic recording (3). The deflection was measured after 3 min. To 0.5 ml of platelet suspension, 0.1 ml of human fibrinogen (5 g/liter) + 0.1 ml of either ADP (4×10^{-5} *M*), epinephrine (5.5×10^{-5} *M*), or buffered saline + 0.1 ml of Mg Cl_2 (0.01 *M*) were added. Due to small amounts of ADP spontaneously released, fibrinogen and magnesium alone produced moderate aggregation which normally decreased the optical density by 29%, whereas the addition of ADP and epinephrine decreased it 44 and 41%, respectively. ADP released spontaneously at 4 and 37°, as well as ADP released by kaolin, latex particles, bentonite, or distilled water were

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TABLE I. Hemostatic Data, Platelet ATP and Fibrinogen in the Three Thrombasthenic Patients Examined.

Patient	ADP-induced platelet aggregation	Clot retraction	Platelet	
			ATP	Fibrinogen
1	Very weak	3+	Normal	Normal
2	None	2+	Diminished	Subnormal
3	None	±		
Control		4+		

estimated according to Cronberg and Caen (3).

Samples of PRP were tested for acid phosphatase activity according to the method described by Kubisz and Caen (6), which uses as substrate disodium 4-nitrophenylphosphate (Merck). Platelet factor 4 was determined in each test tube according to a modification of the technique described by Poplawski and Niewiarowski (7). Portions (1.9 ml) of citrated PRP were incubated at 37° with constant magnetic stirring for 0 to 10 min with: (i) 0.1 ml of buffered saline, pH 7.4; (ii) 0.1 ml of ADP; (iii) 0.1 ml of epinephrine; or (iv) 0.1 ml of collagen. After incubation at 37° for 0, 3, 5, or 10 min, 0.4 ml of each sample was withdrawn. To this, 0.08 ml of heparin (1.2–1.4 U/ml) and 0.1 ml of thrombin (28-30 NIH U/ml) were added and the coagulation time was determined. Total (100%) platelet factor 4 was obtained by incubating 1.9 ml of PRP with 0.05 ml of 20% Triton-×-100 for 3 min at 37°. The percentage of platelet factor 4 activity in each sample was calculated from a standard dilution curve, drawn from results obtained by determining platelet factor 4 activity in serial dilutions of Triton-treated PRP with PPP.

Results. Availability of platelet acid phosphatase and platelet factor 4. In contrast to normal platelets, almost no acid phosphatase was made available by ADP or epinephrine in thrombasthenic platelets (Table II). Collagen liberated much more activity, although the thrombasthenic platelets were less active

TABLE II. Platelet Acid Phosphatase Availability Induced by ADP, Epinephrine, and Collagen in Thrombasthenic Platelets.

Results expressed in mμmoles/ml/hr of liberated *p*-nitrophenol.

Case no.	Time (min)	Tris-buffered saline	ADP 4.6×10^{-6} *M*	Epinephrine 6.2×10^{-6} *M*	Collagen 20 μg/ml of PRP
1	1		0	0	0
	3		0	0	0
	5		0	0	0
	15		0	10	65
2	1		10	10	30
	3		20	10	45
	5		35	10	65
	15		40	20	75
3	1		20	10	10
	3		25	10	40
	5		40	20	50
	15		40	35	105
Control	1	5	35	30	13
(mean of 15)	3	10	70	92	65
	5	15	100	121	95
	15	25	196	210	191

TABLE III. Platelet Factor 4 Activity Induced by ADP, Epinephrine, and Collagen in Thrombasthenic Platelets.

Results expressed in percent.

Case no.	Time (min)	Tris-buffered saline	ADP 4.6×10^{-6} M	Epinephrine 6.2×10^{-6} M	Collagen 20 μg/ml of PRP
1	0	5	5	6	6
	3	5	15	7	63
	5	6	17	7	66
	10	6	17	7	71
2	0	8	8	6	7
	3	8	11	8	34
	5	9	15	8	48
	10	9	16	8	52
3	0	5	8	6	6
	3	5	10	7	36
	5	4	13	6	92
	10	4	13	9	90

than the controls.

Similar results were obtained when platelet factor 4 activity was measured (Table III).

Platelet aggregation. Platelet aggregation tested in platelet-rich plasma (PRP) at 37° was not induced by epinephrine in any of the patients. In patient no. 1, moderate doses (2×10^{-7}–2×10^{-6} M) of ADP induced a slight deflection of 2–3%. Phase microscopy revealed that small, loose aggregates had been formed. In patient no. 3, increased turbidity was noted, possibly signifying platelet swelling, but no aggregation occurred. Nor did the platelets of patient no. 2 aggregate with ADP. In patients no. 1 and 3, collagen induced a moderate modification of optical density (10–19%) after a normal latency of 1–2 min. However, the curve was very flat. Phase microscopy revealed that small round aggregates had been produced.

In patient no. 3, bovine fibrinogen aggregated the thrombasthenic platelets as previously shown in platelets from other thrombasthenic subjects (8). Human fibrinogen was ineffective at the same concentration. Aggregation by bovine fibrinogen could be prevented if ADP (4×10^{-6} M) was added 5 min in advance. Adrenaline, unlike ADP, had no such inhibiting effect. Bovine fibrinogen aggregated washed thrombasthenic or normal platelets in suspension without added magnesium. In a washed platelet suspension of thrombasthenic platelets, no aggregation could be elicited by ADP or adrenaline (Table IV). Unlike those in platelet-rich plasma, thrombasthenic platelets washed and suspended in buffer spread on glass.

As shown in Table III, more ADP was released by thrombin from the platelets in PRP of thrombasthenic subjects than from those of normal controls. In platelet suspensions, more ADP was released by kaolin and other particulate matter in thrombasthenic patients than in normal controls. More ADP was also liberated by distilled water indicating that the platelets contain more releasable nucleotides.

Discussion. Whereas epinephrine did not produce any aggregation or release from the thrombasthenic platelets, ADP induced very weak aggregation in one patient and a slight increase of turbidity in platelet-rich plasma of another, suggesting swelling of the platelets. The concentrations at which these changes were induced were the same as would normally induce aggregation. These findings are in agreement with those of Zucker *et al.* (9) and demonstrated that the absence of aggregation was not due to primary insensitivity to ADP. Responsiveness to ADP was also demonstrated by the observation that pretreatment with the nucleotide prevented the response to bovine fibrinogen. The appar-

TABLE IV. Aggregability of Washed Platelets in Presence of Mg^{2+}, Fibrinogen ± ADP, or Epinephrine (above); Release of nucleotide spontaneously or in presence of various inducers (below).

	Case 1	Case 2	Case 3	Control
	Aggregability of washed platelets (%)			
Mg^{2+} + Fibrinogen	0	3	0	29
+ ADP	0	3	0	44
+ Epinephrine	0	0	0	41
	10^{-9} moles of ADP released/10^9 platelets			
Spontaneously 4°	2	3	10	4
37°	4	8	15	9
Distilled water 37°	90	108	108	58
Kaolin	70	68	74	48
Latex		36	32	28
Bentonite		96	68	34
Thrombin (in PRP)	26		32	16

ent slight aggregation induced by collagen might not be true aggregation but rather signify adhesion of platelets to collagen polymers. This possibility casts some doubt on the measurement of collagen-induced aggregation by optical density change.

In washed platelet suspensions, kaolin and other particulate material induced normal or increased release of ADP as did collagen for platelet factor 4 in thrombasthenic platelets. These different effects of collagen particles or ADP and adrenaline on the release phenomenon (3) support our previous investigations (10). It would appear that unless the platelets are brought into close contact with one another (11), ADP and epinephrine will not induce release. Different reactions can take place inside the large aggregates as contrasted to individual platelets in the plasma. The inability of ADP and adrenaline to induce release in thrombasthenic platelets was therefore probably a consequence of the absence of aggregation. The same mechanism can explain the abnormal platelet factor 3 availability in platelet-rich plasma induced by kaolin (12) and ADP (1, 13). In PRP, kaolin or collagen can induce primary release of ADP due to adhesion or phagocytosis, followed by secondary release from normal platelets as aggregates are formed. If the release abnormalities in thrombasthenia are secondary to the aggregation defect, the mechanisms of defective aggregation and weak clot retraction remain to be explained. These mechanisms must be clearly distinguished from one another since in thrombasthenia the defective clot retraction presumably results from abnormal adhesion of platelets to fibrin (1). The effect of adrenaline on both activities of thrombosthenin (14), ATPase, and superprecipitation, and also on clot retraction, further support a role of thrombosthenin in clot retraction. If thrombosthenin is a prerequisite for the action of epinephrine, the unresponsiveness of thrombasthenic platelets to epinephrine might be due to some structural or behavioral anomaly of their contractile protein. This possibility is reinforced by the defect of microtubules found in pseudopods of thrombasthenic platelets (15). Perhaps an abnormal fibrinogen–thrombosthenin affinity also exists.

Fibrinogen is often decreased in thrombasthenic platelets and is an important cofactor for aggregation in a washed normal platelet suspension. When clot retraction occurs, thrombosthenin could be extruded and becomes attached to fibrin fibers; when the thrombosthenin then contracts, the clot will shrink. An abnormal thrombosthenin which does not become attached to fibrin could explain the defective clot retraction. Formation of fibrinogen bridges has been proposed for platelet aggregation (16). It has been proposed by Booyse and Rafelson (17) that thrombosthenin can be directly involved in

platelet aggregation by a dissociation and reassociation of actin and myosin between adjacent platelets. Thrombosthenin is known to occur near the platelet surface. ADP may act by inducing cell changes (18) which open up some hidden sites. The fibrinogen attached to the thrombosthenin of one platelet might then become attached to that of another. Bovine fibrinogen, which directly aggregates both normal and thrombasthenic platelets, may be able to attach directly to these sites (8). Conversely, pretreatment with ADP may induce structural changes such that bovine fibrinogen can no longer be attached. It is postulated that defective thrombosthenin unable to react properly with fibrin, fibrinogen or adrenaline could be responsible for the defects occurring in thrombasthenia.

Summary. Little platelet acid phosphatase or platelet factor 4 was made available from thrombasthenic platelets by adrenaline or ADP. Conversely, collagen induced marked but subnormal availability of platelet acid phosphatase and normal availability of platelet factor 4. Normal or increased amounts of ADP were released from thrombasthenic platelets in plasma by thrombin and by kaolin. It was concluded that the absence of release by ADP and epinephrine was the direct consequence of the lack of formation of large aggregates.

An hypothesis is suggested to explain the abnormalities found in thrombasthenia which is a defective thrombosthenin responsible for abnormal fibrinogen–thrombosthenin affinity, involved in platelet aggregation and clot retraction.

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