PRELIMINARY COMMUNICATION

Hereditary Giant Platelet Syndrome: A Disorder of a New Aspect of Platelet Function

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Summary

The platelets of three patients with the hereditary giant platelet syndrome of Bernard and Soulier failed to aggregate in response to either ristocetin or bovine fibrinogen. The results of aggregation experiments using mixtures of platelets and plasma suggest that a reaction between a plasma factor deficient in von Willebrand's disease and a platelet component lacking in our patients, and leading to platelet aggregation independently of adenosine diphosphate (ADP), is essential for normal haemostasis.

Introduction

Bernard and Soulier (1948) described an hereditary bleeding disorder characterized by a prolonged bleeding time, normal numbers of platelets with many giant forms, and defective prothrombin consumption. Thrombocytopenia has been an inconstant feature of the otherwise similar cases reported subsequently (Hirsch *et al.*, 1950; Alagille *et al.*, 1964; Cullum *et al.*, 1967; Kurstjens *et al.*, 1968; Gröttum and Solum, 1969; Bithell *et al.*, 1972), but the severity of the haemorrhagic symptoms is characteristically disproportionate to the platelet count. Few such cases have yet been studied by means of the newer tests of platelet function, and the mechanism of the haemostatic failure has not been satisfactorily explained. Bithell *et al.* (1972) reported the failure of platelets from four such cases to aggregate in response to bovine fibrinogen or to undergo the normal shape change on exposure to adenosine diphosphate (ADP). The first of these findings, though not the second, has been independently confirmed (J. P. Caen, personal communication, 1972). We describe here the results of investigations on three patients with the hereditary giant platelet syndrome which suggest that their haemorrhagic tendency is due to a failure of their platelets to react normally with a plasma factor deficient in von Willebrand's disease (von Willebrand factor).

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Case Reports

Cases 1 and 2 were two sisters from Kuwait, born in 1957 and 1963 respectively, of a first cousin marriage. Both had suffered since infancy from a serious haemorrhagic tendency, characterized chiefly by epistaxes, bleeding from the gums, and prolonged haemorrhage from superficial cuts and scratches. One of them (case 1) suffers from severe menorrhagia, for which transfusion was necessary, and the other (case 2) had to be transfused for anaemia resulting from prolonged bleeding after tooth extraction. Neither sister has had joint or muscle bleeds. Neither of their parents nor any of their four sisters and two brothers had any haemorrhagic symptoms. Investigation in Kuwait showed, in each girl, a mild thrombocytopenia (platelets 80-90,000/mm^3) with the presence of giant platelets and a very prolonged bleeding time, and they were therefore referred to London for further study.

Case 3 is an English girl born in 1967. Neither of her parents, who are unrelated, nor her two brothers suffer from any haemorrhagic tendency. She has suffered since infancy from repeated episodes of mucous-membrane haemorrhage, chiefly in the form of epistaxes and bleeding from the gums and gastrointestinal tract. Haematemesis necessitated transfusion on two occasions. She has also had multiple superficial bruises and recurrent crops of petechiae since her second year of life, but no deep haematomata or haemarthroses.

Materials and Methods

Platelet aggregation was studied by the turbidimetric method of Born (1962); none of the patients or controls had received aspirin for at least seven days. Since all patients had thrombocytopenia, platelet-rich plasma from cases 1 and 2 was tested at a platelet count of 50,000/mm^3, and that from case 3 at 150,000/mm^3. Control platelet-rich plasma was adjusted to the same platelet count in each case by dilution with autologous platelet-poor plasma. For aggregation experiments on mixtures of platelets and plasma from different subjects, platelets were separated from their own plasma by a combination of albumin-density centrifugation and gel filtration (Hutton and Howard, 1973), and resuspended at 150,000/mm^3 in the plasma to be tested.

Ristocetin (kindly supplied by H. Lundbeck and Co., Denmark) and bovine fibrinogen were dissolved in Owren's barbitone-buffered saline (pH 7·4) and used in final concentrations of 1·0 mg/ml and 1·7 mg/ml respectively. ADP was used at 1-2 μmol/l., human thrombin (Lister Institute) at 0·17 U/ml and bovine tendon collagen (Sigma) as a concentrated suspension.

Platelet size was determined on May-Grünwald-Giemsa-stained blood films: the diameter of 200 platelets from each patient was measured by each of two observers, using a micrometer eyepiece and a magnification of × 1,200. Platelet adhesion to collagen was studied by the method of Spaet and Lejnieks (1969), and platelet shape change in response to ADP as described by Born (1970). Platelet nucleotides were determined by the firefly luciferase method of Holmsen *et al.* (1966). Other investigations were performed by standard methods.

Results

As shown in the table, all three patients had thrombocytopenia associated with a high proportion of large platelets. No deficiency of plasma clotting factors or of platelet factor 3 availability was detected, yet prothrombin consumption was defective in all the patients. The platelets aggregated normally with ADP, collagen, and thrombin, adhered normally to collagen in the absence of free calcium ions, and were retained normally in a glass bead column. The platelets of all three patients contained adenine nucleotides

in normal proportions; the total amount per platelet was increased in the two sisters and normal in case 3. Platelet shape change was studied after the addition of ADP to edetic acid-platelet-rich plasma from case 1, in whom it occurred normally.

No aggregation occurred after the addition of ristocetin to platelet-rich plasma from any of the patients: typical tracings are shown in fig. 1A. Aggregability by ristocetin was not restored when the patients' platelets were washed and resuspended in platelet-poor plasma from a normal subject or that of a patient with severe von Willebrand's disease (fig. 1B). In contrast, the defect of ristocetin aggregation in von Willebrand's disease was corrected when the washed platelets were resuspended in platelet-poor plasma from either a normal subject or a patient with the giant platelet syndrome. The defect of ristocetin aggregation thus resides in the platelets of our patients but in the plasma of those with von Willebrand's disease.

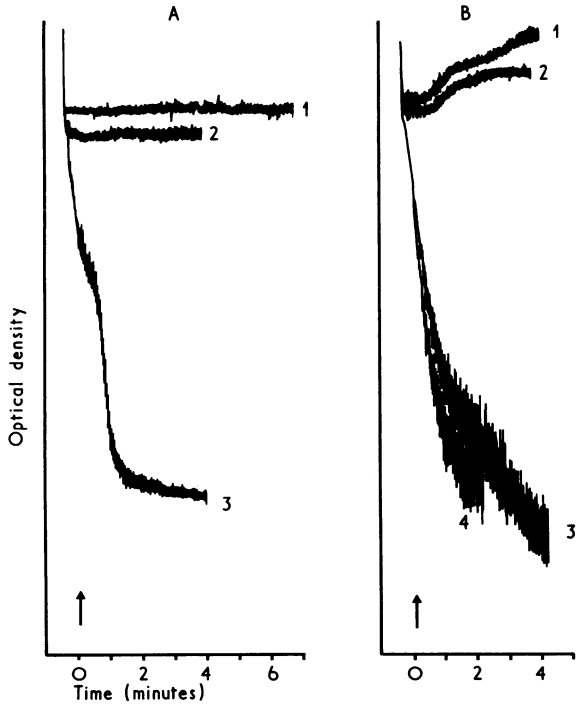

FIG. 1—Aggregation curves after addition of ristocetin at zero time. Platelet-rich plasma: A. 1=case 1; 2=von Willebrand's disease; 3=normal control. B. Platelet-plasma mixtures: 1=case 3 platelets, control plasma; 2=case 3 platelets, von Willebrand plasma; 3=von Willebrand platelets, control plasma; 4=von Willebrand platelets, case 3 plasma.

Our patients' platelets failed to aggregate on the addition of bovine fibrinogen, whether in their own plasma (fig. 2A), normal plasma, or plasma from a patient with von Willebrand's disease (fig. 2B). Von Willebrand's disease platelets aggregate normally with bovine fibrinogen, both in their own plasma and in that of a patient with the giant platelet syndrome.

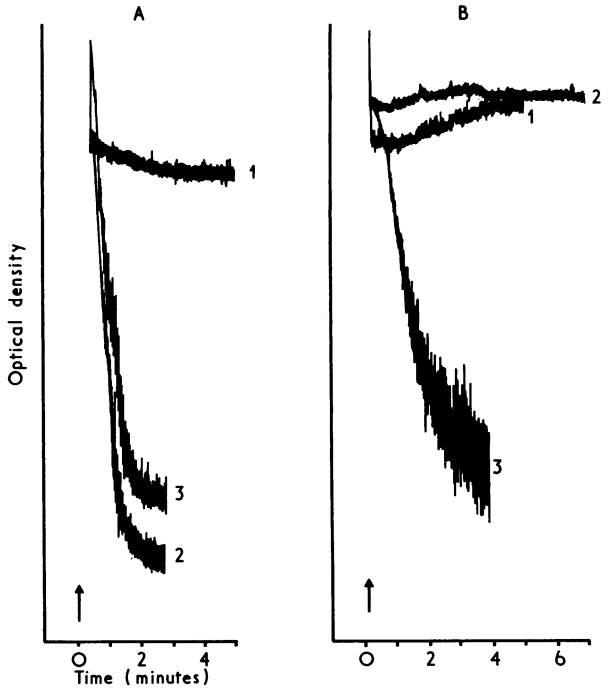

FIG. 2—Aggregation curves after addition of bovine fibrinogen at zero time. A. Platelet-rich plasma: 1=case 1; 2=von Willebrand's disease; 3=normal control. B. Platelet-plasma mixtures: 1=case 3 platelets, control plasma; 2=case 3 platelets, von Willebrand plasma; 3=von Willebrand platelets, case 3 plasma.

Investigations of Haemostatic Mechanism

Test	Case 1	Case 2	Case 3	Control or Normal Range
Bleeding time (Ivy; min)	>12	>12	12	<7
Platelet count (thousands/mm³)	18–38	22–27	91–120	150–400
Prothrombin time (sec)	15	14	13	14
Partial thromboplastin time (sec)	31	34	35	30–37
Factor VIII (one-stage; %)	144	80	80	50–200
Giant platelets (% > 2·5 μm diam.)	71	68	84	5
Prothrombin consumption index (%)	120	135	113	<30
Platelet retention by glass beads (%)	27	—	40	25–50
Platelet factor 3 availability (%)	150	130–160	120	40–150
Platelet adhesion to collagen	Normal	Normal	–	–
Platelet aggregation by ADP, collagen, and thrombin	Normal	Normal	Normal	–
Platelet nucleotides:				
ATP (nmol/10¹¹ platelets)	11·40	13·75	6·60	6·91
ADP (nmol/10¹¹ platelets)	8·06	7·50	4·40	4·21
ATP : ADP ratio	1·48	1·83	1·5	1·68

ADP = Adenosine diphosphate. ATP = Adenosine triphosphate.

Discussion

The results presented in the table and fig. 2 confirm many of the findings previously described in similar cases, including the absence of bovine-fibrinogen-induced aggregation (Bithell *et al.*, 1972; J. P. Caen, personal communication, 1972). We were unable to confirm the absence of shape change in response to ADP reported by Bithell *et al.* (1972).

The antibiotic ristocetin induces biphasic aggregation of normal platelets. The first phase is independent of ADP (Howard and Firkin, 1971), but requires the presence of von Willebrand factor (Howard *et al.*, 1973). Thus platelets from patients with von Willebrand's disease suspended in their own plasma fail to aggregate in response to ristocetin unless normal plasma or its antihaemophilic fraction is added. The platelets of our patients completely failed to aggregate in response to ristocetin, but the defect was not corrected by the addition of normal plasma, while the patients' plasma was able to correct the corresponding defect of platelet-rich plasma from a patient with von Willebrand's disease. It thus appears that the response to ristocetin depends on an interaction between a platelet component, defective in our patients, and a plasma factor deficient in von Willebrand's disease. Ristocetin, unlike collagen and ADP, is clearly an unphysiological reagent. The physiological importance of this platelet-plasma interaction may be inferred, however, from the fact that both von Willebrand's disease and the present platelet disorder result in significant bleeding symptoms associated with a long bleeding time, though the pathway of platelet adhesion to collagen, release of ADP, and subsequent aggregation is normal in each case. While bovine fibrinogen

(containing factor VIII, which is probably the active principle concerned—Forbes and Prentice, 1973; Donati *et al.*, 1973) fails to aggregate the platelets of patients with the giant platelet syndrome, it aggregates von Willebrand's disease platelets normally in their own plasma—perhaps because it also contains the von Willebrand factor.

The exact nature of this platelet-plasma interaction and its place in the haemostatic mechanism—that is, the physiological stimulus for which ristocetin substitutes in vitro—have yet to be discovered. For the present, our findings merely serve to emphasize that it is as necessary for normal haemostasis as is ADP-mediated aggregation.

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Formylation of Folate as Step in Physiological Folate Absorption

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Summary

Oral administration of folate analogues to rats is followed by a rise in plasma folate detectable only by micro-biological assay with Lactobacillus casei, suggesting methylation of folate during absorption. When using the everted rat gut technique with folate (PteGlu), dihydrofolate (H_2PteGlu), or tetrahydrofolate (H_4PteGlu) on the mucosal surface formyl folate (10-CHO-PteGlu, 10-CHO-H_4PteGlu, 5-CHO-H_4PteGlu) and methylfolate (5-CH_3-H_4PteGlu) are recovered from the serosal fluid. This indicates that absorption of PteGlu is followed by formylation and that the formyl group is further reduced to methyl, 5-CH_3-H_4PteGlu passing on to portal blood.

Introduction

Oral doses of reduced forms of folate monoglutamates such as dihydropteroylglutamic acid (H_2PteGlu), and tetrahydropteroylglutamic acid (H_4PteGlu) in man are followed by a rise in plasma methyltetrahydrofolate (5-CH_3-H_4PteGlu) (Baker *et al.*, 1965; Cohen, 1965; Chanarin and Perry, 1969; Perry and Chanarin, 1970; Pratt and Cooper, 1971; Whitehead *et al.*, 1972; Nixon and Bertino, 1972). In man, reduction and methylation of folate occurs in the small gut (Chanarin and Perry, 1969; Perry and Chanarin, 1970). Olinger *et al.* (1972), using isolated rat gut, reported that ^{3}H-PteGlu was converted to methyltetrahydro folate and that this conversion was interfered with by methotrexate.

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In this paper we report evidence that folate is formylated as a first step and that methylation presumably arises from reduction of the formyl group.

Materials and Methods

Pteroylglutamic acid labelled with tritium on the 3rd and 5th positions of the para-aminobenzoyl ring (Amersham) was used. H_2PteGlu and H_4PteGlu were prepared from ^{3}H-folate by reduction with ascorbate and dithionite (Blakley, 1960; Silverman and Noronha, 1961).

5-Formyl-tetrahydrofolate (Lederle) was generally labelled with tritium by an exchange with tritiated water in the presence of a platinum catalyst (Amersham) and purified by chromatography on A25 DEAE-Sephadex followed by G-15 Sephadex (Nixon and Bertino, 1971). Tritium-labelled 10-formyl-H_4PteGlu and 5-CH_3-H_4PteGlu were prepared from the 5-formyl compound (Rabinowitz, 1963; Chanarin and Perry, 1967).

Folate analogues were identified by chromatography on A25 DEAE-Sephadex using a 0·9 by 27 cm column and elution by a gradient system consisting of 1·0 mol/l. phosphate buffer in the reservoir and 0·1 mol/l. phosphate buffer in the mixing chamber. Both buffers were pH 6·0 with 0·1 mol/l. 2-mercaptoethanol. Five-millilitre fractions were collected, radioactivity was counted, and microbiological activity determined using *Lactobacillus casei*, *Streptococcus faecalis*, and *Pediococcus cerevisiae*. Marker compounds as prepared above were used for further identification of folate analogues. Radioactivity was counted on a Wallac liquid scintillation counter.

Male Sprague-Dawley rats weighing 250 g were used to prepare everted sacs (Wilson and Wiseman, 1954). Three 15-cm segments, starting from the pyloric end of the gut, were prepared from each animal. One millilitre of Krebs Ringer bicarbonate solution pH 6·1 was introduced into each sac and the sacs were incubated in 25 ml Krebs Ringer bicarbonate containing glucose 28 mmol/l. The folate compounds under test were added to the mucosal fluid at a concentration of 10 nmols, and the sacs exposed to O_2-CO_2 (95/5%) for three minutes and incubated for one hour at 37°C. At the end of this period ascorbate (pH 6·0) was added to both serosal and mucosal fluids to give a 1% concentration. Folate analogues in these fluids were separated by chromatography and identified.

Results

When 5 μg H_2PteGlu were given orally to three rats there was