

Platelet Aggregation in Whole Blood From Patients With Glanzmann's Thrombasthenia

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We examined platelet aggregation in platelet-rich plasma (PRP) and in whole blood from two patients with Glanzmann's thrombasthenia. In PRP, aggregation was measured by monitoring the changes in light absorbance that occurred in response to aggregating agents; to measure platelet aggregation in whole blood, we used a platelet counting technique. In PRP, the patients' platelets showed defective aggregation in response to ADP, adrenaline, arachidonic acid (AA), and collagen, but normal agglutination occurred in response to ristocetin. In whole blood, however, platelet aggregation in response to the aggregating agents appeared to be either very similar to that which

occurred in blood from normal subjects or only slightly reduced. There was a reduced response to all concentrations of ADP and to low concentrations of collagen but a normal response to all concentrations of adrenaline, AA, and higher concentrations of collagen. Conversely, there seemed to be an increased agglutination response to ristocetin. The abnormality in our two patients with Glanzmann's thrombasthenia probably lies in the inability of their platelets to form large, macroscopic aggregates rather than in platelet aggregation per se.

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GLANZMANN'S thrombasthenia is a rare bleeding disorder characterized by defective platelet aggregation but with a normal platelet count.¹ Platelets from patients with this condition either do not aggregate or aggregate very poorly in response to agents such as ADP, adrenaline, arachidonic acid (AA), and collagen. Platelets from such patients agglutinate normally in response to ristocetin,^{2,3} and this has been used to aid the diagnosis of the condition. Defective platelet aggregation is believed to result from a markedly reduced amount of a complex of glycoproteins (the glycoprotein IIb/IIIa complex) on the platelet surface.⁴ This glycoprotein complex is essential for normal platelet aggregation but plays no part in ristocetin-induced platelet agglutination.

Previous studies in which platelet aggregation in Glanzmann's thrombasthenia was compared with platelet aggregation in healthy controls involved the use of platelet-rich plasma (PRP), in which aggregation is measured by following the changes in light absorbance that occur. With this technique, macroscopic platelet aggregates must form before the light absorbance of the PRP falls. It is now possible to measure platelet aggregation in whole blood by counting the number of single platelets (ie, unaggregated platelets) using a whole-blood platelet counter.^{5,6} Because the technique relies on the determination of the number of single platelets that remain in the blood rather than the size of the platelet aggregates that form, it is an extremely sensitive means of detecting platelet aggregates composed of relatively few platelets. In the present investigation, we compared platelet aggregation in PRP (measured with light absorbance) and

whole blood (measured with the platelet counting technique) from patients with Glanzmann's thrombasthenia. The results obtained using whole blood were compared with those obtained for healthy controls.

MATERIALS AND METHODS

Materials. Adenosine 5'-diphosphate (ADP, sodium salt) adrenaline, and arachidonic acid (AA, grade 1 from porcine liver) were obtained from Sigma Chemical (St Louis). ADP and adrenaline were dissolved in saline before use; AA was converted to the sodium salt before use by dissolving it in 0.1 mol/L of sodium carbonate. Collagen (suspended equine collagen fibrils) was obtained from Hormon-Chemie and used as directed. Ristocetin sulphate was obtained from H. Lundbeck and dissolved in saline. Saline was 150 mmol/L of sodium chloride from Travenol Laboratories. The solution of citrate that was used to anticoagulate blood was 3.8% (wt/vol) trisodium citrate dihydrate. M148 (a monoclonal antibody directed against the glycoprotein IIb/IIIa complex) was a gift from Professor R.M. Hardisty, Institute of Child Health, London, and was used at a final dilution of 1:2,000. A control murine antibody 791T/36 (human osteogenic sarcoma cell-line) was a gift from Dr M. Price, Cancer Research Laboratories, University of Nottingham, England.

Subjects. Blood samples were taken from a sister and brother, designated as patient 1 and patient 2 throughout, aged 17 and 19 years, respectively, who had a similar history of mild bruising and bleeding. The diagnosis of Glanzmann's thrombasthenia had been made by Dr F.E. Preston (Department of Haematology, Royal Hallamshire Hospital, Sheffield, England) on the basis of clinical history and the pattern of platelet aggregation in PRP characteristic of the condition. When examined, the patients exhibited prolonged bleeding times (30 and 21 minutes, respectively), but clot retraction was normal as were the whole blood platelet counts.

Blood samples were also taken from six healthy controls (three men and three women with an age range of 18 to 42 years) to obtain a set of normal values for platelet aggregation in whole blood. The tests on these samples were carried out using the same protocol as that used for the two patients.

Blood collection. Blood was collected with minimum venous stasis from the antecubital vein into polypropylene syringes using a 19 gauge, 2-inch needle, and aliquots (9 mL) were dispensed into polystyrene tubes that contained 1 mL of citrate. Some of the citrated blood was centrifuged at 180 g for 10 minutes to prepare PRP, and the blood from which the PRP had been removed was then centrifuged at 1,200 g for 20 minutes to obtain platelet-poor plasma. The latter was used to adjust the platelet count in the PRP to 300×10^9 platelets/L.

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Measurements of platelet aggregation. Platelet aggregation in PRP was studied using the aggregometer and technique described by Adams and colleagues.⁷ Changes in light absorbance were recorded using a pen recorder.

Platelet aggregation in whole blood was assessed by determining the number of single platelets that remained in the blood after adding an aggregating agent to a sample. The number of single platelets present was counted using an Ultra-Flo 100 Whole Blood Platelet Counter and the technique described by Fox and co-workers.⁶ In brief, samples (500 μ L) of blood were placed in small polystyrene tubes, and the tubes were placed in a water bath at 37 °C. After 2 minutes, a small aliquot of the blood was removed for platelet counting, a metal stirrer bar was placed in each tube, and the blood was stirred at 1,000 rpm. After a further 2 minutes, another small aliquot was removed for platelet counting, and a solution of the aggregating agent under investigation was then added. Stirring was then continued, and further small aliquots were removed for platelet counting at 2-minute intervals up to 6 minutes after addition of the aggregating agent. The results obtained were expressed as the percentage of single platelets lost from the blood relative to the number of platelets present immediately prior to the addition of aggregating agent.

Inhibition of platelet aggregation by M148. The monoclonal antibody, M148, binds to the glycoprotein IIb/IIIa complex on the surface of normal platelets and inhibits aggregation induced by ADP, adrenaline, AA, and collagen in both PRP and in whole blood.⁸ In some experiments, M148 (at a final dilution of 1:2,000) was added to blood from the two patients with Glanzmann's thrombasthenia 2 minutes before the aggregating agent; aliquots were then removed for platelet counting at 20-second intervals up to 2 minutes after addition of the aggregating agent. To facilitate this quicker subsampling technique, the aliquots (15 μ L) were taken into fixative as described by Bevan and colleagues⁹ to be counted later on the Ultra-Flo 100. The results were compared with those obtained using a control murine antibody (791T/36).

Measurement of glycoprotein expression. Expression of the glycoprotein IIb/IIIa complex on the surface of platelets from the patients was measured using the monoclonal antibody, M148,¹⁰ combined with the technique of flow cytofluorimetry. After 240 μ L samples of PRP were incubated with M148, aliquots containing 2×10^6 platelets were removed to tubes containing a fluorescent-labeled rabbit anti-mouse immunoglobulin as described previously.¹¹ After a further incubation, immunofluorescence analysis was carried out using the FACS IV Flow Cytofluorimeter.¹²

Microscopic examination of platelet aggregates. After samples of blood from both patients with Glanzmann's thrombasthenia and samples of blood from a healthy control were stirred with ADP (10 μ mol/L), adrenaline (10 μ mol/L), AA (0.2 mmol/L), collagen (6 μ g/mL), and ristocetin (2 mg/mL), each for 6 minutes, the blood was smeared on a glass slide and stained with May-Grunwald-Giemsa stain. The tails of the blood smears were then examined for platelet/platelet aggregates under a light microscope. The effect of preincubating blood from the patients and controls with M148 or control antibody prior to adding 5 μ mol/L of ADP was also determined.

RESULTS

Platelet aggregation in PRP. The light absorbance of samples of PRP from the patients with Glanzmann's thrombasthenia either did not fall or fell only slightly when adrenaline, ADP, AA, or collagen was added to the PRP. In contrast, adding ristocetin to PRP from the patients induced a large fall in light absorbance; this was accompanied by the appearance of macroscopic platelet aggregates in the sample.

Examples of the results obtained for the two patients are given in Fig 1, these results having been seen on more than one occasion.

Platelet aggregation in whole blood. In contrast to the results obtained in PRP, platelet aggregation did occur when ADP, adrenaline, AA, or collagen was added to whole blood from the patients with Glanzmann's thrombasthenia. Table 1 shows the percentage loss of single platelets 6 minutes after the addition of aggregating agents to samples of whole blood obtained from the two patients and the controls. There was a reduced response in both patients, as compared with the mean control value, to all concentrations of ADP (Fig 2) and to the lower concentrations of collagen (0.3 and 1.0 μ g/mL) and AA (0.1 mmol/L) but a normal response to all concentrations of adrenaline and higher concentrations of collagen (3 and 6 μ g/mL) and AA (0.2 mmol/L). There was an increased agglutination response to the lower concentration of ristocetin used.

When samples of whole blood from the patients with Glanzmann's thrombasthenia were preincubated with M148, the platelet aggregation induced by ADP, adrenaline, and collagen, was virtually abolished (Fig 3).

Glycoprotein expression. After immunofluorescence analysis of platelets incubated with M148 followed by a fluorescent-labeled immunoglobulin, the number of glyco-

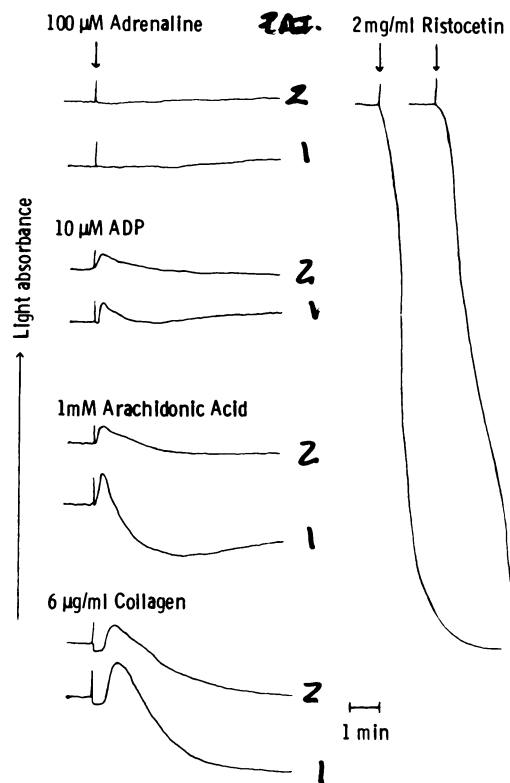


Fig 1. The changes in light absorbance that occurred in platelet-rich plasma (PRP) (480 μ L) that had been stirred at 37 °C for 2 minutes followed by the addition of various aggregating agents (20 μ L). Typical traces obtained from both of the patients on one occasion are shown. For each aggregating agent, the first of each pair of traces is that obtained for patient 2 and the second is that for patient 1.

Table 1. Percentage of Single Platelets Lost Six Minutes After Addition of Aggregating Agents to Samples of Whole Blood Stirred at 37 °C

Aggregating Agent	Patient 1	Patient 2	Controls
Saline	2	4	10 ± 6
ADP (0.5 μmol/L)	9	8	22 ± 5
ADP (1.0 μmol/L)	4	6	30 ± 14
ADP (2.0 μmol/L)	26	15	52 ± 22
ADP (10.0 μmol/L)	40	28	90 ± 9
Adrenaline (0.2 μmol/L)	16	10	10 ± 7
Adrenaline (0.5 μmol/L)	18	19	16 ± 9
Adrenaline (1.0 μmol/L)	21	25	20 ± 9
Adrenaline (2.0 μmol/L)	24	38	26 ± 10
Adrenaline (5.0 μmol/L)	30	39	33 ± 17
AA (0.1 mmol/L)	4	2	43 ± 33
AA (0.2 mmol/L)	92	93	95 ± 3
Collagen (0.3 μg/mL)	11	7	20 ± 14
Collagen (1.0 μg/mL)	31	18	80 ± 29
Collagen (3.0 μg/mL)	89	90	96 ± 1
Collagen (6.0 μg/mL)	92	94	97 ± 1
Ristocetin (1.0 mg/mL)	96	99	20 ± 13
Ristocetin (2.0 mg/mL)	97	99	95 ± 2

Control values are mean ± SD (n = 6).

protein IIb/IIIa complexes on the surface of platelets from patients 1 and 2 were calculated to be 450 and 1,600, respectively. This compares with a mean value of 17,300 for platelets from normal subjects.⁸ We have already shown that these patients have platelets that are deficient in glycoprotein

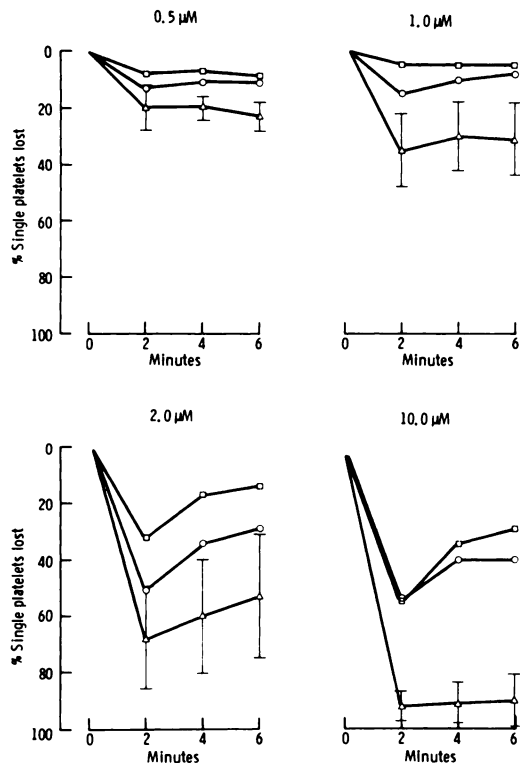


Fig 2. The percentage of single platelets lost from whole blood stirred at 37 °C following the addition of different concentrations of ADP at time 0. The results shown are for patient 1 (O) and patient 2 (□). The control values (Δ) are mean ± SD (n = 6).

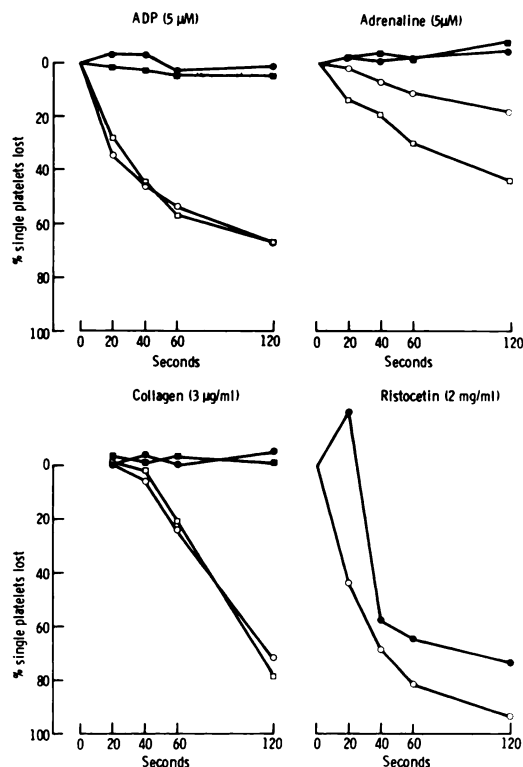


Fig 3. The percentage of single platelets lost from whole blood stirred at 37 °C following the addition of various aggregating agents at time 0. The blood had been preincubated for 2 minutes with either M148 or a control antibody. The results are for patient 1 in the presence of either M148 (●) or control antibody (○) and for patient 2 in the presence of either M148 (■) or control antibody (□). Unfortunately, only data for patient 1 were obtained for ristocetin-induced agglutination.

IIb/IIIa and that the deficiency does not reside in a subpopulation of cells.¹¹

Microscopy. Samples of blood to which aggregating agents had been added were examined for the presence of platelet aggregates by light microscopy. Platelet aggregates were present in whole blood from the patients with Glanzmann's thrombasthenia as well as in blood from the normal control. When blood from the patients or controls was incubated with M148 before adding ADP, only single platelets were seen.

DISCUSSION

The results of our studies on platelet aggregation in PRP in which we used light absorption as a means of measuring the platelet aggregation that occurred are entirely consistent with those obtained by other workers who have investigated patients with Glanzmann's thrombasthenia.¹⁻³ Aggregation was clearly defective in PRP from the two patients that we investigated. We were therefore surprised to find that the loss of single platelets induced by the addition of aggregating agents to whole blood from the patients appeared to be very similar to the loss obtained from healthy controls or was only slightly reduced. The reduced response to ADP was >1 SD at lower concentrations and >2 SDs at the highest concentra-

tion (10 $\mu\text{mol/L}$), whereas the reduced responses to collagen and AA were only seen at the lower concentrations tested.

By preincubating whole blood from the patients with M148, we were able to confirm that the single platelets that were lost when an aggregating agent was added to whole blood were indeed taking part in aggregate formation. The addition of this monoclonal antibody virtually abolished the platelet loss that occurred following addition of ADP, adrenaline, or collagen to blood from the patients with Glanzmann's thrombasthenia. As further confirmation that platelet aggregation was occurring in whole blood, we looked directly for platelet aggregates in blood from the patients using light microscopy. Platelet aggregates were observed in all the samples to which an aggregating agent had been added. In addition, M148 prevented aggregate formation.

We consider that the apparent discrepancy between results obtained in whole blood and PRP in this investigation relates to the differences in the two techniques that were used to measure the aggregation that occurred. The light absorbance of PRP falls only when very large platelet aggregates (that are visible to the naked eye and must contain many thousands of platelets) form. Conversely, the number of single platelets in whole blood falls as soon as the single platelets take part in aggregate formation whatever the size of the aggregates that develop. The defective platelet aggregation in PRP from patients with Glanzmann's thrombasthenia may occur because the aggregates that form are relatively small. We believe that it is unlikely that the different results in PRP and whole blood relate to the presence of RBCs in the latter, but this possibility has not been excluded.

Although the patients we investigated had platelets that contained markedly reduced amounts of surface-expressed glycoprotein IIb/IIIa complex (3% and 9% of normal for patients 1 and 2, respectively), the platelets were not completely devoid of the glycoprotein complex. In addition, clot retraction occurred in coagulated whole blood from these patients. Thus, according to the definition of Caen,¹³ our patients probably have type II Glanzmann's thrombasthenia. Platelets from patients with type I Glanzmann's thrombasthenia have undetectable amounts of the glycoprotein complex and clot retraction is absent in coagulated blood.

Although Nurden and colleagues¹⁴ recently reported the presence of measurable amounts of glycoprotein IIb and/or glycoprotein IIIa on the platelets of patients with type I Glanzmann's thrombasthenia, they proposed that it was the number of functional glycoprotein IIb/IIIa complexes expressed on the surface of the platelet that was important in discriminating between the two subgroups. It is probable that the small amounts of complex that are present on our patients' platelets allow partial platelet aggregation that is detectable in our whole blood system but not in PRP. In view of the reduced platelet aggregation that occurs in response to ADP and low concentrations of collagen in whole blood from these patients, it will be interesting to discover if platelet aggregation is further reduced in whole blood from patients with a more severe glycoprotein IIb/IIIa complex deficiency. It must be noted that in our investigation platelets from patient 1 aggregated slightly more extensively in both PRP and whole blood than did platelets from patient 2, despite having a lesser amount of the glycoprotein IIb/IIIa complex available and a longer bleeding time. This lack of correlation between glycoprotein expression, platelet aggregation, and bleeding time in these two patients may be caused by the close similarity of the patients with regard to both clinical and laboratory parameters, however.

In the cases of Glanzmann's thrombasthenia we have studied, only relatively small amounts of glycoprotein IIb/IIIa were expressed, and the deficiency resides in all the platelets rather than in a subpopulation.¹¹ We have suggested that it is the amount of glycoprotein IIb/IIIa on the surface of platelets that determines the size of the aggregates that are produced in response to an aggregating agent and that the true deficiency in Glanzmann's thrombasthenia relates to the size of the aggregates produced rather than to aggregation per se. The precise relationship between aggregate size and glycoprotein IIb/IIIa expression needs to be explored by further experimentation.

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