Biochemical Studies of Two Patients with the Gray Platelet Syndrome

SELECTIVE DEFICIENCY OF PLATELET ALPHA GRANULES

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ABSTRACT The biochemistry of platelets from two unrelated patients with the gray platelet syndrome, a deficiency of platelet α-granules, has been evaluated. Ultrastructural studies of their platelets revealed the number of α-granules to be <15% of normal, whereas the number of dense bodies was within normal limits. Platelets from both patients had severe deficiencies of platelet factor 4 and β-thromboglobulin (<10% of normal). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed a marked deficiency of thrombin-sensitive protein in both patients. Analysis of the platelet-derived growth factor in one patient showed it was also markedly reduced. Levels of lysosomal enzymes, adenine nucleotides, serotonin, and catalase, and conversion of arachidonic acid by the lipoxygenase and cyclo-oxygenase enzymes, were within normal limits. The results provide important evidence to define the contents of α-granules, and to differentiate these contents from the contents of lysosomal granules, dense bodies, and peroxisomes. Functional studies of these platelets showed deficiencies in ADP, thrombin, and collagen aggregation. The results suggest that α-granules or their contents make a contribution to normal platelet aggregation.

INTRODUCTION

Platelets are circulating blood cells primarily involved in hemostatic and thrombotic functions (1, 2). Disorders of platelet function may predispose to bleeding or to thrombotic and atherosclerotic disorders (3, 4). Understanding of the biology of this cell has been facilitated by careful study of congenital abnormalities of platelets as in thrombasthenia, a deficiency of platelet surface glycoproteins (5–7), and the Hermansky-Pudlak syndrome or storage pool deficiency in which a selective absence of platelet-dense granules occurs (8–11). These natural experiments provide important clues to the role of the missing structures in cellular physiology. The gray platelet syndrome, first described by Raccuglia in 1971 (12), is a disorder characterized by large platelets that contain few granules and, therefore, appear gray when viewed under the light microscope, the characteristic that gives the disorder its name. Electron microscopic studies of this disorder have revealed a relative deficiency of α-granules, and normal numbers of dense bodies and peroxisomes in these platelets (3, 12–14). We now report an evaluation of the biochemistry of these platelets, including analysis of adenine nucleotides and serotonin constituents of platelet-dense bodies, hydrolytic enzymes present in platelet lysosomes, catalase believed to be in the platelet peroxisomes, and various polypeptides present in α-granules. The selective absence of α-granules and their contents in this disorder provides a new approach to subcellular localization of certain platelet proteins, and provides a natural experiment to probe the role of α-granules in platelet function.

METHODS

Patients. Two patients with the gray platelet syndrome were studied. The first was a 22 yr-old male originally described by Raccuglia (12). This patient had petechiae and ecchymoses as a newborn and a bruising tendency with recurrent knee pain, presumably intraarticular bleeding, throughout childhood. His platelet count during this period ranged from 25,000–150,000/mm³. It increased temporarily on two occasions after prednisone and permanently after splenectomy at age nine, though it has been slightly below normal in the last few years (range 100,000–150,000/mm³). He has had only one severe bleeding episode (a large hematoma) since then, after a football injury, although his bleeding times have remained slightly longer than normal. The second patient studied was a girl with Goldenhar's syndrome (oculo-auriculovertebral dysplasia). She developed a recurrent petechial rash associated with frequent bruises at about 8 mo of age, a time when she was frequently falling in her efforts to learn to walk. This bruising tendency continued. About 2 yr of age she developed a large black eye after her face had been hit lightly. She was then referred for evaluation and found to have large gray platelets on a Wright stained blood smear. This patient is now age four. She has continued to have a bruising tendency, and on one occasion, after a small laceration to one toe, she bled for a "long time" before it stopped. Her platelet count has ranged between 100,000 and 150,000/mm³.

Experimental. For most experiments, blood drawn from the antecubital vein from patients or normal donors was anticoagulated with citrate-citric acid dextrose, pH 6.5, to achieve final concentrations of 9.3 mM sodium citrate, 0.7 mM citric acid, and 14 mM dextrose. For study of platelet polypeptides and surface glycoproteins, 17.2 ml of blood was drawn from each normal donor and each patient and mixed with 2.8 ml of acid citrate dextrose (15). The blood was then centrifuged immediately at 100 g for 20 min at room temperature to obtain platelet-rich plasma. Platelet aggregation studies were performed using a Payton dual channel aggregometer (Payton Associates, Buffalo, N. Y.) as described previously (16). Samples for study of platelet ultrastructure were fixed, embedded, sectioned, stained, and viewed under a Phillips 301 electron microscope (Philips Co., Mount Vernon, N. Y.) as described previously (17). Quantitative morphometry of platelets was performed on randomly sampled electron micrographs (×30,000) using an overlain grid of intersecting lines 0.6 cm apart. Point counts of structures beneath intersects were performed to evaluate the contribution of various platelet structures to total platelet volume (18). For each sample, 1,200–1,500 intersects representing 30–50 platelets were counted. Analysis of platelet size was performed using a Coulter counter model ZB channelyzer C-1000 (Coulter Electronics Inc., Hialeah, Fla.) (19). Platelet serotonin levels were measured spectrophotofluorometrically (20). Platelet adenine nucleotides were measured using high pressure liquid chromatography (21). Platelet fibrinogen was measured on gel-filtered platelets by radioimmunoassay (22, 23). Catalase and lysosomal enzymes were measured using standard assays (24, 25). The conversion of arachidonic acid by washed platelets was assessed after stirring of the cells with the fatty acid for 5 min, using both quantitative gas chromatography mass spectrometry, and by assessing conversion of labeled [¹⁴C]arachidonic acid, using thin layer chromatographic separation of products as described earlier (26).

Platelet-derived growth factor was assayed using fibroblast 3T3 cells as described previously (27, 28). To release the platelet-derived growth factor from the platelets, the cells were frozen-thawed three times, the homogenate centrifuged at 16,000 rpm in a Sorvall SS-34 rotor (DuPont Instruments-Sorvall Biomedical Div., DuPont Co., Newtown, Conn.) for 30 min, the pellet resuspended in 1.09 M NaCl, 0.01 M Tris, pH 7.4, and spun again for 30 min using the same conditions. The combined supernates were dialyzed against phosphate-buffered saline for 24 h. The frozen-thawed supernate was equivalent to 0.34×10^9 platelets/ml, and the salt extract equivalent to 0.70×10^9 platelets/ml.

Platelet factor 4 (PF4)[1] and β-thromboglobulin were measured by radioimmunoassay. The procedure used in Lab B for both PF4 and β-thromboglobulin has been described elsewhere (29). The assay for PF4 used in Lab A involved its purification according to the procedure of Hermondson et al. (30), then preparation of antisera by injecting 3-kg New Zealand white rabbits with 1 mg purified protein mixed with complete Freund's adjuvant. Serum, collected after antibody was demonstrated by a precipitin line on agar gel electrophoresis, was stored in 0.3-ml aliquots in a −70° freezer. For the radioimmunoassay, the PF4 was labeled using lactoperoxidase, by combining 10 μg of purified PF4 with 1 mCi of ¹²⁵I in 40 μl of 0.4 M Na acetate. 10 μl of H_2O_2 (~3×10^{-6}g) was added to the mixture for 5 min at room temperature, after which an additional 5 μl of H_2O_2 was added. At 8 min total time, the reaction mixture was run over a heparin agarose column and washed with phosphate-buffered saline, pH 6.4, to remove the unbound ¹²⁵I and to stop the lactoperoxidase reaction. When the eluate had <1,000 cpm/10 μl, the ¹²⁵I-PF4 was eluted with 2 M NaCl and collected in 0.5-ml fractions in plastic tubes coated with 0.2% bovine serum albumin. The ¹²⁵I-PF4 had about 6×10^7 counts/μg. The labeled fraction with the greatest activity was used in the assay after dilution with standard buffer to give 20,000 cpm/20 μl and stored at −70°. ¹²⁵I-PF4 was stable for 3–4 mo.

For the radioimmunoassay itself, standard unlabeled PF4 was diluted in buffer (0.1 M Tris, 0.15 M NaCl, 0.2% Na azide, 0.5 U heparin/ml, and 2% dog plasma) to the PF4 concentrations in the standard curve that included 10 ng, 3 ng, 1 ng, 300 pg, 100 pg, 30 pg, and 10 pg. A zero point, representing maximal precipitation of ¹²⁵I-PF4 by antibody with no added PF4, was also included in the standard curve. The antibody, diluted 20,000–40,000-fold in buffer, gave ~50% binding of the ¹²⁵I-PF4. Excess antibody bound 95% of the labeled PF4.

The assay was then performed in triplicate as follows: 300 μl of diluted antihuman PF4 antibody was mixed with 100 μl of PF4 standard or 100 μl of patient sample, to which 20 μl of radiolabeled PF4 (20,000 cpm) was added. This combination was mixed and incubated overnight at room temperature. Then 50 μl of goat antirabbit immunoglobulin (Ig)G in 0.9% saline and 100 μl of carrier rabbit IgG (0.1 mg) in 0.9% saline were added, mixed, incubated for 2 h, after which 1 ml of 0.01 M Tris, 0.15 M NaCl was added. The tubes were centrifuged at 7,000 g for 20 min. The unbound ¹²⁵I-PF4 in the supernate was decanted and the precipitate counted. Samples for determination of serum levels were prepared by dilution of serum with standard buffer. The 1:500 and 1:1,000 dilutions were placed in the assay and the results of the two dilutions averaged for the final result. Also included in each assay was a normal control pool sample derived from 20 healthy subjects and stored in aliquots at −20°. By measuring the amount of ¹²⁵I-PF4 without added antibody, the patient samples and all dilutions showed nonimmune precipitations of 2.4–4.0% of total counts. Results were calculated as percent inhibition

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[1] *Abbreviations used in this paper*: HETE, 12L-hydroxy-5,8,10,14-eicosatetraenoic acid; HHT, 12L-hydroxy-5,8,10-heptadecatrienoic acid; PF4, platelet factor 4; SDS, sodium dodecyl sulfate; TSP, thrombin sensitive protein.

of binding. The sample without added PF4 (the zero point) represented ~100% binding. Log percentage of inhibition was plotted against log PF4 concentration in the standard; the unknown samples were calculated as percent inhibition of binding and results read from the standard curve.

Secretion of serotonin and thrombin-sensitive protein. Platelets were labeled with [^{14}C]serotonin by adding the radioactive serotonin (0.45 μM final concentration) to the platelet-rich plasma and incubating the solution for 1 h at 18° with occasional agitation. After washing, the platelets were suspended to a concentration of 10^9/ml in ETS buffer (0.154 M sodium chloride, 0.01 M tris, and 1 mM EDTA, pH 7.4). We then added 1 U thrombin to 1-ml samples to initiate the secretion reaction and incubated the samples for 5 min at 37°. The percentage of serotonin secreted was determined on centrifuged samples according to the method described by Massini and Luscher (31). The secretion of thrombin-sensitive protein was monitored by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis of both pellet and supernatant fractions.

SDS-polyacrylamide gel electrophoresis. Platelets were separated from platelet-rich plasma and washed by the method previously described (31). The washed platelets were then suspended in ETS buffer. The washed platelets and the thrombin-induced secreted proteins were solubilized in SDS by the procedure previously described (32). These polypeptides were then separated by electrophoresis through a slab gel with a 5–20% exponential gradient of acrylamide similar to the one originally described by Laemmli (33). The details used for obtaining electrophoretic separations of reduced samples have been reported previously (34). The polypeptides in the gels were stained with Coomassie Blue. Surface glycoproteins were analyzed after lactoperoxidase-catalyzed iodination (33).

Chemicals. [^{14}C]serotonin (55 Ci/mole) was purchased from Amersham/Searle Corp., Arlington Heights, Ill., [1-^{14}C]-arachidonic acid from New England Nuclear, Boston, Mass. Bovine thrombin was purified as previously reported (35) to a specific activity of 1,800 U/mg protein. Acid soluble collagen was obtained from Worthington Biochemical Corp., Freehold, N. J. All other chemicals were reagent grade.

RESULTS

Platelet morphology and size. Detailed qualitative electron microscopy has been reported previously, showing a defect in α-granules in these platelets (13). For the purpose of the present paper, quantitative morphometry was performed and is shown in Table I. Gray platelets contained fewer α-granulelike structures than controls (~10–15% of the control numbers). In addition, gray platelets contained an increased amount of surface-connected canalicular system compared with controls. The number of mitochondria and dense bodies in gray platelets were within the normal range for control platelets. The size of the gray platelets was increased with a mean platelet volume of 11.1 μm^3 for patient R.K. compared with the normal range of 5.7–8.9 μm^3. The platelets from M.P. were not sized quantitatively but, when viewed using the electron microscope, appeared large and identical to those of R.K.

Platelet serotonin, adenine nucleotides, lysosomal enzymes, and catalase. Measurement of platelet ATP and ADP were within normal limits, as were measurements of platelet serotonin (Table II). Platelet lysosomal enzymes were measured only in R.K., with acid phosphatase, β-glucuronidase, and β-N-acetylglucosaminidase, all well within the normal range ($\pm$3 SD) when considered in terms of platelet number or platelet protein (Table III). Catalase activity was also normal in R.K., with a value of 8.8 U/mg platelet protein, and control values ($n = 3$) of 3.9–9.5 U/mg platelet protein.

Platelet fibrinogen, β-thromboglobulin, PF4, and platelet-derived growth factor. When compared with control levels of platelet fibrinogen (7.1 μg/10^8 platelets; range$\pm$3SD = 4.1–10.1), gray platelets were markedly decreased (R.K. = 0.6 μg/10^8 platelets; M.P. = 0.3 μg/10^8 platelets). Plasma fibrinogen levels (R.K. = 2,300 μg/ml, M.P. = 3,300 μg/ml) were not decreased (normal range$\pm$3SD = 1,900–3,200 μg/ml). Levels of the platelet-specific proteins β-thromboglobulin and PF4 were very low when measured in frozen-thawed platelet-rich plasma or on the released substance, after

TABLE I

Morphometric Analysis of Normal and Gray Platelets

	Percent contribution to total platelet volume			
	Mitochondria	Surface-connected canalicular system	Dense granules	α-Granulelike structure*
Normals				
1	2.85	9.26	1.97	11.2
2	3.02	10.34	2.78	10.3
3	2.73	9.52	1.60	8.52
4	3.50	10.10	0.97	13.2
5	3.37	11.90	2.14	11.5
Mean$\pm$1SD	3.09$\pm$0.30	10.22$\pm$0.92	1.89$\pm$0.60	10.9$\pm$1.6
Gray platelets				
R.K.	3.43	13.3	2.71	1.39
M.P.	2.43	22.4	2.24	0.99

* Includes α granules and lysosomes.

TABLE II
Platelet Serotonin and Adenine Nucleotides

		Adenine nucleotides	
	Serotonin	ATP	ADP
	$ng/10^9$ platelets	$\mu mol/10^{11}$ platelets	
Patients			
R.K.	469	7.09	5.46
M.P.	353	6.28	3.84
Normal donors (n = 10) mean	488	7.54	3.92
range (±3SD)	138–838	5.7–9.3	2.1–5.7

thrombin treatment of platelets (Table IV). However, levels of these polypeptides in platelet-poor plasma were higher than normal. The platelet-derived growth factor (Fig. 1) was essentially undetectable in growth curves in which material released from R.K. platelets was compared with that from controls.

Platelet prostaglandin and thromboxane synthesis. Platelets from R.K. were studied for their ability to convert arachidonic acid to thromboxane B_2 using quantitative gas chromatography mass spectrometry. His gray platelets converted 33 nmol arachidonic acid into 6.5 nmol thromboxane B_2 (normals 7.2±2.4 nmol (mean±SD, n = 3)). Platelets from M.P. were evaluated for their ability to convert [^{14}C]arachidonic acid to 12L-hydroxy-5,8,10,14-eicosatetraenoic acid (HETE), 12L-hydroxy-5,8,10-heptadecatrienoic acid (HHT), and thromboxane B_2. Her platelets converted 41.4% of labeled arachidonic acid to HETE (normals 33.2±14.6), 25.4% to HHT (normals 29.8±7.7), and 25.7% to thromboxane B_2 (normals 21.6±6.3). Her metabolism of labeled arachidonic acid by both the lipoxygenase (HETE) and cyclo-oxygenase pathways (HHT and thromboxane B_2) was well within normal limits.

Polypeptide composition of normal and gray platelets. Fig. 2 shows the SDS electrophoresis of the polypeptides from the platelets of the two patients with the gray platelet syndrome. The gels of whole platelets (gels 1–3) showed that the gray platelets were markedly deficient in one polypeptide with a reduced apparent

molecular weight of 185,000. Previous studies (35) have shown that this glycoprotein is a "thrombin sensitive protein" (TSP), a polypeptide described by Majerus and coworkers (36, 37). Electrophoresis of these samples without reduction (disulfides intact) showed that this glycoprotein was of an apparent molecular weight of 400,000. TSP is released by thrombin stimulation of platelets (34). Gels 7–9 show the polypeptides released from platelets after thrombin stimulation. The released material from control platelets (gel 7) clearly contains TSP as well as β-thromboglobulin, PF4, and several other uncharacterized polypeptides. The SDS gel of the platelets obtained after thrombin stimulation in the same experiment (gel 4) revealed that most of the TSP had been secreted. The protein that remains in gel 4 in the region containing β-thromboglobulin and PF4 probably represents other platelet proteins of a similar molecular weight. Gels 2 and 3 of whole platelets from the two patients with the gray platelet syndrome show a marked deficiency in TSP in these patients. Some material is present in the region of the gel that contains PF4 and β-thromboglobulin, but this may in fact represent other platelet proteins of a similar molecular weight. The gels of the supernates of thrombin-treated gray platelets (gels 8 and 9) did not contain enough TSP, β-thromboglobulin, or PF4 to stain on the acrylamide gel. The action of thrombin on the gray platelets was sufficient, however, to cause the secretion of serotonin as the amount

TABLE III
Platelet Lysosomal Enzymes

	Acid phosphatase		β-Glucuronidase		β-N-acetylglucosaminidase	
	Per gram platelet protein	Per 10^{11} platelets	Per gram platelet protein	Per 10^{11} platelets	Per gram platelet protein	Per 10^{11} platelets
	$\mu mol/min$		$\mu mol/h$		$\mu mol/min$	
R.K.	26.2	7.66	6.18	1.81	4.57	1.34
Normals (10)	36.4±10.5*	6.92±2.0	7.31±2.30	1.39±0.44	7.58±1.47	1.44±0.28

R.K. platelets contained 293 mg/10^{11} platelets, whereas normals averaged 190 mg/10^{11} platelets.
* ±1SD.

TABLE IV
TABLE IV
PF4 and β-Thromboglobulin Levels in Gray Platelets

	Platelet-poor plasma			Platelets			
	Patient	Control(s)	Ratio patient/ control	Patient	Control(s)	Ratio patient/ control	Method
		ng/ml			*ng/10⁵ platelets*		
PF4							
Patient R.K.							
Lab A	4.4	1.0±0.7	4.4	170	2,170±390	0.08	Freeze-thawing
				180	2,040±320	0.09	Thrombin released substance
Lab B	7.8	2.98±1.04	2.6	44	943(2)*	0.05	PRP‡ freeze-thawed × 3
Patient M.P.							
Lab B	5.6	2.98±1.04	1.9	25	943(2)	0.03	PRP freeze-thawed × 3
β-Thromboglobulin							
Patient R.K.	72.5	25.8±4.1	2.8	178	3,744(2)	0.05	PRP freeze-thawed × 3
Patient M.P.	51.8	25.8±4.1	2.0	70	3,744(2)	0.02	PRP freeze-thawed × 3

* The number in parentheses represents the number of controls. Where this number is only two, these are controls obtained, processed, transported, and analyzed at the same time as the patient. In each case, these controls were well within the normal range for the laboratory.

‡ PRP, platelet-rich plasma.

of [¹⁴C]serotonin secreted from the gray platelets was similar to that secreted from control platelets.

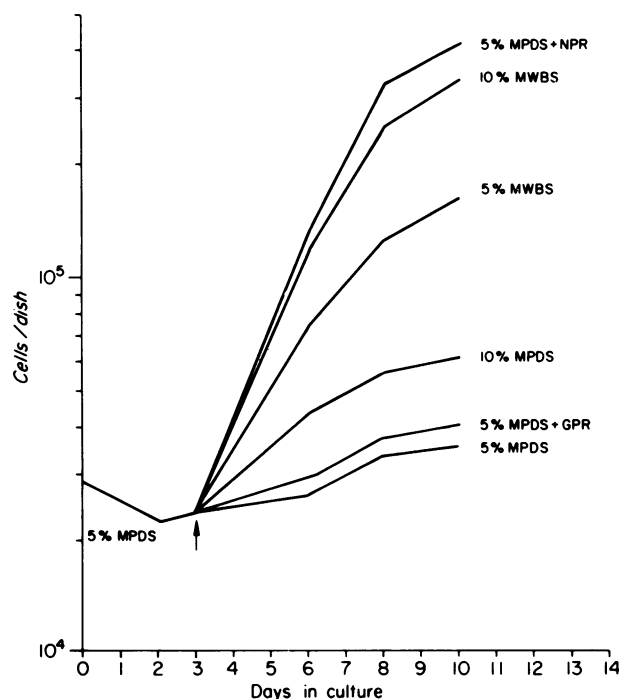Studies of platelet surface glycoproteins using lacto-

FIGURE 1 The influence of the released material from normal and gray platelets (patient R.K.) on the growth of fibroblast 3T3 cells in culture. At 3 d in culture (arrow), monkey whole blood serum (MWBS) or monkey plasma derived serum (MPDS), alone or together with the normal platelet release (NPR) material or gray platelet release (GPR) material, was added.

peroxidase catalyzed iodination showed no major differences from normal platelets (data not shown).

Platelet aggregation studies. Aggregation of gray platelets induced by arachidonic acid and ristocetin was within normal limits (Table V). Platelet aggregation in response to epinephrine was normal in R.K. and slightly delayed in M.P. Aggregation in response to acid-soluble collagen and thrombin were consistently less than normal in both R.K. and M.P., as was ADP aggregation. The abnormal aggregation patterns have been consistent over an 8-yr period for R.K. and a 2-yr period for M.P. In addition to these agents, normal aggregation with R.K. platelets was also observed using phorbol myristate acetate (1 μM), polylysine (0.1 mg/ml), polybrene (0.1 mg/ml) latex beads, and staphylococcus 502A.

DISCUSSION

The present investigation has studied the biochemistry of gray platelets and found them to be selectively deficient in platelet fibrinogen, PF4, platelet-derived growth factor, β-thromboglobulin, and TSP. Electron microscopic studies had previously shown that these platelets were selectively deficient in α-granules (13). This deficiency has been confirmed and quantitated in the present report. Thus, our results provide important confirmation of earlier evidence using platelet subcellular fractionation techniques and studies of patients with combined deficiencies of α-granules and dense bodies that had suggested that PF4, β-thromboglobulin, platelet fibrinogen, and the platelet-derived growth factor were in one granule population (38–44). Our results show that platelet TSP is also in this granule
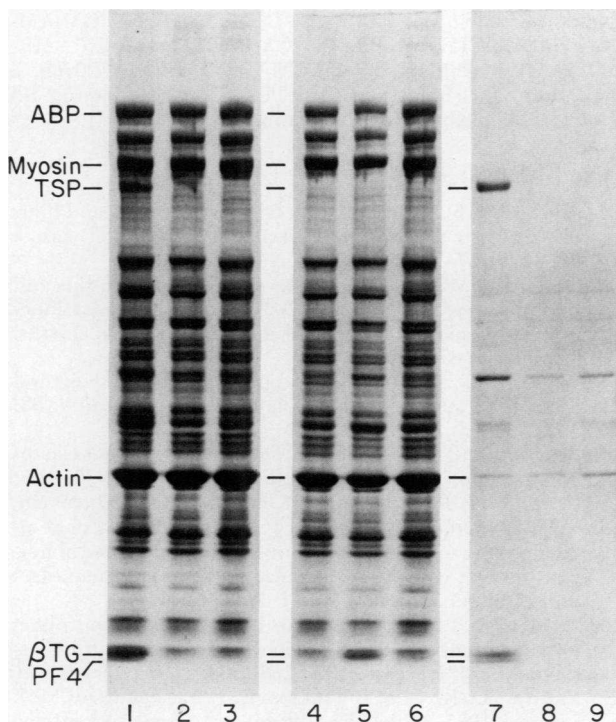
FIGURE 2 SDS gels of platelets from control donors (gels 1, 4, and 7) and from two patients with the gray platelet syndrome (R.K. gels 2.5 and 8; M.P. gels 3, 6, and 9). Washed platelets were solubilized by SDS and electrophoresed after reduction with β-mercaptoethanol (gels 1–3). Washed platelets were also treated with thrombin and removed from suspension by centrifugation. The platelets (gels 4–6) and supernatant fractions (gels 7–9) were solubilized by SDS and electrophoresed on the acrylamide gel. The positions of the labeled protein bands were identified either by comparison with pure proteins (PF4, β-thromboglobulin) or from earlier studies (34). ABP, actin binding protein, βTG, β-thromboglobulin.

population. The α-granules can be seen to be completely distinct from dense granules, which were normal in both numbers and in their composition in

the gray platelets studied. The α-granules are also distinct from lysosomes containing acid phosphatase, β-glucuronidase, and β-N-acetylglucosaminidase. The lysosomal granules were indistinguishable from the α-granules on routine electron microscopic studies; however, with special stains for acid phosphatase and aryl sulfatase, this granule population could be identified (13) and has been shown to be only a very small proportion of the α-granulelike structures in platelets. Thus, the number of lysosomes accounts for some of the α-granulelike structures seen in gray platelets (Table I); the finding of 10% of normal α-granules in gray platelets may, therefore, be on the high side of the actual figure. The normal value for catalase in gray platelets is consistent with the earlier findings that the gray platelets had normal numbers of peroxisomes (13), the organelle believed to contain platelet catalase.

One component deficient in gray platelets is TSP, a major glycoprotein of human platelets (32). TSP is polymeric, consisting of disulfide-linked identical subunits. The nonreduced apparent molecular weight of this glycoprotein on SDS gels is ~400,000–500,000, whereas the reduced apparent molecular weight is 185,000–190,000 (34, 35, 45). The subcellular localization of TSP has been examined and been suggested to be associated with the α-granules (45). When platelets are stimulated with thrombin or other agents, TSP is secreted with other macromolecules and the contents of the dense bodies (32, 46, 47). The present results provide further evidence that TSP is located in the α-granules, and gray platelets may eventually provide useful cells to evaluate the role of this glycoprotein in platelet function.

The fundamental importance of α-granules is uncertain. However, the degree of prolongation of the bleeding time in patient R.K., and the amount of susceptibility to bruising in both patients, suggests that the α-granule or its contents does add to some extent to the platelet response in vivo. Certain functions of the gray

TABLE V
Platelet Aggregation Studies in the Gray Platelet Syndrome

| | Epinephrine 5.5 μM | | | ADP | Collagen | | | Thrombin | | Arachidonic acid | Ristocetin |
	1st wave	2nd wave	Time to 50% aggregation	Concentration to yield 90% aggregation	30 μg/ml	100 μg/ml	Particulate	0.2 U/ml	Concentration to yield 90% aggregation	0.8 mM	1.5 mg/ml
			s	μM					U		
R.K.	+(8)	+(8)	110±40(9)	6.1±2.6(7)	−(2)*	+(1)	+(4)	−(5)	1.6(3)	+(1)	+(3)
M.P.	+(3)	+(3)	295(3)	9.5(2)	−(3)	+(1/2)	+(1)	−(2)	>0.4(1)	+(1)	+(1)
Normal donors	+(30)	+(28/30)	95±21(16)	3.1±1.6(19)	+(15/16)	+(16)	+(16)	+(9/10)	0.18±0.07(10)	+(10)	+(10)

Number in parentheses represents the number of different experiments on different days for R.K. and M.P. or the number of different normal donors tested.
* No aggregation, except for thrombin where it signifies <20% aggregation.

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platelets were normal, including aggregation in response to arachidonic acid and ristocetin. Aggregation in response to epinephrine and ADP showed the presence of both primary and secondary waves in samples of gray platelets, though a higher than usual concentration of ADP was necessary to achieve the ADP aggregation response. Aggregation in response to thrombin and collagen was also decreased when compared with normal donors. This decrease was particularly marked in the case of thrombin. These findings suggest that the contents of α-granules exert a potentiating effect on normal platelet aggregation in response to thrombin, collagen, and ADP.

The low platelet numbers seen in patients with the gray platelet syndrome suggests either that these platelets are being destroyed more readily than normal platelets in the circulation or that megakaryocyte production is decreased. Alternatively, there may be an abnormality in separation of platelets from megakaryocytes similar to Mediterranean macrothrombocytosis with decreased numbers of large platelets representing a normal platelet mass (48).

The reason for the α-granule deficiency in these patients is not entirely clear, but certain findings are suggestive. The deficiency of many proteins at the same time suggests that the primary deficiency might be a regulator gene that governs synthesis of all α-granule proteins, or in a granule membrane or phospholipid component responsible for binding or assembly. The apparent increase in the channels of the surface-connected canalicular system in gray platelets may be due to the presence of granules that are empty and therefore, appear to be channels of the surface-connected canalicular system; this increase would be consistent with either possibility. The raised levels of PF4 and β-thromboglobulin found in the platelet-poor plasma of patients R.K. and M.P., relative to normals, suggests that the proteins are synthesized, supporting the concept that the defect is in the binding or assembly of these proteins into granules. It is very unlikely that the α-granule proteins are low in these patients solely because of release in vivo during the circulation, because examination of the megakaryocytes from these patients has shown them to be deficient in α-granules similar to the platelets. Furthermore, the selective release of >90% of the platelet α-granule contents while the dense bodies remain intact seems unlikely without some underlying specific α-granule defect. A defect in assembly or binding of these proteins into the α-granules, therefore, seems most probable.

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