IMMUNODIAGNOSIS OF PLATELET MEMBRANE GLYCOPROTEIN DEFICIENCIES: GLANZMANN'S THROMBOASTHENIA, THE BERNARD-SOULIER SYNDROME, AND PROTEIN GMP-140 DEFICIENCY

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Two of the chief reactions of platelets, namely adhesion and aggregation, are mediated by membrane glycoproteins (GP). Inherited deficiencies of membrane GP are the cause of a number of hematologic diseases, characterized by disturbances of platelet hemostasis and a hemorrhagic syndrome. Among the best known and best studied deficiencies are Glanzmann's thromboasthenia and the Bernard–Soulier syndrome. In Glanzmann's thromboasthenia the platelets cannot aggregate (except the Willebrand factor-dependent aggregation-agglutination induced by ristocetin), and cannot bind fibrinogen, which is due to a deficiency of the GP IIb-IIIa complex, a fibrinogen receptor. In the Bernard–Soulier syndrome the ability of the platelets to adhere is sharply reduced. The platelets do not aggregate in response to addition of ristocetin (when all other forms of aggregation are preserved), and they cannot interact with Willebrand factor. Functional defects of the platelets in these patients are determined by a deficiency of the GP Ib-IX complex. Deficiencies of other GP have been described, but mainly in single cases.

In the investigation described below immunologic methods of diagnosis of deficiencies of platelet membrane GP, using specific monoclonal and polyclonal antibodies, are suggested and tested. The methods were tested on patients with Glanzmann's thromboasthenia, the Bernard–Soulier syndrome, and granule membrane protein 140 (GMP-140) deficiency.

EXPERIMENTAL METHOD

Three of the patients (G., Sh., and A.) with Glanzmann's syndrome were described previously [13]. Patient M., a girl of 14 years, was studied for the first time. Clinically her state was characterized by a moderately severe hemorrhagic syndrome with petechiae and with a tendency toward bruising and bleeding of the mucous membranes. The bleeding time was greatly lengthened. The patient's platelet count was within normal limits, but ADP-induced aggregation was absent. Aggregation induced by ristocetin was normal. Patient M's mother also was investigated. Her state was clinically normal, and although her ADP-induced aggregation was depressed, it was only slightly so. Patient B., with Bernard–Soulier syndrome, and patient G., with the gray platelet syndrome, associated with GMP-140 deficiency, will be described in detail elsewhere. In brief, the platelets of patient B., with Bernard–Soulier syn-

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drone were enlarged and characterized by inability to undergo ristocetin-induced aggregation, while maintaining normal ADP-induced aggregation. In patient G., with the gray platelet syndrome, a moderate degree of thrombocytopenia was observed, the platelets were enlarged, but the number of α-granules, according to the results of electron-microscopy, was sharply reduced. Both ADP- and ristocetin-aggregation were depressed, and collagen-induced aggregation was absent. The control group consisted of 11 healthy blood donors with no signs of hematologic diseases. Washed platelets were obtained as described previously [2] with certain modifications during washing the enlarged platelets of patients with Bernard–Soulier syndrome and in the patient with the gray platelet syndrome. In both cases the first centrifugation to obtain platelet-enriched plasma was carried out under milder conditions than in the case of healthy blood donors, namely 100*g* for 8 min instead of 180 g for 15 min. The subsequent washing was carried out under identical conditions. After the last sedimentation the platelets were suspended in Tyrode-HEPES solution (137 mM NaCl, 2.7 mM KCl, 0.36 mM NaH_2PO_4, 0.1% glucose, 5 mM HEPES, 2 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.35). Counting of the suspension of washed platelets from healthy blood donors and patients with Glanzmann's thromboasthenia was carried out with the aid of an automatic counter, whereas in patients with enlarged platelets this was done microscopically. The platelet concentration was adjusted with Tyrode-HEPES solution to $(3\text{-}6)\cdot10^8$ platelets/ml. For the study of binding of monoclonal antibodies (MAB) to platelets, bovine serum albumin (BSA), dissolved TyrodeHEPES to a concentration of 1%, was added to the suspension. When the specimen for immunoblotting was prepared no BSA was added.

The properties of VM16a and VM16d MAB, active against GP IIb-IIIa and GP Ib respectively, were described in detail previously [2, 8]. Rabbit polyclonal antibodies against GP IIa and GMP-140 were generously provided by Dr. M. Berndt (Department of Medicine, Sydney University, Sydney, Australia).

MAB were labeled with ^{125}I with the aid of iodogen ("Pierce," USA) by the method in [6] and specific binding of ^{125}I-labeled MAB with platelets was determined as described previously [2].

PMSF (0.5 mM) and leupeptin (0.2 mg/ml) were added to a suspension of washed platelets from the patient with gray platelet syndrome and from healthy blood donors, after which the platelets were solubilized by the addition of an equal volume of 2× the sample buffer – 125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 0.004% bromphenol blue. The concentration of patient's platelets in one experiment was $1.5\cdot10^8$, in the other $3\cdot10^8$/ml. Considering that the patient's platelets were about twice as large, the concentration of platelets of the healthy blood donor in both cases was twice as high, i.e., $3\cdot10^8$ and $6\cdot10^8$/ml in the first and second experiments respectively. SDS-PAG electrophoresis and transfer to nitrocellulose were carried out as described previously [9]. After transfer the nitrocellulose was blocked with 2% BSA, 0.3% gelatin in Tris-salt buffer (TSB), with 0.02% Tween-20 (TSB-Tween) for 1 h at 37°C. Polyclonal antibodies against GMP-140 and GP IIa were added to nitrocellulose strips in a concentration of 5 μg/ml and incubated for 40 min at 20°C. After five washes with TBS-Tween, sheep's antibodies against rabbit IgG ("Biorad," USA), labeled with ^{125}I by the method in [6], were added to the nitrocellulose strips. ^{125}I-secondary antibodies were added in a concentration of 1 μg (10^6 cpm) in 1 ml and incubated with nitrocellulose for 40 min at 20°C. After removal of the secondary antibodies by washing 5 times with TBS-Tween the nitrocellulose was dried and the position of the bands was identified by autoradiography. The intensity of the bands was estimated quantitatively by cutting out the corresponding areas of nitrocellulose and counting them in a γ-counter.

EXPERIMENTAL RESULTS

The quantity of individual GP on the surface of platelets from patients with thrombocytopathies and the healthy control donors was estimated from binding of specific ^{125}I-labeled MAB. Antibody VM16a, directed against the GP IIb-IIIa complex [2] bound effectively with healthy human platelets (Fig. 1, Table 1) and at saturating concentrations the number of binding sites was $(40\text{-}50)\cdot10^3$ VM16a molecules per platelet, which corresponds to the number of copies of GP IIb-IIIa on the platelet surface [10, 14]. The level of binding of ^{125}I-VM16a with platelets

TABLE 1. Binding of MAB VM16a and VM16d with Platelets from Patients with Thrombocytopathics and Healthy Donors (M ± m), n = 11.

Subject under investigation	VM16a	VM16d
	10^3 molecules per platelet	
Healthy donors	49.6±8.0	37.7±3.6
Glanzmann's thromboasthenia (homozygote)		
Sh.	7.2	78.8
A.	3.6	40.4
G.	2.0	60.8
M.	0.5	—
(heterozygote)		
M.	27.0	—
Bernard-Soulier Syndrome (homozygote)		
B.	36.8	0.5
Gray platelet syndrome		
G.	123.2	103.2

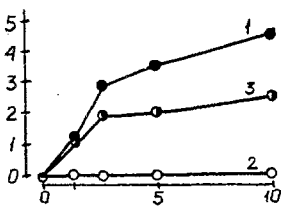

Fig. 1

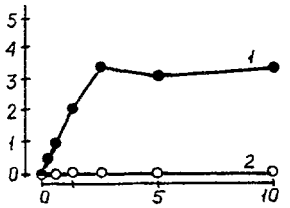

Fig. 2

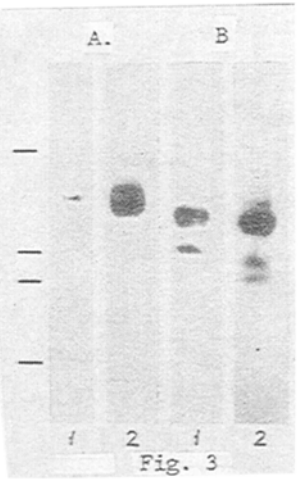

Fig. 3

Fig. 1. Binding of ^{125}I-VM16a with platelets from a healthy blood donor (1), a patient with Glanzmann's thromboasthenia (homozygote, 2), and a heterozygous carrier of the Glanzmann's thromboasthenia gene (heterozygote, 3). Washed platelets, $3 \cdot 10^7$ in 100 μl) were incubated with different concentrations of ^{125}I-VM16a in the absence (total binding) and presence (nonspecific binding) of a 50-fold excess of unlabeled antibody. Specific binding calculated as difference between total and nonspecific binding. Values of specific binding are shown.

Fig. 2. Binding of ^{125}I-VM16d with platelets from a healthy blood donor (1) and a patient with the Bernard–Soulier syndrome (2). Conditions as to Fig. 1.

Fig. 3. Immunoblotting of protein GMP-140 (A) and GP IIa (B) in lysate of platelets from patient with gray platelet syndrome (1) and a healthy blood donor (2). Electrophoresis carried out in 7-12% PAG under nonreducing conditions. Polyclonal antibodies against GP IIb and protein GMP-140 and secondary ^{125}I-labeled antibodies against rabbit immunoglobulins used to identify bands. Results of one of two experiments with a greater protein load are shown. Markers of molecular weights (in kilodaltons), from top to bottom: 200 – myosin, 116 – β-galactosidase, 94 – phosphorylase b, and 68 – BSA.

of patients with Glanzmann's thromboasthenia was significantly lower – in patients M. and G. it was less than 5% of the control level, whereas in patients A. and Sh. it was 7.2 and 14.5% respectively. According to the classification first suggested by Caen and subsequently elaborated in more detail [3, 7], a profound GP IIb-IIIa deficiency, when the GP concentration is below 5% of normal, is diagnosed as type I thromboasthenia, whereas a GP IIb-IIIa deficiency with a protein concentration of about 10% of the normal or higher is diagnosed as type II thromboasthenia. Hence, in patients M. and G. type I thromboasthenia was diagnosed, but type II in patient Sh. (for patient A., occupying an intermediate position, repeated investigation is needed to make an accurate diagnosis). Binding of ^{125}I-VM16a with platelets from patient M's mother, i.e., a heterozygous carrier of the defective gene, was about 50% of the level in control donors (Fig. 1, Table 1). The quantity of GP Ib on the surface of the platelets was estimated from binding of MAB VM16d, the properties of which were described in detail previously [8]. Binding of this antibody with healthy human platelets in saturating concentrations was (30-40) $\cdot 10^3$ molecules per platelet (Fig. 2, Table 1), which is about 1.5-2 times higher than was described previously for other MAB and other groups of donors from other populations (France, USA, Australia) [4, 5, 12]. Binding of ^{125}I-VMl6a with platelets from patients with Glanzmann's thrombo-asthenia was within normal limits or even a little higher than in healthy blood donors (Table 1). Meanwhile, this antibody was virtually not bound with platelets from patient B., with the Bernard–Soulier syndrome (less than 2% of the normal level; Fig. 2, Table 1), evidence of the absence of GP Ib on the surface of this patient's platelets. Binding of patient B's platelets with MAB VM16a was within normal limits (Table 1). The experiments thus demonstrate that by measuring binding of ^{125}I-labeled specific antibodies it is possible to make a rapid diagnosis of platelet membrane GP deficiencies.

The quantities of GP IIb-IIIa and GP Ib were measured also on platelets of patient G, with gray platelet syndrome. Binding of both MAB (VM16a and VM16d), calculated per platelet, not only was not reduced, but was actually increased, possibly due to the large size of this patient's platelets (Table 1). In patient G. the total content of GP IIa and protein GMP-140 in the platelets also was estimated. The Ia-IIa complex is the collagen receptor [15], whereas GMP-140 is a protein specific for membranes of a-granules, appearing on the surface of the platelets only after their activation [11]. These proteins were determined by the immunoblotting method, using specific polyclonal antibodies to detect the bands. As Fig. 3 shows, the intensity of the band corresponding to protein GMP-140 was significantly lower in this patient than in the healthy control. Quantitative analysis of the results of two immuno-blottings showed that the content of GMP-140 in the patient was only 14.5% of its level in the control donor (radioactivity of bands 5143 and 35743 cpm in patient and donor respectively, n = 2). The content of GP IIa in patient G. was about the same as in the control donor (80% of the control, radioactivity of bands 24,661 and 29,955 cpm in the patient and control donor respectively, n = 2) (see Fig. 3). The results of the investigation by quantitative immunoblotting thus revealed a profound deficiency of protein GMP-140 in patient G., with the gray platelet syndrome, but a normal content of GP IIb-IIIa, Ib, and IIa.

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