Short Communication

Role of MRP4 (ABCC4) in Platelet Adenine Nucleotide-Storage

Evidence from Patients with Delta-Storage Pool Deficiencies

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We previously showed that the MRP4 (ABCC4) transporter is expressed in human platelet δ -granules and may be involved in ADP transport. We now demonstrate by immunoblotting and immunofluorescence microscopy that platelet MRP4 is absent in two patients with a platelet δ -storage pool deficiency (δ -SPD)-like phenotype with reduced platelet adenine nucleotide (AN) but normal serotonin levels, whereas their other membrane marker proteins of platelet granules were normally expressed and localized. In these patients, MRP4 was present in lymphocytes, and the coding region of their *MRP4/ABCC4* gene did not show any mutation that explained the lack of expression. In platelets with “classic” δ -SPD (low AN and serotonin levels), MRP4 was quantitatively (immunoblot) normal, but, like other δ -granules membrane marker proteins (eg, LAMP2), was mostly displaced from δ -granules to patches at the plasma membrane, suggesting that platelets with classic δ -SPD have an abnormality that impairs the assembly of normal δ -granules. Thus, defective expression of platelet MRP4 is associated with selective defect in AN storage. The genetic basis of the new δ -SPD phenotype remains to be elucidated. (*Am J Pathol* 2010; 176:1097–1103; DOI: 10.2353/ajpath.2010.090425)

In platelet δ -granules, small molecules such as ADP, ATP, and serotonin are concentrated.¹ These molecules play an important role in hemostasis because, when they are released by activated platelets, they contribute to the formation of platelet aggregates. The high concentration of ADP inside the platelet δ -granules (up to 0.6 mol/L) suggests the involvement of a primary active transport protein. We have identified the multidrug resistance protein MRP4 (ABCC4), a member of the ATP-binding cassette (ABC) transporter superfamily, as a candidate mediating this active transport.² Besides localization in the plasma membrane, MRP4 was also demonstrated to be highly concentrated in δ -granules. This intracellular localization distinguishes the MRP4 expression in platelets from that in other cell types, where it is localized mainly at the plasma membrane. In polarized cells it depends on the tissue whether MRP4 is sorted to the basolateral or to the apical membrane.^{3,4} MRP4 is involved in the ATP-dependent transport of a wide range of amphiphilic anions including steroid conjugates and eicosanoids, as well as cyclic nucleotides and nucleotide analogues,^{3,4} and most likely ADP.² In contrast, serotonin is not a substrate for MRP4.²

Here, we studied platelets of patients with congenital deficiencies of δ -granule constituents (δ -storage pool deficiency [δ -SPD]) to further characterize the role of MRP4 in ADP transport. δ -SPD is a heterogeneous group of platelet disorders, characterized by deficiency of platelet δ -granules, in which adenine nucleotides, serotonin, and other substances are concentrated. δ -SPD may be either syndromic (associated with abnormalities of other cells) or non-syndromic.⁵ The best example of a syndromic δ -SPD is Hermansky-Pudlak syndrome (HPS), a rare disorder in which the development of melanosomes, platelet δ -granules, and lysosomes is impaired.^{6,7} Mutations in

Supported in part by the Deutsche Forschungsgemeinschaft (SFB TR 19).

Accepted for publication November 5, 2009.

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eight different genetic loci have been identified in patients with HPS.^{8–11} In accordance with the impaired formation of δ -granules, we detected MRP4 only in the plasma membrane of platelets from one HPS patient.² A more common platelet-specific storage defect is the non-syndromic form of δ -SPD, with selective defect of platelet δ -granules, the molecular defect(s) of which is/are presently unknown.⁵

Based on the possible role of platelet MRP4 as ADP- but not as serotonin transporter,² we postulated that absence of MRP4 from platelet δ -granules may be associated with a phenotype of platelets with a reduced adenine nucleotide (AN) but normal serotonin content. We identified two unrelated patients with this previously undescribed phenotype of human δ -SPD (" δ -SPD [phenotype-adenine nucleotides (AN)]"). For comparison, we analyzed platelets of patients with nonsyndromic δ -SPD, which, by definition, lack ADP, ATP, and serotonin in their δ -granules ("classic δ -SPD"). The results support the hypothesis that MRP4 plays a major role in platelet AN storage.

Materials and Methods

Patients

Patients with δ -SPD [Phenotype-AN]

Patient 1 is a 43-year-old woman of Caucasian origin, who had a life-long history of easy bruising, menorrhagia, and bleeding after dental extractions and surgery. When she was 17 years old, she was diagnosed with Hodgkin's disease, which relapsed 13 years later, and is now in remission. She reported that both parents had easy bruising and frequent epistaxis. Patient 2, a 42-year-old man of Caucasian origin, had a life-long history of easy bruising and frequent epistaxis. A potential relevant comorbidity was a modest granulocytopenia. He developed chronic HCV infection after blood transfusions on occasion of a bleeding episode in his childhood. His family history was negative for abnormal bleeding manifestations.

Patients with Classic δ -SPD

Patient 3 is a 13-year-old girl of Caucasian origin, with a life-long history of easy bruising and gum bleedings, which were severe enough to cause an iron-deficiency anemia when she was 6 years old. On that occasion, the patient underwent a thorough screening for inherited defects of hemostasis, which revealed nonsyndromic classic δ -SPD. Her family history was negative for abnormal bleedings. Patient 4 is a 21-year-old woman of Caucasian origin, suffering from easy bruising, menorrhagia, and abnormal bleedings after dental extractions. Screening for inherited defects of hemostasis revealed nonsyndromic classic δ -SPD. Her family history was negative for abnormal bleedings.

Complete blood count data were normal for all patients, with the exception of patient 2, who displayed mild granulocytopenia.

Control Subjects

Four healthy subjects, two men and two women, belonging to the laboratory staff in Milan, with normal hemostatic function, served as normal controls. The research was approved by the relevant ethics committees and all human participants gave written informed consent.

Platelet Function Studies

The platelet levels of ADP and ATP were measured with the luciferine-luciferase method, those of serotonin with the orto-phthalaldehyde method, and those of fibrinogen and β -thromboglobulin with ELISA, as previously described.^{12,13} Platelet aggregation and secretion were measured simultaneously by the firefly luciferase method in a lumiaggregometer (Chronolog, Mascia Brunelli, Milano, Italy), using the thromboxane A2 analogue U46619 and collagen (Mascia Brunelli, Milano, Italy) as agonists.¹⁴

Immunoblot Analysis

Platelet-rich plasma was prepared and platelets were washed as described.² Washed platelets were lysed by repeated cycles of freezing and thawing. Pellets of total platelet membrane proteins were loaded onto a 7.5% sodium dodecylsulfate-polyacrylamide gel. Immunoblot analysis was performed as described.² The following primary antibodies were used: polyclonal antibodies SNG and MLP directed against the carboxy- and amino-terminal sequences of human MRP4, respectively^{2,15} (kindly provided by Dr. Dietrich Keppler, Deutsches Krebsforschungszentrum, Heidelberg, Germany; dilution 1:1000 and 1:500); mouse monoclonal antibody against LAMP2 (Lysosome-Associated Membrane Glycoprotein 2; 1:1000), and goat polyclonal antibody against P-selectin (1:2000; Santa Cruz Biotechnology Inc., Santa Cruz, CA). In addition, the monoclonal antibody against MRP4 M₄-I-10 (Axxora Corp., Grünberg, Germany) was used.

Immunofluorescence Microscopy

Aliquots (20 μ l) of platelet suspension (100×10^9 /L) were fixed by formaldehyde on coverslips covered with human collagen type I. Confocal laser scanning immunofluorescence microscopy was performed as described before.² The primary antibodies were used at the following dilutions: anti-MRP4 SNG 1:125; anti-LAMP2 and murine monoclonal anti-P-selectin (Santa Cruz Biotechnology Inc., CA) 1:10; the murine monoclonal antibody Gi5 against the GpIIb/IIIa receptor (epitope formed by both subunits; provided by Dr. Santoso, Justus-Liebig-University Gießen, Germany) 1:10. The respective secondary antibodies, either conjugated to Alexa Fluor488 or Alexa Fluor568 (Molecular Probes, Eugene, OR), were used at dilutions of 1:250 or 1:50, respectively. Localization of CD63 was studied using the FITC-mouse anti-human CD63 (Becton Dickinson, San Diego, CA) and the PE-

Table 1. Characteristics of Platelet Function in Patients with δ -SPD[Phenotype-AN] (Patient 1 and 2) or with Classic δ -SPD (Patient 3 and 4)

	Patient 1	Patient 2	Patient 3	Patient 4	Normal range
Platelet count $\times 10^9/L$	170 270 192	160 154 —	251 244 —	141 236 356	150 to 450
Platelet 5HT, nmoles/ 10^8 plts	0.26 0.35 0.40	0.39 0.60 —	0.06 0.10 —	0.06 0.04 0.06	0.2 to 0.8
Platelet ADP, nmoles/ 10^8 plts	0.53 0.47 0.37	1.00 0.25 —	0.10 0.19 —	0.8 0.85 0.67	1.32 to 3.72
Platelet ATP, nmoles/ 10^8 plts	4.12 5.33 4.13	3.91 1.8 —	3.98 6.34 —	4.05 4.35 4.36	3.57 to 8.05
ATP/ADP ratio	7.78 11.30 11.16	3.91 7.58 —	39.8 33.4 —	5.06 5.11 6.51	1.35 to 3.39
BT, min	20	15	>15	15	<7
β -TG, ng/ 10^6 plts	207 177	38 —	—	—	28 to 152
Fibrinogen, mg/ 10^9 plts	—	—	0.1	0.03 0.03	0.03 to 0.19
Platelet ATP secretion (nmol/ 10^8 plt) induced by					
Collagen 4 $\mu g/ml$	0.29	0.24	0.10	—	0.34 to 0.88
U46619 0.5 $\mu mol/L$	<0.01	<0.01	0.01	—	0.03 to 0.93

All patients were studied in two or three independent occasions. Platelet ATP secretion (which was tested in one occasion only) was not studied in Patient 4 (13-year-old girl), because of denied informed consent, in consideration of the relatively large amount of blood needed for this investigation. The control subjects who were studied on these occasions had normal values of platelet 5HT (serotonin), ADP, ATP, fibrinogen, and β -thromboglobulin (β -TG). Note (especially for ADP and ATP values) that these parameters were measured in total platelets and include the cytosolic fraction.

mouse anti-human CD42b (Becton Dickinson, San Diego, CA).

Partial Sequence Analysis of the *MRP4/ABCC4* Gene

A 1-kb fragment upstream of exon 1 and all exons with adjacent exon-intron-boundaries (≈ 100 bp) were sequenced from the index patients with δ -SPD[phenotype-AN]. Blood DNA was prepared with the QIA-Amp blood kit (Qiagen, Hilden). Fragments of the *MRP4/ABCC4* gene were generated by PCR starting from genomic DNA. Purified PCR amplicons were sequenced by standard sequencing (Seqlab, Göttingen, Germany).

Blood for RNA preparation was taken into PaxGene blood RNA tubes (PreAnalytiX, Hilden, Germany), and RNA was prepared with the PaxGene blood RNA kit and reverse transcribed with the RevertAid First Strand cDNA Synthesis Kits (Fermentas, St. Leon-Rot, Germany). MRP4 transcript fragments were amplified and sequenced. To detect potential splice variants we used several combination sets of the oligonucleotide primers.

Results

Patients' Platelet Function

The two patients from two unrelated families with δ -SPD[phenotype-AN] exhibited prolonged bleeding times, reduced ADP contents, and increased ATP/ADP ratios in their platelets, whereas the concentrations of serotonin were normal (patients 1 and 2, Table 1). The α -granule

marker β -thromboglobulin was normal (patient 2) or even elevated (patient 1). In contrast, platelets from patients 3 and 4, with classic δ -SPD, lacked both ADP and serotonin (Table 1). The concentration of platelet fibrinogen, which was used as a marker of α -granules in these patients, was normal, indicating that their platelet defect was selective for the platelet δ -granules. ATP secretion, measured in a lumiaggregometer after stimulation of PRP samples with the thromboxane A2 analogue U46619 (0.5 $\mu mol/L$) or collagen (4 $\mu g/ml$), was defective in all patients studied (Table 1; Patient 4, a 13-year-old girl, was not studied because of denied informed consent in consideration of the relatively large amount of blood that is necessary for this investigation).

MRP4 Expression and Localization in Platelets with δ -SPD[Phenotype-AN]

Immunoblotting of platelet total membranes with the antibodies SNG and MLP (directed against the carboxy- and the amino-terminal sequence of MRP4, respectively) detected very low amounts of MRP4 in the platelets of the two patients with δ -SPD[phenotype-AN] compared with controls (Figure 1A), whereas the immunodetection of the δ -granule/lysosome marker LAMP2 and the α -granule marker P-selectin was normal. MRP4 expression and subcellular localization were further analyzed by confocal immunofluorescence microscopy studies (Figure 1, B–I). As described before,² MRP4 was detected in normal controls mainly in intracellular granules, colocalizing with LAMP2-positive structures, most corresponding to δ -granules (Figure 1C). Localization of MRP4 in platelet lysosomes has

Figure 1. MRP4 expression and localization in platelets from SPD[phenotype-AN] patients. **A:** Immunoblot analysis of MRP4 in platelets' total membranes (20 μ g protein) from δ -SPD[phenotype-AN] patients (Pt1 and Pt2) or from healthy controls (Co1, Co2) detected with two different antibodies (SNG and MLP) directed against the carboxy- and amino-terminal sequences of MRP4, respectively. Blots were re-exposed to specific antibodies against LAMP2 and P-selectin (Psel) as marker proteins for δ -granular/lysosomal and α -granular membranes, respectively. Bands from MRP4, LAMP2, and P-selectin were detected at 170, 120, and 140 kDa. **B–I:** Double-label immunofluorescence microscopy of platelets from patients with δ -SPD[phenotype-AN]. Platelets of controls (**B, C, F, and G**) and patients (**D, E, H, and I**) were stained with the anti-MRP4 antibody SNG (red fluorescence) and counterstained with antibodies against LAMP2 (**C and E**) or P-selectin (**G and I**; green fluorescence). Images show representative confocal Z-stacks observed with a Nikon inverted microscope and a $\times 100$ oil immersion objective. A CCD camera and "VoxCell" scan software from VisiTech International (Sunderland, UK) were used for analysis. A diminished MRP4 staining was observed in all analyzed platelets of the two patients. Scale bars represent 5 μ m.

been excluded before.² Platelets from the δ -SPD[phenotype-AN] patients revealed a pale diffuse staining by the anti-MRP4 antibody, whereas there were no obvious differences in LAMP2 and P-selectin staining compared with normal controls (Figure 1, E and I), suggesting that they contain normal number of platelet δ - and α -granules and that their δ -granules selectively lack MRP4.

MRP4 Expression and Localization in Platelets with Classic δ -SPD

For comparison, we analyzed two patients who had been diagnosed with classic δ -SPD, who lack ADP and serotonin in their platelet δ -granules (patients 3 and 4). In platelets' total membranes from classic δ -SPD patients, MRP4 was detected in similar amounts as in controls by immunoblot analysis (Figure 2A). The amounts of the δ -granule/lysosome marker LAMP2 and the α -granule marker P-selectin did not show significant differences between patients and controls.

By immunofluorescence microscopy, staining of MRP4 in platelets from the classic δ -SPD patients revealed a strong signal (Figure 2, F and J). However, there was an obvious shift of the MRP4 staining from intracellular structures to patches at the plasma membrane, compared with the control platelets (Figure 2B). This was supported by costaining experiments with P-selectin as intracellular α -granule marker and an antibody detecting the GpIIb/IIIa receptor complex at the plasma membrane (Figure 2, C, G, K, E, I, and M). The latter marker partially colocalized

with MRP4 (Figure 2, I and M). P-selectin was normally localized on α -granules (Figure 2, G and K). In analogy with MRP4, the δ -granules membrane marker LAMP2 preferentially localized at or close to the plasma membrane in patients' platelets but there was still slight granule staining for LAMP2 (Figure 2, D, H, and L). We therefore extended the studies on dense granular structures by using an antibody against granulophysin (CD63). As shown in Figure 2, N–P, CD63 was also detected in intracellular granules in the platelets of the patients with classic δ -SPD; the intensity of the granular staining, however, was reduced (to demonstrate that the CD63 staining is inside the platelets, the plasma membrane protein GpIb was counterstained). These results suggest that in the heterogeneous group of δ -SPD unknown abnormalities are responsible for impaired formation of δ -granules with some remaining dense granules (or dense granule structures) still being present in some patients.

Analysis of the MRP4 Gene and Leukocyte mRNA of Patients with δ -SPD[Phenotype-AN]

To identify a potential genetic cause for the δ -SPD[phenotype-AN] in platelets we sequenced large fragments of the *MRP4/ABCC4* gene of patients 1 and 2 and two healthy individuals. The sequencing strategy included all exons with ≈ 100 bp of the adjacent intron sequences and 500 bp of the 5' upstream region. Our strategy also comprised the entire intron 1 to detect sequence variations potentially contributing to alternative splicing in this

Figure 2. Expression and localization of MRP4 in platelets from patients with classic δ -SPD. **A:** Immunoblot analyses of platelet MRP4, P-selectin (Psel), and LAMP2 were performed as described in the legend to Figure 1. For MRP4, a similar staining profile was also obtained using the MLP antibody as well as a commercially available monoclonal anti-MRP4 antibody (M₄-I-10, not shown). **B–P:** Immunofluorescence microscopy. **B, F, J:** Single-staining of platelets from patients with classic δ -SPD (Pt3 and Pt4) and a healthy control (Co) with the anti-MRP4 antibody SNG (red fluorescence). **C–E, G–I, K–M:** Superimposed images from individual double-stainings of platelets from control (Co: **C–E**) and patients (Pt3: **G–I** and Pt4: **K–M**) with SNG (red fluorescence) and antibodies against P-selectin (Psel: **C, G, and K**) and LAMP2 (**D, H, and L**) as well as against the GpIbIIIa receptor complex (**E, I, and M**; green fluorescence). **N–P:** Costaining of platelets from patients (Pt3 and Pt4) and control (Co) using an antibody against the δ -granule protein granulophysin (CD63; green fluorescence) and an anti-GpIb antibody as plasma membrane marker (red fluorescence). Scale bars 5 μ m.

region. We identified 13 polymorphisms already incorporated in the SNP database (<http://www.ncbi.nlm.nih.gov/SNP>) in noncoding sequences, both in healthy individuals and patients, all occurring in the heterozygous state. None of these variants occurred in both patients simultaneously.

We further analyzed RNA from white blood cells from the δ -SPD[phenotype-AN] patients and controls. MRP4 transcripts were amplified by RT-PCR from blood cell mRNA with numerous primer sets to detect potential splice variants. MRP4 transcripts were detectable in specimens from patients and healthy individuals with no indication for alternative splicing. Sequence analysis of the transcripts revealed no sequence variants in the coding sequence.

Discussion

Platelet δ -granules contain, among other substances, ADP and serotonin, which are important mediators of platelet function. The concentrations of these substances within the granules are so high that active transport processes must be involved for their accumulation. For example, the overall process of serotonin storage results from serotonin transport first across the plasma membrane by a Na⁺-dependent process mediated by the serotonin transporter, which is encoded by the *SLC6A4*

gene,¹⁶ and then across the granule membrane by a second transport system, called vesicular monoamine transporter, which has been characterized functionally as a countertransport with H⁺.¹⁷ We previously showed a high expression of the primary active transporter MRP4 in platelets, which has been recently confirmed by others.¹⁸ Localization of MRP4 in secretory granules² is, as known so far, unique to platelets. The formal proof that the mediator compound ADP is transported into the granules by MRP4 has been hampered by the fact that ADP is both a potential substrate and product of the MRP4-mediated ATP hydrolysis, with ATP being also the energy source of the transport. However, we could demonstrate an orthovanadate-sensitive [³H]ADP uptake in the presence of ATP into δ -granules, which was affected by known MRP4 inhibitors and other substrates as cGMP, supporting the idea that MRP4 is involved in active accumulation of ADP.² We therefore postulated that defects of MRP4 might be associated with decreased levels of adenine nucleotides in platelet δ -granules, but normal levels of other constituents such as serotonin for which other transport proteins are involved. Indeed, we identified two unrelated patients exhibiting a previously undescribed phenotype of δ -SPD (δ -SPD[phenotype-AN]), characterized by decreased platelet ADP content, but normal serotonin. Interestingly, δ -SPD[phenotype-AN] is not restricted to humans: a dog model with selective adenine

nucleotide deficiency has been previously described,¹⁹ but the pathogenic mechanisms in this model have not been elucidated. In accordance with our hypothesis, MRP4 expression in the platelets of the two patients with δ -SPD[phenotype-AN] was severely diminished, whereas the expression and the subcellular localization of LAMP2, another δ -granule membrane protein, were not significantly altered. The diminished expression of MRP4 was observed by both antibodies SNG and MLP, which are directed against the carboxy- and amino-terminal sequences of human MRP4, respectively, indicating that the entire protein was absent from platelet membranes. We cannot rule out that degradation products of MRP4 may be present in the platelet cytosol of these patients, but in the immunofluorescence microscopy only a weak diffuse staining was observed. Thus, expression of functional MRP4 is greatly reduced in δ -SPD[phenotype-AN] platelets compared with healthy controls. Our findings have an additional clinically relevant implication that requires further investigation. Substances that interfere with MRP4 may also diminish the platelet ADP content. This could cause a platelet function defect and might enhance the bleeding risk in patients on therapeutic dose anticoagulant therapy.

Given the reduced MRP4 expression in platelets with δ -SPD[phenotype-AN], we hypothesized that functional mutations in the gene of MRP4 may contribute to a diminished generation of this protein and sequenced essential parts of the *MRP4/ABCC4* gene. However, we could not identify plausible sequence variations explaining the altered expression of the protein. In accordance with the results of our blotting experiments in which we used two different antibodies, we found no evidence for splice variants explaining the molecular phenotype. Theoretically, variants in noncoding sequences of the gene, which have not been explored in our experiments, could lead to shortened mRNA survival and, hence, decreased protein synthesis. Unfortunately, the limited amount of blood that the patients agreed to provide did not allow us to isolate purified RNA from platelets or megakaryocytes. MRP4 expression in lymphocytes of these index patients appeared normal. Thus, mechanisms contributing to the tissue-specific regulation of *MRP4* expression in the megakaryocytic lineage at the transcriptional or posttranscriptional level are most likely candidates for future research into the pathogenic mechanisms of δ -SPD[phenotype-AN].

Analyses of MRP4 expression in platelets of patients with the classic δ -SPD revealed a different pattern. We studied these two patients of the heterogeneous group of classic δ -SPD with unknown pathology primarily as a control to ensure that the reduced ADP levels in these patients are also associated with a deficiency of MRP4 in their dense granules. In these patients, MRP4 was quantitatively well detected by immunoblot and by immunofluorescence microscopy. However, localization had significantly changed compared with normal platelets, because, in classic δ -SPD, MRP4 seemed to be expressed in patches at the plasma membrane (Figure 2). A similar expression has been observed before in an HPS patient.² Our study provides a further intriguing ob-

servation suggesting that δ -SPD are a very heterogeneous group of platelet dense granule defects in which different constituents of the dense granule membrane are mislocalized or even the entire membrane structure is absent, depending on the underlying molecular defect. In the classic δ -SPD assessed we found that LAMP2 appeared to be largely shifted to the plasma membrane, whereas faint CD63²⁰ staining could be detected in intracellular granules in the platelets of these patients. CD63 staining in classic δ -SPD platelets has been reported before but shown to vary between patients of this heterogeneous group of platelet deficiencies.⁵ This suggests that in patients with δ -SPD, several different abnormalities are likely responsible for impaired formation of the complex membrane structures of δ -granules. As shown for MRP4 and LAMP2 in the patients with classic δ -SPD in the present study and for an HPS patient previously, the membrane proteins, which are still synthesized are mislocalized to—and likely fused with—the plasma membrane. As for MRP4, members of the ezrin-radixin-moesin family, which cross-link actin filaments and integral membrane proteins,²¹ have been shown to determine in which membrane compartment MRP4 and related transport proteins are inserted.^{22,23} Further studies are required to unravel the defect(s) leading to the phenotype of classic δ -SPD.

In conclusion, our data indicate an association of the absence from MRP4 from dense granules with a newly described subtype of δ -SPD (δ -SPD[phenotype-AN]), characterized by selective defect of ADP in δ -granules, prolonged bleeding time, and bleeding diathesis. This human phenotype of δ -SPD[phenotype-AN] further supports the important role played by MRP4 in the storage of ADP in platelet δ -granules.

Acknowledgments

The antisera SNG and MLP were kindly provided by Dr. Dietrich Keppler, Deutsches Krebsforschungszentrum, Heidelberg, Germany. The mouse monoclonal antibody Gi5 against the GpIIb/IIIa receptor was a gift from Dr. Sentot Santoso, Department of Clinical Immunology and Transfusion Medicine of the Justus-Liebig-University Gießen, Germany. We acknowledge the excellent technical assistance by Carmen Blumentritt and Ulrike Strobel, Department of Immunology and Transfusion Medicine, University of Greifswald, and by Tina Sonnenberger and Ingrid Geißler, Department of Pharmacology, University of Greifswald.

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