Quantification of a Novel Dense Granule Protein (granulophysin) in Platelets of Patients With Dense Granule Storage Pool Deficiency

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DENSE GRANULES (DG) were originally identified as storage organelles in the platelets of mammalian species containing, in the human, serotonin (0.3 μmol/10^11 platelets), calcium (10 μmol/10^11 platelets), adenosine diphosphate (ADP) (3 μmol/10^11 platelets), adenosine triphosphate (ATP) (2 μmol/10^11 platelets), and pyrophosphate (1.5 μmol/10^11 platelets). Patients with decreased amounts of these substances stored in their platelets-DG storage pool defects- have one of the most common forms of platelet function disorders. Several types of human δ-SPD have already been defined, including two associated with albinism: the Hermansky-Pudlak syndrome and the Chediak-Higashi syndrome. In addition, there are several forms of δ-SPD not associated with albinism, including an autosomal dominant form, a form in which the dense granule defect is associated with absent platelet α granules, and a form in which there is a partial deficiency of α granules. DG deficiency has also been associated with the Wiskott-Aldrich syndrome (WAS), and with the thrombocytopenia absent radii syndrome.

However, the association of δ-SPD with these conditions is inconsistent and, when seen, may be coincidental rather than reflective of a single gene defect. Findings in mice showing platelet dense granule defects, which are associated with at least eight distinct genetic loci, are consistent with the presence of multiple specific forms of inherited dense granule defects.

Recently the platelet protein granulophysin, specific to the membrane of the DG, has been described and defined through the use of monoclonal antibodies (MoAbs). The protein has been shown to migrate in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at about 40 Kd as identified by Western blots. Furthermore, the granulophysin content of platelets from a patient with the Hermansky-Pudlak syndrome lacking dense bodies was found to be very low. It was further demonstrated that a similar protein is present in neutrophils and endothelial cells, and that granulophysin shares some serologic homology with synaptophysin, a well-characterized 38-Kd synaptic vesicle protein that has been postulated to form a fusion pore during synaptic vesicle exocytosis. In the present study, a quantitative sandwich type enzyme-linked immunoassay (ELISA), which allows the detection of as little as 1 to 2 μg DG equivalents of granulophysin per milligram of platelet protein without the need for any purification step, has been developed. We have used this assay to measure the protein in platelets of normals and patients. The results provide new insights into the nature of the granule defects in patients with δ-SPD.

MATERIALS AND METHODS

Samples and Standards

Blood samples were obtained from 42 healthy donors without a bleeding tendency. An additional 22 samples were obtained from the hospital's coagulation laboratory after complete assessment of platelet and coagulation systems and a DG count to determine that the subjects were not storage-pool deficient. This determination was based on quantification of the presence of normal numbers of dense granules by whole mounts and the presence of normal platelet aggregation to collagen and epinephrine.

Platelet homogenate standards from outdated platelets were prepared in the same way as other samples (see below), except that these were diluted in phosphate-buffered saline (PBS) + 1% bovine serum albumin (BSA) and kept frozen at -80°C until use.

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tested. High (1,250 µg/mL) and low (530 µg/mL) protein standards were run with each test.

DG standard curves were prepared from a DG preparation (see below) of known protein content.

Preparation of DG and Samples

DG were prepared using the method of Rendu et al., modified as previously described. The DG pellet was frozen and thawed twice, and then centrifuged at 14,000g for 5 minutes. The supernatant was used as a source of highly enriched granulophysin in a standard curve. DG protein as well as all other proteins were quantitated by the Bradford assay (Bio-Rad kit, Richmond, CA) using BSA as a reference standard.

Platelet samples were prepared from whole blood (10 mL) anticoagulated with 1.9 mL acid/citrate/dextrose (ACD) per 8.1 mL whole blood. The blood was centrifuged at 800g for 5 minutes to obtain platelet-rich plasma (PRP). The PRP was then removed and transferred to another tube containing 2 mL Hank’s balanced salt solution (HBSS) containing 0.5 mmol/L MgCl₂ and 3.4 mmol/L NaHCO₃ and 1 mL of ACD. The suspension was then centrifuged for 15 minutes at 1,000g and the supernatant discarded. Pelleted platelets were resuspended in 1 mL HBSS, transferred to an Eppendorf tube, and centrifuged for 2 minutes in an Eppendorf centrifuge. The washed platelet pellet was stored at -80°C until tested. Thawed samples were resuspended and lyzed in 0.1% SDS in PBS, pH 7.4 (0.3 to 0.6 mL depending on platelet volume) and left on a shaker for 30 minutes at room temperature (RT). The lysate was spun in an Eppendorf centrifuge for 3 minutes and the supernatant transferred to a new tube for protein determinations. When necessary, the platelet homogenate was diluted in 0.1% SDS. The first dilution in the ELISA was always in PBS containing 1% BSA. Samples were kept at 4°C for up to 24 hours or frozen for prolonged conservation.

Antibodies and Conjugates

MoAbs against granulophysin were purified by stepwise precipitation in ammonium sulphate, followed by a G-200 column and then FPLC® using a protein A column. The D545 MoAb was conjugated to peroxidase by the periodate method as described by Nakane and Kawaoi and Tijssen and Kurstak, with 0.1 mL ethylene glycol being added to stop the oxidation step of peroxidase. To select for an optimal combination of capture and conjugated antibody, the conjugated D545 preparation was tested with a number of other MoAbs against granulophysin in a sandwich type assay. The combination of MoAbs used in the present assay (D519 and D545 as capture and conjugated antibodies, respectively) were found to be optimal (Fig 1). Both D519 and D545 have been shown to react with granulophysin using Western blots; reactivity of D519 with granulophysin required an enhanced detection system. D519 was used in part because the epitope recognized by D519 is further separated from the D545 epitope than the epitopes recognized by D503 and D495.

ELISA

Flat bottom flexible microtiter plates (Falcon 3912; Becton Dickinson & Co, Lincoln Park, NJ) were coated with 150 µL/well of 20 µg/mL of the capture antibody (D519) in carbonate/bicarbonate buffer 0.01 mol/L, pH 9.6 overnight at RT. The plates were washed once with wash buffer (PBS-tween 0.05%) and once with distilled water, and PBS 1% BSA (100 µL) was then added to all wells. Most samples were evaluated at least twice; some were tested up to four times for statistical purposes. Quantitation of granulophysin was based on the linear part of the D5 standard curve (normally between 0.4 and 3 µg/well); the equivalent linear part of a semi-log plot of the sample titration with optical density (OD) readings on the y axis, and the logarithm of the protein concentration on the x axis. Although it would have been preferable to use purified platelet and tissue samples were diluted over 4 wells beginning at a protein concentration of 300 to 2,000 µg/mL, depending on measured or expected activity. The plates were left for 75 minutes at RT and washed twice with wash buffer. Peroxidase-conjugated second antibody (D545) at a dilution of 1:500 in PBS-Tween + 1% BSA were added together with 100 µL of the same buffer containing serial dilutions of unconjugated D519 or D545. The plates were then left for 1 hour at RT. Finally, the plates were washed three times with wash buffer and once with distilled water, and 100 µL substrate (O-phenylenediamine dihydrochloride at 1 mg/mL in 0.05 mol/L phosphate citrate buffer pH 5.0 supplemented with 40 µL of 3% H₂O₂ per 10 mL) was added to each plate. The substrate was left to react for 5 to 10 minutes until a clear color was observed; the reaction was then stopped by the addition of 50 µL 3 mol/L H₂SO₄. The results were read in a Flow ELISA reader using a 450-nm filter. The 50% inhibition point for D519 is 6 times that of D545 unlabeled MoAb (2,700:450 µg/mL MoAb).

The left row of the plate was routinely left as a control/blank measure in which all reagents were added except for the antigen sample. The substrate in this row normally remained clear or very weakly yellow and was automatically subtracted from all readings. All tests were performed in duplicate and included DG standards as well as high and low protein platelet homogenate standards. Most samples were evaluated at least twice; some were tested up to four times for statistical purposes. Quantitation of granulophysin was based on the linear part of the DG standard curve (normally between 0.4 and 3 µg/well); the equivalent linear part of a semi-log plot of the sample titration with optical density (OD) readings on the y axis, and the logarithm of the protein concentration on the x axis. Although it would have been preferable to use purified
granulophysin as the standard, difficulties in the routine purification of sufficient pure granulophysin for the assay led us to use the DG preparation as the standard. Our present results suggest that 1 µg DG equivalent equals approximately 40 ng granulophysin.

**ELISA Blocking Assay**

This assay was performed as described above with the following modifications: DG (10 µg/mL) lysate was used as a source of antigen in all wells. Fifty-microliter aliquots of serial dilutions at a ratio of 1:2 of the unlabeled blocking antibody in PBS/1% BSA were added to the wells before adding the peroxidase-conjugated second antibody. The plate was then gently shaken for 1 hour at RT before washing and adding the substrate.

**Counts of Platelet DG**

Quantitation of DG was performed by examination of platelet whole mounts using transmission electron microscopy as described by Israels et al. Twenty to 40 platelets were evaluated and the mean number of DG/platelet was determined using a Philips transmission electron microscope. For some patients, counts of DG were also performed using immunofluorescence microscopy of platelets after incubation in 55 µmol/L quinacrine, or after staining the granules with antigranulophysin antibody D545, using the previously described procedure B.

**Platelet ATP Secretion**

ATP release from DG was measured by the luciferin luciferase assay. Briefly, 0.5 mL of PRP (200 × 10⁹/L) was placed in a cuvette and stirred in a lumiaagrégomètre (Chrono-Log Corp, Scarborough, Ontario, Canada). Twenty microliters of 10 µg/mL luciferase (Boehringer Mannheim, Laval, Québec, Canada), 20 µL of 3.6 mmol/L luciferin (Boehringer Mannheim) and 5 µL of 500 mmol/L MgCl₂ were added. The reaction was started by the addition of 1 U/mL thrombin. Aggregation and luminescence were recorded. When aggregation was complete, an ATP standard (Sigma) 1 µmol/L (final concentration) was added and peak height recorded. ATP release was calculated by comparison of peak luminescence recorded from the patient sample with that of the ATP standard. All patient samples were performed in triplicate.

**Patients**

**Hemansky-Pudlak syndrome.** Thirty-one patients with the typical features of the Hemansky-Pudlak syndrome, including albinism and markedly decreased platelet dense granules, were studied. Twenty-nine of these patients were from Puerto Rico. Two typical patients with no connections to Puerto Rico were also studied. The patient from Winnipeg has been previously described.

**Chediak-Higashi syndrome.** Two patients with the Chediak-Higashi syndrome were studied. JK is a 25-year-old male who has been previously described. MD was a 5-year-old girl with the typical features of the Chediak-Higashi syndrome, including a history of frequent infections and easy bruising, large granules in her neutrophils, abnormal platelet aggregation, and decreased natural killer cell function.

**Family 1.** Three members of a family with an apparent autosomal dominant inherited storage pool deficiency were studied—an 80-year-old man, his 55-year-old sister, and his 43-year-old son. All three members studied have had a lifelong history of excessive bleeding. The platelets of all three have been evaluated at intervals over a period of 11 years. All three show decreased platelet aggregation to collagen and lack the second wave of aggregation to epinephrine. They have been consistently storage-pool deficient as assessed by counts of platelet DG and low levels of platelet ADP and serotonin. This family is unusual in that eight members of the pedigree have developed leukemia.

**Family 2.** Two sisters with a history of a bleeding tendency and findings of a platelet storage pool deficiency were studied. Similar to affected members of Family 1, these sisters show decreased platelet aggregation to collagen and do not undergo second wave aggregation to epinephrine. Platelet ADP and serotonin content were also low. These sisters have had their platelets evaluated at intervals over a period of 7 years with consistent findings of a DG storage pool deficiency condition.

**Statistical Evaluation**

Intra-assay and inter-assay variability were evaluated for both experimental samples and standard homogenates by the reading error (σ²) formula and the nested design anova using the Statistical Analysis System (SAS) program, respectively.

**RESULTS**

**The Assay**

The present assay is an antigen-capture-sandwich ELISA type in which purified platelet DG were used as a highly enriched source of granulophysin. The specificity of the assay has been verified by including specific controls in the assay and by blocking experiments. When the capture antibody and/or conjugate were omitted (test for nonspecific binding or internal peroxidase of the sample), zero or very low background (OD < 0.05) readings were observed. The specificity of the assay is further demonstrated by Fig 1, in which D545 unlabeled antibodies completely abolished the binding of the conjugated antibody to give a zero OD reading at a concentration of 2.5 µg/well (about 5 times the conjugate concentration of 460 ng/well). The capture MoAb (D519) required concentration levels of about 6 times higher than D545 (2,700 ± 450 ng/well) to reach the same 50% inhibition level (Fig 1), suggesting recognition of a related but not identical epitope on the molecule. The DG standard curve followed a sigmoid pattern with a clear linear range between 2.5 and 20 µg/well DG protein (Fig 2). This range was used for quantitation of unknown samples. The dilutions of samples in the assay also followed a linear and parallel pattern equivalent to the DG linear region. Likewise, samples containing higher or lower protein concentrations curved in a similar way to the standard sigmoid curve. Quantitation of granulophysin was routinely performed in the linear and parallel regions of the curves and expressed in terms of micrograms of DG equivalents per milligram of platelet protein.

Occasional samples (about 5%) that read under the linear region or did not follow a linear pattern were repeated or excluded. The reading error (intra-assay variability) for 21 duplicated samples gave a within mean square error of σ² = 1.7 (σ = 1.3). Inter-assay variability of 29 samples (76 readings) gave a within mean square error (σ²) of 2.09. When analyzed according to sources of variability (sum of squares), patient factors accounted for 92% of the variability, with the rest being accounted for by other factors (reproducibility, measuring errors, etc). The means and SE for the high and low protein platelet
homogenate standards were 9.0 ± 0.46 and 10.8 ± 0.57, respectively.

**Solubilization of DG and Samples**

The optimal solubilization solution for DG and other tissues was examined experimentally. Three detergents (SDS, lubrol, and Triton-X100; Sigma Chemical Co, St Louis, MO) were tested. As shown (Fig 3), 0.1% SDS was the most efficient in releasing granulophysin from the platelets leaving practically no activity in the remaining pellet. The choice of 0.1% SDS in PBS as a general solubilization agent for granulophysin in platelets was further examined using a range of SDS concentrations from 0.05 to 1.0% (data not shown), and 0.1% SDS was confirmed as optimal.

**Granulophysin Levels in the Normal Population**

Granulophysin levels in normal volunteers and in subjects from the coagulation laboratory in whom the diagnosis of storage pool deficiency was excluded were 16.4 ± 5.6 (n = 42) and 17.7 ± 6.4 (n = 22) µg DG equivalents/mg platelet protein, respectively. To be sure no patients with SPD were included, all subjects with decreased aggregation to collagen or epinephrine, with secretion of less than 2 µmol/L ATP per 2 × 10⁸ platelets, or with less than 3 DG/platelet on whole mounts were excluded. Because both the distribution and the mean values of these two groups were similar, they were pooled together and resulted in a mean granulophysin value of 17.07 ± 5.96 (n = 64) µg DG equivalents/mg platelet protein. The distribution of granulophysin values in this population appeared to be skewed or possibly bimodal with a higher proportion of about 48% at the 10 to 16 µg/mg level (Fig 4) and an overall range from 8.3 to 30 µg/mg protein.

**Correlation Between Granulophysin and DG Counts**

Analysis of a possible linear correlation between the level of granulophysin and electron microscope DG counts of 51
patients with the Hermansky-Pudlak syndrome had markedly reduced levels of granulophysin and numbers of DGs detected using quinacrine or D545 immunofluorescence. Obligate heterozygotes had levels of granulophysin that were similar to normal controls. Samples from two patients with the Chediak-Higashi syndrome showed low levels of granulophysin and decreased numbers of DG as assessed by D545 and quinacrine immunofluorescence. In five nonalbinos SPD patients, granulophysin levels were normal despite low DG numbers. Three members of one family with an apparently autosomal dominant inherited form of SPD had approximately half the normal number of DG by whole mounts, quinacrine staining, and D545 staining. Members of a second family clearly had "empty sacs" with much higher DG numbers by D545 staining than observed on whole mounts. Platelet granulophysin content in the latter subjects was normal.

**DISCUSSION**

The present study details the development of a quantitative assay for the newly described DG protein, granulophysin, and the use of this assay to characterize the granule defects in patients with δ-SPD. The distribution of granulophysin levels in the normal population was skewed or possibly bimodal: the distribution could represent genetic variability or polymorphism within the population. The reason for this distribution is unknown because there is not a comparable skewed or bimodal distribution of DG numbers. Interference by factors that might affect the accessibility of the epitopes on the protein in the assay also cannot be excluded at this point. In any event, these results indicate that the definition of "normality" for this protein is within a wide range.

Granulophysin levels were markedly lower in δ-SPD patients with albinism (Hermansky-Pudlak and Chediak-Higashi syndromes) than in controls, but within the normal range in patients with SPD who did not have albinism. The studies of δ-SPD patients confirm and extend our initial report of deficient granulophysin content in a patient with the Hermansky-Pudlak syndrome.23 Low granulophysin

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**Table 1. ATP Secretion, DG, and Granulophysin Levels in Storage Pool Deficiency Patients**

<table>
<thead>
<tr>
<th></th>
<th>ATP Secretion (μmol/2 × 10⁸ platelets)</th>
<th>DG Counts*</th>
<th>Granulophysin (μg DG equivalents/mg platelet protein)†</th>
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<tr>
<td></td>
<td></td>
<td>Whole Mounts</td>
<td>Quinacrine</td>
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<tr>
<td><strong>Controls</strong></td>
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<tr>
<td>Winnipeg</td>
<td>n = 42</td>
<td>1.89 - 5.45</td>
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<td>Puerto Rico</td>
<td>n = 3</td>
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<tr>
<td><strong>Hermansky-Pudlak syndrome patients</strong></td>
<td></td>
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<tr>
<td>Winnipeg</td>
<td>n = 1</td>
<td>0.25</td>
<td>0.4</td>
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<tr>
<td>Rochester</td>
<td>n = 1</td>
<td>ND</td>
<td>0.05</td>
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<tr>
<td>Puerto Rico</td>
<td>n = 29</td>
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<td><strong>Obligate</strong></td>
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<td>Heterozygote</td>
<td>n = 13</td>
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<td><strong>Chediak-Higashi syndrome patients</strong></td>
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<tr>
<td>JK</td>
<td>ND</td>
<td>0.8</td>
<td>1.7</td>
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<tr>
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<td><strong>Nonalbino SPD</strong></td>
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<td>Autosomal dominant SPD</td>
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<tr>
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<tr>
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<td>0.34</td>
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<tr>
<td>DM</td>
<td>0.85</td>
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<td>1.5</td>
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*Individual values are based on counts of at least 20 platelets. Normal values are given as the mean ± SD for all controls.
†All samples were solubilized in 0.1% SDS. Values are given as the mean ± SD, or as the mean of duplicate determinations.
‡Whole mounts on these subjects were not done, but previous studies on the same population showed obligate heterozygotes to have normal DG numbers and morphology (J.G.W., C.J.W., unpublished data, 1991).
levels appear characteristic of Hermansky-Pudlak syndrome patients from different genetic backgrounds. Neither the Winnipeg patient studied nor the patient from Rochester, MN are of Puerto Rican background. Levels of granulophysin in 13 obligate heterozygotes were also measured as an approach to try to understand whether granulophysin represented the primary protein defect or was simply one of several dense granule proteins that are low in this condition. The mean value for obligate heterozygotes was close to normal, suggesting granulophysin deficiency in Hermansky-Pudlak syndrome patients is secondary to the absence of granules rather than representing the primary functional defect.

Two patients with the Chediak-Higashi syndrome had low granulophysin levels. A deficiency of a 40-Kd protein in Chediak-Higashi cattle has recently been described by Meyers et al.23 The protein studied by Meyers et al may be the same as granulophysin. It is tempting to speculate that a defect in granulophysin might be the primary protein defect in the Chediak-Higashi syndrome. The presence of D545 immunoreactivity in melanocyte granules and in the membranes of the abnormal giant neutrophil granules in the Chediak-Higashi syndrome (J.M.G., J.G.W, unpublished data, 1991) and a possible role for granulophysin in membrane fusion, a process that appears abnormal in Chediak-Higashi syndrome, might be consistent with such a concept. Further studies will be necessary to prove or disprove such a possibility because it is at least as likely that the low platelet granulophysin content is secondary to the low numbers of DG vesicles as appears to be the case in the Hermansky-Pudlak syndrome.

The evaluation of nonalbino 6-SPD patients suggests that the measurement of granulophysin may facilitate the classification of such patients. Based on our studies, nonalbino 6-SPD can be divided into at least two groups. The first group consists of patients from a family with an autosomal dominant inherited condition in which the number of DG as assessed by whole mount examination, quinacrine, and D545 immunofluorescence is about half normal. Granulophysin levels in these patients fell in the low end of the range when expressed per milligram of platelet protein. However, when granulophysin levels were expressed per dense granule they were at the high end of normal, but still within the normal range because of the skewed distribution of granulophysin concentration in normals. It is likely that the patients from the family with autosomal dominant 6-SPD most likely have a defect in a gene coding for a DG membrane protein that is important for DG formation, such that when only half the normal amount of protein is present only half the usual number of DG are formed. The second group of patients that can be identified are patients with an empty sac syndrome in which the number of DG stained using D545 is normal, and granulophysin levels are normal, but the number of DG seen on whole mounts is very low. Because other forms of 6-SPD are known, particularly forms associated with a granule deficiency,24,27 it will be of interest to evaluate such patients in the future.

The present study may also provide clues to the functional role of granulophysin. The platelet content of a protein with a role in granule formation should correlate closely with the number of DG per platelet. Furthermore, the platelet content of a protein with a role to regulate the accumulation or storage of one or more DG constituents should correlate with the content of the DG. The fact that platelet granulophysin content does not correlate closely with either of the above is most consistent with an alternate function for granulophysin, as for example, in exocytosis. Synaptophysin, a synaptic vesicle protein containing an epitope recognized by antigranulophysin antibodies,25,27 has been postulated to form an exocytotic fusion pore critical to granule secretion.24 Granulophysin could have a related role in platelet DG.

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