Results of an External Proficiency Testing Exercise on Platelet Dense-Granule Deficiency Testing by Whole Mount Electron Microscopy

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Abstract

Performance on specialized diagnostic tests for platelet disorders, including dense-granule deficiency, is rarely evaluated by external quality assessment (EQA). Members of the North American Specialized Coagulation Laboratory Association that evaluate platelet dense-granule deficiency commonly use whole-mount electron microscopy (EM) methods. This observation led us to develop a pilot EQA survey with standardized EM images and clinical samples on grids from a healthy control subject and a subject with dense-granule deficiency. The survey participants were 8 centers, including 2 with no experience in platelet whole mount EM. All participants, including inexperienced sites, correctly interpreted findings for the normal and dense-granule-deficient platelets. Among experienced sites, agreement was excellent (>82%) on platelet structures to count or not count as dense granules. Participants indicated that future EQA challenges should include clinical samples on grids and standardized images. This is the first report that platelet EM can be assessed by EQA.

Diagnostic tests for platelet disorders are essential for the evaluation of many common bleeding disorders.[1,2] At present, most assays for platelet disorders require freshly collected blood samples and complex, time-consuming procedures that are rarely evaluated by internal and external quality control programs.[3] Recent international surveys have highlighted the need for more standardized practice in testing for platelet disorders based on guidelines and recommendations on test uses, performance, and interpretation.[3] To date, the application of internal and external quality assurance (EQA) surveys to diagnostic tests for platelet disorders has been limited.[3] Several organizations have introduced surveys for some tests, such as closure times measured by the PFA-100 (Siemens Diagnostics, Munich, Germany), using control samples obtained at individual centers and spiked with additives.[3-5] However, EQA surveys using clinical samples from people with platelet disorders have never been attempted.[3]

Platelet dense-granule deficiency is recognized as a common type of platelet secretion disorder.[2,6,7] Tests for platelet dense-granule deficiency are performed by some specialized coagulation laboratories because many recognize that platelet dense-granule deficiency may not be detected by screening tests (such as the bleeding time and PFA-100) or by aggregation assays.[1-3,8] In a recent survey of the North American Specialized Coagulation Laboratory Association (NASCOLA), almost all clinical laboratories that offered testing for platelet dense-granule deficiency reported that they used a whole-mount electron microscopy (EM) method.[9] This method was first reported by White[10-12] and Witkop and colleagues[13] and involves an assessment of unfixed platelets (dried on Formvar grids), to evaluate and quantify the numbers of dense granules contained in platelets.[6] This method is

known to detect dense-granule deficiency due to diverse disorders, including Hermansky-Pudlak syndrome, αδ-storage pool deficiency, and many others.[6,13-18]

To investigate whether EQA can be applied to diagnostic tests for platelet disorders, NASCOLA developed and conducted a pilot survey for platelet dense-granule deficiency using diagnostic samples of normal and dense-granule-deficient platelets and standardized images of platelets from whole-mount EM preparations. We report that EQA surveys for platelet dense-granule deficiency are feasible and valued by laboratories that perform these assays.

Materials and Methods

Sample Collection

Blood samples for the proficiency testing exercise were collected with informed consent of donors, in accordance with policies of the Hamilton Regional Laboratory Medicine Program (Hamilton, Canada) on sample collection for EQA, institutional research ethics board guidelines, and the recently revised Helsinki protocol for human research.

The donors for the pilot EQA survey included a healthy control subject and a subject with confirmed dense-granule deficiency. The subject with dense-granule deficiency had a history of abnormal bruising and menorrhagia without pigmentation abnormalities or other health problems to suggest Hermansky-Pudlak syndrome. This subject had abnormal platelet function shown by light transmission aggregation (maximal aggregation below the published reference intervals[19]) with a number of agonists, including 1.25 and 5.0 μg/mL of collagen, 1.6 mmol/L of arachidonic acid, and 1 μmol/L of thromboxane analogue U46619, and also absent secondary aggregation with epinephrine. The subject also had absent to markedly reduced adenosine triphosphate release, assessed by a lumi-aggregometer (Chrono-log, Haverston, PA) using an agonist panel that included 1 U/mL (final) thrombin (Chrono-log) in addition to all agonists routinely used for light transmission aggregation,[19] with the exception of ristocetin and 2.5 μmol/L of adenosine diphosphate. The patient was assessed twice by EM analysis, and both samples contained approximately 0.6 dense core granules per platelet (reference interval, 4.0-8.0 dense granules per platelet; later updated to 5.6-9.0 dense granules per platelet using additional controls). Elemental analysis by energy dispersive spectrometry (EDS)[14] confirmed that the dense granules of this subject contained phosphorus and calcium; however, in a number of the dense granules analyzed, the phosphorus/calcium ratio was inverted, with the calcium peaks higher than the phosphorus peaks.

In accordance with Hamilton Regional Laboratory Medicine Program and NASCOLA policies, the sample shipments were held until the donors were confirmed to test negative for HIV and infectious hepatitis.

Preparation of Platelet Grids for EM

Formvar-coated, 200 mesh copper-palladium grids were prepared for platelet whole-mount EM, as described.[8,14] The platelet-rich plasma samples were harvested from blood anticoagulated with buffered 3.2% sodium citrate and centrifuged as described.[19] Samples (10 μL) were spotted onto Formvar-coated grids, rapidly blotted, rinsed with distilled water, and air dried, as described.[8,14] All of the dried grids for the EQA survey were inspected before placing the samples in gelatin capsules to protect their integrity during shipment.

Preparation of Standardized EM Images

Images of normal platelet whole mounts by EM were acquired using a JEOL 1200 EX TEMSCAN (JEOL, Tokyo, Japan). Tiff files were imported into a PowerPoint file (Microsoft, Redmond, WA) for distribution to participants. Structures in images of 4 platelets were numbered consecutively (1-55) for survey participants to rate as structures that they would or would not count as a dense granule. An additional image of a normal platelet whole mount, without labeled structures, was included in the challenge for participants to estimate the number of dense granules contained in the platelet.

Data Analysis

Survey results were reported online using a standardized form. For the grids, participants were asked to supply their numeric results and interpretation. For the standardized images, data were collated to determine which structures were counted or not counted as dense granules by participants and to determine the number of dense granules reported for the platelet image without labeled structures. To evaluate differences in the numbers of dense granules reported by participants in the EQA survey, participants were asked to supply their reference interval (if determined) for the number of dense granules per platelet. For some analyses, survey participants were stratified as "experienced" or "inexperienced," based on whether they had previously tested clinical samples by platelet whole-mount EM. Participants were also asked to provide feedback on the EQA exercise.

Statistical analysis of the EQA results included calculation of the percentage of agreement, means, and medians.

Results

Grid Challenges

There was fairly good agreement among sites for the findings reported for the dense-granule-deficient sample, which was recognized by all to contain reduced numbers of

dense granules. Six participating sites provided their estimate of the average numbers of dense granules per platelet, which ranged from 0 to 1.0 (mean, 0.4; median, 0.4).

The normal platelet sample was recognized by all sites to contain normal numbers of dense granules. Seven participating sites provided their estimate of the average numbers of dense granules per platelet, which ranged from 3.6 to 8.8 (mean, 5.6; median, 6.0). Six participants (all from experienced sites) indicated that the number was within their reference interval. The lower limits of participants' reference intervals, provided by 3 sites, were 2, 3.5, and 4 dense core granules per platelet.

Only 1 site commented that the grid challenge samples had an appearance different from that of its own preparations.

Standardized Image Challenges

Seven of the participating sites provided an estimate for the number of dense granules contained in the platelet shown in ❙Image 1❙. The number of dense granules reported ranged from 6 for an inexperienced site to 15 to 24 for experienced sites (for experienced sites, mean, 19; median, 18).

The panels in ❙Image 2❙ show the sequentially labeled structures in EM images of platelets that were provided to the EQA survey participants. Seven sites rated these structures, and there was good overall consensus on what to include or not include in counts for dense granules (agreement: average, 83%; median, 86%), which was higher after exclusion of the inexperienced site (average, 84%; median, 100%). Of the 55 structures evaluated (Image 2), there was complete consensus on 20 structures, good consensus on 16 structures (86% agreement), and moderate consensus on 19 structures (57%-71% agreement).

Participant Feedback

Feedback was obtained from 88% of participants, and the majority (86%) indicated that they valued the opportunity to participate in an EQA survey for platelet disorders. The majority (86%) recommended inclusion of grid and image challenges in future EQA exercises for platelet dense-granule testing.

Discussion

Our study illustrates that performance on diagnostic laboratory tests for platelet dense-granule deficiency can be evaluated by EQA. We selected testing for dense-granule disorders for our pilot survey of platelet disorder EQA because the analytic component of the test required air-dried samples, which allowed us to confirm that the donor samples were negative for transmissible viral disease before sample shipment. Nevertheless, we recognize that preanalytic issues have important influences on test findings. Although some
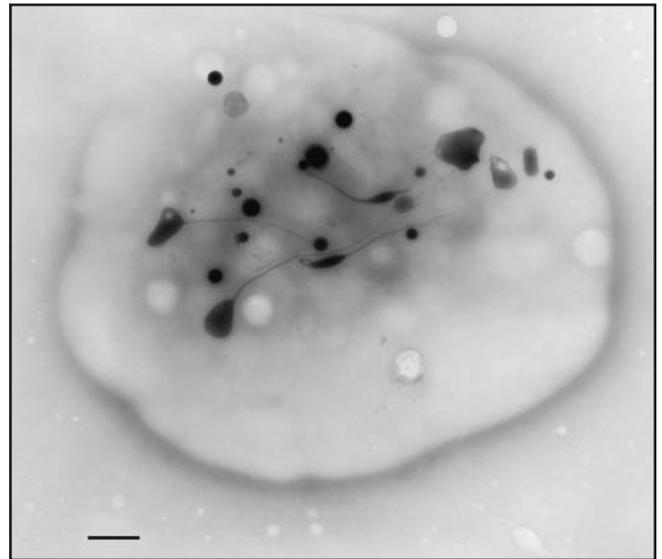

❙Image 1❙ Whole mount electron microscopy image of a normal platelet that was provided to participants to estimate the numbers of dense granules contained in this platelet. Bar indicates 500 nm.

of the sites that participated in our exercise did not have experience with assessing platelet dense-granule deficiency by whole mount EM, the agreement among sites was excellent and all sites reported the correct diagnosis for the normal and dense-granule deficient samples. Furthermore, there was good agreement for the data from standardized image challenges. Nevertheless, the variability in which structures that participants counted or did not count as platelet dense granules emphasizes the importance of establishing a site-specific reference range to guide interpretation of findings, as generally recommended.[3] Participants valued the opportunity to participate in an EQA survey for platelet disorders and suggested that grid and image challenges should be included in future EQA exercises for platelet dense-granule testing.

There are few guidelines and recommendations for diagnostic tests used to assess platelet disorders.[3] Recent international efforts have focused on light transmission aggregometry because it is one of the more commonly performed diagnostic procedures for assessing platelet disorders.[3] Although a number of procedures have been developed to assess platelet dense-granule deficiency,[1-3] few laboratories in North America use serotonin-release assays or mepacrine-uptake methods.[9] Assessments of adenosine triphosphate release are useful to assess platelet secretion defects, and they can be helpful in identifying reduced or absent release in cases of dense-granule deficiency,[1-3] as illustrated by the findings in the abnormal donor case for our pilot EQA survey.

A limitation of our survey was that we assessed the analytic and postanalytic but not the preanalytic component of
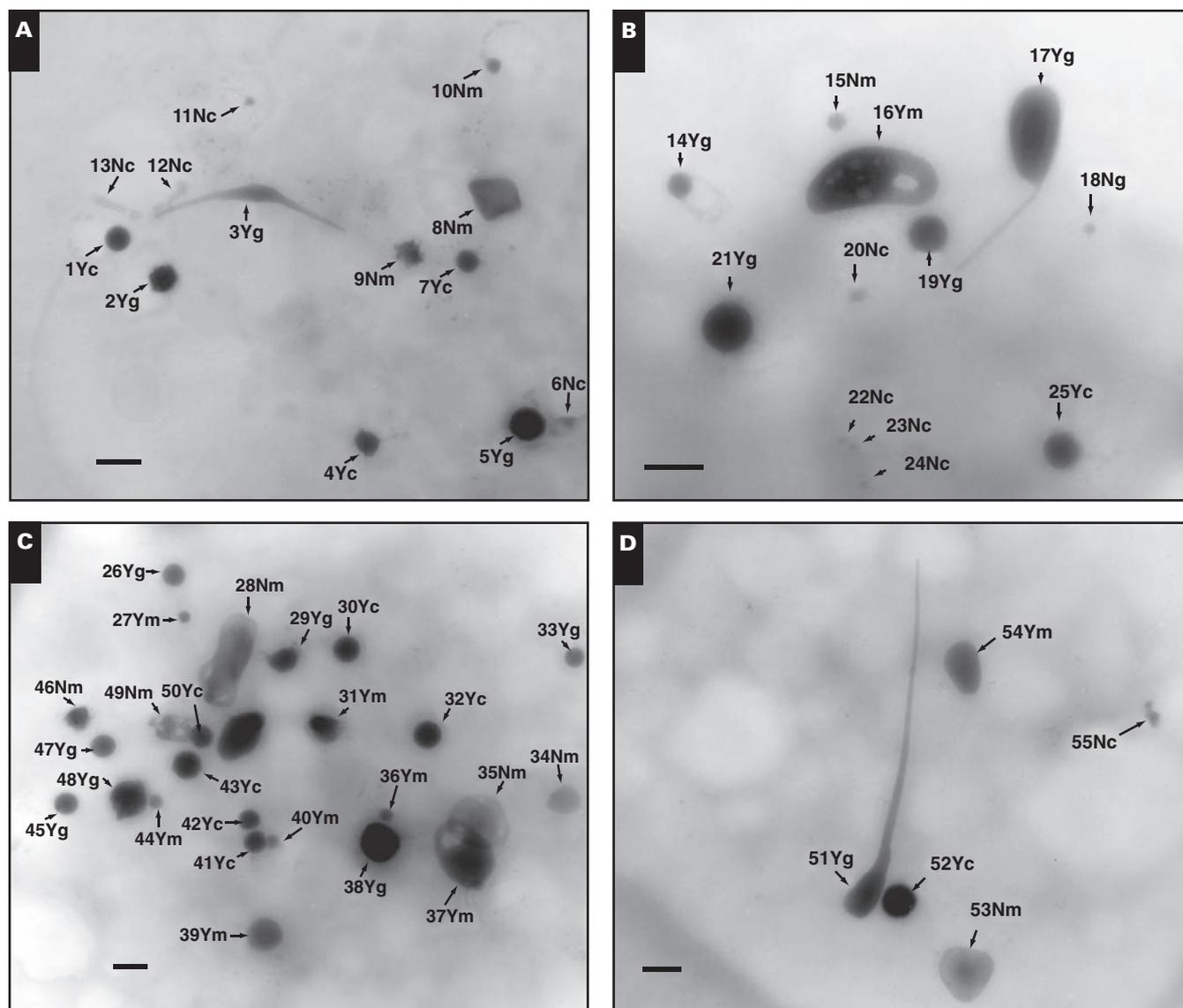
Image 2 **A**, **B**, **C**, and **D**, Electron microscopy images of 4 platelets, with sequentially numbered, labeled structures that were provided to participants to indicate which structures they would count as dense granules. Structures counted (Y) or not counted (N) and the level of consensus (c, complete; g, good or 86%; m, moderate or 57%-71%) are indicated. Bars indicate 200 nm.

platelet whole-mount EM. The reasons were in large part due to regulations on EQA shipments (requiring confirmation of negative tests for infectious viruses before shipping samples), the logistical challenges of shipping fresh blood samples to different sites in North America, and the indication on a pre-survey questionnaire that most sites would not accept shipped blood samples for this assay.

The excellent agreement that we found between experienced laboratories that participated in our survey likely reflects that most participants used the White method for assessing platelet dense-granule deficiency with air-dried, unfixed platelet samples. Only 1 site reported that the grid samples in the challenge had an appearance different from that of its own preparations. EDS analysis[14] has been used to

assess the contents of platelet dense granules. However, only 2 of the 8 sites that participated had the technical capability of performing EDS and only 1 had actually used EDS to evaluate platelet dense-granule contents in clinical samples. This indicates that most sites identified a dense granule based on structural appearance rather than on a determination of the calcium and phosphorus content of a structure. There is a need for morphologic criteria to assess platelet dense granules by whole-mount EM because some chain-like and clustered electron-dense structures are present in similar quantities in control and dense-granule-deficient platelets.[20] Experienced sites were quite similar in what they considered to be a dense granule (based on evaluation of numbered structures), and this likely explains the similar numbers reported for average

numbers of dense granules in grid samples and in the unlabeled platelet image challenge. Nevertheless, the development of guidelines and recommendations and publication of the images and findings from our pilot challenge could prove useful as a reference. This could, for example, be helpful to laboratories with minimal experience in platelet whole-mount EM that want to offer the test for diagnostic purposes.

In general, 2 levels (normal and abnormal) of control samples are recommended for quality assurance of diagnostic laboratory assays.[21] The inclusion of grids containing normal and dense-granule-deficient platelets in our challenge allowed all participants, including those without experience, the benefit of being able to evaluate whether their method correctly identified the confirmed normal and abnormal samples. This is important because there are no commercial controls or international standards to assess this assay.

Our survey on platelet dense-granule deficiency was valued by participants, and it illustrated that it is feasible to do EQA for a laboratory assay used to diagnose an important and fairly common cause of abnormal platelet function. As a result of this survey, a working group was formed to help develop guidelines and recommendations for evaluating platelet dense-granule deficiency and provide input on the needs for future EQA surveys.

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