Diagnosis of Glanzmann thrombasthenia by whole blood impedance analyzer (MEA) vs. light transmission aggregometry

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SUMMARY

Background: Glanzmann thrombasthenia (GT) is a rare inherited platelet disorder that is characterized by spontaneous or postprocedural bleeding. The diagnosis of GT depends on identifying the dysfunction of the platelets.

Aim: The aim of this study was to compare a whole blood impedance Multiplate analyzer (MEA) with the standard method, light transmission aggregometry (LTA) in diagnosis of GT.

Methods: Fifteen patients with GT were assessed on MEA and LTA using arachidonic acid (ASPI: 15 mM), (TRAP: 1 mM), collagen (100 μg/mL), ADP (0.2 mM), and ristocetin (Risto: 10 mg/mL). Whole blood samples were collected in sodium citrate and hirudin vacuum, blood collection tubes and tested within 4 h. Platelet-rich plasma was used for LTA using platelet agonists (ristocetin 1.5 mg/mL) (arachidonic acid 0.5 mg/mL) (ADP 2.5 mg/mL) and (collagen 1 mg/mL).

Results: The platelet count and PFA-100 results were (average and SD) 319 ± 93 × 10⁹ L and 252 ± 34 s, respectively. Flow cytometry analysis showed that all samples are positive for CD42a and CD42b, whereas 9/15 samples were negative for CD61 and CD41. The other six patients had either partial or full expression of CD61/CD41. Aggregation analysis using both methods showed that all samples had no aggregation response to any of the agonists used apart from six samples which, using only the MEA, showed minimal aggregation in response to collagen (average = 14.3 ± 7 μg, which may suggest ability to detect qualitative abnormality of GPIIb/IIIa).

Conclusion: These results suggest that the MEA is sensitive for the detection of Glanzmann thrombasthenia. Furthermore, MEA may also be able to differentiate between the subtypes of Glanzmann thrombasthenia.
INTRODUCTION

Glanzmann thrombasthenia (GT) is an inherited bleeding disorder resulting from either qualitative or quantitative abnormalities in the glycoprotein IIb/IIIa complex located on the platelet membrane. GT was first reported in a series of patients with mucocutaneous bleeding, normal platelet counts, and prolonged bleeding times in 1918 [1]. Later, it was discovered that these patients lacked functional GPIIb/IIa on their platelets [2]. This defect prevents adequate formation of the platelet plug, which then leads to increased bleeding at sites of injury [3]. There are three types of GT, type I, with <5% GPIIb/IIIa and absent clot retraction; and type II, with 10–20% GPIIb/IIa and minimal clot retraction; and type III or GT ‘variants’ where the platelets have normal or near-normal (60–100%) expression of dysfunctional receptors [4, 5]. The genes for glycoprotein IIb and IIIa are on chromosome 17, with many genetic mutations have already been described. GT is not always the result of the same mutation [6, 7]. These mutations have been grouped according to the biochemical consequences and include transcriptional and protein functional defects. Among the functional defects, there are protein stability mutants, glycoprotein complex maturation mutants, and ligand-binding mutants [8, 9]. Treatment of the disorder is limited to local measures such as the application of pressure. Platelet transfusion may be useful, but only on a limited basis as resistance will develop to the infused platelets [10, 11].

Platelet aggregation in vitro has been widely used to test platelet interaction. Platelet aggregation in response to different platelet agonists is absent in patients with GT because binding of fibrinogen to platelets is defective [12–16]. LTA is considered the gold standard method in the detection of GT. In this method, aggregation is assessed in platelet-rich plasma (PRP), by measuring changes that occur in light absorbance. However, measuring platelet function by this method is time-consuming and lacks standardization. Furthermore, it requires relatively large amount of blood which makes it sometimes difficult to use for young children. Thus, a fast, reliable, convenient standardized method to assess platelet function for the diagnosis of patients with GT would be of great value. A new method that has been recently available is the MEA (Dynabyte, Munich, Germany), which rapidly measures platelet aggregation in whole blood [17] and may potentially avoid some disadvantages of the LTA, such as variable reproducibility, lengthy processing time, and requires small blood quantity which make it more practical, especially for young patients. The aim of this study was to compare the gold standard method for measuring platelet function in GT, the LTA, with the MEA.

MATERIALS AND METHODS

Study populations

Blood samples from healthy volunteers (n = 30) have been collected to establish the reference range. Furthermore, 15 patients with bleeding symptoms who had been diagnosed as GT by LTA platelet aggregation, after giving written informed consent, were included in the study. Of the 15 patients, 53.3% (8/15) were females, the mean ± SD age was 14.5 ± 8.70 years, and 46.6% (7/15) were males, the mean ± SD age was 15.28 ± 10.11 years. The study was approved by the research advisory committee at King Faisal Specialist Hospital and Research Center (KFSH&RC). All patients were known cases of GT with typical clinical presentation, and this was also confirmed on the day of sample collection by measuring CD41/CD61 using flow cytometry.

Blood sampling

Blood was drawn from the antecubital vein by puncture without stasis using a 21G butterfly needle into 4.5-mL Vacutainer™ tubes (Becton Dickinson, Oxford, UK) containing 0.105 M sodium citrate (1 : 9 v/v), and another sample was collected in hirudin vacuum blood collection tubes. The blood tubes were then immediately mixed and checked to ensure that it is not over- or under-filled. All samples were kept at room temperature and analysis was made in <4 h from collection, to prevent platelet activation.

Preparation of platelet-rich plasma

Platelet-rich plasma (PRP) was prepared from whole blood by centrifuging at 3000 g for 10 min at room temperature. The resulting top two-thirds of the upper layer of the PRP were transferred to new
polypropylene tubes after adjusting to achieve platelet count $200–400 \times 10^9$ L.

**Platelet function testing**

All clinically suspected patients for platelet function disorder had full blood count and tested for PFA-100 (Siemens Healthcare Diagnostics, Malvern, PA, USA). These patients were further tested by Multiplate analyzer (Dynabyte, Munich, Germany) using whole blood. Multiplate analyzer detects the electrical impedance change due to the adhesion and aggregation of platelets on two independent electrode-set surfaces in the test cuvette (1, 2, 3) using different platelet agonist, that is, arachidonic acid (ASPI: 15 mM), (TRAP: 1 mM), collagen (100 $\mu$g/mL), ADP (0.2 mM), and ristocetin (Risto: 10 mg/mL), whereas platelet-rich plasma was used for LTA using platelet agonists (ristocetin 1.5 mg/mL) (arachidonic acid 0.5 mg/mL) (ADP 2.5 mg/mL) and (collagen 1 mg/mL). MEA has five test cells for parallel testing, and each cell incorporates two independent sensor units. One unit consists of two silver-coated, highly conductive copper wires. Analysis is based on platelet adhesion upon activation, a property that results in aggregation onto the metal sensor wires in the test cell, thus increasing the electrical impedance between the wires. For measurement, 300 $\mu$L hirudin-anticoagulated whole blood was placed into the test cell, and the sample was stirred using a teflon-coated electromagnetic stirrer (800 g) over a 3-min incubation period.

Platelet aggregation was initiated using arachidonic acid (AA) (ASPItest, 0.5 mM) or thrombin receptor-activating peptide (TRAP-6, TRAPtest, 32 $\mu$M) using reagents supplied by the manufacturer. Increased impedance due to the attachment of platelets to the electrodes was continuously measured for each sensor unit over a period of 6 min. Data were transformed to arbitrary AU and plotted as two separate aggregation curves vs. time. Patients were diagnosed as GT if they have absence or abnormal response in the five MEA test cells. Figure 1 shows the Multiplate aggregation curves.

**Flow cytometry analysis**

To study platelet GPIIb/IIIa activation and granule by flow cytometry, a sample of 1.5 mL sodium citrate was collected. Approximately 1 mL of blood transferred to test tubes filled with 3–4 mL of the PBS-EDTA buffer and gently mixed. The tubes spin at 700 g for 20 min at room temperature. The platelets were in suspension throughout the plasma layer above the other cells forming PRP. After washing platelet twice with EDTA–PBS and centrifuged at 3500 g for 2 min, platelets get harvested at the bottom of the tube. The platelets resuspended to a final volume of 1–2 mL with EDTA–PBS and adjusted to platelet count of $100 \times 10^9$/L using the ACT diff cell counter. The washed platelet suspension added to each tube of the monoclonal antibody against platelet glycoproteins for gp IIb (CD41-PE), for gp IIIa

(CD61-FITC), for gp IX (CD42a-FITC), and for gp Ib CD42b-PE from Becton Dickinson. The tubes incubated at room temperature for 30 min and washed again by adding 2–4 mL of EDTA–PBS buffer, and centrifuging the tubes at 3500 $g$ for 2 min, and resuspended with 500 µL of 0.5% PFA Decant, the supernatant. The samples were analyzed in a FACS Caliber flow cytometer instrument (Becton Dickinson). Data with lower intensity than the normal control considered to have abnormally platelet function. The percentage of expression for each of the abnormal platelet glycoproteins was calculated by dividing the Geo Mean patient’s PG by the Geo Mean control PG then multiply by 100. The results were reported as type 1 (severe) if <5% of normal GPIIb-IIIa levels, type 2 (less severe) if 10–20% of normal GPIIb-IIIa levels, and type 3 (variant) when normal levels of GPIIb-IIIa, but abnormal functionally.

Statistical analysis
For all statistical analyses, a value of $P < 0.05$ was considered significant. Analysis was performed using the sss software (Hong Kong).

RESULTS
The platelet count and PFA-100 results were (mean and SD) $319 \pm 93 \times 10^9 \text{L}$ and $252 \pm 34 \text{s}$, respectively. Flow cytometry analysis showed that all samples are positive for CD42a and CD42b, whereas nine of 15 samples were negative for CD61 and CD41. Three patients showed partial and three showed normal expression of CD61 and CD41. Aggregation analysis using both methods showed that all samples had no aggregation response to any of the agonists used apart from six samples which, using only the MEA, showed minimal aggregation in response to collagen (mean = $14.3 \pm 7 \mu g$). Table 1 shows the data from the MEA and LTA, which were in accordance with each other.

DISCUSSION
Glanzmann thrombasthenia is a rare platelet function disorder, characterized by qualitative defect of platelet GPIIb/IIIa with normal platelet count and morphology. Although it is considered rare in most people, it may not be so in some people, their consanguinity is relatively high, such as in Northern Iranians, Iraqi Jews, Southern Indians, and Arabs [18, 19].

Currently, many common and well-established tools for screening and diagnosing GT are available. In a study by Harrison et al. [20], the PFA-100 proved to be sensitive in detecting classical defects by giving prolonged closure times in samples from platelet function defect.

Although the LTA method historically has been the most widely used, there are several disadvantages in its daily use such as sample preparation, the amount of blood needed, and the technical procedure being troublesome and time-consuming. In addition, many sources of variation exist, including type and concentration, and centrifugation procedure.

In the current study, whole blood aggregometry using MEA gave very similar results to LTA in all the patients we studied. Moreover, interestingly, MEA shows minimal aggregation in response to collagen in six patients, of which three patients had partial expression of CD41/CD61 as measured by flow cytometry. In a similar study, Awidi et al. [21] had shown an agreement between both methods when used to diagnosed patients with Glanzmann’s disease. This suggests that the MEA can be used to diagnose Glanzmann’s cases and may be able to differentiate between subtypes of GT although this requires further studies to confirm this suggestion.

This is the first study from the Kingdom of Saudi Arabia that compares platelet aggregation in GT as assessed by MEA in whole blood and comparing it to platelet aggregation by LTA.

The major advantage of MEA over LTA is the elimination of the centrifugation steps to separate platelets. This means that the test is more rapid and would need less standardization compared with LTA. Moreover, as a whole blood method, MEA has the advantage that platelets are measured in their physiological milieu. Also keeping in mind that there is no single test for the correct and absolute diagnosis of platelet function disorder cannot be performed alone by aggregation studies and would require tools such as flow cytometry and molecular diagnosis.

In conclusion, MEA compares well with LTA in the detection of GT, and has some important advantages over which would be of importance in the standardized measurement of platelet function.
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LTA, light transmission aggregation; Neg, negative; Pos, positive; NR, no response; AR, abnormal response.
REFERENCES


