RAPID FLOW CYTOMETRIC TEST USING EOSIN-5-MALEIMIDE FOR DIAGNOSIS OF RED BLOOD CELL MEMBRANE DISORDERS

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Abstract. Conventional diagnosis of hereditary red blood cell (RBC) membrane disorders, in particular hereditary spherocytosis (HS), is labor intensive, time consuming and requires at least 2 ml of blood, which might be impractical in neonatal period. We evaluated the use of eosin-5-maleimide (EMA), a dye that reacts covalently with lysine-430 on the first extracellular loop of band 3 protein, for rapid screening test of patients with HS and Southeast Asian Ovalocytosis (SAO). Fresh RBCs from 142 healthy controls, 50 HS, 17 SAO, 29 hereditary elliptocytosis, 5 autoimmune hemolytic anemia, 66 patients with β-thalassemia/HbE, 31 cases with α-thalassemia (HbH disease) and 4 cases with pyruvate kinase deficiency were stained with EMA, and analyzed for their mean channel fluorescence (MCF) using a flow cytometer. RBCs from patients with HS and SAO expressed a greater degree of reduction in MCF compared to those from normal controls and other hemolytic diseases. These findings showed that the fluorescence flow cytometric-based method is a simple, sensitive and reliable diagnostic test for RBC membrane disorders using a small volume of blood, and results could be obtained within 2 hours. Such method could serve as a first line screening for the diagnosis of HS and SAO in routine hematology before further specific membrane protein electrophoresis and molecular diagnosis are employed.

INTRODUCTION

Red cell membrane composes of several protein structural components, including α- and β-spectrin, ankyrin, band 3, protein 4.1, protein 4.2 and actin. This multiprotein complex interacts with the lipid bilayer to play a critical role in maintaining membrane cohesion and mechanical stability of the erythrocyte (An and Mohandas, 2008). Genetic disorders of various membrane and skeletal proteins that result in either decreased membrane cohesion or membrane mechanical stability lead to membrane surface area loss, decreased red cell life span and resultant anemia in a variety of inherited red cell membrane disorders, such as hereditary spherocytosis (HS), hereditary elliptocytosis (HE), stomatocytosis, pyropoikilocytosis and Southeast Asian ovalocytosis (SAO) (An and Mohandas, 2008).

Diagnosis of hereditary red blood cell membrane disorders, in HS, is based on clinical features, family history, peripheral smear examination and laboratory investigations (Bolton-Maggs *et al*, 2004), such as osmotic fragility from the fresh patient's red blood

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cells (Parpart *et al*, 1947) and autohemolysis test (Selwyn and Dacie, 1954). These methods require at least 2 ml of blood, and are time and labor consuming in order to prepare reagents and equipments for each patient and control. These tests have poor specificity and sensitivity so that milder and atypical cases of HS are often missed (Korones and Pearson, 1989). The confirmatory diagnosis requires sodium dodecyl sulfate polyacrylamide gel-electrophoresis (SDS-PAGE) analysis of red cell membrane proteins to verify the specific protein deficiency (Fairbanks *et al*, 1971). However, this test is not appropriate for routine service.

A flow cytometric-based analysis by measuring fluorescence intensity of red cells labeled with eosin-5-maleimide (EMA) dye, which reacts covalently with lysine-430 on the first extracellular loop of band 3 protein has been developed (King *et al*, 2000). The N-terminal cytoplasmic domain of band 3 interacts with ankyrin and protein 4.2, which interact with the spectrin-based cytoskeleton, and stabilizes the membrane lipid bilayer (Golan *et al*, 1996). Absent or decreased expression of red blood cell membrane proteins found in HS causes a disruption of the cytoskeleton network and reduces normal expression of band 3 protein at the erythrocyte membrane (Mohandas and Chasis, 1993). This results in a reduced binding of EMA to band 3 protein and its fluorescence emission (King *et al*, 2000; Kedar *et al*, 2003; Stoya *et al*, 2006). This study reports the application of this technique in several hemolytic diseases that are prevalent in Thailand including, hereditary elliptocytosis (HE), autoimmune hemolytic anemia (AIHA) commonly presented as microspherocyte in blood smear, thalassemia, pyruvate kinase (PK) deficiency and Southeast Asian ovalocytosis (SAO) (caused by a deletion of amino acids 400-408 located at the N-terminal cytoplasmic domain of band 3 protein (Schofield *et al*, 1992).

MATERIALS AND METHODS

Blood samples

One hundred fourty-two healthy subjects with normal hematological parameters and red cell morphology were investigated as normal control (age ranging 1-60 years). Fifty HS patients were diagnosed based on clinical features (anemia, jaundice and splenomegaly), family history, spherocytes in blood smear, increased autohemolysis and increased osmotic fragility (age ranging 2 weeks - 36 years). Seventeen SAO patients were diagnosed based on peripheral smear examination with stomatocytic ovalocytes and by molecular diagnosis band 3 mutation (Vasuvattakul *et al*, 1999) (age ranging 4 days - 39 years). Twenty-nine HE patients were diagnosed with peripheral blood smears showing prominent elliptocytocytes which exceeded 30% of the red cells (age ranging 1-39 years). There were 5 patients of AIHA with a positive Coombs' reaction (age ranging 2 months - 19 years) and 66 β-thalassemia/HbE subjects (age ranging 2-18 years) and 31 α-thalassemia patients (age ranging 2-17 years) diagnosed based on clinical features, complete blood count and hemoglobin typing. Four patients with PK deficiency (age ranging 1-27 years) were diagnosed based on PK activity. All patients did not have any blood transfusion 3 months prior to their red cell analyses. All blood samples were anticoagulated with EDTA and kept at 4°C. Flow cytometric analysis of intact red cells was performed within 24 hours after blood collection. This study was approved by a local ethics committee at Siriraj Hospital, Thailand.

Fluorescence labeling and flow cytometric analysis of red cells

The method described by King *et al* (2000) was followed. In brief, red blood cells

were washed twice with phosphate buffered saline (PBS), pH 7.4 and 5 µl of packed red blood cells were incubated with 25 µl of EMA (0.5 mg/ml PBS; Fluka, Gillingham, UK) for 1 hour at room temperature in the dark. The cell suspension was centrifuged for 5-10 seconds in a microcentrifuge and the supernatant (containing unbound dye) was removed. The labeled red cells were washed 3 times with 500 µl of PBS-bovine serum albumin (BSA) solution (0.5% BSA in PBS). Red blood cells were suspended in 0.5 ml of PBS-BSA solution and 100 µl aliquot of the cell suspension was added to 1.5 ml of PBS-BSA solution for analysis. Fluorescence intensity, as mean channel fluorescence (MCF), was determined for 15000 events in FL1 channel of Becton Dickinson FACS calibur flow cytometer (Becton Dickinson, San Jose, CA).

Statistical analysis

The mean, standard deviation (SD), minimum value and maximum value were calculated using program SPSS for Windows®, version 15.0. Variance (mean ± SD) was compared using two-tailed Student's *t*-test. A p-value < 0.05 is considered statistically significant. A receiver operator characteristic (ROC) curve was used to determine an optimum cutoff point for discriminating between normal control and HS or SAO patients.

RESULTS

Fluorescence histograms of EMA-labeled red cells from normal and patients with SAO and HS are presented in Fig 1. Fluorescence intensity was determined as mean channel fluorescence (MCF) in FL1 channel. Labeled red cells from both patient groups showed less fluorescence intensity than those of normal control.

The demographic data of normal controls and all patient groups are presented in Table 1. MCF values of red blood cells from patients with HS (n = 50), SAO (n = 17) and HE (n = 29) are significantly lower (72.6 ± 11.2, range 46.0-91.4; 79.3 ± 6.0, range 64.9-87.0 and 108.1 ± 7.0, range 91.6-125.4, respectively) than those of normal controls (114.8 ± 7.7, range 99.2-139.6) (p < 0.001), whereas those of red cells from patients with AIHA (n = 5), β-thalassemia/HbE disease (n = 66), α-thalassemia disease (n = 31) and PK deficiency (n = 4) (119.5 ± 18.7, range 101.3-149.4; 115.8 ± 18.0, range 91.2-191.9; 116.9 ± 11.4, range 98.3-142.9 and 120.9 ± 28.9, range 104.7-164.3, respectively) are not significantly different from those of normal group (Table 1). MCF values of red cells from these latter patient groups are significantly higher than those from HS and SAO groups (p < 0.05). When compared with HE red cells,

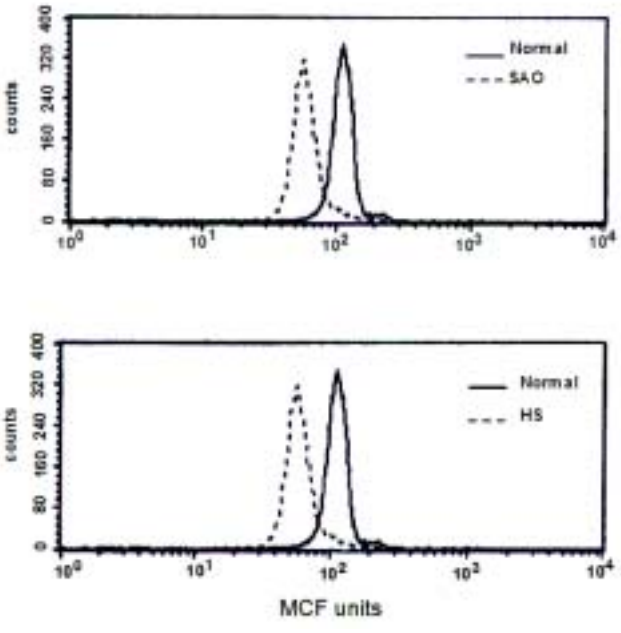

Fig 1–Fluorescence histogram of EMA-labeled red cells from normal and patients with SAO and HS. Red blood cells were stained with EMA dye and measured as MCF using a flow cytometer.

Table 1
Demographic data and flow cytometric analysis of eosin-5-maleimide (EMA) labeled red cells of normal and patient groups.

Group	Case (No.)	Age (yr) (Mean ± SD)	Age (yr) (Range)	Male (%)	MCF unit (Mean ± SD)	MCF unit (Range)	*p*-value
Normal	142	21.4 ± 13.2	1-60	44 (44.9)	114.8 ± 7.7	99.2 - 139.6	
HS	50	11.2 ± 9.3	0-36	25 (50.0)	72.6 ± 11.2	46.0 - 91.4	<0.001
SAO	17	7.4 ± 10.2	0-39	9 (52.9)	79.3 ± 6.0	64.9 - 87.0	<0.001
HE	29	16.2 ± 12.6	1-36	14 (48.0)	108.1 ± 7.0	91.6 - 125.4	<0.001
AIHA	5	11.0 ± 9.7	0.2-19	1 (20.0)	119.5 ± 18.7	101.3 - 149.4	0.600
β-thalassemia/HbE disease	66	9.8 ± 4.2	2-18	29 (43.9)	115.8 ± 18.0	91.2 - 191.9	0.680
α-thalassemia disease	31	8.6 ± 4.7	2-17	17 (54.8)	116.9 ± 11.4	98.3 - 142.9	0.331
PK deficiency	4	8.0 ± 12.6	1-27	1 (25.0)	120.9 ± 28.9	104.7 - 164.3	0.699

HS, hereditary spherocytosis; SAO, Southeast Asian ovalocytosis; HE, hereditary elliptocytosis; AIHA, autoimmune hemolytic anemia; PK, pyruvate kinase; MCF, mean channel fluorescence

MCF readings did not differ in AIHA and PK deficiency but are significantly higher in β-thalassemia/HbE disease and α-thalassemia disease (p=0.004 and 0.001, respectively).

The optimum cutoff point that differentiated HS or SAO patients from normal controls and other hemolytic diseases, identified from ROC curve, was 91.5 MCF units (Fig 2). A positive result for HS or SAO was identified when MCF of labeled red cell was lower than 91.5 units. By using this cutoff point, the fluorescence dye binding method had a sensitivity of 100% and specificity of 99.6%.

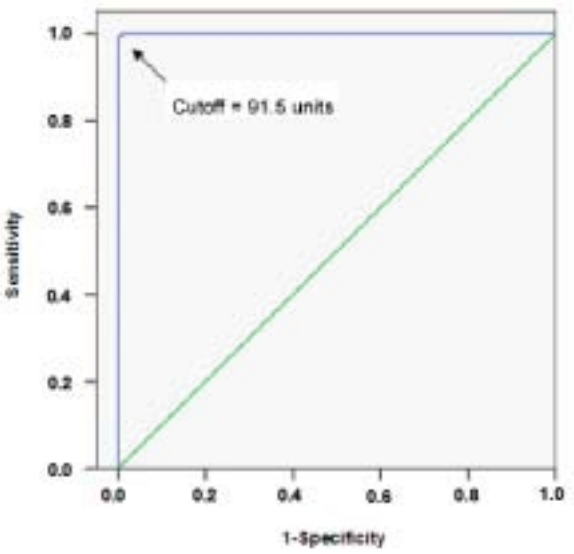

Fig 2–ROC curve for determination of optimum cutoff point of fluorescence method for diagnosis HS and SAO. Cutoff point for discrimination was 91.5 MCF units. The area under ROC curve = 0.99.

DISCUSSION

Diagnosis of HS is generally based on peripheral smear examination for the presence of spherocytes in a patient with anemia, splenomegaly and hyperbilirubinemia, increased osmotic fragility test and increased autohemolysis test (Bolton-Maggs *et al*, 2004). However these methods are time consuming and labor intensive and have poor specificity and sensitivity, resulting in milder and atypical cases of HS often being missed (Korones and Pearson, 1989). A flow cytometric based analysis by measuring EMA fluorescence intensity of labeled red

cells has been developed (King *et al*, 2000). Our results showing a decrease in MCF of HS red cells compared with normal red cells were in agreement with other studies (King *et al*, 2000; Kedar *et al*, 2003; Stoya *et al*, 2006; Girodon *et al*, 2008). This method does not only indicate band 3 deficiency but also spectrin and protein 4.2 deficiency (King *et al*, 2000). The reduction of fluorescence intensity in HS red cells in this study might be due to the loss of membrane surface proteins including band 3 and integral membrane proteins (Rh-associated glycoprotein, Rh protein and CD47). These membrane proteins are present at a lower level in HS than normal red cells (King *et al*, 2004).

The clinical presentation and blood picture of SAO are distinct from those of HS. SAO patients are characterized by the presence of oval-shaped red cells with one or two transverse ridges or a longitudinal slit on blood smears, and clinical manifestations are asymptomatic (Palek and Jarolim, 1993). SAO is caused by a genomic deletion of 27 bp encoding amino acids 400-408 located at the boundary of the cytoplasmic and first transmembrane domain of band 3 (Schofield *et al*, 1992). This leads to a marked increase in membrane rigidity but does not cause band 3 or other membrane protein deficiency (Jarolim *et al*, 1991). From our results, all SAO individuals were confirmed by PCR technique (data not shown) and SAO red cells produced MCF readings similar to those of HS red cells. These findings are in agreement with previous studies (King *et al*, 2000). This reduction of fluorescence intensity may be due to increased band 3 self-association resulting in impaired interaction of EMA with Lysine-430 (Liu *et al*, 1995).

The MCF of HE red cells were slightly lower than those of normal red cells but higher than HS and SAO red cells. Our results are in contrast to those of other investigators (King *et al*, 2000) who found no significant difference in MCF of red cells between common HE and normal control. These discrepancies might be a result of examining populations from different genetic backgrounds. The decrease in MCF of HE red cells might be due to defect in spectrin, which is found in a majority of HE patients (Palek and Jarolim, 1993). MCF readings of HE red cells did not differ from those of AIHA and PK deficient red cells but were lower than β-and α-thalassemia red cells. However, these results were only from 5 subjects of AIHA and 4 subjects of PK deficiency.

By using flow cytometric method at a cutoff of 91.5 MCF units, we could differentiate HS and SAO patients from normal and other hemolytic diseases such as HE, AIHA, β-thalassemia/HbE, α-thalassemia and PK deficiency. Previous reports using this method had shown that the optimum cutoff point for discrimination of HS was variable. King *et al* (2000) reported a cutoff point at 45.5 MCF units and Stoya *et al* (2006) reported a cutoff point at 400 MCF units. It is important that each laboratory sets its own cutoff value.

In summary, this EMA fluorescence dye-based method is a sensitive, reliable and speedy diagnostic test that can be performed in less than 2 hours. It was shown to be very specific for red blood cell membrane proteins found in HS and SAO and helped to distinguish these two conditions from other hemolytic diseases which also might have microspherocytes or ovalocytes. We propose that this assay will serve as a first line screening test for diagnosis of HS and SAO in routine hematology before conducting further specific membrane protein electrophoresis and molecular tests.

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