Usefulness of brilliant cresyl blue staining as an auxiliary method of screening for α-thalassemia

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Hemoglobin (Hb) electrophoresis is widely used in thalassemia screening. Most Hb variants express a specific abnormal band on the cellulose acetate membrane. The technique is useful in the diagnosis of the type of thalassemia but is not sensitive enough to detect α-thalassemia minor because the quantity of the HbH is too small to be expressed on the supporting medium. We used simple staining of blood smears rather than the sophisticated molecular method to detect HbH inclusions. To evaluate the effectiveness of this method, we used brilliant cresyl blue (BCB) staining of red blood cells in 509 patients with microcytosis and erythrocytosis caused by various conditions. The results indicate that BCB staining is useful in the identification of subjects who possess α-thalassemia traits. Coexisting conditions such as β-thalassemia and iron-deficiency anemia did not affect the detection of the HbH inclusions with the use of BCB staining. We conclude that BCB staining is helpful and reliable as an auxiliary method of detecting HbH inclusions in the diagnosis of α-thalassemia traits, especially in places where medical resources are limited. (J Lab Clin Med 2005;145:94–7)

Abbreviations: BCB = brilliant cresyl blue; CBC = complete blood counts; EDTA = ethylenediaminetetraacetic acid; Hb = hemoglobin; HbA = hemoglobin A; HbA_2 = hemoglobin A_2; HbE_1 = hemoglobin E_1; HbF = hemoglobin F; HbG = hemoglobin G; HbH = hemoglobin H; IDA = iron-deficiency anemia; MCV = mean corpuscular volume; RBC = red blood cell; RDW = red cell–distribution width; RDW-SD = SD of RDW

α-Thalassemia is a common genetic disease that is usually caused by the deletion of 1 or more α-globin genes. α-Thalassemia trait that occurs on the deletion of 1 or 2 of 4 α-globin genes is characterized by a lower ratio of α- to β-globin synthesis and results in thalassemia minor by hematologic examination.[1–3] However, it is not unusual for us to encounter "thalassemia-like" RBC indexes with normal hemoglobin fractions on electrophoresis in our medical practice. Traditional screening protocols for thalassemia involve the differentiation of various disorders that impair hemoglobin synthesis. These disorders are characterized by a reduction in the hemoglobin content of erythrocytes and are manifested in laboratory data as a reduction in RBC indexes.[1,2,4] Reduced indexes may be caused by defects in the synthesis of heme, usually resulting from iron deficiency[5]; in the utilization of iron, as in lead poisoning or in globin synthesis, as in α- and β-thalassemia.

For patients with α-thalassemia, disturbed globin production results in a relative excess of β-globin polypeptide chains. The excess β-chain can polymerize to form soluble β4 tetramers – HbH.[1–3,6] Diagnosis of α-thalassemia with the use of molecular analysis is a common practice in places where molecular confirmation is available and affordable. However, in places where medical resources are limited and molecular analysis is not available or is expensive, a simple, easy, and cheap method of screening and diagnosing

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α-thalassemia is important. After supravital staining of RBCs with the redox dye BCB, HbH, being unstable, precipitates and aggregates to form intraerythrocytic inclusions. HbH inclusions occur in approximately 1 of 10,000 RBCs in individuals with α-thalassemia minor, the result of 2 gene deletions on the same chromosome, α-thalassemia-1, which is common in Southeast Asians.[1,6–9] The number of cells with HbH inclusions is influenced by the α-thalassemia genotype, and the number of cells with HbH inclusions increases in syndromes with a greater β-globin excess.[6,7] Because this method of HbH detection is easy to conduct, cheap, and easy to interpret by an experienced and trained laboratory staff, it can be used as a screening procedure for α-thalassemia, especially in areas where the molecular diagnosis of α-thalassemia is not possible. In this study, we evaluated the reliability of BCB inclusion-body determinations for the detection of α-thalassemia in 509 patients with microcytosis resulting from various conditions.

METHODS

A total of 764 venous blood samples taken from patients with microcytic anemia were submitted to the hematologic section of our clinical laboratory for thalassemia screening. Our research was carried out in accordance with the principles of the Declaration of Helsinki, informed consent was obtained, and the institutional review board of Chang Gung Memorial Hospital approved the study.

MCV was less than 70 fL in all cases, and the mean age of the patients was greater than 12 years. Hb electrophoresis, CBC, serum ferritin and iron concentrations, and total iron-binding capacity were determined. BCB staining was performed on all blood samples. Of the 764 patients, only 509 for whom complete data were available were included in the study. Patients were assigned to 1 of several groups on the basis of the results of Hb electrophoresis, serum ferritin and iron concentrations, and total iron-binding capacity. Individuals found to have the α-thalassemia trait had mild microcytic hypochromic anemia with MCVs of less than 70 fL, low RDW-SD, normal hemoglobin fractions on electrophoresis ($HbA > 96.5\%$, $HbA_2 < 3.5\%$, HbF 0%), and no evidence of iron deficiency. Patients with HbH disease are usually more anemic, with abnormal HbH fractions on electrophoresis. We also used 63 venous-blood samples with normal CBC findings as controls.

We performed Hb electrophoresis using a cellulose acetate membrane at a pH of 8.2 to 8.6. Hemolysate was prepared with the use of commercial hemolysin (hemolysate reagent containing 0.005 mol/L EDTA [catalog no. 5127; Helena Laboratory, Beaumont, Texas]). Electrophoresis was carried out on cellulose acetate palate (Titan III-H; Helena Laboratory) with the use of a superheme buffer (Tris–EDTA–boric acid, pH 8.2–8.6) at 350 V for 20 minutes. After staining with Ponceau S, we visualized hemoglobin fractions H, A, F, and A_2 as different bands and quantified them with the use of an Appraise densitometer (Beckman Instruments, Fullerton, Calif).

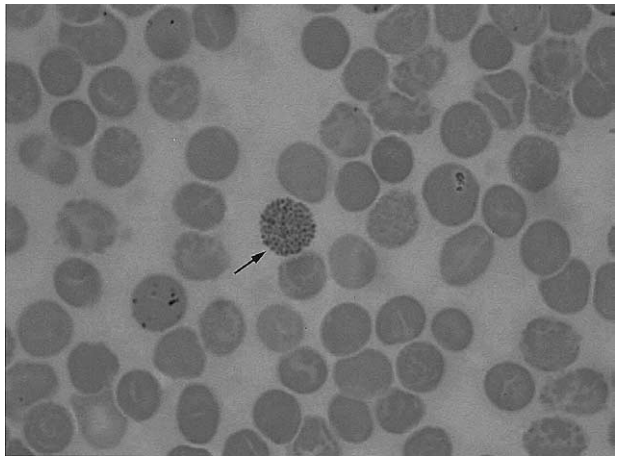

Fig 1. RBCs containing HbH inclusions on a BCB-stained smear in a patient with α-thalassemia trait.

CBC, including MCV and RDW, was determined with the use of a Sysmex NE-8000 automated cell counter (TOA Co, Kobe, Japan) with standard calibration. The RDW-SD is taken at the 20% relation height level, with the histogram peak considered 100%. The RBC histograms of normal blood samples cross this 20% level twice. The distance between the 2 crossed points defined as RDW-SD is reported in femtoliters.

We prepared a 1% BCB-staining solution by dissolving 1.0 g of BCB (Sigma-Aldrich, St Louis, Mo) in 100 mL of 0.85% normal saline solution. K3-EDTA blood and 1% BCB-staining solution were added to a 12 × 75-mm plastic Kohn tube at a ratio of 1:1. We next sealed the mixture with paraffin and incubated it in a 37° water bath for 3 hours, then prepared a BCB smear and observed 10,000 RBCs for HbH inclusions. In the case of a negative result, 50,000 RBCs were observed. Inclusions in a typical preparation are shown in Fig 1.

RESULTS

We observed numerous cells containing HbH inclusions in the BCB-stained smears of all 25 patients with HbH disease (of the total of 509 patients included in this study; Table I). Our most important observation: All 180 patients with the α-thalassemia trait had RBCs containing HbH inclusions. The number of HbH inclusions varied from 0.1 to 30 per 1000 RBCs. Among 304 patients without the α-thalassemia trait, 25 (8.2%) were found to have inclusion-containing cells, including 23 patients with IDA) and 2 with β-thalassemia complicated by IDA. The BCB-stained smears of all patients with δβ-thalassemia, hereditary persistence of fetal Hb, and other hemoglobinopathies, as well as 63 normal controls, consistently showed negative findings. The sensitivity of BCB staining in the detection of the