Journal of Clinical Laboratory Analysis 25 : 223–228 (2011)

Erythrocyte and Reticulocyte Parameters in Iron Deficiency and Thalassemia

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Introduction: Red blood cells (RBCs) extended parameters or erythrocyte subsets are now reported by the new Sysmex XE 5000 analyzer. This study was aimed at establishing a characteristic analytical feature, including the new erythrocyte and reticulocyte parameters, in case of thalassemia trait and iron deficiency (IDA). *Methods*: Ninety healthy individuals, 136 β-thalassemia carriers, 121 mild IDA, and 126 severe IDA patients were analyzed. *Results*: The values obtained for the RBC extended parameters were significantly different ($P < 0.0001$) in the groups; the only exception was %Hypo-He in the case of mild IDA and thalassemia ($P = 0.6226$). %Hypo-He was considerably greater in severe IDA (23.4%) than in mild cases (12.4%), $P < 0.0001$. %MicroR was more increased in thalassemia (38.6 %) than in the mild IDA (16.5%, $P < 0.001$) and in severe IDA (21.6%, $P < 0.001$). Immature reticulocyte fraction (IRF) mean values in the groups were statistically different; the thalassemia group had an intermediate value (8.7%) between healthy (4.4%) and IDA (16.7 and 12.9%). *Conclusions*: Erythrocytosis and severe microcytosis, together with a high percentage of microcytes and a moderate increase in IRF, is the profile of β-thalassemia carriers, whereas anisocytosis and the hypochromic subset correlates with the severity of the anemia in iron-deficient patients. J. Clin. Lab. Anal. 25:223–228, 2011.

Key words: erythrocytes; microcytic anemia; reticulocytes

INTRODUCTION

Microcytic anemia is frequently owing to iron deficiency (IDA) or thalassemia. The differentiation between thalassemic and nonthalassemic microcytosis has important clinical implications (1).

Iron deficiency is one of the leading risk factors for disability and death worldwide, it may result from insufficient iron intake or menstrual loss in women of childbearing age, or chronic blood loss in the gastrointestinal tract in the case of elderly subjects (2).

Microcytic anemia in the case of thalassemia results from impaired globin chain synthesis and decreased hemoglobin (Hb) synthesis (3).

Thalassemia syndromes are among the most common genetic disorders worldwide, with 1.7% of the world's population carrying thalassemic genes (4). Thalassemia is prevalent in some parts of the world where it represents a major public health problem. However, nonendemic countries, such as Northern Europe and North America are also involved in thalassemia-related problems, as a result of demographic changes caused by migration of ethnic minority groups with a high frequency of thalassemic mutations (5,6).

From a practical point of view, it may be useful to make an "at first sight" diagnosis using simple parameters that may be readily obtained from the hemogram.

On the basis of classical hematological parameters subjects with IDA are inappropriately discriminated from subjects with anemia owing to thalassemia or chronic disease (7–9). Iron deficiency develops in sequential changes over a period of negative iron balance. These stages include the iron depletion phase, iron-deficient erythropoiesis, and finally IDA. As the state of iron deficiency proceeds, mean cell volume (MCV), mean cell hemoglobin (MCH), and red blood

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Received 20 December 2010; Accepted 2 March 2011
DOI 10.1002/jcla.20462
Published online in Wiley Online Library (wileyonlinelibrary.com).

cell (RBC) tend to decline, but results in both microcytic anemias overlap.

Automated blood cell counters have changed deeply over the last 20 years and modern hematology analyzers, based on principles of flow cytometry, can provide information about individual cell characteristics.

The quantification of the percentages of microcytic and hypochromic RBCs has proved its clinical usefulness in the differential diagnosis of microcytic anemia (10–14); the measurement of the RBC subpopulations has been restricted to the analyzers of a single manufacturer, ADVIA series Siemens (Siemens Medical Solutions Diagnostics, Tarrytown, NY)

Sysmex XE 2100 analyzer (Sysmex Corporation, Kobe, Japan) is a fully automated hematological analyzer (15). Erythrocyte hemoglobin equivalent (RBC He) and reticulocyte hemoglobin equivalent (Ret He) are parameters reported by this instrument. The role of Ret He and RBC He in thalassemia detection has been evaluated (16–18).

The new analyzer, Sysmex XE 5000 (Sysmex Corporation) by means of the flow fluorescence cytometry technology, enables independent measurement of the volume and Hb content of individual red cells. Derived from this technology, four new RBCs extended parameters (erythrocyte subsets) are now reported by this analyzer.

The reference range of these new RBC extended parameters and their values in anemic patients with red cells abnormalities have been established (19).

IDA anemia and thalassemia have radically different pathophysiology etiologies; so the question is whether these new research parameters could be useful in the diagnosis of both anemias.

The aim of this study was to investigate the erythrocyte and reticulocyte parameters acquired by the Sysmex XE 5000 to assess whether a characteristic profile for these anemias, based on the different analytical results, could be defined.

MATERIALS AND METHODS

Analytical Methods

The Sysmex XE 5000 is a recently introduced fully automated blood cell counter. Measurement of RBC and platelets employs the Sheath Flow DC detection method: the sample flow is focused on the center of the aperture. The precise size distributions of RBC and platelets can then be obtained by changing the generated electrical resistance into pulses, which provides highly precise results.

The percentages of erythrocyte subsets can be calculated and the new parameters %Micro R and %Macro R obtained.

%Micro R indicates the percentage of microcytic red cells with a volume less than 60 fl.

%Macro R indicates the percentage of macrocytic red cells with a volume greater than 120 fl.

In the reticulocyte channel, blood cells are stained by a polymethine dye specific for RNA/DNA, and analyzed by flow cytometry using a semiconductor laser. A bidimensional distribution of forward scattered light and fluorescence is presented as a scattergram, indicating mature red cells and reticulocytes.

Forward scatter correlates with Ret He and RBC He.

A new algorithm calculates the percentages of mature red cells subsets and the new parameters %Hypo He and %Hyper He obtained.

%Hypo He indicates the percentage of hypochromic red cells with an Hb content equivalent to less than 17 pg.

%Hyper He indicates the percentage of hyperchromic red cells with an Hb content equivalent to more than 49 pg.

Fluorescence intensity gives information on RNA/DNA content and, therefore, maturity of the cells. The percentage of reticulocytes with high signals can be obtained and the fraction of immature reticulocytes (IRF) calculated.

Criteria for Selecting the Groups of Patients

Only adults were included in this study, none of them received a transfusion, nor had an acute bleeding in the previous month.

The samples were obtained in the course of routine analysis and collected in EDTA anticoagulant tubes (Vacutainer^{TM} Becton-Dickinson, Rutherford, NJ), and were run in the Sysmex XE 5000 analyzer within 6 hr of collection.

Healthy subjects: samples from healthy adult subjects, with no clinical symptoms of disease. Blood cell counts and biochemical iron tests results were within the reference ranges.

The patients of the IDA group had Hb <120 g/l, serum iron <7.5 μmol/l, Transferrin saturation $<20\%$, serum ferritin <50 μg/l.

The group was divided into mild IDA (Hb ranged 100–120 g/l) and severe IDA (Hb <100 g/l).

β Thalassemia carriers: samples were extracted from patients with a previous diagnosis of the disease.

β thalassemia diagnosis is routinely performed in our Laboratory by means of the measure of their RBC indices and the level of HbA_2. Molecular characterization of mutations is performed with Allele specific oligonucleotide–polymerase chain reaction, PCR–ASO techniques (20,21).

Samples with erythrocytosis (RBC $> 5.5 \times 10^{12}$/l) and microcytosis (MCV <80 fl) are selected for HbA_2

TABLE 1. Hematological and Biochemical Data of the Healthy Group ($n = 90$), β Thalassemia Carriers ($n = 138$), Mild IDA ($n = 121$), and Severe IDA Patients ($n = 126$)

	Healthy mean (SD)	Thalassemia mean (SD)	Mild IDA mean (SD)	Severe IDA mean (SD)
RBC,10^{12}/L	4.95 (0.37)	5.80 (0.53)	4.81 (0.47)	4.17 (0.58)
Hb, g/L	151 (9)	119 (11.3)	110 (7.5)	90 (9.1)
MCV, fL	90.9 (2.9)	64.6 (3.4)	75.3 (4.8)	73.2 (6.7)
MCH, pg	30.5 (0.9)	20.7 (1.1)	21.5 (1.8)	21.7 (2.7)
MCHC, g/L	335 (9)	319 (6.9)	306 (10)	296 (14)
RDW, %	13.1 (0.6)	15.7 (1.0)	17.7 (2.5)	17.7 (2.3)
Ret, 10^9/L	47.8 (14.8)	77.2 (26.1)	53.4 (23.6)	54.9 (27.3)
Reticulocyte, %	0.9 (2.2)	13.4 (4.7)	11.1 (4.7)	13.6 (8)
Ret He, pg	33.7 (1.4)	22.1 (1.6)	25.3 (2.9)	22.3 (3.7)
RBC He, pg	30.9 (1.2)	21.1 (1.3)	23.2 (2.1)	21.5 (3.3)
IRF, %	4.4 (3.3)	8.7 (5)	12.9 (5.8)	16.7 (6.2)
%MicroR	1.1 (0.44)	38.6 (10.9)	16.5 (9.3)	21.6 (13.2)
%Hypo-He	0.3 (0.16)	13.2 (9.9)	12.4 (9.6)	23.4 (16.5)
Iron, μmol/L	17.1 (2.3)	18.0 (0.7)	5.7 (3.7)	3.9 (1.7)
Transf, g/L	2.46 (0.3)	2.41 (3.1)	2.49 (0.6)	3.19 (0.63)
Ferritin, μg/L	103 (54)	116 (97)	17 (22)	13 (15)
Sat, %	28 (5.9)	29 (10.9)	5 (3)	3 (5.5)

SD, standard deviation; RBC, red blood cells; Hb, hemoglobin; MCV, mean cell volume; MCH, mean cell hemoglobin; MCHC, mean cell hemoglobin concentration; RDW, red cell distribution width; Ret, reticulocyte; Ret He, reticulocyte hemoglobin equivalent; RBC He, erythrocyte hemoglobin equivalent; IRF, immature reticulocyte fraction; %MicroR, percentage of microcytic red cells; %Hypo-He, percentage of hypochromic red cells; Transf, transferrin; Sat, % transferrin saturation.

quantification (HPLC HA 8160, Menarini Diagnostics, Firenze, Italy). Increased HbA_2 ($>3.5\%$) is considered to be confirmatory for β thalassemia trait. Molecular analysis is performed if genetic counseling is required.

Biochemical and hematological data of the groups of patients are summarized in Table 1.

Statistical Evaluation of Analytical Results

Statistical software package SPSS (SPSS; Chicago, IL) version 17.0 for Windows was applied for the statistical analysis of the results.

t-Test for independent samples and variance analyses (ANOVA) was performed for the mean comparison between the studied groups; P values less than 0.05 were considered to be statistically significant.

Receiver operating characteristic (ROC) analysis was utilized to illustrate the diagnostic performance for thalassemia screening of the parameters studied.

RESULTS

Ninety healthy adults (45 male and 45 female), 136 β thalassemia carriers, and 247 IDA patients (121 mild and 126 severe) were studied.

Although the differences between the three groups of anemic patients were statistically significant ($P<0.001$), the central 95th percentile ranges showed considerable overlap for Hb, MCV, MCH, MCHC, red cell distribution width (RDW), and absolute reticulocyte count.

The exception were the reticulocyte count expressed in percentages, Ret He, and RBC He. Percent reticulocytes for β thalassemia and severe IDA groups presented no difference ($P = 0.7934$). Ret He and RBC He correlated with the degree of anemia in the IDA groups with no differences between thalassemia carriers and severe IDA ($P = 0.5647$ and $P = 0.1904$, respectively).

The differences of IRF mean values in the four groups were statistically significant; thalassemia had an intermediate value (8.7%) between healthy (4.4%) and IDA anemia (16.7 and 12.9%).

In β thalassemia trait, the RBC count was statistically higher (5.8×10^{12}/l) than in healthy subjects (4.95×10^{12}/l, $P<0.001$), mild IDA patients (4.81×10^{12}/l, $P<0.001$), and severe IDA patients (4.11×10^{12}/l), whereas MCV and MCH values were lower ($P<0.001$).

RDW was increased in anemic patients with respect to the healthy subjects, higher in IDA (17.7%) than β thalassemia (15.7%, $P<0.001$).

The values obtained for the new RBC extended parameters proved to be significantly different ($P<0.0001$) in the different groups; the only exception was %Hypo-He in the case of mild IDA and thalassemia ($P = 0.6226$).

%Hypo-He was considerably greater in severe IDA (23.4%) than in mild cases (12.4%), $P<0.0001$.

%MicroR was more increased in thalassemia (38.6%) than in mild IDA (16.5%, $P<0.001$) and in severe IDA (21.6%, $P<0.001$).

This parameter is the most efficient single measurement (AUC 0.938, 95% CI 0.903–0.964) in the differential diagnosis of microcytic anemia. %MicroR >20% discriminates β thalassemia trait from mild IDA with 93.7% sensitivity and 75.4% specificity. RBC ranked second AUC 0.917 (95% CI 0.878–0.947) cut-off 5.1, with 93.7% sensitivity and 72.5% specificity.

DISCUSSION

Differentiation between thalassemic and nonthalassemic microcytosis has important clinical implications, because each has an entirely different cause, pathogenesis, prognosis, and treatment. An appropriate thalassemia screening, detection of patients, and counseling of couples at risk are the most important procedures for the reduction of morbidity and mortality of the patients (22).

β Thalassemia is characterized by an increase in RBC, as a result of the chronic increase in erythropoiesis. RBC count has been recognized as the most efficient single classical measurement in the differential diagnosis of microcytic anemia, whereas for a certain degree of anemia, MCV and MCH tend to be lower in β thalassemia than in IDA (23).

Another significant difference between these two types of anemia is the level of anisocytosis (24,25).

In iron deficiency states, RBCs are continuously produced in the bone marrow, the iron stores progressively decrease, and they tend to be more microcytic. Because of their long life span, several cohorts of normocytic and microcytic RBCs coexist in the peripheral blood leading to anisocytosis.

On the contrary, the underlying pathogenetic anomaly in β thalassemia has no fluctuations, and as a result, the bone marrow produces a constantly uniform population of microcytic erythrocytes (6).

The measurement of microcytic and hypochromic red cells shows different results in patients with uncomplicated β thalassemia and IDA.

Iron-deficient erythropoiesis is characterized by the production of RBC with a decrease in Hb content, so a high percentage of hypochromic cells is present (26).

The increase in the %Hypo-He values runs in parallel to the severity of the anemic state (23.4% in severe IDA, 12.4% in mild cases).While the anemic state proceeds the number of microcytes increases (16.5% in mild IDA, 21.6% in severe cases).

On the other hand, owing to the impaired globin synthesis, microcytes of β thalassemia have small volume (27) and a high rate of microcytosis is present.

The results suggest that %MicroR >20% are indicative of β thalassemia trait, with 93.7% sensitivity and 72.5% specificity, as determined by ROC curve analysis.

The combination of erythrocytic and reticulocytic parameters could be useful for the understanding the different physiopathologies underlying both anemias (28).

The reticulocyte count and related parameters are clinically important for evaluating the erythropoietic activity of bone marrow and for the diagnosis of anemia. The reticulocyte count in thalassemia carriers correlated with the degree of ineffective erythropoiesis, nevertheless accelerated (29). These data are compatible with an expansion of the erythron to compensate microcytosis and hypochromia and percent reticulocyte counts are higher in the case of thalassemia and severe IDA as well.

The assessment of reticulocyte maturation might be useful for understanding the different pathophysiology of these anemias and can help in differential diagnosis (30). Iron status influences reticulocyte subsets; the erythroid expansion leads to an enhanced immature reticulocyte release from bone marrow to compensate the anemic status. The values observed (12.9% in mild IDA and 16.7% in severe IDA) reflect that the depletion of iron stores induces the elevation of IRF (31).

The moderate increase in IRF in thalassemia carriers, in spite of the chronic increase in erythropoiesis, reflects that it is severely impaired. This can be explained by the fact that erythropoietin levels in β thalassemia minor are significantly lower than in IDA with the same degree of anemia (32). A possible explanation could be that adjustment of capillary circulation owing to life-long anemia would lead to diminished hypoxic stimulus and, consequently, to the secretion of erythropoietin concentrations lower than theoretically expected (16).

The important pathophysiological role played by the deposition of globin chains in erythroid precursors, leading to apoptosis and its correlation with ineffective erythropoiesis, has been assessed in different hemoglobinopathies and thalassemias (33–35). The IRF values observed are not proportionally increased, in spite of marrow erythroid hyperplasia, but are compatible with an ineffective erythropoiesis described by these authors.

According to the results, IRF and the percentages of microcytic and hypochromic red cells may be useful in quantifying different degrees of erythropoietic stimulation and run in parallel to the severity of anemia in iron-deficient patients.

The characteristic features associated with thalassemia, erythrocytosis, and severe mycrocytosis are accompanied in the Sysmex XE 5000 analyzer, with a high percentage of microcytes and a moderate increase of IRF. This set of hematological data reflects the severe impairment of the paradoxically accelerated erythropoiesis in this disease.

The availability of the innovative hematological parameters should provide the basis for further studies comparing the analytical profile found in other types of

anemia, to gain insight into the physiopathology underlying the disease.

A drawback of this study is the fact that only β thalassemia carriers were included. In our geographical area, no nutritional deficiency, chronic infection, or α or δβ thalassemia is present with a high prevalence; nor were patients with Anemia of Chronic Diseases included—only IDA patients were recruited.

Routine laboratory tests are critical in ensuring that physicians are provided with accurate information, so they can be provided with the best patient care. These include parameters reported by automated analyzers and microscopic revision of blood smears (36).

Automated technology and computerization have markedly changed the Hematology Laboratory and the way the results are transmitted to clinicians, with longer and more complicated reports, including new parameters generated by the modern counters.

This excess of data may actually make the comprehension of essential data for the right medical judgment more difficult. Physicians' education about the usefulness of these new parameters is the key (37,38). In addition, Laboratory reports must include not only numerical data (introducing flags to pathological results has proved to be useful (39,40)), but also complex diseases, such as anemia interpretative reports rather than numerical data alone should be reported to the clinicians.

NOMENCLATURE

AUC	= area under curve;
CBC	= complete blood count;
HPLC	= high performance liquid chromatography;
%Hypo-He	= percentage of hypochromic red cells;
IDA	= iron deficiency anemia;
IRF	= immature reticulocyte fraction;
MCH	= mean cell hemoglobin;
MCHC	= mean cell hemoglobin concentration;
MVC	= mean cell volume;
PCR	= polymerase chain reaction;
ROC	= Receiver operating characteristic;
RBC He	= erythrocyte hemoglobin content;
Ret He	= reticulocyte hemoglobin content;
%MicroR	= percentage of microcytic red cells

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