Flow cytometric reticulocyte analysis allows the evaluation of reticulocyte maturity. New reticulocyte parameters have been used in the diagnosis and management of anemias in the bone marrow transplant setting and in the monitoring of iron replacement or erythropoietin therapy. Reticulocyte numbers and maturation levels have been studied in different hemoglobinopathies and the results have been correlated with the degree of ineffective erythropoiesis. In order to verify differences in reticulocyte parameters in various types of anemias and to test the absolute number of immature reticulocytes as a possible discriminating factor among various types of anemias, reticulocyte counts were performed on 219 samples from patients with sickle cell anemia (SS) (n=62), hemoglobin S trait (n=9), Sβthalassemia (n=7), hemoglobin SC disease (n=11), βthalassemia trait (n=33) and iron deficiency anemia (n=47), and non-anemic individuals (n=50). Mean fluorescence index (MFI) was defined as representative of the degree of reticulocyte immaturity and it was evaluated as a percentage and in absolute values. Reticulocyte counts and MFI values were significantly higher in SS, Sβthalassemic and SC groups when compared to controls, but not different among the three anemia groups. Patients with hemoglobin S trait, iron deficiency anemia and βthalassemia trait showed reticulocyte parameters similar to the non-anemic group. There was no difference between the βthalassemic trait and iron deficiency anemia in relation to any parameters. MFI in absolute numbers were significantly higher in anemias that develop with the hemolytic process, although this was not evident in MFI percentage values. Our results showed that the erythroid expansion in sickle cell diseases (SS, SC and Sβthalassemia) leads to an enhanced immature reticulocyte release from bone marrow and that the phenomena is more evident by the MFI counting in absolute figures than in percentages. We concluded that the assessment of reticulocyte maturity provides interesting data about the pathophysiology and erythropoietic response in different types of anemias.
Introduction

The reticulocyte count is clinically important for evaluating the erythropoietic activity of bone marrow and for diagnosis of anemia. Flow cytometry using a fluorescent stain that binds to ribosomal RNA produces more reliable results than the manual method, and provides information about reticulocyte immaturity. The number of immature reticulocytes in peripheral blood reflects an increase in erythropoiesis and in red blood cell turnover or a hyporesponsive marrow. There has not been a standardisation in immaturity reticulocyte expression as of yet, which depends on the equipment used and how it recognises and classifies different stages of reticulocyte subpopulations.

Khuhapinant et al studied the reticulocyte count and maturation levels in various types of thalassemia and correlated the results with the degree of ineffective erythropoiesis. Results supported the concept that ineffective erythropoiesis is more pronounced in β-thalassemia than in α-thalassemia. Different to Paterakis et al, reticulocyte counts in heterozygous β-thalassemia were not higher than in normal controls. Another study has defined as immature reticulocytes the aggregation of middle and high fluorescence ratio provided by the Sysmex R 1000 (Sysmex-TOA Medical Electronics Co., Kobe, Japan). Patients with iron deficiency anemia (IDA) showed reticulocyte maturity within the normal range.

In the present study we outlined two objectives: first, to verify differences of reticulocyte parameters in various types of anemias; second, to test a new approach to immaturity of reticulocytes: mean fluorescence index in absolute number as a possible discriminating factor among different anemias.

Casuistic and Methods

Reticulocyte counts were performed on 219 samples of peripheral blood anticoagulated with EDTA. Fifty of them had normal values for all hematologic parameters and were considered as the control group.

The study included the following hematologic alterations: sickle cell anemia (SS, n= 62); hemoglobin S trait (AS, n= 9); Sβ thalassemia (Sβ thal, n= 7); hemoglobin SC disease (SC, n= 11); β thalassemia trait (β thal, n= 33); and iron deficiency anemia (IDA, n= 47). Specimens were analysed within four hours of collection.

Equipment: The automated haematology analyser Pentra 120-Retic (ABX- Horiba, France) identifies and enumerates reticulocytes based on impedance, fluoro-flow cytometry and argon-ion laser technologies. RNA content is analysed by Thiazole orange fluorescent dye. Maturation classes depending on the RNA content and the intensity of fluorescence are: low (LFR), medium (MFR) and high (HFR). A reticulocyte maturity index (MFI) is obtained by the fluorescence intensity search for the mean reticulocyte channel and represents the medium of immaturity of the reticulocyte population. In a previous study we observed that MFI values are more reproducible than HFR. Then we defined the MFI as representative of the degree immaturity, not only as a percentage but also as absolute values.

Statistical analysis: we used the Kruskal-Wallis non-parametric test for comparison among groups. When the difference was significant (p value < 0.05), groups were compared in pairs. Spearman’s correlation was used to verify the correlation between variables.

Results

The mean value and range for reticulocyte parameters are shown in Table 1. Reticulocyte counts as a percentage and as absolute values, plus MFI values were significantly higher in SS, Sβ thal and SC groups when compared to controls (p < 0.0001), but they were not different among the anemia groups. Only MRV was significantly lower in SC and Sβ thal compared to the SS group.

By comparing the sickle cell trait, β thal and IDA with control group, it is possible to observe that all of them had similar values in reticulocyte parameters, except the MRV value.
that was significantly lower in both microcytic anemias. IDA and βthal patients showed Hb levels significantly lower than normal individuals, but not enough to increase bone marrow activity represented by a rise in reticulocyte release. There was no difference between βthal and IDA in any parameter.

Immaturity of reticulocytes expressed as a MFI percentage value was not different between SS and AS, N and βthal, Sβthal and IDA, SC and IDA. However analysing this parameter as an absolute value, differences were statistically significant, showing that although the proportion of immature reticulocytes was the same in different groups, the number of newly produced reticulocytes per microliter of blood was significant higher in anemias that developed with the haemolytic process (Figure 1).

Table 2 summarises results from correlation tests. MFI count showed a significant positive correlation with HFR percentage in samples both with or without anemia. The correlation was stronger between MFI and HFR percentages. A moderate positive correlation was observed between RTC and MFI percentages; the correlation improved between RTC percentage and MFI absolute count and showed the best values when both parameters were compared in absolute counts.

A weak statistically significant correlation was noted between RTC percentage and Hg only in SS patients (r= -0.405, p= 0.0011) and IDA patients (r= -0.291, p= 0.0465).

**Discussion**

Classification of anemias as non-regenerative or regenerative may be initially assessed by a precise reticulocyte count. A non-responsive bone marrow does not release reticulocytes in sufficient numbers to compensate the degree of anemia. On the other hand, in certain disorders, such as pernicious anemia, thalassemia and sideroblastic anemia, the reticulocyte count is not proportionally increased in spite of marrow erythroid hyperplasia. This situation suggests an ineffective erythropoiesis. Before the advent of the automated reticulocyte counters, the RPI (Reticulocyte Production Index) was defined and widely

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**Table 1**

<table>
<thead>
<tr>
<th>Group</th>
<th>RTC% (x10%)</th>
<th>RTC # (x10%)</th>
<th>MFI%</th>
<th>MFI # (x10%)</th>
<th>MRV (fL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.16 (0.34-2.14)</td>
<td>57.8 (16.3-107.2)</td>
<td>13.2 (6.3-23.2)</td>
<td>6.8 (1.3-21.4)</td>
<td>104.0 (91-118)</td>
</tr>
<tr>
<td>SS</td>
<td>8.27 (1.60-22.80)</td>
<td>248.3 (42.0-487.8)</td>
<td>22.35 (10.3-38.1)</td>
<td>48.49 (8.1-185.3)</td>
<td>117 (80-144)</td>
</tr>
<tr>
<td>AS</td>
<td>0.86 (0.43-2.71)</td>
<td>42.3 (20.8-103.4)</td>
<td>16.20 (6.3-22.8)</td>
<td>7.31 (1.3-18.95)</td>
<td>106 (83-116)</td>
</tr>
<tr>
<td>βthal</td>
<td>7.53 (2.45-14.10)</td>
<td>217 (119.0-670.3)</td>
<td>10.7 (10.1-35.5)</td>
<td>22.81 (12.3-202.4)</td>
<td>88 (78-108)</td>
</tr>
<tr>
<td>SC</td>
<td>3.39 (2.03-6.66)</td>
<td>142.0 (67.9-250.1)</td>
<td>22.3 (7.9-29.0)</td>
<td>27.09 (5.4-74.5)</td>
<td>100 (78-122)</td>
</tr>
<tr>
<td>βthal</td>
<td>1.53 (0.61-4.20)</td>
<td>73.9 (33.7-228.2)</td>
<td>17.5 (10.9-30.5)</td>
<td>10.64 (2.9-60.7)</td>
<td>85 (66-114)</td>
</tr>
<tr>
<td>IDA</td>
<td>1.18 (0.39-2.19)</td>
<td>55.9 (19.3-108.5)</td>
<td>15.3 (5.3-29.1)</td>
<td>7.40 (1.3-27.8)</td>
<td>88 (73-103)</td>
</tr>
</tbody>
</table>

**Fig. 1:** Box plots show MFI as absolute values in analysed groups

RTC%; reticulocyte count as a percentage; RTC #; absolute count; MFI%; mean fluorescence index as a percentage; MFI #: absolute count; MRV (fL); mean reticulocyte volume; SS; sickle cell anemia; AS; sickle cell trait; SC; hemoglobin SC; βthal; heterozygous β thalassemia; IDA; iron deficiency anemia
Reticulocyte parameters in microcytic anemias. Yoldi et al13 observed higher absolute reticulocytes numbers and RPI up 1.97 in thalassemic patients than in β thalassaemia/β thal group was small and we did not determine the types of mutation that occurred and their possible influence on reticulocyte behaviour. The great variability of our results suggests heterogeneity of the patients and should be extended in order to have a more precise evaluation.

There was not difference in reticulocyte parameters between β thal and IDA groups. There is not any consensus about reticulocyte parameters in microcytic anemias. Yoldi et al13 reported a higher HFR value in IDA than in heterozygous β thalassemia. Kojima et al14 studied 8 patients with IDA and observed an increase in the reticulocyte count when compared to normal group, different to Watanabe et al,8 who observed similar values compared to normal group, different to Paterakis et al,7 studying a representative group of heterozygous thalassemia mutation and may be due to anemia as a result of the hemolysis process. Hb C interacts with Hb S and increases the propensity of red cells to sickle.11 Clinical consequences of the sickle cell-β thalassemia interaction depend on the type of β thalassemia mutation and may be very similar to sickle cell anemia.12

Our β thal group was small and we did not determine the types of mutation that occurred and their possible influence on reticulocyte behaviour. The great variability of our results suggests heterogeneity of the patients and should be extended in order to have a more precise evaluation.

There was not difference in reticulocyte parameters between β thal and IDA groups. There is not any consensus about reticulocyte parameters in microcytic anemias. Yoldi et al13 reported a higher HFR value in IDA than in heterozygous β thalassemia. Kojima et al14 studied 8 patients with IDA and observed an increase in the reticulocyte count when compared to normal group, different to Watanabe et al,8 who observed similar values and degree of immaturity of reticulocytes in both groups. Paterakis et al,7 studying a representative group of heterozygous β thalassemic patients (n=102), concluded that these individuals present a slightly higher erythropoietic activity than the normal population, represented by a higher percentage and reticulocyte count. The authors concluded that erythropoietic activity was higher in that type of anemia than in normal population. In our study the absolute number and immaturity fraction were higher in heterozygous β thalassemia than normal individuals, but without statistical significance. In a previous study and using a semi-automated method and new methylene blue as dye, we found no difference between patients with IDA and heterozygous β thalassemia in the highly immature reticulocyte

<table>
<thead>
<tr>
<th>Variables</th>
<th>Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>SS</td>
</tr>
<tr>
<td>MFI# x HFR%</td>
<td>0.734</td>
</tr>
<tr>
<td>MFI% x HFR%</td>
<td>0.754</td>
</tr>
<tr>
<td>RTC% x MFI%</td>
<td>0.322</td>
</tr>
<tr>
<td>RTC% x MFI#</td>
<td>0.749</td>
</tr>
<tr>
<td>RTC% x MFI#</td>
<td>0.783</td>
</tr>
<tr>
<td>RTC# x MFI#</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

Reticulocyte parameters in ... p65
(HFR) population. However, a significant correlation was observed between HFR and the soluble receptor of transferrin in IDA but not in thalassemia (unpublished data). These data are consistent with the accepted view that iron deficiency causes an increase in the rate of transferrin receptor production due to both increased synthesis and increased stability of the transferrin receptor mRNA.15

A strong correlation between reticulocyte and MFI absolute values indicates that the MFI absolute number is better than MFI as a percentage as a useful parameter to evaluate erythropoietic activity in anemias. Other authors obtained similar results using IRF (HFR+MFR) as representative of immature reticulocyte population.3,16

Rowan et al.,17 in a review on reticulocyte count, suggest that absolute reticulocyte count and the reticulocyte percentage indicate different aspects of erythropoietic control: absolute count reflects the rate of red cell production, balanced by maturation, while the percentage indicates the balance of production of red cells in relation to their rate of destruction. Apparently the same concept can be applied to immature reticulocytes. Erythroid expansion in sickle cell diseases leads to an enhanced immature reticulocyte release from bone marrow. These younger cells remain more time in circulation until complete maturation. MFI as an absolute count reflects this process in contrast with MFI as a percentage.

We concluded that assessment of reticulocyte maturation might be useful to understand the pathophysiology of anemia and for helping differential diagnosis. It is necessary to establish an international consensus about the immaturity reticulocyte definition, in order to allow better exchange of information among laboratories and also to compare different results.

**Resumo**

A avaliação de reticulócitos por citometria de fluxo permite que seja analisado o grau dematuração de dessas células. Novos parâmetros reticulocitários têm sido usados no diagnóstico e acompanhamento de anemias, na avaliação de pacientes submetidos a transplante de medula óssea e no monitoramento de reposição terapêutica com ferro ou eritropoetina. O número e grau de maturação dos reticulócitos têm sido estudados em diferentes tipos de anemia e os resultados têm sido relacionados com o grau de eritropoese ineficaz que pode acompanhar essas anemias. Com os objetivos de verificar as possíveis diferenças nos parâmetros reticulocitários em diferentes tipos de anemia e de testar o valor do número absoluto de reticulócitos imaturas como discriminante entre as anemias, estudamos amostras de sangue de 219 indivíduos, sendo 62 com anemia falciforme (SS), 9 heterozigotos para hemoglobinina S (AS), 17 com Sβ talassemia, 11 com hemoglobinopatia SC, 33 com β talassemia híbrida (Hb B0-0) 47 com anemia ferropoia, comparando-os com 50 amostras de indivíduos sem anemia. O índice médio de fluorescência (MFI) foi definido como representativo do grau de imaturidade dos reticulócitos e foi avaliado em números percentuais e absolutos. As contagens de reticulócitos e os valores de MFI foram significativamente mais elevados nos pacientes com anemia falciforme, Sβ talassemia e doença SC quando comparados aos controles, mas não diferiram entre os três grupos de anemia. Pacientes com traço falciforme, anemia ferropoia e β talassemia híbrida mostraram valores de parâmetro reticulocitários semelhantes ao grupo controle. Não houve diferença entre os grupos de anemia. Pacientes com anemia ferropoia e β talassemia híbrida mostraram valores de parâmetro reticulocitário superior nas anemias que cursam com processo hemolítico, embora isso não tenha sido evidenciado nos valores de MFI em porcentagem. Nossos resultados mostraram que a expansão eritróide nas doenças falciformes SS, SC e β talassemia levam a uma maior liberação de reticulócitos imaturas pela medula óssea e que tal fenômeno torna-se mais evidente através da contagem absoluta de MFI do que pelos valores percentuais. Conclusões que a avaliação da maturidade dos reticulócitos fornece dados interessantes sobre a fisiopatologia e a resposta eritróide medular em diferentes tipos de anemias. Rev. bras. hematol. hemoter. 2003;25(2):97-102.

Palavras-chave: Reticulócitos; doenças falciformes; anemia.

**References**


**Palavras-chave:** Reticulócitos; doenças falciformes; anemia.