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REVIEW

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Use of reticulocyte cellular indices in the diagnosis and treatment of hematological disorders

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Abstract Automated counting of reticulocytes has markedly increased the precision and accuracy of this assay compared with the traditional manual counts. In addition, several new reticulocyte parameters are now available to clinicians and pathologists. This review examines the potential role of these parameters in the diagnosis and management of anemias. Reticulocyte maturity can now be assessed based on the staining intensity of reticulocytes, which is proportional to their RNA content. However, the clinical value of the numerical estimate of the immature reticulocyte fraction has not been yet demonstrated. In the bone marrow transplant setting, there is no clear evidence that the use of this index results in improved care of these patients, and many studies have failed to show its superiority compared with the traditional white cell count, especially for autologous transplants. Direct measurement of reticulocyte volume, hemoglobin concentration, and hemoglobin content are now available. Studies have shown that these parameters, and hemoglobin content in particular, allow a real-time assessment of the functional state of the erythroid marrow. In the setting of recombinant human erythropoietin therapy, studies of hemoglobin content have shown that this index allows an early detection of functional iron deficiency. Preliminary studies have also shown that this index may be helpful in the diagnosis of iron deficiency and in the monitoring of iron replacement therapy.

Key words Reticulocytes · Automated hematology · Immature reticulocyte fraction · Hemoglobin content · Mean cell volume

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Introduction

A "real-time" assessment of the functional state of the erythropoietic marrow could significantly improve the management of patients in the settings of bone marrow transplantation (BMT), recombinant human erythropoietin (r-HuEPO) therapy, treatment of iron, folate, or B_{12} deficiencies, and facilitate the differential diagnosis of anemias. We will review here some basic reticulocyte physiology and the possible use of new reticulocyte parameters as indicators of the functional state of erythropoiesis.

A typical reticulocyte matures in 3.5–4 days, and spends only the last 24 h in the circulation [1, 2]. Stress reticulocytes are large reticulocytes produced under conditions of enhanced erythropoietic activity (stress erythropoiesis). They contain more residual RNA than normal reticulocytes, and they stain more intensely. In conditions of severe anemia [hematocrit (Hct) reduced to 25%–30%], the time spent by the reticulocyte in the marrow is markedly reduced (from 3.5 to 1.5 days), while the maturation time in the circulation is increased from the normal 0.8–1.2 days to 1.7–3 days [3]. The survival of reticulocytes and red cells generated by stress erythropoiesis (including r-HuEPO administration) may be reduced. In the mouse, stress reticulocytes disappear from the circulation in 32–36 h, while macroreticulocytes disappear in 4–12 h [4]. The red cells derived from these macroreticulocytes had a reduced survival [5]. In rats, the reticulocytes generated by r-HuEPO have mean cell volume (MCV) values which are almost double the normal values (100 fl vs. 55 fl) and the derived red cells have a substantially reduced life span [6]. Stress reticulocytes undergo extensive remodelling [7] and substantial intravascular hemolysis [8]. In man, the most immature reticulocytes are multilobar, motile [9], and demonstrate a dramatic reduction in membrane deformability and mechanical stability [10]. Extensive membrane remodelling of cytoskeletal proteins leads to the formation of the more-mature reticulocytes. This maturation process takes place in the bone marrow unless the most immature reticulocytes are released into

the circulation by stress erythropoiesis. The spleen may also play a role in the sequestration and maturation of reticulocytes [11].

Macroreticulocytes produced during abrupt erythroid expansion are also characterized by the co-expression of adult and fetal hemoglobin (so-called F reticulocytes) [12, 13]. In states of rapid marrow expansion, accelerated maturation of early erythroid precursors yields a progeny that maintains the capacity for primitive globin expression.

The introduction of flow cytometric methods has greatly improved the precision and accuracy of reticulocyte counting. The manual reticulocyte count has been gradually replaced by automated counting [14–19]. Automated techniques have better precision and reproducibility than the manual count [20–22], and provide several additional parameters. Automated reticulocyte counting methods are based on either fluorescence (thiazole orange, auramine O, or other dyes) or absorbance (ozaxine 750, new methylene blue) of dyes which interact with reticulocyte RNA. In addition to enumerating reticulocytes, these methods can provide information regarding the distribution of staining intensity among the reticulocyte population. Staining intensity is proportional to the RNA content of the reticulocytes. Thus, young, immature, or stress reticulocytes will have higher staining that mature reticulocytes. The staining intensity of reticulocytes has been proposed as a possible indicator of bone marrow erythropoietic activity. We will examine first the studies on the staining intensity of reticulocytes and then those on reticulocyte volume, hemoglobin (Hb) concentration and content.

Reticulocyte maturity

Immature reticulocyte fraction, technical considerations

The initial studies of this parameter used arbitrary fluorescence units to determine a reticulocyte maturity index (RMI) [15, 23, 24]. Dedicated software programs have subsequently been used to calculate this parameter for flow cytometry systems using thiazole orange. This parameter can also be provided directly by some of the dedicated automated reticulocyte analyzers. There have been several alternative definitions of RMI, which have included RNA index or content, and highly immature reticulocyte fraction. The RMI is based on the arbitrary division of reticulocytes into three fractions. These fractions are defined as high, medium, and low staining intensity fractions. Under normal conditions, the vast majority of reticulocytes resides in the low staining intensity fraction. Based on how the immature reticulocytes are defined, they will constitute a certain percentage of the total reticulocyte population. Different studies have used different definitions for immature reticulocytes. Some studies have reported an RMI based only on the high-fluorescence reticulocyte fraction (HFR), while others have used both high- and medium-fluorescence fraction (MFR). Recently, the term immature reticulocyte fraction (IRF) has been proposed to define the fraction of reticulocytes with the highest fluorescence intensity [25]. This value should be calculated using both HFR and MFR. Although a certain degree of consensus seems to have been reached on the definition of IRF, several technical limitations are still unsolved and will be discussed here.

Assay standardization

There are still major obstacles to standardization and cross-correlation of the three major fluorescence-based assays of reticulocytes (ReticCount for FACS/SCAN instrument, Becton Dickinson, Mountain View, Calif.; Sysmex R analyzers, TOA Medical Electronics, Kobe, Japan; Cell-Dyn 4000, Abbott Diagnostics, Santa Clara, Calif., USA) and to the establishment of gating criteria necessary to define the IRF and its normal range values for each of these instruments [26].

Additional serious limitations for this test are represented by the lack of stable and acceptable calibrators and reference materials. This represents a significant problem when data from different laboratories are compared. It is difficult to assess the reliability of a test when there is no agreement on what constitutes the normal range for IRF [27]. Studies of interlaboratory variability of RMI have shown that there is poor correlation among the thiazole orange methods and significant variability among the Sysmex R-1000 instruments [26]. A recent study comparing IRF measurements with three different analyzers in normal and pathological samples has shown the presence of a significant number of unacceptable duplicates when paired precision analysis was performed in 300 samples using the CELL-DYN 4000 and the Sysmex R-3000 analyzers [28].

Reliability of and interferences with IRF measurements

There are serious concerns about the reliability of this assay, which is usually based on a very small number of counted cells. The HFR fraction is less than 1% of the total reticulocyte population. If 50,000 erythrocytes are counted in a sample, 500 of these will be reticulocytes and only 2–4 of these cells may be HFR. In addition, the gating area where the HFR fraction is found is very susceptible to interferences by other cellular blood elements, such as leukocytes or platelets. Some of the leukocytes and platelets may fall within the gating area selected for this assay and be falsely counted as reticulocytes. In addition, any nucleic acid cellular inclusion (nucleated erythrocytes, erythrocytes with malaria parasites, or presence of Howell-Jolly bodies) will produce an artificially high estimate of IRF [27]. These problems are maximized in patients with a reduced reticulocyte count, such as those who have undergone BMT or chemotherapy.

Immature reticulocyte fraction, clinical considerations

We will review here the published studies on IRF in various clinical settings and discuss the potential clinical value of this measurement.

Table 1 Studies of reticulocyte maturity in patients undergoing bone marrow (BMT)/stem cells transplant (*Auto* autologous, *Allo* allogeneic, *RMI* reticulocyte maturity index, *HFR* high-fluorescence reticulocyte fraction, *MFR* medium-fluorescence reticulocyte fraction, *IRF* immature reticulocyte fraction, *ANC* absolute neutrophil count, *pBSCT* peripheral blood stem cell transplant)

Year/Ref.	No. of patients	Type of BMT	Reticulocyte maturity parameter	Time to engraftment (HFR/RMI/IRF)	Time to engraftment (ANC)
1989 [15]	12	12 Auto BMT	RMI	–	–
1989 [23]	20	20 Auto BMT	RMI	15.2±1.0	18.4±1.8
1992 [30]	36	22 Auto BMT	HFR	15.8±9.08	16.1±8.22
		14 Allo BMT	(absolute)	10.7±3.68	16.0±8.48
1993 [31]	42	28 Auto BMT	HFR	10 (6–23)	12 (9–24)
		14 Allo BMT	(%)	14 (9–29)	15 (10–30)
1994 [32]	48	21 Auto BMT/PBSCT	HFR (absolute)	12	15
		27 Allo BMT		12	17
1994 [33]	40	8 Auto BMT	HFR+MFR	13.3±5.5	14.8±5.0
		32 Allo BMT	(%)		
1994 [35]	86	58 Auto BMT	HFR+MFR	12.2±5.8	19.6±9.3
		28 Allo BMT	(%)	17.0±7.0	20.5±6.3
1997 [36]	42	10 Auto PBSCT	HFR+MFR	10.1 (8.7–12.7)	14.7 (11.7–18.2)
		13 Auto BMT	(%)	13.5 (11.2–20.2)	22.6 (16.6–30.4)
		9 Allo PBSCT		10.7 (8.5–11.7)	13.1 (11.7–14.3)
		10 Allo BMT		17.5 (16–23.1)	22.3 (20.2–25.8)

Bone marrow transplantation and recovery following chemotherapy

There have been several reports of the value of RMI and/or IRF in early detection of engraftment after BMT or recovery of bone marrow function following chemotherapy [15, 23, 29–36]. A summary of these studies is provided in Table 1. In the first report [15], 12 patients were studied following autologous BMT for treatment of acute myelogenous leukemia. In this report, RMI closely paralleled the increase in absolute neutrophil counts (ANC) after autologous BMT. A subsequent report from the same group [23] including 19 patients indicated that the average time of engraftment was 15.2±1.0 days using RMI and 18.4±1.8 using ANC. Another study investigated 15 patients with autologous BMT and 8 patients with allogeneic BMT using the thiazole orange technique [29]. The automated reticulocyte count provided an earlier indication of bone marrow recovery than platelet or neutrophil counts (13.3±4.0 vs. 16.8±7.3 and 17.1±3.9 days, respectively). Only 2 patients underwent reticulocyte fluorescence studies, and no additional benefits were demonstrated with these indices. Interestingly, reticulocyte studies seem to provide an early detection of engraftment compared with neutrophil counts only in allogeneic BMT (14.7±4.5 vs. 19.0±2.9) and not in autologous BMT (13.2±4.2 vs. 15.7±4.1).

Another study was carried out using the Sysmex R-1000 reticulocyte counter in 22 autologous and 12 allogeneic BMT patients [30]. The HFR fraction preceded the increase in ANC in 10 autologous BMT and 8 allogeneic BMT patients (mean 5.1 days, 95% confidence interval 3.7–6.5 days). In this study, there was no significant difference in the mean time for engraftment for autologous BMT estimated by the increase in either HFR (15.8±9.08 days) or ANC (16.1±8.22 days). A study of children undergoing BMT showed that the increase in HFR (more than 2%, obtained with the Sysmex R-1000) preceded an ANC of 0.5×10^9/l in the majority of patients [31]. However, there was no significant difference between time to reach HFR of 2% or higher and time to reach an ANC of 0.1×10^9/l. A study from Austria found no difference in autologous BMT patients between the time to reach an HFR of 0.2×10^9/l and an ANC of 0.2×10^9/l [32]. For allogeneic BMT patients, the median times to reach HFR and ANC counts of 0.2×10^9/l were 12 and 17 days, respectively. Engraftment time was significantly shorter for HFR compared with ANC in allogeneic BMT patients given r-HuEPO after BMT. In another study, which used the HFR plus MFR parameters of the Sysmex R-1000 reticulocyte analyzer, a measurable increase in HFR plus MFR was observed at 13.31 days after transplant, compared with 14.82 days for the white blood cell (WBC) count [33]. The Spanish Multicentric Study Group for Hematology Recovery studied 58 autologous BMT patients, 28 allogeneic BMT patients, and 28 patients receiving remission-induction chemotherapy for acute leukemia [35]. Engraftment was defined as an ANC greater than 0.5×10^9/l or an HFR plus MFR greater than 10%. A significant difference was noted in autologous BMT patients, with 84.5% of the patients showing earlier signs of engraftment with the reticulocyte fluorescence studies. There was no significant difference between reticulocyte fluorescence and ANC in the patients receiving allogeneic BMT or chemotherapy. In a more recent study of the same group, the reticulocyte recovery was compared between BMT and peripheral blood stem cell transplant (PBSCT) [36]. HFR plus MFR recovery was significantly faster in the PBSCT than the BMT group.

From the analysis of these studies, some considerations emerge. Different criteria have been used to define immature reticulocytes, and only recent studies have used HFR plus MFR (IRF) as an engraftment parameter.

Different standards have been used for the ANC, with many studies using up to 0.5×10^9 cells/l. These thresholds

were based on the sensitivity of hematology analyzers of the late 1980s/early 1990s. Current instruments can reliably count WBC down to $0.02–0.05\times10^9$ cells/l. No study has evaluated ANC versus reticulocyte fluorescence using the latest generation analyzers. A fair comparison between ANC and IRF should include these new thresholds. Given the fact that an ANC count of 0.2×10^9 cells/l is considered sufficient in many American hospitals for discharging patients who are recovering from neutropenia and infections following chemotherapy, a lower threshold for ANC should also be considered in the recovery from BMT.

No study has clearly demonstrated that the use of the IRF parameter offers a significant improvement in either quality or cost of care for patients undergoing BMT compared with the traditional WBC counts. It is not clear how the data provided by the IRF can affect therapy. In addition, the fact that this parameter is measured in a small number of cells raises concerns about its reliability in this setting, where there is a severe reduction in the number of absolute reticulocytes. There may be a benefit in using the IRF in identifying patients who fail to engraft. Absolute reticulocyte counts of 15×10^9 cells/l and HFR count of 0.5×10^9 cells/l on day 21 post transplant have been shown to be associated with 100% engraftment [30]. There has been one report on the possible use of IRF as a surrogate marker for the appearance of CD34+ cells in the collection of peripheral stem cells [37].

Monitoring of iron or r-HuEPO therapy

Changes in IRF have been reported following r-HuEPO therapy. There is evidence that an early event following administration of r-HuEPO is the release into the circulation of immature reticulocytes with higher staining intensity [38]. This event precedes the increase in bone marrow erythropoietic activity induced by r-HuEPO. A study on the reticulocyte response following r-HuEPO administration in autologous blood donors has shown a dose-dependent increase in both absolute reticulocyte count and absolute HFR reticulocytes [39]. However, the issue in most of the clinical settings requiring use of r-HuEPO is not whether there is a response or not, but rather whether the response is appropriate for a given r-HuEPO dosage. In addition, the balance between r-HuEPO dosage and iron availability should be monitored to promptly identify the appearance of iron-restricted erythropoiesis [40, 41]. We have recently described the effect of intravenous iron on the reticulocyte response to r-HuEPO in normal volunteers [42]. In this study, absolute reticulocyte counts and absolute IRF counts were similar in the control and the IV iron group. Only with the direct measurement of reticulocyte cell Hb content (CHr) and of reticulocyte Hb [43], could the increased production of Hb induced by IV iron be demonstrated. Based on these studies, IRF does not seem to offer any significant advantage in the setting of r-HuEPO therapy and is clearly inferior to the direct measurement of reticulocyte cellular indices.

Diagnosis of anemias

It is not clear whether IRF offers a significant improvement for the diagnosis of anemia. The information provided by the IRF parameter when used in conjunction with the absolute reticulocyte count is the same as the corrected reticulocyte count or the reticulocyte production index [27]. Although several studies have been published on this issue [44–47], none has provided convincing evidence for the additional value of IRF in the differential diagnosis of anemias, especially when the intrinsic limitations of this assay are taken into account.

A careful analysis of the several studies published on reticulocyte maturity suggests that there is no clear evidence for the superiority of this index compared with neutrophil counts in the setting of BMT. Although this index may from time to time provide useful insights into a specific patient, a widespread use of this index is not warranted at present. More basic and clinical work is needed to validate the possible clinical utility of parameters of reticulocyte maturity.

Reticulocyte cellular indices

Reticulocyte size (cell diameter, cell surface area, cell volume)

Reticulocytes are larger than mature erythrocytes. Maturation of reticulocytes is characterized by a progressive decrease in cell size [48, 49]. The first determinations of reticulocyte diameter were carried out with micrometric eyepieces on dry blood smears stained for reticulum with a supravital dye. Normal reticulocytes had a diameter of approximately 8.5 μm, about 1–1.5 μm larger than mature erythrocytes. The diameter of reticulocytes and mature erythrocytes varied considerably in diseases such as megaloblastic and microcytic anemias, but their ratio remained constant.

Thus, a decrease in cell size is a common feature of erythropoietic development. Reticulocytes in bone marrow appeared larger than those in peripheral blood, suggesting that the final stages of maturation took place in the circulation. Reticulocyte diameter progressively decreases throughout the various stages of reticulocyte maturation (Heilmeyer classification), with the greatest changes taking place from stage 0 (orthochromatic erythroblasts) to stage I, because of the loss of the nucleus, and from stage IV to mature erythrocyte [50]. These early studies of reticulocyte size did not have sufficient precision and accuracy, because of the flattening, distortion, and shape changes induced by absorption of supravital dyes and subsequent drying on glass slides.

Photographic methods were used in the 1960 s to study reticulocyte size on wet preparations. Fresh oxalated blood was mixed with brilliant cresyl blue in isotonic saline, placed between a slide and coverslip, and then photographs were taken [51]. In each sample, 30–35 fields containing reticulocytes were photographed. Glossy prints enlarged

to a final magnification of approximately ×4,000 were obtained. The area of reticulocytes and adjacent red cells was determined using an instrument called a Polarplanimeter (coefficient of variation of this method ranged from 0.5% to 1.0%). The area of normal reticulocytes was on average 13.0% larger than that of mature erythrocytes (range 7.7%–22.0%) and the calculated reticulocyte volume was 20% greater. Mean reticulocyte/ erythrocyte area ratio and volume ratio were 1.13 and 1.2, respectively. Since this technique was based on arbitrary units and not actual metric units, only relative measurements could be obtained.

In 1976 Clarkson and Moore [2] using an adapted planimetric method obtained values for reticulocyte/erythrocyte area ratio of 1.125 in 17 normal subjects, a value which is almost identical to that previously reported by Killman [51]. The calculated mean reticulocyte volume was 20% greater than that of mature red cells. Using values for mature erythrocyte MCV of 88 fl (based on Coulter S data), the actual mean reticulocyte volume was estimated to be 106 fl in normal subjects (88×1.20). The mean reticulocyte volume was 79 fl in iron deficiency (erythrocyte MCV=73 fl) and 139 in megaloblastic anemia (erythrocyte MCV=103 fl). The presence of microreticulocytes or macroreticulocytes in the peripheral blood was pointed out as a very early finding during the development of iron deficiency or vitamin B_{12}/folate deficiency, respectively.

Direct determinations of mean volume of reticulocytes using the ratio of packed cell volume to cell number have been attempted in samples with very high reticulocyte concentrations. An enrichment in reticulocytes up to 100% was obtained in experimental animals treated with phlebotomies and/or hemolytic agents. These methods provided values for reticulocyte volumes ranging from 1.2–1.5 to 2–3 times greater than those of mature red cells [52, 53]. However, these methods did not measure the volume of normal reticulocytes, but rather that of the much bigger "stress" reticulocytes, which are produced only under very intense erythropoietic stimulation. Severe hemolytic anemia induced by phenylhydrazine in rats was a popular experimental model in the 1960s [54]. The MCV of reticulocytes produced under such an extreme erythropoietic stimulation was nearly twice that of normal mature red cells. The larger size of these "stress" reticulocytes was attributed to the skipping of cell divisions in the bone marrow, due to the accelerated erythropoiesis and shortening of the interval between differentiation of stem cells and emergence of reticulocytes. These early, oversized reticulocytes had much lower mean cell hemoglobin concentration (MCHC) than normal erythrocytes.

Changes in reticulocyte volume can be indirectly monitored through changes in the red cell size distribution curves obtained with an automated hematology analyzer [55]. In a similar fashion, the count of polychromatophilic red cells has been compared with a "shift reticulocyte count" and has been proposed as an indicator of bone marrow response to anemia [56]. The production of macrocytic reticulocytes was also observed following bleeding, exposure to simulated altitude, injection of erythropoietin [57], and also after successful treatment of iron deficiency anemia [58].

A different experimental model of erythropoietic stimulation was based on inducing a temporary suppression of erythropoiesis with thiamphenicol and phlebotomy [59]. Immature reticulocytes produced in the recovery phase which follows this treatment are larger than normal reticulocytes (mean diameter 9.66±1.10 μm, compared with 7.04±1.10 μm for normal reticulocytes and 6.66±0.34 μm for mature red cells) and have a high content in ribosomes, mitochondria, and other cellular organelles [60].

Stress macroreticulocytes are seen as polychromatophilic erythrocytes on a Romanowsky-stained peripheral blood film. Their average diameter is 27% larger than that of normal erythrocytes [61]. Polychromatophilia is abolished by treatment with ribonuclease. However, only the most immature, Heilmeyer class I and II reticulocytes appear as polychromatophilic red cells on panoptical stains.

Reticulocyte density and cellular Hb concentration

Reticulocytes are less dense than mature red cells. When a sample of blood is centrifuged or allowed to stand, reticulocytes tend to remain in the top layer of cells. According to an old observation, when erythrocyte sedimentation rate is measured in samples with a high reticulocyte count, there is a trail of more slowly sedimenting reticulocytes which gives a characteristic shading at the red cell/plasma interface. The density of reticulocytes from anemic rabbits was estimated to be around 1.105 g/ml cells, compared with 1.122 g/ml cells for the mature anemic red cells [49, 62]. This difference in density is due to the lower Hb concentration and higher water content of reticulocytes. More than 50 years after these studies, it has been shown that the percentage of reticulocytes correlates with the percentage of hypochromic macrocytes [63].

The lower density of reticulocytes permits the recovery of enriched fractions of these cells from mature erythrocytes using centrifugation and density separation on Percoll columns [62] or Stractan [64].

Measurement of reticulocyte cellular indices with automated hematology analyzers

Simultaneous measurement of volume and Hb concentration can be carried out on both red blood cells [65–68] and reticulocytes [69] using laser-based technology. Flow cytometric analysis of red cells allows quantification of their volume, Hb concentration, and Hb content. The oxazine-750 staining method used in the Bayer H*3 and Advia systems (Bayer Diagnostics, Tarrytown, N.Y., USA) allows measurement of reticulocyte staining intensity and provides direct measurements of reticulocyte cellular indices, such as reticulocyte MCV (MCVr) and MCHC (CHCMr) with their respective distribution widths (RDWr and HDWr). Mean Hb content of reticulocytes (CHr) and its distribution width (CHDWr) is calculated from the product of the volume times Hb concentration of single cells.

Table 2 Normal range values for reticulocyte cellular indices (*MCVr* mean reticulocyte cell volume, *RDWr* reticulocyte volume distribution width, *CHCMr* mean reticulocyte cellular hemoglobin concentration, *HDWr* reticulocyte cellular hemoglobin concentration distribution width, *CHr* reticulocyte cell hemoglobin content, *CHDWr* reticulocyte hemoglobin content distribution width)[a]

	Ref. [69]	Ref. [71]	Ref. [72]
MCVr (fl)	88.2–107	103.2–126.3	92.4–120.2
RDWr (%)	10.3– 18.3	–	13.7– 20.1
CHCMr (g/dl)	25.4– 31.0	23.5– 28.7	26.7– 33.0
HDWr (g/dl)	1.9– 4.7	–	2.8– 4.0
CHr (pg)	23.5– 29.9	25.9– 30.6	27.1– 33.9
CHDWr (pg)	2.5– 4.1	–	3.0– 4.7

[a] In ref. [69], data were collected from 110 children (51 males, 59 females) aged 1–10 years; in ref. [71], data were collected from 64 healthy adults (32 males, 32 females); in ref. [72], data were collected from 133 healthy adults

The measurement of reticulocyte indices has shown excellent precision both in normal subjects and in patients with reticulocytosis. Coefficients of variation for measurements of MCVr, CHCMr, and CHr were reported to be 0.8%–1.6% after 21 repeated determinations [69]. The MCVr remains stable after up to 72 h of storage at 4°C, while CHr and CHCMr show a small statistically significant decrease.

Reference values for reticulocyte indices

Following a preliminary report which used a Bayer H*3 early prototype [70], three studies have reported reference intervals of reticulocyte indices in healthy subjects using the Bayer H*3 analyzer (Table 2).

Reference values were obtained studying 32 male and 32 female healthy adults. These subjects had normal complete blood count, WBC differential, and serum ferritin, with no clinical symptoms. [71] All reticulocyte parameters showed a normal distribution and no statistically significant difference between sexes. The average value of MCVr was 111.7 fl, ranging between 103.2 fl and 126.3 fl. MCVr was 24% higher than the MCV of mature erythrocytes. The MCVr/MCV ratio had a mean value of 1.24. The mean CHCMr was 26.3 g/dl (reference range 30.1–33.2 g/dl), compared with a CHCM of 31.6 g/dl, with a mean negative difference of –16.7%. The mean values of the CHCMr/CHCM ratio were consistently lower than 1.0, indicating that the Hb concentration is lower in reticulocytes than in mature erythrocytes. The mean reticulocyte Hb content (CHr) was on average 28.5 pg (reference range 25.9–30.6 pg), very similar to the Hb content (CH) of mature red cells, so that the CHr/CH ratio was close to 1.0 (average value 1.03).

Reticulocyte indices were measured in 110 pediatric outpatients (51 males and 59 females, aged 1–10 years), with normal hematological parameters according to an age-adjusted reference range [69]. This pediatric study (Table 1) shows a good general agreement with the adult studies: although the average volumes are lower, the average MCVr/MCV ratio is again higher than 1.0 (1.24), the average CHCMr/CHCM ratio is lower than 1.0 (0.81), and the CHr/CH ratio is very close to 1.0 (0.96). Thus, in children as in adults, reticulocytes exhibit a larger cell volume, a reduced cell Hb concentration, and a similar Hb content compared with mature red cells. Distribution widths for the reticulocyte indices have also been reported: the RDW of reticulocytes (RDWr), expressed as the coefficient of variation of volumes, is on average very close to that of mature erythrocytes (14.3% vs. 14.0%), while the variability of Hb concentration (HDW), expressed as the standard deviation (HDWr), is greater in reticulocytes than in red cells (3.3 g/dl vs. 2.6 g/dl). Another study has reported reference intervals for reticulocyte indices in 133 adult normal subjects [72]. Table 2 presents the normal range for reticulocyte indices based on these three published reports.

Table 3 Reticulocyte cellular indices in iron deficiency[a]

	d'Onofrio et al. [71] Mean±SD	Buttarello et al. [72] Mean (range)
MCVr (fl)	100.1±4.9	84.7 (67.6–93.4)
RDWr (%)	–	22.3 (20.1–28.8)
CHCMr (g/dl)	20.4±2.1	24.6 (22.2–26.5)
HDWr (g/dl)	–	4.6 (4.1–5.1)
CHr (pg)	19.6±2.5	20.9 (20.1–28.8)
CHDWr (pg)	–	4.1 (2.9–5.0)

[a] In ref. [71], data were collected from 58 patients; in ref. [72], data were collected from 9 patients

Reticulocyte and red blood cell indices have also been measured in anemic patients with abnormalities of red cell size [71]. The microcytic anemia group included 58 patients with overt iron deficiency anemia before iron treatment and 40 with heterozygous β-thalassemia and no iron deficiency. The macrocytic anemia group included 28 patients with anemia of different etiology and MCV above 100 fl. In these subjects the average values of reticulocyte indices were generally very similar to healthy subjects (Table 3). Thus, regardless of the final red blood cell size, reticulocytes appear consistently larger than mature erythrocytes, whereas their Hb concentration is consistently lower. The cellular Hb content is similar in the two cell types. These results are in good agreement with the data obtained using planimetric methods [2, 51]. Another group has also reported data on reticulocyte cellular indices in patients with iron deficiency [72].

Although bone marrow reticulocytes are capable of Hb synthesis [73], the Bayer H*3 measurements indicate that Hb content does not change significantly after these cells are released into the peripheral blood.

Clinical utility of reticulocyte indices

The average survival of red blood cells in the circulating blood is about 4 months in healthy subjects and remains unchanged in most patients with anemia, with the exception of hemolytic anemias. During steady-state erythropoiesis, 20 ml of the total erythrocyte mass is renewed

daily (1% of the circulating red cells). Such relative stability of the erythrocyte population inherently limits the clinical sensitivity of red cell indices MCV, MCHC, and MCH as early indicators of erythropoietic changes. Classical studies on both experimental animals and humans have shown that the first modifications of the MCV do become manifest after at least 6–12 weeks during experimentally induced deficiencies of iron [74] or folic acid [75]. Clarkson and Moore [2] have observed with their planimetric method that experimental induction of iron deficiency by phlebotomy is associated with a sequence of events which begins with an increased production of macroreticulocytes, which represents the first response to increased erythropoietin production. Two days after the fall of the transferrin saturation, the MCVr also abruptly diminishes, while the percentage and absolute reticulocyte count decreases after 1 more week. The MCV does not change for several weeks, then it begins a progressive decline down to values lower than the normal range, which are reached after more than 2 months. With iron supplementation, the MCVr increased rapidly, followed by the reticulocyte count and MCV. Similarly, the administration of methotrexate, a potent inhibitor of dihydrofolate reductase, causes an abrupt and sustained increase in reticulocyte volume (up to 142 fl), while the specific replacement therapy given to patients with macrocytosis and folate or vitamin B_{12} deficiency induces a rapid normalization of reticulocyte size, without any early change in the MCV.

These early studies indicated that the measurement of reticulocyte size, although still inaccurate and imprecise at that time, could provide very-sensitive and early clues to the diagnosis and treatment of patients with abnormal erythropoiesis. Reticulocytes released into the peripheral blood during the last 24 h represent a very timely indicator of marrow erythropoietic activity. The measurement of their size and Hb content using the new cytometric techniques, which are much more precise and rapid than the previous methods, offers a great potential for the diagnosis and treatment of hematological disorders. The following paragraphs report some examples of the clinical value of reticulocyte indices.

r-HuEPO administration and functional iron deficiency

It is known that treatment with r-HuEPO may cause functional iron deficiency in patients with chronic renal failure receiving long-term dialysis [76]. Functional iron deficiency occurs because r-HuEPO stimulates erythropoiesis to such an extent that the demand for iron exceeds the body's ability to release it from stores. Functional iron deficiency is one of the main causes of resistance to r-HuEPO and can be detected by monitoring transferrin saturation or the percentage of hypochromic red blood cells with CHCM lower than 28 g/dl [77]. However, cellular hypochromia is a feature not only of microcytic erythrocytes but also of normal reticulocytes. Therefore, this parameter is not a an exclusive indicator of the presence of hypochromic microcytic erythrocytes. Functional iron deficiency is also observed when r-HuEPO is administered to non-anemic subjects, such as normal subjects undergoing multiple blood donations for autotransfusion schedules [40]. In healthy volunteers the administration of subcutaneous r-HuEPO is associated with a significant increase in the production of reticulocytes, which have an increased MCVr and a decreased CHCMr [78]. In this setting the reticulocyte cell Hb content (CHr, pg/cell) seems to represent a sensitive indicator of functional iron deficiency: in particular, the percentage of reticulocytes with CHr less than 23 pg is an effective indicator of iron-deficient erythropoiesis and is inversely correlated with the log value of baseline serum ferritin [41]. Since oral iron is ineffective in preventing iron-deficient erythropoiesis, IV iron supplementation should be considered in patients with baseline serum ferritin <100 ng/ml. When IV iron saccharate is administer in association with r-HuEPO, no hypochromic reticulocytes are produced and the CHr remains within the normal range [78].

The effect of IV iron on reticulocyte indices has been studied in normal subjects receiving a single dose of r-HuEPO [42]. These data suggest that IV iron potentiates the hematopoietic response to r-HuEPO in normal subjects, which may otherwise be rate-limited. IV iron significantly increased the r-HuEPO-induced production of Hb and prevented the marked decrease in serum ferritin which is usually associated with r-HuEPO administration. While the total number of reticulocytes was not affected by IV iron administration, CHr was increased in the IV iron group compared with the control group. This effect was confirmed by measurement of total reticulocyte Hb, an integrated index which is derived from the absolute reticulocyte count and the CHr [43]. Reticulocyte Hb was significantly increased in the group receiving IV iron, indicating that there was a greater production of Hb when IV iron was used.

Functional iron deficiency may also develop as a consequence of increased endogenous erythropoietin production, as in patients with immuno-hemolytic anemia: in these cases a sudden and unexpected fall of reticulocyte counts associated with the production of microreticulocytes and with an inversion of the MCVr/MCV ratio can be the first manifestation of iron-deficient erythropoiesis.

CHr is a valuable indicator of the iron status in patients undergoing chronic hemodialysis [79]. The group of patients with CHr values lower than red cell mean corpusculare hemoglobin (MCH) had lower transferrin saturation and Hct. In addition, all these patient had a recent increase in r-HuEPO dose. These data suggest that CHr could be an extremely useful index in the management of r-HuEPO therapy for patients on chronic dialysis.

Several different indicators of response are currently used to monitor patients treated with rHuEPO. Increments in Hb after 2–4 weeks have been shown to be a powerful predictor of responsiveness to rHuEPO treatment [80]. However, this parameter may be useless in patients receiving transfusions and/or concomitant chemotherapy. While changes in reticulocyte count after 2 weeks may simply reflect output of shift reticulocytes and not true expansion of erythropoiesis [81], an increase in reticulocyte count of $\geq 40 \times 10^9$/l after 4 weeks was found to be a significant pre-

dictor of response in cancer anemia [81, 82]. Thus, treatment efficacy can be assessed by measuring Hb and reticulocyte count after 4 weeks: patients showing a 4-week change in Hb level ≥1.0 g/dl and/or a change in reticulocyte count $\geq 40 \times 10^9$/l are those most likely to respond to rHuEPO [83]. Future studies should address how reticulocyte indices and CHr in particular can be used to monitor the response to r-HuEPO.

Diagnosis and treatment of iron deficiency and megaloblastic anemia

There are limited data on the use of reticulocyte indices and CHr in the diagnosis of iron deficiency. We have shown, in a group of pediatric patients undergoing routine office visits, that the estimated probability for iron deficiency is >90% when CHr is less than 20 pg. When CHr is close to 23–24 pg, the probability of iron deficiency is approximately 50%, and this probability decreases smoothly for larger values of CHr. Children with CHr above 29 pg have virtually a zero probability of having iron deficiency. Ferritin is not predictive of iron deficiency in this group of patients [84]. CHr was a much stronger predictor of iron deficiency than either the traditional red cell indices (MCV, MCHC, MCH) or erythrocyte zinc protoporphyrin.

In patients undergoing hemodialysis, a value of CHr <26 pg predicts iron deficiency with a sensitivity of 100% and specificity of 80% [79]. Much lower diagnostic accuracy is achieved with the use of serum ferritin, transferrin saturation, or percentage hypochromic red cells [79].

The monitoring of erythropoietic response to iron administration in iron-deficient anemia is also improved by the availability of reticulocyte indices [85]. Changes in MCV and RDW become apparent after weeks or months of iron deficiency and also change slowly during treatment. However, reticulocyte indices may allow a real-time evaluation of iron-deficient erythropoiesis and of the effectiveness of iron replacement therapy, since the lag time for their appearance from the marrow is only 2 days [86]. CHr may also be useful to identify patients who are not responding to oral iron: when abnormally low CHr persists despite oral iron treatment, IV iron therapy is indicated. As shown in Fig. 1, IV iron therapy in severe iron-deficient patients produces a very early increase in CHr, MCVr, and CHCMr well in advance of any other change in red cell indices or total Hb [86]). Figure 2 shows changes in MCH and CHr in another patient treated with IV iron. IV iron therapy leads to normalization of reticulocyte Hb content and subsequent maturation of these reticulocytes into cells with normal MCH. These results provide a direct confirmation and measurement of the changes in reticulocyte size observed following iron replacement therapy in 1977 by Bessman [58] and in 1991 by Patton et al. [87].

The observation that in particular cases reticulocytes may be smaller than the circulating mature red cells was reported for the first time in 1962: by Brecher and Stohlman [57]: "... reticulocytes produced in a megaloblastic

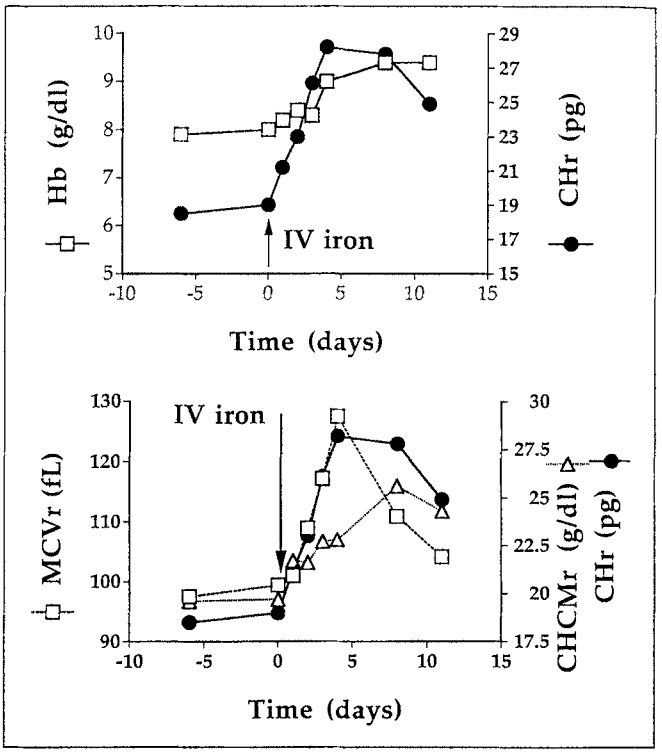

Fig. 1 Changes in reticulocyte cell hemoglobin content (*CHr*) and hemoglobin (*Hb*) (*upper panel*) and in CHr, mean reticulocyte volume (*MCVr*), and mean reticulocyte cellular hemoglobin concentration (*CHCMr*) (*lower panel*) following IV iron administration in a patient with severe iron-deficient anemia

anemia in response to B_{12} are markedly smaller than the predominant megaloblasts circulating at the time." Similar results were reported later by Bessman [88]. An inversion of the MCVr/MCV ratio, with reticulocytes smaller than mature erythrocytes, has been observed with the Bayer H*3 method in a patient with megaloblastic anemia during hematological recovery following vitamin B_{12} administration [71]. In this case, the circulating reticulocytes produced after 17 days of treatment had an MCVr of 108.8 fl, while the MCV was still 109.8 fl, because most of the circulating mature erythrocytes had been formed before the vitamin administration [71].

Sickle cell anemia

The automated measurement of reticulocyte indices can be used to obtain information on the presence and the proportion of dehydrated hyperdense reticulocytes which have CHCMr >38 g/dl [89]. The induction of fetal Hb synthesis by hydroxyurea results in increased cell hydration of sickle reticulocytes, so that the disappearance of dense reticulocytes can be used as an early indicator of the effectiveness of the treatment. Recent studies on the use of oral magnesium (Mg) supplements in patients with sickle cell disease have shown that the reduction in dense erythrocytes induced by Mg is associated with a reduction in the absolute number of high staining intensity reticulocytes [90],

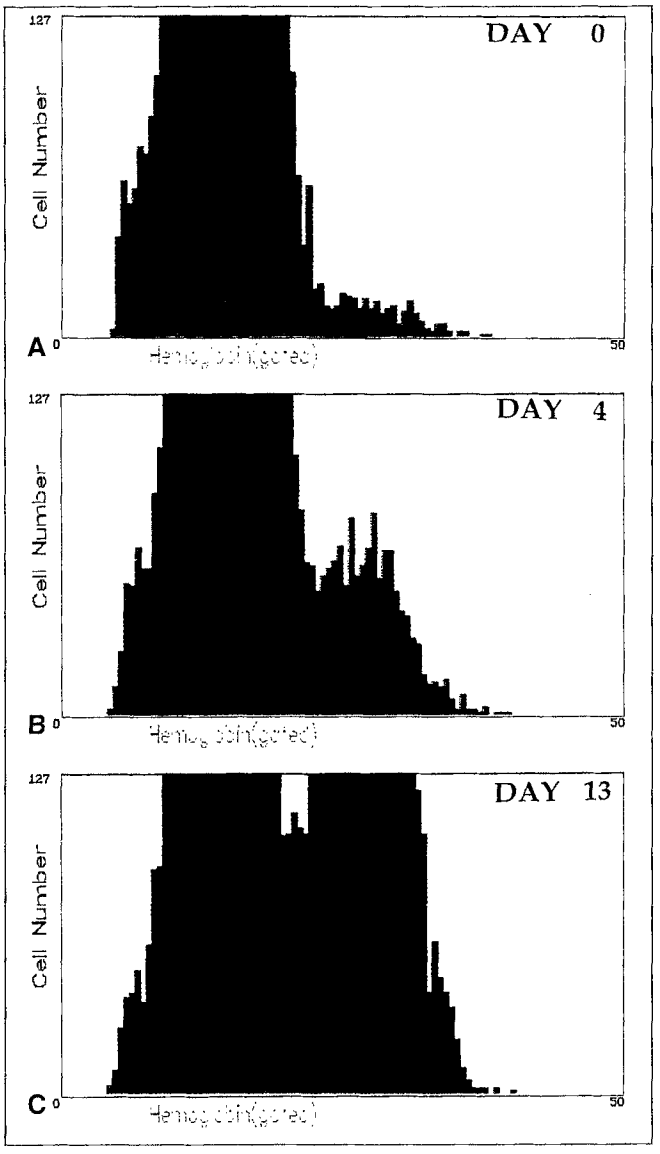

Fig. 2 Frequency distribution histograms of CHr (green) and erythrocyte cell hemoglobin content (CH, blue) in one patient with severe iron-deficient anemia before (**A**) and 4 (**B**) and 13 (**C**) days after IV iron therapy supplementation. The data demonstrate the shift toward normal values of CHr following iron replacement therapy and the subsequent generation of mature erythrocytes with normal mean cellular hemoglobin

and with a reduction in the distribution widths for reticulocyte volume (RDWr) and cell hemoglobin concentration (HDWr) [91].

Bone marrow transplantation

During the follow-up of patients undergoing transplantation of bone marrow or peripheral blood progenitor cells, the succession of suppression and regeneration of erythropoiesis is associated with important changes in MCVr and MCVr/MCV ratio [71]. After conditioning chemotherapy, a severe decrease in reticulocyte percentage and absolute number occurs, which can be associated with a progressive decrease in MCVr and transitory inversion of the MCVr/MCV ratio. Erythroid regeneration, however, is heralded by an abrupt increase of MCVr, up to values above normal [71].

In conclusion, the availability of automated measurements of reticulocyte indices allows a real-time evaluation of the bone marrow erythropoietic activity. Future studies will determine how this information can be translated into clinically effective and cost-effective decisions.

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