

Immature reticulocyte fraction as a useful parameter for blood transfusion assessment in anaemia

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Introduction

Red blood cell production occurs in the bone marrow at steady state levels during health and at altered levels during periods of stress and disease.¹ All mature blood cells originate from haemopoietic stem cells and progenitor cells that respond to haematopoietic growth factors, including erythropoietin in the case of the erythrocyte lineage.

Reticulocytes leave the bone marrow to enter the peripheral vascular system as immature erythrocytes that still contain cellular organelles. As they continue to mature for a further day in the peripheral circulation² their haemoglobin synthesis gradually decreases as their organelles are progressively lost. Thus, detection of immature erythrocyte forms is important in the evaluation of different types of anaemia,³ as it provides information about the ability of the bone marrow to respond to offset deficits in the oxygen-carrying capacity of the blood.

When erythropoiesis is stimulated, the reticulocyte count in the circulation increases due to a reduction in their intramarrow retention time and a correspondingly longer maturation time in the peripheral blood. Methodologies for reticulocyte enumeration have exploited the special staining qualities of these cells, especially the ability of some dyes to bind and crosslink their RNA.^{4,5} Thus, staining heterogeneity reflects different maturation stages in reticulocyte populations.⁶

Development of flow cytometry in the 1980s improved reticulocyte enumeration significantly by providing a rapid, automated, objective, technically easy, accurate and cost-effective method.⁷ In addition, this method counted the most mature reticulocytes (largest fraction).

In 1986, Lee *et al.*⁸ found that the fluorescent dye thiazole orange (TO) provided enhanced resolution in the staining of reticulocytes by flow cytometry. In this way, TO staining provided additional information about individual

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ABSTRACT

During erythropoietic stress (e.g., acute anaemia) the reticulocyte count in peripheral blood normally increases as the bone marrow responds to increased erythropoietin stimulation of erythroid precursors. The efficiency of this process is an indicator of the patient's bone marrow response. This study assesses the utility of the immature reticulocyte fraction (IRF) as a useful parameter of anaemia type, which may inform the decision to treat with red cell transfusion. Moreover, it investigates the value of using IRF as an inexpensive, non-invasive and objective indicator of a patient's bone marrow response. EDTA-treated venous blood specimens were collected from in-patients with a haemoglobin value <100 g/L and analysed to establish the absolute reticulocyte count and IRF using the ABX Pentra 120 Retic analyser. Based on the clinical information provided, the specimens were divided into those with chronic anaemia and those with acute anaemia. Statistical analysis of results showed that there was a significant negative correlation between IRF and haemoglobin level. Importantly, IRF was also found to show a more significant correlation with haemoglobin level than did the absolute reticulocyte count. Furthermore, this correlation was stronger in patients with acute versus chronic anaemia. Thus, this information may aid clinicians in their decisions to recommend blood transfusions for patients with certain types of anaemia.

KEY WORDS: Anemia.

Blood transfusion.

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reticulocytes, with fluorescence level associated with their level of maturity. Flow cytometers are now incorporated commonly into existing haematology analysers and have resulted in a reduction in laboratory expense and the need for additional technical skill.

The observation that the fluorescence intensity of reticulocytes in the peripheral blood was inversely proportional to their RNA content⁹ led to the establishment of the reticulocyte maturation index (RMI).¹⁰ While the vast majority of reticulocytes under normal conditions will reside in the low staining intensity region (i.e., most mature), reticulocytes of differing maturity will constitute certain percentages of the total reticulocyte population.¹¹ To avoid inconsistency in reporting between laboratories, a more defined immature reticulocyte fraction (IRF) was proposed,¹² which included both high- and medium-fluorescence

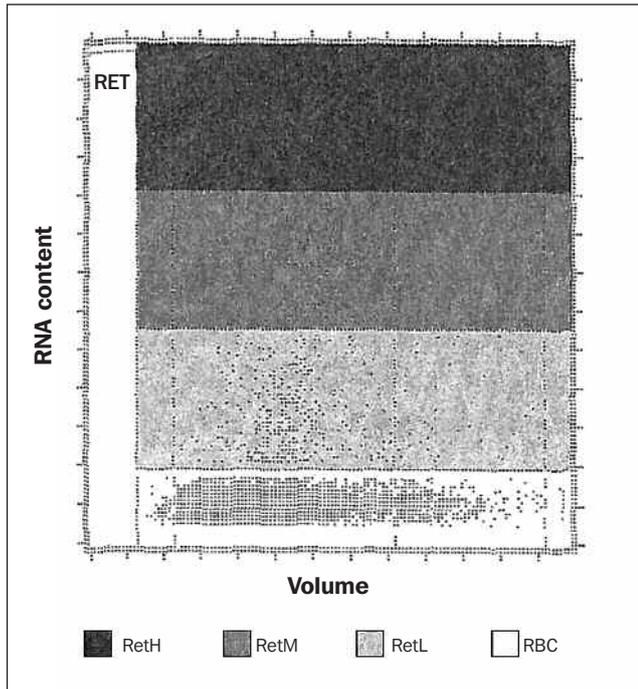


Fig. 1. A reticulocyte matrix from the ABX Pentra 120 analyser showing the areas in which reticulocytes are plotted according to levels of fluorescence.²⁰

reticulocyte fractions into a single score. Thus, an increase in the IRF constitutes an increase in immature reticulocytes recently released from the bone marrow, and is therefore a more reliable indicator of current bone marrow function.

The IRF parameter is of significant clinical benefit in the assessment of a) haematologic recovery in patients following chemotherapy, b) the first sign of engraftment in patients undergoing bone marrow transplantation, and c) the monitoring of patients undergoing recombinant erythropoietin or iron therapy. However, there is also potential use for the IRF parameter in the diagnosis of anaemia (i.e., to determine whether an anaemia is hypoproliferative, ineffective or haemolytic).³ Another potential clinical use of the IRF may be in the assessment of the need for red cell transfusion in patients with anaemia.

There has been little consensus to date on which clinical indications recommend transfusion. Variations in approach often depend upon the particular view of the requesting clinician rather than on defined characteristics of the patient involved. Thus, there may be a risk of inappropriate prescription of blood and blood products. However, there will be a need for red cell transfusion for some patients with anaemia. Currently, the decision to transfuse an anaemic patient is complex and involves multiple factors including age, disease state, ongoing blood loss, clinical symptoms, laboratory findings (e.g., blood oxygen-carrying capacity) and their compensatory capacity and adaptive mechanisms. As clinicians rely too heavily on the haemoglobin concentration, however, there may be an excessive use of blood transfusion.

Specifically, the decision to transfuse can be particularly complex when haemoglobin levels are 70–100 g/L.¹³ Chronic anaemia is much better tolerated than acute anaemia due to the greater time for adaptive mechanisms in the former,

including a shift in the oxygen dissociation curve.^{14,15} However, reticulocytes are a good indicator of the bone marrow's current erythropoietic status,¹⁶ and their number should increase following the development of anaemia¹⁷ to indicate a response from the bone marrow to replace the deficiency in erythrocytes.

The IRF may be a particularly sensitive measure of a patient's erythropoietic status as it includes a count of the most immature reticulocytes and indicates whether or not an anaemic patient may recover from their anaemia without the need for blood products (i.e. if IRF increases). If IRF remains low (i.e. ineffective erythropoiesis¹⁸) then the patient will require a blood transfusion.

There is some controversy over whether or not the erythropoietic response to anaemia is reduced with age,¹⁹ and this should be kept in mind given the age profile in this study. However, the erythropoietic response has been shown to be equal between the sexes.¹⁹

This study investigates IRF as an inexpensive, non-invasive and objective indicator of a patient's bone marrow response by correlating IRF values with haemoglobin levels in patients with chronic or acute anaemia.

Materials and methods

Ethical approval for the study was obtained from the Airedale Research Ethics Committee. Patients who had received blood products for their anaemia were excluded from the study.

Study population

EDTA-treated venous blood samples were taken from 37 anaemia patients at the Airedale General Hospital with haemoglobin (Hb) values <100 g/L, as determined by full blood count (FBC) analysis on an ABX Pentra 120 Retic haematology analyser (Montpellier, France). The normal ranges for adult Hb levels for a patient group of this type are 130–170 g/L (men) and 120–155 g/L (women).² Surplus blood was analysed to assess total reticulocyte count and IRF.

Patients were separated into two groups based on the clinical information given on the request form. Those diagnoses involving blood loss (e.g., GI bleed and post-operative anaemia) were classified as acute anaemia ($n=16$; males 5, females 11). Other diagnoses (e.g., chronic renal failure) were used to define a chronic anaemia group ($n=21$; males 2, females 19). The 30 women were aged 38–95 years (mean: 75.7 years), while the seven men were aged 42–85 years (mean: 60.2 years).

EDTA-treated venous blood samples were also collected from 40 individuals without anaemia (males: $n=16$, age 21–65 years [mean: 46.1 years]; females: $n=24$, age 18–83 years [mean: 41.6 years]) and analysed within six hours of collection to establish reference ranges for the absolute reticulocyte numbers, percentage reticulocytes and IRF.

Analysis

The ABX Pentra 120 Retic analyser (Version 4.5) was used to provide FBC, absolute reticulocyte, percentage reticulocyte and IRF values. A 0.8 μ L aliquot of whole blood was mixed with 2.5 mL proprietary formulation of thiazole orange specific to nucleic acids (under licence from Becton Dickinson, San Jose, CA) and incubated for 25 sec at 35°C.

An aliquot of the solution was transferred to the flow cytometer and cells were analysed sequentially to determine the true volume by resistivity and fluorescence determined by a 20 mW argon ion laser at 488 nm. A maximum of 32,000 cells/blood sample were analysed using customised gating to separate reticulocytes from mature RBCs, WBCs and platelets.

The results were displayed on a reticulocyte matrix with RNA content (y axis) plotted against cell volume (x axis). Reticulocyte maturity was assessed and divided into three classes: low RNA content (RetL), medium RNA content (RetM) and high RNA content (RetH) (Fig. 1). In addition, cells in the immature area (IMM) of the matrix where highly fluorescent elements such as immature reticulocytes and nucleated RBCs are located were also quantitated. The IRF, expressed as a fraction (range: 0.00–1.00), was calculated using the following formula:

$$\frac{(\text{RetH} + \text{RetM} + \text{IMM})}{\text{Total reticulocyte count}}$$

The mean values for each parameter were calculated with standard deviation (SD), and reference ranges were derived from the mean \pm 1.5 SD. Data from each study population (i.e., chronic anaemia and acute anaemia) were analysed separately by Pearson's correlation between Hb, absolute reticulocyte count and IRF, and the significance was noted. Descriptive statistics for each group and variable were calculated (SPSS software program version 12, SPSS, Illinois, USA).

Results and discussion

The reference ranges for absolute reticulocyte numbers, percentage reticulocytes and IRF were established for 40 samples taken from non-anaemic individuals (Table 1). The mean absolute reticulocyte count was $53 \times 10^9/\text{L}$ (SD: 19, reference range: 25–82 $\times 10^9/\text{L}$). This agrees with those reported by Nobes and Carter²¹ (19.4–59.2 $\times 10^9/\text{L}$) and by Cavill²² (25–75 $\times 10^9/\text{L}$). However, they are at variance with those reported for absolute reticulocyte counts by Lewis *et al.*² (50–100 $\times 10^9/\text{L}$). This difference could be explained by the number of different methods now used to achieve absolute reticulocyte values.

The mean IRF was 0.13 (reference range: 0.06–0.20). As this parameter is a relatively new addition to haematology analysers, no single reference range guide exists. However, values reported by Davis and Bigelow²³ (0.05–0.20) and by Chang and Kass²⁴ (0.044–0.228) are similar to the values reported here (0.06–0.20). Variation with other studies may be explained by issues of IRF parameter standardisation in different analyser technologies and the need for the development of IRF calibrants and quality control material.

The iron status of the participants was not determined in this study. Effective erythropoiesis requires sufficient iron stores to prevent the development of iron-deficiency anaemia.²⁵ Reticulocyte fluorescence can be elevated in iron deficiency due to the increase in cytoplasmic levels of transferrin receptor messenger RNA (mRNA).^{25–27} It should be noted that this may lead to a false increase in the IRF, and so distort results. For the purposes of this study, an assumption was made that there was adequate iron present for effective erythropoiesis.

Table 1. Reference ranges calculated for the absolute reticulocyte count and the IRF.

Parameter	Mean	Standard deviation	Reference range (mean \pm 1.5 SD)
Absolute reticulocyte count ($\times 10^9/\text{L}$)	53	19	25–82
IRF	0.13	0.05	0.06–0.20

Participant numbers were limited by the inclusion only of those who were able to provide more than one set of data. This enabled multiple comparisons to be made between a patient's Hb, reticulocyte values and IRF during their episode of acute or chronic anaemia. This provided more accurate data than solely relying upon a single measurement, giving a 'snapshot' of erythropoiesis. However, this reduced the number of eligible participants to those who could contribute sufficient data and were also able to provide informed consent to take part in the study.

Results for each study population (i.e., those with acute anaemia and chronic anaemia) were analysed separately. Pearson's correlation between Hb, absolute reticulocyte count and IRF was calculated and the level of significance determined. For patients with acute anaemia, a stronger negative correlation between Hb level and IRF (-0.464 , $P < 0.001$) was found than for Hb level and absolute reticulocyte count (-0.239 , $P < 0.01$). This observation, though weaker, was also found for patients in the chronic anaemia group with a negative correlation (-0.230 , $P < 0.01$) between Hb level and IRF, and a correlation (-0.150 , $P < 0.05$) for Hb and absolute reticulocyte count. These data suggest that the IRF is a worthwhile parameter to use in the investigation of anaemia, and it may be beneficial to incorporate it with established parameters used for anaemia investigation.

The IRF is more closely linked to Hb level than absolute reticulocyte count, yet the latter is the test more often utilised. Moreover, this relationship with Hb level is stronger for patients with acute anaemia than with chronic anaemia. This reflects the expectation that acute anaemia is more likely to cause erythropoietic stress and so produce a stronger bone marrow red cell response, while chronic anaemia is associated with the development of adaptive mechanisms and tends to be better tolerated.

Descriptive statistics for these patient groups showed that in the acute anaemia group the average Hb was 95.5 g/L (SD: 1.084), the average IRF was 0.30 (SD: 0.199) and the average absolute reticulocyte count was $71.5 \times 10^9/\text{L}$ (SD: 42.18). In

Table 2. Descriptive statistics of the haemoglobin, IRF and absolute reticulocyte parameters for acute and chronic anaemia groups.

Acute anaemia			Chronic anaemia		
Mean Hb (g/L)	Mean IRF	Mean absolute retic count ($\times 10^9/\text{L}$)	Mean Hb (g/L)	Mean IRF	Mean absolute retic count ($\times 10^9/\text{L}$)
95.5	0.30	71.5	93.4	0.32	76.8
SD 1.084	SD 0.119	SD 42.18	SD 0.866	SD 0.125	SD 41.09

the chronic anaemia group the average Hb was 93.4 g/L (SD: 0.866), the average IRF was 0.32 (SD: 0.125) and the average absolute reticulocyte count was $71.6 \times 10^9/L$ (SD: 41.09) (Table 2).

This study provided evidence of a link between IRF and Hb level in anaemic patients, with the link being stronger in patients with acute anaemia. The sensitivity and specificity of the IRF and absolute reticulocyte count parameters show that they are of value when combined with other haematology tests (e.g., FBC) in the investigation of anaemia. Validation of the IRF shows it to be a reliable and accurate method of reticulocyte analysis, but further work should be carried out to provide quality control and calibrant material specifically for this parameter.

Standardisation of the various methods employed currently by automated haematology analysers should be achieved to enable the IRF to become a more frequently used and better understood value for clinicians. To achieve statistically significant results with limited numbers of participants supports the need to investigate this under-utilised parameter further.

The results of this study support the use of the IRF when assessing a patient's need for a blood transfusion, especially if Hb level is 70–100 g/L, a range for which current published guidelines are not definitive in the use of red cells. □

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