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Review Article

Clinical Utility of Immature Reticulocyte Fraction

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ABSTRACT

Automated flow cytometric analysis has led to a significant advance in reticulocyte counting, providing the Immature Reticulocyte Fraction (IRF). IRF is one of the newer parameters of automated hematology analyzers and is a sensitive measure of erythropoiesis. The manual reticulocyte counts enumerate all RNA stained cells and simply puts together immature and mature reticulocytes. It is also laborious and time-consuming. Flow cytometric reticulocyte analysis is more precise and sensitive than the manual count. Besides, the measured fluorescence intensity allows quantification of reticulocyte maturity. IRF gives a basic idea about the marrow erythropoietic activity and its response to drugs and therapy. Moreover, it is simple, quick, cost-effective, reproducible and reliable tool on the automated hematology analyzer. It is also important for the evaluation of aplastic anemia in cases of pancytopenia, as an indicator of post-chemotherapy bone marrow recovery in acute leukemia patients, and in guiding stem cell harvest in autologous peripheral blood stem cell transplant. IRF, in combination with the reticulocyte count, might be useful in improving the classification of anemia, monitoring bone marrow recovery, and monitoring anemia therapies. It is necessary to establish an international consensus about the definition and reference range of IRF, to compare results obtained from different hematology analyzers.

Keywords: Immature reticulocyte fraction; Anemia; Hematopoiesis; Reticulocyte; Leukemia

INTRODUCTION

The reticulocytes are young Red Blood Cells (RBCs) that form a reticulum network or granules on exposure to those supravital stains. The reticulum network or granules represent precipitated rough endoplasmic reticulum with associated polyribosomes [1]. During erythropoiesis, reticulocytes are released into the circulation where they gradually lose their RNA, and evolve into mature RBCs [2]. The International Council for Standardization in Hematology (ICSH) definition states that reticulocytes must have at least 2 blue staining granules, located away from the cell margin to avoid confusion with Heinz bodies [3]. When the late-stage erythroblast loses its nucleus, the cell becomes a reticulocyte that usually remains in the bone marrow for 3 days, traverses the marrow sinusoid and is subsequently released into the circulation, where its maturation is completed in 1 day [4].

The newly formed reticulocytes degrade internal organelles and shed specific plasma membrane proteins and residual portions of the organelles. The existence of the number of reticulocytes in the peripheral blood is a useful clinical indicator of the rate of erythropoiesis. Reticulocyte populations themselves are considerably heterogeneous due to differences in the stage of maturation of individual reticulocytes. Immature reticulocytes have more reticulin, motility, and irregular shapes compared to mature reticulocytes [5]. They are biochemically more active than mature ones, and some activities such as hexokinase, pyruvate kinase, Glucose-6-Phosphate Dehydrogenase (G6PD), and oxygen transport are high in those cells.

The term Immature Reticulocyte Fraction (IRF) was introduced to indicate the less mature reticulocyte fraction. The IRF represents the proportion of young reticulocytes with the highest RNA content [6]. It is defined as the ratio of immature reticulocytes to the total number of reticulocytes. They are larger, having the greatest light scatter properties due to the highest level of Ribonucleic Acid (RNA). They are one of the newer

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parameters of automated hematology analyzers and is a sensitive measure of erythropoiesis [7,8].

IRF is the RBCs equivalent of the "left shift" typically associated with neutrophil White Blood Cells (WBCs), providing additional RBC information that may shorten the time from diagnosis to therapy or therapy itself [9]. It is a new parameter in hematological analysis, which can give an idea on an early morphological change for bone marrow recovery before other tests to be positive after chemotherapy. It is an examination of bright reticulocyte fraction with a high content of RNA. Immature reticulocytes normally constitute less than 5% of the total number of reticulocytes. The normal reference range for IRF is from 3.0%-15.9% in males and 2.3%-13.4% in females IRF more than 5% were taken as bone marrow recovery, but most investigators consider an IRF value greater than 10% to indicate early marrow recovery. Immature reticulocytes are released into the peripheral blood during periods of intense erythropoietic stimulation, such as hemorrhage, certain anemia, or in response to therapy designed to stimulate bone marrow production [10]. The manual reticulocyte counts enumerate all RNA stained cells and simply puts together immature and mature reticulocytes. It is also laborious and time-consuming. IRF replaces other Reticulocyte indices like Absolute Reticulocyte Count (ARC) and Reticulocyte Production Index (RPI) that are important to see the degree of erythropoietic activity. A higher proportion of circulating immature reticulocytes (high RNA content) indicates recovering marrow activity and is quantitated by automated hematology cell analyzers. Flow cytometric reticulocyte analysis is more precise and sensitive than manual reticulocyte counting. Besides this, the measured fluorescence intensity allows the quantification of reticulocyte maturity [11].

Assessment of reticulocyte maturity is based on the intensity of either fluorescence or light scattering/absorbance, which depends on RNA content. Different populations are discriminated by a software-based algorithm that usually clusters reticulocytes into 3 areas according to stain intensity. The fluorescence intensity of the entire reticulocyte population was initially reported as the reticulocyte maturation index or the mean fluorescent index. Reticulocytes have now been grouped into the Low Fluorescent Region (LFR), Middle Fluorescent Region (MFR) or High Fluorescent Region (HFR) corresponding to the lower, middle and higher RNA content, respectively. The percentage of reticulocyte is given as the sum of LFR, MFR and HFR. IRF measures the MFR and HFR populations and is more reproducible than the HFR. Immature fractions have larger amounts of RNA than mature reticulocytes. Thus, the uses of fluorescent probes that label the RNA permit the differentiation and quantification of the IRF. Automated systems provide a graphic display of the different populations according to the size and amount of RNA. Based on this, the indices are calculated as a percentage of total reticulocytes [12].

CLINICAL UTILITY OF IMMATURE RETICULOCYTE FRACTION (IRF)

IRF is a new diagnostic indicator based on flow cytometric determination of RBCs RNA content. It also gives a basic idea about the marrow erythropoietic activity and its response to at an early stage thereby they are useful for monitoring therapy by the physicians without investigating the marrow. IRF just reflects the erythropoietic activity of bone marrow but does not show the actual incorporation of iron in developing RBC's. In certain situations like bleeding, IRF will increase despite no improvement in Hgb. IRF, in combination with the reticulocyte count, might be useful in improving the classification of anemia, monitoring bone marrow recovery, and monitoring anemia therapies.

The clinical utility of IRF has been reported in a variety of conditions such as in the diagnosis of anemia (i.e. to determine whether an anemia is hypo-proliferative, ineffective or hemolytic) and its treatment monitoring, transfusion needs, renal transplant engraftment due to Erythropoietin (Epo) production, the detection of hemorrhages or hemolysis, and assessment of the need for RBC transfusion in anemic patients [13]. They also could potentially be useful in the management of neutropenic cancer patients and in the investigation of antimicrobial therapies [14].

Role of IRF in the evaluation of cases of anemia

Pancytopenia is an important clinical and hematological entity worldwide but with varying patterns in clinical presentations [15]. Bone marrow aspiration is considered as a primary importance in evaluating and diagnosing the cause of pancytopenia. But rather than doing bone marrow aspiration or biopsy, IRF helps to get a picture about the marrow erythropoietic activity. They also help in differentiating aplastic anemia from common nutritional deficiency anemia's that presents as pancytopenia, which is not possible by a reticulocyte count since it is more or less equal in most of the cases. For the diagnosis of various types of anemia, IRF serves as an adjunct to Total Reticulocyte Count (TRC) but is usually not of much help alone in differentiating them all. IRF rises in cases of increased marrow erythropoiesis before the increment of reticulocyte count. Therefore, those cells were found to be the earliest indicator of marrow erythropoietic activity.

IRF differentiates category 1 and 2 cases from category 4 since IRF was zero to normal (<5%) in first two categories but high in category 4. The reticulocyte count was variable in both categories. IRF is also specific in the diagnosis of aplastic anemia cases since IRF is absolute zero where reticulocyte count is variably low. In category 3, there is high IRF with normal to high reticulocyte count, indicating the increased marrow erythropoiesis than normal thus indirectly indicating a peripheral cause of pancytopenia while bone marrow is normal [16].

Peripheral blood reticulocytes from patients with ineffective erythropoiesis have a larger volume and display more RNA content when compared to other causes of macrocytic anemia. Differential diagnosis from several disorders, including mainly MDS, is often challenging. Routine hematological parameters, including reticulocyte counts, do not distinguish these disorders, especially in cases without prominent dysplastic features in peripheral blood smears. However, reticulocytes in MDS are more immature, with a larger size and fluorescence index. This

specific reticulocyte profile in MDS has not been extensively reported and the potential utility of this profile in the differential diagnosis of MDS has not been tested before. It is reasonable to conclude that this circulating, highly immature reticulocyte fraction could reflect abnormal erythropoiesis (with persistence of abnormal cytoplasm structures and elevated amounts of RNA) in the dysplastic clone [17,18].

IRF as an indicator of post-chemotherapy bone marrow recovery in acute leukemia

Acute leukemia is a malignant disorder in which neoplastic White Blood Cells (WBC) arising from hematopoietic stem cell infiltrate blood and bone marrow. Intensive chemotherapy is the available treatment option that can be combined with stem cell transplantation [19]. Absolute Neutrophil Count (ANC) is generally accepted as a primary indicator of successful bone marrow recovery. An increase in ANC ≥ 0.5 × 109/L defines successful myeloid recovery after chemotherapy [20]. The ANC has been used to guide decision making on cessation of antibiotic therapy, discharge from the hospital, and resumption of chemotherapy. Even if it is an easy and practical method for evaluating bone marrow recovery, the count may fall during periods of clinical or subclinical infection. This may provide inaccurate information on the actual status of bone marrow. Reticulocytes are a better parameter for use as a marker of bone marrow recovery in neutropenia patients as they aren't affected by underlying infection.

IRF parameter showed earlier hematopoietic recovery than the current practice of ANC recovery for monitoring in children with Acute Lymphoblastic Leukemia (ALL) after chemotherapy. This early laboratory indicator will guide the clinicians to make important therapeutic decisions, which will be economic and live-saving for the patients [21]. Nowadays, IRF is offered most of the third generation hematology analyzer. Moreover, it is simple, quick, cost-effective, reproducible and reliable tool on the automated hematology analyzer. Thus its potential use as a routine test to see the bone marrow recovery is important.

IRF as a useful parameter for blood transfusion assessment in anemic patients

Another potential clinical use of the IRF may be in the assessment of the need for RBCs transfusion in patients with anemia. Currently, the decision to transfuse an anemic patient is complex and involves multiple factors including age, disease state, ongoing blood loss, clinical symptoms, laboratory findings and their compensatory capacity and adaptive mechanisms. Clinicians rely too heavily on the Hgb concentration and this leads to excessive use of blood transfusion. Specifically, the decision to transfuse anemic patients can be particularly complex when Hgb levels are 7-10 g/dL, a range for which current published guidelines are not definitive in the use of RBCs [13].

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 anemic patient may recover from anemia without the need for blood products and this is confirmed by an increment of IRF. If IRF remains low (i.e. ineffective erythropoiesis) then the patient will require a blood transfusion [2]. RBCs transfusion did not appear to affect the IRF values. However, the time to IRF doubling (IRF-D) was one day longer for patients with lower Hgb levels probably reflecting a poor bone marrow reserve. Even among these patients, the IRF-D still preceded ANC recovery. The IRF normal values (IRF-N) can be particularly useful in patients lacking the daily reticulocyte values needed to calculate the IRF-D.

IRF in guiding stem cell harvest in autologous peripheral blood stem cell transplantation

Autologous peripheral blood stem cell transplant has been widely used as a treatment tool in various hematological disorders. Predicting the timing for stem cell harvesting in autologous peripheral blood stem cell transplant remains a major problem as these patients were previously treated with chemotherapy with variable bone marrow reserve. To predict the optimal timing for stem cell harvesting, various parameters have been used including quantification of Colony-Forming Unit-Granulocytes Macrophages (CFU-GM) and monitoring of WBC count. However, the quantification of CFU-GM requires 2 weeks to obtain the results; thus, this limits its usage as a guide for stem cell harvesting. Peripheral blood CD34+ cells enumeration is currently the most reliable method to guide the timing of stem cell harvest. However, its usage is restricted by being technically challenging, costly, and time-consuming [22].

IRF is important to predict the timing of stem cell collection. The presence of immature hematopoietic cells such as IRF indicates the imminent recovery of erythropoiesis after stem cell transplant therapy. IRF determination, which is simpler and cheaper and has a faster turn-around time, has been proposed for a similar purpose. IRF level is instantly available as it is part of a routine complete blood count. IRF determination appears to be a viable approach in optimizing Peripheral blood stem cell collection. Its increment is also the first response after bone marrow ablation which precedes the increase in the reticulocyte count by several days.

The role of IRF in response to anemia treatment

The IRF value is an early marker for evaluating the regeneration of erythropoiesis. The IRF percentage increases after only a few hours, but the reticulocyte count increases after 2-3 days. It is useful in monitoring the efficacy of therapy in nutritional anemia such as megaloblastic or iron deficiency anemia. After treatment for nutritional anemia (B12, folate or iron deficiency), the increase in IRF occurs several days before an increase in the reticulocyte count. If the IRF value does not increase during the treatment of deficiency anemia with Epo or vitamins, this indicates a lack of response to therapy. Also, it helps to classify hypo-, normo- and hyper-regenerative anemia [23].

IRF as an indicator of erythropoietic response to altitude training in elite cyclists

In sports laboratory medicine, immature reticulocytes are now the subject of studies to detect abnormal bone marrow stimulation. IRF is a sensitive marker of erythropoietic status in athletes undergoing altitude training. The response of IRF has been widely studied among diverse groups of athletes. Among elite athletes, IRF reference ranges were found not significantly different from the control population although some studies have indicated that athletes generally exhibit higher values than the controls although never reaching pathological ranges [24].

Role of IRF in the diagnosis of Hereditary Spherocytosis (HS)

Diagnosis of HS depends on family medical history, clinical manifestations, and laboratory examinations. Laboratory tests used to diagnose HS include RBC morphology examination, osmotic fragility test, RBC membrane protein measurement, and membrane protein mutation detection. There is currently no single index for the diagnostic screening of HS. However, detection of some blood cell parameters by highly automated analyzers may be useful for the early screening of HS. Investigators have reported data concerning the clinical utility of IRF in the diagnosis and monitoring of anemia [25].

Different hypotheses explain the presence of a high reticulocyte count without an equally elevated IRF in HS. The first hypothesis states that there is an insufficient entry of the fluorescence dye into the defective cells and thus staining reaction is abnormal at the time of measurement. This results in decreased staining of RNA. A lower RNA concentration in the RBC implies increased maturity so that immature reticulocytes will be falsely classified as a more mature fraction. Consequently, the highly fluorescent immature reticulocytes containing the most RNA are decreased. The second hypothesis is the early loss of cell surface area during HS. The maturation of reticulocytes into RBC is associated with a loss of intracellular organelles including mitochondria, endoplasmic reticulum, Golgi apparatus, and endocytic vesicles. In HS, the loss of cell surface area at the reticulocyte stage could explain that one or multiple of these compounds normally stained by the fluorescent dye in the reticulocyte channel are less marked in cases of HS.

IRF in leukemic patients and other types of cancer

A normal reticulocyte scatter gram pattern is composed of a region of reticulocytes located between regions of mature RBCs and an Upper Particle (UPP) region. Moreover, these fractions form a continuous non-separated distribution. Thus, UPP values indicate primarily erythroblast numbers and include some immature reticulocytes.

The dye used for reticulocyte analysis stains leukocytes and reticulocytes. Generally, leukocytes do not overlap with reticulocyte regions because of their high fluorescence. Leukocytes of insufficiently stained leukemic patients may have lower fluorescence, and thus may be misidentified as immature reticulocytes and cause erroneously elevated IRF values in leukemia patients. The study shows that in leukemic patients, when a reticulocyte scatter gram pattern is abnormal and the UPP value is high, the IRF value may be erroneously high [16].

IRF in Assessment of Erythroid Regeneration Following Parvo B19 Infection

Parvovirus B19 has a predilection for replication in the bone marrow erythroid progenitor cells including the erythroid Colony-Forming Units (CFU-E) and the Burst-Forming Units (BFU-E). It is an erythrotropic virus which attaches through the 'P' globoside receptor on the surface of human RBCs and precursors. Globoside is found on cells within the bone marrow such as early erythroid progenitor cells, megakaryocytes, and on cells within the placenta, fetal myocardium, kidney, and thyroid. The P antigen, a globoside found on erythroid progenitor cells, serves as cell receptor for viral attachment. This typically benign viral infection can cause a transient aplastic anemia in patients with underlying red cell disorders. A hemolytic anemia associated with transient bone marrow erythroid hypoplasia is a serious complication of Parvovirus B19 infection. Infected cells fail to proliferate and mature thus prohibiting the production of new RBCs. Disturbances in the dynamic equilibrium of erythropoiesis can be monitored clinically by the ARC and the maturity of the subpopulations of reticulocytes quantified by flow cytometry. The quantification of RNA in reticulocytes has generated a useful clinical parameter known as the IRF. This parameter has significant clinical application in assessing regenerative and nonregenerative hematologic conditions [12].

IRF showing erythropoiesis differences in various clinical phases of dengue fever

Dengue is the most rapidly spreading mosquito-borne viral disease in the world. The hematological disorders known in dengue viral infection are temporary thrombocytopenia and leucopenia. Erythropoiesis disorder is also observed in dengue infection, which may be caused by the bone marrow suppression affecting all of the hematopoiesis series. It is hypothesized that a combination of viral infection on hematopoietic progenitor cells, and the viral infection on bone marrow stromal cells and dengue specific Tell activation, both releasing cytokines that suppress the bone marrow. Erythropoiesis suppression during dengue infection was also shown in the form of aplastic anemia by several case reports [9].

CONCLUSION

IRF is a newly routine parameter in hematological analysis, which can give an idea on an early morphological change for bone marrow recovery before other tests to be positive after chemotherapy. The clinical utility of IRF has been reported in a variety of conditions such as in the monitoring of diagnosis of anemia and its treatment, to verify aplastic anemia, and assessment of the need for RBC transfusion in anemic patients. They also could potentially be useful in the management of neutropenic cancer patients and the investigation of antimicrobial therapies. The IRF is a promising parameter that

needs consolidation into clinical practice. It is important to standardize the reference ranges of IRF and further investigations are needed on these indices to make it useful at the most. The utility of IRF as a marker to detect Epo abuse by athletes and their role in monitoring the response to Epo in chronic stable dialysis patients may also need to be explored.

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