Original Research Article

Clinico-hematological correlation of pancytopenia with special reference to the role of IRF (immature reticulocyte fraction) by automated cell counter in a tertiary care hospital

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Received: 03-01-2021 / Revised: 30-01-2021 / Accepted: 20-02-2021

Abstract

Background : Pancytopenia is an important entity worldwide but with varying patterns in clinical presentations. Bone marrow aspiration and biopsy are considered as primary investigation to evaluate and diagnose the causes of pancytopenia. But before doing a bone marrow aspiration or biopsy, a note on the newer reticulocyte indices given by automated cell counters other than reticulocyte percent and absolute reticulocyte count helps us to get a picture about the marrow erythropoietic activity which also bypasses the inter-observer variability. These newer reticulocyte indices are the Immature Reticulocyte Fraction (IRF), Reticulocyte Hemoglobin Content (CHr or Ret-He), difference between the reticulocyte and erythrocyte hemoglobin content (Delta-He).We attempted to emphasize the importance of Immature Reticulocyte Fraction (IRF) over other reticulocyte indices in diagnosis of pancytopenia and assessment of marrow response to therapy.**Methods***:* In this study patient's history were taken. Then EDTA mixed blood examined by Automated Cell Counters (Sysmex XT-4000i) and subsequently bone marrow examination has been done to confirm the etiology. Results were calculated statistically.**Results**: We found that values of IRF were also significant in the diagnoses of Megaloblastic Anaemia, Aplastic Anaemia, early Marrow Recovery from suppression, Hemolytic Disease and Chronic Diseases.**Conclusion***:* It is hypothesized that IRF is an index of acceleration and the absolute reticulocyte count is a quantitative measurement of the effectiveness of erythropoiesis. So after initiation of treatment, repeated observation of both IRF and Reticulocyte Count may be helpful to observe the effectiveness of therapy.

Keywords*:* Pancytopenia, Anaemia, Reticulocyte Count, Immature Reticulocyte Fraction (IRF).

Introduction

Pancytopenia is an uncommon medical condition characterized by a reduction in all the cellular elements of the peripheral blood lineages: leukocytes, platelets, and erythrocytes. It is defined as: hemoglobin < 10 g/dL, total leukocyte count < 4×10^9/L and platelet count < 100×10^9/ L.[1] Though pancytopenia is not very common, fatality is very high.[2]The worldwide incidence of pancytopenia is 2.34 per million per year.[2] Though detailed data about the incidence of pancytopenia is not available from any large scale study, it was around 2.9% in patients aged 1-18 years in India.[3] There are multiple causes of pancytopenia varying widely, ranging from viral transient marrow suppression to marrow infiltration by life-threat-ening malignancy. Pancytopenia requires microscopic examination of a bone marrow biopsy and a marrow aspirate to assess overall cellularity and cellular morphology.The underlying mechanisms are decreased in hematopoietic cell production, marrow replacement by abnormal cells, suppression of marrow growth and differentiation and ineffective hematopoiesis resulting in cell death. Bone marrow examination is extremely useful in the evaluation of pancytopenia. Megaloblastic anaemia is one of the common cause of pancytopenia in India. [4] Megaloblastic Anaemia can be caused by a deficiency of both or either vitamin B12 and folate deficiency (65% pure cobalamin deficiency and 12% combined deficiency). This disease is usually seen in lactovegetarians. [4] Another common cause of pancytopenia is Aplastic anaemia where immune-mediated bone marrow destruction occurs. Relatively acellular fatty aspirate yields in bone marrow aspiration may be obtained according to age. Most common haematological malignancies which cause pancytopenia are AML (>60%), ALL (>25%), plasma cell myeloma, non-Hodgkin's lymphoma, myelodysplastic syndrome (RAEB 2) etc. [5] The immature reticulocyte fraction (IRF) which is a new diagnostic indicator measured in automated cell counters are based on the flow-cytometric determination of erythrocyte-RNA content.[6] IRF gives a basic idea about the marrow erythropoietic activity and its response to drugs way before other findings in peripheral blood like an increase in reticulocyte count or the absolute neutrophil count.[7] This helps in the early administration of therapy and monitoring response without repeated bone marrow examination. A reticulocyte percentage of (0.2-2%) and IRF of <5% is taken as the normal reference range.[8,9] We attempted one of the newer reticulocyte indices (IRF) to categorize the cases of pancytopenia for individualization of management and an easier way to monitor the effect of therapy. [10,11] These newer indices specially IRF is a useful method for evaluating pancytopenia due to its simplicity, low cost, rapid result, high specificity and sensitivity. [12] So here we attempted to emphasize the importance of Immature Reticulocyte Fraction (IRF) in diagnosis the cause of pancytopenia and in the

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assessment of marrow response to therapy without doing repeated bone marrow examination.

Materials and Method

The study was performed in the Department of Pathology in association with the Department of General Medicine and the Department of Pediatric Medicine after obtaining the approval from the Ethical Committee of the Institute of Postgraduate Medical Education and Research, Kolkata. Patients, who presented with symptoms related to pancytopenia, were examined. After obtaining detailed clinical history, peripheral blood smears, bone marrow aspiration smear and bone marrow biopsy specimens were carefully examined in all patients. Sysmex XT-4000i ((Kobe, Japan) hematology auto analyzer was used for peripheral blood

Results

In our single center prospective observational study conducted in the Department of Pathology in association with Department of General Medicine and Department of Pediatric Medicine, IPGME & R, Kolkata over 18 months 190 cases presented with Pancytopenia. Patients were selected from all age groups (1- 85 years) and both sexes. Out of 180 cases, 105 were males and 85 patients were females. We found 45 cases of Aplastic Anaemia, 27 cases of Malignancy (22 hematological and 5 cases of metastasis from non-hematological primary), 13 cases of Hypersplenism, 6 cases of Hemophagocytic Lymphohistiocytosis, 2 cases of DCT +ve Hemolytic Diseases, 46 cases of Megaloblastic Anaemia, 18 cases of Myelodysplastic Syndrome, 25 cases of Early Recovery from Bone Marrow Suppression (16 post cancer chemotherapy & 9 cases of Megaloblastic Anaemia on Treatment), 5 cases of Bone Marrow Infections and 3 cases of Storage Diseases. All the diagnoses were correlated with other ancillary investigations. The common diseases of childhood (<18yrs) were ALL, DCT +ve Hemolytic Anaemia and Storage Disorders. Multiple Myeloma, Metastasis, Hypersplenism and Histoplasma infection were noted in older age groups (>50 yrs). Megaloblastic Anaemia, Myelodysplastic Syndrome, Aplastic Anaemia and AML show a wide range of age at presentation. The most common disease in male patients was Megaloblastic Anaemia (25) followed by Aplastic Anaemia (21). For female patients, it was Aplastic Anaemia (24) followed by Megaloblastic Anaemia (21). The patients affected with Parvo Virus B12 infection (1), Storage Disorders (3) and Hemolytic Disease (2), were all males. [Figure1,2]

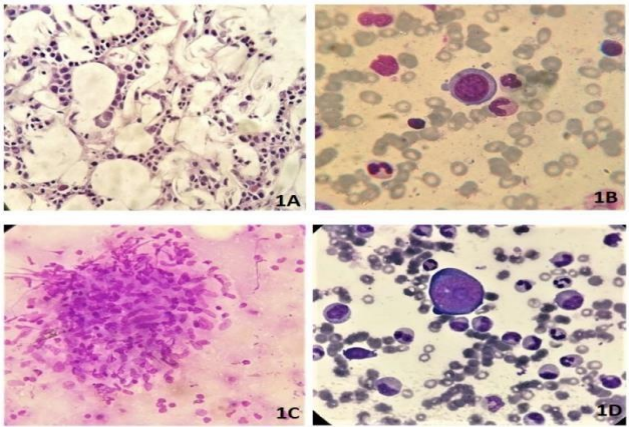


Fig 1; 1A: BMBx from a patient of Aplastic Anaemia of 16 years of old showing hypocellular marrow according to age (x400, H&E Stain);
1B: Megaloblastiod change in an late normoblast with nuclear immaturity (x1000, LG stain)
1C: Granuloma in bone marrow aspiration with collection of epithelioid cells (x400, LG stain);
1D: Parvovirus infected early normoblast with intranuclear inclusion (x1000, LG stain)

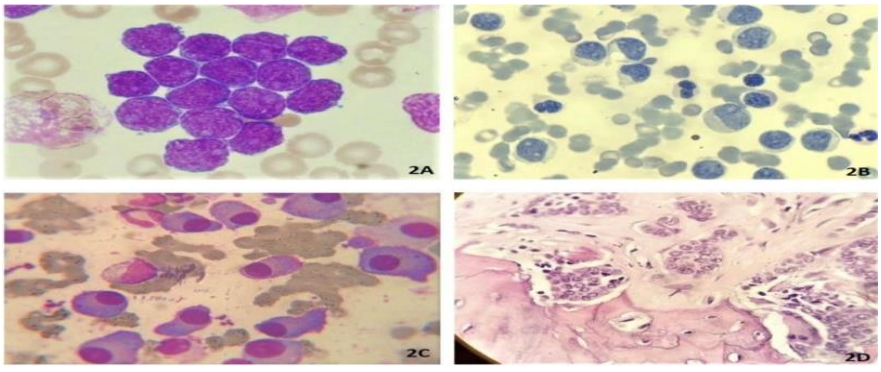


Fig 2; 2A: Lymphoblasts with hyperchromatic nucleus and scanty cytoplasm (x1000, LG Stain);
2B: Myeloblasts with irregular nucleus (x400, LG Stain),
2C: Abnormal Plasma cells in a case of Multiple Myeloma (x400, LG Stain);
2D: BMBx shows Metastatic Deposits (x400, H&E Stain)

Table 1: Comparison between Reticulocyte count and IRF value according to different diagnosis

Cases Of Pancytopenia	Reticulocyte Count		P Value	IRF		P Value
	Median	95% Range		Median	95% Range	
Megaloblsatic Anaemia	0.89	0.23- 2.75	0.169	7.95	0.30- 23.56	**0.036**
Aplastic Anaemia	0.27	0.04- 0.97	0.46	0	0	**0.032**
Hematological Malignancy	2.78	0.13- 7.94	0.255	19.60	2.60- 55.40	0.216
Metastasis	4.12	0.35- 9.12	0.888	12.30	1.53- 24.90	0.714
Early Marrow Recovery	0.74	0.13- 4.13	**0.049**	21.5	9.59- 36.20	**0.047**
Myelodysplastic Syndrome	1.19	0.33- 1.52	0.145	5.8	1.05- 17.60	0.487
Auto Immune Haemolytic Diseases	9.06	1.27- 9.64	0.956	48.70	6.78- 52.20	**0.001**
Hypersplenism	6.53	1.69- 13.02	0.652	41.45	9.03- 44.80	**0.003**
HLH	1.65	0.32- 3.90	**0.002**	17.65	4.41- 26.10	0.649
ParvoVirus B12	0.09	-	-	0.01	-	-
Other Infections	6.25	1.90- 9.80	0.914	38.40	29.60- 47.90	0.968
Storage Diseases	1.70	0.05- 1.70	0.266	12.70	1.94- 13.40	0.569

Then all the findings from the auto-analyzer (Reticulocyte and IRF) were compared with the diagnosis by bone marrow aspiration and biopsy and the results were correlated and tabulated. Based on the result we categorized all cases into five major categories.

Table 2:Categorisation of cases

Category	Criteria	Causes
Category 1	Reticulocyte count – low IRF– zero	Aplastic Anaemia
Category 2	Reticulocyte count – normal IRF – marginally high	Megaloblastic Anaemia Myelodysplastic Syndrome
Category 3	Reticulocyte count – normal to low IRF – high	Early Recovery from Chemotherapy & Megaloblastic Anaemia
Category 4	Reticulocyte count – normal to high IRF – high	Malignancy Hypersplenism Auto-Immune Hemolytic Disease
Category 5	Reticulocyte count – very low IRF - low	ParvoVirus B12 Infection

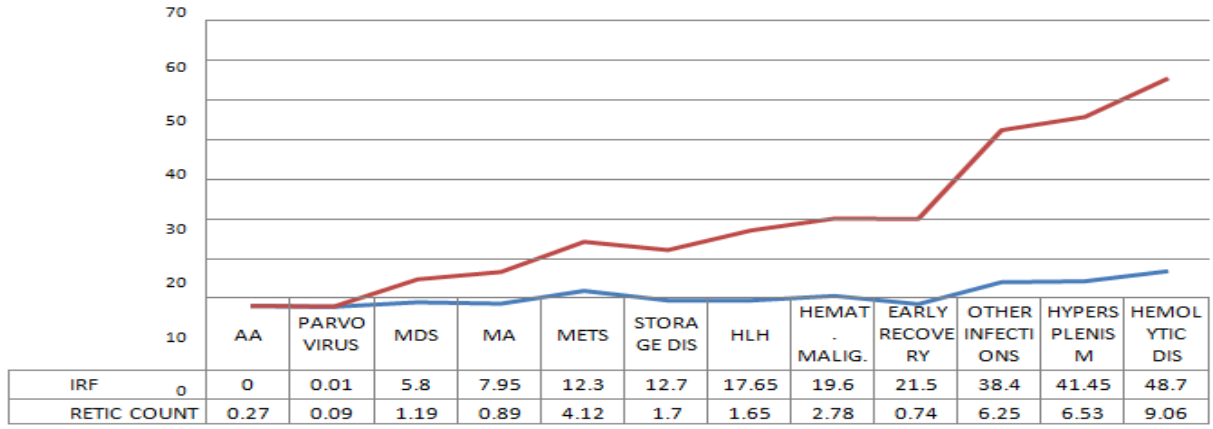
In all cases of category 1, we found that low reticulocyte count with IRF value 0 (Zero) is diagnostic of Aplastic Anaemia and statistically relevant. Aplastic Anaemia is an auto-immune condition, which causes destruction of hemopoietic cells in marrow. It was correlated with various previous studies as well. [13]In category 2, there were 45 cases of megaloblastic anaemia and 15 cases of Myelodysplastic Syndrome. All cases showed reticulocyte count in the low to normal range and IRF was marginally high. The median value of observed IRF (7.95%) for the diagnosis of Megaloblastic Anaemia was also statistically significant. MDS causes ineffective erythropoiesis and hemolysis which cause pancytopenia. There is asynchrony between the maturation of cytoplasm and nuclei leads to macrocytosis, immature nuclei, and hypersegmentation in granulocytes in the peripheral blood. In megaloblastic cells, there is delayed maturation of nuclei with normal cytoplasmicdevelopment. The bone marrow becomes hypercellular and dysplastic mimicking acute leukemia. The ineffective erythropoiesis results in intramedullary hemolysis.

In the category 3, 25 cases of early recovery from megaloblastic anaemia and bone marrow suppression due to Cancer Chemotherapy showed low to normal reticulocyte count but high IRF and were statistically significant. Patients of Megaloblastic Anaemia are treated with dietary or intra-venous supplement of vitamins. Chemotherapeutic agents are medullotoxic by their side effect. This causes severe reduction in bone marrow cellularity or causes fibrosis. Different types of hemopoitic inducers (GM-CSF, G-CSF etc.) are used to treat these patients. Immune pancytopenia is also common with presence of autoantibodies. It resolves by initiation of prednisolone therapy. These treatments cause sudden explosive hematopoiesis.

In the category 4, haemolytic disease and Hypersplenism cases showed statistically significant high Reticulocyte count and very high IRF. All these results were correlated well with the previous study conducted by Sindhu R. et al. [7] In some patients with cirrhosis, chronic hemorrhage into the gastrointestinal tract occurs. Hypersplenism secondary to portal hypertension is another mechanism of anemia in patients with chronic liver disease. Hypersplenism is associated with splenomegaly. In addition to chronic liver disease, thrombosis of the splenic vein may also be a cause of an increase in pressure within the portal venous system, which can lead to secondary hypersplenism. The main characteristics of hypersplenism are those attributable to pancytopenia.

In the Category 5, the case of ParvoVirus B12 infection showed very low Reticulocyte Count and IRF value. Low reticulocyte count due to abnormal early normoblast was correlated with previous publications. [14] Initial IRF values were low and had been shown to increase three to five days prior to the increase in reticulocyte counts after initiation of chemotherapy and therefore can assess marrow erythropoietic activity earlier than the reticulocyte count. [15] The

observations of IRF in our study correlated with previous publications but more follow-up required to observe the therapeutic effectiveness.The following graphical comparison shows the relationship between Reticulocyte count and IRF values in different diagnosis.



	AA	PARVO VIRUS	MDS	MA	METS	STORAGE DIS	HLH	HEMAT. MALIG.	EARLY RECOVERY	OTHER INFECTIONS	HYPERSPLENISM	HEMOLYTIC DIS
IRF	0	0.01	5.8	7.95	12.3	12.7	17.65	19.6	21.5	38.4	41.45	48.7
RETIC COUNT	0.27	0.09	1.19	0.89	4.12	1.7	1.65	2.78	0.74	6.25	6.53	9.06

Fig 3: Graphical comparison shows the relationship between Reticulocyte count and IRF values in different diagnosis

Discussion

In 1865, Wilhelm Heinrich Erb described Reticulocytes as "granulated" normal erythrocytes.[16]Then Theobald Smith described that reticulocytes represent young red cells. Edward Bell Krumbhaar found that those erythrocytes had more or less extensive reticulum (granular filamentous substance), observed by the methods of vital staining and named as 'reticulocytes'. He also postulated that variations in the reticulocyte percentage in disease naturally depend on the intensity of the demand and the capacity of the bone marrow to respond.

Properties of Reticulocytes in comparison with mature erythrocytes-

•Greater volume: approximately 24%
•Slightly higher Hb content: approximately 13%
•Lower Hb concentration: approximately 16.7%
•Constant volume ratio between reticulocytes and erythrocytes: approximately 1.24

Ludwig Heilmeyer proposed the classification of reticulocytes maturity in 1932 by Peripheral Blood Film Examination. In 1947, Giovanni Astaldi classified reticulocytes into 3 stages of maturity using the flow cytometry. Over the years Acridine orange, a fluorescent dye, replaced the supravital stains, improved the sensitivity of microscopic counts.Different populations of reticulocytes are separated by auto-analyzers according to the size and the amount of RNA and classified as low (LFR), medium (MFR) or high (HFR) fluorescence reticulocyte fractions. IRF is counted by the combined counts of the HFR and MFR.Grotto H. et al described that rising IRF was the first sign of hematologic recovery preceding the rise in neutrophils and platelets. Thus recurrent bone marrow examination being a painful procedure need not be a standard practice to observe recovery. [17]Gomez T. et al described presence of hypoproliferative anemia with low absolute or corrected reticulocyte counts and low serum cobalamin or folate assays to diagnose MA. They found IRF values of both MA and MDS to be high but they did not find any statistical differences between MA and MDS with respect to reticulocyte maturation parameters. In cases of megaloblastic anemias caused by abnormal lipid metabolic conditions, IRF was much lower. In our study, we found a wide range of values for IRF in cases of MDS but was not statistically significant. However we observed a larger population of MA patients and a high value (median 7.95%) of IRF to be statistically significant. [18]Conventionally, Absolute Neutrophil Count (ANC) is used as predictor of bone marrow recovery following cancer chemotherapy. But it may be influenced by clinical or subclinical infection and may give a false impression of the actual bone marrow status. So, ANC is not accurate in predicting bone marrow recovery. The Reticulocyte Maturity Index (RMI) was used as an independent parameter for assessing erythropoiesis. Reticulocytes are not influenced by infections and therefore would be a better parameter of bone marrow regeneration in patients with persistent neutropenia to guide further management. Flow cytometric reticulocyte analysis is more precise and more sensitive than manual reticulocyte counting. These methods also provide reticulocyte maturation patterns easily. When younger reticulocytes are detected in peripheral blood, it is a better indication of recovery. A Raja et al described the superiority of IRF compared with ANC in predicting bone marrow recovery in patients with acute leukemia post induction chemotherapy.[19] In nutritional anaemias, the increase in IRF precedes the increase in total reticulocyte count by several days following treatment initiation. [20] In ourstudy, we have examined 16 cases of pancytopenia due to cancer chemotherapy and 9 cases of pancytopenia due to Megaloblastic Anaemia on treatment with early recovery. In these cases we found a median range of Reticulocyte count 0.74% (Range 0.13-4.13%) which is statistically significant. But this normal to marginally elevated reticulocyte count overlaps with other conditions and is non-diagnostic. But, as proposed earlier Immature Reticulocyte Fraction was both statistically significant and had a standalone range. We found median value of IRF was 21.50% (Range 9.59- 36.20%).In reticulocytopenia, there is no difference observed in marrow aplasia and in early treatment response. Also, mild reticulocytosis may be seen in healthy individuals. Therefore, IRF and reticulocyte count may vary in an independent way according to the erythropoietic conditions. High reticulocyte count indicates a gradual deceleration of erythropoiesis following an accelerated phase when IRF shows peak value. So, it is hypothesized that IRF is an index of acceleration and the absolute reticulocyte count is a quantitative measurement of the effectiveness of erythropoiesis. [20] Based on these parameters, pancytopenia can be found in the following conditions-

(i)increase in erythropoiesis, like acquired hemolytic anemias or the loss of blood, which produces an increase both in total reticulocytes and in IRF;

(ii)reduced marrow production/ increased destruction (i.e., Aplastic Anemia) in which both values are found to be decreased,
(iii)infections or myelodysplastic syndromes in which there is a dissociation of values between total reticulocyte count and the IRF.
In our study, we also found low values of reticulocyte count in cases of only Early Marrow recovery and Hemophagocytic Lympho-histiocytosis (HLH) was statistically significant. Low Reticulocyte count was noted in Megaloblastic Ananemia and Aplastic Anemia. Moderate to high Reticulocyte count was found in Hematological Malignancy, Metastasis from other primary site, MDS and Storage disease but was not statistically relevant. High Reticulocyte count was found in Infectious diseases (Tuberculosis and Histoplasmosis), Hemolytic diseases and Hypersplenism and was not any statistical significance. In our study statistical comparison between Reticulocyte count and IRF, we noted that IRF increased earlier than reticulocyte count.

Conclusion

Though bone marrow examination is very important for diagnosis and categorization of various hematological diseases, now based on our study, we propose that newer Reticulocyte parameters like Immature Reticulocyte Fraction can help in categorization and follow-up these diseases. We found that low reticulocyte count with IRF value 0 (Zero) is very much diagnostic of aplastic Anaemia. For the diagnosis of Megaloblastic Anaemia, reticulocyte count was in the low to normal range and IRF was marginally high. IRF helps in differentiating Aplastic Anemia from Megaloblastic Anemia which are the two most common causes of pancytopenia in India, so that treatment can be decided. The cases of early recovery from Megaloblastic Anaemia and bone marrow suppression due to Cancer Chemotherapy showed low to normal reticulocyte count but high IRF and were statistically significant. The cases of hemolytic disease and Hypersplenism showed statistically significant high Reticulocyte count and very high IRF.IRF was initially introduced to monitor the hematopoietic treatment in cases of childhood pancytopenia due to cancer chemotherapy. In our study, we found that values of IRF were also significant in the diagnoses of Megaloblastic Anaemia, Aplastic Anaemia, early Marrow Recovery from suppression, Hemolytic Disease and Chronic Diseases. It has already been mentioned that the response to treatment in cases of pancytopenia can be monitored rapidly by evaluation of IRF, rather than Absolute Neutrophil Count.
These newer parameters are non-invasive, early diagnostic, more sensitive and specific than older parameters like Reticulocyte Count. It is hypothesized that IRF is an index of acceleration and the absolute reticulocyte count is a quantitative measurement of the effectiveness of erythropoiesis. So after initiation of treatment, repeated observation of both IRF and Reticulocyte Count may be helpful to observe the effectiveness of therapy.

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Conflict of Interest: Nil
Source of support:Nil