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SIGNIFICANCE OF ADVANCED HEMATOLOGICAL PARAMETERS FOR DIAGNOSIS AND RECLAMATION IN HEMATOLYMPHOID MALIGNANCIES AND INFECTIONS

Pathology

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ABSTRACT

Background: The present study emphasizes the important role of newer hematological variables generated by Automated Cell Counters (ACCs) in early diagnosis and monitoring the response to the treatment in hematological malignancies and infections

Aims: To evaluate the clinical applications of newer hematological parameters for expanding their diagnostic potential in cases of hematolymphoid malignancies and infections

Study design: This cross sectional observational prospective study was conducted in the Department of Laboratory Sciences & and Molecular Medicine at a "Tertiary Care Super-Speciality Hospital" of North India

Subjects & methods: EDTA blood samples of 40 & 10 cases of hematolymphoid malignancies and infections respectively were collected at pre and post treatment stages over a period of 18 months. The results obtained by ACCs and microscopic examination were compared

Statistical Analysis Used: The hematological, parameters obtained from ACCs/microscopic were assessed by Student's t-test, chi-square test (or Fisher's exact test) using the SPSS 21.0 software with considering p value < 0.05 as significant

Results: Immature Granulocytes (IGs) have potential role to predict sepsis and results obtained by ACCs and manual examination showed statistically significant relationship (p value< .05) IGs, Large unstained cells, WBC count from the Peroxidase channel were markedly raised in sepsis & hematolymphoid malignancies and exhibited a downward trends following effective therapy. ACCs have limitation to accurately evaluate % blast suspected in cases of leukemia as compared to PBS, however this issue can be overcome due to inherent flagging property of ACCs to atypical cells which hint the manual examinations of such samples.

KEYWORDS

Automated cell counters (ACCs), Immature Granulocyte% (IGs%), Acute leukemia, Large unstained cells (LUCs), WBC count from the Peroxidase channel (WBCP)

INTRODUCTION

Considering economic factor and non availability of automation (cell counters) at smaller laboratory set ups in developing countries; manual complete blood cell (CBC) count is still performed despite the indispensability of more accurate and efficacious automated instruments. Initial quantitative methods for blood testing were based on careful wet sample preparation on a slide chamber, visualization, and manual counting with the aid of an optical microscope. In 1896, technique of blood cells measurement in a test tube filled with diluted blood was evolved by George Oliver that could be the forerunner of automated blood count. In 1940s, Wallace Coulter developed a simplified blood cell analysis method for quick screening of large number of blood samples.[1] Automated Cell Counters (ACCs) works either on optical method (light scatter) or impedance Coulter method (changes in electrical current induced by blood cells flowing through an electrically charged opening) or a combination of both. In 1968, Dittrich and Goehde coupled a laser beam to flow device discovered by Fulwyer and successfully demonstrated fluorescence based cytometry.[2] Property of thousands cells getting pass through the laser beam per second led to the foundation of high throughput cytometry. This development may be seen as a watershed in the transition from manual to automated blood counting.

The technology of ACCs has advanced and expanded from three part differentials to seven-part differentials system which can count neutrophils, eosinophils, basophils, lymphocytes, monocytes, large unstained cells (atypical lymphocytes; lacks peroxidase activity) and large immature cells (blasts & immature granulocytes) depending upon its configuration. The newer ACCs i.e. Advia® 2120i and Sysmex XT-4000i additionally provide parameters like Immature Granulocytes (IGs), Nucleated Red Blood Cells (NRBCs), Immature Reticulocyte Fraction (IRF), Mean Reticulocyte Volume (MCVr), Mean Reticulocyte Hemoglobin Content (CHr) etc. These recent parameters have a vital role in early diagnosis and monitoring the response of the treatment especially in anemia, sepsis and hematolymphoid malignancies. Numerous flagging systems, in built quality control programs and automated maintenance are other inherent advantages of modern hematological instruments.[3] Progressive improvement in these instruments has allowed the enumeration, evaluation of blood cells with great accuracy, precision, speed and considerable low cost.

Immature granulocytes are maturing granulocytic myeloid cells; comprise of myelocytes, metamyelocyte and band forms[4-6] The manual "band count" used widely in pediatric practice as a marker for bacterial infection; had limitations of accurate and precise measurement.[7] More than 3% IGs count is very specific predictor of sepsis/early bacteremia and indicates microbiologic laboratory evaluation to facilitate timely initiation of antimicrobial therapy thus leading to reduce morbidity, mortality and healthcare costs. However considerable timelag before the blood culture results has necessitated the usefulness of various hematological parameters to predict infection earlier.[8] ACCs like Sysmex XT- 4000i has inbuilt technology that combines cytochemistry, focused flow impedance and light absorbance; thus enumerates IGs with better precision, turnaround time, accuracy and has potential to predict positive blood culture; can be utilized in routine clinical practice.[9-11] Increased IGs with neutrophilia is seen in bacterial infections, myeloproliferative diseases, acute inflammatory diseases and metastatic BM cancers etc:[4]

ACCs also improve patients care by providing highly accurate and precise results for quantification and identification of normal WBCs however, not much sensitive at identifying/classifying all types of abnormal or immature WBCs. To overcome this problem, ACCs has been provided with property of flagging samples having possible

abnormal WBCs populations thus indicating further need for peripheral smear examination (PBS) by trained personnels to identify abnormal cells.[4]

Nucleated Red Blood Cells (NRBCs) are precursors of reticulocytes, mature erythrocytes. They are primarily formed, stored in bone marrow (BM) in response to erythropoietin with their normal presence in the circulation of fetuses and newborn infants upto 3rd or 4th day of life. Their prolonged existence in blood may indicate intrauterine growth restriction, fetal anemia, or congenital TORCH infections (Toxoplasma, Other viruses, Rubella, Cytomegalovirus, Herpes), ABO isoimmunisation and acute chorioamnionitis etc. Presence of NRBCs in an adult's PBS is pathogenic that lead to raised demand for bone marrow to produce & release immature RBCs into the circulation. Causes include anemia, severe infection, hematological malignancy (leukemia and myelodysplastic syndromes, few lymphoma), bone marrow metastases of solid tumours, extramedullary haematopoiesis, severe hypoxic stress or massive acute hemorrhage. Due to similar sizes of NRBC and lymphocytes, ACCs can misclassify NRBC and generates a wrong WBCs and high lymphocyte count. Inherent flagging system of ACCs indicate microscopic analysis of such samples to get Corrected WBC = Total WBC x [100 ÷ (NRBC + 100)]. The reference range of mean NRBC by manual PBS is 0/100WBC and by Advia^{R} 2120i range is 0-0.2x10^{3} cells/cumm respectively.

The reticulocyte count is an important indicator of effective erythropoiesis.[12] Usually reticulocytes circulate in peripheral blood for 1-2 days prior to develop in to mature RBCs. Increased erythropoietic demand is associated with prolonged reticulocyte life span in peripheral blood owing to premature release of immature reticulocytes from the BM. An increased reticulocyte count reflects ongoing or recent RBC production activity as in acute bleeding, hemolytic anemia response to therapy iron/vitamin B-12/folic acid/erythropoietin supplementation or BM recovery following chemotherapy/bone marrow transplantation) whereas decreased reticulocyte count reflects reduced RBCs production as in nutritional anemia, chronic renal failure, post radiation therapy, BM failure due to metabolic storage diseases/infection or malignant processes like leukemia/lymphomas/ metastatic tumors. The recent development of automated reticulocyte counts by ACCs has permitted their more precise, quantitative counting of Immature Reticulocyte Fraction. IRF is the quantitative proportion of all younger reticulocytes; derived as a ratio of immature reticulocytes to total number of reticulocytes. IRF is a very early and sensitive index of marrow erythropoietic activity and its fraction in excess of 5% is a reliable marker for hemopoietic recovery.[13] Reticulocyte Production Index (RPI) represents functional iron available for incorporation into hemoglobin within RBCs can be calculated as Reticulocyte Production Index = (Reticulocyte Index) X (1/maturation time). An increased RPI of >3 seen in hemolytic anemias, recent hemorrhage and marrow response to therapy[14] whereas a decreased RPI of < 2 noted in hypoproliferative disorder.[15]

IRF	Absolute reticulocyte count	Clinical conditions
Decrease	Decrease	Aplastic anemia, chronic renal failure
Decrease to normal	Decrease	Early erythropoietic response after anemia
Increase	Decrease	Repopulating bone marrow
Increase	Increase	Response to erythropoietin treatment or early acute hemorrhage or hemolytic anemia

Anemia is one of the most frequent side effects of chemotherapy drugs used to treat acute leukemia. After chemotherapy CBCs falls within a week and may take some time to recover thus necessitates extensive monitoring of BM recovery by reliable hematological parameters. Effective erythropoiesis can be monitored by quantitative measurement of reticulocyte indices therefore serial measurement of IRF and MRD is useful to monitor bone marrow regenerative function. The monitoring of BM regeneration after chemotherapy/radiotherapy is further vital as high risk of infection is associated by prolonged time of aplasia.[16]

Hematopoietic precursors (blasts) are immature cells that give rise to lymphoblasts or myeloblasts. These blasts are normally absent in peripheral blood, however their presence in blood is a harbinger of acute leukemia. Lymphoblasts differentiate to form mature lymphocytes whereas myeloblasts (unipotent stem cell) which differentiate into one of the effectors of the granulocyte series under stimulation of Granulocyte-Colony Stimulating Factor (GCSFs) and other cytokines Lymphoblasts can be distinguished microscopically from myeloblasts by having less distinct nucleoli, more condensed chromatin and an absence of cytoplasmic granules. However these morphologic distinctions are not absolute and a definitive diagnosis relies on specific cytochemical stain and detection of unique Cluster of Differentiation (CD) receptors by antibody immunostaining.

Determination of percentage of blasts in PBS and BM is essential for diagnosis and classification into Acute lymphoblastic leukemia (ALL), Acute Myeloblastic Leukemia (AML), Myelodysplastic Syndromes (MDS) and lymphoproliferative disorders like Chronic lymphobcytic leukemia (CLL), Chronic myelocytic leukemia (CML). Acute leukemic or MDS patients with higher percentage of blasts in BM than PBS have better prognosis than patients with reversed relative proportion of blasts. The percentage of blasts in PBS & BM are not of much important for the diagnosis of ALL, because presence of any clonal blast population is diagnostic. However, post-therapy PBS blast percentage is an important prognostic index that reflects the outcome in ALL.[17] Chemotherapy is the main stay of treatment in acute leukemia & it should be monitored by IRF & presence of blasts in bone marrow (Minimal Residual Disease).[18]

MATERIAL AND METHOD

This cross sectional observational prospective study was conducted in the Department of Laboratory Sciences & and Molecular Medicine at a "Tertiary Care Super-Speciality Hospital" of North India during Oct 2015 to Mar 2017. The study population comprises of 40 cases of hematolymphoid malignancies and 10 clinical cases of infections having positive serum markers for sepsis with the aim to analyze multiple traditional and recent hematological parameters in pre and post treatment stages. The relevant clinical data was accrued from the data register in the blood collection centre/wards/concerned departments. PBS with >10% of blasts were selected to assess their patterns using ACCs. Twenty out of forty cases had blast counts of >10% on routine PBS manual differential counts and reported as acute leukemia whereas remaining twenty cases were reported as chronic leukemia. Blood samples were collected in EDTA vacutainers; under aseptic precautions and processed within 2 hours of collection through two new generation hematology analyzers i.e. ADVIA^{R} 2120i and SYSMEX XT- 4000i. All samples were analyzed with three levels of commercial quality control specimens (Sysmex e-Check). Sysmex XT-4000i differentiates among neutrophils, eosinophils, monocytes, and lymphocytes on the basis of their light scatter and fluorescence emission characteristics using electronic cluster analysis protocols. Additional tests like NRBCs, IGs, IRF, ANC, reticulocyte count, cytochemical evaluation, Hb electrophoresis, flow cytometry or molecular analysis were performed wherever needed. For the validation of the ACCs results, 200-cells manual differential counts were obtained by two experienced technologists using the corresponding Leishman- Giemsa stained PBS and these results were confirmed and compared by a hematopathologist. The association between various hematological, morphological and clinical features were tested using Student's t-test for continuous variables and the chi-square test for qualitative variables. All statistical analyses was performed using the SPSS 21.0 software considering p value <0.05 as significant.

RESULTS

Immature granulocytes results were analyzed in forty hematolym phoid malignancy patients (20 cases each of myeloid leukemia & lymphoid leukemia) and ten patients having infection. IG% was increased both types of patients in sepsis & myeloproliferative malignancies and the results were validated with PBS finding. Mean IGs % in sepsis (12.41%) and control (0.36%) cases were analyzed by Advia^{R} 2120i with reference range of 0.21% to 0.49%. These cases on follow up were found to have decreased IG% number on remission. Pre-chemotherapy mean IGs % was maximally raised in CML (26.8%) followed by AML (10.8%), ALL (1.0%), CLL (0.8%) with control value of 0.4% as analyzed by Advia^{R}2120i.

Mean value of White Blood Cells in Perox channels (WBCP) increased to 19.56x10^{3} cells/ uL in sepsis as compared to control value of 0.0x10^{3} cells/uL. Mean value of WBCP among hematolymphoid malignancies was maximally raised in CLL 62.9 x10^{3} cells/uL followed by ALL 56.2 x10^{3} cells/uL, AML 26.9 x10^{3} cells/uL and CML

26.0 with control value of 0.0 x10^3 cells/uL.

Mean value of Large Unstained Cells (LUCs) increased to 0.876x10^3 cells/uL in sepsis as compared to control value of 0.108x10^3 cells/uL with cut off value of >0.31 x10^3cells/uL. Mean value of LUCs among hematolymphoid malignancies was maximally raised in ALL 28.3 x10^3 cells/uL followed by AML 20.9 x10^3 cells/uL, CML 13.5 x10^3 cells/uL and CLL 2.2 x10^3 cells/uL with control value of 0.1 x10^3 cells/uL.

Forty cases of hematolymphoid malignancy comprised 10 cases each of ALL, AML, CML and CLL. Mean % of blasts suspected in ALL, AML, CML and CLL as measured by Advia^R 2120i were 11.39%, 12.09, 0.34 and 0.35 respectively with control value of 0.4%. Corresponding % of blasts counted by PBS were 64.3%, 70.8%, 0.8% and 0% in ALL, AML, CML and CLL respectively with control value of 0%. There was a huge difference in calculation of blasts% in ALL and AML as counted by Advia^{R}2120i & PBS and their comparison was statistically significant (p value <0.05).

On 28th day post-chemotherapy mean IGs% results were drastically reduced to 1% of each from pretreatment value of 10.8% in AML and 26.8% in CML respectively as measured by Advia^R 2120i. Similarly WBCP & LUCs were also significantly subsided in sepsis and hematolymphoid malignancies.

On 28th day post-chemotherapy remission phase, manually there were no blasts seen in acute leukemia, whereas measured mean% blast suspected as measured by Advia^{R}2120i for ALL, AML, CML and CLL were 1.41%, 2.33%, 0.8% and 0% respectively with control value of 0%.

DISCUSSION

This study was performed to access the patterns and role of hematological variables in the course of hematological recovery in hematolymphoid malignancies and infections.

Immature Granulocytes (IGs) have potential role to predict sepsis in hospitalized patients and may be a sign of inflammatory response to an injury or autoimmune condition malignancy in the body/bone marrow infiltration.[19] IGs in PBS reflect active bone marrow response to bacterial infection and myeloproliferative malignancies. 7 & 3 infectious cases were Gram-positive and Gram-negative whereas 6 & 4 cases were with and without toxic granules respectively. Irrespective of the cause; increased IGs% with raised sepsis markers like C-reactive proteins and procalcitonin were found. IG% counts by ACCs will reduce smear reviews, manual differentials and is less time-consuming, less expensive than routine examination of blood smear. ACCs examine thousands of WBCs whereas PBS typically screens 100-200 WBCs. We compared the IGs% results as measured by manual microscopic method and Advia^R 2120i. We found the significant relationship between the two methods of counting IGs (p value< .05) and is in concordance with Mathias Bruegel et al. This validates the replacement of the traditional manual microscopic IG count by the Advia^R 2120i.

An increase in Absolute Neutrophil Count (ANC) of ≥0.5 x10^9/L defines successful myeloid recovery after chemotherapy.[20,21] Some studies have suggested that IRF was the first sign of hematological recovery in 80% of the patients, preceding the rise in ANC on 14th day. The complete reticulocyte picture, total reticulocyte, IRF, RET-H_e, provide less variation than acute phase reactants in patients with inflammation or infection so are direct cellular measurements for a faster indication of patient response.

Manual differential counting is the gold standard procedure for the accurate identification of cells in the peripheral blood.[22] However low cell counts following chemotherapy frequently complicate attempts to obtain a sufficient number of cells to render a meaningful manual differential count.[23] In addition PBS examination of all routine samples for CBCs and WBCs differentials is laborious and time-intensive therefore ACCs can be for the initial screening and detection of hematologic abnormalities in modern hospitals and clinics.[24,25] ACCs are capable of detecting the presence of abnormal cell populations and provide cautionary flags.[26,27] When the preliminary diagnosis of leukemia is made with CBC and manual slide reviews, clinical hematologists generally require crude distinctions between AML and ALL. In our study, Advia^R 2120i can detect the presence of blasts as % suspected blast, besides determination of the lineage of leukemia unlike in previous studies.[27] Though we have not included that parameter in our study but Advia^R 2120i has peroxidase channel which utilize the presence of peroxidase activity in the myeloid blasts. However from the best of our knowledge no study has done so far to differentiate between the acute leukemia lineages.

Advia^{R}2120i e, calculated most of the lymphoblasts as lymphocytes or lymphocytes mixed with neutrophils or monocytes in ALL cases and counted myeloblasts as monocytes, neutrophils or lymphocytes in AML cases. Our study shows % suspected blast in automated analyzers are not analogized with the blast seen in PBS (p value>0.05). The blasts exhibit irregularly shaped nuclei relative to the lymphoblasts in ALL cases and many of them had abundant cytoplasm with many vacuoles. This means Advia^{R}2120i reflects the morphology and not the origins of the cells. However percentage of blast suspected were decreased in remission phase as decreased in PBS (p value<.05). In chronic leukemia, % blast suspected by Advia^R 2120i and in PBS blast was negligible and corroborated. Moreover, determination of the lineage of leukemia via immunophenotypic, molecular, or cytogenetic analyses is a time-consuming process. If the automated differential results vary according to the type of blasts, the CBC can provide us with valuable data to make provisional decisions about the lineage of leukemia. In this study, we assessed the patterns of blasts counted using Advia^R 2120i and compared the results with manual differential counts. However, we can conclude that the manual differential counts and the judicious slide review criteria remain essential whenever a hematologic disorder is suspected even in cases in which blast flags are not generated. Further studies with more specimens will be necessary to determine whether the type of blast is related to the blast flag sensitivity or simply related to their morphology. Our data showed that differential WBC reports from ACCs should be interpreted with great care, with extra attention paid to suspected blasts and flags because the majority of leukemic blasts may be counted as monocytosis, lymphocytosis, or neutrophilia. LUCs are the aberrant number of larger than normal cells which are unstained on Leishman-Giemsa staining.

LUCs are larger than normal lymphocytes and may be atypical lymphocytes, myeloperoxidase deficient cells or MPO negative blasts. Advia^R 2120i showed increased LUCs in leukemia patients (except CLL), while in remission phase their numbers was decreased. Thus our study has emphasized the importance of newer parameters generated by ACCs as cost effective, sensitive, specific and faster mode in diagnosis and follow-up cases of hematolymphoid malignancies and infections.

CONCLUSION

Based on our results, immature granulocytes may be considered a useful marker for bacterial infection and myeloproliferative malignancies, therefore IG% should form part of CBCs. In addition monitoring of WBCP and LUCs will also assist to know recovery in sepsis patients. IRF being non-invasive, inexpensive, and objective indicator can also been used with the reticulocyte count to access patient's bone marrow response.

For leukemia cases percentage of suspected blast may provides the helpful information about differential diagnosis and recovery of the acute leukemia, however Advia^R 2120i does not correlates with the PBS report given by the pathologist. Monitoring of WBCP and LUCs are also helpful to know recovery in leukemia patients. More efforts are needed in respective parameters having variable results by ACCs and PBS examination. Moreover despite the essential role of automation, microscopic examination of blood sample by the pathologist remains Gold standard.

Informed consent: A written consent in the language the patients understands was taken from all the subjects being enrolled after explaining the objectives and benefits of the study to them.

Ethical clearance: The study was then undertaken after due approval of the hospital ethics committee.

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