REVIEW ARTICLE

The Clinical Relevance of Nucleated Red Blood Cell Counts

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INTRODUCTION

The pattern across laboratory medicine during the past thirty years has been one of increasing demand for tests combined with a need for cost containment. The latter has now become an over-riding consideration. It is no longer more work for the same number of technologists but more work for fewer individuals since staff represents the major expenditure in most clinical laboratories. Fortunately, incremental advances in instrumentation, particularly in the area of blood cell counting, have accompanied these increasing economic pressures and this has resulted in a reduction in the need for manual and semi-automated processing. The most recent addition is the ability, with some instruments, to enumerate nucleated red blood cells (NRBC) (***Fig.1***). This inevitably poses the question: *is there clinical value in counting NRBC ?*
At the moment examination of blood cell morphology by microscopy remains one of the major labour intensive non-automated procedures conducted in the haematology laboratory. It is a perpetual challenge to reduce the number of peripheral blood films examined but at the same time not miss valuable diagnostic pointers. Some laboratories cling to the hallowed tradition that examination of the peripheral blood is incomplete without a blood film report. This luxury becomes impossible in the high throughput laboratory where a film review rate greater than 10 – 15 % becomes impossible to manage. This low review rate does not appear to result in significant loss of screening or diagnostic capability[1-3]. The blood film is examined for a number of reasons: to explain an unexpected blood count finding, to examine red cell morphology, to verify an unexplained automated differential leukocyte count or to undertake an extended differential count. For the last, the most frequently occurring findings are the presence of immature granulocytes and NRBC. It is therefore important to recognise the pres-

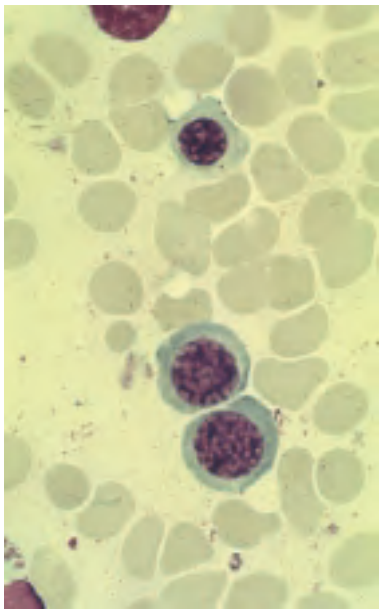

Fig. 1 *Nucleated red blood cells on a conventional stained peripheral blood film*

ence of NRBC with confidence but is it necessary to produce an accurate NRBC count? The simple answer is affirmative if for no other reason than the ability to produce a correct total leukocyte count and a correct differential leukocyte count.

NRBC IN THE PERIPHERAL BLOOD FILM

Thus far it has proved virtually impossible for the automated blood cell counter to distinguish the small mature lymphocyte from the NRBC. The result has been a combined count, which, at times, especially in neonatology, has proved misleading clinically. Small numbers of NRBCs are found in the cord blood of normal neonates. The neonatologist will always argue that there is a need to count NRBCs accurately. This is particularly so where there is a history of fetal hypoxia[4] or asphyxia, in growth retarded infants[5] and in premature infants[6]. The importance of this measurement in predicting risk of cerebral accidents in neonates is well defined[7]. Large numbers of circulating NRBCs most of which derive from sites of extra-medullary haemopoiesis in the liver and spleen, are very characteristic of haemolytic disease of the newborn.
There are also well-recognised adult situations where it is useful to produce an accurate NRBC count separate from the total leukocyte count. NRBC can occur in virtually any patient with severe anaemia although they are unusual in aplastic anaemia. NRBCs in the peripheral blood of adults may be present in Hb-SS disease patients particularly during painful crises and also in thalassaemia. Other causes of erythroblastaemia include leukaemia, myeloproliferative syndromes, particularly myelosclerosis, and carcinomatosis due to either extra-medullary haemopoiesis or perturbation of bone marrow architecture or both. In these last disorders the numbers of circulating NRBCs may be comparatively low and associated with circulating immature granulocytes resulting in the so-called leuko-erythroblastic blood picture. Additionally any condition producing haemopoietic stress such as severe infection, hypoxia or massive acute haemorrhage can result in the appearance of circulating NRBCs.
Recently there is interest in the detection of fetal NRBC, which are present in the maternal circulation during the majority of pregnancies, as a target for fetal DNA analysis[8]. Unfortunately they are not detectable (only 1-2 NRBC/mL maternal blood!) by routine haematological procedures even the latest instruments which count NRBCs.
Areas for future study, however, might include increased feto-maternal cell traffic in pre-eclamptic toxaemia[9], and the diagnosis and timing of occurrence of fetal asphyxia[10].

CIRCULATING NRBC

Under normal conditions NRBC are found only in the blood of the fetus and the neonate. The concentration of NRBC in cord blood in health has been reported as ranging from $0.03 - 4.8 \times 10^9$/L[11] and by 8 days of age is $0.03 - 1.1 \times 10^9$/L. Under all other conditions the presence of NRBC is an indicator of pathology, either an increase in erythroid activity or damage to the marrow micro-architecture.

NRBC COUNTING METHODS

Visual microscopy

Until very recently the only method for NRBC enumeration has been microscopy of a peripheral blood film expressing the count as the number of NRBC / 100 WBC. Not only was this a laborious procedure, it was imprecise with reported Coefficients of Variation (CV) ranging from 30 – 110 %.
Electronic counters were unable to distinguish NRBCs from lymphocytes and thus the former were counted at least partly as white cells. A major problem today is knowing if NRBCs are included in, excluded from, or partly included and partly excluded from the instrument count. Not possessing this information may lead to erroneous WBC corrections[12]. The instrument WBC count may therefore require correction and this is achieved by:

Observed WBC × (100 / (100 + NRBC*))

*NRBC ; The count as the number of NRBC pert 100 WBC.

It has been suggested that the WBC count correction is mandatory only when more than 10 NRBC/100 WBC are present[13].

NRBC instrument flags

Many automated blood cell counters already display flags indicating the presence of NRBC however these are limited by low sensitivity and specificity of flagging performance and do not produce a count. One of the major difficulties is to deal with small numbers. The NRBCs occurring in many adult clinical circumstances constitute a comparatively rare event and a major requirement for the automated blood cell counter is therefore a very low limit of detection.

Flow cytometry studies

More recently a number of flow cytometry methods using monoclonal antibody labels have been published and indicate less imprecision with Coefficients of Variation (CV) consistently below 20 %; however, the technique is time-consuming, expensive, requires considerable flow cytometry expertise and the result is not produced in a clinically meaningful time scale. This technology will, however, probably be used as a reference method to verify the results of NRBC obtained from routine automated blood cell counters.

Commercially available counters

One of the first reports on the use of a commercially available counter for NRBC enumeration was published by Paterakis[14] who used the Sysmex R-1000 (Sysmex Corporation, Kobe, Japan) reticulocyte analyser. During

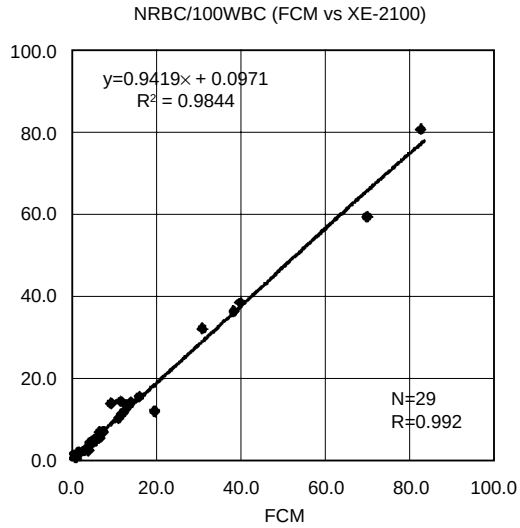

Fig. 2 *Correlation of NRBC results between XE-2100 (y-axis) and flow cytometry (FCM; x-axis)*[15]

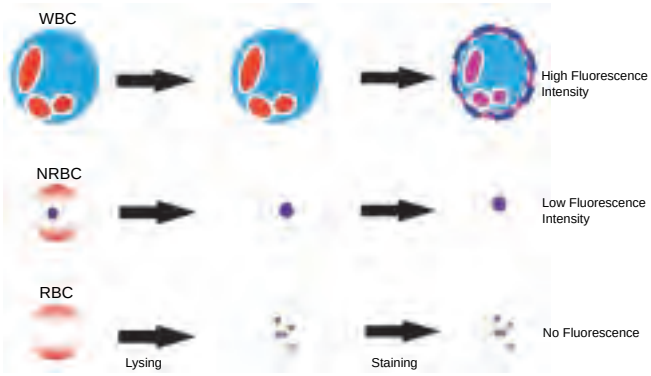

Fig. 3 *Staining mechanism of NRBC in XE-2100*

studies on NRBC interference with leucocyte counts on haematology analysers, Paterakis showed that the upper particle count (UPP), provided by the R-1000 with modified fluorescence-amplification voltage, appeared to produce a direct NRBC count in ß-thalassaemia intermedia when compared to NRBC counts assessed indirectly.

The recently released Sysmex XE-2100 analyser (Sysmex Corporation, Kobe, Japan) incorporates the capability of an accurate NRBC analysis with convincing correlation[15,16] to a flow cytometry reference method (***Fig. 2***)[15]. In this analyser a specific reagent (fluorochrome) completely lyses the RBC and at the same time enucleates, shrinks and slightly stains the nuclei of any NRBC present (***Fig. 3***). The lysing reagent maintains the shape of the WBC while intensely staining their intra-cytoplasmic organelles and nuclei. These different staining intensities between NRBC nuclei and WBCs as well as, their differing volumes are detected by a semiconductor laser using forward-scattered light and fluorescence intensities. Clear separation of the two cell populations occurs (***Fig. 4***).

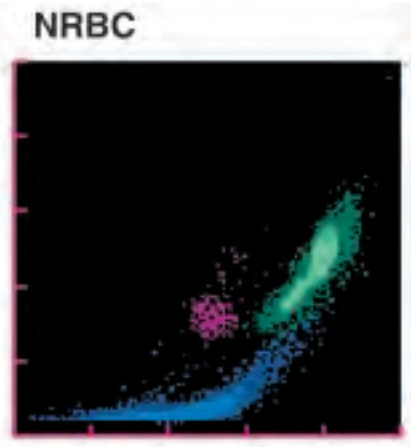

Fig. 4 *The flow cytometry scattergram of the XE-2100 NRBC channel (y-axis=forward scattered light intensity, x-axis=fluorescence intensity). The NRBC are represented in the mauve cluster and WBC in the pale blue cluster.*
The dark blue cluster represents RBC ghosts.
Note the clear separation of orthochromatic NRBC from small lymphocytes.

THE POTENTIAL IMPACT OF AUTOMATED NRBC ANALYSIS

The ability to generate precise and accurate NRBC counts across the entire concentration range in peripheral blood by automated methods offers considerable advantage to the routine laboratory and medical departments of any healthcare institution. While labour saving and eradication of the need to correct total and differential leucocyte counts are immediately obvious, several innovative possibilities arise.

The ability to provide a more comprehensive haematology screening service for neonates is clear. The provision of rapid real-time NRBC counts has implications for earlier diagnosis, more rapid treatment and therefore improved prognosis in this important group of patients[4,5,7,9,10,17]. Screening of children for normoblastaemia has revealed a high probability of underlying disorders with hypoxia (49%) and even malignancies (8%)[17].

Regular screening of routine peripheral blood samples for lower concentrations of NRBC may reveal the onset of a variety of RBC disorders, like congenital dyserythropoietic anaemia (CDA)[18], polycythaemia rubra vera and, of course, megaloblastic anaemia. In conjunction with a low platelet count the presence of even low numbers of NRBCs in peripheral blood may give a first hint for the existence of thrombotic thrombocytopenic purpura[19]. Such screening may also permit detection of myelodysplastic syndromes (MDS) and the chronic myeloproliferative disorders[20].

Non Hodgkin Lymphoma (NHL) treated with radiation,

chemotherapy or both may develop secondary leukaemia (approx. 1%). In that context the appearance of low NRBC counts in the peripheral blood associated with pancytopenia has been described[21].

THE ADDITIONAL BENEFITS OF EXTENDED RED CELL MEASUREMENT

The incremental advance in the available automated *extended red blood cell measurements*, the reticulocyte count[22], the Immature Reticulocyte Fraction (IRF)[23,24] and the NRBC count, add a dynamic dimension to the diagnostic value of the traditional Wintrobe indices. By means of *multivariate measurement combinations* these "state of the art" multi-parameter analysers can now support the diagnostic and patient monitoring requirements of modern medicine, particularly for the new treatment modalities of high dose chemotherapy, growth factor therapy and transplantation for malignant disease. Examples are the bivariate plot of reticulocyte count and immature reticulocytes in diagnosis[25] and that of haemoglobin concentration and NRBC count for monitoring the transfusion requirements of patients with thalassaemia major[26] to name but two. It must be the goal of automated haematology devices to maximise the useful clinical information obtained from a single sample of EDTA blood not only in the interests of good patient care but to reduce the demands on, and costs incurred by the laboratory.

THE ROLE OF AUTOMATED CELL ANALYSERS IN BONE MARROW EXAMINATION

Finally, the role of the routine blood cell counter in bone marrow examination should be considered. Clinical requirements for bone marrow assessment both, for diagnostic but particularly for treatment monitoring purposes, continue to increase yet few changes have taken place in the haematologists approach to this problem. In most instances bone marrow assessment is a laborious cytological procedure by means of microscopy. No automated method is established to accomplish this. Pioneering work in this area has been published by Terstappen and Levin[27] by complex cytofluorimetric techniques using fluorescent nucleic acid dyes and monoclonal antibodies. While the technology is clearly unsuitable for the routine laboratory, this work provides valuable reference data. Two studies on the use of routine blood cell counters have been published but these had only very limited objectives. Bentley and colleagues[28] and Den Ottolander, et al.[29] report an automated method for measuring total marrow cellularity.
More recently, d'Onofrio, et al.[30] report a major study on automated bone marrow assessment. While conceding that automation will not replace the microscope, at least in the foreseeable future, they show, quite unequivocally, that an advanced automated blood cell analyser, capable of measuring the NRBC count, the reticulocyte count and the IRF, has a role in bone marrow examination in a number of ways, provided that interference by fat particles can be avoided:

- First, the total nucleated cell count produced by the instrument correlates well with microscopy assessment of marrow cellularity, particularly with hyper- and hypocellular marrows.
- Secondly, a useful myeloid/erythroid (M/E) ratio can be generated instrumentally.
- Finally, measurement concentration gradients, particularly involving the reticulocyte count and the IRF, between the bone marrow and the peripheral blood have been noted. Perturbation of these gradients may occur in disease and represent an area worthy of further study.

CONCLUSION

The availability of a rapid routine method for accurate NRBC counts on peripheral blood samples, over the entire range of concentrations encountered clinically provides new perspectives for the laboratory and the clinician. This newly automated parameter can be used for the early recognition of a variety of serious diseases in neonates, in children, in adults and perhaps during pregnancy thus enabling earlier, well-targeted therapy resulting in significant improvement of prognosis. Besides the positive consequences for both clinician and patient, a consequent reduction in overall healthcare expenditute for these patients may be anticipated. Additionally, NRBC counts coupled with haemopoietic progenitor cell (HPC) enumeration[32] may have an important role in peripheral blood stem cell transplantation.
In his introduction to a conference on Automation in Hematology[31] in 1979, that doyen of Haematology, Maxwell Wintrobe stated.

" And yet, we must not stop exploring and measuring, for there is always more to learn".

The same holds true today.

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