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Reference method, quality control and automation of reticulocyte count

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ABSTRACT

The reticulocyte count is, conceptually at least, the simplest method to assess erythropoiesis. The traditional method employing supra-vital dyes such as new methylene blue, cresyl blue or azure B and visual microscopy is time-consuming, poorly standardised and extremely imprecise. The major causes of imprecision arise from interobserver variation in the definition of a reticulocyte and the statistical unreliability of the counting procedure. Single measurements at low number level with this method are hardly meaningful. Studies have, however, shown that with repeated but time-consuming procedures both the reliability and the precision can be improved.

The use of fluorescent dyes to precipitate nucleic acids within the reticulocyte offers an alternative method and is increasingly being used in conjunction with flow cytometry counting methods. Of the many candidate fluorescent dyes available, thiazole orange appears popular for use in a variety of flow cytometers. There is, however, a commercially available flow cytometer dedicated to reticulocyte counting which uses auramine O.

INTRODUCTION

The reticulocyte count is familiar to all haematologists and represents in the minds of most, a labour intensive, imprecise laboratory procedure of limited clinical utility. Is this a fair judgement? Regrettably, in routine practice, with widely performed methods using supra-vital stains and with current attitudes the answer must be affirmative. But let us step back and review the requirements which any diagnostic test must fulfil. First, the problem to be solved must be defined in a clear and unambiguous manner establishing proof of clinical utility. Secondly, those properties and specifications which the test must meet require identification and finally benefit-cost analysis must be undertaken. These must be analysed for reticulocyte counting when the procedure can be performed in a standardized reproducible manner.

The clinical usefulness of reticulocyte counting is established and may be more extensive than many believe. It may be summarized as follows: the reticulocyte count demonstrates what is happening to erythropoiesis now and not what happened days, weeks or months ago. The procedure is useful in the following circumstances:

Distinction between hyper- from hypo-proliferative anaemias
Monitoring response to treatment in haematinic deficiency
Monitoring for accidental or therapeutically induced myelosuppression
Monitoring for recovery in myelosuppression for any cause and following bone marrow transplantation
Monitoring of premature infants

The reticulocyte count therefore can produce information which is of value in both diagnosis and treatment.

There is little published information on the frequency with which the reticulocyte count is abnormal in routine practice. A small pilot study in the author's laboratory using a flow cytometry device gives some indication that the reticulocyte count, expressed as an absolute number (x 10^9/L), may be abnormal as often as the platelet count and the latter has long been accepted as a first line haematological measurement. The results of this survey are given in table 1.

There were 100 patients in each group. The normal ranges on which these data were based were calculated using a Gaussian distribution rather than the lognormal distribution reticulocytes are thought to follow. The range found in healthy male subjects was 14-85 x 10^9/L with a mean value of 50 x 10^9/L and in healthy females 27-86 x 10^9/L with a mean value of 57 x 10^9/L (measurements performed using the Sysmex R1000 Reticulocyte Counter).

TABLE 1. Pilot study on the frequency with which the reticulocyte count (absolute number) is abnormal in a University teaching hospital

Unselected new patients	6%
Unselected anaemias	26%
Physician's office	6%
Surgical patients	17%
Medical patients	19%
Renal patients	24%
Haematology patients	29%
Oncology patients	34%

TABLE 2. Morphological definition of reticulocyte stages (ref. 3) and the relative proportions for circulating forms in the healthy individual (ref. 4)

Stage 0:	Orthochromatic normoblast	
Stage I:	Dense coherent reticulum in non-nucleated cell;	0.1%
Stage II:	Extended network of loose reticulum;	7.0%
Stage III:	Scattered granules with residual reticulum;	32.0%
Stage IV:	Scattered granules;	61.0%

SUPRAVITAL STAINING METHODS

Supravital staining and counting by microscopy is widely practised and is the method which has gained for the procedure the poor reputation it currently possesses. Imprecision in routine practice can be judged from results in external proficiency exercises which range from CVs of 30-40% (refs. 1,2). This is clearly unacceptable. The increasing clinical emphasis on reticulocyte counting at low levels demands the development of methods of improved precision and accuracy at these low levels. How can this be improved and accuracy assured? A number of fundamental problems must be overcome.

Definition of reticulocyte

Loosely defined as the penultimate stage in erythroid maturation, no single definition exists for a reticulocyte since it represents a continuum between extrusion of the pyknotic erythroblast nucleus and the final disappearance of mitochondria and ribosomes. The maturation stages of the reticulocyte were described by Heilmeyer in 1932 (ref. 3) and their relative proportions in the circulating blood by Seip in 1953 (ref. 4). These are shown in composite form in table 2.

Stage III and IV reticulocytes which normally circulate in the peripheral blood are the cells of the greatest interest in the count procedure. Paradoxically Stage IV reticulocytes are the most difficult forms to identify with consistency. The situation has improved following the adoption by NCCLS (ref. 5) and ICSH (ref. 6) of the following definition originally proposed by Gilmer and Koepke (ref. 7). The last defines a Stage IV reticulocyte as 'any non-nucleated red cell that contains two or more particles of blue-stained material corresponding to ribosomal RNA'. Universal acceptance of this definition should reduce one of the major sources of error in reticulocyte counting namely that of inter-observer variation in identification. In addition, however, because of premature or delayed release from the bone marrow and different rates of maturation, reticulocyte counts do not always reflect absolute erythroid activity. The supravital staining procedure is therefore fraught with difficulty in anaemia and when 'shift' reticulocytes are present (ref. 5) unless a number of corrections are made.

A reticulocyte count corrected for anaemia is termed the reticulocyte index and is calculated as follows:

$$\text{Reticulocyte index} = \text{observed reticulocyte count (\%)} \times \text{patient PCV}/0.45 \qquad (1)$$

It is recommended that reticulocyte counts be expressed in absolute numbers rather than proportion. So expressed, results are more informative especially low results and additionally correction for anaemia becomes unnecessary.

When the combined proportion of Heilmeyer Stages I and II exceeds 10%, 'shift' correction is also required. A reticulocyte count corrected both for PCV and maturation time is termed the reticulocyte production index. With increasing anaemia associated with increased erythropoietin production, the maturation time of marrow reticulocytes progressively shortens from a normal of 3.5 to 1.5 days or less (ref. 8). Conversely the reticulocytes in the peripheral blood persist for a longer time when anaemia is present.

$$\text{Reticulocyte production index} = \frac{\text{observed retic (\%)} \times \text{patient's PCV}/0.45}{\text{maturation time in peripheral blood}} \qquad (2)$$

Reticulocyte maturation time is taken as one day with a PCV of 0.45 ± 0.5, one and a half days when the PCV is 0.35 ± 0.5, two days with PCV 0.25 ± 0.5 and three days when PCV is 0.15 ± 0.5 (ref. 8).

Reticulocyte staining

It is well recognised that great variability exists among stains used in haematology. Not only does this occur among stains produced by different manufacturers, but lot to lot variability can occur in stains produced by the same manufacturer. Aware of this problem, ICSH (ref. 9) recommends the use of purified stains, particularly Azure B for reticulocyte counting. This has not proved uniformly popular, many laboratories preferring to continue with new methylene blue or brilliant cresyl blue. None-the-less the principles embodied in the ICSH document remain valid. Care in the preparation and storage of the stain for use in the laboratory is important.

Method standardization

Standardization of methodology is required. Scrutiny of existing texts on practical haematology procedures reveal small but possibly important and measurable method differences.

Sample size

The reticulocyte count is normally approximately 1% or 10 reticulocytes per 1000 RBC. It is usually recommended that visual counts are based on evaluation of 1000RBC and it must be conceded that even this modest rule is not always followed in the routine laboratory. Even counts of 1000 red cells are inherently imprecise due to sampling error since reticulocytes are rare events, the statistics of which generally follow the Poisson distribution. The only way in which to improve the statistics of a procedure such as this is to count more cells. The statistical requirements make a strong case for automated methods for reticulocyte counting but at the same time render good comparison between automated procedures and visual counts difficult unless the sample size of the latter approaches that of the former. For statistical validity 30,000 red cell events are desirable. This problem has been addressed by ICSH (ref. 6). Although currently rated as a recommended or selected method, this could be developed into a reference method.

To attain the sample size required for statistical validity a microscope area reducing device is necessary. This device standardizes both the reduced and total areas in which cells should be counted. The 'Miller disc' is recommended by both NCCLS (ref. 5) and ICSH (ref. 6) and contains a small square within a large square. The small square is 1/9th the area of the large square, therefore assuming even distribution of cells on the blood smear, the number of cells in the small square is 1/9th of the number of cells in the large square.

Before use, it is advisable to check the calibration of the graticule by obtaining the average red cell counts in 5 large squares and in 5 small squares to establish that the proportion is 9. This will only apply when blood cells are evenly spread on the slide. Proportional counting is then carried out, i.e. the number of reticulocytes in the entire square divided by the number of red cells in the small square in consecutive fields until the requisite number of red cells is attained.

To determine the total number of red cells to be counted ICSH (ref. 6) recommends a 'target' CV concept. Calculation of the CV is from the following equation:

$$CV = (100/p)\,(p(1-p)/n)^{\frac{1}{2}} \qquad (3)$$

$$n = (100^{2}/CV)\,((1-p)/p)$$

Where CV = coefficient of variation; p = proportion of reticulocytes; n = total number of cells.

Thus the number of cells to be counted to achieve coefficients of variation of 5% and 10% is shown in table 3.

AUTOMATION OF RETICULOCYTE COUNTING

To date automation of reticulocyte counting has developed along two routes. The development of computerized image analysis instruments in the 1970s for peripheral blood smear analysis provided an opportunity to automate reticulocyte counting. This was achieved in three commercial instruments. Reasonable agreement with microscope counts was achieved (ref. 10), but performance was highly stain specific. The introduction of these systems provided an opportunity to standardize methodology with considerable improvement in precision; however, since very few cells were evaluated (up to 500) sampling error continued to exist.

The emergence of flow cytometers using RNA fluorochromes which produced fluorescence proportional to the amount of RNA present in the cell resulted in a more significant advance. The advantages of the flow cytometry method are several-fold and include reduction in imprecision (10,000-30,000 red cells evaluated), improved throughput and reduction in labour costs for the procedure. Statements on improvement in accuracy are still being corroborated because of difficulty in specifying a reference method against which appropriate comparability studies can be performed.

The various fluorescent dyes currently used in flow cytometry devices are listed in Table 4.

TABLE 3. Reticulocyte counting statistics at coefficient of variation of 5% and 10%

Retic count		No. of cells to be counted in small squares	
%	p	CV=5%	CV=10%
1	0.01	4400	1100
2	0.02	2180	550
5	0.05	845	210
10	0.10	400	100
25	0.25	135	(34)

TABLE 4. RNA stains in use for automated reticulocyte counters

Acridine orange
Pyronin Y
Thioflavin T
Dimethyloxacarbocyanine
Thiazole orange
- - - - - - - - - - - -
Auramine O
Ethidium bromide

The first five dyes require at least 30 minutes incubation for uptake thus making the techniques suitable only for batch analyses. Ethidium bromide, on the other hand, at relatively high pH enters intact cells within a few minutes. Likewise, auramine O, the dye used in the Sysmex* R1000, requires only a few seconds, permitting a fast throughput for this instrument.

Since reticulocyte counts are performed on whole blood specimens, careful threshold settings are required to exclude autofluorescing, mature red cells, platelets, white and other nucleated blood cells. Conversion of red cell/reticulocyte scattergram data to a distribution histogram to set a threshold and calculate the proportion of reticulocytes currently is a problem which may account for differences on different systems. This will only be finally resolved when an appropriate distribution model is defined and a satisfactory material standard for reticulocyte counting becomes available.

A major advantage of automated flow cytometry arises from the proportionality of fluorescence to the amount of RNA in the reticulocyte. Increasing fluorescence inversely correlates with the Heilmeyer Stage number, i.e. the most mature reticulocytes have the least fluorescence and vice versa. Clearly increasing fluorescence in the reticulocyte populations can be used to identify 'shift' forms and thus can give a measure of reticulocyte immaturity. Various workers are now using the so-called 'reticulocyte maturity index' although this term is somewhat ambiguous and might be more correctly expressed as the reticulocyte immaturity index. The Sysmex R1000 reticulocyte counting instrument assesses reticulocyte immaturity by dividing the reticulocyte area by two vertical discriminators into low fluorescence ratio (LFR), the most mature forms, middle (MFR) and high fluorescence (HFR) areas, the last being the most immature form. The clinical utility of data on the maturation of the reticulocyte is currently stimulating intense interest.

EVALUATION OF THE SYSMEX R1000

A number of evaluations of this system have now been published (refs. 11,12,13). All stress satisfactory linearity over a good working range; acceptable levels of imprecision (CV of 5% compared with CVs of 30-40% for supravital staining methods in External Quality Assessment surveys); and reasonable agreement with visual reticulocyte counts.

A summary of the evaluation performed on behalf of the UK Department of Health (ref. 13) using the ICSH instrument evaluation protocol (ref. 14) follows. Data is reproduced by kind permission of the Procurement Directorate of the Department of Health, London.

Effect of dilution (linearity)

This is presented in table 5. Each reticulocyte absolute count and reticulocyte percentage represents the mean of four measurements at each dilution.

TABLE 5. Effect of dilution experiment on R1000

Fraction of Original	Absolute Count ($\times 10^9$/L)	R%
1.0	555.9	14.5
0.9	498.5	14.39
0.8	449.6	14.28
0.7	396.8	14.50
0.6	342.9	14.50
0.5	295.2	14.98
0.4	225.3	14.75
0.3	166.2	14.45
0.2	111.2	14.67
0.1	55.8	15.38

TABLE 6. Comparison of reference reticulocyte count (x axis) with R1000 count (y axis)

	Absolute count ($\times 10^9$/L)	Retic %
Correlation (r)	0.947	0.943
Intercept	-54.6	0.44
Slope	1.24	0.88
No. of paired tests	65	65
F-test	1.615 (N.S.)	1.219 (N.S.)
t-test	1.72 NS ($p \equiv 0.09$)	-0.95 NS ($p = 0.35$)
Mean bias	+12.5%	-3.7%

Precision study

Within and between batch precision was assessed using a two-way analysis of variance. In replicate tests 4 specimens were each measured 20 times consecutively.

Within batch			CV 5%
Between batch			CV 5%
Repeatability:	0.3%	- 7×10^9/L	CV 15%
	1.0%	- 17×10^9/L	CV 7%
	5.0%	- 165×10^9/L	CV 4%

A time lapse study with whole blood specimens stored at 4°C and room temperature over a six hour period revealed count stability. Thereafter on storage at room temperature a progressive fall in both absolute and proportional counts occurred (79% of original count at 24h and 66% at 48h with no further deterioration at 72h). This fall in count does not distinguish between maturation in vitro and disintegration of reticulocyte. No significant reduction occurred on storage at 4°C for up to 72h.

Carry-over

No carry-over was detected.

Comparability

Instrument counts were compared with visual counts using the ICSH recommended method (ref. 6). Results are given in table 6. Reference counts were performed by two independent observers and the averaged result compared with those obtained with the R1000.

Comparison of the R1000 with other flow cytometry methods by linear regression (thiazole orange used with (i) Coulter Epics and (ii) the Becton Dickinson Facscan) show similar 'r' values and slope but y axis intercepts are different suggesting that the thresholding of reticulocytes from auto-fluorescing red cells differs in each system.

Interferents

Interferents are well recognised in supravital staining and methods are defined to resolve them. Interferents are a potential problem using flow cytometry techniques. Heinz bodies and HbH have no effect on the reticulocyte count. Nucleated red cells and Howell Jolly bodies are thresholded out. Malarial parasites fluoresce in the R1000 and thus intracellular parasites are counted as reticulocytes.

CONCLUSIONS

There is a clear place for reticulocyte counting in the modern laboratory and automated devices produce counts at rates of up to 60/h with impressively low imprecision. The clinical value of reticulocyte counting particularly at low levels is being increasingly recognised. Counts must be expressed in absolute numbers. Reticulocyte maturation measurements may well have clinical value. Detailed assessment of the frequency with which abnormal counts appear and the clinical actions so stimulated will determine the future of this automated measurement. Whether or not the reticulocyte count becomes a first line haematology test - even to the extent of its incorporation in routine blood cell analyzers, remains to be determined. Development of a reference method and suitable flow cytometry material standards remain important objectives.

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