Reticulocyte counting using flow cytometry

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Abstract

A flow cytometric method for the quantitation of reticulocytes was refined for routine laboratory use. Blood (2 μl) is added to 2 ml of 0·4 μM thiazole orange in phosphate buffered saline, incubated at room temperature for 90 minutes, and analysed on a Coulter EPICS Profile flow cytometer, with gating for red cells on the basis of forward and right angled light scatter. Blood (2 μl) is also incubated with phosphate buffered saline alone as an unstained control. The adult reference range (mean ± 2 SD), established from 30 laboratory personnel, is 19·4 – 59·2 × 10^9/l (0·2 – 1·6%). Comparison of this technique was made on 39 selected patient samples with visual counting of cells stained with brilliant cresyl blue. The correlation between the two methods was 0·99 with slope 0·96 and intercept 0·02. The precision of the automated technique in three subjects with reticulocyte counts of 0·12%, 1·84%, and 14·3% was 33·3%, 7·3%, and 1·4%, respectively (coefficient of variations). In three patients studied serially after intensive chemotherapy, in whom the reticulocyte count quantitated by routine visual methods approached zero (0–0·1%) for eight to 18 days, the automated counts varied between 0 and 0·5%.

Flow cytometric reticulocyte counting is thus a simple and highly reliable methodology for the quantitation of normal and raised reticulocyte counts but cannot be reliably used to quantitate a subnormal level.

Reticulocytes are non-nucleated red cell precursors that contain remnants of the RNA found in earlier nucleated stages of erythrocyte development. The enumeration of reticulocytes in the peripheral blood gives an important insight into the level of bone marrow activity by providing a direct measure of erythropoietic activity.

Traditional methods for the counting of reticulocytes entail staining whole blood with supravital stains such as brilliant cresyl blue or new methylene blue, followed by microscopic analysis of at least 500 red cells. Problems found with this method have been well reported[1,2]—namely, the time required for analysis, the statistical variation among observers, and the poor reproducibility of the method. Although simple in principle, the unbiased counting of large numbers of red cells in a field of a wedge smear is difficult, even with the help of field restrictors and sample dilution.

Thiazole orange is a membrane-permeable basic dye which will bind to RNA, and the complex formed can be excited by light at 488 nm, emitting fluorescence within the green part of the spectrum at 530 nm. Thiazole orange can therefore be used for the demonstration of reticulocytes by flow cytometry.[3] Flow cytometry has the advantage of being able to analyse a larger number of cells in a shorter period of time than visual methods, with a low statistical variation between operators.

We compared our standard manual method[4] with a flow cytometric method and evaluated the use of thiazole orange to stain reticulocyte RNA.

Methods

All blood samples were collected into Vacutainer tubes (Becton Dickinson UK Ltd, Cowley, Oxford) containing tripotassium EDTA as an anticoagulant at a concentration of 1·5 mg/ml.

Normal blood was obtained from 30 healthy laboratory staff and absolute red cell counts were determined by analysis of these samples on a Coulter STKR analyser (Coulter Electronics, Northwell Drive, Luton). Samples were also obtained from 39 patients selected on the basis of clinical details to give a wide range of reticulocyte counts. The provisional diagnosis of these patients is given in table 1.

A stock solution of 0·4 μM thiazole orange was prepared by dissolving 1 mg of thiazole orange power (Molecular Probes, Eugene, Oregon, USA) in 1 ml of methanol. This was kept in the dark at −20°C. A working solution was prepared by adding 6 μl of the stock solution to 30 ml phosphate buffered saline (PBS), pH 7·4. This solution was centrifuged to remove any small particulate matter present and was stable for at least a week if kept at 4°C.

For routine use, 2 μl of whole blood was added to 2 ml of the working thiazole orange solution. Blood (2 μl) was also added to 2 ml

Table 1 Provisional diagnosis of the 39 patient samples

Diagnostic group	*No of patients*
Haemolytic anaemia	15
Haemoglobinopathy	7
Postoperative	10
Haematological malignancy	3
Intensive chemotherapy	4
Total	39

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Accepted for publication
8 February 1990

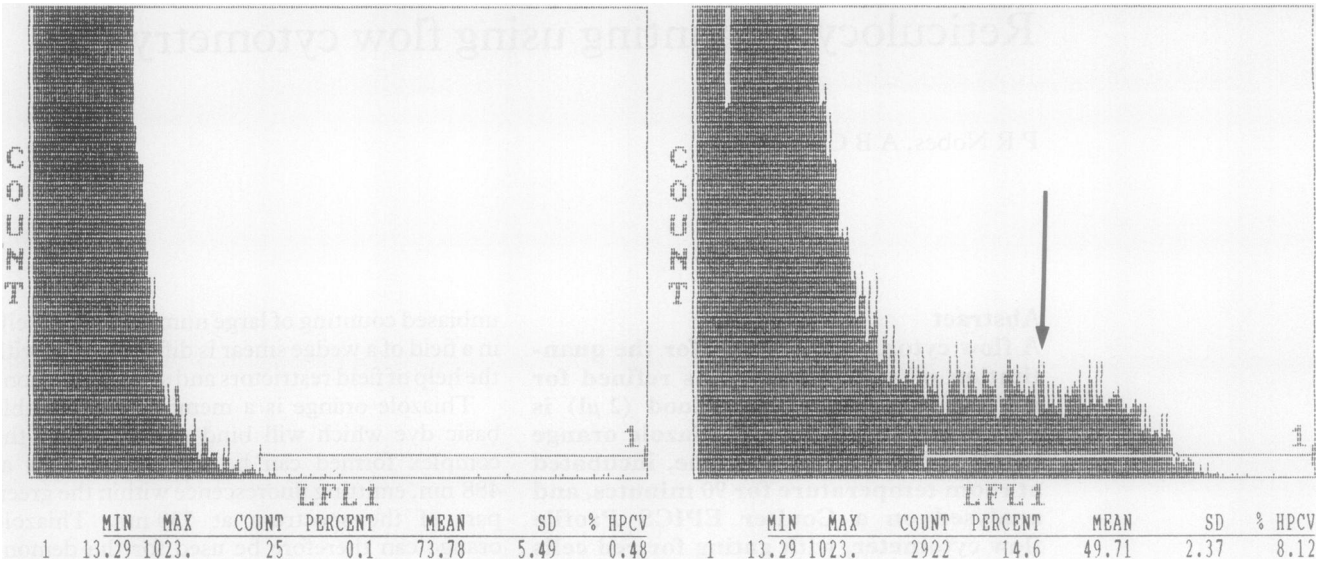

Figure 1 Unstained control (left) and stained (right) obtained by flow cytometry using thiazole orange. The position of reticulocytes is indicated.

PBS alone as an unstained control; both tubes were then incubated for 90 minutes at room temperature in the dark before analysis. Glass tubes were used throughout the staining procedure as thiazole orange is adsorbed by surfaces other than glass.

A total of 20×10^3 erythrocytes were analysed on the Coulter EPICS Profile flow cytometer after gating on a forward scatter against a side scatter plot, to isolate the red cell population, using a 488 nm argon laser at a power level of 15 mW. The filters used included a 525 nm band pass filter in front of the fluorescence detector.

For each sample the unstained control was analysed first and a plot created showing cell number on the Y axis against fluorescence intensity on the X axis. On this plot a cursor was placed on the X axis at the point where the number of positive cells (those cells having fluorescence above the cut off point) equalled 0·1% of the total population. The stained sample was then analysed, while keeping the same cursor position as that used for the unstained control. Any reticulocytes present had a higher fluorescence intensity than the autofluorescence of mature red cells and appeared to the right of the cursor while mature red cells remained on the left (fig 1). The value of the control sample was deducted from the number of positive cells to give the corrected reticulocyte count.

Quality control of the flow cytometric method was maintained by the random selection of samples for visual reticulocyte counts as no biological standards are available for this method at present. Optical alignment was maintained by the daily use of Coulter Immuno-check fluorescent latex spheres.

The precision of the flow cytometric method was determined by five consecutive analyses of each of three samples which had reduced, normal, and raised reticulocyte numbers when counted visually.

Visual reticulocyte counts were performed by mixing one volume of 10 g/l brilliant cresyl blue (BDH Chemicals, Poole, Dorset) in citrate saline with two to three volumes of whole blood. This was incubated at room temperature for 20 minutes before being spread on to clean glass slides. These preparations were examined by two experienced observers who did not know the automated results; each analysed 1000 erythrocytes in accordance with the NCCLS proposed guideline for reticulocyte counting.[5] A mean of the two microscopic counts was used in all subsequent statistical analysis.

Results

Initial studies were performed assessing thiazole orange concentrations of 0·1 μM, 0·2 μM, 0·4 μM and 0·8 μM, and results from each were compared with those of manual counts. The concentration of 0·4 μM performed optimally, giving the best correlation with the manual method (table 2); hence this concentration was used for all subsequent analyses.

The optimal incubation period for flow cytometry was determined by repeated analysis of four samples over a period of time from 0–240 minutes. An incubation time of 90 minutes was chosen (fig 2). In all four cases the stained samples were stable for up to four hours at room temperature.

The adult reference range (mean ± 2SD) for

Table 2 Results of preliminary investigations to find optimal thiazole orange concentration

Concentration of thiazole orange (μM)	*Flow cytometry reticulocyte counts (%)*		
	1	*2*	*3*
0·1	0·3	1·5	8·5
0·2	0·4	1·7	13·3
0·4	0·8	2·0	16·2
0·8	1·3	2·9	18·0
Manual result (%)	0·9	2·4	15·9

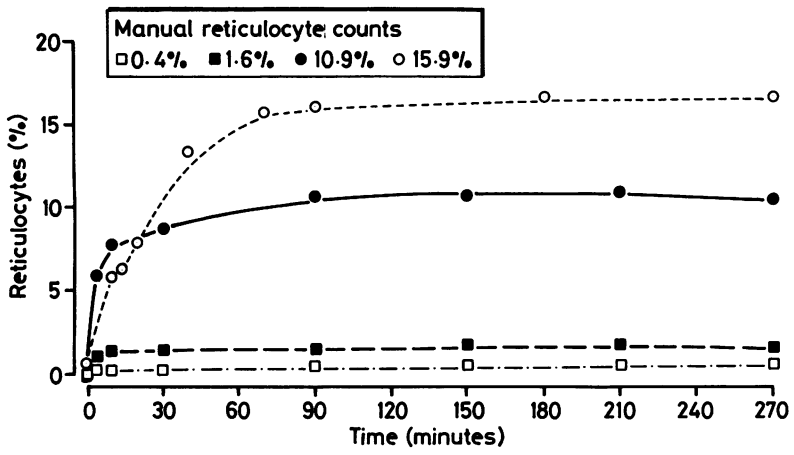

Figure 2 Determination of optimal incubation time.

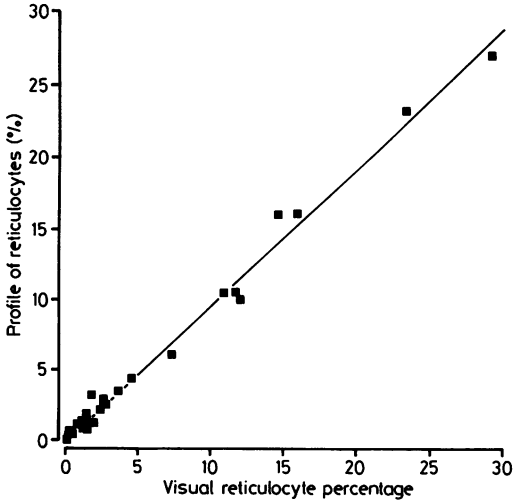

Figure 3 Comparison of manual and profile reticulocyte counts. These data include three patients studied following intensive chemotherapy. Only a single data point on the first day after chemotherapy finished is included (n = 39, r = 0·99, slope = 0·96, intercept = 0·02).

the flow cytometric method in the 30 normal subjects was determined to be 19·4–59·2 × 10^9/l (0·2–1·6%).

Comparison of flow cytometry with the visual technique of 39 samples from patients with a variety of disorders gave a coefficient of correlation of 0·99, with slope 0·96 and intercept 0·02 (fig 3).

To investigate further the accuracy of flow cytometry at low values three patients who had just received intensive chemotherapy were studied serially. Table 3 shows that although these patients had severe reticulocytopenia (0–0·1%) by conventional manual techniques lasting for eight to 18 days, the flow cytometry values were more variable and frequently overlapped with those of the normal range.

Results for the evaluation of precision are given in table 4, showing a coefficient of variation (CV) of 1·42% at a reticulocyte count of 14·3%, a CV of 7·3% at reticulocyte count of 1·8%, and a CV of 33·3% at a reticulocyte count of 0·1%.

Discussion

Reticulocyte counts are an essential technique in the investigation of erythropoietic activity, and it is therefore important that both accurate and precise results are obtained. The investigation of anaemia and the monitoring of treatment and recovery after bone marrow failure all demand a level of reproducibility unavailable by visual methods. Automated flow cytometry, by counting large numbers of cells in suspension and removing observer bias, affords the opportunity to produce more reliable results.

Flow cytometric reticulocyte counting techniques using thiazole orange,[3] Auramine 0,[6] and 1,3′ diethyl-4,2′ quinolyl-thiacyanine iodide[7] have been described. The method of Lee, Chen, and Cheu[3] has been modified here by increasing the incubation time and thiazole orange concentration and decreasing the amount of blood used. With this refined technique the coefficients of variation at a normal and raised reticulocyte count (1·8% and 14·3%) were satisfactory at 7·3% and 1·4%, respectively. Although the CV was considerably greater at a reticulocyte count of 0·1% (CV = 33·3%), these results compare

Table 3 Reticulocyte results obtained during monitoring of three patients after intensive chemotherapy

	Case 1		*Case 2*		*Case 3*	
Day	*Visual*	*Flow cytometry (%)*	*Visual*	*Flow cytometry (%)*	*Visual*	*Flow cytometry (%)*
0	1·2	1·0	0·4	0·6	1·4	0·9
1	0·3	0·5				
2	0·1	0·3				
3			0·2	0·3		
4					0·5	0·5
5	0	0·3				
6					0·1	0·2
7	0·1	0·3			0·1	0·1
8					0	0·2
9	0	0·2				
10			0	0·2		
11					0	0
12	0	0·2	0	0·2	0	0·2
13	0	0			0	0·5
14					0·1	0·3
15			0	0·2	0·1	0·1
16	0·4	0·7			0	0·3
18			0	0	0	0·3
19			0·2	0·3	0·3	0·5
20			0·5	0·6		
21	1·1	1·3	1·2	0·9		

Table 4 Precision analysis of flow cytometry technique

Sample	Flow cytometry results (%)	Mean (%)	CV (%)
1	14·1, 14·2, 14·3, 14·7, 14·3	14·3	1·4
2	1·9, 1·9, 2·0, 1·8, 1·6	1·8	7·3
3	0·1, 0·1, 0·1, 0·2, 0·1	0·12	33·3

favourably with those of the NEQAS analysis of conventional reticulocyte counting, where a sample with a median reticulocyte count of 0·8% gave a CV of 46·3% (n = 397) and a sample with a median reticulocyte count of 13·0% gave a CV of 24·5% (NEQAS, personal communication).

The overall correlation between flow cytometry and manual methods is excellent, giving a coefficient of correlation of 0·99 with slope 0·96 and intercept 0·02. These results are similar to those reported by Lee *et al*[3] and Van Rockstaele and Peetermans.[7]

The automated method was not influenced by low red cell counts, high numbers of leucocytes such as 400 × 10^9/l, or platelets up to 1050 × 10^9/l. Howell-Jolly bodies similarly had no effect.

The thiazole orange stain may be absorbed on to plastic tubing and for this reason the daily cleaning procedure included the cycling of Coulter CLENZ before selection of the shut-down procedure of the instrument. The use of thiazole orange caused no interference with any other application, and separate sample tubing was not required.

At subnormal reticulocyte counts the automated methodology is less accurate and cannot reliably be used. Previous studies of flow cytometric methods have not analysed accuracy at subnormal levels. The reasons for the false positive results at these low levels are not clear, but may be due to limitations in setting the gate for the negative control. Despite this, most requests for a reticulocyte count seek to determine whether the count is normal or raised. The application of flow cytometry to the quantitation of reticulocyte counts will thus be a valuable technique in the routine laboratory.

We thank Dr K Patterson and Professor D Linch for their assistance in the preparation of the manuscript for this paper. NEQAS results are used with the kind permission of Dr S Lewis.

1 Hall RA, Malia RG. *Medical laboratory haematology*. First Edition. London: Butterworths, 1984:107–9.
2 Brecher G, Schneiderman M, Williams GZ. A time saving device for counting reticulocytes. *Am J Clin Pathol* 1950;**20**:1079.
3 Lee LG, Chen C, Chiu LA. Thiazole orange: A new dye for reticulocyte analysis. *Cytometry* 1986;**7**:508–17.
4 Dacie JV, Lewis SM. Preparation and staining methods for blood and bone marrow films. In: *Practical haematology*. Sixth edition. Edinburgh: Churchill Livingstone, 1984:58–60.
5 Finch CA, Marshall PN, Brecher G, *et al*. Methods for reticulocyte counting. NCCLS MIC-P 5;10:1985.
6 Tatsumi N, Tsudu I, Kolima K, *et al*. An automated reticulocyte counting method: Preliminary observations. *Med Lab Sci* 1989;**46**:157–60.
7 Van Rockstaele DR, Peetermans ME. 1,3′ Diethyl-4,2′-quinolylthiacyanine iodide as a "Thiazole Orange" analogue for nucleic acid staining. *Cytometry* 1989;**10**:215–6.