Evaluation of flow cytometric counting procedure for canine reticulocytes by use of thiazole orange

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SUMMARY

An automated reticulocyte counting method that used a flow cytometer and the nucleic acid staining dye, thiazole orange, was developed. Anticoagulated (EDTA) blood specimens were suitable for flow cytometric reticulocyte counting when stored at 4 C for 96 hours after collection. Thiazole orange-stained samples were stable for 5.5 hours after staining when stored capped at 20 C and protected from light. Flow cytometric and manual microscopic reticulocyte counts were compared for counts in the 0.27 to 5.32% range (as determined by flow cytometry) and 0.10 to 4.90% range (as determined by 1 technician). Although the results of flow cytometric analysis generally correlated well ($r = 0.821$) with manual counts, there was poor correlation between the procedures for counts $\leq 2.0\%$ ($r \leq 0.272$). Linearity of flow cytometric counts over the range 0.27 to 14.46% was excellent ($r = 0.999$). Within-run precision of flow cytometric counts (% coefficient of variation [CV] = 3 to 5) was superior to manual microscopic counts obtained by one technician (% CV = 19 to 23) and to manual microscopic counts, which were an average of counts done by 3 technicians (% CV = 8 to 18). Comparable flow cytometric counts were obtained by counting 50,000 or 100,000 blood cells in the flow cytometer.

Reticulocytes are immature erythrocytes. They contain residual RNA and are approximately the same size as mature erythrocytes. They can be distinguished microscopically by precipitation of RNA with supravital stains, such as new methylene blue and brilliant cresyl blue. Measurement of the reticulocyte count is useful in the classification of anemias and for assessing bone marrow function.

Reticulocytes are counted microscopically in an area containing 1,000 erythrocytes, and the resultant count is usually expressed as a percentage. The area of the slide containing 1,000 erythrocytes is frequently estimated for specimens with low reticulocyte counts. In our laboratory, reticulocyte counts are routinely low (< 2%) for Beagles. Thus, a high degree of bias can exist in the microscopic procedure. Flow cytometric counting presents an opportunity to eliminate the statistical pitfalls of microscopic counting by analysis of a larger sample population.

The purpose of the study reported here was to evaluate a flow cytometric method that uses a dye that binds to RNA and fluoresces when exposed to an intense light (laser) of the appropriate wavelength. The resulting emission of light is collected, directed to a signal intensifier, amplified, and used to determine the relative number of reticulocytes.

Dyes that have been used for reticulocyte counting in the flow cytometer are acridine orange,[1] pyronine Y,[2] propidium iodide,[3] and thioflavin T.[4,5] Pyronine Y and propidium iodide require fixation prior to staining. Propidium iodide stains double-stranded RNA only. Acridine orange adsorbs to fluidics systems, increases background signal, and causes maintenance problems. Thioflavin T stains reticulocytes well and does not require a complicated mathematic analysis method. The low-quantum yield, however, of thioflavin T requires precisely timed dilution and sampling methods, which are prohibitive for routine clinical laboratory work.

Thiazole orange (TO),[6,a] a thioflavin T-derived dye, was chosen for this study because it has a high-quantum yield and good stokes shift. It does not require fixation, nor does it adsorb to the fluidics of the flow cytometer. Thiazole orange has been used in the flow cytometer to count human reticulocytes.[6,7]

Materials and Methods

Specimen collection—Blood was collected in tripotassium EDTA-evacuated tubes from Beagles from which food had been withheld for 12 hours. The dogs used were 112 untreated clinically normal dogs, 1 dog with hereditary nonspherocytic hemolytic anemia, and 7 dogs from a phlebotomy study. Samples were collected 10 times from all dogs from the phlebotomy experiment. Two samples were collected from the dog with hemolytic anemia. One sample was collected from each of the other dogs.

Manual microscopic counts—The new methylene blue N (0.5% new methylene blue N, 1.6% potassium oxalate in deionized water) method was used to stain slides for manual counting.[8] Reticulocyte counts were obtained on each smear by 3 technicians, were averaged, and were recorded as mean manual counts. Counts obtained by 1 specific technician were also recorded. These counts are referred to as single manual counts.

Flow cytometric counts—Reticulocyte counts were obtained on a flow cytometer,[b] which used a 2-W capacity argon laser. Laser power of 200 mW was used with an excitation wavelength of 488 nm. The laser was focused

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[a] Jacobberger JW. Flow cytometric reticulocyte counts. Presented at Analytical Cytology XII, Cambridge, England, 1987.

[b] EPICS C, Epics Division, Coulter Electronics Inc, Hialeah, Fla.

on the cell stream by a combination of lenses to provide a 16-μm vertical × 160-μm wide elliptical beam.

Forward light scatter between 0.5 and 19.5° was collected. A neutral density filter (1.0 optical density) was used to exclude extraneous light from the forward light scatter detector. Forward light scatter detector amplification setting was a gain of 20. Green fluorescence light scatter was collected by use of a 488-nm laser-blocking longpass, 515-nm longpass, and 550-nm narrow-band shortpass optical filter combination. Photomultiplier settings for the green fluorescence detector were gain of 2 and high voltage of 1,300. Detected light signals are converted to voltage pulses proportional to the amount of light emission from sample cells. These integrated signals are processed and stored by the instrument's computer.

A two-parameter log green fluorescence vs log forward light scatter histogram was collected on all samples. A total blood cell count of 100,000 was accumulated for each 2-parameter histogram. The X/Y intensity coordinates (bits) were used to define specific cell population areas on the histograms. A polygon (bit map) defining these areas was used to limit analysis to a specific cell population containing erythrocytes.

A clearly defined histogram area for erythrocytes was necessary to prevent analytic interference from platelets and lymphocytes.[9] Washed erythrocytes, platelets,[10] and lymphocytes were separated from EDTA-anticoagulated blood and were compared with samples of nonanticoagulated blood from the same animal. Platelet, lymphocyte, and erythrocyte bit map areas were determined to exclude platelets and lymphocytes from the erythrocyte bit map. The erythrocyte bit map contains reticulocytes.

Staining and analysis—A stock solution of TO[c] was prepared in methanol (1 mg/ml). The stock solution was diluted 1:10,000 in phosphate buffered saline solution (PBSS)[d] pH 7.4 to prepare a working solution of 0.1 μg/ml. The working solution was prepared daily. Stock and working solutions were stored at 20 C in the dark.

Two 12 × 75-mm glass test tubes were used for each blood sample. Three milliliters of PBSS was added to 1 tube (blank). Three milliliters of TO stain was added to the other tube (test). Five microliters of blood was added to each of the 2 tubes. Tubes were vortexed and incubated in the dark for 30 minutes at 20 C. Following incubation, reticulocytes were counted on the flow cytometer, using a bit map previously shown to contain erythrocytes. The counting rate was approximately 5,000 blood cells/s. A one-parameter 1,024-channel log green fluorescence histogram was gated on the bit map. A lower threshold cursor (LTC) was set at the channel where the x axis and the right shoulder of the blank histogram intersected. This channel number was recorded by the computer (Fig. 1). An upper cursor was set at the highest channel number. The percentage of total histogram area between the LTC and the highest channel was recorded for the blank tube. This was designated blank percent. Fluorescence emission of stained reticulocytes occurs between the histogram cursors set with the blank tube and is recorded as test percent (Fig 2). The blank percent was subtracted from the test percent for each sample to calculate percentage of reticulocytes in that sample.

[c] Jacobberger JW, Case Western University, Cleveland, Ohio, and Molecular Probes Inc, Eugene, Ore.

[d] *Sigma* Chemical Co, St Louis, Mo.

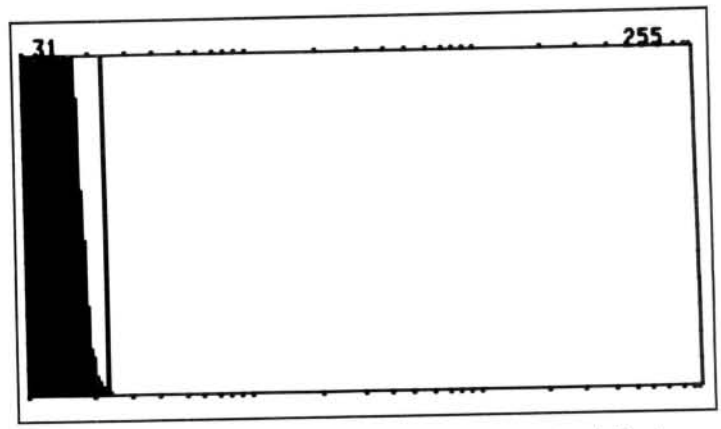

Figure 1—Forward light scatter histogram of blank (unstained) blood sample from a dog. Cursors are set at the edge of erythrocyte peak and highest channel of the histogram.

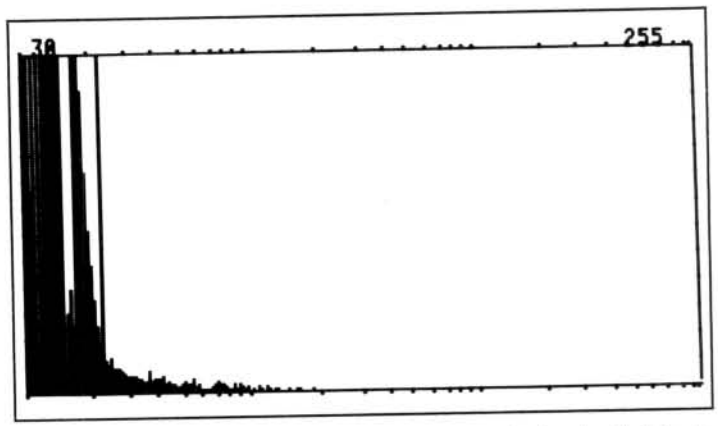

Figure 2—Forward light scatter histogram of test (stained with thiazole orange) blood sample from a dog. Channel range was established by its corresponding blank.

Sample stability—Sample stability at various postcollection time points was determined by comparing reticulocyte counts from the clinically normal dogs. Reticulocyte counts were obtained at 0.5 (n = 86), 1.5 (n = 72), 2.5 (n = 72), 4.0 (n = 72), 6.0 (n = 72), 24.0 (n = 72), 48.0 (n = 48), 72.0 (n = 42), and 96.0 (n = 42) hours after collection. Samples for the 0.5- and 1.5-hour periods were stored at 20 C. Samples for the remaining periods were stored at 4 C until 15 minutes prior to staining. All samples were mixed for 15 minutes prior to staining.

Stain stability—Stain stability was evaluated by performing serial reticulocyte counts on stained samples from 22 clinically normal dogs. Samples were stained at 0.5 hour after collection and stored in the dark at 20 C. Samples were vortexed immediately prior to analysis. Reticulocyte counts were obtained at 0.0, 1.0, 2.0, 3.5, 5.5, and 24.0 hours after staining.

Precision—Within-run precision was determined by counting replicate stained samples from each of 3 dogs. Samples were selected from dogs with low, intermediate (2 dogs from phlebotomy study), and high (dog with he-

reditary hemolytic anemia) reticulocyte counts. Ten reticulocyte counts were obtained on the flow cytometer for each sample. The same samples were also counted manually by 3 technicians. The counts obtained by these technicians were averaged and recorded as mean manual reticulocyte counts. Additionally, the counts of 1 specific technician were recorded as single manual reticulocyte counts.

Correlation between procedures—Correlation between the flow cytometric and manual reticulocyte counts was determined by use of samples from the clinically normal dogs and dogs from the phlebotomy experiment.

Linearity—Linearity was determined for a 0.27 to 14.46% reticulocyte range. Samples with high (dog with hereditary hemolytic anemia) and low (clinically normal dog) reticulocyte counts were mixed together in different proportions and the counts of the mixtures were calculated. Reticulocyte counts for the mixtures were then determined flow cytometrically. These counts were compared with the calculated counts to determine linearity.

Number of blood cells used for reticulocyte counting—Blood samples from 15 clinically normal dogs were used to compare blood cell accumulations of 50,000 and 100,000 for reticulocyte counting. Flow cytometric reticulocyte counts were obtained by use of both total blood cell accumulations for each sample.

Statistical methods—Dunnett *t* test[11] was used for comparison of sample and stain stability. Correlations of flow cytometric vs manual counts, linearity of flow cytometric counts, and counts obtained by use of different total blood cell accumulations were determined by least-squares regression and Pearson correlation coefficient.[12] Student *t* test[13] was used for comparison of reticulocyte counts obtained by use of different total blood cell accumulations.

Results

Blood samples stored for 96 hours at 4 C were stable (Table 1). Stained samples (Table 2) were stable for 5.5 hours when stored in the dark at 20 C. By 24 hours, reticulocyte counts had decreased in these samples.

The precision of the flow cytometric procedure (% CV = 3 to 5) was five- to sixfold better than that for single manual (% CV = 19 to 23) and three- to fourfold better than that for mean manual (% CV = 8 to 18) for low, intermediate, and high counts (Table 3).

Table 1—Comparison of flow cytometric canine reticulocyte counts at various postcollection times

Hours after collection	Mean	SD	N
0.5	0.98	0.45	86
1.5	0.89	0.48	72
2.5	0.90	0.52	72
4.0	0.98	0.50	72
6.0	1.07	0.54	72
24.0	0.87	0.39	72
48.0	0.79	0.36	48
72.0	0.86	0.40	42
96.0	0.97	0.51	42

N = No. of animals.

Table 2—Comparison of flow cytometric canine reticulocyte counts at various poststaining times

Hours after staining	Mean	SD	N
0.0	1.11	0.23	22
1.0	1.06	0.30	22
2.0	1.13	0.37	22
3.5	1.12	0.36	22
5.5	1.00	0.33	22
24.0	0.67*	0.22	22

* Significantly $P \leq 0.05$ different, two-tailed Dunnett *t* test.

Table 3—Comparison of within-run precision of flow cytometric and manual canine reticulocyte counts

Statistical parameters	Reticulocyte counts (%)		
	Flow cytometric	Mean manual*	Single manual†
Mean	0.83	1.24	1.07
SD	0.04	0.22	0.25
% CV	5	18	23
Mean	3.18	3.36	3.33
SD	0.11	0.51	0.65
% CV	3	15	19
Mean	16.41	23.18	20.11
SD	0.51	1.93	3.85
% CV	3	8	19

* Average of counts obtained on the same sample by 3 technicians. † Counts obtained by 1 specific technician.
% CV is the % coefficient of variation.

Table 4—Correlation between flow cytometric (FC) and manual reticulocyte counts

Range of FC reticulocyte counts (%)	Correlation coefficient*		
	FC vs mean manual†	FC vs single manual‡	N
0.27 to 1.00	0.314	0.272	81
1.01 to 2.00	0.233	0.198	55
2.01 to 5.32	0.829	0.780	20
0.27 to 5.32	0.876	0.821	156

* Pearson correlation coefficient. † Average of counts by 3 technicians. ‡ Count obtained by 1 specific technician.

Correlation between flow cytometric and single manual reticulocyte counts was poor for counts < 1.00% (r = 0.272) and for counts between 1.01 and 2.00% (r = 0.198; Table 4). The correlation for counts between 2.01 and 5.32% was good (r = 0.780). The correlation (r = 0.821) was also good when all counts were combined (0.27 to 5.32%). The correlation between flow cytometric and mean manual counts was slightly better than that for single manual counts for all levels examined. Linearity of flow cytometric reticulocyte counts was excellent (r = 0.999) for counts in the 0.27 to 14.46% range (Fig 3).

Reticulocyte counts (Table 5) that included 50,000 or 100,000 total blood cell accumulations were not statistically different ($P \geq 0.05$), and correlated well (r = 0.989) with each other.

Discussion

The difficulties in obtaining manual results with enough accuracy to validate automated procedures are well documented.[14] Variables in manual reticulocyte counting include slide homogeneity,[15] mathematical problems with

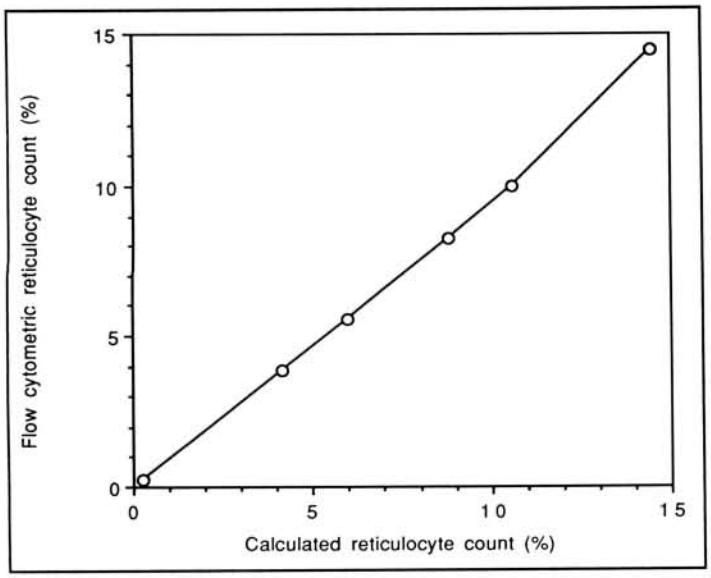

Figure 3—Flow cytometric vs calculated reticulocyte counts (r = 0.999).

Table 5—Comparison of mean flow cytometric reticulocyte counts (%) from 15 dogs obtained by counting different numbers of blood cells

Cells counted	Mean*	SD	Correlation coefficient (r)†
50,000	0.95	0.36	0.989
100,000	0.90	0.36	

* Means not significantly different $P > 0.05$, Student t test. † Pearson correlation coefficient.

low counts, and subjectivity of technicians. The extremely mature reticulocyte is hardest to identify, comprising almost two-thirds of the reticulocyte population in man.[16,17] Reticulocyte counts in Beagles are often < 2%, which makes them a poor model for comparing manual reticulocyte counts with automated methods.

The stability of samples for flow cytometric reticulocyte counting through 96 hours after collection is advantageous if immediate counting is not possible or practicable. The stability of stained samples through 5.5 hours after staining makes batch processing of samples possible. The poor stability of stained cells beyond 5.5 hours after staining is probably related to cell degradation resulting from prolonged exposure to the staining solution. Neither the storage of stained samples at 4 C nor the addition of sodium azide was observed to improve stained cell stability (data not shown). Adding serum albumin or homologous plasma to PBSS throughout the staining procedure might improve stained cell stability.

The precision of TO flow cytometry reticulocyte counts was five- to sixfold better than that for single manual counts. Averaging the counts of 3 technicians did not materially improve the precision of the manual procedure relative to the flow cytometric procedure. The fivefold improvement (% CV = 5, compared with 23) in precision for samples with reticulocytes < 1.0% is especially significant for canine reticulocyte counts. Dogs are frequently used experimentally to assess the effects of cytoreductive agents on erythropoiesis. A reticulocyte counting procedure that is precise and accurate for counts ≤ 1% greatly facilitates early detection of erythroid suppression or recovery associated with administration of cytoreductive agents without having to resort to bone marrow examination.

The correlation of flow cytometric reticulocyte counts with mean manual and single manual counts was good (r = 0.829 and 0.780, respectively) for samples with counts ranging from 2.01 to 5.32%. Correlation was poor for counts in the 0.27 to 1.00% range (r = 0.314 and 0.272, respectively) and for counts between 1.01 and 2.00% (r = 0.233 and 0.198, respectively). The poor correlation between flow cytometric and manual reticulocyte counts ≤ 2.0% is most likely a function of the poor precision of low manual counts.

The linearity (r = 0.999) of flow cytometric counts in the 0.27 to 14.46% range suggests that the procedure is accurate and precise for low and high reticulocyte counts.

Although time-consuming, the preparation and running of a blank for each dog was required to set the LTC and to accurately differentiate TO-stained reticulocyte fluorescence from the autofluorescence of mature erythrocytes. A mathematical mechanism of some kind for setting the LTC might make running a blank sample for each dog unnecessary.

Because TO also reacts with DNA, micronucleated erythrocytes (Howell-Jolly bodies) would give spuriously high reticulocyte counts. Because small lymphocytes and large platelets approach the erythrocyte in size, these could be included in the bit map and result in falsely high reticulocyte counts. The LTC of the blank varied by 1 to 2 channels between samples in each run. This variation could increase or decrease the reticulocyte count by as much as 0.2%.

On the basis of our findings, we suggest that the use of TO in flow cytometric reticulocyte counting is a viable alternative for canine samples, and that it is preferable to microscopic methods if the monitoring of subtle changes in erythrocyte production is of critical importance.

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