Determination of differential cell counts in feline bone marrow by use of flow cytometry

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Objective—To evaluate the potential usefulness of 2 flow cytometric methods for determination of differential cell counts in feline bone marrow.

Sample Population—10 bone marrow specimens from client-owned cats.

Procedure—Bone marrow specimens were stained with 3,3'-dihexyloxacarbocyanine iodide (DiOC₆) and evaluated by use of flow cytometry. Differential counts were also determined by analysis of scatterplots of forward-angle versus side-angle light scatter of unstained specimens, obtained by use of flow cytometry (scatterplot method). Results of both flow cytometric methods were compared with differential cell counts determined by manually counting 1,000 cells on slides of Wright-stained smears.

Results—Staining with DiOC₆ resulted in identification of mature and immature erythroid and myeloid cells and lymphocytes. Use of the scatterplot method resulted in identification of mature and immature erythroid and myeloid cells and metamyelocytes. However, to identify lymphocytes by use of the scatterplot method, bone marrow specimens were first labeled with an anti-major histocompatibility class-II antibody. Comparison of results of the scatterplot method with manual counts yielded higher correlation coefficients and more similar mean values than did comparison of results of the DiOC₆ method.

Conclusions and Clinical Relevance—The scatterplot method provided more accurate and precise results than the DiOC₆ method for determination of bone marrow differential cell counts in cats by use of flow cytometry. When combined with fluorescent labeling of lymphocytes, the scatterplot method has potential to provide rapid semiquantitative assessment of bone marrow differential cell counts in cats. (Am J Vet Res 2001;62:474–478)

Examination of bone marrow has been used for evaluation of hematologic disorders in cats. Qualitative assessments of bone marrow aspiration smears and core biopsy specimens are typically performed clinically, whereas manual differential cell counts (ie, counting 500 or 1,000 cells on Wright-stained bone marrow aspiration smears) are not. However, manual counts have been performed in research and preclinical toxicity studies. Three flow cytometric methods have been described for determination of differential cell counts in bone marrow. In 1 technique, the fluorescent compound 2',7'-dichlorofluorescein (DCF) was used to detect peroxidase activity in rat bone marrow cells. In another technique, 3,3'-dihexyloxacarbocyanine (DiOC₆) was used to differentially stain bone marrow cells from rats, mice, dogs, and monkeys. 3,3'-Dihexyloxacarbocyanine is a lipophilic cationic dye, and the degree of binding of DiOC₆ is dependent on the membrane potential of cells.

Results of staining with DCF or DiOC₆ were compared with differential counts determined by analysis of scatterplots of forward-angle versus side-angle light scatter (scatterplot method) of unstained bone marrow specimens from dogs. Staining with DCF failed to identify distinct cell populations in canine bone marrow. When compared with manual differential cell counts, the scatterplot method was more precise than the DiOC₆ method.

The purpose of the study reported here was to evaluate the potential usefulness of the DiOC₆ and scatterplot methods for determination of differential cell counts in feline bone marrow by use of flow cytometry. Flow cytometric results were compared with results of manual differential cell counts.

Materials and Methods
Bone marrow specimens—Bone marrow was obtained from 10 cats evaluated for hematologic disorders at the University of Minnesota Veterinary Teaching Hospital. Clinical diagnoses included lymphocytic leukemia (n = 2), feline leukemia virus infection (2), myelodysplastic syndrome (2), nonregenerative anemia (2), hemolytic anemia (1), and pure red cell aplasia (1). Bone marrow was aspirated directly into syringes that had been rinsed with 2% EDTA solution. Marrow was aspirated through 20-gauge needles at least 3 times to disperse cell clusters. Wedge-type aspiration smears of bone marrow specimens were prepared on slides for determination of manual differential cell counts.

In preliminary studies, using the scatterplot method, forward- and side-angle light scatter of nonnucleated erythrocytes overlapped with that of mature nucleated erythroid cells. This resulted in overestimation of the latter cell population. To eliminate this discrepancy, nonnucleated erythrocytes were lysed before analysis. Ten microliters of marrow was placed into a 6-ml sterile plastic tube, and 2 ml of erythrocyte lysis buffer was added. After 10 minutes at room temperature (22 to 25 C), tubes were centrifuged, and the cell pellet was washed and resuspended in Dulbecco phosphate-buffered saline solution (DPBS) containing 2% sheep serum. Manual differential cell counts were performed on 5 bone marrow specimens before and after lysis of erythrocytes. Cell counts were similar before and after lysis, indicating that only non-nucleated erythroid cells were lysed.

Manual differential cell counts—Five hundred cells on each of 2 slides of Wright-stained bone marrow aspiration smears were counted. Cells enumerated included mature (rubricytes and metarubricytes) and immature (rubriblasts and prorubricytes) erythroid cells, immature myeloid cells (myeloblasts and progranulocytes), metamyelocytes, mature...
Flow cytometric analysis by use of the scatterplot method—A scatterplot template was developed for determination of bone marrow differential cell counts (Fig 1). Locations of subpopulations of myeloid and erythroid cells were determined on the basis of results of previous studies evaluating canine and feline bone marrow. A gate was set around a distinct population of cells with forward-angle and side-angle light scatter properties consistent with mature myeloid cells. Two additional gates were set to detect cells larger than mature myeloid cells (ie, cells with greater forward-angle light scatter). One of these populations had a low degree of granularity (ie, low side-angle light scatter) and presumably comprised immature myeloid cells. The other population had a high degree of granularity (ie, high side-angle light scatter) and presumably comprised metamyelocytes. Mature and immature erythroid cells were identified as a compact group of small- to medium-sized cells with a low degree of granularity. Two gates were set to differentiate the smaller mature erythroid cells from the larger immature erythroid cells. Distinct lymphocyte or megakaryocyte populations could not be identified on the basis of side- and forward-angle light scatter alone. Using 3 bone marrow specimens obtained from healthy cats, the size and location of scatterplot gates were adjusted to improve agreement with cell percentages obtained by use of manual counting. The final scatterplot template was used to determine differential counts in bone marrow specimens from 10 client-owned cats.

To specifically identify lymphocytes in 4 bone marrow specimens, 10 µl of marrow was incubated with a monoclonal antibody against feline major histocompatibility class-II (MHC-II) antigen for 1 hour at room temperature. Cells were then washed and incubated with fluorescein-labeled sheep anti-mouse IgG for 30 minutes at room temperature. Labeled cells were diluted to 1.0 ml with DPBSS and analyzed by use of flow cytometry. Scatterplots of forward-angle light scatter (x-axis) and log of green fluorescence intensity (y-axis) were obtained, which permitted analysis of cell populations with differing fluorescence intensity (y-axis) were obtained, which permitted analysis of cell populations with differing fluorescence intensity. Labeled cells were diluted to 1.0 ml with DPBSS containing 2% sheep serum, stored at 4°C, and analyzed by use of flow cytometry within 2 hours.

Results were displayed as scatterplots, with log green fluorescence intensity on the x-axis and forward-angle light scatter on the y-axis (Fig 3). A template was developed to detect populations of immature and mature myeloid cells, immature and mature erythroid cells, and lymphocytes on the basis of the described locations of these cell populations.

Flow cytometer—Flow cytometry was performed, using a commercially available cytometer. Forward scatter was set at 0.1 A, side scatter at 444 V, and amp gain at 9.54. The forward scatter threshold was set at 50 to eliminate platelets and small particulate debris. For samples in which erythrocytes were

Flow cytometric analysis by use of DiOC6 staining—Ten microliters of bone marrow was suspended in 100 µl of DPBSS containing 2% albumin and incubated with 300 µl of 133 nM DiOC6 for 30 minutes at 37°C in the dark. A 2.5 mM stock solution of DiOC6 was prepared in dimethyl sulfoxide, and the stock solution was diluted to 133 nM with DPBSS just before use. After incubation, samples were diluted to 1.0 ml with DPBSS containing 2% sheep serum, stored at 4°C, and analyzed by use of flow cytometry within 2 hours.

Results were displayed as scatterplots, with log green fluorescence intensity on the x-axis and forward-angle light scatter on the y-axis (Fig 3). A template was developed to detect populations of immature and mature myeloid cells, immature and mature erythroid cells, and lymphocytes on the basis of the described locations of these cell populations.

Flow cytometer—Flow cytometry was performed, using a commercially available cytometer. Forward scatter was set at 0.1 A, side scatter at 444 V, and amp gain at 9.54. The forward scatter threshold was set at 50 to eliminate platelets and small particulate debris. For samples in which erythrocytes were
lysed, 30,000 cells were analyzed. For samples in which erythrocytes were not lysed, 50,000 cells were analyzed.

Statistical analyses—Differential cell counts determined by use of scatterplot and DiOC₆ methods were compared with manual differential cell counts. Mean values and correlation coefficients for each cell type were determined. To determine significance of correlation coefficients, a Fisher r-to-z transformation was performed. This allowed transformation of correlation coefficients to a variable with a standard normal distribution.¹ A P value was calculated for the null hypothesis that results were not correlated (ie, z = 0), and P ≤ 0.05 was considered significant.

Results

Scatterplot method—Differential cell counts obtained by use of the scatterplot method were compared with manual differential cell counts (Fig 4). With the exception of mature erythroid cells, correlation coefficients (r or z) exceeded 0.9. Correlations were significant, indicating that cell counts obtained by use of the 2 methods were similar. Results of the scatterplot method overestimated the percentage of immature and mature erythroid cells, most likely because we could not differentiate lymphocytes from mature erythroid cells by use of light-scatter properties alone. When the percentage of lymphocytes determined manually was added to the percentage of mature erythroid cells determined manually, mean percentages of mature erythroid cells were similar (z = 0.93) between the 2 methods.

Because lymphocytes could not be differentiated from mature erythroid cells on the basis of light scatter alone, bone marrow specimens from 4 cats were stained with anti-MHC-II antibody, and fluorescent intensity within the mature erythroid cell population was evaluated (Fig 2). When percentage of fluorescent cells in the mature erythroid gate was compared with percentage of lymphocytes determined manually, results were highly correlated (z = 0.98; P < 0.001), but the mean value for fluorescent cells was slightly greater than mean percentage of lymphocytes determined manually (10.5% vs 7.0%).

DiOC₆ method—Large numbers of non-nucleated erythrocytes were detected in bone marrow specimens...
from 3 of the 10 cats. This resulted in counting of inadequate numbers of nucleated cells to perform differential cell counts. Thus, differential counts determined by use of the DiOC6 method were compared with manual differential counts for only 7 of the 10 cats. Correlation coefficients varied between 0.61 and 0.96 (Fig 5). Correlations for immature and mature myeloid cells and myelocytes were significant. However, correlations for immature and mature erythroid cells (z = 0.61 and 0.74, respectively) were not significant (P = 0.15 and 0.06, respectively), indicating that results for these 2 cell populations were dissimilar between methods.

Discussion

Results of 2 flow cytometric methods for determination of differential cell counts in feline bone marrow were compared with manual differential cell counts. In general, comparison of results of the scatterplot method yielded higher correlation coefficients and more similar mean values than did comparison of results of the DiOC6 method. However, the correlation coefficient for lymphocytes determined by use of the DiOC6 method was high, indicating that the DiOC6 method may provide a convenient way to quantify lymphocytes in feline bone marrow.

Results of the scatterplot method consistently overestimated the percentage of mature erythroid cells. This was probably because lymphocytes were included within the mature erythroid gate. The sum of the percentage of lymphocytes and mature erythroid cells determined manually approximated the percentage of mature erythroid cells determined by use of the scatterplot method. Furthermore, labeling of 4 bone marrow specimens with anti-MHC-II antibodies resulted in distinct fluorescent and nonfluorescent populations within the mature erythroid gate. On the basis of comparison with manual differential cell counts, most bone marrow lymphocytes appeared to be labeled by this antibody. However, in a previous study, anti-MHC-II antibodies only reacted with a subpopulation of lymphocytes in feline lymph nodes.

Megakaryocytes were not identified as a distinct cell population by use of either flow cytometric method. This may be because of the relatively low
number of megakaryocytes in marrow specimens and variability in megakaryocyte size and granularity depending on stage of maturity.

Results of the present study indicate that the scatterplot method was superior to the DiOC₆ method for determining differential cell counts in feline bone marrow. A limitation of the scatterplot method was the inability to differentiate lymphocytes from mature erythroid cells. However, this problem can be addressed by use of monoclonal antibodies that label bone marrow lymphocytes. Unlike manual differential cell counts, flow cytometric methods are simple to perform, and processing and analysis times are brief. Moreover, analysis of flow cytometric data does not require extensive training. Additionally, flow cytometric methods can be readily adapted for batch analysis of specimens. Therefore, flow cytometry is ideally suited for research and preclinical toxicity studies but may also be applied to analysis of individual bone marrow specimens in clinical laboratories.

References