NOTE  Avian Pathology

A New Method for Counting of Quail Leukocytes by Flow Cytometry

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ABSTRACT. An automatic counting method was developed for fish blood cells using a fluorescent dye, 3, 3-dihexyloxacarbocyanine (DiOC6(3)), that selectively stain lipid bilayers in living cells. In the present study, the DiOC6(3) method was applied to quail (Coturnix coturnix japonica) blood cells. After quail blood cells were stained with DiOC6(3), absolute counts and relative proportions of erythrocytes, granulocytes, monocytes, and lymphocytes plus thrombocytes in whole blood were obtained by means of flow cytometry (FC). The number of each cell types by the FC was in good agreement with those counted microscopically. This method will offer new possibilities for routine blood cell counting for avian medicine.

KEY WORDS: avian, flow cytometry, leukocyte count.

In mammals, automatic counting methods for blood leukocytes have been developed and widely used for diagnosis of diseases. However, in non-mammalian vertebrates (avian, reptile, amphibian, and fish), the presence of nucleated erythrocytes and thrombocytes preclude the application of leukocyte counting methods used in mammals. With these animals, leukocyte count and leukocyte differential are mostly microscopically determined [2, 5].

In the previous study, we have developed a rapid and reliable method for counting fish blood cells [1]. After fish blood cells were stained with 3, 3-dihexyloxacarbocyanine (DiOC6(3)), absolute counts and relative proportions of leukocytes in whole blood were obtained by means of flow cytometry (FC). DiOC6(3) is known to be cationic and lipophilic dye that selectively stain lipid bilayers such as the plasma membrane and cell organelle membranes [6, 7]. The DiOC6(3) was good at staining leukocytes and thrombocytes, but not as effective at staining erythrocytes, since erythrocytes had poor cell organelle [3, 8]. In the present study, we applied DiOC6(3) staining to quail (Coturnix coturnix japonica) blood cells to establish a new method for counting avian blood cells.

Two to 12 months old Japanese quails, Coturnix coturnix japonica, each weighing 80–110 g, were obtained from a commercial farm. Blood was collected via the brachial vein with a heparinized syringe.

Quail blood cells were stained with DiOC6(3) and analyzed using FACScalibur (Becton Dickinson, Franklin Lakes, NJ, U.S.A.) as described previously [1]. Briefly, quail whole blood (10 µl) was delivered into a test tube and 1910 µl of Hank’s balanced salt solution (HBSS) and 40 µl of DiOC6(3) solution (50 µg/ml of DiOC6(3)) was added. After incubation for 10 min at room temperature, 40 µl of FITC-labeled beads (5 µm in diameter, 5000 particles/µl) was added. For FC analysis, forward scatter (FSC), side scatter (SSC) and green fluorescence (FL-1) of each cell and FITC-bead were measured. An electronic gate was set to count the FITC-beads. Counting was terminated when a standard number of 5000 beads were counted. The number of each leukocyte in blood was calculated according to the following formula:

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\text{Number of each leukocyte} / \mu l = (\text{cell count of each population identified by a typical gating}) \times 100^*5000** \times 200***
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* FITC-labeled beads were added at a final concentration of 100 particles/µl, ** 5000 FITC-labeled beads were counted for each sample, *** Blood was diluted 200 times

Each blood cell population identified by its typical location in a FSC vs. SSC dot-plot was subjected to sorting procedures. Sorting was carried out by the FACScalibur sorting-module as described previously [1]. Since the number of each leukocyte was too small for cell-sorting from whole blood, the leukocytes including thrombocytes were separated from erythrocytes by Percoll density centrifugation (1.085 g/ml, 400 × g, 10 min) [1]. After a centrifugation, interface layer was collected, adjusted around 10^7 cell/ml, stained with DiOC6(3) and used for the sorting procedure.

Figure 1 shows a typical FC analysis of quail blood cells. When FL-1 vs. SSC was recorded, mainly 2 distinct cell populations termed R1 and R2 were revealed. The number of each leukocyte by FC was in good agreement with those counted microscopically. This method will offer new possibilities for routine blood cell counting for avian medicine.
leukocytes were classified and identified as described by Witkowski and Thaxton [9].

The number of each leukocyte obtained by FC analysis was confirmed by comparing cell counts made from microscopic count. For microscopic count, the total number of leukocytes including thrombocytes was obtained by Natt-Herrick method [4]. Differential leukocyte count was obtained by observation on blood smears stained with M-G. Figure 3 clearly demonstrates the close correlation between the two methods for counting and distinguishing each leukocyte type. The correlation coefficients were $r=0.98$ for thrombocytes plus lymphocytes, $r=0.96$ for granulocytes, and $r=0.98$ for monocytes (Fig. 3).

In the present study, DiOC$_6$(3) staining was proven to be applicable to avian blood cells. Absolute count and relative proportion of erythrocytes, granulocytes, monocytes, and lymphocytes plus thrombocytes were obtained by means of FC analysis. Compared to the microscopic count, DiOC$_6$(3) staining combined with FC analysis was simple and fast. Reliable counts were obtained. This method will offer new
possibilities for routine blood cell counting for avian medicine. However, separate count of thrombocytes, lymphocytes, heterophils, and eosinophils has not been succeeded. Further studies were needed.

REFERENCES


Fig. 3. Comparison of leukocyte counts between flow cytometric (FC) and microscopic count. Close correlation between the two methods were obtained. The correlation coefficients were \( r=0.98 \) for thrombocytes plus lymphocytes, \( r=0.96 \) for granulocytes, and \( r=0.98 \) for monocytes.