Separation of Turkey Heterophils from Blood Using Two-Step Ficoll-Hypaque Discontinuous Gradients

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Research Note—

Separation of Turkey Heterophils from Blood Using Two-Step Ficoll-Hypaque Discontinuous Gradients

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SUMMARY. A method is presented to separate turkey heterophils from anticoagulated whole blood using two-step Ficoll-Hypaque discontinuous gradients and ammonium chloride lysis of contaminating erythrocytes. Heterophils can be isolated from multiple blood samples within 3 to 4 hours. Using this technique, 66.4 ± 18.4% (mean ± standard deviation) of blood heterophils were harvested. Final cell isolates averaged 96.0 ± 2.9% heterophils with few contaminating eosinophils (2.5 ± 2.3%) or basophils (1.6 ± 1.8%). Cell viability, as determined by trypan blue dye exclusion, was 98.0 ± 1.4%.

RESUMEN. Nota de Investigación—Separación de los heterófilos de pavo a partir de la sangre usando gradientes discontinuos de Ficoll-Hypaque.

Se presenta un método para separar heterófilos de pavo a partir de sangre entera con anticoagulante. El método se hace en 2 pasos con gradientes discontinuos de Ficoll-Hypaque. Los eritrocitos contaminantes se lijan con cloruro de amonio. Los heterófilos pueden ser aislados en 3 a 4 horas a partir de varias muestras de sangre. Usando esta técnica, se aisló el 66.4 ± 18.4% (promedio ± desviación estándar) de los heterófilos sanguíneos. El promedio final de aislamiento de células fue de 96.0 ± 2.9% de heterófilos con unos pocos eosinófilos (2.5 ± 2.3%) o basófilos (1.6 ± 1.8%) contaminantes. La viabilidad de las células, determinada por el método de exclusión con azúl trián, fue del 98.0 ± 1.4%.

Investigations of avian heterophil function in disease have been limited to studies of whole blood because of the inability to separate relatively pure populations of heterophils from blood (3,4). Because all avian blood cells (erythrocytes, leukocytes, and thrombocytes) are nucleated, density-gradient separation of these cells is more difficult to achieve than in mammals such as human beings (2) and dogs (6). Previous attempts to isolate avian heterophils on density gradients have been hampered by other contaminating leukocytes that account for a large portion of the isolates (10,11). In addition, methods used to isolate populations of heterophils from chicken blood (1,10) will not work reliably on turkey blood samples. Discontinuous gradients have been used to isolate heterophils from the blood of three turkeys (11), but this cell-isolation technique produced disappointing results in our hands. The purpose of the present study, therefore, was to devise a procedure using commercially available reagents to extract turkey heterophils from blood with high cell yield, purity, and viability.

MATERIALS AND METHODS

Turkeys. Twenty healthy 6-to-8-week-old Nicholas tom turkeys were used as blood donors. The turkeys were placed in a commercial poultry house (16-hour-light, 8-hour-dark cycle) and provided growth ration and water ad libitum.

Sample collection. Blood samples (2–2.5 ml) were collected from the ulnar vein of each turkey (n = 20) into disposable plastic syringes containing 200 µl of 10% disodium ethylenediamine tetraacetic acid (EDTA). A 2-ml sample was used for heterophil iso-
lution, and the remainder of the sample was used for total and differential granulocyte counts.

**Hematologic techniques.** Total granulocyte counts (heterophils, basophils, and eosinophils) were performed manually on blood specimens and isolated cell suspensions using Thoma red-cell pipettes, Neubauer-ruled hemacytometers, and methyl violet 2B diluent (9). One-hundred-cell granulocyte differential counts were performed on Wright-Leishman-stained blood smears and cytocentrifuge preparations (Cytospin, Shandon Southern Products, Ltd., Cheshire, England) of cell suspensions. Granulocytes were identified microscopically by accepted criteria (7). The total number of heterophils in the 2-ml blood samples and cell suspensions then was calculated based upon the total and differential granulocyte counts.

**Heterophil isolation.** Turkey heterophils were isolated from blood using modifications of published techniques (1,6,11). Reagents for cell isolation and suspension were filter-sterilized (0.22-μm pore diameter) and equilibrated at room temperature (24–25°C). In addition, all glassware was siliconized (Sigmacote, Sigma Chemical Co., St. Louis, Missouri) to reduce adherent heterophil loss.

Two-step discontinuous Ficoll-Hypaque gradients were made using commercially available reagents (Histopaque-1077 and -1119; Sigma). The gradients were constructed by placing 3 ml of Ficoll-Hypaque with specific gravity of 1.119 in a 13 × 100-mm test tube. This material was overlaid with 3 ml of Ficoll-Hypaque with a specific gravity of 1.077.

All centrifugation steps were performed at room temperature with a tabletop centrifuge equipped with a swinging bucket rotor (Beckman model TJ-6R centrifuge and TA-4 rotor; Beckman Instruments, Inc., Palo Alto, Calif.). The brake was set in the off position to minimize disturbance of the gradients.

The EDTA blood samples were centrifuged at 200 × g for 10 minutes at room temperature. The plasma layer was removed, discarded, and replaced with 1 ml of Hanks' balanced salt solution (HBSS; Ca++ and Mg++ free, pH adjusted to 7.3 with NaHCO₃). The samples were mixed and layered onto the preconstructed discontinuous Ficoll-Hypaque gradients, and the gradient tubes were centrifuged at 225 × g for 20 minutes.

Following centrifugation, the top layer, consisting of HBSS and residual plasma and a white-colored cell band (at the HBSS/Ficoll-Hypaque 1.077 junction) containing thrombocytes, mononuclear leukocytes, eosinophils, and basophils, was discarded. The remaining layers (residual 1.077, 1.119, and red cell pellet) were collected and washed twice in HBSS. Centrifugation at 225 × g for 10 minutes was adequate to pellet the cell suspensions. After the supernatant was decanted, erythrocytes were lysed by the addition of seven volumes of freshly prepared 0.87% NH₄Cl in 0.1% KHCO₃ (pH 7.2) to one volume of packed cells. The tube was placed on a mechanical rotator (Adams Nutator model 1105; Clay Adams Division of Becton, Dickinson Co., Parsippany, N.J.) for 10 minutes for erythrocyte lysis to occur.

Following erythrocyte lysis, the samples again were centrifuged at 200 × g for 10 minutes. The supernatant was discarded and the cell pellet was washed twice in HBSS to remove soluble erythrocyte lysis products. After the second washing, the cell pellets were resuspended with HBSS to a final volume of 2 ml. Portions of the isolates were removed for total granulocyte counts (as detailed above) and viability determination.

**Calculation of heterophil yield, purity, and viability.** Heterophil yield was calculated using absolute heterophil numbers in the blood and final isolates (2-ml volumes). Heterophil purity of the isolates was determined by 100-cell granulocyte differential counts of the final cell isolates. Heterophil viability was determined by the trypan blue dye exclusion test. Wet-mount preparations were made by mixing one drop of the cell isolate and one drop of 0.5% trypan blue dye in saline solution. Heterophils were identified by their characteristic refractile, unstained granules. Viable cells lacked staining, whereas dead cells had blue-staining nuclei. One hundred consecutive heterophils were examined for viability (8). Data concerning heterophil yield, cell isolate purity, and heterophil viability were expressed as percentages (group mean ± standard deviation).

**RESULTS**

The average heterophil yield from blood was 66.4 ± 18.4%, with a range from 34.9% to 97.6%. Approximately 12,499 ± 5,076 × 10⁶ heterophils could be isolated from a single 2-ml blood sample, with total heterophil numbers ranging from 5,626 × 10⁶ to 23,400 × 10⁶ cells/2 ml of blood. Heterophil purity averaged 96.0 ± 2.9%, and overall purity ranged from 90% to 100%. Contaminating leukocytes consisted of low numbers of eosinophils and basophils in some samples. Eosinophil contamination averaged 2.5 ± 2.3% and ranged from 0% to 7%. Basophil contamination was infrequent and averaged 1.6 ± 1.8%, ranging from 0% to 7%. Heterophil viability in the final cell isolates averaged 98.0 ± 1.4%, ranging from 95% to 100%.

Turkey heterophils were distributed throughout the 1.077 and 1.119 Ficoll-Hypaque layers as well as within the red cell pellet. Contamination with basophils and eosinophils was minimized by relatively short centrifugation times.
and by discarding the upper portion of the 1.077 layer. Although most of the erythrocytes pelleted in the bottom of the tube, erythrocytes were present throughout the 1.077 and 1.119 layers in various concentrations. Most of the less-dense erythrocytes were reticulocytes (polychromatophilic erythrocytes) based upon examination of Wright-Leishman-stained cytocentrifuge preparations. Intact reticulocytes, distinguished from erythrocyte ghost cells by the presence of blue-gray cytoplasm, also were present in the final isolates, indicating innate resistance to NH₄Cl lysis. Reticulocytes constituted 5.0 ± 4.9% of the total cell population isolated and ranged from 0% to 16% in the final cell isolates.

Using this technique, heterophils could be isolated from several blood samples simultaneously. The total time required to isolate turkey heterophils from blood ranged from 3 to 4 hours.

**DISCUSSION**

We have modified our neutrophil isolation technique for dogs (6) and heterophil isolation technique for chickens (1) to achieve reliable separation of heterophils from blood samples of 6-to-8-week-old turkey pouls. Heterophil isolates obtained with this modified procedure have exceptional yield, purity, and viability. Although chicken heterophils have been isolated from blood using Ficoll (10) and Ficoll-Hypaque gradients (1), we were unable to achieve the desired cell yield and/or purity using these techniques with turkey blood. A Ficoll discontinuous gradient technique has been used to separate heterophils from the blood of three turkeys (11), but this procedure was not reliable in our experience. The heterophil yield, purity, and viability of the earlier method averaged 45%, 63%, and 85%, respectively (11). In contrast, improved heterophil yield (66%), purity (96%), and viability (98%) were achieved using our method. In addition, heterophil numbers that are adequate for most cell-function studies (12.5 × 10⁶ cells) can be obtained from a 2-ml blood sample.

The presence of erythrocyte nuclear debris and unlysed erythrocytes is a consistent problem when isolating avian heterophils. Erythrocyte debris can be minimized by not collecting the red cell pellet, but the percentage of heterophil yield will decline markedly (1). Reticulocyte contamination of the final heterophil isolates is expected because these young red cells are present in low numbers in health (≤0.6%), have increased cell membrane surface area, and are more resistant to induced lysis (5). A higher percentage of contaminating reticulocytes may be expected in extremely young pouls and in turkeys with regenerative anemia. In each instance, increased reticulocytosis is the result of enhanced bone-marrow erythropoiesis.

**REFERENCES**