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Source: Journal of Wildlife Diseases, 36(3) : 535-540

Published By: Wildlife Disease Association

URL: https://doi.org/10.7589/0090-3558-36.3.535

SHORT COMMUNICATIONS

Journal of Wildlife Diseases, 36(3), 2000, pp. 535–540

Use of a Two-step Percoll® Gradient for Separation of Loggerhead Sea Turtle Peripheral Blood Mononuclear Cells

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ABSTRACT: In order to determine a suitable procedure for isolating peripheral blood mononuclear cells (PBMCs) from loggerhead sea turtles (*Caretta caretta*), blood was collected using three different anticoagulants (sodium heparin, sodium citrate or potassium EDTA) and separated using a single step commercially-prepared arabinogalactan gradient of 1.077 g/ml density or multiple step Percoll gradients between 1.053 and 1.076 g/ml density (40–60% stock isotonic Percoll suspension). Heparinized blood centrifuged over a two-step 45/55% (1.059/1.070 g/ml) Percoll gradient yielded 99 to 100% mononuclear cells at the 45/55% interface. Mononuclear cell viability ranged from 85 to 97% with cell yields up to 9.2×10^6 cells/mL. An unexpected finding was a population of low density granulocytes migrating to 40% (1.053 g/ml) and 45% Percoll layers in the multiple step gradients. These granulocytes could be eliminated from the PBMC preparation by use of the two-step 45/55% Percoll gradient. Isolated PBMCs can be used for cellular immunology and toxicology studies on these threatened marine organisms for which other tissues can usually be obtained only sporadically from post-mortem specimens.

Key words: *Caretta caretta*, density gradient centrifugation, loggerhead turtle, peripheral blood mononuclear cells.

Sea turtles are listed as threatened or endangered throughout their ranges under the U.S. Endangered Species Act, and all marine turtles are listed on Appendix I of the Convention on International Trade in Endangered Species of Flora and Fauna (Pritchard, 1997). Among threats to sea turtle population viability are chemical pollutants in the form of oil spills or agricultural, industrial or urban run-off (Campbell, 1996b; George, 1997; Lutcavage et al., 1997), and diseases with a possible immunosuppressive component such as fibropapillomatosis (Herbst, 1994; Herbst et al., 1998). Because of their regulatory status, sample collection from live sea turtles is usually limited to relatively non-invasive procedures, including venipuncture. Studies of sea turtle toxicology and cellular immune function in response to environmental contaminants and infectious disease would be facilitated by well-characterized blood cell separation techniques.

Separation of peripheral blood mononuclear cells (PBMCs; lymphocytes and monocytes) from granulocytes (eosinophils, basophils, and heterophils or neutrophils) and erythrocytes is required for many cellular immune function assays (Waterstrat et al., 1988). Single layer 1.077 g/mL gradients commonly employed to separate mammalian leukocyte populations (Coligan et al., 1992), have been used to isolate PBMCs from green sea turtle (*Chelonia mydas*) blood (McKinney and Bentley, 1985). Application of techniques standardized for mammalian leukocytes to non-mammalian systems, however, may not always yield ideal results. Gradient separation techniques for leukocytes have been optimized for some teleost fish to permit improved targeting of desired leukocyte populations which migrate at densities different from their mammalian counterparts (Waterstrat et al., 1988). The

objective of the current study was to determine a suitable method for isolation of loggerhead sea turtle (*Caretta caretta*) PBMCs.

Juvenile loggerhead sea turtles were captured in pound nets from Core Sound (North Carolina, USA) in the course of population and behavioral studies. They were held for a maximum of 5 days in flow-through 1,000 L seawater tanks, during which time five turtles (48–55 cm straight carapace length) were bled from the dorsal occipital venous sinus. Blood from the first turtle (turtle #1) was divided between evacuated tubes (Vacutainer, Becton, Dickinson & Co., Rutherford, New Jersey, USA) containing the anticoagulants sodium heparin (14.4 U/ml final concentration), sodium citrate (14 mm final concentration) or potassium EDTA (4 mm final concentration), and thin blood smears were made immediately with non-anticoagulated blood. Blood from three other turtles (turtles #2–4) was collected directly into heparin tubes only. Blood was transported on wet ice to the laboratory, where processing began within 4 hr of collection. Cells and solutions were kept near 4C throughout processing. Blood from a fifth turtle (turtle #5) was collected into heparinized tubes, held at 4C, and processing was attempted following a 48 hr delay.

Heparinized and citrated blood was centrifuged at 400 × g for 5 min at 4C, and the buffy coats were harvested and resuspended in sterile cell culture medium (RPMI 1640 medium Gibco BRL, Rockville, Maryland, USA plus 10% heat-inactivated fetal bovine serum [Sigma, St. Louis, Missouri, USA], 100 U/ml penicillin and 100 μg/ml streptomycin [Gibco BRL], and 2 mM EDTA [Sigma] to reduce cell aggregation; subsequently referred to as complete RPMI) at 50% or 100% of the original blood volume. Plasma was harvested and saved at −80C. Hemolysis completely obscured the buffy coat in the EDTA tube from turtle #1, so for that sample, whole blood was diluted 1:2 (original : final volume) with complete RPMI.

In 15 ml conical tubes, buffy coat suspensions (2–5 ml) or diluted whole blood (8–12 ml) were underlaid with either a single layer of arabinogalactan (1.077 g/ml density; Cellsep™, Larex, Inc., St. Paul, Minnesota, USA; superseded by Arabi-NIM™, Cardinal Associates, Santa Fe, New Mexico, USA), a commercial product designed for separation of mammalian lymphocytes, or with multiple 2 ml layers of colloidal PVP-coated silica (Percoll, Sigma) introduced in increasing order of density beneath the preceding layer using a 14 gauge 12 cm blunt-tipped canula. Stock isotonic Percoll suspension was prepared by diluting nine parts Percoll with one part sterile 10× saline (1.5 M NaCl), and further dilutions (40, 45, 50, 55, and 60% of stock suspension) were made with sterile 1× saline (0.15 M NaCl). Percoll dilutions were made fresh before each use. Final Percoll dilutions corresponded, by calculation, to the following densities: 40% = 1.053 g/ml, 45% = 1.059 g/ml, 50% = 1.064 g/ml, 55% = 1.070 g/ml, 60% = 1.076 g/ml (Pharmacia Biotech, 1995). Cells were centrifuged over their respective gradients at 400 × g for 5 min at 4C, followed immediately by 800 × g for 20 min at 4C, and stopped with no brake to avoid disrupting boundary layers. Cell layers were harvested by pipette from gradient interfaces, and resuspended and washed twice with complete RPMI (400 × g for 5 min at 4C). Cell counts and viability were determined by hemocytometer examination of cells incubated in 0.2% trypan blue/complete RPMI.

Cytospin slides were prepared from 1×10^5 cells in 100 μl complete RPMI with a cytocentrifuge (Cytospin 2, Shandon Inc., Pittsburgh, Pennsylvania, USA) set at 450 rpm for 5 min. Cytospin and blood smear slides were stained with Leukostat™ (Fisher Scientific, Pittsburgh, Pennsylvania, USA), and differential counts were performed by a single observer (CAH) for consistency. Cellular identification was

TABLE 1. Cell yields and differential counts for blood from loggerhead sea turtle #1, using three different anticoagulants on single-step Cellsep gradient or multi-step Percoll gradient. Cells were collected at the indicated gradient interface.

Anticoagulant	Gradient	Live WBC/ml blood	% Viable WBC	% of Total WBC	Number of RBC/ 100 WBC	G[a] (%)	L[b] (%)	M[c] (%)	MC[d] (%)	T[e]
Blood smear						52	38	10	48	
EDTA	Plasma/Cellsep	1.7×10^7	14	100	132	8	53	39	92	3+
	Plasma/Percoll 40%	8.3×10^6	52	54	12	3	66	31	97	3+
	Percoll 40/50%	7.1×10^6	91	46	32	0	80	20	100	2+
	Percoll Total	1.5×10^7								
Citrate	Plasma/Cellsep	1.5×10^7	98	100	16	2	79	19	98	2+
	Plasma/Percoll 40%	3.8×10^5	48	4	25	31	54	15	69	2+
	Percoll 40/45%	7.5×10^5	80	9	12	18	26	56	82	3+
	Percoll 45/50%	2.9×10^6	84	33	15	1	65	34	99	2+
	Percoll 50/55%	4.5×10^6	73	51	25	0	97	3	100	1+
	Percoll 55/60%	3.3×10^5	13	3	250	1	97	2	99	1+
	Percoll Total	8.9×10^6								
Heparin	Plasma/Cellsep	6.8×10^6	91	100	56	13	71	16	87	3+
	Plasma/Percoll 40%	3.7×10^5	26	7	0	31	54	15	69	3+
	Percoll 40/45%	1.1×10^6	47	21	0	21	29	50	79	3+
	Percoll 45/50%	4.6×10^5	85	9	0	3	91	6	97	2+
	Percoll 50/55%	2.5×10^6	90	47	0	0	91	9	100	1+
	Percoll 55/60%	8.3×10^5	43	16	25	0	98	2	100	1+
	Percoll Total	5.3×10^6								

[a] Granulocytes.
[b] Lymphocytes.
[c] Monocytes.
[d] Mononuclear cells (lymphocytes plus monocytes).
[e] Thrombocytes, subjectively graded due to non-random distribution and tendency to aggregate (see text).

based on published criteria (Campbell, 1996a; Cannon, 1992; Hawkey and Dennett, 1989; Samour et al., 1998; Wood and Ebanks, 1984; Work et al., 1998). These sources differ in both their terminology and criteria for sea turtle acidophilic granulocytes (heterophil versus eosinophil), but for the purposes of the present study the distinction was inconsequential, as the objective was elimination of granulocytes. Thrombocytes were distinguished from the similar lymphocytes by their smaller size, denser nuclei, clearer cytoplasm and tendency to aggregate. Because thrombocytes were distributed non-randomly and formed aggregations composed of multiple superimposed cell types, they were not included in the differential counts, but rather were subjectively graded on a scale of 1+ to 3+. A grade of 1+ indicated that thrombocytes formed aggregations of fewer than 10 cells, and aggregations were present in fewer than 50% of high power (400×) fields; a grade of 2+ indicated that aggregations of 20 or more cells were present in fewer than 50% of high power fields; and a grade of 3+ indicated that aggregations of 20 or more cells were present in greater than 50% of high power fields. Osmolality of plasma and complete RPMI was determined by an osmometer (Advanced Micro-osmometer Model 3MO, Advanced Instruments, Inc., Needham Heights, Massachusetts, USA).

White blood cell counts and viability, erythrocyte contamination and differential counts for blood fractions from turtle #1 are shown in Table 1. Cells from the plasma/Cellsep, plasma/40% Percoll and the 40/45% Percoll interfaces tended to form aggregations when harvested, regardless of the anticoagulant used, presumably due to

TABLE 2. Yields and differential counts of heparinized blood cells from loggerhead sea turtles #2–4, centrifuged over a two-step 45/55% Percoll gradient, and harvested from the 45/55% interface.

Sea turtle #	Live WBC/ ml blood	% Viable (WBC)	# RBC/ 100 live WBC	Hetero- phils (%)	Lympho- cytes (%)	Monocytes (%)	MC[a] (%)	Thrombo- cytes[b]
2	9.2×10^6	97	3	0	82	18	100	2+
3	7.4×10^6	95	5	0	83	17	100	2+
4	1.1×10^6	85	150	1	79	20	99	3+

[a] Mononuclear cells (lymphocytes + monocytes).
[b] Thrombocytes subjectively graded due to non-random distribution and tendency to aggregate (see text).

the greater number of thrombocytes in those fractions. Smudge cells, indicative of cell destruction, were abundant in all EDTA samples, and in top layers of heparin and citrate samples in both Cellsep and Percoll gradients, but were rare in samples below the 40/45% Percoll interface. Erythrocytes that appeared at the 55/60% Percoll interface from heparinized and citrated samples tended to be small immature cells. Granulocytes from heparinized and citrated blood were common in lower density Percoll layers (18 to 31% granulocytes at the plasma/40% Percoll and 40/45% Percoll interfaces), but declined to negligible representation below the 40/45% Percoll interface. Mononuclear cells comprised 97 to 100% of the leukocytes at the 45/50% and 50/55% interfaces (Table 1). Mononuclear cells also predominated at the 55/60% layer, but viability was lower and erythrocyte contamination was higher at this interface (Table 1).

Based on results from turtle #1, the best combination of high cell viability and yield, normal cell morphology, reduced erythrocyte and thrombocyte contamination, and high mononuclear cell percentage, was found from heparinized blood cells harvested at the top of the 45/50% and 50/55% Percoll interfaces. A two-step 45/55% Percoll gradient was therefore employed for further evaluation on heparinized blood from turtles #2 to 4 (Table 2). This technique yielded 99 to 100% PBMCs from all three loggerheads. Thrombocyte and erythrocyte contents were moderate to low in two of the three samples. Despite even a second centrifugation, one of the three loggerheads yielded a high level of thrombocyte and erythrocyte contamination.

Heparinized blood from a fifth loggerhead, processed after a 48 hr delay and centrifuged over a multi-step Percoll gradient, yielded high levels of erythrocyte and thrombocyte contamination at all layers, numerous smudge cells on cytospin slides, and markedly altered cellular morphology.

Osmolality for plasma from turtles #1 to 4 was 346 ± 8 mOsm (mean ±SD). This was compared with 287 mOsm for complete RPMI and 290 mOsm for 1× saline.

A two-step 45/55% Percoll gradient utilizing heparinized blood is suitable for harvesting loggerhead sea turtle PBMCs of high viability, minimal granulocyte and erythrocyte content, and reduced thrombocyte content. Sodium citrate anticoagulant could also be used in the same protocol. EDTA causes extensive hemolysis of sea turtle and other reptile erythrocytes (Campbell, 1996a, b; but see Wood and Ebanks, 1984 for contrary report in green sea turtle), which might have offered some benefit for a technique designed to isolate PBMC's. Unfortunately, the extensive erythrocyte damage interfered with PBMC isolation in the EDTA blood sample by completely obscuring the buffy coat following initial centrifugation. Mononuclear cells could still be harvested from the EDTA sample by centrifuging diluted whole blood over the gradient. The quantity of EDTA in commercial blood collection tubes is designed to produce a final

concentration of approximately 4 mM when filled. While EDTA at 4 mM concentration caused extensive erythrocyte lysis in whole blood, the lower concentration of 2 mm EDTA used in the complete RPMI to reduce cell aggregation did not appear to damage PBMCs from buffy coat suspensions, nor did it lyse and eliminate the contaminating erythrocytes noted in turtle #4.

A single step gradient with Cellsep or other gradient medium of 1.077 g/ml density (e.g., Ficoll-Hypaque) used for separation of mammalian PBMC's (Coligan et al., 1992), provides a simpler and somewhat quicker technique as long as a more heterogeneous cell population is acceptable (Table 1, and e.g., McKinney and Bentley, 1985 for green sea turtle blood). In comparison, however, a two-step gradient increases the purity of the PBMC population.

An unexpected finding in the present study was the substantial proportion of granulocytes (18–31%) harvested from the plasma/40% and 40/45% Percoll interfaces (1.053 g/ml and 1.059 g/ml). Granulocyte isolation procedures typically utilize gradient densities greater than 1.077 g/ml (Coligan et al., 1992; Smith et al., 1998; Tell et al., 1998). The low-density granulocytes found at 40% and 45% Percoll were present in a lower proportion than overall granulocytes of peripheral blood (52% for turtle #1), thus implying that some granulocytes of greater density settled below the 60% Percoll (1.076 g/ml) surface, as would be expected. The finding of low-density granulocytes in loggerhead sea turtle blood supports using a two-step gradient to provide an upper screen to reduce granulocyte content of the PBMC preparation.

Minimizing delay from blood collection to processing is important. Blood from turtle #5 was processed 48 hr after collection into heparinized tubes, and proved unsatisfactory due to marked alterations in cell morphology.

Blood from turtle #4 yielded an undesirable high degree of thrombocyte and erythrocyte contamination by the two-step 45/55% Percoll method. Based on the extensive cell aggregation observed, thrombocytes were likely activated, though the cause of activation was not apparent. This method has subsequently been used without visible erythrocyte contamination or cell aggregation complications on 12 additional loggerhead sea turtle samples in the course of an ongoing field toxicology investigation (JMJ) at a facility lacking cytospin capability for more detailed characterization.

The plasma osmolalities reported here were comparable to previous reports for sea turtles (270–370 mOsm, depending on species and population sampled) reviewed in Lutz (1997). Sea turtles and, by extension, their blood cells can tolerate substantial changes in plasma osmolality (Lutz, 1997), so the more dilute (49–62 mOsm lower) standard mammalian cell culture medium used in this study does not appear detrimental for the short term exposure experienced during PBMC isolation.

The two-step 45/55% Percoll method for separation of PBMCs may be suitable for other species of sea turtle. Cell types and distribution vary between sea turtle species (George, 1997), however, so testing on an individual species basis is recommended.

We thank S. Epperly, J. Braun-McNeil, L. Avens, M. A. Stamper, and E. J. Chittick for blood collection, and E. J. Chittick for comments on the manuscript. Financial support came, in part, from a U.S. Environmental Protection Agency, Science to Achieve Results graduate fellowship (CAH), an NCSU College of Veterinary Medicine State Research Support grant (CAH, SKS), and the Oak Foundation (JMJ).

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Received for publication 13 May 1999.