Analysis of Salmonid Leukocytes Purified by Hypotonic Lysis of Erythrocytes

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Abstract.—A technique that uses hypotonic lysis of erythrocytes was optimized for the purification of leukocytes from the peripheral blood and anterior kidney (pronephros) of rainbow trout *Oncorhynchus mykiss*. Comparisons of initial blood dilution (1:2, 1:4, and 1:6) and the time of exposure to hypotonic conditions (10, 20, and 40 s) revealed that a dilution of 1:2 provided the most complete hemolysis after 20 or 40 s in a hypotonic solution. For pronephros, a 1:5 (w:v) dilution and lysis in hypotonic solution for 10–40 s was effective in eliminating erythrocytes. Total leukocyte yield from the blood and pronephros by use of the hypotonic lysis method was comparable with that obtained by use of typical density gradient centrifugation, and cell viability was 97% or greater. Differential cell counts showed that hypotonic lysis resulted in a distribution of leukocyte cell types similar to that of density gradient separation. Hypotonic lysis of erythrocytes is a simple, rapid, and inexpensive method of purifying leukocytes from salmonid fish blood and pronephros.

Interest in the study of functional teleost immunology necessitated the development of a method to rapidly separate leukocytes from erythrocytes. Typically, teleost leukocytes from peripheral blood or tissues are purified by continuous or discontinuous density gradient centrifugation through separation media such as Histopaque, Ficoll-Paque, or Percoll (Blaxhall 1981; Blaxhall and Sheard 1985; Braun-Nesje et al. 1981; Huffman et al. 1997). Density gradient preparations are tedious, time consuming, and expensive and may cause unwanted stimulation of immune cells. The use of Ficoll has been shown to alter morphology and function in heterophils, as well as to activate some leukocyte subsets (Ogle et al. 1985; Verhoef and Waldvogel 1985). The separation of pure, viable, and quiescent leukocytes is essential for subsequent use of cells in in vitro immunological assays.

Classically, mammalian leukocytes have been purified by lysis of the nonnucleated erythrocytes with hypertonic ammonium chloride solutions. However, Rowley (1990) was not successful in removing erythrocytes from fish leukocyte suspensions by the use of ammonium chloride or by rouleaux formation of erythrocytes induced by dextran. The presence of nuclei in teleost eryth-

rocytes was thought to preclude lysis. The present work describes a technique by which teleost erythrocytes can be removed from peripheral blood and pronephric samples by changes in the isotonicity of the surrounding medium. Hypotonic lysis allows inexpensive purification of leukocytes and the rapid processing of more blood samples than traditional gradients permit. Leukocytes can be separated and purified from as little as 0.5 mL of blood, allowing nonlethal, repeat sampling of the same animal, and the technique does not affect leukocyte viability or recovery yield.

Methods

Trout

Adult rainbow trout *Oncorhynchus mykiss* weighing 350–1,000 g were maintained at the Center for Salmon Disease Research, Oregon State University, Corvallis, Oregon. The trout were held in 500-L tanks supplied with flowing 12°C well water and were fed daily with dried trout pellets (BioProducts, Astoria, Oregon). The trout were cared for in accordance with approved Oregon State University animal care guidelines. The studies were done between the months of December 1998 and July 1999.

Blood and Pronephros Collection

Trout were caught in a net and immediately killed by immersion in 1 g benzocaine/L. Blood samples from 10 fish were individually collected from the caudal vein into heparinized Vacutainer tubes and kept on ice throughout the isolation process. Pronephric tissue aseptically collected from 10 individual fish was placed into separate stomacher bags, weighed, and diluted 1:5 in isolation medium consisting of equal amounts of Alsevers solution (0.1 M dextrose, 70 mM sodium chloride, and 30 mM sodium citrate) and Hank's balanced salt solution. The tissues were then homogenized for 10–20 s in a stomacher, and the resulting cell suspension was placed into 15-mL tubes. Tissue clumps that settled after a 10-min incubation on ice were removed. Blood and pronephric leukocytes were then separated either by discontinuous gradient centrifugation or by hypotonic lysis of erythrocytes.

Isolation of Leukocytes from Peripheral Blood

Hypotonic lysis.—To compare the effect of blood dilution on hypotonic lysis of erythrocytes, 0.5 mL of blood from each fish was diluted with 0.5 mL (1:2 dilution), 1.5 mL (1:4 dilution), or 2.5 mL (1:6 dilution) of cold isolation medium and placed into a 15-mL centrifuge tube. Regardless of initial blood dilution, a constant 9 mL of cold, sterile distilled water (dH_2O) was added per tube to lyse erythrocytes. The effect of lysis time was determined by mixing each blood dilution for 10, 20, or 40 s by inversion of the tube several times to ensure that all erythrocytes remained suspended. The cells were returned to isotonicity by adding 1 mL of 10× phosphate-buffered saline (PBS) to each tube. The suspension was centrifuged at 750 × gravity (g) for 10 min at 4°C, and the supernatant fluid was discarded. The viscous mass, resulting from cell debris and release of nuclear material, was gently pulled from the top of the leukocyte cell pellet with a Pasteur pipet and discarded. The cell pellet was resuspended in Eagle's minimum essential medium (MEM; Gibco, Grand Island, New York). After incubation on ice for approximately 5 min, any remaining small viscous debris clumps settled out, and the leukocyte-containing supernatant was collected and transferred to a fresh tube. The cell suspension was centrifuged at 750 × g for 10 min at 4°C, and the supernatant was discarded. The cell pellet was resuspended in fish tissue culture medium (FTCM: MEM buffered with sodium bicarbonate and supplemented with 5% fetal calf serum [Invitrogen, Carlsbad, California], 200 units penicillin/mL [Sigma Chemical Co., St. Louis, Missouri], 0.2 mg streptomycin/mL [Sigma], and 2 mM L-glutamine [Gibco]).

Histopaque discontinuous gradient.—Peripheral blood from 10 trout was individually collected, diluted 1:12 in isolation medium, and overlaid on an 8-mL cushion of Histopaque-1077 (Sigma). Gradients were centrifuged at 400 × g for 40 min at 4°C. The cells at the interface were harvested, washed in PBS, and then resuspended in 1 mL of FTCM.

Isolation of Leukocytes from Pronephros

After homogenization and removal of clumps from the collected tissue, 0.5-mL aliquots of the cell suspension were placed into 15-mL centrifuge tubes containing 1.5 mL of isolation medium. Nine milliliters of cold dH_2O were added, and the cell suspension was mixed for 20 s, followed immediately by the addition of 1 mL of 10× PBS to return the solution to isotonic equilibrium. The cell suspension was centrifuged at 750 × g for 10 min at 4°C. The supernatant was discarded, and 4 mL of MEM was gently added. The viscous mass, resulting from cell debris and release of nuclear material, was gently pulled from the top of the cell pellet and carefully pipetted repeatedly through the

orifice of a sterile 5-mL pipet to release cells trapped in the mass back into the suspension. The cells from the pronephros were very sticky, and this step was required to ensure a high yield of leukocytes. The remaining viscous mass was discarded. After incubation for 5 min on ice, debris clumps at the bottom of the tube were removed with a sterile Pasteur pipet, and the volume of MEM was increased to 10 mL. The cell suspension was centrifuged ($750 \times g$, 10 min, 4°C), the supernatant was discarded, and the cell pellet was resuspended in 1 mL of FTCM.

Percoll Discontinuous Density Gradient

The cell suspension was overlaid on a discontinuous Percoll (Pharmacia Biotech AB, Uppsala, Sweden) gradient of 1.048 g/mL (34%) over 1.070 g/mL (51%) in a 15-mL tube. Gradients were centrifuged at $2,000 \times g$ for 40 min at 4°C. The cells at the 34−51% interface were harvested and washed once in PBS, and the cell pellet was resuspended in 1 mL of FTCM.

Cell Viability and Quantitation

Except for the evaluation of long-term viability in culture, cell viability was determined by 0.1% trypan blue exclusion, and cells were counted in a hemocytometer. Cell counts were expressed as cells per milliliter of blood or gram of pronephric tissue and represent the mean number of cells from triplicate counts. For the evaluation of long-term viability in culture, cells were resuspended at 5×10^6 cells/mL, 100μL were placed into each well of a 96-well tissue culture plate, and the plate was cultured at 12°C for 7 d. After being incubated, the cells were rinsed in PBS twice and resuspended in PBS plus RNAse (10 μg/mL). Propidium iodide (100 μg/mL) was added, and cells were incubated for 30 min on ice prior to analysis. Cells were then analyzed by flow cytometry. The results were expressed as the mean percentages of cells from triplicate samples of 10 trout in each of two separate experiments.

Differential Giemsa Staining

To quantify the number of leukocyte cell types in the blood or pronephric cell preparations, 5×10^6 cells were dropped onto a glass slide and allowed to air dry. Slides were stained with a Hema 3 stain set (Biochemical Sciences, Inc., Swedesboro, New Jersey) by dipping the slide in sequence for 30 s in the fixative solution, 10 s in solution I, and 40 s in solution II. Data are presented as the mean percentage of cells from triplicate counts.

Sudan Black B and Neutral Red Staining

Cells were stained with Sudan black B by using a modification of the method described by Blaxhall and Daisley (1973). To quantify the number of granulocytes in the blood or pronephric cell preparations, 5×10^6 cells were dropped onto a glass slide and allowed to air dry. Slides were immersed for 30 min in a saturated solution of Sudan black B (Sigma) in 70% ethanol in a moist chamber at 17°C. Slides were rinsed for 1–3 min in 50% ethanol, followed by a rinse in dH_2O. Slides were counterstained for 1 min in a 0.33% neutral red solution in PBS and then rinsed in dH_2O and air dried. The neutral-red-uptake inhibition test has previously been used to measure cell viability by lysosomal integrity in fish (Babich et al. 1991; Borenfreund and Puerner 1985; Gagne and Blaise 1998). We used neutral red as a background stain for demarcation of all cells without diminishing the Sudan black B staining. This combined usage has not been described previously. Cells containing Sudan black B granules (heterophils) and cells stained with neutral red were counted microscopically. Data are presented as the mean percentage of cells staining positive for Sudan black B from triplicate counts.

Flow Cytometric Analysis

Fluorescence, forward scatter (FSC), and side scatter (SSC) characteristics of the cell preparations purified by gradient and hypotonic lysis were analyzed on a FACScan (Becton Dickinson, San Jose, California) equipped with an argon ion laser tuned to 488 nm. A minimum of 20,000 events were acquired from each sample, and the resulting list-mode data were analyzed with Cell Quest software (Becton Dickinson).

Statistics

Data obtained on the effect of lysis time and sample dilution were analyzed by analysis of variance, and cell yields and cell types were analyzed by t-tests (SigmaStat version 2.0, Jandel Corporation). All tests used a 5% significance level.

Results

Effects of Lysis Time and Blood Dilution

Both the initial dilution of blood and the length of time blood cells were exposed to a hypotonic solution (lysis time) affected purification of leukocytes. For the 20- and 40-s lysis times, the yield of leukocytes remained constant regardless of dilution (Figure 1A). Comparisons of the dilution
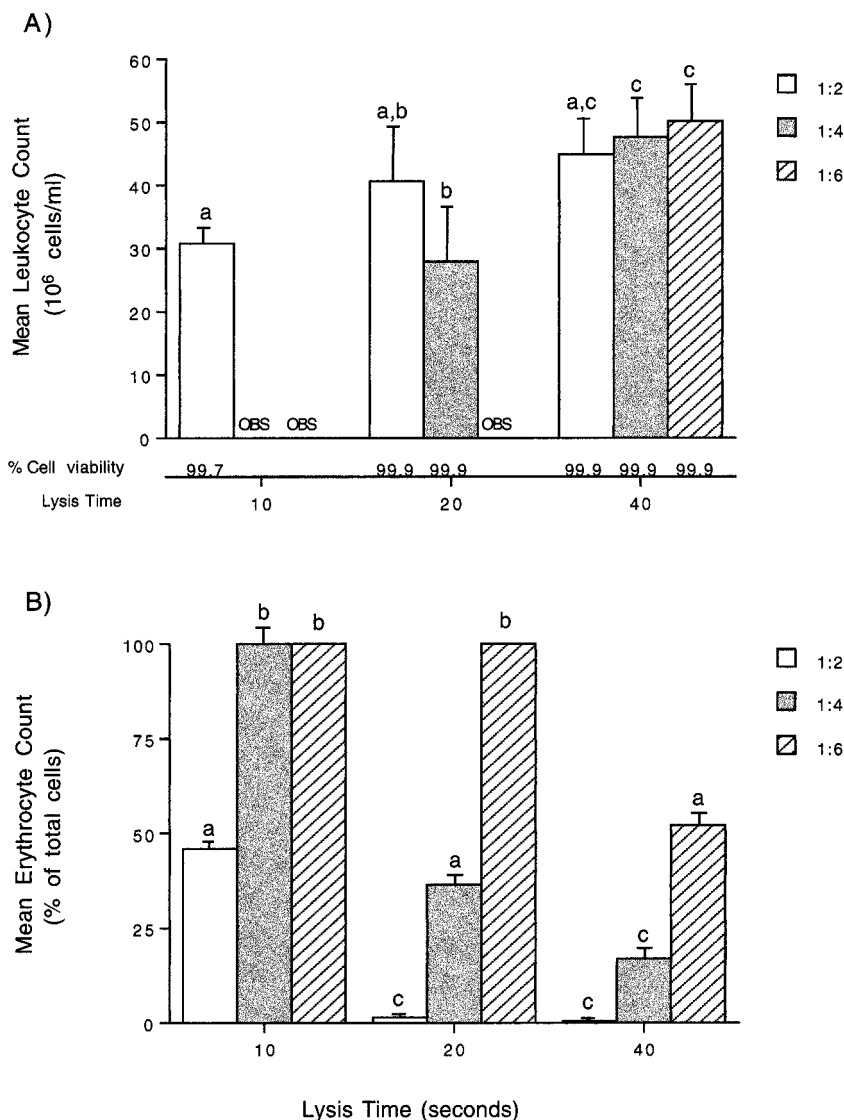
FIGURE 1.—(A) The effects of time of lysis and dilution of blood on leukocyte concentration. Peripheral blood was diluted 1:2, 1:4, or 1:6 in isolation medium and then lysed under hypotonic conditions for 10, 20, or 40 s. In some dilutions, leukocytes were obscured by the high density of erythrocytes (OBS); therefore, accurate counts could not be determined. Leukocyte concentrations are expressed as the mean of 10 fish + SE of the mean. A comparison of the lysis time effect on the 1:2 dilution was performed; mean leukocyte counts labeled with a common letter (a) are not significantly different ($P > 0.05$). A comparison of the dilution effect on 20-s lysis was performed; mean leukocyte counts labeled with a common letter (b) are not significantly different ($P > 0.05$). A comparison of the dilution effect on 40-s lysis was performed; mean leukocyte counts labeled with a common letter (c) are not significantly different ($P > 0.05$). (B) The effects of time of lysis and dilution of blood on erythrocyte concentration. Peripheral blood was diluted 1:2, 1:4, or 1:6 in isolation medium and then lysed under hypotonic conditions for 10, 20, or 40 s. Values of 100% average erythrocytes represent samples in which erythrocytes were too dense to allow counting of leukocytes. Erythrocyte counts are expressed as the mean of 10 fish + SE of the mean. Mean erythrocyte counts labeled with common letters (a–c) are not significantly different ($P > 0.05$).
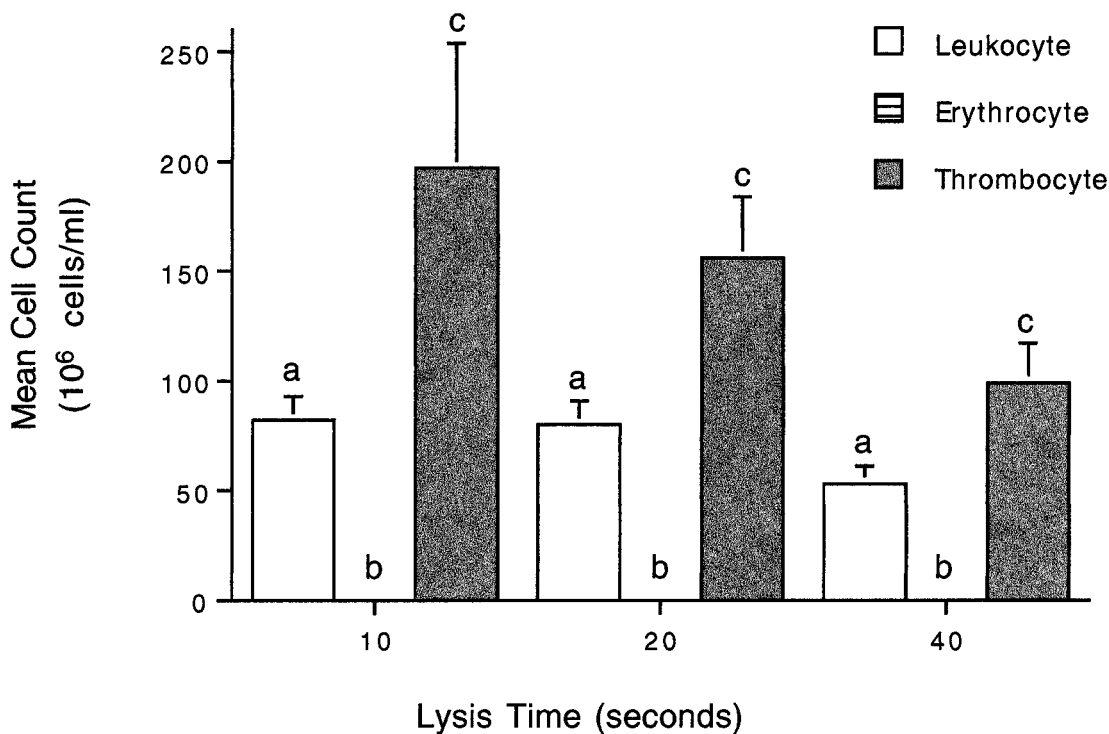
FIGURE 2.—The effects of time of lysis on cell concentration in pronephros. Pronephric tissue was diluted 1:5 (w/v) in isolation medium and then lysed under hypotonic conditions for 10, 20, or 40 s. The total leukocyte concentrations are expressed as the mean of 10 fish + SE of the mean. Mean leukocyte counts labeled with a common letter (a) are not significantly different ($P > 0.05$). Mean erythrocyte counts labeled with a common letter (b) are not significantly different ($P > 0.05$). Mean thrombocyte counts labeled with a common letter (c) are not significantly different ($P > 0.05$).

effect on a 10-s lysis time could not be made because leukocytes were obscured by the high density of erythrocytes remaining in the 1:4 and 1:6 dilutions; therefore accurate counts could not be determined. In addition, leukocyte counts for each lysis time after a 1:2 dilution of blood were not significantly different. There were, however, marked differences in erythrocyte yield (Figure 1B). Blood diluted 1:4 or 1:6 and lysed for 10 s and blood diluted 1:6 and lysed for 20 s did not result in sufficient lysis of erythrocytes, and this obscured the ability to count leukocytes. Regardless of lysis time, the number of erythrocytes was always significantly ($P < 0.05$) lower in blood diluted 1:2 compared with the number in blood diluted 1:6. Increasing the lysis time from 10 to 20 s resulted in a significant reduction in erythrocyte numbers for the 1:2 and 1:4 blood dilutions, and a further increase in time from 20 to 40 s significantly decreased erythrocyte counts further in blood diluted 1:4 and 1:6 but did not affect the 1:2 dilution, which had almost complete lysis at

20 s. At all blood dilutions and lysis times, cell viability remained greater than 99%. In summary, the most complete hemolysis occurred at a 1:2 dilution of blood and a lysis time of 20–40 s. This treatment did not affect total leukocyte recovery (Figure 1A).

Effects of Lysis Time on Pronephric Tissue

Leukocytes from the pronephros were successfully purified by the use of hypotonic lysis of erythrocytes. Exposure of pronephric tissue that had been diluted 1:5 (w:v), to hypotonic conditions for 10, 20, or 40 s each resulted in 100% lysis of erythrocytes and did not significantly affect the recovery of leukocytes or thrombocytes (Figure 2). Cell viability remained at 98% or better (data not shown).

Comparisons of Lysis and Gradient Purification of Leukocytes

We compared our lysis technique with the standard density gradient centrifugation techniques

TABLE 1.—Comparison of total cell yield, cell viability, and percentage of cell type after purification of leukocytes from peripheral blood and pronephros by hypotonic lysis or density gradient. Asterisks indicate significant ($P < 0.05$) differences between purification methods.

	Peripheral blood		Pronephros	
	Lysis	Gradient	Lysis	Gradient
Total cell yield[a]				
(10^6 cells/mL)	21.6 ± 3.4	23.5 ± 2.6	197.3 ± 43.2*	124.8 ± 34.6
Cell viability[a] (%)	97.1 ± 0.9	90.3 ± 1.9	99.7 ± 0.3	100.0 ± 0.0
Cell type[b] (%)				
Lymphocytes	91.9 ± 0.6	93.1 ± 0.7	794 ± 0.7*	75.1 ± 1.0
Granulocytes	7.4 ± 0.5	6.0 ± 0.5	14.3 ± 0.6	18.8 ± 0.7*
Monocytes	0.4 ± 0.1	0.5 ± 0.1	6.0 ± 0.3	5.3 ± 0.4
Thrombocytes	0.3 ± 0.1	0.5 ± 0.2	0.3 ± 0.1	0.8 ± 0.3
Erythrocytes	0.2 ± 0.1	3.6 ± 0.7*	0.5 ± 0.1	0.9 ± 0.2

[a] Mean value of triplicate samples of 10 trout ± SE.
[b] Mean value of duplicate samples of 10 trout ± SE.

used for the separation and purification of fish leukocytes as a result of their density (Table 1). Leukocyte yields resulting after peripheral blood was diluted 1:2 and exposed to hypotonic conditions for 20 s were not significantly different from yields obtained after isolation of cells from an equivalent volume of blood on Histopaque-1077 gradients. The total yield of leukocytes averaged 21.6×10^6 cells/mL after lysis and 23.5×10^6 cells/mL after isolation by the gradient. In contrast, a 20-s hypotonic lysis of pronephric tissue diluted 1:5 resulted in a significantly ($P < 0.05$) higher average total yield of leukocytes (197.3×10^6 cells/mL) than did cell separation by Percoll gradients (124.8×10^6 cells/mL).

To determine whether there were differences in the cell types isolated by lysis or gradients, slides stained for differential cell counts were examined. For blood, the distribution of leukocyte types was equivalent for the two separation techniques, but the mean percentage of erythrocytes was significantly ($P < 0.05$) lower for the lysis method (Table 1). For both separation techniques, the majority of the cells were lymphocytes (>91%); the remainder were granulocytes (6.0–7.4%) and monocytes and thrombocytes (<1%). In contrast to blood, the percentage of lymphocytes in the pronephros was slightly higher after lysis (79.4%) than after Percoll gradients (75.1%), and the percentage of granulocytes was slightly lower after lysis (14.3%) than after gradients (18.8%). However, the percentage of monocytes and thrombocytes remained equivalent between the two techniques (Table 1). The ability to remove erythrocytes from the pronephros was not significantly different; both techniques efficiently removed the erythrocytes.

Quantitation of Granulocytes by Sudan Black B Staining

Sudan black B staining of leukocyte smears allowed the clear identification of cells of the granulocyte lineage. After Sudan black B staining, granulocytes contained black cytoplasmic granules (Figure 3, top). Counterstaining with neutral red (Figure 3, bottom) allowed enumeration of total cell number without disturbing the Sudan black B staining in granulocytes. Immature and mature granulocyte yields from the pronephros and peripheral blood were not affected by hypotonic lysis treatment. The total percentage of cells staining Sudan black B positive after lysis of peripheral blood (6.5%) did not differ significantly from that of gradient-isolated cells (9.3%), nor was there a significant difference in cell yield between lysed (27.6%) and density-gradient-isolated (32.6%) granulocytes from pronephric tissue (Table 2).

Differences in the ease of identification of granulocytes by the two staining methods are exemplified by the significantly ($P < 0.05$) higher percentage of granulocytes identified by Sudan black B in the pronephros after lysis (27.6%) than when enumerated by Geimsa (14.3%). The same was true of granulocytes isolated by gradient and identified by staining with Sudan black B (32.6%) or Geimsa (18.8%).

Comparison of Relative Size and Scatter Characteristics of Purified and Unpurified Leukocytes

Separation and elimination of erythrocytes from leukocytes in trout whole blood and pronephric tissue was achieved by use of gradient and lysis techniques. Mature fish erythrocytes are oval nu-
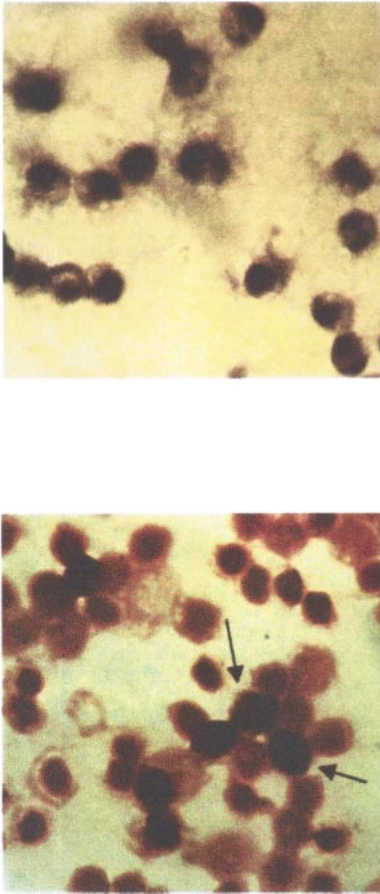
FIGURE 3.—Photographs of purified leukocytes isolated by hypotonic lysis and stained with Sudan black B alone (top) or stained with Sudan black B and then counterstained with neutral red (bottom). All cells took up the vital dye, neutral red, staining red, and granulocyte granules took up Sudan black B, staining black. Magnification was 1,300×.

TABLE 2.—Percentage of cells staining positive with Sudan black B that were purified by hypotonic lysis or density gradient. Means are from triplicate samples of 10 trout. Mean leukocyte counts with common letters are not significantly different ($P > 0.05$).

Percentage of cells	Peripheral blood		Pronephros	
	Lysis	Gradient	Lysis	Gradient
Mean	6.5 z	9.3 z	27.6 y	32.6 y
SE	0.6	0.7	1.3	1.4

separated, and whole pronephric tissue also showed an increase in events in the area between channels 150 and 500 and a decrease in events in areas out of these channels (Figure 5B). Leukocytic events between channels 150 and 500 increased from 72% of the population in pronephros to 94% in gradient-separated blood to 95% in lysed blood. These data are representative of the results obtained from 10 individual fish.
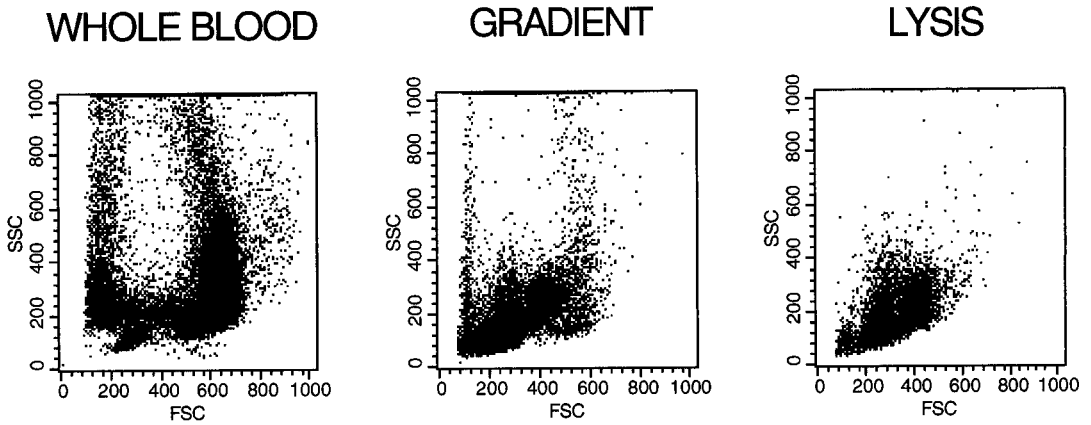
Evaluation of Long-Term Viability in Culture

After isolation by lysis, leukocytes were maintained in culture over a 7-d period, and viability was assessed by measurement of membrane integrity as determined by exclusion of the fluorescent indicator propidium iodide. Cell viability decreased steadily from 99% immediately after isolation to approximately 60% after 7 d in culture (Table 3).

Discussion

In the present study, a rapid, inexpensive, and simple hemolytic technique was developed for obtaining the maximum recovery of viable, pure leukocytes. The cells isolated by hypotonic lysis maintained good viability over 7 d in culture. Rainbow trout were used throughout this study; however, hypotonic lysis has since been used with great success on peripheral blood from chinook salmon *O. tshawytscha* (T. L. Crippen, unpublished data).

The efficiency of hypotonic erythrocyte lysis was affected by blood dilution and time of exposure to hypotonic conditions. Removal of over 95% of the erythrocytes occurred when a 1:2 dilution of peripheral blood was exposed to hypertonic conditions for 20 or 40 s. The increased volume of diluent above a 1:2 concentration interfered with the efficiency of hemolysis of fish blood. Lysis of erythrocytes by exposure to hypotonic solutions has been used previously for mammalian blood (Bramley et al. 1971; Walker et al. 1967). Mammalian erythrocytes placed into distilled water will swell until at some critical point the rising
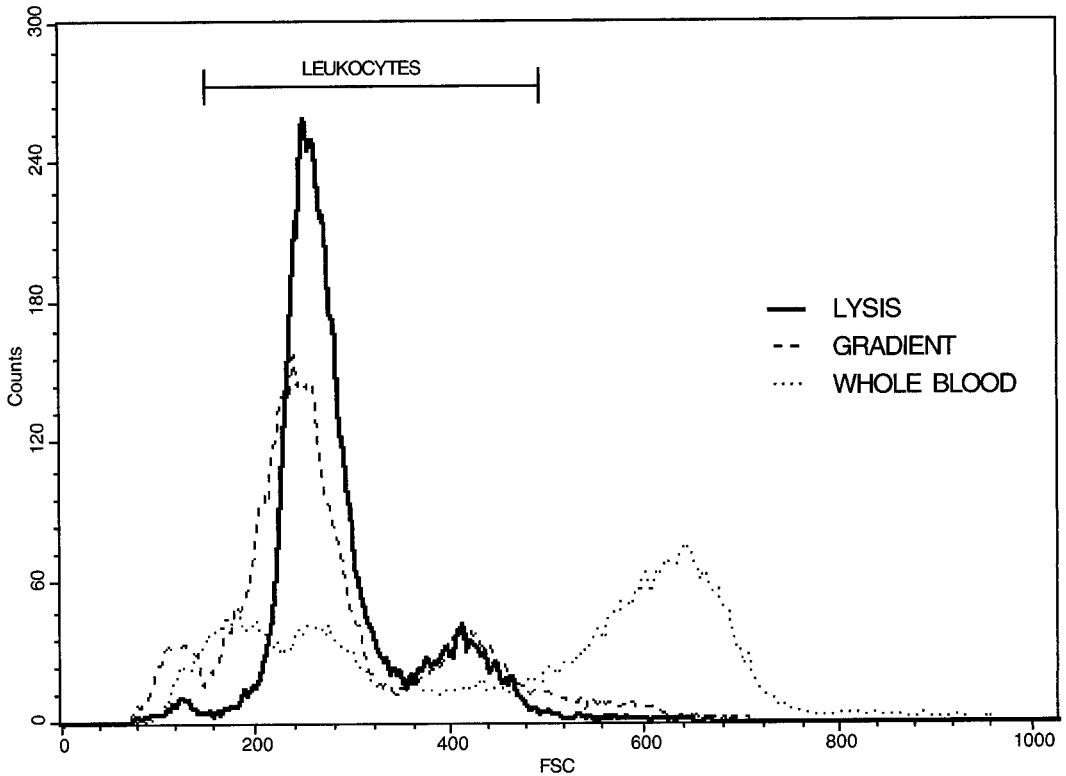
cleated cells of 13–16 × 7–10 m (Yasutake and Wales 1983). Erythrocytes can be observed in FSC and SSC dot plots as two populations on the FSC axis with peak channels at 171 and 648 for whole blood (Figure 4A) and approximately 191 and 635 for pronephros (Figure 5A). Overlapping histograms of lysed, gradient-separated, and whole blood showed an increase in events in the area between channels 150 and 500 and a decrease in events in areas out of these channels, associated with increasing leukocyte purity (Figure 4B). Leukocytic events between channels 150 and 500 increased from 52% of the population in whole blood to 87% in gradient-separated blood to 97% in lysed blood. Overlapping histograms of lysed, gradient-
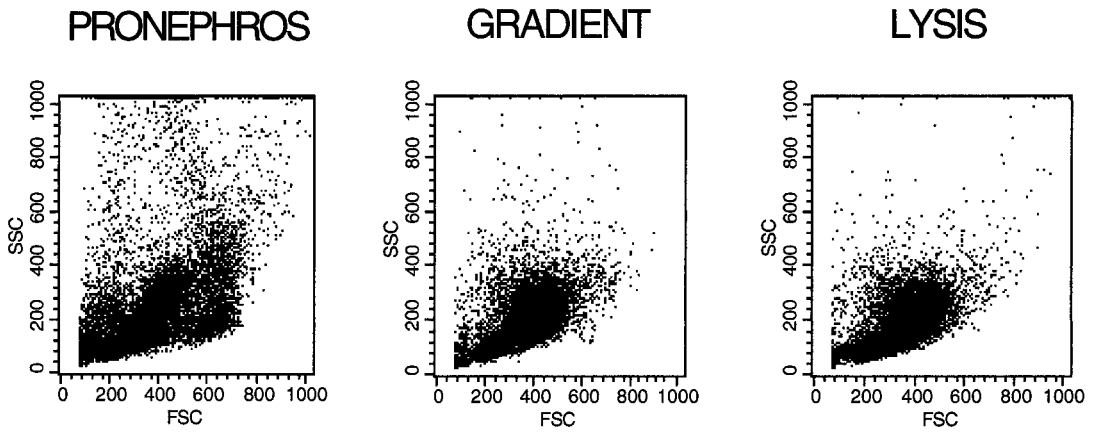
A)

B)

FIGURE 4.—**(A)** Dot plots of forward scatter (FSC) events versus side scatter (SSC) events measured by flow cytometry for peripheral blood samples from a single rainbow trout. Samples measured before purification (WHOLE BLOOD), after density gradient centrifugation (GRADIENT), and after hypotonic lysis (LYSIS) are shown. **(B)** Cytometric analysis of FSC characteristics of a lysed and gradient-purified cell preparation is overlaid on that of whole blood and shown in the frequency histogram.
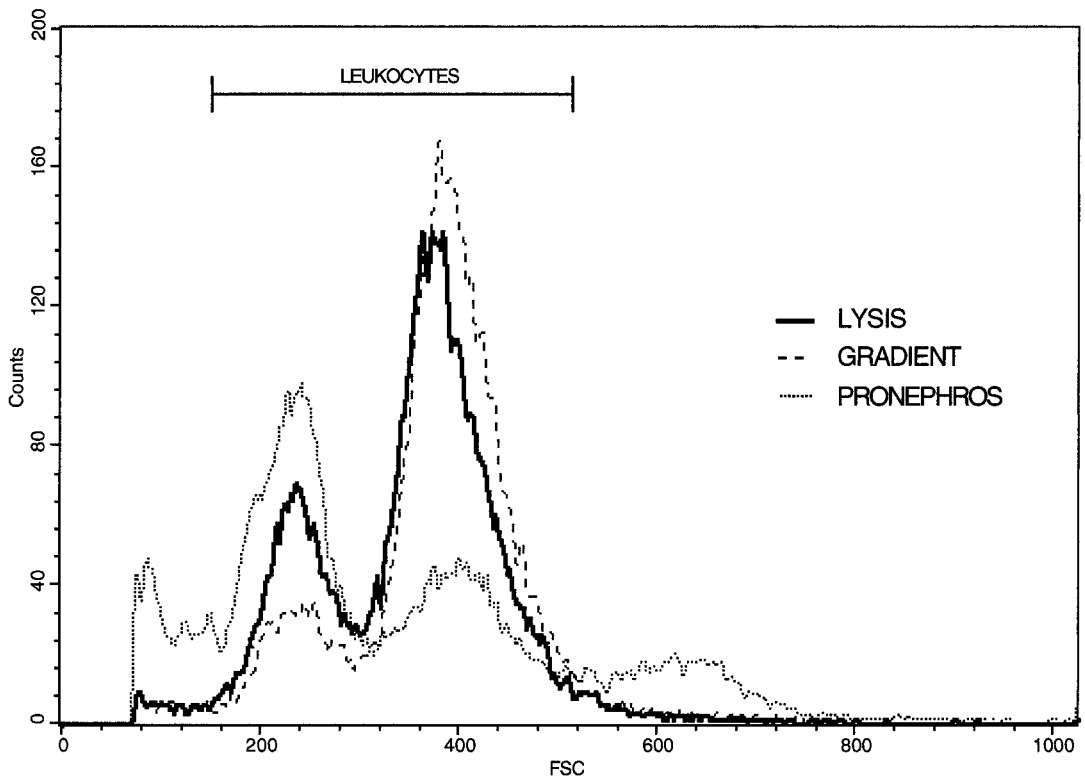
A)

B)

FIGURE 5.—(**A**) Dot plots of forward scatter (FSC) events versus side scatter (SSC) events measured by flow cytometry for pronephric tissue samples from a single rainbow trout. Tissue measured before purification (PRO-NEPHROS), after density gradient centrifugation (GRADIENT), and after hypotonic lysis (LYSIS) are shown. (**B**) Cytometric analysis of FSC characteristics of a lysed and gradient-purified cell preparation is overlaid on that of whole pronephric tissue and shown in the frequency histogram.

TABLE 3.—Percentage of viable peripheral blood cells in culture after purification by hypotonic lysis. Means are from triplicate samples of 10 trout in two separate experiments.

Percentage of cells	Day							
	0	1	2	3	4	5	6	7
Mean	99.8	80.6	75.3	65.9	61.3	60.2	59.6	59.9
SE	0.2	0.3	0.7	0.7	0.6	0.4	0.6	0.6

pressure in the cell interior overwhelms the tensile strength of the cell membrane and hemoglobin escapes (hemolysis). Delano (1995) developed a model to predict the critical set of physical conditions that result in osmotic rupture of mammalian erythrocytes. The model determined that the fate of the erythrocyte in a hypotonic solution depends on tonicity, the initial sphericity of the cell, the initial fraction of its volume not occupied by water, and the maximum fractional amount by which the surface area can be stretched. Fish blood diluted 1:4 and 1:6 and then exposed to hypotonic conditions for up to 40 s resulted in less than complete removal of erythrocytes from the sample. This may have been the result of an insufficient amount of water being added to the samples. The initial blood volume of 0.5 mL was diluted with 0.5, 1.5, or 2.5 mL of isolation medium, and then, regardless of the initial blood dilution, a constant 9 mL of water was added to initiate lysis. Hence, the tonicity after adding water increased with the initial blood dilution due to the higher volume of isolation medium present. When samples are diluted more than 1:2, increasing the lysis time to over 40 s or increasing the amount of water may improve erythrocyte lysis. It is possible to use larger initial blood volumes, because leukocytes were successfully purified from a 2-mL blood volume diluted 1:2 and lysed for 20 s[2] (data not shown). It may be desirable to keep the plasma from blood samples for use in assays such as antibody detection. In this case, whole blood can be centrifuged, the plasma can be removed, and the blood cell pellet can be resuspended in isolation medium to the initial blood volume. This diluted sample can then be lysed as described above for whole blood.

[2] The large-blood-volume protocol was as follows: 2.0 mL of blood was diluted with 2 mL of isolation medium and placed into a 50-mL centrifuge tube. An aliquot of 36 mL of dH_2O was added, and the cell suspension was mixed for 20 s. Immediately after mixing, 4 mL of 10× PBS was added and mixed thoroughly. The analysis continued as described in the small-volume protocol.

Traditionally, discontinuous gradient centrifugation has been the method of choice for isolation of pure populations of leukocytes from the peripheral blood and the pronephros of teleostean species. The present study showed that hypotonic lysis is a viable alternative method for the elimination of erythrocytes and purification of leukocytes. Although blood dilution and lysis time affected erythrocyte lysis, these two variables did not affect leukocyte yield or viability, demonstrating that exposure to hypotonic conditions for these short durations was not disruptive to the leukocytes. In addition, treatment of pronephric tissue with these same short-duration hypotonic conditions resulted in complete hemolysis with no reduction in total leukocyte recovery or viability. Differential cell analysis confirmed that no classes of leukocytes were lost from peripheral blood or pronephric samples, and their relative frequencies were maintained during induced hemolysis. The efficiency of hypotonic elimination of erythrocytes was easily visualized when samples were analyzed by flow cytometry. Thus, hypotonic lysis was as effective as density gradients for elimination of erythrocytes from the blood and pronephric tissue. However, the lysis technique proved to be more rapid than gradients for leukocyte purification.

Granulocytes of fish have been shown to be extremely diverse in morphology and function, making appropriate identification difficult (Ainsworth 1992). In addition, the total number of circulating granulocytes varies considerably from fish to fish (Ellis 1977). After exposure to hypotonic conditions, staining of cells with Sudan black B and counterstaining with neutral red allowed identification of cells of the granulocyte lineage and an assessment of whether mature or immature granulocytes were sensitive to hypotonic conditions. Sudan black B stains lipids, including phospholipids, neutral fats, and sterols. Teleost thrombocytes and lymphocytes have been found to be negative for Sudan black B, whereas the granules of neutrophils are positively stained (Blaxhall and Daisley 1973; Burrows and Fletcher 1987; Doggett et al. 1987; Ellis 1976, 1977; Ellsaesser et al. 1985;

Stoskopf 1993). Neutral red is a vital stain readily absorbed and stored in the lysosomal compartment. The compromising of the integrity of lysosomes and other cell organelles during cell death allows the dye to leak into the cytoplasm and stain the entire cell red. We used neutral red as a background stain to allow enumeration of all cells without diminishing Sudan black B staining in granulocytes.

The number of granulocytes identified in the pronephros was higher when enumerated after Sudan black B staining than after Giemsa-like staining. The pronephros is considered to be comparable to mammalian bone marrow as the primary hematopoietic tissue in teleosts. It contains a range of developing blood cells, but this range of maturing cells has not been well characterized in fish (Pedrera et al. 1993). Granulocytes purified by lysis and by density gradient were often difficult to distinguish in Giemsa-stained slides. The use of Sudan black B staining made it much easier to distinguish granulocytes from other cells types by the distinctive black-staining granules. The definitive black granules removed the subjective component in the determination of the granulocyte cell type, especially immature granulocytes, which tended to be approximately the same size as lymphocytes and difficult to identify due to lack of established distinguishing characteristics. The Sudan-black-B-positive granule staining varied from very intense to light. Blaxhall and Daisley (1973) described staining of immature granulocytes as faint localized cytoplasmic sudanophilia, which increased rapidly to a strong, heavy overall positivity in mature cells. Quantitation of granulocytes by Sudan black B staining verified that the yield of this cell type was not reduced by exposure to hypotonic conditions in the leukocytes isolated from peripheral blood or pronephric tissue.

The present study demonstrates the use of hypotonic lysis to remove erythrocytes and isolate leukocytes from the peripheral blood and the pronephros of trout. It was established that a 20–40-s exposure to hypotonic conditions of peripheral blood diluted 1:2 resulted in the most complete elimination of erythrocytes without effect on total leukocyte recovery. A 1:5 dilution of pronephric tissue followed by hypotonic lysis for 10–40 s also eliminated erythrocytes without reduction of leukocyte yield. Cellular viability remained very high (>98%) in all lysed samples, and total leukocyte recovery was unaffected. This technique is rapid, inexpensive, and simple and allows the separation and isolation of leukocytes from many samples in a shorter period than can be done by discontinuous density gradient centrifugation techniques.

Acknowledgments

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