Published in final edited form as:

Fish Shellfish Immunol. 2008 July ; 25(1-2): 188–190.

An Improved method for separation of leucocytes from peripheral blood of the little skate (*Leucoraja erinacea*)

Mitsuru Tomana^{a,b}, Angela Parton^b, and David W. Barnes^b

^aDepartment of Applied Biological Science, Nihon University, 1866 Kameino, Fujisawa, Kanagawa 252-8510, Japan

^bMount Desert Island Biological Laboratory, Salisbury Cove, ME, USA 04672

Abstract

Cartilaginous fish, especially sharks, rays and skates (elasmobranchs) hold interest as comparative models in immunology because they are thought to be among the organisms most closely related to the ancestor animal that first developed acquired immunity. The aim of this study was to improve methods used for the purification of viable leucocytes from peripheral blood of elasmobranchs. Here we describe modifications of density gradient centrifugation and medium formulation that improve isolation and analysis of highly-purified leucocytes from peripheral blood of a model elasmobranch, *Leucoraja erinacea*, the little skate. These techniques contribute to the preparation of elasmobranch immune cells that can be reliably analyzed by a variety of means, including the study of immune function.

Keywords

Elasmobranch; Little skate; *Leucoraja erinacea*; Leucocytes; Erythrocytes; Density gradient centrifugation

Elasmobranchs (sharks, rays or skates) are common cartilaginous fish models for the study of evolution of the immune system. Among elasmobranchs several species of skates (superorder Batoidea, family Rajidae) have been used for this and other research purposes. Best characterized are the clearnose skate (*Raja eglanteria*), and the little skate (*Leucoraja erinacea*) [1,2]. Recently the National Human Genome Research Institute of the National Institutes of Health approved the whole genome sequencing of the little skate [3,4]. Nevertheless, serious deficiencies exist in available methods for use of immune cells of skates *in vitro*.

A major problem has been separation of leucocytes from erythrocytes in these animals, in which the erythrocytes remain nucleated [5,6]. Mainwaring and Rowley [7] have carried out a careful analysis using the spotted dogfish shark, *Schliorhinus canicula*, in which continuous density gradient centrifugation and differential adhesion to glass coverslips were examined as a means of separation of leucocytes. Thrombocytes and several types of granulocytes could be separated by single-step methods, while other cell types required an additional step of

Author for contact: David W. Barnes, Ph.D., Mount Desert Island Biological Laboratory, P.O. Box 35, Salisbury Cove, ME 04672, Telephone: 207-288-9880 ext 472, FAX: 207-288-2130, dbarnes@mdibl.org.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

adherence to glass for purification. Our experience is that additional improvements are required to rapidly produce a population of cells with high viability. We have developed a purification procedure based on a discontinuous, two-gradient separation procedure and adjustment of the osmolarity of all solutions to correspond to that of peripheral blood of the little skate. Leucocytes of high purity and viability were isolated through centrifugation in the discontinuous gradient.

Peripheral blood was collected from the caudal vein after the fish had been anaesthetised by tricaine methane sulphonate in sea water. For experiments, NaCl was added to the routine 300 mOsm salt solutions to increase osmolarity to 1000 mOsm. RediGrad (Amersham Biosciences), a gradient reagent used for cell separation, was modified by mixing nine volumes of RediGrad with one volume of 5 M NaCl (RediGrad stock solution). A skate RediGrad solution of 1.095 g ml⁻¹ (skate RediGrad-I) was prepared by mixing 6.5 volumes of skate RediGrad stock solution with 3.5 volumes of 0.5 M NaCl. A skate RediGrad solution of 1.050 g ml⁻¹ (skate RediGrad-II) was prepared by mixing 3 volumes of skate RediGrad stock solution with 7 volumes of 0.5 M NaCl. Skate phosphate-buffered saline was prepared by mixing 9.3 volumes of standard phosphate-buffered saline with 0.7 volume of 5M NaCl.

Peripheral blood cells were washed twice by centrifugation at 340 X g for 5 min at 4°C in skate phosphate-buffered saline containing 2% foetal bovine serum (FBS). The washed blood cell suspension in 4.5 ml of this solution was layered carefully onto discontinuous skate RediGrad-I (3 ml)/RediGrad-II (3 ml) in 15 ml polypropylene conical tubes. After centrifugation at 2500 X g for 15 min at 18°C with slow acceleration and reduced braking, three fractions were collected (Fig. 1). Each fraction was washed once by centrifugation at 450 X g for 5 min at 4°C in skate phosphate-buffered saline containing 2% FBS, and twice at 340 X g for 5 min at 4°C. Cells from each fraction were resuspended to the appropriate cell density and observed under the microscope.

The fraction representing the interface between the two density solutions, interface fraction II, contained an enriched population of leucocytes with few erythrocytes (Fig 2). Erythrocytes and few leucocytes were observed in the pellet, fraction III. Nonviable cells and debris, with a few large leucocytes, predominated at the interface of fraction I and the cell loading solution (density 1.050 g/ml). The percentage of leucocytes present in this fraction was less than 5% of that found at the interface between the two densities (Fig 2).

At least 95% of cells isolated from interface fraction II and fraction III, were viable. Analysis of these fractions demonstrated successful removal of dead cells and debris from the samples; propidium iodide staining (nonviable cells) was high in interface fraction I and low in the other fractions. Small cells appearing microscopically to be leucocytes predominated at interface fraction II. Taken together, the results indicate that a gradient of more than 1.095 g ml⁻¹ is best for separation of erythrocytes. This is higher than reported for nurse sharks (1.066-1.077 g ml⁻¹) [8], and may be due to species difference related to lipid composition of animals living in very different water temperatures. The results indicate that the gradient density applied for isolation of cells from other elasmobranchs should be carefully determined before experimental analysis.

While peripheral blood leucocytes represent only about 8% of peripheral blood cells, flow cytometry indicated that viable leucocytes, the major population of these cells, were purified 10-fold through the discontinuous gradient centrifugation technique described here. In this study, our evaluation of various NaCl-modified media adjusted to skate cell osmolarity led to more reliable results in maintenance of cell viability. The modification of density gradient separation methodology reported here improve methods to exclude erythrocytes and dead cells/debris, generating a highly-purified leucocyte population. We anticipate that these techniques

can be further refined for application to elasmobranchs to allow leucocyte separation into lymphocytes, macrophages, granulocytes or other subpopulations.

Acknowledgements

This work was supported by National Institutes of Health (NIH) Grants RO1-RR-019732 and RR016463, with core support from ES03828.

References

1. Cannon JP, Haire RN, Mueller MG, Litman RT, Eason DD, Tinnemore D, Amemiya CT, Ota T, Litman GW. Ancient divergence of a complex family of immune-type receptor genes. *Immunogenetics* 2006;58:362–73. [PubMed: 16738934]
2. Mattingly C, Parton A, Dowell L, Rafferty J, Barnes D. Cell and Molecular Biology of Marine Elasmobranchs: *Squalus acanthias* and *Raja erinacea*. *Zebrafish* 2004;1:111–20. [PubMed: 18248223]
3. <http://www.genome.gov/13014443>
4. Barnes D, Mattingly CJ, Parton A, Dowell LM, Bayne CJ, Forrest JN. Marine organism cell biology and regulatory sequence discovery in comparative functional genomics. *Cytotechnol* 2005;46:123–137.
5. Luer, CA.; Walsh, CJ.; Bodine, AB. The Immune System of Sharks, Skates, and Rays. In: Carrier, J.; Musick, J.; Heithaus, M., editors. *The Biology of Sharks and Their Relatives*. Boca Raton: CRC Press; 2004. p. 369-95.
6. Walsh, CJ.; Luer, CA. Elasmobranch Hematology: Identification of Cell Types and Practical Applications. In: Smith, M.; Warmolts, D.; Thoney, D.; Hueter, R., editors. *Elasmobranch Husbandry Manual: Captive Care of Sharks, Rays and their Relatives*. Columbus: Ohio Biological Survey Inc; 2004. p. 309-26.
7. Mainwaring G, Rowley AF. Separation of leucocytes in the dogfish (*Schliorhinus canicula*) using density gradient centrifugation and differential adhesion to glass cover slips. *Cell and Tissue Research* 1985;241:283–290. [PubMed: 4028127]
8. McKinney EC, Haynes L, Droese AL. Macrophage-like effector of spontaneous cytotoxicity from the shark. *Dev Comp Immunol* 1986;10:497–508. [PubMed: 3817246]

Fig. 1.

Separation of skate peripheral blood by density gradient centrifugation. Skate RediGrad-I (1.095 g ml^{-1}) was prepared by mixing 6.5 volumes of skate RediGrad stock solution with 3.5 volumes of 0.5 M NaCl . Skate RediGrad-II (1.050 g ml^{-1}) was prepared by mixing 3 volumes of skate RediGrad stock solution with 7 volumes of 0.5 M NaCl . The cell loading solution was skate phosphate-buffered saline (1000 mOsm), prepared by mixing 9.3 volumes of standard phosphate-buffered saline with 0.7 volume of 5 M NaCl .

Fig. 2. Light microscopy of samples after May-Grunwald Giemsa staining. (a), whole blood prior to centrifugation; (b) interface Fraction II after density gradient centrifugation.