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Cell Separation by Centrifugal Elutriation

EVELYNE BACHÈRE, DOMINIQUE CHAGOT, AND HENRI GRIZEL

Institut Français de Recherche pour l'Exploitation de la Mer
Laboratoire de Pathologie et de Génétique des Invertébrés Marins
17390 La Tremblade, France

Abstract.—There is an ongoing need to isolate and purify cells in bivalve mollusc disease research. Many separation techniques differentiate cells solely by a single factor and usually require additives in the medium that may damage live cells. Centrifugal elutriation is a technique that separates particles by size and density simultaneously and can be conducted in physiological media without additives. Living cells can be separated without chemical damage and, because the procedure can be conducted aseptically, cells can be maintained in vitro after elutriation. A description of the technique, methods to determine flow rates and rotor speeds, and a preliminary separation of oyster hyalinocytes is presented.

In molluscan pathology and immunology, methods to isolate and purify cells are needed, be they hemocytes involved in the immunity processes, cells susceptible to infection by parasites, or the parasites themselves. These methods must be simultaneously quantitative and qualitative and must preserve the structural and functional integrity of the cells for in vitro studies.

Classical methods of cell isolation are generally based on differential and isopycnic centrifugations in a density (pressure) gradient. The techniques are efficient but present some disadvantages related to the chemical nature of the products used (sucrose, cesium chloride, Percoll, meglumine diatrizoate, metrizamide) and the physicochemical changes they induce (e.g., in ionic force, osmotic pressure, or viscosity) at the concentrations used (Rickwood 1984). Moreover, these methods generally require several stages of centrifugation and collection.

An alternative method, counterstreaming centrifugation, was originated by Lindahl (1948) and later developed by Beckman Instruments under the name of centrifugal elutriation (McEwen et al. 1968). This procedure, which can eliminate the disadvantages previously cited, has been used to prepare different vertebrate cells, e.g., whole blood and hemopoietic cells, cells from brain and liver, transformed and tumor cells, cells from testis and ovary (Pretlow and Pretlow 1979), and also to purify protozoan parasites such as *Plasmodium* sp. (Russman et al. 1982) or *Eimeria* sp. (Stotish et al. 1977). This technique is efficient because it is rapid and because a large quantity of cells can be purified at once.

Principles of Centrifugal Elutriation

Centrifugal elutriation is a form of velocity sedimentation in which cells are separated according to their rates of sedimentation. Separation is proportional to the size of the cells and to the difference between the densities of the cells and the medium. Thus, velocity sedimentation is particularly useful in separating cells of the same density (Pretlow and Pretlow 1982). In centrifugal elutriation, cells are exposed to a centrifugal force while suspended in a selected medium which flows continuously in a centripetal direction (Pretlow and Pretlow 1979). The Beckman Instruments system designed for this technique is shown schematically in Figure 1.

In the elutriation chamber, each cell tends to migrate to a zone where its sedimentation rate is exactly balanced by the flow rate of the fluid. Because the geometry of the chamber produces a gradient of flow rates from one end to the other, cells with a wide range of sedimentation rates can be held in suspension. By increasing the flow rate of the elutriating fluid in steps, or by decreasing the rotor speed, successive populations of homogeneous cell sizes can be washed from the chamber (Figure 2). The major theoretical aspects of elutriation were developed by Lindahl (1948), McEwen et al. (1968), Pretlow et al. (1975) and Sanderson et al. (1976).

Elutriation Methodology

Meistrich (1983) defined and studied the various methodological factors of centrifugal elutriation.

Preparation of Single-Cell Suspension

Some cell systems, such as those of peripheral blood, are already in single-cell suspension and may require only treatment to avoid aggregation (Mackler et al. 1977). However, cells in organs and tissues have to be dissociated, either by

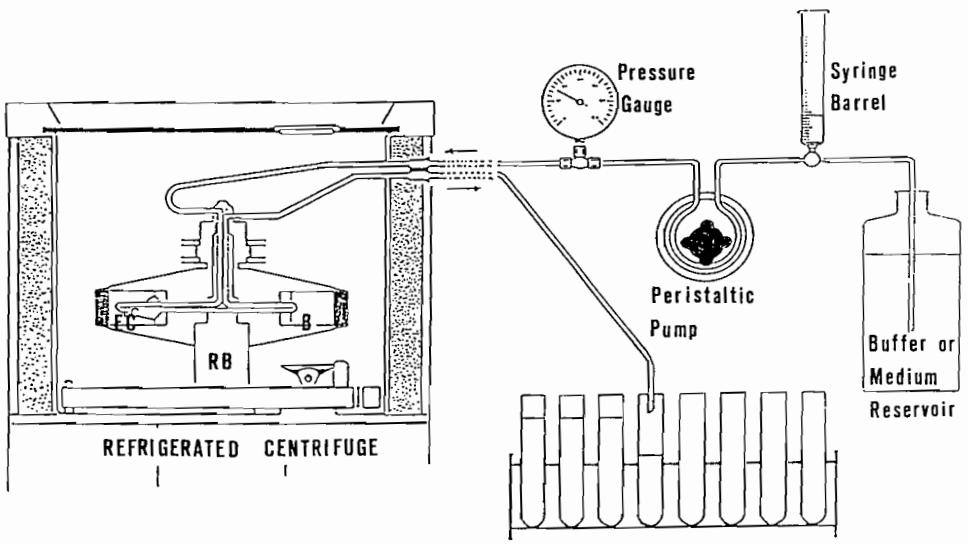

FIGURE 1.—The elutriator system with the loading setup. EC = elutriation chamber; RB = rotor body; B = bypass.

gentle mechanical methods or by different enzymes (trypsin, hyaluronidase, etc.) sometimes together with EDTA treatment (Meistrich 1972).

Temperature Choice and Regulation

The choice of temperature for elutriation should be compatible with cell physiology. Generally, 4°C is recommended to lessen the phenomenon of aggregation. However, Sanderson et al. (1977) claimed that aggregation of mouse spleen cells was greater at 4°C than at 30°C and that temperature consistency was a more important factor.

Choice of Medium

Any buffer and culture medium can be used for elutriation. The essential criterion in the choice of buffer or medium is its capacity to maintain the physiological integrity of the cells and to prevent cell aggregation.

Loading System

The Beckman Instruments system for loading cells into the rotor is designed to avoid passing cells through the peristaltic pump. However, this system is cumbersome because the loading time is long and cell loss is not negligible (Meistrich 1983). In a simpler loading system (Figure 1), such as one by Meistrich (1983), cells pass through the peristaltic pump but without apparent cell damage. A three-way valve with a syringe barrel is inserted between the buffer reservoir and the pump. A small volume of cells in suspension is poured into the barrel and then pumped into the rotor. This loading system reduces the loading time and the formation of aggregates. A wide range of cells, from less than 10^7 to more than 10^9 mL^{-1}, can be used, but the number will vary according to the nature and volume of the cells.

Flow Rates and Rotor Speeds

Cell volume and distribution are the only factors that need to be known before new cell types are separated by elutriation. Cell volume can be used to compute the sedimentation rate (S, mm/h) by a generalized form of Stokes' law (Wyrobek et al. 1976),

$$S = (\rho c - \rho o)V^{2/3}a/\eta f;$$

ρc and ρo are the densities of the cell and the suspending medium, respectively; V is the cell volume; a is the acceleration due to centrifugation; η is the viscosity of the fluid; and f is a coefficient dependent only on the particle shape.

For the standard Beckman chamber, and given $\rho c - \rho o = 0.05$ g/cm^3, $\eta = 1.43 \times 10^{-2}$, and $f = 11.7$, the preceding equation can then be simplified to

$$S/a = 0.105\ V^{2/3}.$$

The sedimentation time S/a can thus be predicted solely on the basis of cell volume (V), which can be estimated either by Coulter counter

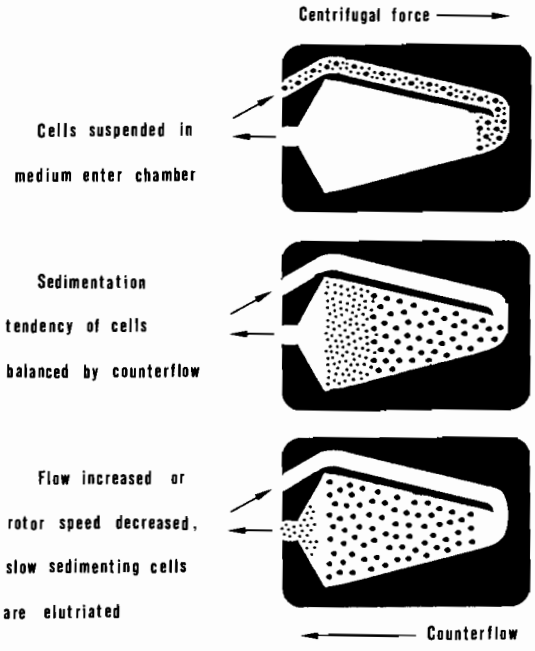

FIGURE 2.—Elutriation process within the separation chamber. The elutriation actually occurs at the wide point of the chamber. The cell population collected will contain cells that are larger or more dense than those of the previous fraction. (From Beckman Instruments applications data.)

or by microscopic measurements. Rotor speed k (1,000 revolutions/min) and flow rate r (mL/min), can be chosen to determine elutriation conditions corresponding to the point of elutriation, i.e., $S/a = 1.93r/k^2 = 0.105\ V^{2/3}$. Thus, when separation of a new cell mixture is desired, values of r and k are chosen for the smallest and largest cells in the population. If there are wide ranges of cell size and sedimentation rate in a given population, two runs will be needed to obtain cell separation.

Collection of Cells

Cells can be eluted by changing either the flow rate or the rotor speed. In some cases, alternately decreasing rotor speed and increasing flow rate during the same run may be best for collecting the different fractions.

Separation of Oyster Hyaline Cells

Ultrastructural studies of hyaline cells in molluscs have shown considerable polymorphism, the ontogenic and physiological significance of which is not well known. To determine the specificities of different morphological types, we conducted separation experiments of hemocytes from the Pacific oyster *Crassostrea gigas* using the technique of elutriation.

In the methodology developed for these experiments, the hemolymph is withdrawn by intrapericardiac puncture and diluted 2:3 in Alsever's solution (glucose, 20.8 g/L; sodium citrate, 8 g/L) to which is added EDTA (3.36 g/L) as a chelating agent and Tween 80 (0.1%) as an emulsifier. Sodium chloride is used to make the solution isotonic for the hemocytes; osmotic pressure is adjusted to 1,000 mosmol by means of a cryoosmometer. Under these conditions, cell aggregation is substantially avoided.

The hemocyte suspension is submitted to isopycnic centrifugation in a Percoll discontinuous gradient to separate hyalinocytes and granulocytes by their density differences. A homogeneous population of granulocytes is distributed in high-density fractions (60–70%); the less-dense fractions (10%, 20%, 30%) contain a heterogeneous population of hyaline cells. We hope that this potentially harmful step with Percoll can be eliminated in the future.

Centrifugal elutriation is performed to separate hyalinocyte subpopulations on the basis of their size differences. The elutriation medium is Alsever's solution (12°C), and the rotor is equipped with a Sanderson chamber. Hyalinocytes are eluted in eight fractions according to the nomogram presented in Figure 3. This protocol is effective and reproducible; cell recovery is about 60%. Nearly 90% of the cells fractioned in this manner were viable when tested by the trypan blue exclusion method. Cell integrity was verified by examination with an electron microscope.

The ultrastructural features of elutriated cells led to the identification by size and morphology of an evolutive series. This series was characterized in the first fractions by cells measured 5–6 μm and having a high nucleocytoplasmic ratio and rough endoplasmic reticulum, often located near the nucleus. In the last fractions collected, the hemocytes measured 11–14 μm and had a low nucleocytoplasmic ratio, numerous small vesicles within the cytoplasm, a considerable system of lamellae and ergastoplasmic cisternae, and some glycogen granules.

In vitro maintenance of these elutriated cells revealed that their capacity for adhesion and displacement remained intact. Moreover, long-term in vitro culture can be attempted because the

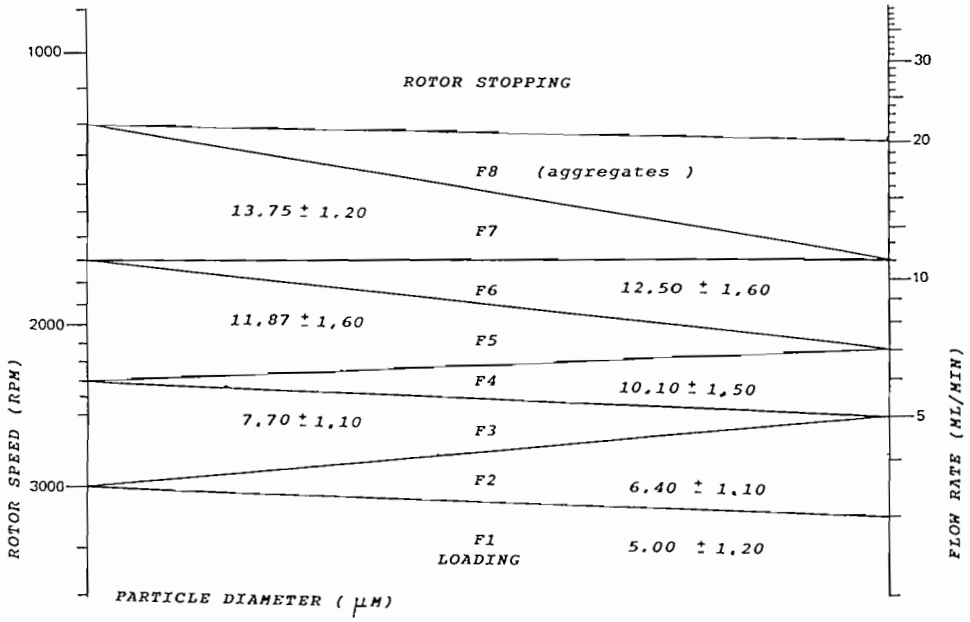

FIGURE 3.—Nomogram of the elutriation of Pacific oyster hyalinocytes. Each successive fraction (F) yields a larger particle diameter as the rotor speed is decreased or the flow rate is increased. RPM = revolutions/min.

system can be sterilized with 70% ethanol prior to an aseptic separation run.

Discussion

The method of elutriation to separate viable hyaline cells of Pacific oysters is new to malacology; it makes possible certain investigations in molluscan immunology and pathology. The ability to obtain consistently homogenous populations of hyalinocytes that can be maintained in vitro should enable us to investigate their biochemical characteristics and their functions in immune mechanisms. Furthermore, this method is now available for the study of cells of other species of bivalves.

Bivalve hemocytes are associated with diseases caused by viruses, e.g., iridovirus in *Crassostrea angulata* (Comps and Duthoit 1976) and retrovirus in softshells *Mya arenaria* (Oprandy and Chang 1983), and by protozoa, e.g., *Bonamia ostreae* in edible oysters *Ostrea edulis* (Pichot et al. 1980). Because these diseases are economically important, we need to understand the relationships of host and parasite at the cellular level. We presently are studying the relationships between oyster hemocytes and the pathogen *B. ostreae*.

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