PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 157, 599–604 (1978)

Granulocyte Seperation by Modified Centrifugal Elutriation System (40105)

MAXIM D. PERSIDSKY AND LYNDA S. OLSON

Department of Cryobiology, The Institutes of Medical Sciences, San Francisco, California 94115

Current laboratory methods for the separation of granulocytes are, to a greater or lesser degree, damaging to the cells. The methods most frequently used are isopyknic density-gradient centrifugation and separation by the reversible adhesion to nylon or glass surfaces. The harmful effects of these procedures have been reported by a number of investigators (1, 2). These methods subject the cells to the physical trauma of repeated centrifugation and packing, are laborious and giving low granulocyte yields. Clearly, there is a need for a better technique for separation of granulocytes.

A new process, CE,[1] has been recently introduced and successfully used for the isolation of a number of different cell types (3–7). In this process sedimentation of cells by the centrifugal force is counteracted by the opposing force of liquid flow. The cell separation results when a portion of the cell population has a sedimentation rate slower than the fluid velocity and is washed out from the separation chamber, while more rapidly sedimenting cells remain suspended in the chamber. The retained cells are eluted either by increasing the flow rate or by decreasing the rotor speed. During this separation process, cell packing and prolonged contact with foreign surfaces are avoided. This method allows the use of any physiologically suitable medium, produces a high yield of cell fractions with a high degree of purification, and requires a relatively short processing time.

In an attempt to utilize CE for separation of granulocytes from whole human blood, McEwen *et al.* (8) reported that it was necessary first to enrich the leukocyte population by Dextran-sedimentation of blood before the lymphocyte-free granulocyte preparation could be obtained by elutriation. Because potential advantages of CE promise to eliminate many shortcomings of the previous techniques, we have investigated the feasibility of employing CE for the one-step separation of granulocytes from whole human blood. This investigation and our earlier studies (9, 10) show that, with proper modification, the CE process is most effective for separation of granulocytes from whole human blood without the need for the leukocyte enrichment step.

Materials and Methods. Blood collection. Blood (5 ml) was collected by venipuncture from healthy volunteers. It was immediately diluted tenfold with HBSS[2] which effectively prevents blood clotting for several hours. This method was adapted from the procedure reported by Chodirker *et al.* (11) in which blood clotting was prevented by twentyfold dilution with saline containing 0.1% bovine serum. For comparison, blood was anticoagulated by heparin (10 U/ml), and then used either undiluted or diluted tenfold.

Cell separation system. The Beckman JE-6 elutriator rotor was used in the Beckman J-21 preparative centrifuge. In order to achieve maximal cell yield and minimal dilution of the purified granulocytes, the flow system of the Beckman elutriator has been substantially modified by the senior author. The main modification is in providing the means for reversing the direction of fluid flow through the separation chamber in the rotor while the system is in operation. This is accomplished by using a compound valve which reverses both the direction of fluid flow and at the same time changes the exit line from waste to sample collection. The diagramatic representation of this new flow system is shown in Fig. 1. Changing the centripetally oriented flow after the completion of elutriation to the centrifugal direction aligns the action of both the fluid drag and the centrifugal force. This facilitates the evacuation of granulocytes from the separation chamber. As a result, the

This work was supported by the NIH Grant No. CA 12754-05.

[1] CE refers to centrifugal elutriation.

[2] HBSS refers to Hanks' balanced salt solution.

0037-9727/78/1574-0599$01.00/0

concentrated suspension of granulocytes can be collected in aliquots of small volume (5 to 8 ml). In contrast, elution of cells by the original Beckman system yields collected cells in aliquots of much larger volume (150 ml). Once the cells are eluted, the system is ready for the next run, so that many blood samples can be successively processed. By reversing back and forth the direction of flow, it is possible to unclog the separation chamber from cell pellet while the system is in operation should cell packing occur. Another modification of the Beckman system is that the mixing chamber was replaced with the inverted sample bottle as shown in Fig. 1. During the loading step the diluted blood is displaced by air from the sample bottle and driven along the fluid line into the rotor, and into the separation chamber.

CE procedure. The CE procedure starts by purging air from the assembled flow system which is facilitated by reversing the direction of flow several times. The rotor speed is then set and maintained at 2500 rpm throughout the entire operation. Rotor speeds lower or higher than 2500 rpm have been found less suitable, as will be discussed later. During the loading of the diluted blood, the rate of the fluid flow is maintained at 11 ml/min. Following completion of the blood loading the flow rate is increased to 14 ml/min. After approximately 15 min, the separation chamber begins to clear at its centripetal side while remaining turbid at the other side (Fig. 2). The visible turbid zone upon microscopic examination was found to consist predominantly of granulocytes mixed with some red cells. To speed up the purification process the flow rate is increased until the boundary of the turbid zone is displaced to the top of the wide side of the chamber. Based on a series of 68 experiments, optimal flow rate for separation of granulocytes was found to vary from 14 to 35 ml/min, depending upon the individual donor. The flow rates from 62 out of a total of 68 runs were in the range of 14–25 ml/min. Blood specimens used for this study were obtained from eight subjects. No correlation was found between the individual subjects and the flow rate variations when measured on different days. However, the flow rates were highly reproducible from run to run, when measured on the same day with blood from the same subject. Following 15 min of elutriation the granulocyte suspension becomes virtually free of red cells and lymphocytes. The concentrated granulocytes are then collected by reversing the direction of the flow through the separation chamber. The entire system was maintained at room temperature throughout the operation.

Dextran–sedimentation. For comparison, leukocytes were collected by the dextran-sedimentation technique (12). Five ml of heparinized (10 U/ml) blood were mixed with equal volume of 3% Dextran 5000 (Pharmacia, Uppsala, Sweden) in saline and allowed to sediment at room temperature for 45 min. The leukocyte-rich supernatant was withdrawn, centrifuged at 80*g* for 6 min, and washed twice with HBSS. The number of granulocytes per ml was determined by using a hemacytometer.

Evaluation of granulocyte function. The

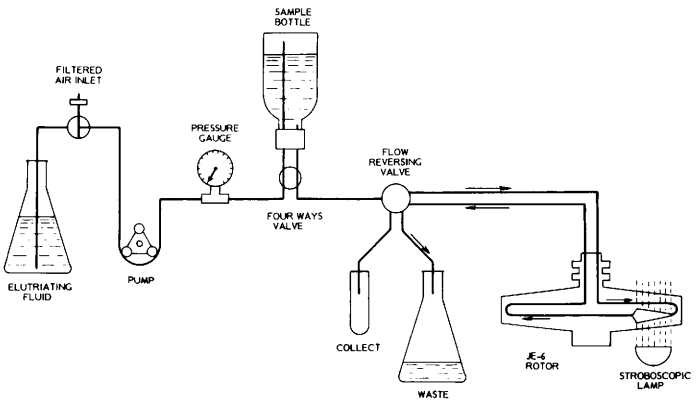

FIG. 1. Flow diagram of the modified centrifugal elutriation system.

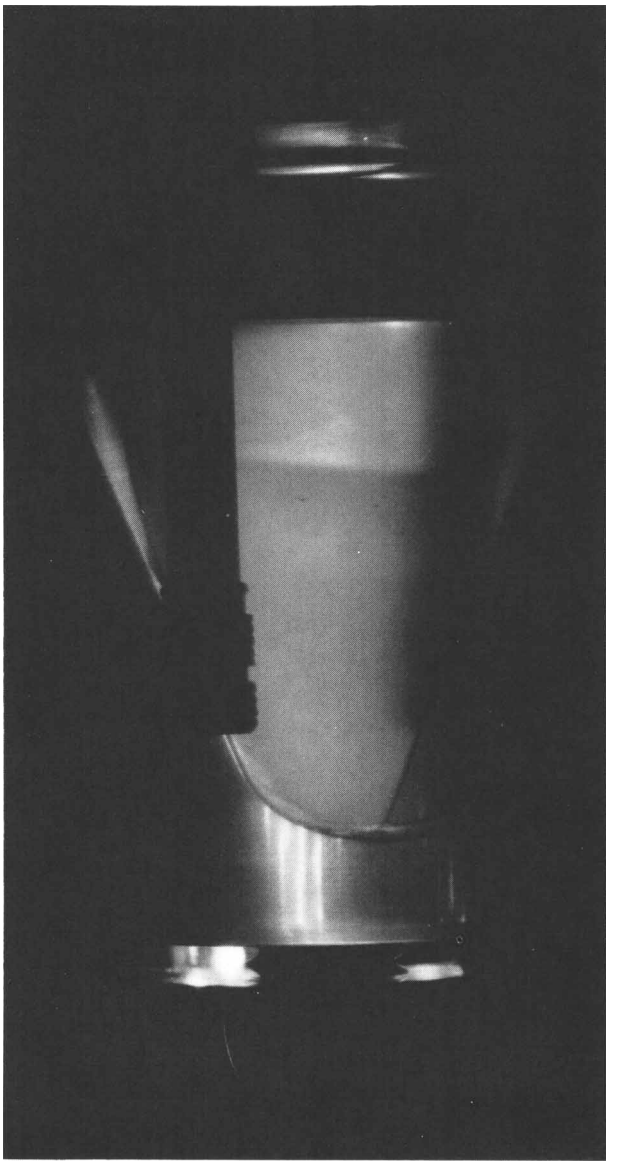

FIG. 2. Beckman's separation chamber in the process of elutriation, photographed with the strobe light. The bottom two thirds of the chamber contains a suspension of purified granulocytes separated from 5 ml of whole blood. Clotting was prevented by tenfold dilution of the blood. 2 × enlargement.

function of granulocytes isolated by CE at different rotor speeds (2500, 3000, 4000 and 5000 rpm) was evaluated for their bactericidal and phagocytic abilities and their membrane integrity. The ability of granulocytes to kill *S. aureus* was compared to that of dextran-sedimented leukocytes from the same donor. Each sample tube contained 0.1 ml cell suspension (1×10^6 granulocytes), 0.1 ml AB+ serum, 0.025 ml bacterial suspension (1×10^6 bacteria), and 0.775 ml HBSS. Samples were incubated at 37° for 120 min. Then, they were plated on agar, incubated at 37° for 24 hr and analyzed for bacterial growth. Results were expressed as the number of bacteria surviving after 120 min of incubation with granulocytes (13). Phagocytic function and membrane integrity of granulocytes were analyzed by our method (14) involving the combined use of fluorescein diacetate, ethidium

bromide, and zymosan particles. This method allows easy distinction between phagocytizing cells, nonphagocytizing cells, and dead cells.

Results. Granulocyte separation. The results of this study on the use of the modified CE system for separation of granulocytes from blood are based on daily experimental runs conducted over a period of two years. The systematic study of different flow rates during the loading of whole blood revealed that the flow rate must initially be kept low (11 ml/min). To obtain maximal granulocytes yield the volume of loaded blood should not exceed 5 ml. Loading larger volume of whole blood than 5 ml results in considerable loss in recovery of granulocytes. Apparently, this is due to overcrowding of cells in the chamber. When tenfold diluted blood is used then a total of 10 ml whole blood can be loaded without significantly reducing the granulocyte yield. The initial dilution allows red blood cells to be continuously eluted from the separation chamber during loading which relieves the overcrowding and improves the condition for retention of granulocytes.

The entire procedure for separating granulocytes from 5 ml of whole blood takes between 30 and 35 min at 2500 rpm, depending upon donor. The collected granulocyte preparation contains approximately 2:1 ratio of erythrocytes to granulocytes. At this point very few lymphocytes are present. From 5 ml of whole blood, the number of lymphocytes and granulocytes from five separate experiments averaged $2.0 \times 10^5 \pm 1.9 \times 10^5$ and $137 \times 10^5 \pm 46 \times 10^5$ cells respectively. The few lymphocytes present constituted only about 1.7% of the total. A complete removal of both lymphocytes and erythrocytes can be achieved after about 90 min of elutriation. Larger blood samples of up to 30 ml after tenfold dilution with HBSS can also be processed with fair yields of granulocytes.

Granulocyte yield. Yields of granulocytes separated by this procedure from diluted blood can be as high as 100%. Typically, the yield approaches 90%, with an average value of 87%. Lymphocytes present in the separated granulocyte samples gave an average value of $1.2 \pm 1.1\%$. The calculation of granulocyte yield was based on differential WBC counts in the samples of whole blood before elutriation and in the collected granulocytes after elutriation.

Cell evaluation. Granulocytes isolated by CE at different rotor speeds and different flow rates were evaluated for their bactericidal function, phagocytic activity and membrane integrity. The results of these tests are presented in Table I. It is evident from these data that, at a rotor speed of 2500 rpm and a flow rate of 21 ml/min, the ability of granulocytes to kill *S. aureus* is comparable to that of Dextran-sedimented control cells. However, with the increase of rotor speed up to 5000 rpm and higher flow rates up to 80 ml/min, there is a significant decrease in the bactericidal ability of granulocytes. Phagocytic function and membrane integrity tests show an apparent decrease in phagocytic capacity up to 4000 rpm while membrane integrity did not display significant changes with increase in rotor speeds. Phagocytic function of granulocytes obtained at 5000 rpm appears to approach the level observed with cells elutriated at 2500 rpm. At this high rotor speed the elutriation rate is very high (80 ml/min), therefore, a much shorter time (10 min) is required to separate granulocytes almost free of lymphocytes and red cells than that at 2500 rpm (35 min). The shorter processing time could be one of the reasons for the recovery of phagocytic function of gran-

TABLE I. EFFECT OF ROTOR SPEED AND ELUTRIATION RATE ON FUNCTIONS OF GRANULOCYTES

Rotor speed rpm	Elutriation rate ml/min	% Phagocytizing cells[a]	% Nonphagocytizing cells	% Dead cells	% Killing of *S. aureus*[c]
2500	21	56 ± 6[b]	41 ± 4	3 ± 0.5	92 ± 3.1
3000	38	38 ± 4	44 ± 5	18 ± 3	84 ± 1.3
4000	48	28 ± 17	60 ± 19	12 ± 2	80 ± 1.6
5000	80	50 ± 16	41 ± 16	9 ± 0.7	62 ± 5.5

[a] As control experiments for phagocytic function, Dextran-sedimented leukocytes were used and the numbers of phagocytizing cells were between 50 and 63%.

[b] Standard deviations.

[c] The percent killing of *S. aureus* by Dextran-sedimented leukocytes was 91 ± 4.4

ulocytes (Table 1). Rotor speed below 2500 rpm requires longer than 35 min of elutriation time and therefore is less desirable.

Granulocytes isolated by this procedure from blood diluted tenfold without anticoagulant have a lesser tendency to clump, particularly upon incubation at 37°, than those isolated from heparinized blood. Also they have less tendency to clump than do Dextran-sedimented cells.

Discussion. The modifications of the Beckman centrifugal elutriator concern mainly with its flow system. By adding new means for reversing the direction of flow through the separation chamber and by making different provisions for loading cell samples, this system becomes more effective for the separation of granulocytes than was the original system as described by McEwen (8). The advantages resulting from these modifications are that with this system one can: (a) separate granulocytes from whole human blood in one-step procedure; (b) collect concentrated cells in small volume; (c) obtain almost 100% yield of purified granulocytes; and (d) unclog the separation chamber without stopping the rotor, should cell packing occur, by simply reversing direction of flow. Based on the evaluation of the granulocytes' bactericidal and phagocytic function, and on their membrane integrity, it appears that our CE procedure is less traumatizing to the cells than are the other available methods. It is simple to perform, requires a relatively short time, and is suitable for serial processing of many blood samples. In addition, granulocytes can be separated without the use of anticoagulants. Therefore, this procedure appears to have a greater potential than other available methods for obtaining granulocytes in the least damaged condition for a variety of clinical tests, such as chemotaxis, migration inhibition, NBT reduction, etc., as well as for a host of scientific studies on the biochemical nature of granulocytes. Although some of these clinical tests do not require pure granulocytes, the use of intact and purified cells should make the tests more efficient, specific and sensitive.

The order in which the blood cell types are distributed in the separation chamber during elutriation appears, paradoxically, to be the reverse of that commonly observed during the centrifugation of blood. During elutriation, granulocytes occupy the centrifugal side of the chamber while red cells and lymphocytes the centripetal side. It is interesting to note that Maupin (15) showed that when blood is entensively diluted with normal saline and then centrifuged, granulocytes are sedimented first, followed by lymphocytes and red cells. Since the CE process also involves extensive dilution of blood, this may be a reason for the analogous distribution of the blood cells within the elutriation chamber.

Of special interest is the observed reproducibility in the flow rates required to maintain granulocytes at a specified level within the separation chamber when measured on the same day with the blood from the same donor. This reproducibility suggests that flow rate variations are not due to the experimental error, but rather due to actual variations in the granulocyte volume or bouyant density. Thus, CE may offer a new way for measuring in bulk this important cell parameter.

Summary. The Beckman centrifugal elutriation system has been modified for a one-step separation of granulocytes from whole human blood. A special valve for reversing the direction of flow permits the collection of concentrated granulocytes in small volumes and allows unclogging of the separation chamber should cell packing occur during centrifugal elutriation. Blood diluted tenfold prevents coagulation, reduces the cell trauma and makes separation more efficient. Maintenance of the precise flow rates by visual monitoring of the granulocyte level in the chamber during elutriation, when the chamber begins to clear of red blood cells, is essential for optimal separation. Granulocytes isolated at rotor speed of 2500 rpm are indistinguishable in their membrane integrity, bactericidal and phagocytic abilities from those collected by Dextran-sedimentation. Cell yields close to 100%, high purty, minimal cell traumatization, and short processing time make this technique highly effective in collecting granulocytes for clinical and laboratory studies.

1. Rothfield, L., and Romeo, D., *in* "Structure and Function of Biological Membranes" (L. I. Rothfield,

ed.), p. 251. Academic Press, New York (1971).
2. Klock, J. C., and Bainton, D. F., Blood **48,** 149 (1976).
3. McEven, C. R., Johns, E. Th., Stallata, K. W., Schell, J. V., Siddigui, W. A., and Geiman, Q. M., J. Parasitology **57,** 887 (1971).
4. Glick, D., Von Redlich, D., Johns, E. Th., and McEwen, C. R., Exp. Cell. Res. **65,** 23 (1971).
5. Flangas, A. L., Prep. Biochem. **4,** 165, (1974).
6. Grabske, R. J., Lake, S., Gledhill, B. L., and Meistrich, M. L., J. Cell. Phys. **86,** 177 (1975).
7. Knook, D. L., and Sleyster, E. Ch., Exp. Cell. Res. **99,** 444 (1976).
8. McEwen, C. R., Stallard, R. W., and Juhos, E. Th., Anal. Biochem. **23,** 369 (1968).
9. Persidsky, M. D., and Milligan, J. O., Abstract. Cryobiology **13,** 656 (1975).
10. Persidsky, M. D., and Olson, L. S., Abstract. Cryobiology **13,** 666 (1976).
11. Chodirker, W. B., Bock, G. N., and Vaughan, J. H., J. Labor. Clin. Med. **71,** 9 (1968).
12. Kelly, M. T., Martin, R. R., and White, A., J. Clin. Invest. **50,** 1044 (1971).
13. Hirsch, J. G., and Strauss, B., J. Immunol. **92,** 145 (1964).
14. Dankberg, F., and Persidsky, M. D., Cryobiology **13,** 430 (1976).
15. Maupin, B., Le Sang **26,** 328 (1955).

Received April 13, 1977. P.S.E.B.M. 1978, Vol. 157.