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A Centrifugal Elutriation System of Separating Small Numbers of Cells

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One of the major disadvantages of centrifugal elutriation (CE) is the relatively large volume (150 ml) of the various fractions, especially if small numbers of cells have to be separated and the fractions contain few cells.

To reduce the volume of the fractions 2 elutriator rotors were coupled in series. Since the rotor speed of the second rotor was always kept 750 rpm higher than that of the first rotor, cells elutriated from rotor 1 were collected in rotor 2. After elutriation of a complete fraction from rotor 1, and collection in rotor 2, the cells were harvested from rotor 2. This was achieved by means of a flow distribution unit (FDU), which made it possible to disconnect the flow of both rotors and simultaneously reverse the flow of the second rotor.

It is demonstrated that $40-95 \times 10^6$ mononuclear leukocytes may be fractionated without loss of resolution in fractions of only 9 ml. The lymphocyte (> 99%) and monocyte subpopulations (88-94%) obtained were as pure as with CE carried out with only 1 rotor. In addition, the cells in rotor 2 could be washed and suspended in culture medium prior to harvesting by means of the FDU. In this way loss of cells by additional centrifugation steps was avoided.

Erythrocytes (RBC) present in certain lymphocyte fractions were lysed with NH_4Cl and after lysis of the RBC and elution of ghosts and debris, the cells were washed and harvested. This procedure did not affect cell viability and the PHA response of the lymphocytes.

The versatile system described made it possible to apply CE for the separation of small numbers of cells without loss of resolution, and demonstrated that CE is ideally suitable for concentration and washing of cells, and removal of contaminating RBC, not affecting the recovery, viability and function of the cells.

Key words: *centrifugal elutriation - human monocytes/lymphocytes - small numbers of cells - development of the technique*

Introduction

Centrifugal elutriation (CE) is widely used for the separation of cells (Pretlow and Pretlow, 1979). It is well suited to separation of large numbers of mononuclear cells into lymphocytes and monocytes (Norris et al., 1979; Contreras et al., 1980; Weiner and Shah, 1980; Figdor et al., 1981). Recently we demonstrated that CE may also be

used to isolate subpopulations of monocytes differing in function and maturation/differentiation stage (Figdor et al., 1982a). These monocyte subpopulations were isolated from relatively large numbers of cells ($5-10 \times 10^8$) derived from volumes of 500 ml of human peripheral blood.

To extend these studies we attempted to isolate monocyte subpopulations from the blood of cancer patients. However, the volumes of blood that can be obtained from patients are rather low, usually about 50 ml. This 10-fold reduction in the number of cells available for separation required isolation procedures combining high resolution with optimal cell recovery. In principle CE is well suited to the separation of small numbers of cells (Sanderson et al., 1977; De Mulder et al., 1981). However, a serious drawback of the technique is the relative large volume (150 ml) of the various fractions, irrespective of whether small or large numbers of cells are being separated. If functional assays are involved, fractions containing few cells ($< 5 \times 10^6/150$ ml) have to be concentrated, which itself inevitably leads to cell loss.

We describe here a new, versatile system in which 2 elutriator rotors are coupled in series. The first rotor is used to separate the mononuclear cells, while the second serves several purposes viz., harvesting of the cells of the various fractions in small volumes, washing of the fractionated cells and lysis of contaminating red blood cells. In this way maximal cell recoveries were obtained since additional centrifugation steps were unnecessary.

Materials and Methods

Media and reagents

Phosphate-buffered saline (PBS) supplemented with 0.15% bovine serum albumin (BSA) (fraction V; Sigma, St. Louis, MO), penicillin (100 IU/ml) and streptomycin (50 μ g/ml) was used as medium for CE (referred to as CE medium). Cells harvested from the blood cell separator (BCS) were suspended in PBS containing 15% acidic citrate dextrose (ACD) (Figdor et al., 1982b). The same medium was used to introduce these cells into the rotor in order to prevent attachment of platelets to monocytes. Red blood cells (RBC) were lysed with a solution containing 0.15 M NH_4Cl , 12×10^{-3} M NaHCO_3 and 1×10^{-4} M EDTA. The density of NH_4Cl containing medium (lysis medium) was adjusted to that of the CE medium by adding 1.2% Ficoll (Pharmacia, Uppsala) to prevent turbulence in the separation chamber when the medium was changed during CE.

Mitogenic stimulation was carried out in Dulbecco's modification of Eagle's minimal essential medium (DMEM) supplemented with NaHCO_3 (3.8 g/ml), 10% FCS, glutamine (2 mM), fungizone (2.5 μ g/ml), penicillin (100 IU/ml) and streptomycin (100 μ g/ml), all obtained from Gibco-Biocult, Glasgow, Scotland. This medium is referred to as culture medium. Phytohemagglutinin (PHA; purified batch HA17) was purchased from Wellcome Reagents, Beckenham, England and [^3H]thymidine ([^3H]TdR, 6.7 Ci/mmol) was obtained from New England Nuclear, Boston, MA.

Cell preparations

Volumes of 50 ml human peripheral blood were obtained from healthy donors. Blood was defibrinated or collected in flasks containing 7.5 ml acidic citrate dextrose (ACD). Mononuclear cell suspensions were prepared either by centrifugation over a Ficoll-Hypaque mixture (Böyum, 1968) or by means of a blood component separator (BCS) as described previously (Figdor et al., 1982b). Buffy coats prepared by the BCS were fractionated and counted by means of a Coulter counter. The fractions containing mononuclear cells were pooled and used for CE. The erythrocyte/leukocyte ratio in these preparations was 10 ± 5 .

Description of the CE system

A schematic representation of the elutriation system is shown in Fig. 1. Before the start of each experiment a vessel of volume approximately 120 l was pressurized with air until the manometer (m) read 0.9 bar. The air was led to the medium reservoirs

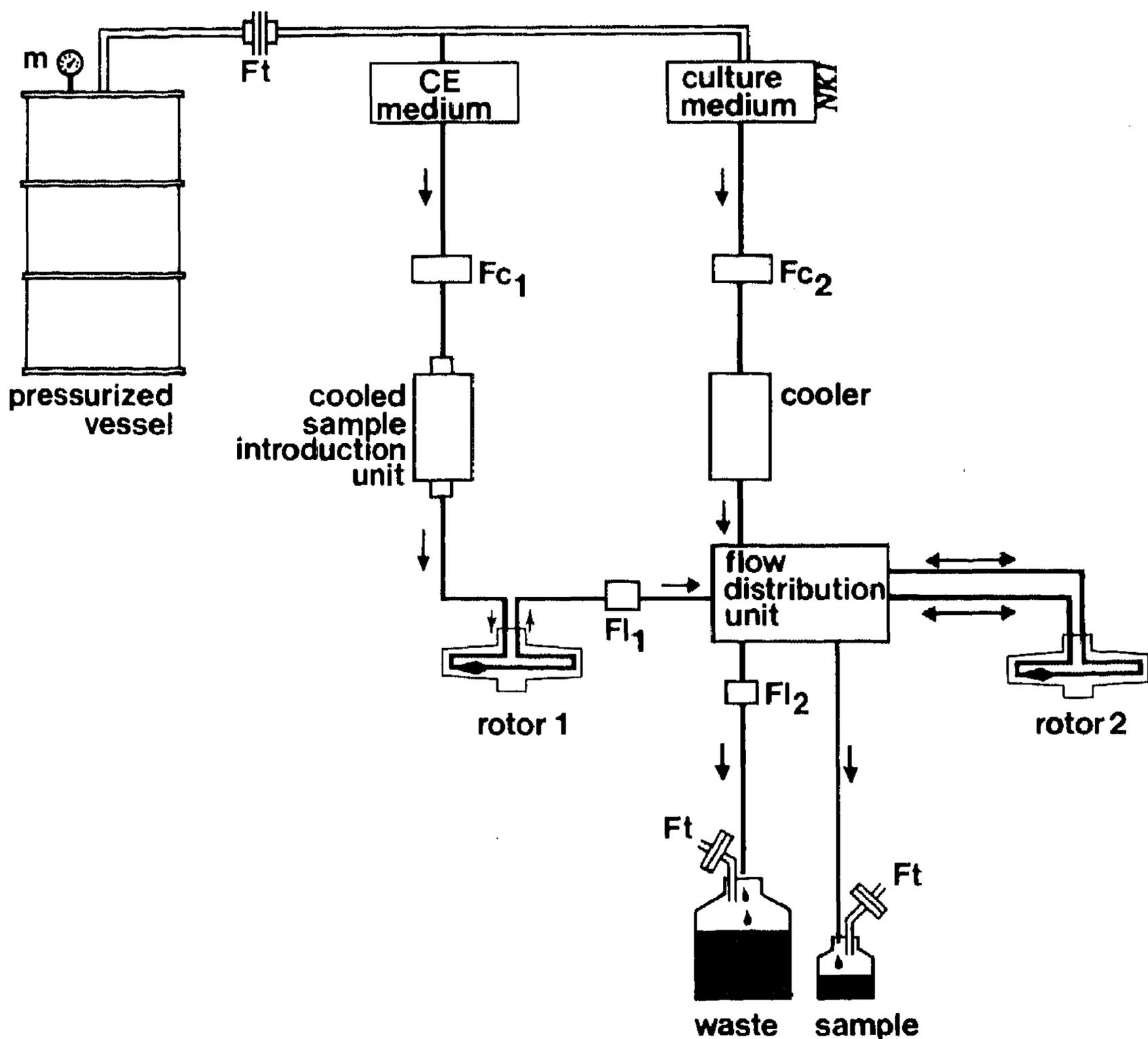


Fig. 1. Schematic representation of the CE system. Fc = flow controller; Fl = flow meter; Ft = sterilization filter; m = manometer; → = direction of flow.

via a sterilization filter (Ft) and used to generate a flow. The flow rate was set and held constant with a flow controller (Fcl) (Brooks Instruments, Veenendaal) which eliminated downstream variations.

CE medium is cooled in the sample introduction unit and led to rotor 1. The flow rate is monitored by flow meters, F11 and F12. The FDU leads the CE medium to rotor 2, to waste, or to sample (for details see below). The flow rate of the culture medium is regulated with a second flow controller (Fc2) cooled and led to the FDU. All parts were connected with each other with silicone tubing (2 mm inner diameter).

Sample introduction

Monocytes are strongly adherent if kept at temperatures above 20°C. Therefore we designed a sample introduction unit that allowed cooling of both the cell sample and the CE medium (Fig. 2). Cooled ethanol (−5°C) was pumped continuously

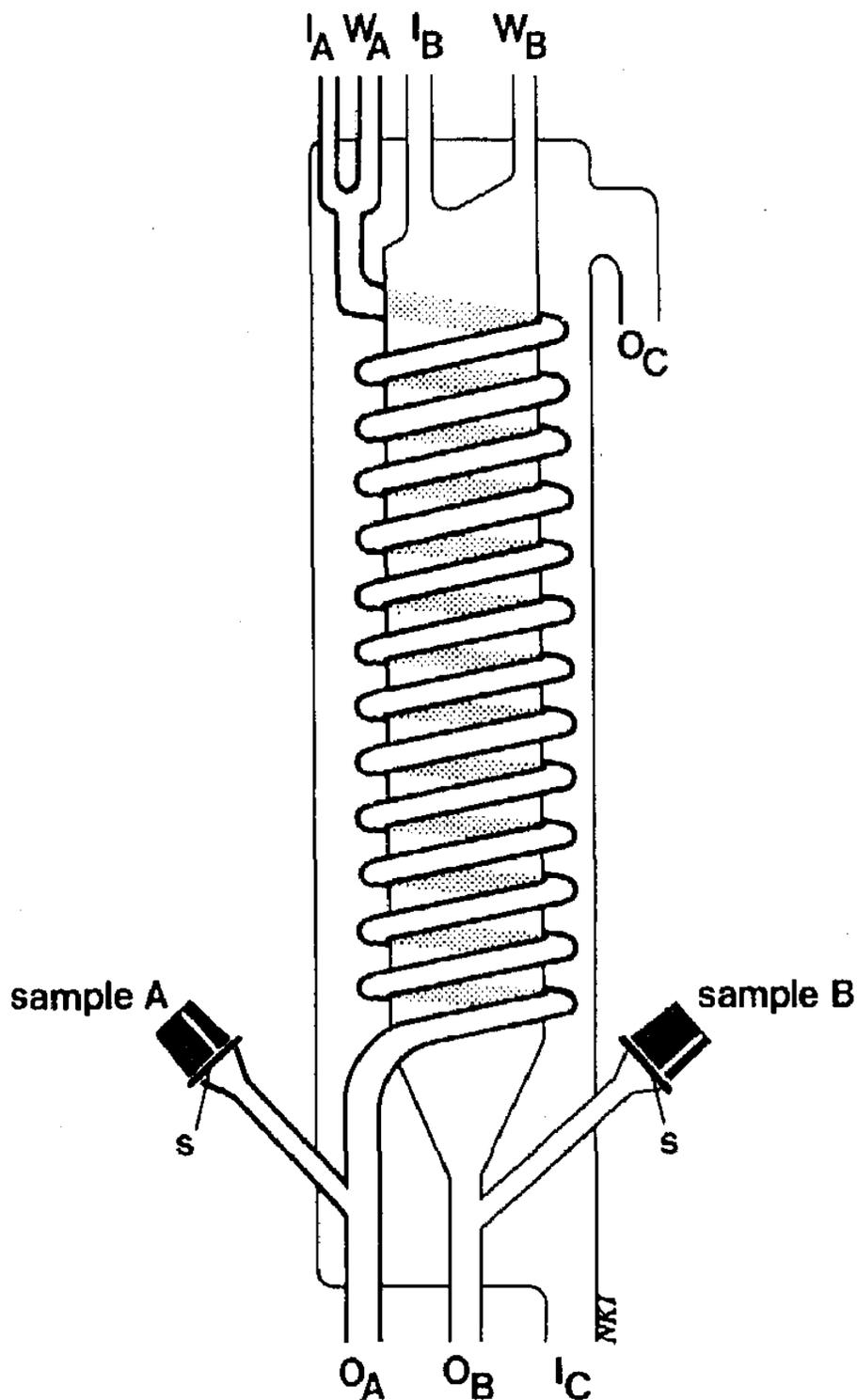


Fig. 2. Sample introduction unit, consisting of 2 systems. System A to introduce small sample volumes (<15 ml) and system B for the introduction of larger sample volumes (15–100 ml). I_c = inlet cooling; O_c = outlet cooling; W_A, W_B = waste system A/B; I_A, I_B = inlet system A/B; O_A, O_B = outlet system A/B; S = self sealing rubber septum.

through the introduction unit via inlet I_c and outlet O_c . CE medium was cooled by passing through the glass spiral or the glass cylinder. Cell samples of volumes up to 15 ml were introduced directly into the glass spiral (system A). Larger volumes, up to 100 ml, were introduced into the glass cylinder (system B). Before injecting the cell sample both the inlet (I_A or I_B) and the outlet (O_A or O_B) were closed by means of clamps. The cell sample was introduced by means of a syringe after opening the waste (W_A or W_B), and owing to its higher density replaced the CE medium without mixing. After injection of the cell sample, W_A or W_B was closed and the inlet and outlet were opened. Since the total flow rate is constant, the rate of introduction of the cell sample into the elutriator rotor may be varied by changing the relative flow rates of systems A and B.

Centrifugal elutriation

CE was carried out by means of 2 JE-6 elutriator rotors in series, both equipped with a standard elutriation chamber and installed in a Beckman JB-6 and J21-C refrigerated centrifuge (Beckmann Instruments, CA). $40-95 \times 10^6$ cells were injected in the cooled sample introduction unit and loaded into rotor 1 at a flow rate of 12 ml/min and at a rotor speed of 3800 rpm. The rotor speed of rotor 2 was set at 4500 rpm. After introduction of the cell sample the flow rate was set at 18 ml/min and kept constant. Fractionation was obtained by a stepwise decrease of the rotor speed of rotor 1, while the speed of rotor 2 was maintained 750 rpm higher to collect cells elutriated from rotor 1. After elutriation of a complete fraction from rotor 1, taking 8 min, and subsequent collection in rotor 2, the cells were washed and harvested in a volume of 9–15 ml. In addition red blood cells (RBC) contaminating the first lymphocyte fractions were lysed with $NH_4 Cl$ before harvesting. A reservoir containing medium to lyse the RBC (not shown) was connected to Fc2 (Fig. 1); a valve selected for either culture/wash or lysis medium. The subsequent lysis of RBC, washing and concentration of the cells was carried out in rotor 2 and controlled by the flow distribution unit.

During CE the temperature of the medium was monitored at 5 different points of the equipment: at the inlet (1) of the cooled sample introduction vessel, at the inlet (2) and outlet (3) of rotor 1 and at the inlet (4) and outlet (5) of rotor 2. After cooling the temperature maintained was $12 \pm 2^\circ C$.

All experiments were carried out under sterile conditions. Tubing, cooler, cooled sample introduction vessel, and flow distribution unit were autoclaved as a unit for 30 min at $121^\circ C$. The whole flow system, including introduction vessel, cooler, tubing and the FDU, were fixed on a board by metal clips and could be easily removed for sterilization. Autoclaving of the shaft, and extensive rinsing of the other parts of the elutriation rotor with alcohol (70%) permitted adequate long-term culture of the fractionated cells.

The flow distribution unit

The flow distribution unit (Fig. 3) has 3 functions: (1) to harvest the cells collected in rotor 2 in the smallest possible volume, (2) to wash the cells collected in rotor 2 and suspend them in culture medium, and (3) to lyse contaminating RBC.

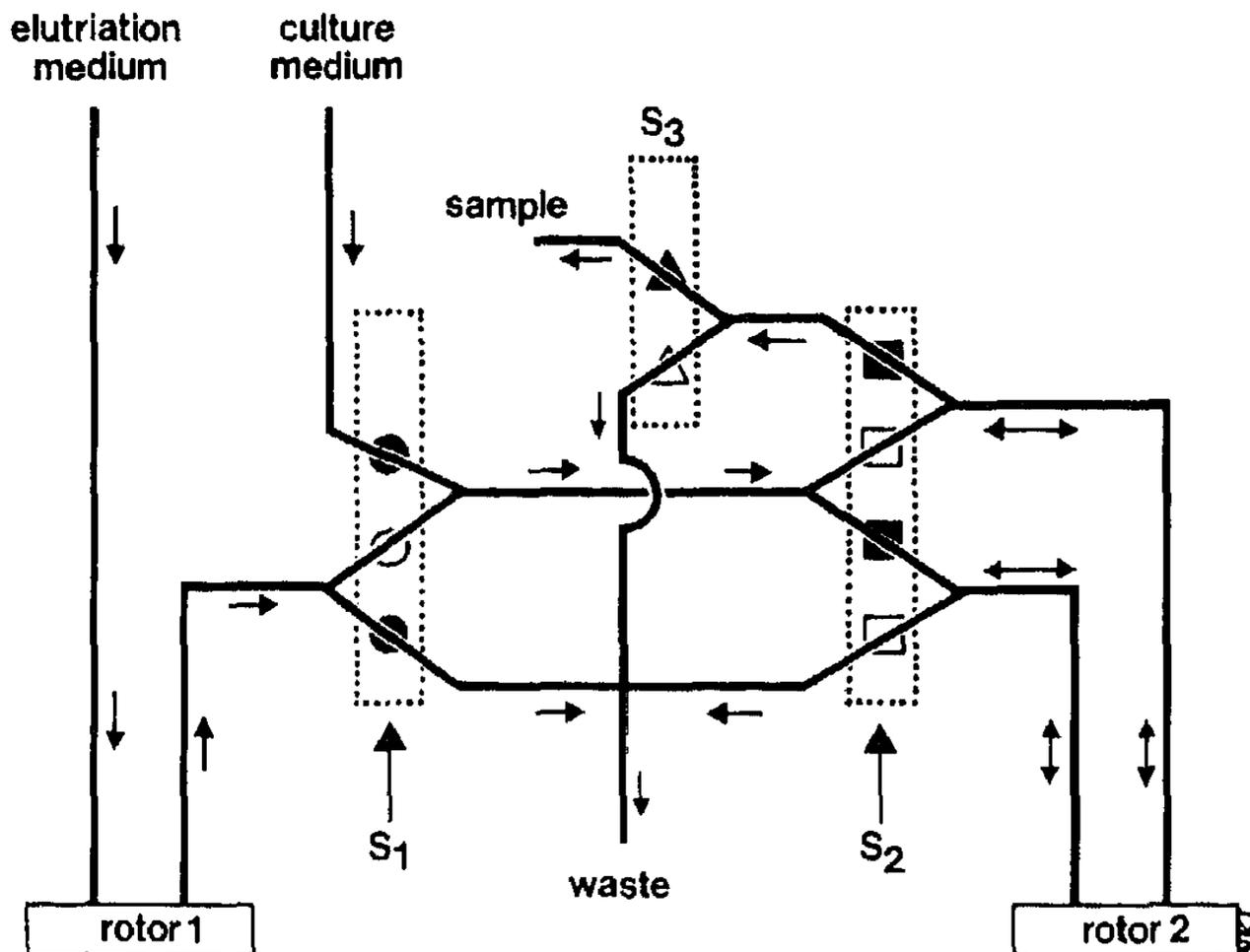


Fig. 3. Flow diagram of the flow distribution unit (FDU). The FDU consists of 3 switches: S1, S2, S3. The switches open 1 or more lines, and simultaneously close 1 or more other lines. The symbols indicate which tubes are closed and opened. According to the position of the switch the open symbols represent open lines and the closed symbols represent closed lines, or vice versa. See also Table I.

The flow distribution unit consists of 3 identical switches placed in series that simultaneously open and close various tubes led through the switch (Fig. 4). The direction of flow for the various positions of the 3 switches is shown in Fig. 3. The function of each switch is shown in Table 1. The first switch is used to collect cells in rotor 2 (○ = open, ● = closed) and to wash the cells with culture medium (● = open, ○ = closed). If the switch is positioned to wash the cells with culture medium

TABLE I

THE POSITIONS OF THE SWITCHES OF THE FLOW DISTRIBUTION UNIT TO EXECUTE VARIOUS FUNCTIONS

The symbols of the switches are those in Fig. 3. The isolation of a fraction consists of an elutriation phase; cells are eluted from rotor 1 and collected in rotor 2, followed by a phase in which contaminating RBC are lysed. Subsequently the cells are washed and suspended in culture medium (phase 3) and harvested in a small volume.

Function	Medium	S ₁	S ₂	S ₃	Time (s)
Elutriation	CE medium	○	□	—	480
Lysis of RBC	lysis medium	●	□	—	240
Washing	culture medium	●	□	—	60
Harvesting	culture medium	●	■	▲ ^a	120
	CE medium	○	■	△ ^a	120

^a S3 is used to reduce the volume of the fraction to a minimum (see Results). The cells could also be harvested directly after collection in rotor 2 in CE-medium

simultaneously, rotor 1 is disconnected from rotor 2 and the exit line of rotor 1 is led to the waste bottle. Further, contaminating RBC could be lysed by NH_4Cl which then replaced the culture medium. Selection for medium either to lyse or wash cells

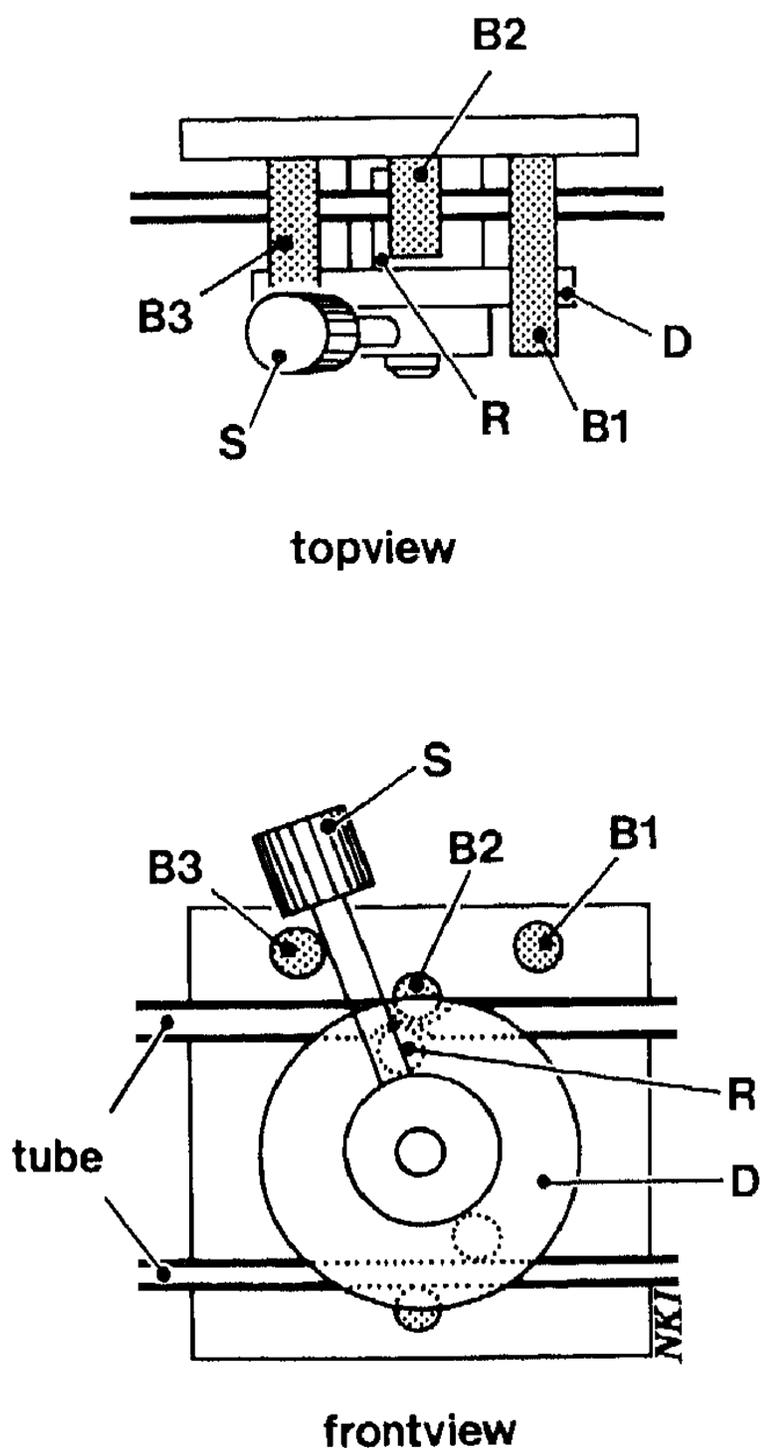


Fig. 4. Top and front view of a switch of the FDU. B = bar; D = disc; R = roller; S = switch handle. The switch (a compound clamp) was made of stainless steel. Up to 3 tubes could be closed and 3 others opened simultaneously (or vice versa). When the handle of the switch was screwed out of the disc, the disc could be pulled off the central bar and the tubes removed.

is by a valve near Fc2 (Fig. 1) as described above.

The second switch, S2, is used either to collect cells eluted from rotor 1 in rotor 2 (\square = open, \blacksquare = closed) or to harvest the cells collected in rotor 2 by reversing the direction of the flow in rotor 2 (\square = closed, \blacksquare = open) and simultaneously leads the cells to the third switch instead of to the waste bottle.

The third switch selects either for waste (\blacktriangle = closed, \triangle = open) or for sample bottle (\blacktriangle = open; \triangle = closed) and is used to minimize the volume of the fractions.

Morphologic characterization of the fractionated cells

The fractionated cells were characterized according to the following criteria: (1)

May Grünwald Giemsa (MGG) staining of cytocentrifuge preparations; (2) staining for non-specific esterase (Lawrence and Grossmann, 1979); (3) electronic sizing with a Coulter counter ZF with a pulse height analyzer, Channelyzer model C-1000.

Mitogen stimulation

Mitogen stimulation assays were carried out as described previously (Figdor et al., 1981). Briefly, 1×10^5 lymphocytes were seeded per well in 200 μ l culture medium in flat-bottomed microtiter plates (Falcon no. 3040). Maximal proliferative responses with 1 μ g PHA were measured after 4 days incubation. Three hours before termination of the culture 0.4 μ Ci [3 H]TdR was added per well to quantitate DNA synthesis. The cells were harvested on glass fiber filters with a MASH-II harvester (Microbiological Associates, Walkerville, MD). The filters were air-dried and counted in a liquid scintillation counter (Packard Instrument Company, Downers Grove, IL).

Results

Determination of minimal fraction volume

CE has hitherto been carried out with a single elutriator rotor and fractions collected in volumes of 150 ml. The introduction of a second rotor in the CE system permits isolation of cells in much smaller volumes. In order to determine the optimal conditions for harvesting fractions in the smallest possible volumes, 110×10^6 RBC were introduced into the second rotor. These cells were harvested under variable conditions into 16 fractions of 3 ml each. The number of cells present in each fraction was determined by Coulter counter. The results in Fig. 5 show that if the rotor is stopped without braking (Fig. 5A) volumes of 40 ml were required to harvest > 95% of the cells. By switching on the brake, the volume of the fractions was reduced to approximately 30 ml (Fig. 5B). Much smaller volumes were obtained if the direction of the flow was reversed, and it appeared that reversing the flow at high rotor speed gave the best results. At a rotor speed of 4000 rpm 95% of the cells was recovered in a volume of only 9 ml (Fig. 5C), whereas at rotor speeds of 3000 and 2000 rpm respectively, 91% (Fig. 5D) and 87% (Fig. 5E) of the cells were obtained in a volume of 9 ml. The RBC were harvested at a flow rate of 18 ml/min (Fig. 5A–E). Increasing the flow rate to 40 ml/min did not lead to a further reduction in the volume of the fractions (Fig. 5F). At decreased flow rates (10 ml/min) larger volumes were required to collect the cells (not shown). Although these experiments were carried out with RBC, results did not differ significantly in experiments in which leukocytes (granulocytes) were used instead of RBC (not shown).

From these results it was concluded that under optimal conditions (reversing the flow at high rotor speed) 95% of the cells could be harvested in a volume of 9 ml. The remaining 5% of the cells could be eluted only after extensive washing. A small percentage of the cells probably pelleted to the bottom of the separation chamber when the direction of the flow was reversed. To prevent contamination of the subsequent fractions the following schedule was used to harvest the cells from rotor 2: (1) after the flow had been reversed, the first 3 ml of medium were discarded by

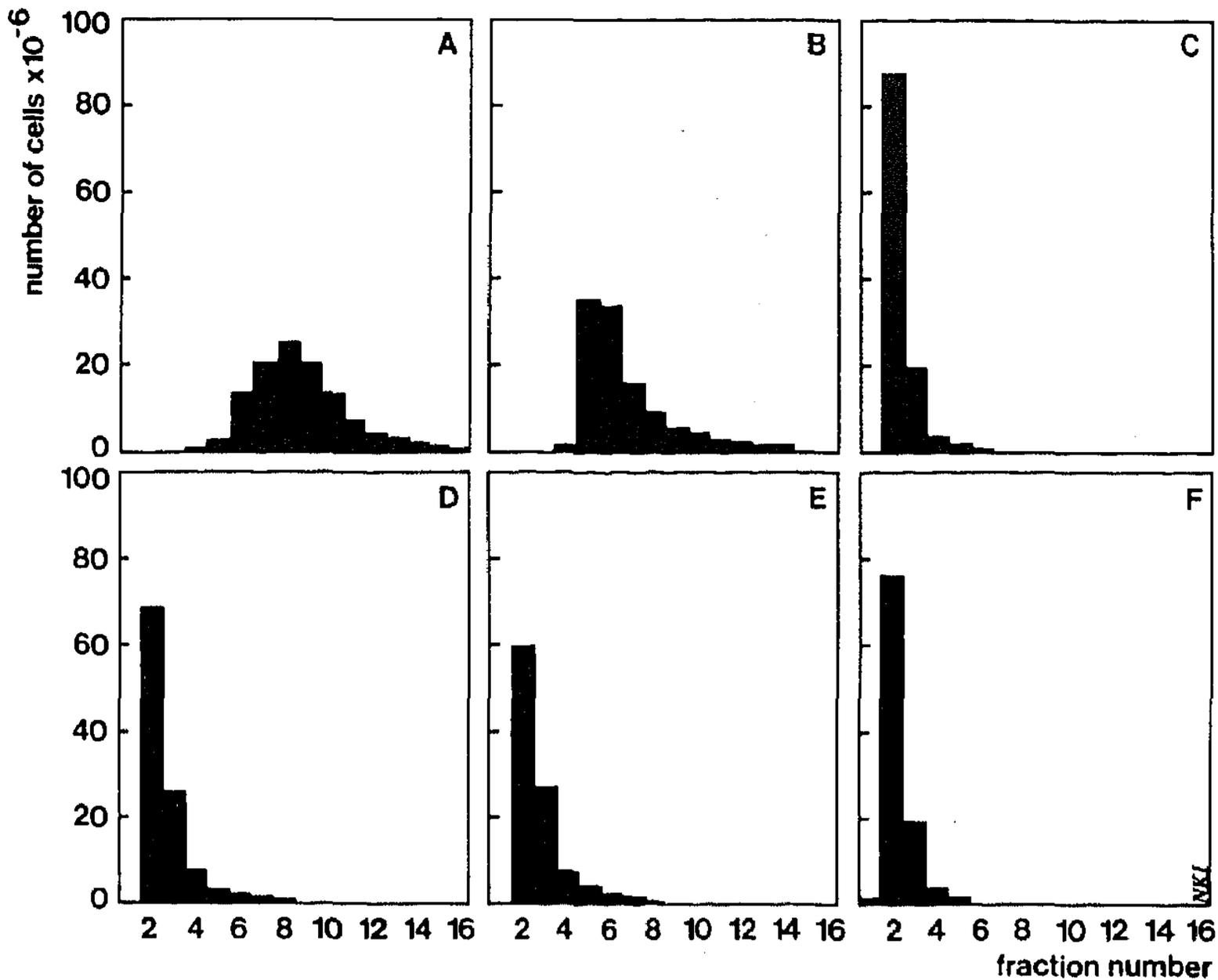


Fig. 5. Distribution profile of RBC harvested from rotor 2 in 16 fractions of 3 ml under varying conditions. A: rotor stopped without brake; B: rotor stopped with brake; C: direction of flow reversed at 4000 rpm; D: direction of flow reversed at 3000 rpm; E: direction of flow reversed at 2000 rpm; F: direction of flow reversed at 4000 rpm. The flow rate was 18 ml/min (A-E) and 40 ml/min (F).

using S3 (S3 = Δ , see Fig. 3); (2) the cells were harvested in a volume of 9 ml (S3 = \blacktriangle); (3) S3 was switched back to the waste (S3 = Δ) and the channels and the separation chamber were cleared by changing the direction of flow several times with S2 to suspend and remove the pelleted cells. The whole procedure took less than 2 min.

Separation of mononuclear cells

To investigate whether the installation of a second elutriator rotor in the CE system affected the resolution and/or the yield, a number of separations was carried out with 1 rotor and with 2 rotors coupled in series. When 2 rotors were used, the fractions were washed, suspended in culture medium and harvested in volumes of 9 ml. In experiments with only 1 rotor the fractions were collected in volumes of 150 ml.

Mononuclear cell suspensions were prepared as described under Material and Methods either by centrifugation over a Ficoll-Hypaque mixture or with the BCS. $45-95 \times 10^6$ nucleated cells were introduced at 3800 rpm at a flow rate of 12 ml/min. After introduction the flow rate was increased to 18 ml/min and kept constant. Cells were elutriated by a stepwise decrease (8 min intervals) in rotor speed as indicated in Table II.

TABLE II

CHARACTERIZATION OF THE ISOLATED FRACTIONS

Comparison of mononuclear cells (MNC) carried out in 1 rotor or in 2 rotors coupled in series. MNC were prepared by density centrifugation or by the BCS. For each type of separation the mean \pm SD of 4 experiments is shown.

Fraction	Rotor speed rpm	Ficoll-Hypaque isolated MNC								BCS isolated MNC			
		1 rotor				2 rotors				2 rotors			
		Cell number $\times 10^6$	% Ly	% Mo	% Gr	Cell number $\times 10^6$	% Ly	% Mo	% Gr	Cell number $\times 10^6$	% Ly	% Mo	% Gr
1	3800 3500 3300	6.7 ± 0.2	> 99	< 1	—	8.0 ± 4.0	> 99	< 1	—	12.8 ± 4.1	> 99	< 1	—
2	3200 3100 3000	40.8 ± 8.1	99 ± 1	1 ± 1	—	42.1 ± 8.8	99 ± 1	1 ± 1	—	38.5 ± 10.1	99 ± 1	1 ± 1	< 1
3	2950	7.0 ± 4.9	84 ± 8	16 ± 6	< 1	3.0 ± 1.5	89 ± 7	10 ± 5	1 ± 1	6.4 ± 2.2	84 ± 8	11 ± 5	5 ± 2
4	2900	2.3 ± 0.7	49 ± 19	59 ± 22	2 ± 2	2.0 ± 0.4	61 ± 17	37 ± 20	2 ± 2	3.3 ± 2.0	54 ± 18	32 ± 13	16 ± 6
5	2850	1.5 ± 0.3	15 ± 14	83 ± 15	2 ± 1	2.2 ± 1.4	16 ± 11	82 ± 14	2 ± 2	2.0 ± 0.7	14 ± 9	79 ± 12	7 ± 5
6	2800	2.1 ± 0.7	6 ± 3	93 ± 3	1 ± 1	3.5 ± 2.1	10 ± 5	88 ± 6	2 ± 1	2.4 ± 0.9	6 ± 3	91 ± 3	3 ± 2
7	2750	2.0 ± 1.3	4 ± 2	95 ± 4	1 ± 1	1.5 ± 0.8	4 ± 2	93 ± 3	3 ± 2	3.1 ± 1.4	5 ± 3	92 ± 4	3 ± 2
8	2700	1.8 ± 0.9	3 ± 3	90 ± 4	4 ± 4	2.1 ± 0.5	3 ± 2	99 ± 4	4 ± 3	2.8 ± 0.6	4 ± 3	90 ± 3	6 ± 4
9	2650	0.9 ± 0.5	3 ± 2	90 ± 6	7 ± 5	1.4 ± 0.9	2 ± 2	92 ± 5	6 ± 4	1.5 ± 0.5	4 ± 2	82 ± 5	14 ± 6
10	0	3.5 ± 2.8	3 ± 5	8 ± 4	89 ± 8	4.7 ± 1.8	4 ± 2	10 ± 6	86 ± 9	4.8 ± 2.9	3 ± 2	6 ± 4	91 ± 7
Recovery		94 ± 4				92 ± 3				91 ± 4			

Lymphocytes with a purity of $> 99\%$ were elutriated in the range 3800–3000 rpm and were collected in 2 fractions (6 fractions of 150 ml if 1 rotor was used). Two intermediate fractions containing both lymphocytes and monocytes were obtained at 2950 and 2900 rpm respectively. Five monocyte fractions were isolated by decreasing the rotor speed with 5 subsequent steps of 50 rpm. The results in Table II show that resolution was not affected by the incorporation of a second rotor into the CE system. The lymphocyte and monocyte fractions isolated with 1 or with 2 rotors were of equal purity. The purity of the lymphocytes and monocytes derived from mononuclear cell suspensions isolated with the BCS did not differ significantly from those derived from Ficoll-Hypaque isolated mononuclear cells. Granulocyte contamination of mononuclear cells prepared with the BCS was only slightly higher than of mononuclear cell suspensions isolated by Ficoll-Hypaque. Apparently the granulocytes contained a subset of cells with a relative low density since part of the granulocytes were preferentially elutriated at about 2900 rpm. Microscopy of these fractions showed that all the granulocytes present (up to 24% in some experiments) were basophil granulocytes.

The cell suspensions prepared with the BCS still contained a considerable number of RBC. Since these cells elutriate in the range 3700–3200 rpm, they contaminate the first lymphocyte fractions. RBC were removed by lysing them with NH_4Cl prior to collection. After lysis the cells were washed and harvested. Three to 4 min were usually sufficient to lyse all RBC. Lysing RBC with NH_4Cl did not affect lymphocyte viability as judged by trypan blue exclusion.

To investigate whether the functional properties of the lymphocytes were altered, proliferation of cells on stimulation with PHA was measured. Ten percent pooled monocytes (fractions 5–9) was added to optimize the proliferative response. The results in Table III show that lymphocytes exposed to NH_4Cl and lymphocytes isolated from mononuclear cell suspensions prepared by density centrifugation (not exposed to NH_4Cl) responded equally well to PHA.

TABLE III

PHA RESPONSE OF LYMPHOCYTE FRACTIONS ISOLATED BY CE WITH AND WITHOUT NH_4Cl

Mean \pm SD of the [^3H]thymidine incorporation by isolated lymphocytes. Lymphocytes isolated by the BCS were treated with NH_4Cl . 10% monocytes were added for optimal proliferation.

	[^3H]thymidine incorporation (mean \pm SD of 3 different donors)	
	Fraction 1	Fraction 2
Lymphocytes isolated by density centrifugation	68 200 \pm 8 400	57 300 \pm 12 700
Lymphocytes isolated by the BCS	73 600 \pm 6 100	55 900 \pm 6 300

Discussion

Centrifugal elutriation has shown to be a very efficient procedure for the separation of large numbers of lymphocytes and monocytes. Recently we demonstrated (Figdor et al., 1983) that under optimal conditions also small numbers of cells could be separated by CE. However, a major disadvantage, especially when few cells are available is the relatively large volume of the fractions required to obtain good resolution. It was demonstrated by Keng et al. (1981) that the resolution obtained with CE is directly related to the volume of the fractions, i.e., the period during which cells are eluted at a preset rotor speed. In the present experiments the separations were carried out by decreasing the rotor speed at a constant flow rate of 18 ml/min. At this flow rate a volume of 150 ml was sufficient to obtain good resolution; if only small volumes (50 ml) of blood were separated most fractions contained less than 5×10^6 cells (Table II) and additional centrifugation steps were required to concentrate the cells for further analysis. This procedure led to considerable cell losses, especially with regard to monocytes. Furthermore we observed that after pelleting the cells the size of the monocytes increased, perhaps indicating that the cells were damaged (Figdor et al., 1983). The new versatile CE system described allows subpopulations of monocytes and lymphocytes to be separated from small samples of blood (50 ml) in small volumes without the need for an additional centrifugation step to concentrate the cells.

The CE system used consists of 2 rotors coupled in series. This modification nearly doubled the total resistance of the system, so that increased pressure was required to obtain a constant flow rate of 18 ml/min. We previously used hydrostatic pressure to generate the flow (Figdor et al., 1981), but air pressure is more suitable if, as here, higher pressure (0.9 bar) is required and different media are used during a single run. Fluctuations in air pressure were eliminated by using a large (120 l) vessel pressurized before the start of each experiment. During CE the pressure decreased by less than 0.009 bar/h. However, the flow distribution unit caused variations in the resistance of the system which affected flow rate. When both rotors were used in the elutriation position, systemic resistance was rather high. If the flow of the second rotor was reversed, systemic resistance decreased. A much stronger decrease of systemic resistances was seen if both rotors were disconnected to wash the cells before collection. To avoid these variations in resistance, which cause unacceptable fluctuations of flow rate, a flow controller was incorporated. This so-called needle flow control valve maintained constant mass flow of fluid at constant upstream pressure and variable downstream pressure. It appeared that a pressure of 0.9 bar was sufficient to maintain a constant flow rate of 18 ml/min, at both low and high systemic resistance.

A new introduction unit was developed to facilitate sample introduction. This unit has several advantages compared with direct injection of the sample with a syringe or with the original Beckman injection system (Manual). In the first place, the cell sample was cooled during the whole introduction period, which prevented adherence and activation of monocytes. Secondly, the rate of introduction could be regulated by diluting the sample with CE medium. The 2 injection ports of the

introduction unit permit the introduction of both small sample volumes that generally contained few cells (up to 15 ml), and large sample volumes (up to 100 ml; Fig. 2). The glass spiral in which the small samples are injected has an inner diameter of only 3 mm. The resulting high flow density in the spiral prevented loss of cells by sedimentation during introduction.

The FDU (Fig. 3) which was developed to carry out various manipulations with the cells in the second rotor consists of 3 individual switches (Fig. 4) that each controlled a different function. The first switch was used to suspend the cells in culture medium instead of CE medium just before harvesting, and/or to lyse contaminating red blood cells by means of NH_4Cl . The second switch was used to reverse the flow of the second rotor in order to harvest the cells in small volumes.

Harvesting cells by reversing the flow was first described by Persidsky and Olson (1978) who isolated granulocytes from whole blood by CE. The granulocytes finally remaining in the separation chamber were harvested by reversing the direction of the flow by a flow reversing valve.

In our system we use compound clamps to close one or more fluid lines and simultaneously open other fluid lines. The clamps are constructed in such a way that the tubes may easily be removed from the bars of the clamps (Fig. 4). This allows the whole flow system to be autoclaved as a single unit of tubing without the need to disassemble it.

The third switch was used to further reduce the volume in which the cells were harvested from the second rotor. It was demonstrated that under optimal conditions, at a rotor speed of 4000 rpm, 95% of the cells could be harvested in volumes as small as 9 ml. Higher rotor speeds did not result in a further decrease of the fraction volume.

In some experiments, the Sanderson separation chamber (Sanderson et al., 1977), instead of the standard Beckman chamber, was placed in the second rotor to harvest the cells. However, reversing the flow often resulted in clogging of the channel at the bottom of the chamber (results not shown), which made this type of separation chamber less suitable for this purpose.

The use of 2 elutriator rotors in series did not affect the resolution of separation of mononuclear cells, nor the viability of the cells, as compared with separations carried out with 1 rotor. The yields obtained in the present set up were slightly lower, since 5% of the cells were discarded during collection of the fractions. However, concentration of the cells, if only 1 rotor was used, resulted in a much larger cell loss. In a number of experiments separations were carried out with cell suspensions prepared with the BCS. This apparatus made it possible to isolate highly enriched mononuclear cell suspensions from whole blood without exposure of the mononuclear cells to foreign substances such as Percoll or Ficoll-Hypaque. Furthermore, this cell separator has the advantage that the cells may be introduced directly into the rotor without washing the cells. The composition of mononuclear cell suspensions prepared with the BCS is comparable with that obtained by density centrifugation. In addition, equally pure lymphocyte and monocyte fractions were obtained. To our surprise the intermediate fractions (3 + 4) contained a relatively high percentage of basophil granulocytes which apparently have a different sedimen-

tation velocity from that of neutrophil and eosinophil granulocytes.

Mononuclear cell suspensions derived from 500 ml of blood and prepared with the BCS contained only few RBC; the WBC/RBC ratio is 2.4 (Figdor et al., 1982b). However, mononuclear cell suspensions of 50 ml of blood contained considerably more red blood cells; WBC/RBC ratio 0.1. Some of these RBC have an elutriation profile similar to that of the lymphocytes and contaminated the first lymphocyte fractions. In several experiments the RBC were removed from the lymphocyte fractions by lysis with NH_4Cl . To prevent non-specific loss of lymphocytes from the separation chamber during lysis, the following precautions were taken. First, to prevent disturbance of the sedimentation process, the density of the medium used to lyse the RBC was adapted to that of the CE medium. Secondly, lysis of RBC was carried out at a high rotor speed (4700 rpm) since the release of intracellular material into the medium increased the density of the medium which might have caused elutriation of some of the lymphocytes.

Lysis of RBC contaminating the lymphocyte fractions did not affect the viability of the cells nor the capacity of the lymphocytes to proliferate after stimulation with PHA.

The monocyte subsets isolated have been shown to display a similar ADCC pattern to monocyte subsets isolated from 500 ml of blood with 1 rotor (Figdor et al., 1982a). These results (not shown) indicate that the present CE system does not affect either viability or function.

Until now the large fraction volumes of CE has prevented this technique from being used routinely for separation of small numbers of cells into lymphocytes and monocyte subpopulations. The CE equipment described offers the unique possibility of separating and washing cells and removing contaminating RBC in a single apparatus. Small numbers of cells ($40-95 \times 10^6$) were efficiently separated in fractions of only 9 ml volume. The procedure is time saving and reduces the risk of contamination due to complex handling. The time during which lymphocytes are exposed to NH_4Cl to lyse RBC is short. Moreover, monocytes were not exposed to NH_4Cl in this system. Studies are in progress to investigate whether this lysis technique may be used to separate T and B cells. Rosetted T or B lymphocytes may be separated from non-rosetted cells and after separation RBC may be removed with NH_4Cl .

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