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CRYOGENIC PRESERVATION OF MONOCYTES FROM HUMAN BLOOD AND PLATELET--ETC(U)

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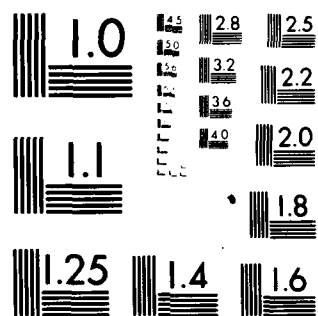
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CONTRACT N00014-80C-0239

Task No. 105-707

TECHNICAL REPORT No. 1

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N00014-80-C-0239, N00014-79-C-0168

CRYOGENIC PRESERVATION OF MONOCYTES FROM HUMAN
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Prepared for publication in BLOOD, The
Journal of the American Society of Hematology.

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11/2X December 20, 1980

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CRYOGENIC PRESERVATION OF MONOCYTES FROM HUMAN BLOOD
AND PLATELETPHERESIS CELLULAR RESIDUES.

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This work was supported by Office of Naval Research Contracts No. N00014-80-C-0239
(F.J.L.) and N00014-79-C-0168 (C.R.V.).

The opinions or assertions contained herein are those of the authors and are
not to be construed as official or reflecting the views of the Navy Department
or Naval Service at large.

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in Medical Laboratory Sciences at Northeastern University's Graduate School
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Short Title: MONOCYTE CRYOPRESERVATION

ABSTRACT

→ A separation procedure was devised for the isolation of human monocytes from buffy coat of whole blood and the cellular residues in platelet bags after plateletpheresis of donors with the Haemonetics Model 30 Cell Processor. The mononuclear cells were isolated using Ficoll-Isopaque (Lymphoprep) and monocytes were then separated from lymphocytes by centrifugal cytography using two separation chambers in a Beckman JE-6 rotor.

^{About 30,000,000}

~~Approximately 30×10^6~~ monocytes were obtained from 100 ml of whole blood.

→ These were 93% myeloperoxidase positive (MPO), while 98% showed intact membranes in the fluorescein diacetate (FDA) ethidium bromide (EB)

test. Eighty-seven percent ingested 1-5 or more opsonized latex particles and 93% ingested 1 or more ethidium-treated zymosan particles. In three procedures ^{1,000,000,000} ~~1×10^9~~ monocytes were obtained from cellular residues in platelet bags.

Monocytes were frozen using 5% dimethylsulfoxide (DMSO), 6% hydroxyethyl starch (HES), 4% albumin, and 56 mM glucose in Normosol-R, pH 7.1. Two ml aliquots containing 6.7×10^6 - 7.2×10^6 monocytes were cooled at 4°C per minute to -80°C, transferred and stored for 1 to 3 months in liquid nitrogen. A total of 25×10^6 - 30×10^6 monocytes were frozen in each of five separate experiments. Monocytes from platelet bags were frozen identically using 2.0 ml aliquots containing ~~60×10^6 - 70×10^6 cells~~. After storage for periods up to 3 months, they were thawed. In some studies the monocytes were diluted to reduce the DMSO to 1.25 percent and in others the monocytes were washed with a 4-fold volume of the solution used to freeze them, but without

→ next page

cont
DMSO. Ninety-eight percent of monocytes frozen were recovered morphologically intact, 90 percent were MPO positive, and 87 percent were FDA positive. The diluted monocytes were incubated at 37°C for one-half hour with latex or yeast and washed twice to remove excess particles. Ninety-four percent ingested latex and 89 ⁹⁰ percent ingested yeast. Slightly higher fluorescence tests and similar ingestion indices were obtained with the larger numbers of monocytes derived from plateletpheresis cellular residues. The results demonstrate the utility of centrifugal cytography as a means to isolate monocytes, which can be cryogenically preserved for months with small loss of function.

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INTRODUCTION

The study of human monocytes is encumbered because of their relatively small numbers in peripheral blood, and difficulty with their isolation due primarily to their adhesiveness to wettable surfaces.¹ The fundamental role of monocytes in the immune response to soluble and particulate antigens could be investigated more efficiently and better understood with improved methods for their isolation and long term preservation. Principles employed for separation include gradient centrifugation on Ficoll-Isopaque^{2,3}, albumin⁴, sucrose⁵, adherence to glass⁶ or plastic⁷, selective adsorption to nylon⁸ and counterflow centrifugation⁹. Preservation has been limited to brief periods of storage at 4°C while cryogenic methods for mononuclear cells stressed the preservation of lymphocytes for antigen typing or antibody testing and did not resolve the lymphocytes from the monocytes prior to freezing¹⁰.

We have employed counterflow centrifugation (elutriation) to isolate granulocytes in high yield and purity from guinea pig¹¹, baboon¹², and human¹³ whole blood and have preserved them using a combination of HES and DMSO. Likewise, we found that counterflow centrifugation of the buoyant fraction obtained with Ficoll-Isopaque after centrifugation of whole blood or the residual cells in platelet bags after plateletpheresis adapted easily to the separation of monocytes from the smaller lymphocytes. Pheresis procedures for the collection of platelets provide a source of large numbers (1×10^9 - 1×10^{10}) of mononuclear cells which occur as contaminating cells in the platelet suspensions and are removed from the bags as by-products. Monocytes subjected to the cryogenic procedure for granulocytes¹², we found, could be stored for months in liquid nitrogen with small losses of cells or diminution of function. This paper describes the isolation of monocytes by centrifugal cytography and their cryogenic preservation.

MATERIALS AND METHODS

Blood Collection.

One hundred ml of whole blood was collected from healthy donors into two 50 ml plastic bottles each containing 7.2 ml of citrate-phosphate-dextrose (CPD) and aseptically transferred into four 50 ml polypropylene screw cap Corning Centrifuge tubes. They were centrifuged in an International centrifuge at 350 x g for 28 minutes at room temperature ($22 \pm 2^{\circ}\text{C}$). The plasma was removed aseptically, put into a separate 50 ml tube, and 4 to 6 ml of buffy coat and upper packed cell layer aspirated into separate tubes using Siliclad treated Pasteur pipettes. The plasma was added back, the tubes mixed in a Vortex mixer for 10 seconds, and the mixture centrifuged a second time for 20 minutes at 350 x g. Four ml of the buffy coat were removed and added to the cells from the first centrifugation. Residual blood cells in platelet bags after plateletpheresis of normal health donors with a Haemonetics Model 30 Cell Separator were obtained. The residual 10-15 ml of pelleted blood cells was the starting material for monocyte isolation.

Isolation of Monocytes from Whole Blood.

Buffy coats were diluted three-fold with one part autologous plasma and one part isotonic saline containing 0.01 M phosphate, pH 7.1. Thirty-five ml of the diluted buffy coat was layered on 15 ml of Lymphopren (Ficoll-Isopaque, Nyegaard & Co., A/S, Oslo, Norway, specific gravity of 1.077 g/ml), the buoyant mononuclear layer isolated, the mononuclear cell fraction pelleted, and resuspended in 20-25 ml buffered NaCl (0.15M), pH 7.1, with glucose (0.028M) and human albumin (1.2 g per dl).

Separation of monocytes from lymphocytes was effected by counterflow centrifugation in a Beckman JE-6 rotor mounted in a Beckman J-21B centrifuge as has

previously been described for the isolation of granulocytes¹³. However, for monocytes, two separation chambers were used and a slightly hypertonic medium, resulting in less cell loss and finer control of separation. Flow rates of buffer and cells passing through the rotor were controlled by a Cole-Palmer #7545 Masterflex pump with a ten turn external potentiometer which produced flow rates from 7 to 20 ml per minute in 0.2 ml increments. The 20 to 25 ml of mononuclear cell suspension were mixed in an external chamber and pumped through the rotor at a flow rate of 8.2 ml per minute. Lymphocytes exited the rotor when the flow rate was increased to 8.8 ml per minute after 20 minutes, and to 9.4 ml per minute after another 20 minutes for approximately 60 minutes. Monocytes remained in the separation chambers and pelleted when the buffer flow and centrifuge was stopped. Monocytes were aspirated into a 10 ml plastic syringe.

Monocytes from Pheresis Residues.

Residues (10-15 ml) were diluted three fold with two volumes of AB plasma and PBS buffer (1:1). The buoyant mononuclear fraction was obtained with Lymphoprep and subjected to counterflow centrifugation as with whole blood. From two chambers in the rotor, 7.0-8.0 ml of monocyte suspension was obtained, diluted 1:2 with counterflow buffer, and divided into two ml aliquots containing 60×10^6 - 70×10^6 monocytes.

Size Distributions.

Size distributions were obtained with a Coulter Counter ZH particle counter with Channelyzer and X-Y plotter^{12,13}. Monocytes were counted by integration of the cell numbers within each population. Platelet, red cell and lymphocyte contaminations were determined in the same way on the monocyte samples in the chambers and cells in the effluent during the isolation. Cell suspensions

counted were a) 9.9 ml of buffer plus 0.1 ml of monocytes, b) 9.8 ml of preservative solution plus 0.2 ml of prefrozen monocytes or thawed monocyte suspension, and c) 9.2 ml of a 1:3 dilution of the freezing solution plus 0.8 ml of thawed diluted cells. Cells counts were also made with a 1:500 dilution of monocyte suspensions before and after lysis of contaminating red cells with Zap-isoton in a Coulter Counter, Model F. Red cells were counted as the difference between non-Zap-isoton treated and Zap-isoton treated aliquots.

Morphology.

Cellular identifications were made from Wright's and fresh myeloperoxidase stained smears¹⁴.

The recovery of monocytes in the different preparations was derived from the total white cell count, the percentage of myeloperoxidase positive cells and the percentage of granulocytes in the differential white cell count.

Microfluorescence observations were made with an Olympus Vanox transmission fluorescence microscope. Thin wet mounted preparations were incubated in a dye solution containing a combination of fluorescein diacetate (Sigma) (FDA) and ethidium bromide (Sigma) (EB) as described¹². Membrane integrity was estimated as green fluorescence in cells where FDA entered the cytoplasm and produced visible fluorescein in intact cells¹⁵. The integrity of nuclear membranes was assessed as the percentage of monocytes which produced intense red nuclear fluorescence when EB, normally excluded, entered nuclei and interacted with DNA.

Particle Ingestion by Monocytes.

Phagocytic properties of monocytes were measured as their ability to ingest opsonized latex and zymosan particles¹⁶. Fluorescent latex (Fluolite DS-5005) was obtained from ICI, Finland and used as described¹². At a particle to cell ratio

of 150:1, a dependence of ingestion on serum opsonization has been shown¹⁷. Wet mounts were prepared and 200 cells counted. The percentage of cells which ingested particles was graded as: zero, no particles; low, 1 to 5; medium, 50 to 15 and high, more than 15 particles per cell.

Zymosan (Sigma, S. cerevisiae) ingestion was carried out in a similar manner¹⁶. Ingestion and fluorescence of 200 cells were scored. Zymosan ingestion was rated as: zero, none; medium, those with 1 to 5 particles, and high; more than five particles. Controls with PBS substituted for serum gave little or no reaction with either zymosan or latex¹⁷.

Cryopreservation of Monocytes.

Monocyte suspensions isolated from whole blood or platelet bags were preserved by freezing using the procedure for granulocytes previously described¹². Briefly, a volume of cryoprotectant solution at 4°C equal to the volume of suspension obtained from each chamber (3.5-4.0 ml) was added dropwise with stirring. The final composition of the mixture was 5% DMSO, 6% HES, (McGaw, M.W. 150,000) 4% human serum albumin, and 56 mM glucose in Normosol-R (Abbott), pH 7.1. The mixture was divided into 2 ml aliquots in 17 x 100 mm polypropylene tubes, held at 2°C for 5-10 minutes, frozen at 4°C per minute to -80°C for 40 minutes, and stored in liquid nitrogen at -197°C for an average of 37 days. Unfrozen monocytes were reserved as controls for cell counts, volume distributions, myeloperoxide activity, microfluorescence and particle ingestion studies.

Frozen monocytes were thawed at 49°C per minute in a 42°C water bath. The tubes were shaken manually until all ice melted except for a pellet of approximately 3 mm diameter. The tubes were removed and swirled until the pellet melted at which time the thawed samples were cool to the touch (8 to 10°C). They

were then maintained at 2°C in ice for washing and testing.

Thawed monocytes were diluted 1:4 dropwise (one drop per 4-5 seconds for 3 minutes) with 3 volumes of a solution at room temperature composed of 6% HES, 4% human serum albumin (HSA) and 28 mM glucose in Normosol-R, pH 7.1. The thawed diluted suspension was assayed for counts, size distribution, microfluorescence, myeloperoxidase activity and particle ingestion as described above.

RESULTS

Volume distributions of mononuclear cells from whole blood subjected to incremental flow counterflow centrifugation are shown in Figure 1. The first distribution at time zero (marked Lymphoprep) defines the composition of the sample obtained with Lymphoprep. Platelets (P, median channel 4, plus red cells, R), lymphocytes (L, median channel 34), and monocytes (M, median channel 69, the shaded area in Figure 1), were the major components. At 8.2, 8.8 and 9.4 ml per minute, platelets, red cells, and lymphocytes exited, accompanied by small numbers of monocytes. After 60 minutes at 9.4 ml per minute, the first chamber in the rotor contained 89 percent of available monocytes in the Ficoll-Isopaque buoyant cell layer. None were found in the second chamber under these conditions. However, its use permitted recovery of monocytes which spilled over from chamber 1 when large numbers in plateletpheresis cell residues were processed. The volume spectrum was one primarily of monocytes (shaded area, median channel number 72). The small peak at channel number 7 consisted of cell fragments and red cells, while the shoulder to the left of the monocyte distribution consisted of large lymphocytes.

Monocytes were isolated in five experiments with whole blood. Of $127 \pm 4.0 \times 10^6$ (av. \pm S.E.) monocytes available in 100 ml of whole blood, $69 \pm 12 \times 10^6$ (65%) were recovered from buffy coat, $33 \pm 16 \times 10^6$ (26%) were recovered with Ficoll-Isopaque, and $25 \pm 9.0 \times 10^6$ (20%) recovered in the chamber after incremental flow centrifugation. The greatest losses of monocytes occurred in preparing buffy coats and buoyant monolayer while only a small loss of monocytes occurred in the JE-6 rotor. Wright's stained smears showed isolated monocytes to be 99% mononuclear. The midrange of the volume distribution (71) was consistent with a population of large mononuclear cells. Contamination with PMN's or other cells was less than 1 percent. All of the cells (98%) produced fluorescein in cytoplasm from fluorescein diacetate and few were ethidium positive (2%), indicating intact cytoplasmic and nuclear membranes. Ninety-three percent were myeloperoxidase positive while the median peak channel (71 ± 3) was unchanged showing that cell volumes remained constant during the isolation. Nearly all were capable of ingesting opsonized latex and zymosan (see Table, control).

Monocytes isolated from platelet bags after pheresis of three donors were present in mixed leukocytes which ranged from 2.0×10^9 - 7.5×10^9 . Of $4.3 \pm 1.7 \times 10^9$ leukocytes available in the average concentrate, 94% were mononuclear, 5% were granulocytes and 28% were myeloperoxidase positive, indicating 1.1×10^9 of the total were monocytes. Recoveries of monocytes at these large magnitudes were much higher than from whole blood. Ninety-one percent of monocytes were recovered after centrifugation with Ficoll-Isopaque and nearly quantitative recovery was attained after separation by counterflow centrifugation. In the

JE-6 dual chamber rotor, 0.6×10^9 cells were recovered in Chamber #1 and 0.4×10^9 in chamber #2. The cells isolated were $99 \pm 10\%$ mononuclear (approx. 4% lymphocytes), of which 95% were myeloperoxidase positive indicative of a small contamination (ca. 5%) with lymphocytes. Nearly all ($97 \pm 1\%$) were "viable" in the fluorescein diacetate test. This was also reflected by the median channel numbers which did not vary, showing maintenance of volume during the separation.

Preservation of monocytes with HES and DMSO in hyperosmolar media was undertaken with the same protocol previously employed for human¹⁸, baboon¹², and guinea pig¹¹ granulocytes. Volume plots of monocytes as collected, mixed with cryoprotective media, frozen, thawed, diluted to reduce DMSO and incubated with particles to assess ingestion, revealed monocytes to be remarkably stable (Figure 2). The constancy of the shape of the volume plots during the steps of freezing, thawing, dilution of DMSO and incubation with particles gives evidence that monocyte volume was well maintained during the osmotic stresses incurred in the various steps in the procedure. The volumes were made to be smaller than normal with hyperosmolal buffer (300 mOs per kg H₂O) monitored by median channel numbers. The values of 59 for thawed: 59 for thawed and diluted; and 62 for the incubated cells were close to 61, the value for monocytes as isolated. The number of cells within the monocyte distribution indicated that 97% of monocytes separated were recovered after being frozen, thawed, diluted, incubated, and washed. These remained morphologically intact without swelling or shrinking. In contrast, of monocytes frozen similarly without cryoprotectant (insert), very few cells survived freezing and thawing. This

is evident from the absence of a cell population at channel #60, the presence of a large population of cell fragments at channel #20, and the inability to detect intact monocytes in stained smears.

Recoveries of monocytes preserved by freezing estimated from volume plots, white cell numbers, differentials counts, and myeloperoxidase positive cells are summarized in the Table. Of $25 \pm 9 \times 10^6$ total monocytes in 4 tubes, 100% were recovered after freeze preservation. Therefore, the only significant loss of cells occurred during their isolation from whole blood, i.e., the preparation of buffy coat and mononuclear cell fraction.

The stability of monocyte membranes determined by fluorescein production from FDA and exclusion of EB from nuclei, as well as phagocytic functions also manifested the stability of monocytes after freeze preservation (Table). Comparison of the control cells, those mixed with cryoprotectants (prefrozen), and thawed cells revealed small changes in cell numbers, FDA and EB reactivity. Thawed monocytes incubated with particles and washed incurred no loss in cell numbers, but a decrease in cytoplasmic fluorescence (FDA) to 87% and an increase in nuclear permeability to EB to 13%. Of the incubated twice washed monocytes, 94% ingested opsonized latex ($50 \pm 21\%$ high, $35 \pm 12\%$ medium, $9 \pm 9\%$ low). These magnitudes were similar to those of the unfrozen controls ($37 \pm 18\%$ high, $39 \pm 20\%$ medium, and $9 \pm 7\%$ low). Likewise 89% of opsonized ethidium treated zymosan (yeast) was ingested by frozen thawed monocytes ($31 \pm 21\%$ high, $58 \pm 21\%$ low) compared to 94% for fresh monocytes ($64 \pm 15\%$ high, $30 \pm 15\%$ low). The major difference due to freezing was observed with yeasts where the high capacity for ingestion of yeast dropped from 64% high in fresh

to 31% in thawed cells indicative of a reduction in avidity for particles due to freezing. Phagocytosis could not be measured in DMSO at the 5% concentration. As reported for granulocytes¹⁸, myeloperoxidase was also inhibited.

Results of freezing experiments with monocytes from plateletpheresis cellular residues were similar to those obtained with whole blood. Because of the greater numbers of monocytes available (1×10^9 - 5×10^9) they were divided into aliquots containing 50×10^6 monocytes in order to be frozen with the same method. The contents of both chambers of the rotor were preserved in an identical manner with similar results. The recoveries of monocytes at each stage showed small losses occurring after thawing and diluting (to reduce the DMSO concentration) and after incubation with particles and subsequent washing. In vitro function tests of thawed monocytes from pheresis cell residues were routinely in the nineties percentile range in all tests (not shown), offering evidence that human mononuclear cells are strongly resistant to cryoinjury to which polymorphonuclear cells are susceptible.

DISCUSSION

Studies of monocyte physiology have been limited by the relatively small numbers that occur, that can be isolated practically from blood, and their innate adhesiveness, which has made it difficult to isolate them in suspension. Gradient isolated monocytes are often few in number and have variable purity, while the Ficoll-Hypaque treated adherent cells are purer, but difficult to detach from the adhering surface without damage or altered function. The counterflow centrifugation elutriation method, on the other hand, isolated monocytes in suspension, as described by Sanderson *et al.*⁹. This utilized a single chamber in which 1.5×10^6 - 3.0×10^6 monocytes of 90% purity and 99% functionality were isolated from 10 ml of whole blood. The methods employed in this paper were similar except for minor modifications. A second separation chamber was added for use with larger volumes of buffy coat and higher numbers of monocytes. Minor changes in g-forces and buffer flow rates were used to effect the separation. Major advantages of counterflow centrifugation were that mononuclear cells were separated on the basis of size at low net g force and the monocytes were in suspension, minimizing adhesion forces frequently used to achieve their isolation.

Monocytes isolated from whole blood or plateletpheresis cellular residues using the centrifugal counterflow elutriation principle were found to have a high proportion with intact cytoplasmic and nuclear membranes as tested by fluorescein diacetate and ethidium bromide respectively. There was also high reactivity of myeloperoxidase and ingestion assays revealed high values for ingestion of yeast and latex.

Dean and Strong¹⁰ demonstrated the feasibility of cryopreservation of mononuclear phagocytes with 7.5% dimethylsulfoxide in 10% heat inactivated fetal bovine serum. However, the population frozen was approximately 85% lymphocytes and only 15% (1×10^6 - 2×10^6) monocytes. In our studies with relatively pure monocytes we employed a lesser concentration of DMSO combined with HES, causing less cytotoxicity and effecting better preservation. We showed previously with granulocytes that DMSO between 5-10% caused swelling, dependent on concentration and temperature¹⁹. This was accompanied by losses of membrane integrity, and cytoplasmic fluorescein, an increase in nuclear fluorescence with ethidium, and a cessation of particle ingestion. Additionally, DMSO in excess of 1.25% inhibited granulocytic phagocytosis and myeloperoxidase¹⁸.

In this study with monocytes, minimization of swelling was facilitated by monitoring volume (size) changes of the cells. Sizing was done before freezing to determine rates of admixture with cryoprotective solutions and after thawing to effect washing with minimal volume changes. Use of hyperosmolar media maintained the cells at smaller volumes thereby reducing swelling effects of DMSO, and the ensuing cellular damage and loss of viability after freezing. By combining HES and DMSO, effective cryopreservation was achieved at lower DMSO concentrations (5%) without permitting monocytes to exceed initial volumes as collected, preventing the deleterious effects of swelling. Although high recoveries and function of monocytes after freezing reflect an inherent stability greater than that of granulocytes, use of principles and procedures developed for granulocyte isolation and cryopreservation are in part responsible for the results described here. These emphasize hyperosmolar media, combined

cryoprotectants HES and DMSO, and concentrations of DMSO adequate for cryopreservation without membrane disruption and loss of function.

In our approach it is highly advantageous that plateletpheresis as performed with the Haemonetics Model-30 processor concentrates billions of monocytes in the cellular residues. Using this starting material, it was possible to isolate 1.0×10^9 mononuclear leukocytes that were 95% myeloperoxidase positive consisting of 94-95% monocytes. This is 20 times that which we isolated from 100 ml of whole blood and 200 to 400 times the number of monocytes isolated by Sanderson et al.⁹ from 10 ml of whole blood.

The availability of a large number of homogeneous monocytes renders their preservation desirable, primarily for future use or study. Fortunately, this can be accomplished with existing technology for preserving granulocytes^{4,5,6}. Monocytes from one donor can be isolated, divided into aliquots, preserved and used for analytical purposes or cell studies. The opportunity to perform monocyte transfusions when indicated may now be contemplated. Studies are now in progress which seek to evaluate in vivo in baboons the therapeutic effectiveness of cryopreserved monocytes.

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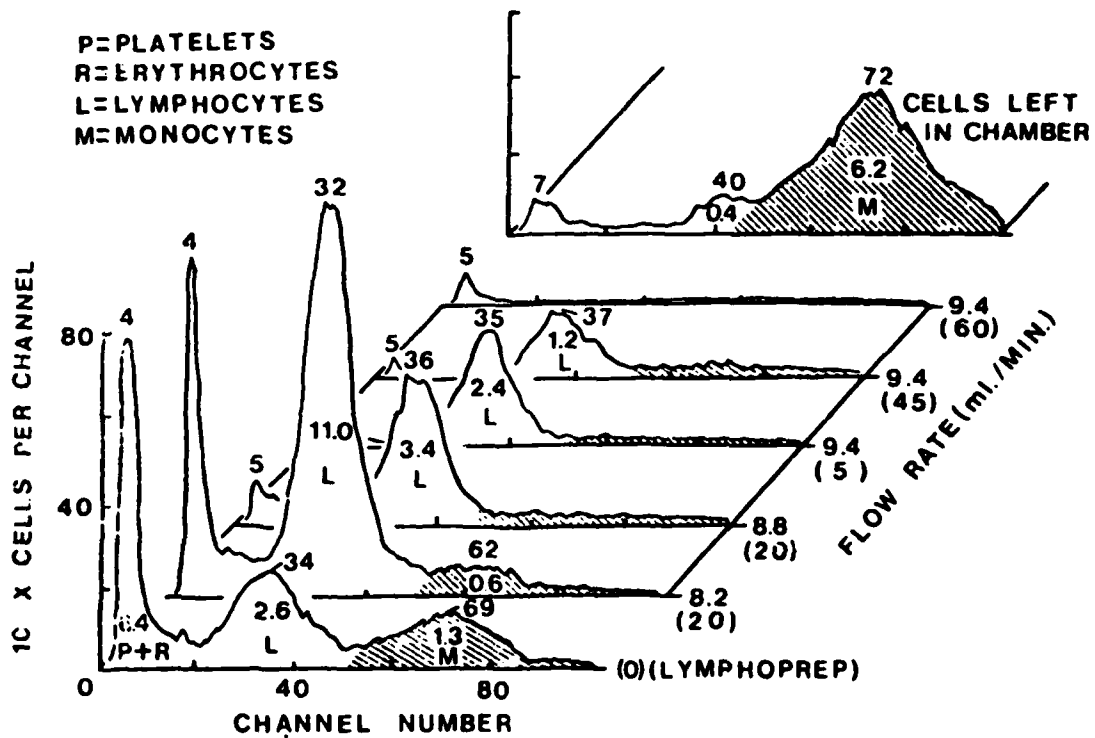


Fig. 1.

Figure 1. The isolation of monocytes from the buoyant mononuclear cell layer of whole blood isolated with Ficoll-Isopaque. Volume plots of the number and types of cells at various flow rates of buffer through the rotor are shown. The numbers in parenthesis below each flow rate are the times in the separation procedure (in minutes) at which the samples were taken. Numbers above the peaks are median channel numbers (midranges). The numbers under the peaks are the cell numbers (in thousands) within the distribution. P + R are platelets plus red cells, and the areas delineated by L are lymphocytes. The final plot shows the distribution of cells left in the chamber. The shaded area is monocytes.

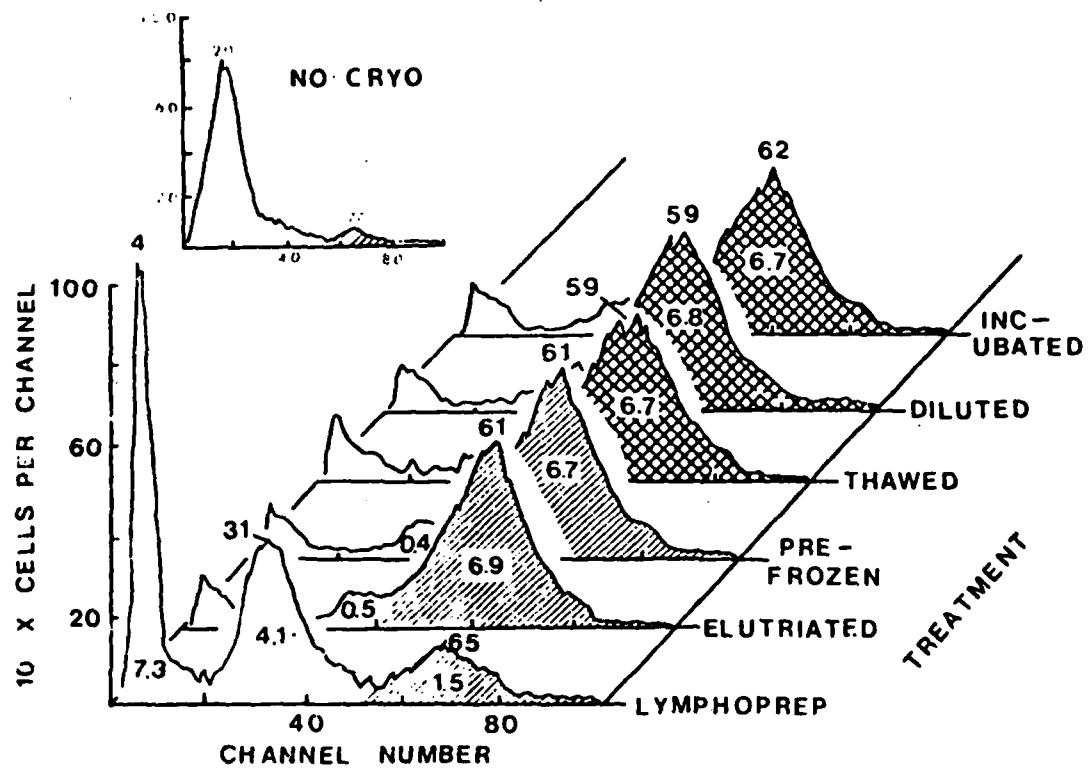


Fig. 2

Figure 2. Cryopreservation of monocytes. Volume distributions of monocytes at various stages of cryopreservation (on the right) are shown. Lymphoprep is the Ficoll-Isopaque buoyant population. Elutriated is the distribution of monocytes isolated by Counterflow centrifugation. Prefrozen are cells mixed with cryoprotectant solution. Thawed-cells, after thawing; Diluted-cells, diluted 1:4 with wash solution; and Incubated, washed-cells incubated at 37°C for 30 minutes. The diagonal hatched area is that of the monocytes before freezing, and the cross-hatched areas are monocytes after freezing. The numbers above the peaks are median channel numbers (midranges) of the population, the numbers under the peaks are the number of cells in the populations (in thousands). The insert in the upper left represents volumes of a monocyte suspension frozen without cryoprotectants showing extreme fragmentation and loss of monocytes.

TABLE - RECOVERY AND FUNCTION OF CRYOPRESERVED MONOCYTES FROM WHOLE BLOOD

Sample	Total Monocytes x 10 ⁶ (n = 5)	Recovery %	Myeloperoxidase Positive (%)	Microfluorescence %	Ingestion (% of 200 cells)					
					LATEX			YEAST		
					FDA	EB	High	Med	Low	Zero
Pre-frozen	25+11 (16-37)	98+5 (88-100) 96+5 (87-100)	89+11 (78-98)	96+2.0 (93-99) 4+2 (1-7)						
Thawed	25+10 (17-39)	98+4 (98-100) 98+3 (90-100)	89+10 (77-97)	96+2.0 (90-96) 4+2 (4-10)						
Thawed and Diluted	25+10 (15-40)	99+1 (96-100) 97+4 (95-100)	94+5 (77-97)	95+2.0 (92-98) 5+2 (2-8)						
Thawed Diluted Incubated Washed*	24+10	98+4 (93-100)	90+11 (77-96)	87+5.0 (80-94) 13+5 (6-20)	50+21 (13-62)	35+12 (27-56)	9+9 (1-25)	6+2 (4-10)	31+21 (16-68)	58+21 (21-74) 11+3 (9-16)
Control	25+9.0 (16-29)	—	93+5 (90-97)	98+1.0 (97-99) 2+1 (1-3)	37+18 (9-58)	39+20 (19-67)	9+7 (2-20)	13+13 (4-24)	64+15 (52-80)	30+15 (10-46) 7+3 (2-10)

The mean + standard error and the range (parenthesis) is given.

Recovery was calculated from counts with the Counter, Counter Model F (upper number) also from counts of cells within the monocyte population obtained with the Coulter ZH Channelizer (lower number). Recoveries are based on the pre-frozen value as 100%. The recovery pre-frozen is the percentage of monocytes isolated.

*Particles were incubated with the thawed, diluted suspensions at 37°C for 30 minutes and the cells washed twice to remove excess particles. Other tests were made on unwashed suspensions.

Latex phagocytosis; High = more than 15, Med = 5-15, Low = 1-5 and Zero = no particles/cell.

Yeast phagocytosis; High = more than 5, Low = 1-5, Zero = no particles/cell.

Phagocytosis was low or absent from suspensions containing DMSO in excess of 1.3%.

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REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER 1	2. GOVT ACCESSION NO. AD-A093447	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) CRYOGENIC PRESERVATION OF MONOCYTES FROM HUMAN BLOOD AND PLATELETPHERESIS CELLULAR RESIDUES		5. TYPE OF REPORT & PERIOD COVERED TECHNICAL-ANNUAL 12/1/79-11/30/80
		6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) Fabian J. Lionetti C.R. Valeri S.M. Hunt A.B. Callahan		8. CONTRACT OR GRANT NUMBER(s) N00014-80C-0239
9. PERFORMING ORGANIZATION NAME AND ADDRESS Center for Blood Research 800 Huntington Ave., Boston, Massachusetts 02115		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS NR 105-707
11. CONTROLLING OFFICE NAME AND ADDRESS Office of Naval Research		12. REPORT DATE 20 December 1980
		13. NUMBER OF PAGES 28
14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office) DCASR- Boston Army Base 666 Summer St., Boston, Massachusetts 02210		15. SECURITY CLASS. (of this report) Unclassified
		15a. DECLASSIFICATION/DOWNGRADING SCHEDULE
16. DISTRIBUTION STATEMENT (of this Report) Distribution of this report is unlimited within the government.		
17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report) Same as 16.		
18. SUPPLEMENTARY NOTES None		
19. KEY WORDS (Continue on reverse side if necessary and identify by block number) monocytes, counterflow centrifugation, plateletpheresis residues, cryopreservation phagocytosis, membrane		
20. ABSTRACT (Continue on reverse side if necessary and identify by block number) A separation procedure was devised for the isolation of human mono- cytes from buffy coat of whole blood and the cellular residues in platelet bags after plateletpheresis of donors with the Haemonetics Model 30 Cell Processor. The mononuclear cells were isolated using Ficoll-Isopaque (Lymphoprep) and monocytes were then separated from lymphocytes by cen- trifugal cytography using two separation chambers in a Beckman JE-6 rotor.		

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1 JAN 73

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Approximately 30×10^6 monocytes were obtained from 100 ml of whole blood. These were 93% myeloperoxidase positive (MPO), while 98 percent showed intact membranes in the fluorescein diacetate (FDA) ethidium bromide (EB) test. Eighty-seven percent ingested 1-5 or more opsonized latex particles and 93 percent ingested 1 or more ethidium-treated zymosan particles. In three procedures 1×10^9 monocytes were obtained from cellular residues in platelet bags.

Monocytes were frozen using 5 percent dimethylsulfoxide (DMSO), 6 percent hydroxyethyl starch (HES), 4 percent albumin, and 56 mM glucose in Normosol-R, pH 7.1. Two ml aliquots containing 6.7×10^6 - 7.2×10^6 monocytes were cooled at 4°C per minute to -80°C , transferred and stored for 1 to 3 months in liquid nitrogen. A total of 25×10^6 - 30×10^6 monocytes were frozen in each of five separate experiments. Monocytes from platelet bags were frozen identically using 2.0 ml aliquots containing 60×10^6 - 70×10^6 cells. After storage for periods up to 3 months, they were thawed. In some studies the monocytes were diluted to reduce the DMSO to 1.25 percent and in others the monocytes were washed with a 4-fold volume of the solution used to freeze them, but without DMSO. Ninety-eight percent of monocytes frozen were recovered morphologically intact, 90 percent were MPO positive, and 87 percent were FDA positive. The diluted monocytes were incubated at 37°C for one-half hour with latex or yeast and washed twice to remove excess particles. Ninety-four percent ingested latex and 89 percent ingested yeast. Slightly higher fluorescence tests and similar ingestion indices were obtained with the larger numbers of monocytes derived from plateletpheresis cellular residues. The results demonstrate the utility of centrifugal cytography as a means to isolate monocytes, which can be cryogenically preserved for months with small loss of function.

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