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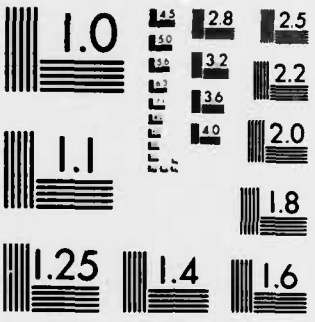

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FINAL REPORT

STABILITY AND FUNCTION OF GRANULOCYTES ISOLATED BY COUNTERFLOW CENTRIFUGATION AND DISCONTINUOUS DENSITY GRADIENTS OF FICOLL-HYPAQUE

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STABILITY AND FUNCTION OF GRANULOCYTES ISOLATED BY COUNTERFLOW CENTRIFUGATION AND DISCONTINUOUS DENSITY GRADIENTS OF FICOLL-HYPAQUE.

INTRODUCTION

Previous studies from our laboratory (1-6) and by others (7,8,9) on the function and preservation of animal and human granulocytes were based on cell preparations isolated by counter flow centrifugation elutriation (CCE). This technique produced granulocytes of high purity and function (1,3,4,5,7). However, CCE requires the use of a Beckman JE-6 elutriator rotor, a J-21B centrifuge and takes 3-4 hours for the procedure.

Recently Berkow et al. (10) showed that the method of neutrophil preparation affected the cellular responses of the cells. Their data suggested that CCE isolated cells more accurately mimicked <u>in vivo</u> characteristics of human neutrophils than those isolated with Ficoll-Hypaque and hypotomic lysis. We therefore investigated two alternative methods for the isolation of human granulocytes, one that employed two densities of Ficoll-Hypaque in discontinuous density gradients (DFDG) (11) and the Ficoll-Hypaque-hypotonic lysis method (SFWL) (12), and compared these cells to those obtained by CCE. The parameters of cell function studied were superoxide production (O_2^-), phagocytosis of opsonized Fluolite particles, cell volume and cell membrane integrity (3,4). These measurements were made immediately after isolation and again after storage at 4°C for 24, 48 and 72 hours. We report here that cells obtained by CCE and SFWL and then stored at 4°C up to 3 days were similar. DFDG cells, while similar at time Zero (except for O_2^-), declined rapidly and by 72 hours were

non-functional. These studies indicate that cells isolated by SFWL are similar and to CCE granulocytes therefore suggest that SFWL cells are preferable because of the simplicity of the method.

METHODS

The methods employed were identical to those given in the references (1, 11, 12). Whole blood was collected into ACD by the American Red Cross, Northeast Services. We divided it into 50 ml plastic tubes, and centrifuged them and removed platelets. Buffy coat white cells plus the top 25.0 ml of packed red cells were used as the starting material for the CCE, SFWL and DFDG methods of granulocyte isolation.

Superoxide anion was determined with the method of Cohen et al. (13).

Immediately after isolation (0-hr) and at 24, 48 and 72 hrs, superoxide production, particle ingestion, volume (measured as median channel number) and membrane integrity (assayed microfluorimetrically) were determined.

RESULTS

The data in Table 1 show CCE isolated cells to undergo similar but lesser increases in volume during storage as compared to SFWL, except at 72 hrs (3.1% vs. 6.9% at 24 hrs, 7.9% vs. 10.5% at 45 hrs, and 28% vs. 18% at 72 hrs; CCE vs. SFWL, respectively). CCE isolated cells also showed a lesser loss of the capacity to produce O_2^- and in the number of non-phagocytosing cells at all time periods examined. Additionally, greater than 90% of all cells tested (CCE, SFWL or DFDG) at 0, 24 and 48 hours, produced fluorescein from fluorecein diacetate and excluded ethidum bromide (data not shown) indicative of viable cells.

Similar results were obtained in separate experiments which compared CCE to DFDG cells (Table 2). CCE cells underwent less swelling during storage (lesser increases in median channel numbers) except after 72 hr. CCE cells also had lesser percentage increments of non-phagocytosing cells and lesser decreases in O_2^- procuction at all intervals tested.

In order to compare the properties of granulocytes isolated simultaneously, the three methods were applied to one unit of blood. Isolated cells were sampled at the same intervals as above (0, 24, 48 and 72 hrs).

The results depicted in Figure 1 revealed DFDG, CCE, and SFWL cells to be similar immediately after isolation. A major difference was the rate of O_2^- production by DFDG isolated cells. This was markedly lower than CCE or SFWL cells (7.2, 12.0 and 13.2nmoles O_2^- per min. per 10^6 cells; DFDG, CCE and SFWL cells respectively). Similarly, DFDG cells stored for 3 days at 4°C also exhibited rapid losses of ingestion of opsonized Fluolite particles, O^- synthesis, and also displayed a greater loss of cell numbers when compared to SFWL or CCE isolated cells. In Figure 1 it is also evident that SFWL cells and CCE cells were similar in production and particle ingestion. However, CCE cells were the most stable over the 72 hour period studied.

DISSCUSSION

Our data shows that CCE and SFWL isolated granulocytes are more stable than DFDG isolated cells. In the comparisons (Table 1 and 2), of cells isolated by both types of density gradients exhibited similar properties to CCE isolated cells. However, when all three methods were simultaneiouly appled to the same unit of blood, we observed that DFDG cells were less stable over 72 hr. than either CCE or SFWL cells. Berkow et al. (10) hypothesized that the CCE technique is less injurious than density gradient centrifugation because of trauma

associated with Ficoll-Hypaque and hypotonic lysis. They suggest these steps damage the cell membranes leading to a reduction in the activation mechanism of granulocytes. Our results show Ficoll-Hypaque-hypotonic lysis prepared cells to be comparable in function and stability. The discrepency is unexplained but may be due to inherit differences in CCE protocols in the two laboratories. We routinely sediment whole blood with dextran to reduce the concentration of red cells submitted to counterflow in the separation chamber. This effects a faster separation of PMN's from contaminating RBC, platelets and mononuclear cells.

Our results based on the similarities of SFWL and CCE cells over 73 hr of storage at 4°C suggest the SFWL method is preferred, because of its relative simplicity and high quality and purity of the isolated granulocytes.

TABLE 1

COMPARISON OF CCE AND SFHL ISOLATED CELLS STORED AT 4°C

TEST	STORAGE TIME (HRS)			
	0	24	48	72
Median Channel (% increase)				
CCE	-	3.1 ± 1.1 (8)	7.9 ± 1.8 (5)	27.9 ± 11.9 (3)
SFHL	-	6.9 ± 1.1 (8)	10.5 ± 2.0 (5)	18.0 ± 5.1 (3)
Fluolite Ingestion (% non-ingesting)				
CCE	4.6 ± 1.4 (7)	12.3 ± 4.1 (8)	24.5 ± 8.5 (4)	21.3 ± 4.4 (3)
SFHL	6.3 ± 2.9 (7)	18.4 ± 5.9 (8)	27.2 ± 9.8 (4)	28.0 ± 12.0 (2)
Superoxide Production (% decrease)				
EBC	-	23.4 ± 9.5 (4)	55.9 ± 6.7 (2)	55.1 (1)
SFHL	-	47.3 ± 11.5 (3)	58.2 (1)	62.7 (1)

n ± SE, (n), equals number of observations

CCE - Counterflow Centrifugation Elutriation
SFHL - Single Ficoll Hypaque Water Lysis

TABLE 2

COMPARISON OF CCE AND DFDG ISOLATED CELLS STORED AT 4°C

TEST	STORAGE TIME (HRS)			
	0	24	48	72
Median Channel (% increase)				
CCE	-	1.7 ± 1.7 (7)	6.6 ± 2.0 (5)	17.8 ± 10.9 (2)
DFDG	-	6.4 ± 1.6 (7)	10.2 ± 2.1 (5)	15.5 ± 1.9 (2)
Fluolite Ingestion (% non-ingesting)				
CCE	5.4 ± 1.6 (7)	14.0 ± 4.5 (7)	27.3 ± 6.9 (4)	19.3 ± 5.5 (3)
DFDG	4.3 ± 1.4 (7)	15.2 ± 5.3 (6)	33.3 ± 8.7 (4)	26.5 ± 11.5 (2)
Superoxide Production (% decrease)				
CCE	-	12.2 ± 5.8 (5)	44.4 ± 12.1 (3)	60.6 ± 5.5 (2)
DFDG	-	34.9 ± 12.1 (4)	51.6 (1)	82.8 (1)

n ± SE, (*n*) equals number of observations.

CCE - Counterflow Centrifugation Elutriation
DFDG - Double Ficoll Density Gradient

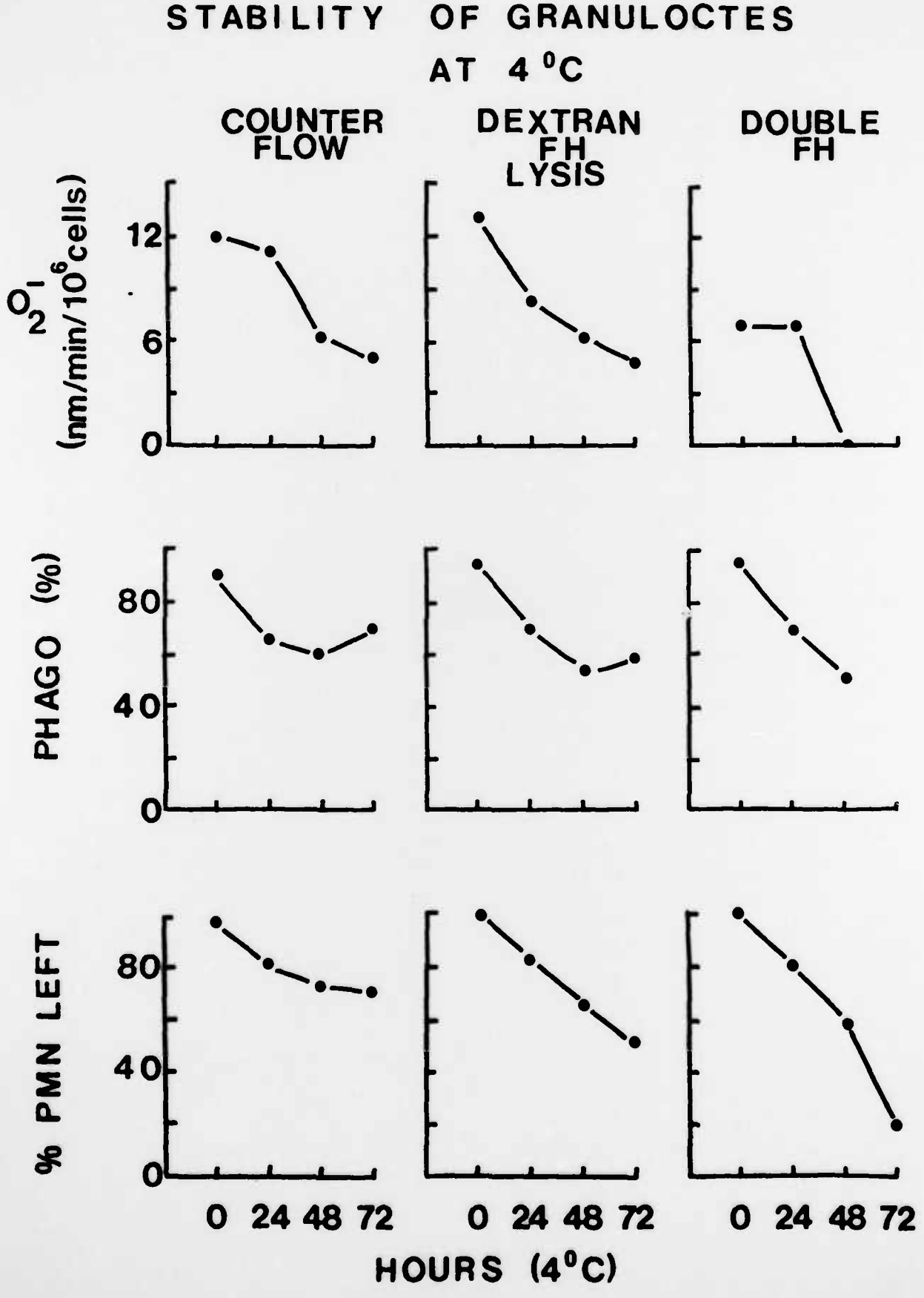
STABILITY OF GRANULOCTES
AT 4 °C
COUNTER FLOW
DEXTRAN FH LYSIS
DOUBLE FH
$^{2}O_{1}$ (nm/min/10^{6}cells)
12
6
0
PHAGO (%)
80
40
0
% PMN LEFT
80
40
0
0 24 48 72
0 24 48 72
0 24 48 72
HOURS (4°C)

FIG. 1

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number) Granulocytes isolated by counterflow centrifugation elutriation (CCE) were compared with those isolated by dextran sedimentation, centrifugation with Ficoll-Hypaque, and lysis of residual red cells (SFWL). They were also compared with granulocytes isolated on discontinuous density gradients using two densities of Ficoll-Hypaque (DFDG). The cells were stored at 4°C for 72 hours and aliquots measured for phagocytic indices (particle ingestion), membrane integrity (fluorescein-diacetate-ethidium bromide) volume (electronic sizing) and membrane function (superoxide anion).		

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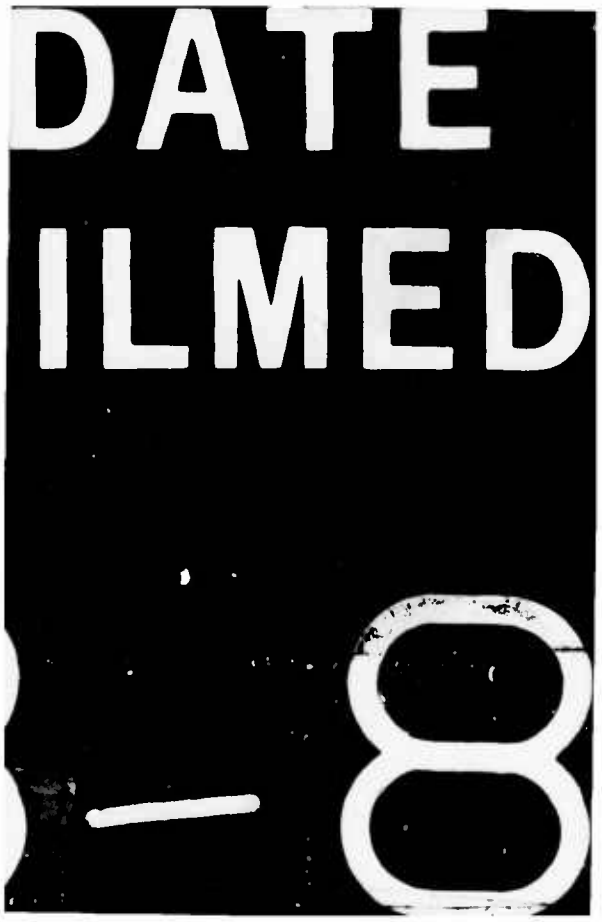
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