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IN VITRO STUDIES OF CRYOPRESERVED GRANULOCYTES.(U)
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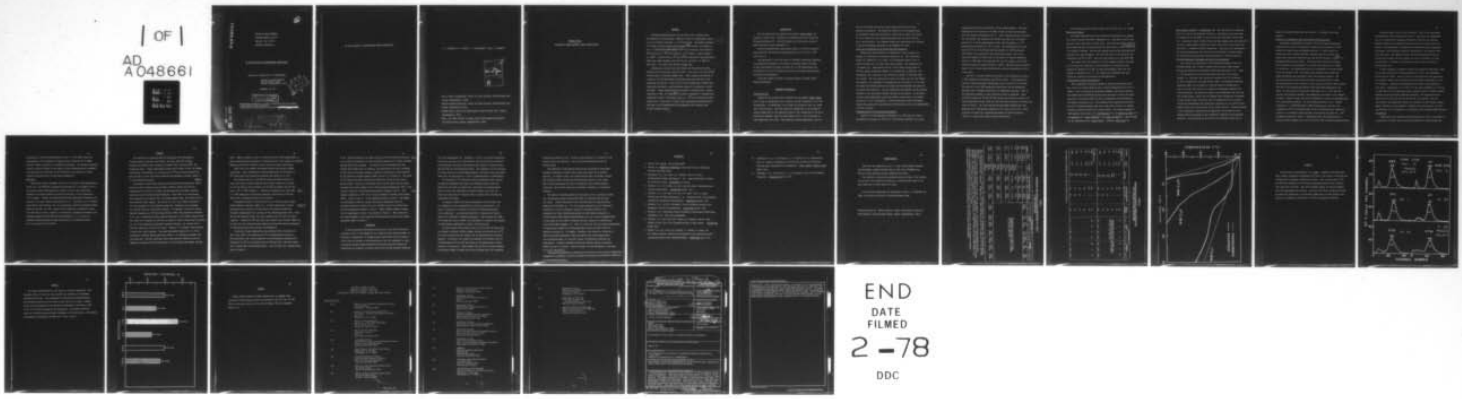

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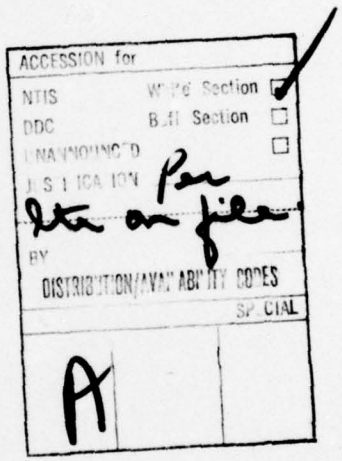
IN VITRO STUDIES OF CRYOPRESERVED BABOON GRANULOCYTES

F. J. LIONETTI,* S. M. HUNT,+ R. J. MATTALIANO,++ AND C. R. VALERI**



*Ph.D., Senior Investigator, Center for Blood Research, 800 Huntington Ave., Boston, Massachusetts 02115

+Senior Research Technician, Center for Blood Research, 800 Huntington Ave., Boston, Massachusetts 02115

++Summer Fellow, Center for Blood Research, 800 Huntington Ave., Boston, Massachusetts 02115

**Capt., MC, USNR, Officer in Charge, Naval Blood Research Laboratory, 615 Albany Street, Boston, Massachusetts 02118

RUNNING TITLE:

ELUTRIATED FREEZE-PRESERVED BABOON GRANULOCYTES

ABSTRACT

Granulocytes were isolated from the buffy coat of baboon blood by counterflow centrifugation. They were frozen in polypropylene tubes in 2.0 ml volumes containing 1 x 10^7 granulocytes. The medium consisted of 5% DMSO, 6% HES, 4% human serum albumin, and 6 mM glucose in Normosol-R, pH 7.1. Granulocytes were cooled to 4°C for 30 minutes, then cooled at 4°C per minute to -80°C and stored for 1 to 3 weeks in liquid nitrogen at -197°C. Cooling rates of 1°C and 10°C per minute were less efficacious. Tubes were thawed manually with swirling for 130 seconds at 42°C in a water bath. The yield after thawing was 98 ± 14%.

Granulocytes isolated from fresh blood all fluoresced green when incubated with fluorescein diacetate (FDA). Less than 1% of cells showed nuclear uptake of ethidium bromide (EB). After freezing and thawing, an average of 79 ± 8% granulocytes fluoresced green with FDA and 21 ± 8% fluoresced red with EB. About 98 ± 2% of the granulocytes isolated from fresh blood exhibited a serum dependent capacity for ingestion of latex and yeast. Thawed granulocytes were diluted 1:4 dropwise with a medium containing 7% HES, 4% albumin, and 6 mM glucose in Normosol-R, incubated with fluorescent latex or fluorescent zymosan at 37°C for 30 minutes and then washed twice. After this, 75 ± 9% of the granulocytes fluoresced green with FDA, 76 ± 5% demonstrated serum-dependent latex ingestion and 67 ± 8% ingested zymosan.

INTRODUCTION

The red blood cells and platelets of baboons (Papio anubis) are similar to those of man. Likewise are conditions for their liquid and freeze-preservation (1). The white blood cell differential counts of baboon and man are also comparable (2).

We have preserved human granulocytes using 10 to 100 ml volumes of human blood for the isolation and preservation of 2×10^7 to 1×10^8 cells (3,4,5).

The opportunity to use the baboon to evaluate circulatory properties of granulocytes motivated us to attempt to preserve them by freezing. We have found that the periodic isolation of 1×10^8 granulocytes from the buffy coat of 30 ml of baboon blood can be routinely achieved using counterflow centrifugation.

This paper reports results of *in vitro* studies of frozen thawed baboon granulocytes.

METHODS AND MATERIALS

Blood Collection

Twenty-five ml of blood was collected from the baboons (Papio anubis, 19 to 24 kg) by venepuncture into a plastic syringe containing 5 ml of CPD anticoagulant. An additional 5 ml of blood was allowed to clot in a glass tube to obtain serum. The whole blood was transported in the syringe at ambient temperature to the laboratory where it was transferred to two 50 ml polystyrene (Nalgene) tubes and centrifuged at 350 x g for 25 minutes at room temperature ($22 \pm 2^{\circ}C$). The plasma was removed aseptically, and 8 to

10 ml of the buffy coat and the upper packed red blood cell layer removed by aspiration. The plasma was added back to the packed cells, the suspension mixed and centrifuged a second time at 350xg for 20 min, 5-7 ml of buffy coat were removed and pooled with the cells from the first centrifugation. The combined mixture was transferred via a syringe to the mixing chambers connected to the Beckman JE-6 rotor.

Isolation of Granulocytes by Counterflow Centrifugation.

Granulocytes were isolated in a Beckman JE-6 rotor attached to a Beckman J-21 centrifuge as described previously (5). The counterflow buffer was composed of 0.15 M NaCl, 0.01 M phosphate buffer at pH 7.1. 0.028 M glucose and 1.2% (W/V) human serum albumin. The osmolality was 325 mOsm/kg H_2O. Approximately 800 ml of this medium refrigerated at $10^{o}C$ was flowed through the chamber (4.5 ml capacity) at 2080 rpm. The granulocytes were retained in the elutriation chamber as the other cells were eluted. The time required to transfer the buffy coat into the chamber and complete the separation of granulocytes was 50 minutes. At this time the buffer and effluent tubes were clamped and simultaneously the pump and the centrifuge turned off. The centrifuge head was removed, and the cellular contents of the elutriation chamber recovered by aspiration. On the basis of whole blood differential and Coulter Model F white counts, the average yield was 5.3×10^{7} granulocytes. Volume distributions and dried smears showed the granulocytes contained about 2% mononuclear cells, and approximately 5 to 10% red cells.

Cryopreservation of Elutriated Granulocytes.

Three ml of cell suspension containing 1.4×10^{7} cells per ml were maintained in ice water at $4^{o}C$ for 15 to 20 minutes, and then 3 ml of the

cryopreservation solution chilled at 4°C was added dropwise. The final composition of the mixture was 5% DMSO, 6% HES, 4% human serum albumin, and 6 mM glucose in Normosol-R, pH 7.1. After complete mixing by swirling for 180 seconds, the contents were divided into three two ml aliquots placed in 17 x 100 mm polypropylene tubes, each containing 1.4×10^7 granulocytes and placed in a 4°C ice bath. The granulocytes were maintained at 4°C for 5-10 minutes and then placed in a -80°C mechanical refrigerator in an improvised two-step freezing system. Two of the tubes were placed in a metal rack in the bottom of a Harris mechanical freezer at -80°C where they were kept for 30 to 40 minutes, after which they were placed in liquid nitrogen at -197°C. The third tube was used for assays of cell counts, volume distributions, and microfluorescence of pre-frozen cells.

The rate of cooling achieved by storage of the polypropylene tube in a -80°C mechanical refrigerator was 4°C per minute. It was measured by placing a copper-constantan thermocouple wire in the center of the tube that was put in the -80°C mechanical refrigerator and the temperature monitored with a Honeywell Electronik III temperature recorder. Most of the granulocyte samples in this study were frozen as described. Also, controlled cooling of 1°C and 10°C per minute were carried out using a GV Planer programmed freezer R-201 that utilized liquid nitrogen to control the rates of cooling. The rates achieved by programmed freezing were also monitored with a thermocouple inserted in the center of the tube. After freezing, the tubes were stoppered and placed in liquid nitrogen at -197°C in a Linde LR-40 liquid nitrogen refrigerator.

The storage period in liquid nitrogen for all tubes was 1 to 3 weeks.

Thawing and Washing.

The tubes containing the frozen granulocyte suspensions were removed from liquid nitrogen and placed immediately in a water bath at 42°C, with the water above the level of frozen cells. The tubes were shaken manually while submerged with a circular motion for 120 seconds or until all but an ice pellet of 3 mm diameter remained. The tube was removed and swirling continued until the pellet was just dissolved. The tubes were cool to the touch and the temperature was 8°C to 10°C. They were then placed in an ice water bath.

The thawed cells were diluted 1:4 by the dropwise addition (one drop per 2 seconds for 3 minutes) of three volumes of a solution at room temperature composed of 7% HES, 4% human serum albumin (HSA) and 6 mM glucose in Normosol-R, pH 7.1. The diluted cell suspension was then tested for stability and function as described below.

Determination of Size Distributions.

Cell suspensions containing 100,000 to 150,000 granulocytes per ml were sized in a Coulter Model ZH with a C-1000 Channelyzer and an X-Y recorder. Prior to testing the granulocyte samples, the Coulter aperture was flushed with the buffered solution that was used to dilute the granulocytes or the solution that was used to cryopreserve the granulocytes. The sample volume was 0.1 ml. All solutions were rendered particle-free by prior filtration through both 0.45 u and 0.20 u filters in a Coulter filtration system or with 0.22 u Falcon filters. The counter employed a 7084 aperture and was set at an amplification of 1; an aperture current of 2; an exclusion of 4; lower threshold 1; and upper threshold 99. The settings for the channelyzer were: count control - external, count range 1K,

base channel threshold 1, window width 100. The instrument was calibrated with 2.0 u and 4.6 u polystyrene particles. Granulocyte counts were made by integration of the number of cells within the granulocyte distribution (5). Granulocyte counts in whole blood and other samples were also made with a Coulter Counter Model F blood cell counter after lysis of red cells with Zap-Isoton, a reagent of Coulter Diagnostics. Red blood cell contamination of isolated granulocytes was counted as the difference in counts between the non-treated and Zap-Isoton-treated aliquots at a 500 fold dilution.

Microfluorescence of Cytoplasm and Nuclei of Granulocytes.

The reaction of granulocytes with fluorescein diacetate (FDA) and ethidium bromide (EB) in Hank's balanced salt solution (HBSS) without calcium and magnesium was performed as described previously (5,6). About 1×10^6 granulocytes from elutriated buffy coat (EBC) and variously treated i.e., prefrozen (PF), prefrozen incubated (PFI) for 20 minutes at 37°C, postthaw (PT), postthaw diluted (PTD), and postthaw diluted and incubated (PTDI) for 20 minutes at 37°C, were mixed in proportions of 0.05 to 0.25 ml with 0.50 ml of a mixture of fluorescein diacetate and ethidium bromide. Wet mounts were made at room temperature and the cells were viewed within 1 minute with an Olympus Vanox transmission microscope. A green exciter filter (G533) was used for identification of the granulocytes and fluorescence was viewed after switching to a UV exciter filter (Schott BG-12) and a blue barrier filter (Schott OG-530). Esterase activity in the cytoplasm of granulocytes was measured as the percent of cells showing green fluorescence of the fluorescein liberated from fluorescein diacetate. The percentage of cells with red fluorescent nuclei due to

uptake of ethidium bromide was also recorded. Two hundred cells were counted.

Ingestion of Fluorescent Latex and Ethidium Treated Zymosan.

Elutriated granulocytes and thawed, diluted and washed granulocyte suspensions were tested for phagocytic properties by assessment of their capacity to ingest latex and Zymosan particles. A stock solution of fluorescent latex (Fluolite DS-5005), obtained from ICI, Finland, was washed three times with distilled water and diluted serially (1:1) with 0.0 1 M phosphate-buffered saline (PBS). Following each dilution the particles were recovered by centrifugation in a Sorval centrifuge spun at 7,700xg for 20 minutes. The washed particles were resuspended in an equal volume of PBS and stored at $4^{o}C$. The washed latex particles were mixed with human granulocytes and autologous serum, and ingestion measured. The particle mixture was then diluted serially and re-tested with various PMN concentrations until a particle to cell ratio was found at which greater than 90% of the granulocytes isolated from fresh blood phagocytized the latex. A 1:16 dilution of latex and a PMN number of 1.5×10^{6} gave full activity when serum was present, and essentially no reaction in the absence of serum. Details of this test and its dependence on serum and immunoglobulins will be described elsewhere. For the ingestion testing, 0.5 ml samples containing 1.5×10^{6} granulocytes in HBSS containing Ca++ and Mg++ were incubated at $37^{o}C$ for 30 minutes with 0.5 ml of autologous serum containing 0.025 ml of fluorescent latex particles as previously described (5). The incubation volume was 1.025 ml. Granulocytes were then washed twice by dilution slowly dropwise with 2.05 ml of cold 7% HES, 4% HSA and Normosol buffer

and centrifuged at 250 x g for 10 minutes. Most of the supernatant containing free latex particles was removed. The pellet was resuspended in a 0.5 ml HBSS buffer containing fluorescein diacetate and ethidium bromide as before, wet mounted slides were prepared, and 200 cells were counted. The percentages of granulocytes demonstrating green cytoplasmic fluorescein, red fluorescent ethidium stained nuclei, and ingested latex were determined. The numbers of latex particles ingested by the granulocytes were divided into four groups: (1) zero, no particles, (2) low, 1-5 particles, (3) medium, 5-15 particles, and (4) high, more than 15 particles per cell.

A stock suspension of Zymosan (Sigma) was prepared by suspending 1 gram of the yeast and 200 mg of ethidium bromide in 2 ml of 0.01M phosphate-buffered saline (PBS). After 0.5 hours at room temperature the suspension was washed 5 times with 10 volumes of PBS and finally resuspended in 10 ml of PBS buffer. Ingestion by granulocytes was studied in the same way as with latex. Granulocytes (1.5×10^6 in 0.5 ml) were incubated for 0.5 hours at $37^{\circ}C$ with 0.5 ml of autologous serum to which 0.025 ml of ethidium treated Zymosan had been previously mixed and incubated. The granulocytes were washed twice by dilution with 7% HES, 4% HSA, and Normosol; they were centrifuged, the supernatant solution was removed; the FDA-ethidium reagent added; and 200 cells were counted. Ingestion was categorized as (1) zero, no particles; (2) M, phagocytic, 1-5 particles, and H, phagocytic >5 particles.

Incubations.

Granulocytes were incubated for twenty minutes at $37^{\circ}C$ at two stages of preservation to assess their stability as measured by volume changes and

percentages of FDA and ethidium positive cells. In the tables these are represented as PFI (incubated in cryopreservative solution with 5% DMSO) and PTDI (thawed, diluted to 1.3% DMSO and incubated). For particle ingestion studies, separate aliquots of the thawed, diluted suspensions were incubated with particles for 30 minutes at 37°C followed by two washings to remove exogenous particles prior to microscopic examination.

Materials.

Hydroxyethyl starch (CryoHES, 40% W/V in 0.15 M NaCl, McGaw Laboratories, Irvine, CA., Lot #P02303C), prepared by dissolving 42.7 g of powder in 70 ml of distilled water and adjusting the volume to 100 ml, was diluted with Normosol-R to prepare freezing and washing solutions, 6% for freezing and 7% for washing. Albumin was obtained from Hyland Laboratories (Buminate, 25% solution, salt poor, normal serum albumin U.S.P.). Normosol-R, pH 7.1 (Abbott Laboratories, North Chicago, IL) is an electrolyte solution containing cations, mEq/liter, Na-140, K-5, Mg-3, Anions Cl-98, Acetate-27, gluconate-23, total 148, mOsm 295 per liter. Zymosan (S. cerevisiae), fluorescein diacetate, and ethidium bromide were obtained from Sigma Chemical Co., St. Louis, MO. Phosphate buffered saline was made by diluting 0.11 M phosphate buffer , pH 7.1,10 times with 0.15 M NaCl.

RESULTS

The results of 17 separate freezing experiments with granulocytes isolated from five baboons (four female, one male) show good *in vitro* recovery and function after freezing and thawing (PT), dilution (PTD), and incubation (PTDI). Baboon granulocytes frozen with 5% DMSO, 6% HES, 4% albumin, 6 mM glucose, and Normosol, at a cooling rate of 4°C per minute produced the best recovery of cells with stable membranes and phagocytic function (Table I) of the formulations tested. Table I

The yields of cells after freezing were estimated from granulocyte counts before and after freezing using the Model F Coulter Counter and from the integration of the counts in volume distributions before and after freezing using the Coulter ZH Counter and Channelyzer. About 98% of the granulocytes were recovered in the thawed (PT), the thawed diluted (PTD), and thawed diluted and incubated (PTDI) samples. The nuclei 21 ± 8% of the thawed granulocytes (PT) were permeable to the ethidium bromide dye, suggesting this percentage of recovered cells was irreversibly damaged. On subsequent dilution to reduce DMSO and incubation at 37°C for twenty minutes (PTDI) this percentage increased to 25 ± 9%. Alternatively after separate incubation of the thawed, diluted cells with latex for 30 minutes at 37°C and two washes to remove extracellular particles, 76 ± 5% of the recovered granulocytes ingested particles, the average sum of high (H), medium (M), and low (L) values. Moreover, 54 % showed a high capacity (H and M) for latex ingestion. The yeast experiments showed that 67 ± 8% of granulocytes ingested Zymosan particles (Table I) in reasonable agreement with the latex data. The only conditions under which particle ingestion could be reliably estimated were for granulocytes in the elutriated and thawed, diluted

state. DMSO in excess of 1.25%, or conditions which caused granulocytes to swell, produced high percentages of ethidium positive cells incable of ingestion. Omissions of entries in Tables I and II are due to these restrictions.

About 80% of the thawed, and thawed diluted granulocytes produced fluorescein. After incubation of diluted suspensions for 20 minutes at 37°C reactivity with fluorescein diacetate was reduced to 75 ± 9%.

The cooling rates of 1 and 10°C per minute performed with the GV Planer and liquid nitrogen produced curves with a plateau of 9 minutes for 1°C per minute, and no plateau for the 10°C per minute rate for the range + 4°C to -80°C (Figure 1). Storage of granulocytes in test tubes in a -80°C mechanical refrigerator achieved a cooling rate of 4°C per minute with a freezing plateau of four minutes. Fig. 1

Granulocytes frozen at 1°C per minute or at 10°C per minute showed reduced membrane stability (Table II). In three experiments in which granulocytes were cooled at 1°C per minute, the thawed, diluted and incubated granulocytes had an average of 32% ethidium positive cells. About 76% had the capacity (H+M+L) for latex ingestion and 54% (H+L) for yeast ingestion. Freezing at 10°C per minute was clearly less efficient (Table II) as less cells were recovered after thawing and these had greater percentages of ethidium positive cells and were less phagocytic. Table II

Granulocyte volume distributions were measured after elutriation of buffy coats (EBC), after addition at 4°C of cryoprotective solution to the granulocytes (PF), after incubation of the granulocyte-cryoprotective suspension at 37°C for 20 minutes prior to freezing (PFI), and when thawed (PT), diluted (PTD) and incubated (PTDI). They are shown for a typical experiment in Figure 2.

In the typical experiment the shape and area under the volume distribution curve reflect the stability of the granulocyte population to osmotic stresses applied during the procedure. The volume of the granulocytes was kept smaller than normal prior to freezing, and granulocytes that were incubated at 37°C prior to freezing showed an increase in granulocyte volume, measured by the increase in median channel number from 49 to 53 (Figure 2). The value 47 (PT) means that after freezing and thawing the volume was the same as before frozen. Dilution at controlled rates with the wash solution medium increased the volume somewhat of the thawed-diluted granulocytes (PTD) to the original volume of the elutriated granulocytes (Figures 2 and 3). Subsequent incubation at 37°C for 20 minutes produced no further volume change (PTDI) (Figure 2 and 3). In the experiment shown in Figure 2, the numbers of granulocytes within the volume distribution demonstrate that 97% survived freezing and thawing (8.7/9.0) and 88% survived the freeze-thaw-dilution-incubation procedure (7.0/9.0). Median channel numbers averaged for all experiments in Table I are plotted in figure 3. They verify that the volume changes at each stage were substantially the same as the typical experiment shown in figure 2.

DISCUSSION

We have previously observed swelling and cell loss during liquid preservation at 4°C (3) and damage due to a swelling effect caused by DMSO (7). Swelling of granulocytes to volumes greater than normal can be presumed to result from an increase in the permeability of the cell membranes to water. In previous studies volume distributions had been especially helpful for determining the stability of murine marrow cells and the optimum conditions

for their preservation (8). Therefore, a goal of the research described here was to maintain cell volume smaller than normal during elutriation of granulocytes and during the manipulations required to preserve them. This was done by using hypertonic media. The importance of the principle of volume control is clearly demonstrated by the data of this paper which shows that its application is a key to cryopreservation of granulocytes.

The magnitude of 70% functional baboon granulocytes after thawing and washing approached that found with sedimented human cells when measured by exclusion of trypan blue, percentages of myeloperoxidase positive cells, and inhibition of growing cultures of E. Coli (4). French and associates (9) reported similar results after freezing dog granulocytes with DMSO.

In separate studies with human granulocytes, we have found that all components of the cryopreservative solution contributed to the overall efficiency of freezing and thawing of baboon granulocytes (to be published). As previously observed (4) Normosol was found to improve the stability of thawed granulocytes. When Normosol was added to 5% DMSO, HES, and 4% albumin, an improvement in stability and function of thawed baboon granulocytes of about 10% was observed.

Our data suggest that cooling rates of $1^{o}C$ and $10^{o}C$ per minute were not optimum to prevent cellular damage, whereas the cooling rate of $4^{o}C$ per minute produced the best results for the cryopreservative solution employed. Leontovich (10) studied the influence of different rates of freezing between $1^{o}C$ and $10^{o}C$ per minute on the preservation of granulocytes in 15% glycerol. Rates between $3^{o}C$ and $5^{o}C$ per minute produced the greatest number of thawed cells which excluded eosin and freezethaw

granulocyte recovery of 79%. At these rates plateaus of 3-4 minutes in the cooling curves were observed. Our rate agrees substantially with the Russian study.

The compositon of the washing solution was determined by experiments designed to maintain a smaller than normal cell volume and to prevent swelling. In Figures 2 and 3 the thawed diluted (PTD) and thawed, diluted and incubated (PTDI) granulocytes showed minimal change in cell volume. When volume measurements are used in conjunction with ethidium bromide reactivity, they provide sensitive and reliable indicators of granulocyte stability.

The uptake of ethidium bromide by granulocyte nuclei, producing intense red fluorescence defines granulocytes which are nonfunctional and thus non-viable. Similar observations have been described for granulocytes by Dankberg and Persidsky (6). In a later paper describing use of fluorescein diacetate as a test for membrane integrity, Persidsky and Bailli (11) commented that frozen thawed granulocytes with 90% membrane integrity in the microscopic FDA-ethidium bromide-zymosan test were inactive phagocytically. In this paper we were unable to measure particle ingestion by thawed granulocytes when DMSO exceeded 1.3%. Also, in a previous study with human granulocytes, we demonstrated inhibition of myeloperoxidase activity by DMSO, which was removed by washing (4). We suggest, therefore, that removal or reduction of DMSO from thawed granulocytes, which we monitor with volume measurements and microfluorescence, is a critical factor in maintaining stability and phagocytosis. Swelling introduced during cell washing results in ethidium uptake and loss of function. This can account for the discrepancy in the data of the two laboratories.

Granulocytes preserved by freezing can be used for in vitro tests of granulocyte compatibility necessary to ensure the safety and efficacy of granulocyte transfusion to alloimmunized patients.

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Address Reprints To: Fabian Lionetti, Senior Investigator, Center for Blood Research, 800 Huntington Avenue, Boston, Massachusetts 02115.

TABLE I

PRESERVATION AND FUNCTION OF BABOON GRANULOCYTES (P. Anubis)
4 C/MIN COOLING RATE

SAMPLE	CELL # X 10^7	RECOVERY (%)	MICROFLUORESCENCE FDA (%)	MICROFLUORESCENCE ETH	MEDIAN CHANNEL #	PHAGOCYTOSIS (%) LATEX H	LATEX M	LATEX L	LATEX 0	ZYMOSAN H	ZYMOSAN M	ZYMOSAN 0
EBC n=8	5.3±1.3 (3.6–6.8)	--	99±1 (98–100)	1±1 (0–2)	48±2.0 (45–51)	86±5 (77–92)	4±2 (1–3)	7±5 (1–18)	3±2 (1–7)	96±2 (94–98)	2±1 (0–3)	.3±2 (1–5)
PF n=8	1.4±0.3 (1.0–1.8)	-- --	99±1 (99–100)	1±1 (0–1)	46±2 (42–48)							
PFI n=8	1.3±0.3 (0.9–1.5)	*91±15 (62–109) **98±5 (90–105)	98±1 (96–100)	2±1 (0–4)	51±2 (47–55)							
PT n=13	1.4±0.3 (0.9–1.7)	98±14 (71–125) 97±4 (91–101)	79±8 (64–90)	21±8 (10–36)	45±2 (43–48)							
PTD n=13	1.4±0.3 (1.1–1.9)	99±15 (73–120) 95±5 (86–105)	81±8 (65–97)	19±8 (3–35)	48±2 (45–51)							
PTDI n=13	1.4±0.3 (1.0–1.8)	100±25 (80–122) 94±6 (82–100)	75±9 (61–95)	25±9 (5–39)	47±2 (44–50)	25±11 (8–45)	29±12 (13–52)	22±15 (8–59)	24±5 (15–36)	44±13 (36–66)	23±10 (10–36)	33±8 (27–46)

PHAGOCYTOSIS WAS LOW OR ABSENT FROM SUSPENSIONS CONTAINING DMSO IN EXCESS OF 1.3% (PF, PFI, PT).

EBC = Elutriated buffy coat, PF = Prefrozen, PFI = Prefrozen incubated, PT = Postthawed, PTD = Postthawed diluted, PTDI = Postthawed diluted, incubated. Incubations (PFI, PTDI) were carried out for 20 minutes at 37 C to test PMN stability.

The mean ± SD and the range (parenthesis) of each value are reported. FDA and ETH are the percentages of PMN's fluorescing with Fluorescein diacetate and Ethidium. PHAGOCYTOSIS: The percentages of PMN's containg Latex or Yeast Particles. The letters H, M, L, O represent the number of particles of Latex or Yeast per cell, where Latex H > 15, M = 5-15, L = 1-5, O = none; Yeast H > 5, M = 1-5, O = none.

*Recovery is determined by counting cells with the Model F before freezing (PF) and each subsequent procedure.

**Recovery obtained by integration of the volume distribution with the ZH counter and Channelyzer.

TABLE II

PRESERVATION AND FUNCTION OF BABOON GRANULOCYTES
AT 1 C/MIN AND 10 C/MIN COOLING RATE

SAMPLE	CELL # x 10^7	RECOVERY	MICROFLUORESCENCE FDA (%)	ETH.	MEDIAN CHANNEL #	PHAGOCYTOSIS (%) LATEX H	M	L	O	ZYMOSAN H	L	O
1 C/MIN n = 3												
EBC	4.68	--	100	0	50	87	7	2	4	79	6	15
PF	1.15	--	99	1	48							
PFI	1.10	*96				PHAGOCYTOSIS WAS LOW OR ABSENT FROM SUSPENSION CONTAINING DMSO IN EXCESS OF 1.3% (PF, PFI, PT).						
		**100	99	1	48							
PT	1.19	103										
		97	71	29	50							
PTD	1.15	100										
		95	76	24	49							
PTDI	1.20	104										
		99	68	32	46	18	40	18	24	16	38	46
10 C/MIN n = 2												
EBC	10.90	--	100	0	51	66	15	5	14	88	7	5
PF	2.94	--	100	0	47							
PFI	2.88	98										
		97	99	1	49							
PT	2.70	92										
		96	51	49	49							
PTD	2.70	92										
		97	52	48	49							
PTDI	2.72	93										
		97	44	56	47	2	8	43	47	3	20	77

The numbers and symbols are the same as those of Table I.

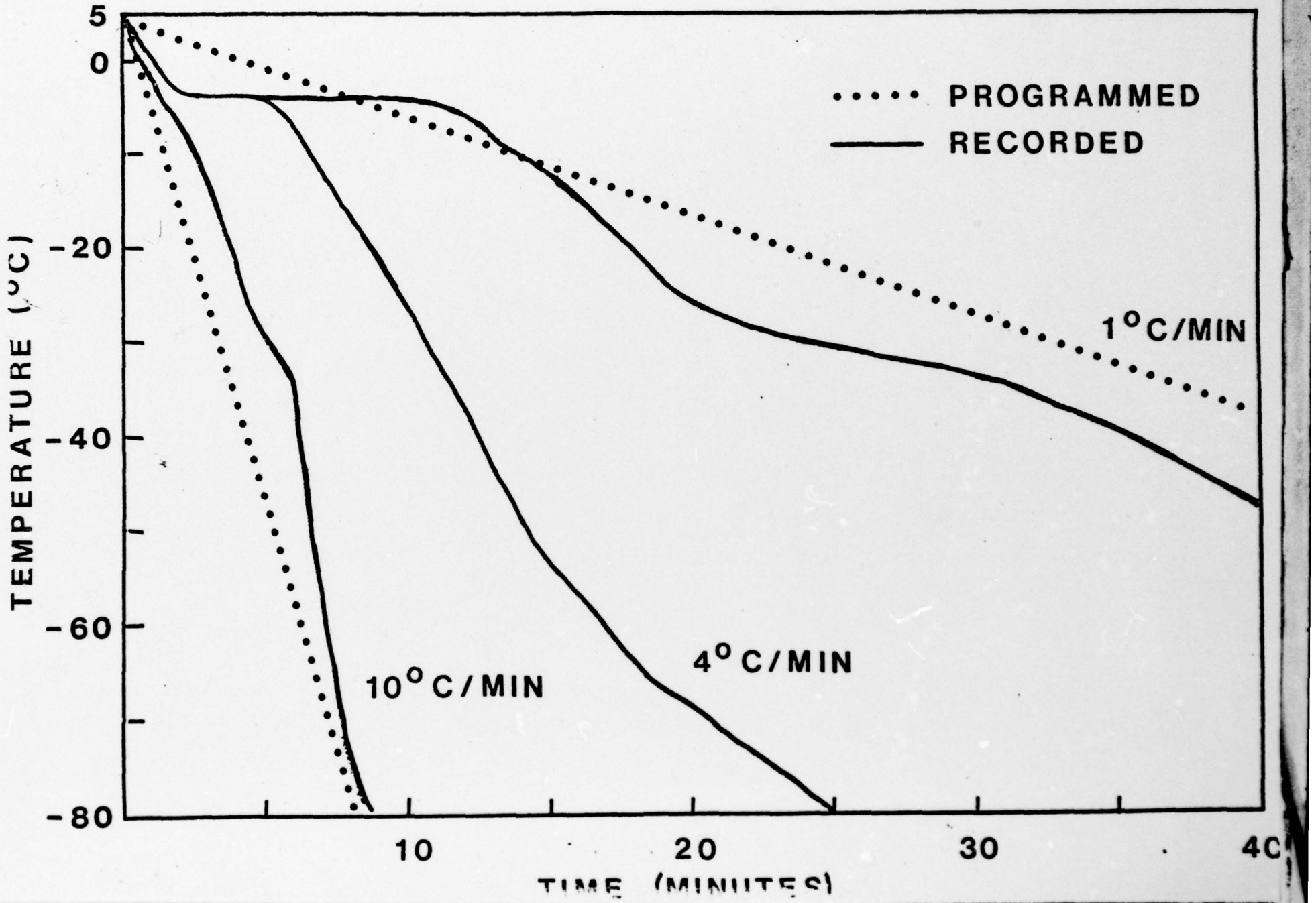
5
0
-20
-40
-60
-80
TEMPERATURE (°C)
10
20
30
TIME (MINUTES)
PROGRAMMED
RECORDED
1°C/MIN
4°C/MIN
10°C/MIN

FIGURE 1

Cooling rates of granulocytes of P. anubis. Temperature was monitored with a copper constantan thermocouple wire fixed in the center of the tubes and recorded with a Honeywell temperature recorder. The freezing rates of 1°C, and 10°C were achieved in the GV Planer programmed freezing rate controller that used liquid nitrogen. The rates recorded (solid) are plotted together with the programmed rates (dotted). The curve for 4°C per minute was derived by monitoring temperature in granulocyte suspensions in tubes placed in the bottom of a mechanical refrigerator at -80°C as described in Methods.

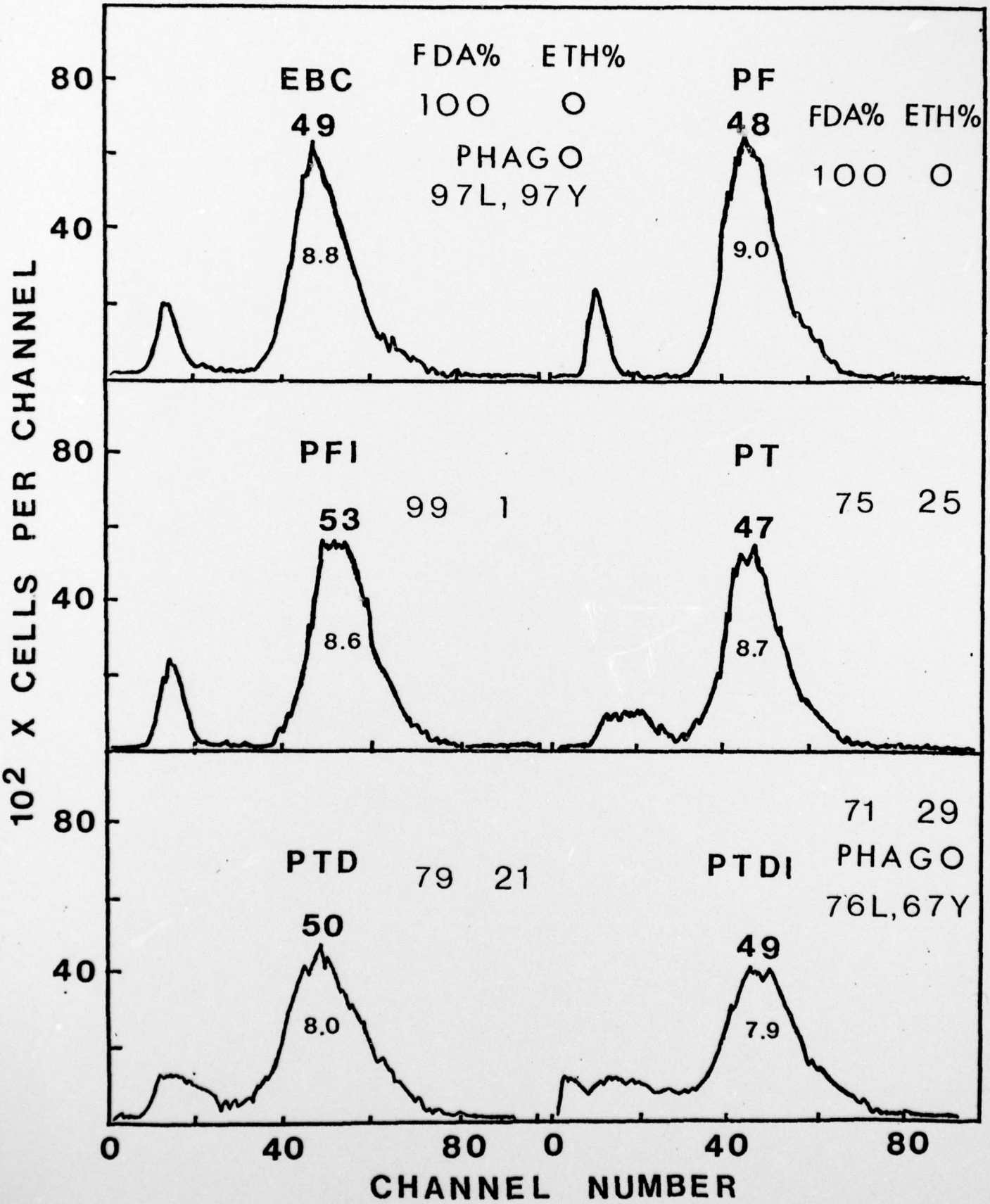
FDA% ETH%
EBC
49
100 0
PHAGO
97L, 97Y
PF
48
FDA% ETH%
100 0
8.8
9.0
PFI
53
99 1
PT
47
75 25
8.6
8.7
71 29
PTD
79 21
PTDI
PHAGO
76L, 67Y
50
49
8.0
7.9
80
40
0
40
80
10^2 X CELLS PER CHANNEL
CHANNEL NUMBER

FIGURE 2

The volume distributions for the stages of a typical experiment. The treatments (EBC, PF, PFI, PT, PTD, AND PTDI are described in the Methods and Materials Section. The percentages of fluorescein diacetate-positive and ethidium-positive cells are given to the right of the curves. Numbers within the distributions are the numbers of granulocytes (divided by 1,000) in the 0.1 ml aliquot counted by the Channelyzer. The numbers above the peaks are the median channel numbers (midrange) of the populations. Percentages of phagocytic granulocytes are shown for L latex, Y yeast.

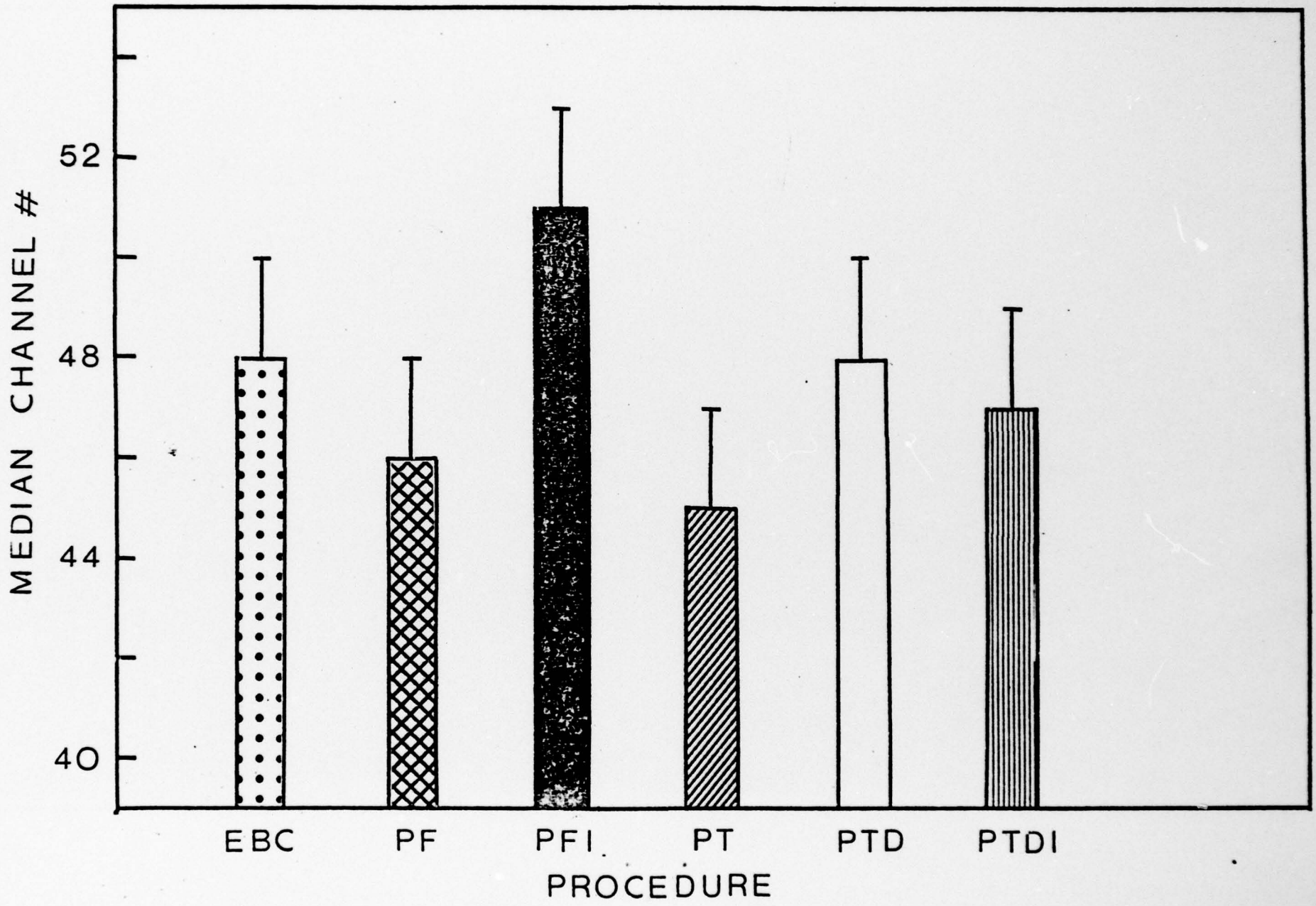
MEDIAN CHANNEL #
52
48
44
40
EBC
PF
PFI
PT
PTD
PTDI
PROCEDURE

FIGURE 3

Median channel numbers of baboon granulocytes (P. anubis) after isolation by elutriation and after each procedure; EBC, PF, PFI, PT, PTD, PTDI are the same as given in the text and tables. The bars represent means ± S.D.

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Granulocytes were isolated from the buffy coat of baboon blood by counterflow centrifugation. They were cooled at 4°C per minute to -80°C in 2.0 ml volumes containing 1 x 10^7 granulocytes and stored for 1 to 3 weeks in liquid nitrogen. The medium consisted of 5% DMSO, 6% hydroxyethylstarch (HES), 4% human serum albumin and 6 mM glucose in Normosol-R, pH 7.1. Tubes were thawed manually at 42°C in a water bath. The yield after thawing was 9% ± 14%. Granulocytes isolated from fresh blood all fluoresced green with fluorescein diacetate (FDA). Less than 1% of cells showed nuclear uptake of ethidium

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bromide (EB). After freezing and thawing, an average of 79 ± 8% granulocytes fluoresced green with FDA and 21 ± 8% fluoresced red with EB. About 98 ± 2% of granulocytes isolated from fresh blood ingested latex or yeast. Of thawed granulocytes diluted dropwise with a medium containing 7% HES, 4% albumin and 6 mM glucose in Normosol-R, 75 ± 9% fluoresced green with FDA. When incubated with latex or zymosan at 37°C for 30 minutes and then washed twice, 76 ± 5% demonstrated serum dependent latex ingestion and 67 ± 8% ingested zymosan.