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RECOVERY, STRUCTURE AND FUNCTION OF DOG GRANULOCYTES AFTER FREE--ETC(U)
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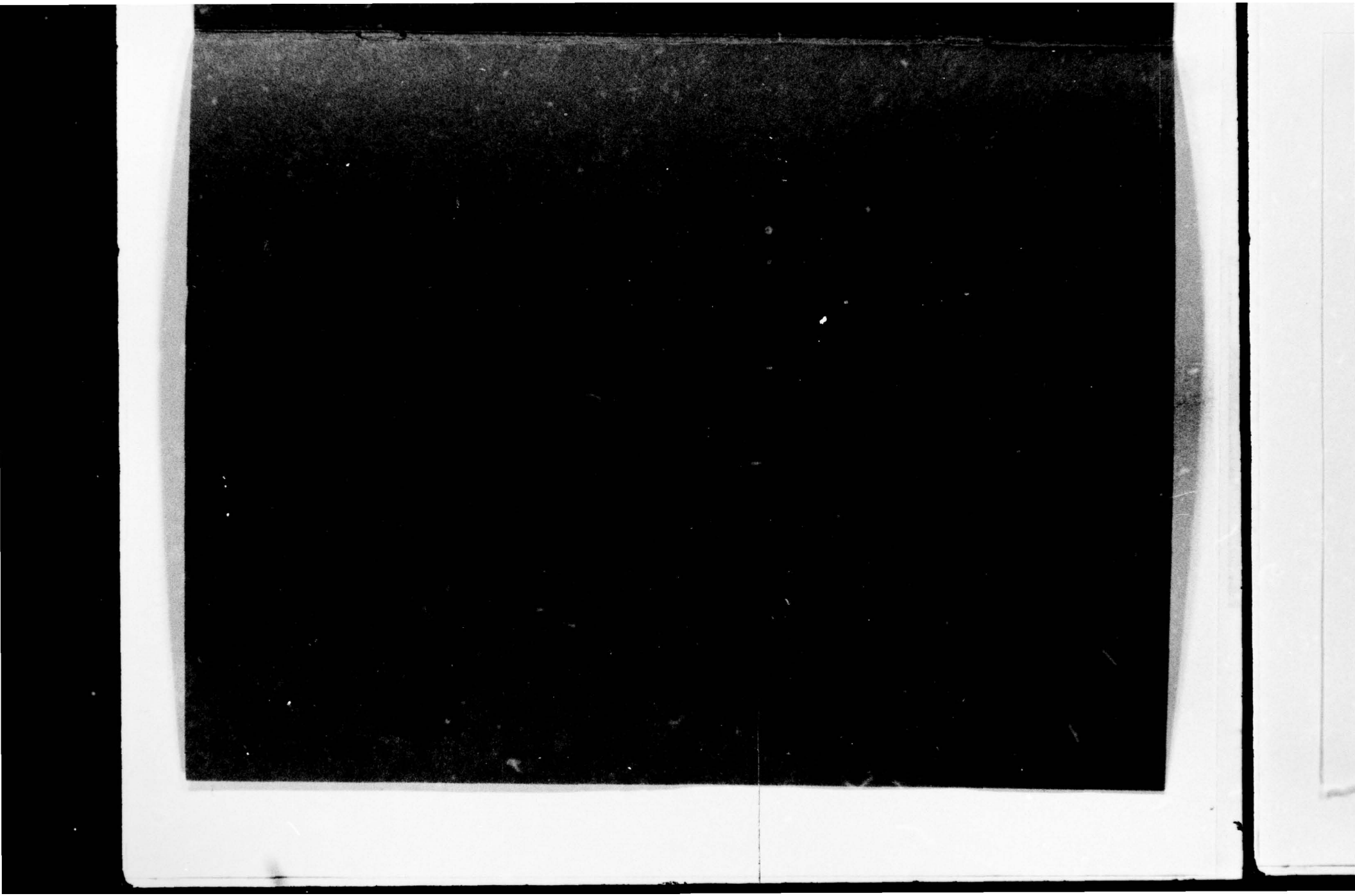

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The recovery, structure and function of dog granulocytes were determined before and after freeze preservation. Leucocytes were isolated from defibrinated or anticoagulated whole blood and subsequent erythrocyte sedimentation on a column of 2:1 dextran (6 percent)-Isopaque (33.9 percent). Granulocytes isolated by these procedures were examined for changes in O_2 consumption associated with phagocytosis, in vitro directed migration (chemotaxis), bactericidal activity, and ultrastructure

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20. ABSTRACT (continued)

before and after freezing. Granulocytes were frozen in DMSO (7.5 percent) and autologous serum or HBSS and 20 percent autologous serum at the rate of -1°C/min to -80°C and stored in liquid N_2 vapor. After freeze preservation, O_2 consumption associated with phagocytosis was decreased by 54 and 64 percent for granulocytes isolated from defibrinated or from ACD anticoagulated blood, respectively. Bactericidal activity is only slightly depressed in samples from either isolation method after freeze preservation when compared to the prefreeze controls, but granulocytes isolated from defibrinated blood are significantly less effective in killing bacteria than those from ACD anticoagulated blood. Chemotactic response after freeze preservation was completely inhibited in granulocytes isolated from defibrinated blood. Exposure of granulocytes to ACD inhibited chemotaxis prior to freezing, but the granulocytes responded chemotactically after freeze-thaw and additional washing. The ultrastructure of granulocytes observed before and after freeze-thaw was similar for cells isolated by both methods. However, nuclear, cytoplasmic and granular changes observed were slightly greater in granulocytes isolated from defibrinated blood. Dog granulocytes isolated by either method withstood freeze preservation in DMSO to a degree not previously reported. It is concluded that dog granulocytes freeze preserved by these methods are functional in vitro, but that phagocytic, directed migration, and bactericidal functions and ultrastructure are impaired to different degrees, according to the method of isolation and preparation for storage. These results indicate the need for continued investigation on the effects of storage variables on the preservation of granulocytes. The increased efficacy of granulocyte transfusions in conjunction with antibiotic therapy against microbial infection during agranulocytosis or granulocytopenia has been well documented. An effective method for granulocyte preservation would minimize logistical problems (typing, preservation, matching and transportation) in increasing the availability of this therapy to combat casualties. In order to meet such a requirement, this model system was developed for studying granulocyte preservation methods.

PREFACE

This work was supported in part by National Institutes of Health Contract 1-Y01-HB-5-0025-00.

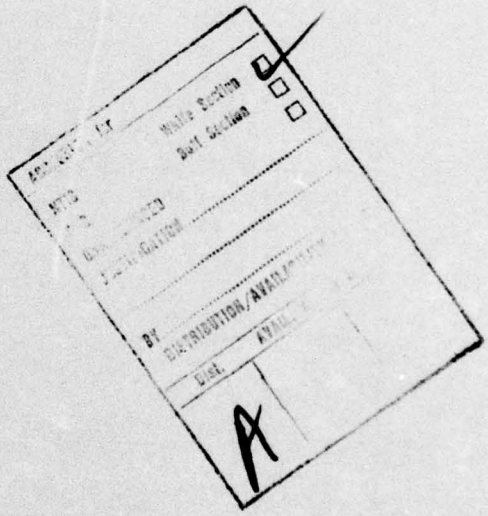

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

The need for a readily available supply of viable granulocytes has become increasingly apparent in recent years[18] due to the use of these phagocytes in the supportive management of several clinical conditions.[17,20] A reduction in the number of circulating granulocytes or a decrease in bone marrow granulocyte production is seen in leukemic individuals, in cases of radiation-induced or fibrous bone marrow aplasia, in patients given immune suppressive drugs for prevention of organ graft rejection, and in cases of cancer chemotherapy. In all of these granulocytopenic crises, the transfusion of clinically effective granulocytes could elevate the concentration of functional white blood cells and aid in combatting sepsis or bacteremia.[15,17,33]

The freeze preservation of small volumes of human peripheral blood leucocytes has been reported by several investigators.[6,7,29] The method most frequently employed was a graded slow freezing process utilizing dimethylsulfoxide (DMSO) as a cryoprotectant with a rapid thaw at a later time. Data indicating successful survival in one case, using the above technique, were based on the resumption of phagocytosis of yeast particles by the granulocytes.[29]

In contrast, Crowley et al.,[9] using different criteria for viability but a similar freezing technique, have reported a low recovery of human granulocytes. Marked reduction in motility and metabolic stimulation during phagocytosis was characteristic of these freeze-preserved granulocytes. Furthermore, Malinin has stated that human granulocytes cannot withstand freezing in the presence of DMSO.[23] Thawed cells did not regain motility, developed large cytoplasmic bubbles, and eventually disintegrated when incubated up to 24 hours. Strong emphasis was placed on vital dye uptake and exclusion as well as cytological and cytochemical observation on fixed cells at the light microscopic level.

More recently, Lionetti et al.[21] have reported the development of a new system compatible with current blood banking techniques, where human granulocytes were frozen in the presence of residual hydroxyethyl starch (HES) and 5 percent DMSO. Postthawed and recovered cells obtained under these

procedures were considered viable by the in vitro assays, trypan blue dye exclusion, myeloperoxidase and bactericidal activity. These results have encouraged our belief that freeze preservation methods can be devised that yield functional cells after preservation.

The importance of ice crystal formation on granulocyte structural integrity as well as the importance of solution effects should be ascertained and demonstrated[22,24] as it has for other cell types. It has also been recognized that anticoagulants, i.e., ACD, heparin, EDTA, sodium citrate, etc. adversely affect O_2 consumption and chemotaxis.[2,16,27,28] However, McCullough and colleagues did not find any difference in bactericidal function or nitroblue tetrazolium (NBT) activity until 96 hours storage at 4°C with various anticoagulants.[25] Also, it is known that the cryopreservative DMSO is toxic to blood cells and exhibits a temperature dependent effect.[3] Crowley and associates[10] have also demonstrated that granulocytes are sensitive to washing by centrifugation. These lesions can occur at several steps in the isolation, storage and preparation of granulocytes for clinical use. The summation of these effects results in the present poor yield and viability of stored granulocytes. If these multiple effects on granulocyte structure-function by isolation, freezing, thawing and recovery methods can be identified, controlled or eliminated, successful methods for preservation could be formulated.

The neutrophil is a highly specialized cell that requires the complete integration of its subcellular systems for function, i.e., the recognition, migration, phagocytosis and neutralizing of foreign organisms and other biological material. Synchronized structure-function assays (in vitro) are required for maximum information concerning the viability of its integrated systems before and after preservation: (1) in vitro directed migration (chemotaxis), (2) phagocytosis, and (3) bactericidal activity. Therefore, we have integrated a number of in vitro techniques to obtain information on how this cell is affected by isolation, storage and preparation for use (Figure 1). Synchronized electron microscopic, physiological and biochemical assays have been utilized.

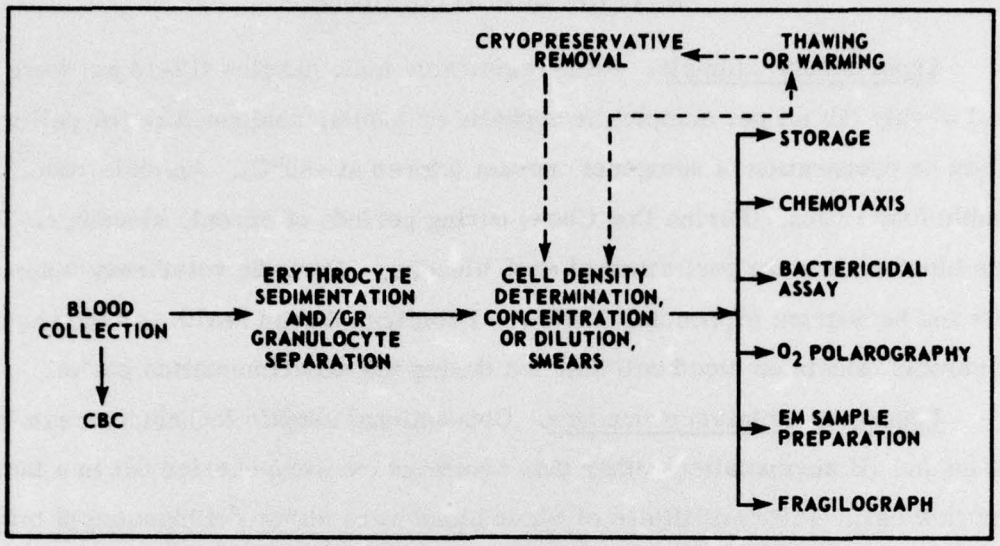

Figure 1. Flow diagram of synchronized in vitro viability assays, storage and handling sequences for dog granulocytes

While the disruptive effects of ice crystals are best demonstrated by ultrastructural freeze-substitution studies, effects due to anticoagulants, cryoprotectants, physical trauma, dehydration, increased solute concentration, changes in pH, and precipitation of solutes can be defined best by in vitro functional assays. In vitro tests of viability used most frequently in the past have been dye exclusion techniques, pseudopod formation, motility and phagocytosis.[7]

It was the objective of this investigation to study an isolation system which eliminated exposure to anticoagulants and washing by centrifugation, which would serve for base-line structure and function assays for lesion identification. Defibrination of whole blood on unsiliconized glass beads with minimum gentle shaking and subsequent erythrocyte sedimentation on dextran-Isopaque was chosen as a minimally manipulated base-line reference in order to eliminate exposure to anticoagulants and centrifugal washing. Anticoagulation of whole blood with acid citrate dextrose (ACD; NIH Formula A), commonly used in blood banking, followed by erythrocyte sedimentation on dextran-Isopaque column, was chosen as a standard human cell handling technique for reference comparison.[5]

MATERIALS AND METHODS

Experimental animals. AKC-registrable male beagles (12-15 kg) were bled weekly (50 ml per sample) by cephalic or jugular venipuncture for cell isolation or preparation of autogeneic serum (stored at -80°C). Animals received double food rations (Purina Dog Chow) during periods of chronic bleedings. Routine blood tests were performed at each bleeding. Periodic veterinary inspection and hemogram (Appendix, Table A-1) confirmed good health and the absence of chronic effects on blood cell kinetics during the experimentation period.

Leucocyte isolation procedure. Conventional aseptic techniques were followed and all manipulations other than venipuncture were carried out in a laminar flow unit. Fifty milliliters of whole blood were either defibrinated (5 min gentle shaking with fifty 5-mm nonsiliconized glass beads) or anticoagulated with acid citrate dextrose at 15 percent (v/v) (ACD, NIH Formula A).

Leucocytes in serum or plasma were isolated by sedimentation (room temperature (RT) 60 min) of the erythrocytes on 2:1 dextran (6 percent w/v)-Isopaque (33.9 percent v/v) (Dextran T-500, Pharmacia, Upsala, Sweden; Isopaque, Winthrop Laboratories, New York, N. Y.).[5] After cell density determination (Model B Coulter Counter, Coulter Electronics, Hialeah, Florida), the WBC rich serum or plasma was maintained at room temperature (RT, 21-22°C) until stored or until synchronized assays were carried out at approximately 4 hours postisolation before freezing and 2 hours postrecovery after thawing. If required, repeated washing was carried out by centrifugation at RT (50-100 x g for 10-15 min) using Hanks' balanced salt solution minus Ca^{++} and Mg^{++} (HBSS-minus) (Gibco, Grand Island, New York). Leucocytes were resuspended in autologous serum, HBSS-minus or a mixture of 40 percent Eagles' minimum essential medium (MEM) (Microbiological Associates, Rockville, Maryland), 40 percent HBSS-minus and 20 percent autologous serum (4:4:2 medium) depending upon the assay to be performed.

Freeze preservation. After isolation and concentration (1-2 x 10^7 leucocytes/ml) in 100 percent autologous serum or HBSS-minus with 20 percent

autologous serum, 1-ml volumes of concentrated leucocytes were frozen in 7.5 percent (v/v) dimethylsulfoxide (DMSO) in 2-ml freezing vials (Nunc, Sweden) at -1°C/min from ambient to -80°C in a Linde BF-4 controlled-rate freezing apparatus (Union Carbide Corporation, Indianapolis, Indiana). Frozen vials were stored in a liquid nitrogen vapor in a cryogenic storage container for 96 hours.

Thawing. Vials were rapidly thawed (~130°C/min) by agitation in a 37°C water bath. DMSO was removed by a slow single step tenfold dilution (unless otherwise indicated) in HBSS-minus (37°C). Cell suspensions were pooled, maintained at room temperature (21-22°C) and centrifuged (RT, 100 x g for 15 min). The supernatant fluid was aspirated and the leucocytes were resuspended in autologous serum or 4:4:2 medium.

O2 consumption during bacterial ingestion. O_2 consumption before and after freeze preservation was measured with a YSI model 53 Oxygen Polarograph (Yellow Springs Instrument Company, Yellow Springs, Ohio) fitted with a Teflon-covered platinum oxygen electrode anaerobically submerged in a 3-ml leucocyte suspension (4×10^6 leucocytes/ml in either autologous serum or 4:4:2 medium). The suspension was gently agitated for 4 min at 37°C in a siliconized chamber. Basal O_2 consumption was determined for 5-10 min before challenge with Escherichia coli (ATCC 25922) (Bacterium-granulocyte ratio 0.75 ± 0.25). The O_2 consumption increase associated with phagocytosis was measured for an additional 5-10 min. Samples were run in duplicate. Data are expressed in nanoatoms $O_2/10^6$ leucocytes or $/10^6$ granulocytes per hour for basal and stimulated rates, respectively.[19]

Chemotaxis. Chemotaxis (in vitro directed migration) was measured in Sykes-Moore Chambers (Schleicher and Schuell, Inc., Keene, New Hampshire). In controls, the lower chamber contained 80 percent MEM and freshly thawed 20 percent autologous serum and was divided from the upper chamber by an 8-μm pore diameter Selectron membrane filter. The experimental, treated lower chambers contained 300 μg Salmonella typhosa lipopolysaccharide (0901, Difco Laboratories, Detroit, Michigan) to activate the serum containing

chemotactic factors. Upper chambers were loaded with 4×10^6 leucocytes in fresh autologous serum or 4:4:2 medium (prefreeze controls) or freshly thawed autologous serum or 4:4:2 medium (postthawed controls). Chambers were incubated for 3 hours at 37°C in a 95 percent air-5 percent CO_2 humidified atmosphere. Filters were removed, rinsed, stained, and cleared according to Boyden[4] and mounted in Pro-Texx (Lerner Laboratories, Stamford, Connecticut) air side down. Polymorphonuclear granulocytes (PMNG) were counted in five high power fields (HPF) (450X) from the filter surface of the chemotactic factor side to 20 μm deep in the membrane. A chemotaxis index was calculated based on the mean number PMNG/HPF that migrated across the 8-μm membrane in response to serum activation by 300 μg S. typhosa endotoxin, divided by the mean number PMNG/HPF that migrated in control chambers to the same depth.

Bactericidal assay. The bactericidal assay technique used was a modification of that used by Shoji and Vogler.[30] Escherichia coli (ATCC 25922) in the form of a Bactrol Disk (Difco Laboratories) were incubated overnight in trypticase-soy broth (TSB) with rotary shaking on a variable speed shaker in a 37°C environmental room. The bacteria were centrifuged at 1500 x g for 10 min at room temperature. The TSB supernatant fluid was aspirated and the bacterial pellet was resuspended in fresh TSB and incubated for an additional hour with shaking in the 37°C room. The culture was washed two times in sterile isotonic saline (1500 x g, 10 min), resuspended in approximately 2 ml of HBSS-minus, and transferred to a cuvette for turbidity reading in a B & L Spectronic 20 (Bausch and Lomb, Inc., Rochester, New York) at 620 nm using a blank of HBSS-minus. The OD was then adjusted to 0.6 by dilution with HBSS-minus. This suspension was diluted 1:1 with HBSS-minus resulting in a bacterial concentration of approximately 2.5×10^7 colony forming bacteria (CFB)/0.1 ml.

A 0.4-ml volume of this diluted bacterial suspension was added to 4 ml of leucocyte concentrate and incubated with shaking at 37°C (treated culture). Also 0.2 ml of the diluted bacterial suspension was added to 1.8 ml MEM and 0.2 ml autologous dog serum (control). At time zero, 1 ml each of these initial diluted

suspensions was carried through several dilutions (10^5-10^8) for precise CFB density determination by standard pour plate technique. After 24 hours incubation at 37°C, CFB were determined using a Quebec colony counter (American Optical Corporation, Buffalo, New York).

In addition, at time zero, 0.1 ml from each control and treated culture tube was diluted and mixed with 1 ml of sterile double distilled water in order to check the initial CFB concentration after hypotonic lysis of leucocytes. The contents of these tubes were diluted to 10^6. For determination of total viable and total intracellularly viable bacteria, at time zero and after 120 min of incubation, control and treated tubes were centrifuged five times at 900 x g for 2 min, and washed using filter sterilized HBSS-minus. The final pellet was resuspended in 1.1 ml double distilled water, mixed, diluted to 10^6 and the CFB density determined as above. A final specific plate count was made at 140 min using the control bacteria. For each experimental count, a final dilution of 10^6 was plated in triplicate. Specific plate counts were determined from four dilutions (10^5-10^8). The percent bacterial inhibition was determined by dividing the total number intracellular viable bacteria by the total number viable bacteria, subtracting the quotient from one and multiplying by 100.

Electron microscopy. Leucocytes for electron microscopic examination were prepared from suspensions in autologous serum or in 4:4:2 medium. Samples were either centrifuged gently (approximately 50 x g) and resuspended in 0.5 N cacodylate-buffered 7.5 percent sucrose before fixation or fixed directly in the suspending medium by a dropwise addition of an equal volume of 4.5 percent distilled glutaraldehyde in cacodylate-buffered 7.5 percent sucrose over a 2- to 3-min period with gentle agitation. Leucocytes were allowed to fix in suspension for 30-45 min, then pelleted (approximately 800 x g), and fixation continued for an additional 15 min before replacement of fixative with cacodylate-buffered sucrose and storage at 4°C until processing. Specimens were dehydrated in graded alcohols, stained in block with uranyl acetate, and embedded by standard methods in either Luft's epon or in Spurr low viscosity

resin. Ultrathin sections were cut on a Porter-Blum MT-2 ultramicrotome (Ivan Sorvall Corporation, Norwalk, Connecticut) stained with uranyl acetate and lead citrate, and examined in a Siemens Elmiskop I electron microscope. Cacodylic acid, sucrose, uranyl acetate, lead citrate and glutaraldehyde were purchased from Fisher Chemical Company, Fairlawn, New Jersey. Epon 812 and Spurr low viscosity resin were purchased from Ladd Research Industries, Inc., Burlington, Vermont, and Polysciences, Inc., Rydal, Pennsylvania, respectively.

RESULTS

Cell isolation and recovery. The effect of anticoagulation by defibrination with glass beads or ACD on the leucocyte population composition is illustrated in Table 1. The percent composition of leucocytes was not altered by

Table 1. Summary of Leucocyte Differentials after Isolation by Defibrination and Sedimentation on 2:1 Dextran (6 percent)-Isopaque (33.9 percent)*

Method	No. of Observations	Granulocytes				Agranulocytes	
		Neutrophils		Eosinophils	Basophils	Lymphocytes	Monocytes
		Bands	Segmented				
Peripheral Whole Blood	67	2 ± 2	65 ± 7	2 ± 2	1 ± 1	27 ± 8	3 ± 2
Defibrination							
Pre-Sedimentation	5	1 ± 1	69 ± 3	1 ± 1	0	27 ± 5	1 ± 1
Post-Sedimentation	9	1 ± 1	57 ± 13	1 ± 1	0	40 ± 14	1 ± 1
Frozen and Thawed†	5	1 ± 1	36 ± 2	3 ± 1	0	57 ± 3	3 ± 1
Frozen and Thawed‡	5	1 ± 1	71 ± 5	1 ± 1	0	27 ± 4	1 ± 1
ACD							
Post-Sedimentation & Washing	6	1 ± 1	64 ± 7	2 ± 1	0	31 ± 5	2 ± 1
Frozen and Thawed‡	6	1 ± 1	72 ± 7	2 ± 1	0	23 ± 8	2 ± 1

*Each differential per observation was examined in duplicate with a mininum of 200 cells counted per slide. Values ($\bar{x}$ ± SEM) were adjusted to 100%.

†Initial observations were determined after frozen leucocyte concentrates were thawed rapidly to 37°C and diluted rapidly with cold HBSS-minus and washed by centrifugation (4°C).

‡These observations were determined after frozen leucocyte concentrates were thawed rapidly to 37°C and diluted slowly with HBSS-minus previously warmed to 37°C and washed by centrifugation at room temperature.

either method of anticoagulation prior to sedimentation when compared to peripheral whole blood values. However, after sedimentation the mean percent polymorphonuclear granulocytes (%PMNG) isolated from defibrinated whole blood declined, but not significantly. There were no differences in composition in leucocytes from ACD-anticoagulated whole blood. Table 1 also indicates that after freezing and thawing, %PMNG composition in preparations from defibrinated whole blood decreased greatly (45 percent total loss of PMNG) when thawed cells were rapidly diluted tenfold with cold HBSS-minus and washed by centrifugation at 4°C. A slow tenfold dilution of thawed samples in 37°C HBSS-minus and washing by centrifugation at room temperature prevented this loss of PMNG isolated by either method of anticoagulation. Total recovery of leucocytes and PMNG is tabulated in Table 2. Prior to freezing there was a significant loss of cells by either method, but the greatest number of cells were

Table 2. Percent of Total Leucocytes and Polymorphonuclear Granulocytes (PMNG) after Isolation from Defibrinated or ACD-Anticoagulated Dog Blood, Followed by Sedimentation on 2:1 Dextran (6 percent)-Isopaque (33.9 percent)

Method	Percent of Total Cells Recovered			
	Pre-Freezing		Post-Freezing and Thawing	
	Leucocytes	PMNG	Leucocytes	PMNG
Defibrination*	33 ± 5	31 ± 5	42 ± 3	43 ± 4
ACD*	57 ± 5	59 ± 3	47 ± 7	51 ± 6

* n=5

recovered from the ACD-anticoagulation method. After freezing, thawing and washing, the magnitude of the cell loss was similar between each method. Recovery of PMNG paralleled that of total leucocyte recovery in either method.

O_2 consumption. The functional status of isolated granulocytes was determined before freezing and after freezing and thawing. Initial basal O_2 consumption studies of leucocytes isolated by defibrination gave rates of 225 ± 29 n-atoms $O_2/10^6$ leucocytes per hour when tested within 2 hours of isolation. Time course studies indicated that this value fell to 82 ± 13 and 35 ± 5 n-atoms $O_2/10^6$ leucocytes per hour after 3 and 5 hours postisolation, respectively.

Since blood from several animals was frequently pooled, values were statistically examined for individuals, and for pairs and triplets of donor animals. Although individual variations were sometimes large, they were also time-dependent.

Table 3 shows basal and stimulated rates of O_2 consumption. The mean basal O_2 consumption rate before freezing and thawing appeared to have been

Table 3. The Effect of Freeze Preservation on Dog Granulocyte Basal* and Stimulated† O_2 Consumption Rates after Isolation from Defibrinated or ACD-Anticoagulated Dog Blood, Followed by Sedimentation on 2:1 Dextran (6 percent)-Isopaque (33.9 percent)

Method	Pre-Freezing		Post-Freezing and Thawing	
	Basal	Stimulated	Basal	Stimulated
Defibrination‡	56 ± 26	527 ± 24	44 ± 8	262 ± 17
ACD‡	71 ± 18	445 ± 54	39 ± 4	177 ± 27

*At resting rate, in n-atoms $O_2/10^6$ leucocytes/hr.

†During phagocytosis of live Escherichia coli (ATCC 25922) (Bacterium/granulocyte ratio = 1) in n-atoms $O_2/10^6$ PMNG per hr. The bacterial O_2 consumption rate was found to be negligible and was not considered in these calculations.

‡n = 5.

greater in leucocytes isolated by ACD-anticoagulation, but it was not significant. After freezing and thawing the difference in rate was not as great between the two methods or significantly different from the prefreezing values. After stimulation with live E. coli, the difference between basal and stimulated rates was

greatest in PMNG isolated from defibrinated blood, either before or after freeze preservation. These values were not significantly different before freezing, but exhibited a significant increase ($p < 0.01$) over PMNG isolated from ACD-anticoagulated blood after freezing. The mean percent loss of activity after freeze-preservation was 54 percent for PMNG isolated from defibrinated blood and 64 percent from PMNG isolated from ACD-anticoagulated blood.

Chemotaxis. PMNG isolated by the defibrination method exhibited a 2.3 times greater chemotactic response than controls did before freezing (Table 4).

Table 4. The Effect of Freeze Preservation on the Chemotactic Activity of Dog Granulocytes Isolated from Defibrinated or ACD-Anticoagulated Dog Blood, Followed by Sedimentation on 2:1 Dextran (6 percent)-Isopaque (33.9 percent)

Method	Chemotaxis Index*	
	Pre-Freezing	Post-Freezing and Thawing
Defibrination†	2.3 ± 0.3	1.2 ± 0.1
ACD†	1.1 ± 0.2	1.8 ± 0.3

*$\bar{x}$ ± SEM of the number of stimulated granulocytes per high power field(HPF), divided by the number of control(unstimulated) granulocytes per HPF that migrate across an 8 µm membrane. Stimulation medium contained 300 µg/ml Salmonella typhosa lipopolysaccharide in MEM plus 20% autologous dog serum.

†n = 5.

After freezing-thawing, there was only an insignificant increase relative to controls. The total number of these PMNG migrating after freeze preservation was reduced to 62 percent. Prior to freezing, chemotactic response was absent after ACD exposure and washing in these experiments. However, after freezing and thawing, the washed PMNG exhibited a 1.8-fold increase in directed migration.

Bactericidal activity. PMNG isolated from both defibrinated and ACD-anticoagulated blood exhibited bactericidal activity of similar magnitude before and after freeze preservation (Table 5). Bactericidal activity after freeze preservation was significantly reduced ($p < 0.01$) in PMNG isolated by the defibrination method, but was not reduced by the ACD-anticoagulation method. Bactericidal activity was also significantly ($p < 0.01$) different between treatments after freeze preservation.

Table 5. The Effect of Freeze Preservation on the Dog Granulocyte Bactericidal Activity after Isolation from Defibrinated or ACD-Anticoagulated Dog Blood, Followed by Sedimentation on 2:1 Dextran (6 percent)-Isopaque (33.9 percent)

Method	% Inhibition of Bacterial Growth*	
	Pre-Freezing	Post-Freezing and Thawing
Defibrination†	93.5 ± 2.2	83.1 ± 5.9
ACD†	93.0 ± 4.8	92.6 ± 4.4

* $\bar{X}$ ± SEM, after 2 hrs exposure at 37°C with a bacteria to granulocyte ratio of 2.7 ± 0.9.

† n = 4.

Ultrastructure. Granulocytes isolated from defibrinated blood are normal in appearance (Figure 2) prior to freezing and exhibit well-segmented nuclei with electron-dense heterochromatin condensed along the nuclear envelope with less electron-dense euchromatin medially and extending to the nuclear pores. The cytoplasm contains small mitochondria, Golgi material, glycogen, microtubules and the characteristic electron-dense neutrophil granules in a cytoplasmic matrix of moderate electron density characteristic of mature neutrophils. The cell surface is irregularly populated with small projections, and frequently a well-defined pseudopod can be observed, which is relatively devoid of larger cytoplasmic organelles. Occasionally, membrane bound vacuoles are observed

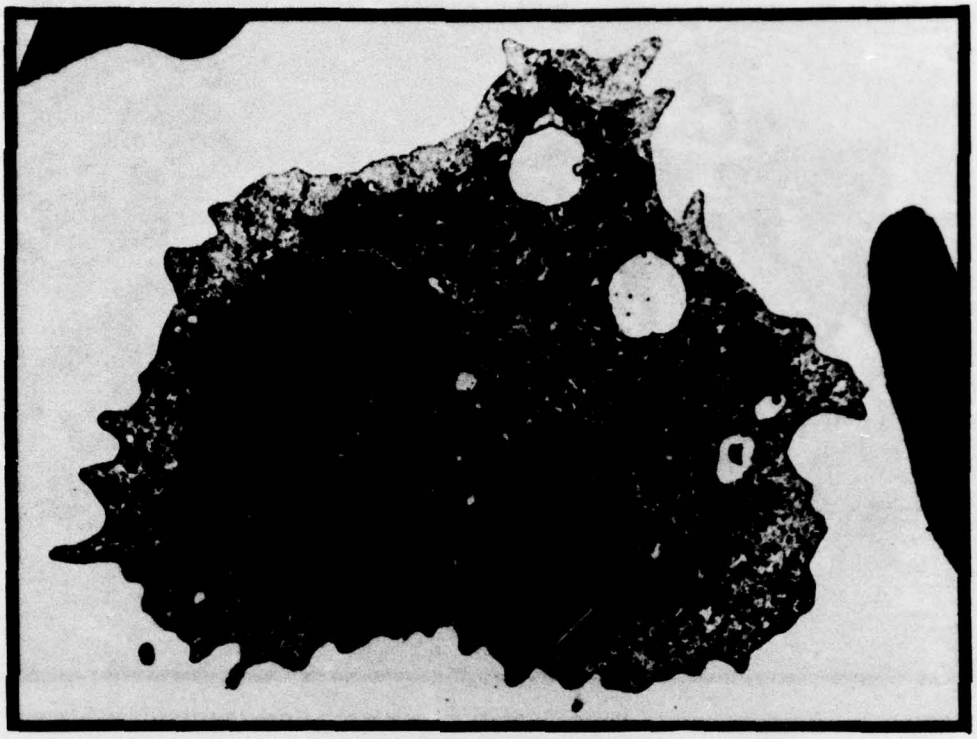

Figure 2. Representative morphology of a normal dog granulocyte after isolation from defibrinated blood and prior to freezing. The nucleus is well-segmented, with normal chromatin patterns. The cytoplasm contains small mitochondria, Golgi material, glycogen, microtubules, and the characteristic electron-dense neutrophil granules (see text). X 11,500.

that appear empty or contain bits of cellular debris. Vacuoles devoid of any electron-dense material may be due to the plane of section through extracellular space and therefore may not be membrane-limited intracytoplasmic vacuoles.

In this study, PMNG isolated from defibrinated blood and subjected to freezing and thawing are significantly more varied morphologically than pre-freeze control PMNG (Figure 3). The changes observed include rounding of cell surfaces, mottled and more electron-lucent cytoplasm with condensed

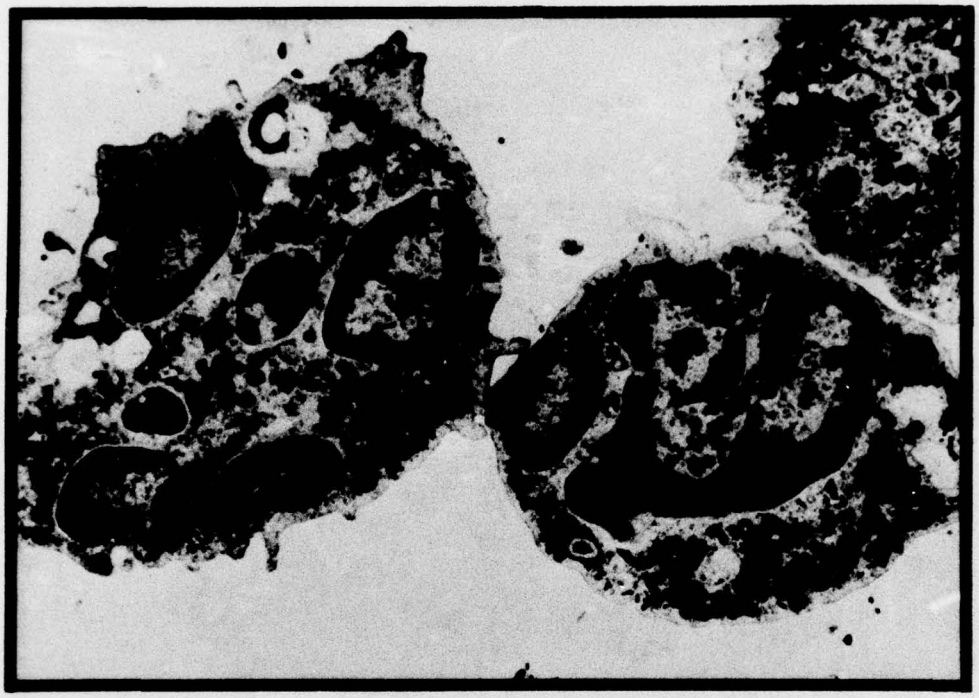

Figure 3. Appearance of representative dog granulocytes (isolated from defibrinated blood) after freezing in 7.5 percent DMSO and thawing. General cell morphology is good, but more varied than in control (prefreeze) cells, and more subtle changes can be observed, including granules which are more heterogeneous in size and in electron density, more common cytoplasmic vacuoles, occasional cytoplasmic lipid figures, and more rounded and less active cell profiles. X 9,700.

organelles, pyknotic and homogeneous nuclei, swollen or ruptured cells and cellular debris. However, there are still numerous structurally intact PMNG in these freeze-thaw preparations, although subtle changes are observed in many of them. These PMNG are often more rounded and the granules are heterogeneous in both size and electron density; some appear to have lost a portion of their contents. These changes may indicate damage to the plasma membranes and osmotic imbalance. Membrane-bound cytoplasmic vacuoles are more common relative to the prefreeze controls and possibly contain ingested cellular

debris. Some of these vacuoles contain small lipid figures, others may be swollen, homogeneous, and less electron-dense lysosomal granules. A mottled appearance, characterized by small, unbounded and irregularly shaped clear patches, is observed in the cytoplasmic matrix, particularly in areas of pseudopod formation. Increased lucency of the euchromatin, swelling and rounding of nuclei, and/or homogenization of the electron density of the nucleoplasm may indicate osmotic imbalance.

Fresh isolated PMNGs from ACD-anticoagulated blood exhibit excellent morphology (Figure 4). The cells display active surfaces, well-segmented nuclei of good electron density, tightly arranged cytoplasmic organelles and matrix, well-fixed mitochondria and only infrequent cytoplasmic membrane-bounded vacuoles. Occasionally, as in all preparations studied, mottled cytoplasm, electron-lucent cells, and some engulfment of cell debris are observed.

The morphology of ACD-exposed PMNG after freezing and thawing was similar to that of the PMNG isolated from defibrinated blood and freeze-thawed (Figure 5). Most of the PMNG appeared intact but significant numbers of disrupted cells or cells with rounded profiles, peripheral cytoplasmic vacuoles, mottled cytoplasmic matrix and dense or swollen organelles were observed. The presence of large amounts of cell debris possibly stimulated the activity of the intact PMNG, and frequent clusters of PMNG around debris were observed. In this preparation, enlarged and more electron-lucent neutrophil granules were a more common finding.

DISCUSSION

We have shown that the method of isolation used significantly influenced the recovery, structure and function of granulocytes before and after cryogenic preservation. Furthermore, the importance of synchronized structure-function assays is illustrated by comparing dog granulocytes isolated from defibrinated blood to dog granulocytes isolated from conventionally ACD-anticoagulated blood.

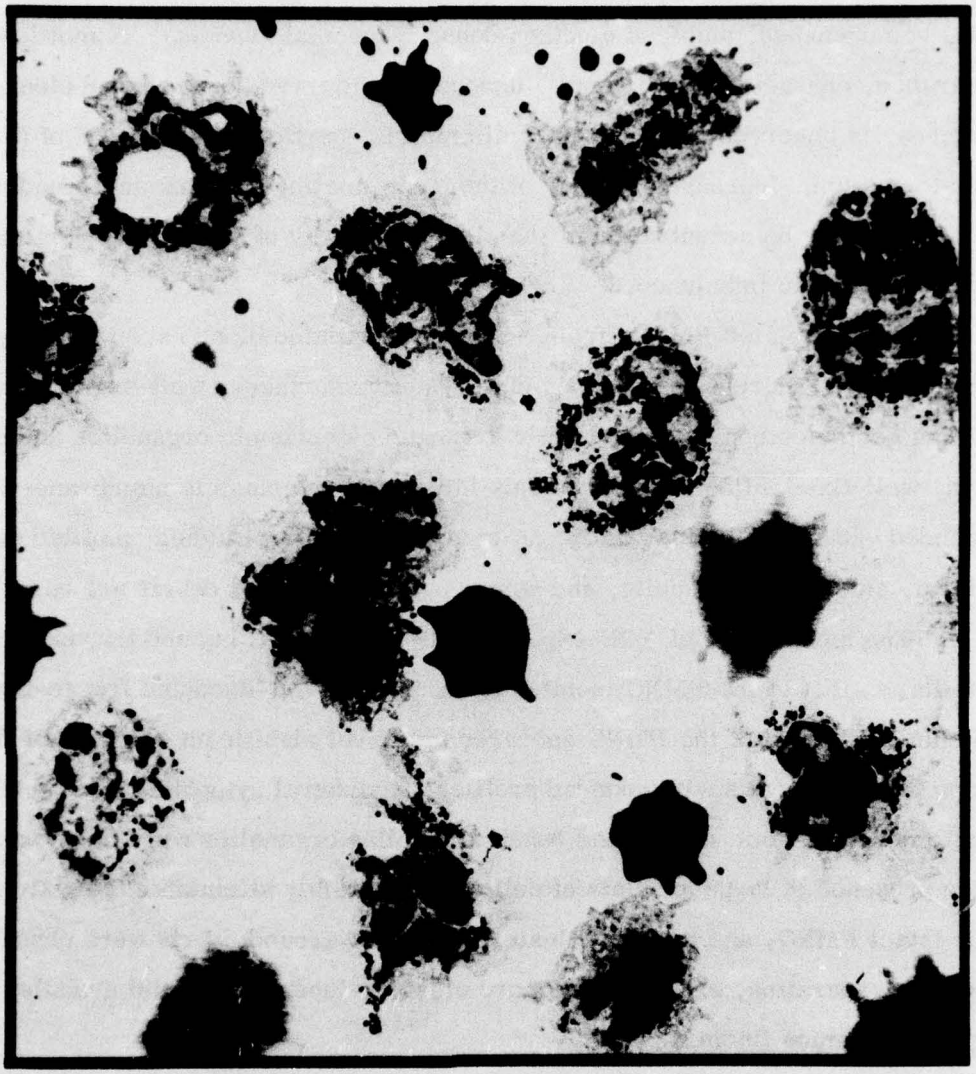

Figure 4. Appearance of representative dog granulocytes isolated from blood anticoagulated with ACD and subsequently washed free of ACD by serial centrifugation prior to freezing. The morphology of these PMNG is uniformly very good and similar to that demonstrated in Figure 2 for control granulocytes from defibrinated blood. X 3,750.

The defibrination process causes the greatest loss of total leucocytes and PMNG, but does not change the percent PMNG in the leucocyte population.

Figure 5. Appearance of a representative dog granulocyte (isolated from ACD anticoagulated blood) after freezing in 7.5 percent DMSO in 4:4:2 medium (see text) and thawing. Morphology is characteristic of mature neutrophils, with some increase in the heterogeneity of granule profiles and occasional mottling of the cytoplasmic matrix. X 13,100.

Total leucocyte and total PMNG recovery consistently paralleled each other before and after freeze preservation. This indicates that PMNG loss was random and not selective as reported by other investigators for other systems.[9,21]

Electron microscopic evidence indicates that cellular debris (and possibly associated humoral factors) generated during the isolation of leucocytes by defibrination induced an increased O_2 consumption. This O_2 consumption decreased with time after isolation or washing.

Granulocytes isolated by either method lost a significant amount of O_2 consumption activity after freeze preservation. However, the significant

difference in O_2 consumption associated with phagocytosis in granulocytes isolated from defibrinated blood, both before and after freeze preservation, indicates a detrimental effect of ACD exposure and/or washing by centrifugation.

The degree of stimulated activity in the dog granulocytes isolated by ACD-anticoagulation of whole blood correlates well with those data reported by Crowley et al.[9] for human PMNG isolated and preserved at -80°C in the presence of 6-10 percent DMSO.

Patten and colleagues[28] found that certain anticoagulants (citrate, oxalate, EDTA) inhibit the formation of chemotactic factors in human serum, whereas heparin apparently inhibits chemotaxis by another mechanism, even after its removal by washing. It is possible that certain anticoagulants may reversibly or irreversibly induce lesions in this system that may complicate PMNG storage[2,27,28] as evidenced by their effects on this in vitro functional test. Our data support such a thesis, since exposure to ACD initially inhibits (reversibly) chemotaxis of dog granulocytes, but after freeze preservation and additional washing granulocytes are capable of in vitro directed migration. In contrast, the defibrination process increases the sensitivity to injury during freeze preservation.

The defibrination process also increases the sensitivity of bactericidal function to injury during freeze preservation. This may be due to partial degranulation during the defibrination process, but apparently not to the extent that granulocyte function is compromised when exposed to bacteria in the range used in these experiments. These data agree with previous reports that leucocytes resumed phagocytosis and bactericidal activity after freeze preservation.[1,6,7,21,29] Various bacteria-PMNG ratios have been examined[1,21,30,31] in order to determine that optimum range for maximum sensitivity. The mean ratio used in this study followed those recommended values (2-4 bacteria/PMNG) but dose-response studies should be performed for each model system in order to determine that ratio which may allow a clearer distinction between isolation and storage variables.

Our electron microscopic observations correlate well with previous light and electron microscopic studies that have noted rounding of human granulocytes with perinuclear swelling,[23,26] degranulation, vacuolation and cell membrane damage.[9] However, it should be noted that under the conditions employed in this study, dog granulocytes survived freezing and thawing and retained their normal structural integrity[32,34] in most cells. PMNG isolated from ACD-anticoagulated blood were more structurally unchanged than PMNG isolated from defibrinated blood.

It is clear that PMNG isolated by either method and then frozen, thawed and recovered are altered morphologically and functionally. It is emphasized that each procedure induces damage to different subcellular systems. For example, freeze-preserved granulocytes isolated from defibrinated blood for base-line reference showed the greatest stimulated O_2 consumption differences after phagocytosis, yet were characterized by reduced chemotactic and bactericidal activity and structural integrity when compared to granulocytes isolated from ACD-anticoagulated blood. Exposure to ACD and serial washing by centrifugation prior to freezing apparently inhibits the chemotactic response, even after ACD removal, but PMNGs respond chemotactically after freezing, thawing and additional washing. It is obvious that neither method yielded granulocytes which are functionally unaltered by all assays employed in this study. Most studies on the freeze preservation of granulocytes[6,7,9,23,29] have indicated little resumption of granulocyte function after preservation by methods similar to those employed in our investigations. To our knowledge, only this study and the recent work of Lionetti et al.[21] indicate even a partial survival of complete in vitro granulocyte function after freeze preservation. This further illustrates the importance of combined assays for complete granulocyte function, especially when attempting to determine the effects of handling and storage techniques on these complex cells.

It is necessary to conduct experimental investigations in an animal model that offers good correlation with man in order to exert maximum experimental

control over in vitro and in vivo efficacy tests and to be able to extrapolate the results to use with human cells. The dog model has been used for in vitro and in vivo studies of transfusion of fresh or frozen bone marrow or fresh granulocytes with some success.[8,12-14] Maximizing the in vitro effectiveness of dog granulocytes after storage should increase the probability of improving the stored granulocytes in vivo effectiveness.

The ability to store large quantities of HL-A typed peripheral granulocytes for clinical use in neutropenic disorders rather than relying upon the availability of fresh compatible cells has obvious advantages. Also, extending the shelf life of granulocytes by improvements in liquid storage methods merits attention. Although liquid stored ($4^{o}C$) granulocytes are thought to have a short shelf life (2-3 days), recent evidence suggests considerably longer in vitro viability[11] (also French, unpublished observations). However, for long-term storage, freeze preservation appears to be the most promising means of extending the short shelf life of these cells. The progress reported in the present study appears to set the stage for the achievement of this task.

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APPENDIX

Table A-1. Hemogram for Male Beagles Used from 1 July 1974 to 1 March 1975

	n	$\bar{x} = \hat{\mu}$	R	$s = \hat{\sigma}$
RBC ($x10^9$/ml)	67	7.093	5.8- 8.6	0.711
WBC ($X10^6$/ml)	67	8.954	4.7-16.8	2.388
HGB (g/100ml)	63	15.680	4.9-18.9	1.900
HCT (%)	66	47.700	17.6-55.0	5.148
SEG (%)	67	64.851	46.0-83.0	7.440
LYM (%)	67	27.716	12.0-48.0	7.594
EOS (%)	67	2.373	0 - 9.0	1.969
BND (%)	67	1.612	0 - 8.0	1.592
MON (%)	67	2.985	0 -10.0	1.832
BAS (%)	67	.015	0 - 1.0	0.121
WT (KGM)	70	13.303	9.1-15.7	2.290
VOL (ML)	92	38.179	0 -52.0	12.587