A FLOW CYTOMETRIC ANALYSIS OF DAMAGE AND EFFECT OF ADDITIVES ON THE MEMBRANE STRUCTURE OF FROZEN RED BLOOD CELLS

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ABSTRACT

Kalman Salata, Research Advisor

Cryopreservation of erythrocytes (RBC) became possible when it was discovered that when mixed with glycerol, RBC could be successfully frozen and thawed. The disadvantages of this method were that glycerol had to be removed from the RBC because it is harmful to the patient and the expense involved. A simple, inexpensive wash procedure has not been developed. The ideal cryoprotectant should be safe for infusion. A one-step technique that eliminates the wash process would have significant impact on transfusion medicine. Protection of RBC from freeze injury and acceptable in vivo survival must also be achieved. The present study was designed to develop a one-step technique using phospholipid-like additives to achieve less than 2% supernatant hemolysis and to develop a flow cytometric assay for quantitation of membrane damage. Cryopreservatives used were hydroxyethyl starch (HES), Viastarch, and CellSep. L-carnitine and urea were used as phospholipid-like additives. Supernatant hemolysis was calculated from total and supernatant hemoglobin concentrations. RBC count and mean

corpuscular volume (MCV) were determined on all samples before and after cryopreservation to further evaluate hemolysis. Cryopreservation with CellSep resulted in the least amount of hemolysis, but the goal of less than 2% hemolysis, with and without additives, was not achieved. The RBC count did not change significantly, but the MCV was slightly increased in all samples post-thaw. The use of flow cytometric analysis was investigated to quantitate RBC membrane damage. Fluorescent labeled antibodies to intracellular antigens were used to label cells with sufficiently large holes to allow entry and attachment of the labeled antibody. Cells without holes should not allow the labeled antibody to enter and attach. There was no difference in fluorescense of cells that had not been frozen and those that had, disallowing the use of these assays to quantitate membrane damage.

This work is dedicated to my sons, Chris and Daniel, my parents Dick and Marge, and my Mother-in-law Marion whose patience, support, motivation and inspiration made this project possible.

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INTRODUCTION

Cryopreservation of Erythrocytes

Red blood cell (RBC) components are indicated for transfusion to patients with symptoms of chronic anemia that can result from malignancy, renal failure, or diseases such as sickle cell anemia and thalassemia. Other indications for red blood cell component therapy include surgical procedures, truama cases, and battlefield injuries. The need for transfusion should be determined based on the clinical status of the patient along with laboratory testing results. Transfusion of RBC results in improved oxygen carrying capacity and an increase in RBC mass.[1] Twenty percent of all United Nations casualities were transfused with whole blood during the Korean Conflict, with 5.54 pints issued for each soldier wounded.[2]

The majority of transfusions during the 1980s and 1990s have been with packed RBC (PRBC).[3] PRBC are the preferred component for most transfusion therapy because of the reduced volume of the product. This is especially important for patients who cannot tolerate the volume overload associated with transfusion of whole blood products. These patients would include those with cardiac problems, elderly patients, and neonates.

PRBC are prepared from a unit of whole blood by centrifugation in a refrigerated centrifuge or by allowing the cells to sediment under refrigeration. The plasma is then expressed into an integrally attached satellite bag leaving just the RBC. The PRBC should have a final hematocrit of 70-80%.[1] When prepared in a closed system, the expiration date is 21-35 days depending on the anticoagulant used or 42 days if additive solutions are used.

The American Association of Blood Banks (AABB) *Standards* defines frozen/thawed deglycerolized red cells as "RBC stored in the frozen state at optimal temperatures in the

presence of a cryoprotective agent, which is removed by washing before transfusion."[4] There are 3 methods of RBC cryopreservation using glycerol that are approved by the Food and Drug Administration (FDA) and the AABB. The methods are 1) high glycerol (40% w/v glycerol), 2) agglomeration (40% w/v glycerol), and 3) low glycerol (20% w/v). Frozen/deglycerolized RBC have qualities not found in whole blood or PRBC. Specifically, the absence of incompatible antibodies present in the plasma, reduced plasma proteins that cause allergic reactions, reduced exposure to platelets and white blood cells (WBC), reduced human leukocyte antigen (HLA) alloimmunization, and the absence of citrates and other additives.[5] Because all these components are washed out of the product during the deglycerolization process, frozen RBC are one alternative for patients with rare phenotypes, with antibodies to high incidence antigens or patients who are IgA deficient (Table 1).[6] The value of freezing RBC is the preservation and long term storage of autologous units, rare units and group O units.

Table 1
Indications for Freezing Red Blood Cells

1) autologous units for patients with rare phenotypes or multiple alloantibodies
2) rare and selected units lacking antigens that commonly cause sensitization
3) units free of citrate and vasoactive substances
4) units free of WBC, platelets, plasma protein, and reduced microaggregates
5) preservation of universal donor Group 0 red cells

The risk of transfusion-transmitted viral disease from allogeneic blood is negated by using autologous blood, making it the safest blood product available today. The ability to freeze and store rare and autologous units provides an inventory of blood for patients in need of rare units in civilian and military hospitals. The ability to stockpile large volumes of O negative

and O positive blood at military depots around the world provides an inventory for military hospitals and field units during contingency operations or war. The military frozen blood supply is intended to handle the mass casualties during contingency or war, until liquid blood is shipped from the United States.

There are disadvantages associated with the use of glycerol as a cryopreservative. The processing and storage are costly in both time and money. The cost of a unit of frozen/deglycerolized RBC is about 2 to 3 times that of a unit of liquid red blood cells. The washing process is also extremely laborious and expensive. The deglycerolization of red cells takes an hour from start to finish before the product is ready for transfusion. This product is prepared in an open system; a process in which the sterile seal has been broken, resulting in a 24 hour shelf life.[7] Criteria must be met before a unit that has been frozen and deglycerolized can be used for transfusion (Table 2).[8]

Table 2
Criteria for Transfusion of Frozen Red Blood Cells

1) residual hemolysis less than 1%
2) red cell recovery greater than 80%
3) residual glycerol concentration less than 1 gram percent

A technique that provides a product that can be frozen, thawed, and immediately infused without further processing would eliminate the tedious and costly wash step and have significant patient care and military advantages over the current methods approved for use. A method of this nature that provides an acceptable product has not been successful to date. To meet AABB standards, a method for frozen blood must produce a unit of blood with an acceptable supernatant hemoglobin level, RBC recovery, post-transfusion RBC survival and must be safe for infusion to the patient. Previous studies have been done to determine the

optimum volume and concentration of cryopreservative, freeze time, and storage temperatures for various cryoprotective agents. The effects of the freezing process on the product have also been studied and have concentrated on the measurement of post-transfusion cell viability and free hemoglobin in the supernatant.[9]

Attempts have been made to preserve living tissue by cryopreservation for many years. The earliest attempts involved the freezing of eels in the 1940s by Luyet.[5] It was discovered that the eels could be successfully recovered after thawing. Experiments leading to feasible methods for the cryopreservation of blood did not begin until after World War II.[10] The use of glycerol as a cryoprotectant was discovered by accident. Studies were being conducted in a British laboratory using fructose to freeze spermatozoa. A laboratory technician thought he was adding fructose but after investigation, it was discovered that the labels on reagent bottles were illegible and he had actually used glycerol.[5] It was also discovered that contaminating RBC had tolerated the freezing process as well as the spermatozoa. Sperm cells were frozen in glycerol and thawed without lysis in 1949.[11] The spermatozoa were also found to be actively motile after thawing. In 1950, the freezing of human RBC using glycerol was reported. A small volume of RBC were cryopreserved in glycerol and successfully stored at -79°C for 3 months.[5] In 1951, a patient with leukemia was the first patient ever to receive red cells that had been frozen and there were no ill effects.[10]

Many cryoprotective agents have been tested, including polyvinylpyrrolidone (PVP), starches, sugars, dextran, and dimethylsulfoxide (DMSO). Investigators began using hydroxyethyl starch (HES) in the 1960s because it has features consistent with those of an ideal cryoprotective reagent. The ideal method of red cell cryopreservation would be one

that prevents the formation of ice crystals by allowing enough water to leave the cell resulting in a moderate intracellular hypertonicity, but not enough to cause cellular dehydration.[3] HES is a colloid that can be injected intravenously to a patient without harm and is sometimes used to improve granulocyte harvest in leukopheresis.[7]

RBC that are frozen undergo biochemical and structural changes resulting in damage to the cell membrane. This damage is the result of osmotic and biochemical effects of dehydration and the formation of ice crystals inside or outside of the cell during freezing.[12] Cryoprotective agents are necessary to prevent the damage that results from the freezing process. The major drawback to using frozen RBC is the damage and resulting hemoglobin release that occurs. This is part of the phenomenon referred to as freeze injury. Cryoprotective agents other than glycerol have been shown to limit this damage, but do not meet AABB guidelines with respect to acceptable levels of supernatant hemoglobin.[4] As mentioned previously, HES is safe for intravenous injection. Viastarch is the HES portion of a solution manufactured by Dupont which has been approved for use in the perfusion of organs for transplant.[13] CellSep is a cell separation medium manufactured by Larex, Inc., that has been tested as a potential cryoprotectant because it is a starch. Previous experiments using CellSep have shown that it causes less hemolysis and more consistent results than HES.[13] CellSep is not licensed by the FDA for injection and the effect of CellSep if injected intravenously is unknown.

Intracellular cryoprotective agents enter the cell and displace the water in the cell while in a concentration equilibrium with the solute prior to freezing. Intracellular agents that have been investigated are glycerol, dimethylsulfoxide (DMSO), ethylene glycol, ethanol,

methanol, trimethylammonium acetate, and ammonium acetate. Glycerol acts by displacing the water in the cell, thus preventing the formation of ice crystals in the cell when the temperature is sufficiently reduced to induce freezing. The inhibition of ice formation results in lower concentration of solutes which reduces the effect of freeze injury. "In its simplest form, an intracellular cryoprotective agent acts as an antifreeze."[12]

Although the first transfusion of previously frozen red blood cells was done in 1951, this product was not widely used until 1972. The problems included an absence of understanding of the principles of washing; along with the absence of support by industry to develop a system to wash RBC; and the expense associated with freezing and washing. The impact of cost as well as the time required to thaw and deglycerolize a unit resulted in the limited use of this product.

Extracellular cryoprotective agents work by creating a complex of starch and water, resulting in reduced formation of ice crystals by preventing the loss of water and reducing dehydration. Extracellular agents are macromolecules that do not penetrate the cell but form a shell around it.[7] The possibility of toxic effects and excess supernatant hemoglobin in RBC frozen using extracellular agents led researchers to the use of intracellular additives. Extracellular agents that have been considered include polyvinylpyrolidone (PVP), HES, lactose, and glucose.[12] Extensive studies using PVP with liquid nitrogen to freeze RBC revealed several problems. After thawing, red cells showed 3% hemolysis, and the infused PVP remained in the reticuloendothelial tissue and in the circulation for an extended period of time.[14] Cells were washed with crystalloid solutions in an attempt to reduce hemolysis but the opposite effect was observed. A one-step method of cryopreservation is needed which

uses an extracellular additive in the optimum concentration, as well as freeze/thaw times and temperatures without requiring washing.[12]

AABB *Standards* require that red blood cells be frozen within 6 days of collection, unless they are rejuvinated to provide red cells with optimum oxygen carrying capacity. The expiration date of frozen RBC according to AABB *Standards* is 10 years from the date of phlebotomy if stored at -65°C or colder. The AABB *Standards* require that "a method of frozen RBC preparation shall ensure minimal free hemoglobin in the supernatant solution. At least 80% of the original red cells should be recovered after deglycerolization and at least 70% viability of the transfused cells 24 hours after transfusion".[4] The problem with excess supernatant hemoglobin is the associated in vivo hemoysis and the determination that the in-vivo hemolysis is the result of immune or non-immune cause. It would be difficult to distinguish between hemolysis that was already present in the product and hemolysis caused by transfusion reaction. Another concern is the possibility that the transfusion of lysed RBC may trigger disseminated intravascular coagulation (DIC).[15] DIC is a condition in which there is an accentuated activation and consumption of coagulation factors which overwhelms inhibitory mechanisms causing ischemic tissue damage, micorangiopathic hemolytic anemia, and small thrombi within the vascular system.[16] These concerns led researchers to the use of glycerol because the extracellular agents yielded a product with more than 3% hemolysis.

RBC Membrane Structure

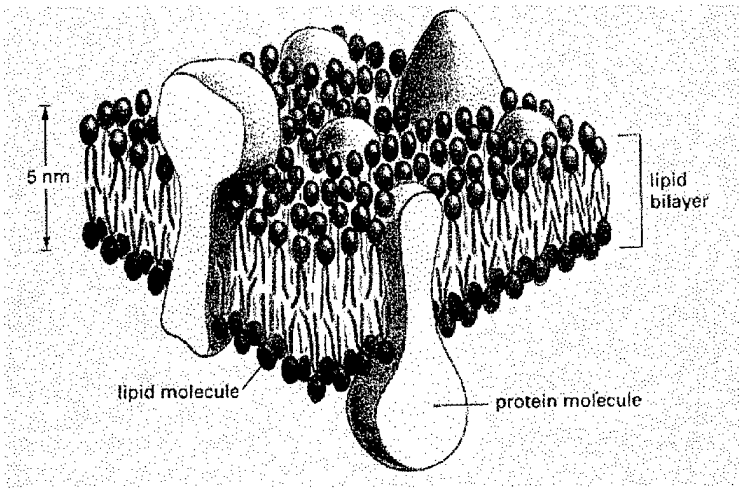
The cell membrane acts as an external boundary of the cell that regulates substances entering or leaving the cell. The membrane separates the cell from the outside world, divides organelles from other parts of the cell, segregates processes, develops

concentration gradients, and is involved in cellular communication. Membranes are characterized by tough, flexible, self-scaling (i.e., exocytosis, endocytosis, cell division), selectively permeable, active barriers. The membranes are usually two layers thick, referred to as a lipid bilayer. Membranes consist of varying amounts of protein, lipid, and carbohydrate.[17]

The lipid composition of RBC plasma membrane is 20% sphingolipids, 25% cholesterol, less than 5% glycolipids, and about 53% phosphoglycerides. The phosphoglycerides are made up of 6% phosphotidyl-inositol, 6% phosphotidyl-serine, 19% phosphotidyl-choline, and 22% phosphotidyl-ethanolamine.[18]

In 1925, Dutch biochemists, Gorder and Grendel, conducted experiments which led them to the conclusion that biological membranes were bilayers.[19] Biologic membranes, both plasma and organelle, have the same basic structure and function.[20] The fluid mosaic model is the most reasonable and widely accepted explanation of membrane structure (Fig. 1). In this model, fatty acid chains on the interior face of the bilayer form a fluid hydrophobic region with polar head groups to the exterior on each face of the bilayer. Integral membrane proteins are those proteins that pass through the membrane, such as Rh, and Band 3, and can move about freely in this fluid region. Integral proteins are held in place by a weak interaction of hydrophobic forces with their non-polar amino acid side chains, also called hydrophobic anchors. Peripheral proteins, such as Duffy blood group antigens, are located extrinsically and associate reversibly with the membrane. Peripheral proteins are held in place by ionic linkages. Integral

Figure 1. Fluid Mosaic Model, Lipid Bilayer*



*Reproduced with permission from Garland Publishing Inc., *Molecular Biology of the Cell,* 3rd Edition by Bruce Alberts, Dennis Bray, Julian Lewis, Martin Raff, Keith Roberts, and James D. Watson, 1994, page 477.[18]

transmembrane glycoproteins, such as glycophorin A and glycophorin B transverse the membrane only once.[20]

Both proteins and lipids are free to move laterally within the plane of the bilayer, but movement from one face to the other is restricted because it takes a great deal of energy to manipulate the polar head group on one side through the hydrophobic region to flip, and therefore, is not favored. Carbohydrate moieties are attached to some of the proteins and lipids of the membrane and are exposed only on the extracellular face of the membrane. Sphingomyelin and phosphatidyl-choline are found in the outer layer of the membrane. Phosphatidyl-serine and phosphatidyl-ethanolamine are found in the inner membrane layer.

The cytoskeleton (also called the spectrin membrane skeleton) is comprised mostly of proteins and maintains the biconcave disc shape of the RBC. The cytoskeleton also provides membrane stability and controls the movement and location of transmembrane proteins. It is made up primarily of spectrin, actin, and protein 4.1.

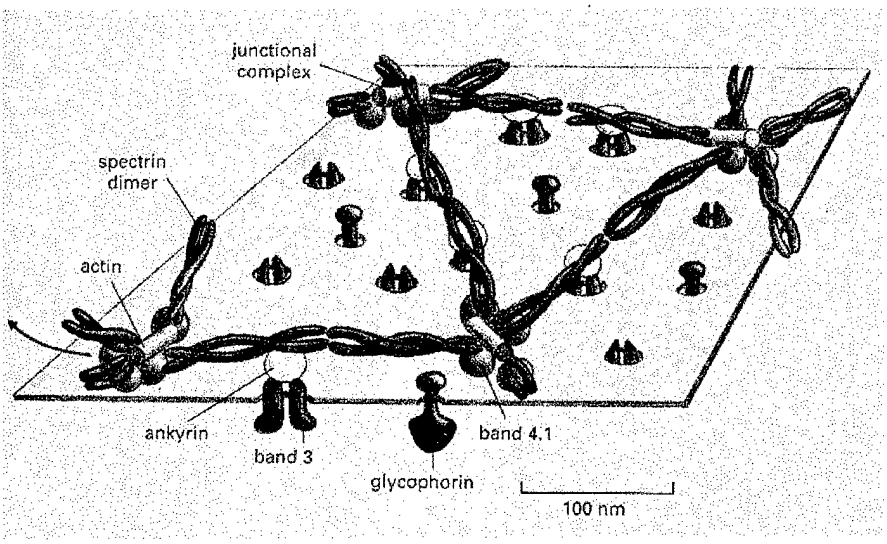
Spectrin is in the form of a tetramer formed by head-to-head interactions of the antiparallel $\alpha\beta$ spectrin heterodimer. There is an actin binding site at each end of the spectrin tetramer where the spectrin cross-links the actin filaments forming a two-dimensional lattice. This lattice covers the cytoplasmic side of the bilayer. The actin filaments are short strings of about fourteen actin monomers. Tropomyosin stabilizes the actin filaments. Each actin filament holds six spectrin tetramers forming a hexagonal complex. Protein 4.1 is bound to the ends of the spectrin tetramer and also provides stability to the structure. Protein 4.1 is also attached to glycophorin which penetrates the

bilayer linking the spectrin complex to the bilayer. The cytoskeleton is bound to the membrane bilayer by ankyrin and protein 4.1. Ankyrin is attached to the β-subunit of the spectrin heterodimer which links spectrin to the amino terminus of band 3 in the cytoplasm (Fig. 2).[20]

The fluidity of the bilayer is important in membrane transport processes and enzyme activities required for the cell to survive. Membrane fluidity depends on the composition of the bilayer and temperature. The fatty acid composition of the membrane changes in response to the environment in order to maintain relative fluidity. Cis-double bonds cause kinks in the fatty acyl chains which make it more difficult to pack them together making the bilayer more fluid. When the temperature decreases, fatty acid chains with more cis-double bonds are produced to prevent the bilayer from becoming more rigid and maintain fluidity.[18]

Greenwalt, et al., established that during storage, the red cell membrane loses cholesterol, phospholipids, and proteins by the phenomenon of microvesiculation.[21] During this process, tiny 50-200 nm, hemoglobin-containing vesicles with membranes lacking spectrin and ankyrin are shed from the stored red cells. The quantity of microvesiculation increases as length of storage increases.[22] Studies indicated that cholesterol is lost during the first weeks of storage followed by loss of phospholipids. The process of microvesicle formation is secondary to this disruption of the membrane lipids.[23] The membranes of microvesicles contain glycophorins, lipids, band 3 and 4.1 proteins, and actin and have also been shown to demonstrate blood group

Figure 2. Spectrin-Based Cytoskeleton *



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antigens.[24] The proteins band 3 and 4.1 and glycophorin A have been identified on vesicle membranes using immunoblotting and periodic acid Schiff staining.[25]

Flow Cytometry

The physical characteristics of cells can be measured using flow cytometry. The principle of flow cytometry is based on cells passing single file through an intense beam of light at a specific wavelength. The light source is usually a laser. Numerous cells can be analyzed individually and multiple parameters such as size, complexity, and presence of specific antigens can be measured using flow cytometry. The distinguishing feature of flow cytometry is that the cells pass one at time through the beam of light.[26] Laminar flow is generated by forcing sheath fluid under pressure through a nozzle forming a fine stream. The sample is aspirated into an insertion rod that passes through the middle of the fine stream of sheath fluid. The cells then pass single file through this focused stream (Fig. 3).[26] As the cell passes through the beam of light, light is scattered through reflection and refraction from cell surfaces and internal structures. Forward angle scatter is detected by a photo-diode located just off the axis of the excitation beam. Forward scatter measures cell size. A large cell will scatter more light than a small cell. Side scatter or right angle scatter is detected by an orthogonal detector located 90° to the axis of the excitation beam. Side scatter detects light reflected by internal organelles and is a measure of the cell's internal complexity. A highly complex or granulated cell such as a neutrophil will reflect more light than a cell with a single simple nucleus such as a lymphocyte (Fig. 4).[26]

Figure 3. Flow Cell and Interrogation Point*

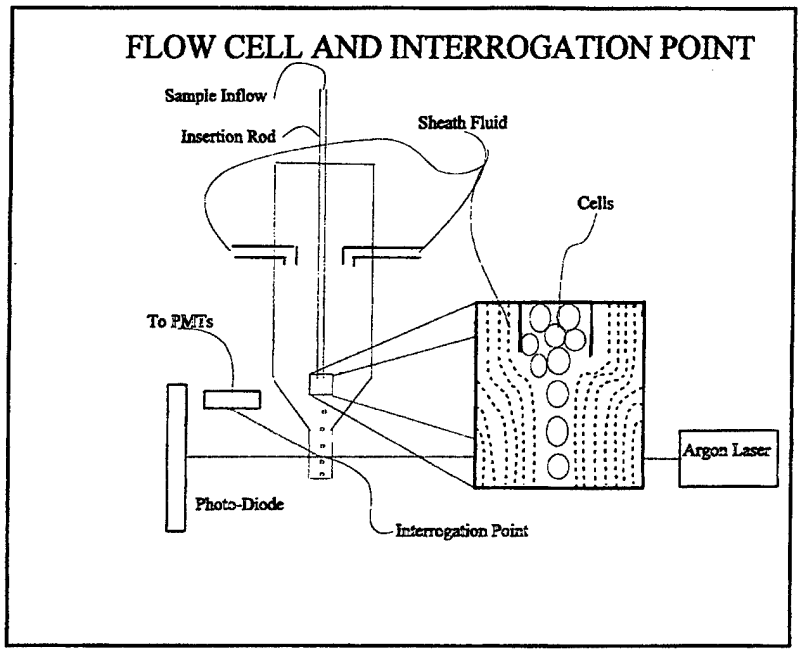

*Diagram adapted from Study Guide for Flow Cytometry, D. Anderson, R. Doe, R. Hensley, and Dr. I Frank showing how laminar flow is produced using pressure and sheath fluid.[26]

Figure 4. Forward Angle Scatter*

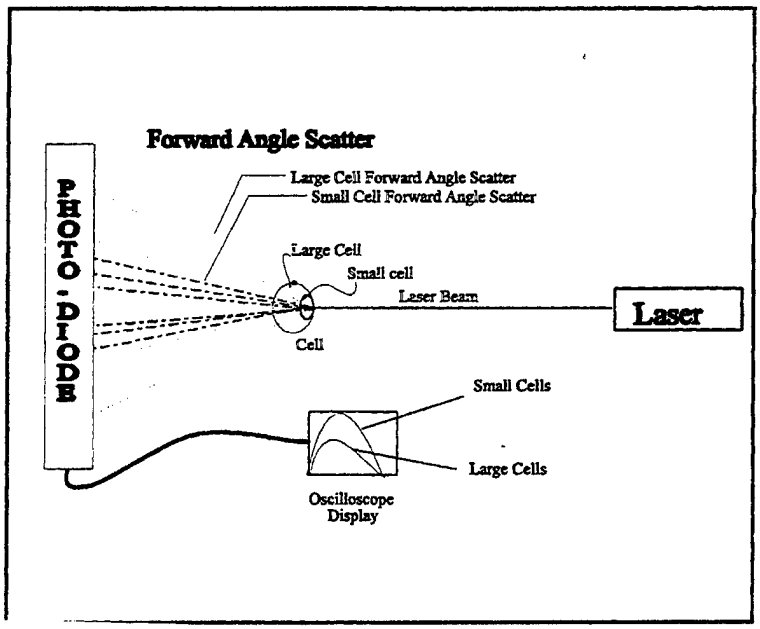

*Diagram adapted from Study Guide for Flow Cytometry, D. Anderson, R. Doe, R. Hensley, and Dr. I Frank[26] showing forward angle scatter which is related to the size of the particle and is detected by the photo-diode.

Gating is the process of selecting a specific population of cells for study. The specific population can be identified using an antibody specific to the population to be studied which has been labeled with a fluorochrome. Fluorescein isothiocyanate (FITC) is one of the most commonly used fluorochromes.[26] The gate for the population to be studied is made by drawing a circle around that population (Fig. 5).[26]

Flow cytometry is most often used in the detection and quantitation of specific antigens on the surface of cells. Monoclonal antibodies with a fluorescent compound attached are usually used to detect these cell surface markers. As cells with fluorescent antibody attached pass through the beam of light they absorb light and immediately produce fluorescence at a slightly lower wavelength. Photomultiplier tubes (PMT) or a photo-diode collects the fluorescence and translates the light into electrical signals that can be captured and recorded.[27]

In the present study, flow cytometry was used to assess membrane integrity and to determine the number of cells with membrane damage in samples that have been frozen and thawed using the extracellular preservatives HES, CellSep, and Viastarch. The damage may be small holes in a major fraction of the cells, large holes in a smaller fraction of the cells, or complete lysis of some cells. Phospholipid-like compounds added to the cells may provide some protective effect.[28] The hypothesis was that the addition of phospholipid like additives, such as L-carnitine and urea, would provide protection of the membrane from freeze injury. The protection would come from integration of the additives into the membrane structure to prevent or repair damage because they are similar to the compounds that make up the membrane.

Figure 5. Gating Technique*

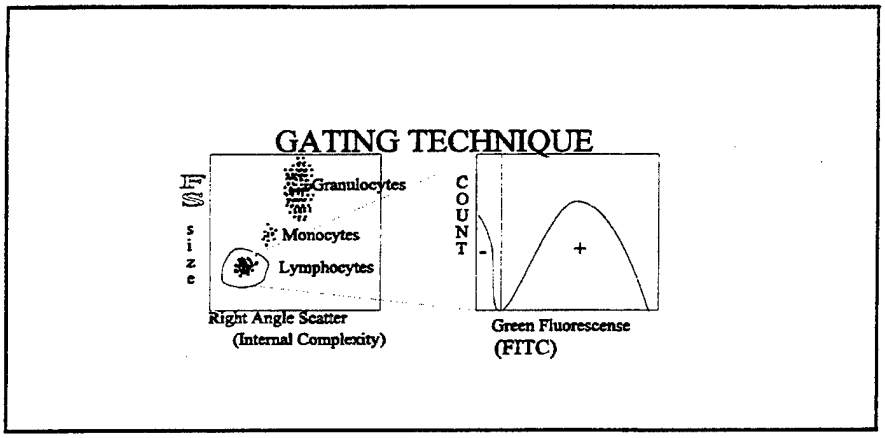

*Diagram adapted from Study Guide for Flow Cytometry, D. Anderson, R. Doe, R. Hensley, and Dr. I Frank showing how the gating technique is used to isolate the cell population to be studied by flow cytometric analysis.[26]

MATERIALS and METHODS

RBC Collection and Preparation

Informed consent was obtained before collecting whole blood from human volunteers acceptable by FDA and AABB *Standards*. The units were drawn for platelet studies and were collected by the donor collection section staff at the Walter Reed Army Institute of Research (WRAIR) Annex according to established procedure. A standard blood collection bag containing citrate phosphate adenine-1 anticoagulant (CPDA-1, Baxter Travenol, Chicago, IL) was used to collect approximately 450 cc of whole blood from each volunteer. The units were centrifuged to separate the platelets and plasma from the red cells. The packed red cells were used for this study. Samples were held at room temperature (20-24° C) for a maximum of 6 hours during preparation for cryopreservation. Packed red blood cells were prepared by centrifugation at 2500 x g for 10 minutes in a Beckman Accuspin-FR centrifuge at 22° C.

Cryoprotectant Preparation

Cryopreservation of RBC was accomplished with hydroxyethyl starch-5 (HES-5), Viastarch, or CellSep, Cell Separation Medium (Larex, Inc., St. Paul, MN). The HES-5 was prepared by Robert Williams of the Red Blood Cell Preservation Research Division, WRAIR. Viastarch, the HES portion of a solution manufactured by Dupont for perfusion of transplant organs, was provided by Dr. Harold Meryman. The HES-5 and Viastarch were prepared as 30% w/v solutions in phosphate buffered saline (PBS). The Cellsep was prepared as 35% w/v solution in PBS. PBS was prepared using 1 tablet (SIGMA Chemical Company, St.Louis, MO) dissolved in 200 mL deionized water to yield a

0.01M sodium and potassium phosphate buffer containing 0.0027 M potassium chloride and 0.137 M sodium chloride.

Cryopreservation

One mL of RBC was added to 12 X 75 Sarstedt plastic tubes using a 1 mL tuberculin syringe. For samples cryopreserved with HES-5 and Viastarch, 0.6 mL of cryoprotectant was added to the 1 mL of cells, and samples frozen with CellSep (Larex, Inc., St. Paul, MN) were mixed with 0.8 mL of cryoprotectant. All cryoprotectants were added to the packed red cells using a tuberculin syringe. The test tubes were vortex mixed with a Vortex Genie (Scientific Industries, Inc., Bohemia, NY, Model K-550-G) set at the lowest setting on the mixer until the RBC and cryoprotectant were well mixed. Polypropylene microtubes, 400 uL (Evergreen Scientific, Los Angeles, CA) were filled with aliquots of the RBC/cryoprotectant mixture. The samples were frozen in liquid nitrogen by floating them on a small wood raft with holes drilled for the tubes suspended by a string (Fig. 6). The samples were left in the liquid nitrogen for a minimum of one minute.

Thawing

All samples were immediately removed from the liquid nitrogen after being submerged for 1 minute and thawed. The samples were thawed immediately after freezing by placing the tubes in the wood raft directly in a circulating 40° C Exacal EX-100 water bath (NESLAB Instruments, Inc., Newington NH) for 2 minutes.

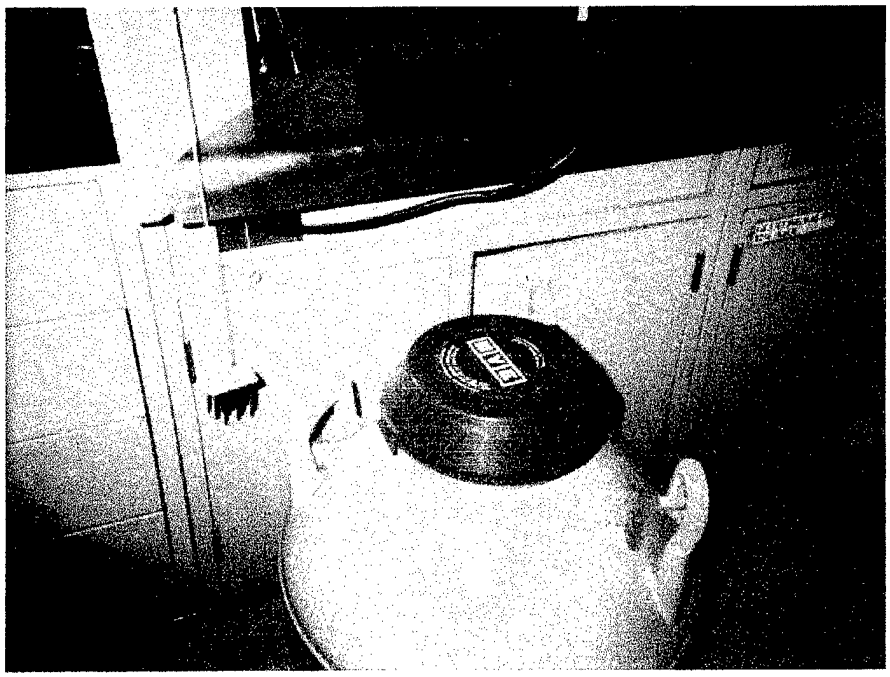

Figure 6. Photograph of Wood Raft. The small wood raft with sample tubes is on a string used to lower tubes in the liquid nitrogen tank

Resuspension

Post-thawing, 50uL aliquots of the frozen/thawed RBC were resuspended in 2 mL of seventy-six (76), a solution of distilled water and additives (Table 3) supplied by the Red Cell Preservation Section, WRAIR in 5 mL, 12 X 75 polypropylene tubes (Sarstedt, Newton, NC). Five tubes for each sample were set up in this manner.

Table 3
Composition of 76 Solution

Ingredient	Grams Percent
Glucose	1.25
Sodium citrate	0.90
Dibasic sodium phosphate (Na_2HPO_4)	0.17
Monobasic sodium phosphate (NaH_2PO_4)	0.04
Adenine	0.028

The ingredients listed above are dissolved in distilled water at the concentrations listed in grams percent.

Phospholipid-Like Additives

Phospholipid-like compounds urea and L-carnitine were used to evaluate the effect on supernatant hemolysis. The L-carnitine (SIGMA Chemical Company, St. Louis, MO) was added directly to an aliquoit of RBC in a 0.2M concentration and allowed to incubate in the refrigerator for two weeks to allow time for the L-carnitine to integrate into the membrane. The urea (Mallinckrodt, Paris, KY) was added directly to the cryopreservative in a 0.2M concentration and the cryopreservative-additive mixture added to the RBC immediately before immersion in liquid nitrogen.

Supernatant Hemoglobin Determination

Hemoglobin values in mg/dL were determined using a modification of the cyanmethemoglobin method. A 1: 400 dilution of the RBC mixture was made by adding 1 mL of the mixture to 3 mL of Drabkin's reagent. Triton X, a detergent added to the Drabkin's reagent, dissolved the microvesicles in the sample resulting in a clearer supernatant for more accurate hemoglobin determination. Total hemoglobin determination was performed for each sample using a hemoglobinometer (Coulter Electronics, Hialeah, FL). Each day of use, the hemoglobinometer results were verified using controls (Coulter Hb-5 and Hb-10, Coulter Corporation, Miami, FL) intended for the quality control of the Coulter hemoglobinometer. One dilution was made from each of 2 of the resuspended RBC samples. The remaining samples were combined in 1 tube and the total hemoglobin determination done on that sample. Total hemoglobin was run in triplicate and the mean used in the calculation of percent supernatant hemolysis. The remaining three tubes were incubated at room temperature for 2 hours.

After the room temperature incubation, the RBC samples were sedimented by centrifugation at 1000 x g in a Serofuge II (Clay Adams Division of Bectin Dickinson and Company, Parsippany, NJ, Model No. 0541) for 3 minutes. Supernatant (1 mL) was then added to Drabkin's reagent (3 mL) and the hemoglobin of the supernatant determined using the Coulter Hemoglobinometer. Supernatant hemoglobin was run in triplicate and the mean used to calculate percent supernatant hemolysis. The supernatant percent hemolysis was calculated using the following equation:

(Supernatant Hgb/total Hgb) X 100 = % Hemolysis

RBC Count and MCV Determination

The RBC count and mean corpuscular volume (MCV) were determined using a Baker 9110 automated cell counter (Biochem Immunosystems Subsidiary of Biochem Pharma Inc.). This instrument provided both the RBC count and the MCV. The extremely high concentration of cells made it necessary to dilute the samples before testing them on the cell counter. A 20 uL aliquot of the RBC mixture (RBC plus cryopreservative) was added to 10 mL of Baker diluent in vials manufactured for analyzing prediluted samples on the Baker 9110. Cell count and MCV were determined on every sample after mixing with cryopreservative before freezing for the pre-freeze and after freezing and thawing for the post-thaw. The post-thaw samples were incubated at room temperature (20-24°C) for 2 hours to afford time for the lysis of RBC damaged during the freeze-thaw process.

Isolation of Microvesicles

Microvesicles were isolated by centrifugation at 2000 x g for 10 minutes of a well-mixed aliquot from a unit on day 42 because vesiculation increases with age of the unit. The supernatant fluid was removed and then centrifuged at 38,000 x g for 1 hour at 4°C. The pellet was washed twice in PBS then resuspended in 0.5 mL PBS for flow cytometry testing.

Flow Cytometry

RBC were stained with a FITC labeled anti-glycophorin A (GPA) monoclonal antibody (IMMUNOTECH, A Coulter Company) and analyzed using the Coulter Epics XL-MCL flow cytometer. FITC has an absorption maximum or excitation wavelength of 490 nm and maximum emission at about 530 nm which is slightly yellow green.

RBC were mixed with anti-spectrin (Sigma Chemical Company, St. Louis, MO) and anti-actin antibodies (Sigma Chemical Company, St. Louis, MO) in an attempt to quantitate membrane damage. The antibodies used were unlabeled anti-mouse IgG1 antibodies. An anti-mouse IgG1 antibody labeled with phycoerythrin (PE) was used as the secondary antibody (Sigma Chemical Company, St. Louis, MO). PE absorbs strongly at 488 nm and the maximum emission is about 580 nm which is in the orange range. Mouse IgG1 (MOPC-21, Sigma Chemical Company) was used as the isotype control to distinguish between the non-specific binding of IgG and the specific binding of anti-actin and anti-spectrin.

Samples were prepared for testing using 6 tubes for each sample that had been frozen and thawed along with a control sample that had not been frozen. All 6 tubes had 5 uL of cells (2.2×10^4) and were brought to a final volume of 200 uL with PBS. The first tube was a negative control containing cells and PBS. The second tube was an IgG control containing 10 uL of anti-IgG1 PE, cells, and PBS. The third tube, isotype control, contained 10 uL MOPC-21, cells, and PBS. The fourth tube contained 10 uL monoclonal anti-actin antibody, cells, and PBS. The fifth tube contained 10 uL monoclonal anti-spectrin antibody, cells, and PBS. The sixth tube contained 5 uL monoclonal anti-GPA FITC, cells, and PBS. All tubes were incubated at 1-6° C protected from light for 30 minutes. The samples were washed once using 2.5 mL of PBS. The samples were centrifuged at 1000 x g for 5 minutes in a JOUN C4-12 centrifuge. All but 200 uL of the supernatant was aspirated and the cells were resuspended in PBS. Monoclonal anti-mouse IgG1 PE, 10 uL, (Sigma Chemical Company), was added to tubes 3,4, and 5 only.

All tubes were incubated again at 1-6° C protected from light for 30 minutes. All samples were washed twice in 2.5 mL PBS. After each wash the samples were centrifuged at 1000 x g for 5 minutes. After the last wash, the supernatant was discarded and the samples were resuspended in 1 mL of PBS. The samples were then analyzed using the flow cytometer.

Before cryopreserved samples were tested, fresh red blood cells untreated and treated with ionomycin were used to determine the optimum dilution of antibodies for the flow cytometric analysis. The ionomycin was added to produce holes in the cells. Ionomycin had been used at a concentration of 1 ug/mL to produce channels in lymphocytes for calcium studies.[33] The fresh red cells were treated with 4 uL of ionomycin at a concentration of 1 ug/ml in the tubes that had anti-actin and anti-spectrin to deliberately punch holes in the cells, then processed as previously described.

The Coulter Epics XL-MCL flow cytometer was turned on and allowed to warm up for 30 minutes. A sample of deionized water was run through the instrument first to flush it and then the instrument was primed. Flow Beads (Immunotech Division Coulter Electronics, Westbrook ME) were used to verify the alignment of the instrument. The tube of Flow Beads was vortex mixed before flow cytometery analysis. The coefficient of variation (CV) for each of the cytosettings was two or less.

Statistical Analysis

Total hemoglobin, supernatant hemoglobin, and percent hemolysis were compared between cryopreservatives using repeated measures analysis of variance. Pairwise

comparisons among the preservatives were made using a Bonferroni adjustment to reduce the chance of type I error.

The results of the pre-freeze and post-thaw RBC counts and MCV determinations were compared using a paired samples test. The paired samples test was used to establish the presence or absence of a statistically significant difference between the pre-freeze and post-thaw values. Multivariate tests were used to compare the pre-freeze and post-thaw RBC counts and MCVs among the three cryopreservatives also using a Bonferroni adjustment to reduce the chance of type 1 error.

RESULTS

Hemoglobin Determination/Percent Hemolysis

Mean total hemoglobin for samples frozen in 30% viastarch with no additive ranged from 368.7 mg/dL to 430 mg/dL with a mean of 394.8 mg/dL and median of 393 mg/dL. Mean supernatant hemoglobin values ranged from 20.7 mg/dL to 31 mg/dL with a mean of 25.1 mg/dL and median of 25.0 mg/dL. The range of percent supernatant hemolysis for samples cryopreserved with viastarch was 5.4% to 7.8% with a mean of 6.4% and median of 6.4% (Table 4).

Mean total hemoglobin for samples frozen in 35% CellSep with no additive ranged from 331.3 mg/dL to 386.7 mg/dL with a mean of 361.7 mg/dL and median of 360.7 mg/dL. Mean supernatant hemoglobin values ranged from 14.3 mg/dL to 28.7 mg/dL with a mean of 19.7 mg/dL and median of 18.7 mg/dL. The range of percent supernatant hemolysis for samples cryopreserved with CellSep was 3.9% to 7.6% with a mean of 5.5% and median of 5.0% (Table 5).

Mean total hemoglobin for samples frozen in 30% HES-5 with no additive ranged from 331.3 mg/dL to 398.3 mg/dL with a mean of 369.3 mg/dL and median of 375.7 mg/dL. Mean supernatant hemoglobin values ranged from 23.0 mg/dL to 36.3 mg/dL with a mean of 27.8 mg/dL and median of 27.3 mg/dL. The range of percent supernatant hemolysis for samples cryopreserved with HES-5 was 5.9% to 10.3% with a mean of 7.6% and median of 7.3% (Table 6).

Table 4
Supernatant Hemoglobin/Percent Hemolysis uisng 30% Viastarch, No Additive

Sample # Number	Total Hemoglobin (mg/dL)	Supernatant Hemoglobin (mg/dL)	Supernatant Hemolysis (%)
1	428.3	27.3	6.4
2	408.7	28.3	6.9
3	380.0	25.7	6.8
4	398.7	28.3	7.1
5	403.3	23.7	5.9
6	390.0	25.0	6.4
7	393.3	21.7	5.5
8	410.3	22.0	5.4
9	400.3	22.3	5.6
10	426.7	31.0	7.3
11	384.7	30.3	7.8
12	400.0	29.3	7.3
13	430.0	27.3	6.4
14	373.0	27.7	7.4
15	384.0	25.7	6.7
16	401.7	25.3	6.3
17	392.3	21.7	5.5
18	393.0	24.7	6.3
19	393.0	21.3	5.4
20	373.0	21.0	5.6
21	372.7	20.7	5.5
22	368.7	24.7	6.7
23	374.7	22.0	5.9
Mean	394.8	20.7	6.4
SD	17.8	3.2	0.7
SEM	3.7	0.7	0.2

Table 5
Supernatant Hemoglobin/Percent Hemolysis using 35% CellSep, No Additive

Sample # Number	Total Hemoglobin (mg/dL)	Supernatant Hemoglobin (mg/dL)	Supernatant Hemolysis (%)
1	381.7	19.0	5.0
2	379.7	20.3	5.4
3	366.0	16.7	4.6
4	357.7	23.0	6.4
5	359.0	22.7	6.3
6	355.0	24.0	6.8
7	359.0	27.0	7.5
8	376.0	28.7	7.6
9	365.7	22.3	6.1
10	366.3	15.3	4.2
11	360.7	14.7	4.1
12	344.3	15.3	4.5
13	342.0	19.0	5.6
14	331.3	22.0	6.6
15	331.7	21.7	6.5
16	354.0	18.7	5.3
17	350.0	17.0	4.9
18	352.0	17.7	5.0
19	368.7	14.3	3.9
20	373.7	17.7	4.7
21	372.0	18.7	5.0
22	386.7	18.0	4.7
23	385.7	18.7	4.8
Mean	361.7	19.7	5.5
SD	15.6	3.8	1.1
SEM	3.3	0.8	0.2

Table 6
Supernatant Hemoglobin/Percent Hemolysis using 30% HES-5, No Additive

Sample # Number	Total Hemoglobin (mg/dL)	Supernatant Hemoglobin (mg/dL)	Supernatant Hemolysis (%)
1	377.3	23.3	6.2
2	359.3	25.3	7.1
3	350.7	26.7	7.6
4	356.7	26.7	7.5
5	347.7	26.7	7.7
6	389.7	33.0	8.5
7	387.7	27.3	7.0
8	378.3	27.7	7.3
9	375.7	26.3	7.0
10	381.0	27.0	7.1
11	398.3	23.3	5.9
12	382.0	29.0	7.6
13	378.3	23.0	6.1
14	331.3	28.0	8.6
15	337.7	26.0	7.7
16	382.3	28.0	7.3
17	395.0	28.7	7.3
18	354.0	36.3	10.3
19	354.7	31.0	8.7
20	352.3	28.7	8.1
21	375.0	34.3	9.2
22	372.3	27.3	7.3
23	375.7	26.3	7.0
Mean	369.3	27.8	7.6
SD	18.2	3.3	1.0
SEM	3.8	0.7	0.2

Mean total hemoglobin for samples frozen in 35% CellSep with 0.2M urea ranged from 315.0 mg/dL to 352.7 mg/dL with a mean of 332.3 mg/dL and median of 333.9 mg/dL. Mean supernatant hemoglobin values ranged from 17.0 mg/dL to 24.0 mg/dL with a mean of 20.1 mg/dL and median of 20.5 mg/dL. The percent supernatant hemolysis ranged from 5.1% to 6.9%. with a mean of 6.1% and a median of 6.0% (Table 7).

Since Viastarch is HES and limited data was available, the data from the 30% Viastarch and 30% HES-5 with L-carnitine added were combined. Mean total hemoglobin for these samples ranged from 395.3 mg/dL to 437.3 mg/dL with a mean of 418.9 mg/dL and median of 416.3 mg/dL. Mean supernatant hemoglobin values ranged from 20.3 mg/dL to 26.7 mg/dL with a mean of 23.5 mg/dL and median of 23.5 mg/dL. The percent supernatant hemolysis ranged from 4.8% to 6.6%. with a mean of 5.6% and a median of 5.7% (Table 8). Limited data were obtained on samples with additives because of a problem with the liquid nitrogen tank. Almost two weeks worth of data could not be used because the level of liquid nitrogen in the tank was insufficient to immerse the tubes completely. The results were spurious with some extremely elevated hemoglobin results in total and supernatant hemoglobin values. Given the nature of the tank, it took several attempts at retesting to determine the cause of spurious results.

Comparison of CellSep, Viastarch, and HES-5, No Additives

Multivariate tests on all 3 parameters for each cryopreservative indicated a statistically significant difference ($P=<0.0005$) among the cryopreservatives. In

Table 7
Supernatant Hemoglobin/Percent Hemolysis using CellSep, 0.2M Urea

Sample # Number	Total Hemoglobin (mg/dL)	Supernatant Hemoglobin (mg/dL)	Supernatant Hemolysis (%)
020	352.7	22.0	6.2
020	337.0	23.0	6.8
020	330.7	20.3	6.1
020	323.7	20.3	6.3
020	324.0	19.7	6.1
020	330.0	18.3	5.6
020	336.3	17.0	5.1
014	347.3	24.0	6.9
014	315.0	20.0	6.5
014	342.3	18.0	5.3
014	328.7	19.7	6.0
014	322.0	20.3	6.3
014	330.0	18.3	5.6
Mean	332.3	20.1	6.1
SD	10.6	2.0	0.5
SEM	2.9	0.6	0.2

Table 8
Supernatant Hemoglobin/Percent Hemolysis using Viastarch and HES-5, L-Carnitine

Cryopreservative	Total Hemoglobin (mg/dL)	Supernatant Hemoglobin (mg/dL)	Supernatant Hemolysis (%)
Viastarch	425.0	23.3	5.5
Viastarch	430.3	23.3	5.4
Viastarch	437.3	22.0	5.0
Viastarch	420.7	20.3	4.8
HES-5	395.3	25.3	6.4
HES-5	405.0	26.7	6.6
Mean	418.9	23.5	5.6
SD	15.9	2.3	0.7
SEM	6.5	0.9	0.3

comparison of viastarch vs HES-5, there was not a significant difference (P=0.154); comparison of viastarch vs CellSep and CellSep vs HES-5 revealed a significant difference, P=0.001 and P=<0.0005, respectively. Of the 3 cryopreservatives, CellSep produced the least hemolysis in the frozen samples.

RBC count and MCV

Samples from 3 different units were frozen using each of the three cryoprotectants and thawed. There were no additives in these samples. A complete blood count (CBC) was performed on each sample to obtain the RBC count in million/cubic mm and MCV in femtoliters (fL) (Table 9).

Comparison of Pre-freeze and Post-thaw RBC Count and MCV

Results of paired samples test of the RBC counts indicate that there was not a statistically significant difference in pre-freeze and post-thaw RBC counts, P=0.156. However, there was a significant difference in the pre-freeze and post-thaw MCV, P=<0.0005. There was an average increase of 4.7 fL or about 5%, from the pre-freeze to the post-thaw sample results.

Comparison of Pre-freeze and Post-thaw RBC Count, MCV and Cryopreservatives

A comparison between RBC counts of the three different cryopreservatives showed a significant difference in RBC counts between the three groups, P=0.009. The MCV results did not indicate a significant difference between groups, P=0.809.

Multiple comparisons among cryopreservatives and pre-freeze vs post-thaw RBC counts were made using a Bonferroni adjustment. Results indicated that the difference in pre-freeze and post-thaw RBC counts for samples cryopreserved in viastarch was

Table 9
Comparison of Pre-Freeze and Post-Thaw RBC and MCV

Cryo	RBC (mill/cu mm) Pre-freeze	RBC (mill/cu mm) Post-thaw	MCV (fL) Pre-freeze	MCV (fL) Post-thaw
CellSep	4.54	4.60	85.6	91.3
CellSep	4.58	4.60	85.6	91.3
CellSep	4.22	4.20	86.8	90.1
CellSep	4.24	4.20	85.5	89.7
CellSep	4.12	4.20	91.7	96.6
CellSep	4.04	4.20	91.5	95.0
CellSep	3.92	4.10	91.9	95.0
CellSep	3.76	3.78	91.7	97.1
CellSep	3.48	3.82	91.7	97.3
CellSep	3.70	3.66	91.4	98.5
Viastarch	4.64	4.80	87.1	90.4
Viastarch	4.76	4.70	85.8	90.0
Viastarch	4.44	4.46	92.0	98.2
Viastarch	4.36	4.28	91.6	97.3
Viastarch	4.38	4.32	91.9	97.2
Viastarch	5.16	4.98	85.9	89.9
Viastarch	5.06	4.80	85.9	90.5
Viastarch	5.24	4.98	85.9	89.9
Viastarch	5.09	4.76	85.9	90.4
Viastarch	5.26	4.96	85.9	90.6
HES-5	4.30	4.48	87.1	92.0
HES-5	4.70	4.70	85.6	90.7
HES-5	4.38	4.32	91.9	97.5
HES-5	4.06	4.12	91.9	96.7
HES-5	3.96	4.22	91.9	96.7
HES-5	4.08	4.24	91.8	97.2
HES-5	4.26	4.34	92.4	96.4
HES-5	4.64	4.76	87.2	91.4
HES-5	4.88	5.04	84.8	89.9
HES-5	4.14	4.26	91.6	97.6
Mean	4.33	4.37	89.3	94.1
SD	0.44	0.37	2.9	3.3
SEM	0.08	0.07	0.5	0.6

significantly different than CellSep, and HES-5, P=0.026 and P=0.015, respectively. Comparison of the difference between pre-freeze and post-thaw RBC counts of samples cryopreserved in CellSep vs HES-5 indicated there was no significant difference between these two groups, P=1.000.

Multiple comparisons among cryopreservatives and pre-freeze vs post-thaw MCV values were made using Bonferroni adjustment. There was no significant difference between the three groups (P=1.000) for all three sets of comparisons.

Flow Cytometry

Fresh RBC, untreated and treated with ionomycin, were used. There was no more fluorescense in test samples than negative controls when tested with 1:10, 1:50, 1:100 dilutions of antibody. When tested using undiluted antibody, the samples treated with ionomycin produced more fluorescense in the PE range with both the anti-spectrin and anti-actin than the negative control. There was not more fluorescence in the PE range with anti-actin and anti-spectrin in the untreated samples than in the negative control as was expected. The fluorescense histogram showed the negative response or lack of fluorescense of the untreated red cells (Fig. 7). The fluorescense histogram of the ionomycin treated cells indicated more fluorescense of those cells with the anti-actin, anti-spectrin, and anti-IgG PE (Fig. 8). Similar results were not seen with the frozen samples. In all cases the non-frozen control samples and frozen samples showed no more fluorescense than the negative control, except for 1 non-frozen control sample. One non-frozen control sample produced a very weak fluorescense with the anti-actin, anti-spectrin and anti-IgG PE. The sample was severely hemolyzed. It was not known what

happened to the sample to cause the excessive hemolysis or the weak positive fluorescense in a sample that had not been frozen. There was a negative response, no more fluorescense in the PE range than negative and isotype controls of control samples that were not frozen (Fig. 9-13) and samples that had been frozen and thawed. A postive response, more fluorescense than the negative control was seen for all samples with anti-GPA antibody (Fig. 14). In general, The results from the frozen samples looked the same as those from the non-frozen samples.

The sample from the microvesicle pellet also showed more fluorescense than the negative control with the glycophorin A antibody; however, the sample had very few particles and the flow cytometer was stopped at only 1,000 events. It took 5 minutes for the flow cytometer to count 1,000 events. Therefore, time and sample volume would not allow further analysis. A total of 5,000 or 10,000 events was desired but was not possible with the microvesicle samples.

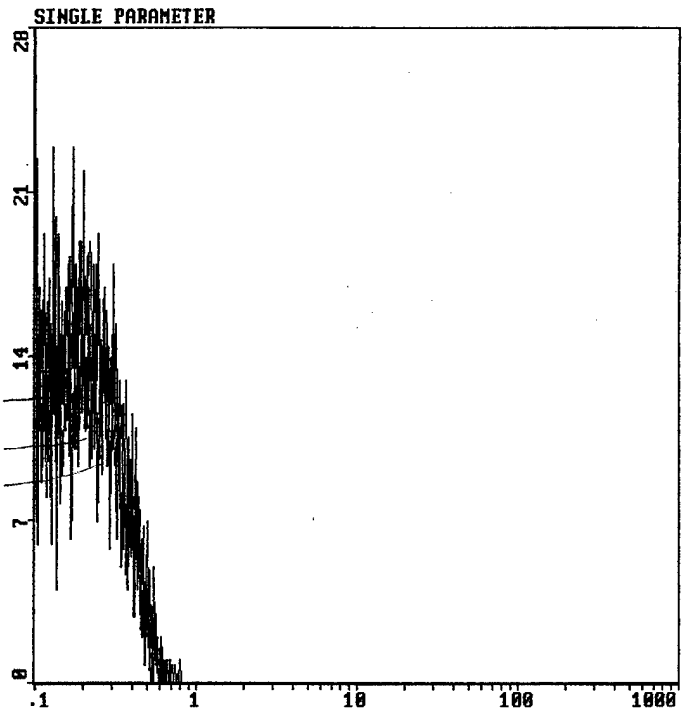

Figure 7. Multigraph Overlay, Untreated Cells. Fresh whole blood sample not treated with ionomycin. The x-axis is fluorescense and the y-axis is the number of events counted. The multigraph overlay of the negative control, anti-IgG control, and, anti-actin, anti-spectrin and anti-IgG PE shows no difference in fluorescense in all the samples. The fluorescense histogram shows that the fluorescense produced by all the samples is superimposable.

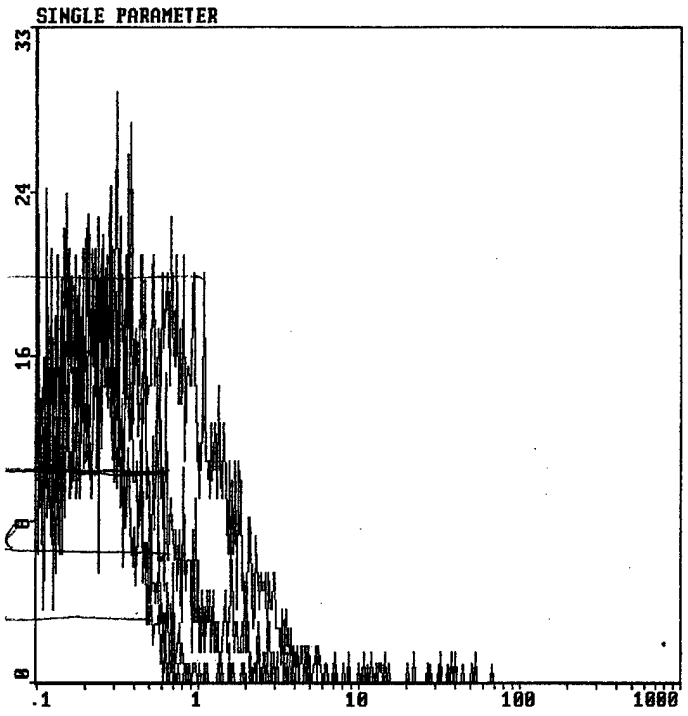

Figure 8. Multigraph Overlay, Treated Cells. Fresh whole blood sample, treated with ionomycin. The x-axis indicates fluorescense of the samples and the y-axis is the number of events counted. The multigraph overlay of the negative control, and anti-IgG control, anti-actin, anti-spectrin and anti-IgG PE shows slightly more fluoresecense in the samples with anti-actin and anti-spectrin and anti-IgG PE.

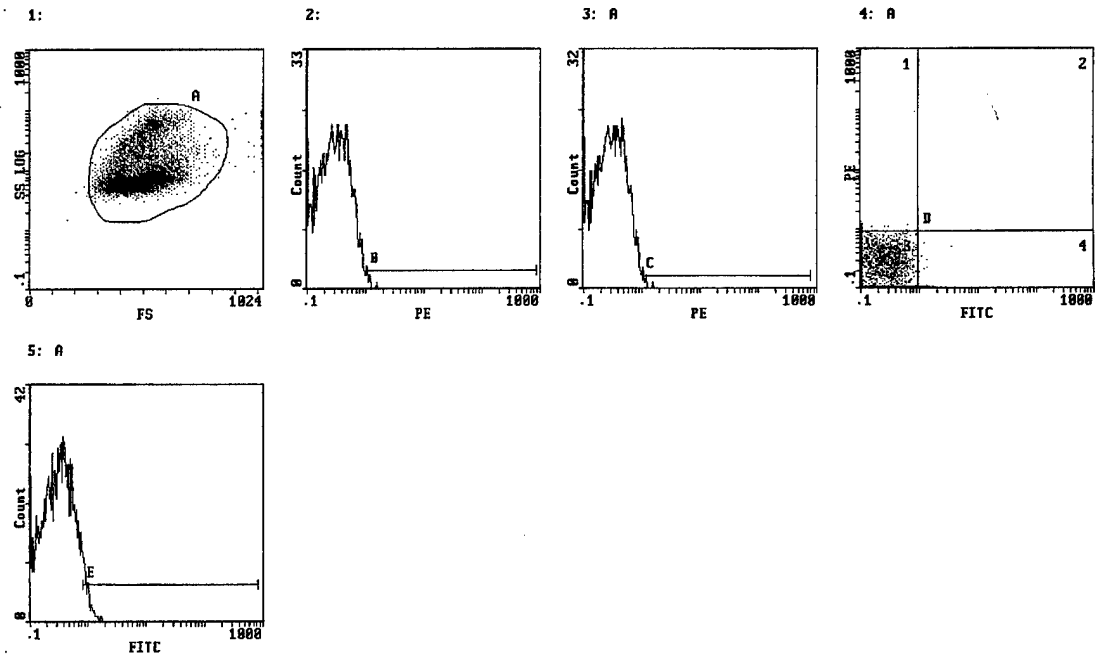

Figure 9. Negative Control. The negative control consisted of cells and PBS with no antibody added. The dotplot 1 shows the gated area of the population being studied.. Histogram 2 shows the fluorescense, in the PE range, of all cells in the sample. Histogram 3 shows the fluorescense, in the PE range, of cells within the gated area. The scattergram 4 shows the lack of fluorescense from the cells inside the gated area. Histogram 5 shows the lack of fluorescense of cells in the gated area in the FITC range. Because this was the negative control sample, there was no fluorescense.

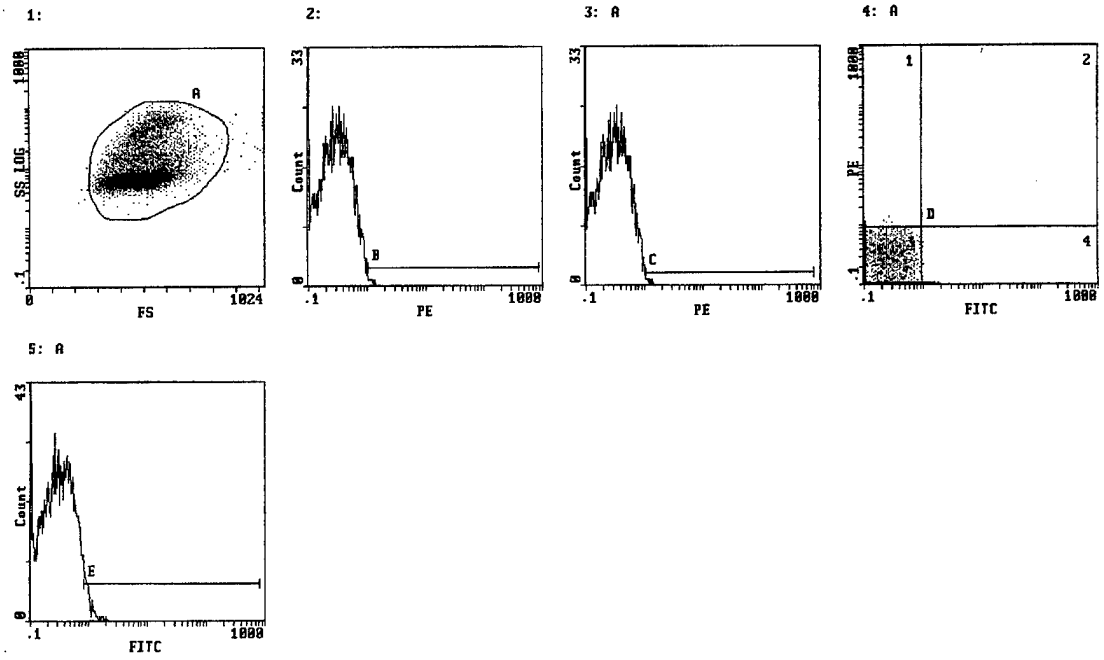

Figure 10. Anti-IgG Control. The anti-IgG control consisted of cells, anti-IgG PE and PBS. The dotplot 1 shows the gated area of the population to be studied.. Histogram 2 shows the lack of fluorescense in the PE range of all cells in the sample. Histogram 3 shows the lack of fluorescense in the PE range of cells within the gated area. The scattergram 4 shows the gated population in area 3 of the scattergram is negative for PE fluorescense. Histogram 5 shows no flourescense in the FITC range of cells in the gated area.

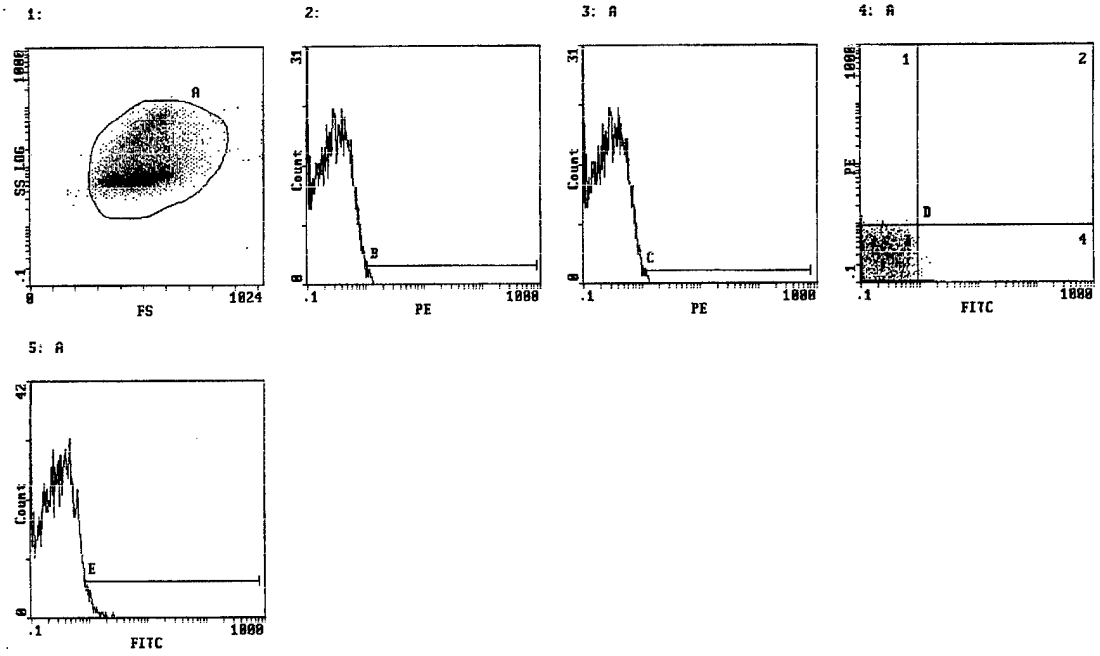

Figure 11. Anti-IgG/MOPC Isotype Control. The isotype control consisted of cells, anti-IgG PE, MOPC 21, and PBS. Dotplot 1 shows the gated area of the population to be studied. Histogram 2 shows the lack of fluorescense in the PE range of all cells in the sample. Histogram 3 shows the lack of fluorescense in the PE range of the cells within the gated area. The scattergram 4 shows the gated population in area 3 of the scattergram are negative for PE fluorescense. Histogram 5 shows the lack of fluorescense in the FITC range of the cells within the gated area.

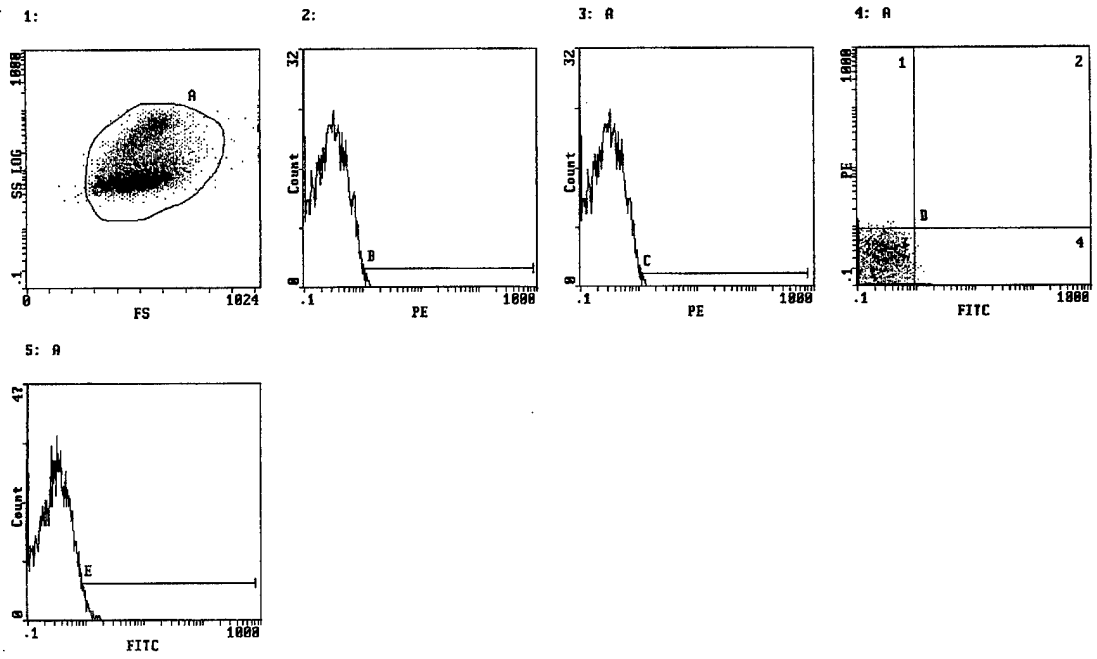

Figure 12. Anti-IgG/Anti-Actin. The sample consisted of cells, anti-actin, anti-IgG PE and PBS. Dotplot 1 shows the gated area of the population of cells to be studied. Histogram 2 shows the lack of fluorescense in the PE range of all cells in the sample. Histogram 3 shows the lack of fluorescense in the PE range of cells within the gated area. The scattergram 4 shows the lack of fluorescense in the PE range of the gated population in area 3 of the scattergram. Histogram 5 shows the lack of fluorescense in the FITC range of the cells within the gated area.

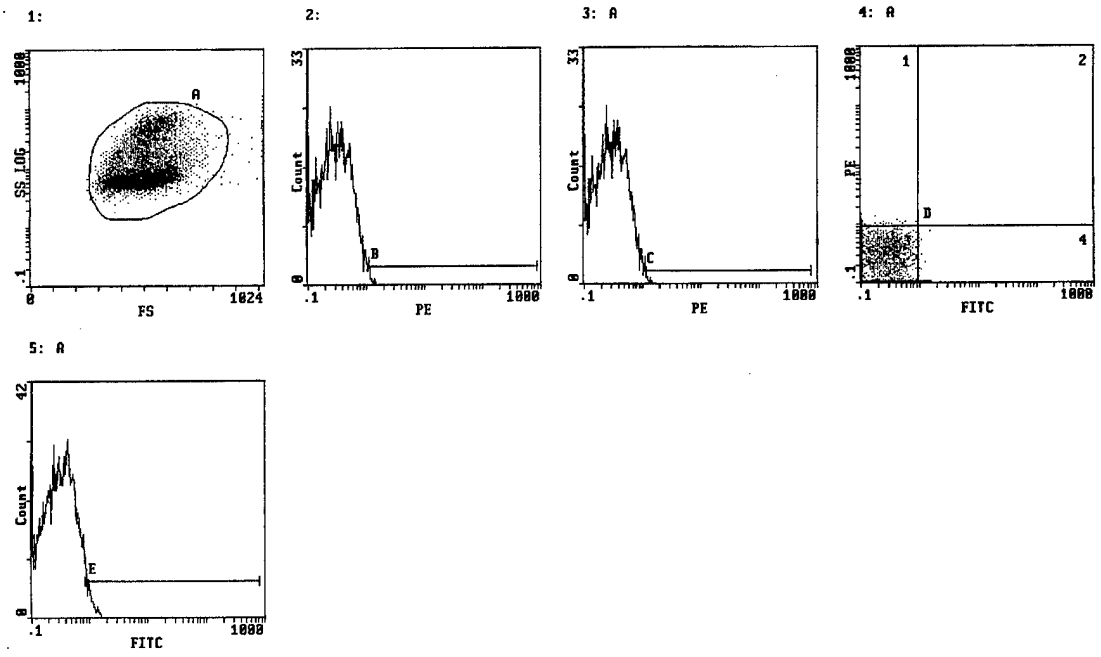

Figure 13. Anti-IgG/Anti-Spectrin. The sample consisted of cells, anti-spectrin, anti IgG PE, and PBS. Dotplot 1 shows the gated area of the population of cells to be studied. Histogram 2 shows the lack of fluorescense in the PE range of all cells in the sample. Histogram 3 shows the lack of fluorescense in the PE range of cells within the gated area. Scattergram 4 shows the lack of fluorescense in the FITC range of the gated population in area 3 of the scattergram. Histogram 5 shows the lack of fluorescense in the FITC range of the cells within the gated area.

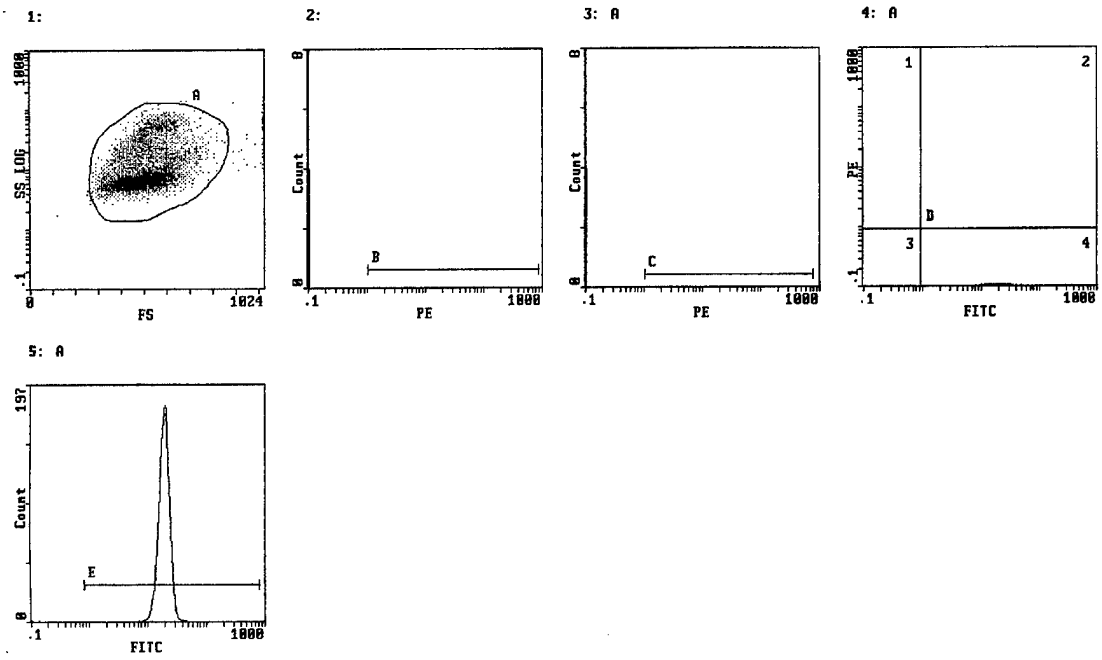

Figure 14. Anti-Glycophorin A. The sample consisted of cells, anti-GPA FITC, and PBS. Dotplot 1 shows the gated area of the population of cells to be studied. Histogram 2 shows the lack of fluorescense in the PE range of all cells in the sample. Histogram 3 shows the lack of fluorescense in the PE range of cells within the gated area. Scattergram 4 shows the lack of fluorescence in the PE range in area 3 and flourescense in the FITC range in area 4 of the scattergram. Histogram 5 shows fluorescense in FITC range of cells within the gated area.

DISCUSSION

The most common method for freezing red blood cells in this country was developed by Meryman and Hornblower in 1972 using approximately 40% glycerol in isotonic saline.[34] Two other approaches to red cell cryopreservation were developed in the 1950s and 1960s because of the disadvantages of glycerolization and deglycerolization processes. The first was the low glycerol procedure which uses 20% glycerol instead of 40% and requires an accelerated freezing rate using liquid nitrogen to freeze and store the samples. The second method, called agglomeration, uses a high concentration of glycerol in a solution of glucose-fructose rather than saline and does not require the use of liquid nitrogen.. After thawing, the cells are diluted with hypertonic followed by isotonic solution causing the cells to agglomerate and fall to the bottom of the container. The cells are then resuspended in isotonic saline which reverses the agglomeration.[14] Then the cells are centrifuged and the supernatant discarded.

Neither of these techniques totally alleviates the obstacles to the processes of glycerolization and deglycerolization. For these reasons investigators have continued studies using alternative cyropreservatives such as high concentrations of sugars, polymers, i.e., PVP and HES, and even reduced concentrations of glycerol. One-step freezing methods using HES, PVP, and dextran have been troubled with 5 to 15 % intravascular hemolysis following transfusion.[14] It has been shown that these cryoprotectants cause a modification of the interfacial tension between the suspending medium and hemoglobin preventing the hemoglobin from leaking out of the cell despite membrane damage from freezing. When these cells are then transfused, the polymer is diluted, the protective effect vanishes and the

cells will lyse.[14] Another problem associated with transfusion of products prepared by one-step techniques using polymers is the increased viscosity of the cell suspension. Because the cell suspension is so thick, it must be infused at a very slow rate increasing the time required for transfusion.

The hemolysis that occurs after cryopreservation is the result of the formation of ice crystals inside the cells which causes excess concentrations of solutes in very small pools inside the cell. These intracellular pools are hypertonic and produce irreversible damage to the cell membrane resulting in hemolysis when the cells are thawed. The goal of all red cell cryopreservation is protection against this irreversible membrane damage.[35]

In this paper, we discussed the use of cryoprotectants and additives and their effect on red cells. Techniques included post-thaw total and supenatant hemoglobin determinations, from which percent supernatant hemolysis was calculated, pre-freeze and post-thaw RBCcount and MCV were compared, and flow cytometry was performed for quantitation of membrane damage using intracellular markers.

Results of the present study showed that of the 3 cryoprotectants tested, CellSep produced the least hemolysis. The samples cryopreserved in viastarch and HES had about the same amount of hemolysis, which was significantly greater than that in samples cryopreserved in CellSep. One possible explanation for the lower hemolysis in samples cyopreserved in CellSep is that CellSep is an arabinogalactan which is a very small molecule with a molecular weight of only 20 daltons. The HES cryopreservatives are made from starchs which are very large molecules ranging from 200-450 daltons molecular weight. Because the arabinogalactan in CellSep is so small, it dissovled quickly and easily and made a much

less viscous preparation than the HES cryopreservatives. Unfortunately, HES is already approved for human use intravenously, and viastarch, which is also HES, has been approved for use in a solution for the perfusion of transplant organs. The in-vivo effects of CellSep are unknown and the approval process of products for intravenous use through the FDA is difficult and time consuming. Washing the cells and removing the supernatant fluid is one solution to this problem with CellSep, but this contradicts the main purpose of this research which was the development of a one-step technique that eliminates the wash step. We did not reach the goal of this study which was supernatant hemolysis of less than 2% in samples cryopreserved with CellSep, HES-5, or viastarch with or without additives.

In the present study, the comparison of RBC counts pre-freeze and post-thaw revealed that there was not a significant difference statistically or clinically in the count before and after freezing . This indicated that leakage of hemoglobin may not be from the total destruction of a few cells but is probably from small leaks in many cells. The MCV of all of the samples after freezing was higher than samples before freezing which indicated that the cells are about 5% larger after the freeze thaw process.

Although a concentration of antibody that would label ionomycin treated cells using fluorescently labeled antibodies and flow cytometry was determined, this procedure did not work with the samples that were cryopreserved and thawed. Therefore, this technique could not be used to quantitate the damage to the RBC membrane as a result of cryopreservation. One of the major hurdles to the development of a suitable one step freeze process is the inability to accurately assess the damage to the red cell membrane.[36]

The development of an assay that could do this would greatly enhance the one-step freeze research effort and should continue.

Flow cytometry results of samples frozen and thawed did not differ from those of samples that were not frozen when stained with the anti-IgG PE and anti-actin and anti-spectrin antibodies. There were holes in some or all of the cells after they had been frozen and thawed because of the presence of hemoglobin in the supernatant. The holes produced in the ionomycin treated cells may have been larger than those created when the RBC were cryopreserved and thawed. This may explain why the inomycin treated cells stained and the frozen cells did not. Another explanation could be that the hemoglobin leaks were transient. The holes may have closed up at some point or reduced in size small enough that an antibody to an intracellular antigen could not get through the membrane.

L-carnitine and urea were added to samples to evaluate the potential protective effect of phospholipid-like additives and the effect on supernatant hemolysis results. In a previous study, it was determined that the addition of L-carnitine to additive solution suspended RBC stored at 4°C resulted in a beneficial effect. L-carnitine in the RBC was shown to increase by fourfold at day 42, was taken up irreversibly by the RBC, and resulted in reduced in-vitro hemolysis and improved in-vivo viability.[28] The results of the present study indicated lower supernatant hemolysis results in samples cryopreserved with L-carnitine added. The mean supernatant hemolysis of samples cryopreserved with viastarch and L-carnitine was 5.2% and was 6.4% in samples without L-carnitine added. For samples cryopreserved in HES-5 with L-carnitine added, the mean supernatant

hemolysis was 6.5% and was 7.6% without L-carnitine. The addition of urea did not reduce superntant hemolysis results. In fact, mean supernatant hemolysis went from 5.5% in samples cryopreserved in CellSep without urea to 6.9% in samples cryopreserved in CellSep with urea added.

Previous studies involving microvesicles indicated that during storage, cholesterol and phospholipids are lost which disrupts the membrane structure and causes the formation of the tiny hemoglobin-containing vesicles. Glycophorin A has been demonstrated on microvesicle membranes by immunoblotting and periodic acid Schiff staining.[25] In the present study we were able to demonstrate the presence of glycophorin A on microvesicle membranes using anti-glycophorin A antibody labeled with FITC and flow cytometry.

The advantages of cryopreserved RBC are important enough to continue the research for a one-step technique. These advantages represent the main clinical circumstances that should be considered for the use of this product. The first is the ability to store RBC for 10 years and to make readily available, units from donors with rare phenotypes, which are needed for patients who are alloimmunized to high frequency blood group antigens or patients who have multiple alloimmunization. The second is to allow long term storage of autologous units for patients with rare phenotypes, provided they have intervals where they are healthy and can have autologous units drawn. These situations make use of the only unique characteristic of cryopreserved-stored RBC, their ability to be stored for 10 years while remaining viable and physiologically sound to sustain the life of a patient with life threatening anemia.[14] Lastly, large inventories of universal donor type O negative and O positive blood for civilian mass casualties and military

contingencies or war would result in the ability to save the lives of patients who have been critically injured and need blood.

Alternatives to the cryopreservation of RBC are under investigation. Because of the problems associated with cryopreservation, studies are ongoing for extended additive solutions that will improve the viability and biochemical processes of stored RBC. Dumaswala, et al., published results from a study that suggest an experimental additive solution (EAS) with ammonium and phosphate can support the biochemical integrity and viability of red cells for up to 84 days.[37] This would impact the autologous transfusion programs at most facilities but does not greatly effect the storage of rare phenotypes and large inventories of universal donor type O blood for civilian or military contingencies or war

Another attempt at resolving the problems associated with current accepted cryopreservation techniques was the development of automated instruments for the glycerolization and the deglycerolization of red cells. One sytem in development at this time deglycerolized a unit in less than 30 minutes using only two wash solutions.[14] Although an automated system for glycerolization and deglycerolization would improve the currently approved method, it still would not be as efficient and effective in mass casualty or wartime situations as an approved one-step technique.

Still another alternative to cryopreservation of RBC receiving attention, time, and money is blood substitutes. There are 3 types of blood substitutes being developed, 1) stroma-free hemoglobin solution (SFHS), 2) perfluorochemicals (PFC), and 3) hemoglobin encapsulation. All three types share the common feature of the ability to carry oxygen without the presence of intact RBC.[15] The only truly synthetic blood substitutes are the PFC

because they do not depend on human or other animal protein as substrates. For this reason, PFC are the only preparations that will not transmit infectious disease. The first commercially available frozen PFC blood substitute was Fluosol.[39] The main disadvantages of PFC are the requirement for frozen storage and the administration of a high-oxygen environment along with the PFC transfusion.

SFHS blood substitutes are prepared by hemolyzing outdated red cells and then removing all of the red cell stroma. The advantages of SFHS include a much longer shelf life than PRBC and storage in almost any environment. SFHS are very stable, eliminate antigenicity, and are biocompatible with all blood types because blood groups reside on the RBC membrane which is absent in SFHS.[15] Disadvantages of SFHS include high oxygen affinity, renal toxicity, and a short intravascular half-life. A human SFHS was recently produced by genetic engineering. Recombinant Hb1.1 was manufactured by Somatogen, Boulder, CO.[40] This particular SFHS has good oxygen carrying capacity and unloading capacity and is not toxic to the kidneys. Results in animal studies were very promising and clinical trials are now being performed.[40]

The present study provided data that indicated cryopreservation of RBC in CellSep results in less hemolysis than cryopreservation in HES and viastarch and that addition of L-carnitine improved the supernatant hemolysis levels,thus improved integrity of the RBC membrane. The arguments that support the need for cryopreservation of red cells are strong and the need for an improved technique still exists. It is unlikely that the one-step technique will ever compete with the existing methods for the cryopreservation of RBC unless there is some new major breakthrough.[14] The advantages of an approved one-step freeze technique using

additives would have such a significant impact on the use of frozen RBC. For this reason, the search for a dramatic breakthrough should continue. Future studies will likely concentrate on improving currently approved methods, development of an acceptable one-step technique, extended storage solutions, and blood substitutes.

REFERENCES

1. American Association of Blood Banks (AABB), Blood Transfusion Therapy A Physicians Handbook, 5th Edition, 1996

2. Rutman, R. C.; Miller, W.V., Transfusion Therapy Principles and Procedures, 2nd Edition, 1985

3. Quinley, E.D., Immunohematology Principles and Practice, J.B. Lippincott Company, 1993

4. Standards for Blood Banks and Transfusion Services, 17th Edition, American Association of Blood Banks, Bethesda, MD, 1996

5. Gottlieb, A.M., A Pictorial History of Blood Practices and Transfusion, Arcane Publications, 1992

6. Rossi, E.C.; Simon, T.L.; Moss, G.S., Principles of Transfusion Medicine, 1991

7. AABB Technical Manual, 12th Edition, 1996

8. Pamphilon, D.H., Modern Transfusion Medicine, CRC Press, 1995

9. Fitzpatrick, G.M., Effects of Low Molecular Weight Hydroxyethyl Starch, Thermal Hysteresis Proteins, Anticoagulants, and Storage Temperature/Time on Cryopreservation of Erythrocytes, Dissertation, Bowling Green State University, Bowling Green, Ohio, August 1997

10. Dutcher, J.P., Modern Transfusion Therapy, Vol. I, CRC Press, 1990

11. Mollison, P.L., Blood Transfusion in Clinical Medicine, 9th Ed., Blackwell Scientific Publications, 1993

12. Valeri, C.R., Blood Banking and the Use of Frozen Blood Products, CRC Press, 1976

13. Meryman, H.T., personal communication, 1998

14. Petz, L.; Swisher, S., Clinical Practice of Transfusion Medicine, 2nd Edition, Churchill Livingstone, 1989

15. Harmening, D.M., Modern Blood Banking and Transfusion Practices, 3rd Edition, F.A. Davis Company, 1994

16. Rudman, S.V., Textbook of Blood Banking and Transfusion Medicine, 1995

17. Lehninger,A.L.; Nelson, D.L.; Cox, M.L., Principles of Biochemistry, 2nd Edition, 1993

18. Alberts, B.; Bray, D.; Lewis, J.; Raff, M.; Roberts, K.; Watson, J.D., Molecular Biology of the Cell, 3rd Edition, Garland Publishing, Inc., 1994

19. Goodman, S.R., Medical Cell Biology, J.B. Lippincott Company, 1994

20. Jamieson, G.A.; Greenwalt, T.J., Red Cell Membrane Structure and Function, JB Lippincott Company, 1969

21. Greenwalt, T.J.; Sostok, C.Z.; Dumaswala, U.J., Studies in Red Blood Cell Preservation: Effects of the Other Formed Elements, Vox Sang 1990; 58: 85-89

22. Greenwalt, T.J.; Bryan, D.J.; Dumaswala, U.J., Studies in Red Blood Cell Preservation: Erythrocyte Membrane Vesiculation and Changes in Membrane Composition during storage in citrate-phosphate-adenine-1, Vox Sang 1984; 47: 261-170

23. Greenwalt, T.J.; Sostok, C.Z.; Dumaswala, U.J., Comparison of Vesicle Formation, Morphology, and Membrane Lipids During Storage in AS-1 amd CPDA-1, Vox Sang 1990; 58: 90-93

24. Oreskovic, R.T.; Dumaswala, U.J.; Greenwalt, T.J., Expression of Blood Group Antigens on Red Cell Microvesicles, TRANSFUSION 1992 - Vol 32, No. 9

25. Dumaswala, U.J.; Dumaswala, R.U.; Devin, D.S.; Greenwalt, T.J., Improved Red Blood Cell Preservation Correlates with Decreased Loss of Bands 3, 4.1, Acetylcholinesterase and Lipids in Microvesicles, Blood, Vol 87 No. 4 (February 15)1996: pp1612-1616

26. Anderson, D.; Doe, R.; Hensley, R.; Frank, I., Study Guide for Flow Cytometry Workshop, Society of Armed Forces Medical Laboratory Scientists (SAFMLS), San Antonio, TX, 1998

27. Shapiro, H.M., Practical Flow Cytometry, Second Edition, 1988

28. Arduini, A.; Holme, S.; Sweeney, J.D.; Dottori, S.; Sciarroni, A.F.; Calvani, M., Addition of L-carnitine to additive solution suspended red cells stored at 4C reduces in vitro hemolysis and improves in vivo viability. Transfusion, Vol. 37, No. 2, February 1997

29. Meryman, H.T.; Hornblower, M., A method for freezing and washing red blood cells using a high glycerol concentration, Transfusion, 12:145, 1972

30. Huestis, Bove, Busch, Practical Blood Transfusion, 2nd Edition, 1976

31. Hornblower, M., personal communication, 1998

32. Dumaswala, U.J.; Oreskovic, R.T.; Petrosky, T.L.; Greenwalt, T.J., Studies in Red Blood Cell Preservation: Determining the Limiting Concentrations of NH_4CL and Na_2HPO_4 Needed toMaintain Red Blood Cell ATP during Storage, Vox Sang 1992;62:136-140

33. Habler, O.P.; Kleen, M.S.; Hutter, J.W.; Podtschaske, A.H.; Tiede, M.; Kemming, G.I.; Welte, M.V.; Corso, C.O.; Batra, S.; Keipert, P.E.; Faithfull, N.S.; Messmer, K.F.W., Hemodilution and intravenous perflbron emulsion as an alternative to blood transfusion: effects on tissue oxygenation during profound hemodilution in anesthetized dogs, TRANSFUSION 1998;38:145-155

34. Stetter, M.N.; Baerlocher, G.M.; Meiselman, H.J.; Reinhart, W.H., Influence of a recombinant hemoglobin solution on blood rheology, TRANSFUSION 1997;37:1149-1155

35. van Oss, C.J., Transfusion Immunology and Medicine, MARCEL DEKKER, INC., 1995

36. Zehner, C.S., Changes in Erythrocyte Membrane Composition and Vesicle Formation During Storage in ADSOL and CPDA-1

37. Coulter Epics XL/XCL system product reference manual. Coulter Electronics INC

38. Baker System 9110 Plus Operator's Manual, BioChem Immunosystems, Rev. .02, Sept 1996

39. Darzynkiewicz, Z.; Crissman, H.A., Methods in Cell Biology, Vol 33, 1990

40. Turabian, K.L., A Manual for Writers of Term Papers, Theses, and Dissertations, 4th Edition, 1973