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RED BLOOD CELL MEMBRANE MICROVISCOSITY CORRELATES WITH POSTTRANSFUSION SURVIVAL

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SUMMARY: The cholesterol/phospholipid and sphingomyelin/phospholipid ratios of red blood cells stored for 42 days were unchanged after storage. However, the total phospholipid concentration in the red cells decreased suggesting a loss of red cell membrane during storage. The 24 h survival of the stored red cells was assessed by the ^{51}Cr technique in homologous donors. A decrease in fluorescence polarization of diphenylhexatriene incorporated into the membrane was observed following storage which correlated with survival. Thus, molecular defects in the lipid bilayer are associated with long-term storage of red blood cells.

Several reports have focused on the membrane alterations associated with long-term storage of erythrocytes. Changes in red cell shape, osmotic fragility, and surface-area to volume ratios have been observed (1-3). These changes are related, in part, to a loss of membrane components during storage. A decrease in the fluidity of the overall cell membrane has been assessed by changes in cell deformation under shear stress (4). These measurements reflect the physical properties of the entire red cell and are primarily a function of membrane cytoskeletal proteins. Few studies have addressed the molecular basis of the defect in stored cells. An abnormality of red cell endocytosis in stored erythrocytes (5) and an altered spectrin-actin interaction (6) have been reported. These changes appear to be the result of cytoskeletal defects. However, it is not clear what changes occur, if any, in the lipid bilayer itself.

In the present report, the microenvironment of the lipid in erythrocyte membranes is examined by measurements of the polarization of the bilayer fluorescent probes/diphenylhexatriene (DPH) before and after 42 days of

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[1]Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene

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storage. In addition, survival of the cells returned to their original donors is measured. These polarization data and measurements of bilayer lipid composition show that the microviscosity of the membrane is correlated with survival of stored red blood cells.

MATERIALS AND METHODS

Storage of red blood cells. Donors were screened in accordance with standard blood banking procedures (7). Protocols for the studies were approved by human experimentation committees. Informed consent was obtained from donors. Normal healthy males donated one unit (450 ml) of blood which was collected into 63 ml of citrate-phosphate-dextrose (CPD) anticoagulant (8). Red blood cells were separated by centrifugation in the primary collection bag and packed to hematocrits of 85±5%, plasma was transferred to a satellite bag, sealed and removed. From a third satellite bag, 100 ml of 2.2% dextrose - 0.9% NaCl - 0.75% mannitol - 0.03% adenine was added to the packed red blood cells, mixed gently and stored at 1-6°C. Once each week the units were mixed by gentle inversion. After 42 days of storage, samples were removed from the bag for testing.

Analytical techniques. Hemolysis was calculated by dividing the plasma hemoglobin by the total hemoglobin concentration of the unit. Hematocrits were measured by microcentrifugation. Red blood cell lipids were extracted by the method of Bligh and Dyer (9). Phospholipids were separated by thin layer chromatography on silica gel and developed in chloroform-methanol-acetic acid-water (25:15:4:2) according to Skipski et al. (10). The lipids were extracted from the silica gel in chloroform-methanol (2:1) and concentrations were measured by phosphorous analysis (11). Cholesterol concentrations were measured on extracted red cell lipids by gas-liquid chromatography (OV-17 column) employing 5-α-cholestane as internal standard.

Fluorescent labeling of red blood cells. Red blood cells were labeled with DPH by adding 20 µl of 2 mM DPH in tetrahydrofuran to approximately 6 x 10^8 red blood cells in 50 ml of Tris-buffered saline (0.15 M NaCl, 10 mM Tris-HCl, pH 7.4). The molar ratio of phospholipid to fluorescent probe was ~500 to 1. The samples were incubated at 37°C until the fluorescence intensity was maximal (~30 min). The red cells were then washed 3X with 50 ml of Tris-buffered saline by spinning at 2800 rpm for 5 min, decanting off the supernatant fraction and repeating. Fluorescence measurements were made after suspending the samples in Tris-buffered saline.

Fluorescence polarization measurements. Polarization measurements on fluorescently-labeled red blood cells were made on a Perkin-Elmer MPF-44A spectrofluorometer equipped with a thermostatted cell compartment maintained to ±0.1°C. The contribution of scattering to the fluorescence measurement was minimized by use of a long wavelength pass filter with cut-on at 430 nm. An excitation wavelength of 360 nm and an emission wavelength of 460 nm was used. The excitation and emission slits were set for a 10 nm bandpass. The correction factor for the instrument's detection system, $G=I_{HV}/I_{HH}$, was measured with the excitation polarizer oriented horizontally and the emission polarizer oriented vertically (I_{HV}) or horizontally (I_{HH}). Polarization measurements were made with a vertical excitation polarizer and vertical (I_{VV}) or horizontal (I_{VH}) emission polarizer. After subtracting the contribution to the intensity from unlabeled cells, the polarization was calculated from $P=(I_{VV}-GxI_{VH})/(I_{VV}+GxI_{VH})$.

Determination of survival of stored red blood cells. The survival of stored cells was determined by a single label method which determines the 100% level of ^{51}Cr infused by measuring radioactivity in the circulation during the period immediately following infusion (12). Cells were labeled with ^{51}Cr-sodium chromate (5-20 µCi in 15 ml of cells at a hematocrit >70%) and

returned to their original donors within 30 min of labeling. Samples of blood were taken 5, 7.5, 10, 12.5, and 15 min after injection and the zero time 100% level was determined by extrapolation. A final sample was taken after 24±4 hours to determine the percent survival.

RESULTS

The lipid composition of the red blood cells before and after storage is shown in Table I. Storage results in a significant decrease (to 80% of initial) in the concentration of phospholipids in red blood cells. This loss of lipid does not appear to be related to hemolysis which averages 0.5% after storage. Storage does not significantly alter either the cholesterol/phospholipid or the sphingomyelin/phospholipid ratios in the red cell membranes.

To examine the relationship between survival and the microviscosity of the red blood cell membrane, the membranes were labeled with fluorescent probe molecules within the lipid bilayer. Over the temperature range 15-45°C, the difference in polarization of the fluorescence probe molecules before and after storage is maintained. The relationship between survival and fluorescence polarization of DPH is shown in Table II. A significant decrease in fluorescence polarization following storage and a decrease in polarization in red cell populations with lower survival percentages is evident.

DISCUSSION

The molecular basis of the decreased microviscosity of red blood cell membranes with storage and its relationship to decreased 24 h survival times is not clear. In membranes, the mole ratio of sphingomyelin and cholesterol to phospholipid plays a significant role in regulating the dynamic properties of the acyl chain region. A decrease in the sphingomyelin/phosphatidylcholine ratio in sheep red blood cells results in an increase in lipid microviscosity and an increase in osmotic fragility (13). Conversely, osmotic fragility is decreased following addition of cholesterol to red blood cells (14). Cooper and coworkers (14,15) have suggested that the addition of cholesterol to red cell membranes is associated with an expansion of the membrane surface area which leads to a decrease in membrane microviscosity and a decrease in osmotic fragility. An increase in osmotic and mechanical fragility of red blood cells has been associated with an increase in microviscosity of the bilayer when

TABLE I
EFFECT OF STORAGE ON RED BLOOD CELL LIPID COMPOSITION

	Before storage	After storage
Phospholipid (μg/10^6 cells)	31.3 ± 3.2	25.1 ± 1.3*
Cholesterol/Phospholipid (mol/mol)	0.89 ± 0.13	0.96 ± 0.16
Sphingomyelin/Phospholipid (mol/mol)	0.32 ± 0.02	0.37 ± 0.06
24-hour survival	100	78.6 ± 2.8***

*Significantly different at $p < 0.05$.

TABLE II
RELATIONSHIP BETWEEN 24 SURVIVAL AND FLUORESCENCE POLARIZATION OF RED BLOOD CELL MEMBRANES

Survival (%)	DPH Polarization
Before storage	.306 ± .005 (16)
90-99%	.287 ± .014 (5)
80-89%	.277 ± .027 (3)
70-79%	.275 ± .014 (4)
60-69%	.266 ± .019 (4)

cells are enriched in cholesterol relative to phosphatidylcholine (14,16) or in phosphatidylcholine relative to sphingomyelin (13). However, no significant changes in red cell membrane sphingomyelin/phospholipid or cholesterol/phospholipid ratios are evident with storage (Table I).

Several mechanisms for the decrease in red cell bilayer microviscosity with storage may be proposed. One is an alteration of lipid-protein or protein-protein interactions in the membrane. This may be secondary to a defect in the membrane cytoskeleton (1,6). Alternatively, lipid oxidation may lead to a decrease in bilayer microviscosity as reported for lipid dispersions and other biological membranes (17). Hydrolysis of membrane lipids by phospholipases may also result in changes in cell membrane shape which may be interpreted in terms of microviscosity changes (18) and possibly alterations in cell viability. Substantial oxidative damage or lipolysis of the bilayer lipids is expected to alter the lipid composition of the membrane. However, the present data do not support an altered lipid composition following storage, and unsaturated fatty acids in red cells are not decreased with storage (19). The present experiments provide the first evidence for molecular defects in the lipid bilayer of red cells during storage.

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